Roles and Regulation of Sphingosine Kinase 2 in Cancer

Heidi Anne Neubauer

Discipline of Biochemistry, School of Biological Sciences
University of Adelaide

A thesis submitted for the degree of
Doctor of Philosophy

July 2017

Table of Contents

List of Figures

Chapter 1: Introduction

Chapter 3: Validating Sphingosine Kinase 2 Antibodies

Chapter 4: An Oncogenic Role for Sphingosine Kinase 2

Chapter 5: Characterising the Interaction between Sphingosine Kinase 2 and Dynein Intermediate Chain

Chapter 6: The Role of Phosphorylation in Sphingosine Kinase 2 Regulation

Chapter 7: General Discussion

List of Tables

Chapter 1: Introduction

Chapter 4: An Oncogenic Role for Sphingosine Kinase 2

Chapter 6: The Role of Phosphorylation in Sphingosine Kinase 2 Regulation

Abstract

The two mammalian sphingosine kinases, SK1 and SK2, produce the bioactive signalling lipid sphingosine-1-phosphate (S1P), which generally promotes cell survival, proliferation and migration. In line with this, SK1 is often found to be upregulated in human cancers, and overexpression of SK1 leads to neoplastic transformation of cells and tumour growth. However, despite generating the same product, most evidence to date suggests that SK2 acts in an opposing manner to promote cell death. In contrast, knockout mouse models indicate that there is at least some functional redundancy between the two SK isoforms, and targeting SK2 via genetic or pharmacological approaches in cancer models results in reduced tumour growth. Clearly, the roles of SK2 are poorly understood, but it is apparent that these unique and complex functions of SK2 are largely dictated by its differential subcellular localisation. SK1 is generally a cytoplasmic protein, but it can also be translocated to the plasma membrane where it mediates cell survival, proliferation and oncogenic signalling through the production of S1P. SK2, however, has been reported to localise to the nucleus, endoplasmic reticulum and mitochondria, and at these locations it appears to possess anti-proliferative and pro-apoptotic functions. SK2 has also been reported to localise to the plasma membrane, but its specific roles here have not been well characterised. Therefore, the main aims of this study were to explore the roles of SK2 in cancer, and to characterise novel mechanisms that regulate SK2 subcellular localisation, such as interacting proteins and post translational modifications, in order to gain a better understanding of this complex enzyme and the potential benefits of targeting SK2 in cancer.

To explore the roles of SK2 in cancer, we examined the expression of SK2 in various human tumour samples using publically available datasets, and found that SK2 shows statistically significant upregulation in many cancers, but only to modest levels up to 2.5-fold over normal tissues. As high-level SK2 overexpression has been previously shown to cause cell death, we explored the effects of low, close to physiological levels of SK2 overexpression. By engineering a series of human and mouse cell lines overexpressing graded levels of SK2, we found that low-level SK2 overexpression increased cell survival and proliferation, and activated oncogenic signalling pathways. Notably, low-level SK2 overexpression (5- to 10-fold over endogenous levels) was sufficient to induce neoplastic transformation of mouse fibroblasts, resulting in efficient tumour formation *in vivo*. These findings coincided with decreased nuclear localisation and increased plasma membrane

localisation of SK2, as well as increases in extracellular S1P formation. Hence, we have shown for the first time that SK2 can have a direct role in promoting oncogenesis.

Furthermore, the Pitson laboratory previously identified a novel SK2-interacting protein, cytoplasmic dynein 1 intermediate chain 2 (IC-2), through a yeast two-hybrid screen, and characterising this interaction formed another part of these studies. We confirmed that SK2 interacts physiologically with the dynein complex in cells via the IC subunit, and being a retrograde-directed transport motor complex, we found that dynein mediates the translocation of SK2 away from the plasma membrane. Interestingly, although IC-2 was identified in the yeast two-hybrid screen, SK2 interacts more robustly with the highly-related IC-1 isoform, which is abundantly expressed in the brain. Strikingly, we found that IC-1 is downregulated 17-fold in glioblastoma multiforme (GBM) patient samples, which correlated with poorer survival of patients with this form of brain tumour. In line with a role for dynein in transporting SK2, low IC-1 expression in GBM cells coincided with more SK2 localised to the plasma membrane, where we had found it to accumulate in an oncogenic setting. Re-expression of IC-1 in these cells reduced plasma membrane localised-SK2 and extracellular S1P formation, and notably, decreased tumour growth and tumour-associated angiogenesis *in vivo*. Thus, these findings demonstrate a novel tumour-suppressive function of dynein IC-1, and uncover new mechanistic insights into SK2 regulation.

Through previous mass spectrometric analyses performed by the Pitson laboratory, it is evident that SK2 contains multiple uncharacterised phosphorylation sites that are not shared with SK1. We explored the function of one such site, Ser363, and found it to potentially regulate nuclear localisation of SK2. Furthermore, we identified SK2 as a *bona fide* substrate of glycogen synthase kinase 3 (GSK3) *in vitro* and in cells, involving residues Ser437 and Ser441, and we found that other phosphorylation events may act to regulate SK2 catalytic activity.

Overall, the studies outlined here have revealed a previously unreported role for SK2 in driving oncogenesis, and have described the characterisation of novel mechanisms that regulate the subcellular localisation of SK2. Therefore, these findings support the use of SK2 inhibitors as promising anti-cancer therapeutic agents. Furthermore, as the opposing functions of SK2 are largely dictated by it subcellular localisation, these findings may also assist in the development of new strategies to target oncogenic SK2 in cancer.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Heidi Anne Neubauer

Acknowledgements

Firstly, I would like to express my sincere thanks to my supervisor Professor Stuart Pitson for allowing me to complete my doctoral studies in the Molecular Signalling Laboratory. I have learned so much through your guidance and mentorship throughout my postgraduate studies. Many thanks for all of your support and of course, for your assistance with the preparation of this thesis. I would also like to thank my co-supervisor Associate Professor Claudine Bonder, I really appreciate all of your advice and assistance with experiments and your general ongoing support. Thank you to the University of Adelaide for accepting my candidature and the Centre for Cancer Biology for allowing me to conduct my research. I am also appreciative of the Australian Postgraduate Award and Dawes Scholarship for providing financial support throughout my studies.

Thank you also to everyone in the Molecular Signalling Laboratory, both past and present. Over the years there have been some great discussions, a lot of laughs, a lot of help and advice, and generally just a great environment to work in. Thanks for being like a second family, I will miss you all and I wish you all the best. Also thank you to everyone at the Centre for Cancer Biology, especially within the department of Human Immunology, for your help and support, friendship, and general chats in the tea room.

A special thank you to my dear friend, Julia, who is arguably the reason I pursued a career in research. In the lab, you were a fantastic supervisor during Honours and your patience, advice and encouragement helped me to grow into a more critically-thinking, independent scientist. Outside the lab, you are a wonderful friend who has always provided unconditional support, which honestly means the world to me. I would also like to acknowledge and thank my PhD buddy Layla who, through the good and tough times, was always there to listen and provide support when I really needed it. We finally made it!

Finally, I would like to thank my family and friends for supporting me through this long journey. Especially to my parents and sister, Amanda, thank you for your love, patience, thoughtfulness, and for always showing an interest in my work, it means a lot. And thank you to Greg, for always being there, for your love and support, for listening, for keeping me sane and making me laugh, for being understanding of my work and always giving me the time I need. This journey would've been so much harder without you.

Abbreviations

Aβ	Amyloid β
ABC	ATP binding cassette
AC	Adenylyl cyclase
AD	Alzheimer disease
ADP	Adenosine-5'-diphosphate
ALL	Acute lymphoblastic leukaemia
AMPK	AMP-activated protein kinase
Ang II	Angiotensin II
ATP	Adenosine-5'-triphosphate
BACE1	Beta-secretase 1
BH3	Bcl-2 homology 3
BRSK	Brain-specific serine/threonine kinase
BSA	Bovine serum albumin
CaM	Calmodulin
CerS	Ceramide synthase
CIB	Calcium- and integrin-binding protein
CMV	Cytomegalovirus
COX-2	Cyclooxygenase 2
DAPI	4', 6-diamidino-2-phenylindole
DAPK	Death-associated protein kinase
DBS	Donor bovine serum
DEPC	Diethylpyrocarbonate
dhS1P	Dihydrosphingosine-1-phosphate
DMEM	Dulbecco's modified eagle medium
DMS	*N*, *N*-dimethylsphingosine
Dox	Doxycycline
DTT	Dithiothreitol
EB	Extraction buffer
ECL	Enhanced chemiluminescence
eEF1A	Eukaryotic elongation factor 1A
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase 1/2
ERM	Ezrin-radixin-moesin
EV	Empty vector
FACS	Fluorescence associated cell sorter
FBS	Fetal bovine serum
FcεRI	High-affinity receptor for IgE
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde phosphate dehydrogenase
GBM	Glioblastoma Multiforme
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GSH	Glutathione-sepharose
GST	Gluthathione-*s*-transferase
GTP	Guanosine triphosphate
H&E	Haematoxylin and eosin
HA	Hemagglutinin
HC	Heavy chain
HDAC	Histone deacetylase
HEK293	Human Embryonic Kidney 293 cells
HRP	Horseradish peroxidase
hTERT	Human telomerase reverse transcriptase
IB	Immunoblot
IBD	Inflammatory bowel disease
IC	Intermediate chain
IF	Immunofluorescence
IL	Interleukin
IP	Immunoprecipitation
IPTG	Isopropyl 1-thio-β-D-galactopyranoside
IRES	Inter-ribosomal entry site
JNK	Jun N-terminal kinase
KLH	Keyhole limpet hemocyanin

KSR	Kinase suppressor of ras
LC	Light chain
LIC	Light intermediate chain
MAPK	Mitogen-activated protein kinase
MAPKAPK	Mitogen-activated protein kinase (MAPK)-activated protein kinase
miRNA	microRNA
MEF	Mouse embryonic fibroblast
MEM	Minimum essential media
MLK	Mixed lineage kinase
MTOC	Microtubule-organising centre
NES	Nuclear export signal
NLS	Nuclear localisation signal
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
NSCLC	Non-small cell lung cancer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PECAM-1	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidylinositol 3-kinase
PIM	Proto-oncogene serine/threonine-protein kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D
PLA	Proximity ligation assay
PLC	Phospholipase C
PM	Plasma membrane
PMA	Phorbol 12-myristate 13-acetate
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
PS	Phosphatidylserine
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RARβ	Retinoic acid receptor β
ROCK	Rho-associated protein kinase
RSK	Ribosomal s6 kinase $p90^{rsk}$

S1P	Sphingosine-1-phosphate
$S1P_{1-5}$	Sphingosine-1-phosphate receptors 1-5
S1PP	Sphingosine-1-phosphate phosphatase
SAC	Spindle assembly checkpoint
SDS	Sodium dodecyl sulphate
SGK	Serum/glucocorticoid regulated kinase
siRNA	Small interfering RNA
SK	Sphingosine kinase
SMP	Skim milk powder
Sph	Sphingosine
SREBP	Sterol regulatory element binding protein
TGF	Transforming growth factor
TLC	Thin-layer chromatography
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	Vascular endothelial growth factor
WT	Wildtype

Chapter 1:
Introduction

Aspects of this Chapter have been published as:

Neubauer HA and Pitson SM (2013) Roles, regulation and inhibitors of sphingosine kinase 2. *FEBS Journal* **280:** 5317-5336.

See Appendix 1 for author contributions and reprint

Chapter 1. Introduction

Sphingolipids have emerged as important signalling molecules that can regulate a vast range of cellular processes, including cell survival, proliferation, migration, differentiation and inflammatory responses (Hannun & Obeid, 2008; Maceyka et al, 2009; Pitson & Pebay, 2009). Although many enzymes are involved in regulating the relative levels of the sphingolipids, the sphingosine kinases (SKs), SK1 and SK2, are of particular interest as their activity can be dynamically regulated by external stimuli (Pitson, 2011). As a result, altering the levels or activity of the SKs can play a key role in controlling cell fate, making them attractive targets in the development of therapeutics for many diseases, particularly cancer (Cuvillier et al, 2010; Edmonds et al, 2011; Gault & Obeid, 2011; Newton et al, 2015; Pitman & Pitson, 2010; Pitson, 2011; Pyne & Pyne, 2010). Indeed, given its unequivocal role in promoting cell survival and proliferation, targeting SK1 has proven effective in attenuating tumour growth and progression in a range of cancer models (reviewed in Pitman & Pitson (2010) and Pitman et al (2016)). However, investigating the therapeutic potential of targeting SK2 in cancer has only recently gained momentum, and although the findings are promising, the mechanisms behind SK2 function and regulation remain poorly understood. Indeed, the current literature focuses largely on SK1, and although the exact cellular roles of SK2 are yet to be fully deciphered, what is currently known about this isoform suggests that its functions and mechanisms of regulation are extremely complex. Therefore, this thesis examines the function of SK2, specifically in the context of neoplastic transformation, and also how SK2 is regulated within the cell by interacting proteins and post-translational modifications.

1.1 Sphingosine-1-phosphate and sphingolipid signalling

Sphingosine-1-phosphate (S1P) is a biologically active phospholipid that is derived from a network of sphingolipid synthesis and degradation pathways (Pitson, 2011). Ceramide resides at the centre of this network and can be deacylated by ceramidases to form sphingosine, which can in turn be phosphorylated by SKs to produce S1P (Figure 1.1). The aforementioned reactions are reversible and the only exit point from the cycle is the irreversible degradation of S1P to hexadecenal and phosphoethanolamine.

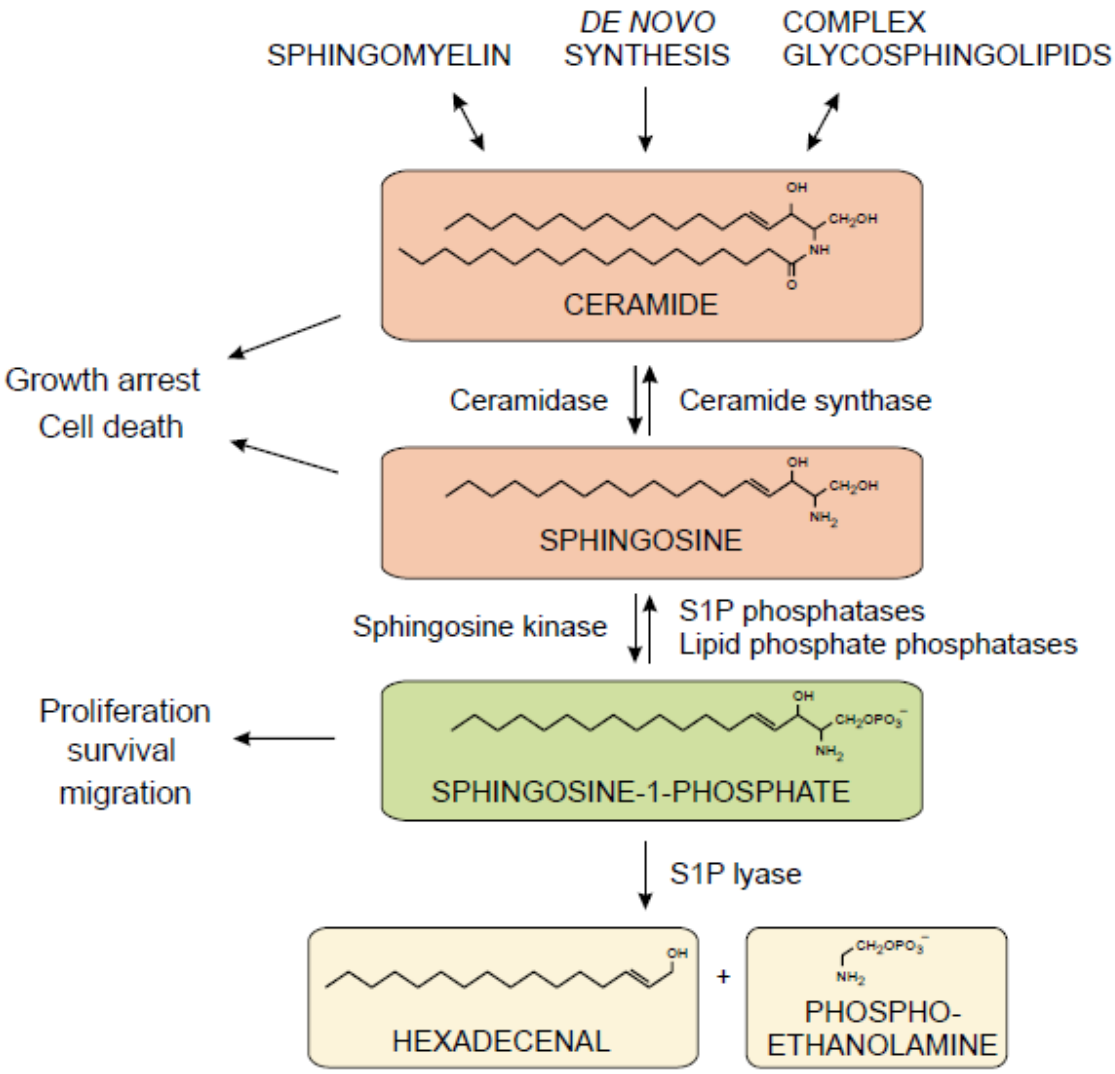


Figure 1.1: Sphingolipid synthesis and degradation pathways

Sphingolipid structures are shown with the key enzymes that catalyse their synthesis and degradation. Figure taken from Neubauer & Pitson (2013).

S1P acts as both an extracellular and intracellular signalling molecule through a range of different pathways (Strub et al, 2010). Although export of the SKs from cells has been reported (Soldi et al, 2007; Venkataraman et al, 2006; Waters et al, 2003; Weigert et al, 2010), the vast majority of S1P appears to be formed within cells (Tani et al, 2007). Intracellular S1P can then be transported out of the cell where it exists in high nanomolar concentrations in the blood (Sensken et al, 2010; Yatomi et al, 1997). This export is mediated by the transporter spinster homolog 2 (Spns2) (Hisano et al, 2012), as well as by members of the ABC transporter family (Sato et al, 2007; Takabe et al, 2010). Upon exit from the cell, S1P can bind to and activate a family of five G protein coupled receptors (GPCRs), referred to as $S1P_{1-5}$ (Chun et al, 2010), to elicit autocrine or paracrine signalling (Figure 1.2). Interestingly, evidence suggests that S1P may engage its GPCRs by lateral diffusion within the outer leaflet of the plasma membrane, rather than directly from the extracellular space (Hanson et al, 2012). The S1P receptors are coupled to various heterotrimeric G proteins, which subsequently activate or inhibit a number of downstream signalling pathways via the modulation of extracellular signal-regulated kinases 1/2 (ERK1/2), small GTPases (Rac and Rho), the phosphoinositide 3-kinase (PI3K)/AKT pathway, adenylyl cyclase (AC)/cyclic AMP, Jun N-terminal kinase (JNK), and phospholipase C (PLC) (Figure 1.2), ultimately promoting cell proliferation, survival and migration (Meyer zu Heringdorf & Jakobs, 2007; Nagahashi et al, 2014; Pyne & Pyne, 2010). These responses can vary in a cell- or tissue-specific manner as a result of the differential expression of the S1P receptors and the various G proteins they couple to (Rosen et al, 2009). Notably, although S1P is present at high levels in blood, it is in relatively low abundance in tissues, resulting in an S1P gradient owing to differences in levels of S1P metabolic enzymes and substrate availability (Hla et al, 2008).

In addition to its extracellular signalling roles, S1P can also act directly on a number of intracellular targets. Specifically, S1P has been shown to directly inhibit histone deacetylase (HDAC) 1/2 activity (Hait et al, 2009), bind to and stabilise human telomerase reverse transcriptase (hTERT) (Panneer Selvam et al, 2015), function as a co-activator to the transcription factor peroxisome proliferator-activated receptor (PPAR)γ (Parham et al, 2015), interact with prohibitin 2 to mediate mitochondrial respiration (Strub et al, 2011) and possibly modulate the activity of p21-activated kinase 1 (Maceyka et al, 2008). Furthermore, upon induction of endoplasmic reticulum (ER)-stress, S1P was found to mediate NF-κB activation by directly binding to the heat shock proteins HSP90α and

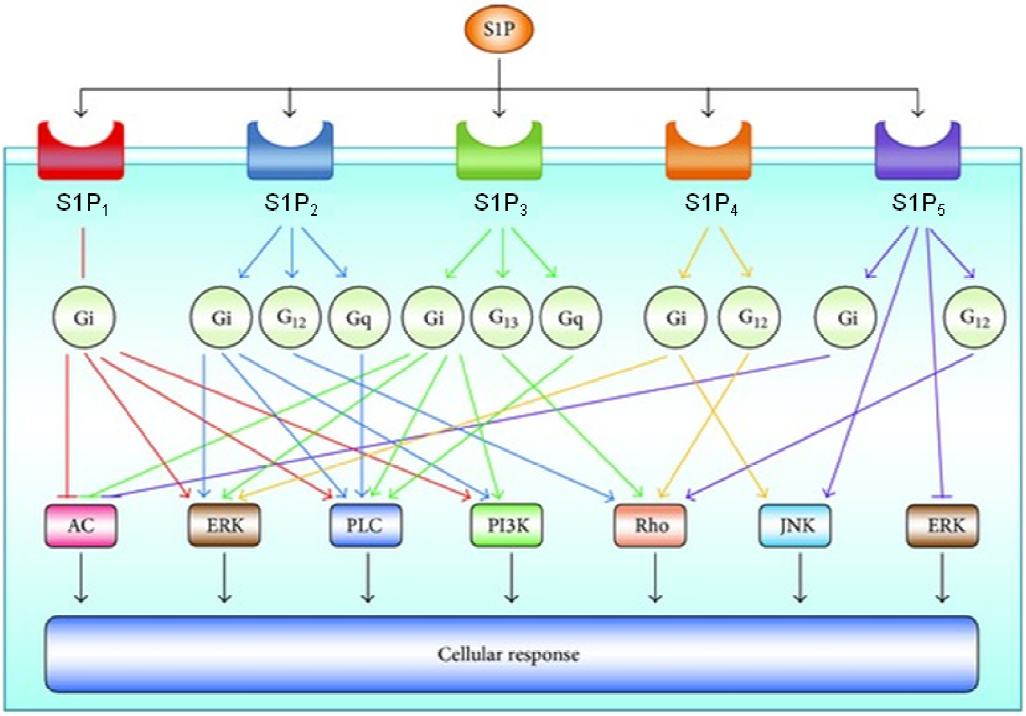


Figure 1.2: Sphingosine-1-phosphate receptors

Downstream signalling pathways initiated by the sphingosine-1-phosphate (S1P) receptors. S1P is a ligand for five G protein coupled receptors: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$. Each S1P receptor is coupled to different G-proteins, which individually regulate different downstream signalling pathways, including adenylyl cyclase (AC)/cyclic AMP, extracellular signal-regulated kinase (ERK), phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3K), Rho family small GTPases, and Jun N-terminal kinase (JNK). Figure adapted from Nagahashi et al (2014).

GRP94, an interaction dependent on the presence of IRE1α (Park et al, 2016). It has also been described that S1P produced specifically by SK1 can directly bind to and activate the E3 ubiquitin ligase activity of TNF receptor-associated factor 2 (TRAF2), leading to the activation of NF-κB signalling (Alvarez et al, 2010). However, recent studies have since reported that SK1 genetic knockout does not recapitulate the disruption of TNF and NF-κB signalling observed as a result of the genetic deletion of TRAF2 in fibroblasts and keratinocytes (Etemadi et al, 2015; Xiong et al, 2013). Additionally, other studies were unable to detect an interaction between S1P and TRAF2 (Park et al, 2016). Therefore, the true function of S1P as a co-activator of TRAF2 will require further examination, as this role may be dependent on cell/tissue type and specific signalling events. Another study has suggested that S1P may contribute to cytochrome c release from mitochondria via modulation of BAK, although it should be noted that in this study a direct interaction of S1P with BAK was not demonstrated (Chipuk et al, 2012).

Through the activation of these varied signalling pathways, S1P can act to promote cell survival, proliferation and migration, as well as regulate differentiation, angiogenesis and inflammation (Pebay et al, 2007; Pyne & Pyne, 2010; Spiegel & Milstien, 2003). Interestingly, an intracellular role for the $S1P_5$ GPCR has also been proposed, where the co-localisation of the receptor and SKs to centrosomes may indicate a novel function in regulating cell division (Gillies et al, 2009). Somewhat surprisingly, exogenously-expressed $S1P_2$ was found to translocate to the nucleus of MDA-MB-231 breast cancer cells and consequently reduce cell growth, but only when SK2 or $S1P_4$ were blocked (Ohotski et al, 2014), suggesting that SK2-mediated signalling through $S1P_4$ can block this anti-proliferative nuclear function of $S1P_2$.

In contrast to S1P, the sphingolipids ceramide and sphingosine, which both lie directly upstream of S1P in the sphingolipid biosynthetic pathway (Figure 1.1), are promoters of apoptosis. Ceramide has been found to directly modulate a number of protein targets that appear to facilitate its pro-apoptotic functions, including protein phosphatases PP1 (Chalfant et al, 2004), PP2A (Chalfant et al, 2004) and PP2C (Perry et al, 2012), protein kinase C (PKC)ζ (Muller et al, 1995; Wang et al, 2005), the kinase suppressor of ras (KSR) (Zhang et al, 1997), cathepsin D (Heinrich et al, 2004), mixed lineage kinase (MLK) (Sathyanarayana et al, 2002), and inhibitor 2 of PP2A (I2PP2A/SET) (Mukhopadhyay et al, 2009; Saddoughi et al, 2013). Through these interactions, ceramide has been found to activate the stress-

activated protein kinase pathway, mediate the dephosphorylation of Bcl-2, AKT, retinoblastoma protein and c-Jun, and trigger caspase-3 activation and mitochondrial-mediated apoptosis (Hannun & Obeid, 2008; Modrak et al, 2006). It has also been suggested that ceramide itself can form pores in mitochondrial membranes, resulting in membrane permeabilisation and release of pro-apoptotic proteins (Siskind, 2005). Furthermore, the pro-apoptotic effects of sphingosine have, to date, been attributed to its ability to strongly inhibit PKC (Taha et al, 2006b), as well as to bind to the pro-survival adaptor protein 14-3-3 and promote its phosphorylation and inactivation by kinases such as PKA and a cleaved fragment of PKCδ (Woodcock et al, 2010). Sphingosine has been shown to mediate inhibition of the ERK and AKT pathways, as well as induce caspase-dependent apoptosis via Bid cleavage, cytochrome c release and effector caspase activation (Taha et al, 2006b).

Given these opposing functions, the current dogma is that a delicate balance must be maintained between the relative levels of pro-apoptotic ceramide and sphingosine and pro-survival S1P, with this equilibrium, referred to as the 'sphingolipid rheostat', playing an important role in dictating cell fate (Cuvillier et al, 1996; Newton et al, 2015; Pyne & Pyne, 2010). Sphingosine kinase is a central regulator of this equilibrium, residing at the critical junction between pro-proliferative, pro-survival S1P and pro-apoptotic sphingosine and ceramide (Figure 1.1). Therefore, understanding how the SKs are regulated is important to understand not only their role in controlling cell fate, but also in aberrant survival and proliferative signalling in disease states like cancer. Indeed, there are a multitude of studies that implicate S1P and the SKs in cancer development, survival and metastasis, and this field has been extensively reviewed (Cuvillier et al, 2010; Edmonds et al, 2011; Gault & Obeid, 2011; Newton et al, 2015; Pitman et al, 2016; Pitman & Pitson, 2010; Pitson, 2011; Pitson et al, 2011; Pyne & Pyne, 2010; Taha et al, 2006a).

1.2 Sphingosine kinases

Two SKs exist in mammalian cells, namely SK1 and SK2. In humans they are generated from two distinct genes, *SPHK1* and *SPHK2*, which are located on chromosome 17 (17q25.2) and 19 (19q13.2), respectively. All known eukaryotic SKs share five highly conserved regions within their sequence (termed C1 to C5; see Figure 1.3), which appear to encompass the regions necessary for ATP binding and catalysis

A

```
SK1    1 ------------------------------------------------------------ 1
SK2    1 MAPPPPPLAASTPLLHGEFGSYPARGPRFALTLTSQALHIQRLRPKPEARPRGGLVPLAE 60

SK1    1 ------------------------------------------------------------ 1
SK2   61 VSGCCTLRSRSPSDSAAYFCIYTYPRGRRGARRRATRTFRADGAATYEENRAEAQRWATA 120
                                          C1
SK1    1 ----------MDPAGGPRGVLPRPCRVLVLLNPRGGKGKALQLFRSHVQPLLAEAEISFT 50
SK2  121 LTCLLRGLPLPGDGEITPDLLPRPPRLLLLVNPFGGRGLAWQWCKNHVLPMISEAGLSFN 180
                                      C2                        
SK1   51 LMLTERRNHARELVRSEELGRWDALVVMSGDGLMHEVVNGLMERPDWETAIQKPLCSLPA 110
SK2  181 LIQTERQNHARELVQGLSLSEWDGIVTVSGDGLLHEVLNGLLDRPDWEEAVKMPVGILPC 240
            C3                                                  
SK1  111 GSGNALAASLNHYAGYEQVTNEDLLTNCTLLLCRRLLSPMNLLSLHTASGLRLFSVLSLA 170
SK2  241 GSGNALAGAVNQHGGFEPALGLDLLLNCSLLLCRGGGHPLDLLSVTLASGSRCFSFLSVA 300
                  C4                                            
SK1  171 WGFIADVDLESEKYRRLGEMRFTLGTFLRLAALRTYRGRLAYLPVGRVGSKT-PA----- 224
SK2  301 WGFVSDVDIQSERFRALGSARFTLGTVLGLATLHTYRGRLSYLPATVEPASPTPAHSLPR 360

SK1  224 ------------------------------------------------------------ 224
SK2  361 AKSELTLTPDPAPPMAHSPLHRSVSDLPLPLPQPALASPGSPEPLPILSLNGGGPELAGD 420

SK1  225 --------------------SP------------------------------VVVQQGPV 234
SK2  421 WGGAGDAPLSPDPLLSSPPGSPKAALHSPVSEGAPVIPPSSGLPLPTPDARVGASTCGPP 480

SK1  235 DAHLVPLEEPVPSHWTVVPDEDFVLVLALLHSHLGSEMFAAPMGRCAAGVMHLFYVRAGV 294
SK2  481 DHLLPPLGTPLPPDWVTL-EGDFVLMLAISPSHLGADLVAAPHARFDDGLVHLCWVRSGI 539
                                                  C5
SK1  295 SRAMLLRLFLAMEKGRHMEYECPYLVYVPVVAFRLEPKDGKGVFAVDGELMVSEAVQGQV 354
SK2  540 SRAALLRLFLAMERGSHFSLGCPQLGYAAARAFRLEPLTPRGVLTVDGEQVEYGPLQAQM 599

SK1  355 HPNYFWMVSG---CVEPPPSWKPQQMPPPEEPL 384
SK2  600 HPGIGTLLTGPPGCPGREP-------------- 618
```

B

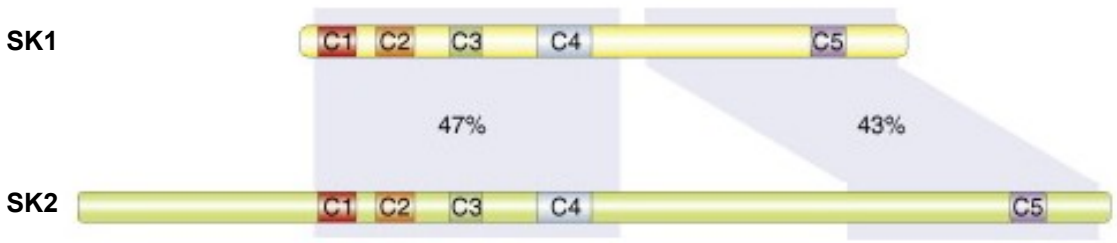


Figure 1.3: Sequence alignment of SK1 and SK2

A, Amino acid sequence alignment of the human SK1a and SK2a isoforms, with the five evolutionarily conserved regions, C1 to C5, indicated by black lines. Sequence alignment was performed using the Clustal Omega program via UniProt. *B,* The sphingosine kinases share 45% overall sequence identity within the regions that are conserved in both isoforms (grey shaded area). Unshaded regions represent sequences unique to each isoform. Figure adapted from Pitson (2011).

(Leclercq & Pitson, 2006; Pitman et al, 2015; Pitson, 2011). Although human SK1 and SK2 vary considerably in size (384 and 618 amino acids for SK1a and SK2a, respectively) (Liu et al, 2000), they share 80% similarity and 45% overall sequence identity, with almost all of the polypeptide sequence of SK1 aligning with regions of the larger SK2 (Figure 1.3). The additional residues present in SK2 result from both its extended N-terminus and additional central proline-rich region not found in SK1, or in any other protein.

Both SK1 and SK2 have multiple splice variants. Human SK1 has three known isoforms; SK1a, SK1b and SK1c, possessing 384, 402 and 470 amino acids, respectively. SK1a is the best characterised of the SK1 isoforms, and it is the main isoform referred to in the literature. SK1b and SK1c both contain N-terminal extensions with respect to SK1a, and are less well characterised. Human SK2 has four reported isoforms, two of which are well documented (SK2a and SK2b) and two are only putative (SK2c and SK2d). The SK2 splice isoforms will be discussed in more detail later in the chapter.

The crystal structure for SK1 was only recently solved (Wang et al, 2013), but there is currently no crystal structure available for SK2, making comparisons between the two SKs difficult. However, the central proline-rich region of SK2 appears to coincide with the sphingosine-binding region of these enzymes (Pitson, 2011), and consistent with this, biochemical information regarding substrate specificity and inhibition of the two SKs suggest significant differences exist in their sphingosine binding regions. As discussed below, SK2 is more promiscuous than SK1 in the substrates it can phosphorylate, and several isoform-selective inhibitors that target the sphingosine binding site of these enzymes have been developed (French et al, 2010; Gao et al, 2012; Patwardhan et al, 2015). Interestingly, despite the high degree of sequence conservation in the putative ATP-binding regions of the SKs (Pitman et al, 2015), an ATP competitive inhibitor has been reported that shows high selectivity for SK1 over SK2 (Gao et al, 2012). Experimental evidence has revealed that the single point mutant G212E completely abolishes SK2 catalytic activity (Maceyka et al, 2005) just like the comparable G82D mutation in SK1 (Pitson et al, 2000b), via interfering with ATP binding (Pitson et al, 2002).

While the activity of both SK isoforms can be enhanced by various cytokines and growth factors (discussed further below), both enzymes also possess intrinsic catalytic activity independent of eukaryotic post-translational modifications, that results in cellular SK

activity even in the absence of agonist stimulation (Kono et al, 2002; Pitson et al, 2000a). This basal SK activity has been proposed to facilitate a housekeeping role in maintaining cellular sphingosine and ceramide levels (Chan & Pitson, 2013). Although both SK isoforms are ubiquitously expressed in all human tissues and cells, some differential expression is apparent, with SK1 most highly expressed in lung, spleen and leukocytes (Melendez et al, 2000) whereas SK2 is highest in the kidney and liver (Liu et al, 2000). Furthermore, while both SKs are expressed throughout embryonic development in the mouse, SK1 expression peaks earlier (E7-E11), with SK2 being more strongly expressed in the later stages (E15-E17) (Liu et al, 2000).

1.2.1 Roles and regulation of SK1

Of the two SKs, SK1 is by far the most well studied. SK1 has been widely described as a pro-survival, pro-proliferative enzyme, demonstrated initially by studies showing that overexpression of SK1 increases cell survival and proliferation (Olivera et al, 1999) and induces neoplastic transformation (Xia et al, 2000). Numerous studies have implicated SK1 in cancer development and progression, where expression levels of SK1 are found to be upregulated in a number of human solid tumours (Bayerl et al, 2008; French et al, 2003; Johnson et al, 2005; Kawamori et al, 2006; Li et al, 2008; Malavaud et al, 2010; Van Brocklyn et al, 2005), and higher SK1 expression in tumours is correlated with poor patient prognosis (Facchinetti et al, 2010; Li et al, 2008; Li et al, 2015; Li et al, 2009; Liu et al, 2010; Long et al, 2010; Meng et al, 2014; Pan et al, 2011; Ruckhaberle et al, 2008; Sinha et al, 2011; Van Brocklyn et al, 2005; Zhang et al, 2014). Notably, targeting SK1 by chemical inhibition or genetic ablation has successfully reduced the growth of a wide variety of tumours in mice (Endo et al, 1991; French et al, 2003; French et al, 2006; Kawamori et al, 2009; Kohno et al, 2006; Park et al, 1994; Pchejetski et al, 2008; Ponnusamy et al, 2012; Shirai et al, 2011; Sinha et al, 2011).

The oncogenic signalling mediated by SK1 is dependent upon its activation and translocation to the plasma membrane (Pitson et al, 2005). SK1 is a cytoplasmic protein that, upon agonist stimulation, can be phosphorylated by ERK1/2 at Ser225, which results in a 14-fold increase in its catalytic activity (Pitson et al, 2003). SK1 can be transported to the plasma membrane via binding to calcium- and integrin-binding protein 1 (CIB1) (Jarman et

al, 2010). Interestingly, other mechanisms of SK1 translocation to the plasma membrane have also been documented, involving Gq-coupled receptor activation (ter Braak et al, 2009) and constitutively-active oncogenic K-RasG12V (Gault et al, 2012). At the plasma membrane, SK1 catalyses the formation of S1P from plasma membrane-associated sphingosine, which appears to facilitate both the efficient release of S1P to act extracellularly on cell surface S1P receptors, as well as interaction with intracellular targets to mediate downstream signalling and promote cell survival, proliferation, migration, differentiation, angiogenesis and inflammation (Maceyka et al, 2009; Pitson & Pebay, 2009; Pyne & Pyne, 2010).

1.2.2 Roles of SK2

1.2.2.1 Apoptosis and cell cycle arrest

Contrary to the roles of SK1 in pro-proliferative, pro-survival signalling, many of the early studies examining SK2 function found that its over-expression induced cell cycle arrest and apoptosis (Igarashi et al, 2003; Maceyka et al, 2005; Okada et al, 2005). A putative BH3 domain was identified within SK2, which has been proposed to mediate its pro-apoptotic functions, at least in part, through interaction with Bcl-x_L, a pro-survival Bcl-2 family member (Liu et al, 2003). Since this SK2-Bcl-x_L interaction was only demonstrated following SK2 overexpression, however, the physiological significance of this proposed association remains unclear. More recently, mitochondrial localisation of SK2, and specifically S1P generation at this site, was shown to contribute to BID-mediated activation of BAK, and subsequent mitochondrial membrane permeabilisation and cytochrome c release (Chipuk et al, 2012). Another study demonstrated that nuclear S1P and dihydro-S1P produced specifically by SK2 inhibited HDAC1/2 activity, leading to increased histone acetylation at distinct promoters, and resulting in enhanced transcription of cyclin-dependent kinase inhibitor p21 and transcriptional regulator c-fos (Hait et al, 2009). While this is likely to contribute to the growth arrest previously associated with SK2, the notion that SK2 can act as an epigenetic and transcriptional regulator suggests that there may be other downstream effects of its nuclear activity yet to be elucidated. Indeed, as outlined below (section 1.2.2.2), S1P-mediated regulation of HDAC activity may also contribute to cell survival and proliferation.

While a number of the initial studies describing pro-apoptotic effects of SK2 are based on data from forced overexpression of this protein, several studies also suggest this role for endogenous SK2. For example, siRNA-mediated knockdown of endogenous SK2 in HEK293 cells or mouse embryonic fibroblasts prevented the induction of apoptosis by TNFα (Chipuk et al, 2012; Okada et al, 2005), while mesangial cells taken from *Sphk2*$^{-/-}$ mice displayed greater resistance to staurosporine-induced apoptosis than wildtype or *Sphk1*$^{-/-}$ cells (Hofmann et al, 2008). Thus, these studies support the notion that SK2 can have an opposite role to SK1 in the control of cell survival. Indeed, the tissue distribution and developmental expression of the SKs would also suggest that they may have at least some differing roles.

1.2.2.2 Cell survival, proliferation and cancer

Although, as outlined above, there is compelling evidence to suggest that SK2 can have a physiological role in inducing apoptosis, there are now many studies also supporting a role for SK2 in promoting cell survival and proliferation, much like SK1. Indeed, there appears to be some functional redundancy between the two enzymes, as deletion of either *Sphk1* or *Sphk2* in mice produces no gross phenotype (Allende et al, 2004; Mizugishi et al, 2005; Zemann et al, 2007), and yet a double knockout of both genes is embryonic lethal as a result of defects in neurological and vascular development (Mizugishi et al, 2005). siRNA-mediated knockdown of SK2 has been shown to enhance apoptosis and decrease chemotherapeutic resistance in a number of cancer cell types (Nemoto et al, 2009; Sankala et al, 2007; Schnitzer et al, 2009; Sun et al, 2015; Van Brocklyn et al, 2005). Notably, targeting SK2 in some cancer cell lines can have more of an anti-cancer effect than targeting SK1 (Gao & Smith, 2011; Van Brocklyn et al, 2005). Furthermore, a number of *in vivo* studies have reported that tumour growth can be significantly attenuated following the genetic ablation of SK2 in MCF-7 breast tumour xenografts (Weigert et al, 2009), or the pharmacological inhibition of SK2 in a range of cancer models in mice, including leukaemia (Liu et al, 2013; Wallington-Beddoe et al, 2014), multiple myeloma (Venkata et al, 2014), breast (Antoon et al, 2012; Antoon et al, 2011; French et al, 2010; Liu et al, 2013), kidney (Beljanski et al, 2011a), pancreatic (Beljanski et al, 2011a), liver (Beljanski et al, 2011b), lung (Panneer Selvam et al, 2015) prostate (Schrecengost et al, 2015) and colon cancer (Chumanevich et al, 2010; Xun et al, 2015). Increased SK2 expression levels have also been

shown to correlate with disease progression in non-small cell lung cancer (NSCLC) (Wang et al, 2014b) and multiple myeloma (Venkata et al, 2014), and poorer survival in NSCLC patients (Wang et al, 2014b).

More recently, functional studies are beginning to uncover the mechanisms whereby SK2 may be mediating cell survival and proliferation. It has been proposed that nuclear S1P, produced by SK2, can act as an antagonist to the retinoic acid receptor β (RARβ), attenuating ligand-stimulated tumour suppressor effects of this nuclear receptor in human colon carcinoma cells (Sun et al, 2012). Furthermore, the known role of SK2 in epigenetic regulation via HDAC1/2 inhibition has recently been shown to facilitate the upregulation of c-Myc mRNA and protein levels in acute lymphoblastic leukaemia (ALL) (Wallington-Beddoe et al, 2014). Notably, S1P produced by nuclear-localised SK2 has also recently been proposed to bind to and stabilise hTERT, preventing telomere damage, delaying cell senescence and promoting tumour development (Panneer Selvam et al, 2015).

SK2 has also been shown to play a role in TGFβ-induced migration of esophageal cancer cells (Miller et al, 2008), epidermal growth factor (EGF)-induced migration of breast cancer cells (Hait et al, 2005), and the migration and invasion of colorectal cancer cells (Zhang et al, 2016), suggesting a potential role for SK2 in metastasis. Indeed, it was recently demonstrated that SK2 is required for EGF-induced cancer cell invasion, by facilitating activation of ezrin-radixin-moesin (ERM) proteins through intracellular S1P production (Adada et al, 2015). Interestingly, a recent study proposed that the transcription factor CREB binds to the 5' promoter region of SK2, and under conditions of serum-deprivation, SK2 is upregulated at the mRNA and protein level as a result of increased CREB binding, in order to promote colon cancer cell proliferation in the absence of serum (Mizutani et al, 2015).

1.2.2.3 Inflammation and immune cell regulation

The role of the SKs in inflammation and immune cell function has been widely investigated, with most studies focusing on SK1 and its role in promoting inflammation. SK1 can be both post-translationally activated and transcriptionally upregulated by a number of inflammatory signalling molecules such as TNF-α, IL-1β, IFN-γ, IgE and C5a (Billich et al, 2005; Snider

et al, 2010; Xia et al, 1998), and shown to regulate monocyte, macrophage and neutrophil function during the inflammatory response (Snider et al, 2010). However, despite SK1 having a clear role in promoting/enhancing inflammation, the role of SK2 in the inflammatory response is controversial, with many of the studies suggesting that SK2 may in fact be anti-inflammatory. For example, it was demonstrated, using a breast cancer xenograft model, that MCF-7 cells with stable shRNA-mediated SK2 knockdown had increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory IL-10, which coincided with a decrease in tumour growth (Weigert et al, 2009). Furthermore, unlike SK1, siRNA-mediated knockdown of SK2 in a murine collagen-induced arthritis model led to more aggressive disease and production of pro-inflammatory cytokines (Lai et al, 2009). Notably, Samy et al employed an adoptive transfer model of inflammatory bowel disease (IBD) where T cell-deficient C.B-17 *scid* mice were injected with *Sphk2*$^{-/-}$ T cells. They found that mice receiving these cells had increased levels of pro-inflammatory cytokines and worsened intestinal inflammation than those receiving wildtype T cells, seemingly due to enhanced IL-2 responsiveness and increased expression of activated pSTAT5 (Samy et al, 2007). Therefore, SK2 may play a role in negatively regulating IL-2 signalling by attenuating STAT5 activation.

In contrast to the studies described above, the pharmacological inhibition of SK2 by ABC294640 demonstrated anti-inflammatory effects in murine models of IBD (Maines et al, 2008; Maines et al, 2010) and in rodent models of inflammatory arthritis (Fitzpatrick et al, 2011a), suggesting that SK2 can promote inflammation. However, it is intriguing that opposite effects are observed only with the pharmacological inhibition of SK2 when compared to genetic ablation and RNAi, suggesting either that the loss of SK2 protein elicits a different effect than its inhibition, or that the purported 'SK2-specific' inhibitor may be having off-target effects on other pathways. Indeed, as discussed further below, the commonly used SK2 inhibitor ABC294640 is now known to modulate other targets in addition to SK2 (Pitman et al, 2016), suggesting that it may not be an ideal tool to interrogate SK2 function.

It also appears that SK2 may have opposite roles to SK1 in mast cell function, but interestingly these roles seem to vary across species despite the sequences for both SK1 and SK2 being highly conserved between human and mouse. Upon IgE-mediated cross-linking of the FcεRI receptor, the Src family kinases Lyn and Fyn facilitate the activation and

translocation of the SKs to the plasma membrane (Olivera et al, 2006). In murine mast cells, SK2 has emerged as the major producer of intracellular S1P and, unlike SK1, appears to mediate calcium influx and initiate downstream activation of PKCα, PKCβ, and NF-κB, leading to degranulation and the production of eicosanoids and cytokines by these cells (Olivera et al, 2007). However, in human mast cells SK1 appears to be more important, initiating degranulation, migration and cytokine production, with the roles of SK2 seemingly limited to the production of TNF-α and IL-6 (Oskeritzian et al, 2008). This variation in SK function between species is intriguing, in particular as exogenous S1P strongly induces degranulation of human mast cells (where SK1 is more important) but only weakly in murine mast cells (where SK2 plays more of a prominent role) (Olivera et al, 2007; Oskeritzian et al, 2008). It has, therefore, been suggested that SK2 may function as an intrinsic regulator of mast cell responses, independent of the S1P receptors, whereas SK1, which is largely responsible for the production of circulating S1P, may regulate extrinsic mast cell responsiveness (Olivera et al, 2007; Oskeritzian et al, 2008). Indeed, sphingosine is reported to inhibit calcium influx (Blom et al, 2005; Mathes et al, 1998) and so SK2 may, at least in murine mast cells, function by decreasing the intracellular levels of sphingosine, thus allowing for calcium entry and the subsequent activation of downstream signalling pathways.

S1P plays an important role in immune cell function by regulating lymphocyte egress from lymphoid tissues, and it appears that SK2 may mediate this response by regulating the transport and circulation of S1P between tissues. A study by Sensken et al (2010) demonstrated that the uptake of blood-borne S1P into peripheral tissues is SK2-dependent, and suggested that intracellular SK2 may play a role in importing S1P into cells and directing it to S1P lyase for degradation and maintenance of an S1P gradient. In agreement, *Sphk2*$^{-/-}$ mice resist lymphopenia induced in wildtype mice by the inhibition of S1P lyase in lymphoid tissues (Sensken et al, 2010). This is an intriguing concept, particularly as a number of groups have reported that *Sphk2*$^{-/-}$ mice, surprisingly, have significantly increased levels of plasma S1P compared to wildtype mice (Kharel et al, 2012; Liang et al, 2013; Olivera et al, 2007; Sensken et al, 2010; Zemann et al, 2006). Another obvious explanation for this phenomenon is that SK1 may be upregulated as a compensatory mechanism for SK2 ablation, and indeed this has been examined with somewhat conflicting results. Some studies have found no differences in SK1 mRNA levels or activity in *Sphk2*$^{-/-}$ mice, compared to wildtype mice (Sensken et al, 2010; Zemann et al, 2006). Conversely, a more

recent study found increased SK1 mRNA and protein levels in *Sphk2*$^{-/-}$ mice (Liang et al, 2013), which was proposed to arise from a compensatory mechanism involving the reduction of HDAC1/2 inhibition by SK2, leading to a decrease in histone acetylation and an indirect transcriptional upregulation of SK1 (Hait et al, 2009; Liang et al, 2013). Notably, however, transgenic mice with ubiquitous overexpression of SK1 (approximately 20-fold over endogenous) did not show a significant increase in blood S1P levels (Takuwa et al, 2010).

Interestingly, murine SK2 was found to interact with the cytoplasmic region of the murine IL-12 receptor β1, mediating downstream IL-12 signalling and production of interferon-γ (Yoshimoto et al, 2003). It still remains to be elucidated if this interaction and function of SK2 also occurs in humans.

1.2.2.4 Other diseases

SK2 also appears to be influential in a number of other disease states. It has been demonstrated that the inhibition or downregulation of SK2 resulted in decreased proteolytic activity of beta-secretase 1 (BACE1), the rate-limiting enzyme for amyloid β (Aβ) peptide production (Takasugi et al, 2011). Targeting SK1 also had the same effect, although the role of SK2 in this process appeared more prominent. Interestingly, SK2 activity was also found to be upregulated in the brain of Alzheimer's disease patients, suggesting a role for SK2 in this disease (Takasugi et al, 2011).

There are conflicting reports on the role of SK2 in ischemia-reperfusion (IR) injury, although generally it appears to play a protective role. The genetic deletion of SK2, but not SK1, in mice significantly increased kidney damage following renal IR (Jo et al, 2009), and SK2 was found to mediate the protective effects of ischemic preconditioning in cerebral (Wacker et al, 2009; Yung et al, 2012) and myocardial (Gomez et al, 2011; Ng et al, 2010; Vessey et al, 2011) IR injury. Interestingly, the protective effects of hypoxic preconditioning in cerebral ischemia appear to result, at least in part, from SK2-dependent upregulation of junctional proteins and concomitant increases in blood-brain barrier integrity, which is lost in *Sphk2*$^{-/-}$ mice (Ishizawa et al, 2015). Furthermore, cerebral ischemia was found to increase SK2 mRNA levels in the brain (Blondeau et al, 2007) and

hypoxic preconditioning rapidly and transiently upregulated SK2 activity and protein levels in cerebral microvessels (Wacker et al, 2009). Conversely, however, SK2 inhibition greatly reduced liver injury and improved survival following hepatic IR, coinciding with a reduction in liver S1P levels and mitochondrial permeabilisation (Shi et al, 2012), suggesting that the role of SK2 in IR injury may be tissue-specific.

It has also been reported that the pharmacological inhibition of SK2 resulted in reduced disease severity in rodent models of osteoarthritis (Fitzpatrick et al, 2011b), rheumatoid arthritis (Fitzpatrick et al, 2011a), Crohn's disease (Maines et al, 2010), ulcerative colitis (Maines et al, 2008) and diabetic retinopathy (Maines et al, 2006). It, therefore, appears that the roles of SK2 are complex and as such, the dysregulation of SK2 can facilitate the development of a number of highly varied disease states. In this regard, SK2 is emerging as a promising therapeutic target in many of these diseases.

1.2.3 SK2 isoforms

Two isoforms of SK2 have been described and characterised (Liu et al, 2000; Okada et al, 2005). Unless otherwise specified, in the literature 'SK2' generally refers to the shorter isoform (SK2a or SK2-S), which is consequently the best characterised. The larger isoform (SK2b or SK2-L) has an additional 36 amino acids at the N-terminus and appears to arise from the use of an alternate start codon (Figure 1.4). While not expressed in mice (Okada et al, 2005), SK2b appears the predominant form of SK2 in several human cell lines and tissues. This may suggest that in fact SK2b is physiologically the more important human SK2 isoform, although very few studies have specifically examined its functions. It has been demonstrated that serum deprivation leads to an increase in SK2b expression and promotes the translocation of SK2b to the nucleus where it can inhibit DNA synthesis (Okada et al, 2005). Furthermore, SK2b appears to phosphorylate some of its substrates at a higher rate than SK2a, including sphingosine (1.3-fold increase) and the sphingosine-like immunomodulatory pro-drug FTY720 (4-fold increase) (Billich et al, 2003). This trend would suggest that the N-terminal extension of SK2b may introduce a conformational change that promotes SK2 catalytic activity or stability (Billich et al, 2003).

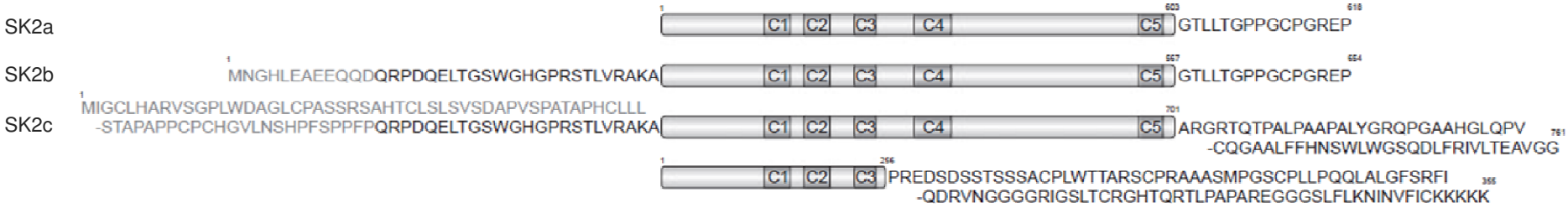


Figure 1.4: Human sphingosine kinase 2 isoforms

All known SKs possess five evolutionarily conserved regions (labelled C1 to C5) important for catalytic activity (Pitson et al, 2002). There are two confirmed human SK2 isoforms (SK2a and SK2b) and two further putative or predicted SK2 isoforms. Compared to SK2a (Genbank™ accession number AF245447), SK2b (RefSeq NM_020126) has an additional 36 amino acids at the N-terminus and appears to be the most abundant human isoform (Okada et al, 2005). SK2c (Genbank™ accession number EF107108) has an additional N-terminal extension as well as an extended C-terminus, however this putative isoform has only been detected at the mRNA level in some human cells (Alemany et al, 2007). A fourth SK2 isoform (bottom) has been predicted by *in silico* analyses (Genbank™ accession number AK000599) which varies considerably at the C-terminus compared to SK2a. The expression of this isoform has not been examined, but since it does not possess the conserved C4 and C5 regions it is unlikely to have SK activity.

A third isoform of SK2 (SK2c; Genbank™ accession number EF107108) has been reported, which possesses an N-terminal extension and an additional C-terminal sequence (Alemany et al, 2007) (Figure 1.4). SK2c mRNA has been reported to be detected in some human cells, but no other analysis of this putative SK2 isoform has been described (Alemany et al, 2007). *In silico* analysis has predicted a fourth SK2 splice variant (Genbank™ accession number AK000599) that differs from SK2a at the C-terminus (from residue 256; see Figure 1.4), although there is yet no physical evidence for the existence of this SK2 isoform. Notably, this putative SK2 variant does not possess the C4 and C5 regions proposed to contain the sphingosine binding and catalytic residues, respectively (Pitson, 2011; Yokota et al, 2004), which have previously shown to be essential for SK activity (Pitson et al, 2002). Therefore, even if this SK2 variant was expressed it is unlikely to have SK activity.

1.2.4 SK2 substrate specificity

Both SK1 and SK2 can utilise D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine as substrates, however SK2 appears to be more promiscuous and can phosphorylate a range of other biological and synthetic lipids with much greater efficiency than SK1 (Pitman & Pitson, 2010). These substrates include phytosphingosine (Liu et al, 2000), ω-biotinyl D-*erythro*-sphingosine (Roberts et al, 2004), and surprisingly, the SK1-specific inhibitor D,L-*threo*-dihydrosphingosine (Liu et al, 2000). SK2 is also responsible for phosphorylating the immunosuppressive agent FTY720, converting it to its active form FTY720-P (Billich et al, 2003; Paugh et al, 2003; Sanchez et al, 2003). Although SK1 can also phosphorylate FTY720 *in vitro,* albeit less efficiently than SK2 (Billich et al, 2003; Kharel et al, 2005; Paugh et al, 2003), levels of FTY720-P in *Sphk2*$^{-/-}$ mice administered FTY720 are negligible, indicating that SK1 does not significantly contribute to FTY720 phosphorylation *in vivo* (Kharel et al, 2005). The ability of SK2 to phosphorylate a larger pool of substrates would suggest that the sphingosine binding pockets of SK1 and SK2 differ slightly in conformation. Although the functional significance of this is currently unknown, it has allowed for these subtle structural differences to be exploited in the generation of isoform-specific SK inhibitors.

1.2.5 SK2-specific inhibitors

A number of SK inhibitors have been generated that show potential for development as therapeutics for cancer and some other diseases (Pitman et al, 2016; Pitman & Pitson, 2010). The majority of these either specifically inhibit SK1 or both SK isoforms. Within the last seven years, however, several SK2-selective inhibitors have emerged, some of which demonstrate promising therapeutic properties. These inhibitors have been previously reviewed in detail (Neubauer & Pitson, 2013; Pitman et al, 2016), and so here, those that have been well characterised, show promising therapeutic effects and/or closely mimic genetic depletion of SK2 will be discussed.

The first described SK2-selective small molecule inhibitor, ABC294640, acts in a sphingosine-competitive manner and has been shown to significantly decrease tumour growth *in vivo* in an array of different tumour models in mice (Antoon et al, 2012; Antoon et al, 2011; Beljanski et al, 2011a; Beljanski et al, 2011b; Chumanevich et al, 2010; French et al, 2010; Panneer Selvam et al, 2015; Schrecengost et al, 2015; Venant et al, 2015; Venkata et al, 2014; Wallington-Beddoe et al, 2014; Xun et al, 2015). There are conflicting reports on the mode of action whereby ABC294640 induces cell death, with some studies demonstrating that apoptotic pathways are activated (Antoon et al, 2012; French et al, 2010), and others describing the presence of autophagy markers (Beljanski et al, 2010; Gao et al, 2012). However, it has been noted that, in addition to SK2 inhibition, ABC294640 can act as a weak antagonist to the estrogen receptor (ERα) (Antoon et al, 2010), and more recently, inhibit dihydroceramide desaturase (Des1) activity and induce its proteasomal degradation (McNaughton et al, 2016; Venant et al, 2015). Furthermore, ABC294640 has been reported to promote the degradation of SK1, c-Myc, androgen receptor and Mcl-1 (McNaughton et al, 2016; Venant et al, 2015; Venkata et al, 2014), although the mechanisms by which it achieves this are unclear. Most notably, administration of ABC294640 *in vivo* resulted in reduced levels of circulating S1P (Beljanski et al, 2011b), which is in stark contrast to the striking increase in circulating S1P levels observed in *Sphk2*$^{-/-}$ mice (Kharel et al, 2012; Liang et al, 2013; Olivera et al, 2007; Sensken et al, 2010; Zemann et al, 2006). Therefore, the anti-tumour effects of ABC294640 may not be entirely attributed to SK2 inhibition, and its off-target actions will need to be considered when interpreting data from studies using ABC294640 as an SK2-specific inhibitor.

Despite this, ABC294640 also seems to have therapeutic potential for a number of other diseases, where attenuated disease progression was observed in rodent models of osteoarthritis (Fitzpatrick et al, 2011b), rheumatoid arthritis (Fitzpatrick et al, 2011a), Crohn's disease (Maines et al, 2010), ulcerative colitis (Maines et al, 2008) and diabetic retinopathy (Maines et al, 2006). Hence, ABC294640 appears to have significant therapeutic potential and as such, this compound has successfully passed Phase I clinical trials in patients with advanced solid tumours, and is now commencing Phase I/II clinical trials for the treatment of refractory/relapsed diffuse large B-cell lymphoma. Additional Phase II clinical trials using ABC294640 are also planned to commence for the treatment of multiple myeloma and advanced hepatocellular carcinoma.

A more recently developed SK2-specific inhibitor, K145, is also demonstrating promising anti-cancer properties. K145 also acts in a competitive manner with respect to sphingosine, and shows no significant inhibition of SK1 or ceramide kinase at concentrations up to 10 μM (Liu et al, 2013). Cellular studies demonstrated that K145 can inhibit growth and induce apoptosis in U937 human leukaemia cells. *In vivo,* K145 was found to reduce tumour volume in JC mammary adenocarcinoma and U937 leukaemia xenograft models (Liu et al, 2013), with comparable anti-tumour activity by both oral administration (50 mg/kg) and i.p. injection (15 mg/kg).

SLR080811 has also been described as a sphingosine-competitive SK2-specific inhibitor (Kharel et al, 2012). Interestingly, SLR080811 was not found to have significant effects on survival or proliferation of U937 human leukaemia cells, despite reducing S1P generation and total S1P levels in these cells (Kharel et al, 2012). The reasons for this unexpected finding remain unclear, with further studies required to examine if this is specific to this cell line or a more general effect. Notably, unlike other purported SK2-specific inhibitors, administration of SLR080811 *in vivo* resulted in an increase in blood S1P levels in wildtype mice, which is the same phenomenon observed in *Sphk2*$^{-/-}$ mice (Kharel et al, 2012). Therefore, SLR080811 may be a useful tool to study SK2 biology as it seems to more closely recapitulate the genetic deletion of SK2. Recently, the same group reported another SK2-specific inhibitor, SLP120701, which also caused decreased S1P levels and increased sphingosine levels in U937 cells, as well as increased circulating S1P levels *in vivo* (Patwardhan et al, 2015). However, again this inhibitor was not found to induce any cytotoxic effects on U937 cells *in vitro* (Patwardhan et al, 2015).

The Pitson Laboratory also recently developed a dual SK1/SK2 inhibitor, MP-A08, which is a first-in-class ATP-competitive small molecule inhibitor (Pitman et al, 2015). MP-A08 inhibits SK2 with somewhat higher affinity than SK1, with K_i values of 6.9 μM and 27 μM, respectively. *In vitro*, MP-A08 decreased the proliferation and neoplastic growth of a number of human cancer cell lines, whilst not affecting the growth of untransformed human fibroblasts (Pitman et al, 2015). Furthermore, MP-A08 significantly reduced tumour burden *in vivo* in A549 human lung adenocarcinoma xenografts in mice, owing to increased apoptosis and decreased angiogenesis within the tumour (Pitman et al, 2015).

Interestingly, recently developed potent SK1-selective inhibitors have demonstrated surprisingly limited anti-cancer properties compared to less isoform-specific SK inhibitors (Gao et al, 2012; Kharel et al, 2011; Schnute et al, 2012). This notion, coupled with the promising anti-cancer effects of the current SK2-specific inhibitors, suggests that in some cancers SK2 may be a more important target for cancer therapy.

1.2.6 Regulation of SK2

1.2.6.1 SK2 activation

Like SK1, the catalytic activity of SK2 can be rapidly increased upon stimulation by a number of agonists, including EGF (Hait et al, 2005), TNFα (Mastrandrea et al, 2005), IL-1β (Mastrandrea et al, 2005), cross-linking of the IgE receptor FcεRI (Olivera et al, 2006), and phorbol esters (Hait et al, 2005). Hypoxia was also found to rapidly activate SK2 *in vivo* (Wacker et al, 2009) and in cultured lung cancer cells (Schnitzer et al, 2009). Like SK1, the mechanism of SK2 activation involves phosphorylation by ERK1/2 (Hait et al, 2007). In cells, agonist-induced activation of SK2 increases its catalytic activity by 2- to 6-fold (Hait et al, 2005), which is comparable to that observed for SK1 (Pitson et al, 2003). Interestingly, however, *in vitro* phosphorylation of SK2 by ERK1 resulted in a modest doubling of catalytic activity of this enzyme (Hait et al, 2007), which is less than the 14-fold increase observed for the same analysis of SK1 (Pitson et al, 2003).

1.2.6.2 Post-translational modifications of SK2

Although the activating phosphorylation site in SK1 (Ser225) occurs within a region that is divergent in SK2, studies have shown that SK2 catalytic activity also increases following phosphorylation by ERK1/2 (Hait et al, 2007). This activation of SK2 has been suggested to involve the phosphorylation of Ser351 and/or Thr578 on SK2a (Ser387 and Thr614 on SK2b) (Hait et al, 2007), although direct evidence for this is lacking. For example, in this study, Hait and colleagues demonstrated that mutating Ser351 or Thr578 to alanine reduced ERK1-mediated phosphorylation of SK2, and they showed that ERK1 can modestly activate SK2 *in vitro*, but they did not then examine or compare the ability of SK2 S351A or T578A mutants to be activated by ERK1 (Hait et al, 2007). Furthermore, mutation of Ser351 to alanine resulted in the same level of activity as wildtype SK2 (Hait et al, 2007), and Ser351 is not conserved in mouse or rat SK2, making its role in SK2 activation doubtful. Therefore, further characterisation of the activating phosphorylation site on SK2 is still required. Phosphorylation of SK2 also appears to regulate its nuclear/cytoplasmic shuttling, with protein kinase D (PKD)-mediated phosphorylation of either Ser419 or Ser421 within the NES of SK2 promoting its nuclear export (Ding et al, 2007).

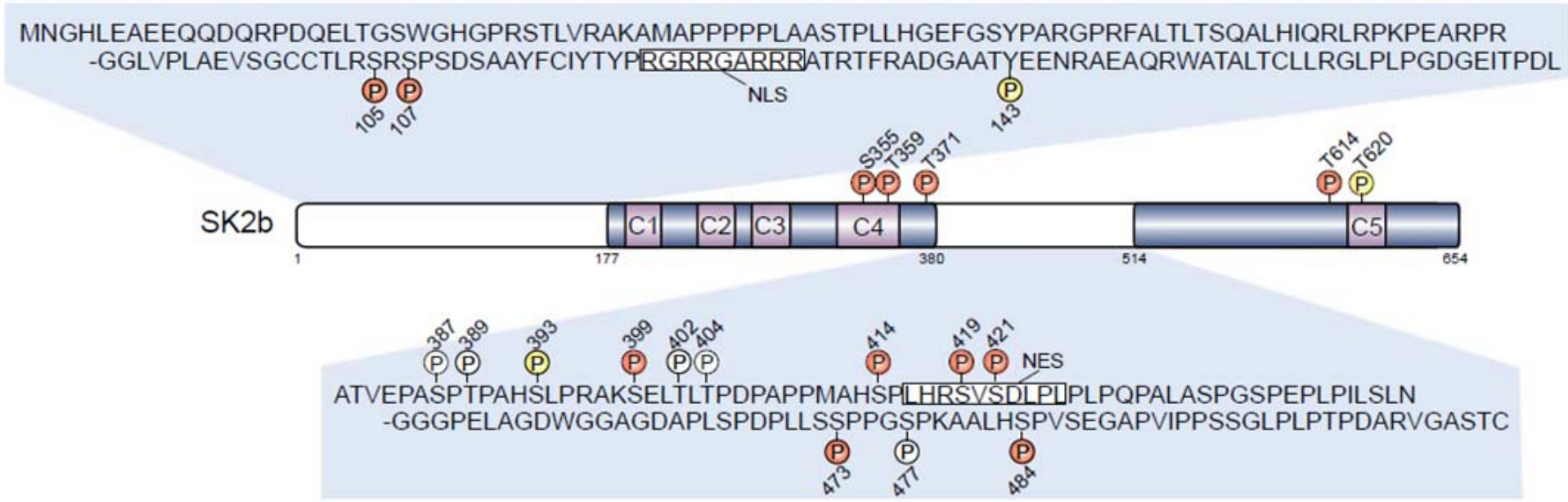
Mining of global phosphoproteome studies and the PhosphositePlus resource (Hornbeck et al, 2012) has revealed 13 other Ser/Thr phosphorylation events in endogenous human SK2, most of which occur within unique regions of SK2 not conserved in SK1 (Figure 1.5). Nine of these residues are conserved in mouse and rat, while another three phosphorylations have been detected in mouse and rat SK2 (see Figure 1.5) but have yet to be confirmed in human (Huttlin et al, 2010). No functional studies have been performed to define the regulatory significance of these novel phosphorylation sites. However, it is possible that these modifications play a role in the isoform-specific regulation of SK2 and may therefore provide an elegant yet undoubtedly complex mechanism to allow for the emerging functional complexity of SK2.

1.2.6.3 SK2 subcellular localisation

The subcellular localisation of the SKs, and hence the compartmentalisation of generated S1P, is widely accepted to play an important role in dictating their function

Figure 1.5: SK2 post-translational modifications and regulatory domains

SK2 contains two unique regions within its sequence not conserved in SK1, or in any other protein. These regions, the N-terminus and the central proline-rich region (white), have been expanded to show the amino acid sequence. The five evolutionarily conserved regions, C1 to C5, are shown in purple. The SK2b isoform is shown here. SK2 possesses a nuclear localisation signal (NLS) within the N-terminus and a nuclear export signal (NES) within the central region. Data from direct analysis of SK2 or from global phosphoproteomic studies have identified a number of SK2 phosphorylation sites. Phosphorylation events detected in human SK2 where the site is conserved in mouse or rat SK2 sequence are shown in red, while those not conserved in rodent SK2 are shown in white. Phosphorylation events detected in mouse or rat SK2 where the site is conserved in the human sequence are shown in yellow. Ser355, Thr359 and Thr371 were identified in untreated HeLa cells (Hornbeck et al, 2012). Ser105 and Ser107 were identified in HeLa cells treated with rapamycin and EGF (Chen et al, 2009). Ser387, Ser389, Thr404, Ser414 and Ser484 were identified in HeLa cells treated with double thymidine block (G_1- or S-phase) or nocodazole (M-phase) (Daub et al, 2008; Dephoure et al, 2008). Ser399, Ser414, Ser473 and Ser477 were identified in HEK293 cells stably transfected with angiotensin II (Ang II) type 1 receptor treated with Ang II ligand (Christensen et al, 2010). Thr402 was identified in Jurkat cells treated with calyculin and pervanadate (Hornbeck et al, 2012). Ser419, Ser421, Ser387 and Thr614 were identified by direct analysis of overexpressed SK2 and validated by mutagenesis (Ding et al, 2007; Hait et al, 2007).


MNGHLEAEEQQDQRPDQELTGSWGHGPRSTLVRAKAMAPPPPPLAASTPLLHGEFGSYPARGPRFALTLTSQALHIQRLRPKPEARPR
-GGLVPLAEVSGCCTLRSRSPSDSAAYFCIYTYPRGRRGARRRATRTFRADGAATYEENRAEAQRWATALTCLLRGLPLPGDGEITPDL
NLS
105
107
143
S355
T359
T371
T614
T620
SK2b
C1
C2
C3
C4
C5
1
177
380
514
654
387
389
393
399
402
404
414
419
421
NES
ATVEPASPTPAHSLPRAKSELTLTPDPAPPMAHSPLHRSVSDLPLPLPQPALASPGSPEPLPILSLN
-GGGPELAGDWGGAGDAPLSPDPLLSSPPGSPKAALHSPVSEGAPVIPPSSGLPLPTPDARVGASTC
473
477
484

(Siow & Wattenberg, 2011; Wattenberg, 2010). It is well established that in order for SK1 to mediate pro-survival, pro-proliferative and oncogenic signalling it must re-localise from the cytoplasm to the plasma membrane (Pitson et al, 2005). By comparison, the subcellular localisation of SK2 appears to be much more complex than for SK1, in line with its more complex functions. SK2 possesses nuclear localisation (NLS) and export (NES) signals (Ding et al, 2007; Igarashi et al, 2003), with the NLS positioned within the N-terminus and the NES located within the central proline-rich region that is not conserved in SK1 (Figure 1.5). Interestingly, when localised to the nucleus, SK2 has been shown to inhibit DNA synthesis (Igarashi et al, 2003), as well as regulate epigenetic modifications via interaction with and modulation of HDAC1/2, to increase p21 and c-fos transcription (Hait et al, 2009). SK2 localisation also appears to vary according to both cell type and cell density. For example, SK2 predominantly localises to the nucleus in HeLa cells whereas in HEK293 cells it is mainly cytoplasmic (Igarashi et al, 2003). Moreover, it has been described that as COS-7 fibroblasts became more confluent in culture, the proportion of SK2 localised to the nucleus increased (Igarashi et al, 2003), suggesting this may represent a response to contact inhibition to decrease cell proliferation.

It has also been demonstrated that under stress conditions SK2 localises to the ER (Maceyka et al, 2005). S1P production at the ER can fuel the sphingosine salvage pathway driven by ER-localised S1P phosphatases and ceramide synthases, to ultimately generate pro-apoptotic ceramide (Maceyka et al, 2005). Interestingly, artificially targeting SK1 to the ER or nucleus can allow the otherwise pro-survival enzyme to promote apoptosis (Igarashi et al, 2003; Maceyka et al, 2005). Moreover, mitochondrial localisation of SK2 has been shown to promote apoptosis via S1P and BAK-dependent membrane permeabilisation and cytochrome-c release (Chipuk et al, 2012; Strub et al, 2011). It should be noted that a lipid binding domain has been identified within the N-terminal region (residues 1-175) of SK2, not conserved in SK1 (Don & Rosen, 2009). This domain was shown to be a requirement for SK2 to interact with phosphoinositides, which may facilitate its differential localisation at internal membranes (Don & Rosen, 2009).

As such, it has long been accepted in the field that the pro-apoptotic functions of SK2 not shared with SK1 seem to be mediated by its localisation to intracellular membranes like the ER, mitochondria and nucleus. However, this notion is now being challenged by more recent studies to demonstrate that nuclear SK2 can seemingly also promote cancer cell

survival and proliferation. Specifically, S1P produced by nuclear-localised SK2 has been implicated in hTERT stabilisation and delayed cell senescence in A549 lung cancer cells (Panneer Selvam et al, 2015), maintaining MYC expression through epigenetic regulation in ALL (Wallington-Beddoe et al, 2014), and antagonising the tumour-suppressive effects of all-trans retinoic acid through retinoic acid receptor β (RARβ) down-regulation in HT-29 colon cancer cells (Sun et al, 2012). Whether this role for nuclear SK2 in promoting cell survival and proliferation is restricted to a cancer setting, or is cell and tissue specific, remains to be elucidated.

The subcellular localisation of SK2 also influences substrate availability, and recent findings suggest that this may be another major contributor to the pro-apoptotic function of SK2. Both SK1 and SK2 can utilise dihydrosphingosine (dhSph) as a substrate (Liu et al, 2000; Pitson et al, 2000a). Since dhSph is generated at internal membranes such as the ER, SK1 targeted to the plasma membrane cannot utilise this substrate pool (Siow et al, 2011; Siow et al, 2010). Unlike S1P, the specific role of dihydro-S1P (dhS1P) in oncogenic signalling is not well characterised. Interestingly, however, Hait et al (2009) demonstrated that dhS1P and S1P generated by SK2 in the nucleus can both inhibit HDAC1/2 and regulate epigenetic modifications to inhibit proliferation. Furthermore, a recent study reported that the anti-tumour efficacy of 'PhotoImmunoNano' therapy is directly attributed to the production of dhS1P specifically by SK2 (Barth et al, 2013). This technology utilises tumour-infiltrating nanoparticles loaded with indocyanine green (ICG), which can be excited by near-infrared light to produce damaging oxidation and cell death. Use of this novel technology in murine models of breast cancer, pancreatic cancer and metastatic osteosarcoma resulted in dhS1P production by SK2, leading to a reduction in immune suppressive myeloid cells expanded in response to tumour-associated inflammation (Barth et al, 2013). Notably, this study also demonstrated that administering dhS1P alone to tumour-bearing mice prevented tumour growth and increased survival of the mice, as opposed to S1P which increased tumour growth (Barth et al, 2013). Therefore, dhS1P and S1P appear to have opposing roles in oncogenic signalling, dependent on their differential cellular compartmentalisation. Again, this highlights the importance of the subcellular localisation of SK2 and consequent access to substrates in relation to its function.

Interestingly, only one study has shown SK2 to mediate pro-proliferative S1P receptor signalling when localised to the plasma membrane (Weigert et al, 2010). This process,

however, appears linked to the activation of caspase-1 in apoptotic cells, which cleaves the N-terminus of SK2 and allows it to be exported from the cell, potentially mediated by the flipping of phosphatidylserine following interaction of the enzyme with the plasma membrane (Weigert et al, 2010).

Overall, the roles of SK2 are clearly complex (as illustrated in Figure 1.6), and much work is still required to uncover and understand the true functions of SK2, particularly in regards to its involvement in cancer. Interestingly, all of the studies demonstrating a functional role for SK2 in promoting survival and proliferation have been conducted in a cancer setting, using cancer cell lines or *in vivo* xenograft models in mice. This begs the question of whether the true physiological role of SK2 in normal cells and tissues is to mediate cell death, but it is somewhat hijacked by transformed cells to drive survival and proliferation. This would have clear implications in targeting SK2 as a cancer therapeutic, and would stress the importance of understanding how the dual functions of SK2 are regulated. Clearly SK2 function is largely dictated by its subcellular localisation, however the mechanisms by which SK2 is localised with the cell, and the factors involved in regulating those mechanisms, are virtually unknown. Hence, the studies outlined in this thesis aim to better understand the true functions of SK2 and how it is regulated.

1.3 Cytoplasmic dynein 1 intermediate chain 2 is a novel SK2-interacting protein

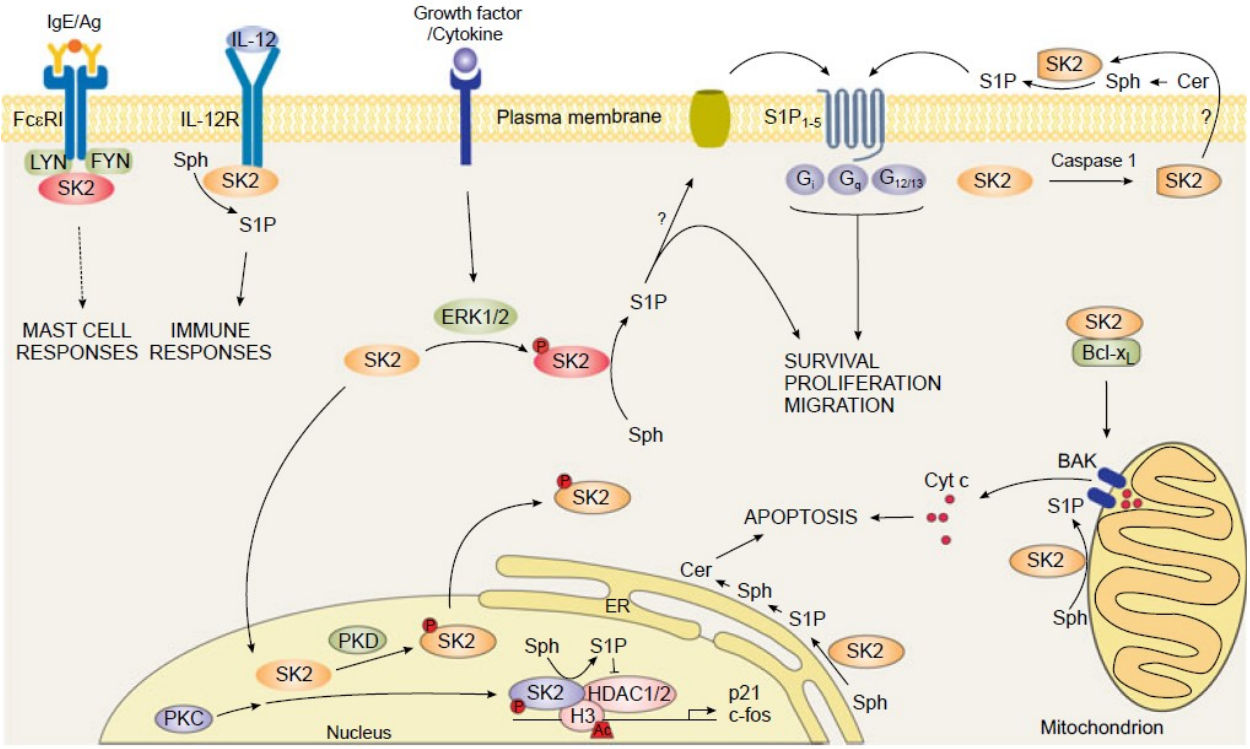
In order to identify novel SK2-interacting proteins, the Pitson Laboratory previously performed a yeast two-hybrid screen using full-length SK2b as the bait and a C-terminal human normalised cDNA library as the prey, under stringent conditions. One putative SK2-interacting protein was discovered from this screen and identified as cytoplasmic dynein 1 intermediate chain 2. As the characterisation of this interaction is a major focus of this thesis, cytoplasmic dynein has been reviewed in detail below.

1.4 Cytoplasmic Dynein

Cytoplasmic dynein is a microtubule minus end-directed transport motor that has a diverse range of cellular functions. Two isoforms of cytoplasmic dynein have been identified;

Figure 1.6: Signalling and regulation of SK2

Activation of SK2 is mediated by ERK1/2 phosphorylation in response to a range of growth factors and cytokines. SK2 can undergo nuclear-cytoplasmic shuttling, regulated by nuclear localisation and nuclear export signals. The latter is regulated by PKD-mediated phosphorylation, which promotes the nuclear export of SK2. In the nucleus, SK2 can interact with histone H3-HDAC1/2 complexes following phorbol ester treatment of cells, and S1P produced by SK2 here can inhibit HDAC1/2-mediated deacetylation of histone H3 and promote transcription of cyclin-dependent kinase inhibitor p21 and the transcriptional regulator c-fos. SK2 can also localise to the ER in response to serum deprivation or cell density, and S1P produced here can fuel a sphingolipid 'salvage' pathway that ultimately results in the generation of pro-apoptotic ceramide via ER-localized S1P phosphatase and ceramide synthase. Mitochondrial localisation of SK2, and subsequent S1P production, can mediate apoptosis via BAK-dependent membrane permeabilisation and cytochrome-c release. SK2 contains a putative BH3 domain through which it can interact with, and presumably sequester, the pro-survival molecule Bcl-x_L, to induce apoptosis. The release of active SK2 from the cell can occur following cleavage at the N-terminus by caspase-1, which can then allow for the production of extracellular S1P. Upon IgE-mediated cross-linking of the FcεRI receptor, Lyn and Fyn can mediate the activation and translocation of SK2 to the plasma membrane to initiate downstream mast cell responses. Murine SK2 has also been found to interact with the IL-12 receptor β1 (IL-12R), mediating downstream IL-12 signalling and production of interferon-γ.


IgE/Ag
IL-12
Growth factor
/Cytokine
FcεRI
IL-12R
Plasma membrane
S1P1-5
SK2
S1P
Sph
Cer
?
LYN
FYN
SK2
Sph
SK2
S1P
Gi
Gq
G12/13
SK2
Caspase 1
SK2
MAST CELL
RESPONSES
IMMUNE
RESPONSES
ERK1/2
SK2
P
SK2
S1P
?
Sph
SURVIVAL
PROLIFERATION
MIGRATION
SK2
Bcl-xL
BAK
Cyt c
APOPTOSIS
S1P
SK2
Sph
P
SK2
Cer
Sph
S1P
SK2
ER
PKD
P
SK2
Sph
S1P
SK2
SK2
HDAC1/2
p21
c-fos
Sph
PKC
P
H3
Ac
Nucleus
Mitochondrion

however, cytoplasmic dynein 2 is almost exclusively found within cilia and flagella to function in retrograde intraflagellar transport for axoneme maintenance and assembly (Allan, 2011; Hook & Vallee, 2006). Therefore, all microtubule minus end-directed transport of cargoes through the cytoplasm, as well as other important mitotic and structural functions, are carried out solely by cytoplasmic dynein 1 (herein referred to simply as dynein) (Cianfrocco et al, 2015). This is quite unique, as the other cytoskeletal motors (plus end-directed kinesins and actin-based myosins) have evolved as part of large families of up to 45 genes (in the case of mammalian kinesin), each specific to different cargoes and cellular functions (Hirokawa et al, 2010). As discussed further below, the vast array of functions achieved by this single dynein motor are thought to be made possible through the use of multiple isoforms of various subunits, post-translational modification of specific subunits, and through differential binding to a range of interacting regulatory proteins/complexes (Cianfrocco et al, 2015; Kardon & Vale, 2009; Pfister, 2015).

1.4.1 Functions of dynein

Dynein transports a diverse range of cargoes in a retrograde direction along microtubules, accumulating at the microtubule-organising centre (MTOC) (Figure 1.7). For fibroblasts, the MTOC is next to the nucleus in the cell centre, but for some other cell types, like polarised epithelial cells and neuronal dendrites, the plus ends can be oriented towards the nucleus or they can have mixed polarity (Allan, 2011). Dynein cargoes can include proteins, RNA/protein complexes, vesicles and membranous organelles such as endosomes, peroxisomes, autophagosomes, lysosomes and mitochondria (a list of various dynein cargoes is provided in Table 1.1) (Allan, 2011; Flores-Rodriguez et al, 2011; Tan et al, 2011). Viruses - specifically adenovirus, the α herpes viruses HSV-1 and PrV, and the retrovirus HIV-1 - are also able to hijack the dynein motor for retrograde-directed transport towards the nucleus (Dodding & Way, 2011). Dynein also has important functions during mitosis, including the transport of chromosomes along microtubules required for chromosome alignment during metaphase (Li et al, 2007; Yang et al, 2007), as well as removing spindle assembly checkpoint (SAC) proteins from kinetochores to initiate anaphase (Wang et al, 2014c), both of which involve a number of dynein-interacting proteins described below. The ability of dynein to attach to a ‘fixed’ cellular structure, like the cell cortex, and use it as leverage to pull on microtubules can facilitate correct

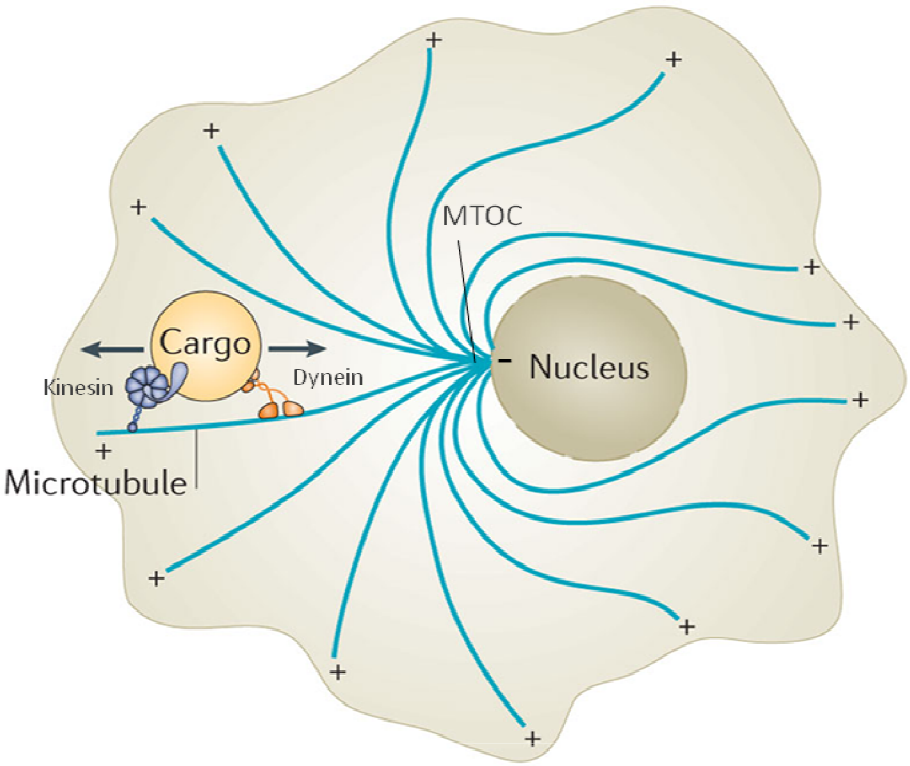


Figure 1.7: Dynein transports its cargo in a retrograde direction

Cytoplasmic dynein can transport various cargoes, such as proteins, vesicles and organelles, through the cytoplasm. It achieves this by binding to microtubules and moving in a retrograde direction (towards the minus ends of the microtubules), ultimately accumulating at the microtubule-organising centre (MTOC) which is generally located in close proximity to the nucleus. Cargoes can also be transported in an anterograde direction (towards microtubule plus ends) by kinesin motors. Figure adapted from Hancock (2014).

Table 1.1: Selected dynein-cargo interactions

Key examples of direct interactions between dynein subunits and various cargoes are listed. Adapted from Allan (2011).

Dynein subunit	Cargo	Reference
IC	Lysosomes/autophagosomes in neurons	(Cai et al, 2010)
IC, LIC	Adenovirus particles	(Bremner et al, 2009)
IC	β-catenin	(Ligon et al, 2001)
IC	Kinetochores	(Whyte et al, 2008)
IC	PLAC-24	(Karki et al, 2002)
LIC, LC8	Endosomes	(Horgan et al, 2010; Traer et al, 2007)
LIC1	Centrosomes	(Tynan et al, 2000)
LIC2, LC8	Cell cortex	(Manneville et al, 2010; Schmoranzer et al, 2009)
LIC	Nuclei	(Malone et al, 2003)
LC8	Ribonucleoproteins	(Navarro et al, 2004)

positioning of the mitotic spindle as well as reorganisation of the cell during cell migration (Allan, 2011). Furthermore, dynein can facilitate the sliding of intermediate filaments along microtubules as well as the sliding of microtubules with respect to each other, for instance within the mitotic spindle (Allan, 2011). It has also been well described that dynein binds to the nuclear envelope to maintain correct cellular positioning of the nucleus through association with the MTOC, as well as to mediate nuclear envelope breakdown during mitosis by essentially peeling open the nuclear membranes (Burke & Roux, 2009; Starr, 2009).

In order for dynein to perform retrograde-directed transport, it often appears to require microtubule plus end localisation to collect its cargo (Cianfrocco et al, 2015). Significant research has been undertaken to understand how this is achieved, and it is apparent that multiple mechanisms are at play. Firstly, it has been shown that dynein can interact, either directly (Ligon et al, 2004) or indirectly via other proteins (Yamada et al, 2010), with the plus end-directed kinesin motor. It appears that dynein and kinesin can engage in a 'tug-of-war' along the microtubules, resulting in overall anterograde-directed transport of dynein as a consequence of kinesin recruiting factors that increase its processivity (Roberts et al, 2014). Secondly, dynein can be recruited to the microtubule plus ends through the coordinated assembly of microtubule plus end binding proteins (+TIPs), such as cytoplasmic linker protein 170 (CLIP-170), end-binding proteins (EBs), and dynactin (Moughamian et al, 2013). These +TIPs bind to the plus ends of polymerising microtubules and can initiate dynein-mediated retrograde transport, although this mechanism seems to only be utilised in specific cell types, such as neurons and *Xenopus* melanophores, where perhaps high-flux trafficking events are required (Lomakin et al, 2009; Moughamian et al, 2013). Interestingly, it has also been suggested that the mRNA of dynein subunits and regulators can be transported to the microtubule plus ends as messenger ribonucleoprotein particles (mRNPs), where they can then be locally translated (Cianfrocco et al, 2015; Preitner et al, 2014).

1.4.2 Dynein complex structure

The large ~1.4 MDa dynein complex is made up of multiple subunits: heavy chains (HC), light intermediate chains (LIC), intermediate chains (IC) and light chains (LC) (Figure 1.8).

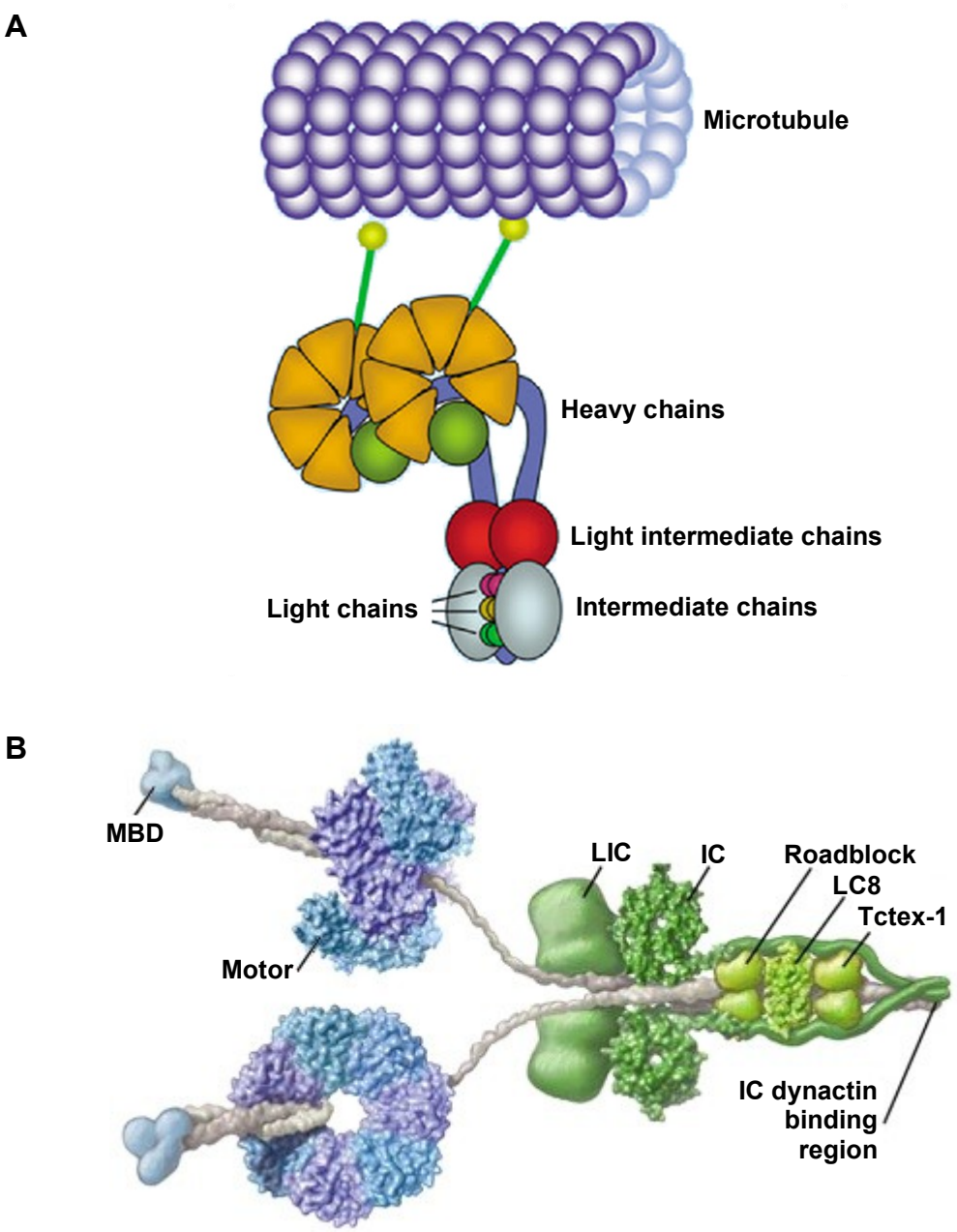


Figure 1.8: The cytoplasmic dynein complex

The cytoplasmic dynein complex is composed of multiple subunits bound as dimers. These subunits include the heavy chains (HC), light intermediate chains (LIC), intermediate chains (IC), and the three light chain subunits (LC; Roadblock, LC8 and Tctex-1). The HC dimers contain ring-shaped motor domains, microtubule-binding domains (MBD), and a long tail domain where all other subunit dimers bind. *A,* Schematic of the dynein complex bound to microtubules, adapted from Hook & Vallee (2006). *B,* Surface features are rendered based upon atomic resolution structures where available, and appear as smooth images for domains of unknown structure. The IC spans a large portion of the HC tail, making contacts with the three LCs as well as interacting with the dynein regulator, dynactin, at the N-terminal end. Adapted from Vale (2003).

Two copies of the HC form the backbone of the complex, and importantly, possess the C-terminal microtubule binding domains and motor domains. The HC is the largest subunit (~500 kDa) and is a member of the ATPases associated with various cellular activities (AAA+) superfamily (Neuwald et al, 1999). The AAA+ ATPase motor domain of the HC is comprised of six AAA+ modules that fold to form a hexameric ring structure, and the HC is a unique member of this superfamily in that its AAA+ domains are linked in a single large polypeptide (Neuwald et al, 1999). Interestingly, of the six AAA+ domains, AAA1 is the predominant site of ATP hydrolysis and is essential for dynein motility (Kon et al, 2004). Although AAA2 is able to bind to ATP, it has lost the residues required for ATP hydrolysis (Cianfrocco et al, 2015). Mutations in the ATP binding site of AAA3 also reduced dynein motility, as did mutations in AAA4 but to a lesser extent (Cho et al, 2008; Kon et al, 2004), suggesting that these domains may also be important for the motor function of the HC. The remaining two AAA+ modules, AAA5 and AAA6, are poorly conserved and have lost the residues required for ATP binding (Neuwald et al, 1999), however they have been shown to play an important structural role in generating force and movement of the motor by transmitting conformational changes through the AAA+ ring (Schmidt et al, 2015).

The tail domain at the N-terminus of the HC allows for the binding of the five non-catalytic dynein subunits, and it is this end of the dynein complex that is responsible for cargo binding and specificity. Binding as dimers, the LICs and the ICs interact directly with the HC, and three LC subunits (t-complex testis-expressed - Tctex1, LC8 and Roadblock/LC7) then bind as dimers to the ICs (see Figure 1.8). Interestingly, where dynein usually recruits the large multi-subunit complex, dynactin, to facilitate in cargo binding and transport (discussed below), there is evidence that dynein can also bind directly to some cargoes via the LICs, ICs and LCs. In vertebrates, there are two genes for each of the five non-catalytic subunits (Pfister et al, 2006). The two LICs, LIC1 and LIC2, are only able to homodimerise within the dynein complex, resulting in two distinct subsets of dynein: LIC1-containing complexes and LIC2-containing complexes (Tynan et al, 2000). There is evidence in favour of the idea that the distinct dynein subsets containing either LIC1 or LIC2 may provide some cargo specificity, as LIC1 has been shown to selectively interact with the centrosomal protein pericentrin (Tynan et al, 2000), whereas only LIC2 can bind to the polarity protein Par3 (Schmoranzer et al, 2009). However, there also appears to be some functional redundancy between the two isoforms in binding to other cargoes, as well as functioning in spindle assembly and organelle positioning (reviewed in Allan, 2011; Jones et al, 2014).

The dynein LC subunits are unique in that they are the only subunits that have been described to have additional functions independent of the dynein motor. The LC8 family have over 60 validated protein interacting-partners (Rapali et al, 2011), including the myosin 5a heavy chain (Espindola et al, 2000), and as such it was thought that the LCs must play an important role in the direct tethering of cargo to the dynein and myosin 5a complexes. However, it has since been noted that LC8 has many interacting partners that are not involved in intracellular transport (Rapali et al, 2011), and it seems to be structurally unlikely that LC8 could link cargo to these complexes (Williams et al, 2007). Furthermore, LC8 is highly evolutionarily conserved and is expressed in some lineages (e.g. plants) where all other dynein components have been lost (Wickstead & Gull, 2007). Indeed, it is now emerging that the true function of LC8 is to act as a 'molecular glue', binding to many cellular proteins in inherently disordered regions to promote their dimerisation and stabilisation, and the dynein complex is one such example (Barbar, 2008). Nevertheless, the dynein light chains are essential components of the dynein complex (Dick et al, 1996), and loss of either LC8 or Tctex from the complex, or mutations in Roadblock/LC7 have been shown to disrupt a number of dynein-mediated cellular processes (Bowman et al, 1999; Varma et al, 2010).

As the dynein ICs are a major focus of this thesis, they are discussed in greater detail below.

1.4.3 Dynein intermediate chains

The dynein ICs act as scaffolds within the complex, binding both the HCs and the LCs (see Figure 1.8) as well as playing critical roles in cargo binding and specificity. Many of the regulator proteins/complexes that mediate dynein function bind to the motor via the ICs (Pfister, 2015). There have also been reports of the ICs directly binding to cargoes, such as β-catenin (Ligon et al, 2001), PLAC-24 (Karki et al, 2002), adenovirus (Bremner et al, 2009), circovirus (Cao et al, 2015), and kinesin light chains 1 and 2 (Ligon et al, 2004). Somewhat surprisingly, the characterisation of the ICs has been best studied in rat, not human, and consequently the majority of the literature documenting IC distribution and expression, as well as structural domains and protein interactions refers to rat IC. This may be due to the relative ease of obtaining rat tissues, particularly brain where dynein function

is important and highly studied. However, as rat IC shares 97% sequence identity with human IC, it is likely to be a good representation of human IC.

The N-terminal coiled-coil region of the IC is where binding to dynein regulators dynactin and NudE occurs (see Figure 1.9) (McKenney et al, 2011; Vaughan & Vallee, 1995). Downstream of the coiled-coil domain are the binding sites for the three LCs as well as the IC dimerisation site (Lo et al, 2006; Lo et al, 2001; Mok et al, 2001; Susalka et al, 2002). The remainder of the C-terminus is composed of seven WD40 repeat domains that form a beta propeller structure, and it is this region of the IC that is responsible for binding to the HC (Figure 1.9) (Pfister, 2015).

There are two vertebrate IC genes: IC-1 and IC-2 (Pfister et al, 2006). They are highly conserved, sharing 76% amino acid sequence identity, and they are able to both homo- and heterodimerise as determined by overexpression as well as endogenous systems (Lo et al, 2006; Zhang et al, 2013). Both genes are alternatively spliced at two conserved regions within the N-terminus (Figure 1.9) to generate a subset of splice isoforms, but the number of isoforms expressed for each gene seems to be species specific. In human and rat, there are three splice isoforms of IC-1 and three of IC-2 (see Figure 1.10), as each has a full-length transcript as well as two shorter transcripts that are spliced at one or both of the two conserved regions (Myers et al, 2007). Interestingly, a large scale analysis of mouse IC isoforms revealed five IC-1 isoforms and six IC-2 isoforms (Kuta et al, 2010). Although it was found that splicing in the mouse IC genes still occurs at the same two regions as in human and rat, it appears that the size of the spliced regions can vary (Kuta et al, 2010).

Notably, in mouse and rat, the only isoform that is ubiquitously expressed in all cell types and tissues is the IC-2C isoform, whereas all other isoforms seem to be restricted to brain and nervous tissues, except for IC-1C, which is also detected in ovary and testis (Kuta et al, 2010; Myers et al, 2007). This suggests that the IC-2C isoform is sufficient for dynein to achieve its functions in most cells, whereas cells in the brain and CNS require the increased complexity of multiple dynein IC isoforms. In rat, IC-2B was also reported to be expressed in many tissues (Myers et al, 2007). Interestingly, however, although less well studied, it has been reported that in human cells and tissues IC-1 may actually be more widely expressed, with the highest levels found in brain, heart and skeletal muscle, and low but significant levels of IC-1 mRNA detected in pancreas, kidney, liver, lung, placenta, colon, small

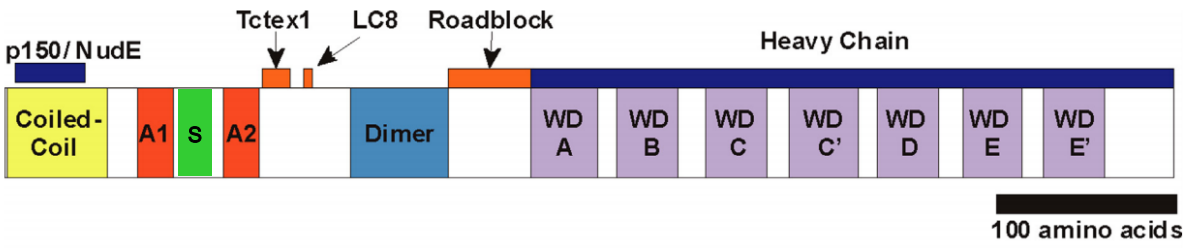


Figure 1.9: Domain and interaction map of the dynein intermediate chain

Domain map shown is of the dynein intermediate chain (IC), with the N-terminus on the left. Domains and interactions were characterised using rat IC, however rat IC shares 97% sequence identity with human IC. Characterised domains include: coiled-coil motif (yellow), two alternative splicing regions (red; A1 and A2), serine-rich region (S; green), IC dimerisation domain (blue), and seven WD40 domain repeats (purple; A-E'). The IC interaction sites for the three light chains (Tctex-1, LC8 and Roadblock) are depicted above the map (orange), as are the heavy chain binding site (dark blue; C-terminus) and the region with which the dynein regulators, p150 (dynactin) and NudE, interact (dark blue; N-terminus). Figure adapted from Pfister (2015).

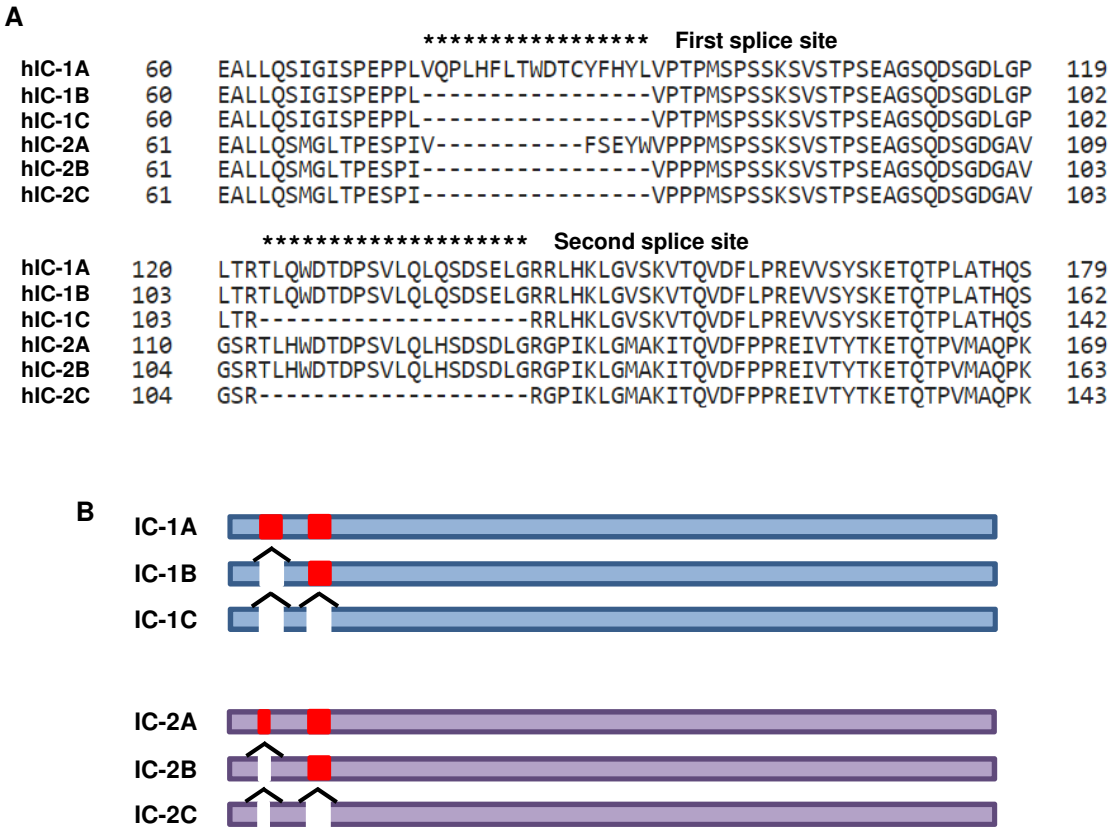


Figure 1.10: Alternative splice isoforms of human dynein intermediate chain genes

Various splice isoforms are shown for human IC-1 and IC-2. *A,* Sequence alignment of an N-terminal portion of the three IC-1 splice isoforms (IC-1A, IC-1B and IC-1C which are 645, 628 and 608 amino acids in length, respectively) and three IC-2 splice isoforms (IC-2A, IC-2B and IC-2C which are 638, 632 and 612 amino acids in length, respectively), revealing the regions that are alternatively spliced. IC-1A and IC-2A proteins arise from unspliced, full-length transcripts, IC-1B and IC-2B proteins arise from splicing at the first splice site, and IC-1C and IC-2C proteins arise from splicing at both the first and second splice sites. Alignment was performed using the Clustal Omega program via UniProt. *B,* Schematic summarising the alternate splice sites (red) at the N-terminal ends of IC-1 and IC-2, resulting in three splice isoforms for each gene. Figure adapted from Myers (2007).

intestine, ovary, testis, prostate, thymus, spleen, and mammary epithelial cells (Horikawa et al, 2001). Therefore, the expression pattern of IC-1 may vary between humans and other species.

Like the LICs, the IC isoforms seem to give rise to distinct dynein complexes with isoform-specific cargoes and functions. Interestingly, in axons only dynein containing the IC-2C isoform was associated with fast anterograde axonal transport of membrane bounded organelles linked to kinesin, whereas all other IC isoforms were associated with the slow component b (Dillman et al, 1996). This may suggest that dynein composed of IC-2C may function in neurons to provide a reverse motor during fast anterograde transport in the event that obstructions are encountered (Pfister, 2015). Furthermore, dynein complexes containing IC-1B specifically interact with and transport neurotrophic factor receptor TrkB-containing signalling endosomes, whereas IC-2C does not associate with TrkB (Ha et al, 2008). Furthermore, dynein with IC-1B was more likely to co-localise with Rab7-positive endosomes than dynein with IC-2C, whereas the opposite was true for mitochondria, which had a greater association with dynein containing IC-2C (Mitchell et al, 2012). Again, these differential functions of IC isoform-specific dynein subsets are only relevant to neuronal type cells where more than one isoform is expressed, demonstrating the importance of intracellular transport and the high level of regulation and functional diversity of dynein required in these long, polarised cells. This is reiterated by the fact that neurological defects are generally the predominant phenotype of mutations in the dynein complex, emphasising that the long distance transport within axons is already pushing dynein to maximal capacity (Hafezparast et al, 2003; Ilieva et al, 2008; Pfister, 2015).

Another important aspect of dynein regulation is through phosphorylation of the ICs. Adjacent to the N-terminal coiled-coil domain, there is a serine rich region near the alternative splicing sites on the ICs (Figure 1.9). Here, a number of phosphorylation events have been described. Phosphorylation has been shown to occur on Ser80 of IC-1B and the equivalent residue Ser81 of IC-2C, which is stimulated by EGF (Pullikuth et al, 2013) and was found to be phosphorylated by ERK1/2 (Mitchell et al, 2012). This modification seems to promote binding of dynein to signalling endosomes, as the corresponding alanine mutations significantly reduced co-localisation of the ICs to these organelles. Interestingly, phosphorylation at Ser81 of IC-2C does not appear to regulate binding of dynein to

mitochondria, suggesting that this site does not regulate binding of dynein to all cargoes (Mitchell et al, 2012).

Furthermore, Ser84 phosphorylation on IC-2C appears to disrupt binding of the IC to the p150 subunit of the dynactin complex, which may contribute to a reduction in dynein-mediated transport (Vaughan et al, 2001). Phosphorylation of Thr89 also prevents binding of dynein to dynactin p150, but appears to be stimulated during early mitosis and concomitantly increases dynein binding to another adaptor protein, ZW10 (Whyte et al, 2008). This event then promotes the localisation of dynein to kinetochores during mitosis, and Thr89 is subsequently dephosphorylated during metaphase to allow dynein to re-interact with dynactin and translocate towards the spindle poles to initiate anaphase (Whyte et al, 2008).

Interestingly, a recent comprehensive study performed to identify IC phosphorylation events by mass spectrometry, using neuronal cells and tissues, identified a total of six sites on IC-2 and eight sites on IC-1 (unpublished work from 'Zhang et al., in preparation', described in Pfister (2015)). The six IC-2 phosphorylation sites were reported to reside within the N-terminal serine rich region where the previously characterised sites lie (Figure 1.9). Fascinatingly, however, it was documented that only four of the identified IC-1 phosphorylation sites are conserved with IC-2 and lie within the serine rich region, whereas the other four sites are unique to IC-1 and are located further downstream: one is near the LC8 binding site, one is within the IC dimerisation domain and one is located at the C-terminus (Zhang et al., in preparation). As this study is not yet published, the exact location of these sites was not disclosed, but this information will undoubtedly provide insight into the differential regulation of the IC isoforms by phosphorylation.

1.4.4 Regulators of dynein

Dynein could not achieve many of its cellular functions by acting alone; in fact, most of its roles require a myriad of different proteins and complexes. As such, important regulators of dynein – dynactin, Lis1 and NudE/Nudel – are described below. Although some of these regulators are not directly examined by the studies undertaken in this thesis, knowledge of their roles provides greater context to the functions of dynein in the cell.

1.4.4.1 Dynactin

Dynactin is an essential regulator of dynein as, since its discovery, it has been implicated in almost all of the cellular functions dynein performs (Schroer, 2004). Interestingly, dynactin itself is a large 1.2 MDa multi-subunit complex, making it almost as large as the dynein complex (see Figure 1.11). It interacts with the N-terminus of the dynein IC via its p150 subunit. As discussed previously, dynactin was found to be involved in recruiting dynein to the microtubule plus-ends to initiate retrograde-directed transport of cargoes, and this process is driven by the recruitment of dynactin to plus-end binding proteins via the CAP-Gly domain of its p150 subunit (Figure 1.11) (Lloyd et al, 2012; Moughamian & Holzbaur, 2012; Moughamian et al, 2013). Upon initiation of dynein-driven retrograde transport, dynactin has also been implicated in activating dynein motility and increasing dynein processivity (Cianfrocco et al, 2015; Kardon & Vale, 2009). Although it appears that dynactin alone can achieve increases in dynein processivity along microtubules (Ayloo et al, 2014; Kardon et al, 2009), recent detailed structural studies have revealed how the combinatorial binding of dynactin and another cargo adaptor (BICD2) to dynein can induce changes in symmetry and conformation of the complexes to promote dynein activity and processivity (Cianfrocco et al, 2015).

Another fundamental role of dynactin is to act as an adaptor to tether cargo to the dynein complex for transport. The ARP1 filament of dynactin (Figure 1.11) binds directly to βIII spectrin, which is a protein found on the cytosolic surface of the Golgi and other cellular membranes transported by dynein (Holleran et al, 2001; Muresan et al, 2001). The p150 subunit of dynactin also mediates dynein-cargo interactions via direct binding to a number of membrane proteins associated with lysosomes, endosomes and transport vesicles budding from the ER (Allan, 2011; Kardon & Vale, 2009). Furthermore, dynactin p50 has been implicated in binding to, and facilitating the transport of secretary organelles, as well as mediating recruitment of dynactin, and hence dynein, to the kinetochore (Allan, 2011; Kardon & Vale, 2009)

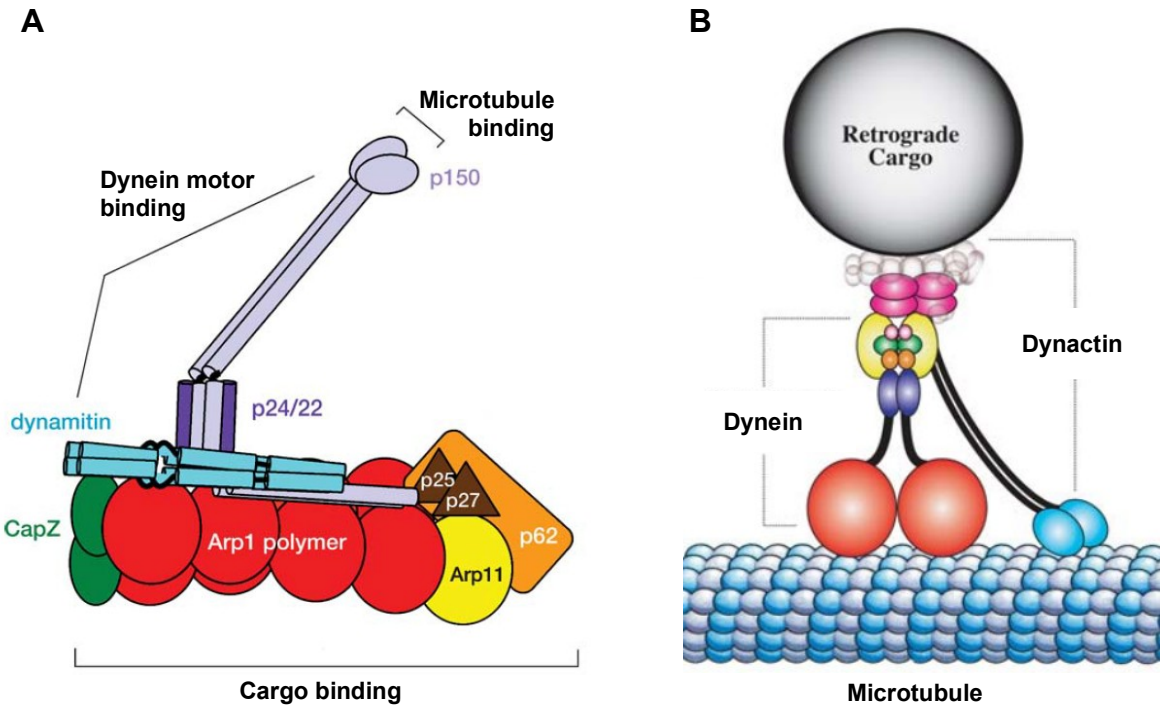


Figure 1.11: The dynactin complex

A, Schematic illustrating the subunit composition of the dynactin complex. The actin-related protein 1 (Arp1) filament (red) forms a central, actin-like scaffold and can interact with many cellular cargoes. CapZ (green) and Arp11 (yellow) act as actin-capping proteins at the Arp1 plus and minus ends, respectively. Arp11 also interacts with the p25/p27 (brown) and p62 (orange) subunits to form a heterotetrameric complex at the Arp1 minus end. Dynamitin (p50) exists as a tetramer and is important for complex structure as well as forming interactions with dynein adaptor proteins. The p24/22 subunit binds directly to dynamitin and p150. The p150 subunit dimer contains N-terminal microtubule binding domains, and it is also the subunit through which dynactin interacts with dynein. Figure adapted from Schroer (2004). *B,* Representation of how the dynein and dynactin complexes interact to transport cargo along microtubules in a retrograde direction. The two complexes interact via the dynein intermediate chain (yellow) and dynactin p150 subunit (double black lines). Dynactin binds to the cargo via the Arp1 filament (transparent circles). Figure adapted from Duncan & Goldstein (2006).

1.4.4.2 Lis1 and NudE/Nudel

Lissencephaly 1 (Lis1) and Nuclear distribution protein E (NudE)/NudE-like (Nudel) are important, ubiquitous regulators of dynein and have been implicated in regulating many dynein functions, including nuclear and centrosome positioning, spindle pole organisation during mitosis, as well as organelle and mRNA transport (Dix et al, 2013; Liang et al, 2004; Moon et al, 2014; Moughamian et al, 2013; Tsai et al, 2007). Lis1 forms a homodimer and is the only known dynein regulator to bind to dynein via the HC motor domain (Cianfrocco et al, 2015). Upon binding to the motor domain, Lis1 sterically alters the normal progression of dynein's mechanochemical cycle such that it prevents a detachment signal and thus promotes prolonged microtubule attachment (Huang et al, 2012; Toropova et al, 2014).

NudE and Nudel are highly homologous proteins that interact at their N-terminus with the dynein IC and interact at their C-terminus with Lis1 (Cianfrocco et al, 2015). Hence, they are thought to predominantly play a tethering role to regulate and maintain the interaction between dynein and Lis1. Indeed, overexpression of Lis1 can partially rescue defects owing to a loss of NudE (Efimov, 2003; Li et al, 2005). Lis1 has been implicated in recruiting dynein to microtubule plus ends, as well as in initiation of dynein motility towards the minus ends, but the exact role and mechanism by which this occurs seems to be species-dependent and requires further characterisation (Cianfrocco et al, 2015; Kardon & Vale, 2009). Lis1-NudE and Lis1-Nudel complexes have also been shown to play important roles in recruiting dynein to the mitotic kinetochore, as NudE/Nudel and the Rod/ZW10/Zwilch (RZZ) complex can bind directly to kinetochore proteins to then recruit other dynein adaptors, and dynein itself (Kardon & Vale, 2009).

Indeed, the number of different regulator proteins and complexes that bind to, tether, recruit and regulate dynein is rather complex. However, it is interesting to note that purified mammalian dynein is largely inactive *in vitro*, but in the same system the addition of dynactin plus one of the many cargo-specific adaptor proteins known to interact with dynein was sufficient to form a highly processive motile complex, as seen in cells (McKenney et al, 2014). Therefore, the system of retrograde-directed transport by dynein is highly regulated, and depending on the cargo to be transported, appropriate adaptor proteins will recruit dynein to their specific cargo, as well as dynactin, to form an active complex capable of initiating motility.

1.5 Aims

The roles of SK2 are complex and still not entirely understood. There is evidence in the literature to suggest that the physiological and pathophysiological roles of SK2 may differ, where it generally seems to promote cell death and yet in a cancer setting it can be pro-tumourigenic. However, unlike SK1, high level overexpression of SK2 does not induce neoplastic transformation, highlighting the importance of better understanding this enzyme in order to effectively target SK2 as a therapeutic option to treat cancer. As discussed, the subcellular localisation of the SKs, and hence the localisation of S1P production, appear critical in determining their function. Hence, the studies outlined in this thesis are aimed at examining the true functions of SK2 in cancer, and characterising novel mechanisms that regulate the subcellular localisation of SK2.

Specifically, the aims of these studies were to:

1) Investigate the role of SK2 in neoplastic transformation and tumourigenesis
2) Characterise and examine the functional relevance of the novel interaction between SK2 and DYNC1I2.
3) Examine the regulation of SK2 by previously uncharacterised phosphorylation sites.

Chapter 2:
General Materials and Methods

Chapter 2. General Materials and Methods

2.1 Cell culture, transfection and lysate preparation

Human embryonic kidney (HEK293, HEK293-C18 and HEK293T) cells and human cervical cancer (HeLa) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), containing 10% heat-inactivated fetal bovine serum (FBS; Bovogen, East Keilor, VIC, Australia), 1 mM HEPES, penicillin (1.2 mg/ml) and streptomycin (1.6 mg/ml). Primary mouse embryonic fibroblasts (MEFs) were generated from both wildtype (WT) C57/Bl6 and *Sphk2*$^{-/-}$ C57/Bl6 (Mizugishi et al, 2005) mouse embryos at 14.5 days *post coitum*. The fibroblasts were isolated and cultured as described above. NIH3T3 mouse fibroblasts were also cultured as above, except using 10% heat-inactivated donor bovine serum with iron (DBS; Gibco, Thermo Fisher Scientific). U-87 MG and U-251 MG human glioblastoma cells were cultured in Minimum essential media (MEM; Gibco, Thermo Fisher Scientific), containing 10% FBS, 2mM L-glutamine, 1mM sodium pyruvate, 1% non-essential amino acids, penicillin (1.2 mg/ml) and streptomycin (1.6 mg/ml). All cells were grown at 37°C with 5% CO_2 in a humidified incubator, except the primary MEFs which were grown in 10% CO_2.

Cells were transiently transfected at 80% confluence using Lipofectamine™ 2000 (Thermo Fisher Scientific) as per the manufacturer's protocol. 24-48 hr after transfection, cells were harvested by scraping into cold phosphate buffered saline (PBS), and unless otherwise indicated, cell pellets were resuspended in extraction buffer (EB; 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.05% Triton X-100, 2 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM DTT and complete protease inhibitor cocktail (Roche)). Cells were lysed by sonication (four cycles of 30 sec on/off; high setting) using a Bioruptor bath sonicator (Diagenode, Seraing, Liège, Belgium). Lysates were clarified by centrifugation at 17,000 x *g* for 15 min and 4°C. Total protein concentration of cell lysates was determined by a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA), using bovine serum albumin (BSA) as a standard.

2.2 Mouse tissue lysate preparation

Mouse tissue was harvested into a 1.5 ml tube on ice and manually homogenized in cold extraction buffer using a pestle (Axygen, Corning, Corning, NY, USA). Tissue was then subjected to freeze/thawing in liquid nitrogen and sonication, and lysates were then clarified by centrifugation at 17,000 × *g* for 15 min at 4°C.

2.3 Cloning of mammalian expression constructs

The following mammalian expression constructs used in these studies were previously generated in the Pitson Laboratory: pcDNA3-SK2(FLAG), pcDNA3-SK2 Δ10P(FLAG), pcDNA3-SK2 Δ9P(FLAG), pcDNA3-SK2 Δ6P(FLAG), pcDNA3-SK2 Δ4P(FLAG), pcDNA3-SK2 S363A(FLAG), pcDNA3-SK2 S398A(FLAG), pcDNA3-SK2 S437A(FLAG), pcDNA3-SK2 S441A(FLAG), pcDNA3-SK2 S448A(FLAG), pcDNA3-DYNC1I2(HA), pcDNA3-DYNC1I1(HA), pcDNA3-DYNC1I2 1-250(HA), pcDNA3-DYNC1I2 251-612(HA), pcDNA3-DYNC1I2 1-439(HA), pcDNA3-DYNC1I2 440-612(HA), pET17b-DYNC1I2(His), pCX4^{NEO}-DYNC1I1:EGFP. More detailed methods for any constructs generated by myself during the scope of my PhD are included in the specific methods section of each results chapter.

2.4 Immunoprecipitation

For immunoprecipitation of over-expressed protein, cells were lysed in IP lysis buffer (50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10% glycerol, 1% Triton X-100, 10 mM Na_3VO_4, 10 mM NaF, and protease inhibitor cocktail (Roche, Basel, Switzerland)). Lysates were sonicated and clarified (as above). 100 µl of lysate was aliquoted and made up to 170 µl in IP lysis buffer. 20 µl of diluted lysate was removed and kept for protein expression levels. The remaining 150 µl of lysate was used for immunoprecipitation, using the µMACS magnetic system (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 2-4 µg of antibody (specific antibody details are given below and in figure legends) was added to the lysate, in addition to 50 µl Protein G µbeads. The lysate was gently mixed and left on ice for 30 min. µMACS

columns were mounted to magnetic stands and equilibrated with 200 µl IP lysis buffer. Lysate was then applied to the columns and bound µbeads were washed with 4 x 200 µl IP lysis buffer. A final wash with 100 µl low salt buffer (20 mM Tris-HCl (pH 7.5)) was performed. 20 µl hot 1x SDS sample buffer (1 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 mM DTT, 1% (w/v) SDS, 10% glycerol and 0.02% (w/v) bromophenol blue) was applied to the columns and incubated for 5 min. Proteins were then eluted from the µbeads by addition of 50 µl hot 1x SDS sample buffer, and collected in eppendorf 1.5 ml tubes ready for SDS-PAGE analysis.

To perform immunoprecipitation of overexpressed FLAG-tagged SK2 from lysates co-expressing DYNC1I1, a pre-clear of the lysate was required (to reduce non-specific binding of DYNC1I1 to the µbeads) prior to performing co-immunoprecipitation. Briefly, after preparation of the diluted lysate (170 µl), 35 µl of Protein G µbeads were added (without antibody), mixed and incubated on ice for 30 min. Lysate was then applied to equilibrated columns and flow-through was collected in an eppendorf tube. 20 µl of this pre-cleared lysate was removed and kept for protein expression levels, and the remaining lysate was used for co-immunoprecipitation, as above.

For immunoprecipitation of endogenous SK2, cells were lysed in extraction buffer (without DTT), sonicated and clarified. 1 mg of lysate was then incubated with 4 µg anti-SK2 antibody and 50 µl each of Protein G and Protein A µbeads (100 µl total beads). The protocol was then continued as above, with extraction buffer (without DTT) being used for column equilibration and 4 x 200 µl washes. Immunoprecipitation of endogenous SK2 from cleared mouse brain tissue lysate was performed essentially as described above for cultured cells, except the 4 x 200 µl column washes were performed using a high salt wash buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1% NP-40) to reduce non-specific protein binding, followed by a single wash with 100 µl low salt buffer.

2.5 Immunoblotting

SDS sample buffer was added to lysates of equal protein, giving a final concentration of 1 mM Tris-HCl (pH 8.0), 1 mM EDTA, 20 mM DTT, 1% (w/v) SDS, 10% glycerol and 0.02% (w/v) bromophenol blue. Samples were then heated to 100ºC for 5 min, briefly

centrifuged and separated by SDS-PAGE on either Criterion™ XT Bis-Tris 4-12% gradient gels (Bio-Rad Laboratories) or freshly-poured 10% SDS-polyacrylamide gels. Kaleidoscope™ Precision Plus protein standards (Bio-Rad Laboratories) were used to estimate protein molecular mass. Proteins were then transferred to nitrocellulose membrane (Pall Life Sciences, Pensacola, FL, USA). Membranes were blocked using Odyssey® Blocking Buffer (LI-COR, Lincoln, NE, USA) prior to incubation with appropriate primary antibodies. Proteins were visualised using IRDye® secondary antibodies and the Odyssey® CLx infrared imaging system (LI-COR). Alternatively, membranes were blocked in PBS containing 5% (w/v) skim milk powder and 0.1% Triton X-100, and subsequent to primary antibody incubation, proteins were visualised using horseradish peroxidise (HRP)-conjugated secondary antibodies and enhanced chemiluminescent (ECL) substrate (GE Healthcare Life Sciences, Parramatta, NSW, Australia) on the ImageQuant LAS 4000 imaging system (GE Healthcare Life Sciences).

2.6 Antibodies

The following commercial primary antibodies were utilised at dilutions recommended by the manufacturer, unless otherwise stated: mouse monoclonal anti-FLAG (Clone M2 #F3165, Sigma-Aldrich, St. Louis, MO, USA), rabbit polyclonal anti-FLAG (#2368, Cell Signaling Technology, Danvers, MA, USA), rabbit monoclonal anti-FLAG (#14793, Cell Signaling Technology), mouse monoclonal anti-HA (#H3663, Sigma-Aldrich), rabbit anti-SK2 (#17096-1-AP, Proteintech, Rosemont, IL, USA), rabbit anti-SK2 (#SP4621, ECM Biosciences, Versailles, KY, USA), rabbit anti-SK1 (#SP1621, ECM Biosciences), mouse anti-α-tubulin (#ab7291, Abcam, Cambridge, MA, USA), goat anti-GFP (#600-101-215, Rockland Immunochemicals, Limerick, PA, USA), rabbit anti-phospho-p44/42 MAPK (ERK1/2) Thr202/Tyr204 (#9101, Cell Signaling Technology), rabbit anti-p44/42 MAPK (ERK1/2) (#9102, Cell Signaling Technology), rabbit anti-phospho-AKT Ser473 (#9271, Cell Signaling Technology), rabbit anti-AKT (#9272, Cell Signaling Technology), mouse anti-Ki-67 (#VP-K452, Vector Labs, Burlingame, CA, USA), mouse monoclonal anti-DYNC1IC (clone 74.1, #MAB1618, Millipore, Bayswater, VIC, Australia), rabbit anti-DYNC1I1 (#13808-1-AP, Proteintech), rabbit anti-DYNC1I2 (#ab96288, Abcam), rabbit monoclonal anti-DYNC1LIC1 (#ab157468, Abcam), mouse monoclonal anti-dynactin p150 (#SC-135890, Santa Cruz Biotechnology, Dallas, TX, USA) and goat anti-PECAM-1

(CD31; #SC-1506 Santa Cruz Biotechnology). IRDye® secondary antibodies (1:10,000, 800CW and 680RD; LI-COR) were utilised in conjunction with the Odyssey® CLx infrared imaging system (LI-COR), and HRP-conjugated secondary antibodies (10,000; Pierce, Thermo Fisher Scientific) were used with the ImageQuant LAS 4000 imaging system (GE Healthcare Life Sciences).

2.7 RNA preparation and cDNA synthesis

To isolate RNA, cultured cells were pelleted and lysed in 1 ml TRIzol (Thermo Fisher Scientific). Lysate was centrifuged at 12,000 x *g* for 10 min at 4˚C, and supernatant was collected. After a 5 min incubation at room temperature, 200 µl chloroform was added and mixed by shaking vigorously for 15 sec. Samples were left at room temperature for 5 min, and phases were then separated by centrifugation at 12,000 x *g* for 15 min at 4˚C. The top aqueous phase was collected, 500 µl isopropanol was added and RNA was then precipitated at -20˚C for 1 hr. Samples were centrifuged at 12,000 x *g* for 10 min at 4˚C to pellet the RNA. The supernatant was discarded, the pellet washed with fresh 75% ethanol (RNase-free) and then reconstituted in RNase-free water. RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and 1 µg RNA was then used as a template to synthesise cDNA using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany).

2.8 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed using a QuantiTect SYBR Green PCR kit (Qiagen). Primer sequences are as follows: hDYNC1I1 forward primer TAAAGTTGGCCAGGACTCAG, hDYNC1I1 reverse primer CCAGGGCTCTTTCAATTACC, hDYNC1I2 forward primer CCAGTTATGGCTCAACCCAA, hDYNC1I2 reverse primer TCAGAGTGCAAGATTTGTTGC, hSK2 forward primer TGGCAGTGGTGTAAGAACCA, hSK2 reverse primer CAGTCAGGGCGATCTAGGAG, hGAPDH forward primer ACCCAGAAGACTGTGGATGG, hGAPDH reverse primer CAGTGAGCTTCCCGTTCAG. Each reaction contained 2 µl RNase-free H_2O, 5 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), 0.5 µl forward primer (at 100 ng/µl),

0.5 µl reverse primer (at 100 ng/µl), and 2 µl cDNA (diluted 1:5 in RNase-free H_2O). qPCR reactions were performed on a Rotor-Gene 6000 RealTime PCR machine (Corbett Life Sciences, Qiagen), using the following protocol: 50°C for 2 min, 95°C for 15 min, 48 cycles of denaturing at 95°C for 30 sec, annealing at 58°C for 21 sec, and extension at 72°C for 15 sec, and finally 72°C for 30 sec. Melt curves were then determined by increasing temperature from 72°C to 99°C, increasing by 1°C every 5 sec. Relative levels of target genes were analysed using the Rotor-Gene software (Qiagen) and normalised to the levels of GAPDH. Comparative quantitation was performed using an internal calibrator cDNA which was included in each experiment.

2.9 Generating and purifying recombinant proteins

To make recombinant full-length IC-2 protein, *Escherichia coli* BL21 cells transformed with pET17b-DYNC1I2(His) plasmid were grown overnight in Luria broth containing 100 µg/ml ampicillin and 50 µg/ml chloramphenicol at 37°C with shaking. The cultures were then diluted 1 in 10 into the same medium and grown at 37°C with shaking for 1 hr or until reaching an OD_{600} of ~0.6 - 0.8. Expression of the IC-2(His) protein was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 200 µM, and the culture was incubated for an additional 3 hr at 30°C with shaking. The cells were harvested by centrifugation at 6000 × *g* for 15 min at 4°C, resuspended in Buffer A (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole, 10% glycerol, complete protease inhibitor cocktail) containing 1% Triton X-100, left on ice for 20 min and then lysed by probe sonication (six pulses 10 sec on/off at 50% amplification). The lysate was clarified by centrifugation at 17,000 × *g* for 20 min at 4°C to remove cell debris. Nickel-Sepharose™ 6 Fast Flow beads (GE Healthcare Life Sciences) were added to the clarified lysate as a 50% slurry in Buffer A, and the mixture was incubated at 4°C for 1 hr with constant agitation. The nickel-sepharose beads were collected in an equilibrated 20 ml chromatography column (BioRad), washed three times with cold Buffer A, and IC-2(His) protein was then eluted from the beads in sequential elution fractions using Buffer B (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 500 mM imidazole, 10% glycerol, complete protease inhibitor cocktail). The amount of IC-2(His) protein in each elution was quantified with Coomassie Brilliant Blue staining following SDS-PAGE using BSA as a standard.

To generate and purify SK2 protein, HEK293 cells were transiently transfected with pCDNA3-SK2(FLAG) vector, and 24 hr later cells were harvested in cold PBS. Cell pellets were resuspended in EB containing 1% Triton X-100, and lysed by three rounds of freeze/thawing in liquid nitrogen. Lysates were clarified by centrifugation at 17,000 × *g* for 15 min at 4°C. To immunoprecipitate SK2(FLAG) proteins, 500 µl clarified lysate was added to 25 µl equivalent volume of packed FLAG-agarose beads (Sigma-Aldrich), and the mixture was incubated at 4°C for 1 hr with constant agitation. FLAG-agarose beads were pelleted by centrifugation at 6,000 × *g* for 30 sec and washed three times in EB containing 1% Triton X-100. SK2(FLAG)-bound agarose beads were subsequently used in pull-down assays or, to quantify the amount of bound protein, 2x SDS sample buffer was added to the beads and proteins were subjected to SDS-PAGE analyses with BSA standards and staining with Coomassie Brilliant Blue.

2.10 Pull-down assays

Pull-down analyses were performed by incubating 1 µg recombinant IC-2(His) protein (described above) diluted in EB with ~500 ng of purified SK2(FLAG) protein bound to FLAG-agarose beads. The proteins were incubated for 2 hr at 4°C with constant agitation. The FLAG-agarose beads were pelleted by centrifugation at 6,000 × *g* and washed three times in EB, and bound proteins were then resolved by SDS-PAGE and visualised by immunoblotting. SK2 was detected using anti-FLAG antibodies whilst recombinant IC-2 was detected using anti-6xHis antibodies.

2.11 Sphingosine kinase activity assays

SK2 activity was determined using D-*erythro*-sphingosine (Biomol, Hamburg, Germany) and [γ^{32}P]ATP (Perkin Elmer, Waltham, MA, USA) as substrates under isoform-selective assay conditions, as previously described (Pitman et al, 2012). Briefly, 20 µl of cell lysate (containing 5 µg of overexpressed protein or 50 µg of endogenous protein, and Triton X-100-free EB) was mixed with 80 µl of assay buffer (100 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 1 M KCl) containing 1 mM ATP, 1 µCi [γ^{32}P]ATP, 100

μM BSA-solubilised sphingosine, and 0.5 mM 4-deoxypyridoxine. Samples were vortexed and incubated at 37°C for 30 min. The reaction was stopped by the addition of 270 μl chloroform/methanol/conc. HCl solution (100:200:1, v/v), followed by 70 μl chloroform and 20 μl 5 M KCl. Samples were vortexed and the aqueous/methanol and chloroform phases were separated by centrifugation at 17,000 x *g* for 5 min. The upper aqueous/methanol layer was removed by aspiration and 50 μl of the remaining chloroform phase was spotted onto a thin layer chromatography (TLC) plate (Silica gel 60; Sigma-Aldrich). The plate was developed in butanol/ethanol/acetic acid/water (8:2:1:2, v/v) solvent, dried and exposed to a phosphor screen (Amersham Biosciences, GE Healthcare Life Sciences). The spots containing radiolabel-incorporated sphingosine-1-phosphate were imaged using a Typhoon phosphorimager (GE Healthcare Life Sciences), and quantified using ImageQuant software (GE Healthcare Life Sciences). A unit (U) of SK2 activity was defined as the amount of enzyme required to produce 1 pmol S1P/min/mg protein.

2.12 Focus formation assays

Focus formation assays were performed as described previously (Zhu et al, 2017). Briefly, NIH3T3 mouse fibroblasts were cultured to form monolayers in 6-well plates in DMEM with 1% DBS (Gibco, Thermo Fisher Scientific), and media was replenished every 2-3 days for a total of 3 weeks. Cells were then fixed in methanol, foci were stained with bromophenol blue, and images were taken using the Odyssey® CLx infrared imaging system (LI-COR), using the 680 nm (red) channel.

2.13 Colony formation in soft agar

Colony formation assays in soft agar were performed as previously described (Pitman et al, 2015). Briefly, cells were trypsinised, resuspended in complete growth media and mixed with DMEM containing low gelling temperature agarose (0.33% (w/v) final; Sigma-Aldrich). This was overlaid onto 0.5% (w/v) low gelling temperature agarose in DMEM, pre-set in 6-well plates. Plates were then incubated at 37°C with 5% CO_2, and an additional layer of growth media/0.33% agarose gel was added weekly. After 14-21 days, colonies

were imaged using an Olympus MVX10 microscope (Tokyo, Japan) and quantified either visually using light microscopy, or using ImageJ software.

2.14 Immunofluorescence

Cells in complete growth media were seeded onto poly-L-lysine (Sigma-Aldrich) coated coverslips in 12-well plates, or into poly-L-lysine coated 8- or 16-well chamber slides, and incubated overnight at 37°C with 5% CO_2. After the appropriate treatments (transfection, serum-deprivation, etc), the media was then aspirated from the cells, the cells washed with PBS, and fixed with 4% paraformaldehyde for 10 min. Fixed cells were then washed twice with 0.1% Triton X-100 in PBS and permeabilised by a further 10 min incubation in Triton X-100/PBS. Cells were then blocked with 3% BSA (in 0.1% Triton X-100/PBS) for 10-30 min. Primary antibodies diluted in BSA/Triton X-100/PBS were added to the cells and incubated for 1 hr at room temperature. Cells were washed four times with Triton X-100/PBS. Secondary antibodies diluted in BSA/Triton X-100/PBS were added to the cells and incubated in the dark for 1 hr at room temperature. Cells were washed four times with Triton X-100/PBS and once more with PBS. For nuclear staining, DAPI (0.2 μg/ml in PBS) was added to the cells for 5 min in the dark. Finally, the cells were washed twice with PBS and mounted onto slides in fluorescent mounting medium (Dako, Agilent Technologies, Santa Clara, CA, USA). Cell images were taken using a Zeiss LSM 700 Confocal microscope (Jena, Germany), and gains were kept constant within each experiment.

2.15 Immunohistochemistry on tumour tissue sections

For immunohistological staining of PECAM-1 (CD31) expression, tumours were paraffin-fixed and sectioned, and citrate buffer antigen retrieval was performed, followed by quenching endogenous peroxidase activity with 3% hydrogen peroxide. Sections were then blocked with rabbit serum/PBS for 60 min at room temperature, and incubated with 133 ng/ml goat polyclonal antibody to PECAM-1 (CD31; Santa Cruz Biotechnology), or goat IgG isotype control, overnight at 4°C. Biotinylated rabbit anti-goat secondary antibody (1:500; Abcam) was incubated for 35 min at room temperature, followed by incubation with VECTASTAIN Elite ABC Reagent (Vector Labs) for 30 min at room temperature, and

peroxidase substrate solution. Sections were counterstained with Mayer's haematoxylin, mounted using DPX mounting medium (Sigma-Aldrich) and imaged on an EVOS XL light microscope (Thermo Fisher Scientific) at 20x magnification.

Immunohistological staining of overexpressed FLAG-tagged SK2 within tumour sections was performed as above, with the following modifications. Sections were blocked with goat serum/PBS and incubated with 150 ng/ml rabbit polyclonal FLAG antibody (Cell Signaling Technology), or rabbit IgG isotype control, overnight at 4°C. Biotinylated goat anti-rabbit secondary antibody (1:500; Vector Labs) was incubated for 35 min at room temperature.

2.16 Animal ethics

Experiments involving mice were conducted according to the guidelines from the Australian code of practice for the care and use of animals for scientific purposes 7th Edition, 2004, and with approval from the SA Pathology/CALHN Animal Ethics Committee (approval #39/15) and the University of Adelaide Animal Ethics Committee (approval #S-2015-048).

Chapter 3:
Validating Sphingosine Kinase 2 Antibodies

Most of the data from this chapter has been published as:

Neubauer HA and Pitson SM (2017) Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence. *F1000Research,* 5: 2825; doi: 10.12688/f1000research.10336.2.

See Appendix 2 for author contributions and reprint

Chapter 3. Validating Sphingosine Kinase 2 Antibodies

3.1 Abstract

Sphingosine kinase 2 (SK2) is a ubiquitously expressed lipid kinase that has important, albeit complex and poorly understood roles in regulating cell survival and cell death. In addition to being able to promote cell cycle arrest and apoptosis under certain conditions, it also appears to play a role in cancer. Therefore, well validated and reliable tools are required to study and better understand the true functions of SK2. In-house polyclonal SK2 antisera raised against amino acids 35-52 of human SK2a had previously been generated by the Pitson Laboratory, and this chapter describes the affinity-purification and subsequent testing of this in-house SK2 antibody. Furthermore, two commercially available SK2 antibodies were examined and compared: a rabbit polyclonal antibody from Proteintech that recognizes amino acids 266-618 of human SK2a, and a rabbit polyclonal antibody from ECM Biosciences that recognizes amino acids 36-52 of human SK2a. The performance of these antibodies was examined for use in immunoblotting, immunoprecipitation and immunofluorescence staining of endogenous SK2, using human HEK293 and HeLa cell lines, as well as mouse embryonic fibroblasts (MEFs). Furthermore, the specificity of these antibodies to the target protein was assessed through the use of siRNA-mediated SK2 knockdown and SK2 knockout (*Sphk2*$^{-/-}$) MEFs. The findings of this study demonstrate that the in-house SK2 antibody can detect endogenous SK2 by immunoblot, but also has extensive cross-reactivity to other proteins, making it not ideal for this application. Furthermore, the in-house SK2 antibody was not able to immunoprecipitate endogenous SK2, and it produced substantial non-specific staining by immunofluorescence, making it unsuitable for these applications. Notably, the Proteintech anti-SK2 antibody reproducibly displayed sensitivity and selectivity towards SK2 in immunoblot analyses, whilst the detection of non-specific proteins made it unsuitable for immunoprecipitation and immunofluorescence. Conversely, the ECM Biosciences anti-SK2 antibody was reproducibly superior for SK2 immunoprecipitation and detection by immunofluorescence staining, whereas it is not ideal for immunoblotting as it produces various non-specific bands. Overall, from this validation study, suitable tools were identified to enable reproducible and robust detection of endogenous SK2 for each application tested.

3.2 Introduction

Sphingolipids are an important family of cellular molecules that form critical structural components of cell membranes, as well as perform numerous signaling functions (Pitson, 2011). Of the many enzymes responsible for the biosynthesis and metabolism of sphingolipids, the sphingosine kinases (SKs) are of particular interest to study as they catalyze the formation of sphingosine-1-phosphate (S1P), and in doing so can promote cell survival, proliferation, migration and angiogenesis (Pyne & Pyne, 2010). Both sphingosine kinases, SK1 and SK2, have been shown to be upregulated in various human cancers and both have documented roles in mediating oncogenesis (Neubauer et al, 2016; Xia et al, 2000). However, where SK1 and its roles in cancer development are relatively well characterized, SK2 remains somewhat enigmatic as, in addition to the pro-cancer functions it shares with SK1, SK2 can also facilitate cell cycle arrest and cell death (Chipuk et al, 2012; Okada et al, 2005).

SK2 is ubiquitously expressed in all cells and tissues, but is expressed most highly in the liver, kidney and brain (Liu et al, 2000). At the mitochondria, SK2-generated S1P has been proposed to facilitate the activation of Bak and subsequent mitochondrial membrane permeabilisation and cytochrome c release (Chipuk et al, 2012). Notably, SK2 can also function as an epigenetic regulator, where S1P produced by nuclear-localized SK2 can inhibit the activity of histone deacetylases 1/2 resulting in increased transcription of specific genes, such as cyclin-dependent kinase inhibitor *p21* and transcriptional regulator *c-fos* (Hait et al, 2009). As SK1 does not appear to localize as prominently to these internal organelles, it is believed that the subcellular localization of SK2 is critical for the additional functions it performs. However, the mechanisms regulating the localization and functions of SK2, allowing it to switch between pro-apoptotic and pro-survival under certain conditions, remain poorly understood.

In order to study SK2 and better characterize its roles in normal cells as well as in cancer, reliable and properly validated tools are required. Antibody-based methods, such as immunoblotting (IB), immunoprecipitation (IP) and immunofluorescence (IF), are particularly useful as tools to examine and visualize important aspects of SK2 biology, like subcellular localization, expression and interaction with regulatory proteins. At the commencement of these studies, there were no validated commercially available SK2-

specific antibodies in our Laboratory that were able to detect endogenous levels of SK2, and many published studies describing the detection of endogenous SK2 using antibody-based methods had generated and affinity-purified their own in-house SK2 antibodies (Hait et al, 2005; Igarashi et al, 2003). As the detection and isolation of endogenous SK2 was required, it was of interest to validate specific SK2 antibodies that were suitable for use in various applications. As such, this chapter describes the affinity-purification of SK2 antisera generated by the Pitson Laboratory, and the subsequent validation of this in-house SK2 antibody, as well as two recently available commercial SK2 antibodies, for use in IB, IP and IF using various human and mouse cells lines. The in-house antibody is a rabbit polyclonal SK2 antibody raised against a synthetic peptide corresponding to amino acids 35-52 of human SK2a. The two commercially available antibodies examined were a rabbit polyclonal SK2 antibody from Proteintech, which was raised against amino acids 266-618 of recombinant human SK2a, and a rabbit polyclonal SK2 antibody from ECM Biosciences, which was raised against a synthetic peptide corresponding to amino acids 36-52 of human SK2a. Although these antibodies have not previously been properly validated, the Proteintech SK2 antibody has been previously utilized in one publication for IB (Liu et al, 2016b), and the ECM Biosciences SK2 antibody has been used in multiple publications for IB (Bruno et al, 2015; Liu et al, 2016a; Sun et al, 2016a; Wallington-Beddoe et al, 2014) and for IF (Reid et al, 2015).

3.3 Specific Materials and Methods

3.3.1 Affinity purification of SK2 antisera

Polyclonal anti-SK2 antibodies, previously generated by the Pitson Laboratory, were raised in rabbits against the SK2 peptide H-CSQALHIQRLRPKPEARPR-OH (amino acid residues 35-52 of human SK2a) conjugated to maleimide-activated keyhole limpet hemocyanin (KLH; Pierce). To affinity purify these antibodies from the rabbit serum, an SK2 peptide affinity column was prepared by adding 1 ml SulfoLink™ Coupling Gel 50% slurry (Pierce) to a disposable column (BioRad) and equilibrating with 50 mM Tris/HCl (pH 8.5) containing 5 mM EDTA. 1 mg SK2 peptide H-CSQALHIQRLRPKPEARPR-OH (ChinaPeptides, Shanghai China) in 50 mM Tris/HCl (pH 8.5) containing 5 mM EDTA was added to the drained and capped column, mixed by gentle inversion for 15 min at room

temperature, and incubated for a further 30 min at room temperature to allow the iodoacetyl groups on the SulfoLink resin to react with the N-terminal cysteine of the peptide. The column was then drained and washed with 50 mM Tris/HCl (pH 8.5) containing 5 mM EDTA. To quench unreacted iodoacetyl groups on the SulfoLink resin, 1 mg cysteine was added to the capped column, mixed by gentle inversion for 15 min at room temperature, and incubated for a further 30 min at room temperature. The column was drained and washed with 50 mM Tris/HCl (pH 7.5) containing 1 M NaCl, then stored in 50 mM Tris/HCl (pH 7.5) containing 0.05% NaN_3 at 4°C overnight.

To purify the antisera, the column was equilibrated with cold 10 mM Tris/HCl (pH 7.5), drained and capped, and 2 ml 10 mM Tris/HCl (pH 7.5) along with 2 ml serum were then added and mixed by gentle inversion at 4°C for 1 hr. The column was drained and washed with 10 mM Tris/HCl (pH 7.5) (eluate discarded). An additional wash with 10 mM Tris/HCl (pH 7.5) containing 0.5 M NaCl was performed and discarded. The column was then washed with 5 ml 100 mM glycine (pH 2.5) and the eluate was collected in a tube containing 2 ml 1 M Tris/HCl (pH 8.0) (purified anti-SK2 antibodies). The column was then washed firstly with 10 mM Tris/HCl (pH 8.8) (eluate discarded), then with 5 ml 100 mM Na_2PO_4 (pH 11) (eluate collected in tube containing purified anti-SK2 antibodies), and lastly with 10 mM Tris/HCl (pH 7.5) (eluate discarded). Total collected purified anti-SK2 antibodies were precipitated by diluting 1:2 in 100% saturated ammonium sulphate (pH 7.4), mixing by gentle inversion, and storing at 4°C overnight. Antibodies were then centrifuged at 46,000 x *g* for 45 min at 4°C, supernatant was discarded, and remaining purified anti-SK2 antibodies were resuspended in 500 µl 100% saturated ammonium sulphate (with 0.05% NaN_3) and stored at 4°C.

3.3.2 Commercial antibodies

The following commercially available SK2 antibodies were utilised: rabbit polyclonal anti-SK2 (ECM Biosciences; anti-Sphingosine Kinase 2 (N-terminal region); #SP4621, lot #1) and rabbit polyclonal anti-SK2 (Proteintech Group, Inc; anti-SPHK2; #17096-1-AP, lot #00010361). The ECM Biosciences SK2 antibody was raised against a synthetic peptide coupled to keyhole limpet hemocyanin (KLH), corresponding to amino acids 36-52 of human SK2a, and was affinity purified with the SK2 peptide (without KLH). It is reported

by the manufacturer to have cross-reactivity with rat and mouse SK2 [human, mouse and rat SK2 share 100% sequence identity in this region (determined using the align tool and protein sequences from www.uniprot.org)], and has been assessed by the manufacturer for use in IB and enzyme-linked immunosorbent assay (ELISA). The Proteintech SK2 antibody was raised against truncated recombinant GST-tagged human SK2a (amino acid residues 266-618 generated in *Escherichia coli* using the PGEX-4T plasmid). The SK2 target antibodies were then affinity purified using 6xHis-tagged antigen protein (to remove GST-specific antibodies) and then again with the immunising GST-tagged antigen protein. It is reported to have cross-reactivity with rat and mouse SK2 [80.2% sequence identity between human and mouse SK2, and 80.2% sequence identity between human and rat SK2 in this region (determined using the align tool and protein sequences from www.uniprot.org)], and according to the manufacturer can be employed for IB, ELISA, IP and immunohistochemistry. Mouse anti-α-tubulin (DM1A; Abcam; #ab7291) is a mouse monoclonal antibody, which was used as a loading control for IB analyses, at a dilution of 1:5,000.

3.3.3 siRNA knockdown of SK2

siRNA-mediated knockdown of SK2 was performed using human SPHK2 siGENOME SMARTpool siRNA (Dharmacon), which targets the following sequences: CCACUGCCCUCACCUGUCU, GCUCCUCCAUGGCGAGUUU, GAGACGGGCUGCUCCAUGA, CAAGGCAGCUCUACACUCA. Cells were seeded and grown to a cell density of approximately 50%, and cells were then transfected with 30 nM (final concentration) of either hSK2 siRNA or siGENOME non-targeting siRNA pool, using Lipofectamine RNAiMAX as per the manufacturer's protocol (Life Technologies). Cells were incubated with the siRNA complexes at 37°C for 48 hr.

3.4 Results

3.4.1 Testing the in-house affinity-purified SK2 rabbit polyclonal antibody

The SK2 antisera previously generated and collected by the Pitson Laboratory was raised against a peptide sequence corresponding to amino acids 35-52 of SK2a, as this peptide sequence had been used to generate SK2-specific antibodies by other groups in published studies detecting endogenous SK2 (Hait et al, 2005; Igarashi et al, 2003). The antisera was affinity-purified using an SK2 peptide affinity column, and the resulting in-house SK2 antibody (herein referred to as HN12) was utilised in IB, IP and IF.

3.4.1.1 Testing the SK2 antibody HN12 for the detection of endogenous SK2 by immunoblot

Firstly, the HN12 SK2 antibody was utilised in IB analyses to examine its specificity and selectivity towards endogenous SK2. As shown in Figure 3.1, HN12 was able to detect overexpressed SK2 very well, and importantly, HN12 demonstrated selectivity for SK2 as it did not detect overexpressed SK1. An additional band was detected at the same size as SK2 (~69 kDa) in lysates from cells transfected with empty vector as well as lysates from cells overexpressing SK1, suggesting that HN12 may have detected endogenous SK2 (Figure 3.1). To validate if this band was indeed endogenous SK2, HEK293 and HeLa cells were subjected to siRNA-mediated knockdown of SK2, which resulted in 50% knockdown of SK2 mRNA levels in both cell lines (Figure 3.2A). IB analyses were then performed on these lysates using HN12. In agreement with Figure 3.1, a band was detected in HEK293 lysate at the correct size for SK2, but this band was, at most, only slightly reduced in the SK2-knockdown lysate (Figure 3.2B). Conversely, no detectable endogenous SK2 band was present in the HeLa lysates, however this may not be surprising as HeLa cells have less SK2 than HEK293 cells, at least at the mRNA level (Figure 3.2A).

To further examine the ability of HN12 to detect endogenous SK2, lysates from wildtype and *Sphk2*$^{-/-}$ MEFs were also subjected to IB analyses using HN12, negating the issues of incomplete knockdown often observed with siRNA. Indeed, a band was detected in the wildtype MEF lysate that was completely absent in the *Sphk2*$^{-/-}$ MEF lysate (Figure 3.2C),

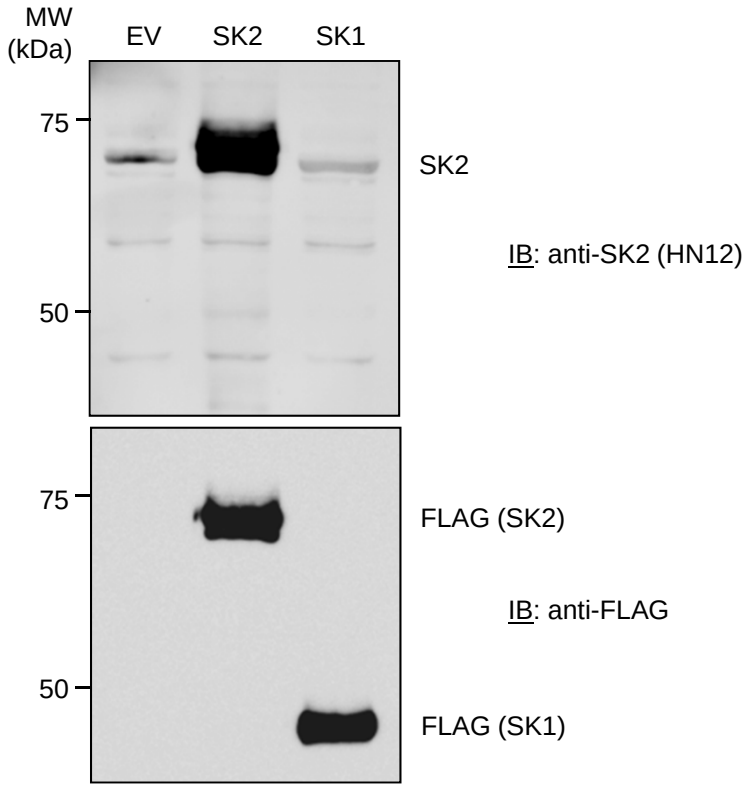


Figure 3.1: Affinity purified HN12 SK2 antibody detects overexpressed, and potentially endogenous, SK2 by immunoblot

HEK293T cells were transfected with vectors encoding FLAG-tagged SK2, FLAG-tagged SK1, or empty vector (EV). The cells were harvested 24 hr later and the lysates were subjected to SDS-PAGE and immunoblot analyses using affinity purified in-house anti-SK2 antibodies (HN12), or anti-FLAG antibodies. Images shown are representative of three independent experiments.

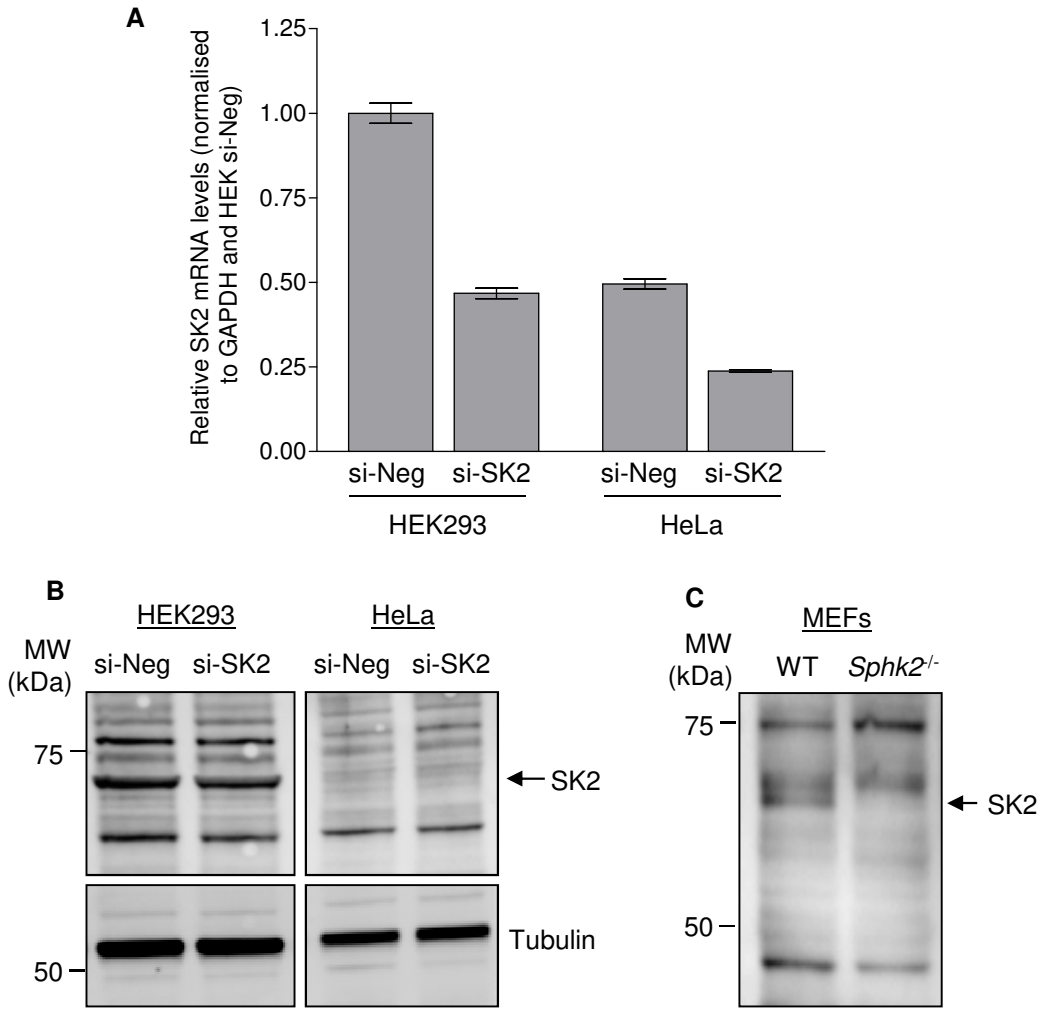


Figure 3.2: Affinity purified HN12 SK2 antibody detects endogenous SK2 by immunoblot in some cell lines, but is nonspecific

A and *B,* HEK293-c18 and HeLa cells were transfected with either negative control siRNA (si-Neg) or SK2 siRNA (si-SK2). After 48 hr, half of the cells were used for (A) qRT-PCR analyses of SK2 mRNA levels (comparative quantitation; normalised to GAPDH and HEK293 si-Neg), and the other half of the cells were used for (B) lysate preparation, SDS-PAGE and immunoblot analyses using affinity-purified in-house HN12 anti-SK2 antibodies, or anti-tubulin antibodies. Data in (A) were collected and analysed by Wenying Zhu and Elferaan Quatermass. Images shown are representative of 1-2 independent experiments. *C,* Lysates made from wildtype (WT) or *Sphk2*$^{-/-}$ MEFs were subjected to SDS-PAGE and immunoblot analyses using affinity-purified in-house HN12 anti-SK2 antibodies. Blot shown is representative of two independent experiments.

confirming that HN12 can detect endogenous SK2. However, in all three cell types tested, a substantial number of non-specific bands were present, some of which were more intense than the SK2 band (Figure 3.2B and C). Therefore, although HN12 may detect endogenous SK2 by IB, it is not an ideal antibody to use for this application.

3.4.1.2 Testing the SK2 antibody HN12 for use in the immunoprecipitation of endogenous SK2

In testing the use of HN12 in other applications, it was of interest to determine if this SK2 antibody could IP endogenous SK2 from cell lysates. IP was performed using HN12 in HEK293 cell lysates, followed by IB analyses with HN12. A faint band at the correct size for SK2 appeared to be immunoprecipitated by HN12, and this band was somewhat weaker in lysate from cells subjected to siRNA-mediated knockdown of SK2 (Figure 3.3). However, the same band was present even in the absence of HN12 (Figure 3.3), suggesting that the bands observed may be endogenous SK2 non-specifically binding to the beads. Given that there was no enrichment of SK2 observed above non-specific levels by IP with HN12, despite a large amount of antibody being used, it is apparent that HN12 is not suitable for IP of endogenous SK2.

3.4.1.3 Testing the SK2 antibody HN12 for the detection of endogenous SK2 by immunofluorescence

IF is a very useful and commonly used technique to visualise the subcellular localisation of a target protein, and given that the localisation of SK2 seems to dictate its function, validating a specific SK2 antibody for use in IF was critical. As such, HeLa cells were subjected to either scrambled control siRNA or SK2-specific siRNA, and then analysed by IF with HN12 and confocal microscopy. As seen in Figure 3.4A, the 'SK2' staining appeared predominantly cytoplasmic, with strong peri-nuclear puncta. However, these puncta were also present in cells with SK2 knockdown, although the cytoplasmic staining was diminished (Figure 3.4A). To negate issues of incomplete knockdown by siRNA, wildtype and *Sphk2*$^{-/-}$ MEFs were also analysed by IF as above. In these cells, SK2 seemed to be evenly localised across the cytoplasm and nucleus, with strong puncta residing within

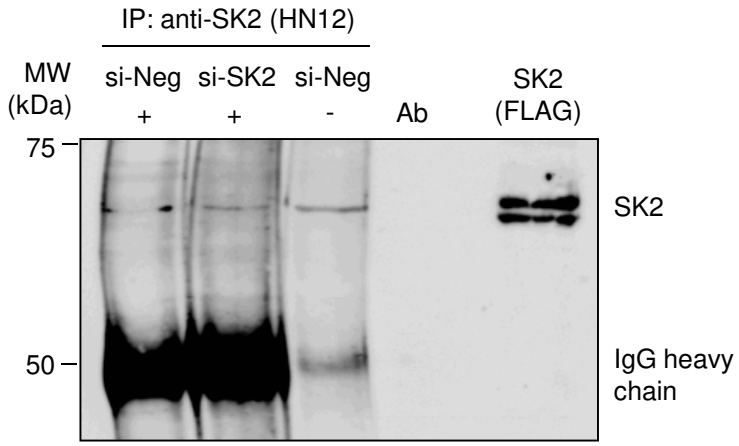


Figure 3.3: Affinity-purified SK2 antibody HN12 cannot immunoprecipitate endogenous SK2

HEK293-c18 cells were transfected with either negative control siRNA (si-Neg) or SK2 siRNA (si-SK2). After 48 hr, SK2 was immunoprecipitated from lysates using affinity-purified HN12 anti-SK2 antibodies. The resulting immunoprecipitate, as well as overexpressed SK2 lysate as a positive control [SK2(FLAG)], were subjected to SDS-PAGE and immunoblot analyses using HN12 anti-SK2 antibodies. Image shown is representative of two independent experiments.

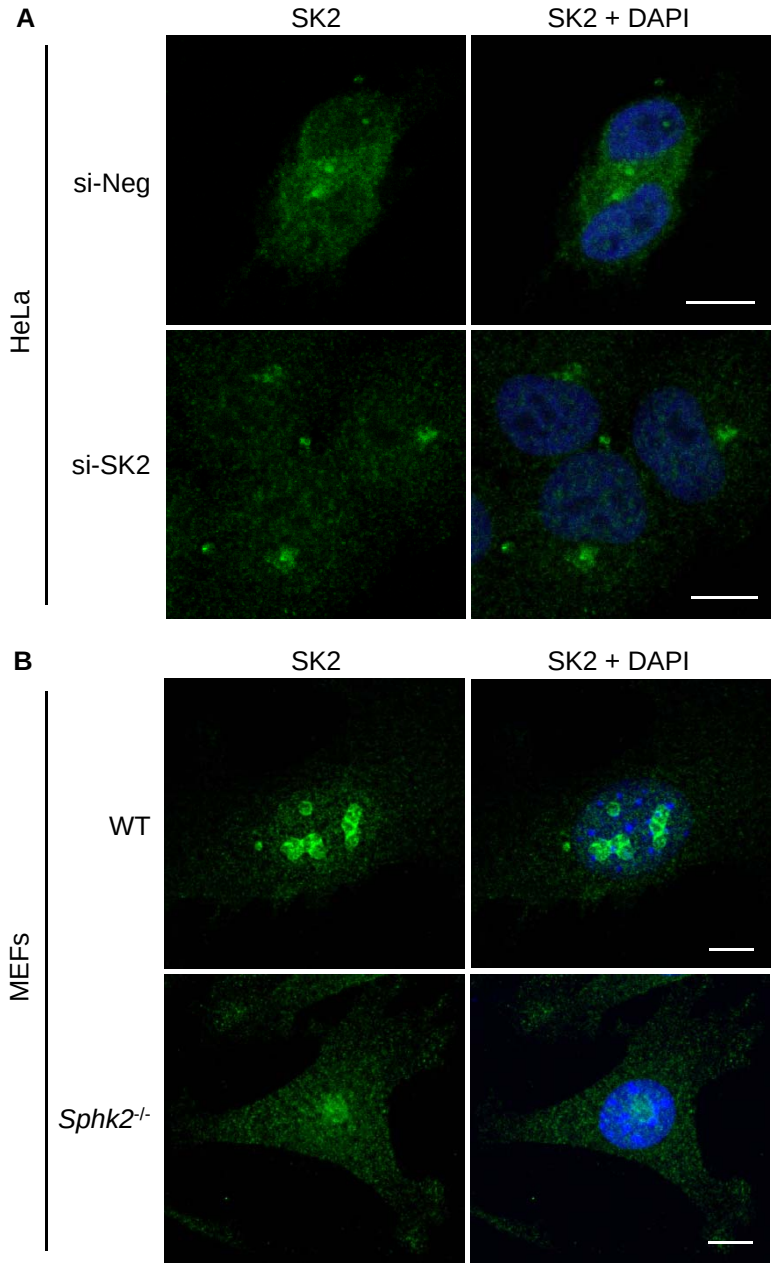


Figure 3.4: Affinity-purified SK2 antibody HN12 is not suitable for use in immunofluorescence

A, HeLa cells were transfected with either negative control siRNA (si-Neg) or SK2 siRNA (si-SK2). After 48 hr, endogenous SK2 was visualised by immunofluorescence and confocal microscopy, using anti-SK2 HN12 antibodies. *B,* Wildtype (WT) or *Sphk2*$^{-/-}$ MEFs were seeded and endogenous SK2 was visualised by immunofluorescence and confocal microscopy, using anti-SK2 HN12 antibodies. Nuclei were stained with DAPI (blue). Images shown are representative of more than 100 cells, from 1-2 independent experiments. Scale bars = 10 µm.

the nucleus (Figure 3.4B). However, very little difference in the cytoplasmic and nuclear staining was observed in the *Sphk2*$^{-/-}$ MEFs, and the nuclear puncta staining was slightly decreased but not absent (Figure 3.4B). Therefore, it cannot be concluded that the staining detected by HN12 is specifically endogenous SK2, and as such, it was concluded that this antibody would not be ideal to use for IF studies.

3.4.2 Validating commercially-available SK2 polyclonal antibodies

As it was important to possess a validated SK2 antibody to detect endogenous SK2 in multiple applications, and given that the in-house SK2 antibody HN12 was only able to detect endogenous SK2 by IB with low specificity, two additional newly available commercial SK2 antibodies were examined.

3.4.2.1 Proteintech SK2 antibody demonstrates target specificity and sensitivity by immunoblot

Both commercial SK2 antibodies examined in the present study are reported by their respective manufacturers to be able to detect endogenous SK2 by IB. To determine the selectivity of the anti-SK2 antibodies, IB analyses were performed using two human cell lines (HEK293 and HeLa) that had been treated with either scrambled control or SK2-directed siRNA, as well as WT and *Sphk2*$^{-/-}$ MEFs. The Proteintech anti-SK2 antibody detected a single prominent band at the correct molecular weight for SK2 (~65 kDa), which was decreased or absent in the knockdown and knockout lines (Figure 3.5A). Some faint non-specific bands were also detected in both the WT and *Sphk2*$^{-/-}$ MEF lysates by this antibody, which were not observed in the human cell lines. The ECM Biosciences anti-SK2 antibody did not appear to be very sensitive towards SK2, as no band was detected at the expected size in the HeLa lysates, and only very faint bands were present in the HEK293 and MEF lysates that were reduced or absent in the knockdown or knockout lines (Figure 3.5B). Furthermore, numerous prominent non-specific bands were present in all lysates, particularly in the MEF lines, indicating a lack of selectivity of this antibody towards SK2. Therefore, the Proteintech anti-SK2 antibody appears to be superior for use in IB,

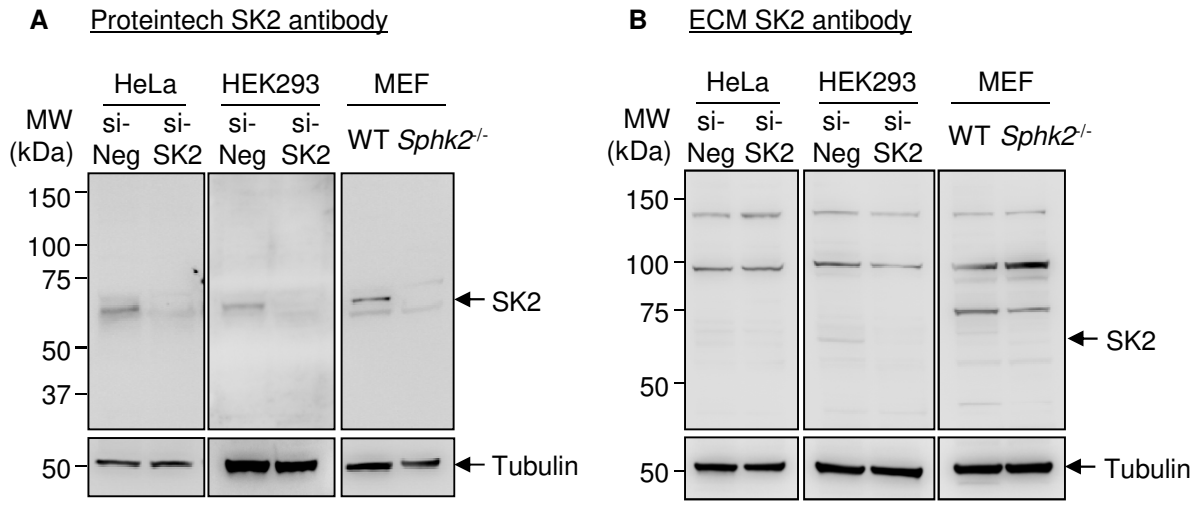


Figure 3.5: Immunoblot analyses of endogenous SK2 in multiple cell lines using two commercially available rabbit polyclonal anti-SK2 antibodies

Immunoblot analyses of lysates from HEK293 and HeLa cells treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), and lysates from wildtype (WT) or *Sphk2*$^{-/-}$ MEFs. An equal amount (40 µg) of total protein from each sample was run in duplicate. After transferring to nitrocellulose and blocking, the membrane was separated and duplicate samples were probed with either (*A*) Proteintech rabbit anti-SK2 antibody or (*B*) ECM Biosciences rabbit anti-SK2 antibody. SK2 membranes were imaged using a 4 min exposure. The expected band size for SK2 is ~65 kDa. Membranes were re-probed with mouse anti-α-tubulin antibodies as a loading control (2 min exposure), and α-tubulin was detected at 55 kDa as expected. Consistent results were observed from 2-3 (HEK293 and MEF) or 3-4 (HeLa) independent experiments for each antibody.

demonstrating both selectivity and sensitivity in the detection of endogenous SK2, particularly in the human cell lines tested.

3.4.2.2 ECM Biosciences SK2 antibody is able to specifically immunoprecipitate SK2

It was also examined whether either of the commercial anti-SK2 antibodies could immunoprecipitate SK2 from cell lysates. The Proteintech anti-SK2 antibody is suggested by the manufacturer to be useful for IP, whereas to our knowledge the ECM Biosciences anti-SK2 antibody has not been previously tested for use in this application. Initially, using lysates from HEK293 cells, it was found that the Proteintech anti-SK2 antibody was sometimes able to IP a band at the correct size for SK2 (Figure 3.6A); however this was not consistent with each experimental repeat and other proteins were also immunoprecipitated to a varying extent by this antibody that were not present in the IgG isotype control.

Conversely, the ECM Biosciences anti-SK2 antibody was able to consistently and cleanly IP a protein of the same size as SK2 from cell lysates, with almost no non-specific bands observed (Figure 3.6B). The protein immunoprecipitated by the ECM Biosciences antibody was considerably enriched from the cell lysate and was strongly detectable by this antibody, which was unable to detect SK2 in the lysate input sample, consistent with Figure 3.5B. To determine if this band was in fact SK2, the ECM Biosciences anti-SK2 antibody was then used to immunoprecipitate SK2 from HEK293 lysates treated with either scrambled control or SK2-directed siRNA. SK2 knockdown consistently resulted in reduced intensity of the band enriched by this antibody (Figure 3.6C), confirming that the ECM Biosciences anti-SK2 antibody can selectively IP endogenous SK2.

3.4.2.3 ECM Biosciences SK2 antibody can specifically detect SK2 by immunofluorescence staining

Finally, it was examined whether these commercially available SK2 antibodies could selectively detect SK2 by IF. Neither antibody has been reported to be tested for use in IF by their respective manufacturers; however, the Proteintech SK2 antibody is recommended for immunohistochemistry. Using IF staining methods routinely performed in the Pitson

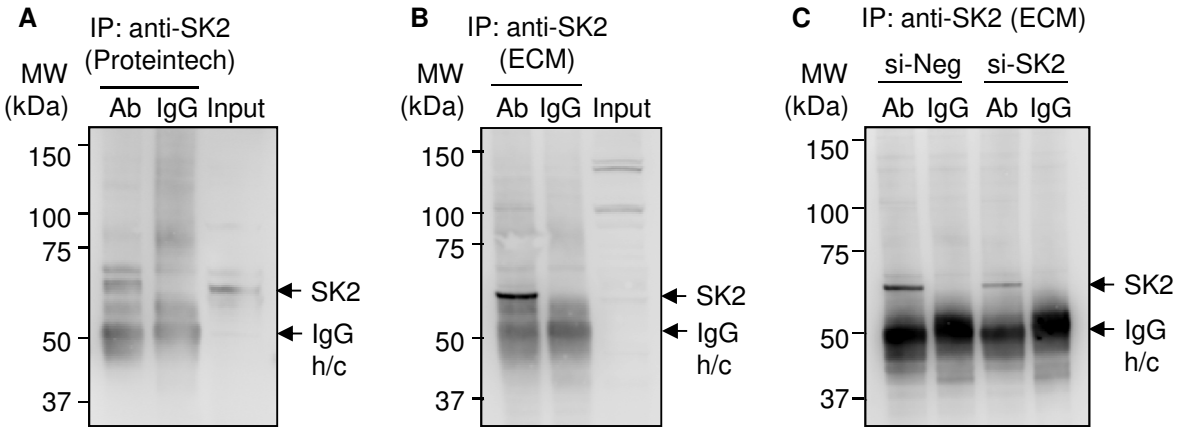


Figure 3.6: Comparison of two commercially available rabbit polyclonal anti-SK2 antibodies for immunoprecipitation of SK2 from HEK293 cell lysates

SK2 was immunoprecipitated from HEK293 cell lysate using either (*A*) Proteintech rabbit anti-SK2 antibody or (*B*) ECM Biosciences rabbit anti-SK2 antibody. Normal rabbit IgG antibody was used as an isotype control. Immunoprecipitates (and 40 µg lysate input) were subjected to immunoblot analyses and probed with (*A*) Proteintech rabbit anti-SK2 antibody or (*B*) ECM Biosciences rabbit anti-SK2 antibody. Membranes were imaged using a 4 min exposure. Images are representative of three independent experiments for each antibody. *C,* SK2 was immunoprecipitated from HEK293 cell lysates (of equal protein) that had been treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), using ECM Biosciences rabbit anti-SK2 antibody. Immunoprecipitates were subjected to immunoblot analyses and probed with ECM Biosciences rabbit anti-SK2 antibody. Membrane was imaged using a 4 min exposure. Image is representative of three independent experiments. IgG h/c = IgG heavy chain.

Laboratory, the two anti-SK2 antibodies were compared using HeLa, HEK293 and MEF cell lines. The Proteintech anti-SK2 antibody produced minimal staining in all cell lines tested (Figure 3.7), and consequently there was no observable differences between the control cells and those with SK2 knockdown (in the human cell lines) or SK2 knockout (in the *Sphk2*$^{-/-}$ MEF line).

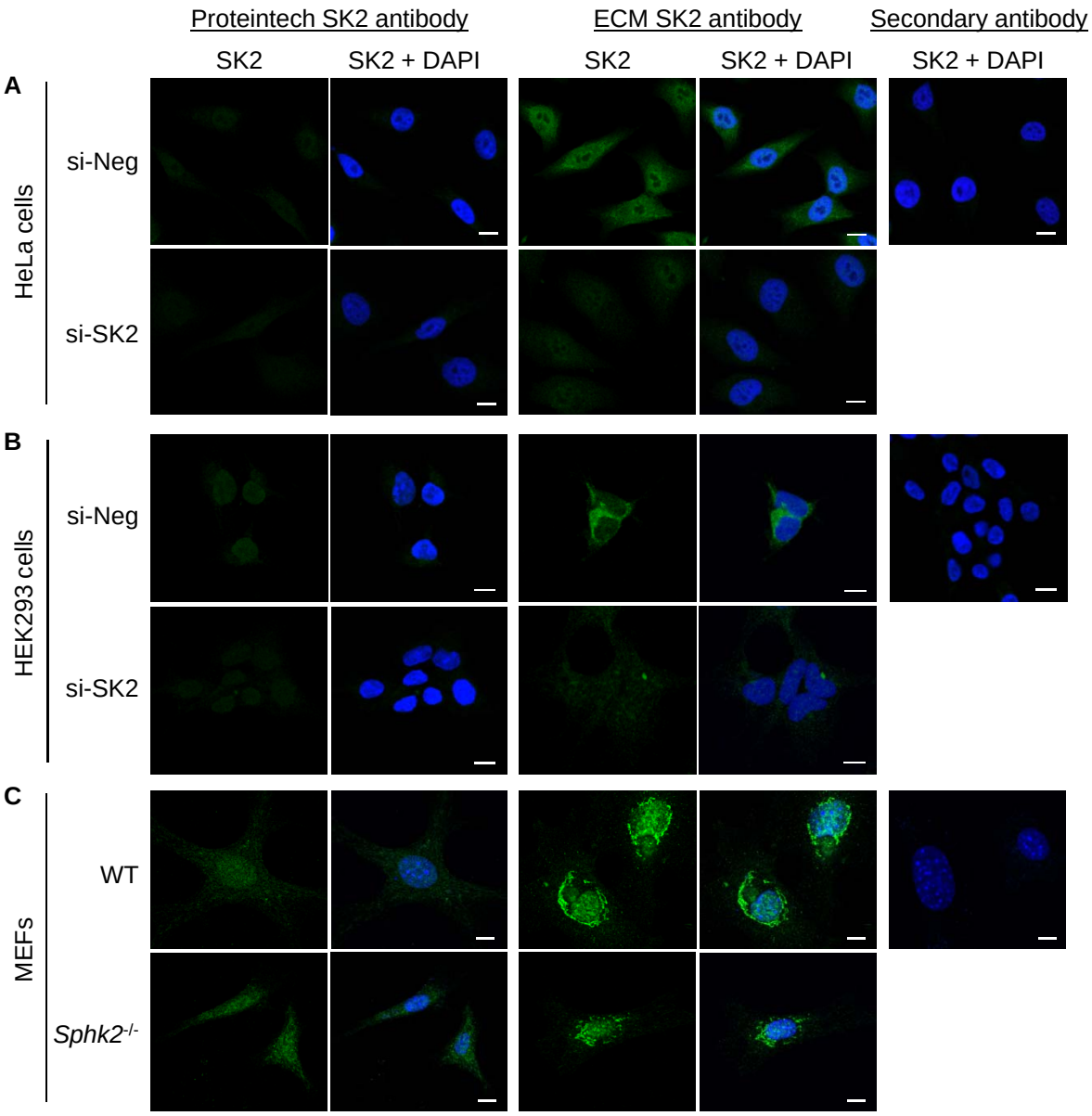
The ECM Biosciences anti-SK2 antibody did result in consistently observable staining in HeLa and HEK293 cells, which was substantially reduced upon knockdown of SK2 (Figure 3.7A and B). Hence, in these cells the ECM Biosciences antibody was able to selectively detect SK2 by IF. Interestingly, in HeLa cells SK2 detected by the ECM Biosciences antibody was predominantly nuclear with some peri-nuclear/cytoplasmic localization, whereas in HEK293 cells SK2 was cytoplasmic and nuclear-exclusion, which is consistent with previous reports for these cell lines (Igarashi et al, 2003). However, the ECM Biosciences anti-SK2 antibody produced very strong peri-nuclear staining/puncta in both the WT and *Sphk2*$^{-/-}$ MEF lines (Figure 3.7C), suggesting that this staining was not specific for SK2 and represents non-specific binding to other proteins in this cell type. Increased non-specific binding of both SK2 antibodies to other proteins in the MEF lines was also observed when used for IB, so this cell type may not be suitable for use with these antibodies. It will remain to be determined if the same level of non-specificity is also observed in other mouse cell lines and tissues.

3.5 Discussion

It can be difficult to find commercially available antibodies that are sensitive, but also selective enough to detect or isolate endogenous levels of a protein of interest. The Pitson Laboratory had previously sought to generate in-house SK2 polyclonal antisera in order to provide a tool with which to detect, purify and examine the localisation of endogenous SK2. The SK2 peptide antigen used to generate the polyclonal SK2 antisera had previously been used and published by other groups, and the resulting purified SK2 antibodies had been utilised in a number of applications, including IB, IP and IF (Hait et al, 2005; Igarashi et al, 2003). This would seem to be an optimal sequence within SK2 to raise an antibody against as it is one of the regions in SK2 not conserved in SK1, which provides isoform selectivity. Furthermore, there is 94% sequence identity between human and mouse SK2 within this

Figure 3.7: Immunofluorescence staining analyses of endogenous SK2 in multiple cell lines using two commercially available rabbit polyclonal anti-SK2 antibodies

(*A*) HeLa or (*B*) HEK293 cells were treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), and endogenous SK2 (green) was visualised by immunofluorescence staining and confocal microscopy, using Proteintech rabbit anti-SK2 antibody or ECM Biosciences rabbit anti-SK2 antibody. *C,* Wildtype (WT) or *Sphk2*$^{-/-}$ MEFs were seeded, and endogenous SK2 (green) was visualised by immunofluorescence staining and confocal microscopy, using Proteintech rabbit anti-SK2 antibody or ECM Biosciences rabbit anti-SK2 antibody. Nuclei were stained with DAPI (blue). For each cell line, background staining was examined by staining cells (si-Neg or WT cells) with secondary antibody and DAPI only, and collecting images using both 488nm and 405nm lasers (SK2 + DAPI). Images were taken at 40x magnification; scale bars = 10 µm. Images shown are representative of more than 100 cells from each experiment, and these results were consistent over three independent experiments for each cell line.


Proteintech SK2 antibody
ECM SK2 antibody
Secondary antibody
SK2
SK2 + DAPI
SK2
SK2 + DAPI
SK2 + DAPI
A
HeLa cells
si-Neg
si-SK2
B
HEK293 cells
si-Neg
si-SK2
C
MEFs
WT
Sphk2-/-

region, and no post-translational modifications have been reported within this sequence (Hornbeck et al, 2015), which may otherwise block antibody binding in physiological settings. In the studies described here, the in-house SK2 antiserum was affinity-purified (HN12) and found to detect endogenous SK2, amongst other non-specific bands, by IB. However, HN12 could not successfully IP SK2, nor could it cleanly detect SK2 by IF. Additional affinity-purification of the SK2 antisera may be beneficial in an attempt to remove/reduce the non-specific antibodies present in the polyclonal pool, which may increase the sensitivity and selectivity of this antibody. Similarly, Hait et al. used their affinity-purified SK2 antibody for IB analyses, and multiple bands were detected, but a ~70 kDa band was observed and confirmed to be endogenous SK2 by siRNA-mediated knockdown (Hait et al, 2005). Igarashi and colleagues validated the specificity of their polyclonal SK2 antibody for use in IP by demonstrating that the immunoprecipitate had SK activity, and co-incubation of the lysate with the SK2 antibody as well as immunogen peptide ablated this activity (Igarashi et al, 2003). However, IB analyses of the immunoprecipitates using their SK2 antibody resulted in many prominent bands, one of which ran at the correct size for SK2 and was thus deemed to be endogenous SK2, without further validation. Furthermore, no validation of their SK2 antibody was performed when utilising it for IF detection of endogenous SK2 (Igarashi et al, 2003). Therefore, it is difficult to conclude whether the affinity-purified SK2 antibody generated by this group, which was raised against the same peptide sequence as HN12, is in fact suitable for use in the published applications.

At the commencement of these studies, very few commercially available antibodies claimed to be able to detect endogenous levels of SK2. Furthermore, commercially available antibodies raised against the SKs can be notorious for not being very sensitive or selective. However, more recent published studies have reported the use of different commercial SK2 antibodies, sometimes without proper controls or validation of selectivity. Two commercial SK2 antibodies were obtained and their selectivity towards SK2 was examined in multiple applications using siRNA-mediated SK2 knockdown or *Sphk2*$^{-/-}$ MEF lines.

The SK2 antibody from Proteintech was able to consistently detect a prominent band at the correct molecular weight by IB, and this band was confirmed to be SK2 by knockdown and knockout analyses, confirming the specificity of this antibody. The Proteintech antibody also resulted in virtually no non-specific detection of any other proteins in the HEK293 and

HeLa lysates, but some additional faint bands were present in the MEF lines. This antibody has been tested by IB on various mouse tissue lysates by the manufacturer and many of these also gave rise to non-specific bands, so this will need to be considered and further validation may be required if this antibody is intended for use with mouse cells or tissues. Occasionally more than one band was detected in the human cell lines by the Proteintech SK2 antibody, but these bands also seemed to be reduced by SK2 knockdown. There are two characterized human SK2 isoforms (Neubauer & Pitson, 2013), so these bands may represent different SK2 variants and/or post-translationally modified forms of SK2.

In contrast, the present results revealed that the sensitivity of the ECM Biosciences antibody towards SK2 by IB was poor, with a faint band detected only in the HEK293 and MEF lines that was not present in the knockdown/knockout lysates. Furthermore, the ECM Biosciences SK2 antibody produced many intense non-specific bands in all cell lines tested, demonstrating poor selectivity. This antibody has been used for IB analyses in multiple publications (Bruno et al, 2015; Liu et al, 2016a; Sun et al, 2016a; Wallington-Beddoe et al, 2014), suggesting that it may be more suitable with other cell/tissue systems or conditions not tested here. However, in agreement with the current findings, the IB analysis performed by the manufacturer also showed various prominent non-specific bands in HeLa lysates. Therefore, these data demonstrate that the ECM Biosciences SK2 antibody is not ideal for this application.

However, the ECM Biosciences anti-SK2 antibody was superior for the IP of endogenous SK2, as it was able to cleanly and substantially enrich the protein from lysates and was confirmed by SK2-specific knockdown to be selective for SK2 in this application. This antibody will therefore be a useful tool to study SK2 function and regulation, as it can be applied to other applications requiring IP, such as chromatin-IP (ChIP) and rapid immunoprecipitation mass spectrometry of endogenous protein (RIME). In the present study, the Proteintech anti-SK2 antibody was inconsistent in its ability to IP protein at the correct size for SK2, and other bands of equal intensity were sometimes present.

Similarly, the ECM Biosciences anti-SK2 antibody was able to selectively detect endogenous SK2 by IF staining in two human cell lines, HeLa and HEK293 cells. Furthermore, the observed localization of SK2 in these two cells lines was consistent with previous reports (Igarashi et al, 2003). The selectivity of this antibody was validated by

knockdown of SK2 in these cell lines, where most of the staining was reduced. A very small level of staining was still visible after SK2 siRNA treatment, possibly owing to the inherently incomplete nature of siRNA-mediated knockdown. However, this could not be corroborated with SK2 knockout using the MEF lines, as considerable non-specific staining was present in this cell type, as was found for IB. Using identical methods, there was minimal staining observed using the Proteintech anti-SK2 antibody for IF, and therefore the selectivity of this antibody towards SK2 in this application could not be properly examined.

During this study, methods routinely used in the Pitson Laboratory were employed, and where applicable, recommendations from the manufacturers for antibody dilutions and concentrations were followed. It is possible that further optimization for these antibodies may allow them to perform better in the applications where they were deemed not optimal. However, as one of the main aims of this study was to directly compare the performance of these two antibodies, and given at least one of the antibodies performed well for each application using the standard methods, further optimization was not performed.

Overall, based on the data from this study, the Proteintech SK2 antibody was optimal and suitable for use in IB, as it demonstrated selectivity and sensitivity towards endogenous SK2 in the human cell lines tested. Furthermore, the ECM Biosciences SK2 antibody is suitable for IP of endogenous SK2 and for visualizing SK2 by IF methods. Both antibodies detected non-specific proteins by IB and IF in the mouse fibroblasts used, and hence further validation will be required to determine if this is the case for other mouse cells or tissues. In line with these results, all subsequent results chapters utilising IB analyses to detect endogenous SK2 were performed using the Proteintech SK2 polyclonal antibody, and all IP and IF analyses of endogenous SK2 were carried out using the ECM SK2 polyclonal antibody.

Chapter 4:
An Oncogenic Role for Sphingosine Kinase 2

Most of the data from this chapter has been published as:

Neubauer HA, Pham DH, Zebol JR, Moretti PAB, Peterson AL, Leclercq TM, Chan H, Powell JA, Pitman MR, Samuel MS, Bonder CS, Creek DJ, Gliddon BL and Pitson SM (2016) An oncogenic role for sphingosine kinase 2. *Oncotarget*, 7(40): 64886-64899.

See Appendix 3 for author contributions and reprint

Chapter 4. An Oncogenic Role for Sphingosine Kinase 2

4.1 Abstract

While both human sphingosine kinases (SK1 and SK2) catalyse the generation of the pleiotropic signalling lipid sphingosine-1-phosphate, these enzymes appear to be functionally distinct. SK1 has well described roles in promoting cell survival, proliferation and neoplastic transformation. The roles of SK2, and its contribution to cancer, however, are much less clear. Some studies have suggested an anti-proliferative/pro-apoptotic function for SK2, while others indicate it has a pro-survival role and its inhibition can have anti-cancer effects. Our analysis of gene expression data revealed that SK2 is upregulated in many human cancers, but only to a small extent (up to 2.5-fold over normal tissue). Based on these findings, we examined the effect of different levels of cellular SK2 and showed that high-level overexpression reduced cell proliferation and survival, and increased cellular ceramide levels. In contrast, however, low-level SK2 overexpression promoted cell survival and proliferation, and induced neoplastic transformation *in vivo*. These findings coincided with decreased nuclear localisation and increased plasma membrane localisation of SK2, as well as increases in extracellular S1P formation. Hence, we have shown for the first time that SK2 can have a direct role in promoting oncogenesis, supporting the use of SK2-specific inhibitors as anti-cancer agents.

4.2 Introduction

The sphingosine kinases (SKs) catalyse the conversion of sphingosine to sphingosine-1-phosphate (S1P). Given that sphingosine and its precursor, ceramide, are pro-apoptotic molecules, and S1P mediates cell survival and proliferation (Pitson, 2011; Pyne & Pyne, 2010), the SKs are considered critical regulators of the balance between cell death and cell survival, and represent promising targets for anti-cancer therapies (Pitman & Pitson, 2010). The two mammalian SKs, SK1 and SK2, share high sequence similarity and both possess constitutive catalytic activity, but generally show distinct subcellular localisation (Neubauer & Pitson, 2013).

The role of SK1 in cancer is well characterised and has been extensively reviewed (Heffernan-Stroud & Obeid, 2013; Pitson, 2011; Pyne & Pyne, 2010), with high SK1 expression observed in many different cancers (Bayerl et al, 2008; French et al, 2003; Kawamori et al, 2006; Malavaud et al, 2010) and often correlating with poorer patient survival (Li et al, 2008; Ruckhaberle et al, 2008; Van Brocklyn et al, 2005). SK1 overexpression promotes neoplastic transformation and tumourigenesis (Xia et al, 2000), and notably, targeting SK1 has been shown to attenuate tumour growth in numerous animal models (French et al, 2003; Ju et al, 2016; Nagahashi et al, 2012; Pchejetski et al, 2008; Ponnusamy et al, 2012). In contrast, the contribution of SK2 to cancer is unclear. Surprisingly, despite both enzymes catalysing the same reaction, most studies examining SK2 function have found that it has an opposite role to SK1, and can promote cell cycle arrest and apoptosis (Chipuk et al, 2012; Igarashi et al, 2003; Liu et al, 2003; Maceyka et al, 2005; Okada et al, 2005). Although most of these studies utilised high-level overexpression systems, functional analysis of endogenous SK2 has supported this role in promoting cell death (Chipuk et al, 2012; Hofmann et al, 2008; Okada et al, 2005). Most notably, nuclear-localised SK2 has been shown to act as an epigenetic regulator, through S1P-mediated inhibition of HDAC1/2 activity and increased transcription of p21 and c-fos (Hait et al, 2009).

Despite the general notion that SK2 is pro-apoptotic, a number of studies have emerged that demonstrate a role for SK2 in promoting cancer. Knockdown of SK2 expression has been shown to enhance apoptosis and chemosensitise many cancer cell types (Nemoto et al, 2009; Sankala et al, 2007; Schnitzer et al, 2009; Van Brocklyn et al, 2005). In fact, targeting

SK2 in a range of cancer cell lines appears to have more of an anti-cancer effect than targeting SK1 (Gao & Smith, 2011; Van Brocklyn et al, 2005). Strikingly, several *in vivo* studies have reported that targeting SK2 significantly attenuated tumour growth in a range of human xenografts models in mice (Chumanevich et al, 2010; French et al, 2010; Liu et al, 2013; Wallington-Beddoe et al, 2014; Weigert et al, 2009). Increased SK2 expression levels also correlate with disease progression in NSCLC (Wang et al, 2014b) and multiple myeloma (Venkata et al, 2014), and poorer survival in NSCLC patients (Wang et al, 2014b). Recent work also suggests that SK2 can play a role in increasing telomerase activity (Panneer Selvam et al, 2015), promoting the upregulation of c-Myc via regulation of HDAC1/2 (Wallington-Beddoe et al, 2014), and facilitating the activation of ERM proteins to promote EGF-induced cancer cell invasion (Adada et al, 2015), all of which may contribute to cancer development and progression.

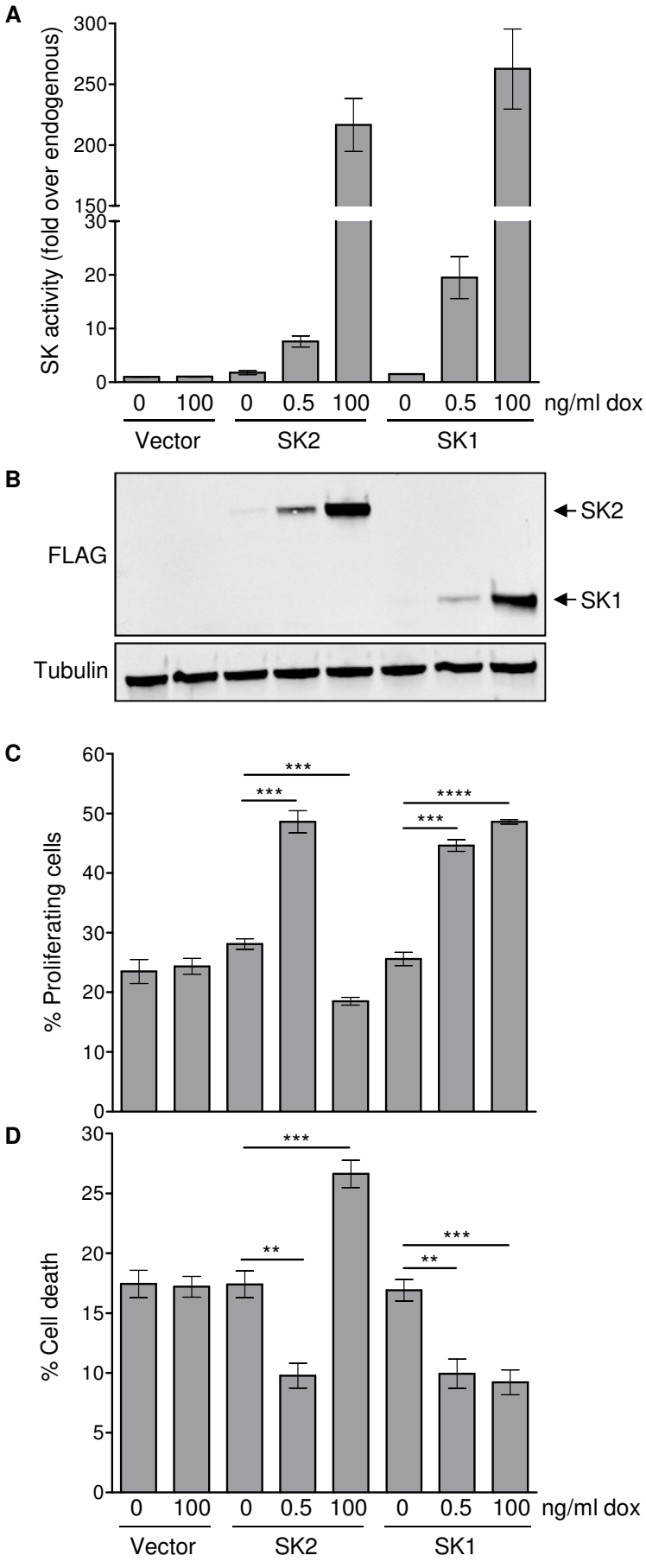
Although there is an emerging body of evidence suggesting that SK2 can play a role in cancer development, this is complicated by the known role of SK2 in facilitating cell death, and that, unlike SK1, SK2 overexpression has never been shown to promote neoplastic transformation and tumourigenesis. Here, I demonstrate for the first time that low-level SK2 overexpression, similar to that observed in numerous cancers, can promote cell proliferation, survival and neoplastic transformation, and that these levels of SK2 overexpression alone can drive tumourigenesis *in vivo*.

4.3 Preliminary data leading to this study

Previously in the Pitson Laboratory, HEK293 Flp-In T-Rex cells had been engineered to express FLAG-tagged SK2 or SK1 in a doxycycline-inducible, concentration-dependent manner (Pham et al, 2008). Therefore, by using different doxycycline concentrations in the culture media, low and high SK2 and SK1 overexpression could be achieved, as determined by isoform-specific activity (Figure 4.1A) and protein expression (Figure 4.1B). These cells had then been utilised to assess the effect of varying levels of SK overexpression on cell proliferation and survival. In agreement with previous studies, overexpression of SK2 at high levels (over 200-fold) in this system resulted in decreased cell proliferation and an increase in cell death (Figure 4.1C and D). Strikingly, however, when SK2 was overexpressed to much lower levels (8-fold over endogenous), this induced a marked

Figure 4.1: Low-level SK2 overexpression promotes cell survival and proliferation, whereas high-level SK2 overexpression promotes cell death

A, SK1 and SK2-specific activity upon doxycycline-induced low- and high-level overexpression in HEK293 Flp-In TRex cells. Data shown are mean (± range) of a representative experiment (of more than three independent experiments) with duplicate data points. *B*, Lysates from the HEK293 Flp-In TRex cells with doxycycline-induced low- and high-level overexpression of SK1 or SK2, or empty vector, were subjected to immunoblot analyses with antibodies against FLAG and α-tubulin. Blots shown are representative of at least three independent experiments. *C*, Measurement of cell proliferation (by BrdU incorporation) in HEK293 Flp-In TRex cells with doxycycline-induced low- and high-level overexpression of SK1 or SK2, or empty vector. Data shown are mean (± SEM), n = 3-4. *D*, Measurement of cell death (by DAPI staining and quantification of nuclear fragmentation) in HEK293 Flp-In TRex cells with doxycycline-induced low- and high-level overexpression of SK1 or SK2, or empty vector. Data shown are mean (± SEM), n = 4-5. Data shown in *C* and *D* were generated by Dr Duyen Pham (Centre for Cancer Biology, Adelaide).


A
SK activity (fold over endogenous)
300
250
200
150
30
20
10
0
0 100 0 0.5 100 0 0.5 100 ng/ml dox
Vector
SK2
SK1
B
FLAG
SK2
SK1
Tubulin
C
% Proliferating cells
60
50
40
30
20
10
0
***
***
***
****
D
% Cell death
30
25
20
15
10
5
0
***
**
**
***
0 100 0 0.5 100 0 0.5 100 ng/ml dox
Vector
SK2
SK1

increase in cell proliferation and a decrease in cell death (Figure 4.1C and D). Notably, these findings were unique to SK2, with both low- and high-level overexpression of SK1 resulting in a consistent increase in cell survival and proliferation (Figure 4.1C and D).

Given that SK1 has known roles in promoting neoplastic transformation and oncogenesis (Xia et al, 2000), and that low-level overexpression of SK2 appeared to phenocopy the survival and proliferative advantages of SK1 overexpression, it was then examined if low-level SK2 overexpression could also induce neoplastic transformation. In contrast to mouse cells, neoplastic transformation of human cells is well known to require multiple oncogenes (Hahn et al, 1999), meaning their use in these type of studies is problematic. Thus, to examine the oncogenic potential of SK2, NIH3T3 mouse fibroblasts were transfected with a pcDNA3 vector encoding SK2 as well as green fluorescent protein (GFP) via an internal ribosome entry site (IRES) such that GFP and SK2 expression were linked. A series of cell lines stably expressing different levels of SK2 were then isolated through the sorting of cells for differential GFP expression. The resulting stable cell lines were then validated through the analysis of SK2-specific activity and exogenous SK2 protein levels, which revealed overexpression of SK2 at 5-fold, 10-fold, 15-fold and 370-fold over endogenous levels, designated ‘very low’, ‘low’, ‘mid’ and ‘high’ level SK2 overexpression, respectively (Figure 4.2A).

These SK2 cell lines, and their respective GFP-alone control cell lines, were then utilised in focus formation assays to assess their ability to overcome contact inhibition as a measure of neoplastic transformation. Strikingly, cell lines with ‘very low’, ‘low’ and ‘mid’-level SK2 overexpression formed foci, whereas cells with ‘high’-level SK2 overexpression, or vector control cells, did not (Figure 4.2B). Therefore, it appeared that low, close to physiological levels of SK2 overexpression could indeed promote neoplastic transformation *in vitro*. It was then of interest to subcutaneously inject these cell lines into the flanks of NOD/SCID mice to determine if the low SK2-overexpressing cells could display full neoplastic transformation through tumour formation *in vivo*. However, it became apparent that the CMV promoter driving the stable overexpression of SK2 from the pcDNA3 vector was being silenced over time in these mouse fibroblast cells, as previously observed (Haase et al, 2010; Meilinger et al, 2009). As such, the precise levels of low SK2 overexpression required could not be maintained long enough to test *in vivo* tumourigenesis. Therefore, to truly interrogate the role of SK2 in driving tumourigenesis *in vivo*, a new system was required to

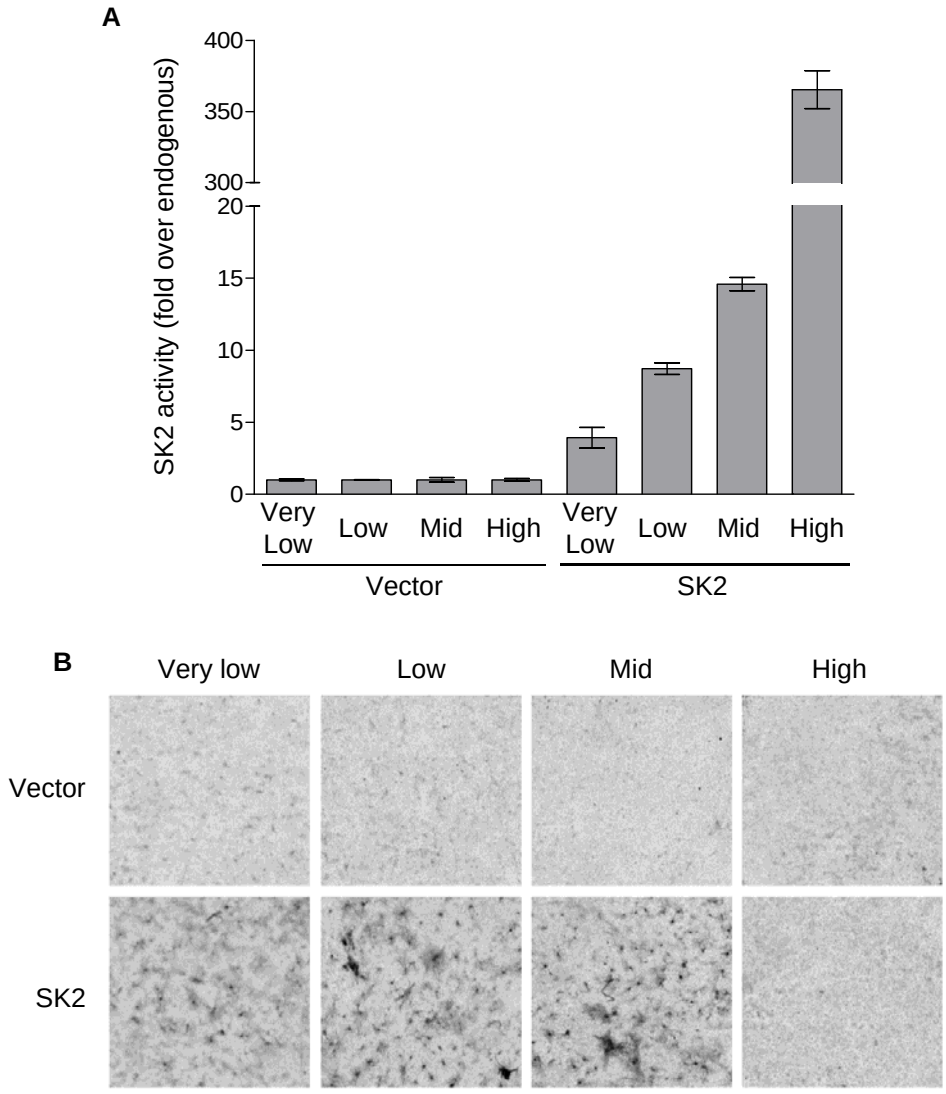


Figure 4.2: SK2 overexpressed at low levels can promote neoplastic transformation *in vitro*

A, SK2-specific activity of NIH3T3 pcDNA3 cell lines stably expressing 'very low' (5-fold), 'low' (10-fold), 'mid' (15-fold) or 'high' (370-fold) levels of SK2 overexpression (above endogenous levels), or corresponding empty vector controls. Data shown are mean (± range) of duplicate data points from a representative experiment. *B,* Focus formation assays testing contact inhibition of the NIH3T3 vector or SK2 overexpressing cell lines. Images shown are representative of at least three independent experiments, each performed in duplicate, using at least three independently generated sets of stable lines. Data were generated by Julia Dobbins, Dr Huasheng Chan and Dr Tamara Leclercq (Centre for Cancer Biology, Adelaide).

enable long-lasting stable overexpression of SK2 in NIH3T3 mouse fibroblasts, which is the focus of this chapter.

4.4 Specific Materials and Methods

4.4.1 Generation of expression constructs

The pCX-EGFP construct was obtained from (Okabe et al, 1997), and was modified by Paul Moretti (Pitson Laboratory). Initially, the EGFP was replaced with a polylinker following digestion with EcoRI and ligation of annealed kinased oligonucleotides 5'-AATTCGGTACCGAGCTCGCTAGCGCGGCCGCCTCGAGC-3' and 5'-AATTGCTCGAGGCGGCCGCGCTAGCGAGCTCGGTACCG-3', to produce pCX4. pCX4-IRES EGFP was then generated by subcloning the IRES EGFP cassette from pcDNA3-IRES EGFP (Pham et al, 2014) with EcoRI and NotI. I then generated pCX4-SK2(FLAG) IRES EGFP by cloning in FLAG-tagged human SK2a (Roberts et al, 2004) following digestion with EcoRI.

To generate pCX4^{NEO} IRES EGFP, a blunted SalI and EcoRI cassette from pCX4 (described above), encompassing the chicken β-actin promoter, was used to replace the CMV promoter of pcDNA3-IRES EGFP (Pham et al, 2014) following blunted BglII and EcoRI digestion. pCX4^{NEO}-SK2(FLAG) IRES EGFP was then produced by cloning in FLAG-tagged human SK2a (Roberts et al, 2004) with EcoRI.

4.4.2 Generating cell lines

To generate NIH3T3 stable cell lines with varying levels of constitutive SK2 overexpression, NIH3T3 mouse fibroblasts were transfected with an SK2 expression construct (pCX4-SK2(FLAG) IRES EGFP or pCX4^{NEO}-SK2(FLAG) IRES EGFP), or the corresponding empty vector, using Lipofectamine™ 2000 (Invitrogen) as per the manufacturer's protocol. 48 hr after transfection, the cells were sorted for GFP-positive cells using a MoFlo Astrios cell sorter (Beckman Coulter). For the pCX4 vector, a stable GFP-positive cell population was obtained by sorting for GFP another two times. For the

pCX4^{NEO} vector, a stable pooled cell line was obtained by selection with G418 (0.8mg/ml). The stable GFP-positive pooled line was then sorted on four separate narrow gates of varying GFP intensity, to produce new stable lines depicted as 'very low', 'low', 'mid' and 'high', for both the SK2 and empty vector lines. The average SK2 expression levels of each line were determined by SK2-specific activity assays, and if the desired level of SK2 overexpression for any line had not been achieved, the line was resorted on four separate narrow gates of varying GFP intensity, as above, until suitable lines had been generated. These new stable lines were then analysed by flow cytometry to confirm that the desired narrow GFP-expression levels were obtained as expected.

4.4.3 *In vivo* subcutaneous tumour model

The NIH3T3 cell lines expressing varying levels of SK2 were trypsinised and washed in PBS, and 1x10^6 cells were injected in 200 µl of PBS subcutaneously into the flank of 8-week old female NOD/SCID mice. The empty vector cells with low GFP expression were selected as a representative control group. Mice were left for 18 days and were examined daily to monitor tumour formation. On day 19, all mice were humanely killed and tumours were excised. Half of each tumour was fixed in 10% formalin, paraffin embedded and sectioned. The remaining half was homogenised using a pestle (Axygen) in extraction buffer (recipe described in Chapter 2), subjected to freeze/thawing in liquid nitrogen and sonication, and lysates were clarified by centrifugation at 17,000 × g for 15 min at 4°C.

4.4.4 Extracellular S1P formation assay

The rate of extracellular S1P formation from intact vector control 'low', SK2 'low' and SK2 'high' NIH3T3 stable cell lines was determined essentially as previously described (Sun et al, 2016b). Briefly, cells were seeded in equal numbers at 80% confluence (in 20 cm^2 dishes), and media was replaced with DMEM containing 0.5% DBS and incubated for a further 16 h. Cells were then labeled with 0.5 µCi of [^{3}H]-sphingosine (Perkin-Elmer, Rowville, VIC, Australia) for 30 min, after which the conditioned media was collected. Extracellular [^{3}H]-S1P generated in the conditioned medium was extracted and analysed by scintillation counting.

4.4.5 Immunofluorescence

For dual immunofluorescence staining of overexpressed FLAG-tagged SK2 and Ki-67 on the formalin-fixed paraffin-embedded tumour tissue samples, sections were de-waxed, rehydrated, and antigen retrieval was performed by boiling in citrate buffer for 30 min. Sections were blocked in 10% goat serum diluted in CAS-Block (Thermo Fisher Scientific) for 30 min. Following blocking, sections were incubated overnight at 4°C with mouse monoclonal anti-Ki-67 antibody (1:20; Vector Labs) and rabbit polyclonal anti-FLAG antibody (1:100; Cell Signaling Technology) diluted together in 10% goat serum/CAS-Block. Sections were then incubated for 1 hr at room temperature with goat anti-mouse AlexaFluor 488 (1:400) and goat anti-rabbit AlexaFluor 594 (1:400) secondary antibodies (Thermo Fisher Scientific) diluted together in 10% goat serum/CAS-Block. Labelled sections were then mounted in Vectashield mounting medium containing DAPI (Vector Labs) and were imaged using a Carl Zeiss LSM 700 confocal microscope (Jena, Germany).

To examine the subcellular localisation of overexpressed FLAG-tagged SK2, NIH3T3 SK2 'low' and 'high' stable cell lines were seeded onto 8-well glass chamber slides (Nalge Nunc International) coated with poly-L-lysine (Sigma-Aldrich) at $4x10^4$ cells/well, and grown overnight in DMEM with 10% DBS. Media was then removed and replaced with DMEM containing 0.5% DBS, and cells were cultured for a further 16 h. Immunofluorescence staining protocol was then performed as detailed in chapter 2 (General Methods), with the following modifications. After fixing and permeabilising, cells were blocked in 5% goat serum in PBS with 0.1% Triton X-100 for 60 min. Rabbit anti-FLAG antibody (1:200; #14793, Cell Signaling Technology) and mouse anti-γ-catenin antibody (1:500; BD Biosciences) were incubated for 1.5 hr at room temperature, followed by goat anti-rabbit AlexaFluor 594 and goat anti-mouse AlexaFluor 488 secondary antibodies (1:500; Thermo Fisher Scientific) for 1 h.

4.4.6 Sphingolipid analyses by liquid chromatography–mass spectrometry

Vector control 'low', SK2 'low' and SK2 'high' NIH3T3 stable cell lines were grown in DMEM with 10% DBS to 80% confluence, media was replaced with DMEM containing 0.5% DBS and cells were cultured for 16 h. Cells were trypsinised, quenched and washed in

PBS. Cells were pelleted in quadruplicate with $8.8x10^6$ cells per sample, and intracellular sphingolipid species were analysed by LC-MS, as previously described (Aurelio et al, 2016) with the following minor modifications. Prepared samples were injected onto an Ascentis Express C18 column (Supelco Analytical, Bellefonte, PA, USA), and non-natural sphingolipid internal standards were added to each sample to allow relative quantification. Data analysis was performed using Tracefinder (Thermo Fisher Scientific).

4.5 Results

4.5.1 Generation of NIH3T3 cell lines with varying levels of stable SK2 overexpression

Given that the NIH3T3 mouse fibroblast cells utilised for neoplastic transformation studies were able to silence protein expression driven by a CMV promoter over time, I needed to develop a system whereby SK2 expression could be driven by a different, more stable promoter that would not be silenced in murine cells. The pCX vector drives protein expression via a chicken β-actin promoter and contains a CMV enhancer and an intron, and has been shown to provide stable transgene expression in mice (Okabe et al, 1997). I therefore subcloned cDNA encoding human SK2a as well as EGFP driven by an IRES into the pCX4 vector, which was then further modified to contain neomycin resistance to assist with stable cell selection ($pCX4^{NEO}$-SK2). Stable NIH3T3 SK2 or empty vector pooled cell lines were made in the presence of G418, and then cell lines with varying levels of SK2 overexpression, or GFP alone, were generated by gating on different GFP expression levels as described above (Figure 4.3). These lines were then assessed using SK2-specific activity assays to determine if the desired levels of SK2 overexpression had been achieved. Surprisingly, where previous attempts using the pcDNA3 vector had yielded SK2 overexpression levels as low as 5-fold over endogenous (Figure 4.2A), the lowest level of SK2 overexpression obtained using the $pCX4^{NEO}$ construct was around 80-fold over endogenous levels (Figure 4.4), even after an attempt to re-sort on the lowest possible levels of GFP.

As the previously generated stable pooled pcDNA3 cell lines had not utilised antibiotic selection, but rather simply relied on three consecutive sorts for GFP-positive cells, it was

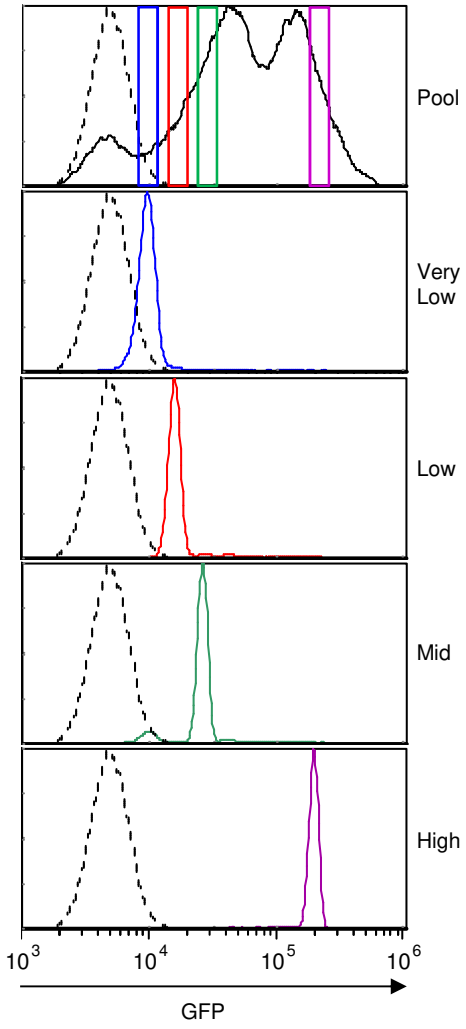


Figure 4.3: Generating cell lines with differential SK2 overexpression by gating on varying levels of GFP intensity using FACS

The NIH3T3 pooled stable cell lines expressing SK2 and GFP, or GFP alone (empty vector), were sorted on four separate narrow gates of varying GFP intensity (colored boxes), to produce new stable lines depicted as 'very low', 'low', 'mid' and 'high'. These new stable lines were then analysed by flow cytometry to confirm that the desired narrow GFP-expression levels were obtained as expected. GFP-negative control cells are depicted by a dotted line.

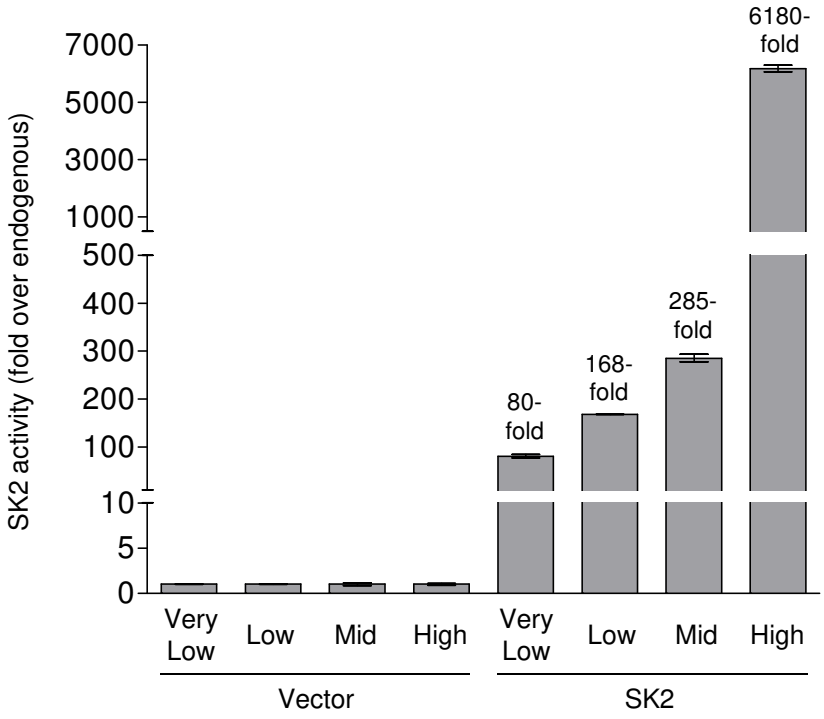


Figure 4.4: SK2 activity of pCX4^{NEO} NIH3T3 stable cell lines

SK2-specific activity of NIH3T3 pCX4^{NEO} cell lines stably expressing SK2 or empty vector. Data shown are mean (± range) from one experiment with duplicate data points.

hypothesised that the G418 selection may have been preferentially selecting for cells with a higher copy number integration of the plasmid, and hence, greater resistance to the antibiotic and consequently higher GFP and SK2 expression levels. The antibiotic resistance was not a requirement for the process of generating the cell lines, and so a new stable NIH3T3 pooled cell line was made using the pCX4 plasmid (containing SK2 or vector alone) without G418 selection, but just by sorting three times for GFP-positive cells. The pooled lines were then gated as described in Figure 4.3, and this time SK2 overexpression was achieved as low as 10-fold over endogenous levels, ranging up to 20-fold, 40-fold and 575-fold overexpression (Figure 4.5).

To ensure that these pCX4 cell lines would be more suitable than the previous pcDNA3 cell lines for long-term stable SK2 overexpression, I examined the changes in SK2 activity over one month of culturing (approximately 12 passages), and also after freezing and then thawing from liquid nitrogen. The freeze/thawing of the cell lines did somewhat affect SK2 expression, decreasing SK2-specific activity by only 13% for the 'high' cell line but decreasing SK2 activity by 42% for the 'very low' cell line (Figure 4.6). Passaging the cells for one month further decreased exogenous SK2 expression, from as little as 12% for the 'high' cell line to as much as 43% for the 'mid' cell line (Figure 4.6). However, these final levels of SK2 overexpression (5-fold, 10-fold, 20-fold and 440-fold over endogenous levels) were still very suitable for examining the role of SK2 in oncogenesis *in vitro* and *in vivo*. Furthermore, a similar length of passaging of the pcDNA3 cell lines had previously resulted in virtually undetectable SK2 overexpression, making these pCX4 NIH3T3 stable cell lines far superior and suitable for further *in vivo* studies.

4.5.2 SK2 can elicit oncogenic signalling and promote neoplastic transformation *in vitro*

Biochemical analysis of the pCX4 NIH3T3 stable cell lines revealed that 'low' SK2 overexpression resulted in the activation of oncogenic signalling pathways, as demonstrated by an increase in phospho-AKT and phospho-ERK1/2 levels (Figure 4.7). Conversely, the 'mid' and 'high'-level SK2 overexpression caused a downregulation of phospho-ERK1/2 signalling (Figure 4.7), in agreement with high-level SK2 overexpression attenuating cell proliferation. Importantly, endogenous SK1 protein levels were unchanged in the multiple

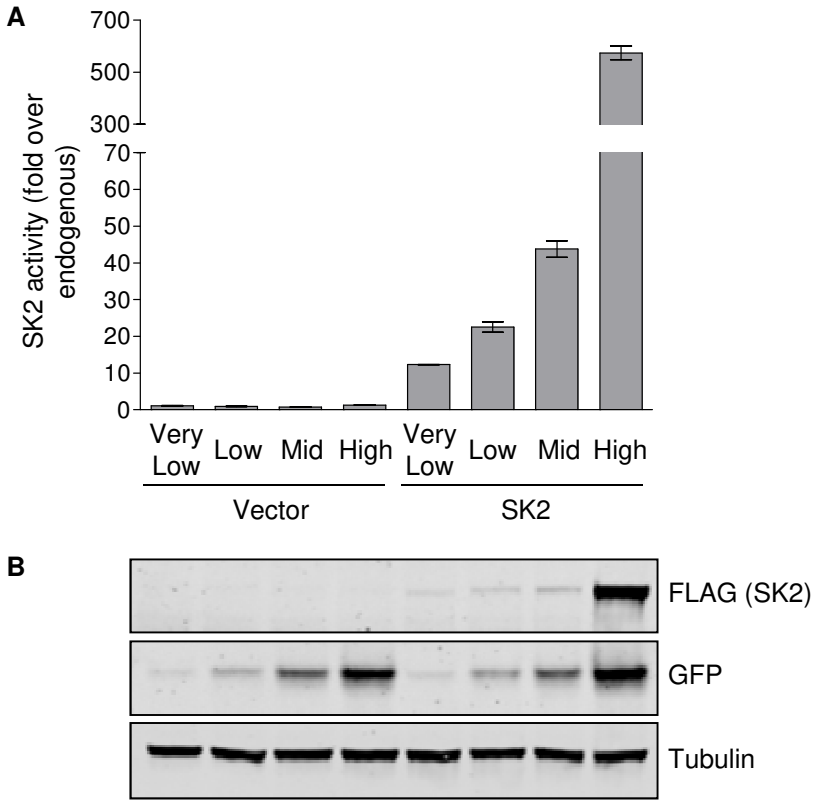


Figure 4.5: SK2 activity and protein levels in the pCX4 NIH3T3 stable cell lines

A, SK2-specific activity of NIH3T3 pCX4 cell lines stably expressing 'very low' (10-fold), 'low' (20-fold), 'mid' (40-fold) or 'high' (575-fold) levels of SK2 overexpression (above endogenous levels), or corresponding empty vector controls. Data shown are mean (± range) of duplicate data points from a representative experiment (of three independent experiments). *B,* Lysates from the NIH3T3 empty vector or SK2-overexpressing pCX4 cell lines were subjected to immunoblot analyses with antibodies against FLAG, GFP and α-tubulin. Blots shown are from the same lysates assayed for activity in (*A*).

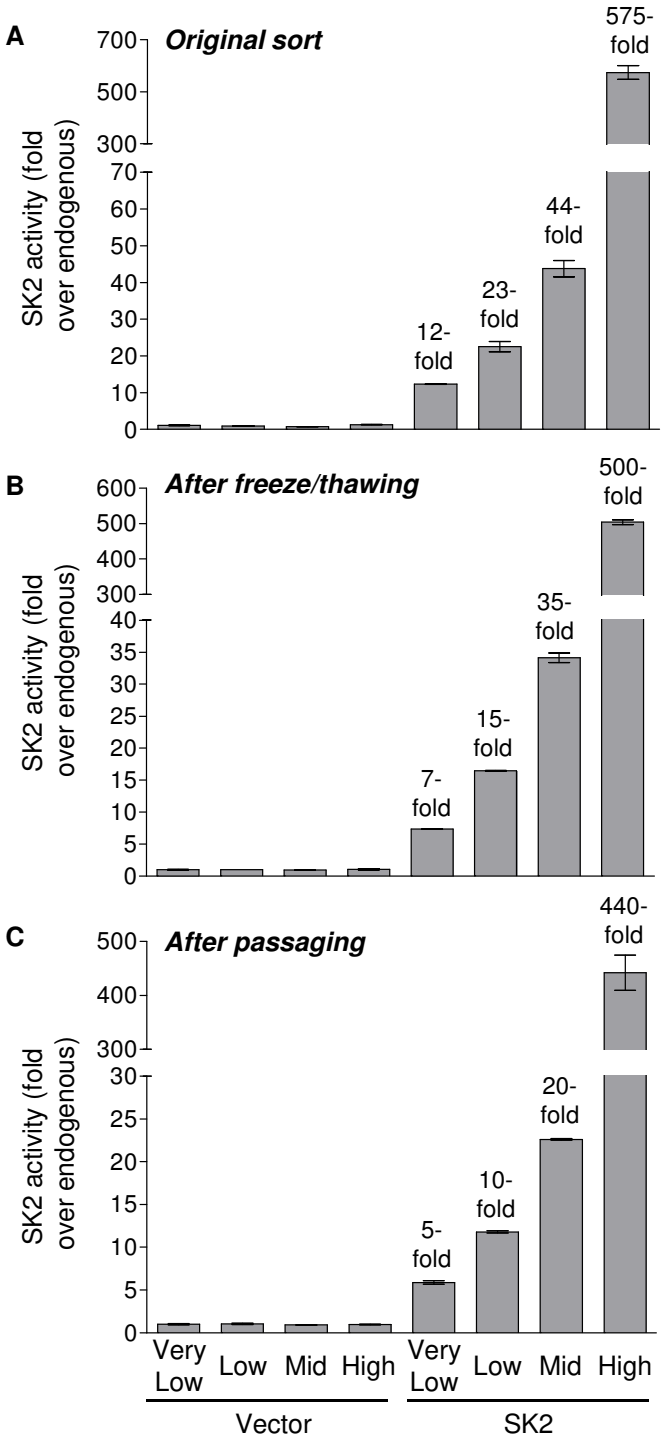


Figure 4.6: Stability of SK2 overexpression in the pCX4 stable cell lines after freeze/thawing and passaging

SK2-specific activity of the NIH3T3 empty vector or SK2-overexpressing pCX4 cell lines after the original sort (*A*), then after thawing cells from storage in liquid nitrogen (*B*) and then after one month of passaging in culture (*C*). Data shown for all graphs are mean (± range) of one experiment with duplicate data points.

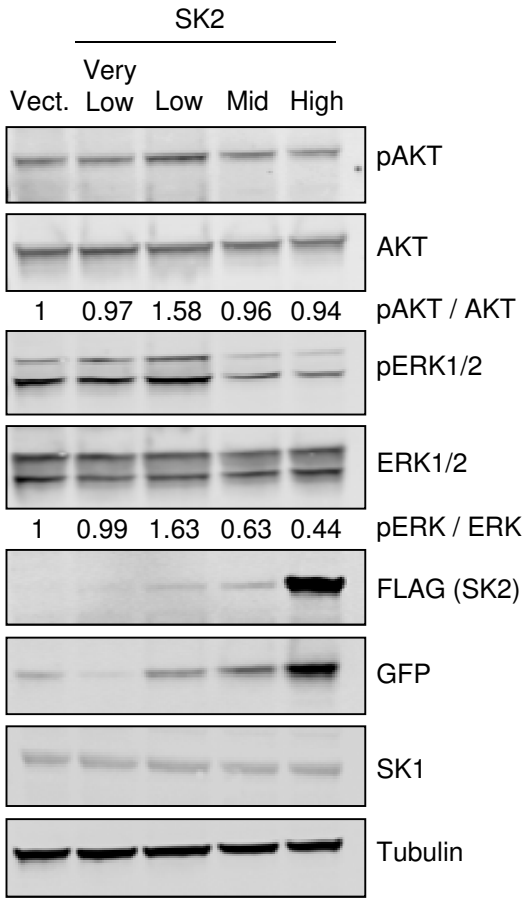


Figure 4.7: SK2 overexpressed at low levels can elicit oncogenic signalling

Lysates from the NIH3T3 vector or SK2-overexpressing cell lines were subjected to immunoblot analyses and probed with antibodies against phospho-AKT, total AKT, phospho-ERK1/2, total ERK1/2, FLAG, GFP, SK1 and α-tubulin. Vect = empty vector with 'low' level GFP expression, chosen as a representative control. Densitometry was performed to quantify phospho-AKT and phospho-ERK band intensities, and is presented as a ratio of total AKT and ERK levels, respectively, and is normalised to vector. Blots shown are representative of three independent experiments.

SK2-overexpressing NIH3T3 cell lines, as compared to vector control cells (Figure 4.7), confirming that these observed changes to oncogenic signalling pathways were not a consequence of altered SK1 expression.

As it had been previously shown using the pcDNA3 NIH3T3 cell lines that low-level SK2 overexpression could allow cells to overcome contact inhibition and become transformed (Figure 4.2), I then examined these new pCX4 NIH3T3 stable cell lines in another *in vitro* assay of neoplastic cell transformation, testing the anchorage-independent growth of cells through their ability to form colonies in soft agar. In this more stringent assay, only the 'very-low' SK2-overexpressing cells were able to form colonies (Figure 4.8). Together, these data confirm that low-level SK2 overexpression can elicit oncogenic signalling and induce neoplastic transformation of cells.

4.5.3 Low level SK2 overexpression can drive tumour formation in vivo

Given that SK2 could promote neoplastic growth *in vitro*, I next examined if this represented full neoplastic transformation through analysing the ability of these cells to form tumours *in vivo*. Hence, the series of cell lines with differential levels of SK2 overexpression were subcutaneously engrafted into the flanks of NOD/SCID mice, and the development of tumours assessed. Consistent with the *in vitro* data, cells with either 'very low' or 'low' SK2 overexpression resulted in efficient tumour formation in mice (Table 4.1). In stark contrast, however, cells with either 'mid' or 'high' SK2 overexpression showed minimal tumour growth (Table 4.1). All tumours were vascularised, as determined by CD31 staining, and showed morphology characteristic of fibrosarcoma (Figure 4.9). Notably, the tumours that developed from the 'very low' SK2 cells were significantly larger than all other tumours formed (Figure 4.10). Overall, these results demonstrate for the first time that through low-level overexpression, SK2 can drive tumourigenesis *in vivo*.

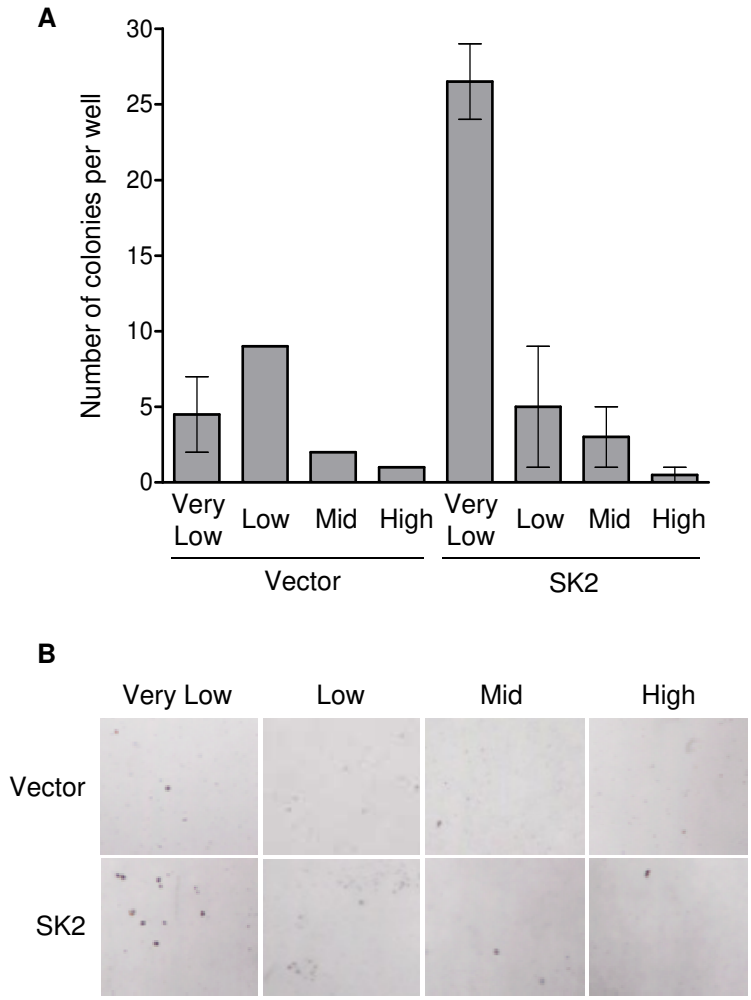


Figure 4.8: 'Very low'-level SK2 overexpression can promote anchorage-independent growth of cells

Anchorage-independent growth of the NIH3T3 pCX4 vector or SK2-overexpressing cell lines were tested using colony formation assays in soft agar. *A,* Total number of colonies per well were quantified and the average number of colonies for duplicate wells from one experiment was graphed (± range). *B,* Representative images are shown from experiment quantified in (*A*).

Cell Line	No. of mice with tumours
Vector	0/5
SK2 very low	4/6
SK2 low	5/6
SK2 mid	1/6
SK2 high	1/6

Table 4.1: Low-level SK2 overexpression results in efficient tumour formation

Table summarising the number of mice with tumours per cell line, 18 days post-cell injection. Mice were injected with NIH3T3 cell lines stably overexpressing 'very low' (5-fold), 'low' (10-fold), 'mid' (20-fold) or 'high' (440-fold) levels of SK2 (above endogenous levels). Empty vector cells with 'low' level GFP expression were chosen as a representative control.

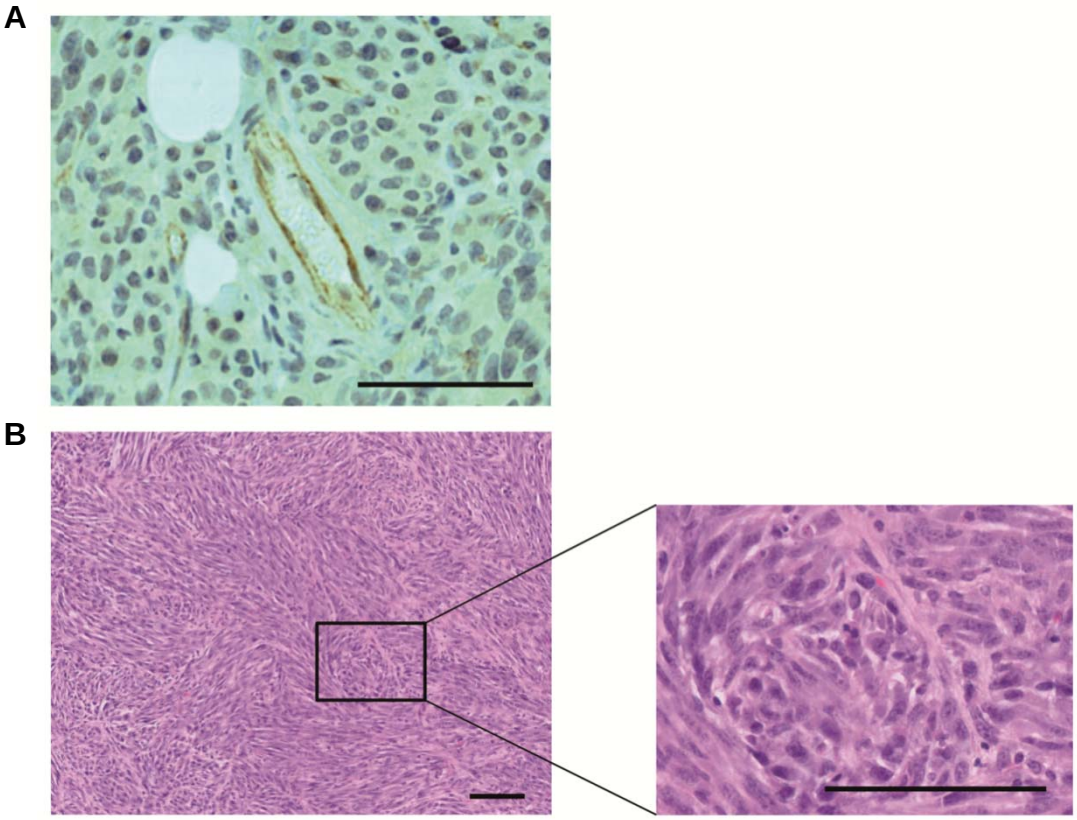


Figure 4.9: Tumours formed from the *in vivo* transformation of NIH3T3 cells overexpressing SK2 are vascularised and characteristic of fibrosarcoma

A, The presence of vasculature within the tumour tissue sections was examined by PECAM-1 (CD31) staining. Representative image is shown at 20x magnification. Scale bar = 100 µm. *B,* Morphology of the tumour tissue sections is shown by hematoxylin and eosin staining. The image on the right is a 20x magnification of the area framed in the left (5x magnification) image. Scale bar = 100 µm.

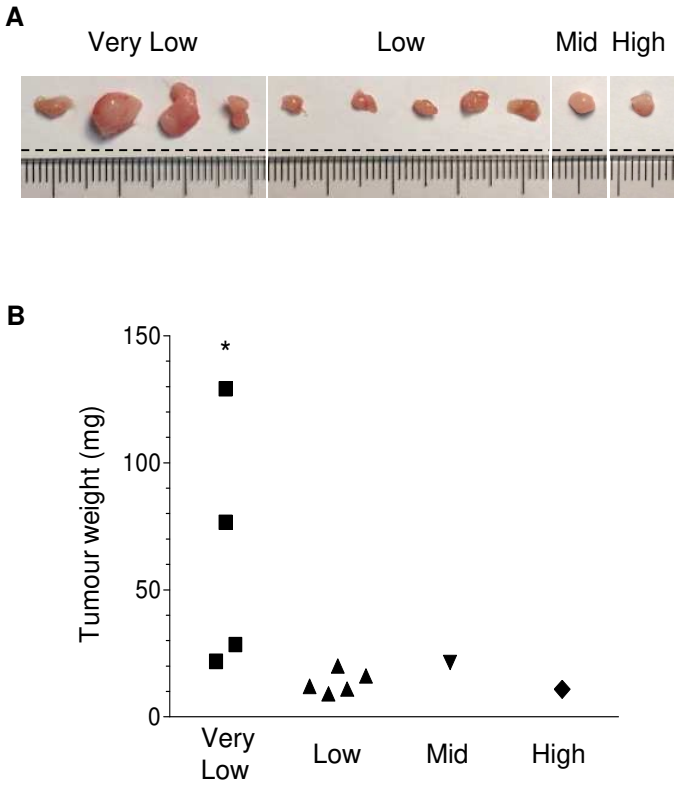


Figure 4.10: 'Very low'-level SK2 overexpression results in the formation of significantly larger tumours

Images (*A*) and weights (*B*) of the excised tumours from each group of NIH3T3 SK2-overexpressing stable cell lines. Statistics denote a significant increase in the weights of SK2 'very low' tumours compared to tumours from the SK2 'low', 'mid' and 'high' groups (* $p<0.05$; Student's unpaired two-tailed t-test).

4.5.4 Less than 5-fold SK2 overexpression is optimal for promoting tumourigenesis, which is observed in many human cancers

Although the SK2-expressing cells engrafted into mice were fractionated based on their GFP (and therefore SK2) expression, these cells remained pools of clones. While this obviates potential defects associated with plasmid integration into the genomes of individual clones, it meant that the level of SK2 overexpression observed for each line was an average of all cells within that line. Thus, I examined the expression levels of SK2 within the resulting tumours. Notably, every tumour that developed from the 'very low', 'low' and 'mid' SK2 overexpressing cells all possessed very similar levels of SK2 protein and catalytic activity (Figure 4.11). Indeed, by comparison to the parental fibroblast cells, it appears that tumour formation resulted preferentially from cells within the pools with less than 5-fold SK2 overexpression (Figure 4.11). This finding suggests that this level of SK2 represents the optimal level to promote oncogenic signalling and tumourigenesis. These results perhaps explain why only one tumour formed from the 'mid' SK2-expressing cells, as within this pool of cells there would likely be fewer low SK2-expressing clones compared with the 'low' and 'very low' groups.

Given that our studies have demonstrated that low, less than 5-fold SK2 overexpression can promote oncogenic signalling, as well as neoplastic transformation *in vitro* and *in vivo*, it was of interest to examine whether SK2 is overexpressed to such levels in human cancers. Despite numerous studies examining the targeting of SK2 in cancer, broad analysis of SK2 expression in cancer had not been previously performed. Thus, I examined SK2 expression in a wide range of human cancers using the public gene expression data sets in the Oncomine database (Rhodes et al, 2004). SK2 was found to be significantly elevated in studies from a broad range of human cancers, including bladder, melanoma, esophageal, breast, lymphoma and leukemia (Figure 4.12A). Interestingly, however, this cancer-associated elevation in SK2 was only modest, with up to 2.5-fold higher levels of SK2 compared with the corresponding normal tissues. This fits nicely with my data demonstrating that similar, low-level SK2 overexpression can promote tumourigenesis. Notably, both SK1 and SK2 were upregulated in three independent datasets for diffuse large B-cell lymphoma (Figure 4.12B), but there was no apparent general correlation between SK2 and SK1 upregulation in the other tumours examined. Indeed, in most other tumour datasets where SK2 was upregulated, SK1 expression was either unaltered or significantly

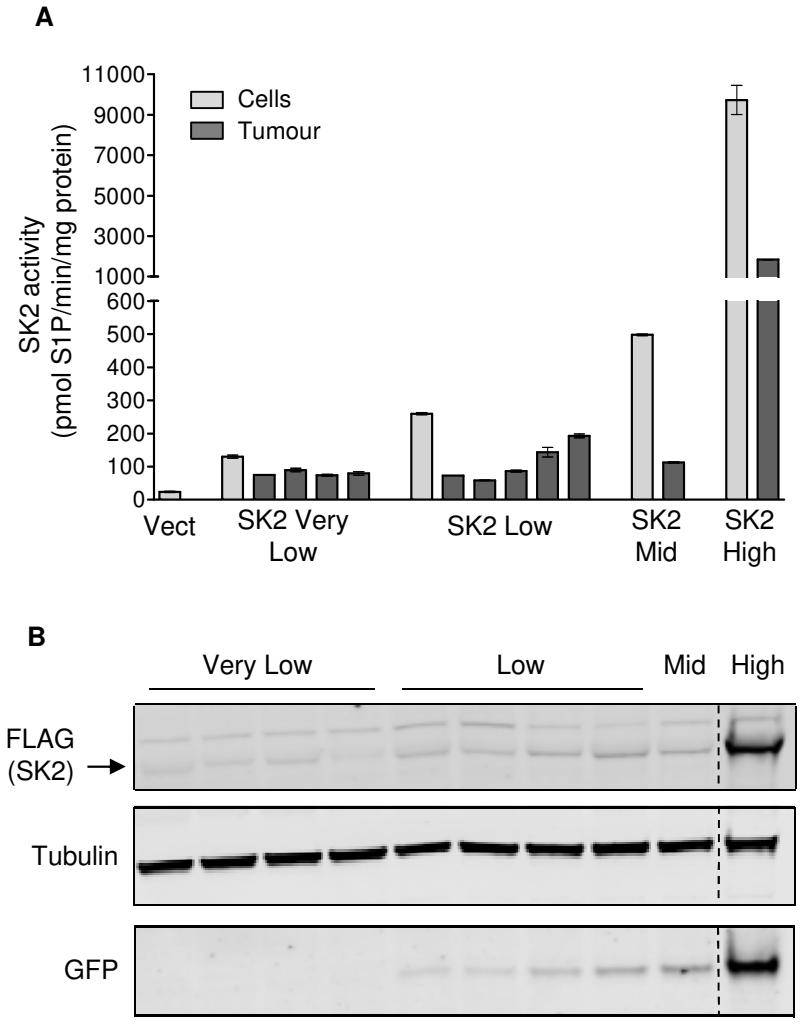


Figure 4.11: Less than 5-fold SK2 overexpression is optimal for promoting tumourigenesis *in vivo*

A, SK2-specific activity from the tumour lysates was measured and graphed as mean (± range) of duplicate samples. These data were plotted alongside data of SK2 activity from the engrafted cell lines, which was transformed from Figure 4.6C as specific-activity (pmol S1P/min/mg protein) for the purposes of comparison. *B,* Tumour tissue lysates were subjected to immunoblot analyses with antibodies against FLAG, α-tubulin and GFP. Dashed lines indicate where lanes from the same immunoblots have been spliced together to aid interpretation.

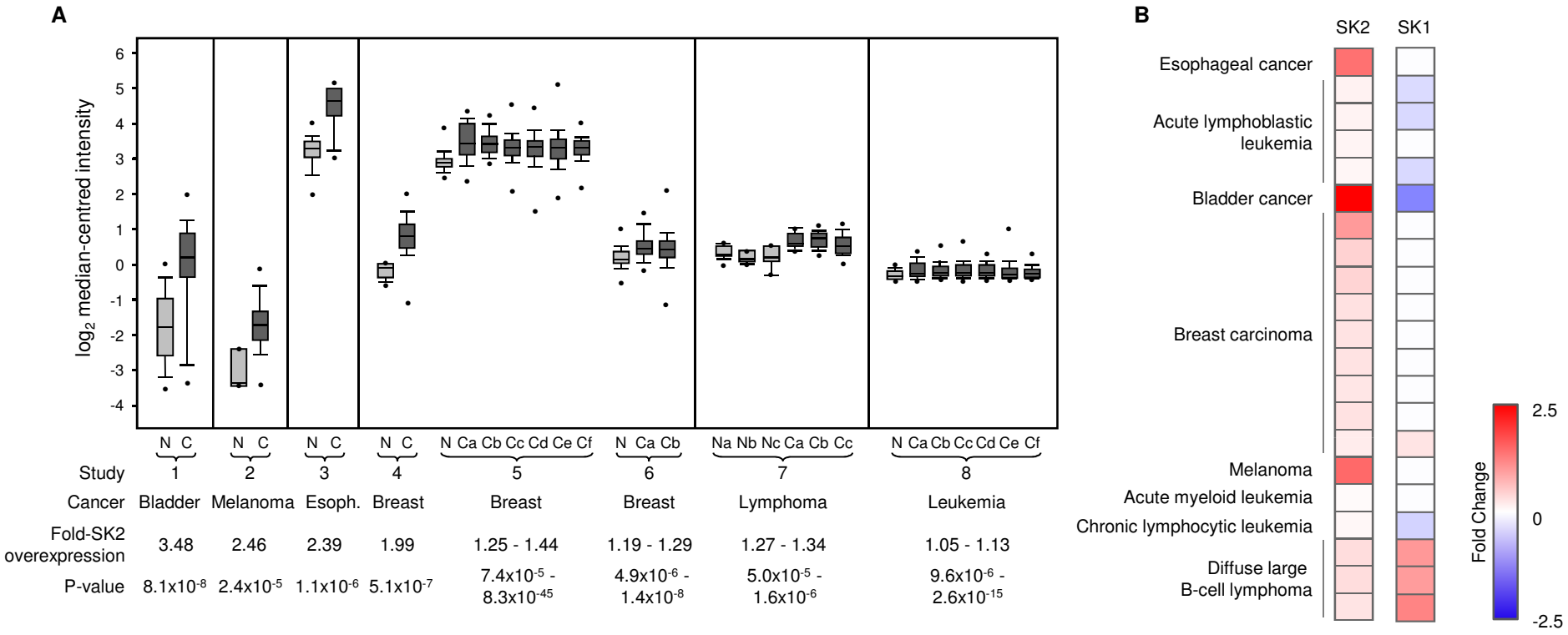


Figure 4.12: SK2 is upregulated in human cancers

A, Box plots showing human cancers with significant ($p<1x10^{-4}$) upregulation of SK2 mRNA levels. N = normal tissue; C = cancerous tissue. Data were extracted from the Oncomine database, from the following studies: study 1: Sanchez-Carbayo Bladder, study 2: Talantov Melanoma, study 3: Kim Esophagus, study 4: Finak Breast, study 5: Curtis Breast, study 6: TCGA Breast, study 7: Compagno Lymphoma, study 8: Haferlach Leukemia. *B,* Heat maps showing significant ($p<1x10^{-4}$) changes in SK2 and SK1 mRNA levels in the human cancer datasets described in (*A*), as compared with corresponding normal tissues.

downregulated (Figure 4.12B).

4.5.5 Examining tumour formation from the 'high' SK2 overexpressing cells

Unexpectedly, the 'high' SK2-overexpressing cells also resulted in one tumour forming. Further analyses, however, revealed that cells within this tumour were not actively proliferating, whereas cells within the tumours generated from 'very low' SK2-expressing cells were highly positive for the proliferation marker Ki-67 (Figure 4.13). Furthermore, the level of SK2 overexpression within the 'high' SK2 tumour was quite heterogeneous, with only small patches of cells with high levels of SK2 protein (Figure 4.14). In fact, the majority of the tumour was comprised of cells with low SK2 overexpression similar in level to that seen in the tumours arising from 'very low' SK2-expressing cells (Figure 4.14). Therefore, again, it is possible that a small population of cells within the original 'high' cell pool drifted to low-level SK2 overexpression and initiated tumour formation, thus supporting the growth of some of the high SK2-expressing cells. Indeed, our previous data demonstrating that low SK2 overexpression supported enhanced cell proliferation and survival, while high SK2 overexpression had the opposite effect suggests that the high SK2-expressing cells would have been under considerable selective pressure towards low SK2 overexpression.

4.5.6 Differential levels of SK2 overexpression alter its subcellular localisation and sphingolipid metabolism

It is well established that the subcellular localisation of the SKs, and hence the compartmentalisation of S1P formation within the cell, plays an important role in the function of these enzymes (Siow & Wattenberg, 2011). The oncogenic role of SK1 requires its translocation to the plasma membrane, a location that results in increased extracellular S1P production (Pitson et al, 2005). Furthermore, the localisation of SK2 to the nucleus, ER or mitochondria appears to promote its anti-proliferative, pro-apoptotic functions (Chipuk et al, 2012; Hait et al, 2009; Igarashi et al, 2003; Maceyka et al, 2005). Thus, I examined the localisation of SK2 when stably overexpressed at low and high levels. In the 'high' SK2-expressing cells, SK2 was strongly nuclear-localised (Figure 4.15),

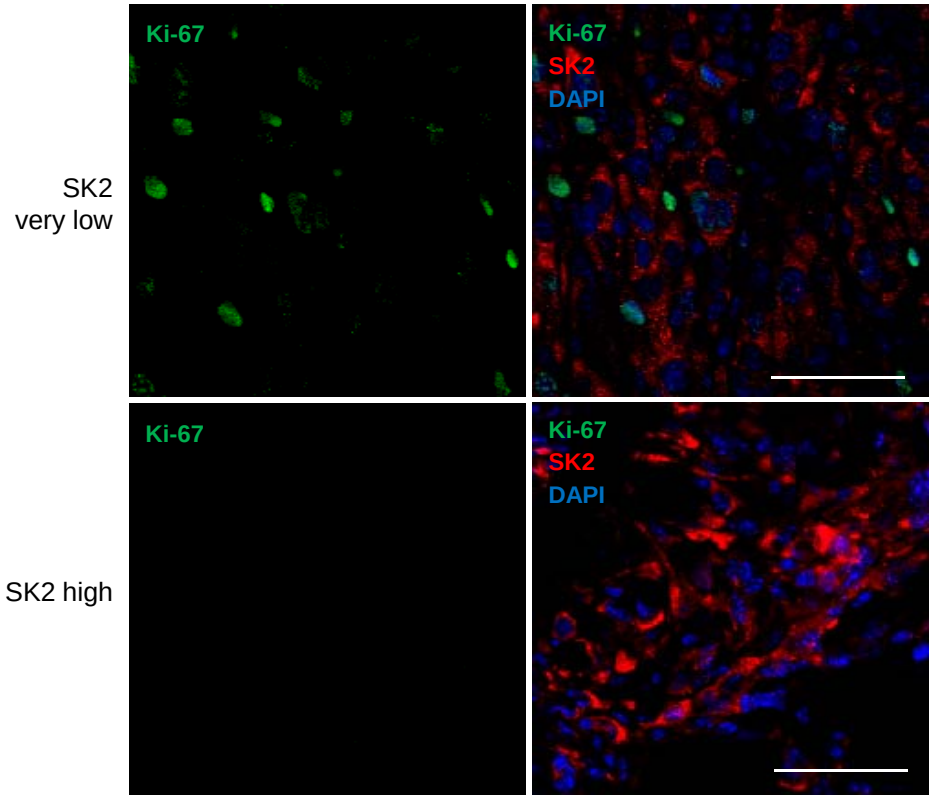


Figure 4.13: Cells within the 'very-low' SK2 tumours are actively proliferating, but cells within the 'high' SK2 tumour are not

Dual immunofluorescence staining of overexpressed FLAG-tagged SK2 (red) and the proliferation marker Ki-67 (green) was performed on the SK2 tumours. Nuclei were stained with DAPI (blue). At least five random fields of view were imaged per tumour and representative images are shown. Scale bar = 50 µm. Staining was performed by Dr Michael Samuel (Centre for Cancer Biology, Adelaide), and imaging was performed by myself.

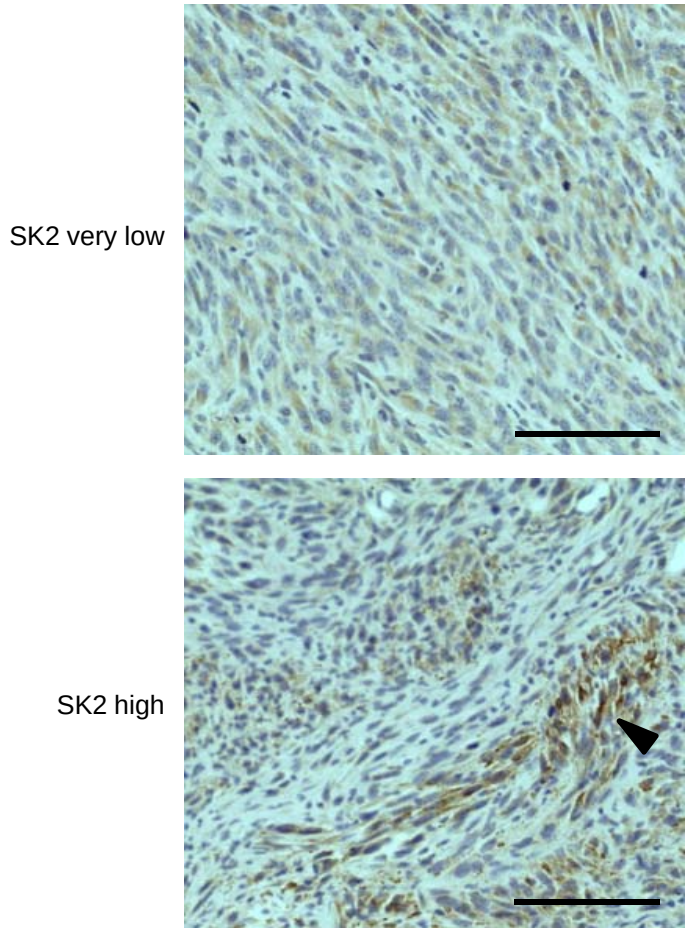


Figure 4.14: Levels of SK2 overexpression are heterogeneous in the SK2 'high' tumour

Expression of ectopically expressed SK2 protein within the tumour tissue sections was examined by immunohistochemical staining for the FLAG epitope of this protein. Multiple images were taken for each tumour, at 20x magnification, and a representative field of view is shown. Scale bar = 100 μm. Arrow denotes a representative area of heterogeneous, intense FLAG (SK2) staining in the SK2 'high' tumour.

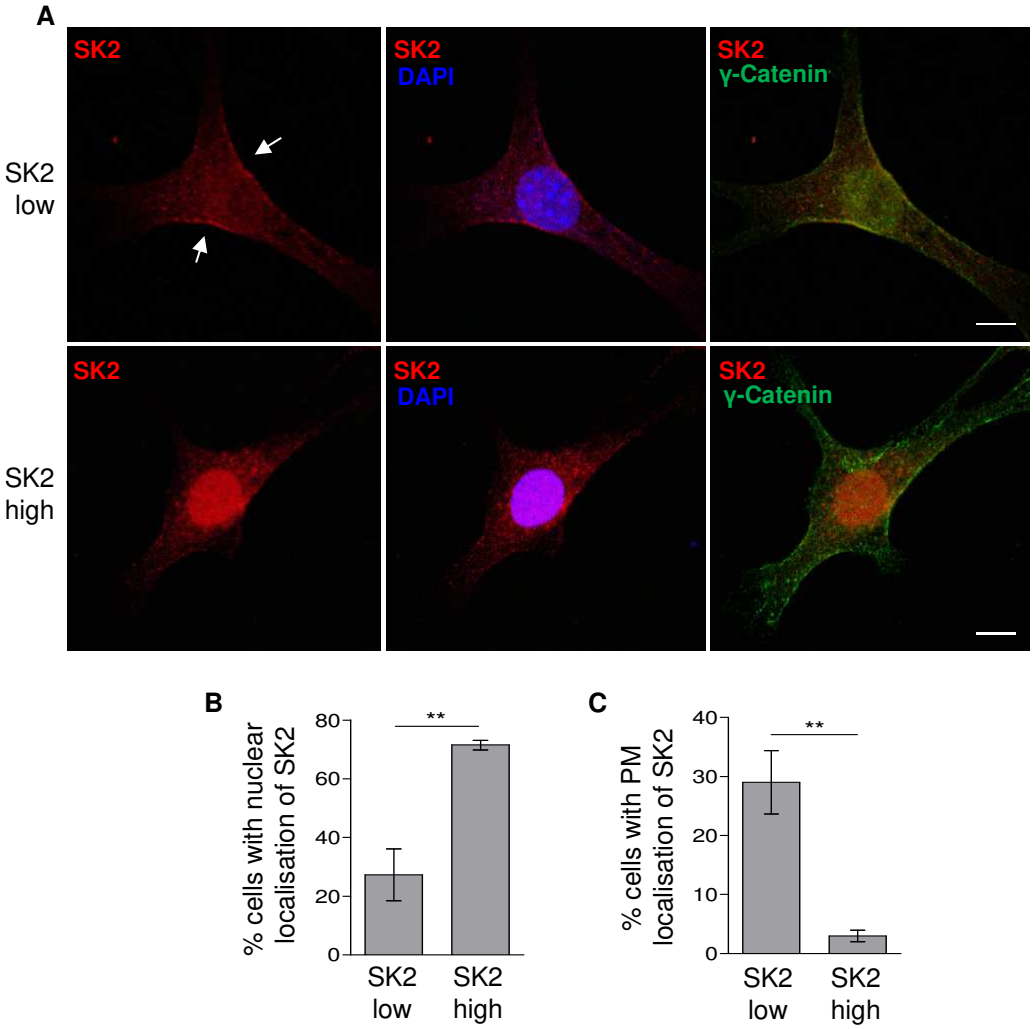


Figure 4.15: Differential levels of SK2 overexpression alter its subcellular localisation

A, The subcellular localisation of FLAG-tagged SK2 (red) in the NIH3T3 stable 'low' and 'high' SK2-overexpressing cells was examined by immunofluorescence staining, using FLAG antibody. Nuclei were stained with DAPI (blue) and cell membranes were stained with an antibody against γ-catenin (green). Images are representative of cells observed from three independent experiments. Arrows denote representative plasma membrane localisation of 'low' SK2. Scale bar = 10 µm. *B and C*, Cells from (*A*) were visualised by confocal microscopy and scored based on the presence or absence of either (*B*) distinct nuclear FLAG-tagged SK2 staining, or (*C*) plasma membrane (PM)-localised FLAG-tagged SK2 staining. A minimum of 200 cells were scored per well. Data were graphed as mean (± SD) of triplicate wells from a single experiment, representative of three independent experiments (** $p<0.01$; Student's unpaired two-tailed t-test).

in agreement with previous reports for this cell type (Igarashi et al, 2003). In the 'low' SK2-expressing cells, however, SK2 was more evenly distributed throughout the cell, and showed a significant increase in its plasma membrane localisation (Figure 4.15). Furthermore, the formation of extracellular S1P was significantly higher from cells with 'low'-level SK2 overexpression compared to vector control cells (Figure 4.16A), consistent with our observations of increased SK2 at the plasma membrane in these cells. Interestingly, 'high'-level SK2 overexpression resulted in a further increase in extracellular S1P formation (Figure 4.16A), but this was only a modest doubling compared with the 'low' SK2 cells, despite these cells having greater than 400-fold more SK2 activity.

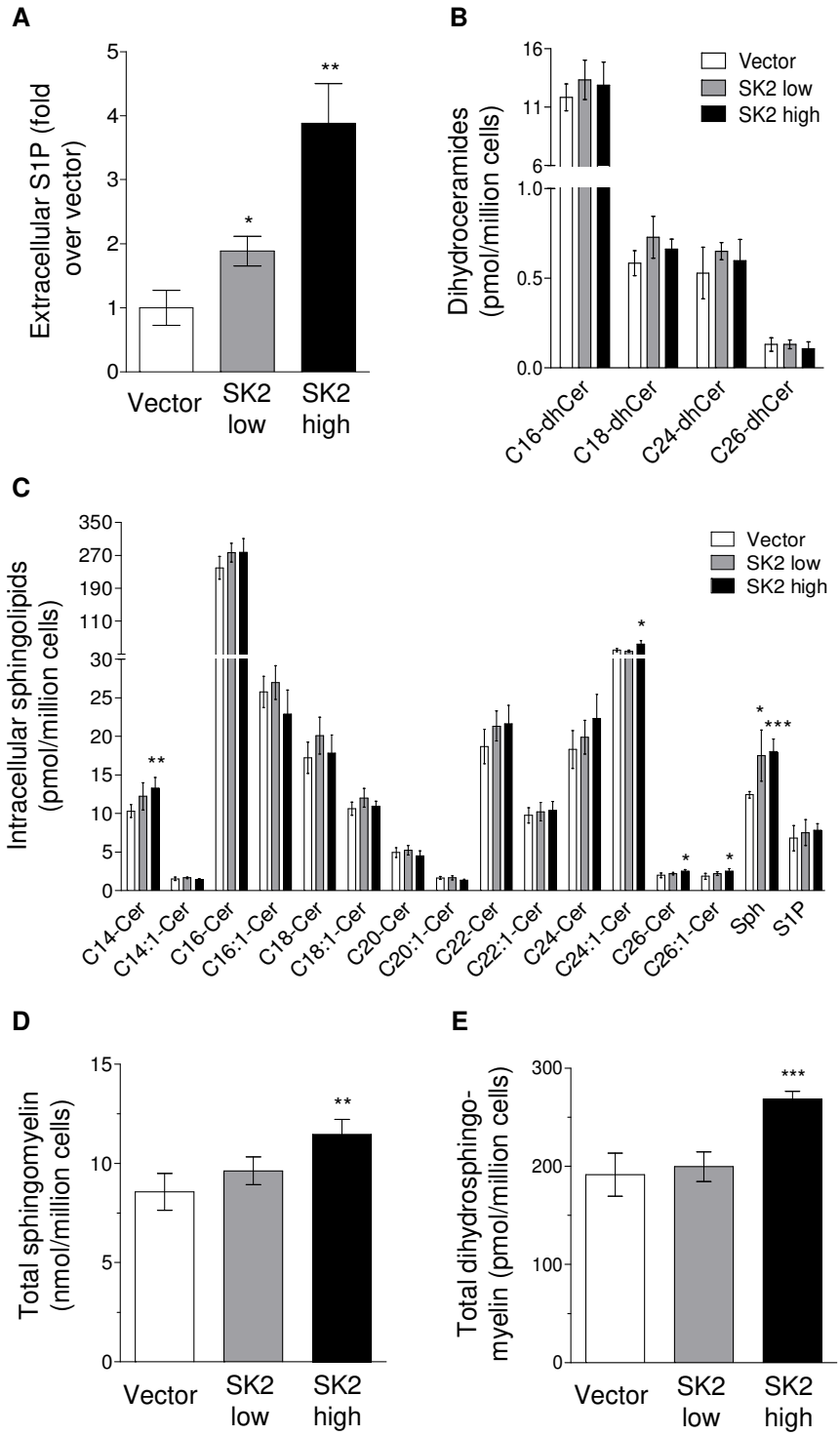
Sphingolipid analysis revealed, somewhat surprisingly, that 'low'-level SK2 overexpression had very little effect on the intracellular levels of S1P, ceramides, dihydroceramides, sphingomyelins or dihydrosphingomyelins (Figure 4.16B-E), with the only change noted being a small increase in sphingosine. In contrast, 'high'-level SK2 overexpression resulted in a significant increase in a range of ceramide species, sphingomyelins and dihydrosphingomyelins, as well as sphingosine (Figure 4.16C-E), in line with a previous report demonstrating that overexpressed SK2 partially localised to the ER, and S1P produced here could feed into an ER/golgi-associated 'salvage pathway' to generate pro-apoptotic sphingosine and ceramide, as well as sphingomyelin (Maceyka et al, 2005). The increase in ceramides and sphingosine are likely to contribute, at least in part, to the anti-proliferative and pro-cell death role of SK2 in these 'high' overexpression cells, with the increase in extracellular S1P formation that was observed in these cells (Figure 4.16A) possibly insufficient to override these effects.

4.6 Discussion

Despite the conflicting data in the literature, the findings from this study demonstrate that, like SK1, SK2 can have a physiological role in promoting cell survival and proliferation, potentially through plasma membrane localisation. This is perhaps not surprising, as the individual genetic deletion of either SK1 or SK2 in mice does not result in any gross phenotypic abnormalities (Allende et al, 2004; Zemann et al, 2007), whereas the double knock-out mice die *in utero* (Mizugishi et al, 2005), suggestive of at least some functional

Figure 4.16: Differential levels of SK2 overexpression alter the levels of extracellular and intracellular sphingolipids

A, The rate of extracellular S1P formation from intact vector control 'low', SK2 'low' and SK2 'high' NIH3T3 stable cell lines was determined. Analyses were performed in triplicate and data are graphed as mean (± SD). Statistics denote significant increases in extracellular S1P compared to vector control cells (* $p<0.05$, ** $p<0.01$; Student's unpaired two-tailed t-test). *B-E*, Intracellular sphingolipid species from the above cell lines were analysed by LC-MS. Data are graphed as mean (± SD) of quadruplicate samples for (*B*) individual dihydroceramide species, (*C*) individual ceramide species, sphingosine (Sph) and sphingosine-1-phosphate (S1P), (*D*) total sphingomyelin levels, and (*E*) total dihydrosphingomyelin levels. Statistics denote significant increases in lipids compared to vector control cells (* $p<0.05$, ** $p<0.01$, *** $p<0.001$; Student's unpaired two-tailed t-test). LC-MS analysis of cell pellets was performed by Dr Darren Creek and Amanda Peterson (Monash University, Melbourne).


A
Extracellular S1P (fold over vector)
Vector
SK2 low
SK2 high
B
Dihydroceramides (pmol/million cells)
Vector
SK2 low
SK2 high
C16-dhCer
C18-dhCer
C24-dhCer
C26-dhCer
C
Intracellular sphingolipids (pmol/million cells)
Vector
SK2 low
SK2 high
C14-Cer
C14:1-Cer
C16-Cer
C16:1-Cer
C18-Cer
C18:1-Cer
C20-Cer
C20:1-Cer
C22-Cer
C22:1-Cer
C24-Cer
C24:1-Cer
C26-Cer
C26:1-Cer
Sph
S1P
D
Total sphingomyelin (nmol/million cells)
Vector
SK2 low
SK2 high
E
Total dihydrosphingo-myelin (pmol/million cells)
Vector
SK2 low
SK2 high

redundancy between the two proteins. Like SK1, there are no mutations in SK2 linked to cancer, however it has been suggested that cancer cells can display a 'non-oncogene addiction' for SK1 (Pyne & Pyne, 2010; Vadas et al, 2008). Given that I have now shown that SK2 can promote neoplastic transformation and tumourigenesis, and is upregulated in many human cancers, coupled with the anti-cancer efficacy of SK2-selective inhibitors leads me to postulate that a non-oncogene addiction may apply for both SKs in cancer. Indeed, targeting both SK isoforms may be the best strategy to overcome tissue and cell type-specific differences in the roles of the SKs in different cancers, and in agreement, dual SK1/SK2 inhibitors show significant decreases in tumour burden *in vivo* (French et al, 2006; Pitman et al, 2015).

One of the most intriguing findings from this study is the observation that SK2 function can dramatically switch, depending on its expression level, from being pro-survival and pro-proliferative to pro-cell death and anti-proliferative. While it could be argued that high-level overexpression is non-physiological, and may generate artefacts, it is notable that previous studies have shown that SK2 can have physiological roles in promoting cell cycle arrest and apoptosis when localised to organelles such as the nucleus and mitochondria (Chipuk et al, 2012; Hait et al, 2009). Clearly, under normal conditions these pro-death roles are likely kept under tight regulation, so it remains possible that high-level overexpression circumvents these regulatory mechanisms. This high-level overexpression of SK2, for example, may lead to altered protein-protein interactions or post-translational modifications, altering the subcellular localisation of SK2, from the plasma membrane to cellular organelles such as the nucleus, in favour of promoting cell death. Indeed, it has been previously reported that when transiently overexpressed, SK2 could interact with and sequester the pro-survival Bcl-x_L protein (Liu et al, 2003), suggesting a possible mechanism for the pro-apoptotic phenotype we observed with high-level SK2 overexpression. However, despite multiple attempts, I was unable to detect an interaction between Bcl-x_L and SK2 in the NIH3T3 stable cell lines (*data not shown*), suggesting that this proposed interaction was unlikely to mediate the observed phenotype. Intriguingly, there appears to be a window whereby low-level SK2 upregulation confers a survival and proliferative advantage to the cell without inducing these pro-apoptotic functions. Whether a different subset of post-translational modifications and/or protein-protein interactions drive the differences in subcellular localisation and function observed with low versus high SK2 overexpression will require further interrogation.

To add further complexity, other studies have demonstrated that nuclear SK2 can contribute to cancer progression through the stabilisation of telomerase and promotion of c-Myc expression (Panneer Selvam et al, 2015; Wallington-Beddoe et al, 2014). In the present study, SK2 was observed in the nucleus when overexpressed at high levels, and yet here it had an opposite, anti-proliferative role, which is also well documented (Hait et al, 2009; Igarashi et al, 2003). It is unclear how SK2 can have such vastly different functions within the same organelle, but it suggests that there must be additional factors regulating these processes. Indeed, the regulatory mechanisms controlling this enzyme remain an important, but currently largely unanswered question (Neubauer & Pitson, 2013). Notably, SK1 remains pro-survival and pro-proliferative even at high-level overexpression, likely due to its different subcellular localisation to SK2 and consequent contribution to different sphingolipid pools within the cell. In line with this, previous studies have shown that artificially targeting SK1 to the ER can render it pro-apoptotic, like SK2 (Maceyka et al, 2005).

Interestingly, while cells with 20-fold ('mid') SK2 overexpression were able to form foci *in vitro*, they had decreased levels of phospho-ERK1/2 and did not efficiently form tumours *in vivo*. This may indicate that this level of SK2 overexpression is at the upper limit of the 'window' whereby SK2 switches from being tumourigenic to having predominantly anti-proliferative functions. Conversely, 5-fold ('very low') SK2 overexpression resulted in no appreciable changes in phospho-ERK1/2 or phospho-AKT levels, and yet these cells developed the largest tumours *in vivo*. It is therefore possible that S1P-mediated angiogenesis and tumour vascularisation played more of an important role in the development of these tumours, given that low-level overexpression of SK2 resulted in plasma membrane localisation and increased formation of extracellular S1P, which is a key regulator of angiogenesis (Takabe & Spiegel, 2014). These observed differences also highlight the importance of employing *in vivo* models for assessment of full neoplastic transformation. Furthermore, it was surprising that 'high'-level SK2 overexpression resulted in a doubling in extracellular S1P production as compared to the 'low'-SK2 overexpressing cells, despite 'high' SK2 overexpression resulting in decreased survival and proliferative signalling, and increases in pro-apoptotic sphingolipid species. Notably, Weigert *et al.* previously reported that transient overexpression of SK2 resulted in a substantial increase in S1P released from apoptotic cells as a result of SK2 being cleaved by caspase-1 and secreted from the cell (Weigert et al, 2010). This may, in part, explain our observed increase

in extracellular S1P in the SK2 'high' cells, which was clearly not able to facilitate any overall pro-survival or proliferative stimulus in these cells.

It should be noted that Liang *et al.* found a significant increase in colitis-associated tumour development in SK2 knockout mice, when compared to wildtype mice (Liang et al, 2013), suggesting that SK2 may function as a tumour suppressor in this model. However, these findings are likely to be an indirect effect as it was also shown in the study that the global genetic loss of SK2 caused an upregulation of both S1P receptor 1 and SK1 levels in the colon, with a concomitant increase in circulating and colonic S1P (Liang et al, 2013). SK1 has been previously shown to contribute to colon carcinogenesis (Kawamori et al, 2009) and indeed, the increase in severity of colitis in the SK2 knockout mice was ablated by the SK1-specific inhibitor SK1-I (Liang et al, 2013). Furthermore, the proposed tumour-suppressive role of SK2 in negatively regulating pro-tumourigenic SK1 levels is not recapitulated with SK2-selective inhibitors, which show efficacy in decreasing tumour burden in murine xenograft models (Chumanevich et al, 2010; French et al, 2010; Liu et al, 2013; Wallington-Beddoe et al, 2014).

Evidently the true functions of SK2 are complex and are also likely to be tissue- and cell type-specific. However, from the findings of this study it is clear that SK2 represents an important target in cancer and future work to better understand how SK2 is regulated will be important for the generation of more efficacious SK2-targeting anti-cancer drugs.

Chapter 5:
Characterising the Interaction between Sphingosine Kinase 2 and Dynein Intermediate Chain

Chapter 5. Characterising the Interaction between Sphingosine Kinase 2 and Dynein Intermediate Chain

5.1 Abstract

While the two mammalian sphingosine kinases, SK1 and SK2, both catalyse the generation of pro-survival sphingosine-1-phosphate, their roles vary dependent on their different subcellular localisation. SK1 is generally found in the cytoplasm or at the plasma membrane where it can promote cell proliferation and survival. However, where SK2 appears to have a similar function to SK1 at the plasma membrane, when localised to internal organelles such as the nucleus, endoplasmic reticulum or mitochondria, SK2 can mediate cell death. Although SK2 has recently been implicated in cancer initiation and progression, it is currently unclear how the subcellular localisation of SK2 is regulated. Here, a novel interaction between SK2 and the intermediate chain subunit of the retrograde-directed transport motor complex, cytoplasmic dynein (DYNC1I; IC), is reported. This interaction between SK2 and dynein is shown to have a physiological role in facilitating transport of SK2 away from the plasma membrane. Furthermore, the dynein intermediate chain isoform IC-1 is dramatically downregulated in patient samples of glioblastoma multiforme (GBM), where low expression of IC-1 correlates with poorer patient survival. Notably, low IC-1 expression in GBM cells coincided with more SK2 localised to the plasma membrane, where it has been implicated in oncogenesis. Strikingly, re-expression of IC-1 in these cells reduced plasma membrane localised-SK2 and extracellular S1P formation, and decreased tumour growth and tumour-associated angiogenesis *in vivo*. Thus, these findings demonstrate a novel tumour-suppressive function of dynein IC-1, and uncover new mechanistic insights into SK2 regulation, which may have implications in targeting this enzyme as a therapeutic strategy in GBM.

5.2 Introduction

Sphingosine-1-phosphate (S1P) is an important signalling lipid that can regulate a vast number of cellular processes, including cell survival, proliferation, apoptosis, migration and differentiation (Pitson, 2011; Pyne & Pyne, 2010). S1P is produced in the cell from the phosphorylation of sphingosine by the sphingosine kinases (SKs), SK1 and SK2. Many of the extracellular and intracellular signalling pathways activated by S1P promote cell survival and proliferation, whereas sphingosine, and its precursor ceramide, are both pro-apoptotic molecules (Newton et al, 2015). Therefore, by modulating the relative levels of these lipids, the SKs play an important role in determining cell fate. Interestingly, despite both enzymes catalysing the formation of the same product, S1P, SK1 and SK2 appear to have both overlapping as well as divergent functions within the cell (Neubauer & Pitson, 2013; Pitson, 2011), with these differing functions seemingly dictated by their differential subcellular localisation (Siow & Wattenberg, 2011).

SK1 is largely a cytoplasmic protein, but it has previously been shown that upon phosphorylation and activation, SK1 can be translocated to the plasma membrane (Pitson et al, 2003) where it can facilitate pro-survival, pro-proliferative signalling (Jarman et al, 2010; Pitson et al, 2003; Pitson et al, 2005; Zhu et al, 2017). As such, targeting SK1 has demonstrated anti-tumour effects (recently reviewed in (Pitman et al, 2016)). Similarly, I demonstrated in Chapter 4 that SK2 can play a role in mediating tumourigenesis, and it too was found to be localised to the plasma membrane to increase S1P levels in this setting. The role of SK2 in cancer is still being elucidated; many studies have implicated SK2 in promoting tumour growth, and the molecular mechanisms involved are beginning to be revealed (reviewed in (Neubauer & Pitson, 2013)). However, unlike SK1, SK2 can also promote cell cycle arrest and cell death under certain conditions, and these functions appear to require changes to the subcellular localisation of this enzyme. SK2 possesses both nuclear localisation and export sequences (NLS and NES) (Ding et al, 2007; Igarashi et al, 2003), and is in fact localised to the nucleus in many cell types. Here, SK2 can promote cell cycle arrest and cell death (Igarashi et al, 2003; Okada et al, 2005), potentially through HDAC1/2 inhibition and the subsequent transcriptional upregulation of p21 and c-fos (Hait et al, 2009). Furthermore, it was reported that S1P production by SK2 at the ER resulted in the induction of apoptosis (Maceyka et al, 2005), and S1P produced by mitochondrial-localised

SK2 has been implicated in Bid-mediated Bak activation and cytochrome c release (Chipuk et al, 2012).

Therefore, it is evident that the subcellular localisation of the SKs, and hence the compartmentalisation of S1P production within the cell, can alter their function and impact on the fate of the cell. Specifically, SK2 localisation to the nucleus and internal organelles within the cell seems to confer pro-apoptotic, anti-proliferative functions, whereas localisation to the plasma membrane drives pro-proliferative and oncogenic signalling. However, the mechanisms regulating the transport of SK2 between various cellular compartments in order to effect changes in its functions are currently unexplored.

In this chapter, I examined cytoplasmic dynein 1 intermediate chain (DYNC1I; IC) as a novel SK2-interacting protein, and demonstrated that SK2 physiologically interacts with the cytoplasmic dynein motor complex in cells and is transported by dynein in a retrograde direction away from the cell periphery. Furthermore, I report a dramatic downregulation of dynein intermediate chain IC-1 in glioblastoma multiforme (GBM), which correlates with poorer patient survival, and demonstrate that lower IC-1 expression in GBM cells coincides with more SK2 localised to the plasma membrane. Notably, re-expression of IC-1 in GBM cells reduced plasma membrane-localised SK2 and extracellular S1P formation, and strikingly, decreased tumour growth and tumour-associated angiogenesis *in vivo,* highlighting a novel tumour-suppressive function of dynein IC-1 that may be linked with regulating SK2 subcellular localisation.

5.3 Preliminary data leading to this study

It was of interest to identify novel mechanisms of SK2 regulation, and as such, the Pitson Laboratory previously performed a yeast two-hybrid screen to examine SK2-interacting proteins. Using full-length SK2b as the bait and a C-terminal human normalised cDNA library as the prey, under stringent conditions, a putative SK2-interacting protein was detected and identified as cytoplasmic dynein 1 intermediate chain 2 (IC-2). The interaction between SK2 and IC-2 was subsequently confirmed in mammalian cells by co-immunoprecipitation of the overexpressed proteins (Figure 5.1). Hence, the focus of this chapter was the functional characterisation of this interaction.

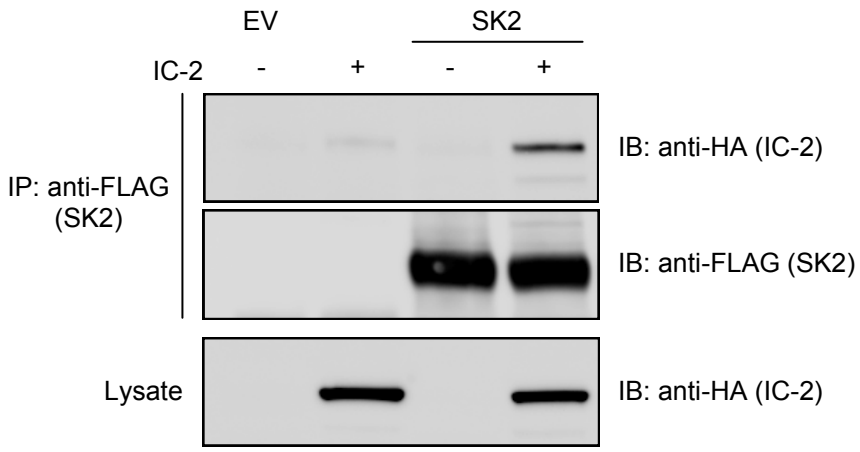


Figure 5.1: SK2 and IC-2 interact when overexpressed in mammalian cells

HEK293-C18 cells were transfected with empty vector (EV) or HA-tagged IC-2, either alone or in combination with FLAG-tagged SK2. SK2 was immunoprecipitated from cell lysates with anti-FLAG antibodies, and co-immunoprecipitated IC-2 was detected by immunoblotting with anti-HA antibodies. Expression levels of IC-2 in the lysates were confirmed by immunoblotting with anti-HA antibodies (*Lysate*). Immunoprecipitates were also probed with anti-FLAG antibodies to confirm pull-down of SK2. Blots shown are representative of at least five independent experiments.

5.4 Specific Materials and Methods

5.4.1 Yeast two-hybrid screen

Yeast two-hybrid screening was performed using the Matchmaker Gold Gal4 Two-Hybrid System (Clontech, Takara, Mountain View, CA, USA) according to the manufacturer's instructions. The bait construct, containing full-length human SK2b cDNA cloned into pGBKT7 in-frame with the Gal4 DNA-binding domain, was transformed into *Saccharomyces cerevisiae* strain AH109. Mating was performed using a Mate & Plate™ universal human normalised cDNA library in pGADT7 (Clontech), transformed into *S. cerevisiae* strain Y187. A total of 1×10^6 clones were screened for growth on yeast synthetic dropout (SD) medium lacking adenine, tryptophan, leucine and histidine. Total plasmid DNA was then isolated from surviving His^+Ade^+ colonies, transformed into *E. coli* JM109, amplified and screened for false positives by transformation of these isolated library plasmids into *S. cerevisiae* AH109 containing either pGBKT7-SK2b or pGBKT7 vector alone. Confirmed positive interactions were identified as those enabling yeast growth on SD medium lacking adenine, tryptophan, leucine and histidine only in the presence of pGBKT7-SK2b, as well as enabling expression of β-galactosidase. The identity of positive SK2b-interacting proteins was confirmed by digestion of plasmid DNA with EcoRI and XhoI, followed by sequencing of the isolated cDNA.

5.4.2 siRNA knock-down of IC-2

siRNA-mediated knockdown of IC-2 was performed using human DYNC1I2 ON-TARGETplus SMARTpool siRNA (Dharmacon), which targets the following sequences: GUA AAG CUU UGG ACA ACU A, GAU GUU AUG UGG UCA CCU A, GCA UUU CUG UGG AGG GUA A, and GUG GUU AGU UGU UUG GAU U. Cells were seeded and grown to a cell density of approximately 50%, and cells were then transfected with 30 nM (final concentration) of either hDYNC1I2 siRNA or ON-TARGETplus non-targeting siRNA pool, using Lipofectamine RNAiMAX as per the manufacturer's protocol (Thermo Fisher Scientific). Cells were incubated with the siRNA complexes at 37°C for 48 hr.

5.4.3 Duolink® protein interaction assay

HEK293 cells were seeded at $1x10^4$ cells/well into 16-well Nunc™ Lab-Tek™ chamber slides (Thermo Fisher Scientific) that had been coated with poly-L-lysine (Sigma-Aldrich). Cells were incubated in DMEM growth medium for 48 hr. Protein-protein interactions were then visualized using the Duolink® *in situ* proximity ligation assay (PLA) kit, as per the manufacturer's instructions (Sigma-Aldrich). Briefly, cells were washed in PBS, fixed in 4% paraformaldehyde for 10 min, and permeabilised in PBS with 0.1% Triton X-100 for 10 min. Blocking was performed using the blocking reagent provided with the kit. Cells were incubated with an antibody recognizing SK2 (1:300; ECM Biosciences) and an antibody specific to the dynein intermediate chains (1:300; Millipore) for 1 hr at room temperature. PLA was then performed, slides were dried and coverslips were mounted using mounting medium with DAPI (provided with the kit). Interactions were visualized and imaged using a Carl Zeiss LSM 700 confocal microscope, with Zen 2011 (Black Edition) version 8.1.5.484 software.

5.4.4 Generating stable cell lines

To generate stable cell lines expressing IC-1 as a GFP-fusion protein, U-251 cells were transfected with an IC-1:GFP expression construct ($pCX3^{NEO}$-DYNC1I1:EGFP), or the corresponding empty vector, using Lipofectamine™ 2000 (Thermo Fisher Scientific) as per the manufacturer's protocol. 48 hr after transfection, the cells were sorted for low-level GFP-positive cells using a FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). Stable GFP-positive pooled cell populations were obtained by sorting for GFP another two times over three weeks. The stable U-251 cell lines were then analysed by immunoblotting with GFP antibodies to confirm the expression of IC-1:GFP (or GFP alone).

5.4.5 *In vivo* subcutaneous tumour model

The U-251 cell lines stably-expressing IC-1:GFP or GFP alone (vector) were trypsinised and washed in PBS, and $5x10^6$ cells were injected in 200 µl of PBS subcutaneously into the

flank of 9 week old female NOD/SCID mice. Mice were examined daily to monitor tumour formation, and calliper measurements of palpable tumours were taken. On day 30 post cell injection, all mice were humanely killed and tumours were excised. A portion of each tumour was fixed in 10% formalin, paraffin embedded and sectioned. The remaining tumour tissue was homogenized using a pestle (Axygen) in extraction buffer, subjected to freeze/thawing in liquid nitrogen and sonication, and lysates were clarified by centrifugation at 17,000 × *g* for 15 min at 4°C.

5.5 Results

5.5.1 Characterising the SK2 binding site on dynein IC-2

Apart from the dynein HC, all other characterised interacting partners of the dynein ICs bind within the N-terminal portion of the protein (Cai et al, 2010; Pfister, 2015; Whyte et al, 2008). However, it was the C-terminal 173 amino acid portion of IC-2 (residues 440-612) that was identified from the yeast two-hybrid screen to interact with SK2. Therefore, it was of interest to interrogate the binding site of SK2 on IC-2. Initially to examine this, a C-terminal truncation of IC-2 identical to the fragment identified in the yeast two-hybrid was co-expressed with SK2 in cells (440-612), and the remaining N-terminal portion of the protein was also co-expressed with SK2 as a control (1-439). SK2 was then immunoprecipitated and, surprisingly, both the N- and C-terminal fragments of IC-2 were strongly and cleanly co-immunoprecipitated, the N-terminal fragment slightly more so (Figure 5.2).

The ICs contain seven WD40 domain repeats spanning the C-terminal half of the protein, and these domains are known to mediate protein-protein interactions (Stirnimann et al, 2010). Therefore, it was speculated that, because the 1-439 and 440-612 truncations of IC-2 each contain three of these WD40 repeats, if SK2 does bind to the C-terminus via these domains then perhaps this is why it can interact with both fragments. Thus, to determine if SK2 does interact with the WD40 repeat domains of IC-2, new IC-2 truncations were generated to separate the C-terminal half of the protein with all seven WD40 repeats (251-612) from the N-terminus that contains no WD40 repeats (1-250). However, upon repeating the co-immunoprecipitation of these fragments with SK2, SK2 was still able to cleanly

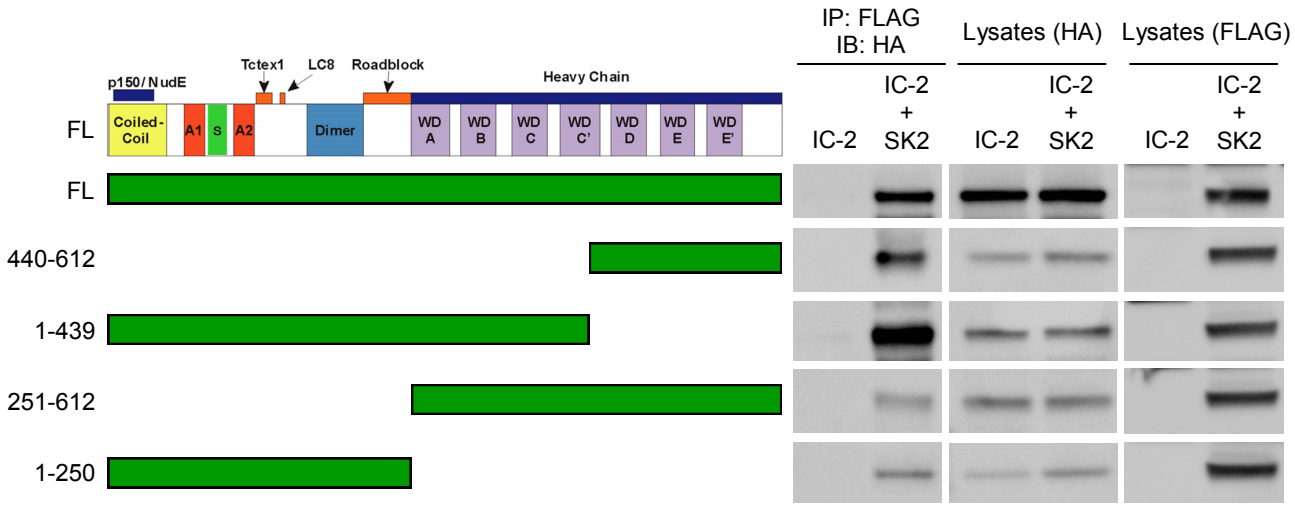


Figure 5.2: Mapping the SK2 interaction site on dynein intermediate chain 2

The domain map of full length dynein intermediate chain 2 (IC-2) is shown at the top of the figure (from Figure 1.9). Along the left are the amino acid sequences of the IC-2 truncations, or full-length (FL). In green are graphical representations of the lengths of the IC-2 truncations, to scale with the full-length domain map above. On the right are immunoblots showing interactions between FLAG-tagged SK2 and the HA-tagged IC-2 polypeptides. HEK293 cells were transfected with HA-tagged IC-2 fragments alone (IC-2), or in combination with FLAG-tagged SK2 (IC-2 + SK2). SK2 was immunoprecipitated from lysates with anti-FLAG antibodies, and co-immunoprecipitated IC-2 fragments were detected by immunoblot with anti-HA antibodies (left panels). Green bars indicate successful co-immunoprecipitation of each HA-tagged IC-2 fragment with FLAG-tagged SK2. Expression levels of full-length IC-2 or truncations in the lysates are shown in the middle panels (Lysates (HA)), and equal expression of SK2 in the co-transfected lysates is shown in the panels on the right (Lysates (FLAG)). Images shown are representative of 3-4 independent experiments.

interact with both regions of the IC-2 protein, again albeit slightly more with the N-terminal fragment given there was less input protein compared to the C-terminal 251-612 fragment (Figure 5.2). Therefore, from these data, identifying the SK2 binding site on IC-2 is inconclusive.

Another possibility is that, because dynein is a large complex made up of various dimeric subunits, the N-terminal fragments of IC-2 expressed in cells may be dimerising with endogenous full-length IC-2 protein (the dimerisation domain is within residues 151-211 of IC-2C (Lo et al, 2006); Figure 5.2), and the C-terminal mutants may still interact with the dynein HC. Therefore, SK2 may still be binding to a full-length endogenous IC-2 and consequently appear to be interacting with any truncated mutants that are also bound within the complex. So, in order to examine the direct binding of SK2 with IC-2 and the truncation mutants, *in vitro* recombinant pull-down assays were employed. Initially, SK2 was overexpressed in HEK293 cells and immunoprecipitated under stringent conditions such that no other proteins were bound (Figure 5.3A), and full-length IC-2 was generated in bacteria and purified (Figure 5.3B). The SK2-bound beads were then incubated with recombinant IC-2 to determine if an interaction would occur. However, no discernible level of IC-2 could be detected from the SK2-bead complexes (Figure 5.3C). Therefore, although these recombinant pull-downs are preliminary and would need to be repeated and optimized, these data may suggest that SK2 and IC-2 require more physiological conditions to form an interaction (i.e. in intact cells), or that the interaction may require other subunits of the dynein complex and/or the dynactin complex.

5.5.2 SK2 interacts more strongly with the IC-1 intermediate chain isoform

There are two mammalian cytoplasmic dynein IC isoforms, the ubiquitously expressed IC-2 identified in the yeast two-hybrid, and IC-1 which is expressed most abundantly in the brain (Horikawa et al, 2001; Kuta et al, 2010; Myers et al, 2007). Thus, the interaction of SK2 with IC-1 was also examined by co-immunoprecipitation. Interestingly, compared to IC-2, the interaction between SK2 and IC-1 was more prevalent (Figure 5.4), potentially suggesting an important isoform-specific role for IC-1 in interacting with SK2.

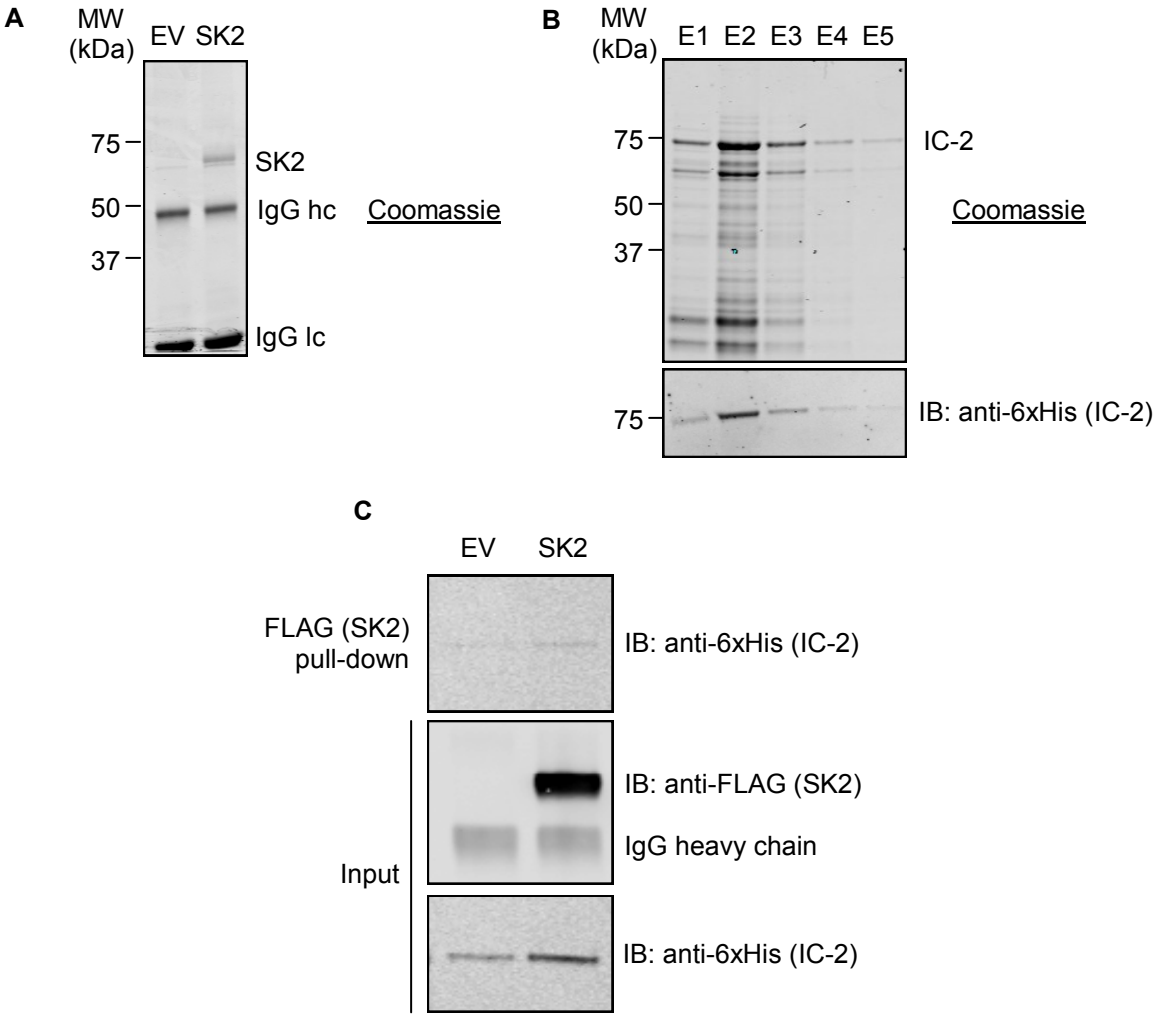


Figure 5.3: SK2 and IC-2 do not appear to directly interact *in vitro*

A, HEK293 cells were transfected with FLAG-tagged SK2 or empty vector. SK2 was immunoprecipitated from lysates with FLAG antibody-conjugated agarose beads, and protein bound to beads was then subjected to SDS-PAGE and stained with coomassie blue. *B,* His-tagged recombinant IC-2 made in bacteria was purified using nickel-sepharose, and was then repeatedly eluted from the beads (E1-E5). Samples from each elution were subjected to SDS-PAGE and either stained with coomassie blue, or analysed by immunoblot with anti-His antibodies. *C,* HEK293 cells were transfected with FLAG-tagged SK2 or empty vector. SK2 was immunoprecipitated from lysates with FLAG antibody-conjugated agarose beads, and SK2-bound beads were then incubated with recombinant His-tagged IC-2. Proteins bound to the beads were then subjected to SDS-PAGE, and any IC-2 bound was detected by immunoblot with anti-His antibodies. The presence or absence of SK2 pulled-down by the beads was confirmed by immunoblot with anti-FLAG antibodies, and samples of recombinant IC-2 equivalent to those added to the beads were also analysed by immunoblot using anti-His antibodies (input). Experiment was only performed once; hc = heavy chain; lc = light chain.

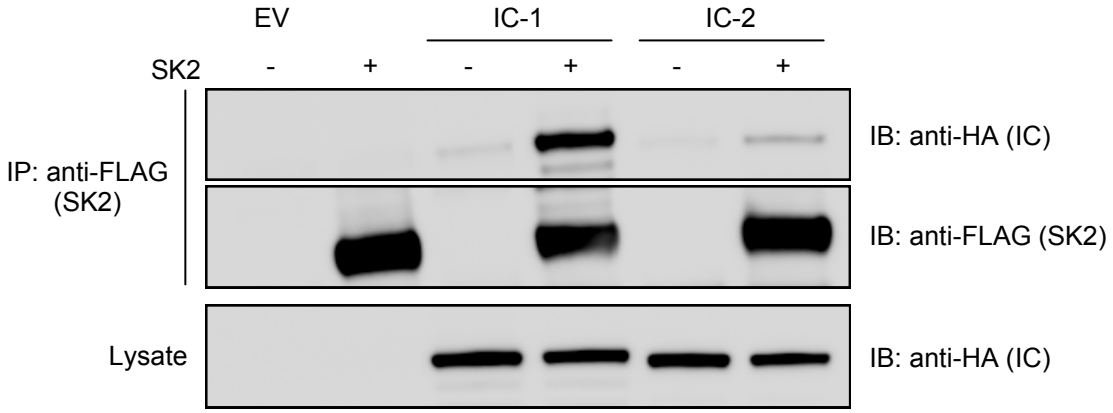


Figure 5.4: SK2 interacts more strongly with the dynein IC-1 isoform

HEK293-c18 cells were transfected with empty vector (EV) or a vector encoding FLAG-tagged SK2, either alone or in combination with vectors encoding HA-tagged IC-1 or HA -tagged IC-2. Lysates were pre-cleared with Protein G µbeads. SK2 was immunoprecipitated from cell lysates with anti-FLAG antibodies, and co-immunoprecipitated IC was detected by immunoblotting with anti-HA antibodies. Expression levels of IC in the lysates were confirmed by immunoblotting with anti-HA antibodies (*Lysate*). Immunoprecipitates were also probed with anti-FLAG antibodies to confirm pull-down of SK2. Blots shown are representative of three independent experiments.

5.5.3 The interaction between SK2 and dynein is physiological

Next, it was important to move to an endogenous system to confirm that the SK2-IC interaction is physiologically relevant. As such, endogenous SK2 was immunoprecipitated from mouse brain lysate, which demonstrated a clear co-immunoprecipitation of endogenous dynein ICs with SK2 (Figure 5.5). Given that the ICs are found physiologically as dimeric subunits of the dynein complex, the presence of these proteins within the SK2 immunocomplexes was examined. Indeed, this showed an association of SK2 with the dynein light intermediate chain (LIC) subunit, as well as the p150 subunit from the dynactin complex (Figure 5.5), a regulatory complex that plays an important role in facilitating almost all dynein functions (Schroer, 2004). Therefore, it would appear that SK2 can physiologically interact with the dynein complex.

Immunofluorescence staining of endogenous SK2 and dynein ICs in HEK293 cells was then performed to examine their localisation, and consistent with their physiological interaction, both proteins showed considerable subcellular co-localisation which was mainly at cytoplasmic and peri-nuclear regions (Figure 5.6A). Further confirmation of the physiological interaction of endogenous SK2 and dynein ICs was also obtained by *in situ* proximity ligation assays (PLAs) which demonstrated a clear SK2-IC interaction, again, mainly at cytoplasmic and peri-nuclear regions (Figure 5.6B). Appropriate single antibody controls were used to confirm that detection of both proteins together was required to produce the PLA signals (Figure 5.6B). Taken together, these data demonstrate that SK2 interacts with dynein IC as a part of the physiological dynein complex in cells.

5.5.4 SK2 subcellular localisation is regulated by dynein

The interaction and subcellular localisation of SK2-IC complexes is consistent with the role of dynein in transporting its cargo in a retrograde direction along microtubules, which eventually accumulate at the MTOC located in close proximity to the nucleus (Barker et al, 2016; Kardon & Vale, 2009). Given that SK2 appears to co-localise with dynein IC mainly in peri-nuclear regions of the cell, it was of interest to determine if dynein is involved in the retrograde-directed transport of SK2. To examine this, siRNA-mediated knockdown of IC-2 was performed using HeLa cells, as knockdown of this single subunit has been shown to

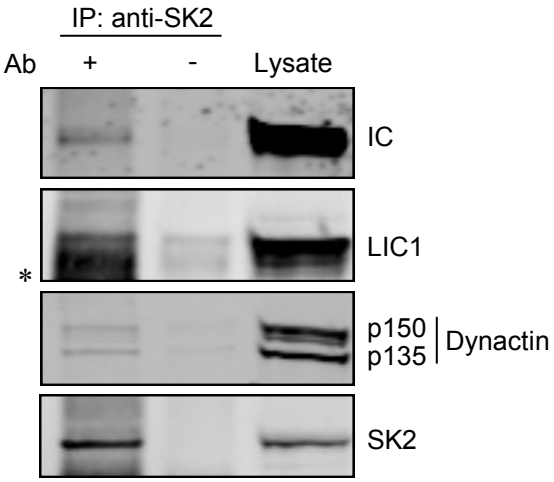


Figure 5.5: Endogenous SK2 interacts with dynein and dynactin complexes in mouse brain

SK2 was immunoprecipitated from whole mouse brain lysate using anti-SK2 antibodies (ECM Biosciences). Co-immunoprecipitated dynein intermediate chains (IC), light intermediate chain 1 (LIC-1) and dynactin p150 were detected by immunoblotting with anti-IC, anti-LIC1 and anti-dynactin p150 antibodies, respectively. Expression levels of these proteins in the mouse brain lysate were confirmed by immunoblot analyses with their respective antibodies (*Lysate*). Lysates and immunoprecipitates were also probed with anti-SK2 antibodies to confirm expression and pull-down of SK2. Asterisk designates the heavy chain IgG band. Blots shown are representative of three independent experiments.

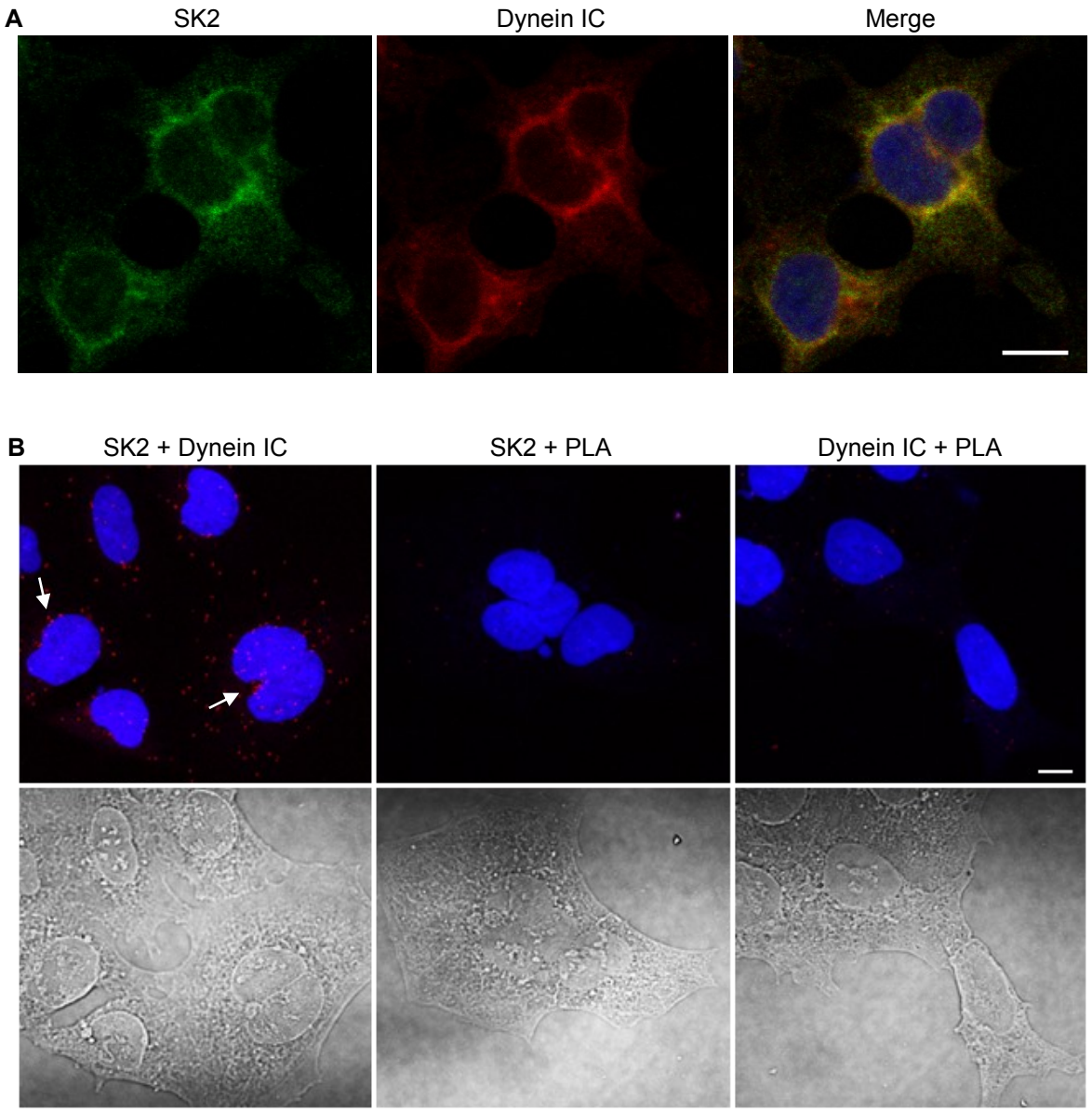


Figure 5.6: Co-localisation of endogenous SK2 and IC

A, Immunofluorescence staining and confocal microscopy demonstrating co-localisation of SK2 and dynein IC in HEK293 cells grown in 10% serum. SK2 (green) was detected using anti-SK2 antibodies (1:300; ECM Biosciences) and dynein IC (red) was detected using anti-IC antibodies (1:300; Millipore). Nuclei were stained with DAPI (blue). Images are representative of at least 100 cells, from three independent experiments. Scale bar = 10 μm. *B,* Immunofluorescence staining and confocal microscopy demonstrating direct interactions between SK2 and dynein IC, using the Duolink® *in situ* PLA system with anti-SK2 (1:300; ECM Biosciences) and anti-IC antibodies (1:300; Millipore) in HEK293 cells grown in 10% serum (top panels). Each red dot indicates a single direct interaction, and arrows denote peri-nuclear clusters of the interacting proteins. Nuclei were stained with DAPI (blue). Differential interference contrast images are also shown (bottom panels). Images are representative of at least 100 cells, from three independent experiments. Scale bar = 10 μm.

disrupt the dynein complex and its functions, and HeLa cells contain no detectable IC-1 to compensate for the loss of IC-2 protein (Palmer et al, 2009). Interestingly, low levels of IC-1 mRNA could be detected in HeLa cells (Figure 5.7A), but no IC protein was detectable by immunoblot with pan-IC antibodies upon IC-2 knockdown (Figure 5.7B), suggesting that IC-1 is not translated, and thus, not functional in these cells.

Consistent with the localisation of endogenous SK2 in HEK293 cells, FLAG-tagged SK2 overexpressed in HeLa cells appeared to have a predominantly peri-nuclear localisation, which again seemed to co-localise with endogenous IC-2 (Figure 5.8A). Upon RNAi-mediated knockdown of IC-2, the peri-nuclear localisation of SK2 was lost in the majority of cells (Figure 5.8B and C), and interestingly, strong plasma membrane localisation of SK2 was observed upon IC-2 knockdown in a subset of cells (Figure 5.8B and D). Therefore, these data suggest that SK2 is transported as a dynein cargo in a retrograde direction, as disruption of dynein through the loss of IC-2 results in a loss of peri-nuclear SK2 and, in some cases, an accumulation of SK2 at the cell periphery.

5.5.5 Dynein IC-1 is downregulated in glioblastoma multiforme and correlates with poor patient survival

At the plasma membrane, S1P produced by the SKs can be exported from the cell where it can act on a family of five S1P receptors to promote cell survival and proliferation, as well as contribute to oncogenic signalling pathways (Pitson, 2011; Pyne & Pyne, 2010). This role is well established for SK1 (Pitson et al, 2005), and I have now demonstrated a role for SK2 in mediating oncogenesis, which coincided with increased localisation of SK2 at the plasma membrane and increased extracellular S1P formation (Chapter 4). Therefore, it was intriguing to observe an increase in plasma membrane-localised SK2 upon dynein IC knockdown, raising the question of whether downregulation of the dynein ICs could contribute to an oncogenic phenotype.

To explore this, a broad gene expression analysis of both IC-1 and IC-2 was performed on a panel of different human cancers, using public gene expression data sets available from the Oncomine database (Rhodes et al, 2004). Strikingly, IC-1 was found to be significantly

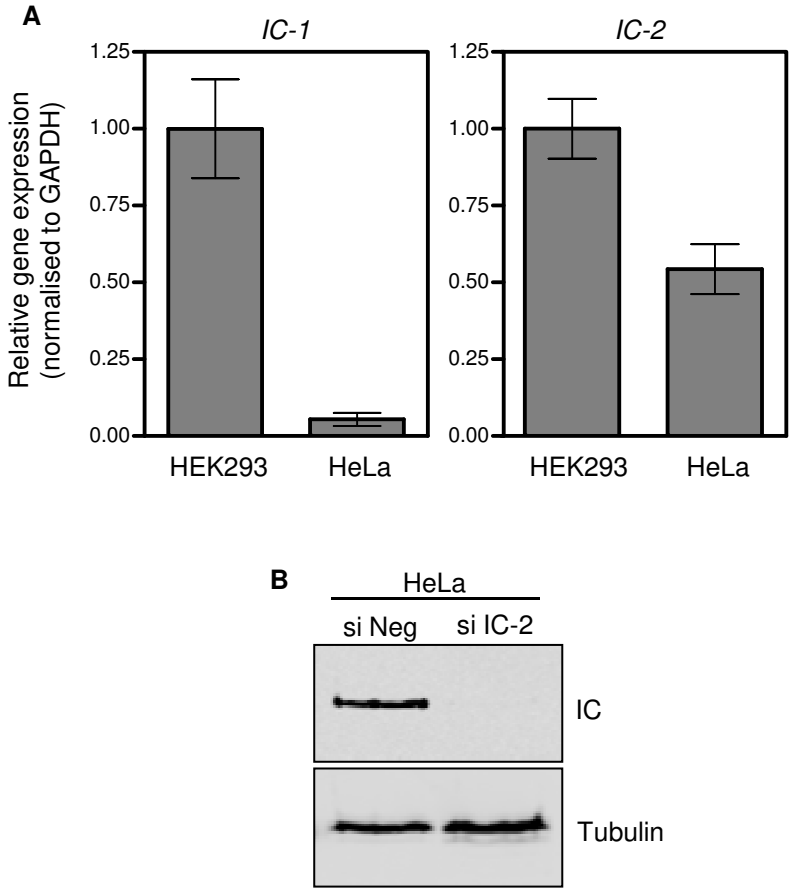


Figure 5.7: Expression of IC-1 in cultured human cell lines

A, Relative IC-1 and IC-2 mRNA levels in HEK293-c18 and HeLa cells, as determined by qRT-PCR (comparative quantitation, normalised to GAPDH). Graphs are plotted as mean (± SEM) of data from three independent experiments. *B,* Immunoblot analyses of pan dynein IC protein expression in lysates from HeLa cells transfected with negative control siRNA (si Neg) or IC-2 siRNA (si IC-2) for 48 hr, using anti-IC (Millipore) antibodies. Immunoblotting for α-tubulin was performed as a loading control. Blots shown are representative of two independent experiments.

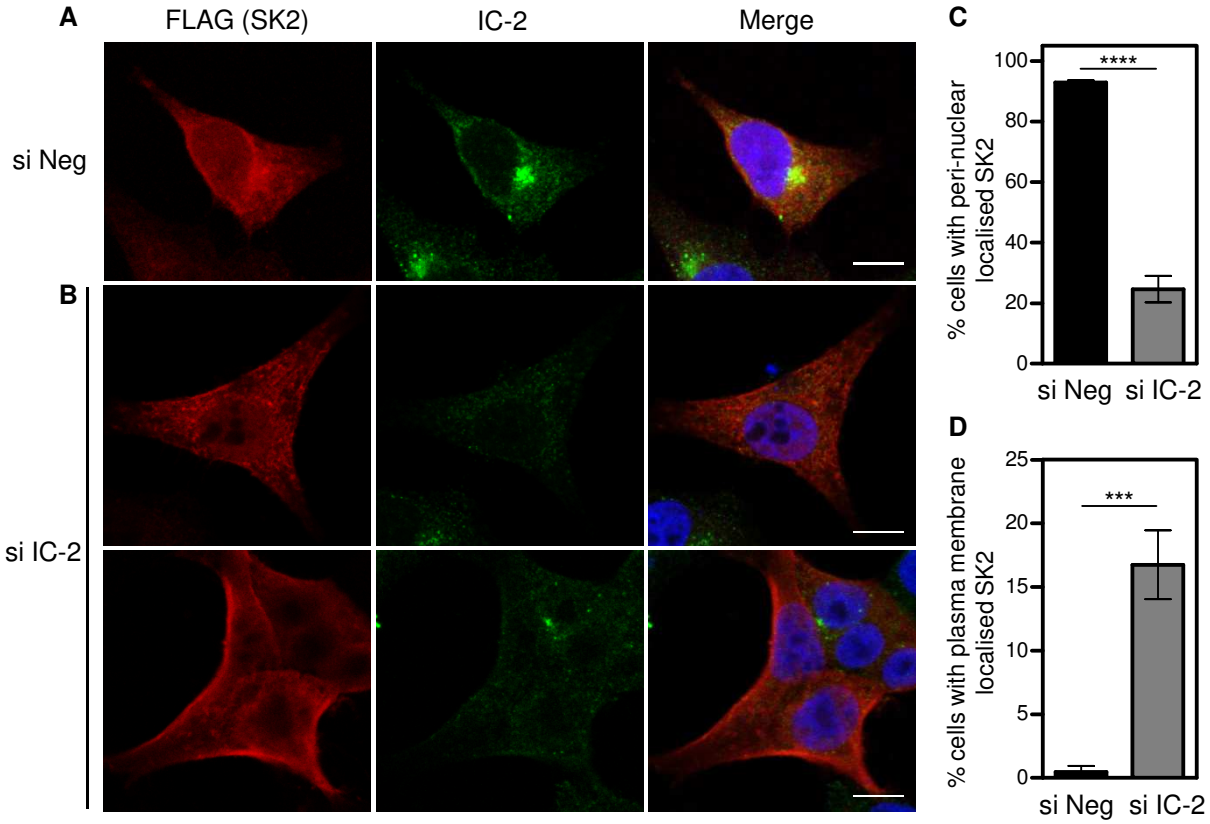


Figure 5.8: Dynein regulates the subcellular localisation of SK2

Immunofluorescence staining and confocal microscopy of HeLa cells transfected with FLAG-tagged SK2 and either (**A**) negative control siRNA (si Neg) or (**B**) IC-2 siRNA (si IC-2) for 48 hr. SK2 (red) was detected using anti-FLAG antibodies (1:4000; Sigma) and IC-2 (green) was detected using anti-IC-2 antibodies (1:250; Abcam). Nuclei were stained with DAPI (blue). Images are representative of at least 200 cells observed from three independent experiments. Scale bar = 10µm. (**C,D**) Cells described above were visualised by confocal microscopy and scored based on the presence or absence of either (C) distinct peri-nuclear FLAG-tagged SK2 staining, or (D) plasma membrane (PM)-localised FLAG-tagged SK2 staining. A minimum of 200 cells were scored per treatment. Data were graphed as mean (± SD) of triplicate wells from a single experiment, and were representative of three independent experiments (*** p<0.001; **** p<0.0001; Student's unpaired two-tailed t-test).

downregulated, compared to the corresponding normal tissues, in a vast range of different cancers, including brain, ovarian, bladder, prostate, colon, breast, uterine, cervical and lymphoma (Figure 5.9A). Importantly, IC-2 gene expression levels in these cancer data sets were generally unaltered, compared to the corresponding normal tissues (Figure 5.10), demonstrating that there is no substantial compensation for IC-1 loss across these cancer types. As IC-1 expression is found to be highest in brain tissues [Figure 5.9A and (Horikawa et al, 2001; Myers et al, 2007)], it was particularly notable that a dramatic 17-fold downregulation in IC-1 expression occurred in glioblastoma multiforme (GBM) patient samples compared to normal brain tissue (Figure 5.9B). Interestingly, this was not specific to a particular GBM subtype, as IC-1 expression was similarly downregulated in all four subtypes of GBM (Verhaak et al, 2010), whereas IC-2 expression was unchanged across all (Figure 5.11). Kaplan-Meier survival analysis of patients with GBM also demonstrated that low expression of IC-1 correlated with poorer overall survival compared to patients with high IC-1 expression (Figure 5.9C). Together, these analyses suggest that IC-1 may play a tumour suppressor role in cancer, and particularly in GBM.

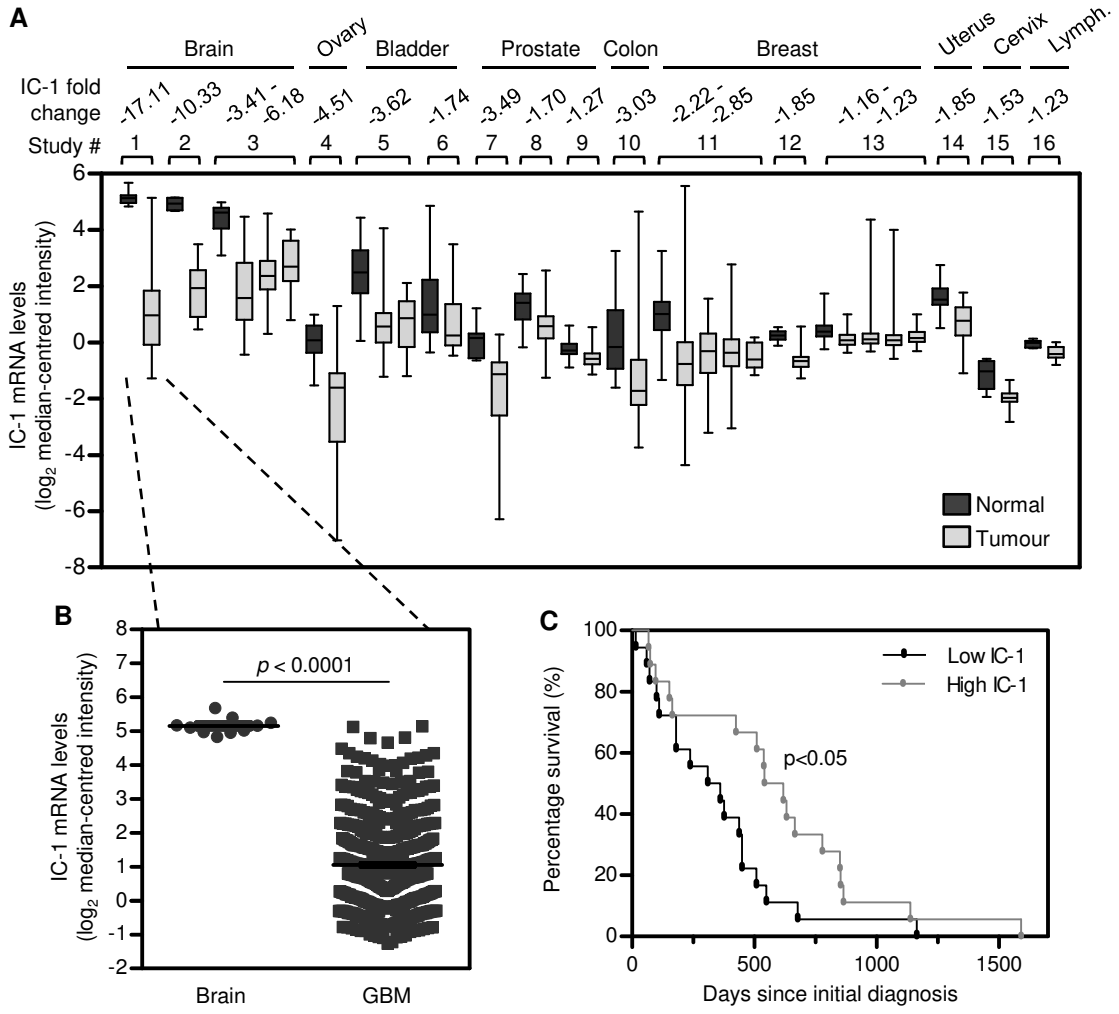
5.5.6 Decreased IC-1 expression levels in U-251 cells correlates with plasma membrane localisation of SK2

To examine the mechanisms behind the potential role of IC-1 as a tumour suppressor in GBM, the expression of IC-1 was initially examined in commonly used GBM cell lines, U-87 and U-251. Notably, U-251 cells had significantly lower levels of IC-1 mRNA (Figure 5.12A) and protein (Figure 5.12B) compared with U-87 cells, whereas SK2 protein levels were largely unchanged. Therefore, using these cell lines with varying levels of IC-1, the role of this protein in regulating the subcellular localisation of SK2 in GBM was examined.

Since I observed SK2 localised to the plasma membrane with IC-2 knockdown (Figure 5.8B and D), I reasoned that the low levels of IC-1 observed in the U-251 cells may result in increased and/or prolonged localisation of SK2 at the plasma membrane. Indeed, examining the localisation of endogenous SK2 by immunofluorescence staining demonstrated prominent plasma membrane localisation of SK2 in U-251 cells (Figure 5.12C and D). In contrast, SK2 was not detected at the plasma membrane in U-87 cells, consistent with higher levels of IC-1 in these cells (Figure 5.12C and D) and further suggesting a role for

Figure 5.9: Dynein IC-1 is downregulated in GBM, which correlates with poorer patient survival

A, Box plots showing human cancers with significant ($p<1x10^{-4}$) downregulation of IC-1 mRNA levels. Data were extracted from the Oncomine database, from the following studies (left to right): 1, TCGA Brain (Glioblastoma); 2, Murat Brain (Glioblastoma); 3, Sun Brain (Glioblastoma, Oligodendroglioma, Anaplastic Astrocytoma); 4, Yoshihara Ovarian (Ovarian Serous Adenocarcinoma); 5, Sanchez-Carbayo Bladder 2 (Infiltrating Bladder Urothelial Carcinoma, Superficial Bladder Cancer); 6, Lee Bladder (Superficial Bladder Cancer); 7, Welsh Prostate (Prostate Carcinoma); 8, Lapointe Prostate (Prostate Carcinoma); 9, Taylor Prostate 3 (Prostate Carcinoma); 10, TCGA Colorectal (Colon Adenocarcinoma); 11, TCGA Breast (Invasive Ductal Breast Carcinoma, Invasive Lobular Breast Carcinoma, Invasive Breast Carcinoma, Mixed Lobular and Ductal Breast Carcinoma); 12, Finak Breast (Invasive Breast Carcinoma Stroma); 13, Curtis Breast (Mucinous Breast Carcinoma, Invasive Lobular Breast Carcinoma, Invasive Ductal Breast Carcinoma, Tubular Breast Carcinoma); 14, Crabtree Uterus (Uterine Corpus Leiomyoma); 15, Pyeon Multi-cancer (Cervical Cancer); 16, Compagno Lymphoma (Activated B-Cell-Like Diffuse Large B-Cell Lymphoma). *B,* TCGA brain dataset showing 17-fold downregulation of IC-1 mRNA levels in human glioblastoma multiforme (GBM) patients, compared with levels in normal brain tissue. Significance ($p<0.0001$) was determined using a Student's unpaired two-tailed t-test. (**C**) IC-1 mRNA expression levels and survival data from human GBM patients were obtained from the REMBRANDT dataset, and Kaplan-Meier survival curves were plotted for patients in the bottom (low IC-1) and top (high IC-1) 10% of IC-1 expression levels. Significance ($p<0.05$) was determined using a two-sided log-rank test.


A
Brain
Ovary
Bladder
Prostate
Colon
Breast
Uterus
Cervix
Lymph.
IC-1 fold change
-17.11
-10.33
-3.41 - -6.18
-4.51
-3.62
-1.74
-3.49
-1.70
-1.27
-3.03
-2.22 - -2.85
-1.85
-1.16 - -1.23
-1.85
-1.53
-1.23
Study #
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
IC-1 mRNA levels (log2 median-centred intensity)
Normal
Tumour
B
p < 0.0001
IC-1 mRNA levels (log2 median-centred intensity)
Brain
GBM
C
Low IC-1
High IC-1
p<0.05
Percentage survival (%)
Days since initial diagnosis

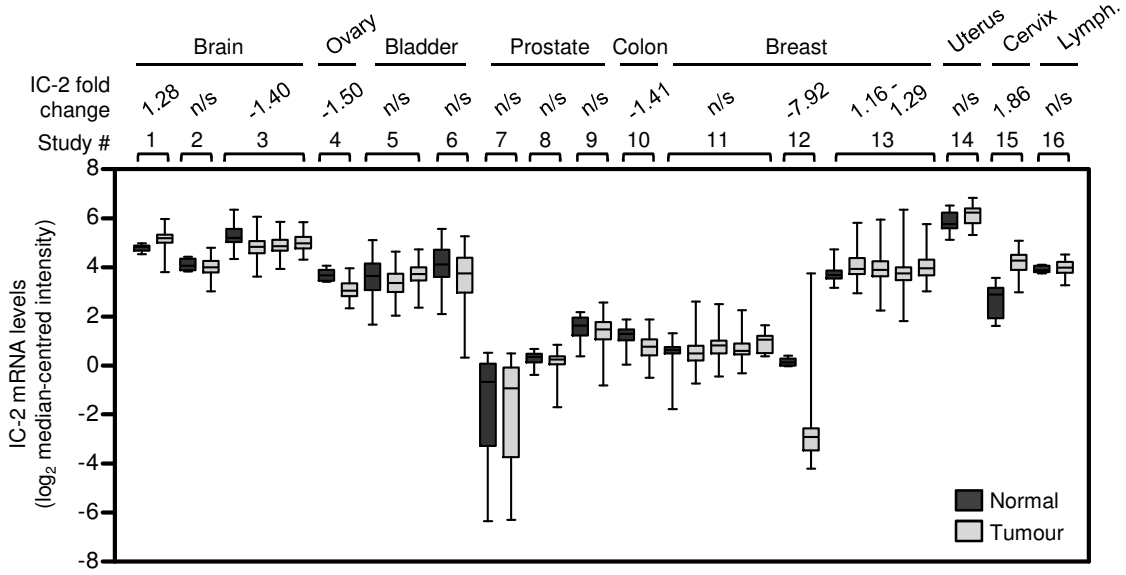


Figure 5.10: IC-2 expression does not vary considerably in human cancer patient data sets where IC-1 is downregulated

Box plots showing human cancers with significant (p<1x10^{-4}) changes in IC-2 mRNA levels, as compared to normal tissues. Data were extracted from the Oncomine database (details of studies used are described in Figure 5.9). n/s denotes no significant change in gene expression compared to normal tissues.

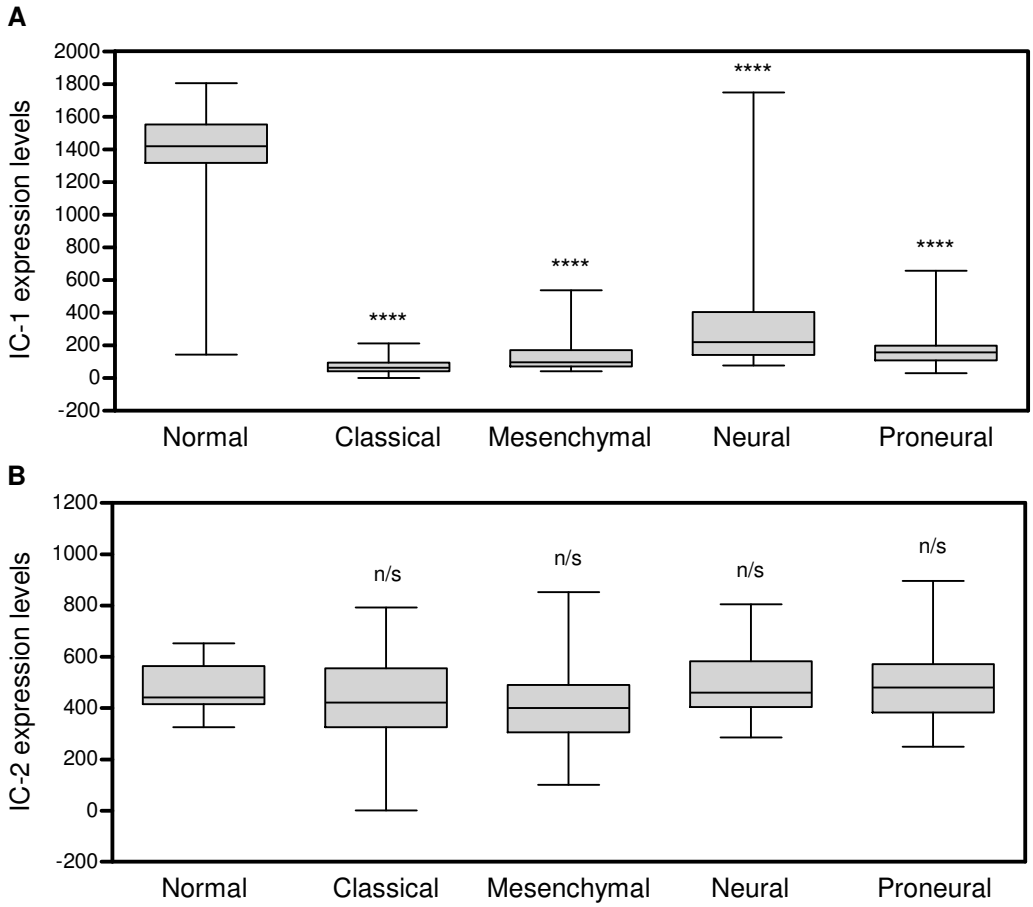
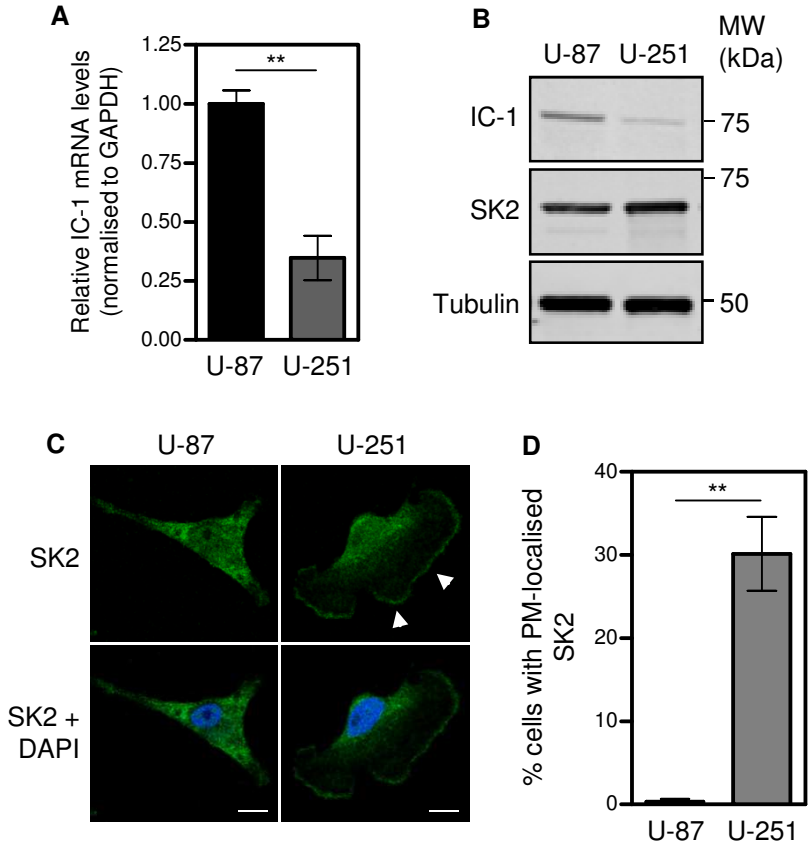


Figure 5.11: IC-1 is significantly downregulated in all GBM subtypes, whereas IC-2 expression is unchanged

Box plots showing (A) IC-1 and (B) IC-2 mRNA levels in human patient samples from the four GBM subtypes: Classical, Mesenchymal, Neural and Proneural, as compared to normal brain tissue. Data were extracted from the TCGA Glioblastoma data set, from the expression box plot (Affymetrix Human Exon 1.0 ST) platform available from the Project Betastasis database (**** p<0.0001; Student's unpaired two-tailed t-test; n/s denotes no significant change in gene expression compared to normal brain tissue).

Figure 5.12: Decreased IC-1 expression levels in U-251 cells correlates with plasma membrane localisation of SK2

A, Relative IC-1 and SK2 mRNA expression levels in U-87 and U-251 human GBM cell lines. Gene expression levels were determined by qRT-PCR (comparative quantitation, normalised to GAPDH). Graphs are plotted as mean (± SEM) of data from three independent experiments (* $p<0.05$; ** $p<0.01$; Student's unpaired two-tailed t-test). *B,* Immunoblot analyses of IC-1 and SK2 protein expression in U-87 and U-251 cell lines, using anti-IC-1 (Proteintech) and anti-SK2 (Proteintech) antibodies. Immunoblotting for α-tubulin was performed as a loading control. Blots are representative of three independent experiments. *C,* Immunofluorescence staining and confocal microscopy showing endogenous SK2 localisation in U-87 and U-251 GBM cells. SK2 (green) was detected using anti-SK2 antibodies (1:300; ECM Biosciences), and nuclei were stained with DAPI (blue). Arrows denote distinct plasma membrane localisation of SK2. Scale bar = 10 μm. *D,* Cells described above were visualised by confocal microscopy and scored based on the presence or absence of distinct plasma membrane (PM)-localised SK2 staining. A minimum of 200 cells were scored per cell type. Data were graphed as mean (± SEM) from three independent experiments (** $p<0.01$; Student's unpaired two-tailed t-test).


A
Relative IC-1 mRNA levels (normalised to GAPDH)
1.25
1.00
0.75
0.50
0.25
0.00
**
U-87
U-251
B
U-87
U-251
MW (kDa)
IC-1
SK2
Tubulin
75
75
50
C
U-87
U-251
SK2
SK2 + DAPI
D
% cells with PM-localised SK2
40
30
20
10
0
**
U-87
U-251

IC-1 in regulating SK2 subcellular localisation. A comprehensive analysis of different GBM models deemed the U-251 cell line to more closely resemble human disease, at least in *in vivo* rodent xenograft models, compared with the U-87 cell line (Jacobs et al, 2011). In line with this, U-251 cells contain genetic alterations commonly associated with human GBM, such as loss-of-function mutant p53 and overexpression of the epidermal growth factor receptor (EGFR) (Verhaak et al, 2010), that are not present in U-87 cells (Jacobs et al, 2011). Hence, U-251 cells were utilized to examine the interplay between plasma-membrane localised SK2 and lower IC-1 expression.

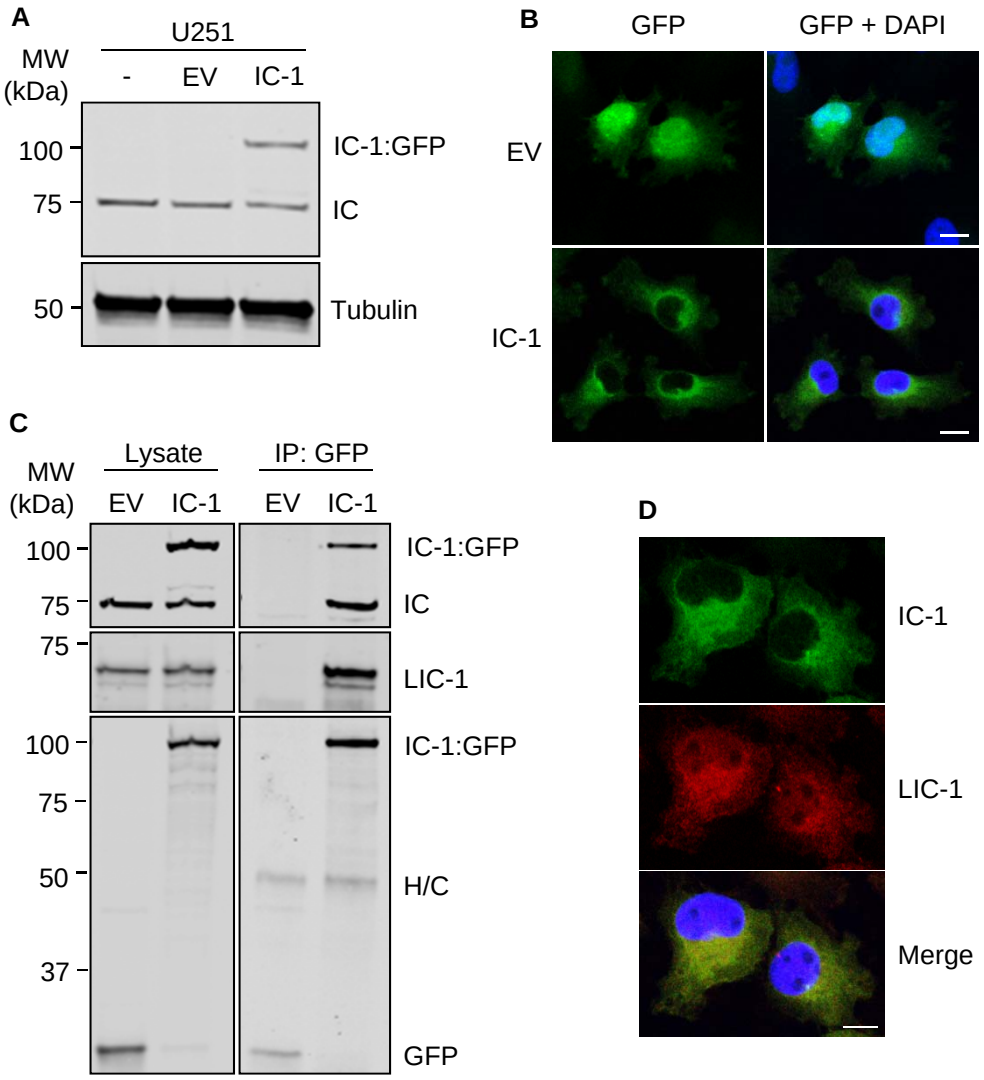
5.5.7 Re-expression of IC-1 in U-251 cells results in reduced plasma membrane localisation of SK2 and decreased extracellular S1P formation

Next, I examined whether re-expression of IC-1 in U-251 cells was sufficient to reduce SK2 localisation at the plasma membrane, and whether this would in turn reduce the oncogenic potential of these cells. Other studies have reported stable expression of GFP-fused IC-1 and have shown that it is still able to incorporate functionally into the dynein complex (Ha et al, 2008; Zhang et al, 2002). As such, I generated U-251 cells with stable, low-level expression of IC-1:GFP, or GFP alone, and confirmed the expression of the fusion protein to be similar to the level of endogenous dynein IC by immunoblot (Figure 5.13A). Furthermore, as shown in Figure 5.13B, the IC-1:GFP fusion protein had a largely cytoplasmic and peri-nuclear localisation pattern, similar to endogenous dynein IC (Figure 5.6A and Figure 5.8A). To confirm that IC-1:GFP was incorporating into endogenous dynein complexes, I immunoprecipitated IC-1:GFP from U-251 cell lysates using its GFP-tag, and was able to show successful pull-down of endogenous dynein IC and LIC-1 subunits (Figure 5.13C). Furthermore, immunofluorescence staining of LIC-1 in the IC-1:GFP-expressing U-251 cells demonstrated a high level of co-localisation between LIC-1 and IC-1:GFP (Figure 5.13D). Therefore, IC-1:GFP expressed in U-251 cells was successfully forming dimers with endogenous IC and incorporating into endogenous dynein complexes.

I next examined the localisation of SK2 in the U-251 GBM cells re-expressing IC-1:GFP. Strikingly, where the GFP control cells had prominent plasma membrane localisation of SK2 as seen previously, this localisation of SK2 was significantly decreased in cells overexpressing IC-1:GFP (Figure 5.14A and B). Concomitant with a loss of SK2 at the

Figure 5.13: Low-level exogenous IC-1:GFP incorporates into endogenous dynein complexes

A, Lysates from parental U-251 cells, or U-251 stable cell lines expressing GFP alone (empty vector; EV) or IC-1:GFP, were subjected to immunoblot analyses with antibodies against dynein IC and α-tubulin. Blots shown are representative of three independent experiments. *B,* U-251 cells stably expressing GFP (EV) or IC-1:GFP were imaged using confocal microscopy, and exogenous proteins were visualised via their GFP tag (green). Nuclei were stained with DAPI (blue). Images are representative of more than 100 cells, from three independent experiments. Scale bar = 10 μm. *C,* Lysates were prepared from U-251 cells stably expressing GFP alone (EV) or IC-1:GFP, and these exogenous proteins were then immunoprecipitated using anti-GFP antibodies. Co-immunoprecipitated endogenous dynein intermediate chains (IC) and light intermediate chain 1 (LIC-1) were detected by immunoblotting with anti-IC and anti-LIC1 antibodies, respectively. Expression levels of these proteins in the lysates were also confirmed by immunoblotting with their respective antibodies (*Lysate*). Lysates and immunoprecipitates were probed with anti-GFP antibodies to confirm expression and pull-down of IC-1:GFP or GFP. Blots shown are representative of three independent experiments. *D,* Co-localisation of IC-1:GFP and endogenous dynein light intermediate chain 1 (LIC-1) in the U-251 stable cell line was demonstrated by confocal microscopy. IC-1:GFP was visualised via its GFP tag (green) and LIC-1 via immunofluorescence staining of the endogenous protein using anti-LIC1 antibodies (red; 1:250; Abcam). Nuclei were stained with DAPI (blue). Images are representative of more than 100 cells, from three independent experiments. Scale bar = 10 μm.


A
U251
MW
(kDa)
-
EV
IC-1
100
75
50
IC-1:GFP
IC
Tubulin
B
GFP
GFP + DAPI
EV
IC-1
C
Lysate
IP: GFP
MW
(kDa)
EV
IC-1
EV
IC-1
100
75
75
100
75
50
37
IC-1:GFP
IC
LIC-1
IC-1:GFP
H/C
GFP
D
IC-1
LIC-1
Merge

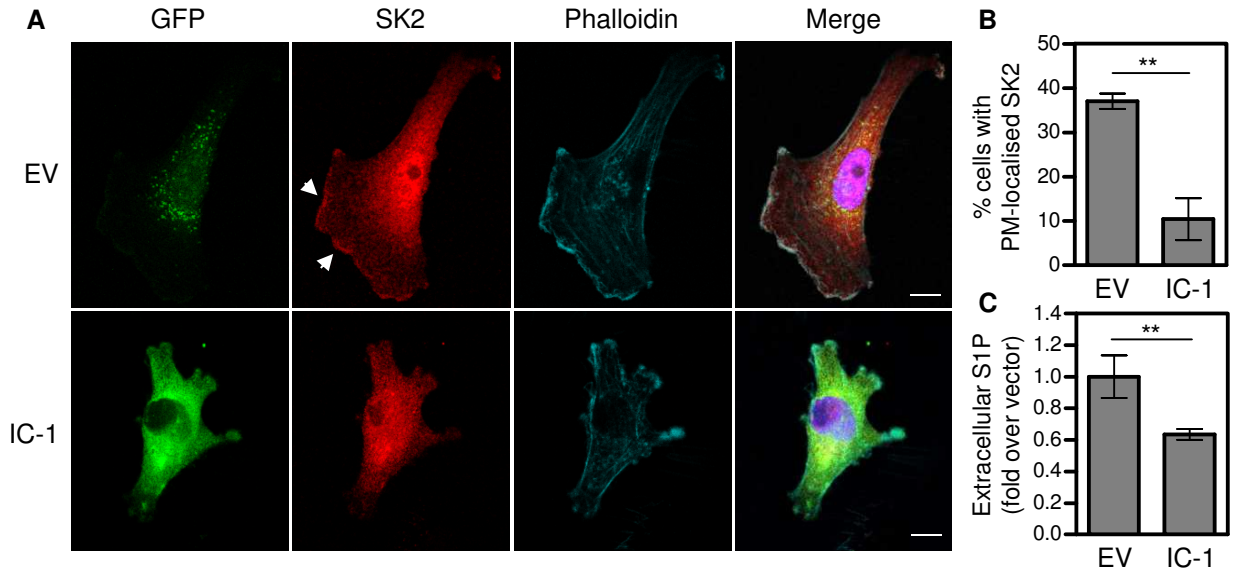


Figure 5.14: Expressing IC-1:GFP in U-251 cells reduces plasma membrane-localised SK2 and extracellular S1P production

A, Localisation of endogenous SK2 in U-251 cells stably expressing GFP alone (empty vector; EV) or IC-1:GFP was examined by confocal microscopy. IC-1:GFP or GFP were visualised via their GFP tag (green) and SK2 via immunofluorescence staining of the endogenous protein using anti-SK2 antibodies (red; 1:300; ECM Biosciences). F-actin was stained using Phalloidin (cyan) to accentuate the cell membrane, and nuclei were stained with DAPI (blue). Arrows denote distinct plasma membrane localisation of SK2. Images are representative of more than 100 cells, from three independent experiments. Scale bar = 10 µm. *B,* Cells described above were visualised by confocal microscopy and scored based on the presence or absence of distinct plasma membrane (PM)-localised SK2 staining. A minimum of 200 cells were scored per cell line. Data were graphed as mean (± SEM) from three independent experiments (** $p<0.01$; Student's unpaired two-tailed t-test). *C,* Rate of extracellular S1P formation was determined from intact U-251 cells stably expressing GFP (EV) or IC-1:GFP, and was normalised to cell number. Analyses were performed in quadruplicate and data are graphed as mean (± SD; ** $p<0.01$; Student's unpaired two-tailed t-test).

plasma membrane, there was also a significant reduction in extracellular S1P formation in the U-251 cells expressing IC-1:GFP compared with control GFP expressing cells (Figure 5.14C). A similar reduction in extracellular S1P levels was observed even in the presence of the highly selective SK1 inhibitor, PF543 (Figure 5.15), confirming that a loss of SK2, and not SK1, from the plasma membrane was responsible for this phenotype. Therefore, collectively these data demonstrate that re-expression of IC-1 in U-251 GBM cells can reinstate dynein-mediated translocation of SK2 away from the plasma membrane, which may be dampened as a result of IC-1 downregulation.

5.5.8 Re-expression of IC-1 in U-251 cells reduces neoplastic growth *in vitro* and *in vivo*

As plasma membrane-localised SK can promote oncogenesis (Pitson et al, 2005; Takabe & Spiegel, 2014), it was intriguing to hypothesize that a loss of SK2 from the plasma membrane, and a consequent decrease in extracellular S1P formation, in U-251 cells re-expressing IC-1 may reduce the oncogenic potential of these GBM cells. Indeed, the U-251 cells re-expressing IC-1 were found to have significantly attenuated neoplastic growth compared to control cells, as assessed by colony formation assays in soft agar (Figure 5.16A). To determine if this reduced oncogenic potential *in vitro* corresponded to a decrease in tumour growth *in vivo,* U-251 cells re-expressing IC-1 (IC-1:GFP), or control U-251 cells expressing GFP only, were subcutaneously engrafted into the flanks of NOD/SCID mice, and tumour growth was measured over time. Strikingly, re-expression of IC-1 decreased the growth of the tumours formed by U-251 cells by 50% compared to that seen with control cells (Figure 5.16B). The presence of IC-1:GFP in the excised tumours was confirmed by immunoblot analyses using GFP antibodies (Figure 5.16C).

Reduced tumour growth can be a consequence of a decrease in infiltrating blood vessels into the tumour, limiting nutrients and oxygen available to the tumour. Since extracellular S1P plays an important role in mediating angiogenesis and blood vessel infiltration into tumour tissue (Takabe & Spiegel, 2014), and given that I observed reduced extracellular S1P upon stable overexpression of IC-1 *in vitro*, tumour vascularisation was assessed by CD31 staining of tumour sections. Notably, there was a significant reduction in the number of

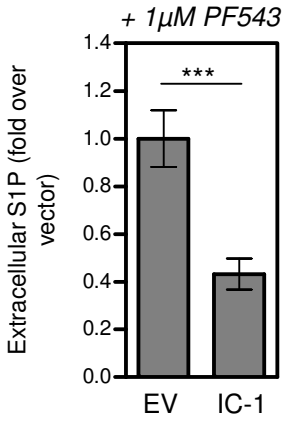


Figure 5.15: Decrease in extracellular S1P formation resulting from IC-1 overexpression is independent of SK1

Rate of extracellular S1P formation was determined from intact U-251 empty vector (EV) or IC-1:GFP stable cell lines incubated with 1 μM PF543 (added to the cells at the same time as 0.5 μCi of [^{3}H]-sphingosine) for 40 min. Analyses were performed in quadruplicate, data were normalised to cell number and graphed as mean (± SD; *** p<0.001; Student's unpaired two-tailed t-test).

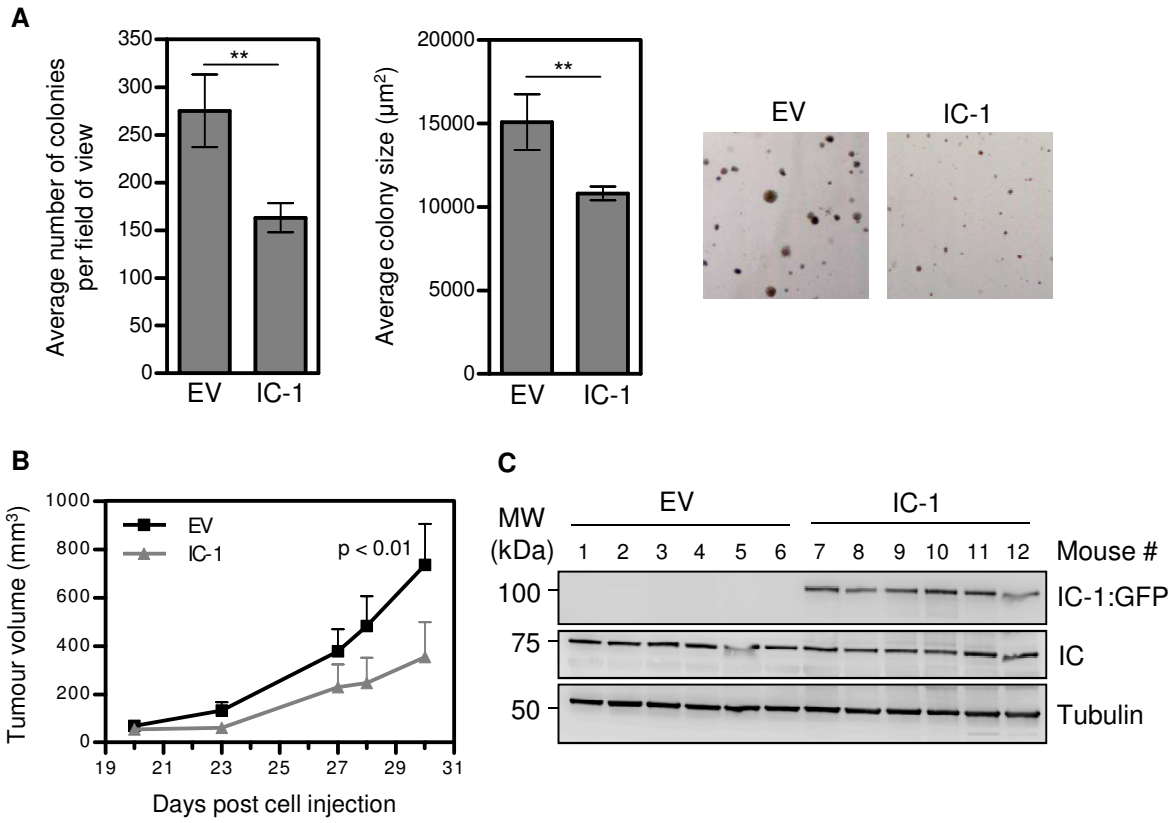


Figure 5.16: Re-expression of IC-1 in U-251 cells reduces neoplastic growth *in vitro* and *in vivo*

A, Anchorage-independent growth of U-251 cells stably expressing GFP alone (empty vector; EV) or IC-1:GFP was tested using colony formation assays in soft agar. Average colony numbers and size were quantified using ImageJ software, and are graphed as mean (± SD) from quadruplicate wells of one experiment, which was representative of three independent experiments (** $p<0.01$; Student's unpaired two-tailed t-test). Representative images of colonies from each group are shown. *B,* NOD/SCID mice were subcutaneously injected with U-251 cells stably expressing IC-1:GFP, or GFP alone (EV), and calliper measurements of palpable tumours were taken over 30 days. Mean tumour volumes are shown (± SEM; n = 6-7 per group). Statistical analysis on all data points was performed by two-way ANOVA. *C,* Equal amounts (30 µg) of total protein from the tumour tissue lysates were subjected to immunoblot analyses with antibodies against GFP, dynein intermediate chains (IC) and α-tubulin. Each lane represents a single tumour sample from one mouse.

CD31-positive vessels present in tumours formed from U-251 cells re-expressing IC-1, compared to the tumours from control U-251 cells (Figure 5.17).

5.6 Discussion

The roles and regulation of SK2 remain poorly understood. In this study I have identified that SK2 interacts with the cytoplasmic dynein complex, via dynein ICs, which transports SK2 in a retrograde direction away from the cell periphery, and thus regulates the subcellular localisation of SK2, which is known to be critical for the function of this signalling enzyme (Siow & Wattenberg, 2011). Furthermore, this newly discovered regulatory mechanism of SK2 appears to have implications in GBM, where I found that the expression of dynein IC-1 subunit is heavily downregulated, and correlates with poorer GBM patient survival. Indeed, my findings demonstrate an interplay between the downregulation of IC-1 in GBM and an increase in SK2 plasma membrane localisation, which was decreased by re-expression of IC-1, resulting in reduced GBM tumour growth *in vivo*. Overall, this work suggests that IC-1 can act as a tumour suppressor in GBM, and this role may involve the removal of SK2 from the plasma membrane to dampen extracellular S1P signalling.

It is well accepted that the subcellular localisation of the SKs, and hence the location of S1P production, can give rise to various somewhat opposing signalling outcomes (Pitson, 2011; Siow & Wattenberg, 2011). Therefore, the cellular functions of the SKs are largely dictated by their subcellular localisation, which has previously been shown to be regulated by various interacting proteins. The calcium and integrin-binding protein CIB1 mediates the translocation of SK1 to the plasma membrane to promote survival and proliferative signalling (Jarman et al, 2010; Zhu et al, 2017), however it has not been confirmed whether this mechanism also regulates the plasma membrane localisation of SK2. Translocation of the SKs to the plasma membrane has also been reported upon IgE-mediated cross-linking of the FcεRI receptor on mast cells, mediated by an interaction with the Src family kinases Lyn and Fyn (Olivera et al, 2006). However, prior to this study, very little was known about the regulation of SK2 to regions of the cell where it is known to promote cell cycle arrest and cell death, such as the nucleus, ER and mitochondria (Chipuk et al, 2012; Hait et al, 2009; Igarashi et al, 2003; Maceyka et al, 2005). Therefore, uncovering mechanisms by which

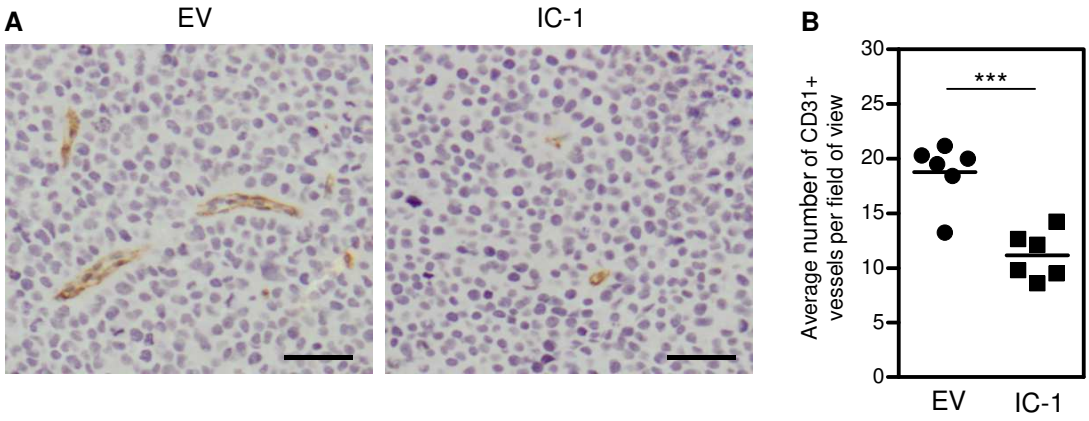


Figure 5.17: IC-1 re-expression in U-251 cells reduces tumour angiogenesis *in vivo*

A, Tumours derived from U-251 cells stably expressing IC-1:GFP, or GFP (empty vector; EV), were sectioned and CD31-positive vessels were examined by immunohistochemistry staining. Representative images for each group are shown. Scale bar = 100 μm. *B,* The number of CD31-positive vessels from tumour sections described in (A) was quantified and averaged from at least four random fields of view per tumour, at 20x magnification, and are graphed with means (n = 6 per group; *** p<0.001; Student's unpaired two-tailed t-test). Data was generated by Associate Professor Claudine Bonder (Centre for Cancer Biology, Adelaide).

SK2 is translocated within the cell, particularly toward these organelles, is critical in understanding how the opposing roles of SK2 are regulated. As such, the studies outlined here have begun to decipher these mechanisms by identifying dynein as a critical regulator of SK2 subcellular localisation, which may therefore control many of the functions of this enzyme.

GBM is the most common and aggressive primary malignant form of brain cancer in adults, where patients have a median survival of less than 15 months after diagnosis and a three-year survival rate of 3-5% (Polivka et al, 2017; Stupp et al, 2005). The current standard of care for GBM patients is surgical resection of the bulk of the tumour, followed by radiotherapy and administration of the chemotherapeutic agent temozolomide (TMZ) (Bush et al, 2017; Stupp et al, 2005). However, GBM remains incurable and there is clearly a desperate need for new targets and more effective treatments for GBM patients. SK inhibitors have been proposed as promising therapeutic agents to use in combination with chemotherapy and/or radiotherapy in GBM (Sordillo et al, 2016), as the role of the SKs in mediating the conversion of pro-apoptotic ceramide, which is produced as a result of these therapies, to pro-proliferative S1P has been implicated in the resistance of GBM tumour cells to death induced by these agents (Abuhusain et al, 2013; Carpinteiro et al, 2008). S1P levels are elevated in GBM tissue samples compared with normal grey matter (Abuhusain et al, 2013), and this bioactive lipid has been shown to mediate GBM cell proliferation and invasiveness (Van Brocklyn et al, 2002; Van Brocklyn et al, 2003). Furthermore, glioma stem-like cells (GSCs), which are thought to be the drivers of disease progression and relapse (Bao et al, 2006), were found to produce higher levels of extracellular S1P than GBM cells, and extracellular S1P was found to promote GSC proliferation and stemness (Marfia et al, 2014; Riccitelli et al, 2013).

The relative importance in GBM of the two SK isoforms that produce S1P is, however, somewhat unclear. While SK1 has been consistently found to be upregulated in GBM and its expression correlates with poor patient survival (Abuhusain et al, 2013; Bien-Moller et al, 2016; Van Brocklyn et al, 2005), highly selective SK1 inhibitors have had surprisingly inconsistent effects on reducing GBM cell proliferation and viability (Abuhusain et al, 2013; Kapitonov et al, 2009). The efficacy of SK2-selective inhibitors has not been examined in GBM, potentially because the role of SK2 in cancer is generally less well understood and, unlike SK1, evidence for an upregulation of SK2 in GBM is inconsistent (Abuhusain et al,

2013; Bien-Moller et al, 2016; Quint et al, 2014). Interestingly, however, RNAi-mediated knockdown of SK2 has been shown to reduce GBM cell proliferation and survival to a greater extent than SK1 knockdown (Van Brocklyn et al, 2005). Notably, SK2 is highly expressed in the brain (Liu et al, 2000); In fact, SK2 expression in the brain is considerably higher than SK1 expression and, as such, SK2 is responsible for the majority of S1P production here (Blondeau et al, 2007).

Here, I have demonstrated that the plasma membrane localisation of SK2 and contribution to extracellular S1P production in GBM cells is negatively regulated by dynein IC-1 (Figure 5.14), which is heavily downregulated in GBM (Figure 5.9B). These findings provide an explanation for why SK2 seemingly contributes to GBM cell proliferation and survival more so than SK1 (Van Brocklyn et al, 2005), despite SK2 expression not being upregulated in GBM. As SK2 can also promote ceramide production via a salvage pathway at the ER (Maceyka et al, 2005), it is perhaps quite logical that SK2 expression would not be upregulated in tissues where it is already highly expressed, such as the brain (Liu et al, 2000), but rather cancer cells would find mechanisms to shuttle SK2 away from cellular compartments where it is pro-apoptotic and toward compartments like the plasma membrane where it can be oncogenic (Neubauer et al, 2016). My work therefore highlights the potential importance of examining SK2 subcellular localisation, and not simply whether it is upregulated, in determining if pharmacological targeting of SK2 is likely to have therapeutic benefits in GBM.

Strikingly, I have also demonstrated that re-expression of dynein IC-1 in GBM cells resulted in reduced tumour growth *in vivo,* potentially due to decreased tumour-associated angiogenesis (Figure 5.16 and Figure 5.17). Drugs that target angiogenesis have emerged as promising anti-cancer therapeutics, and as such, the anti-angiogenic monoclonal vascular endothelial growth factor (VEGF) blocking antibody, bevacizumab (Avastin), has been approved for use in patients with recurrent GBM. Given the important role of extracellular S1P in mediating tumour-associated angiogenesis (Takabe & Spiegel, 2014), and in light of the fact that re-expression of dynein IC-1 resulted in reduced SK2-mediated extracellular S1P formation (Figure 5.14 and Figure 5.15), this suggests that targeting SK2 in GBM may have beneficial anti-angiogenic effects. Indeed, extracellular S1P produced by SK1 and secreted from GBM cells has been shown to mediate angiogenic signals and promote co-cultured endothelial cell sprouting (Abuhusain et al, 2013), and so it is possible that dual

SK1/SK2 inhibitors may be most beneficial at blocking the angiogenic effects of S1P in this cancer.

Another interesting concept to consider is whether the prominent plasma membrane localisation of SK2 observed in GBM cells is seen universally in other cancer types where IC-1 is found to be significantly downregulated. According to our broad gene expression analysis, IC-1 is significantly downregulated in many different human cancers (Figure 5.9), but notably, IC-1 expression levels were reduced in various subtypes of breast cancer across a number of datasets. SK2 has been previously shown to localise predominantly to the plasma membrane in MDA-MB-453 breast cancer cells, where it was found to be required for migration of these cells towards EGF (Hait et al, 2005). Furthermore, SK2 knockdown in MCF-7 breast cancer cells resulted in significantly reduced tumour growth in an *in vivo* xenograft model (Weigert et al, 2009). Therefore, it is tempting to speculate that the important interplay between SK2 and IC-1 observed in GBM may also occur in other cancers, including breast cancer, where loss of IC-1 is observed. However, given that IC-1 is only highly expressed in the brain, heart and skeletal muscle (Horikawa et al, 2001), it will remain to be determined whether the ubiquitous IC-2 isoform would compensate for the decrease in IC-1 in other tissues.

Given the critical cellular functions dynein plays in transporting cargoes and organelles, maintaining cellular organisation, as well as facilitating mitosis (Allan, 2011), it is intriguing that down-regulating a subunit of the dynein complex would be beneficial in cancer. It suggests that a significant reduction in IC-1 levels is not detrimental to the required housekeeping functions of dynein. Indeed, it is thought that the ubiquitously expressed IC-2 isoform is sufficient to maintain the housekeeping functions of dynein in most cells and tissues, whereas IC-1, being most highly expressed in the brain (Horikawa et al, 2001; Myers et al, 2007), facilitates additional functions required by these more specialised cells (Pfister, 2015). Notably, a previous study found IC-1 to play a role in mediating cellular senescence (Horikawa et al, 2001). In this study, it was found that as cells aged, IC-1 message levels increased over time, reaching a maximal level in senescent cells. Furthermore, the authors noted that the *DYNC1I1* gene is located on the long arm of chromosome 7 where tumour suppressor genes and another senescence-inducing gene are mapped, and consistent with our gene expression profiling in cancer patient samples, they found little or no IC-1 expression in MCF-7 breast cancer cells and in BG-1 ovarian cancer

cells (Horikawa et al, 2001). SK2 has also more recently been implicated in regulating cellular senescence, where S1P produced by nuclear-localised SK2 was reported to bind to hTERT and prevent its degradation, thereby stabilising hTERT and preventing cell senescence (Panneer Selvam et al, 2015). No obvious changes in the nuclear localisation of SK2 were evident upon dynein IC knockdown or IC-1 overexpression in these studies (*data not shown*), but it remains to be more closely examined if the roles of IC-1 in cell senescence are in any way mediated by SK2 and its role in promoting hTERT stability.

Furthermore, a mutation found in the dynein heavy chain (F580Y) that gives rise to spinal muscular atrophy with lower extremity predominance was found to negatively affect dynein complex assembly and processivity (Ori-McKenney et al, 2010). Interestingly, Garrett et al found that, upon stimulation of cells with EGF, the reduced dynein function as a result of this mutation led to delayed maturation and retrograde trafficking of EGF-containing endosomes, and consequently, sustained activation of the downstream effector of EGF-mediated signalling, ERK1/2 (Garrett et al, 2014). It is therefore tempting to speculate whether other means of reducing dynein function, such as the downregulation of a component like IC-1, may affect a broader range of signalling pathways and responses in favour of cancer growth, where regulating SK2 subcellular localisation may be one such outcome. It may be particularly interesting, then, to explore the interplay between SK2 and potentially sustained EGF signalling when IC-1 is downregulated in cancer, as EGF can activate SK2 (Hait et al, 2007) and SK2 is required for EGF-induced migration of breast cancer cells (Hait et al, 2005) and EGF-mediated adhesion and invasion of cervical cancer cells (Adada et al, 2015).

Contrastingly, a recent study reported the upregulation of cytoplasmic dynein 2 heavy chain 1 (DHC2) upon treatment of GBM cells with TMZ, and RNAi-mediated knockdown of DHC2 appeared to modestly sensitize primary GBM cells to TMZ-induced cell death (Wang et al, 2016). The cytoplasmic dynein 1 complex, which is the focus of our work, is highly abundant and is found in all microtubule-containing cells where it transports various proteins and organelles throughout the cytoplasm, facilitates cell cycle progression and maintains organelle positioning (Allan, 2011; Hook & Vallee, 2006). Cytoplasmic dynein 2 and its components, on the other hand, are best characterised for their role in retrograde intraflagellar transport (IFT), and consequently are almost exclusively found within and around the base of eukaryotic cilia and flagella to promote their assembly and maintenance

(Hook & Vallee, 2006; Pfister et al, 2006). Therefore, the relevance of DHC2 upregulation in response to TMZ treatment and potential resistance mechanisms in GBM is unclear; it was hypothesised that these phenotypes may be a result of the role DHC2 plays in mediating Hedgehog signalling, which appears to require functional cilia and IFT proteins (Huangfu & Anderson, 2005; Wang et al, 2016), but further work would be required to substantiate this.

Additional work will be required to further understand and characterise the interaction between SK2 and dynein. Retrograde-directed movement of dynein results in its accumulation at the MTOC, and dynein is also found associated with organelles such as the golgi, lysosomes and endosomes (Kardon & Vale, 2009). Dynein, and the cargo that it transports, have been shown previously to exhibit peri-nuclear localisation (Ligon et al, 2001; Lin & Collins, 1992) very similar to that demonstrated here by dynein IC and SK2 (Figure 5.6A). Whether SK2 is being sequestered to specific peri-nuclear organelles, in either a house-keeping shuttling process or in response to certain stimuli, and how it is then transported away from these regions are all questions that will require further attention. Given that here an interaction could be detected between SK2 and dynein IC in cells without any additional stimuli (Figure 5.6B), this might suggest that SK2 is constitutively transported within the cell as a house-keeping function of dynein. Indeed, in some situations this may represent a negative-regulatory mechanism to prevent prolonged signalling of SK2 at the plasma membrane. Given that re-expression of IC-1 is not a feasible therapeutic option, it will be of interest to explore how this interaction is regulated in order to uncover other potential targeting strategies.

Overall, in this study I have identified a novel physiological interaction between SK2 and the cytoplasmic dynein complex, and have uncovered a potential new mechanism of SK2 dysregulation in cancer, whereby modulation of dynein IC-1 expression in GBM can regulate SK2 subcellular localisation and ultimately affect tumourigenic potential. This work highlights the importance of investigating SK2 subcellular localisation, and not simply whether it is upregulated, in determining whether pharmacologically targeting SK2 is likely to have therapeutic benefits in certain cancers. As such, this study provides new mechanistic insights into dysregulated cellular transport and signalling in GBM, and offers a rationale for the use of SK2-specific inhibitors as potential anti-cancer therapeutics in GBM.

Chapter 6:
The Role of Phosphorylation in Sphingosine Kinase 2 Regulation

Chapter 6. The Role of Phosphorylation in Sphingosine Kinase 2 Regulation

6.1 Abstract

The two mammalian sphingosine kinases, SK1 and SK2, are responsible for the formation of the pleiotropic signaling lipid sphingosine-1-phosphate (S1P). It is well known that the subcellular localisation of the SKs, and hence the compartmentalisation of S1P formation within the cell, can alter the function of these enzymes. The SKs are known to be regulated by extracellular as well as intracellular stimuli, and both enzymes can undergo post-translational modifications. Indeed, phosphorylation of SK1 at Ser225 is responsible for its activation, and can also facilitate its localisation to the plasma membrane. Phosphorylation of SK2 at Ser383 and/orSer385 has been shown to regulate its nuclear export, and phosphorylation of Thr578 has been proposed to result in SK2 activation. However, SK2 possesses two regions within its sequence that are not conserved in SK1; an extended N-terminal region and a proline-rich central domain predicted to contain multiple phosphorylation sites. Strikingly, through mass spectrometry analyses, the Pitson Laboratory previously identified ten phosphorylation sites within the unique central region of SK2, and seven of these sites have since been confirmed by global phosphoproteomic studies as being physiological phosphorylation events on endogenous SK2. In this chapter, the role of phosphorylation in the regulation of SK2 was examined. While initial studies suggested phosphorylation at Ser363 may regulate the interaction of SK2 with dynein, further analysis showed that phosphorylation of this site appears to regulate the subcellular localisation of SK2 potentially independently of dynein. Furthermore, Ser437 in SK2 was found to be phosphorylated by GSK3 *in vitro* and in cells, in a manner that appears dependent on a priming phosphorylation at Ser441. Furthermore, these phosphorylation sites may act to regulate the catalytic activity of SK2. These findings suggest that the regulation of SK2 by phosphorylation is likely to be more complex and important to its functions than previously appreciated.

6.2 Introduction

The two mammalian sphingosine kinases, SK1 and SK2, both catalyse the formation of S1P. Despite this, their functions within the cell appear to be somewhat divergent. SK1 is very well characterised as having predominantly pro-survival, pro-proliferative roles, and its role in promoting cancer has been extensively reviewed (Heffernan-Stroud & Obeid, 2013; Pitson, 2011; Pyne & Pyne, 2010). However, as discussed in Chapter 4, the functions of SK2 are much more complex, and although it has physiological roles in mediating cell death (Chipuk et al, 2012; Hofmann et al, 2008), it too appears to be able to promote survival, proliferation and neoplastic transformation.

SK1 and SK2 are likely to have considerably different mechanisms of regulation, and it is evident that subcellular localisation and post-translational modifications are critical in SK regulation (Chan & Pitson, 2013; Siow & Wattenberg, 2011). Indeed, it is well characterized that phosphorylation of SK1 at a single site (Ser225) by ERK1/2 results in its activation (Pitson et al, 2003), and facilitates the localisation of SK1 to the plasma membrane, which is required for its oncogenic signalling (Pitson et al, 2005). However, this SK1 phosphorylation site resides within a region of the protein that is divergent in SK2, and hence, this activating site is not directly conserved (see Figure 6.1). Uniquely, in its central domain SK2 contains a proline-rich sequence of 120 amino acids that are largely absent in SK1. Very little is known about the structure of this region, as it shares no homology with any other known protein domains, and the crystal structure of SK2 is yet to be solved.

Interestingly, quite a number of endogenous SK2 phosphorylation events have recently been reported in global phosphoproteome analyses (reviewed in Neubauer & Pitson (2013)), most of which have not been characterised. Twelve of these sites occur on residues within the unique central region of SK2, not conserved in SK1, indicating that they are likely to be involved in SK2-specific regulation. One of these sites, Ser351, has previously been implicated in SK2 activation (Hait et al, 2007). In this study, it was demonstrated that SK2 catalytic activity is increased upon phosphorylation by ERK1 *in vitro*, and that S351A and T578A mutations ablate agonist-induced phosphorylation of SK2, as well as decrease *in vitro* phosphorylation of SK2 by ERK1. However, the critical experiment examining the catalytic activity of S351A and T578A after *in vitro* phosphorylation by ERK1 was not performed (Hait et al, 2007). Furthermore, Ser351 is not conserved in mouse or rat SK2,

making it unlikely that this residue would be responsible for SK2 activation. Therefore, Thr578 may be more likely involved in SK2 activation (which is not conserved in SK1), however, further characterisation is required to elucidate if other phosphorylation events on SK2 can regulate its activation.

Another two phosphorylation sites within the central region of SK2 have also been previously studied. A novel nuclear export sequence (NES) was reported in SK2, whereby Ser383 and/or Ser385 were found to be phosphorylated within this sequence by PKD, to mediate the nuclear export of SK2 (Ding et al, 2007). Indeed, this study provided clear evidence for the role of PKD in SK2 nuclear export, however, the importance of these two specific phosphorylation sites was less clear. It was nicely demonstrated that alanine mutations at these two sites (S383A/S385A) blocked nuclear export of SK2 driven by PKD overexpression. However, the authors described that S383A alone did not prevent the nuclear export of SK2, whereas S385A could dampen the nuclear export but not provide complete nuclear retention as the double mutant did. Furthermore, the authors noted several other potential NESs within SK2 in addition to the one they characterised. Mutation of two leucine residues within one such sequence (L306A/L308A) also prevented SK2 nuclear export upon PMA stimulation, and yet phosphorylation on Ser383/Ser385 was detected in the nucleus of these cells, demonstrating that phosphorylation of these residues is not the only mechanism of nuclear export. Again, this study provides insight into the regulation of SK2 subcellular localisation by phosphorylation, but it is still a complex and incompletely understood area.

Given that SK2 has complex opposing roles within the cell, it was of interest to better understand how SK2 is regulated, such as by phosphorylation. This will inevitably lead to a better understanding of how to control and manipulate SK2 function in order to exploit it as a therapeutic target. In Chapter 5, the interaction between SK2 and dynein IC was examined, as well as the involvement of dynein in the regulation of SK2 subcellular localisation. Thus, it was also of interest to understand how this interaction is regulated, and whether it is a constitutive interaction mediating the constant shuttling of SK2 around the cell, or whether it is triggered by a specific stimulus, for example, phosphorylation. Therefore, the focus of this chapter was to examine the role of phosphorylation in regulating the interaction between SK2 and dynein, and also to characterise novel SK2-specific phosphorylation sites.

Outlined in this chapter are a series of studies that examine various aspects of SK2 phosphorylation and begin to elucidate the complex regulation of SK2 facilitated by this form of post-translational modification.

6.3 Preliminary data leading to this study

In order to better understand SK2-specific regulation, the Pitson Laboratory previously sought to identify novel SK2 phosphorylation events through mass spectrometric analysis of the overexpressed protein in HEK293T cells treated with phorbol esters. This study yielded the identification of ten phosphorylation events, all of which were located within the unique central domain of SK2a (Figure 6.1A). Of the ten identified sites, only one has since been further studied (Ser351) (Hait et al, 2007). Seven of the phosphorylation sites have been verified as physiological by global phosphoproteomic analyses (Hornbeck et al, 2015), and the remaining three sites (Ser398, Ser401, Ser430) have never been previously detected or documented. To determine whether these ten phosphorylation sites were likely to be the main sites on SK2, the Pitson Laboratory utilised site-directed mutagenesis to mutate all ten identified phosphorylation sites to alanine, to generate a Δ10P mutant. Metabolic labelling of cells expressing the Δ10P mutant with $^{32}P_i$ resulted in no detectable phosphorylation of SK2 (Figure 6.1B), suggesting that these ten phosphorylation sites are either the main sites within SK2, or that any additional sites somehow require, or are regulated by these sites.

Given that these phosphorylation sites are likely to represent novel, uncharacterised SK2-specific regulatory mechanisms, it was of interest to determine if the retrograde-directed translocation of SK2 by dynein (examined in Chapter 5) is regulated by phosphorylation. Given that the retrograde-directed transport of SK2 may result in its localisation to regions of the cell where it is known to have pro-apoptotic roles, like the ER or mitochondria (Chipuk et al, 2012; Maceyka et al, 2005), it was reasoned that such an event would likely be tightly regulated. As such, co-immunoprecipitation experiments were performed using overexpressed IC-2 as well as overexpressed SK2 or various SK2 mutants where some or all of the ten identified phosphorylation sites were mutated to alanine (Δ10P, Δ6P and Δ4P; see Figure 6.2A). Interestingly, less interaction was observed with IC-2 when SK2 was lacking at least six phosphorylation sites, whereas the interaction was comparable to wildtype SK2 when only four phosphorylation sites were mutated (Figure 6.2B). The two extra

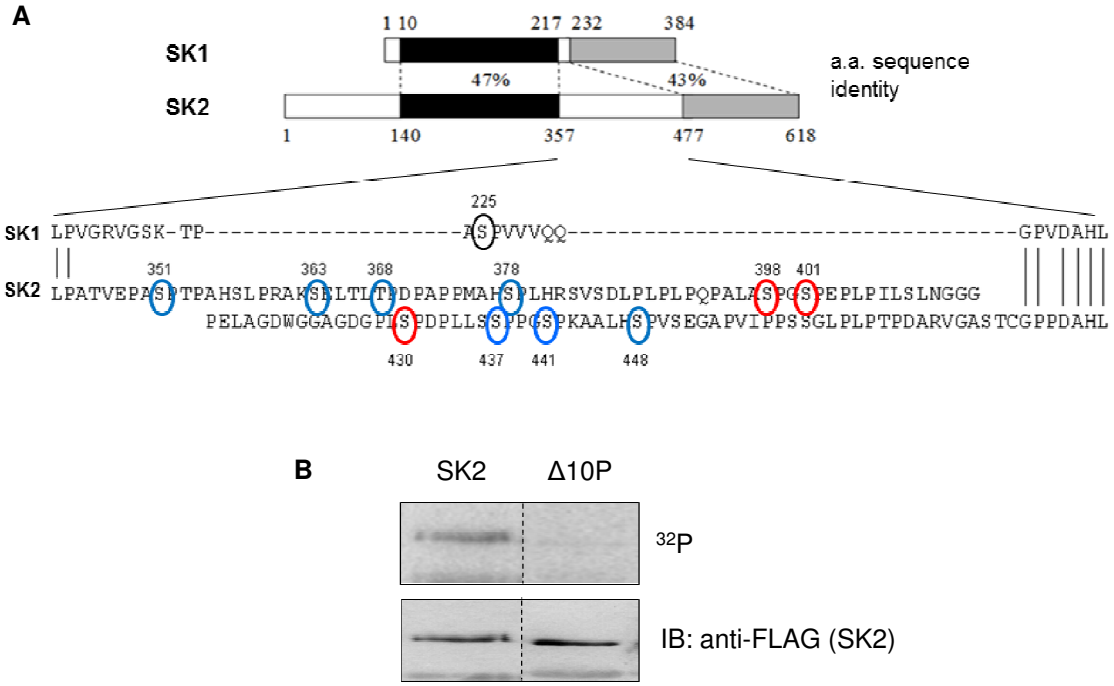


Figure 6.1: Identification of ten SK2 phosphorylation sites by mass spectrometry

A, HEK293T cells overexpressing FLAG-tagged SK2 were treated with 10 ng/ml PMA for 30 min, SK2 was immunoprecipitated using anti-FLAG antibodies, separated by SDS-PAGE, isolated and subjected to tryptic digestion, and analysed by the Adelaide Proteomics Facility using tandem mass spectrometry with a Q-TOF mass spectrometer. Ten serine/threonine phosphorylation sites were identified within the unique central region of SK2a, of which three are novel (red) and seven have since been verified by global phosphoproteomic analyses or direct analysis and mutagenesis of overexpressed SK2 (blue). The known SK1 activating phosphorylation site (Ser225), not conserved in SK2, is depicted (black). *B,* HEK293T cells were transiently transfected with FLAG-tagged SK2 or the phosphorylation deficient mutant, SK2 Δ10P. Cells were cultured in phosphate-free media, incubated with $^{32}P_i$ (0.2 mCi/ml) for 4 hr and then treated with 10 ng/ml PMA for 30 min. SK2 was then immunoprecipitated from lysates with anti-FLAG antibodies, subjected to SDS-PAGE and exposed to a phosphorimager screen. Immunoprecipitates were also subjected to immunoblot analyses with anti-FLAG antibodies, to confirm SK2 expression. Dashed lines indicate where lanes from the same immunoblots have been spliced together to aid interpretation. Data are representative of at least three independent experiments.

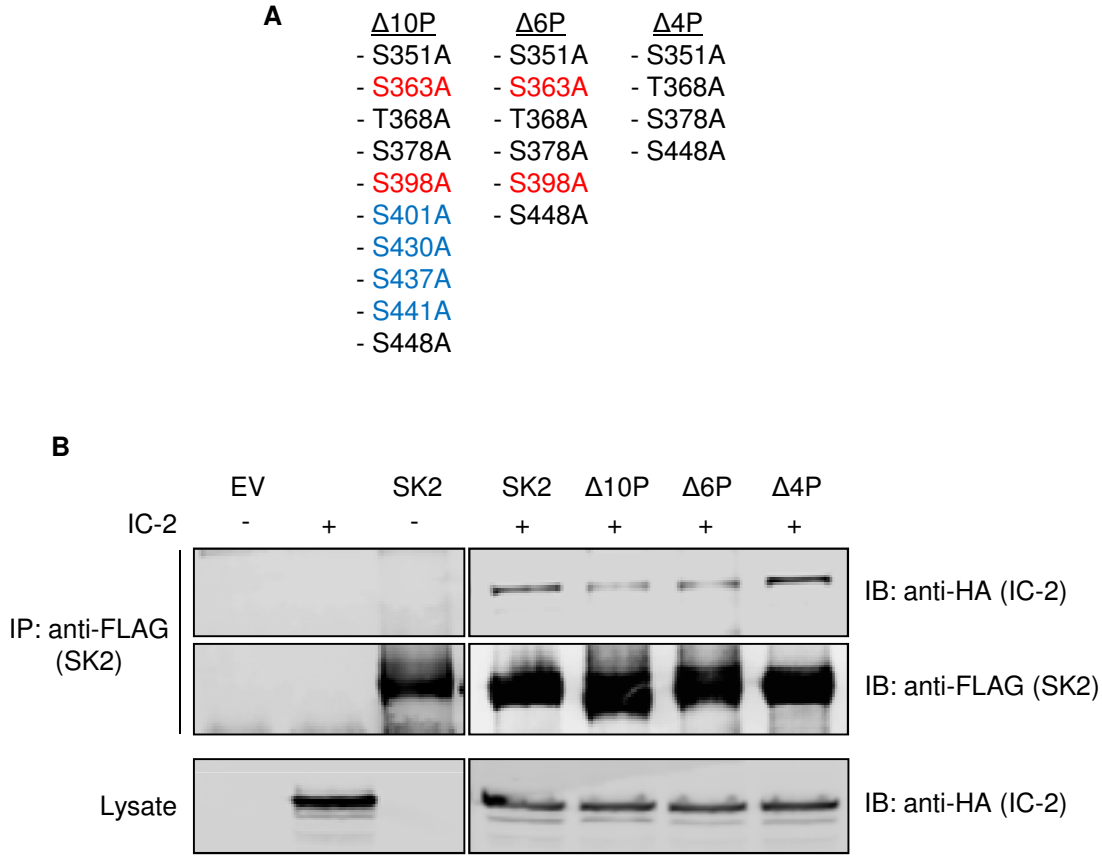


Figure 6.2: Interaction between SK2 and IC-2 appears to be dependent upon SK2 phosphorylation

A, SK2 phosphorylation site mutations present in Δ10P, Δ6P and Δ4P. Those in black are common to all three mutants, those in red are common to Δ10P and Δ6P, and those in blue are present in Δ10P only. *B,* HEK293-c18 cells were co-transfected with HA-tagged IC-2 and either FLAG-tagged SK2, or FLAG-tagged SK2 mutants Δ10P, Δ6P or Δ4P. SK2 (or mutants) were immunoprecipitated from lysates using anti-FLAG antibodies, and co-immunoprecipitated IC-2 was detected by immunoblot analyses using anti-HA antibodies. Expression levels of IC-2 in the lysates were confirmed by immunoblotting with anti-HA antibodies (Lysate). Images shown are representative of three independent experiments.

phosphorylation sites mutated to alanine in the Δ6P mutant that were not mutated in the Δ4P mutant are Ser363 and Ser398. Therefore, it appeared that the interaction between SK2 and IC-2 may be regulated by SK2 phosphorylation, potentially on Ser363 and/or Ser398.

6.4 Specific Materials and Methods

6.4.1 Site-directed mutagenesis

pcDNA3-SK2 S363E(FLAG) was generated by Quikchange PCR with KAPA HiFi DNA polymerase (Kapa Biosystems) using pcDNA3-SK2(FLAG) (Roberts et al, 2004) as template and the following primers: 5'-CTGCCTCGTGCCAAGGAAGAGCTCACCCTAACCCCAGAC-3' and 5'-GTCTGGGGTTAGGGTGAGCTCTTCCTTGGCACGAGGCAG-3'. pcDNA3-SK2 S363D(FLAG) was generated in the same way, using the following primers: 5'-CCATAGCCTGCCTCGAGCCAAGGATGAGCTGACCCTAACC-3' and 5'-GGTTAGGGTCAGCTCATCCTTGGCTCGAGGCAGGCTATGG-3'. Sequencing verified the successful incorporation of the point mutations and the integrity of the human SK2 cDNA sequences.

6.4.2 Kinexus screen

Two peptide substrates (Ser363: RLPRAK**S**ELTLT and S363A: RLPRAK**A**ELTLT) that recapitulate the polypeptide sequence surrounding Ser363 of human SK2 were generated at 90% purity by Kinexus Bioinformatics (Vancouver, Canada). These peptides were then tested by Kinexus in *in vitro* phosphorylation assays against 46 protein kinases. Peptides were screened at 500 μM in duplicate measurements using a radiometric assay method. Strong phosphorylation was deemed to be > 100,000 counts per minute (cpm), and specificity to Ser363 was determined by *in vitro* phosphorylation of the alanine peptide.

6.4.3 TUNEL apoptosis assays

HeLa cells were seeded onto poly-L-lysine coated coverslips in a 12-well plate and grown overnight at 37°C. Cells were then either mock transfected (no DNA) or transfected with pcDNA3-IRES mCherry, FLAG-tagged wildtype SK2, or FLAG-tagged SK2 mutants: S363A, S398A, S437A, S441A or S448A. After 24 hr, media was removed and cells were cultured in serum-free media for another 24 hr, and mock transfected cells were treated for 24 hr with 1 μM Staurosporine as a positive control. Cells were then fixed and apoptotic cells were identified using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) technology with an *in situ* cell death detection kit (Fluorescein; Roche), performed as per the manufacturer's instructions. Secondary labelling of exogenous proteins was achieved by immunofluorescence staining using mouse anti-FLAG antibodies. Cells were visualised by confocal microscopy, and dual TUNEL-positive / transfected cells were quantified in a blinded manner.

6.5 Results

6.5.1 Characterising the regulation of SK2 by Ser363 phosphorylation

6.5.1.1 Ser363 appears to regulate the interaction between SK2 and dynein IC

The previous use of broad phosphorylation-deficient SK2 mutants indicated that Ser363 and/or Ser398 may play a role in regulating the interaction between dynein IC-2 and SK2. Hence, it was of interest to further characterise their importance through the use of single point-mutants. Interestingly, co-immunoprecipitation experiments with co-expressed IC-2 and SK2 S363A or S398A point-mutants clearly demonstrated that an alanine mutation at Ser363 alone could abolish the interaction, whereas mutating Ser398 had only a minimal effect on the interaction (Figure 6.3A). This trend was also confirmed in co-immunoprecipitation experiments with IC-1 (Figure 6.3B), indicating that Ser363 appears to regulate the interaction between SK2 and both IC isoforms.

To confirm that Ser363 alone is responsible for this phenotype, and that the alanine mutation on Ser363 doesn't subsequently interfere with the phosphorylation of another

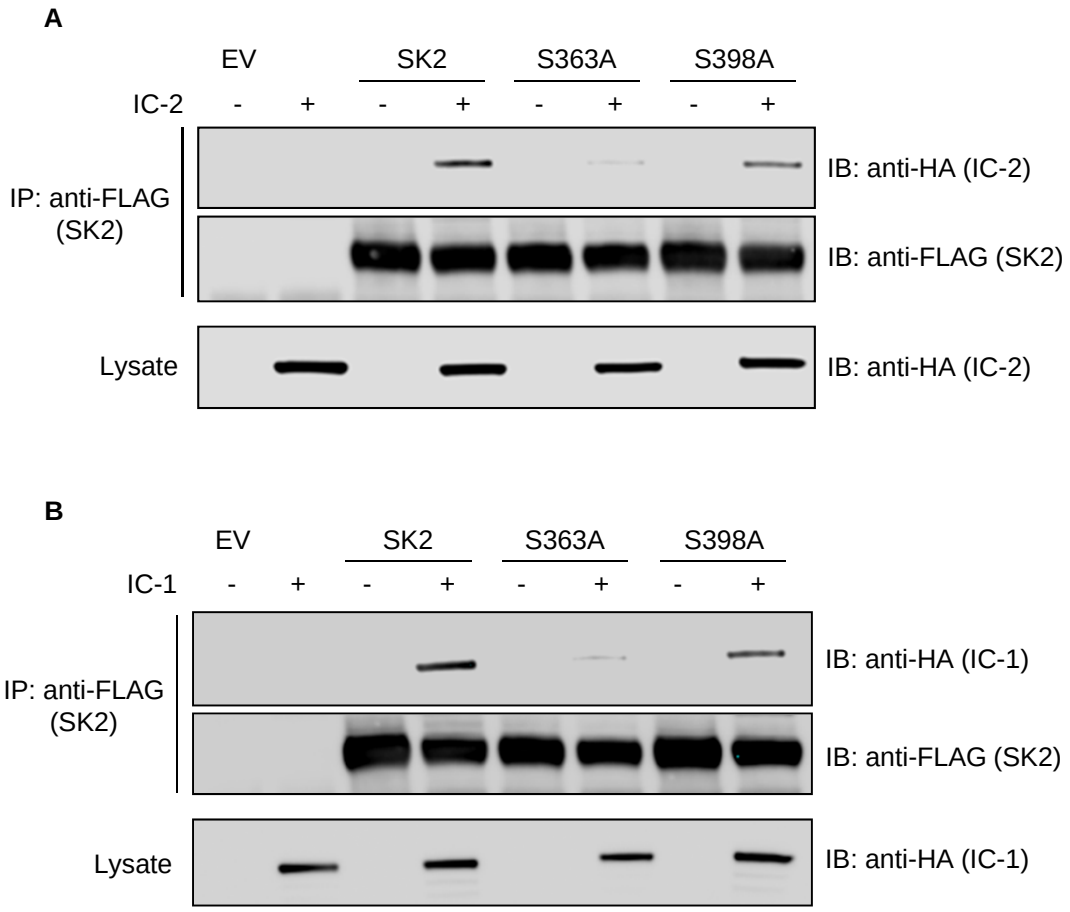


Figure 6.3: Interaction between SK2 and dynein IC appears to be regulated by Ser363

A, HEK293-c18 cells were co-transfected with HA-tagged IC-2 and either FLAG-tagged SK2 or FLAG-tagged SK2 mutants, S363A or S398A. SK2 (or mutants) were immunoprecipitated from lysates using anti-FLAG antibodies, and co-immunoprecipitated IC-2 was detected by immunoblot analyses using anti-HA antibodies. Expression levels of IC-2 in the lysates were confirmed by immunoblot with anti-HA antibodies (Lysate). *B*, Performed as in (A), except with HA-tagged IC-1. All images shown are representative of at least three independent experiments.

important site, a Δ9P mutant was employed where nine of the ten phosphorylation sites previously identified by the Pitson Laboratory were mutated to alanine, leaving only Ser363 intact. Indeed, utilising this SK2 Δ9P mutant in overexpressed co-immunoprecipitation experiments with dynein IC-2 demonstrated that wildtype Ser363 was sufficient for the interaction, suggesting that the other nine phosphorylation sites were not involved (Figure 6.4).

6.5.1.2 Mutating Ser363 to glutamate or aspartate does not appear to function as a phospho-mimetic

In order to further demonstrate the importance of phosphorylation on a particular residue, it is common to substitute the residue for a glutamate or an aspartate in an attempt to constitutively mimic the bulky negative charge of a phosphate group (Dephoure et al, 2013; Hao et al, 1996). Indeed, this method has been previously shown to be successful for SK2, where substitutions of Ser351 and Thr578, the sites implicated in SK2 activation in response to EFG (Hait et al, 2007), to aspartate residues resulted in increased basal adherence levels and increased basal phosphorylation of ERM proteins in the absence of EGF, compared to WT SK2 (Adada et al, 2015). In line with this, both glutamate and aspartate mutants at the Ser363 site (S363E and S363D) were generated for use in co-immunoprecipitation experiments, to determine if this would result in an increase in the interaction between SK2 and dynein IC. However, both of these mutants resembled the S363A mutant, where the interaction between SK2 and IC-1 was decreased (Figure 6.5). Hence, S363E and S363D were not able to mimic the potential role of phosphorylated Ser363 in promoting the interaction between SK2 and dynein IC.

6.5.1.3 Ser363 regulates the subcellular localisation of SK2

Given that Ser363 appeared to regulate the interaction between SK2 and dynein IC, and as examined in Chapter 5, dynein can translocate SK2 in a retrograde direction within the cell, it was therefore hypothesised that modulation of Ser363 would result in changes to SK2 subcellular localisation. Accordingly, wildtype SK2 or various mutants were overexpressed in HEK293 cells and their localisation was visualised by immunofluorescence and confocal

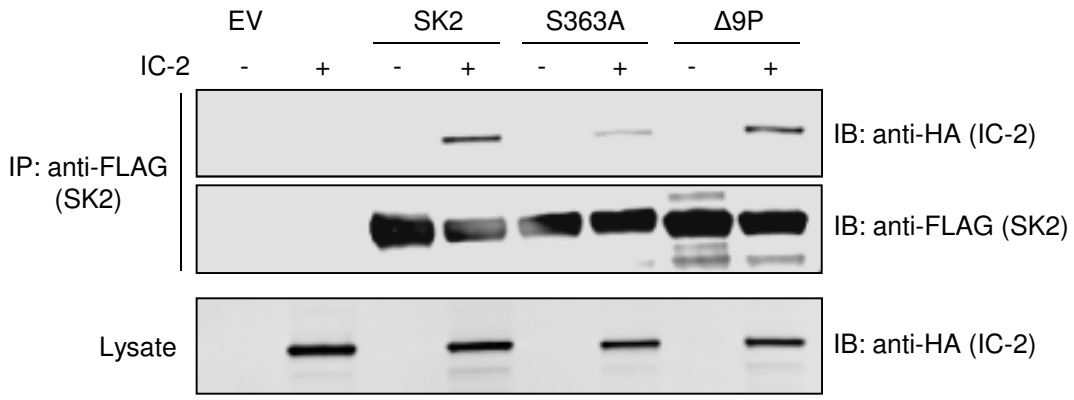


Figure 6.4: Other SK2 phosphorylation sites do not regulate the interaction with IC-2
HEK293-c18 cells were co-transfected with HA-tagged IC-2 and either FLAG-tagged SK2 or FLAG-tagged SK2 mutants, S363A or Δ9P. SK2 (or mutants) were immunoprecipitated from lysates using anti-FLAG antibodies, and co-immunoprecipitated IC-2 was detected by immunoblot analyses using anti-HA antibodies. Expression levels of IC-2 in the lysates were confirmed by immunoblotting with anti-HA antibodies (Lysate). Images shown are representative of two independent experiments.

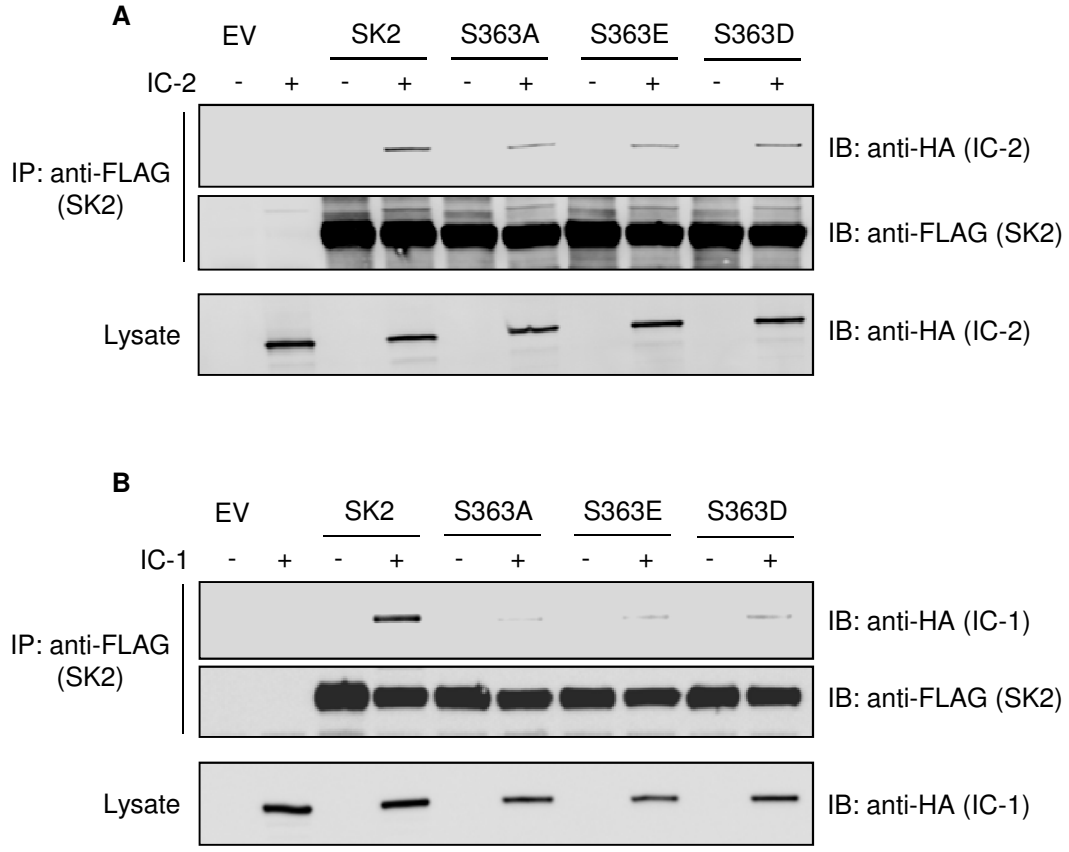


Figure 6.5: Glutamate/aspartate substitutions to Ser363 do not appear to act as phospho-mimetics to enhance the interaction with IC

A, HEK293-c18 cells were co-transfected with HA-tagged IC-2 and either FLAG-tagged SK2 or FLAG-tagged SK2 mutants, S363A, S363E or S363D. SK2 (or mutants) were immunoprecipitated from lysates using anti-FLAG antibodies, and co-immunoprecipitated IC-2 was detected by immunoblot analyses using anti-HA antibodies. Expression levels of IC-2 in the lysates were confirmed by immunoblotting with anti-HA antibodies (Lysate). Images shown are representative of three independent experiments. *B,* Performed as in (A), except with HA-tagged IC-1. Experiment was only performed once.

microscopy. Strikingly, where wildtype SK2 was localised predominantly in the cytoplasm of the cells, the S363A variant was very distinctly nuclear localised (Figure 6.6). The S363E and S363D mutant proteins were also localised mainly to the nucleus like the S363A mutant, demonstrating again that they are not functioning as 'phospho-mimetics', in agreement with the co-immunoprecipitation experiments performed in Figure 6.5. Interestingly, ablation of all ten phosphorylation sites identified in the Pitson Laboratory (Δ10P), which included Ser363, localised SK2 to the nucleus. The conversion, however, of only Ser363 back to wildtype in this mutant (Δ9P) resulted in an almost exclusively cytoplasmic localisation of SK2 (Figure 6.6). These data suggest that Ser363 is an important regulator of SK2 subcellular localisation, such that this site may act to facilitate the nuclear export of SK2, potentially via phosphorylation.

6.5.1.4 The effects of S363A on SK2 binding to dynein IC may be an artefact of its altered localisation

It was expected that if Ser363 regulates the interaction between SK2 and dynein, and dynein can transport SK2 in a retrograde direction (Chapter 5), then modulating Ser363 would result in changes to SK2 localisation. This was in fact observed, but, according to this rationale it would be expected that the S363A mutation would result in a decreased interaction between SK2 and dynein and a consequent decrease in retrograde-directed transport of SK2, potentially leading to an accumulation of SK2 at the plus-ends of the cell (i.e. the cell periphery). However, quite the opposite was observed, with S363A being strongly localised to the nucleus (Figure 6.6). This then posed the following questions: (i) is SK2 S363A retained in the nucleus because it is no longer being transported by dynein, or (ii) is SK2 S363A no longer able to interact with dynein IC because it is trapped exclusively in the nucleus? If the former is true, then it would suggest that dynein transports SK2 out of the nucleus in an anterograde direction, which, based on existing knowledge of dynein function, is unlikely (Allan, 2011; Vale, 2003). However, the latter scenario would suggest that Ser363 may not directly regulate the interaction between SK2 and dynein IC, but rather that mutation of Ser363 to alanine simply results in a physical separation of SK2 and dynein IC to different subcellular compartments so they can no longer interact.

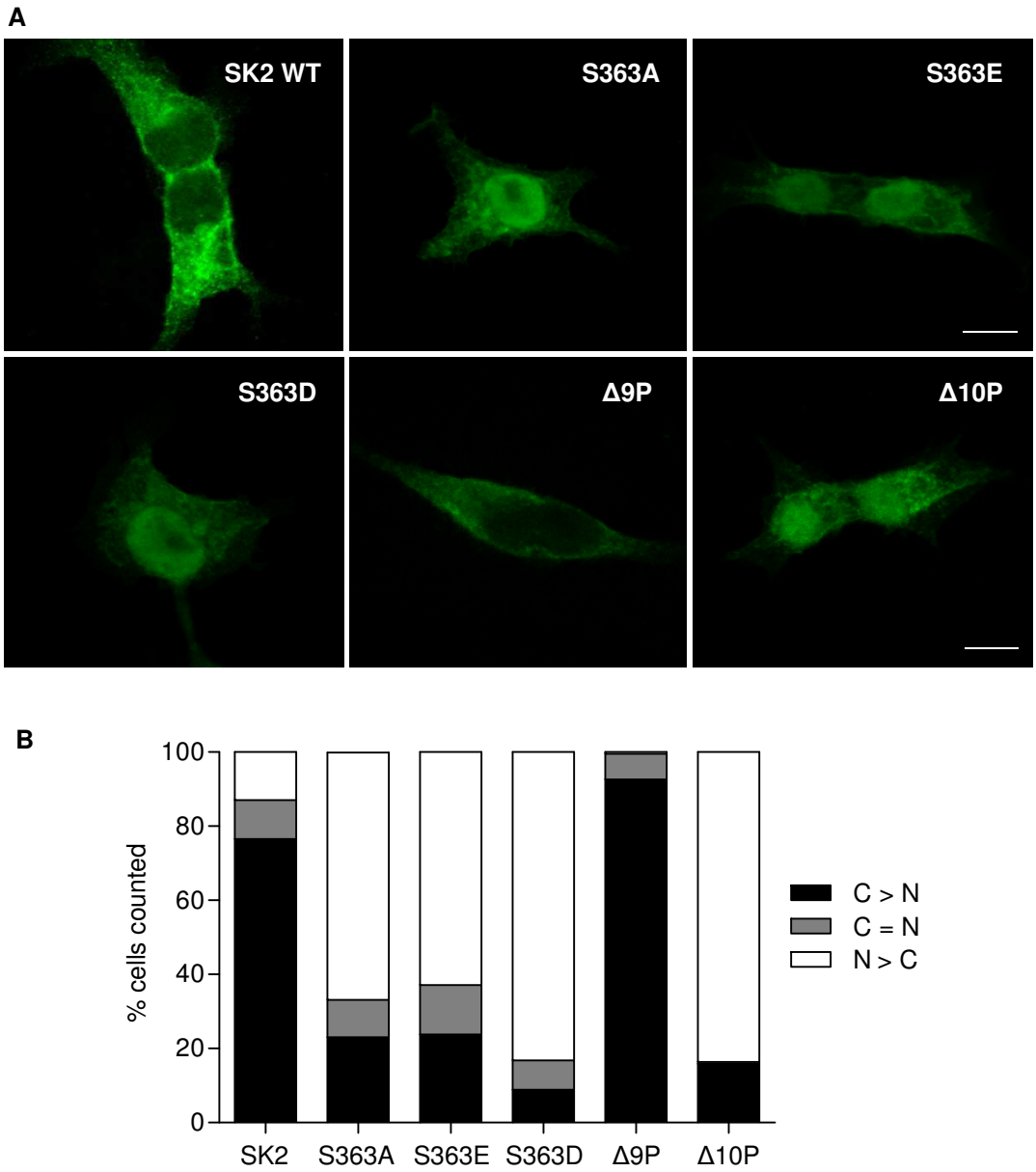


Figure 6.6: Ser363 regulates the subcellular localisation of SK2

A, HEK293 cells were transfected with FLAG-tagged wildtype SK2 or mutants, S363A, S363E, S363D, Δ9P or Δ10P. After 48 hr, exogenous SK2 or mutants were visualised by immunofluorescence and confocal microscopy, using mouse anti-FLAG antibodies. Scale bar = 10 μm. *B,* Using confocal microscopy, cells from (A) were scored visually as having either predominantly cytoplasmic localisation (C > N), equal cytoplasmic and nuclear localisation (C = N), or predominantly nuclear localisation (N > C). A minimum of 200 cells were scored per treatment. Data shown is from one experiment, and is representative of 2-3 independent experiments.

To identify which of these two scenarios is correct, firstly the subcellular localisation of overexpressed IC in the cell was assessed. To that end, HEK293 cells were co-transfected with IC-2 and either wildtype SK2 or S363A mutant, and their localisation was visualized by immunofluorescence and confocal microscopy. IC-2 displayed clear nuclear-exclusion, and there was a high level of cytoplasmic co-localisation between wildtype SK2 and IC-2 (Figure 6.7A). In contrast, the increased nuclear localisation of the S363A mutant meant there was considerably less co-localisation between S363A and IC-2 (Figure 6.7A). This result, therefore, favours the notion that the decrease in binding observed between S363A and dynein IC is due to the physical separation of the two proteins within the cell. However, this theory would imply that there is a need for SK2 and dynein IC to form an interaction within the cell prior to cell lysis and co-immunoprecipitation. Otherwise, upon lysis and disruption of the nuclear membrane, IC and S363A would then be in the vicinity to form an interaction. Thus, to test whether an interaction between SK2 and IC could be formed post cell lysis, HEK293 cells were separately transfected with either SK2 or IC-2, and lysates from these cells were then mixed in a 1:1 ratio and incubated prior to co-immunoprecipitation. Notably, in contrast to the SK2-IC complex observed following co-expression of the proteins, no interaction between the two proteins could be detected when combined *in vitro* following cell lysis (Figure 6.7B).

Although, due to time restraints, this experiment was only performed once and requires further confirmation, such a result may not be surprising, as SK2 appears to interact with IC and the dynein complex in conjunction with the dynactin complex (as shown in Chapter 5). Therefore, an interaction between SK2 and these complexes is quite likely to require an intact cell in order for these proteins to come together physiologically, as dynein-mediated retrograde transport of cargoes generally requires the initial recruitment of dynein to the microtubule plus ends, which can involve the coordinated assembly of many additional components such as kinesis, dynactin and microtubule plus end-tracking proteins (Cianfrocco et al, 2015). If true, it would suggest that the interaction between SK2 and dynein IC is not directly regulated by Ser363, but that Ser363 regulates the subcellular localisation of SK2 via other mechanisms. It therefore remains to be determined if the interaction between SK2 and IC is regulated by other means.

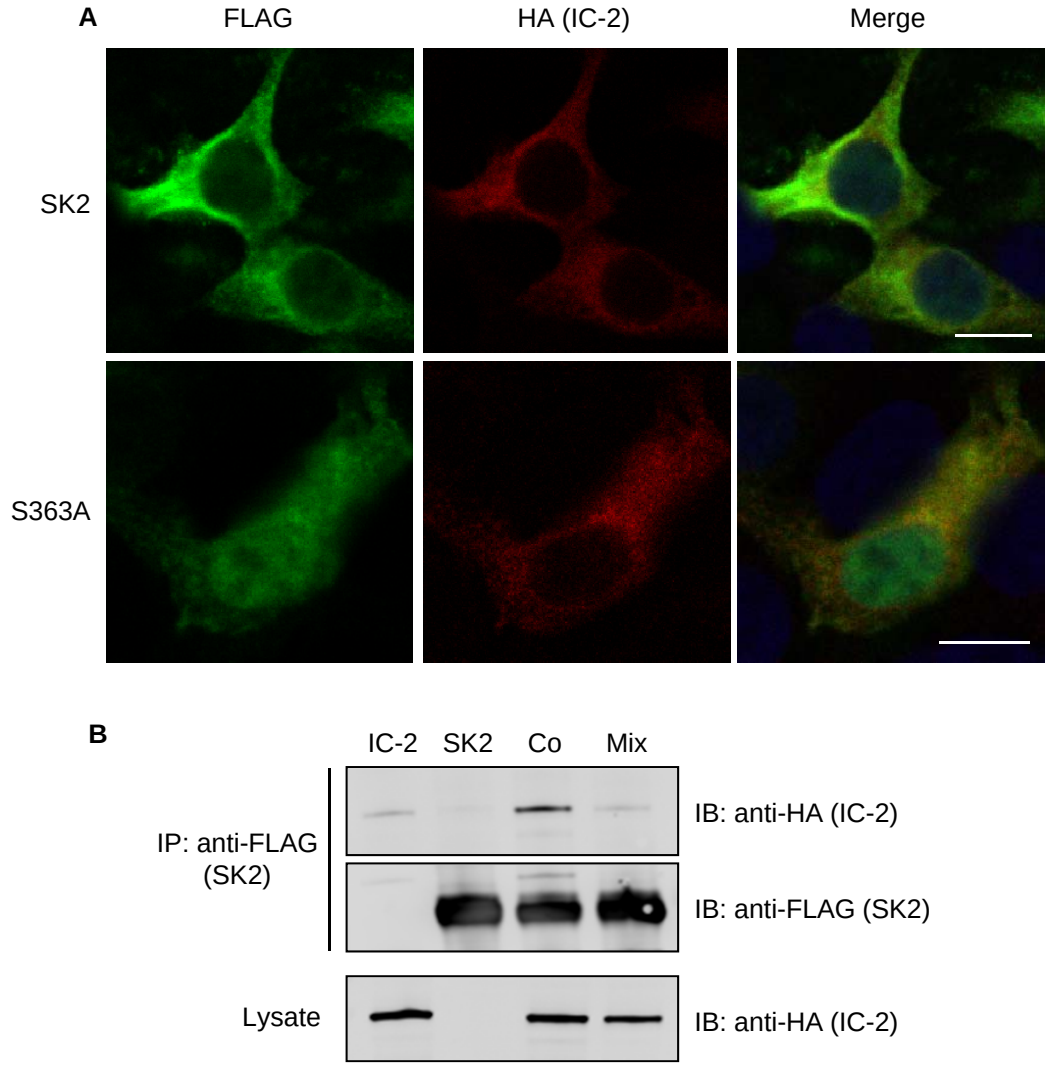


Figure 6.7: Ser363 is likely to regulate the subcellular localisation of SK2 independently of dynein

A, HEK293-c18 cells were co-transfected with HA-tagged IC-2 in combination with either FLAG-tagged wildtype SK2, or S363A mutant. After 48 hr, exogenous proteins were visualised by immunofluorescence and confocal microscopy using mouse anti-FLAG antibodies (to detect SK2 and S363A) and rabbit anti-HA antibodies (to detect IC-2). Scale bar = 10 μm. *B,* HEK293-c18 cells were transfected with HA-tagged IC-2 or FLAG-tagged SK2, or co-transfected with both (Co). 100 μl of lysate from each of the singly-transfected cells were mixed gently for 2 hr at 4°C (Mix). SK2 was then immunoprecipitated from all lysates using anti-FLAG antibodies, and co-immunoprecipitated IC-2 was detected by immunoblot analyses using anti-HA antibodies. Expression levels of IC-2 in the lysates were confirmed by immunoblotting with anti-HA antibodies (Lysate). Experiment was only performed once.

6.5.1.5 Ser363 regulates SK2 catalytic activity

Given that Ser363 clearly regulates the subcellular localisation of SK2, potentially via phosphorylation of this site, it was of interest to determine if this residue could also regulate other aspects of SK2 function, such as its catalytic activity. Overexpression of the S363A mutant in HEK293 cells resulted in a doubling in SK2 catalytic activity compared to wildtype SK2 (Figure 6.8), suggesting that Ser363 phosphorylation may act to negatively-regulate the catalytic activity of SK2 either directly or indirectly via changes in subcellular localisation. Since the proportion of overexpressed wildtype SK2 that was phosphorylated on Ser363 was not known, the S363E and S363D mutants were utilized as potential phospho-mimetics in order to analyse the activity of SK2 when Ser363 was constitutively 'phosphorylated'. However, although this experiment was only performed once, similarly to the modulation of SK2 localisation (Figure 6.6), these mutants phenocopied the S363A mutant and displayed an increase in SK2 activity (Figure 6.8), again implying that they do not function as phospho-mimetics.

6.5.1.6 Screening candidate kinases that phosphorylate Ser363 on SK2

From the studies described above, it was clear that identifying the kinase responsible for phosphorylating Ser363 would be beneficial in understanding SK2 regulation. Unlike the other nine phosphorylation sites identified within the unique central region of SK2 (Figure 6.1), Ser363 is the only site that doesn't conform to the consensus motif for a proline-directed protein kinase [i.e. serine/threonine that is immediately followed by a proline (Lu et al, 2002)]. This gives it a unique set of candidate kinases and also raises the possibility that it may be regulated in a manner different to the other sites.

Given that Ser363 appears to be a physiological SK2 phosphorylation site (Hornbeck et al, 2015), it was of interest to identify the kinase responsible, and so Kinexus Bioinformatics was employed to perform *in vitro* kinase screens. Initially, a list of the top 50 candidate kinases was generated by Kinexus, based on consensus motif algorithms matching the residues surrounding Ser363. Of these 50 kinases, 41 were available for testing by Kinexus. We selected an additional five kinases (PKAc-α, PKAc-β, PKAc-γ, ROCK1 and ROCK2) to screen based on prediction websites and appropriate consensus motifs. These 46 kinases

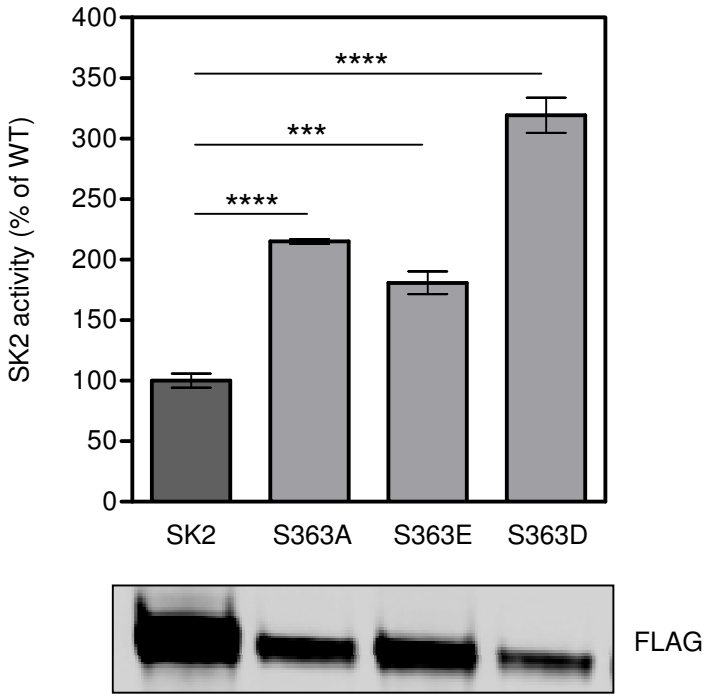


Figure 6.8: Ablation of Ser363 phosphorylation significantly increases SK2 catalytic activity

HEK293 cells were transfected with either wildtype SK2, S363A, S363E or S363D mutants, and 48 hr later lysates were subjected to SK2-specific activity assays. Activity was normalised to expression levels of the exogenous proteins, as determined by immunoblot analyses using anti-FLAG antibodies (panel below). Data are mean (± SD) of experimental triplicates, representative of five (S363A) or one (S363E and S363D) independent experiments.

were then screened by Kinexus in *in vitro* phosphorylation assays for their ability to phosphorylate a synthetic peptide generated from the SK2 sequence containing Ser363 (RLPRAK**S**ELTLT). The 46 kinases were also assayed using a control peptide, with Ser363 converted to a non-phosphorylatable alanine residue, to validate the specificity of the kinases to this site. The ability of each kinase to phosphorylate the wildtype and mutant peptides was quantified and is shown in Figure 6.9. Kinexus deemed greater than 100,000 counts per minute (cpm) as strong phosphorylation of the substrate peptide, and specificity of the kinases to Ser363 phosphorylation was determined by inclusion of the S363A mutant control peptide.

There were eleven kinases from seven different kinase families that demonstrated both strong phosphorylation of Ser363 *in vitro* as well as specificity to this site (see Table 6.1): AMP-activated protein kinase 1 and 2 (AMPK-α1 and AMPK-α2), brain-specific serine/threonine kinase 1 and 2 (BRSK1 and BRSK2), death-associated protein kinase 3 (DAPK3), mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAPK2), proto-oncogene serine/threonine-protein kinase Pim-1 and -3 (PIM1 and PIM3), ribosomal s6 kinase $p90^{rsk}$ (RSK1; also MAPKAPK1), and serum/glucocorticoid regulated kinase 2 and 3 (SGK2 and SGK3). Of these kinases, the strongest phosphorylation of Ser363 was by AMPK-α2, and the greatest specificity to Ser363 was observed for RSK1 (only 3% phosphorylation of the S363A peptide observed). Unfortunately, due to time constraints, further testing of these candidate kinases in *in vitro* phosphorylation assays using full-length SK2 protein was not performed.

6.5.2 The pro-apoptotic role of SK2 may be regulated by phosphorylation

Although many of the studies demonstrating a pro-apoptotic role for SK2 have utilised overexpression systems (Liu et al, 2003; Maceyka et al, 2005), which are likely to alter the normal physiological subcellular localisation of SK2 and possibly override other regulatory mechanisms, it appears the pro-apoptotic function of SK2 is not simply an artefact of overexpression (Chipuk et al, 2012; Hofmann et al, 2008; Okada et al, 2005). Therefore, identifying the mechanisms that allow SK2 to switch from its pro-survival to pro-apoptotic roles would be of interest. For example, it would potentially allow targeting of only the oncogenic form of SK2, and also provide a means to switch on pro-apoptotic SK2 function

Figure 6.9: Kinexus Bioinformatics screen identifying candidate kinases that phosphorylate SK2 at Ser363 *in vitro*

Two peptide substrates (one containing wildtype S363: RLPRAKSELTLT, the other containing the A363 mutation: RLPRAKAELTLT) were tested in *in vitro* phosphorylation assays against 46 protein kinases. Peptides were screened at 500 μM in duplicate measurements using the radiometric assay method. Graph shows level of phosphorylation, in counts per minute (cpm), of both wildtype (blue bars) and mutant (red bars) peptides by the 46 kinases. Kinases resulting in phosphorylation of the wildtype peptide greater than 100,000 cpm were considered to strongly phosphorylate S363 (dotted line). Peptide synthesis and kinase screen were performed by Kinexus Bioinformatics.

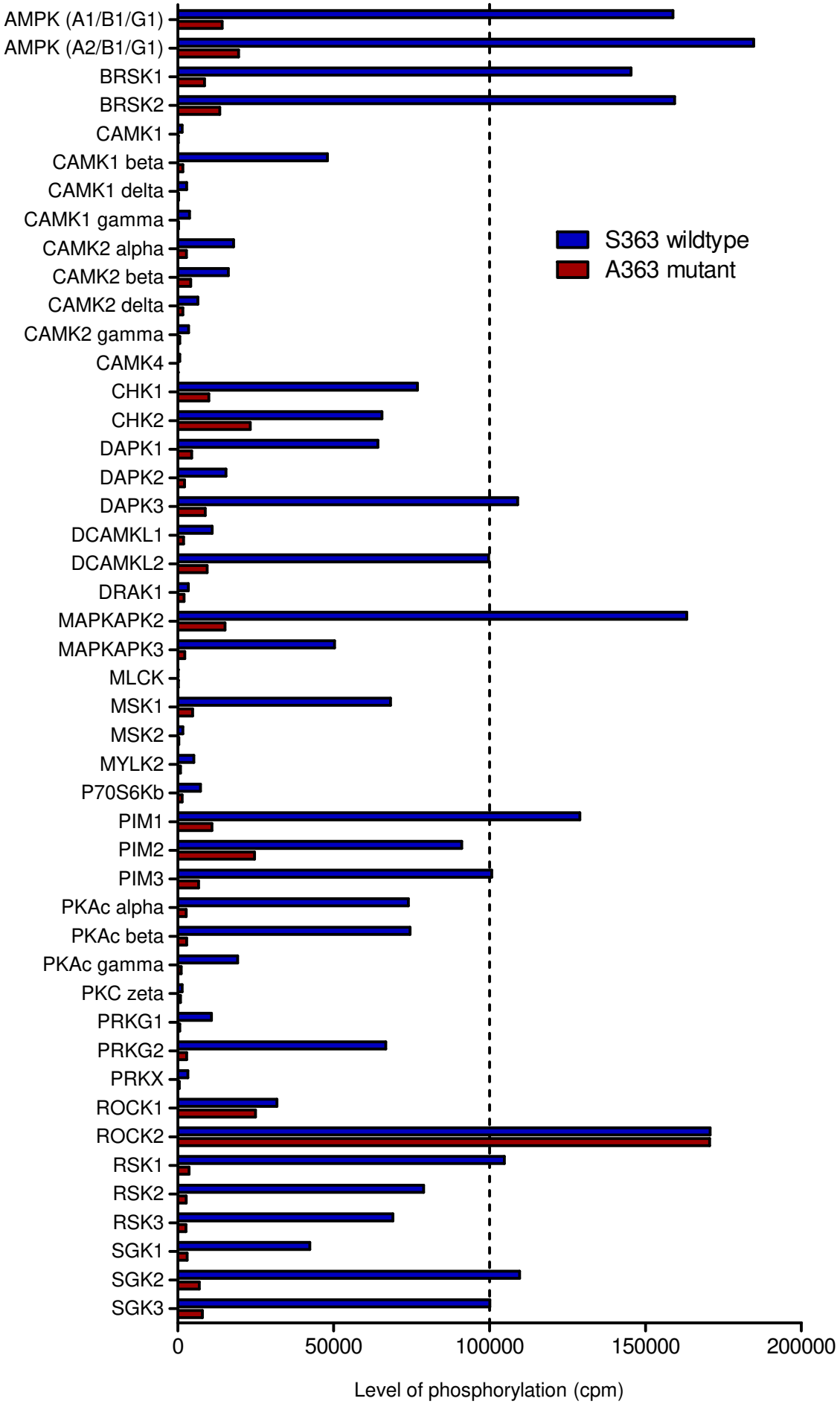

AMPK (A1/B1/G1)
AMPK (A2/B1/G1)
BRSK1
BRSK2
CAMK1
CAMK1 beta
CAMK1 delta
CAMK1 gamma
CAMK2 alpha
CAMK2 beta
CAMK2 delta
CAMK2 gamma
CAMK4
CHK1
CHK2
DAPK1
DAPK2
DAPK3
DCAMKL1
DCAMKL2
DRAK1
MAPKAPK2
MAPKAPK3
MLCK
MSK1
MSK2
MYLK2
P70S6Kb
PIM1
PIM2
PIM3
PKAc alpha
PKAc beta
PKAc gamma
PKC zeta
PRKG1
PRKG2
PRKX
ROCK1
ROCK2
RSK1
RSK2
RSK3
SGK1
SGK2
SGK3
S363 wildtype
A363 mutant
0
50000
100000
150000
200000
Level of phosphorylation (cpm)

Table 6.1: Kinase family members found to strongly and specifically phosphorylate Ser363 *in vitro*

Kinase Family	Specific kinase(s) found to strongly phosphorylate Ser363 *in vitro*
AMP-activated protein kinases	AMPK-α1 and AMPK-α2
Brain-specific serine/threonine kinases	BRSK1 and BRSK2
Death-associated protein kinases	DAPK3
Mitogen-activated protein kinase (MAPK)-activated protein kinases	MAPKAPK2 and MAPKAPK1 (also RSK1)
Proto-oncogene serine/threonine-protein kinases	PIM1 and PIM3
Ribosomal s6 kinase $p90^{rsk}$	RSK1 (also MAPKAPK1)
Serum/glucocorticoid regulated kinase	SGK2 and SGK3

in cancer cells that may be reliant on SK2-driven survival and proliferation. One such mechanism to control SK2 function is the modulation of its subcellular localisation (Siow & Wattenberg, 2011), which, as discussed in Chapter 5, may involve dynein-mediated translocation. It was also of interest to identify whether one or more of the uncharacterised SK2 phosphorylation sites may regulate its pro-apoptotic role. Thus, multiple SK2 point mutants were assessed for their ability to induce apoptosis.

As it has been well documented that overexpression of SK2 results in increased apoptosis compared to vector alone (Liu et al, 2003; Maceyka et al, 2005), overexpression of wildtype SK2 was initially used as a positive control to optimise methods of quantifying apoptosis. Various well validated methods were employed using a number of different cell lines to assess apoptosis, and such methods included quantifying fragmented nuclei by DAPI staining, flow cytometric assessment of annexin V and propidium iodide staining of intact cells as well as caspase 3 activation using NucView™ Caspase 3 substrate, and TUNEL assays. However, despite efforts to optimise these methods, considerable variability was observed between experimental repeats, but the reasons for this are unclear. The expected increase in apoptosis with wildtype SK2 overexpression was best observed using TUNEL apoptosis assays, however due to time constraints, subsequent assays using the full array of SK2 point mutants were only performed once and would therefore need to be repeated.

With this in mind, cells overexpressing SK2 with an alanine mutation at either Ser363 or Ser437 showed increased levels of apoptosis compared with wildtype SK2, whereas overexpression of SK2 with an alanine mutation at Ser398 appeared to abolish the SK2-mediated increase in apoptosis (Figure 6.10). Furthermore, mutation of Ser441 to alanine also reduced the level of apoptosis but to a lesser extent than S398A (Figure 6.10). Therefore Ser398, and possibly Ser441, may act to promote or activate the pro-apoptotic role of SK2, however as these were findings from a single experiment, caution must be taken in interpreting these data.

6.5.3 SK2 is phosphorylated at a novel site by GSK3

The SK2 phosphorylation sites examined above for their roles in apoptosis are either currently uncharacterised or completely novel (Figure 6.1). Interestingly, Ser437 adheres

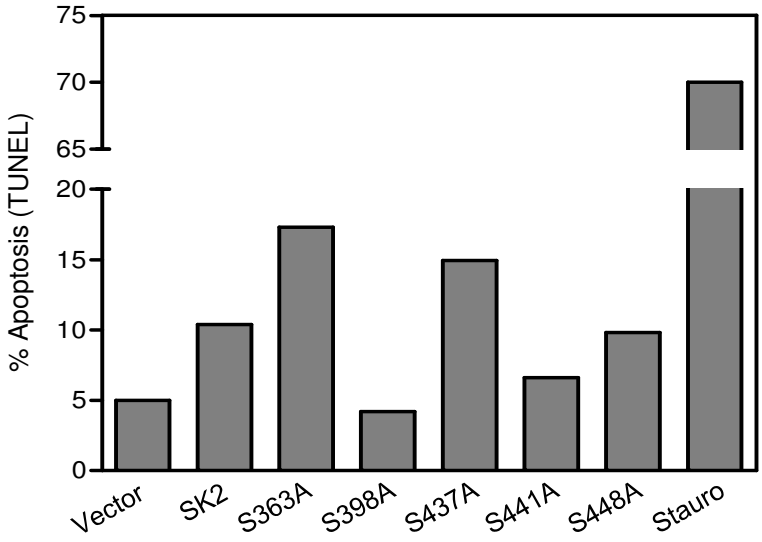


Figure 6.10: Phosphorylation may regulate the pro-apoptotic role of SK2

HeLa cells were transfected with either pcDNA3-IRES mCherry, FLAG-tagged wildtype SK2, or FLAG-tagged SK2 mutants S363A, S398A, S437A, S441A or S448A. Cells were cultured in serum-free media for 24 hr, and untransfected cells were treated for 24 hr with 1 μM Staurosporine as a positive control. Cells were then fixed and a TUNEL apoptosis assay was performed, followed by labelling of exogenous proteins by immunofluorescence using anti-FLAG antibodies. Cells were visualised by confocal microscopy, and dual TUNEL-positive / transfected cells were quantified in a blinded manner. A minimum of 200 cells were scored per treatment. Data are from only one experiment.

perfectly to a consensus motif found on substrates of glycogen synthase kinase 3 (GSK3; Figure 6.11A), whereby a priming phosphorylation event is required four residues downstream in order for GSK3 to dock and subsequently phosphorylate the second N-terminal site (Cole, 2012). Therefore, it was hypothesised that SK2 could be phosphorylated by GSK3 at Ser437, and this phosphorylation would be dependent on a priming phosphorylation event at Ser441. To test this, *in vitro* radiolabel phosphorylation assays were employed using wildtype SK2, or S437A or S441A mutants immunoprecipitated from cell lysates (performed by Dr. Adam Cole, Garvan Institute, Sydney). Indeed, recombinant GSK3 was able to phosphorylate SK2 *in vitro*, and this phosphorylation was significantly reduced when Ser437 or Ser441 were mutated to alanine (Figure 6.11B and C). Notably, when SK2-overexpressing cells were treated with the selective GSK3 inhibitor, CT99021 (Ring et al, 2003), to eliminate any endogenous GSK3 phosphorylation of SK2 prior to cell lysis, a doubling in SK2 phosphorylation by recombinant GSK3 was observed (Figure 6.11B and C). This demonstrates that SK2 is in fact a substrate of endogenous GSK3 and SK2 can be phosphorylated by GSK3 in cells.

In order to further characterise this novel GSK3 phosphorylation site, it was of interest to determine whether Ser437, or the priming site Ser441, had any effect on SK2 catalytic activity. Therefore, cells overexpressing these mutants, or wildtype SK2, were utilised in SK2-specific activity assays. As observed in Figure 6.12, rendering these residues non-phosphorylatable modestly, but significantly, increased SK2 catalytic activity by ~50% as compared to wildtype SK2. Hence, it appears that phosphorylation of SK2 at these sites may result in a decrease in SK2 enzymatic activity.

6.6 Discussion

6.6.1 Involvement of Ser363 in regulating the interaction between SK2 and dynein IC

This chapter examined the role of phosphorylation in SK2 regulation. There are a large number of uncharacterised phosphorylation sites in SK2 that have been identified in the Pitson Laboratory (Figure 6.1) and/or by global phosphoproteome analyses, that are likely to provide complex and potentially coordinated regulatory mechanisms to reflect the emerging functional complexity of SK2. It initially appeared that the phosphorylation site

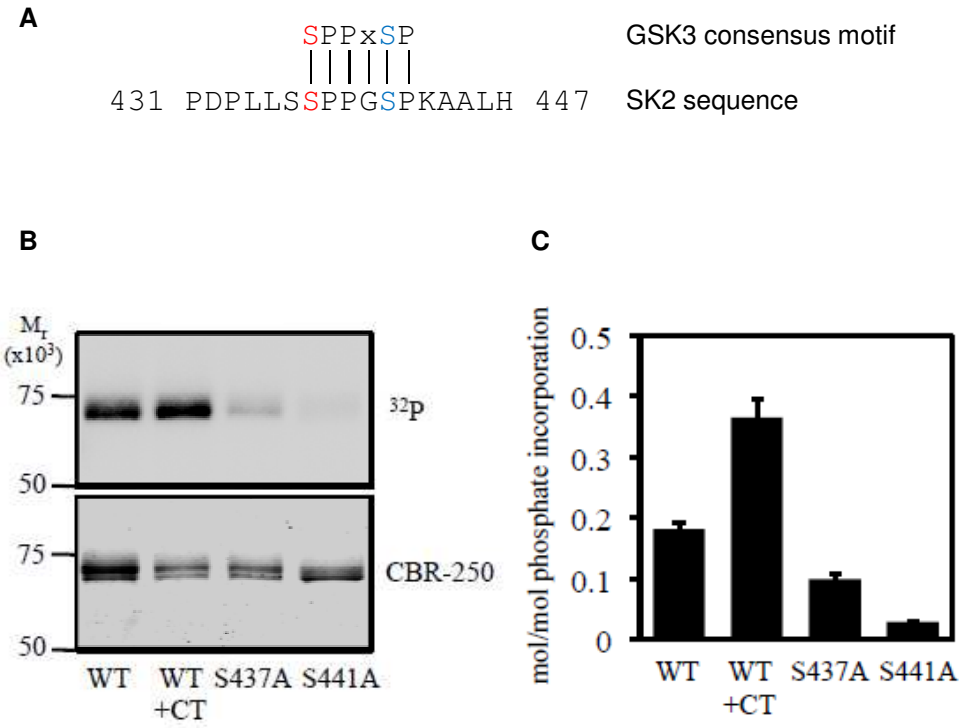


Figure 6.11: GSK3 phosphorylates SK2 on Ser437 *in vitro* and in cells

A, SK2 contains a sequence within its central domain that matches the GSK3 substrate consensus motif. The GSK3 phosphorylation site (red) as well as the required priming site (+4 position; blue) are indicated. *B,* HEK293 cells were transfected with FLAG-tagged SK2 (WT) or FLAG-tagged SK2 mutants, S437A or S441A. Cells transfected with wildtype SK2 were then either left untreated or treated with the GSK3 inhibitor CT99021 (CT). SK2 (or mutants) were then immunoprecipitated from lysates using anti-FLAG antibodies, and subjected to *in vitro* kinase assays with recombinant GSK3 and γ^{32}P-ATP. Expression of SK2 and mutants in the immunoprecipitates was confirmed by SDS-PAGE and staining with coomassie brilliant blue R250 (CBR-250). *C,* Phosphorylation of wildtype or mutant SK2 by GSK3 was quantified and normalised to protein expression. Data was generated and analysed by Dr. Adam Cole (Garvan Institute, Sydney).

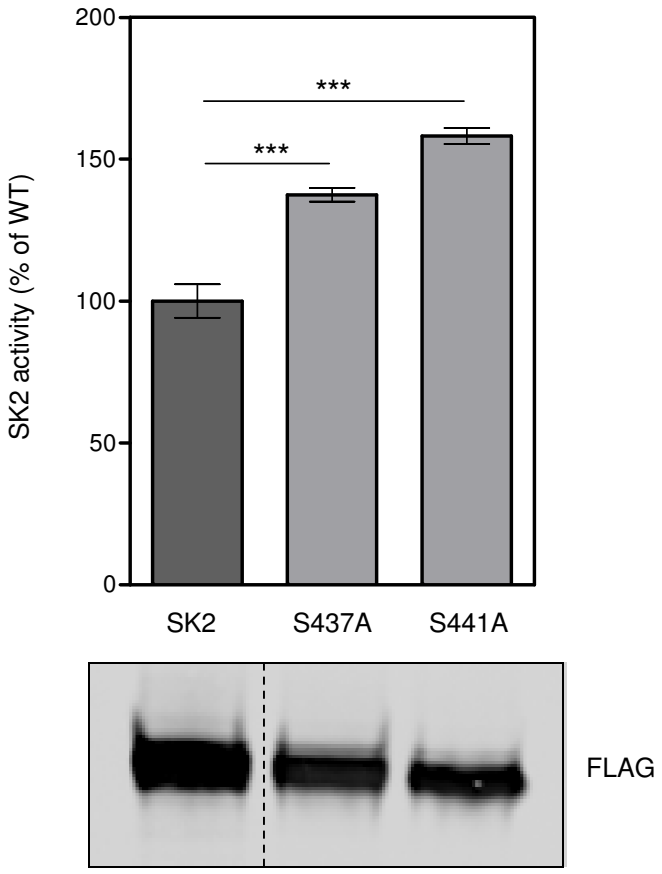


Figure 6.12: Ablation of Ser437 or Ser441 phosphorylation significantly increases SK2 catalytic activity

HEK293 cells were transfected with either wildtype SK2, or SK2 mutants S437A or S441A. After 48 hr, lysates were subjected to SK2-specific activity assays. Activity was normalised to expression levels of the exogenous proteins, as determined by immunoblot analyses using anti-FLAG antibodies (panel below). Dashed lines indicate where lanes from the same immunoblot have been spliced together to aid interpretation. Data are graphed as mean (± SD) of experimental triplicates from one experiment, representative of three independent experiments.

predominantly examined in this chapter, Ser363, was regulating the interaction between SK2 and dynein IC (Figure 6.3). However, further characterisation revealed that the observed reduction in binding of the SK2 mutant S363A with dynein IC was likely due to an indirect mechanism as a result of the isolation of these two proteins in different subcellular compartments (Figure 6.7). This would perhaps explain why some variability in the levels of interaction between IC and SK2 S363A were occasionally observed, ranging from almost completely abolished to only slightly reduced from wildtype (*data not shown*). This observation would likely have coincided with the levels of nuclear localised S363A mutant and consequently, the amount of protein in contact with dynein IC within the cell.

Since Ser363 phosphorylation does not appear to directly control the association of SK2 with dynein, future work will be required to characterise the regulation of this interaction. It remains possible that phosphorylation of SK2 at other sites may play a role in regulating this interaction, and these should be screened by *in vitro* pull-down assays to prevent any artefacts of altered SK2 localisation. However, it is also feasible that phosphorylation of the dynein IC may regulate its interaction with SK2, as a number of phosphorylation sites have been documented within the serine-rich region at the N-terminus of the protein (Figure 1.9). Specifically, phosphorylation of Ser81 and Ser84 on IC-2 appear to regulate the binding of dynein to certain cargoes for transport (Mitchell et al, 2012; Vaughan et al, 2001). On the contrary, the interaction between SK2 and dynein may be regulated by phosphorylation-independent mechanisms, such as by other post-translational modifications.

6.6.2 The role of Ser363 in the regulation of SK2 subcellular localisation

From the data in this study, it appears that Ser363 regulates the subcellular localisation of SK2, such that SK2 is sequestered to the nucleus when this site is mutated to alanine (Figure 6.6). It is tempting to speculate that phosphorylation of this site may be the mechanism regulating SK2 nuclear localisation, although other possibilities exist, such that Ser363 may form a critical residue involved in a kinase binding motif to regulate other SK2 phosphorylation sites, for example. Ser363 may also serve as an important residue in regulating or maintaining a specific conformation of the NES on SK2 through interactions with other residues, or it may regulate binding of SK2 to other proteins involved in regulating its nuclear localisation.

However, SK2 has been previously found to be phosphorylated endogenously on Ser363 in cells (Hornbeck et al, 2015). Indeed, the nuclear-localisation phenotype resulting from the S363A mutant is reminiscent of the previous study characterising the nuclear export of SK2, which was reported to be regulated by PKD-mediated phosphorylation of Ser383 and/or Ser385 within a novel nuclear export sequence (Ding et al, 2007). Similarly to S363A, when these two residues were mutated to alanine, SK2 was almost exclusively nuclear, suggesting that Ser363 may also be involved in SK2 nuclear export. It would therefore be of interest to determine if Ser363 is also phosphorylated to regulate SK2 nuclear export in a similar manner as the residues reported by Ding et al. Although, if Ser363 is being phosphorylated to regulate SK2 nuclear localisation, it cannot be ruled out that it is simply preventing nuclear import, and this would need to be examined in future work. Notably, however, like the SK2 nuclear export regulation site Ser385, Ser363 also perfectly matches the PKD minimal consensus motif of LxRxx(p)S/T (Nishikawa et al, 1997). Furthermore, again like Ser385, Ser363 resides within a sequence that contains a number of hydrophobic leucine residues, a hallmark of the nuclear export sequence motif: $Hx_{2-3}Hx_{2-3}HxH$ (where H is a hydrophobic residue, generally leucine but can also be isoleucine, methionine, phenylalanine, or valine, X is any amino acid, and the subscript numbers indicate the possible number of repeats) (Ishizawa et al, 2015). Although the sequence surrounding Ser363 is missing the second hydrophobic residue in the motif, it has been reported that as little as a third of experimentally-validated NESs match the above motif (la Cour et al, 2003). Therefore, given that PKD was clearly implicated in promoting the nuclear export of SK2, and it was documented that SK2 possesses multiple putative NESs, of which at least two appear to be functional (Ding et al, 2007), it is possible that Ser363 may also be phosphorylated by PKD to regulate the nuclear export of SK2. As discussed further below, PKD was not included in the broad kinase screen, so *in vitro* phosphorylation of Ser363 using PKD will need to be performed. Furthermore, whether the sequence surrounding Ser363 is a *bona fide* NES, and whether it acts redundantly or in cooperation with the NES charactered previously (Ding et al, 2007) requires further investigation.

6.6.3 The role of Ser363 in the regulation of SK2 catalytic activity

Mutating Ser363 to alanine also resulted in an increase in SK2 catalytic activity (Figure 6.8), again potentially due to a loss of phosphorylation at this site. The observations that an increase in catalytic activity and change in subcellular localisation occurred when Ser363

was mutated to alanine, without any further stimulation of the cells, may suggest that there is basal phosphorylation of wildtype SK2. This would fit with the hypothesis of Ser363 being a modulator of the basal nucleo/cytoplasmic shuttling of SK2. However, it is also possible that this basal phenotype could also be an artefact of overexpression, where the high cellular levels of SK2 protein may facilitate its basal phosphorylation at sites normally only phosphorylated under certain circumstances. Therefore, to validate this potential basal effect of Ser363 phosphorylation on SK2 activity and localisation, it would be of interest to generate an inducible cell line where low-level overexpression of SK2 with a S363A mutation could be achieved through titrating levels of doxycycline, as previously achieved in the Pitson Laboratory (Pham et al (2008), and Chapter 3). Furthermore, for a completely physiological setting, CRISPR/Cas9 gene editing could be employed to insert an alanine mutation into Ser363, as well as a C-terminal FLAG-tag, within the endogenous SK2 gene in cells. Therefore, SK2 localisation and activity in these cells could be analysed and compared to wildtype cells to determine the physiological effects of Ser363 phosphorylation.

6.6.4 Substitutions of Ser363 to glutamate and aspartate

Through the use of glutamate and aspartate substitutions, an attempt was made to constitutively mimic phosphorylation of Ser363 in order to further characterise the role of this potential phosphorylation site. However, these mutants (S363E and S363D) displayed a localisation phenotype and activity level similar to the S363A mutant (Figure 6.6 and Figure 6.8). It is possible that Ser363 may be regulating SK2 in a phosphorylation-independent manner, in which case substitution to glutamate or aspartate would likely still abolish this function. However, if Ser363 is regulating SK2 through phosphorylation, then in this case glutamate and aspartate were unsuccessful at recapitulating the appropriate size and charge of a phospho-serine. Although the differences in the size and charge of a phospho-serine compared with a glutamate/aspartate residue are only subtle, and they have been shown to successfully mimic phosphorylation of certain proteins including SK2 (Adada et al, 2015), it has been reported that even small differences in charge and size can lead to changes in hydrogen bonding and overall protein conformation (Ng et al, 2010). Consequently, the hydrogen bonding that occurs due to the presence of a phosphate group can be lost when a glutamate is substituted, and hence the function of the phosphorylation will not be recapitulated. Therefore, in order to further characterise the function of this site in the

future, *in vitro* phosphorylation of recombinant SK2 on Ser363 or stimulating endogenous phosphorylation of this site in cells would be informative, however the identity of the kinase responsible will be required.

6.6.5 Screening candidate kinases responsible for Ser363 phosphorylation

The *in vitro* kinase screen performed provided eleven candidate kinases that were able to strongly, and selectively, phosphorylate Ser363 in SK2-derived peptides. This number is quite high, likely due to the use of a short peptide as opposed to full-length protein and the relatively high concentration of peptide used, undoubtedly resulting in false-positives. Interestingly, AMP-activated protein kinase (AMPK) was the top candidate with the strongest phosphorylation of Ser363 (Figure 6.9). Both isoforms of the catalytic domain (AMPK-α1 and AMPK-α2) were able to phosphorylate Ser363 *in vitro*. AMPK is an important, ubiquitously expressed, energy-sensing kinase that, when the cell is experiencing energy stress and the AMP:ATP ratio is high, becomes activated through binding to AMP to promote catabolic pathways to restore energy homeostasis (Hardie et al, 2015). However, there appear to be other less well characterised roles of AMPK, including a requirement of AMPK for normal cell cycle progression, and conflicting roles in both supporting and preventing cancer progression (Dasgupta & Chhipa, 2015), both of which would be interesting in the context of SK2 regulation by phosphorylation. AMPK has a very well defined substrate recognition motif, where some flexibility can be tolerated but most substrates possess the sequence LxRSx(p)S/TxxxL (Hardie et al, 2015). Aside from the polar residue at the -2 position [which is the least critical residue (Hardie et al, 2015)], Ser363 perfectly matches this motif (LPRAKpSELTL).

Interestingly, another kinase family closely related to AMPK, BRSK, was also able to strongly phosphorylate SK2 at Ser363 *in vitro*. BRSK, also known as SAD or AMPK-related kinase, is not as well characterised as AMPK but has known roles in promoting neuronal cell polarity, likely via its role in phosphorylating tau to regulate microtubule organisation (Kishi et al, 2005; Yoshida & Goedert, 2012). The specific expression of BRSKs in the brain and nervous system makes them an interesting candidate kinase for SK2 phosphorylation, as SK2 is also highly expressed in brain (Liu et al, 2000) and preferentially interacts with the predominantly neuronal-expressed dynein IC-1 (Chapter 5), highlighting

that SK2 may have additional brain-specific functions that require brain-specific regulatory mechanisms.

DAPK would also make an interesting candidate kinase for SK2 regulation, as it is a well characterised tumour suppressor with various roles in mediating interferon-γ induced apoptosis, promoting anoikis and suppressing cell motility (Huang et al, 2014). Intriguingly, DAPK3, the isoform found to strongly phosphorylate Ser363 *in vitro* (Figure 6.9), does not possess the Ca2+/CaM autoregulatory domain found on DAPK1 and DAPK2, but instead possesses an NLS and a leucine zipper, and has unique nuclear functions as well as roles in mitochondrial mediated apoptosis and autophagy (Shiloh et al, 2014). As SK2 has also been shown to mediate cell death at these localisations (Chipuk et al, 2012; Okada et al, 2005), it would be fitting for SK2 to be regulated by DAPK3. However, DAPK1 and DAPK2 could also phosphorylate Ser363 specifically *in vitro*, albeit to a lesser extent than DAPK3 (Figure 6.9), and so if DAPK is the kinase responsible for phosphorylation of this site, it would be of interest to examine the involvement of each isoform.

Another set of candidate kinases include the MAPK-activated protein kinases, which encompass MAPKAPK2 and RSK1. Interestingly, canonical activation of the RSKs occurs via ERK1/2 whereas canonical activation of the MAPKAPKs is mediated by p38 (Gaestel, 2016). As such, RSKs have been shown to promote cell proliferation, cell growth, protein synthesis and cell survival, and they generally translocate to and signal from the nucleus upon mitogenic stimulation (Cargnello & Roux, 2011). Furthermore, MAPKAPK2 is activated by stress stimuli and, in contrast to the RSKs, is exported out of the nucleus to the cytoplasm upon stimulation, where it acts to facilitate cytokine production, cell migration, actin remodelling, cell cycle control and gene expression (Cargnello & Roux, 2011). Given that Ser363 appears to be involved in SK2 transport out of the nucleus, where it has anti-proliferative functions (Hait et al, 2009; Igarashi et al, 2003), it is perhaps more plausible that a pro-proliferative kinase like RSK1 that signals from the nucleus would be a more likely candidate.

The possibility of SK2 regulation by PIM kinases is intriguing, as PIM kinases are proto-oncogenes that mediate cell cycle progression, cell survival, transcriptional upregulation, protein synthesis and cellular metabolism, and are frequently upregulated in various cancers (Alvarado et al, 2012; Bullock et al, 2005). Fascinatingly, PIM kinases do not possess a

kinase regulatory domain, and so they are likely to be constitutively active following translation (Alvarado et al, 2012). In line with this, Ser363 phosphorylation has been detected in 34 different phosphoproteomic studies (Hornbeck et al, 2015), suggesting that it may be a prevalent modification. Furthermore, like RSK1, the pro-proliferative functions of the PIM kinases make them logical candidates for Ser363 phosphorylation. Similarly, the SGKs are also involved in the regulation of important processes such as cell growth, proliferation, survival and migration, with additional functions including involvement in locomotive behaviour, cellular stress, and transport of ions, nutrients and amino acids (Bruhn et al, 2010). Interestingly, the SGKs exhibit structural similarity to AKT and are activated downstream of the PI3K pathway, and they also interact at various levels with the Ras/Raf/ERK pathway (Tessier & Woodgett, 2006) As such, the SGKs have also been found to contribute to tumourigenesis (Bruhn et al, 2010). Unlike the PIM kinases, however, the SGKs translocate to the nucleus upon growth factor stimulation (Buse et al, 1999).

The substrate consensus motifs for each of the seven kinase families demonstrating strong phosphorylation of Ser363 *in vitr*o are listed in Table 6.2. The amino acid sequence surrounding Ser363 on SK2 is also provided to demonstrate how well each consensus motif suits this site. However, it should be noted that some of the kinases' known substrates themselves do not match the consensus motifs well [e.g. DAPK; Lin et al (2010)], and so this does not seem to be a good indicator of whether or not a protein will be a *bona fide* substrate.

Notably, ROCK2 was able to phosphorylate both the wildtype and the alanine mutant peptides very strongly (Figure 6.9), indicating that it must be phosphorylating another residue on the peptides, possibly Thr366 and/or Thr368. Interestingly, ROCK1 was also able to phosphorylate both the wildtype and S363A peptides, but to a lesser extent than ROCK2. As Thr368 lies directly upstream of a proline residue in SK2, the proline residue had been specifically excluded from the peptide sequences used for the *in vitro* kinase assays in order to prevent background phosphorylation of Thr368 by proline-directed kinases (although ROCK is not a member of this family of kinases). Mass spectrometric-based phosphorylation studies have reported Thr366 as being physiologically phosphorylated in Jurkat cells treated with the protein phosphatase inhibitors calyculin and pervanadate (Hornbeck et al, 2015). Thr368 was also found to be endogenously phosphorylated by phosphoproteomic analyses (Daub et al, 2008; Sharma et al, 2014), and

Table 6.2: Substrate consensus motifs of the candidate kinases for Ser363 phosphorylation on SK2

Kinase Family	Consensus Motif	SK2 sequence	Reference
AMPK	LxRSxS/TxxxL	LPRAKSELTL	(Hardie et al, 2015)
BRSK	R/KxxS/T	LPRAKSELTL	(Yoshida & Goedert, 2012)
DAPK	KRRxxS/T	LPRAKSELTL	(Lin et al, 2010)
MAPKAPK	ΦxRxL/NS/TΦ	LPRAKSELTL	(Cargnello & Roux, 2011)
PIM	RxRHxS/T	LPRAKSELTL	(Bullock et al, 2005)
RSK	R/KxRxxS	LPRAKSELTL	(Cargnello & Roux, 2011)
SGK	RxRxxS/T	LPRAKSELTL	(Kobayashi & Cohen, 1999)
PKD	LxRxxS/T	LPRAKSELTL	(Nishikawa et al, 1997)

Underlined residues in the SK2 sequence designate residues that match the consensus motif. Residues in red represent the phosphorylated residue (Ser363). Φ represents a hydrophobic residue.

phosphorylation of this site was also detected previously by the Pitson Laboratory using overexpressed SK2 (Figure 6.1). Neither of these threonine residues match the ROCK substrate consensus motif (R/Kx(p)S/T or R/Kxx(p)S/T) (Amano et al, 2010), however, other *bona fide* ROCK substrates (e.g. Calponin) also deviate from this sequence (Kaneko et al, 2000). Furthermore, neither Thr366 nor Thr368 are conserved in mouse or rat SK2, and hence phosphorylation at these sites would likely represent human-specific regulatory modifications of SK2.

Future work will require the validation of these kinases by *in vitro* phosphorylation assays, using recombinant full-length SK2 and a S363A mutant (or T366A and T368A mutants for ROCK), and ultimately the use of selective inhibitors and activating compounds specific for the kinase of interest in cell-based assays. Unfortunately, PKD was not included in this kinase screen as, surprisingly, it was not selected by Kinexus as being in the top 50 candidate kinases for this site. However, given that PKD mediates the nuclear export of SK2 (Ding et al, 2007), and Ser363 matches the PKD consensus motif (Nishikawa et al, 1997), PKD should also be included in future kinase validation assays.

6.6.6 Examining phosphorylation sites as potential regulators of the pro-apoptotic role of SK2

Any molecule that plays a role in mediating apoptosis must be appropriately regulated to ensure that this function is only activated when cell death is required. However, although SK2 has known roles in mediating cell death (Chipuk et al, 2012; Hofmann et al, 2008; Okada et al, 2005), the mechanisms through which this function is regulated are not clear. SK2 was previously proposed to possess a BH3 domain which can interact with Bcl-x_L to mediate the intrinsic mitochondrial apoptosis pathway (Liu et al, 2003). However, these studies employed high level SK2 overexpression and so the physiological relevance of this domain, and how it may be regulated, remains to be addressed.

It was of particular interest to determine if any of the uncharacterised SK2-specific phosphorylation sites within the unique central region were able to regulate the pro-apoptotic role of SK2. Although preliminary, the results from these studies are suggestive that phosphorylation of Ser363 or Ser437 may act to prevent the pro-apoptotic function of SK2 (Figure 6.10). These assays will need to be repeated to validate these findings,

however, given that the localisation data suggests that Ser363 phosphorylation may promote SK2 nuclear export, it would be quite appropriate for Ser363 phosphorylation to also prevent cell death as SK2 has been previously shown to promote cell cycle arrest in the nucleus (Hait et al, 2009; Igarashi et al, 2003). This is purely speculative and considerable work will be required to validate these hypotheses.

Furthermore, the data also suggest that phosphorylation at Ser398 or Ser441 may drive the pro-apoptotic role of SK2, as when these sites were mutated to alanine the percentage of apoptotic cells resembled that of the empty vector cells (Figure 6.10). Again, this will require further validation. Interestingly, Ser398 phosphorylation was previously detected on overexpressed SK2 by the Pitson Laboratory (Figure 6.1), however phosphorylation of this site has not been detected endogenously and has not been otherwise characterised.

Upon future validation of these results, if the pro-apoptotic role of SK2 is in fact regulated by phosphorylation, it would be intriguing to examine how this regulation occurs. It could be likely to involve changes in subcellular localisation, which is critical for SK2 function (Siow & Wattenberg, 2011), and/or modulation of SK2 interacting with binding partners. It would also be interesting to determine if more than one site is involved in such regulation (as the preliminary data may suggest), and if so, whether they act in a co-ordinated manner or whether individual phosphorylation events could regulate slightly different functions of SK2, dependent on the pro-apoptotic stimuli presented. For example, SK2 is known to mediate cell cycle arrest in the nucleus (Hait et al, 2009; Igarashi et al, 2003), and apoptosis at the ER (Maceyka et al, 2005) and mitochondria (Chipuk et al, 2012; Liu et al, 2003), but it is unknown whether different regulatory mechanisms dictate where SK2-mediated cell death occurs in the cell. It would be very interesting to pursue this further.

6.6.7 Phosphorylation of SK2 by GSK3

Through collaboration with Dr. Adam Cole (Garvan Institute, Sydney), it was demonstrated that SK2 is a *bona fide* substrate of GSK3 *in vitro* and in cells (Figure 6.11). GSK3 is a ubiquitously-expressed proline-directed kinase with particularly high expression in the brain (Cole, 2012). Rather unusually, GSK3 is constitutively active in resting/unstimulated cells, and is in fact inhibited by a number of extracellular stimuli, including growth factors, insulin and serum (Kaidanovich-Beilin & Woodgett, 2011). Furthermore, GSK3 generally

requires another kinase to 'prime' its substrate in order to bind to and phosphorylate it (Kennelly & Krebs, 1991), however an acidic residue in place of a priming phosphorylation site can sometime suffice (Beurel et al, 2015). Previously reported priming kinases include cyclin-dependent kinase-5 (CDK-5), PAR-1 (microtubule affinity-regulating kinase; MARK in mammals), casein kinase 1 and 2 (CK1 and CK2), protein kinase A (PKA), and protein kinase C (PKC) (Kaidanovich-Beilin & Woodgett, 2011).

In agreement with the constitutive activity of GSK3 in cells, there was evidence of basal phosphorylation of SK2 at Ser437 by endogenous GSK3, as application of the GSK3 inhibitor CT99021 to cells, to prevent endogenous phosphorylation, resulted in a doubling of SK2 phosphorylation by recombinant GSK3 *in vitro* (Figure 6.11). This would imply that the 'priming' kinase must also be constitutively active, although it will need to be determined if this observed basal phosphorylation of SK2 is simply an artefact of overexpression. Such high cellular levels of SK2 protein could potentially facilitate basal phosphorylation at sites normally only phosphorylated under certain circumstances, for example when a priming kinase is activated.

The concept of constitutive basal phosphorylation of SK2 by GSK3 is intriguing, and raises the possibility that de-phosphorylation of this site may also be an important regulatory mechanism of SK2. GSK3 is generally considered to be pro-apoptotic, as a number of GSK3 substrates that promote cell proliferation and survival are negatively regulated by GKS3-mediated phosphorylation (Cole, 2012; Sutherland, 2011). Furthermore, GSK3 can promote the intrinsic apoptosis pathway (Beurel & Jope, 2006). Perhaps basal GSK3 phosphorylation suppresses the pro-survival/proliferative, oncogenic role of SK2. Or, if this phosphorylation event is not constitutive, then it is possible that GSK3 phosphorylation of SK2 might be triggered to promote the pro-apoptotic role of SK2. The preliminary apoptosis data is conflicting, where preventing SK2 phosphorylation of the GSK3 priming site Ser441 by mutation to alanine appeared to prevent SK2-mediated apoptosis, whereas alanine mutation of the GSK3 phosphorylation site Ser437 seemed to enhance this function (Figure 6.10). Hence, it will be interesting to validate the apoptosis data in order to examine the potential role of GSK3 in regulating the pro-apoptotic role of SK2.

Notably, as shown in Figure 6.11B, SK2 protein expression levels substantially decreased upon use of the GSK3 inhibitor CT99021, and upon alanine substitution of the two residues

involved in GSK3 phosphorylation, Ser437 and Ser441. This may represent a novel role of GSK3-mediated phosphorylation in maintaining the stability of SK2 protein. Indeed, this has been previously observed for other GSK3 substrates, where phosphorylation of Axin by GSK3 results in increased Axin protein stability (Yamamoto et al, 1999), although the exact mechanisms are unclear but may involve the dissociation of Axin from Adenomatous Polyposis Coli (APC), resulting in less degradation of Axin (Pronobis et al, 2015; Takacs et al, 2008). Furthermore, it was proposed that GSK3-mediated phosphorylation of p130 Retinoblastoma protein increases its stability when cells are undergoing quiescence, but this stabilisation of p130 does not appear to involve changes in proteasome-mediated degradation (Litovchick et al, 2004). It will therefore be of interest to further explore the possibility of GSK3-mediated SK2 protein stabilisation, and determine the mechanisms at play and how SK2 function might be affected. It should also be noted that the non-phosphorylatable priming site mutant, S441A, resulted in 3-fold less phosphorylation of SK2 by GSK3 *in vitro* than the direct GSK3 site mutant S437A (Figure 6.11C). The reason for this is unclear but could potentially involve changes to SK2 conformation.

Interestingly, while the GSK3 phosphorylation site Ser437 is conserved in SK2 across many mammalian species, including human, chimpanzee, dog, cow, mouse and rat, the priming phosphorylation site likely required by GSK3, Ser441 (in human), is divergent in some species (Figure 6.13A). Compared to the minimal consensus motif used by GSK3 (Beurel et al, 2015), mouse and rat SK2 do not possess a functional priming site, whereas bovine SK2 acquired a threonine residue, and canine, chimpanzee and human possess a serine residue at this site (Figure 6.13A). Interestingly, the acquisition of a potentially functional priming phosphorylation site appears to have occurred in line with other evolutionary changes to the SK2 sequence, as demonstrated by a phylogenetic tree showing the relationships between SK2 sequences of the different species (Figure 6.13B). As there are no acidic residues in place of the serine/threonine to mimic a priming phosphorylation in rodents, it is unlikely that the GSK3 site is functional in these species, but this would require further examination. Therefore, similarly to Thr366 and Thr368, phosphorylation of SK2 by GSK3 at Ser437 does not appear to be an evolutionarily conserved regulatory mechanism. Why SK2 has acquired additional phosphorylation sites in higher-order mammals, and whether these pertain to functions specifically involving the longer SK2b isoform not found in rodents, remains to be elucidated.

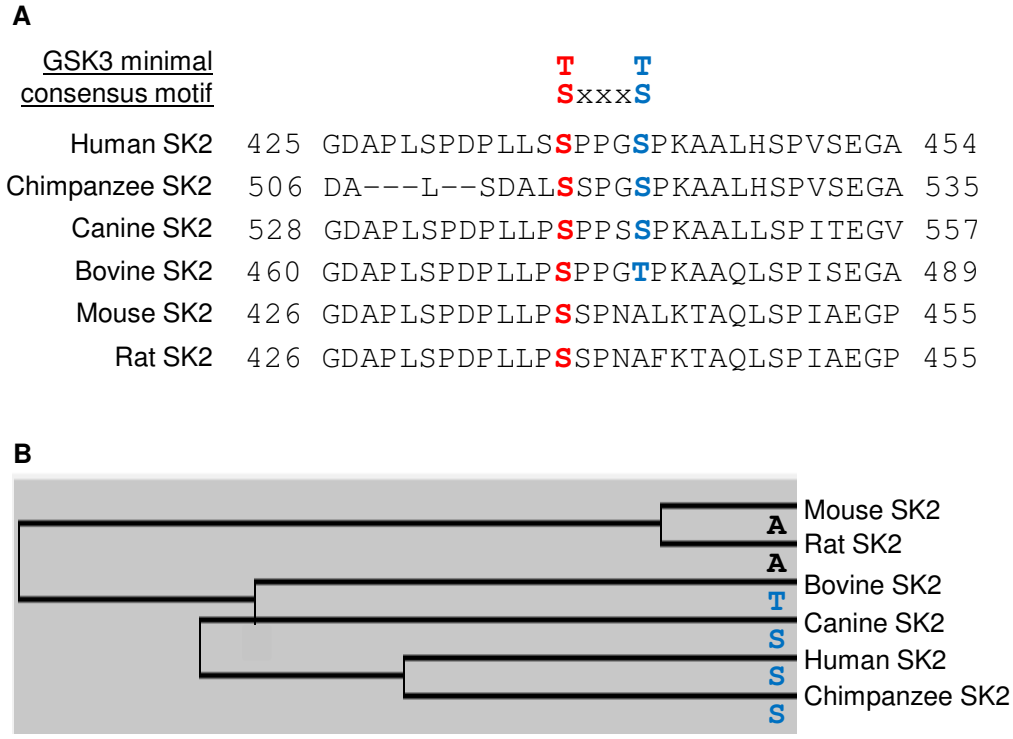


Figure 6.13: Sequence alignment of SK2 from various species showing conservation of the GSK3 phosphorylation and priming sites

A, Amino acid sequence alignment of SK2 is shown from human, chimpanzee, canine, bovine, murine and rat SK2 sequences. The canonical SK2 sequences were used for all species, except human where SK2a was used in order to keep numbering consistent. The GSK3 phosphorylation site and the priming phosphorylation site are highlighted in red and blue, respectively. The GSK3 minimal consensus motif is shown above. *B,* Phylogenetic tree showing the evolutionary relationships of SK2 sequences between different species. The 'priming site' residue for each species (from A) is overlayed. Sequence alignments and phylogenetic tree were generated using the UniProt resource.

Similarly to the S363A SK2 mutant, the non-phosphorylatable S437A and S441A mutants had higher catalytic activity than wildtype SK2 (Figure 6.12). Therefore, these data suggest that phosphorylation at these sites may act to decrease SK2 activity in cells, although further investigation is required to confirm that phosphorylation is in fact involved in these observed changes in SK2 activity. Given that the S363A mutant resulted in a similar phenotype (Figure 6.8), it will need to be determined whether these sites are all acting independently to regulate SK2, or whether there may be some interplay allowing the loss of one site to co-ordinately act on other sites.

It is interesting to note that when overexpressed in cells, SK2 has four-fold less specific activity than SK1 (Roberts et al, 2004). Examining the kinetics of the SKs overexpressed in cells revealed that their K_m values for sphingosine were equivalent, whereas the catalytic efficiency (k_{cat}/K_m) of SK1 is approximately 1.3-fold higher than that of SK2 (Billich et al, 2003). Therefore, it is tempting to speculate that the naturally lower activity of SK2 may be due, at least in part, to this array of SK2-specific phosphorylation events within its unique central region that may act to lower its activity. The basal catalytic efficiency of non-phosphorylated SK1 and SK2 made in *E. coli* have not previously been analysed together for comparison. Furthermore, the crystal structure of SK2 has not yet been solved, and as there is no sequence similarity of the ~130 residue proline-rich central domain to any other known protein, the conformation of this region and whether or not it could physically interact with and modulate the catalytic domains, cannot currently be determined. It is, however, predicted to be a disordered loop (Wang et al, 2014a). Nevertheless, it is rather fascinating to speculate that SK2 may have lower basal activity than SK1 as a result of its collection of regulatory phosphorylation sites. Whether the reason for this potential evolutionary downregulation of SK2 activity is because of its differential subcellular localisation and additional pro-apoptotic roles would require further investigation.

Notably, in contrast to my findings, Hait et al previously reported that the S441A mutant had only 35% of the activity of wildtype SK2 (Hait et al, 2007), suggesting that phosphorylation of this site may increase SK2 activity. However, this previous study did not appear to normalise the activity of SK2 or the S441A mutant to their expression levels in the lysates, and since the S441A mutant does not express as well as wildtype SK2 (Figure 6.11 and Figure 6.12), this may explain the discrepancy.

6.6.8 Conclusions

The studies outlined in this chapter aimed to provide a better understanding of SK2 regulation by phosphorylation. While, due to time constraints, some of these results are preliminary, they touch on the level of complexity of SK2 phosphorylation and provide a number of avenues to further investigate how SK2 localisation, function and activity may all be differentially regulated by different signalling pathways. What is clear is that there is still a substantial amount of work required to truly understand how these regulatory mechanisms are co-ordinated, and how they may be potentially manipulated in order to develop therapeutics to target SK2 in cancer and other diseases.

Chapter 7:
General Discussion

Chapter 7. General Discussion

Despite first being identified almost two decades ago, the functions and regulation of SK2 still remain rather poorly understood. Unlike SK1, which has well established roles in promoting cell survival, proliferation and oncogenesis (Newton et al, 2015; Pitson, 2011; Pyne & Pyne, 2010), SK2 has generally been perceived to be pro-apoptotic and to promote cell cycle arrest (Hait et al, 2009; Igarashi et al, 2003; Liu et al, 2003). However, conflicting evidence has emerged suggesting that SK2 can also play a role in cancer, as knockdown or inhibition of SK2 can reduce cancer cell growth and proliferation, and tumour growth *in vivo* (French et al, 2010; Liu et al, 2013; Panneer Selvam et al, 2015; Weigert et al, 2009). Despite this, at the beginning of the studies outlined in this thesis it still remained to be determined whether, like SK1, SK2 could in fact drive neoplastic transformation and tumourigenesis. Furthermore, the subcellular localisation of SK2 is thought to be critical for its functions, as the production of S1P at different organelles within the cell can induce varying functional outcomes (Siow & Wattenberg, 2011). Indeed, where SK1 is generally localised to the cytoplasm and plasma membrane, SK2 is often found localised to the nucleus, ER and mitochondria, suggesting that its additional functional complexity may arise from its localisation to these organelles. Therefore, given the clearly complex and poorly understood roles of SK2 in cancer, the goal of the studies outlined in this thesis were to interrogate the role of SK2 in promoting oncogenesis, as well as to better understand how SK2 is regulated and localised within the cell.

7.1 SK2 can mediate oncogenesis

Given that SK1 and SK2 both catalyse the production of S1P from sphingosine, and given that there is evidence of functional redundancy between these enzymes as demonstrated by individual genetic knockout of each gene (Allende et al, 2004; Mizugishi et al, 2005; Zemann et al, 2007), it would perhaps be expected that SK2 could mediate oncogenesis through S1P production like SK1. However, where overexpression of SK1 in NIH3T3 mouse fibroblasts leads to neoplastic transformation of these cells (Xia et al, 2000), overexpression of SK2 in these cells had previously been shown to decrease proliferation and promote apoptosis (Igarashi et al, 2003; Liu et al, 2003; Maceyka et al, 2005). This was

not consistent with more recent reports hinting a role for SK2 in promoting cancer, through knockdown or inhibition of the endogenous protein, which suggested that perhaps a more physiological system needed to be employed to interrogate the true roles of SK2 in cancer.

In this study, we were able to demonstrate that low, much closer to physiological levels of SK2 overexpression in NIH3T3 fibroblasts could induce neoplastic transformation *in vitro* and could promote tumour formation *in vivo*. This was a significant finding, as it is the first to finally confirm that SK2 can mediate oncogenesis and that the previously observed anti-cancer effects of SK2 knockdown or inhibition are likely to be specific to SK2 and not the result of off-target effects or other affected downstream mediators. I also demonstrated that, consistent with previous findings, the subcellular localisation of SK2 seemed to change in line with its opposing functions. When overexpressed at low levels (10-fold over endogenous levels), there was a significant increase in plasma membrane-localised SK2, as well as a significant increase in extracellular S1P formation. Whereas when overexpressed at high levels (~400-fold over endogenous levels), SK2 was found more strongly localised to the nucleus.

These findings highlight the importance of using physiologically relevant systems and tools to explore the roles of SK2. However, it is unclear whether the anti-proliferative, pro-apoptotic functions of SK2 that have been proposed from studies using non-physiological high-level overexpression are real or artifacts of these systems. Indeed, there have been studies demonstrating that siRNA-mediated knockdown of endogenous SK2 in HEK293 cells or MEFs prevented apoptosis induced by TNFα (Chipuk et al, 2012; Okada et al, 2005). Similar results were observed upon staurosporine-induced apoptosis in mesangial cells taken from *Sphk2*$^{-/-}$ mice (Hofmann et al, 2008). These studies would suggest that SK2 does have physiological roles in promoting apoptosis, however it cannot be ruled out that this function of SK2 may be cell type dependent (as two of the studies used renal cells) or may be more prevalent earlier in development (since human embryonic kidney cells and mouse embryonic fibroblasts were used). These studies reported endogenous SK2 localisation to the nucleus and mitochondria (Chipuk et al, 2012; Okada et al, 2005), which is consistent, at least for nuclear-localisation, with reports from high-level overexpression studies of SK2 mediating cell cycle arrest and cell death (Igarashi et al, 2003; Okada et al, 2005). However, it is unclear whether reports of overexpressed SK2 promoting apoptosis when localised to the ER (Maceyka et al, 2005), and inducing apoptosis through a putative

BH3 domain and interaction with Bcl-x_L (Liu et al, 2003) are physiological roles of SK2 or artifacts of overexpression. High-level overexpression can mis-localise proteins and can also force protein-protein interactions that may otherwise not occur naturally (Berggard et al, 2007; Lalonde et al, 2008). As many of the initial studies examining and characterising SK2 function and localisation employed high-level overexpression systems, it seems necessary that as the roles of SK2 are appearing more complex, care will need to be taken to ensure that the systems and tools used to dissect SK2 functions are as physiological and relevant as possible.

In this study, we demonstrated that low-level overexpression of the SK2a isoform can induce neoplastic transformation. SK2a is by far the best characterised SK2 isoform and is the isoform most commonly used in the literature. However, it would be of interest to examine whether the larger, human-specific isoform SK2b can also have the same effects. It was reported that SK2b is the predominant form of SK2 in various human tissues and cell lines, and serum deprivation resulted in increased expression of SK2b (Okada et al, 2005). Using high-level overexpression in HEK293 cells, it was shown that serum-withdrawal induced the translocation of SK2b to the nucleus in a small proportion of cells, and this coincided with a decrease in cell proliferation, whereas SK2a decreased cell proliferation in both the presence and absence of serum (Okada et al, 2005). It is tempting to suggest that this role of SK2a is an artifact of overexpression in this cell type, as it mediated cell cycle arrest even in the absence of any anti-proliferative stimuli, and we have shown that SK2a is capable of inducing neoplastic transformation. As SK2b seems to be the predominant isoform found in human cells, and as it only promotes cell cycle arrest upon stressful stimuli, perhaps it is the main isoform mediating cell death. It would therefore be of interest to determine whether SK2b can also localise to the plasma membrane to mediate oncogenesis, or whether this role is unique to SK2a. However, the relevance of SK2b being present in human cells but not in mice (Okada et al, 2005) is still unclear, but this would suggest that SK2a must be capable of fulfilling all of the functions SK2 is required to perform. Additional roles unique to SK2b, if any exist, remain to be elucidated. Of note, it was previously demonstrated, using SK2b, that the N-terminus of SK2 (residues ~1-175) contains a lipid binding domain that can interact with phosphoinositides and facilitate localisation of SK2 to intracellular membranes and the nucleus (Don & Rosen, 2009). Although similar characterisation of lipid binding was not performed using SK2a, it appears that deletion of more than the first 77 amino acids is required to completely abrogate lipid

binding of SK2b (Don & Rosen, 2009), suggesting that the first 36 residues unique to SK2b are alone unlikely to mediate these interactions and that this lipid binding domain may also be present in SK2a.

7.1.1 Managing a double-edged sword in cancer

Since the discovery of the first SK2-selective inhibitor, ABC294640, there has been an interest in targeting SK2 in cancer. Indeed, pharmacologically targeting SK2 has been found to have anti-cancer effects in many different human xenograft models in mice (French et al, 2010; Liu et al, 2013; Panneer Selvam et al, 2015). As such, ABC294640 (Yeliva®) is currently in clinical trials for patients with advanced solid tumours and refractory/relapsed diffuse large B-cell lymphoma, with plans to soon commence trials for patients with multiple myeloma and advanced hepatocellular carcinoma. However, as discussed further below, ABC294640 has since been reported to target other proteins and enzymes in addition to SK2, such as dihydroceramide desaturase (Des1) (McNaughton et al, 2016). Nevertheless, the literature would suggest that targeting SK2 in cancer cells and *in vivo* models is therapeutically beneficial; however there has been very little mechanistic data to explain why such outcomes would be expected from targeting an enzyme that is generally considered to be pro-apoptotic.

Our findings here demonstrating that SK2 can induce neoplastic transformation provide the first solid evidence that SK2 plays an important role in carcinogenesis, and validate the use of SK2-targeting agents in cancer. However, many questions now remain to be elucidated. What is the functional relevance of low-level SK2 overexpression, and why does it mediate oncogenesis when high-level overexpression does not? I found that SK2 is significantly upregulated in some human cancers, although this was generally a rather modest upregulation and certainly SK2 is not found to be upregulated as frequently as SK1 (Rhodes et al, 2004). It is tempting to speculate that due to the additional physiological pro-apoptotic roles of SK2, it would not be advantageous for a cancer cell to have SK2 upregulated too much, unlike SK1 and other known cancer-promoting proteins that have only pro-proliferative roles. It is then of interest to know, what is the critical 'tipping-point' level of SK2, below which it is oncogenic but above which it would induce cell death? My studies would perhaps suggest that this point is somewhere between 10- and 20-fold above

endogenous levels, as 10-fold overexpression induced oncogenic signalling and efficient tumour formation *in vivo*, whereas 20-fold overexpression did not. This is, however, still an artificial system and whether this would translate to a physiological setting is unclear, but such levels of SK2 overexpression have certainly not been reported in human cancer patient samples.

It is interesting to consider that the exact level of SK2 that represents the 'tipping-point' will likely be tissue- and cell-type dependent, based on the basal, normal expression of SK2 in that tissue. In fact, it is rather intriguing to note that there seems to be a correlation between the normal level of SK2 expression in a given tissue and whether SK2 will be up- or down-regulated, or unchanged, in cancers that develop from that tissue. For example, in tissues where SK2 is relatively highly expressed, such as the kidney and liver (Liu et al, 2000), SK2 expression is actually commonly downregulated in cancers of those tissues (Figure 7.1). This could suggest that the pro-apoptotic role of SK2 may be more prevalent or 'primed' in these tissue types. Indeed, the physiological role of SK2 in mediating cell death has been demonstrated using renal cells (Hofmann et al, 2008; Okada et al, 2005). On the other hand, SK2 has been found to be significantly upregulated in cancers that have developed from tissues where SK2 is not as highly expressed, such as breast and esophagus (Figure 7.1). This may suggest that SK2 has more of a pro-proliferative, pro-survival role in these tissue types and may represent a promising target in these cancers.

However, this trend was not observed for all cancers where a significant up- or downregulation of SK2 had been reported (i.e. colon; Figure 7.1). Of course, there is likely to be other factors at play in addition to SK2 expression levels, for example where SK2 is localised within the cell and the concurrent up- or downregulation of proteins involved in SK2 regulation, such as IC-1. Nevertheless, it is likely that the balance between the opposing roles of SK2 may vary in different tissues, and as suggested by Figure 7.1, this may result in different changes to SK2 expression in cancer which could potentially have implications in the benefits of targeting SK2 in certain cancers. Clearly much work is still required to understand the interplay between the opposing roles of SK2 in different tissues, however this knowledge may be the key to understanding which cancers will respond best to SK2 inhibition.

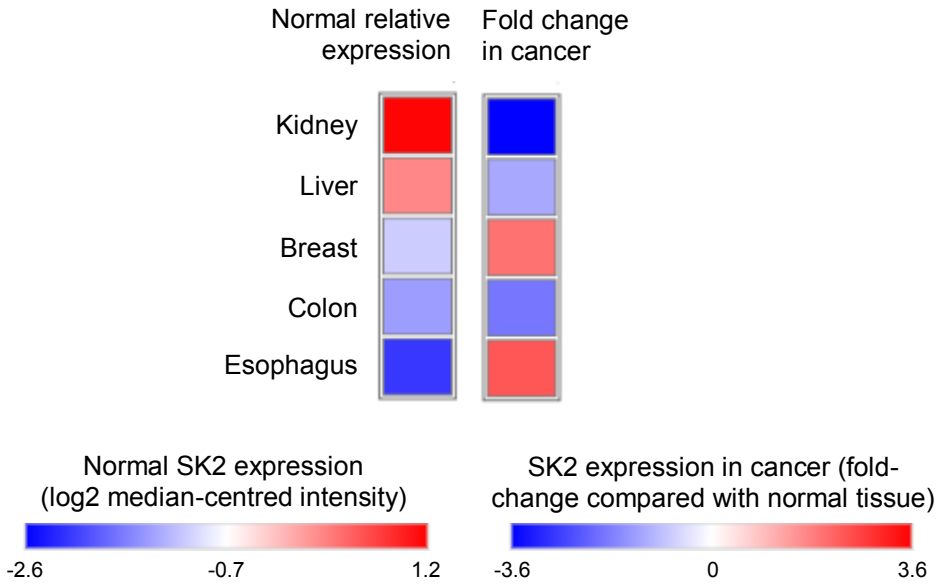


Figure 7.1: Relative expression of SK2 in normal tissues compared with corresponding cancerous tissues

Heat maps showing SK2 mRNA levels in various normal human tissues (left), and fold-changes in SK2 mRNA levels in cancerous tissues (right). Data were extracted from the Oncomine database; median SK2 expression in normal tissues (log2 median-centred intensity) was taken from the Roth Normal 2 dataset, and significant (p<1x10^{-4}) changes in SK2 expression levels in cancerous tissues (fold-change compared with normal tissues) were extracted from the following datasets: Gumz Renal, Mas Liver, Finak Breast, TCGA Colorectal and Kim Esophagus. Heat maps were generated using Morpheus online software (Broad Institute).

Further studies will be required to determine the effects of low- and high-level SK2 overexpression and why they mediate different functional outcomes. Overexpression in this system is of course not physiological, but if we can use it as a tool to identify the changes that are occurring to the regulatory mechanisms governing SK2 function and localisation, in settings where SK2 is oncogenic or pro-apoptotic, then we can further examine these mechanisms in systems that are more physiologically relevant. Although SK2 upregulation in cancer has not been observed as high as 10-fold or even 5-fold over endogenous levels, overexpression at these levels still triggered the oncogenic role of SK2 suggesting that the physiological changes and mechanisms that regulate this role were activated. A change in SK2 subcellular localisation in favour of the plasma membrane was observed, but other mechanisms could potentially include changes to SK2 post-translational modifications and/or protein-protein interactions. Therefore, it would be of interest to use this system of graded SK2 overexpression in the future to isolate SK2 protein from the various cell lines and perform mass spectrometry to identify phosphorylated residues, as well as use methods to identify novel SK2-interacting proteins such as rapid immunoprecipitation mass spectrometry of endogenous protein (RIME).

This knowledge would be very insightful, as I hypothesize that a different subset of the uncharacterised sites on SK2 will become phosphorylated to mediate its oncogenic role, whereas different phosphorylation sites will facilitate its pro-apoptotic role. Similarly, SK2 may interact with specific proteins to mediate oncogenesis but may switch to interact with other proteins when it is triggered to promote cell death. This could potentially explain why high-level overexpression results in the pro-apoptotic role of SK2 becoming dominant, as overexpression can force phosphorylation events and protein-protein interactions to occur in the absence of their normal stimuli. Therefore, in a system with high-levels of SK2, inadvertently triggering cell death would likely override any other potentially pro-proliferative signals that are simultaneously activated. Understanding such regulatory mechanisms governing SK2, and their distinct roles in the interplay between oncogenesis and cell death, would be a major step forward in understanding this complex enzyme, and would uncover new ways in which SK2 function could be targeted in cancer.

It is interesting to note that there are reports of other proteins within the cell that also have anti-proliferative functions, and yet have been found to be physiologically overexpressed in cancer and linked with higher-grade tumours. The $p16^{Ink4a}$ protein is generally considered a

tumour suppressor that has widely studied roles in negatively regulating cell cycle progression, by inhibiting S phase (Romagosa et al, 2011). However, it has been documented that, where anti-proliferative $p16^{Ink4a}$ would normally be expressed at lower levels and localised to the nucleus, in some cancers $p16^{Ink4a}$ has been found overexpressed and localised more to the cytoplasm (Evangelou et al, 2004). This increased ratio of cytoplasmic:nuclear localisation of $p16^{Ink4a}$ has been linked to poorer prognosis in some cancers (Romagosa et al, 2011), and interestingly, the increased accumulation of 'oncogenic' $p16^{Ink4a}$ in the cytoplasm has been attributed to altered post-translational modifications and interactions with other proteins (Nilsson & Landberg, 2006; Souza-Rodrigues et al, 2007). Although this scenario does not involve high-level overexpression like that used for SK2, it does demonstrate that increased levels of a protein can cause it to undergo changes in subcellular localisation, in response to altered modifications and interacting-partners, to perform a completely opposing function, as hypothesized for SK2. Notably, other proteins such as p27, PTEN and protein arginine methyltransferase 5 (PRMT5) have also been documented to undergo translocations to different subcellular compartments in cancer settings, in order to perform other secondary functions (Gu et al, 2012; Kim et al, 2009; Lobo et al, 2008). As SK2 has been shown to have physiological roles in mediating cell death, without the use of high-level overexpression (Chipuk et al, 2012; Hofmann et al, 2008; Okada et al, 2005), it is tempting to speculate that the primary house-keeping function of SK2 may be to mediate cell cycle arrest and cell death but overexpression in cancer may provide additional oncogenic roles in different cellular compartments.

In fact, there are many known examples of 'moonlighting' proteins – proteins that possess a primary function but appear to have evolved to perform additional, diverse secondary functions (Jeffery, 2003). Often these proteins carry out their secondary functions at different subcellular locations. In this sense, SK2 could be considered a moonlighting protein, as it appears to share some functional redundancy with SK1, but unlike SK1, it also appears to have acquired an additional pro-apoptotic function, likely facilitated by the additional unique N-terminal and central domains in its sequence. Often the secondary functions of moonlighting enzymes do not require their primary catalytic function (Jeffery, 2003). However, in the case of SK2, the role of S1P production in mediating its roles in cell death has not been properly interrogated, for example through the use of the catalytically inactive G212D mutation. It has been suggested that catalytic activity is not required for the

induction of apoptosis via the putative BH3 domain of SK2, however disrupting SK2 activity whilst maintaining the BH3 domain via use of an N-terminal truncated form of SK2 reduced the levels of apoptosis by 50% (Liu et al, 2003).

7.2 The role of dynein in regulating SK2 subcellular localisation

In these studies, I have demonstrated that SK2 interacts physiologically with the cytoplasmic dynein motor complex, and further, that this interaction appears to mediate the retrograde-directed transport of SK2. For an enzyme whose subcellular localisation has a significant impact on its functions, uncovering mechanisms by which SK2 is localised within the cell provides a considerable step forward in better understanding SK2.

Despite attempts to characterise the specific binding site of SK2 on dynein IC-2, I was unable to conclusively determine where SK2 interacts. This will require future characterisation, as understanding where SK2 binds to the dynein complex will potentially provide insights into how this interaction is regulated. IC-2 was first identified as a putative SK2-interacting protein from a yeast two-hybrid screen, as a 173 amino acid C-terminal truncated fragment (residues 440-612 of IC-2). Therefore, it would perhaps be expected that SK2 would interact with the C-terminus of IC-2. However, this region of dynein IC contains multiple WD40 repeat domains that fold into a beta propeller structure, and it is where IC forms an interaction with the HC subunit (Vale, 2003). Furthermore, no cargo of the dynein complex have previously been found to bind to the C-terminal end of IC; all previously identified cargo interact with the IC N-terminus or via regulators such as dynactin, which also binds IC at the N-terminus (Pfister, 2015). Indeed, yeast do possess both dynein and dynactin complexes (Pfister et al, 2006; Schroer, 2004), and so it cannot be ruled out that endogenous subunits may have facilitated a bridging of the interaction between the bait, SK2, and the prey, C-terminal IC-2, to produce a positive interaction. As dynein HC subunit dimers bind two IC subunits (Kardon & Vale, 2009), it is possible that a yeast HC dimer may have bound one endogenous full-length IC as well as truncated prey IC-2, where bait SK2 could have then interacted with the N-terminus of the full-length IC to activate transcription (yeast IC protein, known as Pac11, shares 44% amino acid sequence similarity with human IC-2C, and yeast HC shares 62% amino acid sequence similarity with human dynein HC). Hence, optimizing methods to examine the interaction *in vitro* using purified

recombinant proteins will be insightful. Of course, it is also possible that SK2 does interact with the C-terminus of IC, as the beta propeller structure of the WD40 repeat domains is such that it may be able to interact with other proteins as well as the HC (Vale, 2003). This would be a novel finding and will require further investigation.

7.2.1 The involvement of IC-1 versus IC-2: roles in brain function and disease

Intriguingly, I found that SK2 seems to interact more robustly with the IC-1 isoform compared with the IC-2 isoform. This observation may hint at where SK2 interacts within the IC sequence; IC-1 and IC-2 share 76% amino acid sequence identity, but the majority of the differences within their sequences come from the N-termini (Figure 7.2). Therefore, the fact that differential binding was observed between SK2 and these two isoforms may suggest that SK2 interacts with a sequence within the N-terminus of IC-1 that is more divergent in IC-2, and therefore results in a less prevalent interaction. However, it is also possible that the interaction between SK2 and IC may not be constitutive and may be regulated by isoform-specific post-translational modifications or other interacting regulatory proteins, thus altering the preference for SK2 binding to IC-1 over IC-2.

Isoform-specific phosphorylation sites have been reported for the dynein ICs, which may confer selectivity in their binding-partners. A number of phosphorylation events have been detected on IC-2 by mass spectrometry, all of which are restricted to the N-terminal half of the protein and most occur within the serine-rich region (residues 80-105) (Lo et al, 2006), with others found spanning the IC dimerisation domain and light chain binding sites (Hornbeck et al, 2015). IC-1 was also found to be phosphorylated at a number of these conserved sites within the serine-rich region, but interestingly, IC-1 is also phosphorylated at additional sites not conserved in IC-2, found within regions flanking the light chain binding sites and the IC dimerisation domain (Ser148, Thr159, Ser162), and notably, at the C-terminus (Ser618; see Figure 7.2) (Hornbeck et al, 2015). Again, given that SK2 seems to bind differentially to IC-1 and IC-2, it will be of interest to investigate the potential role of isoform-specific IC phosphorylation on SK2 binding. Indeed, isoform-selective cargoes have been reported previously, where dynein complexes containing the isoform IC-1B (see section 1.4.3 and Figure 1.10 in Chapter 1 for a description of IC splice isoforms) were more likely than complexes containing IC-2C to transport Rab7-containing endosomes and

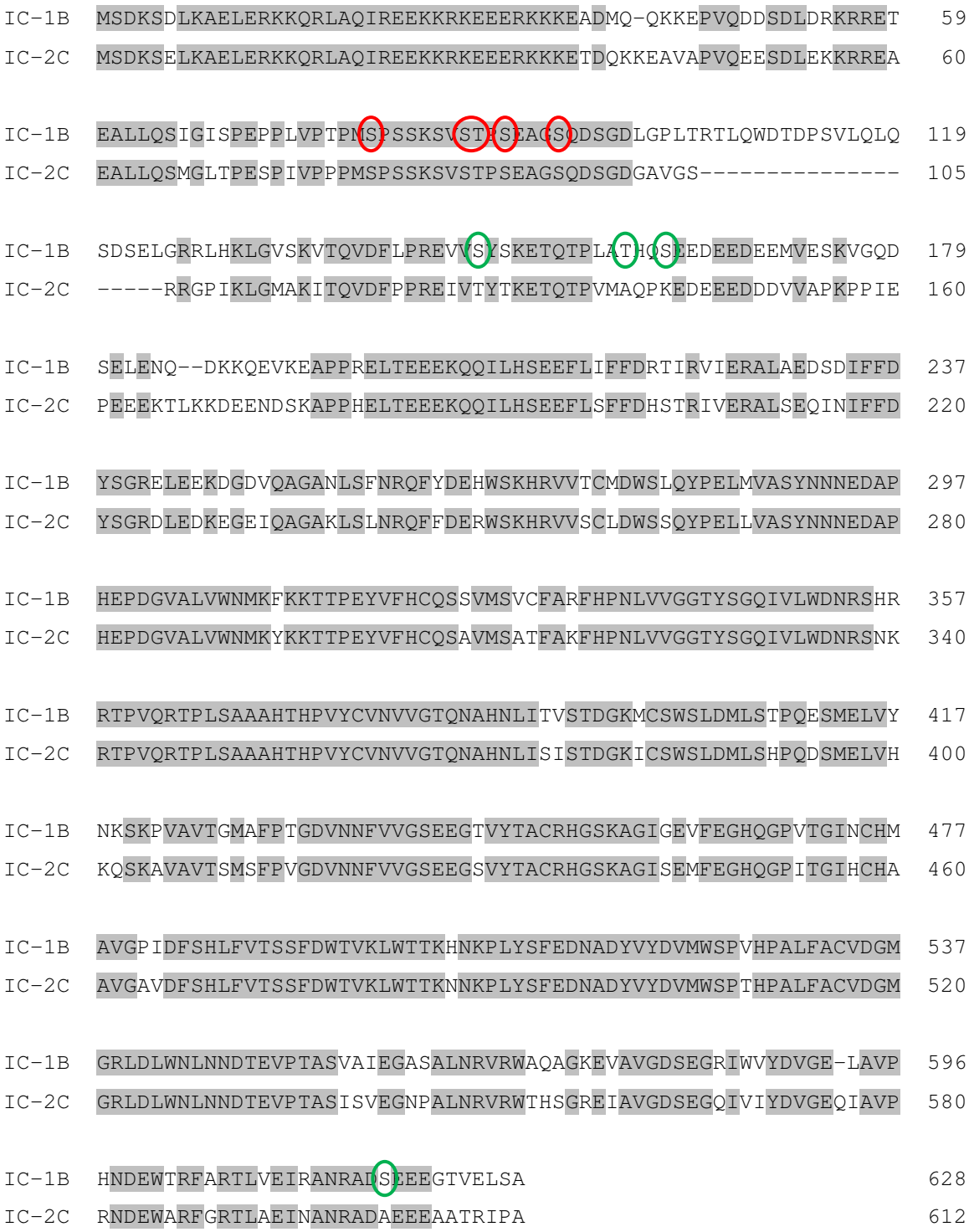


Figure 7.2: Sequence alignment of human IC-1 and IC-2

Amino acid sequence alignment of the human IC-1B and IC-2C isoforms (performed using the Clustal Omega program via UniProt). Regions containing identical residues between the two isoforms are highlighted in grey. Phosphorylation events that have been detected on human IC-1 by mass spectrometry are circled; red denotes those that have also been detected on IC-2, green denotes phosphorylation sites unique to IC-1.

signalling endosomes containing the transmembrane neurotrophin receptor kinase, TrkB (Ha et al, 2008; Mitchell et al, 2012). On the other hand, dynein complexes containing the IC-2C isoform were more likely to associate with mitochondria than complexes containing IC-1B (Mitchell et al, 2012).

The physiological relevance of SK2 interacting more robustly with IC-1 remains to be determined. IC-1 is, at least in mouse and rat, a predominantly neuronal-specific isoform (Kuta et al, 2010; Myers et al, 2007). Very few studies, however, have examined human IC-1 tissue expression and distribution, but IC-1 expression has been detected in many non-neuronal human tissues (Horikawa et al, 2001). Therefore, it is unclear whether the interaction between SK2 and IC-1 has a particularly important function in the brain, or whether it is a ubiquitous interaction also involving IC-2 and occurring equally in all cells and tissues. It is believed that the additional expression of IC-1 isoforms in neuronal cells, in addition to ubiquitously expressed IC-2, is to compensate for the higher demand and longer distances required to travel and transport cargo along axons (Pfister, 2015). Given that SK2 is transported or sequestered by dynein in HeLa cells (Figure 5.8), and that HeLa cells do not have any detectable IC-1 protein (Figure 5.7 and Palmer et al, (2009)), this would suggest that perhaps the interaction between SK2 and dynein is ubiquitous. However, as SK2 is the major SK isoform expressed in the brain and is responsible for the majority of S1P production here (Blondeau et al, 2007; Couttas et al, 2014), it is possible that SK2 may require additional regulation, above that provided by ubiquitous IC-2, in this setting.

Dynein is the major molecular motor responsible for retrograde axonal transport of cargoes, and is indispensable for the maintenance of healthy neurons and the nervous system (Hirokawa et al, 2010). Consequently, defects in dynein complex function have been linked to a plethora of neurological disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease and motor neuron diseases, as well as many other disorders that are caused by defects in other components that regulate dynein-mediated transport (Eschbach & Dupuis, 2011). Notably, a number of different mutations in the cytoplasmic dynein heavy chain gene have been found in human patients that possess a range of neurological abnormalities (Schiavo et al, 2013).

SK2 has also been implicated in neurodegeneration, specifically in Alzheimer's disease, the most common form of dementia. Conflictingly, SK2 activity has been reported as

significantly upregulated in the brains of Alzheimer's disease patients (Takasugi et al, 2011), whereas more in-depth stratification of Alzheimer's disease progression demonstrated a decrease in SK2 activity with increased disease severity (Couttas et al, 2014). The reasons for this discrepancy are unclear, but may reflect the samples and preparation methods used; one study measured SK2 activity normalised to SK2 protein levels from Tris-soluble fractions isolated from frontal cortices of diseased and non-diseased patients (Takasugi et al, 2011), whereas the other study examined SK2 activity from total homogenates of hippocampus and temporal grey matter tissues from patients with varying stages of disease (Couttas et al, 2014).

The pathogenesis of Alzheimer's disease is typically thought to involve two lesions, amyloid plaques – extracellular aggregates containing Aβ peptides – and neurofibrillary tangles composed of intracellular hyperphosphorylated Tau aggregates (Blennow et al, 2006; Scheltens et al, 2016). Interestingly, inhibition or downregulation of SK2 in mouse neuronal cells resulted in decreased proteolytic activity of BACE1, the rate-limiting enzyme for Aβ production, and decreased levels of secreted Aβ (Takasugi et al, 2011). In line with this, S1P was found to directly interact with BACE1 and increase its activity (Takasugi et al, 2011). BACE1 contains a transmembrane domain and shuttles between the cell surface, the endosomal pathway and the trans-Golgi network (Walter et al, 2001). Dynein mediates the retrograde transport of BACE1 to the lysosomes for degradation, hence reducing Aβ production (Ye & Cai, 2014). It is therefore intriguing to speculate that disruption of dynein-mediated retrograde axonal transport may contribute to the development of Alzheimer's disease through both an accumulation of BACE1 protein as well as a reduction in retrograde transport of SK2, potentially resulting in increased SK2 and S1P at the membrane to facilitate BACE1 activity and Aβ production. Hence, SK2 may represent a promising therapeutic target in Alzheimer's disease, but the mechanisms involved and the potential interplay with dynein will require further investigation.

The downregulation of IC-1 in human cancers had not been previously reported, but strikingly, by mining publically available datasets I found IC-1 to be significantly downregulated in a range of difference cancer types. The highest level of downregulation was found in brain cancers, which is perhaps not surprising as it is where it has the highest expression in normal tissue (Horikawa et al, 2001). However, the relevance of IC-1 downregulation in other cancer types is unclear as, although it is found to be expressed in

other human tissues (Horikawa et al, 2001), it has been generally considered to be neuronal-specific in mouse and rat. Therefore, future studies will be required to examine the role of IC-1 in other human tissues, and specifically, to identify whether the interplay between IC-1 downregulation and increased SK2 plasma-membrane localisation observed in GBM is also recapitulated in other cancer types. If it is, then this will add another layer of complexity in identifying cancer types where SK2 may represent a valid therapeutic target, as altered subcellular localisation may be as equally an important factor as overexpression.

7.2.2 Does dynein also interact with and regulate SK1?

The studies described here have focused on the interaction and regulation of SK2 with dynein, as dynein IC was identified in a yeast two-hybrid screen using SK2 as the bait. However, it will be of interest in the future to determine whether dynein can also interact with and regulate SK1. My data demonstrating that re-expression of IC-1 in GBM cells resulted in reduced extracellular S1P formation even in the presence of a potent SK1-specific inhibitor suggests that only SK2 is being translocated by dynein, at least in this system. However, as SK2 is the most highly expressed SK isoform in the brain (Blondeau et al, 2007), it cannot be ruled out that my observations in this system were biased toward SK2, whereas there may be other 'housekeeping' roles of dynein in transporting both SK1 and SK2 in other cell types. Moreover, the roles of dynein in regulating SK2 explored in Chapter 5 may be specific to a cancer setting, whereas other 'housekeeping' roles may exist in normal cells.

7.2.3 Are other cellular processes regulated by this interaction?

Due to time constraints, I was unable to delve further into the characterisation of the interaction between dynein and SK2, the mechanisms that regulate the interaction, and potential additional factors and processes that may be involved. However, from the literature, it appears that there are various overlapping functions shared between SK2/SK1 and dynein, posing the question of whether the interaction between these proteins may act to regulate other cellular processes in addition to, or as a result of, the translocation of SK2.

It would be interesting to investigate the potential involvement of EGF and its receptor EGFR in the functional effects of SK2 and dynein interactions, as both SK2 and dynein are integral to EGFR signalling outcomes. SK2 has been shown to localise to the plasma membrane in MDA-MB-453 breast cancer cells, where it was required for the migration of these cells towards EGF (Hait et al, 2005). Furthermore, SK2 was found to be required for EGF-induced cancer cell invasion, a process involving S1P production and phosphorylation of ERM proteins (Adada et al, 2015). Therefore, SK2 appears to play important roles in mediating EGF-dependent signalling to promote cell migration and invasion, which may have implications in the metastasis of cancer cells. EGF has been found to activate SK2 localised to the membrane fraction in cells (Hait et al, 2005), and notably, EGF stimulated an increase in S1P secretion from GBM stem cells, which was found to play a role in maintaining their proliferation and stemness (Marfia et al, 2014). Approximately 50% of GBM patient tumours possess amplification of EGFR (across all GBM subtypes, for the Classical subtype alone it is as high as 95%) (Verhaak et al, 2010), making EGF/EGFR an important signalling axis in GBM.

Dynein also plays an important role in EGF signalling. Dynein IC-2 is phosphorylated by ERK1/2 at Ser81 in response to EGF (Pullikuth et al, 2013), and this phosphorylation event recruits dynein to signalling endosomes (Mitchell et al, 2012). Dynein mediates the transport of internalised, activated EGFR through the endosomal pathway to the lysosomes for degradation (Garrett et al, 2014; Taub et al, 2007). Indeed, mutations in the dynein heavy chain result in sustained EGF/MAPK signalling (Garrett et al, 2014). It is therefore possible that the highly significant downregulation of IC-1 in GBM patient samples observed in my studies may result in reduced dynein function and sustained signalling of both SK2, which is critical for aspects of EGF signalling, and EGFR at the cell periphery, working in concert with EGFR upregulation to mediate pro-proliferative signalling. Thus, it would be of interest to examine EGF/EGFR signalling and degradation, and EGF-induced cell migration and invasion in the U-251 cell lines ectopically expressing IC-1, or the control cells, to examine potential co-localisation of SK2 with EGFR.

Dynein has well characterised roles in mediating endocytic trafficking and autophagy, which are particularly important for maintaining healthy functioning neurons. Dynein mediates the retrograde-directed transport of late endosomes and enhances endosomal/lysosomal membrane trafficking (Cai et al, 2010). The molecular motor also

mediates important aspects of autophagy, as it is critical for the movement of autophagosomes towards lysosomes, which are usually localised in close proximity to the centrosome (Kimura et al, 2008), and for autophagosome-lysosome fusion (Ravikumar et al, 2005). Notably, many of the neurological diseases described above that develop in response to mutations or defects in dynein function are found to have defective autophagy or protein aggregate-clearance pathways (Eschbach & Dupuis, 2011), and hence, it is critical that these pathways are well understood and characterised.

The SKs have been implicated in regulating endocytic trafficking, although the exact mechanisms involved are still being elucidated. It is accepted that the balance of lipids such as cholesterol and sphingolipids in the cell membrane can affect endocytic trafficking processes, and experimental perturbations to these lipid compositions can induce endocytic trafficking (Shen et al, 2014). Addition of sphingosine and sphingosine-like molecules were found to induce endocytosis and fusion of vesicles to form enlarged late endosomes, and this endosomal fusion was found to be dependent on SK1 but, surprisingly, independent of its catalytic activity (Lima et al, 2017; Young et al, 2016). However, later stages of the endocytic pathway involving endosomal maturation and processing were found to be dependent on SK1 activity (Young et al, 2016). Furthermore, SK1 was found to co-localise with early and late endosomes, as well as autophagosomes in neurons, where it was found to mediate autophagy in a manner dependent on S1P production (Moruno Manchon et al, 2015). In another study, both SK2 and SK1 were found to localise to early endocytic intermediates, and both were shown to be required for recycling of transferrin-containing endosomes (Shen et al, 2014). However, specifically SK2, but not SK1, was implicated in S1P production in multivesicular endosomes to constitutively activate associated S1P receptors to mediate endosome maturation, cargo sorting and exosomal release of vesicles (Kajimoto et al, 2013).

It should be noted that the studies implicating only SK1 in regulating endocytic trafficking did not actually examine SK2, but they did note that SK2 is likely to share compensatory functions (Lima et al, 2017; Young et al, 2016). There is additional literature to suggest an involvement of SK2 in autophagy, as inhibition of SK2 induced autophagic cell death in kidney, prostate and breast cancer cell lines (Beljanski et al, 2010), as well as in T-cell acute lymphoblastic leukemia (T-ALL) cell lines (Evangelisti et al, 2014). The same autophagic mechanism of cell death was also observed in kidney carcinoma cells *in vivo* when an SK2

inhibitor was administered (Beljanski et al, 2010). However, care must be taken in interpreting these data, as both inhibitors used in these studies, ABC294640 and ROME, are, like most other SK inhibitors, sphingosine-like molecules (French et al, 2010; Lim et al, 2011). Therefore, as discussed above, the lipid-like nature of these drugs may perturb the balance of lipids in cellular membranes, resulting in a non-physiological induction of vesicle and endosome formation and trafficking (Lima et al, 2017; Shen et al, 2014; Young et al, 2016). Furthermore, ABC294640 has many known targets in addition to SK2 (Pitman et al, 2016). Nevertheless, it is clear that the SKs play a role in endocytic trafficking and autophagy, and whether there is crosstalk between the SKs and dynein in regulating these processes remains to be examined.

SK2 has also been previously implicated in cell cycle regulation, a cellular process that dynein plays important roles in mediating (Kardon & Vale, 2009). SK2 has been implicated in promoting cell cycle arrest in the nucleus (Igarashi et al, 2003), and it was shown that S1P produced by SK2 in the nucleus can alter epigenetic modifications at the promoter of the gene encoding cyclin-dependent kinase inhibitor p21, resulting in its increased transcription (Hait et al, 2009). However, my co-localisation studies did not demonstrate a specific strong interaction between SK2 and dynein in the nucleus, as one would expect if they interact to perform functions involving the cell cycle. Although, as I did not examine synchronized cells or particular phases of the cell cycle, this may not have been apparent in my system.

In addition, the SKs were previously reported to localise to the centrosome, in conjunction with $S1P_5$, and it was suggested that they may play a role in regulating mitosis here, although this has not specifically been examined (Gillies et al, 2009). Dynein accumulates at the centrosome, being the main microtubule organizing centre in metazoan cells (Barker et al, 2016), and may therefore interact with SK2 there, or transport SK2 there under certain conditions. Indeed, I did observe co-localisation of SK2 and dynein IC at distinct peri-nuclear regions, however due to time constraints I was not able to examine potential co-staining with specific organelle markers. The involvement of $S1P_5$ is intriguing, as its expression is mostly restricted to brain and skin (Anliker & Chun, 2004), and interestingly its expression levels have been found to correlate significantly with poorer survival in GBM patients (Quint et al, 2014). Therefore, the potential intracellular role of $S1P_5$ in modulating cell cycle in GBM, and any involvement of SK2 and dynein in this process, could warrant

further investigation. Indeed, a very recent mechanistic study has reported that S1P, signalling extracellularly via $S1P_{5,}$ can activate the PI3K-AKT pathway and polo-like kinase 1 to promote mitotic progression, which in the case of exogenous S1P addition or SK overexpression, overrides the spindle assembly checkpoint and results in chromosome segregation defects (Andrieu et al, 2017). There is evidence to suggest that dysregulation of the mitotic-spindle checkpoint can promote aneuploidy, a form of genetic instability commonly observed in cancers, termed chromosomal instability (CIN) (Bharadwaj & Yu, 2004; Jallepalli & Lengauer, 2001). Therefore, these findings have interesting implications in cancer, where the SKs are often upregulated (Heffernan-Stroud & Obeid, 2013; Neubauer et al, 2016), and increased S1P production may therefore give rise to increased CIN, a known driver of tumourigenesis (Jallepalli & Lengauer, 2001). In this study it was shown that both SK1 and SK2 can play an equivalent role in this S1P-mediated effect, despite the paper focusing predominantly on SK1 (Andrieu et al, 2017). However, an intracellular role for $S1P_5$ at the centrosome still remains to be explored. Notably, a role for nuclear $S1P_2$ in modulating cell growth in breast cancer cells has previously been reported (Ohotski et al, 2014).

7.3 Regulation of SK2 by phosphorylation

The studies outlined in Chapter 6 aimed to examine SK2 regulation by novel, previously uncharacterized phosphorylation sites. However, there is still a vast amount of work required in this area. Given that there are clearly some functional redundancies between SK1 and SK2, it is fascinating that the main activating phosphorylation site on SK1, Ser225, is not directly conserved in SK2 (Pitson, 2011; Pitson et al, 2003). It is also intriguing that the proposed activating phosphorylation site on SK2, Thr578 (Hait et al, 2007), has never been detected by mass spectrometry on endogenous SK2 (Hornbeck et al, 2015), nor was it detected by our previous mass spectrometry analyses of overexpressed SK2. However, it is possible that the peptide containing this modification may not be suitable for detection by these methods. Nevertheless, further work is required to understand SK2 regulation by phosphorylation.

The Ser363 residue has been shown to be phosphorylated on endogenous SK2 by 37 studies employing mass spectrometry analyses (Hornbeck et al, 2015). It is therefore likely to be a

common, potentially important modification of SK2. While many of these studies were examining a broad range of pathways, cell types and conditions, making it difficult to narrow down the potential function of this modification, it is interesting to note that Ser363 phosphorylation was found to be significantly increased upon use of the AMPK activator AICAR, as determined by SILAC labeling and quantitative phosphoproteomic analysis (Hoffman et al, 2015). Furthermore, another group detected Ser363 phosphorylation by using a phospho-(Ser/Thr) AMPK substrate antibody to purify peptides prior to LCMS (Hornbeck et al, 2015). Notably, AMPK was ranked as the lead candidate kinase for Ser363 phosphorylation from the Kinexus *in vitro* phosphorylation screen, and as such, future studies should validate whether AMPK is the kinase responsible for phosphorylating SK2 at Ser363.

Interestingly, phosphorylation and binding prediction software predicts that, in addition to AMPK being a likely candidate kinase for Ser363 phosphorylation, phospho-Ser363 is also predicted to be a binding site for the pro-survival 14-3-3 chaperone protein (Obenauer et al, 2003). 14-3-3 has known roles in binding to phospho-client proteins and mediating their transport out of the nucleus via a 14-3-3 NES (Rittinger et al, 1999) or by sequestering them in the cytoplasm (Grozinger & Schreiber, 2000), which fits with the hypothesis and my data suggesting that Ser363 is involved in SK2 nuclear export or exclusion from the nucleus. As predicted, phospho-Ser363 fits the requirements of a 14-3-3 binding motif; the most common motif is Rxx(pS/pT)xP, where the +2 proline residue appears to be dispensable in more than half of client proteins (Johnson et al, 2010). Ser363 has an arginine at the -3 position but does not contain the +2 proline residue. Generally 14-3-3 will bind to its clients within a flexible region (Johnson et al, 2010), and indeed Ser363 resides within the unique central domain of SK2 that is predicted to be disordered (Wang et al, 2014a). As 14-3-3 is a dimeric protein, it generally binds to two phospho-residues on the client protein, where the two binding sites need to be at least 15 residues apart to allow for the conformation required to bind each dimer (Johnson et al, 2010). It is tempting to speculate that the second phospho-residue binding site on SK2 may be Ser383 or Ser385, the phosphorylation sites 20-22 residues downstream of Ser363 that were previously reported to regulate SK2 nuclear export (Ding et al, 2007). This would explain why manipulating these sites generates the same phenotype as mutating Ser363 to alanine, if they are both involved in 14-3-3 binding to be exported from the nucleus. The PKD-mediated export of SK2 from the nucleus involving Ser383/Ser385 was shown to be CRM1 dependent (Ding et al, 2007), however

14-3-3 contains an NES and does transport clients from the nucleus in a CRM1-dependent manner (Lopez-Girona et al, 1999; Rittinger et al, 1999).

AMPK is known to shuttle between the cytoplasm and the nucleus (Kodiha et al, 2007), and can therefore phosphorylate nuclear substrates. Notably, another 14-3-3 client protein, CREB-regulated transcription coactivator 2 (CRTC2; also TORC2), can be phosphorylated by AMPK which promotes 14-3-3 binding at this site and sequestration of CRTC2 to the cytoplasm (Koo et al, 2005; Screaton et al, 2004). However, upon dephosphorylation of this site, CRTC2 no longer binds to 14-3-3 and can translocate to the nucleus in order to perform its roles in transcriptional regulation (Koo et al, 2005; Screaton et al, 2004). As SK2 also has nuclear roles in regulating transcription (Hait et al, 2009; Nagahashi et al, 2015; Wallington-Beddoe et al, 2014), this could represent an interesting mechanism of regulating SK2 nuclear localisation and it will be of interest to determine whether SK2 is regulated in this manner involving Ser363, AMPK and 14-3-3.

Through collaboration with Dr. Adam Cole (Garvan Institute of Medical Research, Sydney), we were able to identify SK2 as a novel substrate of GSK3 *in vitro* and in cells. Due to time constraints, however, no further characterisation of the GSK3 phosphorylation site, Ser437, or the associated priming site, Ser441, was performed. This will be a particularly interesting modification to examine in the future, as GSK3 is highly expressed in the brain and, like SK2 and dynein as discussed above, has been implicated in various neurological diseases such as Alzheimer's disease (Cole, 2012). Therefore, again it will be interesting to determine whether there is an interplay between SK2 regulation by GSK3 and its interaction with dynein, and if so, whether this has specific implications in the brain.

7.4 Implications for targeting SK2 in cancer

Evidence is now building to suggest that SK2 is a therapeutically valid target in cancer. Indeed, from the accumulating studies demonstrating reduced tumour growth upon SK2 knockdown or inhibition (French et al, 2010; Liu et al, 2013; Panneer Selvam et al, 2015; Weigert et al, 2009), it doesn't appear that its pro-apoptotic roles are causing any negative or complicating effects upon its targeting in cancer. However, this will require further examination, as the roles of SK2 may vary in different tissues and cell types, and as

discussed previously, there are some cancer types where SK2 does appear to be predominantly downregulated (Figure 7.1). If SK2 switches to having more of an oncogenic role in cancer cells, but in normal cells or certain cell types its pro-apoptotic role is more dominant, then this could have potentially negative implications for blocking SK2 systemically. On the other hand, this may in fact provide a beneficial therapeutic window, where SK2 is more dispensable in normal cells but cancer cells perish because they rely more upon the oncogenic signalling SK2 provides (a non-oncogene addiction as previously described for SK1 (Vadas et al, 2008)). Therefore, better understanding of the complexity of SK2 and its roles and regulation may assist in developing better strategies to target only the oncogenic pool of SK2, which may reduce or prevent any potential side effects of broadly targeting SK2.

The SKs have gained interest as therapeutic targets in GBM. It is well accepted that resistance to radiotherapy and chemotherapy can involve mechanisms to metabolise and clear the excess pro-apoptotic ceramide that is produced from these treatments (Senchenkov et al, 2001; Sordillo et al, 2016), and hence targeting SK in combination with these therapies can overcome this resistance (Bektas et al, 2009; Riccitelli et al, 2013). Notably, elevated levels of S1P are found in GBM tumours compared to normal brain tissue (Abuhusain et al, 2013), and SK1 has been shown to be overexpressed in GBM and its expression correlates with poor patient survival (Abuhusain et al, 2013; Van Brocklyn et al, 2005). However, all of the *in vivo* studies have been performed using either SK1-specific or dual SK1/SK2 inhibitors, but not SK2-selective inhibitors, despite its genetic knockdown inducing GBM cell death *in vitro* (Van Brocklyn et al, 2005). Granted, SK2 is not consistently found to be overexpressed in GBM, however my studies suggest that it may still play an important role in the production of S1P and potentially, therefore, in resistance to current therapies. Again, this may depend heavily on the subcellular localisation of SK2, as it may contribute towards metabolizing ceramide to S1P when at the plasma membrane, when IC-1 is downregulated, but it has also been shown to contribute to ceramide production via a salvage pathway at the ER (Maceyka et al, 2005). Therefore, inhibition of SK2 in GBM may be beneficial, and should be examined in future studies. However, it will also be of interest to explore other avenues of targeting only oncogenic SK2, potentially by manipulating regulators that dictate its subcellular localisation. Unfortunately, increasing cellular IC-1 levels in GBM or manipulating dynein in any way is not feasible as a therapeutic approach, but a better understanding of how the interaction is regulated, for example through phosphorylation,

may lead to new strategies to target kinases or phosphatases to increase the interaction between SK2 and dynein and drive SK2 away from the plasma membrane. Building on my *in vivo* studies, it will also be of interest to employ a more physiological orthotopic model of GBM to explore the therapeutic benefit of targeting SK2 as well as SK2 regulation by dynein.

A current problem in the field is a lack of truly SK2-specific inhibitors. ABC294640, which is currently in clinical trials for various solid and non-solid cancers, was originally pitched as an SK2-selective inhibitor (French et al, 2010). However, it is now known that ABC294640 has a plethora of additional targets that are either inhibited or degraded by the drug, including Des1 and SK1 (McNaughton et al, 2016). Although this complicates the fact of whether or not the anti-tumour properties of this compound are mediated by SK2 inhibition, and care must be taken in linking its effects to SK2 biology, it is likely that the multiple targets it has are what makes it effective. An array of more potent SK2-selective inhibitors have recently been developed that seem to more closely recapitulate phenotypes observed with the genetic knockout of SK2, such as SLR080811, SLM6031434, SLP120701, and the most potent and selective to date, SLC4101431 (K_i = 90 nM, 100-fold SK2 selectivity) (Childress et al, 2017; Kharel et al, 2015; Kharel et al, 2012; Patwardhan et al, 2015). However, these drugs have not yet been utilised in *in vivo* xenograft models. Other as yet biologically uncharacterised potent SK2-selective inhibitors have also been recently reported, namely 27c and 27d (Schnute et al, 2017). Hopefully this range of newly developed tools will assist in exploring the roles of SK2 in cancer.

7.5 Concluding remarks

Overall, the studies outlined in this thesis have contributed new knowledge to the complex field of SK2 biology, and how its roles and regulation may be altered in cancer. There is, however, much still to discover and understand about this enigmatic enzyme, and as highlighted by my work, the field should move away from systems that use high-level overexpression to ensure that correct interpretations are made about SK2 function. From my work, we now know that SK2 can contribute to oncogenesis and can initiate neoplastic transformation when overexpressed at low levels, which coincides with plasma membrane

localisation and extracellular S1P production. Further, a novel interaction between SK2 and dynein IC revealed that dynein can transport SK2 in a retrograde direction, away from the plasma membrane, which appears to have implications in GBM where downregulation of IC-1 correlates with SK2 plasma membrane localisation. Finally, novel SK2 phosphorylation events were explored, where Ser363 appears to regulate SK2 nuclear localisation, and other SK2 phosphorylation sites may control its pro-apoptotic functions. These findings are illustrated in Figure 7.3.

Some of the key questions in SK2 biology that I believe will be critical to explore in the future include: (1) How are the nuclear roles of SK2 regulated, allowing it to mediate cell cycle arrest but also stabilize hTERT and regulate epigenetic modifications and transcription of genes involved in various pathways? (2) What are the tissue-specific roles of SK2 and how does this affect its roles in cancer? (3) How is SK2 intricately regulated by phosphorylation? Are all identified phosphorylation sites physiological and do they act individually or in concert to regulate various SK2 functions? Addressing these questions will bring the field substantially closer to truly understanding SK2 biology.

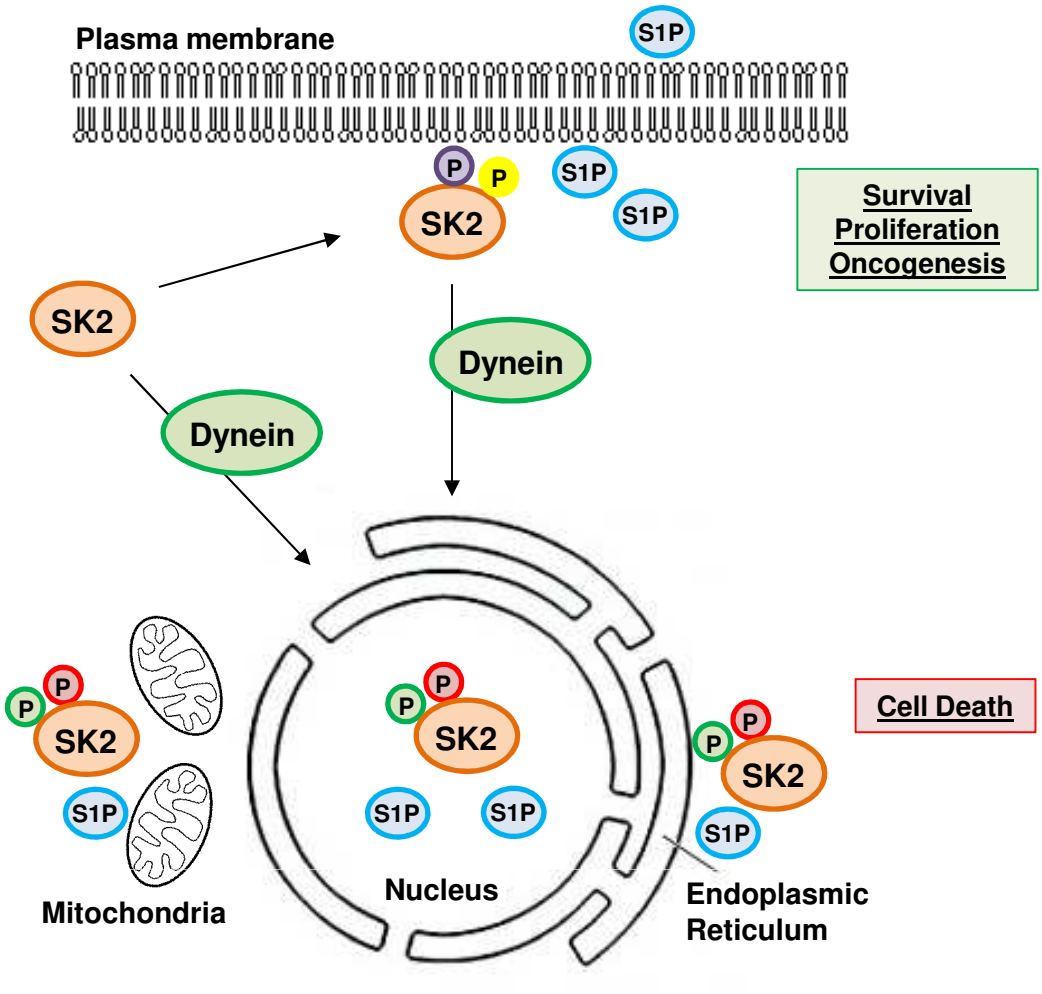


Figure 7.3: Model of SK2 functions and regulation

SK2 can localise to organelles such as the mitochondria, endoplasmic reticulum and nucleus, where it has been reported to have predominantly pro-apoptotic and anti-proliferative functions. I have shown that SK2 can mediate survival and proliferative signalling, and drive neoplastic transformation, and these functions coincide with enhanced plasma membrane localisation of SK2 and increased extracellular S1P production. Furthermore, I have shown that cytoplasmic dynein can mediate the retrograde-directed transport of SK2, from the plasma membrane to peri-nuclear regions of the cell. I hypothesise that SK2 subcellular localisation and function can be regulated by various phosphorylation events, where certain phosphorylation sites will mediate its plasma membrane localisation and proliferative functions, and others will promote its apoptotic roles and localisation to internal organelles.

References

Abuhusain HJ, Matin A, Qiao Q, Shen H, Kain N, Day BW, Stringer BW, Daniels B, Laaksonen MA, Teo C, McDonald KL, Don AS (2013) A metabolic shift favoring sphingosine 1-phosphate at the expense of ceramide controls glioblastoma angiogenesis. *J Biol Chem* **288:** 37355-37364

Adada MM, Canals D, Jeong N, Kelkar AD, Hernandez-Corbacho M, Pulkoski-Gross MJ, Donaldson JC, Hannun YA, Obeid LM (2015) Intracellular sphingosine kinase 2-derived sphingosine-1-phosphate mediates epidermal growth factor-induced ezrin-radixin-moesin phosphorylation and cancer cell invasion. *FASEB J* **29:** 4654-4669

Alemany R, van Koppen CJ, Danneberg K, Ter Braak M, Meyer Zu Heringdorf D (2007) Regulation and functional roles of sphingosine kinases. *Naunyn Schmiedebergs Arch Pharmacol* **374:** 413-428

Allan VJ (2011) Cytoplasmic dynein. *Biochem Soc Trans* **39:** 1169-1178

Allende ML, Sasaki T, Kawai H, Olivera A, Mi YD, van Echten-Deckert G, Hajdu R, Rosenbach M, Keohane CA, Mandala S, Spiegel S, Proia RL (2004) Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J Biol Chem* **279:** 52487-52492

Alvarado Y, Giles FJ, Swords RT (2012) The PIM kinases in hematological cancers. *Expert Rev Hematol* **5:** 81-96

Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, Maceyka M, Jiang H, Luo C, Kordula T, Milstien S, Spiegel S (2010) Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature* **465:** 1084-1088

Amano M, Nakayama M, Kaibuchi K (2010) Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. *Cytoskeleton (Hoboken)* **67:** 545-554

Andrieu G, Ledoux A, Branka S, Bocquet M, Gilhodes J, Walzer T, Kasahara K, Inagaki M, Sabbadini RA, Cuvillier O, Hatzoglou A (2017) Sphingosine 1-phosphate signaling through its receptor S1P5 promotes chromosome segregation and mitotic progression. *Sci Signal* **10:** doi: 10.1126/scisignal.aah4007

Anliker B, Chun J (2004) Cell surface receptors in lysophospholipid signaling. *Semin Cell Dev Biol* **15:** 457-465

Antoon JW, White MD, Driver JL, Burow ME, Beckman BS (2012) Sphingosine kinase isoforms as a therapeutic target in endocrine therapy resistant luminal and basal-A breast cancer. *Exp Biol Med* **237:** 832-844

Antoon JW, White MD, Meacham WD, Slaughter EM, Muir SE, Elliott S, Rhodes LV, Ashe HB, Wiese TE, Smith CD, Burow ME, Beckman BS (2010) Antiestrogenic effects of the novel sphingosine kinase-2 inhibitor ABC294640. *Endocrinology* **151:** 5124-5135

Antoon JW, White MD, Slaughter EM, Driver JL, Khalili HS, Elliott S, Smith CD, Burow ME, Beckman BS (2011) Targeting NFκB mediated breast cancer chemoresistance through selective inhibition of sphingosine kinase-2. *Cancer Biol Ther* **11:** 678-689

Aurelio L, Scullino CV, Pitman MR, Sexton A, Oliver V, Davies L, Rebello RJ, Furic L, Creek DJ, Pitson SM, Flynn BL (2016) From sphingosine kinase to dihydroceramide desaturase: A structure-activity relationship (SAR) study of the enzyme inhibitory and anticancer activity of 4-((4-(4-chlorophenyl)thiazol-2-yl)amino)phenol (SKI-II). *J Med Chem* **59:** 965-984

Ayloo S, Lazarus JE, Dodda A, Tokito M, Ostap EM, Holzbaur EL (2014) Dynactin functions as both a dynamic tether and brake during dynein-driven motility. *Nat Commun* **5:** 4807

Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, Dewhirst MW, Bigner DD, Rich JN (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* **444:** 756-760

Barbar E (2008) Dynein light chain LC8 is a dimerization hub essential in diverse protein networks. *Biochemistry* **47:** 503-508

Barker AR, McIntosh KV, Dawe HR (2016) Centrosome positioning in non-dividing cells. *Protoplasma* **253:** 1007-1021

Barth BM, Shanmugavelandy SS, Kaiser JM, McGovern C, Altinoglu EI, Haakenson JK, Hengst JA, Gilius EL, Knupp SA, Fox TE, Smith JP, Ritty TM, Adair JH, Kester M (2013) PhotoImmunoNanoTherapy reveals an anticancer role for sphingosine kinase 2 and dihydrosphingosine-1-phosphate. *ACS Nano* **7:** 2132-2144

Bayerl MG, Bruggeman RD, Conroy EJ, Hengst JA, King TS, Jimenez M, Claxton DF, Yun JK (2008) Sphingosine kinase 1 protein and mRNA are overexpressed in non-Hodgkin lymphomas and are attractive targets for novel pharmacological interventions. *Leuk Lymphoma* **49:** 948-954

Bektas M, Johnson SP, Poe WE, Bigner DD, Friedman HS (2009) A sphingosine kinase inhibitor induces cell death in temozolomide resistant glioblastoma cells. *Cancer Chemother Pharmacol* **64:** 1053-1058

Beljanski V, Knaak C, Smith CD (2010) A novel sphingosine kinase inhibitor induces autophagy in tumor cells. *J Pharmacol Exp Ther* **333:** 454-464

Beljanski V, Knaak C, Zhuang Y, Smith CD (2011a) Combined anticancer effects of sphingosine kinase inhibitors and sorafenib. *Invest New Drugs* **29:** 1132-1142

Beljanski V, Lewis CS, Smith CD (2011b) Antitumor activity of sphingosine kinase 2 inhibitor ABC294640 and sorafenib in hepatocellular carcinoma xenografts. *Cancer Biol Ther* **11:** 524-534

Berggard T, Linse S, James P (2007) Methods for the detection and analysis of protein-protein interactions. *Proteomics* **7:** 2833-2842

Beurel E, Grieco SF, Jope RS (2015) Glycogen synthase kinase-3 (GSK3): regulation, actions, and diseases. *Pharmacol Ther* **148:** 114-131

Beurel E, Jope RS (2006) The paradoxical pro- and anti-apoptotic actions of GSK3 in the intrinsic and extrinsic apoptosis signaling pathways. *Prog Neurobiol* **79:** 173-189

Bharadwaj R, Yu H (2004) The spindle checkpoint, aneuploidy, and cancer. *Oncogene* **23:** 2016-2027

Bien-Moller S, Lange S, Holm T, Bohm A, Paland H, Kupper J, Herzog S, Weitmann K, Havemann C, Vogelgesang S, Marx S, Hoffmann W, Schroeder HW, Rauch BH (2016) Expression of S1P metabolizing enzymes and receptors correlate with survival time and regulate cell migration in glioblastoma multiforme. *Oncotarget* **7:** 13031-13046

Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N, Baumruker T (2003) Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem* **278:** 47408-47415

Billich A, Bornancin F, Mechtcheriakova D, Natt F, Huesken D, Baumruker T (2005) Basal and induced sphingosine kinase 1 activity in A549 carcinoma cells: function in cell survival and IL-1β and TNF-α induced production of inflammatory mediators. *Cell Signal* **17:** 1203-1217

Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. *Lancet* **368:** 387-403

Blom T, Slotte JP, Pitson SM, Tornquist K (2005) Enhancement of intracellular sphingosine-1-phosphate production by inositol 1,4,5-trisphosphate-evoked calcium mobilisation in HEK-293 cells: endogenous sphingosine-1-phosphate as a modulator of the calcium response. *Cell Signal* **17:** 827-836

Blondeau N, Lai YS, Tyndall S, Popolo M, Topalkara K, Pru JK, Zhang L, Kim H, Liao JK, Ding K, Waeber C (2007) Distribution of sphingosine kinase activity and mRNA in rodent brain. *J Neurochem* **103:** 509-517

Bowman AB, Patel-King RS, Benashski SE, McCaffery JM, Goldstein LS, King SM (1999) Drosophila roadblock and Chlamydomonas LC7: a conserved family of dynein-associated proteins involved in axonal transport, flagellar motility, and mitosis. *J Cell Biol* **146:** 165-180

Bremner KH, Scherer J, Yi J, Vershinin M, Gross SP, Vallee RB (2009) Adenovirus transport via direct interaction of cytoplasmic dynein with the viral capsid hexon subunit. *Cell Host Microbe* **6:** 523-535

Bruhn MA, Pearson RB, Hannan RD, Sheppard KE (2010) Second AKT: the rise of SGK in cancer signalling. *Growth Factors* **28:** 394-408

Bruno G, Cencetti F, Pertici I, Japtok L, Bernacchioni C, Donati C, Bruni P (2015) CTGF/CCN2 exerts profibrotic action in myoblasts via the up-regulation of sphingosine

kinase-1/S1P3 signaling axis: Implications in the action mechanism of TGFbeta. *Biochim Biophys Acta* **1851:** 194-202

Bullock AN, Debreczeni J, Amos AL, Knapp S, Turk BE (2005) Structure and substrate specificity of the Pim-1 kinase. *J Biol Chem* **280:** 41675-41682

Burke B, Roux KJ (2009) Nuclei take a position: managing nuclear location. *Dev Cell* **17:** 587-597

Buse P, Tran SH, Luther E, Phu PT, Aponte GW, Firestone GL (1999) Cell cycle and hormonal control of nuclear-cytoplasmic localization of the serum- and glucocorticoid-inducible protein kinase, Sgk, in mammary tumor cells. A novel convergence point of anti-proliferative and proliferative cell signaling pathways. *J Biol Chem* **274:** 7253-7263

Bush NA, Chang SM, Berger MS (2017) Current and future strategies for treatment of glioma. *Neurosurg Rev* **40:** 1-14

Cai Q, Lu L, Tian JH, Zhu YB, Qiao H, Sheng ZH (2010) Snapin-regulated late endosomal transport is critical for efficient autophagy-lysosomal function in neurons. *Neuron* **68:** 73-86

Cao J, Lin C, Wang H, Wang L, Zhou N, Jin Y, Liao M, Zhou J (2015) Circovirus transport proceeds via direct interaction of the cytoplasmic dynein IC1 subunit with the viral capsid protein. *J Virol* **89:** 2777-2791

Cargnello M, Roux PP (2011) Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev* **75:** 50-83

Carpinteiro A, Dumitru C, Schenck M, Gulbins E (2008) Ceramide-induced cell death in malignant cells. *Cancer Lett* **264:** 1-10

Chalfant CE, Szulc Z, Roddy P, Bielawska A, Hannun YA (2004) The structural requirements for ceramide activation of serine-threonine protein phosphatases. *J Lipid Res* **45:** 496-506

Chan HS, Pitson SM (2013) Post-translational regulation of sphingosine kinases. *Biochim Biophys Acta* **1831:** 147-156

Chen R-q, Yang Q-k, Lu B-w, Yi W, Cantin G, Chen Y-l, Fearns C, Yates JR, III, Lee J-D (2009) CDC25B mediates Rapamycin-induced oncogenic responses in cancer cells. *Cancer Res* **69:** 2663-2668

Childress ES, Kharel Y, Brown AM, Bevan DR, Lynch KR, Santos WL (2017) Transforming sphingosine kinase 1 inhibitors into dual and sphingosine kinase 2 selective inhibitors: design, synthesis, and in vivo activity. *J Med Chem***:** doi: 10.1021/acs.jmedchem.1027b00233

Chipuk JE, McStay GP, Bharti A, Kuwana T, Clarke CJ, Siskind LJ, Obeid LM, Green DR (2012) Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. *Cell* **148:** 988-1000

Cho C, Reck-Peterson SL, Vale RD (2008) Regulatory ATPase sites of cytoplasmic dynein affect processivity and force generation. *J Biol Chem* **283:** 25839-25845

Christensen GL, Kelstrup CD, Lyngso C, Sarwar U, Bogebo R, Sheikh SP, Gammeltoft S, Olsen JV, Hansen JL (2010) Quantitative phosphoproteomics dissection of seven-transmembrane receptor signaling using full and biased agonists. *Mol Cell Proteomics* **9:** 1540-1553

Chumanevich AA, Poudyal D, Cui X, Davis T, Wood PA, Smith CD, Hofseth LJ (2010) Suppression of colitis-driven colon cancer in mice by a novel small molecule inhibitor of sphingosine kinase. *Carcinogenesis* **31:** 1787-1793

Chun J, Hla T, Lynch KR, Spiegel S, Moolenaar WH (2010) International union of basic and clinical pharmacology. LXXVIII. Lysophospholipid receptor nomenclature. *Pharmacol Rev* **62:** 579-587

Cianfrocco MA, DeSantis ME, Leschziner AE, Reck-Peterson SL (2015) Mechanism and regulation of cytoplasmic dynein. *Annu Rev Cell Dev Biol* **31:** 83-108

Cole AR (2012) GSK3 as a sensor determining cell fate in the brain. *Front Mol Neurosci* **5:** 4

Couttas TA, Kain N, Daniels B, Lim XY, Shepherd C, Kril J, Pickford R, Li H, Garner B, Don AS (2014) Loss of the neuroprotective factor sphingosine 1-phosphate early in Alzheimer's disease pathogenesis. *Acta Neuropathol Commun* **2:** 9

Cuvillier O, Ader I, Bouquerel P, Brizuela L, Malavaud B, Mazerolles C, Rischmann P (2010) Activation of sphingosine kinase-1 in cancer: implications for therapeutic targeting. *Curr Mol Pharmacol* **3:** 53-65

Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind JS, Spiegel S (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381:** 800-803

Dasgupta B, Chhipa RR (2015) Evolving lessons on the complex role of AMPK in normal physiology and cancer. *Trends Pharmacol Sci* **37:** 192-206

Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, Korner R, Greff Z, Keri G, Stemmann O, Mann M (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol Cell* **31:** 438-448

Dephoure N, Gould KL, Gygi SP, Kellogg DR (2013) Mapping and analysis of phosphorylation sites: a quick guide for cell biologists. *Mol Biol Cell* **24:** 535-542

Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ, Gygi SP (2008) A quantitative atlas of mitotic phosphorylation. *Proc Natl Acad Sci U S A* **105:** 10762-10767

Dick T, Ray K, Salz HK, Chia W (1996) Cytoplasmic dynein (ddlc1) mutations cause morphogenetic defects and apoptotic cell death in Drosophila melanogaster. *Mol Cell Biol* **16:** 1966-1977

Dillman JF, 3rd, Dabney LP, Karki S, Paschal BM, Holzbaur EL, Pfister KK (1996) Functional analysis of dynactin and cytoplasmic dynein in slow axonal transport. *J Neurosci* **16:** 6742-6752

Ding G, Sonoda H, Yu H, Kajimoto T, Goparaju SK, Jahangeer S, Okada T, Nakamura S (2007) Protein kinase D-mediated phosphorylation and nuclear export of sphingosine kinase 2. *J Biol Chem* **282:** 27493-27502

Dix CI, Soundararajan HC, Dzhindzhev NS, Begum F, Suter B, Ohkura H, Stephens E, Bullock SL (2013) Lissencephaly-1 promotes the recruitment of dynein and dynactin to transported mRNAs. *J Cell Biol* **202:** 479-494

Dodding MP, Way M (2011) Coupling viruses to dynein and kinesin-1. *EMBO J* **30:** 3527-3539

Don AS, Rosen H (2009) A lipid binding domain in sphingosine kinase 2. *Biochem Biophys Res Commun* **380:** 87-92

Duncan JE, Goldstein LS (2006) The genetics of axonal transport and axonal transport disorders. *PLoS Genet* **2:** e124

Edmonds Y, Milstien S, Spiegel S (2011) Development of small-molecule inhibitors of sphingosine-1-phosphate signaling. *Pharmacol Ther* **132:** 352-360

Efimov VP (2003) Roles of NUDE and NUDF proteins of Aspergillus nidulans: insights from intracellular localization and overexpression effects. *Mol Biol Cell* **14:** 871-888

Endo K, Igarashi Y, Nisar M, Zhou QH, Hakomori S (1991) Cell membrane signaling as target in cancer therapy: inhibitory effect of N,N-dimethyl and N,N,N-trimethyl sphingosine derivatives on in vitro and in vivo growth of human tumor cells in nude mice. *Cancer Res* **51:** 1613-1618

Eschbach J, Dupuis L (2011) Cytoplasmic dynein in neurodegeneration. *Pharmacol Ther* **130:** 348-363

Espindola FS, Suter DM, Partata LB, Cao T, Wolenski JS, Cheney RE, King SM, Mooseker MS (2000) The light chain composition of chicken brain myosin-Va: calmodulin, myosin-II essential light chains, and 8-kDa dynein light chain/PIN. *Cell Motil Cytoskeleton* **47:** 269-281

Etemadi N, Chopin M, Anderton H, Tanzer MC, Rickard JA, Abeysekera W, Hall C, Spall SK, Wang B, Xiong Y, Hla T, Pitson SM, Bonder CS, Wong WW, Ernst M, Smyth GK, Vaux DL, Nutt SL, Nachbur U, Silke J (2015) TRAF2 regulates TNF and NF-kappaB signalling to suppress apoptosis and skin inflammation independently of Sphingosine kinase 1. *Elife* **4:** e10592

Evangelisti C, Teti G, Chiarini F, Falconi M, Melchionda F, Pession A, Bertaina A, Locatelli F, McCubrey JA, Beak DJ, Bittman R, Pyne S, Pyne NJ, Martelli AM (2014) Assessment of the effect of sphingosine kinase inhibitors on apoptosis,unfolded protein response and autophagy of T-cell acute lymphoblastic leukemia cells; indications for novel therapeutics. *Oncotarget* **5:** 7886-7901

Evangelou K, Bramis J, Peros I, Zacharatos P, Dasiou-Plakida D, Kalogeropoulos N, Asimacopoulos PJ, Kittas C, Marinos E, Gorgoulis VG (2004) Electron microscopy evidence that cytoplasmic localization of the p16(INK4A) "nuclear" cyclin-dependent kinase inhibitor (CKI) in tumor cells is specific and not an artifact. A study in non-small cell lung carcinomas. *Biotech Histochem* **79:** 5-10

Facchinetti MM, Gandini NA, Fermento ME, Sterin-Speziale NB, Ji Y, Patel V, Gutkind JS, Rivadulla MG, Curino AC (2010) The expression of sphingosine kinase-1 in head and neck carcinoma. *Cells Tissues Organs* **192:** 314-324

Fitzpatrick LR, Green C, Frauenhoffer EE, French KJ, Zhuang Y, Maines LW, Upson JJ, Paul E, Donahue H, Mosher TJ, Smith CD (2011a) Attenuation of arthritis in rodents by a novel orally-available inhibitor of sphingosine kinase. *Inflammopharmacology* **19:** 75-87

Fitzpatrick LR, Green C, Maines LW, Smith CD (2011b) Experimental osteoarthritis in rats is attenuated by ABC294640, a selective inhibitor of sphingosine kinase-2. *Pharmacology* **87:** 135-143

Flores-Rodriguez N, Rogers SS, Kenwright DA, Waigh TA, Woodman PG, Allan VJ (2011) Roles of Dynein and dynactin in early endosome dynamics revealed using automated tracking and global analysis. *PLoS ONE* **6:** e24479

French KJ, Schrecengost RS, Lee BD, Zhuang Y, Smith SN, Eberly JL, Yun JK, Smith CD (2003) Discovery and evaluation of inhibitors of human sphingosine kinase. *Cancer Res* **63:** 5962-5969

French KJ, Upson JJ, Keller SN, Zhuang Y, Yun JK, Smith CD (2006) Antitumor activity of sphingosine kinase inhibitors. *J Pharmacol Exp Ther* **318:** 596-603

French KJ, Zhuang Y, Maines LW, Gao P, Wang W, Beljanski V, Upson JJ, Green CL, Keller SN, Smith CD (2010) Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. *J Pharmacol Exp Ther* **333:** 129-139

Gaestel M (2016) MAPK-activated protein kinases (MKs): novel insights and challenges. *Front Cell Dev Biol* **3:** 88

Gao P, Peterson YK, Smith RA, Smith CD (2012) Characterization of isoenzyme-selective inhibitors of human sphingosine kinases. *PLoS ONE* **7:** e44543

Gao P, Smith CD (2011) Ablation of sphingosine kinase-2 inhibits tumor cell proliferation and migration. *Mol Cancer Res* **9:** 1509-1519

Garrett CA, Barri M, Kuta A, Soura V, Deng W, Fisher EM, Schiavo G, Hafezparast M (2014) DYNC1H1 mutation alters transport kinetics and ERK1/2-cFos signalling in a mouse model of distal spinal muscular atrophy. *Brain* **137:** 1883-1893

Gault CR, Eblen ST, Neumann CA, Hannun YA, Obeid LM (2012) Oncogenic K-Ras regulates bioactive sphingolipids in a sphingosine kinase 1-dependent manner. *J Biol Chem* **287:** 31794-31803

Gault CR, Obeid LM (2011) Still benched on its way to the bedside: sphingosine kinase 1 as an emerging target in cancer chemotherapy. *Crit Rev Biochem Mol Biol* **46:** 342-351

Gillies L, Lee SC, Long JS, Ktistakis N, Pyne NJ, Pyne S (2009) The sphingosine 1-phosphate receptor 5 and sphingosine kinases 1 and 2 are localised in centrosomes: possible role in regulating cell division. *Cell Signal* **21:** 675-684

Gomez L, Paillard M, Price M, Chen Q, Teixeira G, Spiegel S, Lesnefsky EJ (2011) A novel role for mitochondrial sphingosine-1-phosphate produced by sphingosine kinase-2 in PTP-mediated cell survival during cardioprotection. *Basic Res Cardiol* **106:** 1341-1353

Grozinger CM, Schreiber SL (2000) Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. *Proc Natl Acad Sci U S A* **97:** 7835-7840

Gu Z, Li Y, Lee P, Liu T, Wan C, Wang Z (2012) Protein arginine methyltransferase 5 functions in opposite ways in the cytoplasm and nucleus of prostate cancer cells. *PLoS ONE* **7:** e44033

Ha J, Lo KW, Myers KR, Carr TM, Humsi MK, Rasoul BA, Segal RA, Pfister KK (2008) A neuron-specific cytoplasmic dynein isoform preferentially transports TrkB signaling endosomes. *J Cell Biol* **181:** 1027-1039

Haase R, Argyros O, Wong SP, Harbottle RP, Lipps HJ, Ogris M, Magnusson T, Vizoso Pinto MG, Haas J, Baiker A (2010) pEPito: a significantly improved non-viral episomal expression vector for mammalian cells. *BMC Biotechnol* **10:** 20

Hafezparast M, Klocke R, Ruhrberg C, Marquardt A, Ahmad-Annuar A, Bowen S, Lalli G, Witherden AS, Hummerich H, Nicholson S, Morgan PJ, Oozageer R, Priestley JV, Averill S, King VR, Ball S, Peters J, Toda T, Yamamoto A, Hiraoka Y, Augustin M, Korthaus D, Wattler S, Wabnitz P, Dickneite C, Lampel S, Boehme F, Peraus G, Popp A, Rudelius M, Schlegel J, Fuchs H, Hrabe de Angelis M, Schiavo G, Shima DT, Russ AP, Stumm G, Martin JE, Fisher EM (2003) Mutations in dynein link motor neuron degeneration to defects in retrograde transport. *Science* **300:** 808-812

Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA (1999) Creation of human tumour cells with defined genetic elements. *Nature* **400:** 464-468

Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, Luo C, Marmorstein R, Kordula T, Milstien S, Spiegel S (2009) Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science* **325:** 1254-1257

Hait NC, Bellamy A, Milstien S, Kordula T, Spiegel S (2007) Sphingosine kinase type 2 activation by ERK-mediated phosphorylation. *J Biol Chem* **282:** 12058-12065

Hait NC, Sarkar S, Le Stunff H, Mikami A, Maceyka M, Milstien S, Spiegel S (2005) Role of sphingosine kinase 2 in cell migration toward epidermal growth factor. *J Biol Chem* **280:** 29462-29469

Hancock WO (2014) Bidirectional cargo transport: moving beyond tug of war. *Nat Rev Mol Cell Biol* **15:** 615-628

Hannun YA, Obeid LM (2008) Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol* **9:** 139-150

Hanson MA, Roth CB, Jo E, Griffith MT, Scott FL, Reinhart G, Desale H, Clemons B, Cahalan SM, Schuerer SC, Sanna MG, Han GW, Kuhn P, Rosen H, Stevens RC (2012) Crystal structure of a lipid G protein-coupled receptor. *Science* **335:** 851-855

Hao M, Lowy AM, Kapoor M, Deffie A, Liu G, Lozano G (1996) Mutation of phosphoserine 389 affects p53 function in vivo. *J Biol Chem* **271:** 29380-29385

Hardie DG, Schaffer BE, Brunet A (2015) AMPK: An energy-sensing pathway with multiple inputs and outputs. *Trends Cell Biol* **26:** 190-201

Heffernan-Stroud LA, Obeid LM (2013) Sphingosine kinase 1 in cancer. *Adv Cancer Res* **117:** 201-235

Heinrich M, Neumeyer J, Jakob M, Hallas C, Tchikov V, Winoto-Morbach S, Wickel M, Schneider-Brachert W, Trauzold A, Hethke A, Schutze S (2004) Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. *Cell Death Differ* **11:** 550-563

Hirokawa N, Niwa S, Tanaka Y (2010) Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. *Neuron* **68:** 610-638

Hisano Y, Kobayashi N, Yamaguchi A, Nishi T (2012) Mouse SPNS2 functions as a sphingosine-1-phosphate transporter in vascular endothelial cells. *PLoS ONE* **7:** e38941

Hla T, Venkataraman K, Michaud J (2008) The vascular S1P gradient-cellular sources and biological significance. *Biochim Biophys Acta* **1781:** 477-482

Hoffman NJ, Parker BL, Chaudhuri R, Fisher-Wellman KH, Kleinert M, Humphrey SJ, Yang P, Holliday M, Trefely S, Fazakerley DJ, Stockli J, Burchfield JG, Jensen TE, Jothi R, Kiens B, Wojtaszewski JF, Richter EA, James DE (2015) Global phosphoproteomic analysis of human skeletal muscle reveals a network of exercise-regulated kinases and AMPK substrates. *Cell Metab* **22:** 922-935

Hofmann LP, Ren S, Schwalm S, Pfeilschifter J, Huwiler A (2008) Sphingosine kinase 1 and 2 regulate the capacity of mesangial cells to resist apoptotic stimuli in an opposing manner. *Biol Chem* **389:** 1399-1407

Holleran EA, Ligon LA, Tokito M, Stankewich MC, Morrow JS, Holzbaur EL (2001) beta III spectrin binds to the Arp1 subunit of dynactin. *J Biol Chem* **276:** 36598-36605

Hook P, Vallee RB (2006) The dynein family at a glance. *J Cell Sci* **119:** 4369-4371

Horgan CP, Hanscom SR, Jolly RS, Futter CE, McCaffrey MW (2010) Rab11-FIP3 binds dynein light intermediate chain 2 and its overexpression fragments the Golgi complex. *Biochem Biophys Res Commun* **394:** 387-392

Horikawa I, Parker ES, Solomon GG, Barrett JC (2001) Upregulation of the gene encoding a cytoplasmic dynein intermediate chain in senescent human cells. *J Cell Biochem* **82:** 415-421

Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V, Sullivan M (2012) PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res* **40:** D261-270

Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Res* **43:** D512-520

Huang J, Roberts AJ, Leschziner AE, Reck-Peterson SL (2012) Lis1 acts as a "clutch" between the ATPase and microtubule-binding domains of the dynein motor. *Cell* **150:** 975-986

Huang Y, Chen L, Guo L, Hupp TR, Lin Y (2014) Evaluating DAPK as a therapeutic target. *Apoptosis* **19:** 371-386

Huangfu D, Anderson KV (2005) Cilia and Hedgehog responsiveness in the mouse. *Proc Natl Acad Sci U S A* **102:** 11325-11330

Huttlin EL, Jedrychowski MP, Elias JE, Goswami T, Rad R, Beausoleil SA, Villen J, Haas W, Sowa ME, Gygi SP (2010) A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* **143:** 1174-1189

Igarashi N, Okada T, Hayashi S, Fujita T, Jahangeer S, Nakamura S (2003) Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. *J Biol Chem* **278:** 46832-46839

Ilieva HS, Yamanaka K, Malkmus S, Kakinohana O, Yaksh T, Marsala M, Cleveland DW (2008) Mutant dynein (Loa) triggers proprioceptive axon loss that extends survival only in the SOD1 ALS model with highest motor neuron death. *Proc Natl Acad Sci U S A* **105:** 12599-12604

Ishizawa J, Kojima K, Hail N, Jr., Tabe Y, Andreeff M (2015) Expression, function, and targeting of the nuclear exporter chromosome region maintenance 1 (CRM1) protein. *Pharmacol Ther* **153:** 25-35

Jacobs VL, Valdes PA, Hickey WF, De Leo JA (2011) Current review of in vivo GBM rodent models: emphasis on the CNS-1 tumour model. *ASN Neuro* **3:** e00063

Jallepalli PV, Lengauer C (2001) Chromosome segregation and cancer: cutting through the mystery. *Nat Rev Cancer* **1:** 109-117

Jarman KE, Moretti PAB, Zebol JR, Pitson SM (2010) Translocation of sphingosine kinase 1 to the plasma membrane is mediated by calcium- and integrin-binding protein 1. *J Biol Chem* **285:** 483-492

Jeffery CJ (2003) Moonlighting proteins: old proteins learning new tricks. *Trends Genet* **19:** 415-417

Jo SK, Bajwa A, Ye H, Vergis AL, Awad AS, Kharel Y, Lynch KR, Okusa MD (2009) Divergent roles of sphingosine kinases in kidney ischemia-reperfusion injury. *Kidney Int* **75:** 167-175

Johnson C, Crowther S, Stafford MJ, Campbell DG, Toth R, MacKintosh C (2010) Bioinformatic and experimental survey of 14-3-3-binding sites. *Biochem J* **427:** 69-78

Johnson KR, Johnson KY, Crellin HG, Ogretmen B, Boylan AM, Harley RA, Obeid LM (2005) Immunohistochemical distribution of sphingosine kinase 1 in normal and tumor lung tissue. *J Histochem Cytochem* **53:** 1159-1166

Jones LA, Villemant C, Starborg T, Salter A, Goddard G, Ruane P, Woodman PG, Papalopulu N, Woolner S, Allan VJ (2014) Dynein light intermediate chains maintain spindle bipolarity by functioning in centriole cohesion. *J Cell Biol* **207:** 499-516

Ju T, Gao D, Fang ZY (2016) Targeting colorectal cancer cells by a novel sphingosine kinase 1 inhibitor PF-543. *Biochem Biophys Res Commun* **470:** 728-734

Kaidanovich-Beilin O, Woodgett JR (2011) GSK-3: Functional insights from cell biology and animal models. *Front Mol Neurosci* **4:** doi: 10.3389/fnmol.2011.00040

Kajimoto T, Okada T, Miya S, Zhang L, Nakamura S (2013) Ongoing activation of sphingosine 1-phosphate receptors mediates maturation of exosomal multivesicular endosomes. *Nat Commun* **4:** 2712

Kaneko T, Amano M, Maeda A, Goto H, Takahashi K, Ito M, Kaibuchi K (2000) Identification of calponin as a novel substrate of Rho-kinase. *Biochem Biophys Res Commun* **273:** 110-116

Kapitonov D, Allegood JC, Mitchell C, Hait NC, Almenara JA, Adams JK, Zipkin RE, Dent P, Kordula T, Milstien S, Spiegel S (2009) Targeting sphingosine kinase 1 inhibits Akt signaling, induces apoptosis, and suppresses growth of human glioblastoma cells and xenografts. *Cancer Res* **69:** 6915-6923

Kardon JR, Reck-Peterson SL, Vale RD (2009) Regulation of the processivity and intracellular localization of Saccharomyces cerevisiae dynein by dynactin. *Proc Natl Acad Sci U S A* **106:** 5669-5674

Kardon JR, Vale RD (2009) Regulators of the cytoplasmic dynein motor. *Nat Rev Mol Cell Biol* **10:** 854-865

Karki S, Ligon LA, DeSantis J, Tokito M, Holzbaur EL (2002) PLAC-24 is a cytoplasmic dynein-binding protein that is recruited to sites of cell-cell contact. *Mol Biol Cell* **13:** 1722-1734

Kawamori T, Kaneshiro T, Okumura M, Maalouf S, Uflacker A, Bielawski J, Hannun YA, Obeid LM (2009) Role for sphingosine kinase 1 in colon carcinogenesis. *FASEB J* **23:** 405-414

Kawamori T, Osta W, Johnson KR, Pettus BJ, Bielawski J, Tanaka T, Wargovich MJ, Reddy BS, Hannun YA, Obeid LM, Zhou D (2006) Sphingosine kinase 1 is up-regulated in colon carcinogenesis. *FASEB J* **20:** 386-388

Kennelly PJ, Krebs EG (1991) Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem* **266:** 15555-15558

Kharel Y, Lee S, Snyder AH, Sheasley-O'neill S L, Morris MA, Setiady Y, Zhu R, Zigler MA, Burcin TL, Ley K, Tung KS, Engelhard VH, Macdonald TL, Pearson-White S, Lynch KR (2005) Sphingosine kinase 2 is required for modulation of lymphocyte traffic by FTY720. *J Biol Chem* **280:** 36865-36872

Kharel Y, Mathews TP, Gellett AM, Tomsig JL, Kennedy PC, Moyer ML, Macdonald TL, Lynch KR (2011) Sphingosine kinase type 1 inhibition reveals rapid turnover of circulating sphingosine 1-phosphate. *Biochem J* **440:** 345-353

Kharel Y, Morris EA, Congdon MD, Thorpe SB, Tomsig JL, Santos WL, Lynch KR (2015) Sphingosine kinase 2 inhibition and blood sphingosine 1-phosphate levels. *J Pharmacol Exp Ther* **355:** 23-31

Kharel Y, Raje M, Gao M, Gellett AM, Tomsig JL, Lynch KR, Santos WL (2012) Sphingosine kinase type 2 inhibition elevates circulating sphingosine 1-phosphate. *Biochem J* **447:** 149-157

Kim J, Jonasch E, Alexander A, Short JD, Cai S, Wen S, Tsavachidou D, Tamboli P, Czerniak BA, Do KA, Wu KJ, Marlow LA, Wood CG, Copland JA, Walker CL (2009) Cytoplasmic sequestration of p27 via AKT phosphorylation in renal cell carcinoma. *Clin Cancer Res* **15:** 81-90

Kimura S, Noda T, Yoshimori T (2008) Dynein-dependent movement of autophagosomes mediates efficient encounters with lysosomes. *Cell Struct Funct* **33:** 109-122

Kishi M, Pan YA, Crump JG, Sanes JR (2005) Mammalian SAD kinases are required for neuronal polarization. *Science* **307:** 929-932

Kobayashi T, Cohen P (1999) Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. *Biochem J* **339 (Pt 2):** 319-328

Kodiha M, Rassi JG, Brown CM, Stochaj U (2007) Localization of AMP kinase is regulated by stress, cell density, and signaling through the MEK-->ERK1/2 pathway. *Am J Physiol Cell Physiol* **293:** C1427-1436

Kohno M, Momoi M, Oo ML, Paik JH, Lee YM, Venkataraman K, Ai Y, Ristimaki AP, Fyrst H, Sano H, Rosenberg D, Saba JD, Proia RL, Hla T (2006) Intracellular role for sphingosine kinase 1 in intestinal adenoma cell proliferation. *Mol Cell Biol* **26:** 7211-7223

Kon T, Nishiura M, Ohkura R, Toyoshima YY, Sutoh K (2004) Distinct functions of nucleotide-binding/hydrolysis sites in the four AAA modules of cytoplasmic dynein. *Biochemistry* **43:** 11266-11274

Kono K, Sugiura M, Kohama T (2002) Inhibition of recombinant sphingosine kinases by novel inhibitors of microbial origin, F-12509A and B-5354c. *J Antibiot* **55:** 99-103

Koo SH, Flechner L, Qi L, Zhang X, Screaton RA, Jeffries S, Hedrick S, Xu W, Boussouar F, Brindle P, Takemori H, Montminy M (2005) The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* **437:** 1109-1111

Kuta A, Deng W, Morsi El-Kadi A, Banks GT, Hafezparast M, Pfister KK, Fisher EM (2010) Mouse cytoplasmic dynein intermediate chains: identification of new isoforms, alternative splicing and tissue distribution of transcripts. *PLoS ONE* **5:** e11682

la Cour T, Gupta R, Rapacki K, Skriver K, Poulsen FM, Brunak S (2003) NESbase version 1.0: a database of nuclear export signals. *Nucleic Acids Res* **31:** 393-396

Lai WQ, Irwan AW, Goh HH, Melendez AJ, McInnes IB, Leung BP (2009) Distinct roles of sphingosine kinase 1 and 2 in murine collagen-induced arthritis. *J Immunol* **183:** 2097-2103

Lalonde S, Ehrhardt DW, Loque D, Chen J, Rhee SY, Frommer WB (2008) Molecular and cellular approaches for the detection of protein-protein interactions: latest techniques and current limitations. *Plant J* **53:** 610-635

Leclercq TM, Pitson SM (2006) Cellular signalling by sphingosine kinase and sphingosine 1-phosphate. *IUBMB Life* **58:** 467-472

Li J, Guan HY, Gong LY, Song LB, Zhang N, Wu J, Yuan J, Zheng YJ, Huang ZS, Li M (2008) Clinical significance of sphingosine kinase-1 expression in human astrocytomas progression and overall patient survival. *Clin Cancer Res* **14:** 6996-7003

Li J, Lee WL, Cooper JA (2005) NudEL targets dynein to microtubule ends through LIS1. *Nat Cell Biol* **7:** 686-690

Li W, Tian Z, Qin H, Li N, Zhou X, Li J, Ni B, Ruan Z (2015) High expression of sphingosine kinase 1 is associated with poor prognosis in nasopharyngeal carcinoma. *Biochem Biophys Res Commun* **460:** 341-347

Li W, Yu CP, Xia JT, Zhang L, Weng GX, Zheng HQ, Kong QL, Hu LJ, Zeng MS, Zeng YX, Li M, Li J, Song LB (2009) Sphingosine kinase 1 is associated with gastric cancer progression and poor survival of patients. *Clin Cancer Res* **15:** 1393-1399

Li Y, Yu W, Liang Y, Zhu X (2007) Kinetochore dynein generates a poleward pulling force to facilitate congression and full chromosome alignment. *Cell Res* **17:** 701-712

Liang J, Nagahashi M, Kim EY, Harikumar KB, Yamada A, Huang WC, Hait NC, Allegood JC, Price MM, Avni D, Takabe K, Kordula T, Milstien S, Spiegel S (2013) Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. *Cancer Cell* **23:** 107-120

Liang Y, Yu W, Li Y, Yang Z, Yan X, Huang Q, Zhu X (2004) Nudel functions in membrane traffic mainly through association with Lis1 and cytoplasmic dynein. *J Cell Biol* **164:** 557-566

Ligon LA, Karki S, Tokito M, Holzbaur EL (2001) Dynein binds to beta-catenin and may tether microtubules at adherens junctions. *Nat Cell Biol* **3:** 913-917

Ligon LA, Tokito M, Finklestein JM, Grossman FE, Holzbaur EL (2004) A direct interaction between cytoplasmic dynein and kinesin I may coordinate motor activity. *J Biol Chem* **279:** 19201-19208

Lim KG, Sun C, Bittman R, Pyne NJ, Pyne S (2011) (R)-FTY720 methyl ether is a specific sphingosine kinase 2 inhibitor: Effect on sphingosine kinase 2 expression in HEK 293 cells and actin rearrangement and survival of MCF-7 breast cancer cells. *Cell Signal* **23:** 1590-1595

Lima S, Milstien S, Spiegel S (2017) Sphingosine and sphingosine kinase 1 involvement in endocytic membrane trafficking. *J Biol Chem* **292:** 3074-3088

Lin SX, Collins CA (1992) Immunolocalization of cytoplasmic dynein to lysosomes in cultured cells. *J Cell Sci* **101:** 125-137

Lin Y, Hupp TR, Stevens C (2010) Death-associated protein kinase (DAPK) and signal transduction: additional roles beyond cell death. *FEBS J* **277:** 48-57

Litovchick L, Chestukhin A, DeCaprio JA (2004) Glycogen synthase kinase 3 phosphorylates RBL2/p130 during quiescence. *Mol Cell Biol* **24:** 8970-8980

Liu G, Zheng H, Zhang Z, Wu Z, Xiong H, Li J, Song L (2010) Overexpression of sphingosine kinase 1 is associated with salivary gland carcinoma progression and might be a novel predictive marker for adjuvant therapy. *BMC Cancer* **10:** 495

Liu H, Sugiura M, Nava VE, Edsall LC, Kono K, Poulton S, Milstien S, Kohama T, Spiegel S (2000) Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J Biol Chem* **275:** 19513-19520

Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, Sankala H, Payne SG, Bektas M, Ishii I, Chun J, Milstien S, Spiegel S (2003) Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J Biol Chem* **278:** 40330-40336

Liu K, Guo TL, Hait NC, Allegood J, Parikh HI, Xu W, Kellogg GE, Grant S, Spiegel S, Zhang S (2013) Biological characterization of 3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) as a selective sphingosine kinase-2 inhibitor and anticancer agent. *PLoS ONE* **8:** e56471

Liu W, Ning J, Li C, Hu J, Meng Q, Lu H, Cai L (2016a) Overexpression of Sphk2 is associated with gefitinib resistance in non-small cell lung cancer. *Tumour Biol* **37:** 6331-6336

Liu X, Ren K, Suo R, Xiong SL, Zhang QH, Mo ZC, Tang ZL, Jiang Y, Peng XS, Yi GH (2016b) ApoA-I induces S1P release from endothelial cells through ABCA1 and SR-BI in a positive feedback manner. *J Physiol Biochem***:** doi:10.1007/s13105-13016-10504-13106

Lloyd TE, Machamer J, O'Hara K, Kim JH, Collins SE, Wong MY, Sahin B, Imlach W, Yang Y, Levitan ES, McCabe BD, Kolodkin AL (2012) The p150(Glued) CAP-Gly domain regulates initiation of retrograde transport at synaptic termini. *Neuron* **74:** 344-360

Lo KW, Kan HM, Pfister KK (2006) Identification of a novel region of the cytoplasmic Dynein intermediate chain important for dimerization in the absence of the light chains. *J Biol Chem* **281:** 9552-9559

Lo KW, Naisbitt S, Fan JS, Sheng M, Zhang M (2001) The 8-kDa dynein light chain binds to its targets via a conserved (K/R)XTQT motif. *J Biol Chem* **276:** 14059-14066

Lobo GP, Waite KA, Planchon SM, Romigh T, Houghton JA, Eng C (2008) ATP modulates PTEN subcellular localization in multiple cancer cell lines. *Hum Mol Genet* **17:** 2877-2885

Lomakin AJ, Semenova I, Zaliapin I, Kraikivski P, Nadezhdina E, Slepchenko BM, Akhmanova A, Rodionov V (2009) CLIP-170-dependent capture of membrane organelles by microtubules initiates minus-end directed transport. *Dev Cell* **17:** 323-333

Long JS, Edwards J, Watson C, Tovey S, Mair KM, Schiff R, Natarajan V, Pyne NJ, Pyne S (2010) Sphingosine kinase 1 induces tolerance to human epidermal growth factor receptor 2 and prevents formation of a migratory phenotype in response to sphingosine 1-phosphate in estrogen receptor-positive breast cancer cells. *Mol Cell Biol* **30:** 3827-3841

Lopez-Girona A, Furnari B, Mondesert O, Russell P (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* **397:** 172-175

Lu KP, Liou YC, Zhou XZ (2002) Pinning down proline-directed phosphorylation signaling. *Trends Cell Biol* **12:** 164-172

Maceyka M, Alvarez SE, Milstien S, Spiegel S (2008) Filamin A links sphingosine kinase 1 and sphingosine-1-phosphate receptor 1 at lamellipodia to orchestrate cell migration. *Mol Cell Biol* **28:** 5687-5697

Maceyka M, Milstien S, Spiegel S (2009) Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling. *J Lipid Res* **50:** S272-S276

Maceyka M, Sankala H, Hait NC, Le Stunff H, Liu H, Toman R, Collier C, Zhang M, Satin LS, Merrill AH, Milstien S, Spiegel S (2005) SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. *J Biol Chem* **280:** 37118-37129

Maines LW, Fitzpatrick LR, French KJ, Zhuang Y, Xia Z, Keller SN, Upson JJ, Smith CD (2008) Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. *Dig Dis Sci* **53:** 997-1012

Maines LW, Fitzpatrick LR, Green CL, Zhuang Y, Smith CD (2010) Efficacy of a novel sphingosine kinase inhibitor in experimental Crohn's disease. *Inflammopharmacology* **18:** 73-85

Maines LW, French KJ, Wolpert EB, Antonetti DA, Smith CD (2006) Pharmacologic manipulation of sphingosine kinase in retinal endothelial cells: implications for angiogenic ocular diseases. *Invest Ophthalmol Vis Sci* **47:** 5022-5031

Malavaud B, Pchejetski D, Mazerolles C, de Paiva GR, Calvet C, Doumerc N, Pitson S, Rischmann P, Cuvillier O (2010) Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. *Eur J Cancer* **46:** 3417-3424

Malone CJ, Misner L, Le Bot N, Tsai MC, Campbell JM, Ahringer J, White JG (2003) The C. elegans hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. *Cell* **115:** 825-836

Manneville JB, Jehanno M, Etienne-Manneville S (2010) Dlg1 binds GKAP to control dynein association with microtubules, centrosome positioning, and cell polarity. *J Cell Biol* **191:** 585-598

Marfia G, Campanella R, Navone SE, Di Vito C, Riccitelli E, Hadi LA, Bornati A, de Rezende G, Giussani P, Tringali C, Viani P, Rampini P, Alessandri G, Parati E, Riboni L (2014) Autocrine/paracrine sphingosine-1-phosphate fuels proliferative and stemness qualities of glioblastoma stem cells. *Glia* **62:** 1968-1981

Mastrandrea LD, Sessanna SM, Laychock SG (2005) Sphingosine kinase activity and sphingosine-1 phosphate production in rat pancreatic islets and INS-1 cells - Response to cytokines. *Diabetes* **54:** 1429-1436

Mathes C, Fleig A, Penner R (1998) Calcium release-activated calcium current (ICRAC) is a direct target for sphingosine. *J Biol Chem* **273:** 25020-25030

McKenney RJ, Huynh W, Tanenbaum ME, Bhabha G, Vale RD (2014) Activation of cytoplasmic dynein motility by dynactin-cargo adapter complexes. *Science* **345:** 337-341

McKenney RJ, Weil SJ, Scherer J, Vallee RB (2011) Mutually exclusive cytoplasmic dynein regulation by nude-LIS1 and dynactin. *J Biol Chem* **286:** 39615-39622

McNaughton M, Pitman M, Pitson SM, Pyne NJ, Pyne S (2016) Proteasomal degradation of sphingosine kinase 1 and inhibition of dihydroceramide desaturase by the sphingosine kinase inhibitors, SKi or ABC294640, induces growth arrest in androgen-independent LNCaP-AI prostate cancer cells. *Oncotarget* **7:** 16663-16675

Meilinger D, Fellinger K, Bultmann S, Rothbauer U, Bonapace IM, Klinkert WE, Spada F, Leonhardt H (2009) Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. *EMBO Rep* **10:** 1259-1264

Melendez AJ, Carlos-Dias E, Gosink M, Allen JM, Takacs L (2000) Human sphingosine kinase: molecular cloning, functional characterization and tissue distribution. *Gene* **251:** 19-26

Meng XD, Zhou ZS, Qiu JH, Shen WH, Wu Q, Xiao J (2014) Increased SPHK1 expression is associated with poor prognosis in bladder cancer. *Tumour Biol* **35:** 2075-2080

Meyer zu Heringdorf D, Jakobs KH (2007) Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. *Biochim Biophys Acta* **1768:** 923-940

Miller AV, Alvarez SE, Spiegel S, Lebman DA (2008) Sphingosine kinases and sphingosine-1-phosphate are critical for transforming growth factor β-induced extracellular signal-regulated kinase 1 and 2 activation and promotion of migration and invasion of esophageal cancer cells. *Mol Cell Biol* **28:** 4142-4151

Mitchell DJ, Blasier KR, Jeffery ED, Ross MW, Pullikuth AK, Suo D, Park J, Smiley WR, Lo KW, Shabanowitz J, Deppmann CD, Trinidad JC, Hunt DF, Catling AD, Pfister KK (2012) Trk activation of the ERK1/2 kinase pathway stimulates intermediate chain phosphorylation and recruits cytoplasmic dynein to signaling endosomes for retrograde axonal transport. *J Neurosci* **32:** 15495-15510

Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL (2005) Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol* **25:** 11113-11121

Mizutani N, Omori Y, Tanaka K, Ito H, Takagi A, Kojima T, Nakatochi M, Ogiso H, Kawamoto Y, Nakamura M, Suzuki M, Kyogashima M, Tamiya-Koizumi K, Nozawa Y, Murate T (2015) Increased SPHK2 transcription of human colon cancer cells in serum-depleted culture: The involvement of CREB transcription factor. *J Cell Biochem* **116:** 2227-2238

Modrak DE, Gold DV, Goldenberg DM (2006) Sphingolipid targets in cancer therapy. *Mol Cancer Ther* **5:** 200-208

Mok YK, Lo KW, Zhang M (2001) Structure of Tctex-1 and its interaction with cytoplasmic dynein intermediate chain. *J Biol Chem* **276:** 14067-14074

Moon HM, Youn YH, Pemble H, Yingling J, Wittmann T, Wynshaw-Boris A (2014) LIS1 controls mitosis and mitotic spindle organization via the LIS1-NDEL1-dynein complex. *Hum Mol Genet* **23:** 449-466

Moruno Manchon JF, Uzor NE, Dabaghian Y, Furr-Stimming EE, Finkbeiner S, Tsvetkov AS (2015) Cytoplasmic sphingosine-1-phosphate pathway modulates neuronal autophagy. *Sci Rep* **5:** 15213

Moughamian AJ, Holzbaur EL (2012) Dynactin is required for transport initiation from the distal axon. *Neuron* **74:** 331-343

Moughamian AJ, Osborn GE, Lazarus JE, Maday S, Holzbaur EL (2013) Ordered recruitment of dynactin to the microtubule plus-end is required for efficient initiation of retrograde axonal transport. *J Neurosci* **33:** 13190-13203

Mukhopadhyay A, Saddoughi SA, Song P, Sultan I, Ponnusamy S, Senkal CE, Snook CF, Arnold HK, Sears RC, Hannun YA, Ogretmen B (2009) Direct interaction between the inhibitor 2 and ceramide via sphingolipid-protein binding is involved in the regulation of protein phosphatase 2A activity and signaling. *FASEB J* **23:** 751-763

Muller G, Ayoub M, Storz P, Rennecke J, Fabbro D, Pfizenmaier K (1995) PKC zeta is a molecular switch in signal transduction of TNF-alpha, bifunctionally regulated by ceramide and arachidonic acid. *EMBO J* **14:** 1961-1969

Muresan V, Stankewich MC, Steffen W, Morrow JS, Holzbaur EL, Schnapp BJ (2001) Dynactin-dependent, dynein-driven vesicle transport in the absence of membrane proteins: a role for spectrin and acidic phospholipids. *Mol Cell* **7:** 173-183

Myers KR, Lo KW, Lye RJ, Kogoy JM, Soura V, Hafezparast M, Pfister KK (2007) Intermediate chain subunit as a probe for cytoplasmic dynein function: biochemical analyses and live cell imaging in PC12 cells. *J Neurosci Res* **85:** 2640-2647

Nagahashi M, Ramachandran S, Kim EY, Allegood JC, Rashid OM, Yamada A, Zhao R, Milstien S, Zhou H, Spiegel S, Takabe K (2012) Sphingosine-1-phosphate produced by sphingosine kinase 1 promotes breast cancer progression by stimulating angiogenesis and lymphangiogenesis. *Cancer Res* **72:** 726-735

Nagahashi M, Takabe K, Liu R, Peng K, Wang X, Wang Y, Hait NC, Allegood JC, Yamada A, Aoyagi T, Liang J, Pandak WM, Spiegel S, Hylemon PB, Zhou H (2015) Conjugated bile acid-activated S1P receptor 2 is a key regulator of sphingosine kinase 2 and hepatic gene expression. *Hepatology* **61:** 1216-1226

Nagahashi M, Takabe K, Terracina KP, Soma D, Hirose Y, Kobayashi T, Matsuda Y, Wakai T (2014) Sphingosine-1-phosphate transporters as targets for cancer therapy. *Biomed Res Int* **2014:** 651727

Navarro C, Puthalakath H, Adams JM, Strasser A, Lehmann R (2004) Egalitarian binds dynein light chain to establish oocyte polarity and maintain oocyte fate. *Nat Cell Biol* **6:** 427-435

Nemoto S, Nakamura M, Osawa Y, Kono S, Itoh Y, Okano Y, Murate T, Hara A, Ueda H, Nozawa Y, Banno Y (2009) Sphingosine kinase isoforms regulate oxaliplatin sensitivity of

human colon cancer cells through ceramide accumulation and Akt activation. *J Biol Chem* **284:** 10422-10432

Neubauer HA, Pham DH, Zebol JR, Moretti PA, Peterson AL, Leclercq TM, Chan H, Powell JA, Pitman MR, Samuel MS, Bonder CS, Creek DJ, Gliddon BL, Pitson SM (2016) An oncogenic role for sphingosine kinase 2. *Oncotarget* **7:** 64886-64899

Neubauer HA, Pitson SM (2013) Roles, regulation and inhibitors of sphingosine kinase 2. *FEBS J* **280:** 5317-5336

Neuwald AF, Aravind L, Spouge JL, Koonin EV (1999) AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9:** 27-43

Newton J, Lima S, Maceyka M, Spiegel S (2015) Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy. *Exp Cell Res* **333:** 195-200

Ng YW, Raghunathan D, Chan PM, Baskaran Y, Smith DJ, Lee CH, Verma C, Manser E (2010) Why an A-loop phospho-mimetic fails to activate PAK1: understanding an inaccessible kinase state by molecular dynamics simulations. *Structure* **18:** 879-890

Nilsson K, Landberg G (2006) Subcellular localization, modification and protein complex formation of the cdk-inhibitor p16 in Rb-functional and Rb-inactivated tumor cells. *Int J Cancer* **118:** 1120-1125

Nishikawa K, Toker A, Johannes FJ, Songyang Z, Cantley LC (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. *J Biol Chem* **272:** 952-960

Obenauer JC, Cantley LC, Yaffe MB (2003) Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res* **31:** 3635-3641

Ohotski J, Rosen H, Bittman R, Pyne S, Pyne NJ (2014) Sphingosine kinase 2 prevents the nuclear translocation of sphingosine 1-phosphate receptor-2 and tyrosine 416 phosphorylated c-Src and increases estrogen receptor negative MDA-MB-231 breast cancer cell growth: The role of sphingosine 1-phosphate receptor-4. *Cell Signal* **26:** 1040-1047

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* **407:** 313-319

Okada T, Ding G, Sonoda H, Kajimoto T, Haga Y, Khosrowbeygi A, Gao SY, Miwa N, Jahangeer S, Nakamura S (2005) Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis. *J Biol Chem* **280:** 36318-36325

Olivera A, Kohama T, Edsall L, Nava V, Cuvillier O, Poulton S, Spiegel S (1999) Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J Cell Biol* **147:** 545-557

Olivera A, Mizugishi K, Tikhonova A, Ciaccia L, Odom S, Proia RL, Rivera J (2007) The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis. *Immunity* **26:** 287-297

Olivera A, Urtz N, Mizugishi K, Yamashita Y, Gilfillan AM, Furumoto Y, Gu H, Proia RL, Baumruker T, Rivera J (2006) IgE-dependent activation of sphingosine kinases 1 and 2 and secretion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses. *J Biol Chem* **281:** 2515-2525

Ori-McKenney KM, Xu J, Gross SP, Vallee RB (2010) A cytoplasmic dynein tail mutation impairs motor processivity. *Nat Cell Biol* **12:** 1228-1234

Oskeritzian CA, Alvarez SE, Hait NC, Price MM, Milstien S, Spiegel S (2008) Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions. *Blood* **111:** 4193-4200

Palmer KJ, Hughes H, Stephens DJ (2009) Specificity of cytoplasmic dynein subunits in discrete membrane-trafficking steps. *Mol Biol Cell* **20:** 2885-2899

Pan J, Tao YF, Zhou Z, Cao BR, Wu SY, Zhang YL, Hu SY, Zhao WL, Wang J, Lou GL, Li Z, Feng X, Ni J (2011) An novel role of sphingosine kinase-1 (SPHK1) in the invasion and metastasis of esophageal carcinoma. *J Transl Med* **9:** 157

Panneer Selvam S, De Palma RM, Oaks JJ, Oleinik N, Peterson YK, Stahelin RV, Skordalakes E, Ponnusamy S, Garrett-Mayer E, Smith CD, Ogretmen B (2015) Binding of the sphingolipid S1P to hTERT stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation. *Sci Signal* **8:** ra58

Parham KA, Zebol JR, Tooley KL, Sun WY, Moldenhauer LM, Cockshell MP, Gliddon BL, Moretti PA, Tigyi G, Pitson SM, Bonder CS (2015) Sphingosine 1-phosphate is a ligand for peroxisome proliferator-activated receptor-gamma that regulates neoangiogenesis. *FASEB J* **29:** 3638-3653

Park K, Ikushiro H, Seo HS, Shin KO, Kim YI, Kim JY, Lee YM, Yano T, Holleran WM, Elias P, Uchida Y (2016) ER stress stimulates production of the key antimicrobial peptide, cathelicidin, by forming a previously unidentified intracellular S1P signaling complex. *Proc Natl Acad Sci U S A* **113:** E1334-1342

Park YS, Hakomori S, Kawa S, Ruan F, Igarashi Y (1994) Liposomal N,N,N-trimethylsphingosine (TMS) as an inhibitor of B16 melanoma cell growth and metastasis with reduced toxicity and enhanced drug efficacy compared to free TMS: cell membrane signaling as a target in cancer therapy III. *Cancer Res* **54:** 2213-2217

Patwardhan NN, Morris EA, Kharel Y, Raje MR, Gao M, Tomsig JL, Lynch KR, Santos WL (2015) Structure-activity relationship studies and in vivo activity of guanidine-based sphingosine kinase inhibitors: discovery of SphK1- and SphK2-selective inhibitors. *J Med Chem* **58:** 1879-1899

Paugh SW, Payne SG, Barbour SE, Milstien S, Spiegel S (2003) The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. *FEBS Lett* **554:** 189-193

Pchejetski D, Doumerc N, Golzio M, Naymark M, Teissie J, Kohama T, Waxman J, Malavaud B, Cuvillier O (2008) Chemosensitizing effects of sphingosine kinase-1 inhibition in prostate cancer cell and animal models. *Mol Cancer Ther* **7:** 1836-1845

Pebay A, Bonder CS, Pitson SM (2007) Stem cell regulation by lysophospholipids. *Prostaglandins Other Lipid Mediat* **84:** 83-97

Perry DM, Kitatani K, Roddy P, El-Osta M, Hannun YA (2012) Identification and characterization of protein phosphatase 2C activation by ceramide. *J Lipid Res* **53:** 1513-1521

Pfister KK (2015) Distinct functional roles of cytoplasmic dynein defined by the intermediate chain isoforms. *Exp Cell Res* **334:** 54-60

Pfister KK, Shah PR, Hummerich H, Russ A, Cotton J, Annuar AA, King SM, Fisher EM (2006) Genetic analysis of the cytoplasmic dynein subunit families. *PLoS Genet* **2:** e1

Pham DH, Moretti PA, Goodall GJ, Pitson SM (2008) Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3' AU-rich mRNA destabilizing elements. *BioTechniques* **45:** 155-162

Pham DH, Powell JA, Gliddon BL, Moretti PA, Tsykin A, Van der Hoek M, Kenyon R, Goodall GJ, Pitson SM (2014) Enhanced expression of transferrin receptor 1 contributes to oncogenic signalling by sphingosine kinase 1. *Oncogene* **33:** 5559-5568

Pitman MR, Costabile M, Pitson SM (2016) Recent advances in the development of sphingosine kinase inhibitors. *Cell Signal* **28:** 1349-1363

Pitman MR, Pham DH, Pitson SM (2012) Isoform-selective assays for sphingosine kinase activity. *Methods Mol Biol* **874:** 21-31

Pitman MR, Pitson SM (2010) Inhibitors of the sphingosine kinase pathway as potential therapeutics. *Curr Cancer Drug Targets* **10:** 354-367

Pitman MR, Powell JA, Coolen C, Moretti PA, Zebol JR, Pham DH, Finnie JW, Don AS, Ebert LM, Bonder CS, Gliddon BL, Pitson SM (2015) A selective ATP-competitive sphingosine kinase inhibitor demonstrates anti-cancer properties. *Oncotarget* **6:** 7065-7083

Pitson SM (2011) Regulation of sphingosine kinase and sphingolipid signaling. *Trends Biochem Sci* **36:** 97-107

Pitson SM, D'Andrea R J, Vandeleur L, Moretti PA, Xia P, Gamble JR, Vadas MA, Wattenberg BW (2000a) Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes. *Biochem J* **350 Pt 2:** 429-441

Pitson SM, Moretti PA, Zebol JR, Zareie R, Derian CK, Darrow AL, Qi J, D'Andrea RJ, Bagley CJ, Vadas MA, Wattenberg BW (2002) The nucleotide-binding site of human sphingosine kinase 1. *J Biol Chem* **277:** 49545-49553

Pitson SM, Moretti PAB, Zebol JR, Lynn HE, Xia P, Vadas MA, Wattenberg BW (2003) Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *EMBO J* **22:** 5491-5500

Pitson SM, Moretti PAB, Zebol JR, Xia P, Gamble JR, Vadas MA, D'Andrea RJ, Wattenberg BW (2000b) Expression of a catalytically inactive sphingosine kinase mutant blocks agonist-induced sphingosine kinase activation - A dominant-negative sphingosine kinase. *J Biol Chem* **275:** 33945-33950

Pitson SM, Pebay A (2009) Regulation of stem cell pluripotency and neural differentiation by lysophospholipids. *Neurosignals* **17:** 242-254

Pitson SM, Powell JA, Bonder CS (2011) Regulation of sphingosine kinase in hematological malignancies and other cancers. *Anticancer Agents Med Chem* **11:** 799-809

Pitson SM, Xia P, Leclercq TM, Moretti PAB, Zebol JR, Lynn HE, Wattenberg BW, Vadas MA (2005) Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. *J Exp Med* **201:** 49-54

Polivka J, Jr., Polivka J, Holubec L, Kubikova T, Priban V, Hes O, Pivovarcikova K, Treskova I (2017) Advances in experimental targeted therapy and immunotherapy for patients with glioblastoma multiforme. *Anticancer Res* **37:** 21-33

Ponnusamy S, Selvam SP, Mehrotra S, Kawamori T, Snider AJ, Obeid LM, Shao Y, Sabbadini R, Ogretmen B (2012) Communication between host organism and cancer cells is transduced by systemic sphingosine kinase 1/sphingosine 1-phosphate signalling to regulate tumour metastasis. *EMBO Mol Med* **4:** 761-775

Preitner N, Quan J, Nowakowski DW, Hancock ML, Shi J, Tcherkezian J, Young-Pearse TL, Flanagan JG (2014) APC is an RNA-binding protein, and its interactome provides a link to neural development and microtubule assembly. *Cell* **158:** 368-382

Pronobis MI, Rusan NM, Peifer M (2015) A novel GSK3-regulated APC:Axin interaction regulates Wnt signaling by driving a catalytic cycle of efficient betacatenin destruction. *Elife* **4:** e08022

Pullikuth AK, Ozdemir A, Cardenas D, Bailey E, Sherman NE, Pfister KK, Catling AD (2013) Epidermal growth factor stimulates extracellular-signal regulated kinase phosphorylation of a novel site on cytoplasmic Dynein intermediate chain 2. *Int J Mol Sci* **14:** 3595-3620

Pyne NJ, Pyne S (2010) Sphingosine 1-phosphate and cancer. *Nat Rev Cancer* **10:** 489-503

Quint K, Stiel N, Neureiter D, Schlicker HU, Nimsky C, Ocker M, Strik H, Kolodziej MA (2014) The role of sphingosine kinase isoforms and receptors S1P1, S1P2, S1P3, and S1P5 in primary, secondary, and recurrent glioblastomas. *Tumour Biol* **35:** 8979-8989

Rapali P, Szenes A, Radnai L, Bakos A, Pal G, Nyitray L (2011) DYNLL/LC8: a light chain subunit of the dynein motor complex and beyond. *FEBS J* **278:** 2980-2996

Ravikumar B, Acevedo-Arozena A, Imarisio S, Berger Z, Vacher C, O'Kane CJ, Brown SD, Rubinsztein DC (2005) Dynein mutations impair autophagic clearance of aggregate-prone proteins. *Nat Genet* **37:** 771-776

Reid SP, Tritsch SR, Kota K, Chiang CY, Dong L, Kenny T, Brueggemann EE, Ward MD, Cazares LH, Bavari S (2015) Sphingosine kinase 2 is a chikungunya virus host factor co-localized with the viral replication complex. *Emerg Microbes Infect* **4:** e61

Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM (2004) ONCOMINE: a cancer microarray database and integrated data-mining platform. *Neoplasia* **6:** 1-6

Riccitelli E, Giussani P, Di Vito C, Condomitti G, Tringali C, Caroli M, Galli R, Viani P, Riboni L (2013) Extracellular sphingosine-1-phosphate: a novel actor in human glioblastoma stem cell survival. *PLoS ONE* **8:** e68229

Ring DB, Johnson KW, Henriksen EJ, Nuss JM, Goff D, Kinnick TR, Ma ST, Reeder JW, Samuels I, Slabiak T, Wagman AS, Hammond ME, Harrison SD (2003) Selective glycogen synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. *Diabetes* **52:** 588-595

Rittinger K, Budman J, Xu J, Volinia S, Cantley LC, Smerdon SJ, Gamblin SJ, Yaffe MB (1999) Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding. *Mol Cell* **4:** 153-166

Roberts AJ, Goodman BS, Reck-Peterson SL (2014) Reconstitution of dynein transport to the microtubule plus end by kinesin. *Elife* **3:** e02641

Roberts JL, Moretti PA, Darrow AL, Derian CK, Vadas MA, Pitson SM (2004) An assay for sphingosine kinase activity using biotinylated sphingosine and streptavidin-coated membranes. *Anal Biochem* **331:** 122-129

Romagosa C, Simonetti S, Lopez-Vicente L, Mazo A, Lleonart ME, Castellvi J, Ramon y Cajal S (2011) p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. *Oncogene* **30:** 2087-2097

Rosen H, Gonzalez-Cabrera PJ, Sanna MG, Brown S (2009) Sphingosine 1-phosphate receptor signaling. *Annu Rev Biochem* **78:** 743-768

Ruckhaberle E, Rody A, Engels K, Gaetje R, von Minckwitz G, Schiffmann S, Grosch S, Geisslinger G, Holtrich U, Karn T, Kaufmann M (2008) Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. *Breast Cancer Res Treat* **112:** 41-52

Saddoughi SA, Gencer S, Peterson YK, Ward KE, Mukhopadhyay A, Oaks J, Bielawski J, Szulc ZM, Thomas RJ, Selvam SP, Senkal CE, Garrett-Mayer E, De Palma RM, Fedarovich D, Liu A, Habib AA, Stahelin RV, Perrotti D, Ogretmen B (2013) Sphingosine analogue drug FTY720 targets I2PP2A/SET and mediates lung tumour suppression via activation of PP2A-RIPK1-dependent necroptosis. *EMBO Mol Med* **5:** 105-121

Samy ET, Meyer CA, Caplazi P, Langrish CL, Lora JM, Bluethmann H, Peng SL (2007) Cutting edge: Modulation of intestinal autoimmunity and IL-2 signaling by sphingosine kinase 2 independent of sphingosine 1-phosphate. *J Immunol* **179:** 5644-5648

Sanchez T, Estrada-Hernandez T, Paik JH, Wu MT, Venkataraman K, Brinkmann V, Claffey K, Hla T (2003) Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. *J Biol Chem* **278:** 47281-47290

Sankala HM, Hait NC, Paugh SW, Shida D, Lepine S, Elmore LW, Dent P, Milstien S, Spiegel S (2007) Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin. *Cancer Res* **67:** 10466-10474

Sathyanarayana P, Barthwal MK, Kundu CN, Lane ME, Bergmann A, Tzivion G, Rana A (2002) Activation of the Drosophila MLK by ceramide reveals TNF-alpha and ceramide as agonists of mammalian MLK3. *Mol Cell* **10:** 1527-1533

Sato K, Malchinkhuu E, Horiuchi Y, Mogi C, Tomura H, Tosaka M, Yoshimoto Y, Kuwabara A, Okajima F (2007) Critical role of ABCA1 transporter in sphingosine 1-phosphate release from astrocytes. *J Neurochem* **103:** 2610-2619

Scheltens P, Blennow K, Breteler MM, de Strooper B, Frisoni GB, Salloway S, Van der Flier WM (2016) Alzheimer's disease. *Lancet* **388:** 505-517

Schiavo G, Greensmith L, Hafezparast M, Fisher EM (2013) Cytoplasmic dynein heavy chain: the servant of many masters. *Trends Neurosci* **36:** 641-651

Schmidt H, Zalyte R, Urnavicius L, Carter AP (2015) Structure of human cytoplasmic dynein-2 primed for its power stroke. *Nature* **518:** 435-438

Schmoranzer J, Fawcett JP, Segura M, Tan S, Vallee RB, Pawson T, Gundersen GG (2009) Par3 and dynein associate to regulate local microtubule dynamics and centrosome orientation during migration. *Curr Biol* **19:** 1065-1074

Schnitzer SE, Weigert A, Zhou J, Brune B (2009) Hypoxia enhances sphingosine kinase 2 activity and provokes sphingosine-1-phosphate-mediated chemoresistance in A549 lung cancer cells. *Mol Cancer Res* **7:** 393-401

Schnute ME, McReynolds MD, Carroll J, Chrencik J, Highkin MK, Iyanar K, Jerome G, Rains JW, Saabye M, Scholten JA, Yates M, Nagiec MM (2017) Discovery of a potent and selective sphingosine kinase 1 inhibitor through the molecular combination of chemotype-distinct screening hits. *J Med Chem* **60:** 2562-2572

Schnute ME, McReynolds MD, Kasten T, Yates M, Jerome G, Rains JW, Hall T, Chrencik J, Kraus M, Cronin CN, Saabye M, Highkin MK, Broadus R, Ogawa S, Cukyne K, Zawadzke LE, Peterkin V, Iyanar K, Scholten JA, Wendling J, Fujiwara H, Nemirovskiy O, Wittwer AJ, Nagiec MM (2012) Modulation of cellular S1P levels with a novel, potent and specific inhibitor of sphingosine kinase-1. *Biochem J* **444:** 79-88

Schrecengost RS, Keller SN, Schiewer MJ, Knudsen KE, Smith CD (2015) Downregulation of critical oncogenes by the selective SK2 inhibitor ABC294640 hinders prostate cancer progression. *Mol Cancer Res*

Schroer TA (2004) Dynactin. *Annu Rev Cell Dev Biol* **20:** 759-779

Screaton RA, Conkright MD, Katoh Y, Best JL, Canettieri G, Jeffries S, Guzman E, Niessen S, Yates JR, 3rd, Takemori H, Okamoto M, Montminy M (2004) The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. *Cell* **119:** 61-74

Senchenkov A, Litvak DA, Cabot MC (2001) Targeting ceramide metabolism--a strategy for overcoming drug resistance. *J Natl Cancer Inst* **93:** 347-357

Sensken SC, Bode C, Nagarajan M, Peest U, Pabst O, Graler MH (2010) Redistribution of sphingosine 1-phosphate by sphingosine kinase 2 contributes to lymphopenia. *J Immunol* **184:** 4133-4142

Sharma K, D'Souza RC, Tyanova S, Schaab C, Wisniewski JR, Cox J, Mann M (2014) Ultradeep human phosphoproteome reveals a distinct regulatory nature of Tyr and Ser/Thr-based signaling. *Cell reports* **8:** 1583-1594

Shen H, Giordano F, Wu Y, Chan J, Zhu C, Milosevic I, Wu X, Yao K, Chen B, Baumgart T, Sieburth D, De Camilli P (2014) Coupling between endocytosis and sphingosine kinase 1 recruitment. *Nat Cell Biol* **16:** 652-662

Shi YJ, Rehman H, Ramshesh VK, Schwartz J, Liu QL, Krishnasamy Y, Zhang X, Lemasters JJ, Smith CD, Zhong Z (2012) Sphingosine kinase-2 inhibition improves mitochondrial function and survival after hepatic ischemia-reperfusion. *J Hepatol* **56:** 137-145

Shiloh R, Bialik S, Kimchi A (2014) The DAPK family: a structure-function analysis. *Apoptosis* **19:** 286-297

Shirai K, Kaneshiro T, Wada M, Furuya H, Bielawski J, Hannun YA, Obeid LM, Ogretmen B, Kawamori T (2011) A role of sphingosine kinase 1 in head and neck carcinogenesis. *Cancer Prev Res (Phila)* **4:** 454-462

Sinha UK, Schorn VJ, Hochstim C, Chinn SB, Zhu S, Masood R (2011) Increased radiation sensitivity of head and neck squamous cell carcinoma with sphingosine kinase 1 inhibition. *Head & neck* **33:** 178-188

Siow D, Wattenberg B (2011) The compartmentalization and translocation of the sphingosine kinases: Mechanisms and functions in cell signaling and sphingolipid metabolism. *Crit Rev Biochem Mol Biol* **46:** 365-375

Siow DL, Anderson CD, Berdyshev EV, Skobeleva A, Natarajan V, Pitson SM, Wattenberg BW (2011) Sphingosine kinase localization in the control of sphingolipid metabolism. *Adv Enzyme Regul* **51:** 229-244

Siow DL, Anderson CD, Berdyshev EV, Skobeleva A, Pitson SM, Wattenberg BW (2010) Intracellular localization of sphingosine kinase 1 alters access to substrate pools but does not affect the degradative fate of sphingosine-1-phosphate. *J Lipid Res* **51:** 2546-2559

Siskind LJ (2005) Mitochondrial ceramide and the induction of apoptosis. *J Bioenerg Biomembr* **37:** 143-153

Snider AJ, Orr Gandy KA, Obeid LM (2010) Sphingosine kinase: Role in regulation of bioactive sphingolipid mediators in inflammation. *Biochimie* **92:** 707-715

Soldi R, Mandinova A, Venkataraman K, Hla T, Vadas M, Pitson S, Duarte M, Graziani I, Kolev V, Kacer D, Kirov A, Maciag T, Prudovsky I (2007) Sphingosine kinase 1 is a critical component of the copper-dependent FGF1 export pathway. *Exp Cell Res* **313:** 3308-3318

Sordillo LA, Sordillo PP, Helson L (2016) Sphingosine kinase inhibitors as maintenance therapy of glioblastoma after ceramide-induced response. *Anticancer Res* **36:** 2085-2095

Souza-Rodrigues E, Estanyol JM, Friedrich-Heineken E, Olmedo E, Vera J, Canela N, Brun S, Agell N, Hubscher U, Bachs O, Jaumot M (2007) Proteomic analysis of p16ink4a-binding proteins. *Proteomics* **7:** 4102-4111

Spiegel S, Milstien S (2003) Sphingosine-1-phosphate: An enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4:** 397-407

Starr DA (2009) A nuclear-envelope bridge positions nuclei and moves chromosomes. *J Cell Sci* **122:** 577-586

Stirnimann CU, Petsalaki E, Russell RB, Muller CW (2010) WD40 proteins propel cellular networks. *Trends Biochem Sci* **35:** 565-574

Strub GM, Maceyka M, Hait NC, Milstien S, Spiegel S (2010) Extracellular and intracellular actions of sphingosine-1-phosphate. *Adv Exp Med Biol* **688:** 141-155

Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, Maceyka M, Price MM, Chen Q, Simpson DC, Kordula T, Milstien S, Lesnefsky EJ, Spiegel S (2011) Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. *FASEB J* **25:** 600-612

Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* **352:** 987-996

Sun DF, Gao ZH, Liu HP, Yuan Y, Qu XJ (2012) Sphingosine 1-phosphate antagonizes the effect of all-trans retinoic acid (ATRA) in a human colon cancer cell line by modulation of RARβ expression. *Cancer Lett* **319:** 182-189

Sun E, Zhang W, Wang L, Wang A, Ma C, Lei M, Zhou X, Sun Y, Lu B, Liu L, Han R (2015) Down-regulation of Sphk2 suppresses bladder cancer progression. *Tumour Biol***:** doi:10.1007/s13277-13015-13818-z

Sun E, Zhang W, Wang L, Wang A, Ma C, Lei M, Zhou X, Sun Y, Lu B, Liu L, Han R (2016a) Down-regulation of Sphk2 suppresses bladder cancer progression. *Tumour Biol* **37:** 473-478

Sun WY, Dimasi DP, Pitman MR, Zhuang Y, Heddle R, Pitson SM, Grimbaldeston MA, Bonder CS (2016b) Topical application of Fingolimod perturbs cutaneous inflammation. *J Immunol* **196:** 3854-3864

Susalka SJ, Nikulina K, Salata MW, Vaughan PS, King SM, Vaughan KT, Pfister KK (2002) The roadblock light chain binds a novel region of the cytoplasmic dynein intermediate chain. *J Biol Chem* **277:** 32939-32946

Sutherland C (2011) What are the bona fide GSK3 substrates? *Int J Alzheimers Dis* **2011:** 505607

Taha TA, Hannun YA, Obeid LM (2006a) Sphingosine kinase: biochemical and cellular regulation and role in disease. *J Biochem Mol Biol* **39:** 113-131

Taha TA, Mullen TD, Obeid LM (2006b) A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochim Biophys Acta* **1758:** 2027-2036

Takabe K, Kim RH, Allegood JC, Mitra P, Ramachandran S, Nagahashi M, Harikumar KB, Hait NC, Milstien S, Spiegel S (2010) Estradiol induces export of sphingosine 1-phosphate from breast cancer cells via ABCC1 and ABCG2. *J Biol Chem* **285:** 10477-10486

Takabe K, Spiegel S (2014) Export of sphingosine-1-phosphate and cancer progression. *J Lipid Res* **55:** 1839-1846

Takacs CM, Baird JR, Hughes EG, Kent SS, Benchabane H, Paik R, Ahmed Y (2008) Dual positive and negative regulation of wingless signaling by adenomatous polyposis coli. *Science* **319:** 333-336

Takasugi N, Sasaki T, Suzuki K, Osawa S, Isshiki H, Hori Y, Shimada N, Higo T, Yokoshima S, Fukuyama T, Lee VMY, Trojanowski JQ, Tomita T, Iwatsubo T (2011) BACE1 activity is modulated by cell-associated sphingosine-1-phosphate. *J Neurosci* **31:** 6850-6857

Takuwa N, Ohkura S, Takashima S, Ohtani K, Okamoto Y, Tanaka T, Hirano K, Usui S, Wang F, Du W, Yoshioka K, Banno Y, Sasaki M, Ichi I, Okamura M, Sugimoto N, Mizugishi K, Nakanuma Y, Ishii I, Takamura M, Kaneko S, Kojo S, Satouchi K, Mitumori K, Chun J, Takuwa Y (2010) S1P3-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species. *Cardiovasc Res* **85:** 484-493

Tan SC, Scherer J, Vallee RB (2011) Recruitment of dynein to late endosomes and lysosomes through light intermediate chains. *Mol Biol Cell* **22:** 467-477

Tani M, Ito M, Igarashi Y (2007) Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. *Cell Signal* **19:** 229-237

Taub N, Teis D, Ebner HL, Hess MW, Huber LA (2007) Late endosomal traffic of the epidermal growth factor receptor ensures spatial and temporal fidelity of mitogen-activated protein kinase signaling. *Mol Biol Cell* **18:** 4698-4710

ter Braak M, Danneberg K, Lichte K, Liphardt K, Ktistakis NT, Pitson SM, Hla T, Jakobs KH, Meyer zu Heringdorf D (2009) Galpha(q)-mediated plasma membrane translocation of sphingosine kinase-1 and cross-activation of S1P receptors. *Biochim Biophys Acta* **1791:** 357-370

Tessier M, Woodgett JR (2006) Serum and glucocorticoid-regulated protein kinases: variations on a theme. *J Cell Biochem* **98:** 1391-1407

Toropova K, Zou S, Roberts AJ, Redwine WB, Goodman BS, Reck-Peterson SL, Leschziner AE (2014) Lis1 regulates dynein by sterically blocking its mechanochemical cycle. *Elife* **3:** e03372

Traer CJ, Rutherford AC, Palmer KJ, Wassmer T, Oakley J, Attar N, Carlton JG, Kremerskothen J, Stephens DJ, Cullen PJ (2007) SNX4 coordinates endosomal sorting of TfnR with dynein-mediated transport into the endocytic recycling compartment. *Nat Cell Biol* **9:** 1370-1380

Tsai JW, Bremner KH, Vallee RB (2007) Dual subcellular roles for LIS1 and dynein in radial neuronal migration in live brain tissue. *Nat Neurosci* **10:** 970-979

Tynan SH, Purohit A, Doxsey SJ, Vallee RB (2000) Light intermediate chain 1 defines a functional subfraction of cytoplasmic dynein which binds to pericentrin. *J Biol Chem* **275:** 32763-32768

Vadas M, Xia P, McCaughan G, Gamble J (2008) The role of sphingosine kinase 1 in cancer: oncogene or non-oncogene addiction? *Biochim Biophys Acta* **1781:** 442-447

Vale RD (2003) The molecular motor toolbox for intracellular transport. *Cell* **112:** 467-480

Van Brocklyn J, Letterle C, Snyder P, Prior T (2002) Sphingosine-1-phosphate stimulates human glioma cell proliferation through Gi-coupled receptors: role of ERK MAP kinase and phosphatidylinositol 3-kinase beta. *Cancer Lett* **181:** 195-204

Van Brocklyn JR, Jackson CA, Pearl DK, Kotur MS, Snyder PJ, Prior TW (2005) Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: Roles of sphingosine kinase isoforms in growth of glioblastoma cell lines. *J Neuropathol Exp Neurol* **64:** 695-705

Van Brocklyn JR, Young N, Roof R (2003) Sphingosine-1-phosphate stimulates motility and invasiveness of human glioblastoma multiforme cells. *Cancer Lett* **199:** 53-60

Varma D, Dawn A, Ghosh-Roy A, Weil SJ, Ori-McKenney KM, Zhao Y, Keen J, Vallee RB, Williams JC (2010) Development and application of in vivo molecular traps reveals

that dynein light chain occupancy differentially affects dynein-mediated processes. *Proc Natl Acad Sci U S A* **107:** 3493-3498

Vaughan KT, Vallee RB (1995) Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150Glued. *J Cell Biol* **131:** 1507-1516

Vaughan PS, Leszyk JD, Vaughan KT (2001) Cytoplasmic dynein intermediate chain phosphorylation regulates binding to dynactin. *J Biol Chem* **276:** 26171-26179

Venant H, Rahmaniyan M, Jones EE, Lu P, Lilly MB, Garrett-Mayer E, Drake RR, Kraveka JM, Smith CD, Voelkel-Johnson C (2015) The sphingosine kinase 2 inhibitor ABC294640 reduces the growth of prostate cancer cells and results in accumulation of dihydroceramides in vitro and in vivo. *Mol Cancer Ther* **14:** 2744-2752

Venkata JK, An N, Stuart R, Costa LJ, Cai H, Coker W, Song JH, Gibbs K, Matson T, Garrett-Mayer E, Wan Z, Ogretmen B, Smith C, Kang Y (2014) Inhibition of sphingosine kinase 2 downregulates the expression of c-Myc and Mcl-1 and induces apoptosis in multiple myeloma. *Blood* **124:** 1915-1925

Venkataraman K, Thangada S, Michaud J, Oo ML, Ai Y, Lee YM, Wu M, Parikh NS, Khan F, Proia RL, Hla T (2006) Extracellular export of sphingosine kinase-1a contributes to the vascular S1P gradient. *Biochem J* **397:** 461-471

Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, Alexe G, Lawrence M, O'Kelly M, Tamayo P, Weir BA, Gabriel S, Winckler W, Gupta S, Jakkula L, Feiler HS, Hodgson JG, James CD, Sarkaria JN, Brennan C, Kahn A, Spellman PT, Wilson RK, Speed TP, Gray JW, Meyerson M, Getz G, Perou CM, Hayes DN (2010) Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* **17:** 98-110

Vessey DA, Li L, Jin Z-Q, Kelley M, Honbo N, Zhang J, Karliner JS (2011) A sphingosine kinase form 2 knockout sensitizes mouse myocardium to ischemia/reoxygenation injury and diminishes responsiveness to ischemic preconditioning. *Oxid Med Cell Longev* **2011:** doi:10.1155/2011/961059

Wacker BK, Park TS, Gidday JM (2009) Hypoxic preconditioning-induced cerebral ischemic tolerance role of microvascular sphingosine kinase 2. *Stroke* **40:** 3342-3348

Wallington-Beddoe CT, Powell JA, Tong D, Pitson SM, Bradstock KF, Bendall LJ (2014) Sphingosine kinase 2 promotes acute lymphoblastic leukemia by enhancing MYC expression. *Cancer Res* **74:** 2803-2815

Walter J, Fluhrer R, Hartung B, Willem M, Kaether C, Capell A, Lammich S, Multhaup G, Haass C (2001) Phosphorylation regulates intracellular trafficking of beta-secretase. *J Biol Chem* **276:** 14634-14641

Wang G, Silva J, Krishnamurthy K, Tran E, Condie BG, Bieberich E (2005) Direct binding to ceramide activates protein kinase Czeta before the formation of a pro-apoptotic complex with PAR-4 in differentiating stem cells. *J Biol Chem* **280:** 26415-26424

Wang H, Feng W, Lu Y, Li H, Xiang W, Chen Z, He M, Zhao L, Sun X, Lei B, Qi S, Liu Y (2016) Expression of dynein, cytoplasmic 2, heavy chain 1 (DHC2) associated with glioblastoma cell resistance to temozolomide. *Sci Rep* **6:** 28948

Wang J, Knapp S, Pyne NJ, Pyne S, Elkins JM (2014a) Crystal structure of sphingosine kinase 1 with PF-543. *ACS Med Chem Lett* **5:** 1329-1333

Wang Q, Li J, Li G, Li Y, Xu C, Li M, Xu G, Fu S (2014b) Prognostic significance of sphingosine kinase 2 expression in non-small cell lung cancer. *Tumour Biol* **35:** 363-368

Wang Y, Jin F, Higgins R, McKnight K (2014c) The current view for the silencing of the spindle assembly checkpoint. *Cell Cycle* **13:** 1694-1701

Wang Z, Min X, Xiao SH, Johnstone S, Romanow W, Meininger D, Xu H, Liu J, Dai J, An S, Thibault S, Walker N (2013) Molecular basis of sphingosine kinase 1 substrate recognition and catalysis. *Structure* **21:** 798-809

Waters C, Sambi B, Kong KC, Thompson D, Pitson SM, Pyne S, Pyne NJ (2003) Sphingosine 1-phosphate and platelet-derived growth factor (PDGF) act via PDGF beta receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J Biol Chem* **278:** 6282-6290

Wattenberg BW (2010) Role of sphingosine kinase localization in sphingolipid signaling. *World J Biol Chem* **1:** 362-368

Weigert A, Cremer S, Schmidt MV, von Knethen A, Angioni C, Geisslinger G, Brune B (2010) Cleavage of sphingosine kinase 2 by caspase-1 provokes its release from apoptotic cells. *Blood* **115:** 3531-3540

Weigert A, Schiffmann S, Sekar D, Ley S, Menrad H, Werno C, Grosch S, Geisslinger G, Brune B (2009) Sphingosine kinase 2 deficient tumor xenografts show impaired growth and fail to polarize macrophages towards an anti-inflammatory phenotype. *Int J Cancer* **125:** 2114-2121

Whyte J, Bader JR, Tauhata SB, Raycroft M, Hornick J, Pfister KK, Lane WS, Chan GK, Hinchcliffe EH, Vaughan PS, Vaughan KT (2008) Phosphorylation regulates targeting of cytoplasmic dynein to kinetochores during mitosis. *J Cell Biol* **183:** 819-834

Wickstead B, Gull K (2007) Dyneins across eukaryotes: a comparative genomic analysis. *Traffic* **8:** 1708-1721

Williams JC, Roulhac PL, Roy AG, Vallee RB, Fitzgerald MC, Hendrickson WA (2007) Structural and thermodynamic characterization of a cytoplasmic dynein light chain-intermediate chain complex. *Proc Natl Acad Sci U S A* **104:** 10028-10033

Woodcock JM, Ma Y, Coolen C, Pham D, Jones C, Lopez AF, Pitson SM (2010) Sphingosine and FTY720 directly bind pro-survival 14-3-3 proteins to regulate their function. *Cell Signal* **22:** 1291-1299

Xia P, Gamble JR, Rye KA, Wang L, Hii CS, Cockerill P, Khew-Goodall Y, Bert AG, Barter PJ, Vadas MA (1998) Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway. *Proc Natl Acad Sci U S A* **95:** 14196-14201

Xia P, Gamble JR, Wang L, Pitson SM, Moretti PA, Wattenberg BW, D'Andrea RJ, Vadas MA (2000) An oncogenic role of sphingosine kinase. *Curr Biol* **10:** 1527-1530

Xiong Y, Lee HJ, Mariko B, Lu YC, Dannenberg AJ, Haka AS, Maxfield FR, Camerer E, Proia RL, Hla T (2013) Sphingosine kinases are not required for inflammatory responses in macrophages. *J Biol Chem* **288:** 32563-32573

Xun C, Chen MB, Qi L, Tie-Ning Z, Peng X, Ning L, Zhi-Xiao C, Li-Wei W (2015) Targeting sphingosine kinase 2 (SphK2) by ABC294640 inhibits colorectal cancer cell growth in vitro and in vivo. *J Exp Clin Cancer Res* **34:** 94

Yamada M, Toba S, Takitoh T, Yoshida Y, Mori D, Nakamura T, Iwane AH, Yanagida T, Imai H, Yu-Lee LY, Schroer T, Wynshaw-Boris A, Hirotsune S (2010) mNUDC is required for plus-end-directed transport of cytoplasmic dynein and dynactins by kinesin-1. *EMBO J* **29:** 517-531

Yamamoto H, Kishida S, Kishida M, Ikeda S, Takada S, Kikuchi A (1999) Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability. *J Biol Chem* **274:** 10681-10684

Yang Z, Tulu US, Wadsworth P, Rieder CL (2007) Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *Curr Biol* **17:** 973-980

Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N, Satoh K, Ozaki Y, Kume S (1997) Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J Biochem* **121:** 969-973

Ye X, Cai Q (2014) Snapin-mediated BACE1 retrograde transport is essential for its degradation in lysosomes and regulation of APP processing in neurons. *Cell reports* **6:** 24-31

Yokota S, Taniguchi Y, Kihara A, Mitsutake S, Igarashi Y (2004) Asp177 in C4 domain of mouse sphingosine kinase 1a is important for the sphingosine recognition. *FEBS Lett* **578:** 106-110

Yoshida H, Goedert M (2012) Phosphorylation of microtubule-associated protein tau by AMPK-related kinases. *J Neurochem* **120:** 165-176

Yoshimoto T, Furuhata M, Kamiya S, Hisada M, Miyaji H, Magami Y, Yamamoto K, Fujiwara H, Mizuguchi J (2003) Positive modulation of IL-12 signaling by sphingosine kinase 2 associating with the IL-12 receptor beta 1 cytoplasmic region. *J Immunol* **171:** 1352-1359

Young MM, Takahashi Y, Fox TE, Yun JK, Kester M, Wang HG (2016) Sphingosine kinase 1 cooperates with autophagy to maintain endocytic membrane trafficking. *Cell reports* **17:** 1532-1545

Yung LM, Wei Y, Qin T, Wang YM, Smith CD, Waeber C (2012) Sphingosine kinase 2 mediates cerebral preconditioning and protects the mouse brain against ischemic injury. *Stroke* **43:** 199-204

Zemann B, Kinzel B, Muller M, Reuschel R, Mechtcheriakova D, Urtz N, Bornancin F, Baumruker T, Billich A (2006) Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. *Blood* **107:** 1454-1458

Zemann B, Urtz N, Reuschel R, Mechtcheriakova D, Bornancin F, Badegruber R, Baumruker T, Billich A (2007) Normal neutrophil functions in sphingosine kinase type 1 and 2 knockout mice. *Immunol Lett* **109:** 56-63

Zhang J, Han G, Xiang X (2002) Cytoplasmic dynein intermediate chain and heavy chain are dependent upon each other for microtubule end localization in Aspergillus nidulans. *Mol Microbiol* **44:** 381-392

Zhang J, Twelvetrees AE, Lazarus JE, Blasier KR, Yao X, Inamdar NA, Holzbaur EL, Pfister KK, Xiang X (2013) Establishing a novel knock-in mouse line for studying neuronal cytoplasmic dynein under normal and pathologic conditions. *Cytoskeleton (Hoboken)* **70:** 215-227

Zhang K, Chen H, Wu G, Chen K, Yang H (2014) High expression of SPHK1 in sacral chordoma and association with patients' poor prognosis. *Med Oncol* **31:** 247

Zhang L, Liu X, Zuo Z, Hao C, Ma Y (2016) Sphingosine kinase 2 promotes colorectal cancer cell proliferation and invasion by enhancing MYC expression. *Tumour Biol***:** doi:10.1007/s13277-13015-14700-13278

Zhang Y, Yao B, Delikat S, Bayoumy S, Lin XH, Basu S, McGinley M, Chan-Hui PY, Lichenstein H, Kolesnick R (1997) Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell* **89:** 63-72

Zhu W, Gliddon BL, Jarman KE, Moretti PA, Tin T, Parise LV, Woodcock JM, Powell JA, Ruszkiewicz A, Pitman MR, Pitson SM (2017) CIB1 contributes to oncogenic signalling by Ras via modulating the subcellular localisation of sphingosine kinase 1. *Oncogene***:** doi: 10.1038/onc.2016.1428 [in press]

Appendix 1

Roles, regulation and inhibitors of sphingosine kinase 2

Heidi A Neubauer[1,2] and Stuart M Pitson[1,2,3]

FEBS Journal (2013) **280:** 5317-5336

[1]Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, South Australia, Australia;
[2]School of Biological Sciences, University of Adelaide, Adelaide, South Australia, Australia;
[3]School of Medicine, University of Adelaide, Adelaide, South Australia, Australia;

Statement of Authorship

Title of Paper	Roles, regulation and inhibitors of sphingosine kinase 2
Publication Status	Published
Publication Details	Neubauer HA, and Pitson SM (2013) Roles, regulation and inhibitors of sphingosine kinase 2. *FEBS Journal* **280:** 5317-5336

Principal Author

Name of Principal Author (Candidate)	Heidi A Neubauer		
Contribution to the Paper	Wrote the first draft of the manuscript and assisted in preparation of the figures.		
Overall percentage (%)	80%		
Signature		Date	4/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stuart M Pitson		
Contribution to the Paper	Assisted in the preparation and editing of the manuscript.		
Signature		Date	4/5/17

the FEBS Journal
REVIEW ARTICLE

Roles, regulation and inhibitors of sphingosine kinase 2

Heidi A. Neubauer[1,2] and Stuart M. Pitson[1,2,3]

1 Centre for Cancer Biology, SA Pathology, Adelaide, Australia
2 School of Molecular and Biomedical Science, University of Adelaide, Australia
3 School of Pharmacy and Medical Science, University of South Australia, Adelaide, Australia

Keywords
apoptosis; cancer; inhibitors; phosphorylation; sphingolipids; sphingosine kinase 2; sphingosine-1-phosphate; subcellular localization

Correspondence
S. M. Pitson, Centre for Cancer Biology, SA Pathology, Frome Road, Adelaide, SA 5000, Australia
Fax: +61 8 8232 4092
Tel: +61 8 8222 3472
E-mail: stuart.pitson@health.sa.gov.au
Website: www.centreforcancerbiology.org.au

(Received 27 March 2013, revised 29 April 2013, accepted 29 April 2013)

doi:10.1111/febs.12314

The bioactive sphingolipids ceramide, sphingosine and sphingosine-1-phosphate (S1P) are important signalling molecules that regulate a diverse array of cellular processes. Most notably, the balance of the levels of these three sphingolipids in cells, termed the 'sphingolipid rheostat', can dictate cell fate, where ceramide and sphingosine enhance apoptosis and S1P promotes cell survival and proliferation. The sphingosine kinases (SKs) catalyse the production of S1P from sphingosine and are therefore central regulators of the sphingolipid rheostat and attractive targets for cancer therapy. Two SKs exist in humans: SK1 and SK2. SK1 has been extensively studied and there is a large body of evidence to demonstrate its role in promoting cell survival, proliferation and neoplastic transformation. SK1 is also elevated in many human cancers which appears to contribute to carcinogenesis, chemotherapeutic resistance and poor patient outcome. SK2, however, has not been as well characterized, and there are contradictions in the key physiological functions that have been proposed for this isoform. Despite this, many studies are now emerging that implicate SK2 in key roles in a variety of diseases, including the development of a range of solid tumours. Here, we review the literature examining SK2, its physiological and pathophysiological functions, the current knowledge of its regulation, and recent developments in targeting this complex enzyme.

Introduction

Sphingolipids have emerged as important signalling molecules that can regulate a vast range of cellular processes, including cell survival, proliferation, migration, differentiation and inflammation [1–3]. Although many enzymes are involved in regulating the relative levels of the sphingolipids, the sphingosine kinases (SKs) SK1 and SK2 are of particular interest as their activity can be dynamically regulated by external stimuli [4]. As a result, altering the levels or activity of the SKs can play a key role in controlling cell fate, making them attractive targets in the development of cancer therapeutics [4–9]. Indeed, given its unequivocal role in promoting cell survival and proliferation, targeting SK1 has proved effective in attenuating tumour growth and progression in a range of cancer models (reviewed in [5]). However, investigating the

Abbreviations
dhS1P, dihydro-sphingosine-1-phosphate; dhSph, dihydrosphingosine; EGF, epidermal growth factor; ER, endoplasmic reticulum; ERK1/2, extracellular-signal-regulated kinases 1/2; GPCR, G-protein coupled receptor; HDAC, histone deacetylase; IBD, inflammatory bowel disease; IFN, interferon; IL, interleukin; IR, ischemia-reperfusion; NES, nuclear export signal; NF-κB, nuclear factor κB; NLS, nuclear localization signal; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C; $S1P_{1-5}$, S1P receptors 1–5; S1P, sphingosine-1-phosphate; siRNA, small interfering RNA; SK, sphingosine kinase; TGF, transforming growth factor; TNF, tumour necrosis factor; TRAF2, TNF receptor-associated factor 2.

therapeutic potential of targeting SK2 in cancer has only very recently gained momentum, and although the findings are promising, the mechanisms behind SK2 function and regulation remain poorly understood. Indeed, the current literature focuses largely on SK1, and although the exact cellular roles of SK2 are yet to be deciphered, what is currently known about this isoform suggests that its functions and mechanisms of regulation are extremely complex. Here, we review what is currently known about SK2 function and regulation in both physiological and pathophysiological settings, and the recent developments made in targeting this SK isoform.

Sphingosine-1-phosphate (S1P) and sphingolipid signalling

S1P is a biologically active phospholipid that is derived from a network of sphingolipid synthesis and degradation pathways [4]. Ceramide resides at the centre of this network and can be deacylated by ceramidase to form sphingosine, which can in turn be phosphorylated by the SKs to produce S1P (Fig. 1). The aforementioned reactions are reversible and the only exit point from the cycle is the irreversible degradation of S1P to hexadecenal and phosphoethanolamine. S1P acts as both an extracellular and intracellular signalling molecule through a range of different pathways [10]. It can be transported out of the cell where it exists in high nanomolar concentrations in the blood [11,12]. This export is mediated by members of the ABC transporter family [13,14] and, more recently noted, by spinster homolog 2 [15]. Upon exit from the cell, S1P can bind to and activate a family of five G-protein coupled receptors (GPCRs), referred to as $S1P_{1–5}$ [16], to elicit autocrine or paracrine signalling. Interestingly, recent evidence suggests that S1P may engage its GPCRs by lateral diffusion within the outer leaflet of the plasma membrane rather than directly from the extracellular space [17]. The S1P receptors are coupled to various heterotrimeric G proteins, which subsequently activate a number of downstream signalling pathways via the modulation of extracellular-signal-regulated kinases 1/2 (ERK1/2), small GTPases (Rac and Rho), the phosphoinositide 3-kinase (PI3K)/AKT pathway and phospholipase C (PLC), ultimately modulating cell proliferation, survival and migration [6,18]. These responses can vary in a cell- or tissue-specific manner as a result of the differential expression of the S1P receptors and the various G proteins they couple to [19].

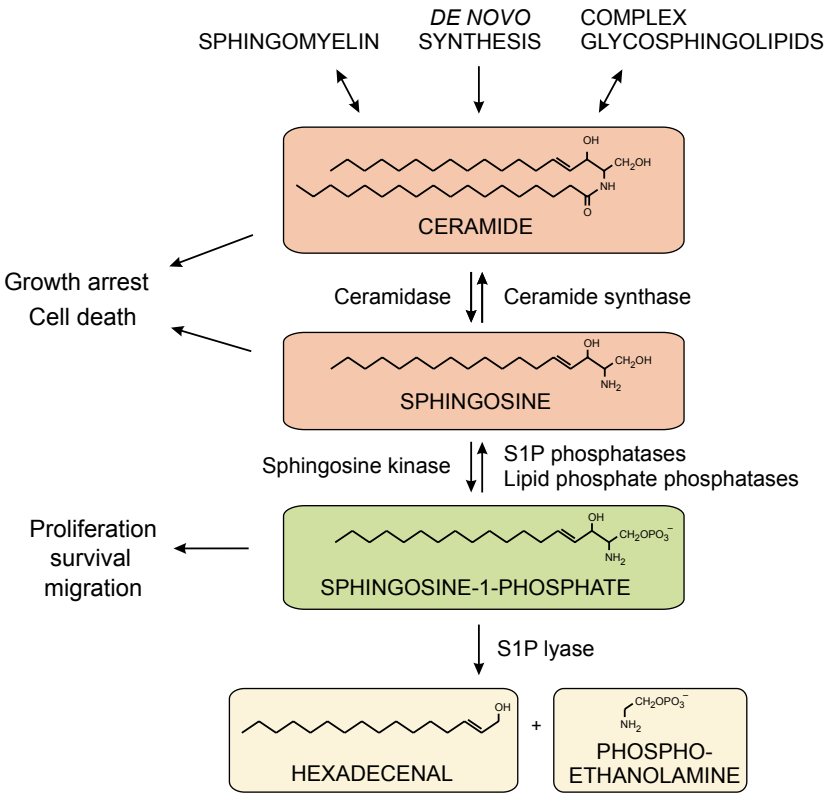


Fig. 1. Sphingolipid synthesis and degradation pathways. Sphingolipid structures are shown with the key enzymes that catalyse their synthesis and degradation.

In addition to its extracellular signalling roles, S1P can also act directly on a number of intracellular targets. Specifically, S1P has been shown to directly inhibit histone deacetylase (HDAC) 1/2 activity [20], activate the E3 ubiquitin ligase activity of tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2) [21], interact with prohibitin 2 to mediate mitochondrial respiration [22] and possibly modulate the activity of p21-activated kinase 1 [23]. A recent study also suggested that S1P may contribute to cytochrome *c* release from mitochondria via modulation of BAK, although the direct interaction of S1P with BAK was not described [24]. Through the activation of these varied signalling pathways, S1P can act to promote cell survival, proliferation and migration, as well as regulate differentiation, angiogenesis and inflammation [6,25,26]. Interestingly, an intracellular role for the $S1P_5$ GPCR has also been proposed, where the colocalization of the receptor and SKs to centrosomes may indicate a novel function in regulating cell division [27].

In contrast to S1P, the sphingolipids ceramide and sphingosine, which both lie directly upstream of S1P in the sphingolipid biosynthetic pathway (Fig. 1), are promoters of apoptosis. Ceramide has been found to directly activate a number of protein targets that appear to mediate its pro-apoptotic functions, including protein phosphatases 1 [28], 2A [28] and 2C [29], protein kinase C (PKC) ζ [30,31], the kinase suppressor of ras [32] and cathepsin D [33]. Furthermore, sphingosine can bind to and initiate the phosphorylation and inactivation of the pro-survival adaptor protein 14-3-3 [34]. Given these opposing functions, the current dogma is that a delicate balance must be maintained between the relative levels of pro-apoptotic ceramide and sphingosine and pro-survival S1P, with this equilibrium, referred to as the 'sphingolipid rheostat', playing an important role in dictating cell fate [6,35]. SK is a central regulator of this equilibrium, residing at the critical junction between pro-proliferative, pro-survival S1P and pro-apoptotic sphingosine and ceramide (Fig. 1). Therefore, understanding how SKs are regulated is important to understand their role not only in controlling cell fate but also in aberrant survival and proliferative signalling in disease states like cancer. Indeed, there are a multitude of studies that implicate S1P and the SKs in cancer development, survival and metastasis, and this field has been extensively reviewed [4–9,36,37].

Sphingosine kinases

Two SKs exist in mammalian cells, namely SK1 and SK2. In humans they are generated from two distinct genes, *SPHK1* and *SPHK2*, which are located on chromosome 17 (17q25.2) and 19 (19q13.2), respectively. Although human SK1 and SK2 vary considerably in size (384 and 618 amino acids for SK1a and SK2a, respectively) [38], they share 80% similarity and 45% overall sequence identity, with almost all of the polypeptide sequence of SK1 aligning with regions of the larger SK2. All known eukaryotic SKs share five highly conserved regions within their sequence (termed C1–C5; see Fig. 2), which appear to encompass the regions necessary for ATP binding and catalysis [39]. The additional residues present in SK2 result from both its extended N-terminus and an additional central proline-rich region not found in SK1 or in any other protein (Fig. 2). While there are currently no crystal structures available to allow for comparisons between the two SKs, the central proline-rich region of SK2 appears to coincide with the sphingosine binding region of these enzymes [4]. Consistent with this, biochemical information regarding substrate specificity and inhibition of the two SKs suggests that significant differences exist in their substrate binding pockets. As discussed below, SK2 is more promiscuous than SK1 in the substrates it can phosphorylate, and several isoform-selective inhibitors that target the sphingosine binding site of these enzymes have been developed [47,48]. Surprisingly, due to the high degree of sequence conservation in the putative ATP binding regions, a recent study has also reported an ATP competitive inhibitor that shows high selectivity for SK1 [47], suggesting that at least some divergence also occurs between SK1 and SK2 in this pocket. Experimental evidence has demonstrated that the single point mutant G212E completely abolishes SK2 catalytic activity [49] just like the comparable G82D mutation in SK1 [50], via interfering with ATP binding [51].

While the activity of both SK isoforms can be enhanced by various cytokines and growth factors (see below), both enzymes also possess intrinsic catalytic activity independent of eukaryotic post-translational modifications that results in cellular SK activity even in the absence of agonist stimulation [52–54]. This basal SK activity has been proposed to facilitate a housekeeping role in maintaining cellular sphingosine and ceramide levels [55].

Although both SK isoforms are ubiquitously expressed in all human tissues, some differential expression is apparent, with SK1 most highly expressed in lung, spleen and leukocyte [56] whereas SK2 is highest in the kidney and liver [38]. Furthermore, while both SKs are expressed throughout embryonic development in the mouse, SK1 expression

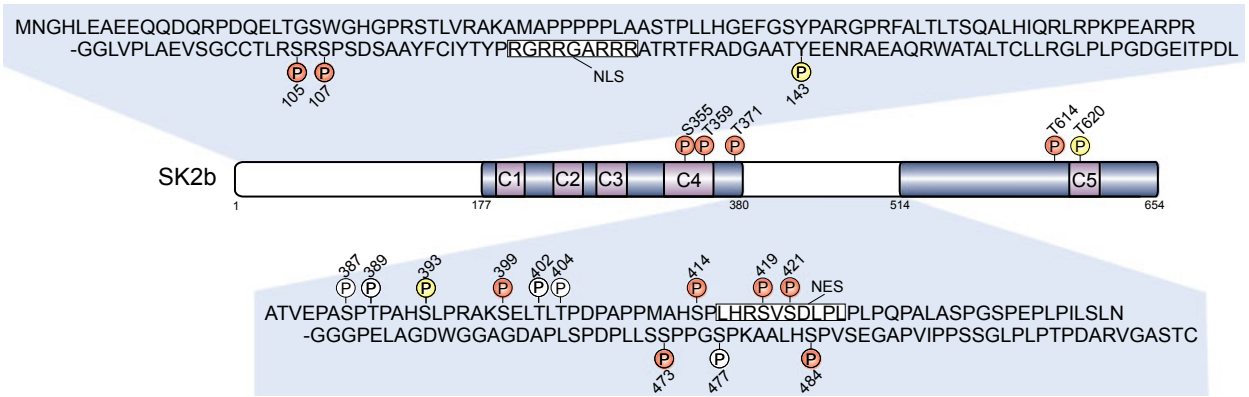


Fig. 2. SK2 post-translational modifications and regulatory domains. SK2 contains two unique regions within its sequence not conserved in SK1, or in any other protein. These regions, the N-terminus and the central proline-rich region (white), have been expanded to show the amino acid sequence. Shaded regions represent sequences that are highly conserved in both SK1 and SK2, including the five evolutionarily conserved regions C1–C5. The SK2b isoform is shown here. SK2 possesses an NLS within the N-terminus and an NES within the central region. A lipid binding domain also spans the N-terminus, allowing SK2 to interact with phosphoinositides. Data from direct analysis of SK2 or from mining of global phosphoproteomic studies and the PhosphositePlus resource [40] identified a number of phosphorylation sites that have been detected on SK2. Phosphorylation events detected in human SK2 where the site is conserved in mouse or rat SK2 sequence are shown in red, while those not conserved in rodent SK2 are shown in white. Phosphorylation events detected in mouse or rat SK2 where the site is conserved in the human sequence are shown in yellow. Ser355, Thr359 and Thr371 were identified in untreated HeLa cells [40]. Ser105 and Ser107 were identified in HeLa cells treated with rapamycin and EGF [41]. Ser387, Ser389, Thr404, Ser414 and Ser484 were identified in HeLa cells treated with double thymidine block (G_1- or S-phase) or nocodazole (M-phase) [42,43]. Ser399, Ser414, Ser473 and Ser477 were identified in HEK293 cells stably transfected with angiotensin II (Ang II) type 1 receptor treated with Ang II ligand [44]. Thr402 was identified in Jurkat cells treated with calyculin and pervanadate [40]. Ser419, Ser421, Ser387 and Thr614 were identified by direct analysis of overexpressed SK2 and validated by mutagenesis [45,46].

peaks earlier (E7–E11), with SK2 being more strongly expressed in the later stages (E15–E17) [38].

Roles and regulation of SK1

Of the two SKs, SK1 is by far the most well studied. SK1 has been widely described as a pro-survival, pro-proliferative enzyme, demonstrated initially by studies showing that overexpression of SK1 increases cell survival and proliferation [57] and induces neoplastic transformation [58]. Numerous studies have implicated SK1 in cancer development and progression, where expression levels of SK1 are found to be upregulated in a number of human solid tumours [59–65] and higher SK1 expression is correlated with poor patient prognosis [62,63,66–72]. Notably, targeting SK1 by chemical inhibition or genetic ablation has successfully reduced tumour growth in mice [59,71,73–80]. The oncogenic signalling mediated by SK1 is dependent upon its activation and translocation to the plasma membrane [81]. SK1 is a cytoplasmic protein that, upon agonist stimulation, can be phosphorylated by ERK1/2 at Ser225, which results in a 14-fold increase in catalytic activity and also facilitates the transport of SK1 to the plasma membrane via binding to calcium- and integrin-binding protein 1 [82,83]. Interestingly, other phosphorylation-independent mechanisms of SK1 translocation to the plasma membrane have also been documented [84,85]. At this location, SK1 catalyses the formation of S1P from plasma-membrane-associated sphingosine, which appears to facilitate both the efficient release of S1P to act extracellularly on cell surface S1P receptors and the interaction with intracellular targets to mediate downstream signalling and promote cell survival, proliferation, migration, differentiation, angiogenesis and inflammation [2,3,6].

Roles of SK2

SK2-induced apoptosis and growth arrest

Contrary to the roles of SK1 in pro-proliferative, pro-survival signalling, many of the early studies examining SK2 function found that its overexpression induced cell cycle arrest and apoptosis [49,86,87]. A putative BH3 domain was identified within SK2, which has been proposed to mediate its pro-apoptotic functions, at least in part, through interaction with Bcl-x_L, a pro-survival Bcl-2 family member [88]. Since this

SK2–Bcl-x_L interaction was only demonstrated following SK2 overexpression, however, the physiological significance of the proposed association remains unclear. More recently, mitochondrial localization of SK2, and specifically S1P generation at this site, was shown to contribute to BID-mediated activation of BAK, and subsequent mitochondrial membrane permeabilization and cytochrome *c* release [24]. Another recent study also demonstrated that nuclear S1P and dihydro-S1P produced specifically by SK2 inhibited HDAC1/2 activity, leading to increased histone acetylation at distinct promoters and resulting in enhanced transcription of cyclin-dependent kinase inhibitor p21 and transcriptional regulator c-fos [20]. While this is likely to contribute to the growth arrest previously associated with SK2, the notion that SK2 can act as an epigenetic and transcriptional regulator suggests that there may be more downstream effects of its nuclear activity yet to be elucidated.

While a number of the initial studies describing pro-apoptotic effects of SK2 are based on data from forced overexpression of this protein, several studies also suggest this role for endogenous SK2. For example, small interfering RNA (siRNA) mediated knockdown of endogenous SK2 in HEK293 or mouse embryonic fibroblasts prevented the induction of apoptosis by TNF-α [24,86], while mesangial cells taken from $SK2^{-/-}$ mice displayed greater resistance to staurosporine-induced apoptosis than wild-type or $SK1^{-/-}$ cells [89]. Thus, these studies support the notion that SK2 can have an opposite role to SK1 in the control of cell survival. Indeed, the tissue distribution and developmental expression of the SKs would also suggest that they may have differing roles.

Interestingly, although there is compelling evidence to suggest that SK2 can have a physiological role in mediating apoptosis, there are now many studies also supporting a role for SK2 in promoting survival and proliferation, much like SK1. siRNA-mediated knockdown of SK2 has been shown to enhance apoptosis and decrease chemotherapeutic resistance in a number of cancer cell types [63,90–92]. Interestingly, a recent study also proposed that nuclear S1P, produced by SK2, can act as an antagonist to the retinoic acid receptor β, attenuating ligand-stimulated tumour suppressor effects of this nuclear receptor in human colon carcinoma cells [93]. This is an intriguing concept, as nuclear-localized SK2 and S1P have been shown in a number of other studies to have exclusively anti-proliferative roles [20,86,87].

Notably, there appears to be at least some functional redundancy between the two enzymes as deletion of either *Sphk1* or *Sphk2* in mice produces no gross phenotype [94–96], and yet a double knockout of the two genes is embryonic lethal from defects in neurological and vascular development [96].

SK2 in cancer

Despite the apparent roles, in some situations, of SK2 in inducing apoptosis and growth arrest described above, a number of studies have emerged that demonstrate a role for SK2 in cancer promotion, similar to SK1. Notably, targeting SK2 in a range of cancer cell lines can have more of an anti-cancer effect than targeting SK1 [63,97]. A number of *in vivo* studies have reported that tumour growth can be significantly attenuated following the genetic ablation of SK2 in MCF-7 breast tumour xenografts [98] or the pharmacological inhibition of SK2 in a range of mouse tumour models, including breast [48,99,100], kidney [101], pancreatic [101], liver [102] and colon cancer [103]. Furthermore, SK2 was shown to play a role in transforming growth factor (TGF) β induced migration of oesophageal cancer cells [104] and epidermal growth factor (EGF) induced migration of breast cancer cells [105], suggesting a potential role for SK2 in metastasis.

SK2 in inflammation and immune cell regulation

The role of SK in inflammation and immune cell function has been widely investigated, with most studies focusing on SK1 and its role in promoting inflammation. SK1 can be both post-translationally activated and transcriptionally upregulated by a number of inflammatory signalling molecules such as TNF-α, interleukin (IL)-1β, interferon (IFN)-γ, IgE and C5a [106–108], and is shown to regulate monocyte, macrophage and neutrophil function during the inflammatory response [108]. However, despite SK1 having a clear role in promoting/enhancing inflammation, the role of SK2 in the inflammatory response is controversial, with many of the studies suggesting that SK2 may in fact be anti-inflammatory. It was demonstrated, using a breast cancer xenograft model, that SK2-deficient MCF-7 cells had increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory IL-10, which coincided with a decrease in tumour growth [98]. Furthermore, unlike SK1, siRNA-mediated knockdown of SK2 in a murine collagen-induced arthritis model led to more aggressive disease and production of pro-inflammatory cytokines [109]. Notably, Samy *et al.* employed an adoptive transfer model of inflammatory bowel disease (IBD) where T-cell-deficient C.B-17 *scid* mice were injected

with $SK2^{-/-}$ T cells, and found that mice receiving these cells had increased levels of pro-inflammatory cytokines and worsened intestinal inflammation than those receiving wild-type T cells, seemingly due to enhanced IL-2 responsiveness and increased expression of activated pSTAT5 [110]. Therefore, SK2 may play a role in negatively regulating IL-2 signalling by attenuating STAT5 activation.

In contrast to the studies described above, the pharmacological inhibition of SK2 demonstrated anti-inflammatory effects in murine IBD models of ulcerative colitis [111] and Crohn's disease [112] and in rodent models of inflammatory arthritis [113], suggesting that SK2 can promote inflammation. However, it is intriguing that opposite effects are observed only with the pharmacological inhibition of SK2 compared with genetic ablation and RNA interference, suggesting either that the loss of SK2 protein elicits a different effect in comparison with its inhibition or that the SK2-specific inhibitor may be affecting different pathways.

It also appears that SK2 may have opposite roles to SK1 in mast cell function. Upon IgE-mediated cross-linking of the FcεRI receptor, the Src family kinases Lyn and Fyn facilitate the activation and translocation of the SKs to the plasma membrane [114]. In murine mast cells, SK2 has emerged as the major producer of intracellular S1P and, unlike SK1, appears to mediate calcium influx and initiate downstream activation of PKCα, PKCβ and nuclear factor κB (NF-κB), leading to degranulation and the production of eicosanoids and cytokines [115]. However, in human mast cells SK1 appears to be more important, initiating degranulation, migration and cytokine production, with the roles of SK2 seemingly limited to the production of TNF-α and IL-6 [116]. This variation in SK function between species is intriguing, in particular as S1P strongly induces degranulation of human mast cells but only weakly in murine mast cells [115,116]. It has therefore been suggested that SK2 may function as an intrinsic regulator of mast cell responses, independent of the S1P receptors, whereas SK1, which is largely responsible for the production of circulating S1P, may regulate extrinsic mast cell responsiveness [115,116]. Indeed, sphingosine is reported to inhibit calcium influx [117,118] and so SK2 may function, at least in murine mast cells, by decreasing the intracellular levels of sphingosine, thus allowing for calcium entry and the subsequent activation of downstream signalling pathways.

S1P plays an important role in immune cell function by regulating lymphocyte egress from lymphoid tissues, and it appears that SK2 may mediate this response by regulating the transport and circulation of S1P between tissues. A study by Sensken *et al*. demonstrated that the uptake of blood-borne S1P into peripheral tissues is SK2-dependent, and suggested that intracellular SK2 may play a role in importing S1P into cells and directing it to S1P lyase for degradation and maintenance of an S1P gradient [12]. In agreement, $SK2^{-/-}$ mice resist lymphopenia induced in wild-type mice by the inhibition of S1P lyase in lymphoid tissues [12]. This is an intriguing concept, particularly as a number of groups have reported that $SK2^{-/-}$ mice, surprisingly, have significantly increased levels of plasma S1P compared with wild-type mice [12,115,119–121]. Another obvious explanation for this phenomenon is that SK1 may be upregulated as a compensatory mechanism for SK2 ablation, and indeed this has been examined with somewhat conflicting results. Some studies have found no differences in SK1 mRNA levels or activity in $SK2^{-/-}$ mice compared with wild-type mice [12,120]. Conversely, a recent study found increased SK1 mRNA and protein levels in $SK2^{-/-}$ mice [121], which was proposed to arise from a compensatory mechanism involving a reduction of HDAC1/2 inhibition by SK2, leading to a decrease in histone acetylation and an indirect transcriptional upregulation of SK1 [20,121]. Notably, however, transgenic mice with ubiquitous overexpression of SK1 (approximately 20-fold over endogenous) did not show a significant increase in blood S1P levels [122].

Interestingly, murine SK2 was found to interact with the cytoplasmic region of the murine IL-12 receptor β1, mediating downstream IL-12 signalling and production of IFN-γ [123]. It still remains to be elucidated whether this interaction and function of SK2 also occurs in humans.

SK2 in other diseases

SK2 also appears to be influential in a number of other disease states. A recent study demonstrated that the inhibition or downregulation of SK2 resulted in decreased proteolytic activity of BACE1, the rate-limiting enzyme for amyloid-β peptide production [124]. Targeting SK1 also had the same effect, although the role of SK2 in this process appeared more prominent. Interestingly, SK2 activity was also found to be upregulated in the brain of Alzheimer's disease patients, suggesting a role for SK2 in this disease [124].

There are conflicting reports on the role of SK2 in ischaemia-reperfusion (IR) injury, although generally it appears to play a protective role. The genetic deletion of SK2, but not SK1, in mice significantly increased

kidney damage following renal IR [125], and SK2 was found to mediate the protective effects of ischaemic preconditioning in cerebral [126,127] and myocardial [128,129] IR injury. Interestingly, cerebral ischaemia was found to increase SK2 mRNA levels in the brain [130] and hypoxic preconditioning rapidly and transiently upregulated SK2 activity and protein levels in cerebral microvessels [127]. Conversely, however, SK2 inhibition greatly reduced liver injury and improved survival following hepatic IR, coinciding with a reduction in liver S1P levels and mitochondrial permeabilization [131], suggesting that the role of SK2 in IR injury may be tissue-specific.

It has also been reported that the pharmacological inhibition of SK2 resulted in reduced disease severity in rodent models of osteoarthritis [132] and diabetic retinopathy [133]. It therefore appears that the roles of SK2 are complex and, as such, the dysregulation of SK2 can facilitate the development of a number of highly varied disease states. In this regard, SK2 is emerging as a promising therapeutic target in many of these diseases.

Regulation of SK2

Activation

Like SK1, the catalytic activity of SK2 can be rapidly increased upon stimulation by a number of agonists, including EGF [105], TNF-α [134], IL-1β [134], cross-linking of the IgE receptor FcεRI [114], and phorbol esters [105]. Hypoxia was also found to rapidly activate SK2 *in vivo* [127] and in cultured lung cancer cells [91]. Like SK1, SK2 activation can occur via phosphorylation by ERK1/2 [46]. In cells, agonist-induced activation of SK2 increases its catalytic activity by 2- to 6-fold [105], which is comparable with that observed for SK1 [83]. Interestingly, however, *in vitro* phosphorylation of SK2 by ERK1 resulted in a modest doubling of catalytic activity of this enzyme [46], which is less than the 14-fold increase observed for the same analysis of SK1 [83]. Enhanced SK2 activity following interaction with eukaryotic elongation factor 1A has also been described, suggesting that alternative mechanisms of SK2 activation may occur in addition to that involving phosphorylation [135].

Subcellular localization

The subcellular localization of the SKs, and hence the compartmentalization of generated S1P, is widely accepted to play an important role in dictating their function [136,137]. It is well established that in order for SK1 to mediate pro-survival, pro-proliferative and oncogenic signalling it must relocalize from the cytoplasm to the plasma membrane [81]. By comparison, the subcellular localization of SK2 appears to be much more complex than for SK1, in line with its more complex functions. SK2 possesses nuclear localization (NLS) and export (NES) signals [45,87], with the NLS positioned within the N-terminus and the NES located within the central proline-rich region that is not conserved in SK1 (Fig. 2). Interestingly, when localized to the nucleus, SK2 has been shown to inhibit DNA synthesis [87], as well as regulate epigenetic modifications via interaction with and modulation of HDAC1/2 [20]. SK2 localization also appears to vary according to both cell type and cell density. For example, SK2 predominantly localizes to the nucleus in HeLa cells whereas in HEK293 cells it is mainly cytoplasmic [87]. Moreover, it has been described that, as COS-7 fibroblasts became more confluent in culture, the proportion of SK2 localized to the nucleus increased [87], suggesting that SK2 may be involved in a contact inhibition response to decrease cell proliferation.

It has also been demonstrated that under stress conditions SK2 localizes to the endoplasmic reticulum (ER) [49]. S1P production at the ER can fuel the sphingosine salvage pathway driven by ER-localized S1P phosphatases and ceramide synthases, to ultimately generate pro-apoptotic ceramide [49]. Interestingly, artificially targeting SK1 to the ER or nucleus can allow the otherwise pro-survival enzyme to promote apoptosis [49,87]. Moreover, mitochondrial localization of SK2 has been shown to promote apoptosis via S1P and BAK-dependent membrane permeabilization and cytochrome *c* release [22,24]. Therefore the additional pro-apoptotic functions of SK2 not shared with SK1 seem to be mediated by its localization to intracellular membranes like the ER, mitochondria and nucleus. It should be noted that a lipid binding domain has been identified within the N-terminal region (residues 1–175) of SK2, not conserved in SK1 [138]. This domain was shown to be a requirement for SK2 to interact with phosphoinositides and may therefore facilitate its differential localization at internal membranes [138].

The subcellular localization of SK2 also influences substrate availability, and recent findings suggest that this may be another major contributor to the pro-apoptotic function of SK2. Both SK1 and SK2 can utilize dihydrosphingosine (dhSph) as a substrate [5]. Since dhSph is generated at internal membranes such as the ER, SK1 targeted to the plasma membrane cannot utilize this substrate pool [139]. Unlike S1P, the specific role of dihydro-S1P (dhS1P) in oncogenic

signalling is not well characterized. Interestingly, however, Hait *et al*. demonstrated that dhS1P and S1P generated by SK2 in the nucleus can both inhibit HDAC1/2 and regulate epigenetic modifications to inhibit proliferation [20]. Furthermore, a very recent study employing photodynamic therapy and nanoparticle technology to treat solid tumours has reported that the anti-tumour efficacy of this novel technology is directly attributed to the production of dhS1P specifically by SK2, leading to a reduction in immune suppressive myeloid cells expanded in response to tumour-associated inflammation [140]. Notably, this study also demonstrated that administering dhS1P in tumour-bearing mice prevented tumour growth and increased survival, as opposed to S1P which increased tumour growth [140]. Therefore, dhS1P and S1P appear to have opposing roles in oncogenic signalling, dependent on their differential cellular compartmentalization. Again, this highlights the importance of SK2 subcellular localization and consequent access to substrates in relation to its function.

While most studies have described SK2 localization to internal organelles to either induce apoptosis or elicit growth arrest, one study has shown SK2 to mediate pro-proliferative S1P receptor signalling when localized to the plasma membrane [141]. This process, however, appears linked to the activation of caspase-1 in apoptotic cells, which cleaves the N-terminus of SK2 and allows it to be exported from the cell, potentially mediated by the flipping of phosphatidylserine following interaction of the enzyme with the plasma membrane [141].

Post-translational modifications

Although the activating phosphorylation site in SK1 (Ser225) occurs within a region that is divergent in SK2, studies have shown that SK2 catalytic activity also increases following phosphorylation by ERK1/2 [46]. This activation of SK2 has been suggested to involve the phosphorylation of Ser351 and/or Thr578 on SK2a (Ser387 and Thr614 on SK2b) [46], although notably Ser351 is not conserved in mouse or rat SK2. Phosphorylation of SK2 also appears to regulate its nuclear–cytoplasmic shuttling, with protein kinase D mediated phosphorylation of either Ser419 or Ser421 within the NES of SK2 promoting its nuclear export [45].

Recent global phosphoproteome analyses have identified 13 other Ser/Thr phosphorylation events in endogenous human SK2, most of which occur within unique regions of SK2 not conserved in SK1 (Fig. 2) [40–44]. Nine of these residues are conserved in mouse and rat, while another three phosphorylations have been detected in mouse and rat SK2 (see Fig. 2) but have yet to be confirmed in human [142]. No functional studies have been performed to define the regulatory significance of these novel phosphorylation sites. However, it is possible that these modifications play a role in the isoform-specific regulation of SK2 and may therefore provide an elegant yet undoubtedly complex mechanism to allow for the emerging functional complexity of SK2.

SK2 isoforms

Two isoforms of SK2 have been described and characterized [38,86]. Unless otherwise specified, in the literature 'SK2' generally refers to the shorter isoform (SK2a or SK2-S), which is consequently the best characterized. The larger isoform (SK2b or SK2-L) has an additional 36 amino acids at the N-terminus and appears to arise from the use of an alternative start codon (Fig. 3). While not expressed in mice, SK2b appears the predominant form of SK2 in several human cell lines and tissues [86]. This may suggest that

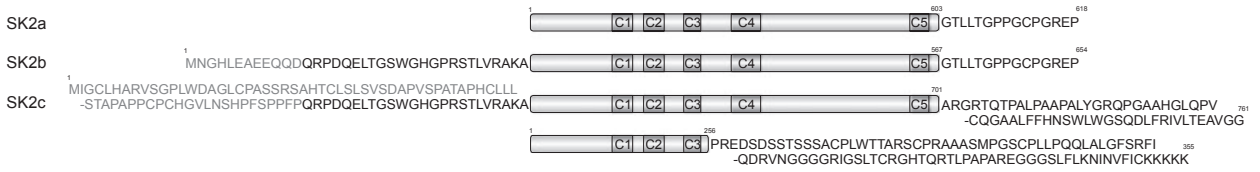


Fig. 3. Human sphingosine kinase 2 isoforms. All known SKs possess five evolutionarily conserved regions (labelled C1–C5) important for catalytic activity [51]. There are two confirmed human SK2 isoforms (SK2a and SK2b) and two further putative or predicted SK2 isoforms. Compared with SK2a (Genbank™ accession number AF245447), SK2b (RefSeq NM_020126) has an additional 36 amino acids at the N-terminus and appears to be the most abundant human isoform [86]. SK2c (Genbank™ accession number EF107108) has an additional N-terminal extension as well as an extended C-terminus; however, this putative isoform has only been detected at the mRNA level in some human cells [143]. A fourth SK2 isoform (bottom) has been predicted by *in silico* analyses (Genbank™ accession number AK000599) and varies considerably at the C-terminus compared with SK2a. The expression of this isoform has not been examined, but since it does not possess the conserved C4 and C5 regions it is unlikely to have SK activity.

in fact SK2b is physiologically the more important human SK2 isoform, although very few studies have specifically examined its functions. It has been demonstrated that serum deprivation leads to an increase in SK2b expression and promotes the translocation of SK2b to the nucleus where it can inhibit DNA synthesis [86]. Furthermore, SK2b appears to phosphorylate some of its substrates at a higher rate than SK2a, including FTY720 (4-fold increase) and sphingosine (1.3-fold increase) [144]. This trend would suggest that the N-terminal extension of SK2b may introduce a conformational change that promotes catalytic activity [144].

A third isoform of SK2 (SK2c; Genbank™ accession number EF107108) has been reported, which possesses an N-terminal extension and an additional C-terminal sequence [143] (Fig. 3). SK2c mRNA has been reported to be detected in some human cells, but no other analysis of this putative SK2 isoform has been described [143]. *In silico* analysis has predicted a fourth SK2 splice variant (Genbank™ accession number AK000599) that differs from SK2a at the C-terminus (from residue 256; see Fig. 3), although there is yet no physical evidence for the existence of this SK2 isoform. Notably, this putative SK2 variant does not possess the C4 and C5 regions proposed to contain the sphingosine binding and catalytic residues, respectively [4,145], which have previously been shown to be essential for SK activity [51]. Therefore, even if this SK2 variant was expressed it is unlikely to have SK activity.

SK2 substrate specificity

Both SK1 and SK2 can utilize D-*erythro*-sphingosine and D-*erythro*-dihydrosphingosine as substrates; however, SK2 appears to be more promiscuous and can phosphorylate a range of other biological and synthetic lipids with much greater efficiency than SK1 [5]. These substrates include phytosphingosine [38], ω-biotinyl D-*erythro*-sphingosine [146] and, surprisingly, the SK1-specific inhibitor D,L-*threo*-dihydrosphingosine [38]. SK2 is also responsible for phosphorylating the immunosuppressive agent FTY720, converting it to its active form FTY720-P [144,147,148]. Although SK1 can also phosphorylate FTY720 *in vitro*, albeit less efficiently than SK2 [144,147,148], levels of FTY720-P in $SK2^{-/-}$ mice administered FTY720 are negligible, indicating that SK1 does not significantly contribute to FTY720 phosphorylation *in vivo* [149]. The ability of SK2 to phosphorylate a larger pool of substrates suggests that the sphingosine binding pockets of SK1 and SK2 differ slightly in conformation. Although the functional significance of this is currently unknown, it has allowed for these subtle structural differences to be exploited in the generation of isoform-specific SK inhibitors.

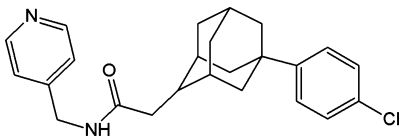
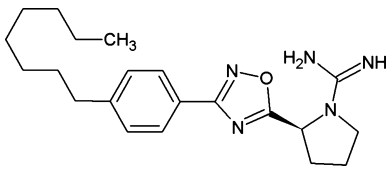
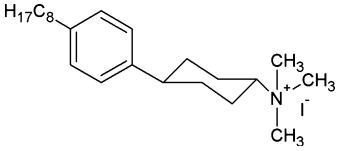
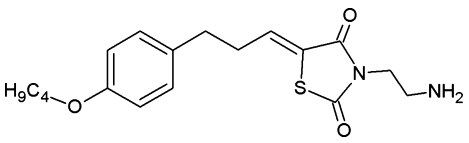
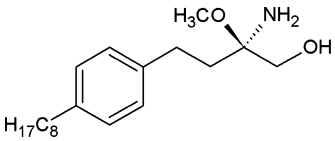
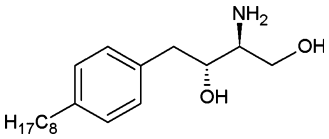
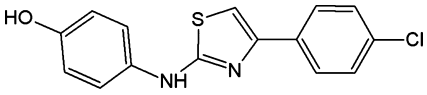
SK2 inhibitors

A number of SK inhibitors have been generated which show potential for development as therapeutics for cancer and some other diseases [5]. The majority of these specifically inhibit either SK1 or both SK isoforms. In the last few years, however, several SK2-specific inhibitors have emerged that demonstrate promising therapeutic properties (Table 1). Notably, recently developed potent SK1-selective inhibitors have demonstrated surprisingly limited anti-cancer properties compared with less isoform-specific SK inhibitors [47,150,151]. This notion, coupled with the promising anti-cancer effects of the current SK2-specific inhibitors (described below), suggests that in some cancers SK2 may be a more important target for cancer therapy.

ABC294640

There is growing interest in the therapeutic effects of the first described SK2-selective small molecule inhibitor ABC294640 [48]. This orally bioavailable agent specifically targets SK2 in a sphingosine-competitive manner with a K_i of 9.8 μM. No effect was observed with ABC294640 on SK1, or the closely related diacylglycerol kinase, at concentrations up to 100 μM [47,48]. There have been a number of studies documenting the effects of this inhibitor in a range of disease models. Notably, ABC294640 has been shown to significantly decrease tumour growth *in vivo* in an array of different tumour models in mice [48,99–103]. Furthermore, ABC294640 also seems to have therapeutic potential for a number of other diseases, where attenuated disease progression was observed in rodent models of osteoarthritis [132], rheumatoid arthritis [113], Crohn's disease [112], ulcerative colitis [111] and diabetic retinopathy [133]. Biochemically, ABC294640 has been reported to decrease S1P and increase ceramide levels in cells [47,48], decrease plasma S1P levels in mice [102], inhibit TNF-α-induced NF-κB activation [99], as well as inhibit the activation of STAT3, AKT and ERK2, and decrease the expression of STAT3 and ERK2 [47]. There are conflicting reports on the mode of action whereby ABC294640 induces cell death, with some studies demonstrating that apoptotic pathways are activated [48,100] and others describing the presence of autophagy markers [47,152]. Perhaps the mechanism by which SK2 inhibition leads to cell death is

Table 1. Structures and inhibitory properties of SK2 inhibitors. ND, not determined.

Inhibitor	Chemical name	Structure	K_i (μM)		Reference
			SK1	SK2	
ABC294640	[3-(4-chlorophenyl)-adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide]		–[a]	9.8 ± 1.4	[48]
SLR080811	[(*S*)-2-[3-(4-octylphenyl)-1,2,4-oxadiazol-5-yl]pyrrolidine-1-carboxamidamide]		12	1.3	[119]
Trans-12a	(1*r*,4*r*)-*N*,*N*,*N*-trimethyl-4(4-octylphenyl) cyclohexanaminium iodide		60 ± 6	8 ± 2	[154]
K145	3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione		ND	6.4 ± 0.7	[155]
(R)-FTY720-OMe	(2*R*)-2-Amino-3-(*O*-methyl)-(2-(4′-*n*-octylphenyl) ethyl)propanol		ND	16.5 ± 1	[156]
SG-12	(2*S*,3*R*)-2-Amino-4-(4-octylphenyl)butane-1,3-diol		ND	ND (IC_{50} = 22)	[158]
SKI-II	2-(*p*-hydroxyanilino)-4-(*p*-chlorophenyl) thiazole		16 ± 1.3	7.9 ± 0.6	[59]

[a] No inhibition detected up to 100 μM.

tissue- and disease-specific, as different diseases may exploit different roles of SK2, and so inhibition of SK2 in each case may affect different pathways. Nevertheless, targeting SK2 with ABC294640 appears to have significant therapeutic potential and, as such, this compound is currently in phase I clinical trials for the treatment of advanced solid tumours.

Notably, in addition to its SK2 inhibitory properties, ABC294640 can also bind to the oestrogen receptor and act as a partial antagonist like tamoxifen [153]. While this additional action of ABC294640 complicates some studies into the role of SK2 in disease, it does suggest that, with dual targeting of SK2 and the oestrogen receptor, this agent may be particularly

beneficial in the treatment of oestrogen-receptor-positive breast cancer.

SLR080811

SLR080811 has been recently described as an SK2-specific inhibitor [119]. This agent is a competitive inhibitor with respect to sphingosine, with a K_i of 1.3 μM for SK2 and a 10-fold weaker affinity for SK1 (K_i = 12 μM). Interestingly, SLR080811 was not found to have significant effects on survival or proliferation of U937 human leukaemia cells, despite reducing S1P generation and total S1P levels in these cells [119]. The reasons for this unexpected finding remain unclear, with further studies required to examine whether this is specific to this cell line or a more general effect.

Notably, use of this inhibitor *in vivo* resulted in an increase in blood S1P levels in wild-type mice [119], which is the same unexpected phenomenon observed in SK2$^{-/-}$ mice [12,115,119,120] but different from that observed with ABC294640 [102]. Again, the reasons for these differences require further analysis.

Trans-12a

Amidine-based compounds have recently been described as SK inhibitors [154]. To this end, a quaternary ammonium salt, labelled trans-12a, was synthesized and found to selectively inhibit SK2 over SK1, with K_i values of 8 and 60 μM, respectively [154]. In U937 human leukaemia cells, trans-12a was reported to significantly reduce the production of FTY720-P,

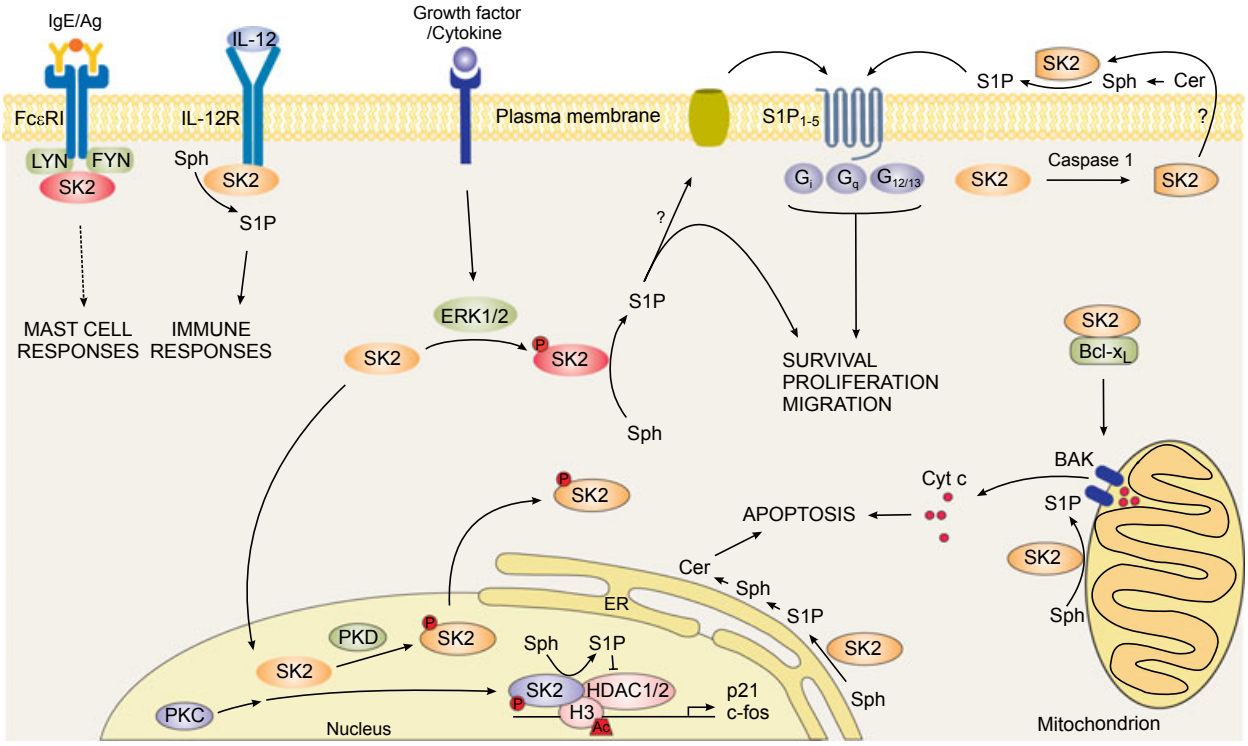


Fig. 4. Signalling and regulation of SK2. Activation of SK2 is mediated by ERK1/2 phosphorylation in response to a range of growth factors and cytokines. SK2 can undergo nuclear–cytoplasmic shuttling, regulated by NLS and NES. The latter is regulated by PKD-mediated phosphorylation, which promotes the nuclear export of SK2. In the nucleus, SK2 can interact with histone H3–HDAC1/2 complexes following phorbol ester treatment of cells, and S1P produced by SK2 here can inhibit HDAC1/2-mediated deacetylation of histone H3 and promote transcription of cyclin-dependent kinase inhibitor p21 and the transcriptional regulator c-fos. SK2 can also localize to the ER in response to serum deprivation or cell density, and S1P produced here can fuel a sphingolipid 'salvage' pathway that ultimately results in the generation of pro-apoptotic ceramide via ER-localized S1P phosphatase and ceramide synthase. Mitochondrial localization of SK2, and subsequent S1P production, can mediate apoptosis via BAK-dependent membrane permeabilization and cytochrome *c* release. SK2 contains a putative BH3 domain through which it can interact with, and presumably sequester, the pro-survival molecule Bcl-x_L, to induce apoptosis. The release of active SK2 from the cell can occur following cleavage at the N-terminus by caspase-1, which can then allow for the production of extracellular S1P. Upon IgE-mediated crosslinking of the FcεRI receptor, Lyn and Fyn can mediate the activation and translocation of SK2 to the plasma membrane to initiate downstream mast cell responses. Murine SK2 has also been found to interact with the IL-12 receptor β1, mediating downstream IL-12 signalling and production of IFN-γ.

suggesting inhibition of SK2, although there was no detectable change in S1P levels in these cells [154].

K145

K145 is another SK2-selective inhibitor that acts in a competitive manner with respect to sphingosine, with a K_i of 6.4 μM [155]. No significant inhibition of SK1 or ceramide kinase was observed by K145 at concentrations up to 10 μM [155]. At 10 μM, K145 also caused a > 40% decrease in the activity of CaMKIIβ, and at 4 μM a significant decrease in phospho-ERK and phospho-AKT was observed, implying that K145 may function as a dual-pathway inhibitor [155]. Cellular studies demonstrated that, in contrast to SLR080811, K145 can inhibit growth and induce apoptosis in U937 human leukaemia cells. *In vivo* K145 was found to reduce tumour volume in JC mammary adenocarcinoma and U937 leukaemia xenograft models [155], with comparable anti-tumour activity by both oral administration (50 $mg \cdot kg^{-1}$) and intraperitoneal injection (15 $mg \cdot kg^{-1}$).

(R)-FTY720-OMe

Lim *et al*. have recently synthesized an SK2-selective inhibitor, (R)-FTY720 methyl ether, based on the structure of the SK2-specific substrate FTY720 [156]. (R)-FTY720-OMe is a competitive inhibitor with respect to sphingosine, inhibiting SK2 with a K_i of 16.5 μM and showing no significant inhibition of SK1 activity at 50 μM [156]. In LNCaP prostate cancer cells, (R)-FTY720-OMe was found to decrease expression of SK2 and stimulate autophagy, but not apoptosis, in these cells [157].

SG-12

SG-12 was synthesized amongst a panel of 15 potential SK2-specific inhibitors and was found to inhibit SK2 with an IC_{50} of 22 μM while not affecting SK1 activity [158]. SG-12 induced cell death in CHO-K1 cells, consistent with SK inhibition, but since it also inhibits PKC the actual mechanism mediating these effects is not clear [158]. Interestingly, it has now been proposed that SG-12 is phosphorylated by SK2 and this modification is critical for its ability to induce cell death [159].

SKI-II

SKI-II is one of the most commonly used SK inhibitors [59]. Among numerous findings, SKI-II has been shown to decrease S1P production and induce apoptosis in tumour cells *in vitro* [59,76] and inhibit tumour growth in a mammary adenocarcinoma xenograft mouse model [76]. SKI-II has been used widely as a direct SK1 inhibitor [133,160–162], although more recent studies suggest it acts mainly to target SK1 through enhancing degradation of this enzyme [163,164]. While commonly stated as an SK1-specific inhibitor, analyses of this compound revealed that SKI-II inhibits SK2 with slightly higher affinity than SK1 (K_i values of 7.9 and 16 μM for SK2 and SK1, respectively) [47], but, contrary to SK1, does not induce degradation of SK2 [157].

Concluding remarks and future perspectives

There is now considerable evidence that SK2 can physiologically perform both pro- and anti-survival functions in cells. It is not currently understood how this is regulated, but it is almost certainly influenced by the subcellular localization of SK2, which may vary according to the cell and tissue type, disease state or environmental stimuli. It is notable, however, that the role for SK2 specifically in cancer and disease, as determined by pharmacological or genetic ablation *in vivo*, is overwhelmingly in support of survival and proliferation. Therefore, despite its ability to promote apoptosis under some conditions, it still appears that targeting SK2 in cancer and other diseases will provide substantial therapeutic benefits. However, as the functional complexity of SK2 becomes more apparent (Fig. 4), it stands to reason that the mechanisms regulating its contrasting roles need to be identified in order to develop therapeutics to specifically target the pro-survival functions of this enzyme.

Acknowledgements

This work was supported by the Fay Fuller Foundation, an Australian Postgraduate Award and Dawes Scholarship (to HAN), and a Senior Research Fellowship and Project Grant (626936) from the National Health and Medical Research Council of Australia (to SMP).

References

1 Hannun YA & Obeid LM (2008) Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol* **9**, 139–150.
2 Maceyka M, Milstien S & Spiegel S (2009) Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling. *J Lipid Res* **50**, S272–S276.

3 Pitson SM & Pebay A (2009) Regulation of stem cell pluripotency and neural differentiation by lysophospholipids. *Neurosignals* **17**, 242–254.
4 Pitson SM (2011) Regulation of sphingosine kinase and sphingolipid signaling. *Trends Biochem Sci* **36**, 97–107.
5 Pitman MR & Pitson SM (2010) Inhibitors of the sphingosine kinase pathway as potential therapeutics. *Curr Cancer Drug Targets* **10**, 354–367.
6 Pyne NJ & Pyne S (2010) Sphingosine 1-phosphate and cancer. *Nat Rev Cancer* **10**, 489–503.
7 Gault CR & Obeid LM (2011) Still benched on its way to the bedside: sphingosine kinase 1 as an emerging target in cancer chemotherapy. *Crit Rev Biochem Mol Biol* **46**, 342–351.
8 Cuvillier O, Ader I, Bouquerel P, Brizuela L, Malavaud B, Mazerolles C & Rischmann P (2010) Activation of sphingosine kinase-1 in cancer: implications for therapeutic targeting. *Curr Mol Pharmacol* **3**, 53–65.
9 Edmonds Y, Milstien S & Spiegel S (2011) Development of small-molecule inhibitors of sphingosine-1-phosphate signaling. *Pharmacol Ther* **132**, 352–360.
10 Strub GM, Maceyka M, Hait NC, Milstien S & Spiegel S (2010) Extracellular and intracellular actions of sphingosine-1-phosphate. *Adv Exp Med Biol* **688**, 141–155.
11 Yatomi Y, Igarashi Y, Yang L, Hisano N, Qi R, Asazuma N, Satoh K, Ozaki Y & Kume S (1997) Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J Biochem* **121**, 969–973.
12 Sensken SC, Bode C, Nagarajan M, Peest U, Pabst O & Graler MH (2010) Redistribution of sphingosine 1-phosphate by sphingosine kinase 2 contributes to lymphopenia. *J Immunol* **184**, 4133–4142.
13 Sato K, Malchinkhuu E, Horiuchi Y, Mogi C, Tomura H, Tosaka M, Yoshimoto Y, Kuwabara A & Okajima F (2007) Critical role of ABCA1 transporter in sphingosine 1-phosphate release from astrocytes. *J Neurochem* **103**, 2610–2619.
14 Takabe K, Kim RH, Allegood JC, Mitra P, Ramachandran S, Nagahashi M, Harikumar KB, Hait NC, Milstien S & Spiegel S (2010) Estradiol induces export of sphingosine 1-phosphate from breast cancer cells via ABCC1 and ABCG2. *J Biol Chem* **285**, 10477–10786.
15 Hisano Y, Kobayashi N, Yamaguchi A & Nishi T (2012) Mouse SPNS2 functions as a sphingosine-1-phosphate transporter in vascular endothelial cells. *PLoS One* **7**, e38941.
16 Chun J, Hla T, Lynch KR, Spiegel S & Moolenaar WH (2010) International Union of Basic and Clinical Pharmacology. LXXVIII. Lysophospholipid receptor nomenclature. *Pharmacol Rev* **62**, 579–587.
17 Hanson MA, Roth CB, Jo E, Griffith MT, Scott FL, Reinhart G, Desale H, Clemons B, Cahalan SM, Schuerer SC *et al.* (2012) Crystal structure of a lipid G protein-coupled receptor. *Science* **335**, 851–855.
18 Meyer zu Heringdorf D & Jakobs KH (2007) Lysophospholipid receptors: signalling, pharmacology and regulation by lysophospholipid metabolism. *Biochim Biophys Acta* **1768**, 923–940.
19 Rosen H, Gonzalez-Cabrera PJ, Sanna MG & Brown S (2009) Sphingosine 1-phosphate receptor signaling. *Annu Rev Biochem* **78**, 743–768.
20 Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, Luo C, Marmorstein R, Kordula T, Milstien S *et al.* (2009) Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science* **325**, 1254–1257.
21 Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, Maceyka M, Jiang H, Luo C, Kordula T *et al.* (2010) Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. *Nature* **465**, 1084–1088.
22 Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, Maceyka M, Price MM, Chen Q, Simpson DC *et al.* (2011) Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. *FASEB J* **25**, 600–612.
23 Maceyka M, Alvarez SE, Milstien S & Spiegel S (2008) Filamin A links sphingosine kinase 1 and sphingosine-1-phosphate receptor 1 at lamellipodia to orchestrate cell migration. *Mol Cell Biol* **28**, 5687–5697.
24 Chipuk JE, McStay GP, Bharti A, Kuwana T, Clarke CJ, Siskind LJ, Obeid LM & Green DR (2012) Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. *Cell* **148**, 988–1000.
25 Spiegel S & Milstien S (2003) Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol* **4**, 397–407.
26 Pebay A, Bonder CS & Pitson SM (2007) Stem cell regulation by lysophospholipids. *Prostaglandins Other Lipid Mediat* **84**, 83–97.
27 Gillies L, Lee SC, Long JS, Ktistakis N, Pyne NJ & Pyne S (2009) The sphingosine 1-phosphate receptor 5 and sphingosine kinases 1 and 2 are localised in centrosomes: possible role in regulating cell division. *Cell Signal* **21**, 675–684.
28 Chalfant CE, Szulc Z, Roddy P, Bielawska A & Hannun YA (2004) The structural requirements for ceramide activation of serine-threonine protein phosphatases. *J Lipid Res* **45**, 496–506.

29 Perry DM, Kitatani K, Roddy P, El-Osta M & Hannun YA (2012) Identification and characterization of protein phosphatase 2C activation by ceramide. *J Lipid Res* **53**, 1513–1521.
30 Muller G, Ayoub M, Storz P, Rennecke J, Fabbro D & Pfizenmaier K (1995) PKCζ is a molecular switch in signal transduction of TNF-α, bifunctionally regulated by ceramide and arachidonic acid. *EMBO J* **14**, 1961–1969.
31 Wang G, Silva J, Krishnamurthy K, Tran E, Condie BG & Bieberich E (2005) Direct binding to ceramide activates protein kinase Cζ before the formation of a pro-apoptotic complex with PAR-4 in differentiating stem cells. *J Biol Chem* **280**, 26415–26424.
32 Zhang Y, Yao B, Delikat S, Bayoumy S, Lin XH, Basu S, McGinley M, Chan-Hui PY, Lichenstein H & Kolesnick R (1997) Kinase suppressor of Ras is ceramide-activated protein kinase. *Cell* **89**, 63–72.
33 Heinrich M, Neumeyer J, Jakob M, Hallas C, Tchikov V, Winoto-Morbach S, Wickel M, Schneider-Brachert W, Trauzold A, Hethke A *et al*. (2004) Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. *Cell Death Differ* **11**, 550–563.
34 Woodcock JM, Ma Y, Coolen C, Pham D, Jones C, Lopez AF & Pitson SM (2010) Sphingosine and FTY720 directly bind pro-survival 14–3-3 proteins to regulate their function. *Cell Signal* **22**, 1291–1299.
35 Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind JS & Spiegel S (1996) Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. *Nature* **381**, 800–803.
36 Taha TA, Hannun YA & Obeid LM (2006) Sphingosine kinase: biochemical and cellular regulation and role in disease. *J Biochem Mol Biol* **39**, 113–131.
37 Pitson SM, Powell JA & Bonder CS (2011) Regulation of sphingosine kinase in hematological malignancies and other cancers. *Anticancer Agents Med Chem* **11**, 799–809.
38 Liu H, Sugiura M, Nava VE, Edsall LC, Kono K, Poulton S, Milstien S, Kohama T & Spiegel S (2000) Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J Biol Chem* **275**, 19513–19520.
39 Leclercq TM & Pitson SM (2006) Cellular signalling by sphingosine kinase and sphingosine 1-phosphate. *IUBMB Life* **58**, 467–472.
40 Hornbeck PV, Kornhauser JM, Tkachev S, Zhang B, Skrzypek E, Murray B, Latham V & Sullivan M (2012) PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse. *Nucleic Acids Res* **40**, D261–D270.
41 Chen R-Q, Yang Q-K, Lu B-W, Yi W, Cantin G, Chen Y-I, Fearns C, Yates JR & Lee J-D (2009) CDC25B mediates rapamycin-induced oncogenic responses in cancer cells. *Cancer Res* **69**, 2663–2668.
42 Daub H, Olsen JV, Bairlein M, Gnad F, Oppermann FS, Korner R, Greff Z, Keri G, Stemmann O & Mann M (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Mol Cell* **31**, 438–448.
43 Dephoure N, Zhou C, Villen J, Beausoleil SA, Bakalarski CE, Elledge SJ & Gygi SP (2008) A quantitative atlas of mitotic phosphorylation. *Proc Natl Acad Sci USA* **105**, 10762–10767.
44 Christensen GL, Kelstrup CD, Lyngso C, Sarwar U, Bogebo R, Sheikh SP, Gammeltoft S, Olsen JV & Hansen JL (2010) Quantitative phosphoproteomics dissection of seven-transmembrane receptor signaling using full and biased agonists. *Mol Cell Proteomics* **9**, 1540–1553.
45 Ding G, Sonoda H, Yu H, Kajimoto T, Goparaju SK, Jahangeer S, Okada T & Nakamura S (2007) Protein kinase D-mediated phosphorylation and nuclear export of sphingosine kinase 2. *J Biol Chem* **282**, 27493–27502.
46 Hait NC, Bellamy A, Milstien S, Kordula T & Spiegel S (2007) Sphingosine kinase type 2 activation by ERK-mediated phosphorylation. *J Biol Chem* **282**, 12058–12065.
47 Gao P, Peterson YK, Smith RA & Smith CD (2012) Characterization of isoenzyme-selective inhibitors of human sphingosine kinases. *PLoS One* **7**, e44543.
48 French KJ, Zhuang Y, Maines LW, Gao P, Wang W, Beljanski V, Upson JJ, Green CL, Keller SN & Smith CD (2010) Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. *J Pharmacol Exp Ther* **333**, 129–139.
49 Maceyka M, Sankala H, Hait NC, Le Stunff H, Liu H, Toman R, Collier C, Zhang M, Satin LS, Merrill AH *et al*. (2005) SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. *J Biol Chem* **280**, 37118–37129.
50 Pitson SM, Moretti PAB, Zebol JR, Xia P, Gamble JR, Vadas MA, D'Andrea RJ & Wattenberg BW (2000) Expression of a catalytically inactive sphingosine kinase mutant blocks agonist-induced sphingosine kinase activation: a dominant-negative sphingosine kinase. *J Biol Chem* **275**, 33945–33950.
51 Pitson SM, Moretti PA, Zebol JR, Zareie R, Derian CK, Darrow AL, Qi J, D'Andrea RJ, Bagley CJ, Vadas MA *et al*. (2002) The nucleotide-binding site of human sphingosine kinase 1. *J Biol Chem* **277**, 49545–49553.

52 Pitson SM, D'Andrea RJ, Vandeleur L, Moretti PA, Xia P, Gamble JR, Vadas MA & Wattenberg BW (2000) Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes. *Biochem J* **350**, 429–441.
53 Pitman MR, Pham DH & Pitson SM (2012) Isoform-selective assays for sphingosine kinase activity. *Methods Mol Biol* **874**, 21–31.
54 Kono K, Sugiura M & Kohama T (2002) Inhibition of recombinant sphingosine kinases by novel inhibitors of microbial origin, F-12509A and B-5354c. *J Antibiot* **55**, 99–103.
55 Chan HS & Pitson SM (2013) Post-translational regulation of sphingosine kinases. *Biochim Biophys Acta* **1831**, 147–156.
56 Melendez AJ, Carlos-Dias E, Gosink M, Allen JM & Takacs L (2000) Human sphingosine kinase: molecular cloning, functional characterization and tissue distribution. *Gene* **251**, 19–26.
57 Olivera A, Kohama T, Edsall L, Nava V, Cuvillier O, Poulton S & Spiegel S (1999) Sphingosine kinase expression increases intracellular sphingosine-1-phosphate and promotes cell growth and survival. *J Cell Biol* **147**, 545–557.
58 Xia P, Gamble JR, Wang L, Pitson SM, Moretti PA, Wattenberg BW, D'Andrea RJ & Vadas MA (2000) An oncogenic role of sphingosine kinase. *Curr Biol* **10**, 1527–1530.
59 French KJ, Schrecengost RS, Lee BD, Zhuang Y, Smith SN, Eberly JL, Yun JK & Smith CD (2003) Discovery and evaluation of inhibitors of human sphingosine kinase. *Cancer Res* **63**, 5962–5969.
60 Bayerl MG, Bruggeman RD, Conroy EJ, Hengst JA, King TS, Jimenez M, Claxton DF & Yun JK (2008) Sphingosine kinase 1 protein and mRNA are overexpressed in non-Hodgkin lymphomas and are attractive targets for novel pharmacological interventions. *Leuk Lymphoma* **49**, 948–954.
61 Malavaud B, Pchejetski D, Mazerolles C, de Paiva GR, Calvet C, Doumerc N, Pitson S, Rischmann P & Cuvillier O (2010) Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. *Eur J Cancer* **46**, 3417–3424.
62 Li J, Guan HY, Gong LY, Song LB, Zhang N, Wu J, Yuan J, Zheng YJ, Huang ZS & Li M (2008) Clinical significance of sphingosine kinase-1 expression in human astrocytomas progression and overall patient survival. *Clin Cancer Res* **14**, 6996–7003.
63 Van Brocklyn JR, Jackson CA, Pearl DK, Kotur MS, Snyder PJ & Prior TW (2005) Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: roles of sphingosine kinase isoforms in growth of glioblastoma cell lines. *J Neuropathol Exp Neurol* **64**, 695–705.
64 Kawamori T, Osta W, Johnson KR, Pettus BJ, Bielawski J, Tanaka T, Wargovich MJ, Reddy BS, Hannun YA, Obeid LM *et al*. (2006) Sphingosine kinase 1 is up-regulated in colon carcinogenesis. *FASEB J* **20**, 386–388.
65 Johnson KR, Johnson KY, Crellin HG, Ogretmen B, Boylan AM, Harley RA & Obeid LM (2005) Immunohistochemical distribution of sphingosine kinase 1 in normal and tumor lung tissue. *J Histochem Cytochem* **53**, 1159–1166.
66 Li W, Yu CP, Xia JT, Zhang L, Weng GX, Zheng HQ, Kong QL, Hu LJ, Zeng MS, Zeng YX *et al*. (2009) Sphingosine kinase 1 is associated with gastric cancer progression and poor survival of patients. *Clin Cancer Res* **15**, 1393–1399.
67 Ruckhaberle E, Rody A, Engels K, Gaetje R, von Minckwitz G, Schiffmann S, Grosch S, Geisslinger G, Holtrich U, Karn T *et al*. (2008) Microarray analysis of altered sphingolipid metabolism reveals prognostic significance of sphingosine kinase 1 in breast cancer. *Breast Cancer Res Treat* **112**, 41–52.
68 Liu G, Zheng H, Zhang Z, Wu Z, Xiong H, Li J & Song L (2010) Overexpression of sphingosine kinase 1 is associated with salivary gland carcinoma progression and might be a novel predictive marker for adjuvant therapy. *BMC Cancer* **10**, 495.
69 Pan J, Tao YF, Zhou Z, Cao BR, Wu SY, Zhang YL, Hu SY, Zhao WL, Wang J, Lou GL *et al*. (2011) An novel role of sphingosine kinase-1 (SPHK1) in the invasion and metastasis of esophageal carcinoma. *J Transl Med* **9**, 157.
70 Facchinetti MM, Gandini NA, Fermento ME, Sterin-Speziale NB, Ji Y, Patel V, Gutkind JS, Rivadulla MG & Curino AC (2010) The expression of sphingosine kinase-1 in head and neck carcinoma. *Cells Tissues Organs* **192**, 314–324.
71 Sinha UK, Schorn VJ, Hochstim C, Chinn SB, Zhu S & Masood R (2011) Increased radiation sensitivity of head and neck squamous cell carcinoma with sphingosine kinase 1 inhibition. *Head Neck* **33**, 178–188.
72 Long JS, Edwards J, Watson C, Tovey S, Mair KM, Schiff R, Natarajan V, Pyne NJ & Pyne S (2010) Sphingosine kinase 1 induces tolerance to human epidermal growth factor receptor 2 and prevents formation of a migratory phenotype in response to sphingosine 1-phosphate in estrogen receptor-positive breast cancer cells. *Mol Cell Biol* **30**, 3827–3841.
73 Kohno M, Momoi M, Oo ML, Paik JH, Lee YM, Venkataraman K, Ai Y, Ristimaki AP, Fyrst H, Sano H *et al*. (2006) Intracellular role for sphingosine kinase 1 in intestinal adenoma cell proliferation. *Mol Cell Biol* **26**, 7211–7223.
74 Endo K, Igarashi Y, Nisar M, Zhou QH & Hakomori S (1991) Cell membrane signaling as target in cancer

therapy: inhibitory effect of N, N-dimethyl and N, N, N-trimethyl sphingosine derivatives on *in vitro* and *in vivo* growth of human tumor cells in nude mice. *Cancer Res* **51**, 1613–1618.
75 Park YS, Hakomori S, Kawa S, Ruan F & Igarashi Y (1994) Liposomal N, N, N-trimethylsphingosine (TMS) as an inhibitor of B16 melanoma cell growth and metastasis with reduced toxicity and enhanced drug efficacy compared to free TMS: cell membrane signaling as a target in cancer therapy III. *Cancer Res* **54**, 2213–2217.
76 French KJ, Upson JJ, Keller SN, Zhuang Y, Yun JK & Smith CD (2006) Antitumor activity of sphingosine kinase inhibitors. *J Pharmacol Exp Ther* **318**, 596–603.
77 Pchejetski D, Doumerc N, Golzio M, Naymark M, Teissie J, Kohama T, Waxman J, Malavaud B & Cuvillier O (2008) Chemosensitizing effects of sphingosine kinase-1 inhibition in prostate cancer cell and animal models. *Mol Cancer Ther* **7**, 1836–1845.
78 Kawamori T, Kaneshiro T, Okumura M, Maalouf S, Uflacker A, Bielawski J, Hannun YA & Obeid LM (2009) Role for sphingosine kinase 1 in colon carcinogenesis. *FASEB J* **23**, 405–414.
79 Ponnusamy S, Selvam SP, Mehrotra S, Kawamori T, Snider AJ, Obeid LM, Shao Y, Sabbadini R & Ogretmen B (2012) Communication between host organism and cancer cells is transduced by systemic sphingosine kinase 1/sphingosine 1-phosphate signalling to regulate tumour metastasis. *EMBO Mol Med* **4**, 761–775.
80 Shirai K, Kaneshiro T, Wada M, Furuya H, Bielawski J, Hannun YA, Obeid LM, Ogretmen B & Kawamori T (2011) A role of sphingosine kinase 1 in head and neck carcinogenesis. *Cancer Prev Res* **4**, 454–462.
81 Pitson SM, Xia P, Leclercq TM, Moretti PAB, Zebol JR, Lynn HE, Wattenberg BW & Vadas MA (2005) Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. *J Exp Med* **201**, 49–54.
82 Jarman KE, Moretti PAB, Zebol JR & Pitson SM (2010) Translocation of sphingosine kinase 1 to the plasma membrane is mediated by calcium- and integrin-binding protein 1. *J Biol Chem* **285**, 483–492.
83 Pitson SM, Moretti PAB, Zebol JR, Lynn HE, Xia P, Vadas MA & Wattenberg BW (2003) Activation of sphingosine kinase 1 by ERK1/2-mediated phosphorylation. *EMBO J* **22**, 5491–5500.
84 Gault CR, Eblen ST, Neumann CA, Hannun YA & Obeid LM (2012) Oncogenic K-Ras regulates bioactive sphingolipids in a sphingosine kinase 1-dependent manner. *J Biol Chem* **287**, 31794–31803.
85 ter Braak M, Danneberg K, Lichte K, Liphardt K, Ktistakis NT, Pitson SM, Hla T, Jakobs KH & Meyer zu Heringdorf D (2009) $G\alpha_q$-mediated plasma membrane translocation of sphingosine kinase-1 and cross-activation of S1P receptors. *Biochim Biophys Acta* **1791**, 357–370.
86 Okada T, Ding G, Sonoda H, Kajimoto T, Haga Y, Khosrowbeygi A, Gao SY, Miwa N, Jahangeer S & Nakamura S (2005) Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis. *J Biol Chem* **280**, 36318–36325.
87 Igarashi N, Okada T, Hayashi S, Fujita T, Jahangeer S & Nakamura S (2003) Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. *J Biol Chem* **278**, 46832–46839.
88 Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, Sankala H, Payne SG, Bektas M, Ishii I, Chun J *et al.* (2003) Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J Biol Chem* **278**, 40330–40336.
89 Hofmann LP, Ren S, Schwalm S, Pfeilschifter J & Huwiler A (2008) Sphingosine kinase 1 and 2 regulate the capacity of mesangial cells to resist apoptotic stimuli in an opposing manner. *Biol Chem* **389**, 1399–1407.
90 Sankala HM, Hait NC, Paugh SW, Shida D, Lepine S, Elmore LW, Dent P, Milstien S & Spiegel S (2007) Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin. *Cancer Res* **67**, 10466–10474.
91 Schnitzer SE, Weigert A, Zhou J & Brune B (2009) Hypoxia enhances sphingosine kinase 2 activity and provokes sphingosine-1-phosphate-mediated chemoresistance in A549 lung cancer cells. *Mol Cancer Res* **7**, 393–401.
92 Nemoto S, Nakamura M, Osawa Y, Kono S, Itoh Y, Okano Y, Murate T, Hara A, Ueda H, Nozawa Y *et al.* (2009) Sphingosine kinase isoforms regulate oxaliplatin sensitivity of human colon cancer cells through ceramide accumulation and Akt activation. *J Biol Chem* **284**, 10422–10432.
93 Sun DF, Gao ZH, Liu HP, Yuan Y & Qu XJ (2012) Sphingosine 1-phosphate antagonizes the effect of all-trans retinoic acid (ATRA) in a human colon cancer cell line by modulation of RARβ expression. *Cancer Lett* **319**, 182–189.
94 Zemann B, Urtz N, Reuschel R, Mechtcheriakova D, Bornancin F, Badegruber R, Baumruker T & Billich A (2007) Normal neutrophil functions in sphingosine kinase type 1 and 2 knockout mice. *Immunol Lett* **109**, 56–63.
95 Allende ML, Sasaki T, Kawai H, Olivera A, Mi YD, van Echten-Deckert G, Hajdu R, Rosenbach M, Keohane CA, Mandala S *et al.* (2004) Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J Biol Chem* **279**, 52487–52492.

96 Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S & Proia RL (2005) Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol* **25**, 11113–11121.
97 Gao P & Smith CD (2011) Ablation of sphingosine kinase-2 inhibits tumor cell proliferation and migration. *Mol Cancer Res* **9**, 1509–1519.
98 Weigert A, Schiffmann S, Sekar D, Ley S, Menrad H, Werno C, Grosch S, Geisslinger G & Brune B (2009) Sphingosine kinase 2 deficient tumor xenografts show impaired growth and fail to polarize macrophages towards an anti-inflammatory phenotype. *Int J Cancer* **125**, 2114–2121.
99 Antoon JW, White MD, Slaughter EM, Driver JL, Khalili HS, Elliott S, Smith CD, Burow ME & Beckman BS (2011) Targeting NFκB mediated breast cancer chemoresistance through selective inhibition of sphingosine kinase-2. *Cancer Biol Ther* **11**, 678–689.
100 Antoon JW, White MD, Driver JL, Burow ME & Beckman BS (2012) Sphingosine kinase isoforms as a therapeutic target in endocrine therapy resistant luminal and basal-A breast cancer. *Exp Biol Med* **237**, 832–844.
101 Beljanski V, Knaak C, Zhuang Y & Smith CD (2011) Combined anticancer effects of sphingosine kinase inhibitors and sorafenib. *Invest New Drugs* **29**, 1132–1142.
102 Beljanski V, Lewis CS & Smith CD (2011) Antitumor activity of sphingosine kinase 2 inhibitor ABC294640 and sorafenib in hepatocellular carcinoma xenografts. *Cancer Biol Ther* **11**, 524–534.
103 Chumanevich AA, Poudyal D, Cui X, Davis T, Wood PA, Smith CD & Hofseth LJ (2010) Suppression of colitis-driven colon cancer in mice by a novel small molecule inhibitor of sphingosine kinase. *Carcinogenesis* **31**, 1787–1793.
104 Miller AV, Alvarez SE, Spiegel S & Lebman DA (2008) Sphingosine kinases and sphingosine-1-phosphate are critical for transforming growth factor β-induced extracellular signal-regulated kinase 1 and 2 activation and promotion of migration and invasion of esophageal cancer cells. *Mol Cell Biol* **28**, 4142–4151.
105 Hait NC, Sarkar S, Le Stunff H, Mikami A, Maceyka M, Milstien S & Spiegel S (2005) Role of sphingosine kinase 2 in cell migration toward epidermal growth factor. *J Biol Chem* **280**, 29462–29469.
106 Xia P, Gamble JR, Rye KA, Wang L, Hii CS, Cockerill P, Khew-Goodall Y, Bert AG, Barter PJ & Vadas MA (1998) Tumor necrosis factor-α induces adhesion molecule expression through the sphingosine kinase pathway. *Proc Natl Acad Sci USA* **95**, 14196–141201.
107 Billich A, Bornancin F, Mechtcheriakova D, Natt F, Huesken D & Baumruker T (2005) Basal and induced sphingosine kinase 1 activity in A549 carcinoma cells: function in cell survival and IL-1β and TNF-α induced production of inflammatory mediators. *Cell Signal* **17**, 1203–1217.
108 Snider AJ, Orr Gandy KA & Obeid LM (2010) Sphingosine kinase: role in regulation of bioactive sphingolipid mediators in inflammation. *Biochimie* **92**, 707–715.
109 Lai WQ, Irwan AW, Goh HH, Melendez AJ, McInnes IB & Leung BP (2009) Distinct roles of sphingosine kinase 1 and 2 in murine collagen-induced arthritis. *J Immunol* **183**, 2097–2103.
110 Samy ET, Meyer CA, Caplazi P, Langrish CL, Lora JM, Bluethmann H & Peng SL (2007) Cutting edge: modulation of intestinal autoimmunity and IL-2 signaling by sphingosine kinase 2 independent of sphingosine 1-phosphate. *J Immunol* **179**, 5644–5648.
111 Maines LW, Fitzpatrick LR, French KJ, Zhuang Y, Xia Z, Keller SN, Upson JJ & Smith CD (2008) Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. *Dig Dis Sci* **53**, 997–1012.
112 Maines LW, Fitzpatrick LR, Green CL, Zhuang Y & Smith CD (2010) Efficacy of a novel sphingosine kinase inhibitor in experimental Crohn's disease. *Inflammopharmacology* **18**, 73–85.
113 Fitzpatrick LR, Green C, Frauenhoffer EE, French KJ, Zhuang Y, Maines LW, Upson JJ, Paul E, Donahue H, Mosher TJ *et al*. (2011) Attenuation of arthritis in rodents by a novel orally-available inhibitor of sphingosine kinase. *Inflammopharmacology* **19**, 75–87.
114 Olivera A, Urtz N, Mizugishi K, Yamashita Y, Gilfillan AM, Furumoto Y, Gu H, Proia RL, Baumruker T & Rivera J (2006) IgE-dependent activation of sphingosine kinases 1 and 2 and secretion of sphingosine 1-phosphate requires Fyn kinase and contributes to mast cell responses. *J Biol Chem* **281**, 2515–2525.
115 Olivera A, Mizugishi K, Tikhonova A, Ciaccia L, Odom S, Proia RL & Rivera J (2007) The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis. *Immunity* **26**, 287–297.
116 Oskeritzian CA, Alvarez SE, Hait NC, Price MM, Milstien S & Spiegel S (2008) Distinct roles of sphingosine kinases 1 and 2 in human mast-cell functions. *Blood* **111**, 4193–4200.
117 Blom T, Slotte JP, Pitson SM & Tornquist K (2005) Enhancement of intracellular sphingosine-1-phosphate production by inositol 1,4,5-trisphosphate-evoked calcium mobilisation in HEK-293 cells: endogenous sphingosine-1-phosphate as a modulator of the calcium response. *Cell Signal* **17**, 827–836.

118 Mathes C, Fleig A & Penner R (1998) Calcium release-activated calcium current (ICRAC) is a direct target for sphingosine. *J Biol Chem* **273**, 25020–25030.
119 Kharel Y, Raje M, Gao M, Gellett AM, Tomsig JL, Lynch KR & Santos WL (2012) Sphingosine kinase type 2 inhibition elevates circulating sphingosine 1-phosphate. *Biochem J* **447**, 149–157.
120 Zemann B, Kinzel B, Muller M, Reuschel R, Mechtcheriakova D, Urtz N, Bornancin F, Baumruker T & Billich A (2006) Sphingosine kinase type 2 is essential for lymphopenia induced by the immunomodulatory drug FTY720. *Blood* **107**, 1454–1458.
121 Liang J, Nagahashi M, Kim EY, Harikumar KB, Yamada A, Huang WC, Hait NC, Allegood JC, Price MM, Avni D *et al*. (2013) Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. *Cancer Cell* **23**, 107–120.
122 Takuwa N, Ohkura S, Takashima S, Ohtani K, Okamoto Y, Tanaka T, Hirano K, Usui S, Wang F, Du W *et al*. (2010) $S1P_3$-mediated cardiac fibrosis in sphingosine kinase 1 transgenic mice involves reactive oxygen species. *Cardiovasc Res* **85**, 484–493.
123 Yoshimoto T, Furuhata M, Kamiya S, Hisada M, Miyaji H, Magami Y, Yamamoto K, Fujiwara H & Mizuguchi J (2003) Positive modulation of IL-12 signaling by sphingosine kinase 2 associating with the IL-12 receptor beta 1 cytoplasmic region. *J Immunol* **171**, 1352–1359.
124 Takasugi N, Sasaki T, Suzuki K, Osawa S, Isshiki H, Hori Y, Shimada N, Higo T, Yokoshima S, Fukuyama T *et al*. (2011) BACE1 activity is modulated by cell-associated sphingosine-1-phosphate. *J Neurosci* **31**, 6850–6857.
125 Jo SK, Bajwa A, Ye H, Vergis AL, Awad AS, Kharel Y, Lynch KR & Okusa MD (2009) Divergent roles of sphingosine kinases in kidney ischemia-reperfusion injury. *Kidney Int* **75**, 167–175.
126 Yung LM, Wei Y, Qin T, Wang YM, Smith CD & Waeber C (2012) Sphingosine kinase 2 mediates cerebral preconditioning and protects the mouse brain against ischemic injury. *Stroke* **43**, 199–204.
127 Wacker BK, Park TS & Gidday JM (2009) Hypoxic preconditioning-induced cerebral ischemic tolerance role of microvascular sphingosine kinase 2. *Stroke* **40**, 3342–3348.
128 Vessey DA, Li L, Jin Z-Q, Kelley M, Honbo N, Zhang J & Karliner JS (2011) A sphingosine kinase form 2 knockout sensitizes mouse myocardium to ischemia/reoxygenation injury and diminishes responsiveness to ischemic preconditioning. *Oxid Med Cell Longev* **2011**, 961059.
129 Gomez L, Paillard M, Price M, Chen Q, Teixeira G, Spiegel S & Lesnefsky EJ (2011) A novel role for mitochondrial sphingosine-1-phosphate produced by sphingosine kinase-2 in PTP-mediated cell survival during cardioprotection. *Basic Res Cardiol* **106**, 1341–1353.
130 Blondeau N, Lai YS, Tyndall S, Popolo M, Topalkara K, Pru JK, Zhang L, Kim H, Liao JK, Ding K *et al*. (2007) Distribution of sphingosine kinase activity and mRNA in rodent brain. *J Neurochem* **103**, 509–517.
131 Shi YJ, Rehman H, Ramshesh VK, Schwartz J, Liu QL, Krishnasamy Y, Zhang X, Lemasters JJ, Smith CD & Zhong Z (2012) Sphingosine kinase-2 inhibition improves mitochondrial function and survival after hepatic ischemia-reperfusion. *J Hepatol* **56**, 137–145.
132 Fitzpatrick LR, Green C, Maines LW & Smith CD (2011) Experimental osteoarthritis in rats is attenuated by ABC294640, a selective inhibitor of sphingosine kinase-2. *Pharmacology* **87**, 135–143.
133 Maines LW, French KJ, Wolpert EB, Antonetti DA & Smith CD (2006) Pharmacologic manipulation of sphingosine kinase in retinal endothelial cells: implications for angiogenic ocular diseases. *Invest Ophthalmol Vis Sci* **47**, 5022–5031.
134 Mastrandrea LD, Sessanna SM & Laychock SG (2005) Sphingosine kinase activity and sphingosine-1 phosphate production in rat pancreatic islets and INS-1 cells: response to cytokines. *Diabetes* **54**, 1429–1436.
135 Leclercq TM, Moretti PAB, Vadas MA & Pitson SM (2008) Eukaryotic elongation factor 1A interacts with sphingosine kinase and directly enhances its catalytic activity. *J Biol Chem* **283**, 9606–9614.
136 Siow D & Wattenberg B (2011) The compartmentalization and translocation of the sphingosine kinases: mechanisms and functions in cell signaling and sphingolipid metabolism. *Crit Rev Biochem Mol Biol* **46**, 365–375.
137 Wattenberg BW (2010) Role of sphingosine kinase localization in sphingolipid signaling. *World J Biol Chem* **1**, 362–368.
138 Don AS & Rosen H (2009) A lipid binding domain in sphingosine kinase 2. *Biochem Biophys Res Commun* **380**, 87–92.
139 Siow DL, Anderson CD, Berdyshev EV, Skobeleva A, Pitson SM & Wattenberg BW (2010) Intracellular localization of sphingosine kinase 1 alters access to substrate pools but does not affect the degradative fate of sphingosine-1-phosphate. *J Lipid Res* **51**, z2546–2559.
140 Barth BM, Shanmugavelandy SS, Kaiser JM, McGovern C, Altinoglu EI, Haakenson JK, Hengst JA, Gilius EL, Knupp SA, Fox TE *et al*. (2013)

PhotoImmunoNanoTherapy reveals an anticancer role for sphingosine kinase 2 and dihydrosphingosine-1-phosphate. *ACS Nano* **7**, 2132–2144.

141 Weigert A, Cremer S, Schmidt MV, von Knethen A, Angioni C, Geisslinger G & Brune B (2010) Cleavage of sphingosine kinase 2 by caspase-1 provokes its release from apoptotic cells. *Blood* **115**, 3531–3540.

142 Huttlin EL, Jedrychowski MP, Elias JE, Goswami T, Rad R, Beausoleil SA, Villen J, Haas W, Sowa ME & Gygi SP (2010) A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* **143**, 1174–1189.

143 Alemany R, van Koppen CJ, Danneberg K, Ter Braak M & Meyer Zu Heringdorf D (2007) Regulation and functional roles of sphingosine kinases. *Naunyn-Schmiedeberg's Arch Pharmacol* **374**, 413–428.

144 Billich A, Bornancin F, Devay P, Mechtcheriakova D, Urtz N & Baumruker T (2003) Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. *J Biol Chem* **278**, 47408–47415.

145 Yokota S, Taniguchi Y, Kihara A, Mitsutake S & Igarashi Y (2004) Asp177 in C4 domain of mouse sphingosine kinase 1a is important for the sphingosine recognition. *FEBS Lett* **578**, 106–110.

146 Roberts JL, Moretti PA, Darrow AL, Derian CK, Vadas MA & Pitson SM (2004) An assay for sphingosine kinase activity using biotinylated sphingosine and streptavidin-coated membranes. *Anal Biochem* **331**, 122–129.

147 Paugh SW, Payne SG, Barbour SE, Milstien S & Spiegel S (2003) The immunosuppressant FTY720 is phosphorylated by sphingosine kinase type 2. *FEBS Lett* **554**, 189–193.

148 Sanchez T, Estrada-Hernandez T, Paik JH, Wu MT, Venkataraman K, Brinkmann V, Claffey K & Hla T (2003) Phosphorylation and action of the immunomodulator FTY720 inhibits vascular endothelial cell growth factor-induced vascular permeability. *J Biol Chem* **278**, 47281–47290.

149 Kharel Y, Lee S, Snyder AH, Sheasley-O'Neill SL, Morris MA, Setiady Y, Zhu R, Zigler MA, Burcin TL, Ley K *et al.* (2005) Sphingosine kinase 2 is required for modulation of lymphocyte traffic by FTY720. *J Biol Chem* **280**, 36865–36872.

150 Schnute ME, McReynolds MD, Kasten T, Yates M, Jerome G, Rains JW, Hall T, Chrencik J, Kraus M, Cronin CN *et al.* (2012) Modulation of cellular S1P levels with a novel, potent and specific inhibitor of sphingosine kinase-1. *Biochem J* **444**, 79–88.

151 Kharel Y, Mathews TP, Gellett AM, Tomsig JL, Kennedy PC, Moyer ML, Macdonald TL & Lynch KR (2011) Sphingosine kinase type 1 inhibition reveals rapid turnover of circulating sphingosine 1-phosphate. *Biochem J* **440**, 345–353.

152 Beljanski V, Knaak C & Smith CD (2010) A novel sphingosine kinase inhibitor induces autophagy in tumor cells. *J Pharmacol Exp Ther* **333**, 454–464.

153 Antoon JW, White MD, Meacham WD, Slaughter EM, Muir SE, Elliott S, Rhodes LV, Ashe HB, Wiese TE, Smith CD *et al.* (2010) Antiestrogenic effects of the novel sphingosine kinase-2 inhibitor ABC294640. *Endocrinology* **151**, 5124–5135.

154 Raje MR, Knott K, Kharel Y, Bissel P, Lynch KR & Santos WL (2012) Design, synthesis and biological activity of sphingosine kinase 2 selective inhibitors. *Bioorg Med Chem* **20**, 183–194.

155 Liu K, Guo TL, Hait NC, Allegood J, Parikh HI, Xu W, Kellogg GE, Grant S, Spiegel S & Zhang S (2013) Biological characterization of 3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) as a selective sphingosine kinase-2 inhibitor and anticancer agent. *PLoS One* **8**, e56471.

156 Lim KG, Sun C, Bittman R, Pyne NJ & Pyne S (2011) (R)-FTY720 methyl ether is a specific sphingosine kinase 2 inhibitor: effect on sphingosine kinase 2 expression in HEK 293 cells and actin rearrangement and survival of MCF-7 breast cancer cells. *Cell Signal* **23**, 1590–1595.

157 Watson DG, Tonelli F, Alossaimi M, Williamson L, Chan E, Gorshkova I, Berdyshev E, Bittman R, Pyne NJ & Pyne S (2013) The roles of sphingosine kinases 1 and 2 in regulating the Warburg effect in prostate cancer cells. *Cell Signal* **25**, 1011–1017.

158 Kim JW, Kim YW, Inagaki Y, Hwang YA, Mitsutake S, Ryu YW, Lee WK, Ha HJ, Park CS & Igarashi Y (2005) Synthesis and evaluation of sphingoid analogs as inhibitors of sphingosine kinases. *Bioorg Med Chem* **13**, 3475–3485.

159 Hara-Yokoyama M, Terasawa K, Ichinose S, Watanabe A, Podyma-Inoue KA, Akiyoshi K, Igarashi Y & Yanagishita M (2013) Sphingosine kinase 2 inhibitor SG-12 induces apoptosis via phosphorylation by sphingosine kinase 2. *Bioorg Med Chem Lett* **23**, 2220–2224.

160 Nishiuma T, Nishimura Y, Okada T, Kuramoto E, Kotani Y, Jahangeer S & Nakamura S (2008) Inhalation of sphingosine kinase inhibitor attenuates airway inflammation in asthmatic mouse model. *Am J Physiol Lung Cell Mol Physiol* **294**, L1085–L1093.

161 Ricci C, Onida F, Servida F, Radaelli F, Saporiti G, Todoerti K, Deliliers GL & Ghidoni R (2009) *In vitro* anti-leukaemia activity of sphingosine kinase inhibitor. *Br J Haematol* **144**, 350–357.

162 Lee C, Xu DZ, Feketeova E, Kannan KB, Yun JK, Deitch EA, Fekete Z, Livingston DH & Hauser CJ (2004) Attenuation of shock-induced acute lung injury

by sphingosine kinase inhibition. *J Trauma* **57**, 955–960.

163 Ren S, Xin C, Pfeilschifter J & Huwiler A (2010) A novel mode of action of the putative sphingosine kinase inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole (SKI II): induction of lysosomal sphingosine kinase 1 degradation. *Cell Physiol Biochem* **26**, 97–104.

164 Loveridge C, Tonelli F, Leclercq T, Lim KG, Long JS, Berdyshev E, Tate RJ, Natarajan V, Pitson SM, Pyne NJ *et al*. (2010) The sphingosine kinase 1 inhibitor 2-(p-hydroxyanilino)-4-(p-chlorophenyl) thiazole induces proteasomal degradation of sphingosine kinase 1 in mammalian cells. *J Biol Chem* **285**, 38841–38852.

Appendix 2

Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence

Heidi A Neubauer[1,2] and Stuart M Pitson[1,2,3]

F1000Research (2017) **5**: 2825; doi: 10.12688/f1000research.10336.2.

[1]Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, South Australia, Australia;
[2]School of Biological Sciences, University of Adelaide, Adelaide, South Australia, Australia;
[3]School of Medicine, University of Adelaide, Adelaide, South Australia, Australia;

Statement of Authorship

Title of Paper	Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence
Publication Status	Published
Publication Details	Neubauer HA, and Pitson SM (2017) Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence. *F1000Research* **5**: 2825; doi: 10.12688/f1000research.10336.2

Principal Author

Name of Principal Author (Candidate)	Heidi A Neubauer		
Contribution to the Paper	Designed and performed experiments, generated all figures and wrote the first draft of the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	4/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stuart M Pitson		
Contribution to the Paper	Conceived and assisted in editing the manuscript.		
Signature		Date	4/5/17

ANTIBODY VALIDATION ARTICLE

REVISED Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence [version 2; referees: 2 approved]

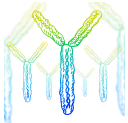
Heidi A. Neubauer [1,2], Stuart M. Pitson [1-3]

[1]Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, Australia
[2]School of Biological Sciences, University of Adelaide, Adelaide, Australia
[3]School of Medicine, University of Adelaide, Adelaide, Australia

v2 **First published:** 06 Dec 2016, **5**:2825 (doi: 10.12688/f1000research.10336.1)
Latest published: 23 Mar 2017, **5**:2825 (doi: 10.12688/f1000research.10336.2)

Abstract

Sphingosine kinase 2 (SK2) is a ubiquitously expressed lipid kinase that has important, albeit complex and poorly understood, roles in regulating cell survival and cell death. In addition to being able to promote cell cycle arrest and apoptosis under certain conditions, it has recently been shown that SK2 can promote neoplastic transformation and tumorigenesis *in vivo*. Therefore, well validated and reliable tools are required to study and better understand the true functions of SK2. Here, we compare two commercially available SK2 antibodies: a rabbit polyclonal antibody from Proteintech that recognizes amino acids 266-618 of human SK2a, and a rabbit polyclonal antibody from ECM Biosciences that recognizes amino acids 36-52 of human SK2a. We examine the performance of these antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence staining of endogenous SK2, using human HEK293 and HeLa cell lines, as well as mouse embryonic fibroblasts (MEFs). Furthermore, we assess the specificity of these antibodies to the target protein through the use of siRNA-mediated SK2 knockdown and SK2 knockout (*Sphk2*$^{-/-}$) MEFs. Our results demonstrate that the Proteintech anti-SK2 antibody reproducibly displayed superior sensitivity and selectivity towards SK2 in immunoblot analyses, while the ECM Biosciences anti-SK2 antibody was reproducibly superior for SK2 immunoprecipitation and detection by immunofluorescence staining. Notably, both antibodies produced non-specific bands and staining in the MEFs, which was not observed with the human cell lines. Therefore, we conclude that the Proteintech SK2 antibody is a valuable reagent for use in immunoblot analyses, and the ECM Biosciences SK2 antibody is a useful tool for SK2 immunoprecipitation and immunofluorescence staining, at least in the human cell lines employed in this study.



This article is included in the Antibody validations channel.

Open Peer Review

Referee Status: ✔ ✔

	Invited Referees 1	2
REVISED version 2 published 23 Mar 2017		
version 1 published 06 Dec 2016	✔ report	✔ report

1 **Maria Laura Allende**, National Institutes of Health (NIH) USA

2 **Dagmar Meyer zu Heringdorf**, Klinikum der Johann Wolfgang Goethe-Universität Germany

Discuss this article

Comments (0)

Corresponding authors: Heidi A. Neubauer (heidi.neubauer@sa.gov.au), Stuart M. Pitson (stuart.pitson@unisa.edu.au)

How to cite this article: Neubauer HA and Pitson SM. **Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence [version 2; referees: 2 approved]** *F1000Research* 2017, **5**:2825 (doi: 10.12688/f1000research.10336.2)

Grant information: This work was funded by an Australian Postgraduate Award, Royal Adelaide Hospital Dawes Scholarship and the University of South Australia (HN), and a National Health and Medical Research Council of Australia Project Grant (#626936) and Senior Research Fellowship (#1042589), and the Fay Fuller Foundation (SP).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 06 Dec 2016, **5**:2825 (doi: 10.12688/f1000research.10336.1)

REVISED **Amendments from Version 1**

Following the reviewers' comments, this revised version of the manuscript now includes additional comments in the Conclusion section addressing the low expression of SK2 protein in many cell lines, which may impact on immunoblotting and immunofluorescence detection. It also includes further discussion on the splice isoforms of human SK2.

See referee reports

Introduction

Sphingolipids are an important family of cellular molecules that form critical structural components of cell membranes, as well as performing numerous signaling functions[1]. Of the many enzymes responsible for the biosynthesis and metabolism of sphingolipids, the sphingosine kinases (SKs) are of particular interest to study as they catalyze the formation of sphingosine 1-phosphate (S1P), and in doing so can promote cell survival, proliferation, migration and angiogenesis[2]. Both sphingosine kinases, SK1 and SK2, have been shown to be upregulated in various human cancers and both have documented roles in mediating oncogenesis[3,4]. However, where SK1 and its roles in cancer development are relatively well characterized, SK2 remains somewhat enigmatic as, in addition to the pro-cancer functions it shares with SK1, SK2 can also facilitate cell cycle arrest and cell death[5,6].

SK2 is ubiquitously expressed in all cells and tissues, but is expressed most highly in the liver, kidney and brain[7]. At the mitochondria, SK2-generated S1P has been proposed to facilitate the activation of Bak and subsequent mitochondrial membrane permeabilisation and cytochrome c release[5]. Notably, SK2 can also function as an epigenetic regulator, where S1P produced by nuclear-localized SK2 can inhibit the activity of histone deacetylases 1/2 resulting in increased transcription of specific genes, such as cyclin-dependent kinase inhibitor *p21* and transcriptional regulator *c-fos*[8]. As SK1 does not appear to localize as prominently to these internal organelles, it is believed that the subcellular localization of SK2 is critical for the additional functions it performs. However, the mechanisms regulating the localization and functions of SK2, allowing it to switch between pro-apoptotic and pro-survival under certain conditions, remain poorly understood.

In order to study SK2 and better characterize its roles in normal cells as well as in cancer, reliable and properly validated tools are required. Antibody-based methods, such as immunoblotting (IB), immunoprecipitation (IP) and immunofluorescence (IF), are particularly useful as tools to examine and visualize important aspects of SK2 biology, like subcellular localization, expression and interaction with regulatory proteins. A number of groups in the field have taken to generating their own in-house SK2-specific polyclonal antibodies[9,10], but to our knowledge there has been no systematic validation of commercially available SK2 antibodies. Here, we compare two commercially available SK2 antibodies, and validate the suitability of their use in IB, IP and IF using various human and mouse cells lines. We have examined a rabbit polyclonal SK2 antibody from Proteintech, which is raised against amino acids 266–618 of recombinant human SK2a, and a rabbit polyclonal SK2 antibody from ECM Biosciences, which is raised against a synthetic peptide corresponding to amino acids 36–52 of human SK2a. The Proteintech SK2 antibody has been previously utilized in one publication for IB[11], and the ECM Biosciences SK2 antibody has been used in multiple publications for IB[12–15] and for IF[16].

Materials and methods

Antibody details

The following SK2 antibodies were assessed: rabbit polyclonal anti-SK2 (ECM Biosciences; anti-Sphingosine Kinase 2 (N-terminal region); #SP4621, lot #1) and rabbit polyclonal anti-SK2 (Proteintech Group, Inc; anti-SPHK2; #17096-1-AP, lot #00010361). The ECM Biosciences SK2 antibody was raised against a synthetic peptide coupled to keyhole limpet hemocyanin (KLH), corresponding to amino acids 36–52 of human SK2a, and was affinity purified with the SK2 peptide (without KLH). It is reported by the manufacturer to have cross-reactivity with rat and mouse SK2 [human, mouse and rat SK2 share 100% sequence identity in this region (determined using the align tool and protein sequences from www.uniprot.org)], and has been assessed by the manufacturer for use in IB and enzyme-linked immunosorbent assay (ELISA). The Proteintech SK2 antibody was raised against truncated recombinant GST-tagged human SK2a (amino acid residues 266–618 generated in *Escherichia coli* using the PGEX-4T plasmid). The SK2 target antibodies were then affinity purified using 6xHis-tagged antigen protein (to remove GST-specific antibodies) and then again with the immunising GST-tagged antigen protein. It is reported to have cross-reactivity with rat and mouse SK2 [80.2% sequence identity between human and mouse SK2, and 80.2% sequence identity between human and rat SK2 in this region (determined using the align tool and protein sequences from www.uniprot.org)], and according to the manufacturer can be employed for IB, ELISA, IP and immunohistochemistry. Mouse anti-α-tubulin (DM1A; Abcam; #ab7291) is a mouse monoclonal antibody, which was used as a loading control for IB analyses, at a dilution of 1:5,000. All antibody details, including information for secondary antibodies used, are provided in Table 1.

Cell culture

Human embryonic kidney (HEK) 293 cells (CellBank Australia; #85120602) and HeLa human cervical cancer cells (ATCC; #CCL-2) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific Inc.), containing 10% heat-inactivated fetal bovine serum (FBS; Bovagen), 1 mM HEPES, penicillin (1.2 mg/ml) and streptomycin (1.6 mg/ml). Cells were grown at 37°C with 5% CO_2 in a humidified incubator. Primary mouse embryonic fibroblasts (MEFs) were generated from both wildtype (WT) C57/Bl6 and *Sphk2*$^{-/-}$ C57/Bl6[17] mouse embryos at 14.5 days *post coitum*. The fibroblasts were isolated and cultured as described above, except they were maintained at 37°C in a humidified atmosphere with 10% CO_2.

RNAi knockdown of SK2

siRNA-mediated knockdown of SK2 was performed using human SPHK2 siGENOME SMARTpool siRNA (Dharmacon), which targets the following sequences: CCACUGCCCUCACCUGUCU, GCUCCUCCAUGGCGAGUUU, GAGACGGGCUGCUCCAUGA, CAAGGCAGCUCUACACUCA. Cells were seeded and grown to a cell density of approximately 50%, and were then transfected

with 30 nM (final concentration) of either human SK2 siRNA or siGENOME non-targeting siRNA control pool (Dharmacon), using Lipofectamine RNAiMAX (Life Technologies), as per the manufacturer's protocol. Cells were incubated with the siRNA complexes at 37°C for 48 h.

Immunoblotting

Specific details for all reagents used can be found in Table 2. Cells were pelleted by centrifugation (400 × *g*, 5 min, 4°C) and washed in cold phosphate buffered saline (PBS). Cell pellets were resuspended in extraction buffer [EB; 50 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.05% Triton X-100, 2 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM dithiothreitol (DTT) and protease inhibitor cocktail (Roche)], and lysed by bath sonication (4× 30 sec on/off). Lysates were clarified (17,000 × *g*, 15 min, 4°C) and equal amounts of protein [as determined by a Bradford protein assay (Bio-Rad Laboratories)] were mixed with 5× Laemmli sample buffer, boiled at 100°C for 5 min, and separated by SDS-PAGE on a Criterion™ XT Bis-Tris 4–12% gradient gel (Bio-Rad Laboratories). Proteins were then transferred to nitrocellulose membrane (Pall Life Sciences) at 400 mA for 1 h. Membranes were blocked with 5% skim milk in PBS containing 0.1% Triton X-100 (PBS-T) for 1 h at room temperature, with gentle rocking. Membranes were probed with rabbit anti-SK2 antibodies diluted in Signal Boost primary antibody diluent at 1:1,000 (ECM Biosciences: 1 µg/ml;

Table 1. Details of primary and secondary antibodies.

Antibody	Manufacturer	Catalogue number	RRID
Rabbit anti-SK2	ECM Biosciences	SP4621	AB_2619719
Rabbit anti-SK2	Proteintech	17096-1-AP	AB_10598479
Mouse anti-α-tubulin	Abcam	ab7291	AB_2241126
Goat anti-rabbit IgG HRP	Thermo Fisher Scientific	31460	AB_228341
Goat anti-mouse IgG HRP	Thermo Fisher Scientific	31430	AB_228307
Normal rabbit IgG	Cell Signaling Technology	2729	AB_2617119
Goat anti-rabbit Alexa Fluor 488	Thermo Fisher Scientific	A-11008	AB_143165

Table 2. Reagents used for immunoblotting.

Process	Reagent	Manufacturer	Catalogue number	Concentration
Protein Concentration Assay	Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories	500-0006	Proprietary
5x Laemmli Protein Loading Buffer	Tris/HCl pH 8.0 EDTA Dithiothreitol (DTT) SDS Glycerol Bromophenol blue	Invitrogen Merck Millipore Sigma Aldrich Sigma Aldrich Chem-Supply Sigma Aldrich	15504-020 108421 D0632 75746 GA010 B5525	50 mM 5 mM 100 mM 5% w/v 50% v/v 0.1% w/v
SDS-PAGE Running Buffer 1×	Tris/HCl pH 7.3 MES SDS EDTA	Invitrogen Amresco Sigma Aldrich Merck Millipore	15504-020 E169 75746 108421	50 mM 50 mM 0.1% w/v 1 mM
SDS-PAGE Transfer Buffer 1×	Tris Glycine Methanol	Invitrogen MP Biomedicals RCI Labscan Ltd	15504-020 04808831 AR1115	25 mM 192 mM 20% v/v
Blocking Buffer, Antibody Diluent, Wash Buffer	Skim milk powder PBS-T (PBS, Triton X-100)	Diploma Merck Millipore	 108643	5% w/v 0.1% v/v
Antibody Diluent	SignalBoost Immunoreaction Enhancer Kit	Calbiochem	407207	Proprietary
Enhanced Chemiluminescence	Clarity Western ECL Substrate	Bio-Rad Laboratories	170-5061	Proprietary

Proteintech: 687 ng/ml) overnight at 4°C with gentle rocking. Alternatively, membranes were probed with mouse anti-α-tubulin antibody diluted in 5% skim milk in PBS-T at 1:5,000 (200 ng/ml) for 1 h at room temperature, with gentle rocking. Following primary antibody incubation, membranes were washed 3 × 5 min in 5% skim milk in PBS-T at room temperature with gentle agitation. Membranes were probed with goat anti-rabbit horseradish peroxidase (HRP) secondary antibody diluted in Signal Boost secondary antibody diluent at 1:10,000 (40 ng/ml), or goat anti-mouse HRP secondary antibody diluted in 5% skim milk in PBS-T at 1:10,000 (40 ng/ml), for 1 h at room temperature, with gentle rocking. Membranes were washed 3 × 5 min in 5% skim milk in PBS-T at room temperature with gentle agitation, and proteins were visualized using enhanced chemiluminescence (ECL) on a LAS-4000 luminescence image analyser (Fujifilm). Exposure times are indicated in the figure legends for each blot.

Immunoprecipitation

HEK293 cell lysates were prepared as for immunoblotting, with the exclusion of DTT in the extraction buffer (EB–DTT). Protein concentration was determined from clarified lysates, and 800 µg total protein was transferred to fresh tubes and made up to 300 µl in EB–DTT. In total, 20 µl of diluted lysate was removed and mixed with 5× Laemmli sample buffer for immunoblot analysis. Immunoprecipitation was performed using the µMacs magnetic system (Miltenyi Biotec; see Table 3 for reagent details). Rabbit anti-SK2 antibodies (4 µg; ECM Biosciences 1:75 or Proteintech 1:52) or rabbit IgG isotype control antibody (4 µg), as well as 50 µl each of Protein A and G µBeads were added to the lysate, mixed gently and incubated on ice for 30 min. µMacs columns were placed onto a magnetic stand, equilibrated with 200 µl EB–DTT, and lysate/antibody/bead complexes were run through the columns. Columns were washed four times with 200 µl EB–DTT, and once with 100 µl low salt wash buffer, before the addition of 20 µl hot 1× Laemmli sample buffer for 5 min. Immunoprecipitates were then eluted with 50 µl hot 1× Laemmli sample buffer and collected in fresh tubes. Samples were boiled and 25 µl was analysed by SDS-PAGE and immunoblotting as described above.

Immunofluorescence staining

Cell lines were seeded onto coverslips coated with poly-L-lysine (Sigma-Aldrich) in a 12-well plate (HEK293: 1.5 $\times 10^5$ cells/well; HeLa: 7.5 $\times 10^4$ cells/well; MEF: 3 $\times 10^4$ cells/well). Cells were allowed to bed down overnight at 37°C in DMEM with 10% FBS. The following day (for HEK293 and HeLa cells), cells were treated with siRNA as described above, and incubated for 48 h. The following immunofluorescence staining protocol was then performed, with all steps carried out at room temperature (see Table 4 for reagent details). Cells were washed once in PBS, fixed in 4% paraformaldehyde for 10 min, washed three times in

Table 3. Reagents used for immunoprecipitation

Process	Reagent	Manufacturer	Catalogue number	Concentration
Magnetic Labelling (µMacs)	Protein A µBeads Protein G µBeads	Miltenyi Biotech	130-071-001 130-071-101	Proprietary
Washing Columns	EB -DTT (described in methods) Low Salt Wash Buffer: Tris/HCl pH 7.5	Invitrogen	15504-020	Various 20 mM
1× Laemmli Protein Loading Buffer	Tris/HCl pH 8.0 EDTA Dithiothreitol (DTT) SDS Glycerol Bromophenol blue	Invitrogen Merck Millipore Sigma Aldrich Sigma Aldrich Chem-Supply Sigma Aldrich	15504-020 108421 D0632 75746 GA010 B5525	10 mM 1 mM 20 mM 1% w/v 10% v/v 0.02% w/v

Table 4. Reagents used for immunofluorescence

Process	Reagent	Manufacturer	Catalogue number	Concentration
Washing	PBS			1x
Fixing	Paraformaldehyde	Santa Cruz Biotechnology	sc-281692	4% in PBS
Permeabilizing, Washing	PBS-T			0.1% Triton X-100 in PBS
Blocking, Antibody Diluent	Bovine Serum Albumin (BSA) PBS-T	Sigma Aldrich	A7906	3% w/v
Nuclear Staining	DAPI (4',6-diamidino-2-phenylindole)	Roche	10236276001	0.2 µg/ml in PBS
Mounting	Fluorescent Mounting Medium	Dako	S3023	Proprietary

PBS-T, and permeabilized for 10 min in PBS-T. Cells were then blocked in 3% bovine serum albumin (BSA) in PBS-T for 30 min, and incubated for 1 h with anti-SK2 antibodies (4 µg/ml; ECM Biosciences 1:250 and Proteintech 1:172) diluted in 3% BSA/PBS-T. Cells were washed five times in PBS-T, and then incubated with goat anti-rabbit AlexaFluor 488 secondary antibody (1:500) for 1 h. After washing five times in PBS-T, cell nuclei were stained with DAPI (0.2 µg/ml) for 5 min. Cells were then washed twice in PBS, coverslips were partially dried and mounted onto slides using fluorescence mounting medium (Dako), and then left to set overnight. Fluorescence microscopy and imaging were performed using a Carl Zeiss LSM 700 confocal microscope, with Zen 2011 (Black Edition) version 8.1.5.484 software. All microscope settings, including gains, were kept constant for each cell line, allowing direct comparison between antibodies.

Controls

Various controls were used in these studies. Immunoprecipitations were performed with IgG isotype control antibody to control for any non-specific binding of proteins to the antibodies. Primary antibodies were also omitted from the immunofluorescence protocol to control for background fluorescence of the secondary antibody alone. siRNA-mediated SK2 knockdown as well as *Sphk2*$^{-/-}$ MEFs were utilized to verify the specificity of the SK2 antibodies to their target.

Results

Proteintech SK2 antibody demonstrates target specificity and sensitivity by immunoblot

Both SK2 antibodies examined in the present study are reported by their respective manufacturers to be able to detect endogenous SK2 by IB. To determine the selectivity of the anti-SK2 antibodies, we performed IB analyses using two human cell lines (HEK293 and HeLa) that had been treated with either scrambled control or SK2-directed siRNA, as well as WT and *Sphk2*$^{-/-}$ MEFs. The Proteintech anti-SK2 antibody detected a single prominent band at the correct molecular weight for SK2 (~65 kDa), which was decreased or absent in the knockdown and knockout lines (Figure 1A; Dataset 1[18]). Some faint non-specific bands were also detected in both the WT and *Sphk2*$^{-/-}$ MEF lysates by this antibody, which were not observed in the human cell lines. The ECM Biosciences anti-SK2 antibody did not appear to be very sensitive towards SK2, as no band was detected at the expected size in the HeLa lysates, and only very faint bands were present in the HEK293 and MEF lysates that were reduced or absent in the knockdown or knockout lines (Figure 1B). Furthermore, numerous prominent non-specific bands were present in all lysates, particularly in the MEF lines, indicating a lack of selectivity of this antibody towards SK2. Therefore, the Proteintech anti-SK2 antibody appears to be superior for use in IB, demonstrating both selectivity and sensitivity in the detection of endogenous SK2, particularly in the human cell lines tested.

Dataset 1. Raw images of all experimental replicates for Figure 1, immunoblotting experiments

http://dx.doi.org/10.5256/f1000research.10336.d145416

This dataset includes uncropped blots for all experimental replicates that are represented in Figure 1. Treatments and immunoblot methods were performed as outlined in Figure 1. Blots were probed with Proteintech rabbit polyclonal anti-SK2 antibody (**A–D**) or ECM Biosciences rabbit polyclonal anti-SK2 antibody (**E–H**). Anti-α-tubulin antibody was used as a loading control. O/E SK2 = lysate from cells overexpressing SK2, used as a positive control to validate the correct size of SK2. Asterisks denote other protein bands that were probed using other antibodies not relevant to this study, prior to anti-α-tubulin.

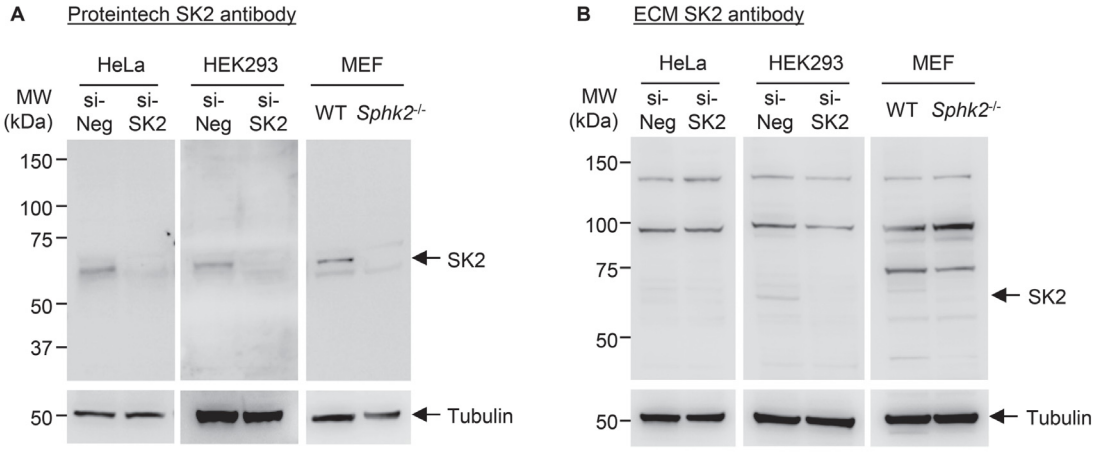


Figure 1. Immunoblot analyses of endogenous SK2 in multiple cell lines using two commercially available rabbit polyclonal anti-SK2 antibodies. Immunoblot analyses of lysates from HEK293 and HeLa cells treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), and lysates from wildtype (WT) or *Sphk2*$^{-/-}$ MEFs. An equal amount (40 µg) of total protein from each sample was run in duplicate. After transferring to nitrocellulose and blocking, the membrane was separated and duplicate samples were probed with either (**A**) Proteintech rabbit anti-SK2 antibody or (**B**) ECM Biosciences rabbit anti-SK2 antibody. SK2 membranes were imaged using a 4 min exposure. The expected band size for SK2 is ~65 kDa. Membranes were re-probed with mouse anti-α-tubulin antibody as a loading control (2 min exposure), which was detected at 55 kDa as expected. Consistent results were observed from 2-3 (HEK293 and MEF) or 3-4 (HeLa) independent experiments for each antibody.

ECM Biosciences SK2 antibody is able to specifically immunoprecipitate SK2

We also examined whether either of the commercial anti-SK2 antibodies could immunoprecipitate SK2 from cell lysates. The Proteintech anti-SK2 antibody is suggested by the manufacturer to be useful for IP, whereas to our knowledge the ECM Biosciences anti-SK2 antibody has not been previously tested for use in this application. Initially, using lysates from HEK293 cells, we found that the Proteintech anti-SK2 antibody was sometimes able to IP a band at the correct size for SK2 (Figure 2A); however this was not consistent with each experimental repeat and other proteins were also immunoprecipitated to a varying extent by this antibody that were not present in the IgG isotype control (Dataset 2[19]).

Conversely, the ECM Biosciences anti-SK2 antibody was able to consistently and cleanly IP a protein of the same size as SK2 from cell lysates, with almost no non-specific bands observed (Figure 2B; Dataset 2[19]). The protein immunoprecipitated by the ECM Biosciences antibody was considerably enriched from the cell lysate and was strongly detectable by this antibody, which was unable to detect SK2 in the lysate input sample, consistent with Figure 1B. To determine if this band was in fact SK2, the ECM Biosciences anti-SK2 antibody was then used to immunoprecipitate SK2 from HEK293 lysates treated with either scrambled control or SK2-directed siRNA. SK2 knockdown consistently resulted in reduced intensity of the band enriched by this antibody (Figure 2C; Dataset 2[19]), confirming that the ECM Biosciences anti-SK2 antibody can selectively IP endogenous SK2.

Dataset 2. Raw images of all experimental replicates for Figure 2, immunoprecipitation experiments

http://dx.doi.org/10.5256/f1000research.10336.d145417

This dataset includes uncropped blots for all experimental replicates that are represented in Figure 2. SK2 immunoprecipitation from HEK293 cell lysate, and subsequent immunoblotting, were performed using either (**A–C**) Proteintech rabbit anti-SK2 antibody or (**D–F**) ECM Biosciences rabbit anti-SK2 antibody. (**G–I**) SK2 immunoprecipitation from HEK293 cell lysates (of equal protein) treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), and subsequent immunoblotting, were performed using ECM Biosciences rabbit anti-SK2 antibody.

ECM Biosciences SK2 antibody can specifically detect SK2 by immunofluorescence staining

Finally, we examined whether these commercially available SK2 antibodies could selectively detect SK2 by IF. Neither antibody has been reported to be tested for use in IF by their respective manufacturers; however, the Proteintech SK2 antibody is recommended for immunohistochemistry. Using IF staining methods routinely performed in our laboratory, we compared the two anti-SK2 antibodies using HeLa, HEK293 and MEF cell lines. The Proteintech anti-SK2 antibody produced minimal staining in all cell lines tested (Figure 3A–C), and consequently there was no observable differences between the control cells and those with SK2 knockdown (in the human cell lines) or SK2 knockout (in the *Sphk2*$^{-/-}$ MEF line).

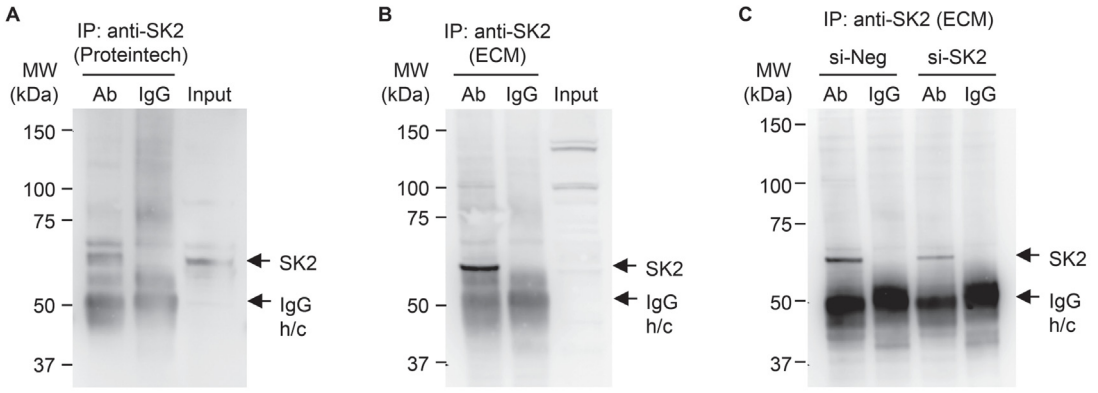


Figure 2. Comparison of two commercially available rabbit polyclonal anti-SK2 antibodies for immunoprecipitation of SK2 from HEK293 cell lysates. SK2 was immunoprecipitated from HEK293 cell lysate using either (**A**) Proteintech rabbit anti-SK2 antibody or (**B**) ECM Biosciences rabbit anti-SK2 antibody. Normal rabbit IgG antibody was used as an isotype control. Immunoprecipitates (and 40 µg lysate input) were subjected to immunoblot analyses and probed with (**A**) Proteintech rabbit anti-SK2 antibody or (**B**) ECM Biosciences rabbit anti-SK2 antibody. Membranes were imaged using a 4 min exposure. Images are representative of three independent experiments for each antibody. (**C**) SK2 was immunoprecipitated from HEK293 cell lysates (of equal protein) that had been treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), using ECM Biosciences rabbit anti-SK2 antibody. Immunoprecipitates were subjected to immunoblot analyses and probed with ECM Biosciences rabbit anti-SK2 antibody. Membrane was imaged using a 4 min exposure. Image is representative of three independent experiments. IgG h/c = IgG heavy chain.

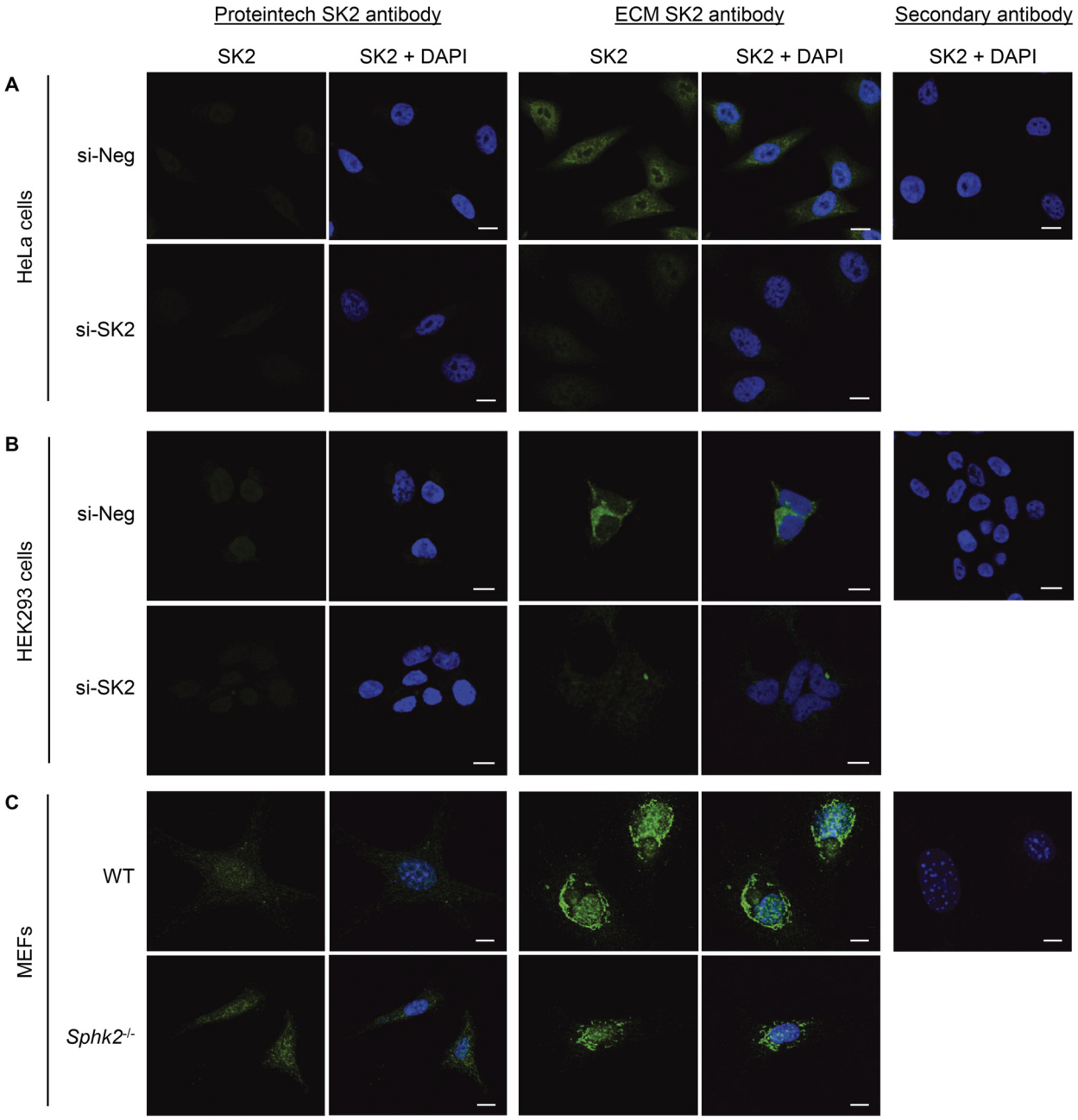


Figure 3. Immunofluorescence staining analyses of endogenous SK2 in multiple cell lines using two commercially available rabbit polyclonal anti-SK2 antibodies. (**A**) HeLa or (**B**) HEK293 cells were treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), and endogenous SK2 (green) was visualised by immunofluorescence staining and confocal microscopy, using Proteintech rabbit anti-SK2 antibody or ECM Biosciences rabbit anti-SK2 antibody. (**C**) Wildtype (WT) or *Sphk2*$^{-/-}$ MEFs were seeded, and endogenous SK2 (green) was visualised by immunofluorescence staining and confocal microscopy, using Proteintech rabbit anti-SK2 antibody or ECM Biosciences rabbit anti-SK2 antibody. Nuclei were stained with DAPI (blue). For each cell line, background staining was examined by staining cells (si-Neg or WT cells) with secondary antibody and DAPI only, and collecting images using both 488nm and 405nm lasers (SK2 + DAPI). Images were taken at 40× magnification; scale bars = 10 μm. Images shown are representative of more than 100 cells from each experiment, and these results were consistent over three independent experiments for each cell line.

The ECM Biosciences anti-SK2 antibody did result in consistently observable staining in HeLa and HEK293 cells, which was substantially reduced upon knockdown of SK2 (Figure 3A and B; Dataset 3[20]). Hence, in these cells the ECM Biosciences antibody was able to selectively detect SK2 by IF. Interestingly, in HeLa cells SK2 detected by the ECM Biosciences antibody was predominantly nuclear with some peri-nuclear/cytoplasmic localization, whereas in HEK293 cells SK2 was cytoplasmic and nuclear-exclusion, which is consistent with previous reports for these cell lines[9]. However, the ECM Biosciences anti-SK2 antibody produced very strong peri-nuclear staining/puncta in both the WT and *Sphk2*$^{-/-}$ MEF lines (Figure 3C), suggesting that this staining was not specific for SK2 and represents non-specific binding to other proteins in this cell type. Increased non-specific binding of both SK2 antibodies to other proteins in the MEF lines was also observed when used for IB, so this cell type may not be suitable for use with these antibodies. It will remain to be determined if the same level of non-specificity is also observed in other mouse cell lines and tissues.

Dataset 3. Raw images of additional experimental replicates for Figure 3, immunofluorescence experiments

http://dx.doi.org/10.5256/f1000research.10336.d145418

This dataset includes additional images from experimental replicates that demonstrate reproducibility of the images presented in Figure 3. Treatments and immunofluorescence staining methods were performed as outlined in Figure 3. Images were taken at 40× magnification; scale bars = 10 µm.

Conclusion

Commercially available antibodies raised against the SKs can be notorious, in our experience, for not being very sensitive or selective. This is accentuated by the apparent low abundance of SK proteins in most cells, which can be in some cases hundreds of fold lower than when overexpressed[4,21]. A number of groups have generated their own SK-specific antibodies; however, many published studies have reported the use of different commercial SK2 antibodies, sometimes without proper controls or validation of selectivity. Hence, we have compared two commercially available SK2 antibodies and evaluated their selectivity towards SK2 in multiple applications using siRNA-mediated SK2 knockdown or *Sphk2*$^{-/-}$ MEF lines.

We found that the SK2 antibody from Proteintech was able to consistently detect a prominent band at the correct molecular weight by IB, and this band was confirmed to be SK2 by knockdown and knockout analyses, confirming the specificity of this antibody. The Proteintech antibody also resulted in virtually no non-specific detection of any other proteins in the HEK293 and HeLa lysates, but some additional faint bands were present in the MEF lines. This antibody has been tested by IB on various mouse tissue lysates by the manufacturer and many of these also gave rise to non-specific bands, so this will need to be considered and further validation may be required if this antibody is intended for use with mouse cells or tissues. Occasionally more than one band was detected in the human cell lines by the Proteintech SK2 antibody, but these bands also seemed to be reduced by SK2 knockdown. There are two characterized human SK2 isoforms[22], so these bands may represent different SK2 variants and/or post-translationally modified forms of SK2. Notably, although a previous report suggested that the mRNA of the 69 kDa SK2b (SK2-L) isoform is the most abundant in many human cell lines, including HeLa cells[6], our studies clearly suggest that the SK2a (SK2-S) isoform is the predominant form of SK2 protein in HeLa and HEK293 cells.

In contrast, the present results revealed that the sensitivity of the ECM Biosciences antibody towards SK2 by IB was poor, with a faint band detected only in the HEK293 and MEF lines that was not present in the knockdown/knockout lysates. Furthermore, the ECM Biosciences SK2 antibody produced many intense non-specific bands in all cell lines tested, demonstrating poor selectivity. This antibody has been used for IB analyses in multiple publications[12–15], suggesting that it may be more suitable with other cell/tissue systems or conditions not tested here. However, in agreement with our findings, the IB analysis performed by the manufacturer also showed various prominent non-specific bands in HeLa lysates. Therefore, at least in our hands, the ECM Biosciences SK2 antibody was not ideal for this application.

However, the ECM Biosciences anti-SK2 antibody was superior for the IP of endogenous SK2, as it was able to cleanly and substantially enrich the protein from lysates and was confirmed by SK2-specific knockdown to be selective for SK2 in this application. This antibody will therefore be a useful tool to study SK2 function and regulation, as it can be applied to other applications requiring IP, such as chromatin-IP (ChIP) and rapid immunoprecipitation mass spectrometry of endogenous protein (RIME). In the present study, the Proteintech anti-SK2 antibody was inconsistent in its ability to IP protein at the correct size for SK2, and other bands of equal intensity were sometimes present.

Similarly, we found that the ECM Biosciences anti-SK2 antibody was able to selectively detect endogenous SK2 by IF staining in two human cell lines, HeLa and HEK293 cells. Furthermore, the observed localization of SK2 in these two cells lines was consistent with previous reports[9]. The selectivity of this antibody was validated by knockdown of SK2 in these cell lines, where most of the staining was reduced. A very small level of staining was still visible after SK2 siRNA treatment, possibly owing to the inherently incomplete nature of siRNA-mediated knockdown. However, we were unable to corroborate these data with SK2 knockout using the MEF lines, as considerable non-specific staining was present in this cell type, as was found for IB. Using identical methods, there was minimal staining observed using the Proteintech anti-SK2 antibody for IF, and therefore the selectivity of this antibody towards SK2 in this application could not be properly examined.

During this study, methods routinely used in our laboratory were employed, and where applicable, recommendations from the manufacturers for antibody dilutions and concentrations were followed. It is possible that further optimization for these antibodies may allow them to perform better in the applications where they were deemed not optimal. However, as our main aim was to directly compare the performance of these two antibodies, and given at least one of the antibodies performed well for each

application using our standard methods, further optimization was not performed.

Overall, based on the data from this study we would recommend the use of the Proteintech SK2 antibody for IB, as it demonstrated selectivity and sensitivity towards endogenous SK2 in the human cell lines tested. Furthermore, we recommend the ECM Biosciences SK2 antibody for IP of endogenous SK2 and for visualizing SK2 by IF methods. Furthermore, both antibodies detected non-specific proteins by IB and IF in the mouse fibroblasts used, and hence further validation will be required to determine if this is the case for other mouse cells or tissues.

Data availability

Dataset 1: Raw images of all experimental replicates for Figure 1, immunoblotting experiments. This dataset includes uncropped blots for all experimental replicates that are represented in Figure 1. Treatments and immunoblot methods were performed as outlined in Figure 1. Blots were probed with Proteintech rabbit polyclonal anti-SK2 antibody (**A–D**) or ECM Biosciences rabbit polyclonal anti-SK2 antibody (**E–H**). Anti-α-tubulin antibody was used as a loading control. O/E SK2 = lysate from cells overexpressing SK2, used as a positive control to validate the correct size of SK2. Asterisks denote other protein bands that were probed using other antibodies not relevant to this study, prior to anti-α-tubulin.

DOI, 10.5256/f1000research.10336.d145416[18]

Dataset 2: Raw images of all experimental replicates for Figure 2, immunoprecipitation experiments. This dataset includes uncropped blots for all experimental replicates that are represented in Figure 2. SK2 immunoprecipitation from HEK293 cell lysate, and subsequent immunoblotting, were performed using either (**A–C**) Proteintech rabbit anti-SK2 antibody or (**D–F**) ECM Biosciences rabbit anti-SK2 antibody. (**G–I**) SK2 immunoprecipitation from HEK293 cell lysates (of equal protein) treated with scrambled control siRNA (si-Neg) or SK2 siRNA (si-SK2), and subsequent immunoblotting, were performed using ECM Biosciences rabbit anti-SK2 antibody.

DOI, 10.5256/f1000research.10336.d145417[19]

Dataset 3: Raw images of additional experimental replicates for Figure 3, immunofluorescence experiments. This dataset includes additional images from experimental replicates that demonstrate reproducibility of the images presented in Figure 3. Treatments and immunofluorescence staining methods were performed as outlined in Figure 3. Images were taken at 40× magnification; scale bars = 10 μm.

DOI, 10.5256/f1000research.10336.d145418[20]

Author contributions

SP conceived the study. HN designed and carried out the experiments, and prepared the first draft of the manuscript. Both authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

This work was funded by an Australian Postgraduate Award, Royal Adelaide Hospital Dawes Scholarship and the University of South Australia (HN), and a National Health and Medical Research Council of Australia Project Grant (#626936) and Senior Research Fellowship (#1042589), and the Fay Fuller Foundation (SP).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We would like to thank Dr Briony Gliddon for generating the primary mouse embryonic fibroblasts used in this study.

References

1. Pitson SM: **Regulation of sphingosine kinase and sphingolipid signaling.** *Trends Biochem Sci.* 2011; **36**(2): 97–107.
PubMed Abstract | Publisher Full Text
2. Pyne NJ, Pyne S: **Sphingosine 1-phosphate and cancer.** *Nat Rev Cancer.* 2010; **10**(7): 489–503.
PubMed Abstract | Publisher Full Text
3. Xia P, Gamble JR, Wang L, *et al.*: **An oncogenic role of sphingosine kinase.** *Curr Biol.* 2000; **10**(23): 1527–1530.
PubMed Abstract | Publisher Full Text
4. Neubauer HA, Pham DH, Zebol JR, *et al.*: **An oncogenic role for sphingosine kinase 2.** *Oncotarget.* 2016.
PubMed Abstract | Publisher Full Text
5. Chipuk JE, McStay GP, Bharti A, *et al.*: **Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis.** *Cell.* 2012; **148**(5): 988–1000.
PubMed Abstract | Publisher Full Text | Free Full Text
6. Okada T, Ding G, Sonoda H, *et al.*: **Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis.** *J Biol Chem.* 2005; **280**(43): 36318–36325.
PubMed Abstract | Publisher Full Text
7. Liu H, Sugiura M, Nava VE, *et al.*: **Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform.** *J Biol Chem.* 2000; **275**(26): 19513–19520.
PubMed Abstract | Publisher Full Text
8. Hait NC, Allegood J, Maceyka M, *et al.*: **Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate.** *Science.* 2009; **325**(5945): 1254–1257.
PubMed Abstract | Publisher Full Text | Free Full Text
9. Igarashi N, Okada T, Hayashi S, *et al.*: **Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis.** *J Biol Chem.* 2003; **278**(47): 46832–46839.
PubMed Abstract | Publisher Full Text
10. Hait NC, Sarkar S, Le Stunff H, *et al.*: **Role of sphingosine kinase 2 in cell migration toward epidermal growth factor.** *J Biol Chem.* 2005; **280**(33): 29462–29469.
PubMed Abstract | Publisher Full Text
11. Liu X, Ren K, Suo R, *et al.*: **ApoA-I induces S1P release from endothelial cells through ABCA1 and SR-BI in a positive feedback manner.** *J Physiol Biochem.* 2016; **72**(4): 657–667.
PubMed Abstract | Publisher Full Text
12. Bruno G, Cencetti F, Pertici I, *et al.*: **CTGF/CCN2 exerts profibrotic action in myoblasts via the up-regulation of sphingosine kinase-1/S1P$_3$ signaling axis: Implications in the action mechanism of TGFβ.** *Biochim Biophys Acta.* 2015;

1851(2): 194–202.
PubMed Abstract | Publisher Full Text

13. Wallington-Beddoe CT, Powell JA, Tong D, *et al.*: **Sphingosine kinase 2 promotes acute lymphoblastic leukemia by enhancing *MYC* expression.** *Cancer Res.* 2014; **74**(10): 2803–2815.
PubMed Abstract | Publisher Full Text

14. Liu W, Ning J, Li C, *et al.*: **Overexpression of Sphk2 is associated with gefitinib resistance in non-small cell lung cancer.** *Tumour Biol.* 2016; **37**(5): 6331–6336.
PubMed Abstract | Publisher Full Text

15. Sun E, Zhang W, Wang L, *et al.*: **Down-regulation of Sphk2 suppresses bladder cancer progression.** *Tumour Biol.* 2016; **37**(1): 473–478.
PubMed Abstract | Publisher Full Text

16. Reid SP, Tritsch SR, Kota K, *et al.*: **Sphingosine kinase 2 is a chikungunya virus host factor co-localized with the viral replication complex.** *Emerg Microbes Infect.* 2015; **4**(10): e61.
PubMed Abstract | Publisher Full Text | Free Full Text

17. Mizugishi K, Yamashita T, Olivera A, *et al.*: **Essential role for sphingosine kinases in neural and vascular development.** *Mol Cell Biol.* 2005; **25**(4): 11113–11121.
PubMed Abstract | Publisher Full Text | Free Full Text

18. Neubauer H, Pitson S: **Dataset 1 In: Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence.** *F1000Research.* 2016.
Data Source

19. Neubauer H, Pitson S: **Dataset 2 In: Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence.** *F1000Research.* 2016.
Data Source

20. Neubauer H, Pitson S: **Dataset 3 In: Validation of commercially available sphingosine kinase 2 antibodies for use in immunoblotting, immunoprecipitation and immunofluorescence.** *F1000Research.* 2016.
Data Source

21. Pitson SM, D'andrea RJ, Vandeleur L, *et al.*: **Human sphingosine kinase: purification, molecular cloning and characterization of the native and recombinant enzymes.** *Biochem J.* 2000; **350**(Pt 2): 429–41.
PubMed Abstract | Publisher Full Text | Free Full Text

22. Neubauer HA, Pitson SM: **Roles, regulation and inhibitors of sphingosine kinase 2.** *FEBS J.* 2013; **280**(21): 5317–5336.
PubMed Abstract | Publisher Full Text

Appendix 3

An oncogenic role for sphingosine kinase 2

Heidi A Neubauer[1,2], Duyen H Pham[1,2], Julia R Zebol[1], Paul AB Moretti[1], Amanda L Peterson[3], Tamara M Leclercq[1], Huasheng Chan[1,2], Jason A Powell[1,4], Melissa R Pitman[1], Michael S Samuel[1,4], Claudine S Bonder[1,2,4], Darren J Creek[3], Briony L Gliddon[1] and Stuart M Pitson[1,2,4]

Oncotarget (2016) **7**: 64886-64899

[1]Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, South Australia, Australia;
[2]School of Biological Sciences, University of Adelaide, Adelaide, South Australia, Australia;
[3]Monash Institute of Pharmaceutical Science, Monash University, Parkville, Victoria, Australia;
[4]School of Medicine, University of Adelaide, Adelaide, South Australia, Australia.

Statement of Authorship

Title of Paper	An oncogenic role for sphingosine kinase 2
Publication Status	Published
Publication Details	Neubauer HA, Pham DH, Zebol JR, Moretti PAB, Peterson AL, Leclercq TM, Chan H, Powell JA, Pitman MR, Samuel MS, Bonder CS, Creek DJ, Gliddon BL and Pitson SM (2016) An oncogenic role for sphingosine kinase 2. *Oncotarget* 7: 64886-64899

Principal Author

Name of Principal Author (Candidate)	Heidi A Neubauer
Contribution to the Paper	Performed many of the experiments and analysed data. Specifically, generated the data presented in Figures 1A, 1C, 2A-C, 3A, 4A-E, 4G, 5A-C, Supplementary Figures 1A-B, 2A-B, 3A-B and 4B, performed quantitation of the data in Figure 3C, performed imaging of sections shown in Figure 4F, and performed all cell preparation work for Figure 6A-E. Also made all figures and wrote the first draft of the manuscript.
Overall percentage (%)	60%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date: 4/5/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Duyen H Pham
Contribution to the Paper	Performed experiments shown in Figure 1B, D and E.
Signature	Date: 2/5/17

Name of Co-Author	Julia R Zebol		
Contribution to the Paper	Performed focus formation assays shown in Figure 3B.		
Signature		Date	2/5/2017

Name of Co-Author	Paul AB Moretti		
Contribution to the Paper	Performed most of the molecular cloning to generate DNA constructs required for experiments.		
Signature		Date	2-5-17

Name of Co-Author	Amanda L Peterson		
Contribution to the Paper	Performed mass spectrometry of cell pellets that contributed to the data shown in Figure 6B-E.		
Signature		Date	26/04/2017

Name of Co-Author	Tamara M Leclercq		
Contribution to the Paper	Performed focus formation assays with an independent set of cell lines that support the data shown in Figure 3B and C.		
Signature		Date	02/05/2017

Name of Co-Author	Huasheng Chan		
Contribution to the Paper	Performed focus formation assays with an independent set of cell lines that support the data shown in Figure 3B and C.		
Signature		Date	02/05/2017

Name of Co-Author	Jason A Powell		
Contribution to the Paper	Assisted Neubauer with mouse experiments shown in Figure 4A-C.		
Signature		Date	2/5/17

Name of Co-Author	Melissa R Pitman		
Contribution to the Paper	Performed extracellular S1P formation assays that generated the data of Figure 6A.		
Signature		Date	2/5/17

Name of Co-Author	Michael S Samuel		
Contribution to the Paper	Performed immunofluorescence staining on the tumour tissue samples that generated the images shown in Figure 4F.		
Signature		Date	2/5/17

Name of Co-Author	Claudine S Bonder		
Contribution to the Paper	Performed immunohistochemistry staining on the tumour tissue samples to generate the data shown in Supplementary Figure 4A, provided intellectual input and supervision.		
Signature		Date	2/5/17

Name of Co-Author	Darren J Creek		
Contribution to the Paper	Supervised the mass spectrometry work and performed data analysis that contributed to the data shown in Figure 6B-E.		
Signature		Date	26/04/2017

Name of Co-Author	Briony L Gliddon		
Contribution to the Paper	Supervised and assisted Neubauer with mouse experiments shown in Figure 4A-C.		
Signature		Date	2/5/17

Name of Co-Author	Stuart M Pitson		
Contribution to the Paper	Supervised the work, helped design experiments, assisted with data analysis and editing of the manuscript.		
Signature		Date	4/5/17

Research Paper

An oncogenic role for sphingosine kinase 2

Heidi A. Neubauer[1,2], Duyen H. Pham[1,2], Julia R. Zebol[1], Paul A.B. Moretti[1], Amanda L. Peterson[4], Tamara M. Leclercq[1], Huasheng Chan[1,2], Jason A. Powell[1,3], Melissa R. Pitman[1], Michael S. Samuel[1,3], Claudine S. Bonder[1,2,3], Darren J. Creek[4], Briony L. Gliddon[1] and Stuart M. Pitson[1,2,3]

[1] Centre for Cancer Biology, University of South Australia and SA Pathology, Adelaide, South Australia, Australia

[2] School of Biological Sciences, University of Adelaide, Adelaide, South Australia, Australia

[3] School of Medicine, University of Adelaide, Adelaide, South Australia, Australia

[4] Monash Institute of Pharmaceutical Science, Monash University, Parkville, Victoria, Australia

Correspondence to: *Stuart M. Pitson* , ***email***: *stuart.pitson@unisa.edu.au*

Keywords: sphingosine kinase 2, neoplastic transformation, oncogenesis, proliferation, tumorigenesis

Received: *June 08, 2016* ***Accepted***: *August 25, 2016* ***Published***: *August 30, 2016*

ABSTRACT

While both human sphingosine kinases (SK1 and SK2) catalyze the generation of the pleiotropic signaling lipid sphingosine 1-phosphate, these enzymes appear to be functionally distinct. SK1 has well described roles in promoting cell survival, proliferation and neoplastic transformation. The roles of SK2, and its contribution to cancer, however, are much less clear. Some studies have suggested an anti-proliferative/pro-apoptotic function for SK2, while others indicate it has a pro-survival role and its inhibition can have anti-cancer effects. Our analysis of gene expression data revealed that SK2 is upregulated in many human cancers, but only to a small extent (up to 2.5-fold over normal tissue). Based on these findings, we examined the effect of different levels of cellular SK2 and showed that high-level overexpression reduced cell proliferation and survival, and increased cellular ceramide levels. In contrast, however, low-level SK2 overexpression promoted cell survival and proliferation, and induced neoplastic transformation *in vivo*. These findings coincided with decreased nuclear localization and increased plasma membrane localization of SK2, as well as increases in extracellular S1P formation. Hence, we have shown for the first time that SK2 can have a direct role in promoting oncogenesis, supporting the use of SK2-specific inhibitors as anti-cancer agents.

INTRODUCTION

The sphingosine kinases (SKs) catalyze the conversion of sphingosine to sphingosine 1-phosphate (S1P). Given that sphingosine and its precursor, ceramide, are pro-apoptotic molecules, and S1P mediates cell survival and proliferation [1, 2], the SKs are considered critical regulators of the balance between cell death and cell survival, and represent promising targets for anti-cancer therapies [3]. The two mammalian SKs, SK1 and SK2, share high sequence similarity and both possess constitutive catalytic activity, but generally show distinct subcellular localization [4].

The role of SK1 in cancer is well characterized and has been extensively reviewed [1, 2, 5], with high SK1 expression observed in many different cancers and often correlating with poorer patient survival [5]. SK1 overexpression promotes neoplastic transformation and tumorigenesis [6], and notably, targeting SK1 has been shown to attenuate tumor growth in numerous animal models [3]. In contrast, the contribution of SK2 to cancer is unclear. Surprisingly, despite both enzymes catalyzing the same reaction, most studies examining SK2 function have found that it has an opposite role to SK1, and can promote cell cycle arrest and apoptosis [7-11]. Although most of these studies utilized high-level overexpression systems, functional analysis of endogenous SK2 has supported this role in promoting cell death [9, 11, 12]. Most notably, nuclear-localized SK2 has been shown to act as an epigenetic regulator, through S1P-mediated inhibition of histone deacetylase 1/2 (HDAC1/2) activity and increased transcription of p21 and c-fos [13].

Despite this general notion that SK2 is pro-apoptotic, a number of studies have emerged that demonstrate a role for SK2 in promoting cancer. Knockdown of SK2 expression has been shown to enhance apoptosis and chemosensitize many cancer cell types [14-17]. In fact, targeting SK2 in a range of cancer cell lines appears to have more of an anti-cancer effect than targeting SK1 [14, 18]. Strikingly, several *in vivo* studies have reported that targeting SK2 significantly attenuated tumor growth in a range of human xenograft models in mice [19-23]. Increased SK2 expression levels also correlate with disease progression in non-small cell lung cancer (NSCLC) [24] and multiple myeloma [25], and poorer survival in NSCLC patients [24]. Recent work also suggests that SK2 can play a role in increasing telomerase activity [26], promoting the upregulation of c-Myc *via* regulation of HDAC1/2 [20], and facilitating the activation of ezrin-radixin-moesin proteins to promote EGF-induced cancer cell invasion [27], all of which may contribute to cancer development and progression.

Although there is an emerging body of evidence suggesting that SK2 can play a role in cancer development, this is complicated by the known role of SK2 in facilitating cell death, and that, unlike SK1, SK2 overexpression has never been shown to promote neoplastic transformation and tumorigenesis. Here, we demonstrate for the first time that low-level SK2 overexpression, similar to that observed in numerous cancers, can promote cell proliferation, survival and neoplastic transformation, and that these levels of SK2 overexpression alone can drive tumorigenesis *in vivo*.

RESULTS

SK2 expression is elevated in a wide range of human cancers

Despite numerous studies examining the targeting of SK2 in cancer, broad analysis of SK2 expression in cancer has not been previously performed. Thus, we examined SK2 expression in a wide range of human cancers using the public gene expression datasets in the Oncomine database [28]. We found that SK2 is significantly elevated in studies from a broad range of human cancers, including bladder, melanoma, esophageal, breast, lymphoma and leukemia (Figure 1A and Supplementary Figure S1A).

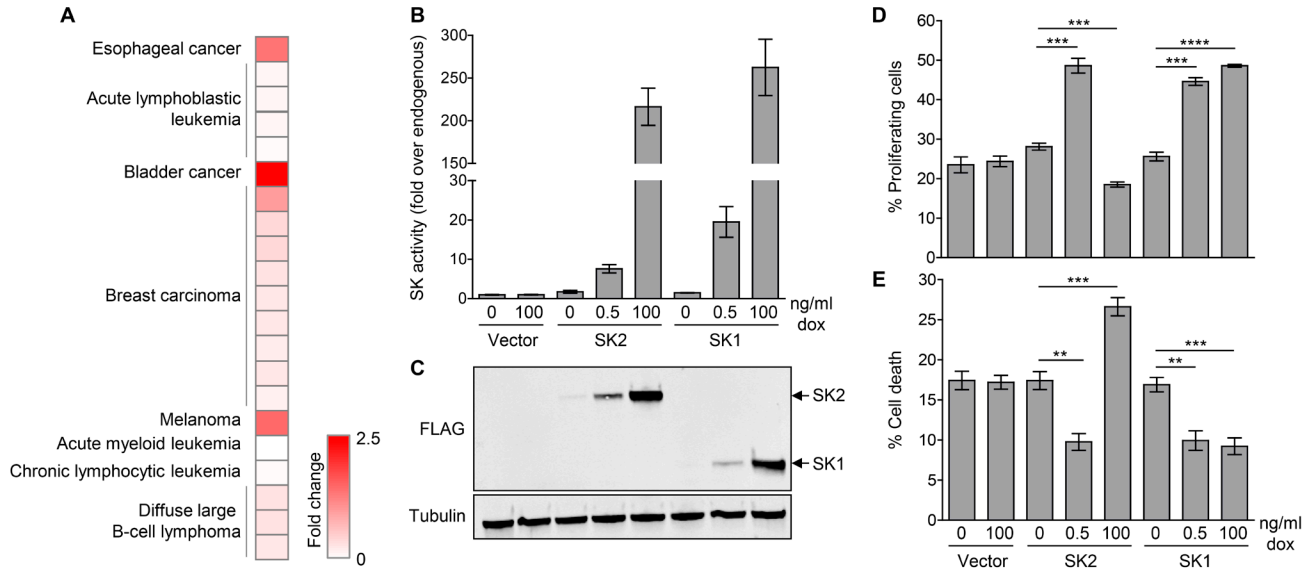


Figure 1: Low-level SK2 overexpression is observed in human cancers, and can promote cell survival and proliferation. **A.** Heat map showing human cancers where significant ($p < 1x10^{-4}$) upregulation of SK2 mRNA levels have been observed in cancerous tissues compared with corresponding normal tissue. Data was extracted from the Oncomine database [28], where each row represents a cancer subtype from an individual dataset. Further detail is presented in Supplementary Figure S1A. **B.** SK1 and SK2-specific activity upon doxycycline (dox)-induced low- and high-level overexpression in HEK293 Flp-In T-Rex cells. Data shown are mean (± range) of duplicate data points from a representative experiment (of more than three independent experiments). **C.** Lysates from the HEK293 Flp-In T-Rex cells with doxycycline-induced low- and high-level overexpression of FLAG-tagged SK1 or SK2, or empty vector, were subjected to immunoblot analyses with antibodies against FLAG and α-tubulin. Blots shown are representative of at least three independent experiments. **D.** Measurement of cell proliferation in HEK293 Flp-In T-Rex cells with doxycycline (dox)-induced low- and high-level overexpression of SK1 or SK2, or empty vector. Data shown are mean ± SEM, n = 3-4. Statistics were performed using an unpaired Student's t-test (two-tailed); ***$p < 0.001$, ****$p < 0.0001$. **E.** Measurement of cell death in HEK293 Flp-In T-Rex cells with doxycycline (dox)-induced low- and high-level overexpression of SK1 or SK2, or empty vector. Data shown are mean ± SEM, n = 4-5. Statistics were performed using an unpaired Student's t-test (two-tailed); **$p < 0.01$, ***$p < 0.001$.

Interestingly, however, this cancer-associated elevation in SK2 was only modest, with up to 2.5-fold higher levels of SK2 compared with the corresponding normal tissues. Notably, both SK1 and SK2 were upregulated in three independent datasets for diffuse large B-cell lymphoma (Supplementary Figure S1B), but there was no apparent general correlation between SK2 and SK1 upregulation in the other tumors examined. Indeed, in most other tumor datasets where SK2 was upregulated, SK1 expression was either unaltered or significantly downregulated (Supplementary Figure S1B).

Low-level SK2 overexpression enhances cell survival and proliferation, whereas high-level overexpression promotes cell death

Many studies have demonstrated that high-level SK2 overexpression can promote cell cycle arrest and cell death [7-10]. However, based on the gene expression analysis showing only low levels of SK2 overexpression in human cancers, we reasoned that more informative functional analysis would be gained by overexpression

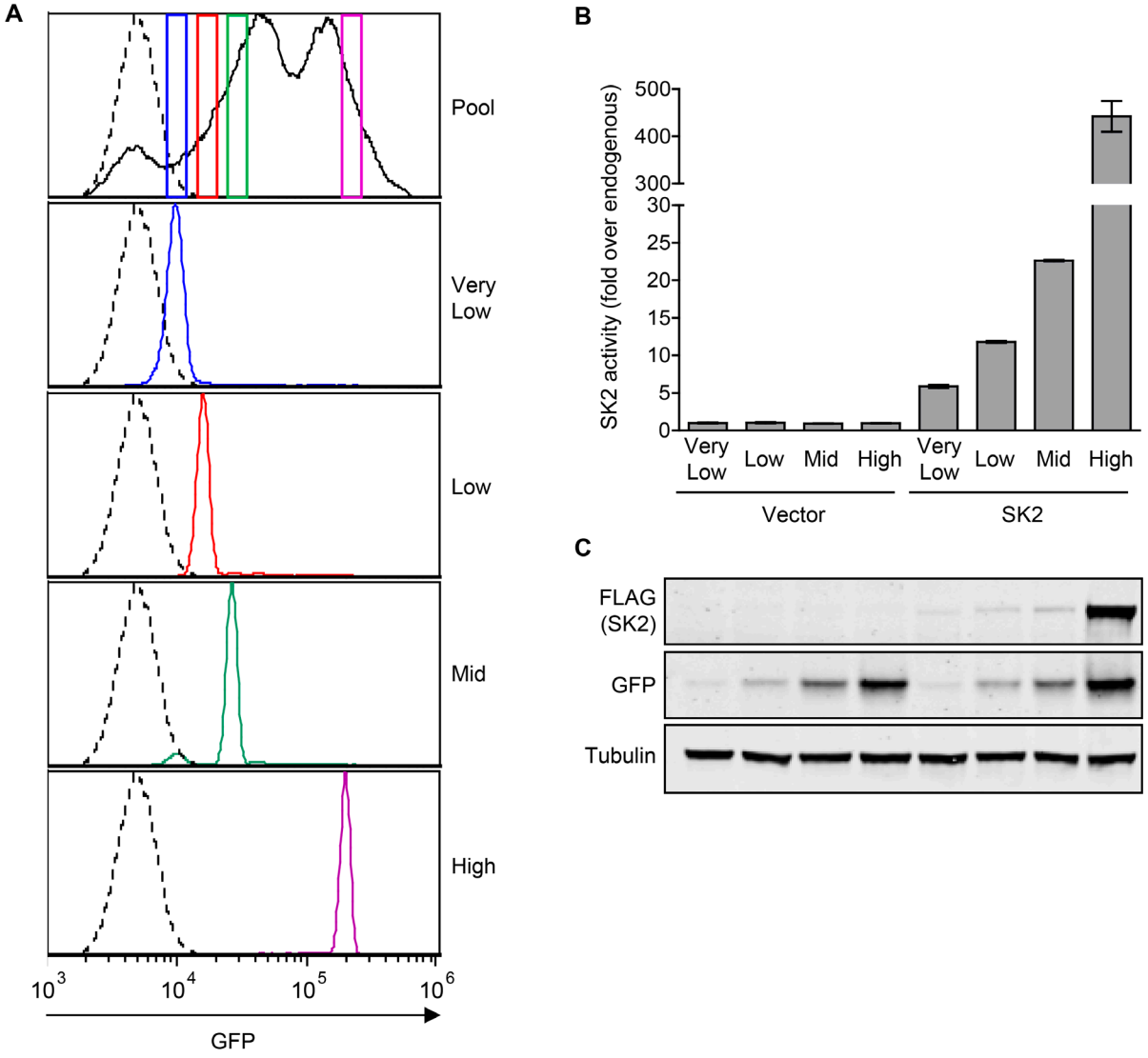


Figure 2: Generation of NIH3T3 stable cell lines with varying levels of constitutive SK2 overexpression. A. The NIH3T3 pooled stable cell line expressing SK2 and GFP, or GFP alone (empty vector), were sorted on four separate narrow gates of varying GFP intensity (colored boxes in top panel), to produce new stable lines depicted as 'very low', 'low', 'mid' and 'high'. These new stable lines were then analyzed by flow cytometry to confirm that the desired narrow GFP-expression levels were obtained as expected. GFP-negative control cells are depicted by a dotted line. **B.** SK2-specific activity of NIH3T3 cell lines stably expressing 'very low' (5-fold), 'low' (10-fold), 'mid' (20-fold) or 'high' (440-fold) levels of SK2 overexpression (above endogenous levels), or empty vector. Data are shown as mean (± range) of duplicate samples from a representative experiment, of at least three independent experiments. **C.** Lysates from the NIH3T3 vector or SK2 overexpressing cell lines were subjected to immunoblot analyses with antibodies against FLAG, GFP and α-tubulin. Blots shown are representative of at least three independent experiments.

of SK2 at much lower levels. To investigate this, we utilized human embryonic kidney (HEK) 293 cells engineered to express FLAG-tagged SK2 or SK1 in a doxycycline-inducible, concentration-dependent manner. Using different doxycycline concentrations in the culture media, we could achieve low and high SK2 and SK1 overexpression, as determined by specific-activity (Figure 1B) and protein expression (Figure 1C). Importantly, endogenous SK2 and SK1 protein levels remained unaltered upon induction of SK1 and SK2 overexpression, respectively (Supplementary Figure S2A and S2B). We then used this system to assess the effect of varying levels of SK overexpression on cell proliferation and survival. In agreement with previous studies, overexpression of SK2 at high levels (over 200-fold) in this system resulted in decreased cell proliferation and an increase in cell death (Figure 1D and 1E). Strikingly, however, when SK2 was overexpressed to much lower levels (8-fold over endogenous), more comparable to that seen in the cancer expression analysis, this induced a marked increase in cell proliferation and a decrease in cell death (Figure 1D and 1E). These contrasting findings clearly demonstrate that the cellular levels of SK2 influence its function. Notably, these findings were unique to SK2, with both low- and high-level overexpression of SK1 resulting in a consistent increase in cell survival and proliferation (Figure 1D and 1E).

SK2 can elicit oncogenic signaling and promote neoplastic transformation *in vitro*

Next, we assessed if low-level overexpression of SK2 could also induce neoplastic transformation, as had been previously observed for SK1 [6]. In contrast to mouse cells, neoplastic transformation of human cells is well known to require multiple oncogenes [29], meaning their use in these type of studies is problematic. Thus, to

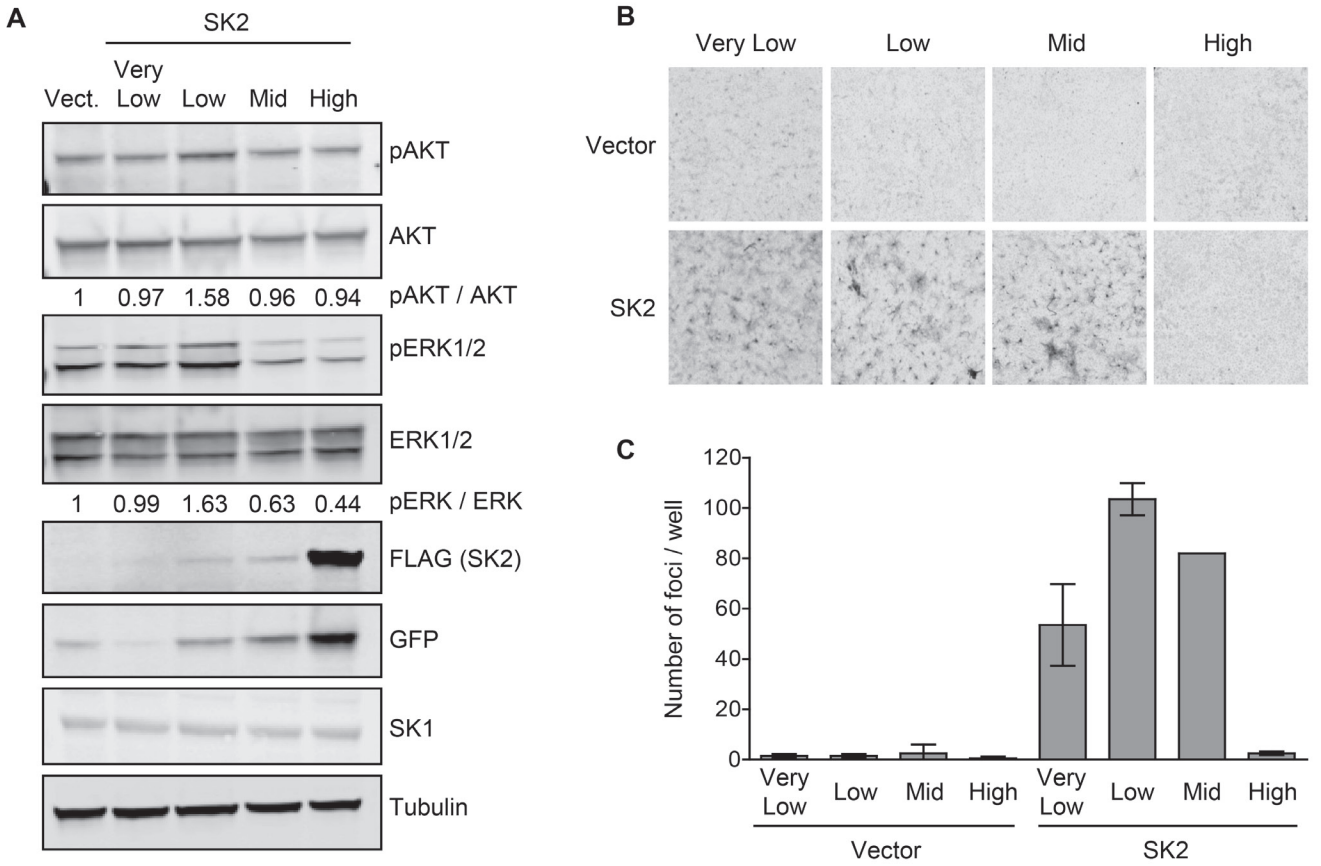


Figure 3: SK2 overexpressed at low levels can elicit oncogenic signaling and drive neoplastic transformation *in vitro*. **A.** Lysates from the NIH3T3 vector or SK2-overexpressing cell lines were subjected to immunoblot analyses and probed with antibodies against phospho-AKT, total AKT, phospho-ERK1/2, total ERK1/2, FLAG, GFP, SK1 and α-tubulin. Vect = empty vector with 'low' level GFP expression, chosen as a representative control. Densitometry was performed to quantify phospho-AKT and phospho-ERK band intensities, and is presented as a ratio of total AKT and ERK levels, respectively, and is normalized to vector. Blots shown are representative of three independent experiments. **B.** Contact inhibition of the NIH3T3 vector or SK2-overexpressing cell lines was tested using focus formation assays. Images shown are representative of at least three independent experiments, each performed in duplicate, using at least three independently generated sets of stable lines. **C.** Number of foci per well from the experiment shown in Figure 3B were quantified and the mean number of foci for duplicate wells was graphed (± range).

examine the oncogenic potential of SK2, we transfected NIH3T3 mouse fibroblasts with a vector encoding SK2 as well as green fluorescent protein (GFP) *via* an internal ribosome entry site (IRES) such that GFP and SK2 expression were linked. We then isolated a series of cell lines stably expressing different levels of SK2 through the sorting of cells for differential GFP expression (Figure 2A). The resulting stable cell lines were then validated through the analysis of SK2-specific activity (Figure 2B) and exogenous SK2 protein levels (Figure 2C), which revealed stable overexpression of SK2 at 5-fold, 10-fold, 20-fold and 440-fold over endogenous levels, designated 'very low', 'low', 'mid' and 'high' level SK2 overexpression, respectively.

Biochemical analysis of these cell lines revealed that 'low' SK2 overexpression resulted in the activation of oncogenic signaling pathways, as demonstrated by an increase in phospho-AKT and phospho-ERK1/2 levels (Figure 3A). Conversely, the 'mid' and 'high'-level SK2 overexpression caused a downregulation of phospho-ERK1/2 signaling (Figure 3A), in agreement with high-level SK2 overexpression attenuating cell proliferation. Also consistent with the HEK293 Flp-In T-Rex overexpression system, endogenous SK1 protein levels were unchanged in the multiple SK2-overexpressing NIH3T3 cell lines, as compared to vector control cells (Figure 3A). These cells were then utilized in *in vitro* assays of neoplastic cell transformation. Notably, in focus formation assays, cell lines with 'very low', 'low' and 'mid'-level SK2 overexpression formed foci, whereas cells with 'high'-level SK2 overexpression did not (Figure 3B and 3C). Similar results were also observed in colony formation assays (Supplementary Figure S3), demonstrating that low-level SK2 overexpression can promote anchorage-independent growth in soft agar. Together, these data demonstrate that low-level SK2 overexpression can elicit oncogenic signaling and induce neoplastic transformation of cells.

Low-level SK2 overexpression can drive tumor formation *in vivo*

Given that SK2 could promote neoplastic growth *in vitro*, we next examined if this represented full neoplastic transformation through analyzing the ability of these cells to form tumors *in vivo*. Hence, the series of cell lines with differential levels of SK2 overexpression were subcutaneously engrafted into the flanks of NOD/SCID mice, and the development of tumors assessed. Consistent with the *in vitro* data, cells with either 'very low' or 'low' SK2 overexpression resulted in efficient tumor formation in mice (Figure 4A). In stark contrast, however, cells with either 'mid' or 'high' SK2 overexpression showed minimal tumor growth (Figure 4A). All tumors were vascularized, as determined by CD31 staining, and showed morphology characteristic of fibrosarcoma (Supplementary Figure S4A and S4B). Notably, the tumors that developed from the 'very low' SK2 cells were significantly larger than all other tumors formed (Figure 4B and 4C). Overall, these results demonstrate for the first time that through low-level overexpression, SK2 can drive tumorigenesis *in vivo*.

Although the SK2-expressing cells engrafted into mice were fractionated based on their GFP (and therefore SK2) expression, these cells remained pools of clones. While this obviates potential defects associated with plasmid integration into the genomes of individual clones, it meant that the level of SK2 overexpression observed for each line was an average of all cells within that line. Thus, we examined the expression levels of SK2 within the resulting tumors. Notably, every tumor that developed from the 'very low', 'low' and 'mid' SK2 overexpressing cells all possessed very similar levels of SK2 protein (Figure 4D) and catalytic activity (Figure 4E). Indeed, by comparison to the parental fibroblast cells, it appears that tumor formation resulted preferentially from cells within the pools with less than 5-fold SK2 overexpression (Figure 4E). This finding suggests that this level of SK2 represents the optimal level to promote oncogenic signaling and tumorigenesis, and is consistent with the low level of SK2 upregulation seen in many human cancers (Figure 1A). These results perhaps explain why only one tumor formed from the 'mid' SK2-expressing cells, as within this pool of cells there would likely be fewer low SK2-expressing clones compared with the 'low' and 'very low' groups. Unexpectedly, the 'high' SK2-overexpressing cells also resulted in one tumor forming. Further analyses, however, revealed that cells within this tumor were not actively proliferating, whereas cells within the tumors generated from 'very low' SK2-expressing cells were highly positive for the proliferation marker Ki-67 (Figure 4F). Furthermore, the level of SK2 overexpression within the 'high' SK2 tumor was quite heterogeneous, with only small patches of cells with high levels of SK2 protein (Figure 4G). In fact, the majority of the tumor was comprised of cells with low SK2 overexpression similar in level to that seen in the tumors arising from 'very low' SK2-expressing cells (Figure 4G). Therefore, again, it is possible that a small population of cells within the original 'high' cell pool drifted to low-level SK2 overexpression and initiated tumor formation, thus supporting the growth of some of the high SK2-expressing cells. Indeed, our data demonstrating that low SK2 overexpression supported enhanced cell proliferation and survival, while high SK2 overexpression had the opposite effect suggests that the high SK2-expressing cells would have been under considerable selective pressure towards low SK2 overexpression.

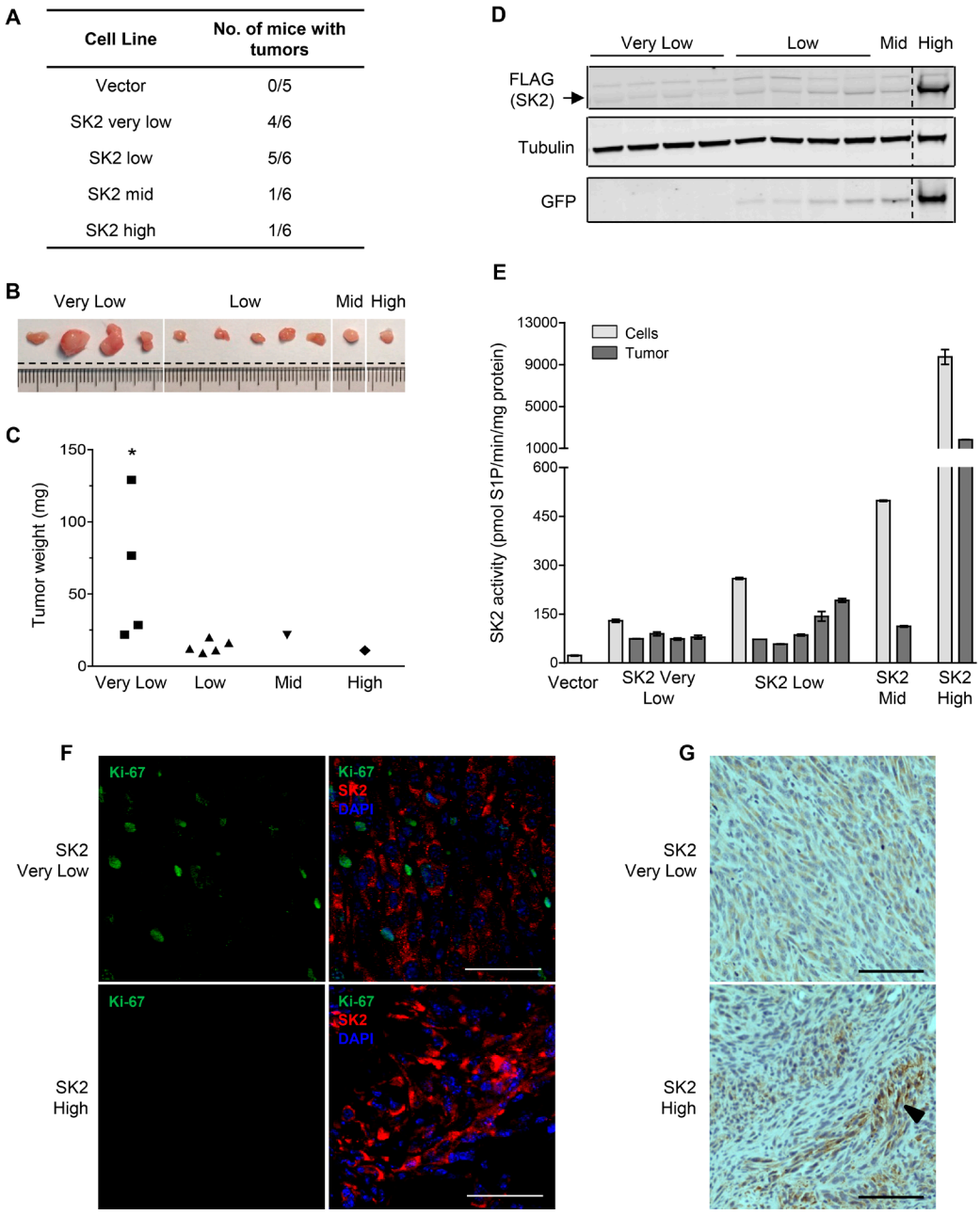


Figure 4: Low-level SK2 overexpression can drive proliferation and tumorigenesis *in vivo*. NOD/SCID mice were injected with NIH3T3 cell lines stably overexpressing 'very low' (5-fold), 'low' (10-fold), 'mid' (20-fold) or 'high' (440-fold) levels of SK2 (above endogenous levels). Empty vector cells with 'low' level GFP expression were chosen as a representative control (Vector). **A.** Table summarizing the number of mice with tumors per cell line, 18 days post-cell injection. **B.** Images of the excised tumors from each group of NIH3T3 stable SK2 cell lines. Dashed lines indicate where the same image has been spliced together to aid interpretation. **C.** Weights of the excised tumors from each group of NIH3T3 stable SK2 cell lines. Statistics denote a significant increase in the weights of SK2 'very low' tumors compared to tumors from the SK2 'low', 'mid' and 'high' groups (* $p < 0.05$; Student's unpaired two-tailed *t*-test). **D.** Equal amounts of total protein from the tumor tissue lysates were subjected to immunoblot analyses with antibodies against FLAG, α-tubulin and GFP. Each lane represents a different tumor sample. Dashed lines indicate where lanes from the same immunoblots have been spliced together to aid interpretation. **E.** SK2-specific activity from the tumor lysates was measured and graphed as mean (± range) of duplicate samples. These data were plotted alongside data of SK2 activity from the engrafted cell lines, which was transformed from Figure 2B as specific-activity (pmol S1P/min/mg protein) for the purposes of comparison. **F.** Dual immunofluorescence staining of overexpressed FLAG-tagged SK2 (red) and the proliferation marker Ki-67 (green) on the SK2 tumors. Tumor sections were counterstained with DAPI to indicate nuclei (blue). At least five random fields of view were imaged per tumor and representative images are shown. Scale bar = 50 μm. **G.** Levels of SK2 overexpression are heterogeneous in the SK2 'high' tumor. Staining of overexpressed FLAG-tagged SK2 was visualized by immunohistological analyses. Multiple images were taken for each tumor and a representative field of view is shown. Arrow denotes a representative area of heterogeneous, intense FLAG (SK2) staining in the SK2 'high' tumor. Scale bar = 100 μm.

Differential levels of SK2 overexpression alter its subcellular localization and sphingolipid metabolism

It is well established that the subcellular localization of the SKs, and hence the compartmentalization of S1P within the cell, plays an important role in the function of these enzymes [30]. The oncogenic role of SK1 requires its translocation to the plasma membrane, a location that results in increased extracellular S1P production [31]. Furthermore, the localization of SK2 to the nucleus, endoplasmic reticulum (ER) or mitochondria appears to promote its anti-proliferative, pro-apoptotic functions [7, 8, 11, 13]. Thus, we examined the localization of SK2 when overexpressed at low and high levels. In the 'high' SK2-expressing cells, SK2 was strongly nuclear-localized (Figure 5A and 5B), in agreement with previous reports for this cell type [7]. In the 'low' SK2-expressing cells, however, SK2 was mostly cytoplasmic, and showed a significant increase in its plasma membrane localization (Figure 5A and 5C). Furthermore, the formation of extracellular S1P was significantly higher from cells with 'low'-level SK2 overexpression compared to vector control cells (Figure 6A), consistent with our observations of increased SK2 at the plasma membrane in these cells. Interestingly, 'high'-level SK2 overexpression resulted in a further increase in extracellular S1P formation (Figure 6A), but this was only a modest doubling compared with the 'low' SK2 cells, despite these cells having greater than 400-fold more SK2 activity.

Sphingolipid analysis revealed, somewhat surprisingly, that 'low'-level SK2 overexpression had very little effect on the intracellular levels of S1P, ceramides, dihydroceramides, sphingomyelins or dihydrosphingomyelins (Figure 6B-6E), with the only change noted being a small increase in sphingosine. In contrast, 'high'-level SK2 overexpression resulted in a significant increase in a range of ceramide species, sphingomyelins and dihydrosphingomyelins, as well as sphingosine (Figure 6B, 6D and 6E), in line with a previous report demonstrating that overexpressed SK2 partially localized to the ER, and S1P produced here could feed into an ER/golgi-associated 'salvage pathway' to generate pro-apoptotic sphingosine and ceramide, as well as sphingomyelin [8]. The increase in ceramides and sphingosine are likely to contribute, at least in part, to the anti-proliferative and pro-cell death role of SK2 in these 'high' overexpression cells, with the increase in extracellular S1P formation that was observed in these cells (Figure 6A) possibly insufficient to override these effects.

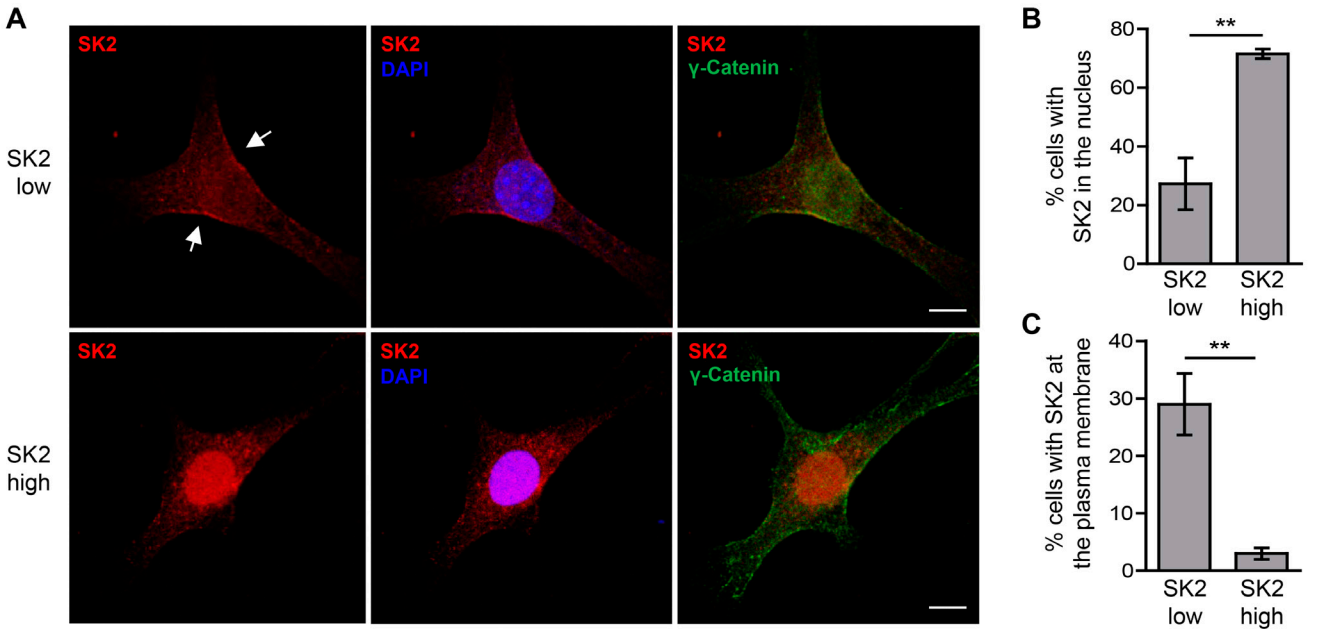


Figure 5: Varying levels of SK2 overexpression affect its subcellular localization. A. The subcellular localization of FLAG-tagged SK2 (red) in the NIH3T3 stable 'low' and 'high' SK2-overexpressing cells was examined by immunofluorescence staining, using FLAG antibody. Nuclei were stained with DAPI (blue) and cell membranes were stained with antibodies against γ-catenin (green). Images are representative of cells observed from three independent experiments. Arrows denote representative plasma membrane localization of 'low' SK2. Scale bar = 10 μm. **B.-C.** Cells from **A.** were visualized by confocal microscopy and scored based on the presence or absence of either **B.** distinct nuclear FLAG-tagged SK2 staining or **C.** plasma membrane-localized FLAG-tagged SK2 staining. A minimum of 200 cells were scored per well, and data were graphed as mean (± SD) of triplicate wells from a single experiment, representative of three independent experiments (** $p < 0.01$; Student's unpaired two-tailed *t*-test).

DISCUSSION

Despite the conflicting data in the literature, our findings demonstrate that, like SK1, SK2 can have a physiological role in promoting cell survival and proliferation, potentially through plasma membrane localization. This is perhaps not surprising, as the individual genetic deletion of either SK1 or SK2 in mice does not result in any gross phenotypic abnormalities [32, 33], whereas the double knock-out mice die *in utero* [34], suggestive of at least some functional redundancy between the two proteins. Like SK1, there are no mutations in SK2 linked to cancer, however it has been suggested that cancer cells can display a 'non-oncogene addiction' for SK1 [2, 35]. Given that we have now shown that SK2 can promote neoplastic transformation and tumorigenesis, and is upregulated in many human cancers, coupled with the anti-cancer efficacy of SK2-selective inhibitors leads us to postulate that a non-oncogene addiction may apply for both SKs in cancer. Indeed, targeting both SK isoforms may be the best strategy to overcome tissue and cell type-specific differences in the roles of the SKs in different cancers, and in agreement, dual SK1/SK2 inhibitors show significant decreases in tumor burden *in vivo* [36, 37].

One of the most intriguing findings of our study is the observation that SK2 function can dramatically switch, depending on its expression level, from being pro-survival and pro-proliferative to pro-cell death and anti-proliferative. While it could be argued that high-level overexpression is non-physiological, and may generate

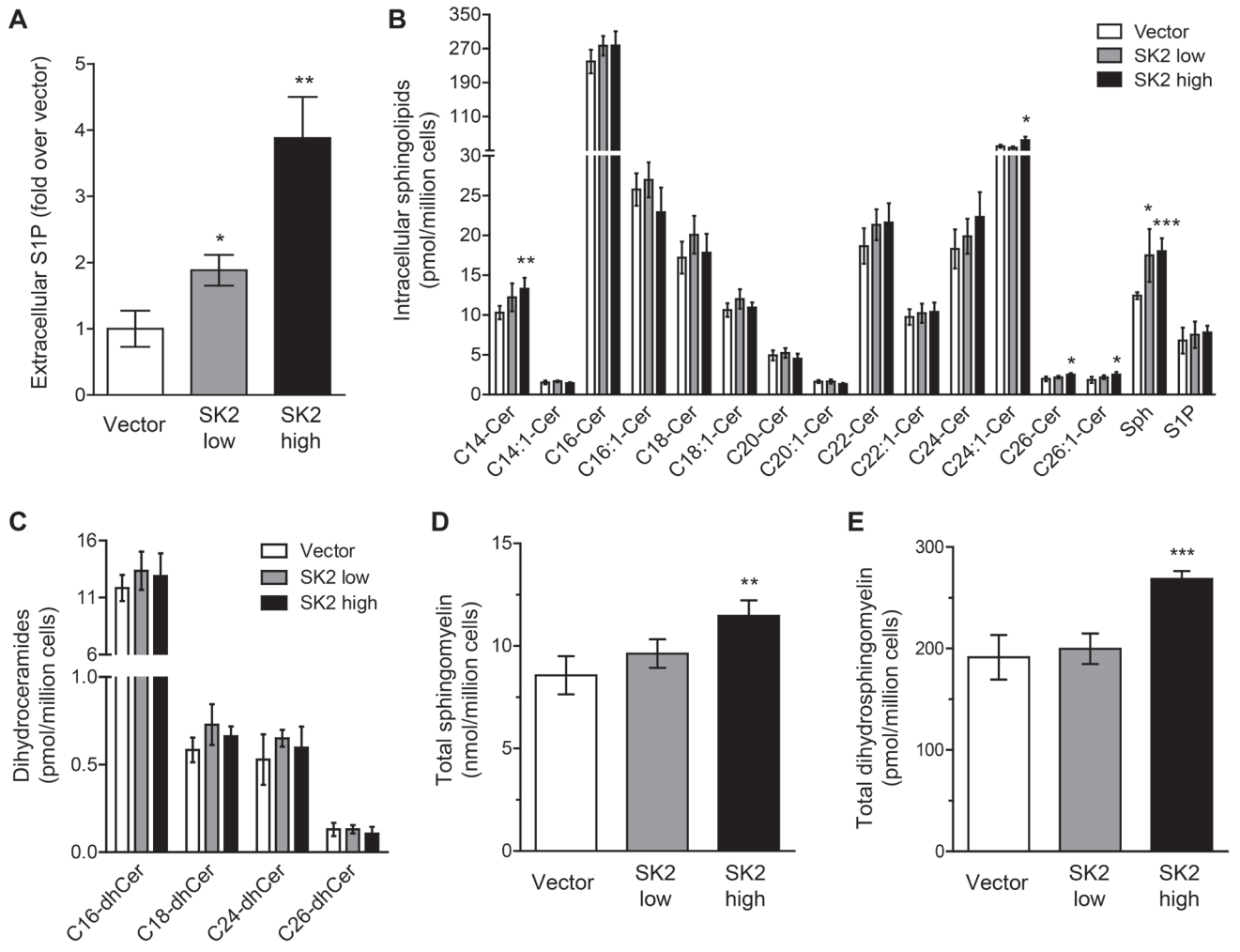


Figure 6: Sphingolipid metabolism is altered when SK2 is overexpressed at varying levels. A. Rate of extracellular S1P formation was determined from intact vector control 'low', SK2 'low' and SK2 'high' NIH3T3 stable cell lines. Analyses were performed in triplicate and data are graphed as mean (± SD). Statistics denote significant increases in extracellular S1P compared to vector control cells (* $p < 0.05$, ** $p < 0.01$; Student's unpaired two-tailed *t*-test). **B.-E.** Intracellular sphingolipid species were analyzed by LC-MS using NIH3T3 vector control 'low', SK2 'low' and SK2 'high' stable cell lines. Data are graphed as mean (± SD) of quadruplicate samples for **B.** individual ceramide species, sphingosine (Sph) and sphingosine 1-phosphate (S1P), **C.** individual dihydroceramide species, **D.** total sphingomyelin levels, and **E.** total dihydrosphingomyelin levels. Statistics denote significant increases in lipids compared to vector control cells (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Student's unpaired two-tailed *t*-test).

artefacts, it is notable that previous studies have shown that SK2 can have physiological roles in promoting cell cycle arrest and apoptosis when localized to organelles such as the nucleus and mitochondria [11, 13]. Clearly, under normal conditions these pro-death roles are likely kept under tight regulation, so it remains possible that high-level overexpression circumvents these regulatory mechanisms. This high-level overexpression of SK2, for example, may lead to altered protein-protein interactions or post-translational modifications, altering the subcellular localization of SK2, from the plasma membrane to cellular organelles such as the nucleus, in favor of promoting cell death. Indeed, it has been previously reported that when transiently overexpressed, SK2 could interact with and sequester the pro-survival Bcl-x_L protein [10], suggesting a possible mechanism for the pro-apoptotic phenotype we observed with high-level SK2 overexpression. However, despite multiple attempts, we were unable to detect an interaction between Bcl-x_L and SK2 in our system, suggesting that this proposed interaction was unlikely to mediate the observed phenotype. Intriguingly, there appears to be a window whereby low-level SK2 upregulation confers a survival and proliferative advantage to the cell without inducing these pro-apoptotic functions. Whether a different subset of post-translational modifications and/or protein-protein interactions drive the differences in subcellular localization and function observed with low *versus* high SK2 overexpression will require further interrogation.

To add further complexity, other studies have demonstrated that nuclear SK2 can contribute to cancer progression through the stabilization of telomerase and promotion of c-Myc expression [20, 26]. In the present study, SK2 was observed in the nucleus when overexpressed at high levels, and yet here it had an opposite, anti-proliferative role, which is also well documented [7, 13]. It is unclear how SK2 can have such vastly different functions within the same organelle, but it suggests that there must be additional factors regulating these processes. Indeed, the regulatory mechanisms controlling this enzyme remain an important, but currently largely unanswered question [4]. Notably, SK1 remains pro-survival and pro-proliferative even at high-level overexpression, likely due to its different subcellular localization to SK2 and consequent contribution to different sphingolipid pools within the cell. In line with this, previous studies have shown that artificially targeting SK1 to the ER can render it pro-apoptotic, like SK2 [8].

Interestingly, while cells with 20-fold ('mid') SK2 overexpression were able to form foci *in vitro*, they had decreased levels of phospho-ERK1/2 and did not efficiently form tumors *in vivo*. This may indicate that this level of SK2 overexpression is at the upper limit of the 'window' whereby SK2 switches from being tumourigenic to having predominantly anti-proliferative functions. Conversely, 5-fold ('very low') SK2 overexpression resulted in no appreciable changes in phospho-ERK1/2 or phospho-AKT levels, and yet these cells developed the largest tumors *in vivo*. It is therefore possible that S1P-mediated angiogenesis and tumor vascularization played more of an important role in the development of these tumors, given that low-level overexpression of SK2 resulted in plasma membrane localization and increased formation of extracellular S1P, which is a key regulator of angiogenesis [38]. These observed differences also highlight the importance of employing *in vivo* models for assessment of full neoplastic transformation. Furthermore, it was surprising that 'high'-level SK2 overexpression resulted in a doubling in extracellular S1P production as compared to the 'low'-SK2 overexpressing cells, despite 'high' SK2 overexpression resulting in decreased survival and proliferative signaling, and increases in pro-apoptotic sphingolipid species. Notably, Weigert *et al*. previously reported that transient overexpression of SK2 resulted in a substantial increase in S1P released from apoptotic cells as a result of SK2 being cleaved by caspase-1 and secreted from the cell [39]. This may, in part, explain our observed increase in extracellular S1P in the SK2 'high' cells, which was clearly not able to facilitate any overall pro-survival or proliferative stimulus in these cells. It should be noted that Liang *et al*. found a significant increase in colitis-associated tumor development in SK2 knockout mice, when compared to wildtype mice [40], suggesting that SK2 may function as a tumor suppressor in this model. However, these findings are likely to be an indirect effect as it was also shown in the study that the global genetic loss of SK2 caused an upregulation of both S1P receptor 1 and SK1 levels in the colon, with a concomitant increase in circulating and colonic S1P [40]. SK1 has been previously shown to contribute to colon carcinogenesis [41] and indeed, the increase in severity of colitis in the SK2 knockout mice was ablated by the SK1-specific inhibitor SK1-I [40]. Furthermore, the proposed tumor-suppressive role of SK2 in negatively regulating pro-tumorigenic SK1 levels is not recapitulated with SK2-selective inhibitors, which show efficacy in decreasing tumor burden in murine xenograft models [20-23].

Evidently the true functions of SK2 are complex and are also likely to be tissue- and cell type-specific. However, from our findings it is clear that SK2 represents an important target in cancer and future work to better understand how SK2 is regulated will be important for the generation of more efficacious SK2-targeting anti-cancer drugs.

MATERIALS AND METHODS

Antibodies

The following primary antibodies were utilized: mouse monoclonal anti-FLAG (Clone M2 #F3165, Sigma-Aldrich), rabbit polyclonal anti-FLAG (#2368, Cell Signaling Technology), rabbit monoclonal anti-FLAG (#14793, Cell Signaling Technology), mouse anti-α-tubulin (#ab7291, Abcam, Cambridge, MA, USA), goat anti-GFP (#600-101-215, Rockland Immunochemicals, Limerick, PA, USA), rabbit anti-SK1 (#SP1621, ECM Biosciences, Versailles, KY, USA), rabbit anti-SK2 (#17096-1-AP, Proteintech, Rosemont, IL, USA), rabbit anti-phospho-p44/42 MAPK (ERK1/2) Thr202/Tyr204 (#9101, Cell Signaling Technology), rabbit anti-p44/42 MAPK (ERK1/2) (#9102, Cell Signaling Technology), rabbit anti-phospho-AKT Ser473 (#9271, Cell Signaling Technology), rabbit anti-AKT (#9272, Cell Signaling Technology), mouse anti-Ki-67 (#VP-K452, Vector Labs, Burlingame, CA, USA), mouse anti-γ-Catenin (#610253, BD Biosciences) and goat anti-PECAM-1 (CD31; #SC-1506, Santa Cruz Biotechnology, Dallas, TX, USA).

Generation of cell lines

HEK293 Flp-In T-Rex cells (Invitrogen, Life Technologies) with doxycycline-inducible FLAG-tagged SK1 or SK2 expression, or empty vector, were generated as previously described [42].

To generate NIH3T3 cells with varying levels of constitutive SK2 overexpression, we obtained a pCX-EGFP construct [43] that we initially modified by replacing the EGFP with a polylinker following digestion with EcoRI and ligation of annealed kinased oligonucleotides AATTCGG TACCGAGCTCGCTAGCGCGGCCGCCTCGAGC-3' and 5'-AATTGC TCGAGGCGGCCGCGCTAGCGAGCTCGGTACCG-3' to produce pCX4. pCX4-IRES EGFP was then generated by subcloning the IRES EGFP cassette from pcDNA3-IRES EGFP [44] with EcoRI and NotI. pCX4-SK2(FLAG) IRES EGFP was then made by cloning in FLAG-tagged human SK2a [45] following digestion with EcoRI. NIH3T3 mouse fibroblasts (ATCC CRL-1658) were transfected with pCX4-SK2(FLAG) IRES EGFP, or empty vector, using Lipofectamine™ 2000 (Invitrogen) as per the manufacturer's protocol. 48 h after transfection, the cells were sorted for GFP-positive cells using a MoFlo Astrios cell sorter (Beckman Coulter). A stable GFP-positive cell population was obtained by sorting for GFP another two times. The stable GFP-positive pooled line was then sorted on four separate narrow gates of varying GFP intensity, to produce new stable lines depicted as 'very low', 'low', 'mid' and 'high'. These new stable lines were then analyzed by flow cytometry to confirm that the desired narrow GFP-expression levels were obtained as expected.

Cell culture

HEK293 Flp-In T-Rex cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen), containing 10% fetal bovine serum (Bovagen), 1 mM HEPES, penicillin (1.2 mg/ml) and streptomycin (1.6 mg/ml). NIH3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen), containing 10% donor bovine serum with iron (DBS; Gibco, Invitrogen), 1 mM HEPES, penicillin (1.2 mg/ml) and streptomycin (1.6 mg/ml). All cells were grown at 37°C, 5% CO_2 in a humidified incubator.

For doxycycline-induced low- and high-level overexpression of FLAG-tagged SK1 and SK2, HEK293 Flp-In T-Rex cells were seeded, and 24 h later media was removed and replaced with serum-free media containing 0.1% bovine serum albumin (BSA; Sigma Aldrich) with either vehicle (methanol, 0.001% v/v final), 0.5 ng/ml or 100 ng/ml doxycycline (Sigma Aldrich). After 24 h induction, cells were harvested and lysates prepared. Cell proliferation and cell death were determined as previously described [36].

Immunoblot analysis

Cells were harvested by scraping into cold phosphate buffered saline (PBS), and cell pellets were resuspended in extraction buffer (50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 10% glycerol, 1 mM EDTA, 0.05% Triton X-100, 2 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM dithiothreitol and protease inhibitor cocktail (Roche)). Cells were lysed by sonication, and total protein concentration was determined by a Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Lysates of equal protein were separated by SDS-PAGE on a Criterion™ XT Bis-Tris 4-12% gradient gel (Bio-Rad Laboratories) under reducing conditions. Proteins were transferred to nitrocellulose membrane (Pall Life Sciences, Pensacola, FL, USA). Membranes were blocked using Odyssey® Blocking Buffer (LI-COR, Lincoln, NE, USA), and subjected to immunoblotting with various primary antibodies. Proteins were visualized using IRDye® secondary antibodies and the Odyssey® CLx infrared imaging system (LI-COR). Densitometry was performed using ImageQuant 5.2 analysis software (Molecular Dynamics).

Focus formation assays

Focus formation assays were performed as described previously [6]. Briefly, cells were cultured to form monolayers in 6-well plates in DMEM with 1% DBS (Gibco, Life Technologies), and media was replenished every 2-3 days for a total of 3 weeks. Cells were then fixed in methanol, foci were stained with bromophenol blue, and images were taken using the Odyssey® CLx infrared imaging system (LI-COR).

Colony formation in soft agar

Colony formation assays in soft agar were performed as previously described [36]. After 14-21 days, colonies were quantified visually using light microscopy. Colonies were imaged using an Olympus MVX10 microscope.

In vivo tumor model

Experiments involving mice were conducted according to the guidelines from the Australian code of practice for the care and use of animals for scientific purposes 7th Edition, 2004, and with approval from the SA Pathology/CALHN Animal Ethics Committee and the University of Adelaide Animal Ethics Committee.

The NIH3T3 cell lines expressing varying levels of SK2 described above were trypsinized and washed in PBS, and $1x10^6$ cells were injected in 200 µl of PBS subcutaneously into the flank of 8 week old female NOD/SCID mice. The empty vector cells with 'low' GFP expression were selected as a representative control group. Mice were examined daily to monitor tumor formation. On day 19, all mice were humanely killed and tumors were excised. Half of each tumor was fixed in 10% formalin, paraffin embedded and sectioned. The remaining half was homogenized using a pestle (Axygen) in extraction buffer, subjected to freeze/thawing in liquid nitrogen and sonication, and lysates were clarified by centrifugation at 17,000 × g for 15 min at 4°C.

Sphingosine kinase activity assays

SK1 and SK2 activity was determined as previously described, using isoform-selective assay conditions [46].

Immunohistological analyses

Immunohistological staining was performed as previously described [36], with the following modifications. For PECAM-1 (CD31) expression, sections were blocked with rabbit serum, incubated with goat polyclonal antibody to PECAM-1 (Santa Cruz Biotechnology) at 133 ng/ml, and biotinylated rabbit anti-goat secondary antibody (1:500; Abcam). For FLAG-tagged SK2 expression, sections were blocked with goat serum, incubated with rabbit polyclonal FLAG antibody (Cell Signaling Technology) at 150 ng/ml, and biotinylated goat anti-rabbit secondary antibody (1:500; Vector Labs). Sections were visualized on an EVOS XL light microscope (Life Technologies) at 20x magnification.

Immunofluorescence analyses

For dual immunofluorescence staining of overexpressed FLAG-tagged SK2 and Ki-67 on the formalin-fixed paraffin-embedded tumor tissue samples, sections were de-waxed, rehydrated, and antigen retrieval was performed by boiling in citrate buffer for 30 min. Sections were blocked in 10% goat serum diluted in CAS-Block (Thermo Fisher Scientific) for 30 min. Following blocking, sections were incubated overnight at 4°C with mouse monoclonal anti-Ki-67 antibody (1:20; Vector Labs) and rabbit polyclonal anti-FLAG antibody (1:100; Cell Signaling Technology) diluted together in 10% goat serum/CAS-Block. Sections were then incubated for 1 h at room temperature with goat anti-mouse AlexaFluor 488 (1:400) and goat anti-rabbit AlexaFluor 594 (1:400) secondary antibodies (Thermo Fisher Scientific) diluted together in 10% goat serum/CAS-Block. Labelled sections were then mounted in Vectashield mounting medium containing DAPI (Vector Labs) and were imaged using a Zeiss LSM 700 confocal microscope (Jena, Germany).

To examine the subcellular localization of overexpressed FLAG-tagged SK2, NIH3T3 SK2 'low' and 'high' stable cell lines were seeded onto 8-well glass chamber slides (Nalge Nunc International) coated with poly-L-lysine (Sigma-Aldrich) at $4x10^4$ cells/well, and grown overnight in DMEM with 10% DBS. Media was then removed and replaced with DMEM containing 0.5% DBS, and cells were cultured for a further 16 h. Cells were then fixed in 4% paraformaldehyde for 10 min, permeabilized for 10 min in PBS with 0.1% Triton X-100, and blocked in 5% goat serum in PBS with 0.1% Triton X-100 for 60 min. Rabbit monoclonal anti-FLAG antibody (1:200; Cell Signaling Technology) and mouse anti-γ-catenin antibody (1:500; BD Biosciences) were incubated for 1.5 h at room temperature, followed by goat anti-rabbit AlexaFluor 594 and goat anti-mouse AlexaFluor 488 secondary antibodies (1:500; Thermo Fisher Scientific) for 1 h. Cells were counterstained with DAPI to identify nuclei (blue). Fluorescence microscopy and imaging were performed using a Zeiss LSM 700 confocal microscope.

Measuring rate of extracellular S1P production

Rate of extracellular S1P formation from intact vector control 'low', SK2 'low' and SK2 'high' NIH3T3

stable cell lines was determined essentially as previously described [47]. Briefly, cells were seeded in equal numbers at 80% confluence (in 20 cm^2 dishes), and media was replaced with DMEM containing 0.5% DBS and incubated for a further 16 h. Cells were then labelled with 0.5 μCi of [^{3}H]-sphingosine (Perkin-Elmer, Rowville, VIC, Australia) for 30 min, after which the conditioned media was collected. Extracellular [^{3}H]-S1P generated in the conditioned medium was extracted and analyzed by scintillation counting.

Intracellular sphingolipid analyses

Vector control 'low', SK2 'low' and SK2 'high' NIH3T3 stable cell lines were grown in DMEM with 10% DBS to 80% confluence, media was replaced with DMEM containing 0.5% DBS and cells were cultured for 16 h. Cells were trypsinized, quenched and washed in PBS. Cells were pelleted in quadruplicate with 8.8x106 cells per sample, and intracellular sphingolipid species were analyzed by LC-MS, as previously described [48] with the following minor modifications. Prepared samples were injected onto an Ascentis Express C18 column (Supelco Analytical, Bellefonte, PA, USA), and non-natural sphingolipid internal standards were added to each sample to allow relative quantification. Data analysis was performed using Tracefinder (Thermo Fisher Scientific).

ACKNOWLEDGMENTS

We thank S. Escarbe and H. Taing for technical assistance, S. Otto and J. Finnie (SA Pathology, Adelaide) for expert assessment of tumor morphology, and M. Okabe (Osaka University, Japan) for generously providing the pCX-EGFP plasmid.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FINANCIAL SUPPORT

This work was supported by an Australian Postgraduate Award and Royal Adelaide Hospital Dawes Scholarship (to H.A.N.), the Fay Fuller Foundation, Project Grant (626936) and Senior Research Fellowships (508098 and 1042589) from the National Health and Medical Research Council of Australia (NHMRC) (to S.M.P.), and a NHMRC Career Development Fellowship (1088855) (to D.J.C.).

REFERENCES

1. Pitson SM. Regulation of sphingosine kinase and sphingolipid signaling. Trends Biochem Sci. 2011; 36: 97-107.
2. Pyne NJ, Pyne S. Sphingosine 1-phosphate and cancer. Nat Rev Cancer. 2010; 10: 489-503.
3. Pitman MR, Pitson SM. Inhibitors of the sphingosine kinase pathway as potential therapeutics. Curr Cancer Drug Targets. 2010; 10: 354-367.
4. Neubauer HA, Pitson SM. Roles, regulation and inhibitors of sphingosine kinase 2. FEBS J. 2013; 280: 5317-5336.
5. Heffernan-Stroud LA, Obeid LM. Sphingosine kinase 1 in cancer. Adv Cancer Res. 2013; 117: 201-235.
6. Xia P, Gamble JR, Wang L, Pitson SM, Moretti PA, Wattenberg BW, D'Andrea RJ, Vadas MA. An oncogenic role of sphingosine kinase. Curr Biol. 2000; 10: 1527-1530.
7. Igarashi N, Okada T, Hayashi S, Fujita T, Jahangeer S, Nakamura S. Sphingosine kinase 2 is a nuclear protein and inhibits DNA synthesis. J Biol Chem. 2003; 278: 46832-46839.
8. Maceyka M, Sankala H, Hait NC, Le Stunff H, Liu H, Toman R, Collier C, Zhang M, Satin LS, Merrill AH, Milstien S, Spiegel S. SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. J Biol Chem. 2005; 280: 37118-37129.
9. Okada T, Ding G, Sonoda H, Kajimoto T, Haga Y, Khosrowbeygi A, Gao SY, Miwa N, Jahangeer S, Nakamura S. Involvement of N-terminal-extended form of sphingosine kinase 2 in serum-dependent regulation of cell proliferation and apoptosis. J Biol Chem. 2005; 280: 36318-36325.
10. Liu H, Toman RE, Goparaju SK, Maceyka M, Nava VE, Sankala H, Payne SG, Bektas M, Ishii I, Chun J, Milstien S, Spiegel S. Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. J Biol Chem. 2003; 278: 40330-40336.
11. Chipuk JE, McStay GP, Bharti A, Kuwana T, Clarke CJ, Siskind LJ, Obeid LM, Green DR. Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. Cell. 2012; 148: 988-1000.
12. Hofmann LP, Ren S, Schwalm S, Pfeilschifter J, Huwiler A. Sphingosine kinase 1 and 2 regulate the capacity of mesangial cells to resist apoptotic stimuli in an opposing manner. Biol Chem. 2008; 389: 1399-1407.
13. Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, Luo C, Marmorstein R, Kordula T, Milstien S, Spiegel S. Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science. 2009; 325: 1254-1257.
14. Van Brocklyn JR, Jackson CA, Pearl DK, Kotur MS, Snyder PJ, Prior TW. Sphingosine kinase-1 expression correlates with poor survival of patients with glioblastoma multiforme: Roles of sphingosine kinase isoforms in growth of glioblastoma cell lines. J Neuropathol Exp Neurol. 2005; 64: 695-705.

15. Sankala HM, Hait NC, Paugh SW, Shida D, Lepine S, Elmore LW, Dent P, Milstien S, Spiegel S. Involvement of sphingosine kinase 2 in p53-independent induction of p21 by the chemotherapeutic drug doxorubicin. Cancer Res. 2007; 67: 10466-10474.

16. Schnitzer SE, Weigert A, Zhou J, Brune B. Hypoxia enhances sphingosine kinase 2 activity and provokes sphingosine-1-phosphate-mediated chemoresistance in A549 lung cancer cells. Mol Cancer Res. 2009; 7: 393-401.

17. Nemoto S, Nakamura M, Osawa Y, Kono S, Itoh Y, Okano Y, Murate T, Hara A, Ueda H, Nozawa Y, Banno Y. Sphingosine kinase isoforms regulate oxaliplatin sensitivity of human colon cancer cells through ceramide accumulation and Akt activation. J Biol Chem. 2009; 284: 10422-10432.

18. Gao P, Smith CD. Ablation of sphingosine kinase-2 inhibits tumor cell proliferation and migration. Mol Cancer Res. 2011; 9: 1509-1519.

19. Weigert A, Schiffmann S, Sekar D, Ley S, Menrad H, Werno C, Grosch S, Geisslinger G, Brune B. Sphingosine kinase 2 deficient tumor xenografts show impaired growth and fail to polarize macrophages towards an anti-inflammatory phenotype. Int J Cancer. 2009; 125: 2114-2121.

20. Wallington-Beddoe CT, Powell JA, Tong D, Pitson SM, Bradstock KF, Bendall LJ. Sphingosine kinase 2 promotes acute lymphoblastic leukemia by enhancing MYC expression. Cancer Res. 2014; 74: 2803-2815.

21. Liu K, Guo TL, Hait NC, Allegood J, Parikh HI, Xu W, Kellogg GE, Grant S, Spiegel S, Zhang S. Biological characterization of 3-(2-amino-ethyl)-5- [3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) as a selective sphingosine kinase-2 inhibitor and anticancer agent. PLoS ONE. 2013; 8: e56471.

22. Chumanevich AA, Poudyal D, Cui X, Davis T, Wood PA, Smith CD, Hofseth LJ. Suppression of colitis-driven colon cancer in mice by a novel small molecule inhibitor of sphingosine kinase. Carcinogenesis. 2010; 31: 1787-1793.

23. French KJ, Zhuang Y, Maines LW, Gao P, Wang W, Beljanski V, Upson JJ, Green CL, Keller SN, Smith CD. Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. J Pharmacol Exp Ther. 2010; 333: 129-139.

24. Wang Q, Li J, Li G, Li Y, Xu C, Li M, Xu G, Fu S. Prognostic significance of sphingosine kinase 2 expression in non-small cell lung cancer. Tumour Biol. 2014; 35: 363-368.

25. Venkata JK, An N, Stuart R, Costa LJ, Cai H, Coker W, Song JH, Gibbs K, Matson T, Garrett-Mayer E, Wan Z, Ogretmen B, Smith C, et al. Inhibition of sphingosine kinase 2 downregulates the expression of c-Myc and Mcl-1 and induces apoptosis in multiple myeloma. Blood. 2014; 124: 1915-1925.

26. Panneer Selvam S, De Palma RM, Oaks JJ, Oleinik N, Peterson YK, Stahelin RV, Skordalakes E, Ponnusamy S, Garrett-Mayer E, Smith CD, Ogretmen B. Binding of the sphingolipid S1P to hTERT stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation. Sci Signal. 2015; 8: ra58.

27. Adada MM, Canals D, Jeong N, Kelkar AD, Hernandez-Corbacho M, Pulkoski-Gross MJ, Donaldson JC, Hannun YA, Obeid LM. Intracellular sphingosine kinase 2-derived sphingosine-1-phosphate mediates epidermal growth factor-induced ezrin-radixin-moesin phosphorylation and cancer cell invasion. FASEB J. 2015; 29: 4654-4669.

28. Rhodes DR, Yu J, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, Pandey A, Chinnaiyan AM. ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia. 2004; 6: 1-6.

29. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA. Creation of human tumour cells with defined genetic elements. Nature. 1999; 400: 464-468.

30. Siow D, Wattenberg B. The compartmentalization and translocation of the sphingosine kinases: Mechanisms and functions in cell signaling and sphingolipid metabolism. Crit Rev Biochem Mol Biol. 2011; 46: 365-375.

31. Pitson SM, Xia P, Leclercq TM, Moretti PAB, Zebol JR, Lynn HE, Wattenberg BW, Vadas MA. Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling. J Exp Med. 2005; 201: 49-54.

32. Allende ML, Sasaki T, Kawai H, Olivera A, Mi YD, van Echten-Deckert G, Hajdu R, Rosenbach M, Keohane CA, Mandala S, Spiegel S, Proia RL. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. J Biol Chem. 2004; 279: 52487-52492.

33. Zemann B, Urtz N, Reuschel R, Mechtcheriakova D, Bornancin F, Badegruber R, Baumruker T, Billich A. Normal neutrophil functions in sphingosine kinase type 1 and 2 knockout mice. Immunol Lett. 2007; 109: 56-63.

34. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol. 2005; 25: 11113-11121.

35. Vadas M, Xia P, McCaughan G, Gamble J. The role of sphingosine kinase 1 in cancer: oncogene or non-oncogene addiction? Biochim Biophys Acta. 2008; 1781: 442-447.

36. Pitman MR, Powell JA, Coolen C, Moretti PA, Zebol JR, Pham DH, Finnie JW, Don AS, Ebert LM, Bonder CS, Gliddon BL, Pitson SM. A selective ATP-competitive sphingosine kinase inhibitor demonstrates anti-cancer properties. Oncotarget. 2015; 6: 7065-7083. doi: 10.18632/oncotarget.3178.

37. French KJ, Upson JJ, Keller SN, Zhuang Y, Yun JK, Smith CD. Antitumor activity of sphingosine kinase inhibitors. J Pharmacol Exp Ther. 2006; 318: 596-603.

38. Takabe K, Spiegel S. Export of sphingosine-1-phosphate and cancer progression. J Lipid Res. 2014; 55: 1839-1846.

39. Weigert A, Cremer S, Schmidt MV, von Knethen A, Angioni C, Geisslinger G, Brune B. Cleavage of sphingosine kinase 2 by caspase-1 provokes its release from apoptotic cells. Blood. 2010; 115: 3531-3540.

40. Liang J, Nagahashi M, Kim EY, Harikumar KB, Yamada A, Huang WC, Hait NC, Allegood JC, Price MM, Avni D, Takabe K, Kordula T, Milstien S, et al. Sphingosine-1-phosphate links persistent STAT3 activation, chronic intestinal inflammation, and development of colitis-associated cancer. Cancer Cell. 2013; 23: 107-120.

41. Kawamori T, Kaneshiro T, Okumura M, Maalouf S, Uflacker A, Bielawski J, Hannun YA, Obeid LM. Role for sphingosine kinase 1 in colon carcinogenesis. FASEB J. 2009; 23: 405-414.

42. Pham DH, Moretti PA, Goodall GJ, Pitson SM. Attenuation of leakiness in doxycycline-inducible expression via incorporation of 3' AU-rich mRNA destabilizing elements. BioTechniques. 2008; 45: 155-162.

43. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett. 1997; 407: 313-319.

44. Pham DH, Powell JA, Gliddon BL, Moretti PA, Tsykin A, Van der Hoek M, Kenyon R, Goodall GJ, Pitson SM. Enhanced expression of transferrin receptor 1 contributes to oncogenic signalling by sphingosine kinase 1. Oncogene. 2014; 33: 5559-5568.

45. Roberts JL, Moretti PA, Darrow AL, Derian CK, Vadas MA, Pitson SM. An assay for sphingosine kinase activity using biotinylated sphingosine and streptavidin-coated membranes. Anal Biochem. 2004; 331: 122-129.

46. Pitman MR, Pham DH, Pitson SM. Isoform-selective assays for sphingosine kinase activity. Methods Mol Biol. 2012; 874: 21-31.

47. Sun WY, Dimasi DP, Pitman MR, Zhuang Y, Heddle R, Pitson SM, Grimbaldeston MA, Bonder CS. Topical application of Fingolimod perturbs cutaneous inflammation. J Immunol. 2016; 196: 3854-3864.

48. Aurelio L, Scullino CV, Pitman MR, Sexton A, Oliver V, Davies L, Rebello RJ, Furic L, Creek DJ, Pitson SM, Flynn BL. From sphingosine kinase to dihydroceramide desaturase: A structure-activity relationship (SAR) study of the enzyme inhibitory and anticancer activity of 4-((4-(4-chlorophenyl)thiazol-2-yl)amino)phenol (SKI-II). J Med Chem. 2016; 59: 965-984.