Dissecting Signalling Contributions of the Alpha and Beta Subunits of the GM-CSF Receptor

Michelle Perugini, B. Med. Pharm. Biotech. (Hons.)

December, 2006

A thesis submitted for the degree of Doctor of Philosophy in the

School of Medicine at the University of Adelaide

Declaration

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and that, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to the thesis being made available for photocopying and loan if accepted for the award of the degree.

Michelle Perugini

December 2006

Acknowledgements

I would like to thank all of the staff and students at the Child Health Research Institute where this research was undertaken. The continuous scientific and non-scientific dialogue was useful and greatly appreciated. Thanks also to the University of Adelaide for their financial support and resources.

A special thankyou to my supervisor Richard D'Andrea who has provided continuous support, encouragement, and scientific advice throughout my project. I am very grateful for your help and for making my PhD such an enjoyable experience. I would also like to thank my co-supervisor Tom Gonda for his helpful discussions and meetings throughout my tenure.

To all the members of the Leukaemia and Molecular Immunology Laboratories, thankyou for your friendships, and useful scientific and non-scientific communications. You made this a wonderful place in which to work and made my PhD experience more than tolerable.

To my parents, sister, and in-laws, thank you for your continuing support throughout this experience. I certainly could not have come this far without your help and encouragement and I love you all dearly.

Lastly, but certainly not least I wish to thank my husband, Don, the one person I have leant on continuously for support and without whom this would have been a far less enjoyable experience. I love you dearly and thank you for being my inspiration and motivation in life.

List of Figures

List of Tables

List of Appendices

Abbreviations

aa	Amino acid
Ab	Antibody
Abs	Absorbance
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
bp	Base pairs
BM	Bone marrow
BSA	Bovine serum albumin
cDNA	Complementary DNA
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleic acid triphosphates
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetra-acetate
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid
ERK	Extracellular regulated kinase
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal bovine serum
FITC	fluorescein isothiocyanate
g	Gram
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMR	Granulocyte-macrophage colony-stimulating factor receptor
GMRα	GMR alpha subunit
GST	Glutathione-S-transferase
$h\beta_c$	Human beta common
hr	Hour
HRP	Horse radish peroxidase
IκB	I-kappa-B
IKKβ	I-kappa-B kinase beta
IMDM	Iscove's modified Dulbecco's medium
IPTG	Isopropylthio-beta-D-galactosidase
JAK2	Janus kinase 2
kDa	Kilo Dalton

LB	Luria broth
M	Molar
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MFI	Mean fluorescence intensity
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
NFκB	Nuclear factor-kappa-B
OD	Optical density
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositol 3 kinase
RIPA	Radioimmunoprecipitation buffer
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDM	Site-directed mutagenesis
STAT5	Signal transducer and activator of transcription 5
TEMED	N,N,N',N'-Tetramethylethylenediamine
μ	Micro (10^{-6})
μg	Micro gram
μL	Micro liter
μM	Micro molar
WCL	Whole cell lysate
WT	Wild-type
w/v	weight to volume

Abstract

Normal tissue homeostasis and appropriate responses to injury and infection are dependent on cellular communication mediated by cell surface receptors that respond to extrinsic stimuli. The GM-CSF receptor was the major focus of this project. This receptor shares a common signalling subunit, β_c, with the IL-3 and IL-5 receptors. The unique GM-CSF receptor α-subunit (GMRα) confers ligand binding specificity to the complex and is essential for GM-CSF receptor signalling, although the full complement of signalling events mediated by GMRα remains elusive. Through cloning of candidate interacting proteins, expression and co-immunoprecipitation studies, we have confirmed interactions for two proteins previously reported to interact with the GMRα, p85 and IKKβ. Additionally, we identified the Src family kinase, Lyn, as a novel direct interacting partner of GMRα and provide insights into possible roles of this kinase in initiating signalling from the GM-CSF receptor. In addition to GMRα associated events we aimed to further characterise the role of the common β_c subunit in GM-CSF mediated signalling. We utilised two classes of consitutively active β_c mutants (extracellular or transmembrane) which transform the bi-potential myeloid FDB1 cell line to either factor-independent growth and survival, or granulocyte-macrophage differentiation, respectively. Here we report a comprehensive biochemical analysis of signalling by these two classes of mutants in this cell line. The two activated GMR mutants displayed distinct and non-overlapping signalling capacity. In particular, expression of a mutant with a substitution in the transmembrane domain (V449E) selectively activated JAK/STAT5 and MAPK pathways resulting in a high level of sensitivity to JAK and MEK inhibitors. In contrast, expression of a mutant with a 37

amino acid duplication in its extracellular domain (FIΔ) selectively activates the PI3K/AKT and IKKβ/NFκB pathways. Cells responding to this mutant display a relative high level of sensitivity to two independent PI3K inhibitors and relative resistance to inhibition of MEK and JAK2. The non-overlapping nature of signalling by these two activated mutants suggests that there are alternative modes of receptor activation that differentially dependent on JAK2 and that act synergistically in the mature liganded cytokine receptor complex. Further detailed analysis of these mutants will facilitate the dissection of the signalling pathways involved in the GM-CSF response that mediate proliferation, survival and differentiation.

Table of Contents

CHAPTER 1

INTRODUCTION

1.1 Haemopoiesis

Haemopoiesis is the process whereby multipotent haemopoietic stem cells give rise to committed progenitors which in turn differentiate to produce functionally diverse mature blood cells (**Figure 1.1**) (Metcalf and Nicola 1995; Evans 1997; Passegue, Jamieson et al. 2003). Haemopoietic stem cells are capable of self-renewal, the ability to produce a daughter cell identical to the parent cell, and thus retaining the same developmental potential. Secondly, haemopoietic stem cells have the potential to give rise to all cells of both lymphoid and myeloid compartments and thus can reconstitute all cells in the blood system in a transplantation setting.

Haemopoiesis involves the replacement of old or damaged cells as a consequence of normal 'steady-state' cell turnover as well as generation of large numbers of specific cell types. Haemopoiesis therefore maintains homeostasis and regulates immune defences against pathogens (Barreda, Hanington et al. 2004). The process of steady-state haemopoiesis is tightly governed by signal transduction processes that are mediated by cytokines and their cognate receptors. Cytokines produce multiple effects dependent on the cell type and stage of differentiation (Kishimoto 1994). Associated with this pleiotropic nature is an ability to activate a variety of signalling pathways which have overlapping outcomes. The transmission of signals from the cell surface to the nucleus through complex signal transduction processes results in changes to biological outcomes including motility, adhesion, survival, growth and differentiation (Kishimoto 1994; Taniguchi 1995). In addition, co-ordination of these cytokine-induced signalling events with the activity of lineage specific transcription factors

results in changes to proliferation and differentiation programs and amplification of particular lineages.

It is notable that as stem cells differentiate their proliferative potential is reduced until they become mature blood cell types at which point they have very limited proliferative or self renewal ability (Reddy, Korapati et al. 2000). Haemopoiesis is therefore often thought of as a balance between differentiation and proliferation/self-renewal capability, both of which can be modulated by cytokines signalling through their cognate receptors. Disruption of this balance can result in pathologies characterised by an overproduction of progenitor cells and/or a lack of differentiation into mature cell lineages. Importantly it has been demonstrated in mouse models that a combination of a differentiation suppression signal along with a growth factor, proliferation/survival signal, can reconstitute leukaemia (Deguchi and Gilliland 2002). This requirement for cooperating lesions is a reflection of the multi-step process that is characteristic of leukaemia and many other cancers.

A great deal of research has been performed on the individual components of receptor complexes and signal transduction pathways. However, linking receptor proximal signalling events with specific biological outcomes has been challenging given the high degree of pleiotropy displayed by cytokines and the complexity and redundancy associated with signal transduction pathways (Bagley, Woodcock et al. 1997; Ozaki and Leonard 2002). It is essential to study this process further to understand and identify key signalling components and to establish how deregulation can lead to a variety of blood and immune disorders including leukaemia. In doing this new targets for therapy will be identified.

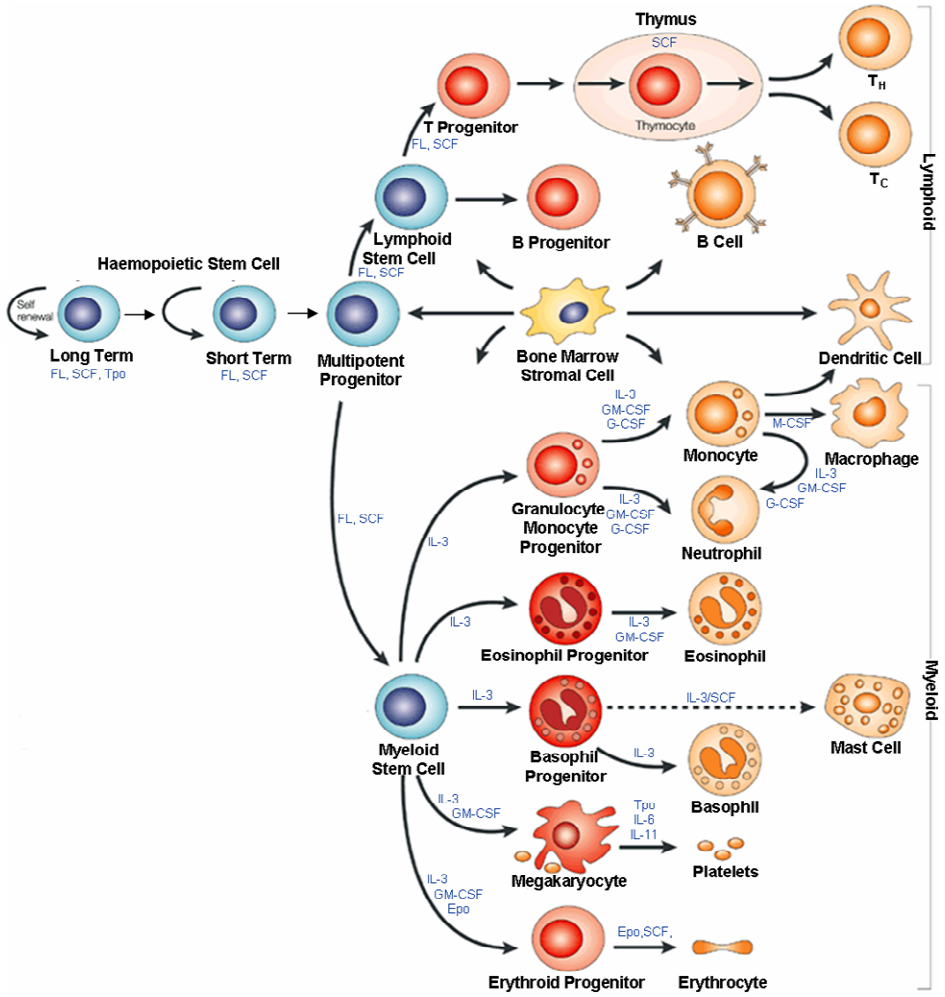


Figure 1.1 Schematic representation of haemopoiesis (adapted from Nabel 2002). Key myeloid and stem-cell cytokines are shown.

1.2 Growth Factors and their Receptors in Haemopoiesis

1.2.1 Receptor Tyrosine Kinases Involved in Haemopoiesis

Receptor tyrosine kinases (RTKs), represent a distinct family of receptors that play important roles in regulating cell proliferation, differentiation and survival signalling (Reilly 2003). Multiple receptor tyrosine kinases including c-Kit and FLT3 are crucial regulators of steady-state haemopoiesis and co-operate with key cytokine receptors to modulate these processes (**Figure 1.1**) (Scheijen and Griffin 2002). FLT3 and c-Kit are

both members of the type III receptor tyrosine kinase subfamily. Other members of this family include the receptors for colony-sitmulating factor (CSF-1), and platelet derived growth factor (PDGF). Unlike cytokine receptors, receptor tyrosine kinases have intrinsic tyrosine kinase domains. They therefore lack motifs including Box 1 and Box 2 which are important for association with cytoplasmic tyrosine kinases.

FLT3 is expressed in pre-B, monocytic and myeloid cell lines (Brasel, Escobar et al. 1995; Meierhoff, Dehmel et al. 1995; Naoe and Kiyoi 2004). The FLT3 ligand, FL, has been demonstrated to enhance the colony stimulating activity of haemopoietic progenitor cells in synergy with G-CSF, GM-CSF, M-CSF, IL-3 IL-6, IL-11, IL-12 or Kit ligand (KL) (Scheijen and Griffin 2002). c-Kit is expressed on haemopoietic stem cells as well as myeloid, erythroid, megakaryocytic and dentritic progenitors, pro-B and pro-T cells, and mature mast cells (Reviewed by (Ashman 1999) (**Figure 1.1**). In early myeloid cells c-Kit influences survival and proliferation responses through phosphatidyl inositol-3 kinase (PI3K) activation (Young, Cambareri et al. 2006). The receptor for CSF-1 is encoded by the c-fms proto-oncogene. CSF-1 is predominantly expressed on cells of the macrophage lineage at late stages of differentiation (Sweet and Hume 2003).

In addition to their roles in regulating steady-state haemopoiesis, mutations in receptor tyrosine kinases are common in haematological malignancies (reviewed in Reilly 2003). Increased expression of FLT3 is detected on most leukaemic samples of acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (B-ALL), but at low levels in T-ALL (Birg, Courcoul et al. 1992; Carow, Levenstein et al. 1996). Additionally, FLT3 internal tandem duplication (ITD) and tyrosine kinase domain (TKD) mutations represent the most common mutations in AML with approximately 30% of AML

patients expressing a FLT3 mutation (Kottaridis, Gale et al. 2001). These mutations result in constitutive activation of signal transducer and activator of transcription (STAT5) and mitogen activated protein kinase (MAPK) pathways and are associated with increased phosphorylation of AKT (Scheijen and Griffin 2002). Mutations of c-Kit are also associated with haematological malignancies. Activating point mutations in c-Kit have been identified in patients with mastocytosis (Nagata, Worobec et al. 1995; Longley, Tyrrell et al. 1996; Longley 1999). Another common c-Kit mutation substituting aspartic acid at codon 816 to valine (D816V) has been identified in patients with myeloproliferative disorders (Beghini, Peterlongo et al. 2000; Ning, Li et al. 2001), AML (Ikeda, Kanakura et al. 1991; Cole, Aylett et al. 1996), mast cell tumours (Butterfield, Weiler et al. 1988), breast cancer (Hines, Litz et al. 1999) and germ cell tumours (Tian, Frierson et al. 1999). More recently, ITD mutations have also been identified in c-Kit (Cairoli, Beghini et al. 2006).

1.2.2 Cytokine Receptor Structure and Classification

Cytokines are regulatory proteins secreted by white blood cells and a variety of other cells in the body; the pleiotropic actions of cytokines include numerous effects on primitive haemopoietic cells and other cells of the immune system and modulation of inflammatory responses (Vilcek 1998; Ozaki and Leonard 2002). Cytokines are polypeptides or glycoproteins that bind to specific high-affinity cell surface receptors. The interaction between a cytokine and its receptor ultimately results in an altered gene expression pattern in the target cells, resulting in modulation of proliferation, differentiation state, and cell survival (Vilcek 1998). Individual cytokines act on multiple cell types, but at least some actions of each cytokine are directed at haemopoietic cells. Structurally dissimilar cytokines can perform similar actions (Arai,

Lee et al. 1990; Nicola 1994). Additionally, individual cytokines can exhibit numerous actions on different cell types and tissues. There is also significant redundancy associated with the signal transduction pathways activated by various cytokines.

The activity of cytokines depends on their interaction with specific receptors. Class I cytokine receptors represent the largest subgroup of the cytokine receptor superfamily and are of particular relevance to this project. Class I receptors include interleukins (IL) -2, -4, -6, -12, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (Epo), growth hormone (GH) and prolactin (Bazan 1989; Kishimoto 1994; Taga and Kishimoto 1995)(**Table 1**). Many cytokine receptors share a common signalling subunit (β or γ) and are further classified into subfamilies based on this shared subunit (**Table 1**) (Taga and Kishimoto 1995).

Table 1. Classification of Cytokines and their Receptors. $^{+}$IL-13 uses IL-4Rα not γ_c. Leptin^^ and IL-12* do not share gp130, but their receptors are related. ^NNT-11/BSF-3 binds IL-12Rβ1 (modified from Vilcek 1998).

Cytokine receptor	Cytokines
Class I Cytokine receptors	
Cytokine receptors that share γ_c	IL-2, IL-4, IL-7, IL-9, IL-13^{+}, IL-15, IL-21,
Cytokine receptors that share β_c	IL-3, IL-5, GM-CSF
Cytokine receptors that share gp130	IL-6, IL-11, IL-12*, IL-23, LIF, Oncostatin M, CNTF,CT-1, G-CSF, Leptin^^, ^NNT-1/BSF-3
Cytokine receptors that are comprised of a single chain only	GH, EPO, PRL, TPO
Class II Cytokine Receptors	
Cytokine receptors utilising two or more distinct subunits	IFN-γ, IFN-αs/β/ω/Limitin, IL-10, IL-19, IL-20, IL-22, mda-7/IL-24, AK155/IL-26, FISP

Class II cytokine receptors are structurally related to Class I receptors (Bazan 1990) and include the heterodimeric receptors for interferon α (IFNα), IFNβ, and IFNγ (Bach, Tanner et al. 1996; Domanski and Colamonici 1996; Pestka, Kotenko et al. 1997) and IL-10 (Ho, Liu et al. 1993) (**Table 1**).

Some common structural features characterise members of the Class I cytokine receptor superfamily. Class I Cytokine receptors are characterised by an extracellular cytokine receptor module (CRM) which contains cytokine receptor domains comprising several beta strands. Within this region lies a membrane proximal, conserved WSXWS motif which has been shown to be critical for folding of the receptor and stabilisation of its structure (**Figure 1.2**) (Bazan 1990; Quelle, Quelle et al. 1992; Bagley, Woodcock et al. 1997) .

Similarities in cytokine responses can in part be explained by the sharing of receptor subunits (Kishimoto 1994), (Ihle, Witthuhn et al. 1995). In addition to the shared signalling subunit, many cytokine receptor complexes are heterodimers including a ligand specific α subunit. The exception to this is the EpoR subfamily of receptors that form functional homodimers (**Figure 1.2**). Ligand binding to the α subunit of the receptor occurs with low affinity, and binding to the signalling subunit converts this complex to a high affinity state as exemplified by the IL-3/IL-5/GM-CSF family of receptors (Hayashida, Kitamura et al. 1990; Kitamura, Sato et al. 1991; Watowich, Wu et al. 1996; Bagley, Woodcock et al. 1997).

Other cytoplasmic motifs, such as the Box 1 and Box 2 motifs (Ihle 1995; Theze, Alzari et al. 1996) and the SH3 binding site PROX-like (SBP) motif (D'Andrea, Sadlon et al.

2004) may also be present in these cytokine receptors, however, these vary between the family members and will be discussed separately in Section 1.2.3 (**Figure 1.2**).

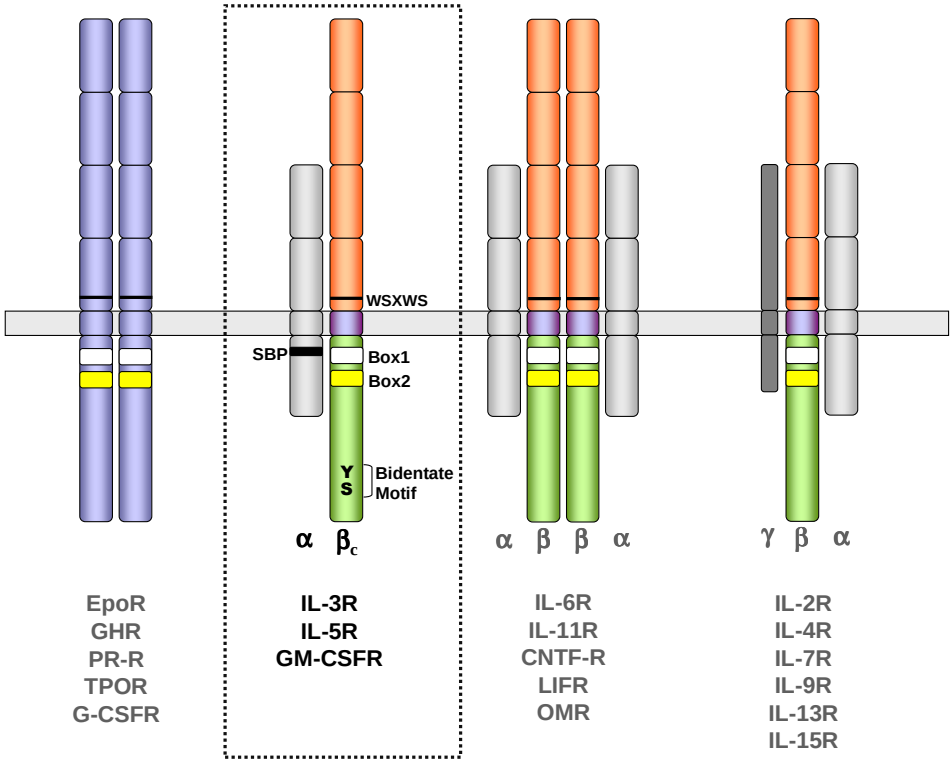


Figure 1.2 Schematic of the Class I Cytokine Receptors. Members of the Class I cytokine receptor family have been divided into subfamilies on the basis of their shared subunits. The IL-3/IL-5/GM-CSF family of receptors is highlighted and conserved WSXWS, Box1 (Tanner, Chen et al. 1995), Box2 (Tanner, Chen et al. 1995), SBP (D'Andrea, Sadlon et al. 2004) and Y577/S585 bidentate (Guthridge, Powell et al. 2006) motifs are indicated. Other receptor subfamilies indicated may also contain Box1, Box2 and WSXWS motifs (labels have been omitted for simplicity). In addition, a hybrid Box1/SBP motif has been identified in some cytokine receptor subunits, however these have not been highlighted (D'Andrea, Sadlon et al. 2004). The mature GM-CSF receptor is most likely a higher order complex (McClure, Woodcock et al. 2001), however, for simplicity this receptor has been represented as an α/β_c heterodimer. This picture was adapted from (Ihle, Witthuhn et al. 1994).

1.2.3 Cytokine Receptor Motifs Important in Receptor Signalling

The ability of signalling molecules to alter cell activity, in response to cytokine, is dependent on their ability to associate specifically with activated cytokine receptors and other molecules to form active complexes. There are various conserved domains that are present in the intracellular portions of cytokine receptors. These domains direct interactions and formation of signalling complexes following stimulation of the receptor. Some of the key domains involved in cytokine receptor signalling that are relevant to this study are discussed below.

Box1 motifs have been extensively characterised and are known to mediate binding to the FERM (Four-point-one, Ezrin, Radixin, Moesin) domain of Janus kinase (JAK) family members (reviewed in Haan, Kreis et al. 2006). This motif is highly conserved among cytokine receptors, and is usually contained within the signalling subunit of heterodimeric receptors (**Figure 1.2**).

The alignment in **Figure 1.3** highlights two common proline rich motifs found in the membrane proximal regions of cytokine receptors (D'Andrea, Sadlon et al. 2004). This includes the canonical Box1 motif, essential for JAK activation, along with a distinct SBP motif. The configuration of these motifs suggest that they cooperate to fully activate the ligand bound receptor (D'Andrea, Sadlon et al. 2004). In some cases these motifs overlap, while in other receptor complexes the two motifs are provided by heterologous receptor subunits (**Figure 1.3**) (D'Andrea, Sadlon et al. 2004). The SBP motif bears structural similarity to the 'PROX' domain in γ_c which is critical for JAK3 activation and for generating JAK3-independent signals including JAK3-independent activation of JAK1 (Ellery and Nicholls 2002). The consensus sequence of the SBP

motif is ϕXX(k/r)(I/L)(F/W)Pp(I/V)PXϕkXXϕX(D/E); where ϕ represents a hydrophobic residue. The core of this motif bears similarity to the Class I SH3 domain binding site (R/K)XϕPXϕP (Mayer and Gupta 1998) (**Figure 1.3**). Given the sequence similarity between the SBP motif and the Class I SH3 binding site, it is likely that the SBP motif may bind SH3-domain containing substrates including Src family kinases (SFKs). Identification of interacting partners of the GM-CSF receptor alpha subunit (GMRα), which are mediated by the SBP motif, formed a major part of this project (discussed further in Chapter 3).

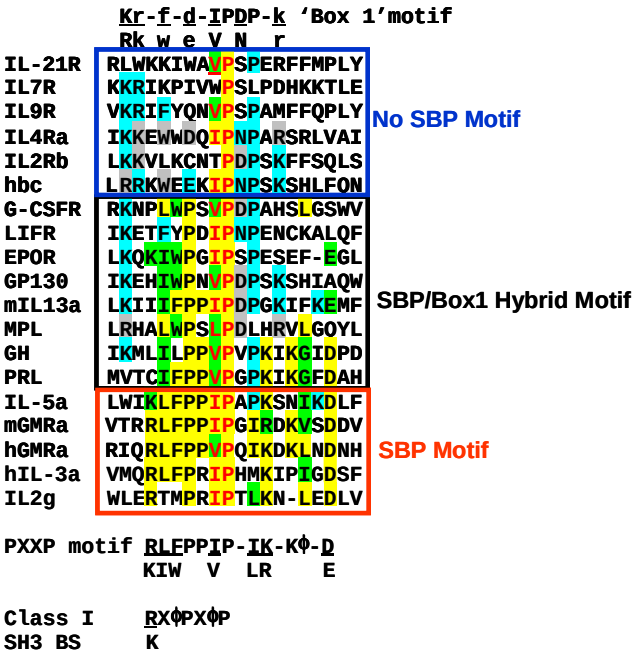


Figure 1.3 Overlapping motifs in the membrane-proximal region of cytokine receptors (adapted from D'Andrea, Sadlon et al. 2004). The receptors outlined in blue do not contain an SBP motif but dimerise with receptor subunits that do contain an SBP motif (red outline). The receptors outlined in black exhibit a hybrid of both Box1 and SBP motifs and can signal independently of the SBP containing subunits.

1.3 Intracellular Signalling by Cytokine Receptors

Class I cytokine receptors lack intrinsic kinase activity and the initial activation event following ligand binding is thought to involve non-receptor protein tyrosine kinases such as members of the JAK, Src, Fps/Fes, Tec/Btk and Syk/ZAP70 families (reviewed in Rane and Reddy 2002). These kinases serve to phosphorylate tyrosine residues on the receptor and other targets and subsequently phosphorylate and activate downstream signalling molecules. Ultimately the signal is transduced to the nucleus where gene transcription profiles are altered. The characteristics and properties of key signalling proteins involved in cytokine receptor responses are discussed below.

1.3.1 Janus Kinase Structure and Function

JAKs are fundamental regulators of cytokine receptor activation and are essential for multiple cytokine responses (Nosaka, van Deursen et al. 1995; Neubauer, Cumano et al. 1998; Parganas, Wang et al. 1998; Rodig, Meraz et al. 1998; Haan, Kreis et al. 2006). The JAK family consists of JAK1, JAK2, JAK3 and Tyk2 (reviewed in (Ihle, Stravapodis et al. 1998; Ward, Touw et al. 2000; Valentino and Pierre 2006). JAK1/2 and Tyk2 are ubiquitously expressed. JAK3 is predominantly expressed in haemopoietic cells and is associated with the IL-2R γ_c where it is involved in IL-2, 4, 7, 9 and 15 signalling (Nelson, Lord et al. 1996; Suzuki, Nakajima et al. 2000). Knockout mice of all four JAKs exist and display immune or haemopoietic phenotypes associated with multiple cytokine signalling deficiencies (Nosaka, van Deursen et al. 1995; Park, Saijo et al. 1995; Thomis, Lee et al. 1997; Neubauer, Cumano et al. 1998; Parganas, Wang et al. 1998; Rodig, Meraz et al. 1998; Karaghiosoff, Neubauer et al. 2000; Shimoda, Kato et al. 2000).

JAKs are approximately 130kDa, comprise a carboxyl kinase domain and a pseudokinase domain, and lack Src homology (SH-), SH2 and SH3, domains (**Figure 1.4**). JAKs have 7 JAK homology domains, JH1-JH7, of which JH1 and JH2 bear structural homology to tyrosine kinase domains (Giordanetto and Kroemer 2002; Giordanetto and Kroemer 2002). It appears, however, that JH1 is the only domain that has kinase activity. JH2 lacks certain critical amino acids that are required for this activity (Giordanetto and Kroemer 2002; Khwaja 2006) and is referred to as a pseudokinase domain.

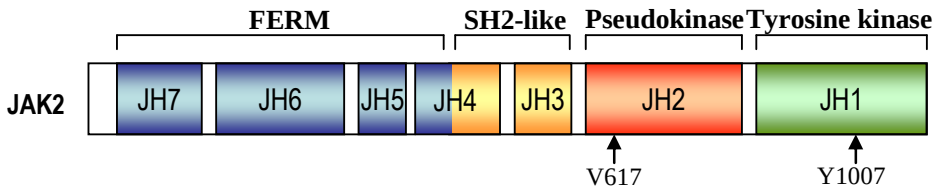


Figure 1.4 Schematic domain structure of JAK kinase (adapted from Valentino and Pierre 2006) The JAK2 mutation (V617F) found in myeloproliferative disorders and the key regulatory phosphorylation site (Y1007) are shown.

The JH2 pseudokinase domain, although not implicated as a functional tyrosine kinase, plays a regulatory role in JAK2 catalytic activity and is important for STAT5 activation by JAK2 (Luo 1997; Liu, Itoh et al. 1999; Saharinen 2000; Rane and Reddy 2002). The JH2 domain has a role in regulating the JH1 domain and deletions in the JH2 domain have been associated with increased JAK2 and JAK3 basal activity (Saharinen 2000). For example, deletions or phosphorylation of the tyrosine in the JH2 domain (Y570), can abolish the JAK2 response to cytokine (Saharinen and Silvennoinen 2002; Saharinen, Vihinen et al. 2003; Feener, Rosario et al. 2004). Most strikingly a mutation in the JH2 domain of JAK2 (V617F) comprising a single amino acid alteration from

valine to phenylalanine has been identified in greater than 90% of Polycythemia vera patients. The role of JAKs in haematological malignancies is discussed further in Section 1.3.3.

The JH3-JH7 domains, comprising the N-terminal region of JAKs, are involved in interactions with cytokine receptors (Zhao, Wagner et al. 1995; Chen, Cheng et al. 1997; Kohlhuber, Rogers et al. 1997; Richter, Dumenil et al. 1998; Cacalano, Migone et al. 1999; Yeh, Dondi et al. 2000). In particular the FERM domain (**Figure 1.4**) of JAKs, has been shown to be pivotal for binding to cytokine receptors (reviewed in Haan, Kreis et al. 2006). JAKs bind to highly conserved Box1 and Box2 motifs on receptor subunits (Ihle, Stravapodis et al. 1998; Ward, Touw et al. 2000; Valentino and Pierre 2006). The proline-rich Box1 motif has approximately 8 amino acids that are required for the interaction with the FERM domain of JAKs (Pellegrini and Dusanter-Fourt 1997; Haan, Is'harc et al. 2001; Haan, Heinrich et al. 2002; Usacheva 2002; Khwaja 2006). The role of the Box2 motif is still not clear, however, it has been shown that this motif facilitates the interaction between JAKs and the Box1 region on cytokine receptors (Usacheva 2002).

Although JAK activation is a critical first step in cytokine receptor signalling, the nature of the trigger that activates JAKs remain unclear. The current dogma suggests that JAKs are constitutively associated (Ihle 2001) with the receptor subunits and receptor oligomerisation induces the close proximity of receptor associated JAKs enabling their transphosphorylation and activation. Evidence that this is always necessary or sufficient is lacking (O'Shea 1997; Rane and Reddy 2002; Khwaja 2006). Evidence for constitutive association of JAKs with cytokine receptors is supported by localisation

studies that show JAKs predominantly localise to the plasma membrane and share a near identical half life to their respective cytokine receptors (Behrmann, Smyczek et al. 2004). Thus, JAK-cytokine receptor complexes may behave as a receptor tyrosine kinase (reviewed in Haan, Kreis et al. 2006).

The site of transphosphorylation of JAK2, is tyrosine 1007 (Y1007) in the JH1 domain (**Figure 1.4**), which is essential for JAK2 regulation and function (Feng, Witthuhn et al. 1997). As a result of JAK activation, cytokine receptors are phosphorylated on tyrosine residues allowing the recruitment of proteins with SH2 or phosphotyrosine binding (PTB) domains (Nosaka and Kitamura 2000). This initiates a signalling cascade which includes the activation of STATs, MAPK, AKT and other signalling pathways.

1.3.2 Signal Transducers and Activators of Transcription

The STAT family of proteins, are transcription factors that, as their name suggests, transduce cytokine-initiated signals from the plasma membrane to the nucleus. There are 7 members of the STAT family that have been identified in mammalian cells since their original discovery in the early 1990s, these include; STAT1, 2, 3, 4, 5A, 5B, and 6 (Benekli, Baer et al. 2003; Wittig and Groner 2005; Khwaja 2006). STAT3 and STAT5 are the most common STATs involved in control of myelopoiesis (Coffer, Koenderman et al. 2000; Smithgall, Briggs et al. 2000; Benekli, Baer et al. 2003).

A critical role for STAT3 in myeloid differentiation has been identified using dominant negative mutants of STAT3 (Minami, Inoue et al. 1996; Nakajima, Yamanaka et al. 1996; Shimozaki, Nakajima et al. 1997). Specifically, STAT3 has been implicated in G-CSF-induced myeloid differentiation of M1 cells, where expression of dominant

negative STAT3 mutants results in maturation arrest (Shimozaki, Nakajima et al. 1997; Ward, Hermans et al. 1999; Benekli, Baer et al. 2003).

STAT5 has also been implicated in myeloid growth and differentiation induced by the IL-3, G-CSF and GM-CSF receptors (Ilaria, Hawley et al. 1999; Nosaka, Kawashima et al. 1999; Kieslinger, Woldman et al. 2000). Dominant negative forms of STAT5 have enabled dissection of STAT5 functions. Specifically, a C-terminally truncated form of STAT5 leads to inhibition of G-CSF-induced granulocytic differentiation of myeloid 32D cells (Ilaria, Hawley et al. 1999). Unlike STAT3, STAT5 has also been implicated in cell proliferation, where C-terminally truncated STAT5 inhibits cell growth in response to IL-3 in factor-dependent cell lines without inducing apoptosis (Ilaria, Hawley et al. 1999). STAT5 is also implicated in self-renewal and in survival (discussed further in Chapter 4) (Wittig and Groner 2005; Khwaja 2006).

Given that STATs play an important role in signal transduction from cytokine receptors, it is not surprising that they have been implicated in the development of malignant transformations. Constitutive STAT activation (STAT1, STAT3, and STAT5) has been detected in numerous primary tumours including, breast cancer, lung cancer, prostate cancer, head and neck tumours, brain tumours, multiple myeloma, leukaemias, lymphomas, renal cell carcinoma, melanoma, and ovarian cancer (reviewed in (Bowman, Garcia et al. 2000; Bromberg 2002; Benekli, Baer et al. 2003). The mechanisms leading to de-regulated STAT activation most commonly involve generation of the C-terminally truncated molecule or activation of the full-length molecule by various oncoproteins including v-Src (Bromberg 2002; Benekli, Baer et al. 2003). STAT isoforms lacking C-terminal domains, act as dominant negatives of STAT

activity/function (Caldenhoven, van Dijk et al. 1996; Chakraborty, White et al. 1996; Wang, Stravopodis et al. 1996).

1.3.2.1 JAK2/STAT5 Signalling

Upon ligand binding, conformational changes in the receptor complex permits transphosphorylation of JAK2 molecules resulting in tyrosine phosphorylation of the cytoplasmic portions of the receptor. Consistent with this mechanism of activation, several unbound receptors have been reported to form dimers with conformations that prevent JAK association. For example EpoR complex in its unbound state has cytoplasmic domains that are predicted to be separated by 24 angstroms (Livnah, Stura et al. 1999). STAT5 monomers then interact with the phosphotyrosine residues on the receptor through their SH2 domains (**Figure 1.5**). This brings them into close proximity with the activated JAK2 which then phosphorylates tyrosine residues in the cytoplasmic domain of STAT5 resulting in its activation (Reviewed in Benekli, Baer et al. 2003; Valentino and Pierre 2006). Once STAT molecules have been phosphorylated, they dissociate from the receptor and form cytoplasmic homo- or heterodimers which translocate to the nucleus. (Aaronson and Horvath 2002; Benekli, Baer et al. 2003) (**Figure 1.5**). In the nucleus STAT proteins regulate multiple genes that control cellular responses including proliferation, differentiation, cell survival/apoptosis, and a variety of immune responses (for reviews see (Wittig and Groner 2005; Khwaja 2006).

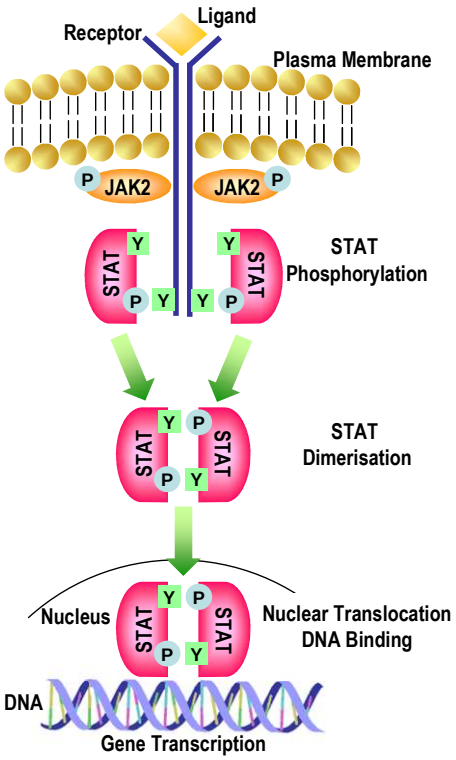


Figure 1.5 The JAK/STAT signal transduction pathway. Adapted from (Benekli, Baer et al. 2003).

Of particular interest to this study is the activation of the JAK2/STAT5 signalling pathway downstream of the GM-CSF receptor. This pathway is differentially regulated by constitutively activated mutants of the common β_c of this receptor and is discussed in detail in Chapter 4.

1.3.2.2 Negative Regulators of JAK/STAT Signalling

The regulation of tyrosine phosphorylation and signalling is critical for controlling biological responses like survival, proliferation and differentiation. The level of tyrosine phosphorylation is controlled by a balance of positive and negative signals. In many cases signal termination is mediated by protein tyrosine phosphatases (PTPases) which act to dephosphorylate kinases and other signalling proteins in order to regulate

their activation (Fischer, Charbonneau et al. 1991; Ostman, Hellberg et al. 2006; Tonks 2006). Thus many phosphatases function as anti-oncogenes or growth suppressive genes. However, some dephosphorylation events are required for the activation of proteins, especially those involved in cell cycle regulation (Tonks 2006). Many cytoplasmic protein tyrosine phosphatases contain SH2 domains, allowing them to be recruited to phosphorylated tyrosine residues and initiate dephosphorylation of proteins in a signalling complex marking the termination of signalling (Neel, Gu et al. 2003).

In most systems the phosphorylation/activation of JAKs and STATs is transient, consistent with key activation events being regulated by PTPases (Shuai 2000; Ostman, Hellberg et al. 2006). The SH2-containing cytosolic protein tyrosine phosphatases, SHP1/2, are examples of PTPases that remove phosphate groups from phosphorylated JAK to effect its downregulation (Ostman, Hellberg et al. 2006). Given the pivotal role of PTPases in regulating cytokine receptor signalling, it is not surprising that perturbation of PTPase function is associated with tumorigenesis in various human cancers (reviewed in Ostman, Hellberg et al. 2006). Down-regulation of SHP1 by hypermethylation of its promoter or SHP1 deficiency has been associated with hyper-responsiveness to cytokines including GM-CSF and IL-3 and hyperphosphorylation of JAK2 (Zhang, Somani et al. 2000; Zhang, Raghunath et al. 2000). SHP1 hypermethylation has been demonstrated in a number of haematological malignancies including myeloma, anaplastic lymphoma, mantle cell and follicular lymphomas, and acute leukemias (Zhang, Somani et al. 2000; Oka, Ouchida et al. 2002; Chim, Fung et al. 2004; Chim, Wong et al. 2004; Khoury, Rassidakis et al. 2004; Johan, Bowen et al. 2005).

Recently another PTPase, CD45, has been identified, which regulates antigen receptor signalling in B and T cells (Irie-Sasaki, Sasaki et al. 2001). CD45 is a membrane-bound PTPase which binds and dephosphorylates JAKs and in doing so negatively regulates IL-3 mediated proliferation and erythropoietin dependent haemopoiesis (Irie-Sasaki, Sasaki et al. 2001). In addition to JAKs, Srcs have also been implicated as targets of CD45 (Irie-Sasaki, Sasaki et al. 2003).

In addition to the tumour suppressor function displayed by some PTPases, others, including SHP2, can positively regulate cytokine receptor signalling and can therefore have oncogenic potential (Feng 1999; Neel, Gu et al. 2003). Consistent with this, activating SHP2 mutations have also been identified in AML, ALL and Juvenile myelomonocytic leukaemia (JMML) (reviewed in Ostman, Hellberg et al. 2006).

JAK2 is also negatively regulated by the ubiquitin-proteasome pathway through the suppressor of cytokine signalling, SOCS1 (Ungureanu, Saharinen et al. 2002). Inactive JAK2 is mono-ubiquitinated and stimulation of cells with cytokine, IL-3 or Interferon gamma (IFNγ), stimulates activation of SOCS1 transcription via STAT, followed by recruitment of a SOCS1/E3 ubiquitin ligase complex to JAK2. This results in polyubiquitination of JAK2 and subsequent targeting to the proteasome for proteolytic degradation (Kamizono, Hanada et al. 2001; Ungureanu, Saharinen et al. 2002). SOCS1 association, as well as proteolysis of JAK2, is dependent upon tyrosine phosphorylation of residue Y1007 in JAK2, which occurs during factor stimulation of cells (Quelle, Sato et al. 1994). This represents a classical negative feedback loop for regulation of JAK activity. SOCS proteins also bind to specific receptor phospho-

tyrosine residues and block STAT binding (Kamura, Sato et al. 1998; Frantsve, Schwaller et al. 2001).

STAT proteins are also regulated directly through the protein inhibitors of activated STATs (PIAS) (Chung, Liao et al. 1997; Liu, Liao et al. 1998; Shuai 2000). These inhibitors bind directly to STATs, inhibiting STAT-DNA binding activity and subsequently blocking STAT-mediated gene transcription (Chung, Liao et al. 1997; Liu, Liao et al. 1998).

1.3.3 JAKs in Haematological Malignancies

JAKs clearly play an important role at the apex of cytokine mediated signal transduction. It is therefore not surprising that they have also been implicated in multiple haematological malignancies (reviewed in Kaushansky 2005; Khwaja 2006). Importantly, a JAK2 mutation at residue Val617 (**Figure 1.4**) in the JH2 domain has recently been shown to be a key event involved in myeloproliferative diseases. This mutation is present in greater than 90% of Polycythemia vera cases and also with varying frequency in other Philadelphia chromosome-negative myeloproliferative disorders (Baxter, Scott et al. 2005; James, Ugo et al. 2005; Kralovics, Passamonti et al. 2005; Levine, Wadleigh et al. 2005; Zhao, Xing et al. 2005). A G-T base pair substitution at position 1443 results in the valine to phenylalanine amino acid change and leads to hyper-responsiveness to cytokine signalling (Baxter, Scott et al. 2005; James, Ugo et al. 2005; Kralovics, Passamonti et al. 2005; Levine, Wadleigh et al. 2005; Zhao, Xing et al. 2005; Kaushansky 2006; Staerk, Kallin et al. 2006). Large screens have also revealed this V617F substitution in low frequency in other haematological disorders, for example in JMML (Tono, Xu et al. 2005).

JAK2 fusion proteins have also been associated with various haematological malignancies including; ALL, T-ALL, chronic myelogenous leukaemia (CML), AML and T lymphoblastic leukaemia (TLL) (Lacronique, Boureux et al. 1997; Peeters, Raynaud et al. 1997; Griesinger, Hennig et al. 2005). Generally, the fusion proteins result in constitutively activated kinase as a result of the fusion with oligomerisation motifs (Khwaja 2006). A number of fusion proteins that result in constitutive JAK2 activation have been reported, including TEL/JAK2 (Lacronique, Boureux et al. 1997; Peeters, Raynaud et al. 1997), breakpoint cluster region (BCR)/JAK2 (Griesinger, Hennig et al. 2005) and pericentriolar material (PCM1)-JAK2 (Bousquet, Quelen et al. 2005; Murati, Gelsi-Boyer et al. 2005; Reiter, Walz et al. 2005; Adelaide, Perot et al. 2006). These fusions are quite rare and involve different lineages.

1.3.4 Ras Signalling

Ras proteins are members of the superfamily of small GTPases (Takai, Sasaki et al. 2001). There are four ubiquitously expressed members of the Ras family encoded by N-Ras, K-Ras (two isoforms), and H-Ras genes (Spandidos, Sourvinos et al. 2002). Ras is a proto-oncogene that is an important regulator of cell survival, proliferation and differentiation and lies at the apex of multiple signalling cascades including Raf/MEK/ERK, Ral/Rac/Rho, and PI3K/AKT. Activating mutations of Ras have been linked to numerous human malignancies (Spandidos and Anderson 1990; Zachos and Spandidos 1997). Activation of Ras occurs following the recruitment of adaptor molecules to phosphorylated tyrosine residues on the receptor. Key adaptor molecules in this process include Grb2, SHP2, and the Grb2 associated binding protein-1 (Gab1) (Shi, Yu et al. 2000; Yart, Laffargue et al. 2001; Cai, Nishida et al. 2002). Grb2 associates with an activated receptor through its SH2 domain and binds to the guanine

nucleotide exchange factor (GEF) son of sevenless (SOS) through its SH3 domain (Reviewed in Nishida and Hirano 2003). The Grb2/SOS complex acts as a guanine nucleotide exchange complex for Ras converting the GDP-form of Ras to the activated GTP-form (**Figure 1.6**). Once activated, Ras is able to associate with multiple downstream effectors including Raf and Ral/Cdc42, initiating multiple biological effects (**Figure 1.6**) (Adjei 2001; Nishida and Hirano 2003). Activation of the Ral/Rac/Rho pathway by Ras results in altered biological responses including changes in the actin cytoskeleton (Adjei 2001). Importantly, Ras is also able to activate the PI3K pathway through an adaptor molecule, Gab, that is phosphorylated and activated upon recruiting to an activated cytokine receptor. Activated Gab subsequently recruits the regulatory subunit of PI3K, p85, and CrkL which mediates activation of anti-apoptotic proteins including, Bcl-XL, and subsequently promotes cell survival (**Figure 1.6**).

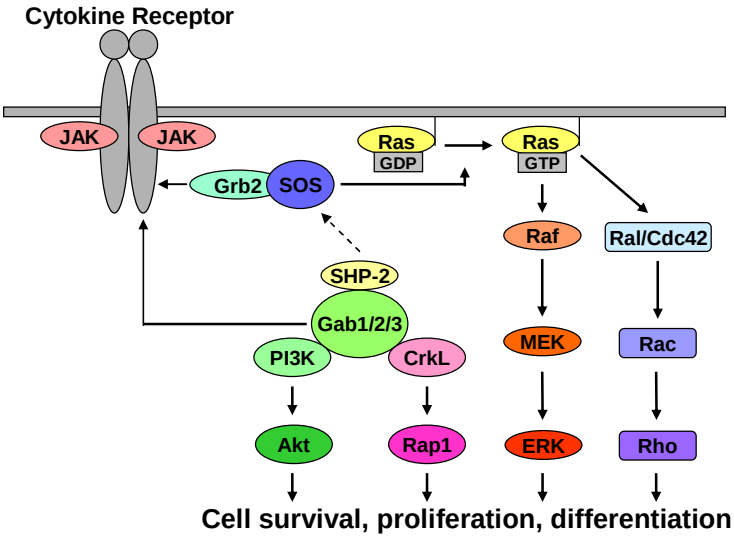


Figure 1.6 Ras Signalling Cascade (adapted from Adjei 2001; Nishida and Hirano 2003). Gab and Grb2 are both associated with the cytoplasmic domain of the cytokine receptor following cytokine binding and receptor activation. Phosphorylation of Gab by receptor associated tyrosine kinase activity leads to the recruitment and activation of p85 PI3K, SHP2 and CrkL. Ras driven Raf/MEK/ERK activation and Ral/Rac/Rho activation are also shown. MAPK can also be activated independently of Ras (see text).

The roles of these Ras-associated pathways have not been characterised in full for all cytokine receptors. Of relevance to this study, and perhaps the best described pathway mediated by Ras, is the activation of MAPK family members (Nishida and Gotoh 1993; Lewis, Shapiro et al. 1998; Chang and Karin 2001; Pearson, Robinson et al. 2001; Torii, Nakayama et al. 2004). There are four members of the MAPK family including, extracellular-signal-regulated-kinase 1/2 (p44/42 MAPK or ERK1/2), c-Jun-amino-terminal kinase (JNK), p38 and ERK5 (Sturgill and Wu 1991; Nishida and Gotoh 1993; Robinson and Cobb 1997; Davis 2000; Kyriakis and Avruch 2001; Wang and Tournier 2006). Activation of the ERK1/2 pathway occurs through the association of activated Ras with the serine/threonine kinase, Raf. Raf subsequently activates MEK and finally ERK (**Figure 1.6**). Once activated, ERK dissociates from MEK allowing it to enter the nucleus and phosphorylate several transcription factors including Elk1 and c-Myc and protein kinases such as ribosomal S6 kinase (RSK) (Nishimoto and Nishida 2006). As a result, immediate early genes like c-Fos and c-Jun are induced (Nishimoto and Nishida 2006). The Ras/Raf/MAPK pathway is therefore an important contributor to cellular processes like proliferation and survival (Lewis, Shapiro et al. 1998). In addition to providing proliferative and survival signals activation of the MAPK pathway has recently been demonstrated to have an important role in suppressing differentiation in AML (Radomska, Basseres et al. 2006). The kinetics of activation of this pathways is also clearly important and it has been demonstrated recently that sustained activation of this pathway leads to the down-regulation of multiple growth-arrest genes (Yamamoto, Ebisuya et al. 2006).

1.3.5 The PI3K/AKT Pathway

The PI3K/AKT signalling pathway, activation of which is initiated by phosphatidylinositol lipid phosphorylation at the cell membrane, coordinates a set of events leading to cell survival, cell cycle entry, cell migration and vesicle trafficking, and is activated in response to stimulation with multiple growth factors (Cantley 2002; Djordjevic and Driscoll 2002; Guthridge, Barry et al. 2004; Engelman, Luo et al. 2006).

PI3Ks are grouped into three classes (I-III) according to their sequence homology and substrate specificities (reviewed in Engelman, Luo et al. 2006). Different classes display different functional roles in signal transduction. Within the Class I PI3Ks there are two subgroups; 1) those that are activated by cytokine receptors and receptor tyrosine kinases (Class IA) and 2) PI3Ks that are activated by G-protein-coupled receptors (Class IB) (Katso, Okkenhaug et al. 2001; Wymann, Bjorklof et al. 2003). Members of the Class IA PI3Ks consist of a p85 regulatory subunit and a p110 catalytic subunit (**Figure 1.7**). Members of Class IB comprise a heterodimer of a p101 regulatory subunit and a p110γ catalytic subunit (Engelman, Luo et al. 2006). Class II PI3Ks consist of only a p110-like subunit (Gaidarov, Smith et al. 2001; Katso, Okkenhaug et al. 2001) and Class III consists of only one member, Vps34 (vacuolar protein-sorting defective 34) (Odorizzi, Babst et al. 2000; Byfield, Murray et al. 2005; Nobukuni, Joaquin et al. 2005). The Class IA PI3Ks are of particular relevance to this work due to their importance in signalling from receptor tyrosine kinases and cytokine receptors.

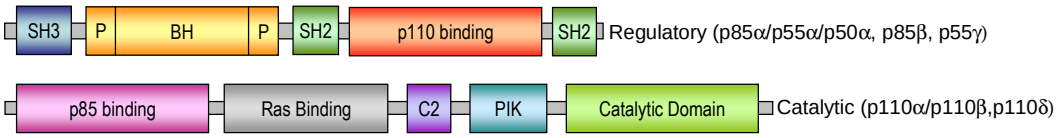


Figure 1.7 Class IA PI3K domain structure (adapted from Engelman, Luo et al. 2006) The Class1A PI3K consists of a p85 regulatory subunit and a p110 catalytic subunit. The class IA p85 regulatory isoforms have a common core structure consisting of a p110-binding domain flanked by two Src-homology 2 (SH2) domains. The other p85 isoforms also contain an N-terminal SH3 domain and BCR homology (BH) domain flanked by two proline-rich (P) regions. The p110N-terminal p85-binding domain that interacts with the p85 regulatory subunit, a Ras-binding domain (RBD) that mediates activation by the small GTPase Ras, a C2 domain, a phosphatidylinositol kinase homology (PIK) domain and a C-terminal catalytic domain.

Class I PI3K activity is generally regulated by p85 binding to phosphotyrosine residues on activated growth factor receptors or adaptor proteins. Serine phosphorylation of $h\beta_c$ has also been shown to recruit the adaptor protein 14-3-3 to the receptor which then binds to and activates PI3K (Guthridge, Stomski et al. 2000; Guthridge, Barry et al. 2004). PI3K activity is further stimulated by direct binding of p110 to activated Ras protein (Cantley 2002). In quiescent cells, p110 is maintained in a low activity state (Cantley 2002). Activated PI3K converts phosphatidyl-4,5-bisphosphate [$PI(4,5)P_2$] to phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$] or PIP_3. The lipid product of PI3K, PIP_3, subsequently recruits to the plasma membrane, a variety of signalling molecules, with pleckstrin homology (PH) domains including; protein serine-threonine kinases (AKT and PDK1), protein tyrosine kinases (Tec family), and exchange factors for GTP-binding proteins (Grp1 and Rac exchange factors) (Cantley 2002). This recruitment to the membrane is a critical activation step, allowing these molecules to be phosphorylated by other receptor-associated kinases.

The serine/threonine kinase, AKT, is a principle target of PIP_3 (Franke, Kaplan et al. 1997; Klippel, Kavanaugh et al. 1997). AKT is recruited to the membrane, through association with PIP3, and is subsequently phosphorylated by mTOR (mammalian target of rapamycin) and by PDK1 (3-phosphoinositide-dependent kinase). The activated AKT then phosphorylates many target proteins regulating a range of cellular responses including; cell survival (Vivanco and Sawyers 2002), cell-cycle entry (Vivanco and Sawyers 2002; Burgering and Medema 2003), and protein synthesis (Richardson, Schalm et al. 2004). Specifically, members of the forkhead (FOXO) family of transcription factors are important targets of AKT. AKT phosphorylation of FOXO proteins results in their inactivation, through cytoplasmic sequestration by binding to the phospho-serine binding proteins comprising the 14-3-3 family (Cantley 2002). One mechanism of AKT survival signalling involves direct phosphorylation of the pro-apototic regulator, BAD, resulting in 14-3-3 binding and BAD sequestration into the cytoplasm (Guthridge, Stomski et al. 2000).

Dephosphorylation of PIP_3 is an important means of terminating PI3K/AKT signalling. This dephosphorylation can be mediated by SH2 domain-containing inositol phosphatases such as SHIP1 and SHIP2, which act by dephosphorylating the inositol ring to produce PIP_2 (Cantley 2002). PTEN (phosphatase and tensin homologue) a tumour suppressor gene that converts PIP_3 to PIP_2) is also a PTPase that regulates PI3K activity by dephosphorylating the inositol ring (although at a position distinct from that of SHIP1/2 (Lynch and Silberg 2002). In many human cancers the gene encoding PTEN is mutated and inactivated resulting in constitutive PI3K activity (Maehama 1999; Vanhaesebroeck and Waterfield 1999; Djordjevic and Driscoll 2002). The importance of PI3K signalling in inflammation and cancer renders it an important

therapeutic target and there is great interest in the development of isozyme-specific inhibitors that might enable inhibition of specific PI3K signalling pathways (Djordjevic and Driscoll 2002). There are currently also numerous PI3K inhibitors that are useful for analysing the role of this pathway. Wortmannin and LY294002, in particular, are cell-permeable, low-molecular-weight compounds used extensively for research applications (Stein 2001).

1.3.6 NFκB Signalling

The transcription factor Nuclear Factor kappa-B (NFκB) plays important biological roles in the regulation of cell survival, proliferation, immune response, and development (Ghosh, May et al. 1998; Silverman and Maniatis 2001; Ghosh and Karin 2002; Guthridge, Barry et al. 2004). NFκB comprises a collection of transcription factors including members of the Rel family including; RelA, RelB, c-Rel, NFκB1/p50 and NFκB2/p52 (Xiao, Rabson et al. 2006). A defining structural property common to each of the family members is the 300-amino-acid Rel homology domain (RHD) which is required for DNA binding, dimerisation and nuclear translocation (Xiao, Rabson et al. 2006). NFκB dimers are maintained in the cytoplasm through sequestration by a family of ankyrin repeat domain (ARD)-containing proteins termed IκB (inhibitor of kappa-B). The IκB proteins interact with NFκB members through the RHD. The canonical NFκB signalling pathway is based on phosphorylation of IκB molecules by IKKs (IκB kinases) which allows IκB dissociation from NFκB proteins and subsequent translocation of NFκB into the nucleus to effect gene expression (reviewed by Xiao, Rabson et al. 2006). Recently, AKT has been shown to regulate NFκB transcriptional activity through direct phosphorylation of IKKs resulting in activation of NFκB and

regulation of genes involved in cell survival including *bcl-2, bcl-x_L* and *A1/bft1* (Barkett and Gilmore 1999; Lee, Dadgostar et al. 1999; Grossmann, O'Reilly et al. 2000; Guthridge, Barry et al. 2004). One mechanism by which AKT regulates NFκB signalling in response to GM-CSF is through a binary switch that involves a tyrosine (Y577) and a serine (S585) residue, on β_c (discussed in Section 1.4.2.2) (**Figure 1.2**). These residues control alternative receptor responses to GM-CSF (Guthridge, Barry et al. 2004). Deregulated function of NFκB has been widely reported in AML (Karin, Cao et al. 2002; Birkenkamp, Geugien et al. 2004) and has also been demonstrated to contribute to the development of other human diseases including immune-related diseases and cancers (Rayet and Gelinas 1999; Baldwin 2001; Sun and Xiao 2003).

1.3.7 Src Family Kinases

Src Family Kinases (SFKs) are cytoplasmic/non-receptor tyrosine kinases. They are regulated by a variety of receptors including cytokine receptors and adhesion and antigen receptors (Thomas and Brugge 1997). The 9 members of the Src family include Src, Lyn, Lck, Hck, Fyn, Blk, Fgr, Yes and Yrk. There is extensive redundancy associated with the actions of SFKs and this is reflected in the structural similarity observed between the family members. All SFKs contain a tyrosine kinase domain in addition to the prototype SH2 and SH3 domains which are found in many signalling proteins (**Figure 1.8**) (Boggon and Eck 2004). The SH3 domain of SFKs mediates binding to proline rich sequences with the core consensus PXXP (**Figure 1.9)** (Boggon and Eck 2004). Additionally, the SH2 domain mediates binding to phosphotyrosine containing motifs (particularly YEEI) (Tatosyan and Mizenina 2000). All family members also contain an N-terminal membrane-targeting region which is myristoylated and sometimes palmitoylated (Koegl, Zlatkine et al. 1994; Resh 1999). SFKs also

contain a 'unique' domain, of 50-70 residues, which is not conserved among family members and is likely to mediate protein interactions with specific substrates (**Figure 1.8**). The Tec family of non-receptor tyrosine kinases share significant sequence homology with Src kinases particularly, in the SH2 and SH3 domain regions; however, these families are structurally distinct in the carboxy- and amino-terminal regions (Reviewed by Mano 1999; Brazin, Fulton et al. 2000).

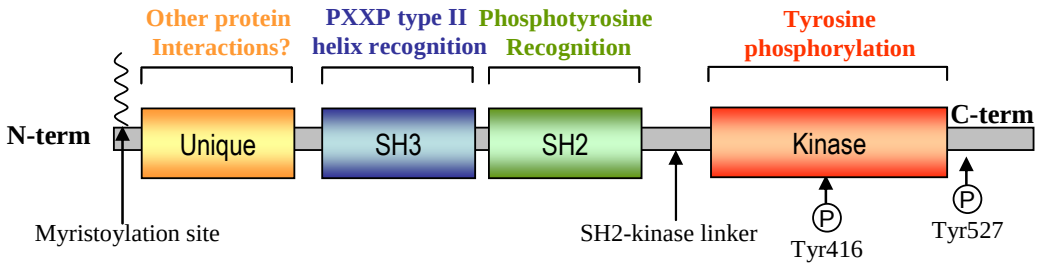


Figure 1.8 Schematic domain structure of Src kinase. The key regulatory phosphorylation sites Tyr416 and Tyr527 are shown. Conserved SH3 and SH3 domains are indicated in addition to the N-terminal myristoylation site and the unique domain which is not conserved amoung SFKs (Adapted from Boggon and Eck 2004).

1.3.7.1 Src-homology Domains

SH2 and SH3 domains were originally identified as conserved domains in Src and multiple other signalling molecules. These domains have been previously shown to be involved in mediating protein-protein interactions important for multiple intracellular signalling pathways. These interactions mediate interactions of receptors with adaptor proteins, recruitment of substrates to enzymes, localisation of proteins to subcellular compartments and the regulation of the catalytic activity of various proteins.

SH2 domains are composed of approximately 100aa and were first identified as a conserved sequence region present in the oncoproteins Src and Fps (Sadowski, Stone et al. 1986). SH2 domains were later characterised in many other signal-transducing molecules and over 100 SH2-domain containing proteins are now listed in the PROSITE database (www.expasy.org/prosite) (Russell, Breed et al. 1992). SH2 domains interact with high affinity to phosphotyrosine-containing target peptides in a sequence-specific and strictly phosphorylation-dependent manner (Mayer and Baltimore 1993; Pawson and Schlessingert 1993; Marengere, Songyang et al. 1994; Pawson 1995). Conventional SH2 domains have a conserved pocket that recognises phosphotyrosine (pY), and a more variable pocket that binds 3-6 residues C-terminal to the pY and confers specificity.

SH3 domains are 60aa modules and were first identified as a conserved sequence in the non-catalytic region of cytoplasmic tyrosine kinases including Src, Abl, and Lck (Mayer, Hamaguchi et al. 1988). SH3 domains are possibly the most widespread of all protein recognition motifs (Murohashi, Tohda et al. 1989; Pawson and Gish 1992; Mayer and Baltimore 1993; Pawson and Schlessingert 1993; Cesareni, Panni et al. 2002) and are contained within approximately 200 proteins to date in the PROSITE database (www.expasy.org/prosite). These SH3 modules have a conserved fold arising from the organisation of two three-stranded β-sheets. One side of this fold is relatively hydrophobic and constitutes the ligand-binding region of the structure (Cesareni, Panni et al. 2002). It is well established that SH3 domains selectively bind to sequences that are rich in proline, however, there are many combinations of proline-rich sequences that the various SH3 domains bind to preferentially, leading to further classification of these motifs into sub-classes (**Figure 1.9**). The most common class of motif is Class 1 which

the consensus RxLPP#P and bind in a NH2-COOH orientation (where # is normally a hydrophobic residue) (Cesareni, Panni et al. 2002). Alternatively, Class II peptides are characterised by the consensus Px#PxR and bind in the opposite orientation (COOH-NH2) (Rickles, Botfield et al. 1995; Cesareni, Panni et al. 2002). Numerous SFKs have Class 1 SH3 domains, including Yes, Src, Hck, Lyn and Fyn (Cesareni, Panni et al. 2002). The PI3K p85 subunit is also a member of this class. Class II family members include p53 binding protein-2 (p53BP2) and Grb2 (Cesareni, Panni et al. 2002).

Although the polyproline core motif contributes to binding specificity, other residues distal to this motif have also been identified as contributing to the specificity of interactions. Moreover, studies using biased phage display libraries, found that flanking sequences not only increase the binding affinity of SH3 domains, but may be primary determinants for specificity (Rickles, Botfield et al. 1995; Lynch, Minor et al. 1999).

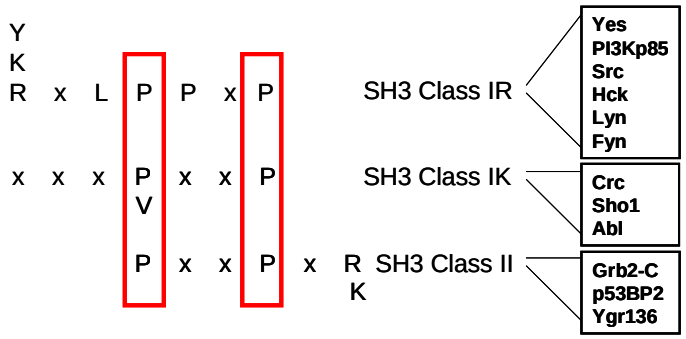


Figure 1.9 Classification of SH3 recognition specificity (Adapted from Cesareni, Panni et al. 2002). Conserved proline residues are outlined in red.

Interactions with both the SH2 and SH3 domain of SFKs are important for modulating activity. Conformational regulation of SFKs occurs through interaction of the phosphorylated tyrosine residue (Y527) in the C-terminal tail and the SH2 domain of

Src, which inhibits kinase activity. Inhibitory interactions also involve the SH3 domain and direct contact with a proline-rich sequence in the N-terminal region of Src (**Figure 1.8**) (Brabek, Mojzita et al. 2002). Activation of SFKs occurs when either of these interactions are perturbed. This can involve dephosphorylation of the C-terminal tyrosine by Csk (Matsuoka, Nada et al. 2004) or competition with other proline-rich sequences as occurs in activation of Hck by the Nef protein of human immunodeficiency virus 1 (HIV-1), which binds to the Hck-SH3 domain leading to constitutive Hck kinase activation (Briggs, Sharkey et al. 1997; Moarefi, LaFevre-Bernt et al. 1997; Lerner, Trible et al. 2005).

1.3.7.2 Signalling by Src Family Kinases

SFKs are activated in response to a variety of cytokines including G-CSF, GM-CSF, stem cell factor (SCF), IL-3, and Epo, demonstrating their potential importance in cytokine mediated signal transduction. Of particular interest to this study is the role of SFK signalling in IL-3, IL-5 and GM-CSF responses. The exact role of SFKs in signalling from these receptors remains unclear, however, β_c phosphorylation by SFKs has been reported and suggests the possibility that SFKs can substitute for JAKs in some instances. One study demonstrated direct binding of Lyn to the β_c through multiple interactions (Dahl, Arai et al. 2000). Lyn is predominantly expressed in the myeloid cell compartment and is likely to be an important regulator of signalling from IL-3, IL-5 and GM-CSF receptors. Furthermore, it has been suggested that Lyn may directly mediate β_c phosphorylation (Dahl, Arai et al. 2000). The interaction between Lyn and the β_c is constitutive (Rao 1995), however, it has been recently shown that following factor stimulation, an increased proportion of the Lyn pool associates with the

β_c (Li 1995; Dahl, Arai et al. 2000). Hck and Fyn binding to the β_c of the IL-3 receptor has also been demonstrated (Burton 1997).

Functionally, a strong connection has been made between SFKs and cytokine induced proliferation, differentiation, and suppression of apoptosis. Specifically, Lyn binding to the GM-CSF receptor is required for the delay, or inhibition of apoptosis, in polymorphonuclear leukocytes, although the mechanism of action remains unclear (Sheng 1996). Additionally, it has been shown that Lyn is required for the induction of DNA synthesis in response to G-CSF (Corey, Dombrosky-Ferlan et al. 1998). Studies conducted with the Src kinase specific inhibitor PP1, indicate that there is a PP1 dose-dependent decrease in DNA synthesis and total abrogation of proliferation (while maintaining viability) in Ba/F3 cells in response to GM-CSF (Dahl, Arai et al. 2000), suggesting a critical role for SFKs in GM-CSF-induced proliferation.

Involvement of SFKs in signalling in the Ras/Raf/MAPK, PI3K/AKT and STAT3 pathways has been documented, however, the contributions of each of these pathways to survival and proliferation is still being resolved. Recently it has become evident that SFKs can activate signalling pathways in response to cytokine, independently of JAKs. Specifically, it has been demonstrated that Src kinases can mediate phosphorylation of STATs independent of JAKs in response to IL-3 (Reddy 2000). The importance of these JAK-independent, Src-dependent pathways is highlighted by another report in which Src was able to initiate signalling events from the growth hormone receptor activating p44/42 MAPK signalling, irrespective of JAK2 (Zhu, Ling et al. 2002). Additionally, activation of c-Src by Prolactin independently of JAK2 has also been reported (Fresno Vara 2000). Other reports of JAK-independent signalling mediated by

SFKs include; SFK mediated AKT (PKB) activation by G-CSF (Dong and Larner 2000) and IL-3 stimulation of STAT3 which is thought to occur through direct interaction of STAT3 with Src (Chaturvedi, Reddy et al. 1998). These studies further support the suggestion that it may be the combined efforts of JAKs and SFKs, following cytokine receptor stimulation, that lead to full receptor activation (Fresno Vara 2000). The role of SFKs in mediating JAK-independent pathways in GM-CSF signalling is discussed further in Section 4.3.1. It will be important to define the specific roles of SFKs in cytokine receptor activation and to address directly the precise relationship between SFKs and JAKs to gain a better insight into signal transduction.

1.4 GM-CSF and its Receptor

1.4.1 GM-CSF Biology

GM-CSF, IL-3 and IL-5 are key regulators of the myeloid lineage. They have overlapping, pleiotropic effects on myeloid cells, including neutrophils, eosinophils, monocytes and early progenitor cells that include mitogenesis, protection from apoptosis, differentiation and functional activation (de Groot, Coffer et al. 1998; Martinez-Moczygemba and Huston 2003). Although there are redundant signalling pathways activated by these receptors, due to the sharing of a common β_c subunit, the expression profiles of the specific alpha subunits differ significantly contributing to their specificity. GM-CSF receptors are expressed in multiple cell lineages including the majority of myeloid progenitors, mature monocytes, neutrophils, eosinophils, basophils and dendritic cells (Nicola 1994). IL-3 receptors are expressed on early haemopoietic progenitor cells, on some committed myeloid progenitors, eosinophils, and basophils (Nicola 1994). Interestingly, the expression of the IL-5 receptor is

limited to eosinophils, reflected by its biological activity being essentially confined to this cell lineage (Nicola 1994). **Table 2** highlights the biological activities of each of these key cytokines. For the purpose of this study we have focussed on the GM-CSF receptor and its structure, function and activation.

Table 2. Heterogenous Biological Activities of IL-5, IL-3 and GM-CSF. Adapted from (Martinez-Moczygemba and Huston 2003).

Cytokine	Effect on Haemopoiesis	Biological Activities
IL-5	Differentiation, activation, and survival of eosinophils	Allergic inflammation; asthma and airway remodelling, parasite immunity
IL-3	Growth, differentiation and survival of progenitor cells	Allergic inflammation; parasite immunity: growth of myeloid, monocytic and lymphocytic malignancies
GM-CSF	Growth and differentiation of dendritic cells, myelomonocyte progenitors and granulocytes	Allergic inflammation, growth of myeloid and monocytic malignancies, surfactant metabolism, anti-GM-CSF autoantibodies cause alveolar proteinosis

GM-CSF was originally identified as a growth factor capable of generating granulocyte and macrophage colonies from precursor cells in mouse bone marrow (Burgess and Metcalf 1980). It has since been shown to have a broad range of effects on mature cells of the myeloid lineage (Handman and Burgess 1979; Hamilton, Stanley et al. 1980; Simon, Yousefi et al. 1997). Knockout mice have failed to identify a major role for GM-CSF in steady-state myelopoiesis as these mice show no obvious perturbation in myeloid cell production and show only a small deficit in dendritic cell number (Vremec, Lieschke et al. 1997). From these *in vivo* studies, the most obvious phenotype is alveolar proteinosis and lymphoid hyperplasia associated with lung airways and blood vessels (Stanley, Lieschke et al. 1994; Robb, Drinkwater et al. 1995). Null animals also

display compromised antigen-specific and lipopolysaccharide (LPS) induced T-cell responses and IFNγ production which may be dendritic cell-mediated (Wada, Noguchi et al. 1997; Noguchi, Wada et al. 1998). Additionally, these mice have some defects in macrophage function (Basu, Dunn et al. 1997; Scott, Hughes et al. 1998; Enzler, Gillessen et al. 2003), and are susceptible to various infectious agents (Zhan, Lieschke et al. 1998; LeVine, Reed et al. 1999; Paine, Preston et al. 2000). These findings suggest that GM-CSF may perform a role that is redundant with other haemopoietic growth factors, but nevertheless is still an important regulator of myelopoiesis.

GM-CSF and its receptor have been implicated in various leukaemic disorders, such as JMML/JCML, AML and ALL (Faderl, Harris et al. 2003). It has been shown that JMML cells are hypersensitive to very low concentrations of GM-CSF and form excessive numbers of colony-forming unit granulocyte macrophage (CFU-GM) colonies in methylcellulose assays when compared to their normal counterparts (Emanuel, Bates et al. 1991). Transduced bone marrow, overexpressing GM-CSF and transplanted into syngeneic mice, induces an acute myeloid disorder similar to JMML (Johnson, Gonda et al. 1989). Interestingly, autocrine production of GM-CSF is also implicated in JCML and ALL as well as AML (Gualtieri, Emanuel et al. 1989; Freedman, Grunberger et al. 1993). To date, no activating mutations of β_c have been identified in AML however, only small numbers of leukaemias have been screened for these mutations (Freeburn, Gale et al. 1996; Freeburn, Gale et al. 1998).

In light of its ability to activate macrophages and influence dendritic cell development, GM-CSF displays adjuvant activity (Armitage 1998). GM-CSF has been demonstrated to increase the immunogenicity of tumours in animal models, and this approach has also

been tested with considerable efficacy in humans (Armitage 1998; Dranoff 2002; Jager, Jager et al. 2003). Additionally, antigen/GM-CSF fusion proteins have been used in immunisations and GM-CSF has also been successfully used as an adjuvant in human vaccine studies (Tao and Levy 1993; Tarr, Lin et al. 1996).

1.4.2 GM-CSF Receptor Structure and Function

The receptors for GM-CSF, IL-3 and IL-5 comprise a common β subunit, β_c and a ligand specific α subunit (Woodcock, McClure et al. 1997). The α subunit of the receptor complex is a low affinity ligand docking site and confers the ligand binding specificity. The β_c subunit is the major signalling subunit and does not direct ligand binding. GM-CSF, IL-3 and IL-5 bind to their respective α subunits with low affinity and further binding to the β_c subunit results in conversion of the complex to a high affinity state (Hayashida, Kitamura et al. 1990; Kitamura, Sato et al. 1991; Bagley, Woodcock et al. 1997; Guthridge, Stomski et al. 1998). It has been demonstrated that a 1:1:1 ratio of GM-CSF:GMRα:β_c represents a high affinity state, however, it is less clear what the stoichiometry of the active complex is. Studies have suggested that a 2:2:2 complex may exist as the activated complex (Guthridge, Stomski et al. 1998). Specifically, it has been demonstrated that β_c dimerisation is required for transphosphorylation and activation of β_c associated JAK2 (Lia, Rajotte et al. 1996; Stomski, Sun et al. 1996).

1.4.2.1 The GMRα Subunit

The α subunit of the GM-CSF receptor is 400aa in length of which 54aa make up a short cytoplasmic domain. In contrast, the β_c cytoplasmic domain is 430aa in length of

a total 881aa. As each α subunit is specific for a different ligand, the α chain is responsible for conferring specificity of ligand binding to the cytokine receptor complex. In addition to being required for ligand binding, the α subunit plays an essential signalling role. Specifically, the α chain cytoplasmic domain is absolutely required for a ligand response and a 29aa internal deletion of the membrane proximal cytoplasmic domain abolishes the proliferation response even though binding of the receptor to the ligand remains unaffected (Polotskaya, Zhao et al. 1993). There is some evidence that sequences in GMRα contribute to specificity of GMR function. Specifically, mutation of the KLN tripeptide in GMRα with the equivalent PIG tripeptide in IL-3Rα generates a receptor that signals for maintenance of the primitive cell phenotype, more like IL-3 than GM-CSF and similarly mutation of IL-3Rα (PIG - KLN) signals for maturation of FDCP-mix cells (Evans, Ariffin et al. 2002).

Although the GM-CSF receptor mediates a variety of phosphorylation events in factor-dependent cells, little is known about the role of the α subunit in signalling. Very few proteins have been identified that bind directly to the α chain of the GM-CSF receptor (**Table 3)**. The GM-CSF receptor α subunit-associated protein (GRAP), has been shown to associate with GMRα, however, this protein contains no known signalling motifs (Tu, Karasavvas et al. 2000). GRAP binds to the intracellular domain of GMRα and has been implicated in glucose transport (Tu, Karasavvas et al. 2000). It is a highly conserved protein, with homologues in yeast, and is likely to have other functions in addition to its GM-CSF-associated role.

Table 3. Known interactors of the GM-CSF receptor alpha subunit.

Name of GMRα Interactor	Experiment Type	Direct/ Indirect	Reference
GRAP	*In vivo; Yeast 2 Hybrid*	Direct	(Tu, Karasavvas et al. 2000)
p85	*In vitro; IP*	?	(Dhar-Mascareno, Chen et al. 2003)
IKKβ	*In vivo; Yeast 2 Hybrid*	Direct	(Ebner, Bandion et al. 2003)
c-Kit	*In vitro; IP*	Direct	(Chen, Carcamo et al. 2006)
Laminin Receptor	*In vitro; IP*	Direct	(Chen, Carcamo et al. 2003)

With regard to known signalling pathways it has been reported that the regulatory subunit of PI3K, p85, binds to the cytoplasmic domain of the GMRα (Dhar-Mascareno, Chen et al. 2003). It is possible that the integral role of PI3K in signalling from the GM-CSF receptor is partly initiated via an interaction between p85 and GMRα. It is also interesting to note that the interaction demonstrated between p85 and GMRα is dependent upon the proline-rich domain in the membrane-proximal region of the α chain (Dhar-Mascareno, Chen et al. 2003). This proline rich sequence contained within the SBP motif is of primary interest to us as it is indispensable for GM-CSF receptor signalling and may form a major docking site in many cytokine receptors. Whether the SH3 domain of p85 interacts directly with the SBP motif is unclear. This nature of this interaction has been investigated further as part of this study and is discussed in Chapter 3.

Additionally, IκB kinase beta (IKKβ) has been recently shown to directly interact with the GMRα, affecting NFκB activation (Ebner, Bandion et al. 2003). IKKβ is a signalling kinase that activates NFκB and in this particular study it was shown that GM-CSF triggers IKKβ activation contributing to NFκB activation and subsequently the

generation of cell survival and proliferation signals (Ebner, Bandion et al. 2003; Guthridge, Barry et al. 2004).

Interestingly, a recent study has identified a functional interaction between c-Kit and and GMRα (Chen, Carcamo et al. 2006). The interaction was found to be mediated by the cytoplasmic domains of both receptors and specifically involved the proline-rich SBP motif of GMRα (Chen, Carcamo et al. 2006). The consequence of this interaction is negative regulation of c-Kit signalling through inhibition of c-Kit auto-phosphorylation normally induced by stem cell factor binding (Chen, Carcamo et al. 2006). The role of this interaction *in vivo* is still unclear.

In addition to c-Kit, GMRα has been demonstrated to interact with the nonintegrin matrix protein, laminin receptor (Chen, Carcamo et al. 2003). This interaction was found to involve the cytoplasmic portions of both receptors and was again dependent upon the proline rich region of GMRα (Chen, Carcamo et al. 2003). Interestingly, the laminin receptor also interacts with β_c (Chen, Carcamo et al. 2003). The interaction between laminin receptor and GMRα prevents complex formation between the GMRα and β_c subunits and therefore inhibits GM-CSF signalling.

An open question with regard to GMR signalling relates to the association of JAK kinases with the membrane-proximal region of the α-subunits for GM-CSF, IL-3 and IL-5. One study indicated an interaction between the α chain of the IL-5 receptor and JAK2 (Ogata, Kouro et al. 1998), however, no such interaction has been reported between JAK2 and the GMRα. Moreover, a separate study reported a lack of association of GMRα and JAK2 (Quelle, Sato et al. 1994).

It is clear from these studies that the GMRα cytoplasmic domain and the SBP motif in particular are important in mediating binding of GMR to various signalling molecules. Characterisation of these interactions and identification of novel GMRα interactors formed a major part of this project and is discussed in Chapter 3.

1.4.2.2 The β_c Subunit of GMR

The common β-subunit, β_c, is an obligatory requirement for signalling. JAK2 is the major kinase thought to activate receptor signalling by binding directly to β_c and phosphorylating tyrosine residues on the receptor and other signalling molecules (Quelle, Sato et al. 1994). In addition to JAK2, SFKs have also been shown to phosphorylate β_c (Chaturvedi, Reddy et al. 1998). Phosphorylation of the β_c is an important part of the signalling response and the phosphorylated residues act as docking sites for phosphotyrosine-binding signalling proteins. There are eight tyrosine residues on the β_c, of which six are conserved between mouse and human (Geijsen, Koenderman et al. 2001). Mutation of all eight tyrosine residues to phenylalanine is able to support survival of Ba/F3 cells in response to GM-CSF, suggesting there are survival signals that cannot be accounted for by receptor tyrosine phosphorylation (Okuda, Smith et al. 1997; Guthridge, Stomski et al. 1998; Itoh, Liu et al. 1998). Roles for several of these tyrosines have been defined revealing a great deal of redundancy associated with the recruitment of signalling molecules to β_c. All 6 conserved cytoplasmic tyrosine residues have been associated with STAT5 recruitment and activation. The mechanism of this activation remains unclear, however, the STAT5 SH2 domain is thought to interact with YXXLP motifs found in Y612, Y750, Y806, and Y866 (Itoh, Liu et al. 1998). Interaction of STAT5 with the other tyrosine residues is likely to occur through

recruitment of other adaptor molecules (Itoh, Liu et al. 1998). In other cases, tyrosine residues can be specific for a particular signalling molecule, as with Y577, which binds to the PTB domain of Shc, and Y612 which interacts with SHP2 (Okuda, Smith et al. 1997; Itoh, Liu et al. 1998; Dijkers, van Dijk et al. 1999). Extensive mutational characterisation of tyrosine residues in the β_c has been carried out and has helped to define specific roles for these residues in signalling and biological outcomes (Okuda, Smith et al. 1997; Itoh, Liu et al. 1998; Brown, Peters et al. 2004).

In addition to specific signalling roles for tyrosine residues in the β_c, Ser585 phosphorylation has also been implicated in mediating binding to 14-3-3 and indirectly to p85 resulting in AKT phosphorylation (Guthridge, Barry et al. 2004). AKT subsequently phosphorylates and activates IKKβ resulting in NFκB activation and survival (Guthridge, Stomski et al. 2000). Ser585 is also part of a bidentate motif in the GM-CSF receptor β_c comprising the Shc binding site, Y577, and Ser585 (Guthridge, Powell et al. 2006). Phosphorylation of these residues is mutually exclusive and they act in concert as a binary switch to activate distinct signalling pathways associated with either survival or proliferation and survival (Guthridge, Barry et al. 2004; Guthridge, Powell et al. 2006). Ser585 is selectively phosphorylated in response to low levels of GM-CSF (<10pM). At high GM-CSF concentrations (>10pM), Y577 is phosphorylated resulting in Shc recruitment and activation of cell survival, proliferation and activation pathways (Guthridge, Barry et al. 2004; Guthridge, Powell et al. 2006). Importantly, in a number of AML samples, constitutive phosphorylation of Ser585 is observed and maintained at high GM-CSF concentrations (Guthridge, Powell et al. 2006).

Mutation studies have also helped characterise the various domains of β_c and their role in signalling. A variety of activating mutants of β_c have been identified that result in constitutive activity of the receptor (**Figure 1.10**) (D'Andrea, Barry et al. 1996). These mutants were isolated by virtue of their ability to confer factor independent growth on the murine myeloid leukaemia cell line, FDC-P1 (McCormack and Gonda 1997). The activating mutants display differential oncogenic potential *in vivo* and allow continuous factor-independent proliferation in factor-dependent haemopoietic cell lines (McCormack and Gonda 1997; Jenkins, Blake et al. 1998). FIΔ and I374N mutants result from a 37aa duplication and an isoleucine-to-asparagine substitution in the extracellular domain respectively (**Figure 1.10**). V449E results in a valine-to-glutamic acid substitution in the transmembrane domain (Jenkins 1995; McCormack 2000) (**Figure 1.10**). In our laboratory a number of these extracellular (I374N, FIΔ) and transmembrane (V449E) mutants have been utilised to analyse β_c involvement in cytokine receptor signalling and to investigate pathways controlling granulocyte/macrophage growth and differentiation. These mutants have been proposed to mimic the dimerisation and signalling events that occur during formation of the mature ligand-receptor complex (Gonda and D'Andrea 1997). The extracellular FIΔ mutant receptor importantly, requires dimerisation with the GMRα subunit for activity. Conversely, the V449E mutant is likely to form active β_c homodimers (D'Andrea and Gonda 2000) .

Consistent with these activated receptors forming alternative configurations, biochemical studies performed to date indicate that the extracellular mutants produce distinct signals when compared to the transmembrane mutant (discussed further in Chapter 4). Furthermore, extracellular and transmembrane mutants promote differential

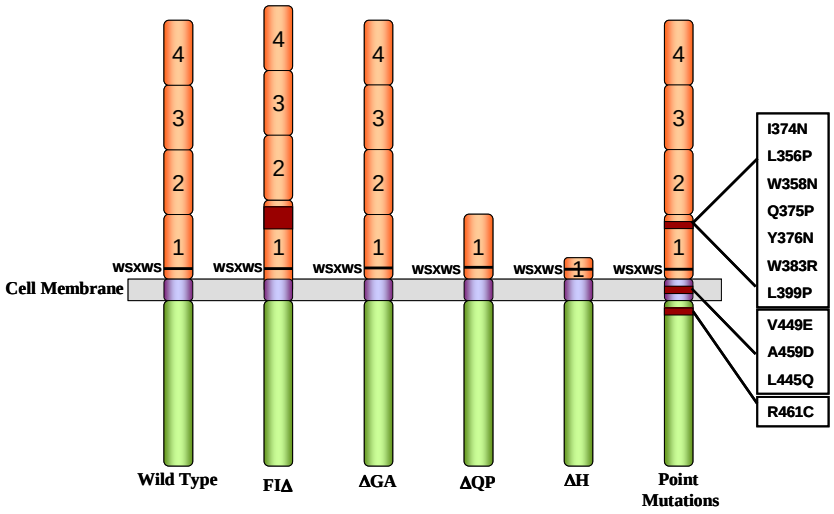


Figure 1.10 hβ_c activating mutations (adapted from D'Andrea, Barry et al. 1996).

biological responses in the bi-potential FDB1 cell line; FIΔ and I374N promote differentiation while V449E elicits a proliferative response (McCormack 2000). Extensive mutational analysis of these mutants has enabled dissection of the regions and specific residues of hβ_c critical for viability, proliferation and differentiation outcomes and is summarised in **Figure 1. 11** (Brown, Peters et al. 2004). Investigating the nature of this differential signalling formed a major part of the work described in Chapter 4.

1.4.3 GM-CSF Receptor Signalling Overview

The primary focus of this work is signalling events associated with the GM-CSF receptor. **Figure 1.12** shows a schematic summarising key signalling events and pathways described to date. Briefly, a number of events occur very rapidly following GM-CSF treatment of factor-dependent cell lines and primary cells (**Figure 1.12**). Upon stimulation with GM-CSF, JAK2 (associated with the Box1 motif on β_c) is phosphorylated and phosphorylates tyrosine residues on the intracellular region of the receptor (Quelle, Sato et al. 1994). Src kinases appear to be involved in activating

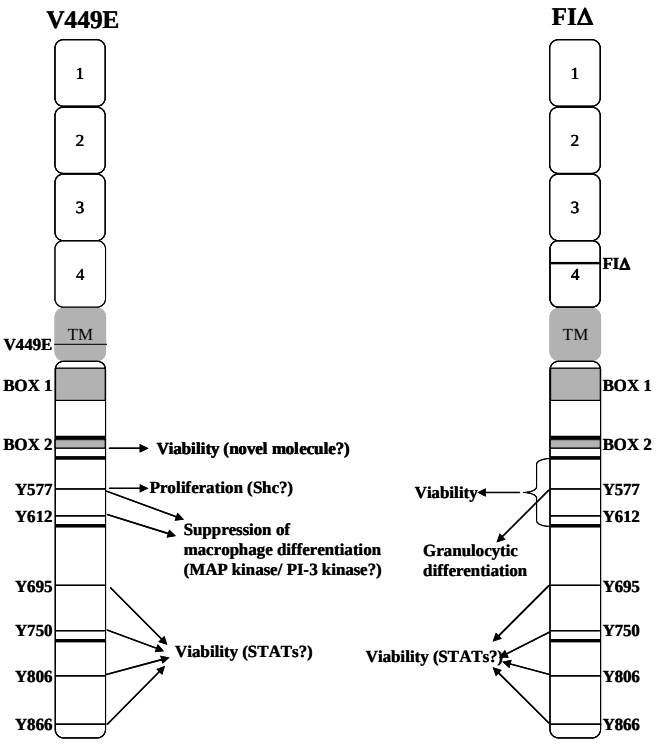


Figure 1. 11 Summary of the effects on FDB1 cells of V449E and FIΔ mutational analysis (Brown, Peters et al. 2004).

signalling from the receptor at this early stage, however, their role is perhaps less defined than that of JAK2 (Chaturvedi, Reddy et al. 1998). The phosphorylated tyrosine residues on the receptor serve as docking sites for SH2 domain or PTB domain containing proteins resulting in their phosphorylation and activation (**Figure 1.12**) (Pawson and Gish 1992). Interactions of signalling molecules with the tyrosine phosphorylated receptor serve to couple the receptor to a number of downstream signalling pathways, ultimately resulting in the control of specific cellular functions (Guthridge, Stomski et al. 1998). The GM-CSF receptor activates numerous signalling cascades including JAK2/STAT5, Ras/Raf/MAPK and PI3K/AKT pathways (discussed in detail in Sections 1.3.2.1, 1.3.4 and 1.3.5) (**Figure 1.12**). The recent study by Guthridge *et al* emphasises the differences in signalling that occur at different levels of

GM-CSF stimulation as well as the role of serine phosphorylation in mediating GM-CSF survival signals (Guthridge, Powell et al. 2006).

A number of other tyrosine kinases have been reported to interact with β_c including Lyn, Btk, Tec, Fyn and Hck (Li 1995; Burton 1997; Geijsen, Koenderman et al. 2001). The role of these kinases in β_c signalling is unclear. It is possible that the restricted expression of these proteins in different cell types may provide a means of attaining alternative β_c signalling responses (Geijsen, Koenderman et al. 2001).

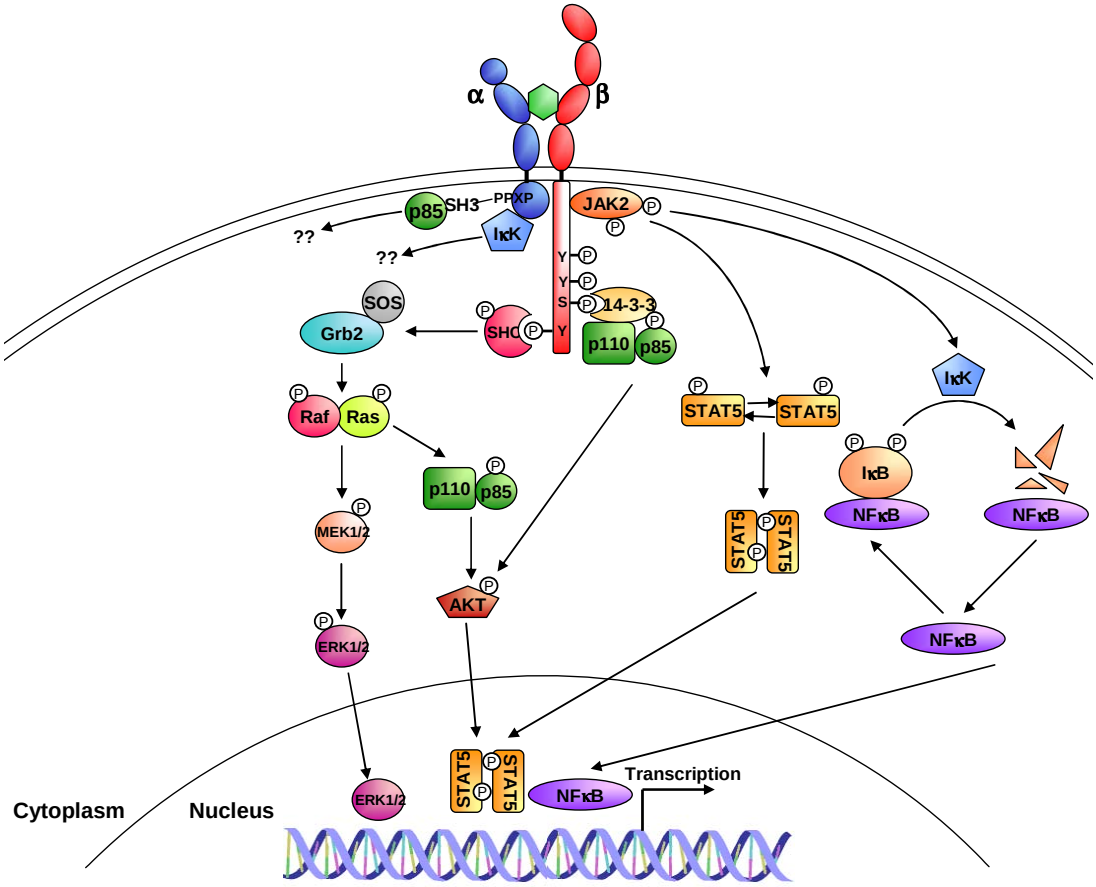


Figure 1.12 Summary of GMR signalling events. Binding of Shc to a membrane distal tyrosine residue is shown. Shc is also able to interact with Y577 located membrane proximally from Ser585. These two residues represent a binary switch that is regulated by the dose of GM-CSF. Shc binding to this residue has been omitted for simplicity. Although signalling pathways are represented separately, there may be significant overlap between these pathways.

1.5 Project Aims

The identification and characterisation of signalling events associated with the GM-CSF receptor requires further characterisation of the cytoplasmic interactions for both receptor subunits and an understanding of the contribution of particular events to activation of specific pathways and downstream cellular responses. The linking of individual signalling events and pathways to myeloid cell survival, mitogenesis, self-renewal, differentiation and leukaemic induction has been hindered by the extensive redundancy and cross-talk associated with signalling. Interacting partners of the GMRα were studied in detail to define better understanding of the role of this subunit in initiation of signalling from the GM-CSF receptor and to provide clues as to which pathways are associated with specific responses involving this subunit. Additionally, we studied signalling associated with constitutive mutants of the common β_c which represent alternative receptor configurations and deliver only some of the proliferative, survival and differentiation signals activated by the normal liganded receptor comples. The non-redundant signalling capacity and capacity to induce alternative responses facilitated the delineation of key events associated with survival during these two alternative responses.

The specific aims of this study were:

Aim 1: To characterise known and novel interaction partners of GMRα (Section 1.4.2.1) with a view to further characterising the role of this subunit in GM-CSF receptor signalling and function.

Aim 2: To dissect the contribution of particular receptor-induced signalling events to survival responses. We made extensive use of a well-characterised bi-potential myeloid cell-line model, FDB1 and the activated GM-CSF receptor mutants (Section 1.4.2.2).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Supplier	**Reagent/Kit**
Ajax Univar – Australia	Potassium dihydrogen orthophosphate Tris base (Tris-hydroxymethylmethylamine) Sodium Chloride Dimethyl Sulphoxide (DMSO) Methanol Sodium Hydroxide
Auspep Ltd - Australia	mGMRα-fluoroscein peptide
BDH AnalaR – Australia	Ethanol Glycine Glycerol Glacial Acetic Acid
BD Biosciences - USA	Anti-human c-Myc monoclonal antibody
Biomol - USA	AG-490 JAK inhibitor
Bio-Rad Laboratories – USA	40% Acrylamide/Bis-Acrylamide Sodium dodecyl sulphate (SDS) Bio-Rad gel drying solution Bio-Rad DC Protein Assay Ponceau S stain
Biosource International – USA	JAK2 $^{[pYpY1007/1008]}$ polyclonal antibody Cat# 44-426G
Boehringer Mannheim – Germany	Ampicillin
Calbiochem- Germany	MEK Inhibitor Set (PD98059/U0124/U0126)
Cayman Chemical - USA	LY294002 – PI3K inhibitor
CSL - Australia	Fetal Bovine Serum

Cell Signalling Technology Inc - USA	p44/42 MAP kinase antibody Cat# 9102 phospho-p44/42 MAPK(Thr^{202}/Tyr^{204}) antibody Cat# 9101 AKT antibody Cat# 9272 Phospho-AKT (Ser^{473}) (193H12) Rabbit mAb Cat# 9271 Phospho-IκBα ($Ser^{32/36}$) antibody Cat# 9246 IκBα antibody Cat# 9242 Phospho-JAK2 ($Tyr^{1007/1008}$) antibody Cat# 3771 Lyn antibody Cat# 2732 STAT5 antibody Cat# 9352 Wortmannin
GE Healthcare Life Sciences – USA	Protein A sepharose CL-4B Coomassie™ Brilliant Blue Glutatione sepharose Reduced Glutathione
JRH Biosciences Inc – USA	Dulbecco's Modified Eagle Medium (DMEM) Iscove's Modified Eagle Medium (IMDM) Phosphate Buffered Saline (PBS)
Invitrogen - Australia	1 Kb Plus DNA ladder (E-Gel® DNA markers) BenchMark™ prestained protein ladder Lipofectamine™ 2000 Platimum Pfx DNA Polymerase Iscove's Modified Dulbecco's Medium (IMDM) - Gibco® Dulbecco's Modified Eagle Medium (DMEM) - Gibco® Phosphate Buffered Saline (PBS) - Gibco®
New England Biolabs – Australia	Restriction Endonucleases

Pierce	SuperSignal WestPico substrate SuperSignal WestDura substrate ImmunoPure Goat Anti-Mouse IgG HRP ImmunoPure Goat Anti-Rabbit IgG HRP B-PER® II bacterial protein extraction reagent
Progen Industries Ltd - Australia	Isopropyl-β-D-1-thiogalactopyranoside (IPTG) T4 DNA ligase Agarose
QIAGEN Pty Ltd	QIAquick gel extraction kit HiSpeed Plasmid Purification kit Omniscript
Roche Germany	Glycogen Pefabloc™ Complete™ protease inhibitor cocktail AmpliTaq® DNA polymerase FS
Sigma Chemical Company - USA	TEMED Ammonium Persulphate Lennox L Agar Lennox L Broth Puromycin EDTA (ethylenediamine-tetraacetic acid) Isoamyl alcohol EGTA (ethyleneglycol-bis-tetraacetic acid) Ethidium Bromide Monoclonal anti-FLAG® M2 antibody 7-Aminoactinomycin D (7-AAD)
Stratagene	QuikChange® Site-Directed Mutagenesis kit
Upstate Cell Signalling Solutions - USA	Phospho-STAT5A/B antibody ($Tyr^{694/699}$) Cat# 05-495 Anti-PI3 kinase p85 antibody Phospho-STAT3 antibody(Tyr^{705}) Anti-JAK2 antibody Cat# 06-255

2.1.1 Oligonucleotide PCR Primers

Gene	Upstream Primer (5' to 3')	Downstream Primer (5' to 3')	Product Size (bp)
hGMRα-APVA	CGGCTGTTGGCGCCAGTTGCACAGATCAAAG	CTTTGATCTGTGCAACAACTGGCGCGAACAGCCG	1841
hUnc119	CCGGGATCCACCATGAAGGTGAAGAAGG	CGGGAATTCTCACTACAAGTCTTCTTCAGAAATGAGTTTTTGTTCCGTGGGATCAGGGTGT	722
mUnc119	CCGGGATCCACCATGAAGGTGAAGAAAGGCGG	CGGGAATTCTCACTACAAGTCTTCTTCAGAAATGAGTTTTTGTTCGGGTGTCCCACTGTAG	722
mLynSH3	CCGGAATTCACCATGGAACAAGGTGACATTGTGTGGC	CGGGGATCCTCAGGTGTTGACCTTGGCCACG	168
mSrcSH3	CCGGAATTCACCATGGGTGGGGTGACCACCTTTGTG	CGCGGATCCTCAGGAGTCGGAGGGCGCCAC	171
mp85SH3	CCGGAATTCACCATGGCAGAGGGCTACCAGTACAG	CGGGGATCCTCACCTTCCAATGTATTCAACGTAAG	216

2.2 Bacterial Techniques

2.2.1 Media and Solutions

All bacterial cell culture media and glassware was autoclaved prior to use and aseptic techniques were used to prevent contamination. Liquid bacterial cell culture was carried out in Luria broth (LB), supplemented with 50μg/ml ampicillin to select for transformed populations. Bacterial cultivation was carried out in an orbital shaker (Paton Industries, Australia) at 37°C overnight. Solid culture was carried out on LB agar plates supplemented with 50μg/ml ampicillin.

Luria Broth (LB): 10g Bacto-tryptone, 5g Bacto-yeast extract and 5g NaCl was made up to 1L with Milli-Q water. The pH was adjusted to 7.6 with HCl and subsequently autoclaved.

LB Agar: 9.1g tryptone, 4.6g yeast extract, 4.6g NaCl and 13.7g agar was made up to 1L with Milli-Q water. The pH was adjusted to 7.6 with HCl and subsequently autoclaved. Plates were poured whilst the agar was still warm.

Psi Broth: 2% Bacto-tryptone (w/v), 0.5% Bacto-yeast extract (w/v), 20mM $MgSO_4.7H_2O$, 10mM NaCl, adjusted to pH 7.6 with KOH and sterilised by autoclaving.

Transformation Buffer I (TfbI): 30mM KAc, 100mM RbCl, 10mM $CaCl_2.2H_2O$, 50mM $MnCl_2.4H_2O$ and 15% glycerol, adjusted to pH 5.8 with acetic acid and sterilised through a 0.2μM filter.

Transformation Buffer II (TfbII): 10mM MOPS, 10mM RbCl, 75mM $CaCl_2.2H_2O$, 15% glycerol (v/v), adjusted to pH 6.5 with KOH and sterilised through a 0.2μM filter.

2.2.2 Preparation of Chemically Competent E. coli

Escherichia coli (*E. coli*) strain DH5α cells were made chemically competent for transformation using a method described by Sambrook *et al* (Sambrook, Fritsch et al. 1989). 20μl of a DH5α glycerol stock was inoculated in 50μl of LB and aseptically streaked onto an LB agar plate. The plate was incubated at 37°C overnight. A colony was picked and inoculated in 50ml Psi broth and incubated at 37°C until it reached an optical density of 0.4 at 550nm. The culture was transferred to ice for 15 minutes and

centrifuged for 5 minutes at 5000g at 4°C. The bacterial cell pellet was resuspended in 80µl of buffer TfbI and incubated on ice for 15 minutes. The cells were centrifuged at 5000g for 5 minute at 4°C. 8ml of buffer TfbII was then used to resuspend the cell pellet followed by a 15 minute incubation on ice. Aliquots of competent cells were snap frozen in liquid nitrogen and stored at -80°C.

2.2.3 Midi-prep Plasmid Purification

Plasmid purifications were carried out using the QIAfilter Plasmid Midi Kit from QIAGEN. This method was used for the purification of DNA to be used in transient transfections, retroviral transductions, and subcloning techniques. For this we followed manufacturers instructions and all DNA obtained was analysed by agarose gel electrophoresis. Concentrations were determined using a DNA spectrophotometer (OD_{260}).

2.2.4 Mini-prep Plasmid Purification

This mini-prep method utilises the buffers supplied with the QIAfilter Plasmid Maxi/Midi Kit as described in Section 2.2.3. 2ml of overnight culture was pelleted and the cell pellet resuspended in 300µl of Buffer P1 (QIAGEN). 300µl of Buffer P2 (QIAGEN) was then added and the tubes were inverted 5 times followed by a 5 minute incubation at room temperature. 300µl of Buffer P3 (QIAGEN) was then added to the mixture and inverted 5 times. The mixture was subsequently centrifuged at 13000rpm for 5 minutes and the supernatant transferred to a new tube. 600µl of isopropanol was added to precipitate the DNA and the mixture was centrifuged at 13000rpm for 15 minutes. The pellet was subsequently washed in 70% ethanol, dried on a 55°C heating

block and resuspended in 30µl of Milli-Q water. This method was used to purify DNA for screening clones and restriction digests.

2.2.5 Transformation

Transformations were carried out using chemically competent DH5α cells prepared as described in Section 2.2.2. The entire ligation was added to 200µl of competent cells and incubated on ice for 30 minutes followed by a heat shock step at 47°C for 90 seconds. The transformation mixture was then transferred to ice for a further 5 minutes. 500µl of LB was added to the mix and incubated at 37°C for 30 minutes. The cells were then plated on an LB agar plate containing 100µg/ml ampicillin and incubated overnight at 37°C.

2.3 Recombinant DNA Techniques

2.3.1 Restriction Digest and DNA ligation

Restriction endonucleases and buffers were obtained from New England Biolabs (NEB). Specifically, 1-1.5µg plasmid DNA was digested for 1-3hrs at 37°C using buffers supplied by NEB and 2 units NEB restriction endonuclease.

DNA ligations were carried out using T4 DNA ligase (Invitrogen) according to manufacturers instructions. Specifically, 50ng of vector DNA, a 3-fold molar ratio of insert DNA, 1 X T4 DNA ligase buffer and 0.1 units of T4 DNA ligase were used in each ligation. Reactions were incubated overnight at 16°C. Control ligations were also set up lacking DNA insert, to determine the level of background from uncut or re-ligated vector.

2.3.2 PCR Amplification

Specific PCR primers are outlined in Section 2.1.1. For all PCR amplification reactions, Platinum® Pfx DNA Polymerase (Invitrogen) was used according to manufacturers conditions. Specifically, PCR reactions contained 100ng of genomic DNA template, 1X PCR buffer, 15µl of 10mM dNTPs, 1µl of 50mM $MgSO_4$, 1.5 µl of each 10µM primer and 1µl of Platinum® Pfx DNA Polymerase in a total volume of 50µl. Thermal cycling was carried out at 94°C for 15 seconds and 30 cycles of [94°C for 15 seconds, 55°C for 30 seconds and 1 minute per kb at 68°C].

2.3.3 Site Directed Mutagenesis

Targeted mutations were introduced using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The following considerations were made when designing mutagenic primers; primer length between 25-40 bases, both mutagenic primers contain the desired mutation and bind to the same sequence on opposite strands, and a minimum GC content of 40% with the primer sequence ending in a G or C. Specific primers for hGMRα-APVA are described in Section 2.1.1. All components and conditions were used according to manufacturers instructions. Thermal cycling was carried out for 18 cycles (for multiple base changes) of [95°C for 30 seconds, 55°C for 1 minute and 1 minute per kb at 68°C]. Following mutagenesis, 1µl of the *Dpn I* restriction enzyme (10U/ml) was added directly to each amplification reaction and incubated at 37°C for 1 hour to digest the parental (ie. methylated) DNA. Correct mutation was confirmed by DNA sequencing.

2.3.4 Sequencing

DNA sequencing was conducted by the IMVS (Institute of Medical and Veterinary Science, Adelaide, Aust) Molecular Pathology Sequencing Centre. Specifically, sequencing reactions were set up using 1500ng of DNA, 6µl of BigDye terminator reaction mix (dye-labelled dedeoxynucleotides, deoxynucleotides, AmpliTaq® DNA Polymerase FS, thermostable pyrophosphatase, Tris-HCl pH 9.0, $MgCl_2$) and 2µl of 10µM primer made up to a total volume of 20µl. Thermal cycling was carried out for 25 cycles of [96°C for 10 seconds, 50°C for 10 seconds and 60°C for 4 minutes]. 80µl of 75% isopropanol was added to the reaction which was then incubated at room temperature for 15 minutes. Following the incubation, the mixture was centrifuged at 10000g for 20 minutes and the supernatant was discarded. The remaining DNA pellet was washed with 75% isopropanol and the tube was re-centrifuged at 16000g for 5 minutes. The DNA pellet was dried on a 100°C heating block for 1 minute. The dried pellet was then sent to the, IMVS for DNA sequencing.

2.4 Tissue Culture

2.4.1 Media and Cytokines

Tissue Culture Media

All Gibco® tissue culture media was obtained from Invitrogen, Australia. Iscove's Modified Dulbecco's Medium (IMDM) was used to culture FDB1 cells and Dulbecco's Modified Eagle Medium (DMEM) was used to culture HEK293T cells. Phosphate Buffered Saline (PBS) was used for washing cells. All cell lines were maintained in a Hotpack CO_2 incubator (Philadelphia, USA). Cell manipulation was carried out in an Oliphant Laminar Flow cabinet in a PC2, OGTR approved facility.

Haemopoietic Cytokines

Recombinant murine IL-3 (mIL-3) was produced from baculoviral vectors supplied by Dr Andrew Hapel (John Curtin School of Medical Research, Canberra, Adelaide).

Recombinant murine GM-CSF (mGM-CSF), was produced in *Eschericia coli* and purified by anion exchange and reverse phase high-performance liquid chromatography and was a kind gift from Professor Angel Lopez, Hanson Institute, Adelaide, South Australia.

2.4.2 Cell Lines and Maintenance

The murine factor-dependent cell line, FDB1 (McCormack and Gonda 2000) was cultured in IMDM supplemented with 10% heat-inactivated fetal bovine serum, 100units/ml penicillin, 100μg/ml streptomycin, and 500 bone-marrow units (BMU) mIL-3 (1 BM unit is the amount that gives 50% maximal colony formation, CFU-GM, using bone marrow cells (Metcalf 1984)). Media was supplemented with 1μg/ml Puromycin to select for infected populations of cells expressing the Pac gene.

The human embryonic kidney cell line, HEK293T cells was cultured in DMEM supplemented with 10% fetal bovine serum, 100units/ml penicillin and 100μg/ml streptomycin.

2.4.3 Transient Transfection of HEK293T Cells

HEK293T cells were grown to 70% confluence in a T25 flask. Prior to transfection, media was removed and replaced with 5ml fresh supplemented DMEM excluding

antibiotics. Lipofectamine™ 2000 was used as a method of DNA transfer following manufacturers instructions. Briefly, 500µl of DMEM was mixed with 20µl of Lipofectamine™ 2000 reagent. In a second tube, 500µl of DMEM was mixed with 8µg of DNA of interest. The two tubes were incubated at room temperature for 5 minutes to allow formation of liposomes. Following this incubation, the contents from the two tubes were mixed and incubated for a further 30 minutes at room temperature before being transferred to the HEK293T cells and incubated at 37°C for a further 48 hours. Transiently transfected HEK293T cells were analysed by flow cytometry for expression of the desired construct.

2.4.4 Retroviral Transduction

DNA was stably introduced into murine FDB1 cells using the retroviral expression vector pRUFpuro (**Appendix A**). Retroviral constructs were co-transfected into HEK293T cells with the pEQ packaging plasmid (Persons, Mehaffey et al. 1998) to produce viral supernatant. Specifically, 4µg of the gene of interest/pRUFpuro and 4µg of pEQ was transfected (refer Section 2.4.3) into a 60% confluent T25 flask of HEK293T cells. Viral supernatant was collected at 24 hours and 48 hours post transfection. For infection of FDB1 cells, this viral supernatant was added neat to 1 X 10^6 cells for 6 hours. This media was then replaced with fresh supplemented IMDM. Appropriate retroviral markers were used to select infected cells and cell sorting was used to isolate a population of cells expressing a retroviral marker (eg. GFP). All stable cell lines were maintained in media supplemented with 1µg/ml puromycin to maintain expression.

2.5 Cell Viability Assays

Trypan Blue Exclusion

Trypan blue exclusion was used to measure cell viability. The dye is taken up by dead or dying cells that have reduced cell membrane integrity. Cells were diluted 1:1 with trypan blue and placed in one chamber of a Neubauer hemacytometer slide. An inverted microscope was used to manually count the viable and non-viable cells using the hemacytometer grid. Viability was expressed as % viable cells.

7-Aminoactinomycin D (7-AAD) Viability Staining

7-AAD staining (Rabinovitch, Torres et al. 1986) was used to measure viability of cells following treatment with pharmacological inhibitors. 7-AAD is a non-permeant dye that does not enter cells with an intact cell membrane or active cell metabolism. The dye enters cells with damaged plasma membranes and binds to intracellular structures producing fluorescent adducts which identify "non-viable" cells.

Following 24 hour inhibitor treatment, 1 X 10^6 cells were diluted in 1ml of DMEM (not supplemented) in a FACS (fluorescence activated cell sorting) tube. 1µl of 7-AAD dye was added to each FACS tube and incubated for 15 minutes at room temperature and subsequently analysed by flow cytometry on a Beckman-Coulter Epics Elite ESP Flow Cytometer/Cell Sorter.

2.6 Differentiation Assay

Differentiation assays were used to examine morphological differentiation of cells following inhibitor treatments. 3 X 10^5 cells of each cell type were washed 3 times with 30ml IMDM (not supplemented), and subsequently seeded at a density of 1 X 10^5cells/ml in 6-well dishes with triplicate wells for each cell type. One of these wells was left without factor and to the other two wells either 1000BMU of mIL-3 or mGM-CSF was added. Trypan blue exclusion was used to assess viability at day 2 and day 5. Cytocentrifugation was carried out using a Shandon Cytospin® 4 Cytocentrifuge. On day 5, 5 X 10^4 cells were centrifuged onto microscope slides at 500rpm for 5 minutes. These slides were stained with May-Grunwald-Giemsa and visualised under a microscope for morphology assessment.

2.7 Immunoprecipitation and Western Blotting

2.7.1 Solutions and Buffers

Modified RIPA Lysis Buffer (MRLB): 50mM Tris-HCl (pH 7.4); 1% NP-40; 0.25% Na-deoxycholate; 150mM NaCl; 1mM EDTA; 2mM Pefabloc™; 1X Complete™ protease inhibitor cocktail; 1mM Na_3VO_4; 2mM NaF.

5X SDS Load Buffer: 0.5M Tris-HCl (pH7.4); 0.8ml glycerol; 1.6ml 10% sodium dodecyl sulfate (SDS); 0.4ml β-mercaptoethanol; 0.2ml 0.5% (w/v) bromophenol blue; 4ml Milli-Q water.

Resolving Gel (8%): 2.7ml 30% Acrylamide/bis-acrylamide; 2.5ml 1.5M Tris (pH8.8); 0.1ml 10% SDS; 0.1ml 10% ammonium persulfate (APS); 6µl N,N,N',N'-Tetramethylethylenediamine (TEMED); 4.6ml Milli-Q water.

Stacking Gel (5%): 0.83ml 30% Acrylamide/bis-acrylamide; 0.63ml 1.5M Tris (pH6.8); 50µl 10% SDS; 10υl 10% APS; 5µl TEMED; 3.4ml Milli-Q water.

10X SDS-PAGE Running Buffer: 30.2g Tris base; 144g glycine; 10g SDS; 1L Milli-Q water.

10X Towbin Buffer: 25mM Tris base; 192mM glycine; 10% methanol

Immunoprecipitation Wash Buffer: 50mM Tris base (pH8.0); 120mM KCl; 1mM EDTA, 0.5% Triton-X-100

Phosphate Buffered Saline (PBS): 16mM Na_2HPO_4; 4mM $NaHPO_4$; to 1L with Milli-Q water.

Phosphate Buffered Saline Tween (PBST): PBS, 0.1% Tween-20

2.7.2 Isolation of Protein from Cell Lines

FDB1 cell lines, at a density of 1 X 10^6 cells/ml, were starved of factor by washing three times in 30ml IMDM (no supplements). Inhibitors were added at the following concentrations unless otherwise indicated: 50µM AG490, 50µM PD98059, 25µM U0126, 25µM U0124, 25µM LY294002, and 0.5µM Wortmannin. These inhibitors

were incubated with the cells for 16 hours, or the length of growth factor starvation, unless otherwise indicated. Additionally, where indicated, cells were pulsed at 37°C with 1000U mGM-CSF or 1000U mIL-3 for 5 min, which was determined to be the optimal time for activation of downstream signalling molecules. Cells were then lysed in 100µl of cold MRLB (1 X 10^6cells/100µl buffer), incubated on ice for 30 minutes and centrifuged at 13000rpm for 5 minutes at 4°C. Protein concentration was determined using the BioRad DC Protein Assay according to the manufacturer's instructions and 5X SDS load buffer was added (except where lysates were to be used in immunoprecipitations), 1:4 (v/v), to each sample and boiled at 95°C for 5 minutes.

2.7.3 Immunoprecipitation

Immunoprecipitations (IPs) were performed using 50µg whole cell lysate from HEK293T cells (as described in Section 2.7.2), 1µg of antibody and 50µl of protein A sepharose CL 4B (50% slurry). Lysates were initially precleared for 2 hr at 4°C, with 50µl of protein A sepharose (50% slurry). The precleared lysate were incubated with 1µg of anti-hGMRα antibody, 4H1, 50µl of a 50% slurry of protein-A-sepharose, and 200ul of 1 X PBS. The IPs were rotated at 4°C for 4 hours. Following binding, the sepharose was washed three times in IP wash buffer (Section 2.7.1). Equivalent concentrations of cell lysate and immunoprecipitation samples were run on a 8% acrylamide gel and were detected by western immunoblot analysis as described in Section 2.7.4.

2.7.4 SDS-PAGE Gel and Western Blotting

Acrylamide SDS page gels (8%) were made according to the recipe in Section 2.7.1. Gels were poured between two glass, 1.5mm thick, spacer plates (MiniPROTEAN® 3 System Plates, Bio-Rad Laboratories, Hercules, CA). Once polymerised, a 5% acrylamide stacking gel was made and poured above the resolving gel and a 10-well comb was inserted. Once the stacking gel was polymerised, the comb was removed and 75μg protein extracts or immunoprecipitations were boiled in SDS load buffer at 95°C for 5 minutes. The samples were then loaded and electrophoresed at a constant voltage of 100V (Bio-Rad power supply, model 200/2.0, Bio-Rad Laboratories, Hercules, CA).

Samples were subsequently transferred to PROTRAN® nitrocellulose transfer membrane (Schleicher & Schuelle, USA) using BioRad wet transfer apparatus at 75 volts for 45 minutes. The membranes were blocked for 1 hour at room temperature in 1 X PBS, 5% non-fat dry milk, and 0.1% Tween-20. The membranes were then incubated overnight at 4°C with the recommended dilutions of primary antibodies, according to manufacturers instructions, in PBST with 5% non-fat dry milk. Three, 10 minute washes in PBST were then performed and the membranes were incubated with the recommended concentrations of HRP-conjugated secondary antibodies in PBST with 5% non-fat dry milk for 1 hour at room temperature. Following three washes with PBST, immunolabeling was detected using SuperSignal West Pico or SuperSignal West Dura detection substrates. Westerns were developed on AGFA Curix Blue HC-S Plus film using a CP1000 AGFA Developer (AGFA, Germany).

2.8 GST Pull-down

2.8.1 GST Fusion Protein Preparation

Murine Lyn-SH3, Src-SH3 and p85-SH3 domains were amplified using the primers described in Section 2.1.1. These products were cloned into the pGEX-2T vector which contains a *tac* promoter for chemically inducible, high-level expression (**Appendix B**). The vector also contains a thrombin cleavage site to enable purified proteins to be dissociated from GST using thrombin cleavage. Following cloning, constructs were transformed into a protease-deficient *E. coli* cell type, BL-21. 50ml overnight cultures were set up in LB. The overnight culture was then inoculated into 500ml Terrific Broth (TB) and incubated for 1.5 hours or until the OD_{600} was approximately 0.5. 500µl of IPTG was subsequently added to each culture and incubated at 37°C for a further 2.5 hours for induction of the protein of interest. Bacteria were then lysed in 10ml B-PER®II (Bacterial Protein Extraction Reagent) at room temperature for 10 minutes followed by centrifugation at 12000rpm for 15 minutes at 4°C. 1ml of a 50% slurry of glutathione sepharose was added to each lysate and rotated at 4°C for 30 minutes. Samples were then spun at 2000rpm for 3 minutes and the pellets were washed 4 times with 1 X PBS + 0.1% Triton-X-100 and finally resuspended in 1ml of 1 X PBS + 2mM dithiothreitol (DTT) + 10% glycerol. To elute the fusion proteins from the sepharose, 1 ml glutathione elution buffer was added to the sepharose and rotated at room temperature for 30 minutes. The sepharose was centrifuged again and the supernatant was retained. 10µl of each eluate was run on an SDS PAGE gel and fusion proteins were visualised by Coomassie Blue staining and protein concentrations were estimated by comparison to BSA standards loaded on the same gel. The eluates were then stored at -20°C for later use.

2.8.2 GST Pull-down

GST pull-down assays (Einarson 2004) were used to assess binding of proteins in whole cell lysates with a protein of interest tagged with GST and immobilised on a glutathione sepharose support. GST pull-downs were set up using 50µg of whole cell lysate, 30µl glutathione sepharose (50% slurry), 25µg of purified GST fusion protein and 200µl of IP wash buffer. The pull-down was rotated at 4°C overnight followed by 3, 10minute washes with IP wash buffer to ensure the removal of any proteins bound non-specifically to the sepharose. The sepharose was subsequently boiled in 30µl SDS load buffer (see recipe in Section 2.7.1) for 2 minutes at 95°C. The entire sample was then run on an SDS-PAGE gel and subjected to western immunoblot analyses using specific antibodies to detect proteins of interest bound to the immobilised GST fusion protein.

2.9 Fluorescence Polarisation

Fluorescence polarisation was used to measure direct interactions between the SH3 domains of murine Lyn, Src and p85 with a fluoroscein labelled mGMRα peptide. GST fusion proteins were prepared as outlined in Section 2.8.1 for mLyn, mSrc and mp85 SH3 domains. Additionally, the mGMRα fluorescent peptide outlined in Section 2.11 was utilised in these experiments.

A black 96-well plate was blocked with casein (200µl per well) for 1 hour at 37°C. Each sample was set up in triplicate wells as follows: 1) 100nM mGMRα-fluoroscein peptide; 2) 100nM hGMRα-fluoroscein peptide and 25µg GST; 3) 100nM mGMRα-fluoroscein peptide and 25µg GST-mp85-SH3; 4) 100nM mGMRα-fluoroscein

peptide and 25µg GST-mLyn-SH3; 5) 100nM mGMRα-fluoroscein peptide and 25µg of GST-mSrc-SH3. Each well was made up to a 100µl volume with 1 X TBS pH 7.4 (6.05g Tris base; 8.86g NaCl, in 1L Milli-Q water). The assay plate was incubated for 5 minutes at room temperature prior to reading on the FLUOstar OPTIMA™ (BMG Labtechnologies) fluorescence plate reader at an excitation wavelength of 520nm. Data was analysed using FLUOstar Galaxy software and polarisation values of the controls, were subtracted from the other sample values to obtain the relative change in polarisation (ie. ΔmP value) for the test samples.

2.10 Intracellular Flow Cytometry

Cells were fixed using a final concentration of 1.6% formaldehyde in 6-well flat bottom tissue culture dishes at 37°C for 15 minutes. Following fixation, cells were permeabilised with 1ml ice cold methanol on ice for 30 minutes (methanol was added whilst vortexing). Three PBS washes were then carried out and the cells were incubated with the 5µg/µl primary antibody (unless otherwise stated) for 30 minutes on ice. Following a single wash in PBS, a 1:100 dilution of the corresponding FITC-conjugated secondary antibodies were added to the samples and incubated on ice for 30 minutes. Approximately 20,000 ungated events were collected for each sample on a Epics Elite ESP Flow Cytometer/Cell Sorter (Beckman Coulter, Fullerton, CA, USA) and Expo™32 Version 1.2 software was used for the analysis of all samples.

2.11 SH3 Membrane Domain Array

The SH3 Domain III (Panomics) is a membrane domain array that consists of 34 SH3-domains of various proteins spotted onto a nitrocellulose membrane. This array was used to screen for potential interactors of the mGMRα. A short mGMRα peptide containing the SBP motif and 5 flanking residues was synthesised with an attached C-terminal fluoroscein group. The membrane was blocked for 1 hour at room temperature in 1 X PBS + 0.1% tween 20 + 2% skim milk. A solution of 1mg/ml mGMRα peptide in 1 X PBS was incubated on the membrane for 2 hours at room temperature. The membrane was then rinsed in 1 X PBS and scanned using a fluorimager (Typhoon™ FluorImager®, Molecular Dynamics). Spot quantitation was performed using ImageQuant™ software taking into account the local background surrounding each spot and comparing binding affinities to GST alone as a measure of background.

2.12 Statistical Analysis and Presentation of Data

Error bars represent the standard error of the mean. Probability and statistical significance were analysed using the two-tailed student *t* test with a confidence interval of either 95% ($p<0.05$) or 99% ($p<0.01$). Experiments were carried out a minimum of three times ($n=3$) unless otherwise stated.

CHAPTER 3

ROLE OF THE GMRα IN GM-CSF INDUCED SIGNALLING

3.1 Introduction

The cytokines; IL-3, IL-5 and GM-CSF display overlapping roles in growth and differentiation of myeloid cell types such as eosinophils, neutrophils, and monocytes. Although these receptors share a common beta subunit, β_c, the ligand specific alpha subunits confer the ligand binding specificity to the complex and thereby direct the activation of multiple signalling pathways which result in specified biological outcomes (Geijsen, Koenderman et al. 2001). In this chapter we aimed to investigate the role of GMRα in signalling from the GM-CSF receptor complex through the identification and characterisation of interacting partners.

The cytoplasmic domain of the GMRα subunit is essential for GM-CSF receptor signalling (Geijsen, Koenderman et al. 2001). Several studies have shown that deletion of the short cytoplasmic domains of the ligand-specific alpha subunits abolishes ligand-induced signalling with no affect on ligand binding (Polotskaya, Zhao et al. 1994; Takaki, Kanazawa et al. 1994; Ronco, Doyle et al. 1995; Matsuguchi, Zhao et al. 1997; Doyle and Gasson 1998). Additionally, sequential deletions from the C-terminus of GMRα have defined the membrane-proximal 29 amino acids as essential for signalling (Takaki, Kanazawa et al. 1994; Doyle and Gasson 1998). Targeted mutation of conserved proline residues (Polotskaya, Zhao et al. 1994; Dhar-Mascareno, Chen et al. 2003) within this region is also consistent with a critical role for the membrane-proximal SBP motif (refer Section 1.4.2.1). In particular this motif has been shown to be essential for critical signalling events such as GM-CSF-induced activation of JAK2 (Doyle and Gasson 1998).

Identifying specific interaction partners of GMRα will give an indication of its role in triggering receptor signalling and reveal which pathways and cellular functions are coupled to GMRα. Comparisons with other α-subunits is also likely to provide clues regarding the specificity conferred by these subunits. Domains that recognise proline-rich motifs are some of the most common modular recognition domains (Rubin, Yandell et al. 2000; Zarrinpar and Lim 2000; Zarrinpar, Bhattacharyya et al. 2003). We focused on the potential SH3 recognition sequence, contained within the SBP motif of the GMRα cytoplasmic domain as this is likely to mediate at least some of the interactions that occur between the GMRα and other signalling proteins. We aimed to screen a number of candidate proteins to identify novel interactors with GMRα as well as characterise some known interactors that have been implicated in GMR signalling.

To date, few interactions between signalling molecules and GMRα have been identified. Dhar-Mascareno *et al* identified an interaction with the regulatory subunit of PI3K, p85, as being important for regulating glucose uptake (Dhar-Mascareno, Chen et al. 2003). This interaction is postulated to occur through the SH3 domain of p85 and the SBP motif of GMRα as the interaction is dependent upon an intact PXXP motif (Dhar-Mascareno, Chen et al. 2003). Additionally, IKKβ has been shown to interact with GMRα in a yeast-2-hybrid screen, however, the nature and role of the interaction between these two molecules is poorly characterised (Ebner, Bandion et al. 2003). It is unlikely that the interaction with IKKβ occurs directly with the proline-rich sequence in GMRα as IKKβ does not contain any proline-rich recognition motifs such as SH3 or WW domains.

Another potential candidate for binding to GMRα is the SFK activator, Unc119. Unc119 is reported to associate with the IL-5Rα and is a potent activator of multiple SFKs (Cen, Gorska et al. 2003; Gorska, Stafford et al. 2004). We sought to determine if Unc119 is an interactor with GMRα given the high sequence similarity between IL-5Rα and GMRα and the crucial role for SFK activation in GMR signalling (Refer Section 1.4.2.1).

As part of this study, we sought to confirm the interactions of IKKβ and p85 with GMRα and further characterise their contribution to GM-CSF induced receptor signalling. Several different techniques were used to confirm these interactions. In addition we also aimed to identify novel interactors of GMRα. Immunoprecipitation and affinity pull-down assays were combined with direct peptide binding to an SH3 domain membrane array and to GST-SH3 fusion protein for fluorescence polarisation assays.

3.2 Results – Characterisation of GMRα Interactions

3.2.1 Identification of Lyn and Src as Interacting Partners of GMRα

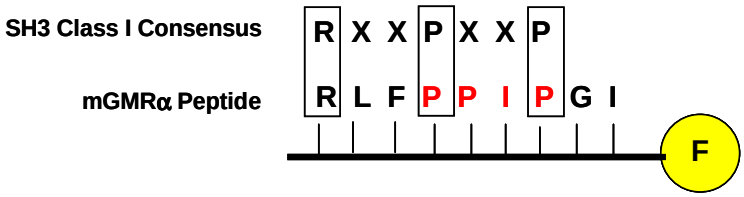
Initially we wished to identify novel interactions of candidate proteins with GMRα. We used an SH3 domain array and fluorescence polarisation techniques as a first screen of candidate proteins and confirmed interactions using GST-pulldowns and immunoprecipitations.

3.2.1.1 Identification of Novel Interactors of GMRα

To assess direct binding of candidate SH3 domains to GMRα we utilised a commercial SH3 Domain III protein array which contained recombinant SH3 domains from 34 known proteins fused to GST and immobilised onto a nitrocellulose membrane in duplicate spots (Panomics). A short mGMRα peptide containing the SBP motif and 5 flanking residues was synthesised with an attached C-terminal fluoroscein group (**Figure 3.1A**). In designing this peptide we used the guidelines determined in a study by Kay *et al* which emphasised the importance of flanking sequences in conferring target specificity in a similar binding assay (Kay, Williamson et al. 2000). We did not use a control peptide for screening normalisation because this peptide array was not designed to be reused. This is a clear limitation of this technique and as such it was used as a primary screen only.

The lyophilised peptide was solubilised in water to a final concentration of 1mg/ml and incubated for 4 hours with the SH3 Domain III protein array. Following three washes in PBS, binding was determined by scanning on a fluorimager. Binding to GST alone was used as a measure of background fluorescence for ImageQuant™ quantitation and the intensity of each spot was compared to this background control. The results generated from this experiment indicated binding of multiple SH3 domains to the GMRα peptide, including SH3 domains derived from Lyn, Src, CRK-D2 and Nck2 (**Figure 3.1B and C**). The graph in **Figure 3.1D** shows the quantitation for the spots of interest. Lyn, CRK-D2 and Nck2 all have volume report values (spot intensity) greater than 2 fold higher than GST alone. We observed a very strong signal with Lyn consistent with a direct interaction. The volume report for Src was not significantly

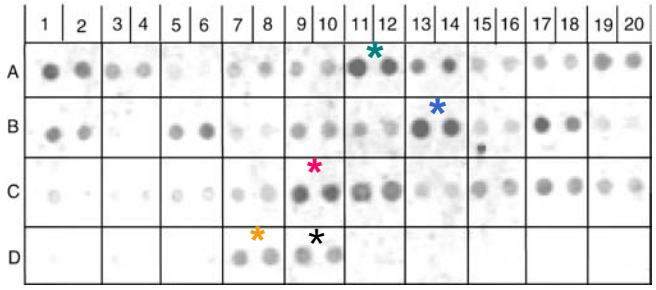
A.



B.

	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18	19 20
A	PRMT2	BMX	BPAG1	CACNB2	CRK-D1	CRK-D2	CRKL-D2	FYB-D2	GAP	Grap-D2
B	Grb2-D1	Grb2-D2	Grap-2-D2	ITSN-D3	ITSN-D3	JIP2	Lyn	ZO2	MATK	MIA
C	MY1E	MY1F	NCF1-D1	NCF1-D2	Nck2-D1	NPH1	OTOR	Dlg1	SH3GL2	SH3GL3
D	SPIN90	Trio	VINE-D2	c-Src	GST					

C.



D.

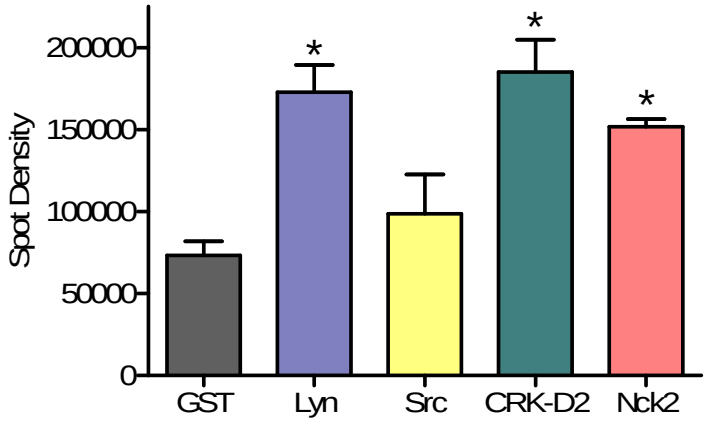


Figure 3.1 SH3 Domain III membrane array. A. Design of a C-terminal fluoroscein-tagged peptide derived from the SBP motif of hGMRα. **B.** Layout of the SH3 Domain III membrane array (Panomics) with SH3 domains spotted in duplicate. D1 and D2 refer to alternative SH3 domains within the same protein. **C.** Fluorimager scan showing binding of the fluorescent peptide to spots on the membrane. **D.** Graph indicating spot intensity calculations (quantitated using ImageQuant software) where an increased intensity suggests higher confidence in the interaction. * represents statistical significance $P<0.01$ for duplicate spots where n=1.

higher than GST alone, however, other studies have suggested a role for Src in signalling from GMR and we therefore chose to further characterise this candidate along with Lyn. Due to the volume of work required to characterise all interactions, we did not persue any of the other interactions identified by this membrane and this will be considered future work.

3.2.1.2 Fluorescence Polarisation Confirms Binding of Lyn and Src SH3 Domains to mGMRα

An alternative method for measuring direct interactions between two proteins of interest in a controlled environment is Fluorescence Polarisation (FP) (Park and Raines 2004). FP measures the polarisation of light caused by molecules rotating in solution (Park and Raines 2004). As larger molecules/complexes spin at a slower rate than smaller molecules in liquid the rate of spin is a function of the size. If a fluorescent-tagged molecule finds and binds a second molecule, the complex spins at a slower rate and its polarisation value therefore increases. FP is used to measure interactions between a tagged molecule and a target molecule in solution. Milli-polarisation (mP) units are used to measure the change (ΔmP) in the rotation of a molecule in solution.

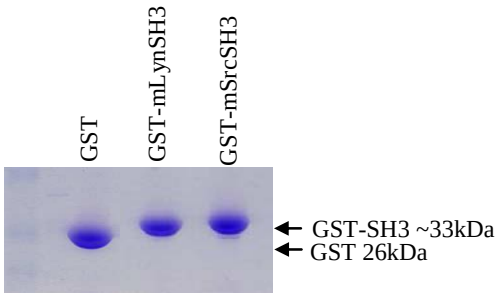
SH3 domains of murine (m) Src and Lyn were amplified from FDB1 cDNA using PCR and primers engineered to contain 3′BamHI and 5′EcoRI restriction sites for cloning into the pGEX2T vector (**Appendix B**). The resultant pGEX2T construct contains the glutathione-S-transferase (GST) coding sequence upstream of the cloned SH3 domain allowing production of GST fusion proteins. Sequence verified clones were transformed into a protease-deficient *Escherichia. coli* strain, BL-21, and clones that showed induction of proteins of the predicted sizes were selected for large scale

purification. Lysates were prepared and proteins were immobilised on glutathione sepharose. Fusion proteins were released from the sepharose with reduced glutathione, passed through a PD-10 desalting column and concentrated by size exclusion chromatography. The purified proteins were then rebound to glutathione sepharose, separated on an SDS-PAGE gel alongside BSA (bovine serum albumin) standards and their concentrations estimated using Coomassie Blue staining. **Figure 3.2A** shows 5μg of the purified GST, GST-LynSH3 and GST-SrcSH3 fusion proteins used as input for subsequent GST fluorescence polarisation and pull-down assays.

To measure interactions with mGMRα we used the previously described fluorescein tagged peptide encompassing the PPVP motif and 5 flanking residues of mGMRα (Section 2.11). The peptide was incubated with GST-SH3 domain fusions of mLyn and mSrc (Section 2.8.1). FLUOstar Galaxy™ software was used to determine the ΔmP values of the molecules in solution (**Figure 3.2B**). The Δmp value for the mGMRα fluorescent peptide alone was used to measure background fluorescence and was set at 0. The ΔmP value for GST + mGMRα peptide was used as baseline fluorescence polarisation. Any specific binding was assumed to have an ΔmP value above this baseline value.

The data presented in **Figure 3.2C** is consistent with direct binding of the SH3 domains of mLyn and mSrc to the mGMRα peptide containing the proline-rich motif. Binding of both mLyn and mSrc SH3 domains was at least two fold higher than with GST alone which was statistically significant (Section 2.12), suggesting a specific interaction with the mGMRα peptide (**Figure 3.2C**).

A.



B.

mGMRα Pep	mGMRα Pep + GST	mGMRα Pep + GST-mLynSH3	mGMRα Pep + GST-mSrcSH3	
98.40	105.13	104.56	115.63	mP
80.72	99.37	102.63	106.75	
89.37	85.74	114.53	116.42	
89.49	96.74	107.24	112.93	Mean mP
0	**7.25**	**17.74***	**23.43***	**Δ mP**

C.

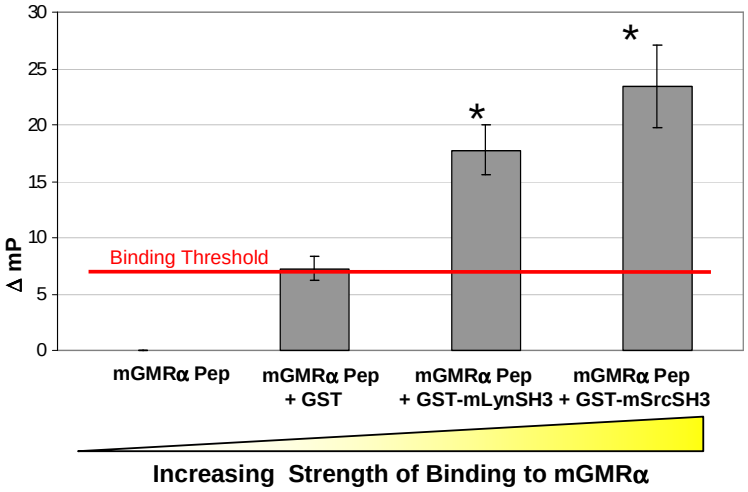


Figure 3.2 Fluorescence polarisation assay identifies GMRα interactors. A. GST-SH3 fusion proteins purified and bound to glutathione sepharose were separated by SDS-PAGE and stained with Coomassie Blue. **B.** Table of ΔmP values calculated for GST alone and mLyn- and mSrc-GST fusions complexed with mGMRα peptide. ΔmP values were calculated by subtracting the mean mP value of the mGMRα peptide alone from all other samples. The ΔmP value for the mGMRα peptide was therefore set at 0. **C.** Graph of ΔmP values where the specific binding threshold was set at the ΔmP value of the mGMRα peptide + GST, representing non-specific binding of the peptide to GST. * represents statistical significance $P<0.05$ for triplicate samples where n=1.

3.2.1.3 GST pull-down confirms interaction of Lyn and Src SH3 domains to full-length hGMRα

We next wished to confirm interactions of hGMRα with the SH3 domain-containing proteins using GST pull-down assays (Einarson 2004). We utilised the GST fusion proteins shown in **Figure 3.2A** as bait for pull-down experiments with lysates from HEK293T cells overexpressing hGMRα.

For expression of hGMRα in HEK293T cells we introduced the full length hGMRα cDNA using a retroviral vector, pRUFpuro (**Appendix A**), by transient transfection using Lipofectamine 2000 (Section 2.4.3). The expression of the transfected HEK293T cells was determined by flow cytometry using the anti-human GMRα antibody, 4H1 (**Figure 3.3A**). This antibody was also used to detect the expression of the transfected HEK293T cells by western blot analysis (**Figure 3.3B**). As the proline residues in the membrane proximal region of hGMRα are essential for GM-CSF-induced signalling we also used site-directed mutagenesis to construct a hGMRα-pRUFpuro construct where two critical prolines in the SBP motif were substituted to alanine (PPVP – APVA) (Section 2.3.3). Mutation of these two outer prolines has been previously shown to render this protein inactive (Matsuguchi, Zhao et al. 1997).

GST pull-down experiments were performed using 25μg of HEK293T lysate expressing hGMRα or hGMRα-APVA, and 5μg of purified GST-SH3 domain fusion protein. Western blot analysis was carried out using the anti-hGMRα antibody, 4H1, to detect pull-down of hGMRα.

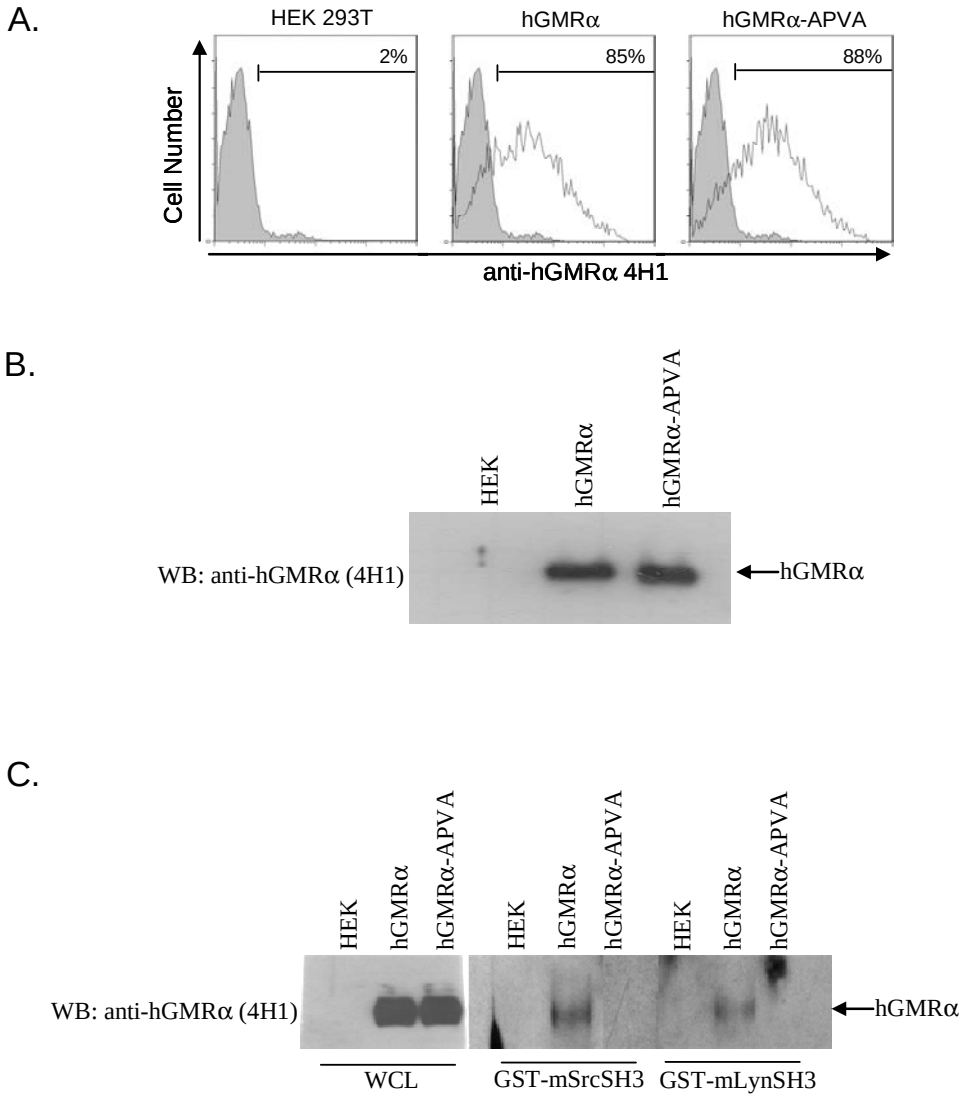


Figure 3.3 GST pull-down assays confirm interactions of Src- and Lyn-SH3 domains with hGMRα. A. Expression profiles of HEK293T cells transiently expressing hGMRα and hGMRα-APVA. Expression was determined 48 hours after transfection by staining with the anti-hGMRα antibody, 4H1. **B.** Western analysis of cell lysates using 4H1 antibody to detect hGMRα and hGMRα-APVA protein. **C.** GST pull-down assays using SH3 domain fusions of mouse Src and Lyn as bait to pull down hGMRα or hGMRα-APVA from whole cell lysates (WCL). Complexes were separated on an SDS-PAGE gel and anti-hGMRα 4H1 antibody was used to detect immunoprecipitated hGMRα.

As shown in **Figure 3.3C** we observed association of GST-mSrcSH3 and GST-mLynSH3 with hGMRα. These interactions were specific to the SH3 domains as GST alone did not pull-down either wild-type or mutated hGMRα. Both interactions were abrogated by mutation of the two outer prolines of hGMRα, consistent with the interactions with mLyn and mSrc being mediated through an intact proline-rich SBP motif. Whilst the interactions in these GST pulldown assays can be mediated through other proteins in the HEK293T cell lystate, the data obtained by the FP analysis in Section 2.9 is also consistent with these interactions being direct.

3.2.1.4 Immunoprecipitations confirm the interaction between full-length Lyn and hGMRα

We next aimed to confirm the interaction between Lyn and hGMRα in immunoprecipitated complexes from HEK293T whole cell lysates. hGMRα was readily detected in whole cell lysates, following transfection of HEK293T cells, and in immunoprecipitations using the anti-hGMRα antibody, 4H1 (**Figure 3.4A**). Endogenous Lyn could be detected in HEK293T cell lysates using a rabbit polyclonal anti-Lyn antibody (**Figure 3.4B**). To measure co-immunopreciptiation we used the 4H1 antibody to capture hGMRα from transiently transfected HEK293T-hGMRα cell lysates and bound Lyn was detected using the anti-Lyn antibody. As shown in **Figure 3.4B** Lyn is detected in complexes precipitated from cells expressing hGMRα but not from untransfected cells or cells expressing the APVA mutant form of hGMRα. Co-immunoprecipitation of Src to hGMRα was also investigated, however, endogenous Src protein levels in HEK293T cells were insufficient to measure co-immunoprecipitation.

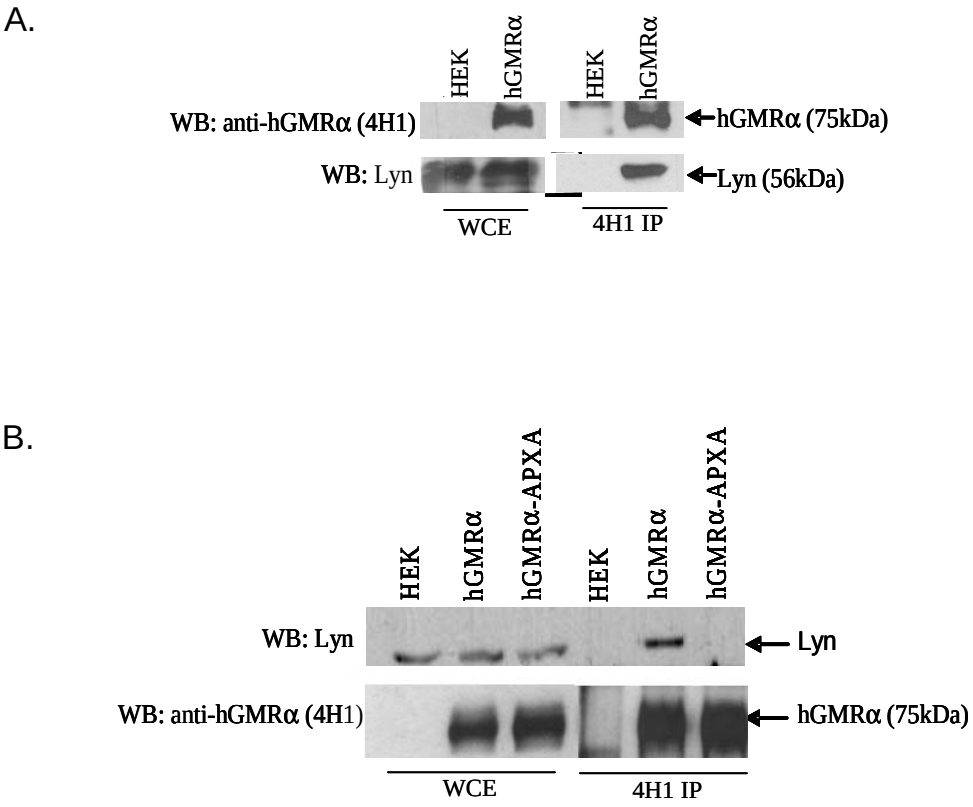


Figure 3.4 hGMRα interacts with full-length Lyn. A. hGMRα immunoprecipitation was performed using the 4H1 antibody and whole cell lysates (WCL) from untransfected HEK293T cells, or cells transfected with pRUFpuro-hGMRα. Co-immunoprecipitation of Lyn with hGMRα was determined using an anti-hLyn antibody. **B.** Abrogation of Lyn binding to hGMRα by mutation of the proline-rich sequence (PPVP - APVA). Co-precipitated proteins were detected with anti-hLyn antibodies by western blot analysis after immunoprecipitation with anti-hGMRα 4H1 antibody.

3.2.2 Confirming Interactions of p85 and IKKβ with hGMRα

As previously mentioned, recent reports have demonstrated both p85 and IKKβ as interaction partners of GMRα (Dhar-Mascareno, Chen et al. 2003; Ebner, Bandion et al. 2003). We wished to confirm these interactions and further characterise their role in GMR signalling.

3.2.2.1 Immunoprecipitations confirm interactions between endogenous p85 and IKKβ with hGMRα

Levels of endogenous human p85 and IKKβ in HEK293T cells were sufficient to allow detection following immunoprecipitation of GMRα as described above. Consistent with the study by Dhar-Mascareno *et al* (Dhar-Mascareno, Chen et al. 2003), we were able to co-immunoprecipitate p85 with hGMRα (**Figure 3.5A**). The interaction appeared to be dependent on the two prolines in the SBP motif as indicated by an inability of p85 to co-immunoprecipitate with the hGMRα-APVA mutant (**Figure 3.5B**).

Endogenous levels of IKKβ were readily detected in HEK293T cells using western immunoblot analysis with a rabbit polyclonal anti-IKKβ antibody (**Figure 3.5A**). Consistent with Ebner *et al* (Ebner, Bandion et al. 2003), we were able to demonstrate co-immunoprecipitation of IKKβ with hGMRα (**Figure 3.5A**). The role of IKKβ and the NFκB signalling pathway has been characterised further in Chapter 4, however the nature of the interaction between IKKβ and hGMRα was not characterised any further as part of this study.

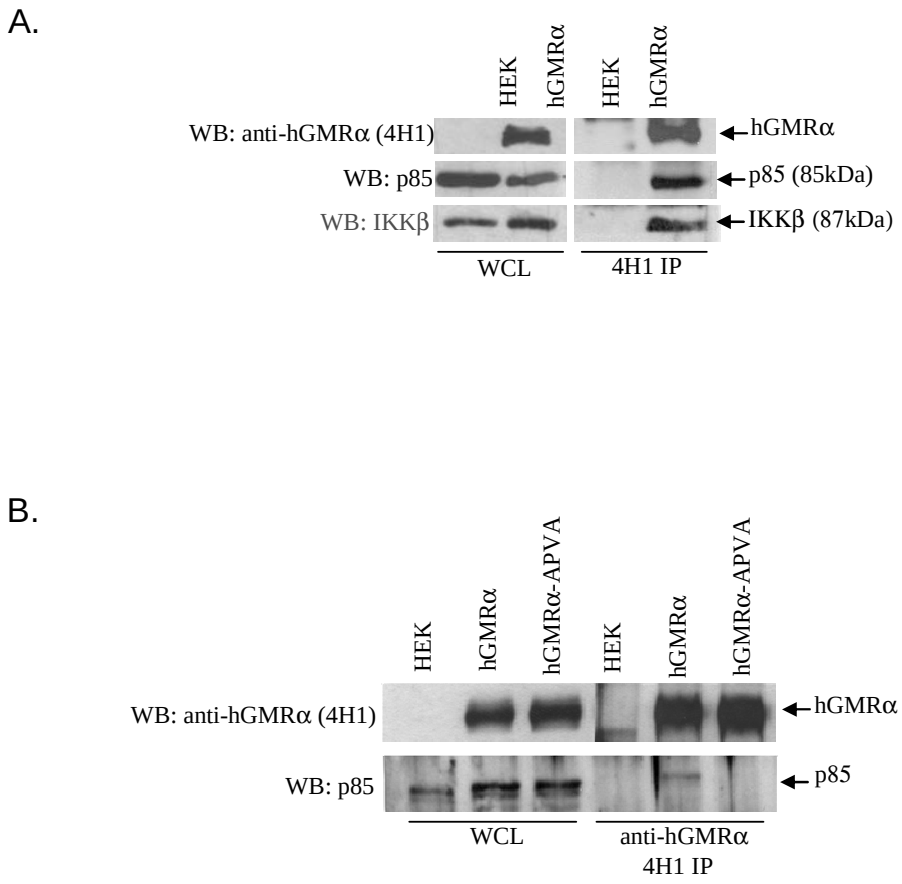


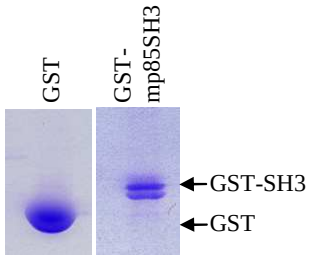
Figure 3.5 hGMRα interacts with p85 and IKKβ. A. hGMRα immunoprecipitation was performed using the 4H1 antibody and whole cell lysates (WCL) from untransfected HEK293T cells or cells transfected with pRUFpuro-hGMRα. Co-immunoprecipitation of p85 and IKKβ with hGMRα was determined using specific antibodies (see Methods 2.7.3). **B.** Abrogation of p85 binding to hGMRα by mutation of the proline-rich sequence (PPVP - APVA) within the SBP motif. Immunoprecipitation was performed as above and co-immunoprecipitated proteins were detected with anti-p85 antibodies by western blot analysis.

3.2.2.2 Nature of the interaction between p85 and hGMRα

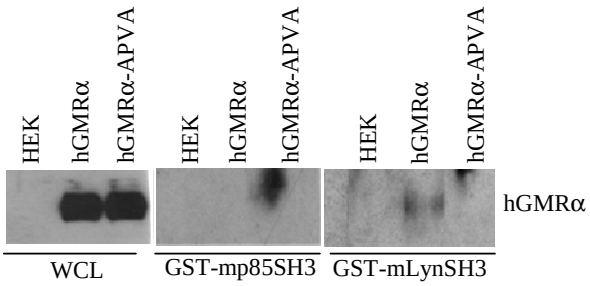
To investigate the nature of the interaction between p85 and GMRα and assess its dependence on the SH3 domain of p85, we performed GST pull-down assays using a GST fusion protein containing the SH3 domain of mouse p85 (mp85). 5μg of GST-mp85SH3 was incubated with cell lysates from transfected HEK293T cells transiently expressing hGMRα or hGMRα-APVA. The fusion proteins were prepared as previously described and bound to glutathione sepharose (Section 2.8.1) (**Figure 3.6A**). Immobilised GST-mp85SH3 was incubated with lysates from HEK293T cells transfected with hGMRα or hGMRα-APVA and pull-down assessed using western immunoblot analysis with the anti-hGMRα, 4H1, antibody. Using this assay we were unable to detect binding of mp85SH3 to hGMRα or hGMRα-APVA (**Figure 3.6B**). Lyn was used as a positive control for the pull-down assay (**Figure 3.6B**).

We also performed a fluorescence polarisation assay (Section 3.2.1.2) to address direct interaction between mp85 and a hGMRα peptide. We did not detect a significant increase in ΔmP upon addition of GST-mp85SH3 when compared to GST alone (**Figure 3.6C and D**). In the same experiment we were able to detect binding of mLyn to the hGMRα peptide. Taken together these experiments suggest that p85 is able to associate with hGMRα indirectly, presumably as a consequence of binding another molecule that interacts with the hGMRα SBP motif.

A.



B.



C.

mGMRα	mGMRα Pep	mGMRα Pep + GST-mp85SH3	mGMRα Pep + GST-mLynSH3	
98.40	105.13	102.95	104.56	mP
80.72	99.37	98.61	102.63	
89.37	85.75	95.29	114.53	
89.49	96.74	98.95	107.24	
0	**7.25**	**9.46**	**17.74**	**ΔmP**

D.

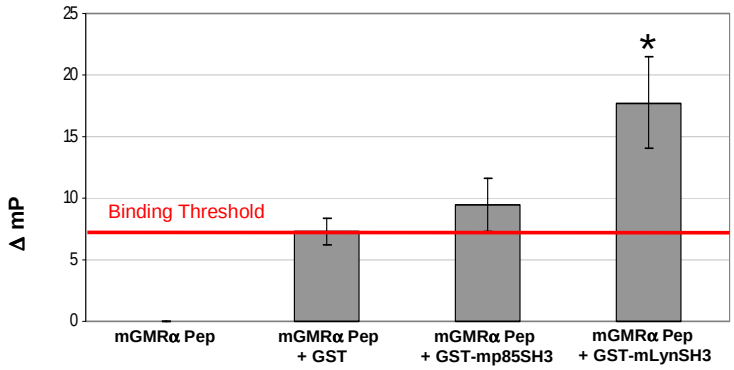


Figure 3.6 p85 does not interact with GMRα through its SH3 domain. A. GST and GST-p85SH3 fusion proteins purified and bound to glutathione sepharose were separated by SDS-PAGE and stained with Coomassie Blue. **B.** Pull-down of hGMRα using GST-mp85SH3 as bait. The Lyn-SH3 interaction with hGMRα was included as a control. Complexes were separated by SDS-PAGE and hGMRα pull-down was detected using anti-hGMRα 4H1 antibody. Binding to Lyn was used as a positive control. **C.** Fluorescence polarisation assay. Table of ΔmP values calculated for GST, mp85-, mLyn- and mSrc-GST fusions incubated with mGMRα peptide. ΔmP values were calculated by subtracting the mean mP value of the mGMRα peptide from all other samples. The ΔmP value for the mGMRα peptide alone was therefore set at 0. Graph of ΔmP values where the specific binding threshold was set at the ΔmP value of the mGMRα peptide + GST, representing non-specific binding of the peptide to GST. * represents statistical significance, $P<0.05$ for triplicate samples where n=1. Lyn and Src samples were included as controls for this experiment.

3.2.3 The SFK Activator Unc119

A potential mechanism for activation of the SFKs associated with α-subunits is suggested by the recent report demonstrating binding of the SFK-activator, Unc119 to IL5Rα (Cen, Gorska et al. 2003; Gorska, Stafford et al. 2004). Unc119 is an adapter protein found in relatively few tissues. It contains an SH2 domain in addition to a proline rich SH3 recognition motif (Cen, Gorska et al. 2003; Gorska, Stafford et al. 2004). In both of these studies, Unc119 was shown to be an activator of SFKs namely Lck, Src, Hck and Lyn and a direct interaction was identified between Lyn and Unc119 (Cen, Gorska et al. 2003; Gorska, Stafford et al. 2004). Unc119 induces the catalytic activity of these SFKs through interaction with SH2 and SH3 domains, which is increased upon IL-5 binding to its receptor (Cen, Gorska et al. 2003). Given these findings we postulated that Unc119 may act as an adaptor molecule binding to Lyn and/or the GMRα and play a role in the activation of Lyn in response to GM-CSF. As IL5Rα and GMRα both contain potential SH3-binding motifs, and Lyn is able to bind to IL5Rα and GMRα (Refer Section 3.2.1) (Adachi, Pazdrak et al. 1999; Adachi, Stafford et al. 1999), we wished to determine if Unc119 was able to interact with GMRα.

3.2.3.1 Unc119 expression in myeloid cells

Unc119 has been shown previously to be preferentially expressed in retina (Higashide and Inana 1999). However, its expression in hematopoietic cells, and more specifically myeloid cells, has only recently been studied (Cen, Gorska et al. 2003). We initially aimed to confirm the expression of *Unc119* in the myeloid/haemopoietic lineages. Human and mouse primers were designed to amplify full-length *UNC119 and Unc119* from a panel of human cell line cDNAs and a panel of mouse and human tissue cDNAs.

(Section 2.3.2). We performed PCR for *β-Actin* as a control for cDNA quality and quantity. Initially we examined the expression of human *UNC119* in a panel of human haemopoietic cell lines and confirmed expression in several myelomonocytic cell lines consistent with a role in myelopoiesis (**Figure 3.7A**). Human *UNC119* was detected in the B cell lymphoma line L428 mRNA but not in any of the other B- or T-cell line cDNAs tested (**Figure 3.7A**). We also measured the expression of *Unc119* in various human and mouse tissue cDNAs. **Figure 3.7B** shows expression of *Unc119* in mouse thymus and fetal liver and in human fetal liver cDNA (**Figure 3.7B**). *Unc119* was also clearly expressed in the FDB1 myeloid cell line and other myelomonocytic cell line including HL-60 and KG1-A cDNAs. We were unable to detect *UNC119* or *Unc119* in total human or mouse bone marrow cDNA samples.

3.2.3.2 Unc119 Interactions

The expression pattern of Unc119 above is consistent with expression in myelomonocytic cells which also express GMRα. We next aimed to investigate whether Unc119 interacts with GMRα. We examined this initially using the GST-pull-down approach described previously (Section 2.8). For this we amplified the coding region of hUnc119 from human thymus cDNA and cloned this into the pGEX2T expression vector resulting in production of GST-hUnc119 fusion protein. Purified GST-hUnc119 fusion protein was prepared as discussed in Methods (**Figure 3.8A**). As shown in **Figure 3.8B** pull-down assays confirmed the reported interaction between Unc119 and Lyn, however, we were unable to demonstrate an interaction between Unc119 and the hGMRα (**Figure 3.8B**). Given that GMRα is not able to bind the Lyn

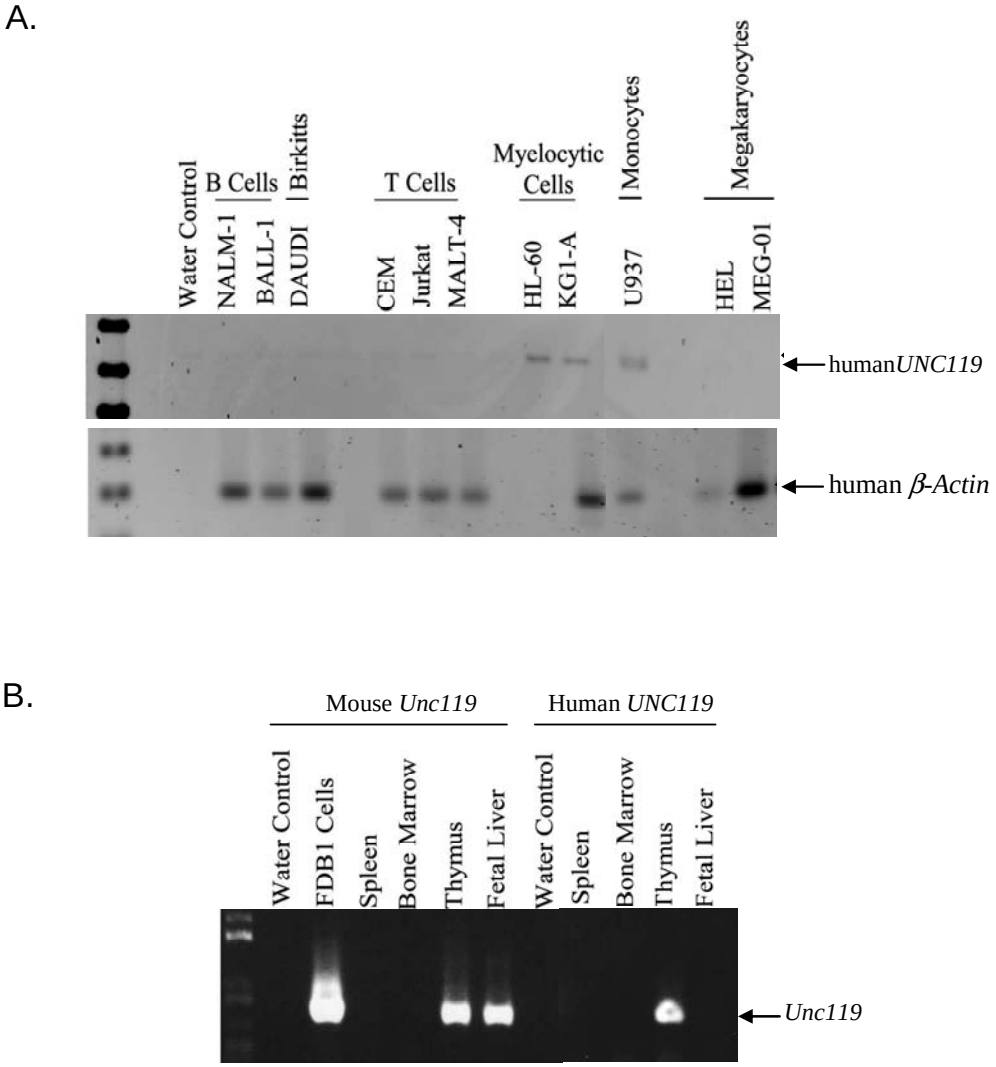


Figure 3.7 Unc119 Expression Profile. A. PCR amplification of human *UNC119* and the housekeeping gene human *β-Actin* from a human cell line cDNA panel (kindly provided by Hamish Scott, Walter and Eliza Hall. Institute). Products were separated by agarose gel electrophoresis and the gel was scanned on a fluorimager **B.** PCR amplification of *UNC119* or *Unc119* from murine FDB1 cells and various human and mouse tissue cDNAs.

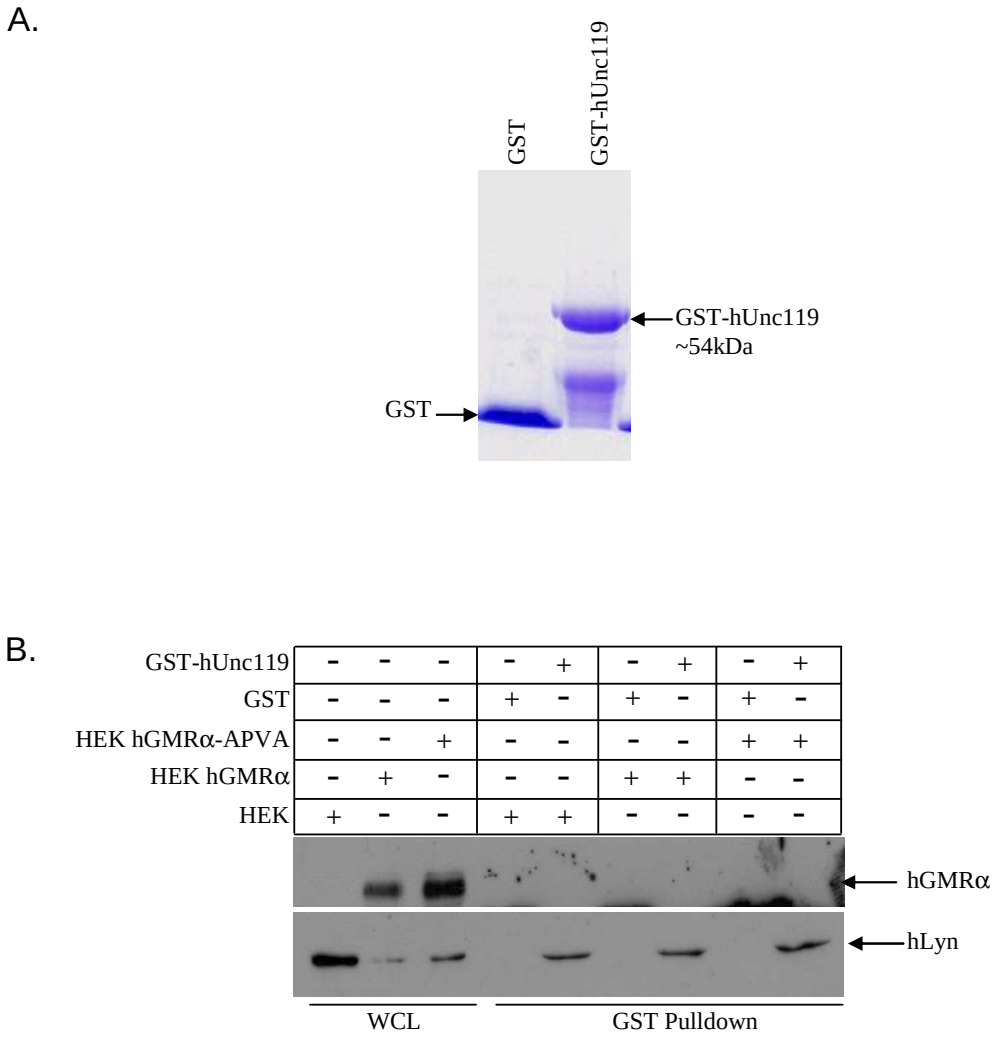


Figure 3.8 hUnc119-GST pull-downs. A. Purified GST-hUnc119 fusion protein separated by SDS-PAGE and stained with Coomassie Blue. **B.** GST pull-down assays were performed using GST-hUnc119 as bait and lysates from HEK293T, HEK293T-hGMRα or HEK293T-hGMRα-APVA cells. Anti-hLyn and anti-hGMRα 4H1 antibodies were used to detect hLyn or hGMRα respectively.

pulled-down by Unc119, we would suggest that the interaction between Lyn and GMRα and that between Unc119 and Lyn are mutually exclusive.

3.3 Discussion

All available evidence suggests that triggering of tyrosine kinases associated with the cytoplasmic portions of the cytokine receptor subunits is a critical first step in signalling. Of particular relevance is the JAK family of kinases (Rawlings, Rosler et al. 2004) which interact with the membrane proximal Box 1 motif of cytokine receptor signalling subunits. However, there is an accumulation of evidence that is consistent with JAK-independent signalling contributing to important outcomes from cytokine receptors including the $h\beta_c$ (Avalos, Parker et al. 1997; Dorsch, Fan et al. 1997; Nelson, McIntosh et al. 1997; Corey and Anderson 1999; Tsujino, Miyazaki et al. 1999; Reddy, Korapati et al. 2000; Tsujino, Di Santo et al. 2000). This suggests an important role for other tyrosine kinases. Cytokine receptors have been shown to associate with SFKs, including Lck, Fyn, Hck and Lyn (for review see Corey and Anderson 1999; Reddy, Korapati et al. 2000) and a substantial body of evidence implicates the SFKs in cytokine receptor signalling including signalling from GMR/IL3R/IL5R (Corey, Eguinoa et al. 1993; Linnekin, Howard et al. 1994; Burton, Hunter et al. 1997; Chaturvedi, Reddy et al. 1998; Adachi, Pazdrak et al. 1999; Adachi, Stafford et al. 1999; Corey and Anderson 1999; Dahl, Arai et al. 2000; Reddy, Korapati et al. 2000; Irie-Sasaki, Sasaki et al. 2001; Suh, Kim et al. 2005). The SFK, Lyn, has been implicated in signalling from IL3R/IL5R/GM-CSF receptors and Lyn is activated by IL3, IL5 and GM-CSF (Corey, Eguinoa et al. 1993; Rao and Mufson 1995; Burton 1997; Adachi, Pazdrak et al. 1999; Adachi, Stafford et al. 1999; Cen, Gorska et al. 2003; Suh, Kim et al. 2005). Lyn can

also physically associate with the $h\beta_c$ subunit and tyrosine residues in $h\beta_c$ are phosphorylated by Lyn *in vitro* (Dahl, Arai et al. 2000). In spite of this the precise role of the SFKs has been difficult to clearly establish given the dominant role of the JAK kinase signal.

Cytokine receptor alpha subunits confer ligand-binding specificity to cytokine receptors. The alpha subunit of the GM-CSF receptor contributes considerable signalling specificity to the complex and recent literature demonstrates a crucial role in signalling (Dhar-Mascareno, Chen et al. 2003; Ebner, Bandion et al. 2003; Dhar-Mascareno, Pedraza et al. 2005; Chen, Carcamo et al. 2006). We postulated that characterisation of GMRα associated events will provide important information with regard to the mechanisms mediating the receptor response.

To better define the role of GMRα in signalling we have characterised interactions of GMRα with known signalling proteins and screened a number of SH3 domains for interaction with the proline-rich region of GMRα. We focussed on the SBP motif as described by D'Andrea *et al* (D'Andrea, Sadlon et al. 2004) as this is known to be critical for signalling in response to GM-CSF and has been shown to be important for signalling in a number of other receptors in the family (D'Andrea, Sadlon et al. 2004). This proline rich sequence shows also strong homology to an SH3 recognition motif and was therefore a likely target of binding to SH3 domain containing proteins such as SFKs (D'Andrea, Sadlon et al. 2004).

This is the first report of a direct interaction between Lyn and GMRα. We substantiated the interaction using a number of different approaches including fluorescence

polarisation, immunoprecipitation and GST pull-downs, which measure both direct and indirect binding. A role for Lyn in GM-CSF signalling is consistent with a substantial body of evidence implicating SFKs in cytokine receptor signalling. In addition to the direct interaction with the GMRα reported here, there are several other lines of evidence that implicate a role for SFKs in GM-CSF signalling. Unpublished data from our laboratory has shown that GM-CSF induced growth and survival of an immature myeloid cell line, FDB1 is sensitive to the SFK inhibitors PP1 and SU66556. Additionally, signalling via a constitutively activated mutant of $h\beta_c$, FIΔ, which requires GMRα for activity (Jenkins, Le et al. 1999) is also highly sensitive to these inhibitors . While, another activated β_c mutant, V449E, which signals independently of GMRα, does not display sensitivity to the SFK inhibitors (unpublished data).

These findings are also consistent with current literature which suggests a functional role for Lyn in GM-CSF signalling. As discussed above Lyn has been implicated in signal transduction from the GM-CSF receptor, but is also known for its activation of inhibitory molecules that attenuate receptor signalling predominantly through activation of SHP-1 and SHIP-1 (Harder, Quilici et al. 2004; Hibbs and Harder 2006). A positive signalling role in GM-CSF responses is consistent with evidence that Lyn participates with JAK2 to mediate EPOR responses (Ingley, McCarthy et al. 2005). However, in macrophages, Lyn deficiency leads to enhanced sensitivity to GM-CSF and M-CSF, impaired inhibitory signalling, enhanced AKT activation and survival (Harder, Parsons et al. 2001; Baran, Tridandapani et al. 2003) suggesting that Lyn may be a negative regulator of macrophage responses to some haematopoietic growth factors. Lyn -/- mice exhibit an age-dependent myeloproliferative defect characterised by an accumulation of myeloid and erythroid progenitor cells (Harder, Quilici et al. 2004)

consistent with dysregulated receptor signalling. These mice also develop macrophage tumours with a long latency consistent with the demonstrated ability of Lyn to regulate a number of inhibitory signalling molecules (SHP-1, SHP-2, SHIP-1, PRI-B and SIRPα) in bone marrow derived macrophages (Harder, Parsons et al. 2001). Despite this tumour-suppressor activity in macrophages Lyn is frequently activated in leukaemic blasts from AML patients (Enzler, Gillessen et al. 2003) and is implicated in Bcr-Abl induction of CML (Zhan 1999). Thus Lyn is likely to be involved in several receptor responses in myeloid cells generating both positive and negative signals with the outcome varying depending on receptor, cell type, stimulus and stage of maturation. It will be important to characterise the role of the Lyn-GMRα interaction further using primary cell populations, including cells from Lyn-/- mice (Beavitt, Harder et al. 2005).

We also studied the previously reported interaction between the regulatory subunit of PI3K, p85, and GMRα using indirect and direct binding techniques. Dhar-Mascareno *et al* described this interaction and related it to a functional increase in glucose uptake demonstrating these binding events are functionally important (Dhar-Mascareno, Chen et al. 2003; Dhar-Mascareno, Pedraza et al. 2005). Although we were able to successfully co-immunoprecipitate p85 with GMRα and showed that the interaction is dependent upon an intact proline recognition site, we were unable to demonstrate a direct interaction between this molecule and GMRα using fluorescence polarisation and GST pull-down assays. A separate study reported an interaction between p85 and Cbl and showed evidence that p85 is able to associate with Lyn indirectly via this association (Dombrosky-Ferlan and Corey 1997). This interaction could potentially provide a mechanism whereby p85 may associate with GMRα (**Figure 3.9**). A role for Cbl in GM-CSF signalling is suggested by a study that found GM-CSF stimulated Cbl

tyrosine phosphorylation and association with the SH3 domain of Grb2 (Odai, Sasaki et al. 1995).

The p85 interaction with GMRα is likely to be important for activation of the PI3K pathway. The p85 regulatory subunit, has been strongly implicated in signalling from the GM-CSF and IL-3 receptors and has also been shown to associate with the β_c through the scaffolding protein 14-3-3 following serine phosphorylation of the receptor (Guthridge, Powell et al. 2006). This provides one mechanism for activation of the downstream kinase AKT which in turn phosphorylates BAD, a pro-apoptotic bcl2 homologue (Guthridge, Powell et al. 2006). In the model in **Figure 3.9** we propose that the indirect interaction between p85 and the GMRα through Lyn and Cbl provides an alternative mechanism for activation of the PI3K/AKT pathway (). Further characterisation of these interactions and pathways are required to identify specific roles in signalling outcomes from GMR.

Ebner *et al* also demonstrated a direct interaction between IKKβ and GMRα which we were able to reproduce using immunoprecipitations with lysate from hGMRα transfected HEK293T cells (Ebner, Bandion et al. 2003). Ebner *et al* showed this interaction to be direct resulting in activation of NFκB through phosphorylation and degradation of the inhibitory molecule IκB (Ebner, Bandion et al. 2003). Furthermore, there is evidence of cross-talk between the PI3K and NFκB pathways as PDK1 (a downstream kinase of AKT) has been reported to activate NFκB via IKKβ (Tanaka, Fujita et al. 2005). We have investigated the role of the p85-AKT and IKKβ-NFκB pathways in GM-CSF receptor signalling in the following chapter.

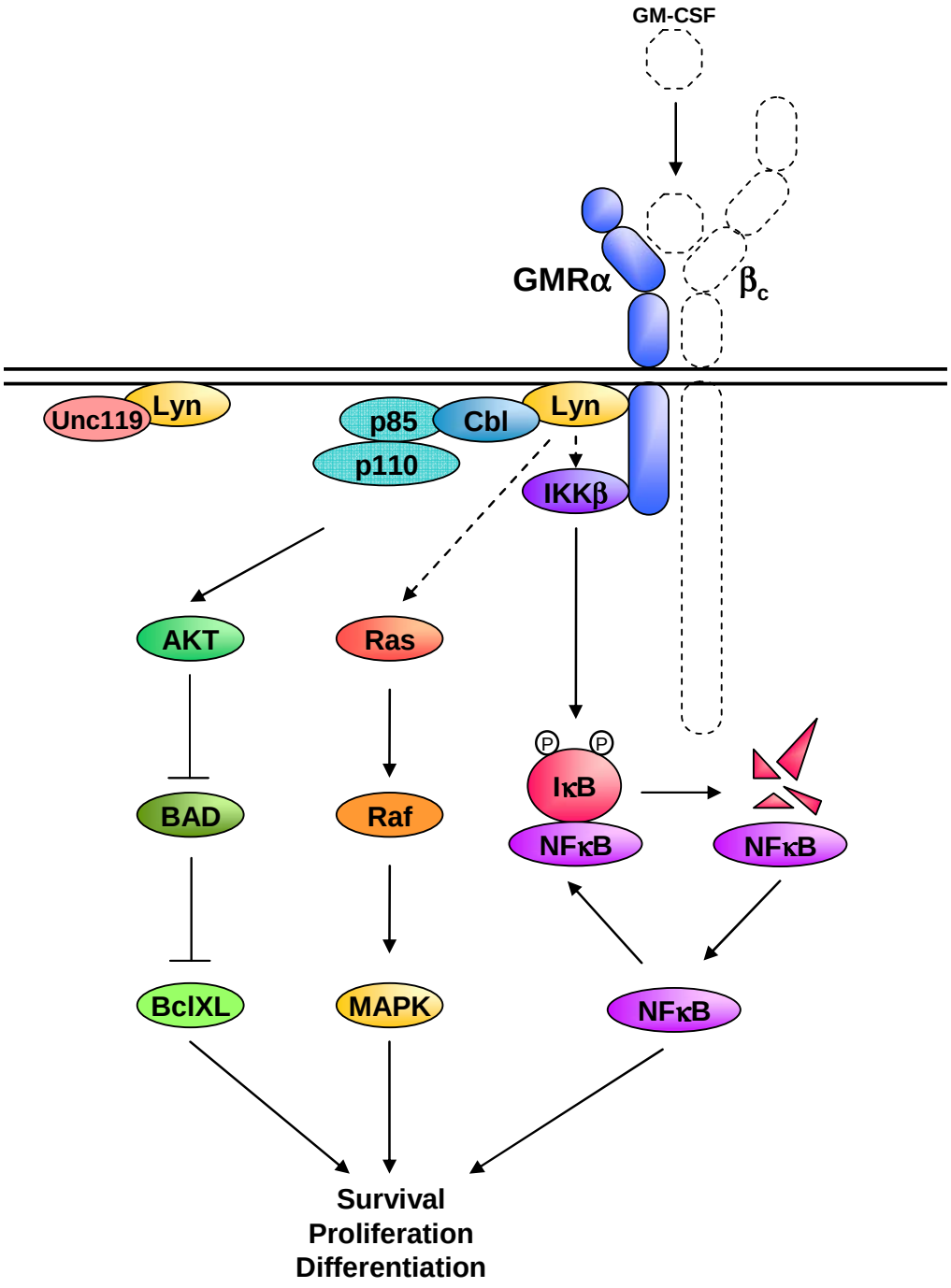


Figure 3.9 Schematic model for GMRα associated signalling and key downstream events.

Finally, we studied the role of a SFK activator, Uncll9 in signalling from GMR. This protein has been implicated in signalling from the IL-5R through direct interaction with IL-5Rα (Cen, Gorska et al. 2003). IL-5Rα and GMRα are structurally similar and both contain the SBP motif which is critical for receptor activity (D'Andrea, Sadlon et al. 2004). We postulated that Unc119 may play a role in GMR signalling through binding to GMRα. Although we were able to demonstrate expression of *UNC119/Unc119* in the haemopoietic compartment, and specifically in the myeloid lineage, we were unable to demonstrate interaction between this protein and GMRα using GST pull-down assays. We did however demonstrate an interaction between Unc119 and Lyn, as previously reported (Cen, Gorska et al. 2003). Further studies are required to establish the role of Unc119 in GMR signalling, however its association with Lyn and its role as a SFK activator suggest it may play a pivotal role.

There are both benefits and limitations to looking at binding to GMRα in isolation as we have done in this study. We have identified proteins that have the potential to interact with GMRα, however, it will be necessary to characterise these GMRα interactions in cells containing both GMRα and β_c subunits, such as the TF-1 cell line, and to determine if the interactions are dependent upon ligand interaction with the receptor.

CHAPTER 4

ACTIVATED β_c MUTANTS LINK SIGNALLING PATHWAYS TO MYELOID CELL SURVIVAL

4.1 Introduction

Many biological systems rely on the signalling cascades initiated by ligand-mediated receptor activation that in turn modulate multiple cellular responses. To understand this fundamental biological process one must examine how cell surface receptors are activated, how these receptors generate multiple intracellular signals and how these signals are integrated to affect cell behaviour.

As with most cytokine and growth factor receptors, multiple signalling events are activated by the receptor complexes for GM-CSF, IL-3 and IL-5. Activation of the Ras-Raf-MAPK, JAK-STAT, PI3K-AKT pathways have been well characterised (for review see de Groot, Coffer et al. 1998). More recently it has become evident that these receptor complexes also activate NF-κB which has been coupled to activation of PI3K and STAT5A/B (Nakamura, Ouchida et al. 2002; Guthridge, Barry et al. 2004). All available evidence suggests that triggering of tyrosine kinases associated with the cytoplasmic portions of the cytokine receptor subunits is a critical first step in signalling. Of particular relevance is the JAK family of kinases (Rawlings, Rosler et al. 2004) which interact with Box1 on cytokine receptor signalling subunits. However, there is an accumulation of evidence that is consistent with JAK-independent signalling contributing to important outcomes from cytokine receptors including hβ_c (Avalos, Parker et al. 1997; Dorsch, Fan et al. 1997; Nelson, McIntosh et al. 1997; Corey and Anderson 1999; Tsujino, Miyazaki et al. 1999; Reddy, Korapati et al. 2000; Tsujino, Di Santo et al. 2000), suggesting important roles for other tyrosine kinases including SFKs. A substantial body of evidence implicates SFKs in cytokine receptor signalling from GMR/IL-3R/IL-5R (Corey, Eguinoa et al. 1993; Linnekin, Howard et al. 1994;

Appleby, Kerner et al. 1995; Burton, Hunter et al. 1997; Chaturvedi, Reddy et al. 1998; Adachi, Pazdrak et al. 1999; Adachi, Stafford et al. 1999; Corey and Anderson 1999; Dahl, Arai et al. 2000; Reddy, Korapati et al. 2000; Irie-Sasaki, Sasaki et al. 2001; Suh, Kim et al. 2005), however the role of SFKs has been difficult to establish given the dominant role of the JAK signal.

While much is known about the signalling events activated by cytokine receptor complexes the redundancy of signalling has made it difficult to link individual pathways to specific outcomes such as cell survival, proliferation and differentiation. Activation of one pathway often feeds into another resulting in a networked signalling response the final outcome of which is influenced by the cell context (for example cell type, stage of differentiation, transformation and microenvironment) (Blake, Jenkins et al. 2002; Ishihara and Hirano 2002; Guthridge, Powell et al. 2006). It is clear that many signalling pathways converge on the same effector molecules and some biological effects can be mediated by multiple effectors. Thus, the extensive signalling redundancy and cross-talk is problematic with regard to dissecting the contribution of signalling events to various cellular outcomes (survival, proliferation and differentiation).

We have made extensive use of a unique myeloid cell line model, FDB1 (see Section 4.2.1) (McCormack and Gonda 2000), that is able to undergo mitogenic or differentiation responses and is amenable to generation of adequate numbers of synchronously behaving cells for biochemical and molecular analysis. We have also utilised a panel of constitutively active hβ_c mutants that display a reduced range of signalling events to address the issue of redundancy in signalling from the GM-CSF

receptor specifically (Gonda and D'Andrea 1997; D'Andrea and Gonda 2000). These mutants fall into two classes that display different *in vivo* properties and leukaemic activity depending on the site of the mutation (extracellular, eg. FIΔ, or transmembrane domain, eg V449E) (Brown, Peters et al. 2004).

In this chapter we characterise the differential signalling displayed by the two classes of activated mutants allowing us to associate signalling events with the alternative cellular outcomes generated by these mutants *in vivo*. In doing so we identify pathways that contribute to the leukaemogenic phenotype exhibited by the V449E mutation and also link specific signalling events to the granulocyte-macrophage differentiation induced by the FIΔ mutation. We make use of pharmacological inhibitors targeting various pathways to further assess the involvement of these specific signalling events in the control of distinct cellular processes. Bearing in mind the varying specificities of small molecule inhibitors we utilised two inhibitors for each target to account for potential off target effects. **Table 4** summarises the inhibitors used in this study and their relative specificities for their intended targets.

Table 4. Specificity and mechanism of action of some commonly used inhibitors

Inhibitor Name	Specificity	Mechanism of Action	References
AG490	JAK2**,** JAK1, JAK3	Protein tyrosine kinase Inhibitor	(Dadi, Ke et al. 1994; Sharfe, Dadi et al. 1995; Meydan, Grunberger et al. 1996)
JAK2 Inhibitor II	JAK2	Inhibits JAK2 autophosphorylation	(Sandberg, Ma et al. 2005)
U0126	MEK1, MEK2, MEK5, ERK1, ERK2, ERK5	Binds to MEK1 and uppresses its activation through dephosphorylation	(DeSilva, Jones et al. 1998; Kamakura, Moriguchi et al. 1999; Davies, Reddy et al. 2000)
PD98059	MEK1, MEK5, ERK1, ERK2 and ERK5	Binds to MEK1 preventing its activation	(Kamakura, Moriguchi et al. 1999; Davies, Reddy et al. 2000)
Wortmannin	PI3K**,** smooth muscle myosin light chain kinase (SmMLCK)	Selective non-competitive PI3K inhibitor	(Nakanishi, Kakita et al. 1992; Davies, Reddy et al. 2000)
LY294002	PI3K**,** Casein Kinase II (CK2)	Selective non-competitive PI3K inhibitor	(Vlahos, Matter et al. 1994; Davies, Reddy et al. 2000)

4.2 Results

4.2.1 Validation of the FDB1 Cell-Line Model

Our laboratory has previously characterised an immature myeloid cell line, FDB1 (McCormack and Gonda 2000; Brown, Peters et al. 2004). This cell line is strictly factor-dependent for survival, and will proliferate continuously in the presence of mIL-3 with a minimal amount of spontaneous differentiation. In the presence of mGM-CSF, FDB1 cells undergo limited proliferation and terminally differentiate to generate neutrophils and monocytes. There are a number of key advantages of this cell line over other myeloid cell lines and primary cells for dissecting the events involved in proliferation and differentiation; firstly, in contrast to primary progenitors, proliferation and differentiation are uncoupled in FDB1 cells greatly facilitating discrimination of signalling events involved in each process. Secondly, FDB1 is not leukaemia derived; it

has a normal karyotype and has not acquired the many co-operating lesions associated with disease progression and is therefore more likely to represent normal receptor signalling (McCormack and Gonda 2000).

Constitutively activated mutants of $h\beta_c$ are able to recapitulate the effects of GM-CSF and IL-3 on FDB1 cells (McCormack and Gonda 2000). There is good correspondence between the outcomes of signalling by these activated GMR mutants in FDB1 cells and the properties associated with these mutants *in vivo* (McCormack and Gonda 2000). For example, the transmembrane mutation V449E induces a myeloid leukaemia *in vivo* and can support the generation of immature myeloid cell lines *in vitro* consistent with its ability to drive cytokine-independent proliferation and maintain the block in differentiation of the FDB1 cell line even in the presence of mGM-CSF (McCormack and Gonda 1999). The 37 amino acid duplication in the extracellular domain of $h\beta_c$ termed FIΔ, induces cytokine-independent colony formation and leads to a myeloproliferative disease *in vivo* (McCormack and Gonda 1999). This is consistent with the ability of this mutant to provide constitutive signals supporting survival and differentiation of FDB1 cells.

Prior to commencing extensive biochemical analyses of the FDB1 cell populations expressing $h\beta_c$ mutants, we characterised the properties of the cell populations. The expression of the $h\beta_c$ mutants in the FDB1 cells was maintained throughout experimentation using puromycin antibiotic selection and was monitored using flow cytometry with an anti-FLAG™ M2 antibody that recognises the FLAG epitope on the C-terminus of the mutant $h\beta_c$ constructs. **Figure 4.1A** shows representative histograms

measuring expression of the mutant receptors. In all the experiments to follow, expression of the mutant receptors was monitored by flow cytometry and maintained above 85% in puromycin.

The morphology of the FDB1 cells cultured in mIL-3 or mGM-CSF was determined by cytocentrifugation of cells onto microscope slides followed by May-Grunwald-Giemsa staining. **Figure 4.1B** demonstrates the promyelocyte morphology of the FDB1 cells when maintained in mIL-3. Differentiation to mature granulocytes and macrophages occurs over a 5 day period in cultures supplemented with GM-CSF (**Figure 4.1B**). Analysis of the V449E cell population confirmed the ability of this mutant to induce factor-independent survival and proliferation and to block differentiation. FDB1 V449E cells displayed promyelocyte characteristics with a high nucleus to cytoplasm ratio (**Figure 4.1B**). The morphology of these cells is similar to that of the parental FDB1 cells cultured in mIL-3. As previously reported, mGM-CSF was not able to induce FDB1 V449E cells to differentiate (data not shown) (McCormack and Gonda 2000). Conversely, analysis of the FDB1 cell population expressing the extracellular FIΔ mutant confirmed that this mutant induces the factor-independent differentiation of FDB1 cells as previously reported. The morphology of these FIΔ cells at day 5 is similar to that of the parental FDB1 cells cultured in mGM-CSF with approximately equal numbers of mature granulocytes and macrophages (**Figure 4.1B**) (McCormack and Gonda 2000).

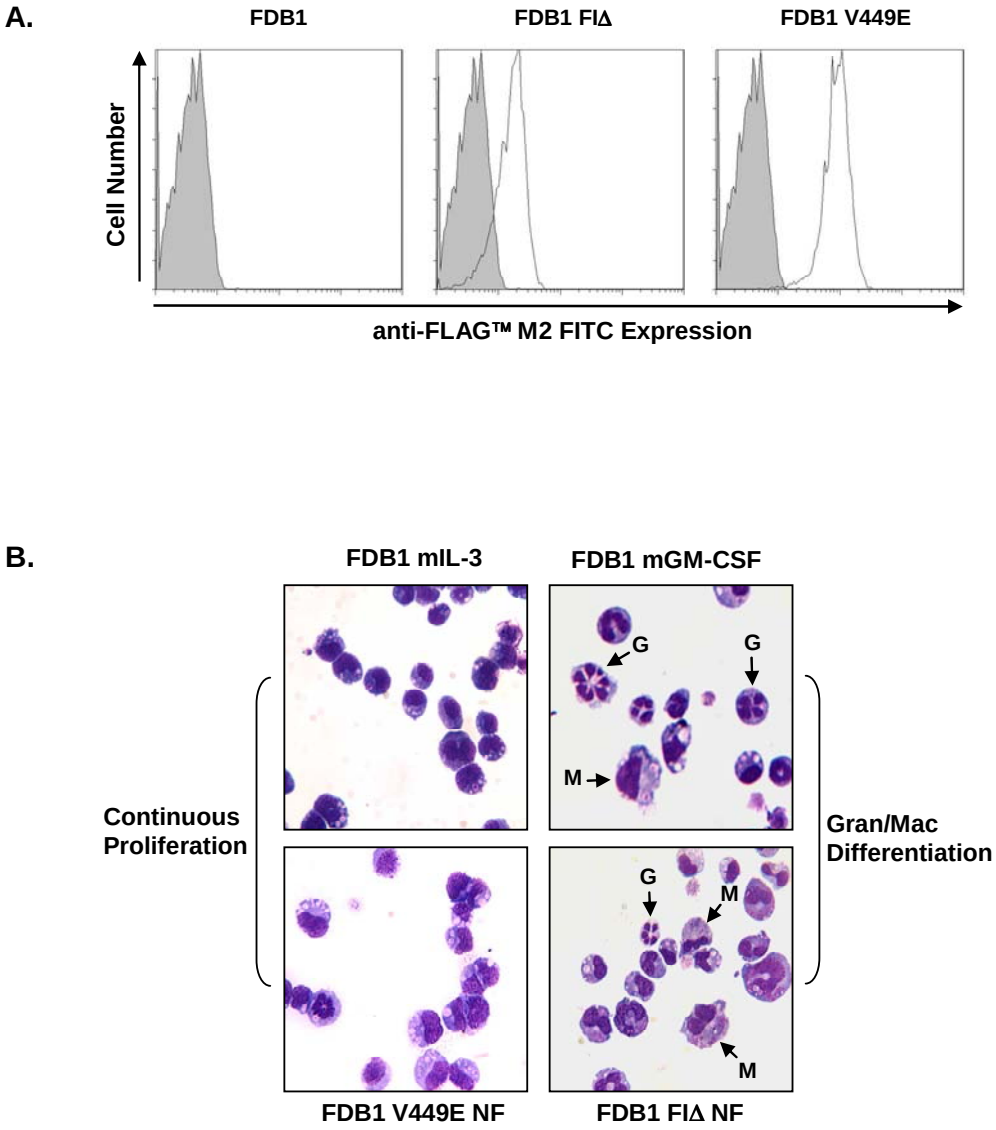


Figure 4.1 Characterisation of FDB1 Cell Populations. A. Expression of the hβ_c mutants FIΔ and V449E was measured by flow cytometry in FDB1 cells stained with anti-FLAG™ M2 antibody directly conjugated to FITC. Grey histograms represent parental FDB1 cells stained with M2 and open histograms show staining of cells expressing hβ_c mutants. **B.** FDB1 cells cultured in mIL-3 and mGM-CSF and FDB1 FIΔ and FDB1 V449E populations grown without factor for 5 days were cytocentrifuged and stained with May-Grunwald-Giemsa. G and M represent granulocytes and macrophages respectively.

4.2.2 Differential Signalling Capacity of Constitutive FIΔ and V449E Mutants in FDB1 Cells

JAK2 is activated in response to GM-CSF, IL-3 and IL-5 binding to its receptor and is thought to be the primary initiating event resulting in receptor activation and phosphorylation (Sakamaki, Miyajima et al. 1992; Watanabe, Itoh et al. 1996). The JAK2-STAT5 signalling cascade has been studied extensively in various systems and results in altered transcription of a variety of genes including, *Pim-1*, *Oncostatin-M*, *Id-1*, *Bcl2* and Bcl_{XL}, involved in the cell cycle, survival, proliferation and differentiation (Mui, Wakao et al. 1996; Guthridge, Stomski et al. 1998). p44/42 MAPK is another key kinase which is a member of the Ras-Raf-MAPK signalling cascade and its activation occurs downstream of JAK2 leading to survival and proliferation (Jenkins, Blake et al. 1998; Dijkers, van Dijk et al. 1999). To characterise signalling events induced by the $h\beta_c$ mutants in FDB1 cells we investigated whether JAK2, STAT5 and p44/42 MAPK activation are integral to the properties of the FIΔ and V449E mutants.

We first assessed the phosphorylation of JAK2, STAT5A/B and p44/42 MAPK in parental FDB1 cells, and cells expressing the constitutively activated FIΔ or V449E mutations. FDB1 cell populations were starved of mIL-3 for 16 hours to reduce the level of signalling via murine receptors, and a fraction of each population was stimulated with a 5 minute pulse of mIL-3 or mGM-CSF as a positive control. Western immunoblot analysis was carried out on the stimulated and unstimulated populations to determine the phosphorylation status of JAK2 and its downstream effector STAT5A/B as well as p44/42 MAPK (**Figure 4.2A**).

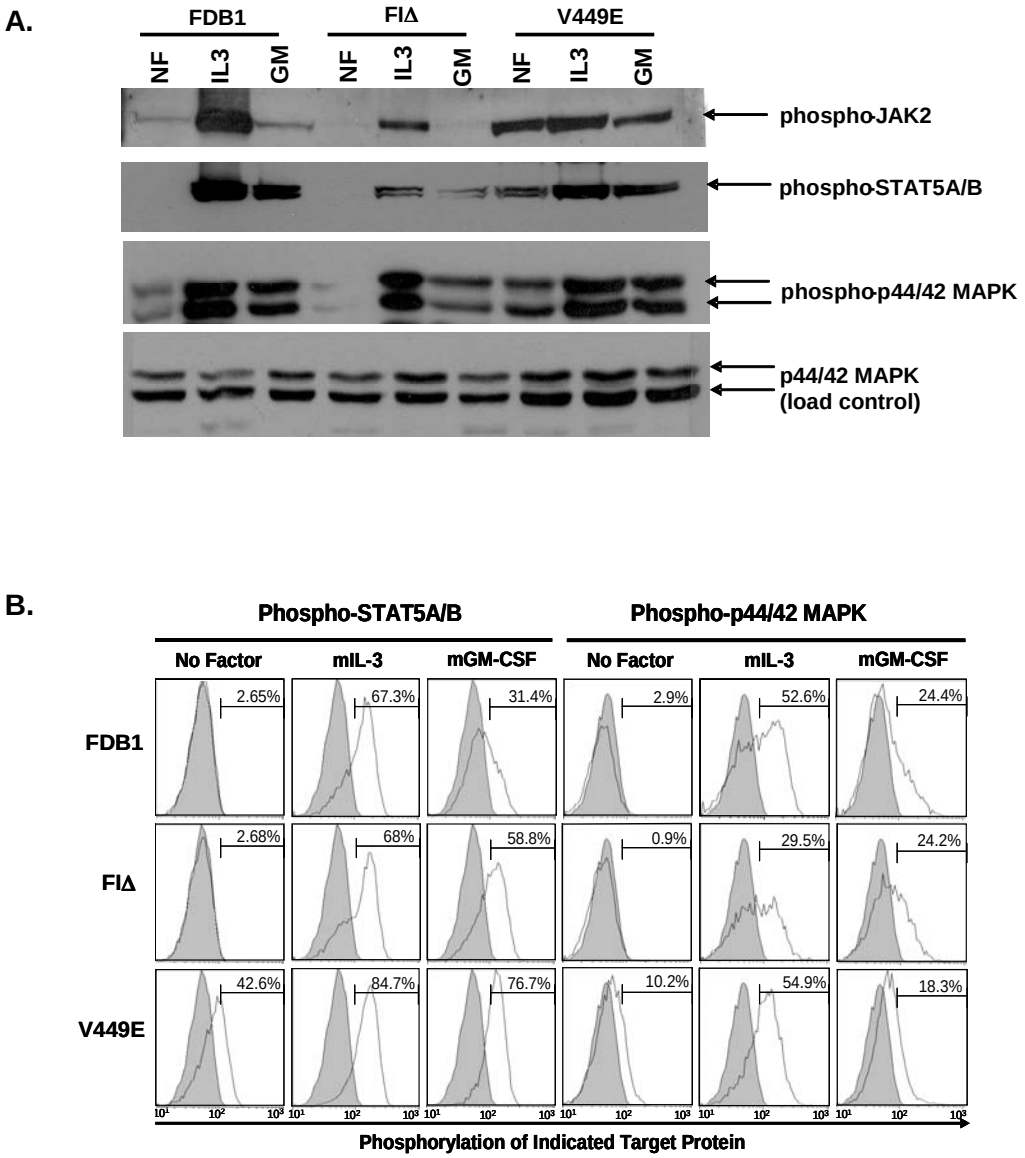


Figure 4.2 Signalling properties of the hβ_c mutants, FIΔ and V449E. A. Murine FDB1 cells expressing the hβ_c mutants, FIΔ or V449E, and parental cells were starved for 16h and subsequently stimulated for 5 min with either mIL-3 or mGM-CSF. Cells were then lysed, separated by SDS-PAGE, and probed with phospho-specific JAK2, STAT5A/B and p44/42 MAPK antibodies. p44/42 MAPK was used as a loading control. **B.** Following starvation and stimulation cells were fixed with formaldehyde, permeabilised in methanol, then stained with phospho-specific STAT5A/B and p44/42 MAPK antibodies, followed by a secondary stain with a FITC conjugated antibody. Phosphorylation was measured in each cell population by analysing the intensity of FITC fluorescence using flow cytometry. Grey histograms represent isotype matched antibody controls and open histograms represent phospho-specific antibody staining.

Following overnight starvation the parental FDB1 cells displayed a reduction in phosphorylation of JAK2 and STAT5A/B (**Figure 4.2A**). Following stimulation with mIL-3 and mGM-CSF, FDB1 cells showed a rapid increase in JAK2 and STAT5A/B phosphorylation, consistent with signalling through murine IL-3 and GM-CSF receptors (**Figure 4.2A**). In the absence of growth factor, expression of the V449E mutant resulted in constitutive JAK2 and STAT5A/B activation as measured by their phosphorylation using phospho-specific antibodies (**Figure 4.2A**). FDB1 V449E cells also displayed constitutive activation p44/42 MAPK.

Surprisingly, FDB1 cells expressing the FIΔ mutation did not display phosphorylation of either JAK2 or STAT5A/B in the absence of growth factor, raising the possibility that the survival and differentiation response of this mutant is JAK2-independent (**Figure 4.2A**). Phosphorylation of JAK2 and STAT5A/B was observed in FDB1 FIΔ cells stimulated with mIL-3 or mGM-CSF similar to the parental cells. Activation of a JAK2-independent pathway by this mutant is consistent with our published analysis of serial truncations of this mutant which indicate that primary signalling is generated distal to the Box 1 region which is thought to mediate binding of JAK2 to the $h\beta_c$ (Brown, Peters et al. 2004). However we cannot rule out that our assays are not sensitive enough to detect low levels of JAK2 activity or that FIΔ may activate another JAK family member. Although JAK2 is the predominant JAK activated by GMR, JAK1 and TYK2 can be activated under some circumstances (de Groot, Coffer et al. 1998).

In multiple experiments we also did not observe constitutive phosphorylation of p44/42 MAPK in FDB1 FIΔ cells (**Figure 4.2A**). Once again, stimulation with mIL-3 and

mGM-CSF resulted in phosphorylation of p44/42 MAPK in these cells through murine receptor signalling (**Figure 4.2A**).

Levels of phosphorylation of STAT5A/B and p44/42 MAPK were also assessed using a more sensitive intracellular single cell phospho-profiling approach (Krutzik and Nolan 2003; Irish, Hovland et al. 2004) to validate the immunoblot analyses above. Such single cell data provides additional information enabling us to determine whether the increase/decrease in phosphorylation reflects increased phosphorylation per cell or an increase in the number of cells responding.

Following stimulation, cells were fixed in formaldehyde, methanol permeabilised and stained with phospho-p44/42 MAPK and/or phospho-STAT5A/B antibodies. Appropriate FITC conjugated secondary antibody was used for detection (**Figure 4.2B**). STAT5A/B phosphorylation in these experiments was used as an indirect measure of JAK2 phosphorylation as there were no commercially available phospho-JAK2 antibodies suitable for use in flow cytometry. Constitutive phosphorylation of both STAT5A/B and p44/42 MAPK was once again readily observed by this method in factor-starved FDB1 V449E cells consistent with immunoblot analyses (**Figure 4.2B**). We did not detect constitutive phosphorylation of either STAT5A/B or p44/42 MAPK in FIΔ, or parental FDB1 cells, upon removal of factor also in agreement with previous biochemical analyses (**Figure 4.1B**). All FDB1 populations displayed phosphorylation of STAT5A/B and p44/42 MAPK following 5 minute stimulation with either mIL-3 or mGM-CSF. However, distinct cell populations are present following stimulation of parental FDB1 and FDB1 FIΔ cells, suggesting that there is a heterogeneous response to the cytokine stimulus. The flow cytometry also indicates that the weak GM-CSF

response of FDB1 and FDB1 FIΔ cells (seen in **Figure 4.2A**) is associated with a small sub-population of the cells responding to this cytokine. It is possible that there is a lag in the response to stimulation with GM-CSF and a longer stimulation time may increase the proportion of cells responding to GM-CSF. Additionally, the V449E expressing population did not display this heterogeneous response, consistent with the constitutive activation of p44/42 MAPK and STAT5A/B. Mean fluorescence intensity (MFI) values from this analysis were also tabulated from three independent experiments (**Appendix C**).

Thus, while the V449E mutant clearly induces activation of JAK2 and its downstream pathways via STAT5 and p44/42 MAPK we could not detect JAK2, STAT5A/B or p44/42 MAPK activation from the $h\beta_c$ mutant, FIΔ. The identification of a mutant which signals predominantly through JAK2-independent mechanisms, provides a useful non-redundant system in which to delineate signalling and biological outcomes associated with JAK2-independent pathways from cytokine receptors.

4.2.3 FIΔ Constitutively Activates AKT and NFκB Pathways

Given that we could not detect JAK2, STAT5A/B or p44/42 MAPK activation in cells responding to the FIΔ mutant we next wished to identify specific signalling pathways, associated with signalling from this mutant, that may contribute to its ability to drive factor-independent survival and differentiation. FIΔ has been shown previously to have an absolute requirement for the GMRα for function (Jenkins, Le et al. 1999). We therefore predicted that a GMRα-associated signalling event may be responsible for the survival signals generated by this mutant. Association with GMRα has been reported

for the regulatory subunit of PI3K, p85, and for IKKβ suggesting that the GMRα-FIΔ complex may induce signalling via the PI3K-AKT and/or IκB-NFκB pathways. We also confirmed these associations in our system (Chapter **Error! Reference source not found.**). We therefore assessed FIΔ and V449E induced phosphorylation of AKT, a downstream effector of p85; and IκB, a signalling molecule involved in activation of NFκB survival pathways.

Whole cell lysates, starved and stimulated with mIL-3 or mGM-CSF were immunoblotted with phospho-AKT and phospho-IκB antibodies to detect signalling via AKT and NFκB pathways. **Figure 4.3** shows that, in the absence of growth factor, FIΔ induces robust phosphorylation of AKT and IκB contrasting with the V449E mutant which leads to much lower level IκB phosphorylation and no detectable AKT phosphorylation. Both of these pathways were activated in parental FDB1 cells and in cells expressing either of the two mutants following stimulation with mIL-3 or mGM-CSF.

4.2.4 JAK2 and MAPK Activity are Required for V449E Survival Signals while FIΔ Survival is Mediated by AKT/PI3K

To link the biochemical analyses above to biological responses in FDB1 cells and to identify the relative contribution of signalling pathways to factor-independent cell survival, we performed viability assays with varying concentrations of the PI3K inhibitors, Wortmannin and LY294002; MEK inhibitors, U0126 and PD98059; and the

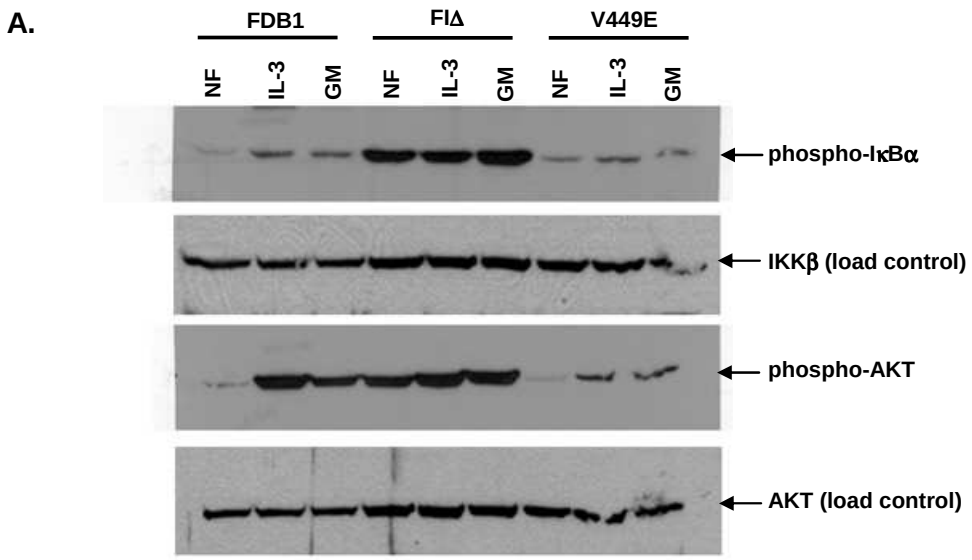


Figure 4.3 FIΔ constitutively activates IκB and AKT. A. Murine FDB1 cells expressing the hβ_c mutants, FIΔ or V449E, or parental FDB1 cells were starved for 16h and subsequently stimulated for 5 min with either mIL-3 or mGM-CSF. Cells lysates were separated by SDS PAGE and probed with phospho-specific IκB and AKT antibodies. IKKβ and AKT were used as a loading control.

JAK inhibitors, AG490 and JAK2 Inhibitor II. A 24 hour time point was chosen as parental FDB1 cells undergo rapid apoptosis following growth factor removal. mIL-3 or mGM-CSF stimulation was also used as a positive control as the transient activation of endogenous murine receptors with cytokines allows a rapid induction of multiple signalling pathways allowing easy detection of phosphorylation events by western immunoblot analysis.

4.2.4.1 Inhibition of JAK2 using Pharmacological Inhibitors

To investigate further the requirement for JAK2 in signalling from the FIΔ mutant we used two pharmacological inhibitors targeting JAK2, AG490 and JAK2 Inhibitor II (Refer to **Table 4** for inhibitor specificity information). We first examined the effect of the AG490 on the survival of FDB1 cells expressing the FIΔ and V449E mutations. An AG490 dose response was carried out using concentrations between 10μM and 50μM. Concentrations over 50μM were toxic to the FDB1 cell line (**Figure 4.4**). At 25μM, AG490 resulted in significant reduction in the levels of phospho-JAK2 and phospho-STAT5A/B, in parental FDB1 cells in mIL-3, as detected by western immunoblot analysis (**Appendix D**). Cells were treated with increasing concentrations of AG490 for 24hrs and viable cells scored by trypan blue exclusion. Parental FDB1 cells displayed limited sensitivity to AG490 at low doses and showed a significant reduction in viability at the highest dose, 50μM (Figure 4.4**A**). FIΔ cells showed limited sensitivity to AG490, only showing a significant decrease in viability at the highest concentration, 50μM,. consistent with FIΔ utilising JAK2-independent pathways for viability (Figure 4.4**B**). In contrast, viability of FDB1 V449E cells was significantly reduced upon treatment with AG490 at all concentrations, in line with constitutive JAK2 activation,

suggesting the factor-independent survival signal from this mutant is predominantly attributed to the JAK2 signalling cascade (n=3, $p< 0.01$) (Figure 4.4**C**). At least one pathway downstream of JAK2 which mediates survival signalling is the MAPK pathway. The role of this pathway in survival is dicussed further in Section 4.3. The effect of AG490 on survival of all cells in mIL-3 was minimal, except at the highest dose where off target effects may be occurring. This is consistent with generation of multiple redundant survival signals in the FDB1 cells in response to growth factor (Figure 4.4).

To confirm the reduced dependence of FIΔ for JAK2 we used a recently described specific JAK2 inhibitor, JAK2 Inhibitor II (**Table 4**) (Sandberg, Ma et al. 2005). Cell viability was determined by 7AAD staining. FDB1 cells in mIL-3 displayed some sensitivity to this inhibitor at all concentrations, with a maximal reduction (25%) in viability at the highest concentration, 30μM (**Figure 4.5A**). In the absence of growth factor the FIΔ cells displayed a dose-dependent decrease in viability and were 50% viable at 30μM JAK2 Inhibitor II at 24hrs (**Figure 4.5B**). However, the viability of FDB1 V449E cells dropped much more dramatically to approximately 13% at the same concentration (**Figure 4.5C**). As predicted the viability of all cells in mIL-3 showed limited sensitivity to treatment with JAK2 inhibitor II consistent with additional overlapping signals generated by the murine receptors (**Figure 4.5**). Interestingly, FDB1 V449E cells cultured in mIL-3 were more sensitive to JAK2 Inhibitor II than the other cell populations suggesting that the presence of the V449E mutant is affecting survival signalling by the murine IL-3 complex. The mechanism of this downregulation remains unclear.

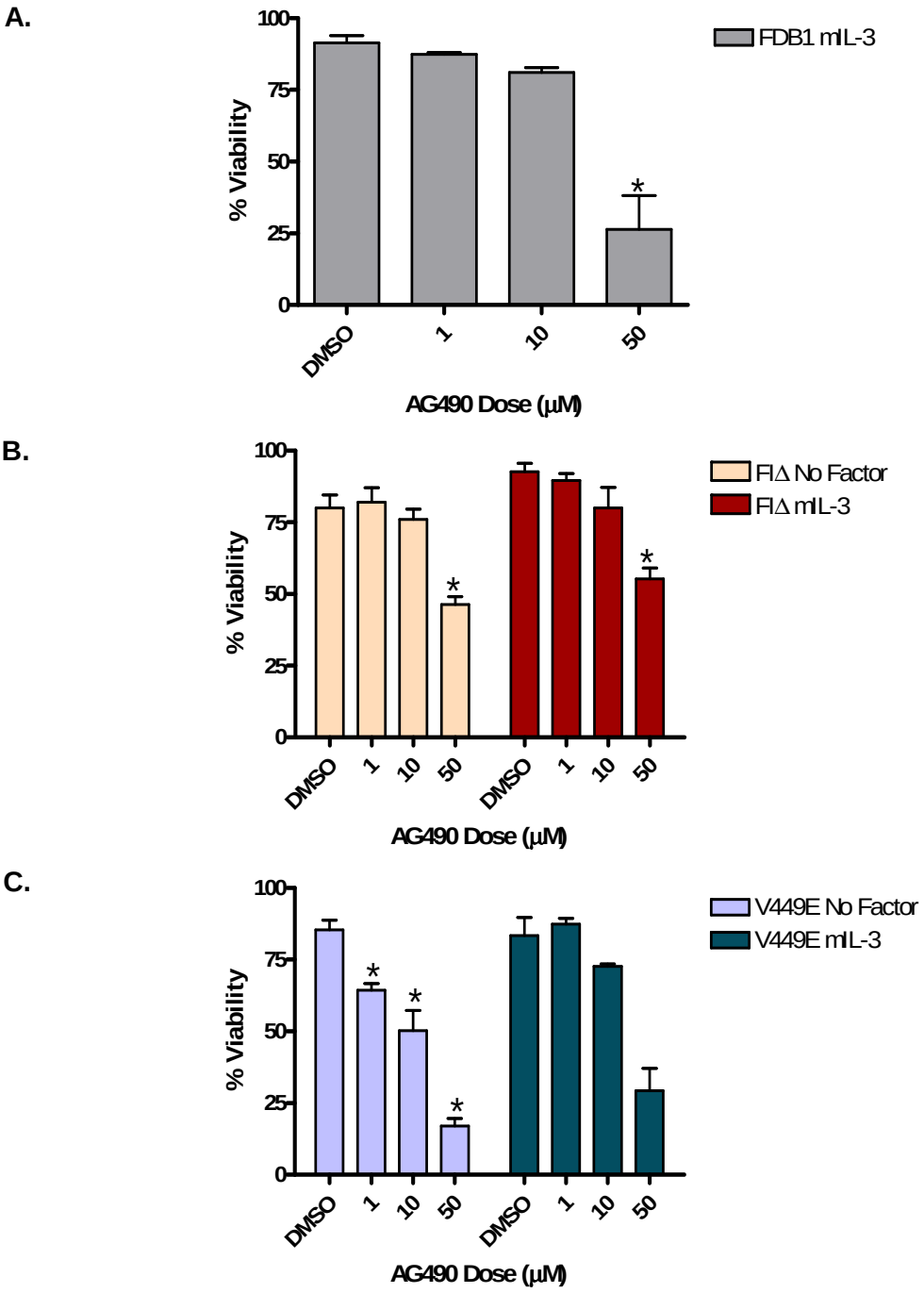


Figure 4.4 Sensitivity of FDB1 V449E to the JAK Inhibitor AG490. FDB1 parental (A), FIΔ (B) and V449E (C) cells were starved of factor and varying concentrations of AG490 were added for 24hrs. DMSO was used as a vehicle control for these experiments. Percentage viability was determined using trypan blue exclusion. Error bars represent SEM. * represents statistical significance compared to DMSO where $p<0.01$ (n=3).

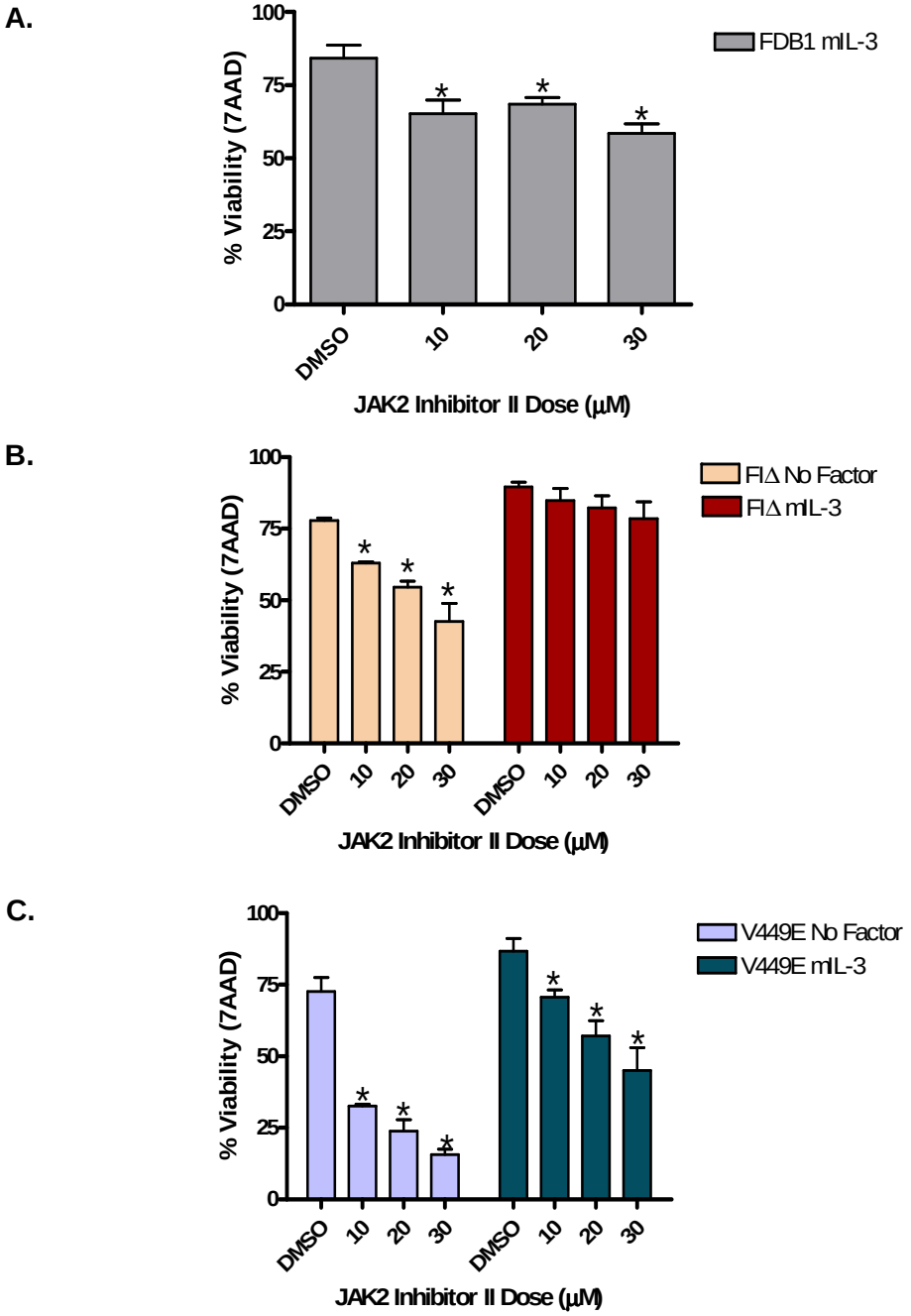


Figure 4.5. Sensitivity of FDB1 V449E to the JAK2 Inhibitor JAK2 Inhibitor II. Parental FDB1 cells (A), or cells expressing FIΔ (B) and V449E (C), were starved of factor and varying concentrations of JAK2 Inhibitor II were added for 24hrs. DMSO was used as a control for these experiments. Percentage viability was determined using 7AAD staining and flow cytometric analysis. Error bars represent SEM. * represents statistical significance compared to DMSO where $P<0.01$ (n=3).

Thus, V449E cells display higher sensitivity to two independent JAK2 inhibitors when compared to cells expressing the FIΔ mutant. The differing sensitivities of the hβ_c mutants to the JAK2 Inhibitor II and AG490 are consistent with predominant survival signalling being mediated by JAK2-dependent pathways in V449E and via JAK2-independent signalling in FIΔ. The fact that FIΔ displays some sensitivity to JAK2 Inhibitor II suggests that JAK2 contributes to survival despite the absence of detectable phosphorylation in FIΔ. This analysis confirms that the FIΔ mutant will be a useful tool for dissecting the role of JAK-independent pathways in survival.

4.2.4.2 Inhibition of MAPK Signalling Using MEK Inhibitors

We next wished to establish the relative contribution of the p44/42 MAPK pathway to survival of FDB1 cells expressing the FIΔ or V449E activated mutants. The V449E mutant constitutively activates the p44/42 MAPK pathway as determined by constitutive phosphorylation of p44/42 MAPK in FDB1 cells (**Figure 4.2**). MEK kinases lie upstream of MAPK (Platanias 2003) and inhibition of MEK1 results in attenuation of p44/42 MAPK activity (Kamakura, Moriguchi et al. 1999). Western immunoblot analysis was carried out on parental FDB1 cells cultured in varying concentrations of the MEK inhibitors, U0126 (or control U0124) or PD98059 (or DMSO control) to determine the appropriate concentration for viability assays (**Appendix D**). U0124 is an inactive analogue of U0126 (DeSilva, Jones et al. 1998) and was used as a negative control for these experiments. For the viability assays we chose concentrations of 25μM U0126 and 50μM PD98059 to ensure complete inhibition of p44/42 MAPK activity.

To determine the relative sensitivity to these inhibitors we measured the viability of parental FDB1, FDB1 FIΔ and FDB1 V449E cells following 24hr incubation with 25μM U0126 and 50μM PD98059. MEK inhibition did not affect the viability of the parental FDB1 cells in IL-3 (**Figure 4.6A**). Additionally, the viability of FDB1 FIΔ cell populations remained unaffected with both MEK inhibitors at 24hrs without factor and in mIL-3 (**Figure 4.6B**). There was a significant decrease in viability for V449E expressing cells upon treatment with both U0126 and PD98059 in the absence of factor (**Figure 4.6C**). The control inhibitor U0124 did not effect the viability of any of the cell populations (**Figure 4.6**). These experiments demonstrate a clear differential sensitivity of the mutants to the two independent MEK inhibitors consistent with the differential activation of p44/42 MAPK observed in Section 4.2.2.

These results are consistent with a central role for p44/42 MAPK signalling, in addition to JAK2, in the survival of the FDB1 cells in response to the V449E mutant. Both STAT5 and p44/42 MAPK are downstream of JAK2 and have been shown to contribute to haemopoietic cell survival (discussed further in Section 3.3). Viability of all FDB1 cell populations in mIL-3 is unaffected by the MEK inhibitors, consistent with mIL-3 activating multiple redundant survival pathways.

4.2.4.3 Inhibition of MAPK Instructs Differentiation of FDB1 V449E Cells

We next wished to investigate whether inhibitory phosphorylation of the lineage-specific transcription factor, C/EBPα, by p44/42 MAPK is important in the block in differentiation in FDB1 cells expressing the V449E mutant. Recent studies have shown that MAPK-mediated phosphorylation of C/EBPα is important for the differentiation

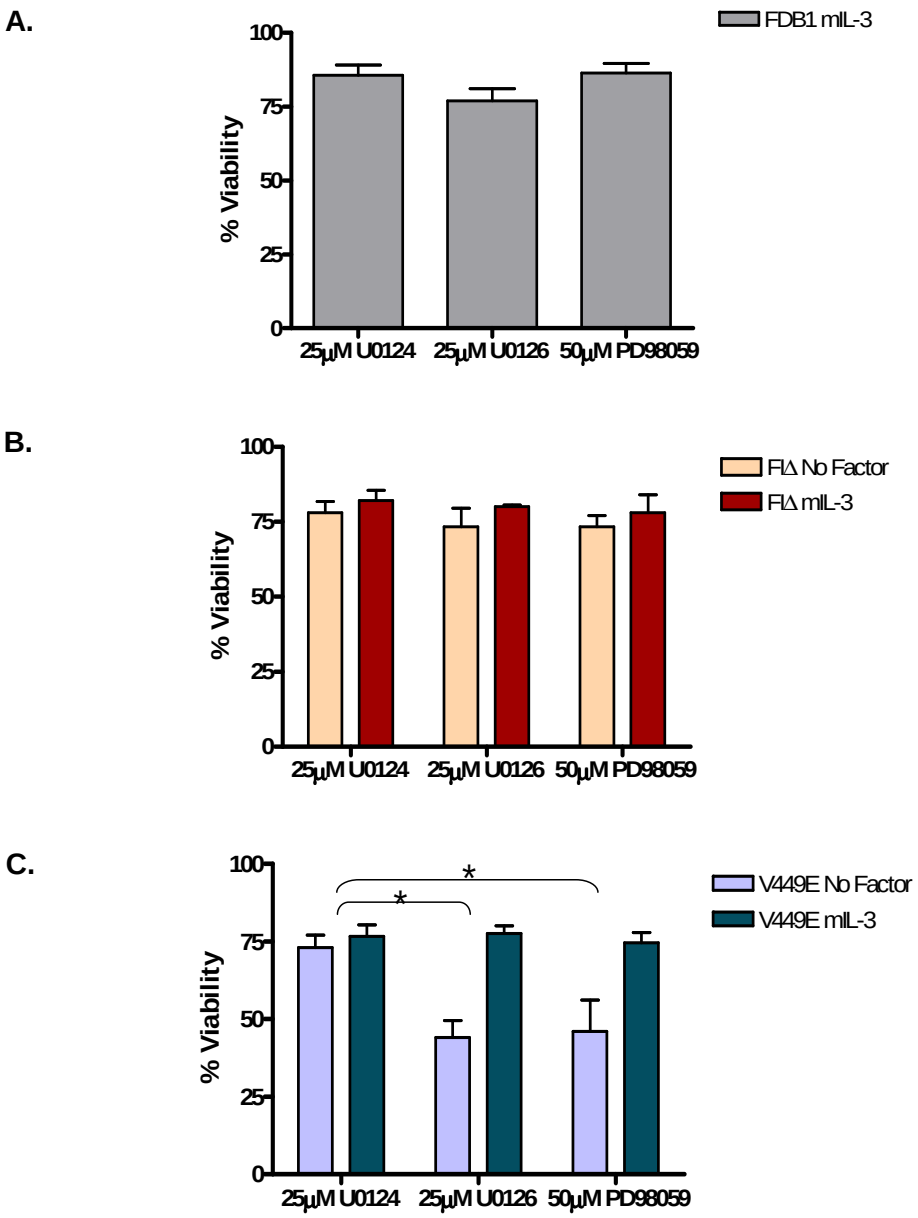


Figure 4.6 Sensitivity of FDB1 Cell Populations to MEK Inhibitors U0126 and PD98059. Parental FDB1 cells (A), or cells expressing FIΔ (B) and V449E (C) were starved of factor and MEK inhibitors U0126, PD98059 and the control, U0124 were added for 24hrs. Percentage viability was determined using trypan blue exclusion. Error bars represent SEM. * represents statistical significance compared to U0124 where $P<0.01$ (n=3).

block in some AML leukaemic cell lines (Radomska, Basseres et al. 2006). Recent findings by Radomska *et al* identified a p44/42 MAPK phosphorylation site in C/EBPα (Ser21), the phosphorylation of which inhibits C/EBPα function (Radomska, Basseres et al. 2006). Furthermore, inhibition of MEK or FLT3 restored morphological granulocytic differentiation of a leukaemic cell line, which contains an activated FLT3-ITD receptor.

We assessed phosphorylation of C/EBPα downstream of the V449E mutant which constititutively activates p44/42 MAPK. Parental FDB1 cells and FDB1 cells expressing V449E were cultured without factor, or in mIL-3, and phosphorylation of C/EBPα Ser21 was detected in whole cell lysates using the Ser21 phospho-specific antibody (Cell Signalling Technology). As shown in **Figure 4.7A**, we observed phosphorylation of C/EBPα Ser21 in parental FDB1 cells cultured in mIL-3 and also in FDB1 V449E cells cultured without factor or in mIL-3 (**Figure 4.7A**). To assess whether the p44/42 MAPK pathway contributes to the differentiation block, characteristic of the V449E mutant, we monitored the morphology of cells following incubation for 48 hrs with the MEK inhibitor U0126, or control U0124. FDB1 cells cultured in mIL-3 in the presence of 25μM U0126 underwent morphological differentiation at 48 hrs whereas the same cells cultured in 25μM U0124 maintained the characteristic FDB1 cell morphology. As reported previously (McCormack and Gonda 1999) FDB1 V449E cells did not differentiate in mIL-3, mGM-CSF, or in the absence of factor when incubated with the control inhibitor, U0124. However, in the presence of U0126 we observed morphological differentiation associated with increased cytoplasm and lobulated nuclei, consistent with granulocytic differentiation (**Figure**

4.7B). We also assessed the surface expression of the granulocytic marker, GR-1, by flow cytometry. The histogram overlays in **Figure 4.7C** show increased GR-1 expression in all of the cell populations treated with U0126, consistent with granulocytic differentiation. These data suggest that in the FDB1 cell line, phosphorylation of p44/42 MAPK, through IL-3 or V449E induced receptor activation, contributes to the differentiation block, most likely via phosphorylation of C/EBPα Ser21.

4.2.4.4 Inhibition of AKT Signalling using PI3K Inhibitors

Activation of the PI3K/AKT signalling pathway is important for survival in response to GM-CSF and IL-3 (de Groot, Coffer et al. 1998). We again wanted to determine the relative contribution of this pathway to signalling/survival from the two activated mutants. To assess the role of this pathway in FDB1 cells their viability was measured following 24hr treatment with the PI3K inhibitors, Wortmannin and LY294002, or DMSO as a negative control. We performed immunoblot analysis to assess inhibition of AKT phosphorylation in the presence of these inhibitors. Concentrations of 10, 25 and 50μM of LY294002, or 0.2-1.0μM Wortmannin were sufficient to prevent phosphorylation of AKT (**Appendix E**). For viability assays, 25μM LY294002 and 0.5μM Wortmannin were used and DMSO was the negative control.

PI3K inhibition did not significantly affect the viability of the parental FDB1 cells in IL-3 (**Figure 4.8A**). In the absence of growth factor FDB1 FIΔ cells were sensitive to both inhibitors and showed a significant decrease in cell viability over a 24 hour period (42% and 28% for LY294002 and Wortmannin respectively; **Figure 4.8B**). This is

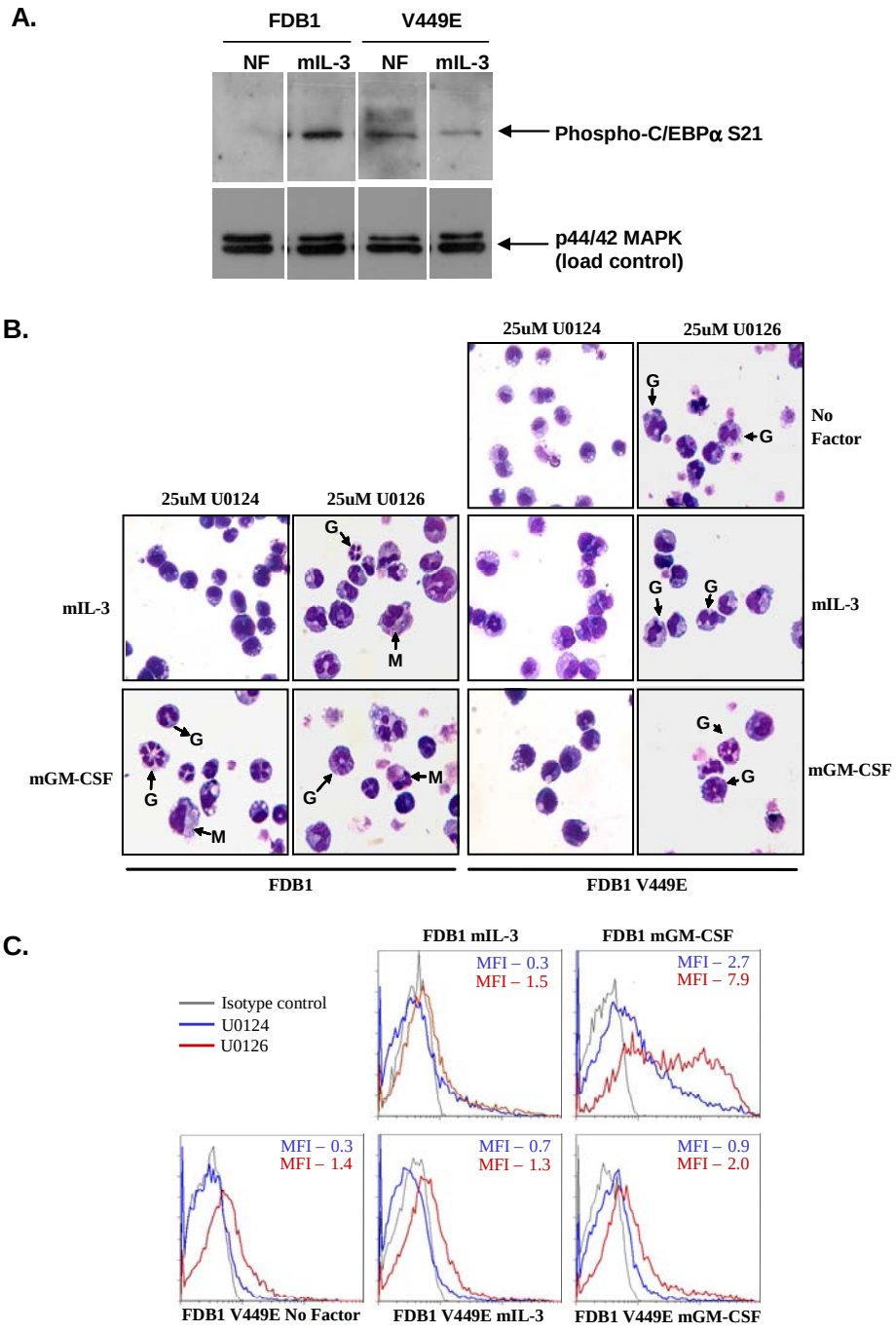


Figure 4.7 FDB1 V449E cell differentiation induced by MEK inhibition and C/EBPα serine phosphorylation. A. Serine phosphorylation of C/EBPα in FDB1 and FDB1 V449E cells, starved of factor or cultured in mIL-3, was determined by immunoblot analysis using a phospho-C/EBPα Ser21 antibody. **B.** FDB1 and FDB1 V449E cells were starved of factor or cultured in mIL-3 or mGM-CSF with the MEK inhibitor, U0126, or control U0124 for 24hr. Cells were centrifuged onto glass slides and stained with May-Grunwald-Giemsa to determine morphology. G and M represent granulocytes and macrophages respectively. **C.** Cells from **B.** were stained with the GR-1 and anti-mouse FITC secondary antibodies and GR-1 surface expression was determined by flow cytometry. MFI values are indicated for each peak.

consistent with the PI3K/AKT pathway playing an important role in FDB1 cell survival in response to FIΔ signalling. In contrast, neither inhibitor affected the survival of FDB1 V449E cells, consistent with the lack of constitutive AKT phosphorylation by this mutant (**Figure 4.8C**). All cells grown in mIL-3 maintained good viability irrespective of inhibitor treatment once again emphasising the high level of redundant signalling in this system (**Figure 4.8**). Differentiation status and proliferation rates of all cell populations remained unaltered in the presence of both of these inhibitors as measured by cell cytocentrifugation and proliferation assays (data not shown).

4.3 Discussion

In this chapter we performed biochemical and inhibitor analysis of FDB1 cells expressing activated GM-CSF receptor mutants, FIΔ and V449E. This analysis has demonstrated the reduced redundancy of signalling associated with these mutants when compared with IL-3. We show activation of JAK2, STAT5, and p44/42 MAPK in parental FDB1 cells responding to GM-CSF or IL-3, and in FDB1 cells expressing V449E in the absence of added cytokine. In contrast a comprehensive biochemical analysis of signalling using Western blotting and intracellular flow cytometry (Krutzik and Nolan 2003) has been unable to detect JAK2, STAT5, and p44/42 MAPK activation during the FIΔ-induced factor-independent response in FDB1 cells. FIΔ in contrast induces robust phosphorylation of AKT and IκB. Additionally, we could not detect activation of AKT or NFκB pathways in V449E expressing FDB1 populations. Thus these two mutants display non-overlapping patterns of signalling.

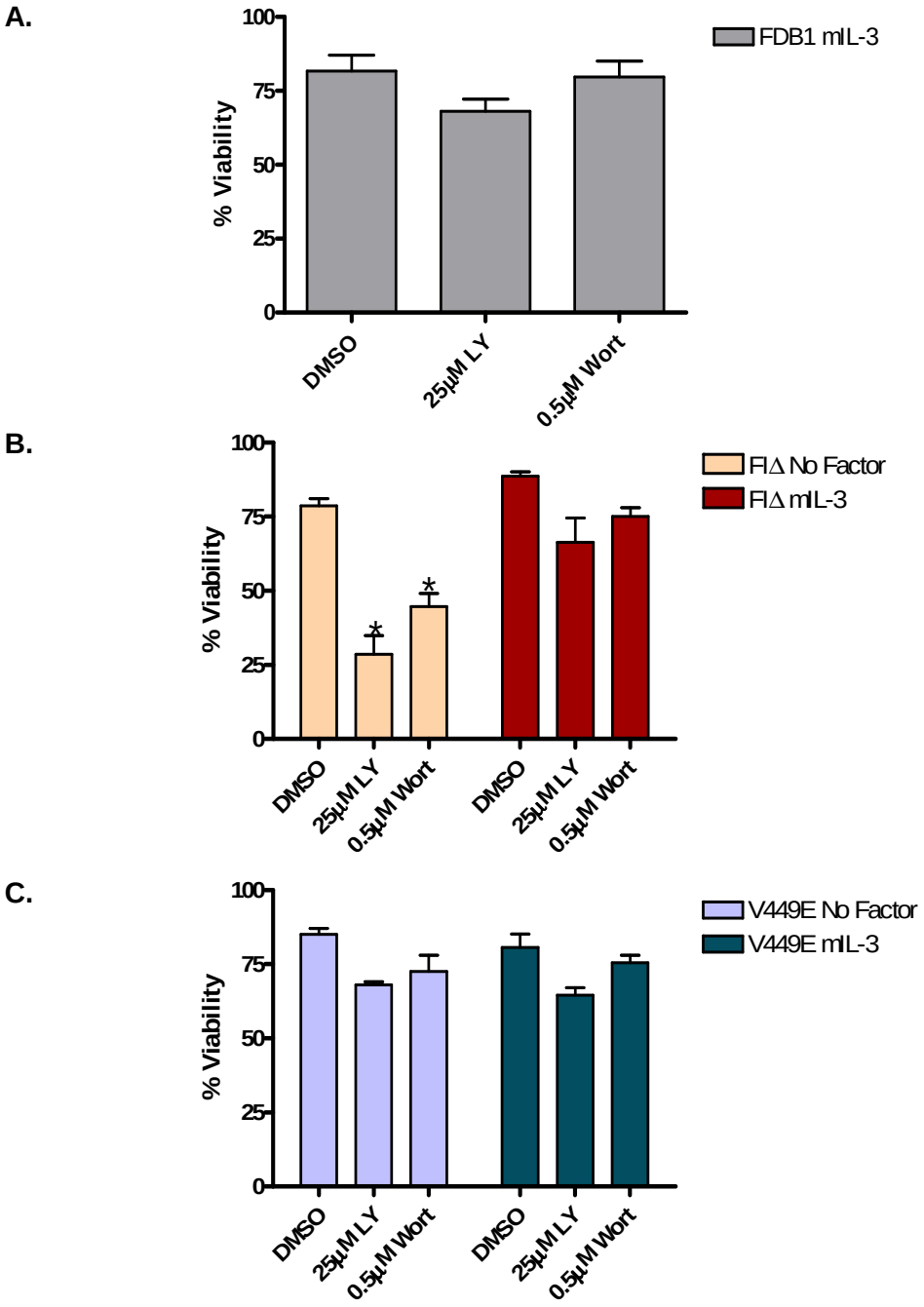


Figure 4.8. Sensitivity of GMR FIΔ to the PI3K Inhibitors LY294002 and Wortmannin. Parental FDB1 cells **(A)**, and cells expressing FIΔ **(B)** and V449E **(C)** were starved of factor and PI3K inhibitors LY294002 and Wortmannin were added for 24hrs. DMSO was used as a vehicle control for these experiments. Percentage viability was determined using trypan blue exclusion and haemocytometer counting. Error bars represent SEM. * represents statistical significance compared to DMSO where $P<0.01$ (n=3).

4.3.1 JAK-Independent Activation of Signalling from the FIΔ Mutant?

The inability to detect JAK2 phosphorylation using multiple methods raises the possibility that signalling by the FIΔ mutant occurs in a JAK2-independent fashion. This is supported by several other lines of evidence. JAK2-independent signalling is consistent with our previous analysis of serial truncations of this mutant which indicate that primary survival signalling is generated distal to the Box1 region responsible for JAK2 association (Brown, Peters et al. 2004). The viability region in FIΔ (from amino acid 545 to 626) includes Y577 and Ser585 which are associated with activation of known survival pathways mediated by MAPK and AKT (Brown, Peters et al. 2004). We suggest that this JAK2-independent pathway is utilised by the liganded GM-CSF receptor complex, although there may be extensive redundancy associated with JAK2 activated pathways. *In vivo* studies suggest that JAK2- independent signalling from $h\beta_c$ will support only limited responses. While myeloid progenitors from the foetal liver of JAK2-deficient mice show a lack of growth responsiveness to IL-3 and GM-CSF (Parganas, Wang et al. 1998), survival responses of JAK2 knockout progenitors in response to these growth factors have not been studied in detail. Deletion of the Box1 region of the $h\beta_c$ prevents all signalling from the GM-CSF receptor (Matsuguchi, Lilly et al. 1998). Finally, mutation of all tyrosine residues in the $h\beta_c$ cytoplasmic domain results in an inability to activate STAT5A/B or the MAPK cascade. However, this mutant is still capable of maintaining cell survival and a weak proliferative response (Okuda, Smith et al. 1997), consistent with an impornat role for other kinases in these responses.

4.3.2 Key Survival Signals in FIΔ Include PI3K and NFκB?

Inhibitor studies suggest that the FIΔ survival response is mediated predominantly by PI3K/AKT and IKKβ/IkB/NFκB pathways consistent with the observed constitutive AKT and IκB phosphorylation (**Figure 4.8**). We observed robust activation of both of these pathways and, given the lack of JAK2 activation, we predict that this is mediated via molecules associated with the GMRα subunit. GMRα is critical for FIΔ activity (Jenkins, Le et al. 1999) and in Chapter 3 we confirmed association of GMRα with the regulatory subunit of PI3K, p85, and with IKKβ, consistent with previous reports (Dhar-Mascareno, Chen et al. 2003; Ebner, Bandion et al. 2003). In addition we describe association of the GMRα subunit with the SFK, Lyn. Mutation of the SBP motif in GMRα showed that both the Lyn and p85 association dependent on this proline-rich motif (**Figure 3.4**, **Figure 3.5**). IKKβ is a key component of the IκB kinase signalosome which acts to phosphorylate IκB (inhibitor of NFκB) leading to its ubiquitination and degradation, and the consequent activation of NFκB target genes. Association of IKKβ with GMRα is consistent with our observed activation of this pathway by FIΔ (**Figure 4.3**) and with several reports of NFκB activation by GM-CSF and IL-3 (Denk, Wirth et al. 2000; Cruz, Duarte et al. 2001; Guthridge, Barry et al. 2004). NFκB activation also plays an important role in modulating survival in myeloid cells (Jordan, Upchurch et al. 2000; Cruz, Duarte et al. 2001; Nakamura, Ouchida et al. 2002; Ebner, Bandion et al. 2003). Experiments with NFκB inhibitors are now needed to confirm the contribution of this pathway to survival. Such experiments could be performed with small molecule or peptide inhibitors that are commercially available (Pierce, Schoenleber et al. 1997; Watanabe, Dewan et al. 2005).

JAK2-independent activation of the PI3K/AKT pathway is also consistent with the observation that this pathway is not activated in Ba/F3 cells expressing a β_c/JAK2 chimera (Liu, Fan et al. 1999). The exact mechanism by which PI3K/AKT is activated is not yet clear. We propose that Lyn or other SFKs play a role in initiating JAK-independent signalling downstream of the GM-CSF receptor (discussed in Chapter 3). A role for SFKs in generating JAK-independent survival signals has been demonstrated previously for a number of receptors (Fresno Vara 2000; Reddy, Korapati et al. 2000; Zhu, Ling et al. 2002).

It is possible that in the absence of JAK2-mediated tyrosine phosphorylation of the receptor, Ser585 phosphorylation may initiate signal transduction by recruiting 14-3-3 and p85 to the receptor mediating their activation (Guthridge, Barry et al. 2004). It has been previously shown that the β_cSer585Gly mutation results in defects in AKT phosphorylation, NFκB activation, Bcl2 induction and subsequently survival (Guthridge, Barry et al. 2004). Interestingly, mutation of Tyr577, which has been shown to contribute to survival signals in our cell system (Brown, Peters et al. 2004), leads defects in Shc, Ras and p44/42 MAPK activation but such a mutant still supports survival in response to GM-CSF (Durstin, Inhorn et al. 1996; Guthridge, Barry et al. 2004). These observations together suggest that FIΔ produces a signal, independent of JAK2 activation and tyrosine phosphorylation, that is potentially initiated by phosphorylation of Ser585 and subsequent activation of pro-survival genes. It will be important to mutate Ser585 and assess the phosphorylation status of this residue in the activated receptor mutants to determine the role of this residue in the responses shown here.

4.3.3 Survival Signals in V449E

The results from this chapter suggest that the V449E mutant constitutively activates a limited subset of signalling molecules including JAK2, STAT5A/B and p44/42 MAPK. Consistent with the activation of these signalling molecules, the survival of FDB1 cells expressing the V449E was markedly reduced by JAK2 and, to a lesser extent, p44/42 MAPK inhibitors. The survival signals generated by the V449E mutant in FDB1 cells are therefore likely to be JAK2 mediated. In particular, our data suggests that JAK2 mediated p44/42 MAPK is a key pathway in the survival of FDB1 cells expressing the V449E mutant. Consistent with our data, MAPK signalling has been previously implicated in survival of myeloid cells (Nishida and Gotoh 1993; Lewis, Shapiro et al. 1998; Chang and Karin 2001; Pearson, Robinson et al. 2001; Torii, Nakayama et al. 2004). Additionally, JAK2-dependent activation of STAT5A/B may also contribute to FDB1 V449E cell survival. STAT5A/B have also been previously implicated in regulating myeloid cell survival (Wittig and Groner 2005; Khwaja 2006). Further clarification of the relative roles of these pathways in the survival of FDB1 cells mediated by the GM-CSF receptor mutant, V449E may require the use of a dominant negative form of STAT5 (eg STAT5aΔ749 which lacks the COOH-terminal transactivtaion domain) (Galsgaard, Friedrichsen et al. 2001).

4.3.4 Summary

The properties of the two hβ_c activated mutants, determined from these biochemical studies and previous molecular analysis are summarised in **Figure 4.9**. These properties are consistent with the previously proposed model that the two classes of mutant represent alternative complexes, possibly intermediates in formation of the mature GM-

complex (D'Andrea and Gonda 2000). Each complex initiates different primary signalling events resulting in activation of complementary pathways and non-redundant signalling. We suggest that the nature of signalling from the FIΔ mutant will facilitate a study of AKT and NFκB activation in the absence of other signals, providing us with a novel tool for analysing further the role of these pathways in myeloid growth and differentiation.

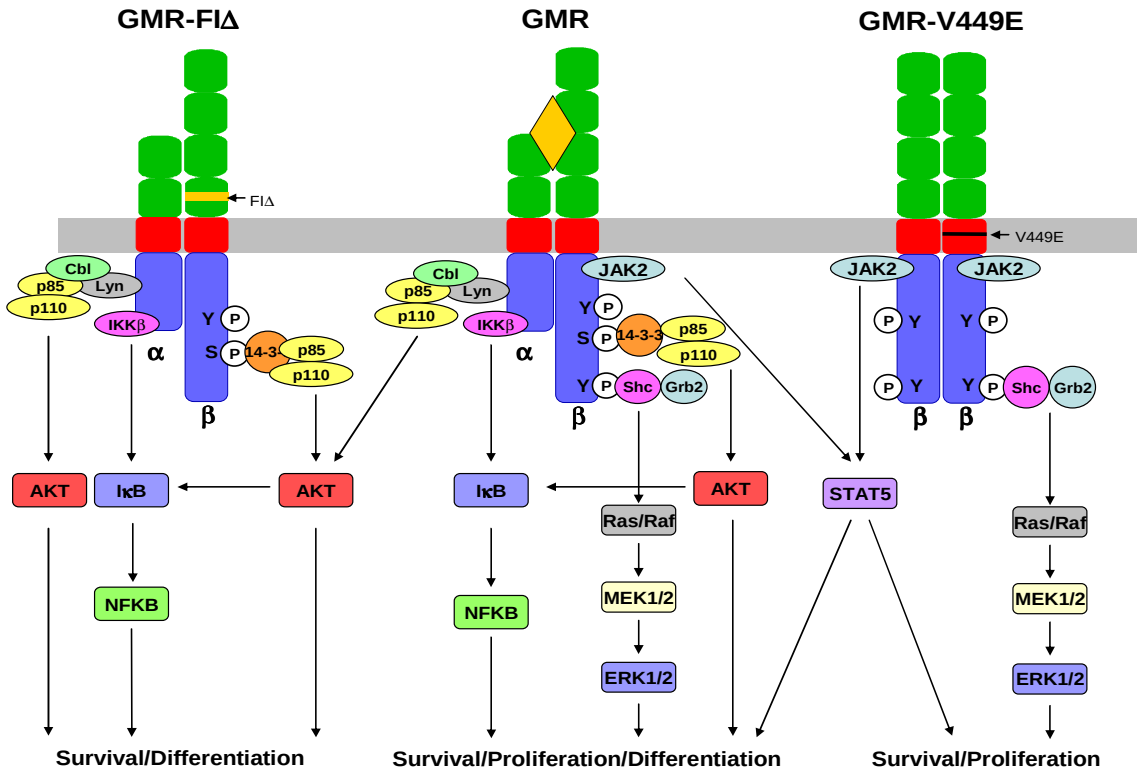


Figure 4.9. Schematic representation of GM-CSF receptor signalling and signalling by constitutive FIΔ and V449E mutants. GMR and GMR-FIΔ activate AKT and NFκB pathways. Activation of AKT is postulated to occur through two mechanisms; 1) association of p85, via Lyn and Cbl, to the GMRα (refer Sections 3.2.1 and 3.2.2) and 2) association of 14-3-3 with Ser585 on hβ_c[214]. Activation of NFκB also occurs through two mechanisms 1) association of IKKβ directly to GMRα [187] (Section 3.2.2) and 2) activation of IKKβ by AKT [135, 136]. Shc is able to associate with numerous phophotyrosine residues on hβ_c including Y577 which is not shown here [195]. The MEK/ERK pathway is shown downstream of GMR and GMR-V449E. We have not measured Ras activity directly in this study and it is possible that Ras-independent activation of ERK1/2 may also occur, however, for simplicity we have only included Ras/MEK/ERK pathways.

CHAPTER 5

DISCUSSION

Cell proliferation, differentiation and survival are processes that are tightly governed by soluble growth factors via their membrane bound receptors. Extensive study of growth factor receptor signalling has revealed overlapping activation of signalling pathways for many haemopoietic growth factors and extensive signalling redundancy (Ozaki and Leonard 2002). This signalling redundancy is problematic with regard to dissecting the contribution of signalling events to various cellular outcomes (survival, proliferation, differentiation). It is important to develop approaches that overcome these difficulties as these will allow the identification of potential therapeutic targets for the treatment of leukaemia and other blood disorders. Further dissection of signalling events that alter cell behaviours is therefore warranted.

The findings described in this thesis provide new insights into the role played by the specific GM-CSF receptor subunit (GMRα), which is an essential component of the functional GM-CSF receptor complex, providing a better understanding of the signalling pathways that contribute to GM-CSF-induced myeloid cell survival. A number of alternative approaches were used to identify novel interaction partners of GMRα and further characterise interactions that were previously reported in the literature. In addition, a panel of constitutively activated GM-CSF receptor mutants that have been characterised previously, was also utilised, to further define the role of the signalling β_c subunit in mediating GM-CSF mediated biological outcomes. This work, and other published studies (McCormack and Gonda 1997; Jenkins, Blake et al. 1998; McCormack and Gonda 1999; Brown, Peters et al. 2004) show that different classes of these mutants deliver a subset of proliferative, survival and differentiative signals. Importantly, the more limited nature of the signalling response from these mutants overcomes the redundancy issues associated with dissecting wild-type receptor

function. We also made use of the unique myeloid cell line, FDB1, which displays a switch between growth and granulocyte-macrophage differentiation depending on the nature of the receptor signalling (McCormack and Gonda 2000). This allowed a detailed exporation of the signalling associated with alternative receptor responses driving survival and continuous proliferation or survival and granulocyte-macrophage differentiation. In doing so, this study also revealed alternative mechanisms with differential dependence on JAK activity.

Below we discuss the key findings from these studies and how these relate to other studies looking at GM-CSF receptor signalling and function. Approaches for further investigation of our findings are also discussed.

4.4 GMRα-Associated Signalling Events

Previous studies have indicated a key role of the membrane-proximal region of GMRα in signalling from the GM-CSF receptor (Jenkins, Le et al. 1999; Geijsen, Uings et al. 2001). In Chapter 3, we confirmed the previously reported association of p85 and IKKβ with the GMRα. Multiple direct binding assays (eg. fluorescence polarisation) were unable to demonstrate a direct interaction between p85 and GMRα. We confirmed that the p85-GMRα interaction is dependent upon an intact SBP motif as reported by Dhar-Mascareno *et al* (Dhar-Mascareno, Chen et al. 2003) and we suggest that the interaction occurs via other adaptor proteins in an indirect fashion. A candidate adaptor protein that may couple p85 to GMRα, is the SFK, Lyn. Lyn has been shown to interact with p85 via association with Cbl (Dombrosky-Ferlan and Corey 1997). Additionally, we

identified a novel and direct interaction between Lyn and GMRα, consistent with a potential role for Lyn in coupling p85 to the GMRα. In contrast to the indirect interaction observed with p85, our data indicate that the interaction between IKKβ and GMRα is direct, consistent with its isolation in a yeast-two-hybrid screen using the GMRα cytoplasmic tail as bait (Ebner, Bandion et al. 2003).

In addition to investigating these previously identified interactions, we wished to identify other interaction partners of GMRα in order to further define the signalling role of this subunit. Using multiple techniques we were able to identify a novel and direct interaction between Lyn and GMRα which was dependent on the proline-rich motif in the membrane proximal region of GMRα. Specifically, the direct nature of the interaction between the SH3 domain of Lyn and the proline-rich SBP motif of GMRα was established by fluorescence polarisation. Additionally, we were able to confirm association of Lyn and GMRα in whole cell lysates using immunoprecipitation and GST pull-down approaches. Mutation of the proline sequence (SBP motif) of GMRα prevented the association with Lyn, further suggesting a role for this motif in mediating the interaction. The association of Lyn with GMRα is consistent with studies demonstrating an important but complex role of Lyn in GM-CSF signalling in the myeloid lineage (see section 1.3.7) (Li 1995; Sheng 1996; Wei, Liu et al. 1996; Dahl, Arai et al. 2000; Harder, Parsons et al. 2001; Hibbs and Harder 2006). Lyn has also been previously shown to interact with the IL-5Rα (Stafford, Lowell et al. 2002), further indicating that Lyn may be important in mediating α-subunit associated signalling events. It will be important to further study the role of Lyn in GM-CSF mediated signalling responses, however, this is associated with difficulties due to the

complex role of Lyn in signalling from multiple receptors and in different cell lineages (Corey, Eguinoa et al. 1993; Rao and Mufson 1995; Burton 1997; Tilbrook, Ingley et al. 1997; Adachi, Pazdrak et al. 1999; Adachi, Stafford et al. 1999; Cen, Gorska et al. 2003; Suh, Kim et al. 2005). A role for Lyn in GM-CSF and IL-3 signalling is evident for some cell types; in particular, published studies suggest a role for Lyn in the response to GM-CSF in macrophages and dendritic cells (Beavitt, Harder et al. 2005; Chu and Lowell 2005). Macrophages from the bone marrow of Lyn -/- mice are hyper-responsive to M-CSF and there are suggestions that this is also true for GM-CSF (Baran, Tridandapani et al. 2003). Lyn -/- mast cells show a hyper-responsiveness to IL-3 (Hernandez-Hansen, Mackay et al. 2004). Lyn may also play a negative role in regulating the activity of other SFKs in some cell types, and hence the observed hyper-responsiveness could relate to constitutive activation of other SFKs in the absence of Lyn (Odom, Gomez et al. 2004). Additionally, GM-CSF is reported play a critical role in dendritic cell growth and maturation as dendritic cells are less mature in Lyn -/- animals (Witmer-Pack, Olivier et al. 1987; Beavitt, Harder et al. 2005).

To define the Lyn-GMRα interaction in more detail it will be important to confirm the interaction in complexes with the β_c in the presence and absence of ligand. For example; Lyn may be constitutively associated with GMRα and activated on ligand binding potentially through interactions with the SFK activator, Unc119, which we and others have shown to interact with Lyn (Cen, Gorska et al. 2003). Our results suggest that Lyn is able to bind GMRα in the absence of ligand, consistent with the suggestion that it is constitutively associated with the receptor and is activated on ligand binding. In addition, the use of Lyn knockout haemopoietic cells (Harder, Parsons et al. 2001) in

conjunction with the activated $h\beta_c$ mutants will further assess the role of this kinase in specific signalling pathways activated by GM-CSF. The analysis of signalling from activated β_c mutants (Chapter 4) also indirectly supports a role for SFKs in GM-CSF receptor signalling (discussed below).

4.5 β_c-Associated Signalling Events

In chapter 4 we characterised signalling events associated with alternative constitutively activated $h\beta_c$ mutants, FIΔ and V449E. This has demonstrated non-overlapping signalling profiles between these two mutants, which are summarised below.

We demonstrate that FIΔ signals in the absence of detectible JAK2, STAT5A/B and p44/42 MAPK phosphorylation. Interestingly, although we were unable to detect FIΔ-induced JAK2 phosphorylation, this mutant displayed limited sensitivity to the JAK2 inhibitor (JAK2 Inhibitor II). In this study, we assessed the phosphorylation of JAK2, and we could therefore not rule out a low level of JAK2 activity or the involvement another JAK family member. For example JAK1 and Tyk2 have been implicated in signalling from the GM-CSF receptor (de Groot, Coffer et al. 1998). Consistent with a key role for JAK-independent pathways, FIΔ also showed limited sensitivity to a second JAK inhibitor, AG490. Given the limitations associated with the use of pharmacological inhibitors, it is important to test further the requirement for JAK2 activation in FIΔ signalling. A number of approaches to this are possible. Based on the analysis outlined in this study, we predict that mutation of Box 1 will not impair the function of FIΔ and this could be tested by targeted mutagenesis of this region.

Truncation studies suggest that the survival signal of FIΔ is generated distal to the Box 1 region consistent with a JAK-independent survival pathway (Brown, Peters et al. 2004). Knockdown of JAK2 using siRNA has also been used effectively (James, Ugo et al. 2005) and could be used as an alternative to inhibitors to further assess the requirement of JAK2 for FIΔ mediated signalling. Another approach used to test the role of JAK signalling is over-expression of SOCS proteins (Li, Metze et al. 2004).

In light of the reduced JAK dependence we raise the possibility that the SFKs, and Lyn in particular, may be critical, in addition to JAK2, for signalling from GMR. Taken together with the high degree of sensitivity that the FIΔ mutant displays to SFK inhibitors (unpublished data) and the strict requirement that this mutant has for GMRα (Jenkins, Le et al. 1999), it is likely that SFKs are key initiators of GM-CSF survival signalling.

In Chapter 4 we also describe constitutive activation of IκB/NFκB and PI3K/AKT pathways by FIΔ and sensitivity to multiple inhibitors of these pathways. We highlight that the association of p85 with GMRα may be of functional importance for the activity of this receptor. Recent studies have also shown that p85 is associated with β_c via the adaptor molecule 14-3-3 (Guthridge, Stomski et al. 2000). This contributes to AKT activation of downstream survival pathways including those involving NFκB and Bcl2 induction (Guthridge, Barry et al. 2004). The relative role of these events for the survival response of the FIΔ mutant is unclear and second site mutagenesis of the 14-3-3 binding site, (Ser585Gly) in the FIΔ mutant will help establish the specific events

required for activation of this pathway. This will in turn lead to insights into the survival signalling pathways associated with the mature liganded GM-CSF receptor.

The V449E mutant was shown, in this study, to activate JAK2, STAT5, p44/42 MAPK pathways consistent with its proposed stoichiometry as a β_c homodimer (D'Andrea and Gonda 2000). We showed that the V449E mutant activates distinct signalling pathways to FIΔ and were unable to detect AKT or NFκB phosphorylation events associated with activation. Consistent with these observations, V449E-induced survival was relatively sensitive to JAK2 and MEK inhibitors but did not display sensitivity to PI3K inhibitors, with respect to survival. Unpublished data from our group has also shown that the V449E mutant is relatviely insensitive to SFK inhibitors (unpublished data) indicating that this mutant generates predominantly SFK-independent survival signals probably mediated by JAK2. Treatment with MEK inhibitors dramatically and selectively affects survival of V449E-expressing FDB1 cells implicating the MAPK pathway in survival signalling. This is consistent with the ability of this pathway to generate survival signals in other systems (Nishida and Gotoh 1993; Lewis, Shapiro et al. 1998; Chang and Karin 2001; Pearson, Robinson et al. 2001; Torii, Nakayama et al. 2004).

We believe that the signalling exhibited by FIΔ and V449E mutants represent a subset of the signalling events induced from the mature liganded GM-CSF receptor complex (D'Andrea and Gonda 2000). These mutants are therefore useful tools for addressing signalling complexity and redundancy issues. While these two distinct activated mutants utilise predominantly different pathways for survival it is likely that both JAK- and SFK-dependent survival pathways are contributing to survival in the mature GM-CSF receptor complex. These pathways may be activated concomitantly or as alternate

states controlled by varying levels of GM-CSF receptor activation by ligand (Guthridge, Barry et al. 2004). Such a model is consistent with the model with these mutants representing alternative configurations or intermediates in the formation of the mature GMR complex (D'Andrea and Gonda 2000).

In summary, the findings in this thesis are in agreement with the current body of evidence that suggests the specific α-subunits of the GM-CSF receptor are important mediators of signalling in addition to conferring ligand binding specificity to the complex. Further characterisation of the signalling role of the GMRα is therefore warranted. This thesis also describes the use of activated β_c mutants in characterising signalling pathways important in achieving specific biological outcomes. We identified signalling differences between two classes of activated mutants which suggest that JAK-dependent and JAK-independent signalling mechanisms are likely to be important in survival signalling from the mature liganded GM-CSF receptor complex. The non-redundant nature of signalling from the activated mutants will greatly facilitate further dissection of these events and is likely to define new pathways involved in GM-CSF signalling. A more comprehensive understanding of how GM-CSF activates its cognate cell surface receptor and, in turn how the resultant intracellular signals impact upon transcriptional programs, is likely to reveal additional targets for therapeutic intervention in diseases of myelopoiesis and in myeloid leukaemia.

References

Aaronson, D. S. and C. M. Horvath (2002). "A road map for those who don't know JAK-STAT." Science **296**(5573): 1653-5.

Adachi, T., K. Pazdrak, et al. (1999). "The mapping of the Lyn kinase binding site of the common beta subunit of IL-3/granulocyte-macrophage colony-stimulating factor/IL-5 receptor." J Immunol **162**(3): 1496-501.

Adachi, T., S. Stafford, et al. (1999). "A novel Lyn-binding peptide inhibitor blocks eosinophil differentiation, survival, and airway eosinophilic inflammation." **163**(2): 939-946.

Adelaide, J., C. Perot, et al. (2006). "A t(8;9) translocation with PCM1-JAK2 fusion in a patient with T-cell lymphoma." Leukemia **20**(3): 536-7.

Adjei, A. A. (2001). "Blocking oncogenic Ras signaling for cancer therapy." J Natl Cancer Inst **93**(14): 1062-74.

Appleby, M. W., J. D. Kerner, et al. (1995). "Involvement of p59fynT in interleukin-5 receptor signalling." Journal of Experimental Medicine **182**: 811-820.

Arai, K.-I., F. Lee, et al. (1990). "Cytokines: coordinators of immune and inflammatory responses." Annu Rev Biochem **59**: 783-836.

Armitage, J. O. (1998). "Emerging applications of recombinant human granulocyte-macrophage colony-stimulating factor." Blood **92**(12): 4491-508.

Ashman, L. K. (1999). "The biology of stem cell factor and its receptor C-kit." Int J Biochem Cell Biol **31**(10): 1037-51.

Avalos, B. R., J. M. Parker, et al. (1997). "Dissociation of the Jak kinase pathway from G-CSF receptor signaling in neutrophils." Exp Hematol **25**(2): 160-168.

Bach, E. A., J. W. Tanner, et al. (1996). "Ligand-induced assembly and activation of the gamma interferon receptor in intact cells." Mol Cell Biol **16**(6): 3214-21.

Bagley, C. J., J. M. Woodcock, et al. (1997). "The structural and functional basis of cytokine receptor activation: lessons from the common beta subunit of the granulocyte-macrophage colony-stimulating factor, interleukin-3 (IL-3) and IL-5 receptors." Blood **89**: 1471-1482.

Baldwin, A. S., Jr. (2001). "Series introduction: the transcription factor NF-kappaB and human disease." J Clin Invest **107**(1): 3-6.

Baran, C. P., S. Tridandapani, et al. (2003). "The inositol 5'-phosphatase SHIP-1 and the Src kinase Lyn negatively regulate macrophage colony-stimulating factor-induced Akt activity." J Biol Chem **278**(40): 38628-36.

Barkett, M. and T. D. Gilmore (1999). "Control of apoptosis by Rel/NF-kappaB transcription factors." Oncogene **18**(49): 6910-24.

Barreda, D. R., P. C. Hanington, et al. (2004). "Regulation of myeloid development and function by colony stimulating factors." Dev Comp Immunol **28**(5): 509-54.

Basu, S., A. R. Dunn, et al. (1997). "Increased tolerance to endotoxin by granulocyte-macrophage colony-stimulating factor-deficient mice." J Immunol **159**(3): 1412-7.

Baxter, E. J., L. M. Scott, et al. (2005). "Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders." Lancet **365**(9464): 1054-61.

Bazan, J. F. (1989). "A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor beta-chain." Biochem Biophys Res Commun **164**(2): 788-95.

Bazan, J. F. (1990). "Structural design and molecular evolution of a cytokine receptor superfamily." Proc Natl Acad Sci USA **87**: 6934-6938.

Beavitt, S. J., K. W. Harder, et al. (2005). "Lyn-deficient mice develop severe, persistent asthma: Lyn is a critical negative regulator of Th2 immunity." J Immunol **175**(3): 1867-75.

Beghini, A., P. Peterlongo, et al. (2000). "C-kit mutations in core binding factor leukemias." Blood **95**(2): 726-7.

Behrmann, I., T. Smyczek, et al. (2004). "Janus kinase (Jak) subcellular localization revisited: the exclusive membrane localization of endogenous Janus kinase 1 by cytokine receptor interaction uncovers the Jak.receptor complex to be equivalent to a receptor tyrosine kinase." J Biol Chem **279**(34): 35486-93.

Benekli, M., M. R. Baer, et al. (2003). "Signal transducer and activator of transcription proteins in leukemias." Blood **101**(8): 2940-54.

Birg, F., M. Courcoul, et al. (1992). "Expression of the FMS/KIT-like gene FLT3 in human acute leukemias of the myeloid and lymphoid lineages." Blood **80**(10): 2584-93.

Birkenkamp, K. U., M. Geugien, et al. (2004). "Constitutive NF-kappaB DNA-binding activity in AML is frequently mediated by a Ras/PI3-K/PKB-dependent pathway." Leukemia **18**(1): 103-112.

Blake, T. J., B. J. Jenkins, et al. (2002). "Functional cross-talk between cytokine receptors revealed by activating mutations in the extracellular domain of the beta-subunit of the GM-CSF receptor." J Leukoc Biol **72**(6): 1246-1255.

Boggon, T. J. and M. J. Eck (2004). "Structure and regulation of Src family kinases." Oncogene **23**(48): 7918-27.

Bousquet, M., C. Quelen, et al. (2005). "The t(8;9)(p22;p24) translocation in atypical chronic myeloid leukaemia yields a new PCM1-JAK2 fusion gene." Oncogene **24**(48): 7248-52.

Bowman, T., R. Garcia, et al. (2000). "STATs in oncogenesis." Oncogene **19**(21): 2474-88.

Brabek, J., D. Mojzita, et al. (2002). "The SH3 domain of Src can downregulate its kinase activity in the absence of the SH2 domain-pY527 interaction." Biochem Biophys Res Commun **296**(3): 664-70.

Brasel, K., S. Escobar, et al. (1995). "Expression of the flt3 receptor and its ligand on hematopoietic cells." Leukemia **9**(7): 1212-8.

Brazin, K. N., D. B. Fulton, et al. (2000). "A specific intermolecular association between the regulatory domains of a Tec family kinase." J Mol Biol **302**(3): 607-23.

Briggs, S. D., M. Sharkey, et al. (1997). "SH3-mediated Hck tyrosine kinase activation and fibroblast transformation by the Nef protein of HIV-1." J Biol Chem **272**(29): 17899-17902.

Bromberg, J. (2002). "Stat proteins and oncogenesis." J Clin Invest **109**(9): 1139-42.

Brown, A. L., M. Peters, et al. (2004). "Constitutive mutants of the GM-CSF receptor reveal multiple pathways leading to myeloid cell survival, proliferation, and granulocyte-macrophage differentiation." Blood **103**(2): 507-516.

Burgering, B. M. and R. H. Medema (2003). "Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty." J Leukoc Biol **73**(6): 689-701.

Burgess, A. W. and D. Metcalf (1980). "The nature and action of granulocyte-macrophage colony stimulating factors." Blood **56**(6): 947-58.

Burton, E. A., Hunter, S., Wu, S.C. & Anderson, S.M. (1997). "Binding of src-like kinases to the beta subunit of the interleukin-3 receptor." Journal of Biological Chemistry **272**: 16189-16195.

Butterfield, J. H., D. Weiler, et al. (1988). "Establishment of an immature mast cell line from a patient with mast cell leukemia." Leuk Res **12**(4): 345-55.

Byfield, M. P., J. T. Murray, et al. (2005). "hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase." J Biol Chem **280**(38): 33076-82.

Cacalano, N. A., T. S. Migone, et al. (1999). "Autosomal SCID caused by a point mutation in the N-terminus of Jak3: mapping of the Jak3-receptor interaction domain." Embo J **18**(6): 1549-58.

Cai, T., K. Nishida, et al. (2002). "Gab1 and SHP-2 promote Ras/MAPK regulation of epidermal growth and differentiation." J Cell Biol **159**(1): 103-12.

Cairoli, R., A. Beghini, et al. (2006). "Prognostic impact of c-KIT mutations in core binding factor leukemias: an Italian retrospective study." Blood **107**(9): 3463-8.

Caldenhoven, E., T. B. van Dijk, et al. (1996). "STAT3beta, a splice variant of transcription factor STAT3, is a dominant negative regulator of transcription." J Biol Chem **271**(22): 13221-7.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-1657.

Carow, C. E., M. Levenstein, et al. (1996). "Expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flk2) in human leukemias." Blood **87**(3): 1089-96.

Cen, O., M. M. Gorska, et al. (2003). "Identification of UNC119 as a novel activator of SRC-type tyrosine kinases." J Biol Chem **278**(10): 8837-8845.

Cesareni, G., S. Panni, et al. (2002). "Can we infer peptide recognition specificity mediated by SH3 domains?" FEBS Lett **513**(1): 38-44.

Chakraborty, A., S. M. White, et al. (1996). "Granulocyte colony-stimulating factor activation of Stat3 alpha and Stat3 beta in immature normal and leukemic human myeloid cells." Blood **88**(7): 2442-9.

Chang, L. and M. Karin (2001). "Mammalian MAP kinase signalling cascades." Nature **410**(6824): 37-40.

Chaturvedi, P., E. P. Reddy, et al. (1998). "Src kinases and not JAKs activate STATs during IL-3 induced myeloid cell proliferation." Oncogene **16**(13): 1749-1758.

Chen, J., J. M. Carcamo, et al. (2003). "The laminin receptor modulates granulocyte-macrophage colony-stimulating factor receptor complex formation and modulates its signaling." Proc Natl Acad Sci U S A **100**(24): 14000-5.

Chen, J., J. M. Carcamo, et al. (2006). "The {alpha} Subunit of the Granulocyte-Macrophage Colony-stimulating Factor Receptor Interacts with c-Kit and Inhibits c-Kit Signaling." J Biol Chem **281**(31): 22421-6.

Chen, M., A. Cheng, et al. (1997). "The amino terminus of JAK3 is necessary and sufficient for binding to the common gamma chain and confers the ability to transmit interleukin 2-mediated signals." Proc Natl Acad Sci USA **94**(13): 6910-6915.

Chim, C. S., T. K. Fung, et al. (2004). "SOCS1 and SHP1 hypermethylation in multiple myeloma: implications for epigenetic activation of the Jak/STAT pathway." Blood **103**(12): 4630-5.

Chim, C. S., K. Y. Wong, et al. (2004). "SOCS1 and SHP1 hypermethylation in mantle cell lymphoma and follicular lymphoma: implications for epigenetic activation of the Jak/STAT pathway." Leukemia **18**(2): 356-8.

Chu, C. L. and C. A. Lowell (2005). "The Lyn tyrosine kinase differentially regulates dendritic cell generation and maturation." J Immunol **175**(5): 2880-9.

Chung, C. D., J. Liao, et al. (1997). "Specific inhibition of Stat3 signal transduction by PIAS3." Science **278**(5344): 1803-5.

Coffer, P. J., L. Koenderman, et al. (2000). "The role of STATs in myeloid differentiation and leukemia." Oncogene **19**(21): 2511-22.

Cole, S. R., G. W. Aylett, et al. (1996). "Increased expression of c-Kit or its ligand Steel Factor is not a common feature of adult acute myeloid leukaemia." Leukemia **10**(2): 288-96.

Corey, S., A. Eguinoa, et al. (1993). "Granulocyte macrophage-colony stimulating factor stimulates both association and activation of phosphoinositide 3OH-kinase and src-related tyrosine kinase(s) in human myeloid derived cells." EMBO J **12**: 2681-2690.

Corey, S. J. and S. M. Anderson (1999). "Src-related protein tyrosine kinases in hematopoiesis." Blood **93**(1): 1-14.

Corey, S. J., P. M. Dombrosky-Ferlan, et al. (1998). "Requirement of Src kinase Lyn for induction of DNA synthesis by granulocyte colony-stimulating factor." J Biol Chem **273**(6): 3230-5.

Cruz, M. T., C. B. Duarte, et al. (2001). "Granulocyte-macrophage colony-stimulating factor activates the transcription of nuclear factor kappa B and induces the expression of nitric oxide synthase in a skin dendritic cell line." Immunol Cell Biol **79**(6): 590-6.

Dadi, H., S. Ke, et al. (1994). "Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor is dependent on protein tyrosine kinase activity." Blood **84**(5): 1579-86.

Dahl, M. E., K. I. Arai, et al. (2000). "Association of Lyn tyrosine kinase to the GM-CSF and IL-3 receptor common betac subunit and role of Src tyrosine kinases in DNA synthesis and anti-apoptosis." Genes Cells **5**(2): 143-53.

D'Andrea, R., T. J. Sadlon, et al. (2004). "Overlapping motifs in the membrane-proximal region of cytokine receptor accessory and signaling subunits." Cytokine and Growth Factor Reviews **15**: 83-85.

D'Andrea, R. J., S. C. Barry, et al. (1996). "Extracellular truncations of hác, the common signaling subunit for Interleukin-3 (IL-3), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) and IL-5, lead to ligand-independent activation." Blood **87**: 2641-2648.

D'Andrea, R. J. and T. J. Gonda (2000). "A model for assembly and activation of the GM-CSF, IL-3 and IL-5 receptors. Insights from activated mutants of the common beta subunit [In Process Citation]." **28**(3): 231-243.

Davies, S. P., H. Reddy, et al. (2000). "Specificity and mechanism of action of some commonly used protein kinase inhibitors." Biochem J **351**(Pt 1): 95-105.

Davis, R. J. (2000). "Signal transduction by the JNK group of MAP kinases." Cell **103**(2): 239-52.

de Groot, R. P., P. J. Coffer, et al. (1998). "Regulation of proliferation, differentiation and survival by the IL- 3/IL-5/GM-CSF receptor family." Cell Signal **10**(9): 619-628.

Deguchi, K. and D. G. Gilliland (2002). "Cooperativity between mutations in tyrosine kinases and in hematopoietic transcription factors in AML." Leukemia **16**(4): 740-4.

Denk, A., T. Wirth, et al. (2000). "NF-kappaB transcription factors: critical regulators of hematopoiesis and neuronal survival." Cytokine Growth Factor Rev **11**(4): 303-320.

DeSilva, D. R., E. A. Jones, et al. (1998). "Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy." Journal of Immunology **160**(9): 4175-4181.

Dhar-Mascareno, M., J. Chen, et al. (2003). "Granulocyte-macrophage colony-stimulating factor signals for increased glucose transport via phosphatidylinositol 3-kinase- and hydrogen peroxide-dependent mechanisms." J Biol Chem **278**(13): 11107-11114.

Dhar-Mascareno, M., A. Pedraza, et al. (2005). "PI3-kinase activation by GM-CSF in endothelium is upstream of Jak/Stat pathway: role of alphaGMR." Biochem Biophys Res Commun **337**(2): 551-6.

Dijkers, P. F., T. B. van Dijk, et al. (1999). "Regulation and function of protein kinase B and MAP kinase activation by the IL-5/GM-CSF/IL-3 receptor." Oncogene **18**(22): 3334-3342.

Djordjevic, S. and P. C. Driscoll (2002). "Structural insight into substrate specificity and regulatory mechanisms of phosphoinositide 3-kinases." Trends Biochem Sci **27**(8): 426-32.

Domanski, P. and O. R. Colamonici (1996). "The type-I interferon receptor. The long and short of it." Cytokine Growth Factor Rev **7**(2): 143-51.

Dombrosky-Ferlan, P. M. and S. J. Corey (1997). "Yeast two-hybrid in vivo association of the Src kinase Lyn with the proto-oncogene product Cbl but not with the p85 subunit of PI 3-kinase." Oncogene **14**(17): 2019-24.

Dong, F. and A. C. Larner (2000). "Activation of Akt kinase by granulocyte colony-stimulating factor (G-CSF): evidence for the role of a tyrosine kinase activity distinct from the Janus kinases." Blood **95**(5): 1656-62.

Dorsch, M., P. D. Fan, et al. (1997). "The thrombopoietin receptor can mediate proliferation without activation of the Jak-STAT pathway." Journal of Experimental Medicine **186**(12): 1947-1955.

Doyle, S. E. and J. C. Gasson (1998). "Characterization of the role of the human granulocyte-macrophage colony- stimulating factor receptor alpha subunit in the activation of JAK2 and STAT5." Blood **92**(3): 867-876.

Dranoff, G. (2002). "GM-CSF-based cancer vaccines." Immunol Rev **188**: 147-54.

Durstin, M., R. C. Inhorn, et al. (1996). "Tyrosine phosphorylation of Shc is not required for proliferation or viability signalling by granulocyte-macrophage colony-stimulating factor in hematopoietic cell lines." Journal of Immunology **157**: 534-540.

Ebner, K., A. Bandion, et al. (2003). "GMCSF activates NF-{kappa}B via direct interaction of the GMCSF receptor with I{kappa}B kinase {beta}." Blood **102**(1): 192-199.

Ebner, K., A. Bandion, et al. (2003). "GMCSF activates NF-{kappa}B via direct interaction of the GMCSF-receptor with I{kappa}B kinase {beta}." Blood**.**

Einarson, M. B. (2004). "Detection of protein-protein interactions using the GST fusion protein pull-down technique." Nature Methods **1**: 275-276.

Ellery, J. M. and P. J. Nicholls (2002). "Alternate signalling pathways from the interleukin-2 receptor." Cytokine Growth Factor Rev **13**(1): 27-40.

Emanuel, P. D., L. J. Bates, et al. (1991). "Selective hypersensitivity to granulocyte-macrophage colony-stimulating factor by juvenile chronic myeloid leukemia hematopoietic progenitors." Blood **77**(5): 925-9.

Engelman, J. A., J. Luo, et al. (2006). "The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism." Nat Rev Genet **7**(8): 606-19.

Enzler, T., S. Gillessen, et al. (2003). "Deficiencies of GM-CSF and interferon gamma link inflammation and cancer." J Exp Med **197**(9): 1213-9.

Evans, C. A., S. Ariffin, et al. (2002). "Identification of primary structural features that define the differential actions of IL-3 and GM-CSF receptors." Blood **100**(9): 3164-74.

Evans, T. (1997). "Developmental biology of hematopoiesis." Hematol Oncol Clin North Am **11**(6): 1115-47.

Faderl, S., D. Harris, et al. (2003). "Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces antiapoptotic and proapoptotic signals in acute myeloid leukemia." Blood **102**(2): 630-7.

Feener, E. P., F. Rosario, et al. (2004). "Tyrosine phosphorylation of Jak2 in the JH2 domain inhibits cytokine signaling." Mol Cell Biol **24**(11): 4968-78.

Feng, G. S. (1999). "Shp-2 tyrosine phosphatase: signaling one cell or many." Exp Cell Res **253**(1): 47-54.

Feng, J., B. A. Witthuhn, et al. (1997). "Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop." Mol Cell Biol **17**(5): 2497-2501.

Fischer, E. H., H. Charbonneau, et al. (1991). "Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes." Science **253**: 401-406.

Franke, T. F., D. R. Kaplan, et al. (1997). "Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate." Science **275**(5300): 665-8.

Frantsve, J., J. Schwaller, et al. (2001). "Socs-1 inhibits TEL-JAK2-mediated transformation of hematopoietic cells through inhibition of JAK2 kinase activity and induction of proteasome-mediated degradation." Mol Cell Biol **21**(10): 3547-57.

Freeburn, R. W., R. E. Gale, et al. (1998). "Activating point mutations in the betaC chain of the GM-CSF, IL-3 and IL-5 receptors are not a major contributory factor in the pathogenesis of acute myeloid leukaemia." Br J Haematol **103**(1): 66-71.

Freeburn, R. W., R. E. Gale, et al. (1996). "The beta subunit common to the GM-CSF, IL-3 and IL-5 receptors is highly polymorphic but pathogenic point mutations in patients with acute myeloid leukaemia (AML) are rare." Leukemia **10**(1): 123-129.

Freedman, M. H., T. Grunberger, et al. (1993). "Autocrine and paracrine growth control by granulocyte-monocyte colony-stimulating factor of acute lymphoblastic leukemia cells." Blood **81**(11): 3068-75.

Fresno Vara, J. A. C., M.V. Geronimo, H. Ballmer-Hofer, K. Martin-Perez, J. (2000). "Stimulation of c-Src by prolactin is independent of Jak2." Biochem. J. **345**: 17-24.

Gaidarov, I., M. E. Smith, et al. (2001). "The class II phosphoinositide 3-kinase C2alpha is activated by clathrin and regulates clathrin-mediated membrane trafficking." Mol Cell **7**(2): 443-9.

Galsgaard, E. D., B. N. Friedrichsen, et al. (2001). "Expression of dominant-negative STAT5 inhibits growth hormone- and prolactin-induced proliferation of insulin-producing cells." Diabetes **50 Suppl 1**: S40-1.

Geijsen, N., L. Koenderman, et al. (2001). "Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family." Cytokine Growth Factor Rev **12**(1): 19-25.

Geijsen, N., I. J. Uings, et al. (2001). "Cytokine-specific transcriptional regulation through an IL-5Ralpha interacting protein." Science **293**(5532): 1136-1138.

Ghosh, S. and M. Karin (2002). "Missing pieces in the NF-kappaB puzzle." Cell **109 Suppl**: S81-96.

Ghosh, S., M. J. May, et al. (1998). "NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses." Annu Rev Immunol **16**: 225-60.

Giordanetto, F. and R. T. Kroemer (2002). "Prediction of the structure of human Janus kinase 2 (JAK2) comprising JAK homology domains 1 through 7." Protein Eng **15**(9): 727-37.

Gonda, T. J. and R. J. D'Andrea (1997). "Activating mutations in cytokine receptors: implications for receptor function and role in disease." Blood **89**(2): 355-69.

Gorska, M. M., S. J. Stafford, et al. (2004). "Unc119, a novel activator of Lck/Fyn, is essential for T cell activation." J Exp Med **199**(3): 369-379.

Griesinger, F., H. Hennig, et al. (2005). "A BCR-JAK2 fusion gene as the result of a t(9;22)(p24;q11.2) translocation in a patient with a clinically typical chronic myeloid leukemia." Genes Chromosomes Cancer **44**(3): 329-33.

Grossmann, M., L. A. O'Reilly, et al. (2000). "The anti-apoptotic activities of Rel and RelA required during B-cell maturation involve the regulation of Bcl-2 expression." Embo J **19**(23): 6351-60.

Gualtieri, R. J., P. D. Emanuel, et al. (1989). "Granulocyte-macrophage colony-stimulating factor is an endogenous regulator of cell proliferation in juvenile chronic myelogenous leukemia." Blood **74**(7): 2360-7.

Guthridge, M. A., E. F. Barry, et al. (2004). "The phosphoserine-585-dependent pathway of the GM-CSF/IL-3/IL-5 receptors mediates hematopoietic cell survival through activation of NF-kappaB and induction of bcl-2." Blood **103**(3): 820-827.

Guthridge, M. A., J. A. Powell, et al. (2006). "Growth factor pleiotropy is controlled by a receptor Tyr/Ser motif that acts as a binary switch." Embo J **25**(3): 479-89.

Guthridge, M. A., F. C. Stomski, et al. (2000). "Site-specific serine phosphorylation of the IL-3 receptor is required for hemopoietic cell survival." Mol Cell **6**(1): 99-108.

Guthridge, M. A., F. C. Stomski, et al. (1998). "Mechanism of activation of the GM-CSF, IL-3, and IL-5 family of receptors." Stem Cells **16**(5): 301-313.

Haan, C., P. C. Heinrich, et al. (2002). "Structural requirements of the interleukin-6 signal transducer gp130 for its interaction with Janus kinase 1: the receptor is crucial for kinase activation." Biochem J **361**(Pt 1): 105-111.

Haan, C., H. Is'harc, et al. (2001). "Mapping of a region within the N terminus of Jak1 involved in cytokine receptor interaction." J Biol Chem **276**(40): 37451-37458.

Haan, C., S. Kreis, et al. (2006). "Jaks and cytokine receptors-An intimate relationship." Biochem Pharmacol **72**(11): 1538-46.

Hamilton, J. A., E. R. Stanley, et al. (1980). "Stimulation of macrophage plasminogen activator activity by colony-stimulating factors." J Cell Physiol **103**(3): 435-45.

Handman, E. and A. W. Burgess (1979). "Stimulation by granulocyte-macrophage colony-stimulating factor of Leishmania tropica killing by macrophages." J Immunol **122**(3): 1134-7.

Harder, K. W., L. M. Parsons, et al. (2001). "Gain- and loss-of-function Lyn mutant mice define a critical inhibitory role for Lyn in the myeloid lineage." Immunity **15**(4): 603-615.

Harder, K. W., C. Quilici, et al. (2004). "Perturbed myelo/erythropoiesis in Lyn-deficient mice is similar to that in mice lacking the inhibitory phosphatases SHP-1 and SHIP-1." Blood **104**(13): 3901-3910.

Hayashida, K., T. Kitamura, et al. (1990). "Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor." **87**(24): 9655-9659.

Hernandez-Hansen, V., G. A. Mackay, et al. (2004). "The Src kinase Lyn is a negative regulator of mast cell proliferation." J Leukoc Biol **75**(1): 143-51.

Hibbs, M. L. and K. W. Harder (2006). "The duplicitous nature of the Lyn tyrosine kinase in growth factor signaling." Growth Factors **24**(2): 137-49.

Higashide, T. and G. Inana (1999). "Characterization of the gene for HRG4 (UNC119), a novel photoreceptor synaptic protein homologous to unc-119." Genomics **57**(3): 446-50.

Hines, S. J., J. S. Litz, et al. (1999). "Coexpression of c-kit and stem cell factor in breast cancer results in enhanced sensitivity to members of the EGF family of growth factors." Breast Cancer Res Treat **58**(1): 1-10.

Ho, A. S., Y. Liu, et al. (1993). "A receptor for interleukin 10 is related to interferon receptors." Proc Natl Acad Sci U S A **90**(23): 11267-71.

Ihle, J. N. (1995). "Cytokine receptor signalling." Nature **377**(6550): 591-594.

Ihle, J. N. (2001). "The Stat family in cytokine signaling." Curr Opin Cell Biol **13**(2): 211-7.

Ihle, J. N., D. Stravapodis, et al. (1998). "The roles of Jaks and Stats in cytokine signaling." Cancer J Sci Am **4 Suppl 1**: S84-91.

Ihle, J. N., B. Witthuhn, et al. (1994). "Cytokine receptors and signal transduction." **7**(1): 17-48.

Ihle, J. N., B. A. Witthuhn, et al. (1995). "Signaling through the hematopoietic cytokine receptors." **13**: 369-398.

Ikeda, H., Y. Kanakura, et al. (1991). "Expression and functional role of the proto-oncogene c-kit in acute myeloblastic leukemia cells." Blood **78**(11): 2962-8.

Ilaria, R. L., Jr., R. G. Hawley, et al. (1999). "Dominant negative mutants implicate STAT5 in myeloid cell proliferation and neutrophil differentiation." Blood **93**(12): 4154-4166.

Ingley, E., D. J. McCarthy, et al. (2005). "Lyn deficiency reduces GATA-1, EKLF and STAT5, and induces extramedullary stress erythropoiesis." Oncogene **24**(3): 336-343.

Irie-Sasaki, J., T. Sasaki, et al. (2001). "CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling." Nature **409**(6818): 349-54.

Irie-Sasaki, J., T. Sasaki, et al. (2003). "CD45 regulated signaling pathways." Curr Top Med Chem **3**(7): 783-96.

Irish, J. M., R. Hovland, et al. (2004). "Single cell profiling of potentiated phospho-protein networks in cancer cells." Cell **118**(2): 217-228.

Ishihara, K. and T. Hirano (2002). "Molecular basis of the cell specificity of cytokine action." Biochim Biophys Acta **1592**(3): 281-96.

Itoh, T., R. Liu, et al. (1998). "Definition of the role of tyrosine residues of the common beta subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor." Mol Cell Biol **18**(2): 742-752.

Jager, E., D. Jager, et al. (2003). "Antigen-specific immunotherapy and cancer vaccines." Int J Cancer **106**(6): 817-20.

James, C., V. Ugo, et al. (2005). "A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera." Nature **434**(7037): 1144-8.

Jenkins, B. J., T. J. Blake, et al. (1998). "Saturation mutagenesis of the beta subunit of the human granulocyte-macrophage colony-stimulating factor receptor shows clustering of constitutive mutations, activation of ERK MAP kinase and STAT pathways, and differential beta subunit tyrosine phosphorylation." Blood **92**(6): 1989-2002.

Jenkins, B. J., D'Andrea, R., Gonda, T.J. (1995). "Activating point mutations in the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors suggest the involvement of beta subunit dimerisation and cell-type specific molecules in signalling." EMBO **14**: 4276-4287.

Jenkins, B. J., F. Le, et al. (1999). "A cell type-specific constitutive point mutant of the common beta-subunit of the human granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5 receptors requires the GM-CSF receptor alpha-subunit for activation." J Biol Chem **274**(13): 8669-8677.

Johan, M. F., D. T. Bowen, et al. (2005). "Aberrant methylation of the negative regulators RASSFIA, SHP-1 and SOCS-1 in myelodysplastic syndromes and acute myeloid leukaemia." Br J Haematol **129**(1): 60-5.

Johnson, G. R., T. J. Gonda, et al. (1989). "A lethal myeloproliferative syndrome in mice transplanted with bone marrow cells infected with a retrovirus expressing granulocyte-macrophage colony stimulating factor." EMBO **8**: 441-448.

Jordan, C. T., D. Upchurch, et al. (2000). "The interleukin-3 receptor alpha chain is a unique marker for human acute myelogenous leukemia stem cells." Leukemia **14**(10): 1777-1784.

Kamakura, S., T. Moriguchi, et al. (1999). "Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus." J Biol Chem **274**(37): 26563-71.

Kamizono, S., T. Hanada, et al. (2001). "The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2." J Biol Chem **276**(16): 12530-8.

Kamura, T., S. Sato, et al. (1998). "The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families." Genes Dev **12**(24): 3872-81.

Karaghiosoff, M., H. Neubauer, et al. (2000). "Partial impairment of cytokine responses in Tyk2-deficient mice." Immunity **13**(4): 549-60.

Karin, M., Y. Cao, et al. (2002). "NF-kappaB in cancer: from innocent bystander to major culprit." Nat Rev Cancer **2**(4): 301-10.

Katso, R., K. Okkenhaug, et al. (2001). "Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer." Annu Rev Cell Dev Biol **17**: 615-75.

Kaushansky, K. (2005). "On the molecular origins of the chronic myeloproliferative disorders: it all makes sense." Hematology (Am Soc Hematol Educ Program): 533-7.

Kaushansky, K. (2006). "Hematopoietic growth factors, signaling and the chronic myeloproliferative disorders." Cytokine Growth Factor Rev.

Kay, B. K., M. P. Williamson, et al. (2000). "The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains." FASEB J **14**(2): 231-241.

Khoury, J. D., G. Z. Rassidakis, et al. (2004). "Methylation of SHP1 gene and loss of SHP1 protein expression are frequent in systemic anaplastic large cell lymphoma." Blood **104**(5): 1580-1.

Khwaja, A. (2006). "The role of Janus kinases in haemopoiesis and haematological malignancy." Br J Haematol **134**(4): 366-84.

Kieslinger, M., I. Woldman, et al. (2000). "Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation." Genes Dev **14**(2): 232-44.

Kishimoto, T. T., T. Akira, S. (1994). "Cytokine signal transduction." Cell **76**: 253-262.

Kitamura, T., N. Sato, et al. (1991). "Expression cloning of the human IL-3 receptor cDNA reveals a shared á subunit for the human IL-3 and GM-CSF receptors." Cell **66**: 1165-1174.

Klippel, A., W. M. Kavanaugh, et al. (1997). "A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain." Mol Cell Biol **17**(1): 338-44.

Koegl, M., P. Zlatkine, et al. (1994). "Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif." Biochem J **303 (Pt 3)**: 749-53.

Kohlhuber, F., N. C. Rogers, et al. (1997). "A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses." Mol Cell Biol **17**(2): 695-706.

Kottaridis, P. D., R. E. Gale, et al. (2001). "The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials." Blood **98**(6): 1752-9.

Kralovics, R., F. Passamonti, et al. (2005). "A gain-of-function mutation of JAK2 in myeloproliferative disorders." N Engl J Med **352**(17): 1779-90.

Krutzik, P. O. and G. P. Nolan (2003). "Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events." Cytometry A **55**(2): 61-70.

Kyriakis, J. M. and J. Avruch (2001). "Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation." Physiol Rev **81**(2): 807-69.

Lacronique, V., A. Boureux, et al. (1997). "A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia." Science **278**: 1309-1311.

Lee, H. H., H. Dadgostar, et al. (1999). "NF-kappaB-mediated up-regulation of Bcl-x and Bfl-1/A1 is required for CD40 survival signaling in B lymphocytes." Proc Natl Acad Sci U S A **96**(16): 9136-41.

Lerner, E. C., R. P. Trible, et al. (2005). "Activation of the Src family kinase Hck without SH3-linker release." J Biol Chem **280**(49): 40832-7.

LeVine, A. M., J. A. Reed, et al. (1999). "GM-CSF-deficient mice are susceptible to pulmonary group B streptococcal infection." J Clin Invest **103**(4): 563-9.

Levine, R. L., M. Wadleigh, et al. (2005). "Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis." Cancer Cell **7**(4): 387-97.

Lewis, T. S., P. S. Shapiro, et al. (1998). "Signal transduction through MAP kinase cascades." Adv Cancer Res **74**: 49-139.

Li, Y., SHen, B., Karanes, C., Snsenbrenner, L. & Chen, B. (1995). "Association between Lyn protein tyrosine kinase (p53/p56Lyn) and the B subunit of granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors in a GM-CSF dependent human megakaryocyte leukemia cell line (MO7e)." Journal of Immunology **154**: 2165-2174.

Li, Z., D. Metze, et al. (2004). "Expression of SOCS-1, suppressor of cytokine signalling-1, in human melanoma." J Invest Dermatol **123**(4): 737-45.

Lia, F., D. Rajotte, et al. (1996). "A dominant negative granulocyte-macrophage colony-stimulating factor receptor alpha chain reveals the multimeric structure of the receptor complex." **271**(45): 28287-28293.

Linnekin, D., O. M. Howard, et al. (1994). "Hck expression correlates with granulocyte-macrophage colony- stimulating factor-induced proliferation in HL-60 cells." Blood **84**(1): 94-103.

Liu, B., J. Liao, et al. (1998). "Inhibition of Stat1-mediated gene activation by PIAS1." Proc Natl Acad Sci U S A **95**(18): 10626-31.

Liu, C. B., T. Itoh, et al. (1999). "Constitutive activation of JAK2 confers murine interleukin-3-independent survival and proliferation of BA/F3 cells." J Biol Chem **274**(10): 6342-9.

Liu, R. Y., C. Fan, et al. (1999). "Constitutive activation of the JAK2/STAT5 signal transduction pathway correlates with growth factor independence of megakaryocytic leukemic cell lines." Blood **93**(7): 2369-2379.

Livnah, O., E. A. Stura, et al. (1999). "Crystallographic evidence for preformed dimers of erythropoietin receptor before ligand activation." Science **283**(5404): 987-90.

Longley, B. J. (1999). "What dermatologists need to know about mast cell disease: a dermatopathologist's view." Cutis **64**(4): 281-2.

Longley, B. J., L. Tyrrell, et al. (1996). "Somatic c-KIT activating mutation in urticaria pigmentosa and aggressive mastocytosis: establishment of clonality in a human mast cell neoplasm." Nat Genet **12**(3): 312-4.

Luo, H. R., P. Barber, D. Hanratty, W.P. Lee, S. Roberts, T.M. D'Andrea, A.D. Dearolf, C.R. (1997). "Mutation in the Jak kinase JH2 domain hyperactivates Drosophila and mammalian Jak-Stat pathways." Molecular Cell Biology **17**(3): 1562-1571.

Lynch, B. A., C. Minor, et al. (1999). "Simultaneous assay of Src SH3 and SH2 domain binding using different wavelength fluorescence polarization probes." Anal Biochem **275**(1): 62-73.

Lynch, J. P. and D. G. Silberg (2002). "To differentiate or proliferate? The interaction between PI3K/PTEN and Cdx2." Gastroenterology **123**(4): 1395-7.

Maehama, T. D., J.E. (1999). "PTEN: a tumor suppressor that functions as a phospholipid phosphatase." Trends in Cell Biology **9**: 125-128.

Mano, H. (1999). "Tec family of protein-tyrosine kinases: an overview of their structure and function." Cytokine Growth Factor Rev **10**: 267-280.

Marengere, L. E., Z. Songyang, et al. (1994). "SH2 domain specificity and activity modified by a single residue." Nature **369**(6480): 502-5.

Martinez-Moczygemba, M. and D. P. Huston (2003). "Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF." J Allergy Clin Immunol **112**(4): 653-65; quiz 666.

Matsuguchi, T., M. B. Lilly, et al. (1998). "Cytoplasmic domains of the human granulocyte-macrophage colony- stimulating factor (GM-CSF) receptor beta chain (hbetac) responsible for human GM-CSF-induced myeloid cell differentiation." J Biol Chem **273**(31): 19411-19418.

Matsuguchi, T., Y. Zhao, et al. (1997). "The cytoplasmic domain of granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor à subunit is essential for both GM-CSF-mediated growth and differentiation." J Biol Chem **272**: 17450-17459.

Matsuoka, H., S. Nada, et al. (2004). "Mechanism of Csk-mediated down-regulation of Src family tyrosine kinases in epidermal growth factor signaling." J Biol Chem **279**(7): 5975-83.

Mayer, B. J. and D. Baltimore (1993). "Signalling through SH2 and SH3 domains." Trends Cell Biol **3**(1): 8-13.

Mayer, B. J. and R. Gupta (1998). "Functions of SH2 and SH3 domains." Curr Top Microbiol Immunol **228**: 1-22.

Mayer, B. J., M. Hamaguchi, et al. (1988). "A novel viral oncogene with structural similarity to phospholipase C." Nature **332**(6161): 272-5.

McClure, B. J., J. M. Woodcock, et al. (2001). "GM-CSF binding to its receptor induces oligomerisation of the common beta-subunit." Cytokine **13**(4): 240-243.

McCormack, M. and T. J. Gonda (1997). "Expression of activated mutants of the human interleukin-3/interleukin-5/granulocyte-macrophage colony-stimulating factor receptor common beta subunit in primary hematopoietic cells induces factor-independent proliferation and differentiation." Blood **90**(4): 1471-81.

McCormack, M. P. and T. J. Gonda (1999). "Myeloproliferative disorder and leukaemia induced in mice by different classes of constitutive mutants of the human IL-3/IL-5/GM-CSF receptor common á subunit." Oncogene **18**: 7190-7199.

McCormack, M. P. and T. J. Gonda (2000). "Novel murine myeloid cell lines which exhibit a differentiation switch in response to IL-3 or GM-CSF, or to different constitutively active mutants of the GM-CSF receptor á subunit." Blood **95**: 120-127.

Meierhoff, G., U. Dehmel, et al. (1995). "Expression of FLT3 receptor and FLT3-ligand in human leukemia-lymphoma cell lines." Leukemia **9**(8): 1368-72.

Metcalf, D. (1984). The haemopoietic colony stimulating factors. Amsterdam, Elsevier/North Holland Biomedical Press.

Metcalf, D. and N. A. Nicola (1995). The hemopoietic colony-stimulating factors: from biological to clinical applications. New York, Cambridge University Press.

Meydan, N., T. Grunberger, et al. (1996). "Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor." Nature **379**(6566): 645-648.

Minami, M., M. Inoue, et al. (1996). "STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line." Proceedings of the National Academy of Science USA **93**: 3963-3966.

Moarefi, I., M. LaFevre-Bernt, et al. (1997). "Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement [see comments]." Nature **385**(6617): 650-653.

Mui, A. L., H. Wakao, et al. (1996). "Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation." Embo J **15**(10): 2425-33.

Murati, A., V. Gelsi-Boyer, et al. (2005). "PCM1-JAK2 fusion in myeloproliferative disorders and acute erythroid leukemia with t(8;9) translocation." Leukemia **19**(9): 1692-6.

Murohashi, I., S. Tohda, et al. (1989). "Specific binding of radioiodinated human GM-CSF to the blast cells of acute myeloblastic leukemia." Leuk Res **13**(7): 599-604.

Nabel, E. G. (2002). "CDKs and CKIs: molecular targets for tissue remodelling." Nat Rev Drug Discov **1**(8): 587-98.

Nagata, H., A. S. Worobec, et al. (1995). "Identification of a point mutation in the catalytic domain of the protooncogene c-kit in peripheral blood mononuclear cells of patients who have mastocytosis with an associated hematologic disorder." Proc Natl Acad Sci U S A **92**(23): 10560-4.

Nakajima, K., Y. Yamanaka, et al. (1996). "A central role for Stat3 in IL-6-induced regulation of growth and differentiation of M1 leukaemia cells." **14**: 3651-3658.

Nakamura, T., R. Ouchida, et al. (2002). "Cytokine receptor common beta subunit-mediated STAT5 activation confers NF-kappa B activation in murine proB cell line Ba/F3 cells" **277**(8): 6254-6265.

Nakanishi, S., S. Kakita, et al. (1992). "Wortmannin, a microbial product inhibitor of myosin light chain kinase." J Biol Chem **267**(4): 2157-63.

Naoe, T. and H. Kiyoi (2004). "Normal and oncogenic FLT3." Cell Mol Life Sci **61**(23): 2932-2938.

Neel, B. G., H. Gu, et al. (2003). "The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling." Trends Biochem Sci **28**(6): 284-93.

Nelson, B. H., J. D. Lord, et al. (1996). "A membrane-proximal region of the interleukin-2 receptor gamma c chain sufficient for Jak kinase activation and induction of proliferation in T cells." Mol Cell Biol **16**(1): 309-17.

Nelson, B. H., B. C. McIntosh, et al. (1997). "Requirement for an initial signal from the membrane-proximal region of the interleukin 2 receptor gamma(c) chain for Janus kinase activation leading to T cell proliferation." Proc Natl Acad Sci USA **94**(5): 1878-1883.

Neubauer, H., A. Cumano, et al. (1998). "Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis." Cell **93**(3): 397-409.

Nicola, N. A. (1994). Guidebook to Cytokines and Their Receptors. New York, Oxford University Press, Sambrook and Tooze Publications.

Ning, Z. Q., J. Li, et al. (2001). "Activating mutations of c-kit at codon 816 confer drug resistance in human leukemia cells." Leuk Lymphoma **41**(5-6): 513-22.

Nishida, E. and Y. Gotoh (1993). "The MAP kinase cascade is essential for diverse signal transduction pathways." Trends Biochem Sci **18**(4): 128-31.

Nishida, K. and T. Hirano (2003). "The role of Gab family scaffolding adapter proteins in the signal transduction of cytokine and growth factor receptors." Cancer Sci **94**(12): 1029-33.

Nishimoto, S. and E. Nishida (2006). "MAPK signalling: ERK5 versus ERK1/2." EMBO Rep **7**(8): 782-6.

Nobukuni, T., M. Joaquin, et al. (2005). "Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase." Proc Natl Acad Sci U S A **102**(40): 14238-43.

Noguchi, Y., H. Wada, et al. (1998). "Regulation of IFN-gamma production in granulocyte-macrophage colony-stimulating factor-deficient mice." Eur J Immunol **28**(12): 3980-8.

Nosaka, T., T. Kawashima, et al. (1999). "STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells." Embo J **18**(17): 4754-65.

Nosaka, T. and T. Kitamura (2000). "Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) in hematopoietic cells." Int J Hematol **71**(4): 309-19.

Nosaka, T., J. M. van Deursen, et al. (1995). "Defective lymphoid development in mice lacking Jak3." Science **270**(5237): 800-2.

Odai, H., K. Sasaki, et al. (1995). "The proto-oncogene product c-Cbl becomes tyrosine phosphorylated by stimulation with GM-CSF or Epo and constitutively binds to the SH3 domain of Grb2/Ash in human hematopoietic cells." J Biol Chem **270**(18): 10800-5.

Odom, S., G. Gomez, et al. (2004). "Negative regulation of immunoglobulin E-dependent allergic responses by Lyn kinase." J Exp Med **199**(11): 1491-502.

Odorizzi, G., M. Babst, et al. (2000). "Phosphoinositide signaling and the regulation of membrane trafficking in yeast." Trends Biochem Sci **25**(5): 229-35.

Ogata, N., T. Kouro, et al. (1998). "JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor alpha and betac subunit, respectively, and are activated upon IL-5 stimulation." Blood **91**(7): 2264-71.

Oka, T., M. Ouchida, et al. (2002). "Gene silencing of the tyrosine phosphatase SHP1 gene by aberrant methylation in leukemias/lymphomas." Cancer Res **62**(22): 6390-4.

Okuda, K., L. Smith, et al. (1997). "Signaling functions of the tyrosine residues in the ác chain of the granulocyte-macrophage colony-stimulating factor receptor." Blood **90**(12): 4759-4766.

O'Shea, J. J. (1997). "Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet?" Immunity **7**(1): 1-11.

Ostman, A., C. Hellberg, et al. (2006). "Protein-tyrosine phosphatases and cancer." Nat Rev Cancer **6**(4): 307-20.

Ozaki, K. and W. J. Leonard (2002). "Cytokine and cytokine receptor pleiotropy and redundancy." J Biol Chem **277**(33): 29355-8.

Paine, R., 3rd, A. M. Preston, et al. (2000). "Granulocyte-macrophage colony-stimulating factor in the innate immune response to Pneumocystis carinii pneumonia in mice." J Immunol **164**(5): 2602-9.

Parganas, E., D. Wang, et al. (1998). "Jak2 is essential for signaling through a variety of cytokine receptors." Cell **93**(3): 385-395.

Park, S. H. and R. T. Raines (2004). "Fluorescence polarization assay to quantify protein-protein interactions." Methods Mol Biol **261**: 161-6.

Park, S. Y., K. Saijo, et al. (1995). "Developmental defects of lymphoid cells in Jak3 kinase-deficient mice." Immunity **3**(6): 771-82.

Passegue, E., C. H. Jamieson, et al. (2003). "Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics?" Proc Natl Acad Sci U S A **100 Suppl 1**: 11842-9.

Pawson, T. (1995). "Protein modules and signalling networks." Nature **373**: 573-580.

Pawson, T. and G. D. Gish (1992). "SH2 and SH3 domains: from structure to function." Cell **71**(3): 359-62.

Pawson, T. and J. Schlessingert (1993). "SH2 and SH3 domains." Curr Biol **3**(7): 434-42.

Pearson, G., F. Robinson, et al. (2001). "Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions." Endocr Rev **22**(2): 153-83.

Peeters, P., S. D. Raynaud, et al. (1997). "Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor- associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia." Blood **90**(7): 2535-2540.

Pellegrini, S. and I. Dusanter-Fourt (1997). "The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs)." Eur J Biochem **248**(3): 615-33.

Persons, D. A., M. G. Mehaffey, et al. (1998). "An improved method for generating retroviral producer clones for vectors lacking a selectable marker gene." Blood Cells Mol Dis. **24**(2): 167-182.

Pestka, S., S. V. Kotenko, et al. (1997). "The interferon gamma (IFN-gamma) receptor: a paradigm for the multichain cytokine receptor." Cytokine Growth Factor Rev **8**(3): 189-206.

Pierce, J. W., R. Schoenleber, et al. (1997). "Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo." J Biol Chem **272**(34): 21096-103.

Platanias, L. C. (2003). "Map kinase signaling pathways and hematologic malignancies." Blood **101**(12): 4667-79.

Polotskaya, A., Y. Zhao, et al. (1994). "Mapping the intracytoplasmic regions of the alpha granulocyte- macrophage colony-stimulating factor receptor necessary for cell growth regulation." **269**: 14607-14613.

Polotskaya, A., Y. Zhao, et al. (1993). "A critical role for the cytoplasmic domain of the granulocyte-macrophage colony-stimulating factor alpha receptor in mediating cell growth." Cell Growth Differ **4**(6): 523-31.

Quelle, D. E., F. W. Quelle, et al. (1992). "Mutations in the WSAWSE and cytosolic domains of the erythropoietin receptor affect signal transduction and ligand binding and internalization." Mol Cell Biol. **12**: 4553-4561.

Quelle, F. W., N. Sato, et al. (1994). "JAK2 associates with the ác chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region." Molecular and cellular Biology **14**: 4335-4341.

Rabinovitch, P. S., R. M. Torres, et al. (1986). "Simultaneous cell cycle analysis and two-color surface immunofluorescence using 7-amino-actinomycin D and single laser excitation: applications to study of cell activation and the cell cycle of murine Ly-1 B cells." J Immunol **136**(8): 2769-75.

Radomska, H. S., D. S. Basseres, et al. (2006). "Block of C/EBP alpha function by phosphorylation in acute myeloid leukemia with FLT3 activating mutations." J Exp Med **203**(2): 371-81.

Rane, S. G. and E. P. Reddy (2002). "JAKs, STATs and Src kinases in hematopoiesis." Oncogene **21**(21): 3334-58.

Rao, P., & Mufson, R.A. (1995). "A membrane proximal domain of th human interleukin-3 receptor beta subunit that signals DNA synthesis in NIH 3T3 cells specifically binds a complex of Src and Janus family tyrosine kinases and phosphatidylinositol 3-kinase." Journal of Biological Chemistry **270**: 6886-6893.

Rawlings, J. S., K. M. Rosler, et al. (2004). "The JAK/STAT signaling pathway." J Cell Sci **117**(Pt 8): 1281-1283.

Rayet, B. and C. Gelinas (1999). "Aberrant rel/nfkb genes and activity in human cancer." Oncogene **18**(49): 6938-47.

Reddy, P. E., Korapati, Anita., Chaturvedi, Priya. & Rane, Sushil. (2000). "IL-3 signaling and the role of Src kinases, JAKs and STATs: a covert liaison unveiled." Oncogene **19**: 2532-2547.

Reilly, J. T. (2003). "Receptor tyrosine kinases in normal and malignant haematopoiesis." **17**(4): 241-248.

Reiter, A., C. Walz, et al. (2005). "The t(8;9)(p22;p24) is a recurrent abnormality in chronic and acute leukemia that fuses PCM1 to JAK2." Cancer Res **65**(7): 2662-7.

Resh, M. D. (1999). "Fatty acylation of proteins: new insights into membrane targeting of myristoylated and palmitoylated proteins." **1451**(1): 1-16.

Richardson, C. J., S. S. Schalm, et al. (2004). "PI3-kinase and TOR: PIKTORing cell growth." Semin Cell Dev Biol **15**(2): 147-59.

Richter, M. F., G. Dumenil, et al. (1998). "Specific contribution of Tyk2 JH regions to the binding and the expression of the interferon alpha/beta receptor component IFNAR1." J Biol Chem **273**(38): 24723-9.

Rickles, R. J., M. C. Botfield, et al. (1995). "Phage display selection of ligand residues important for Src homology 3 domain binding specificity." Proc Natl Acad Sci U S A **92**(24): 10909-13.

Robb, L., C. C. Drinkwater, et al. (1995). "Hematopoietic and lung abnormalities in mice with a null mutation of the common beta subunit of the receptors for granulocyte-macrophage colony-stimulating factor and interleukins 3 and 5." **92**: 9565-9569.

Robinson, M. J. and M. H. Cobb (1997). "Mitogen-activated protein kinase pathways." Curr Opin Cell Biol **9**(2): 180-6.

Rodig, S. J., M. A. Meraz, et al. (1998). "Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses." Cell **93**(3): 373-383.

Ronco, L. V., S. E. Doyle, et al. (1995). "Conserved amino acids in the human granulocyte-macrophage colony- stimulating factor receptor-binding subunit essential for tyrosine phosphorylation and proliferation." Journal of Immunology **154**: 3444-3453.

Rubin, G. M., M. D. Yandell, et al. (2000). "Comparative genomics of the eukaryotes." Science **287**(5461): 2204-15.

Russell, R. B., J. Breed, et al. (1992). "Conservation analysis and structure prediction of the SH2 family of phosphotyrosine binding domains." FEBS Lett **304**(1): 15-20.

Sadowski, I., J. C. Stone, et al. (1986). "A noncatalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130gag-fps." Mol Cell Biol **6**(12): 4396-408.

Saharinen, P. and O. Silvennoinen (2002). "The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction." J Biol Chem **277**(49): 47954-63.

Saharinen, P., M. Vihinen, et al. (2003). "Autoinhibition of Jak2 tyrosine kinase is dependent on specific regions in its pseudokinase domain." Mol Biol Cell **14**(4): 1448-59.

Saharinen, P. T. (2000). "Regulation of the Jak2 tyrosine kinase by its pseudokinase domain." Molecular Cell Biology **20**(10): 3387-3395.

Saharinen, P. T., K. Silvennoinen, O. (2000). "Regulation of the Jak2 tyrosine kinase by its pseudokinase domain." Molecular Cell Biology **20**(10): 3387-3395.

Sakamaki, K., I. Miyajima, et al. (1992). "Critical cytoplasmic domains of the common beta subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation." EMBO J **11**(10): 3541-3549.

Sambrook, J., E. F. Fritsch, et al. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sandberg, E. M., X. Ma, et al. (2005). "Identification of 1,2,3,4,5,6-hexabromocyclohexane as a small molecule inhibitor of jak2 tyrosine kinase autophosphorylation [correction of autophophorylation]." J Med Chem **48**(7): 2526-33.

Scheijen, B. and J. D. Griffin (2002). "Tyrosine kinase oncogenes in normal hematopoiesis and hematological disease." Oncogene **21**(21): 3314-33.

Scott, C. L., D. A. Hughes, et al. (1998). "Functional analysis of mature hematopoietic cells from mice lacking the betac chain of the granulocyte-macrophage colony-stimulating factor receptor." Blood **92**(11): 4119-27.

Sharfe, N., H. K. Dadi, et al. (1995). "JAK3 protein tyrosine kinase mediates interleukin-7-induced activation of phosphatidylinositol-3' kinase." Blood **86**(6): 2077-85.

Sheng, W. (1996). "Critical role of Lyn kinase in inhibition of neutrophil apoptosis by granulocyte-macrophage colony-stimulating factor." Journal of Immunology **157**: 5155-5162.

Sheng, W., Liu, J.H., Epling-Burnette, P.K. et al (1996). "Critical role of Lyn kinase in inhibition of neutrophil apoptosis by granulocyte-macrophage colony-stimulating factor." Journal of Immunology **157**: 5155-5162.

Shi, Z. Q., D. H. Yu, et al. (2000). "Molecular mechanism for the Shp-2 tyrosine phosphatase function in promoting growth factor stimulation of Erk activity." Mol Cell Biol **20**(5): 1526-36.

Shimoda, K., K. Kato, et al. (2000). "Tyk2 plays a restricted role in IFN alpha signaling, although it is required for IL-12-mediated T cell function." Immunity **13**(4): 561-71.

Shimozaki, K., K. Nakajima, et al. (1997). "Involvement of STAT3 in the granulocyte colony-stimulating factor-induced differentiation of myeloid cells." J Biol Chem **272**(40): 25184-9.

Shuai, K. (2000). "Modulation of STAT signaling by STAT-interacting proteins." Oncogene **19**(21): 2638-44.

Silverman, N. and T. Maniatis (2001). "NF-kappaB signaling pathways in mammalian and insect innate immunity." Genes Dev **15**(18): 2321-42.

Simon, H. U., S. Yousefi, et al. (1997). "Anti-apoptotic signals of granulocyte-macrophage colony-stimulating factor are transduced via Jak2 tyrosine kinase in eosinophils." Eur J Immunol **27**(12): 3536-9.

Smithgall, T. E., S. D. Briggs, et al. (2000). "Control of myeloid differentiation and survival by Stats." Oncogene **19**(21): 2612-8.

Spandidos, D. A. and M. L. Anderson (1990). "A role of ras oncogenes in carcinogenesis and differentiation." Adv Exp Med Biol **265**: 127-31.

Spandidos, D. A., G. Sourvinos, et al. (2002). "Normal ras genes: their onco-suppressor and pro-apoptotic functions (review)." Int J Oncol **21**(2): 237-41.

Staerk, J., A. Kallin, et al. (2006). "JAK2, the JAK2 V617F mutant and cytokine receptors." Pathol Biol (Paris).

Stafford, S., C. Lowell, et al. (2002). "Lyn tyrosine kinase is important for IL-5-stimulated eosinophil differentiation." J Immunol **168**(4): 1978-83.

Stanley, E., G. J. Lieschke, et al. (1994). "Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology." Proc Natl Acad Sci U S A **91**(12): 5592-6.

Stein, R. C. (2001). "Prospects for phosphoinositide 3-kinase inhibition as a cancer treatment." Endocr Relat Cancer **8**(3): 237-48.

Stomski, F., Q. Sun, et al. (1996). "Human interleukin-3 (IL-3) induces disulphide-linked IL-3 receptor à and á chain heterodimerization which is required for receptor activation but not high affinity binding." Mol Cell Biol. **16**: 3035-3046.

Sturgill, T. W. and J. Wu (1991). "Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6." Biochim Biophys Acta **1092**(3): 350-7.

Suh, H. S., M. O. Kim, et al. (2005). "Inhibition of granulocyte-macrophage colony-stimulating factor signaling and microglial proliferation by anti-CD45RO: role of Hck tyrosine kinase and phosphatidylinositol 3-kinase/Akt." J Immunol **174**(5): 2712-2719.

Sun, S. C. and G. Xiao (2003). "Deregulation of NF-kappaB and its upstream kinases in cancer." Cancer Metastasis Rev **22**(4): 405-22.

Suzuki, K., H. Nakajima, et al. (2000). "Role of common cytokine receptor gamma chain (gamma(c))- and Jak3-dependent signaling in the proliferation and survival of murine mast cells." Blood **96**(6): 2172-80.

Sweet, M. J. and D. A. Hume (2003). "CSF-1 as a regulator of macrophage activation and immune responses." Arch Immunol Ther Exp (Warsz) **51**(3): 169-77.

Taga, T. and T. Kishimoto (1995). "Signaling mechanisms through cytokine receptors that share signal transducing receptor components." **7**(1): 17-23.

Takai, Y., T. Sasaki, et al. (2001). "Small GTP-binding proteins." Physiol Rev **81**(1): 153-208.

Takaki, S., H. Kanazawa, et al. (1994). "A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor alpha chain and its function in IL-5-mediated growth signal transduction." Mol Cell Biol **14**(11): 7404-7413.

Tanaka, H., N. Fujita, et al. (2005). "3-Phosphoinositide-dependent protein kinase-1-mediated IkappaB kinase beta (IkkB) phosphorylation activates NF-kappaB signaling." J Biol Chem **280**(49): 40965-73.

Taniguchi, T. (1995). Science **268**: 251-255.

Tanner, J. W., W. Chen, et al. (1995). "The conserved box 1 motif of cytokine receptors is required for association with JAK kinases." **270**: 6523-6530.

Tao, M. H. and R. Levy (1993). "Idiotype/granulocyte-macrophage colony-stimulating factor fusion protein as a vaccine for B-cell lymphoma." Nature **362**(6422): 755-8.

Tarr, P. E., R. Lin, et al. (1996). "Evaluation of tolerability and antibody response after recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and a single dose of recombinant hepatitis B vaccine." Vaccine **14**(13): 1199-204.

Tatosyan, A. G. and O. A. Mizenina (2000). "Kinases of the Src family: structure and functions." Biochemistry (Mosc) **65**(1): 49-58.

Theze, J., P. M. Alzari, et al. (1996). "Interleukin 2 and its receptors: recent advances and new immunological functions." Immunol Today **17**(10): 481-6.

Thomas, S. M. and J. S. Brugge (1997). "Cellular functions regulated by Src family kinases." Annu Rev Cell Dev Biol **13**: 513-609.

Thomis, D. C., W. Lee, et al. (1997). "T cells from Jak3-deficient mice have intact TCR signaling, but increased apoptosis." Journal of Immunology **159**(10): 4708-4719.

Tian, Q., H. F. Frierson, Jr., et al. (1999). "Activating c-kit gene mutations in human germ cell tumors." Am J Pathol **154**(6): 1643-7.

Tilbrook, P. A., E. Ingley, et al. (1997). "Lyn tyrosine kinase is essential for erythropoietin-induced differentiation of J2E erythroid cells." EMBO J **16**(7): 1610-1619.

Tonks, N. K. (2006). "Protein tyrosine phosphatases: from genes, to function, to disease." Nat Rev Mol Cell Biol **7**(11): 833-46.

Tono, C., G. Xu, et al. (2005). "JAK2 Val617Phe activating tyrosine kinase mutation in juvenile myelomonocytic leukemia." Leukemia **19**(10): 1843-4.

Torii, S., K. Nakayama, et al. (2004). "Regulatory mechanisms and function of ERK MAP kinases." J Biochem (Tokyo) **136**(5): 557-61.

Tsujino, S., T. Miyazaki, et al. (1999). "Critical role of the membrane-proximal, proline-rich motif of the interleukin-2 receptor gammac chain in the Jak3-independent signal transduction." Genes Cells **4**(6): 363-373.

Tu, J., N. Karasavvas, et al. (2000). "Molecular characterization of a granulocyte macrophage-colony- stimulating factor receptor alpha subunit-associated protein, GRAP." Blood **96**(3): 794-799.

Tu, J., N. Karasavvas, et al. (2000). "Molecular characterization of a granulocyte macrophage-colony-stimulating factor receptor alpha subunit-associated protein, GRAP." Blood **96**(3): 794-9.

Ungureanu, D., P. Saharinen, et al. (2002). "Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1." Mol Cell Biol **22**(10): 3316-26.

Usacheva, A. S., R. Domanski, P. Kotenko, S.V. Nelms, K. Goldsmith, M.A. Colamonici, O.R. (2002). "Contribution of the Box 1 and Box 2 Motifs of Cytokine Receptors to Jak1 Association and Activation." The Journal of Biological Chemistry **277**(50): 48220-48226.

Valentino, L. and J. Pierre (2006). "JAK/STAT signal transduction: regulators and implication in hematological malignancies." Biochem Pharmacol **71**(6): 713-21.

Vanhaesebroeck, B. and M. D. Waterfield (1999). "Signaling by distinct classes of phosphoinositide 3-kinases." Exp Cell Res **253**(1): 239-54.

Vilcek, J. (1998). The Cytokines: An Overview. The Cytokine Handbook. A. Thomson, Academic Press**:** 1-20.

Vivanco, I. and C. L. Sawyers (2002). "The phosphatidylinositol 3-Kinase AKT pathway in human cancer." Nat Rev Cancer **2**(7): 489-501.

Vlahos, C. J., W. F. Matter, et al. (1994). "A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)." J Biol Chem **269**(7): 5241-8.

Vremec, D., G. J. Lieschke, et al. (1997). "The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs." Eur J Immunol **27**(1): 40-4.

Wada, H., Y. Noguchi, et al. (1997). "T cell functions in granulocyte/macrophage colony-stimulating factor deficient mice." Proc Natl Acad Sci U S A **94**(23): 12557-61.

Wang, D., D. Stravopodis, et al. (1996). "Naturally occurring dominant negative variants of Stat5." Mol Cell Biol **16**(11): 6141-6148.

Wang, X. and C. Tournier (2006). "Regulation of cellular functions by the ERK5 signalling pathway." Cell Signal **18**(6): 753-60.

Ward, A. C., M. H. Hermans, et al. (1999). "Tyrosine-dependent and -independent mechanisms of STAT3 activation by the human granulocyte colony-stimulating factor (G-CSF) receptor are differentially utilized depending on G-CSF concentration." Blood **93**(1): 113-24.

Ward, A. C., I. Touw, et al. (2000). "The Jak-Stat pathway in normal and perturbed hematopoiesis." Blood **95**(1): 19-29.

Watanabe, M., M. Z. Dewan, et al. (2005). "A novel NF-kappaB inhibitor DHMEQ selectively targets constitutive NF-kappaB activity and induces apoptosis of multiple myeloma cells in vitro and in vivo." Int J Cancer **114**(1): 32-8.

Watanabe, S., T. Itoh, et al. (1996). "JAK2 is essential for activation of c-fos and c-myc promoters and cell proliferation through the human granulocyte-macrophage colony-stimulating factor receptor in BA/F3 cells." **271**(21): 12681-12686.

Watowich, S. S., H. Wu, et al. (1996). "Cytokine receptor signal transduction and the control of hematopoietic cell development." Annu Rev Cell Dev Biol **12:91-128.**: 91-128.

Wei, S., J. H. Liu, et al. (1996). "Critical role of Lyn kinase in inhibition of neutrophil apoptosis by granulocyte-macrophage colony-stimulating factor." Journal of Immunology **157**(11): 5155-5162.

Witmer-Pack, M. D., W. Olivier, et al. (1987). "Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells." J Exp Med **166**(5): 1484-98.

Wittig, I. and B. Groner (2005). "Signal transducer and activator of transcription 5 (STAT5), a crucial regulator of immune and cancer cells." Curr Drug Targets Immune Endocr Metabol Disord **5**(4): 449-63.

Woodcock, J. M., B. J. McClure, et al. (1997). "The human granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor exists as a preformed receptor complex that can be activated by GM-CSF, interleukin-3, or interleukin-5." Blood **90**: 3005-3017.

Wymann, M. P., K. Bjorklof, et al. (2003). "Phosphoinositide 3-kinase gamma: a key modulator in inflammation and allergy." Biochem Soc Trans **31**(Pt 1): 275-80.

Xiao, G., A. B. Rabson, et al. (2006). "Alternative pathways of NF-kappaB activation: a double-edged sword in health and disease." Cytokine Growth Factor Rev **17**(4): 281-93.

Yamamoto, T., M. Ebisuya, et al. (2006). "Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression." Curr Biol **16**(12): 1171-82.

Yart, A., M. Laffargue, et al. (2001). "A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor." J Biol Chem **276**(12): 8856-64.

Yeh, T. C., E. Dondi, et al. (2000). "A dual role for the kinase-like domain of the tyrosine kinase Tyk2 in interferon-alpha signaling." Proc Natl Acad Sci U S A **97**(16): 8991-6.

Young, S. M., A. C. Cambareri, et al. (2006). "Role of c-KIT expression level and phosphatidylinositol 3-kinase activation in survival and proliferative responses of early myeloid cells." Cell Signal **18**(5): 608-20.

Zachos, G. and D. A. Spandidos (1997). "Expression of ras proto-oncogenes: regulation and implications in the development of human tumors." Crit Rev Oncol Hematol **26**(2): 65-75.

Zarrinpar, A., R. P. Bhattacharyya, et al. (2003). "The structure and function of proline recognition domains." Sci STKE **2003**(179): RE8.

Zarrinpar, A. and W. A. Lim (2000). "Converging on proline: the mechanism of WW domain peptide recognition." Nat Struct Biol **7**(8): 611-3.

Zhan, Y. (1999). "Functional deficiencies of peritoneal cells from gene-targeted mice lacking G-CSF or GM-CSF." J Leukoc Biol **65**: 256-264.

Zhan, Y., G. J. Lieschke, et al. (1998). "Essential roles for granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of Listeria monocytogenes-infected mice." Blood **91**(3): 863-9.

Zhang, J., A. K. Somani, et al. (2000). "Roles of the SHP-1 tyrosine phosphatase in the negative regulation of cell signalling." Semin Immunol **12**(4): 361-78.

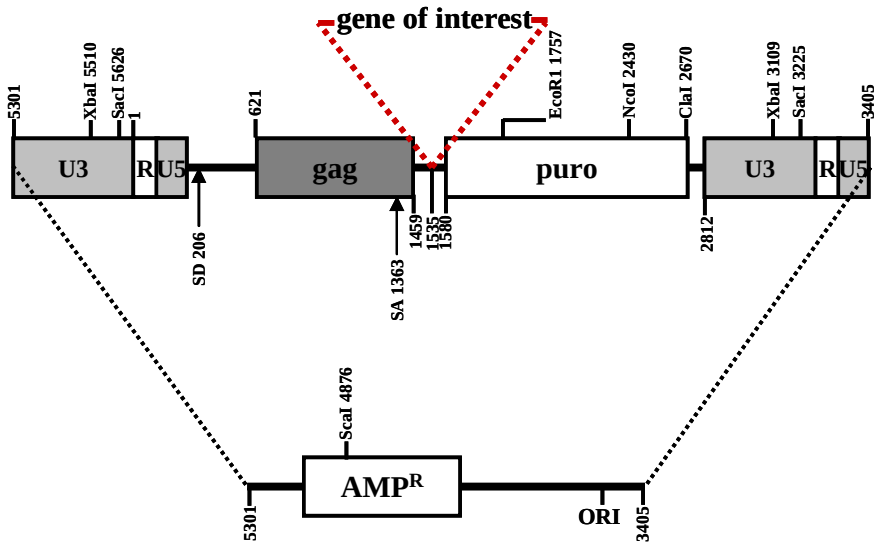
Zhang, Q., P. N. Raghunath, et al. (2000). "Lack of phosphotyrosine phosphatase SHP-1 expression in malignant T-cell lymphoma cells results from methylation of the SHP-1 promoter." Am J Pathol **157**(4): 1137-46.

Zhao, R., S. Xing, et al. (2005). "Identification of an acquired JAK2 mutation in polycythemia vera." J Biol Chem **280**(24): 22788-92.

Zhao, Y., F. Wagner, et al. (1995). "The amino-terminal portion of the JAK2 protein kinase is necessary for binding and phosphorylation of the granulocyte- macrophage colony-stimulating factor receptor beta c chain." J Biol Chem **270**: 13814-13818.

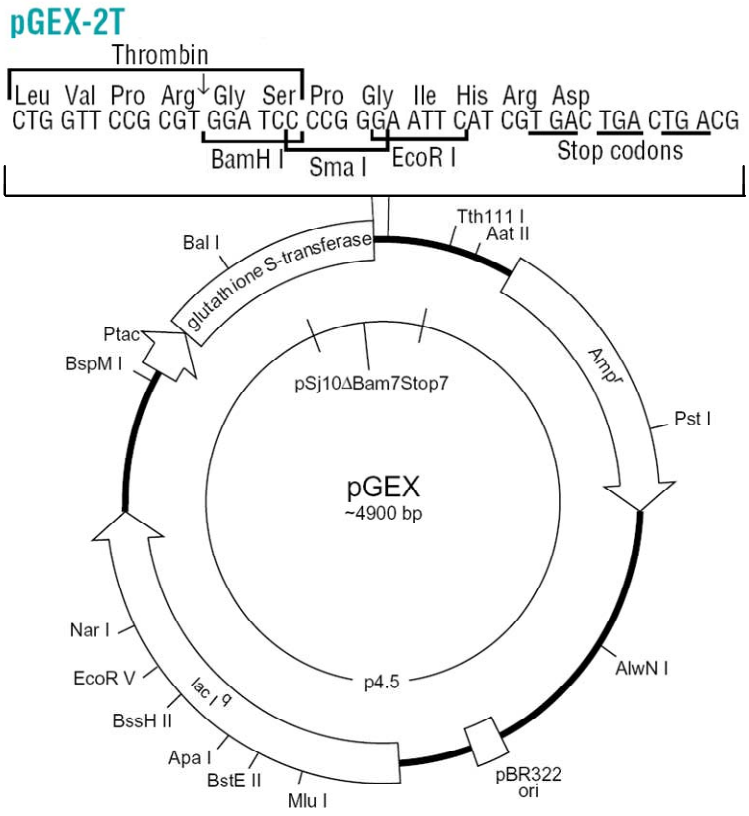
Zhu, T., L. Ling, et al. (2002). "Identification of a JAK2-independent pathway regulating growth hormone (GH)-stimulated p44/42 mitogen-activated protein kinase activity. GH activation of Ral and phospholipase D is Src-dependent." J Biol Chem **277**(47): 45592-603.

Appendix A



Schematic of retroviral vector pRUFpuro. Features of the retroviral expression vector, pRUFpuro include a polylinker, an origin of replication (ORI), an ampicillin resistance gene (AMP^R), viral gag and U3 sequences and a puromycin resistance gene (puro) (adapted from Rayner 1994).

Appendix B



Plasmid map of the glutathione S-transferase fusion vector, pGEX2T. Reading frames and main features are indicated (Adapted from GST Gene Fusion Handbook, Amersham Biosciences).

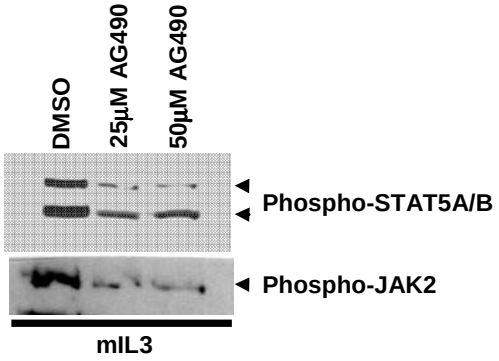
Appendix C

FDB1 Cells	Condition	Mean % P-STAT5A/B	Mean MFI P-STAT5A/B	Mean % P-p44/42 MAPK	Mean MFI P-p44/42 MAPK
FDB1	No Factor	1.81± 0.78	4.43± 0.83	1.22± 1.50	3.57± 0.53
	mIL-3	62.1± 7.44	10.2± 0.8	39.2± 11.9	6.90± 1.8
	mGM-CSF	34.0± 5.65	7.40± 0.9	14.7± 8.65	4.90± 1.32
FIΔ	No Factor	9.78± 12.4	5.43± 1.01	2.13± 2.13	3.89± 0.61
	mIL-3	64.2± 6.53	10.3± 1.11	30.4± 2.61	5.83± 0.52
	mGM-CSF	38.7± 17.6	8.27± 1.40	13.9± 9.22	5.23±1.05
V449E	No Factor	48.9± 9.45	8.87± 0.45	8.07± 3.87	4.87± 0.50
	mIL-3	75.8± 9.62	13.1± 1.65	43.3± 12.8	7.57± 1.75
	mGM-CSF	69.7± 7.37	11.1± 1.25	13.9± 6.69	5.73± 1.00

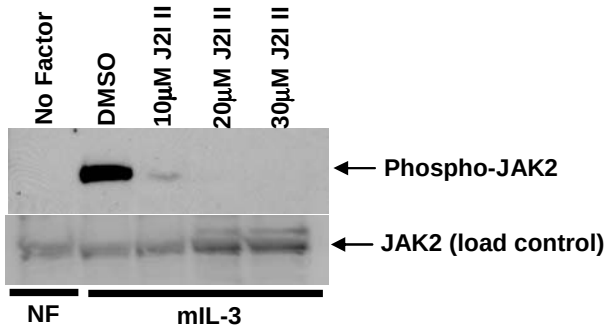
Flow cytometric analysis of FDB1 and FDB1 V449E cells. Cell populations were fixed, permeabilised and stained with phospho-STAT5A/B or phospho-p44/42 MAPK antibodies. Expression of the phosphorylated proteins was detected by flow cytometry. Mean MFI (mean fluorescence intensity) and mean % positive cells are represented. n=3, ± SD.

Appendix D

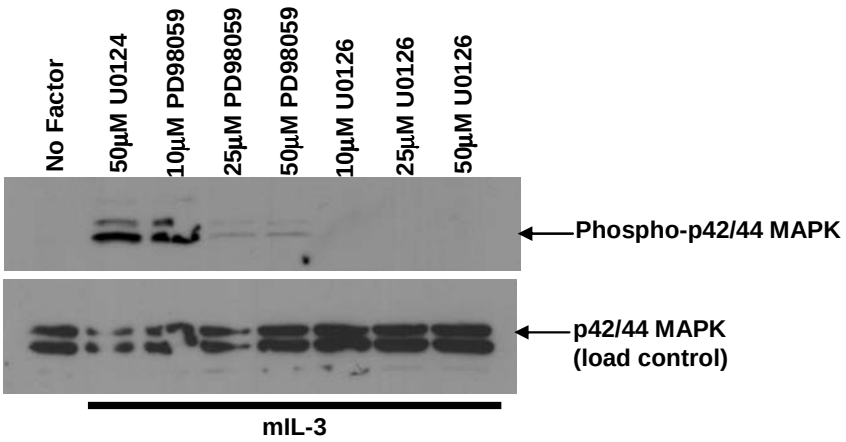
1.



2.



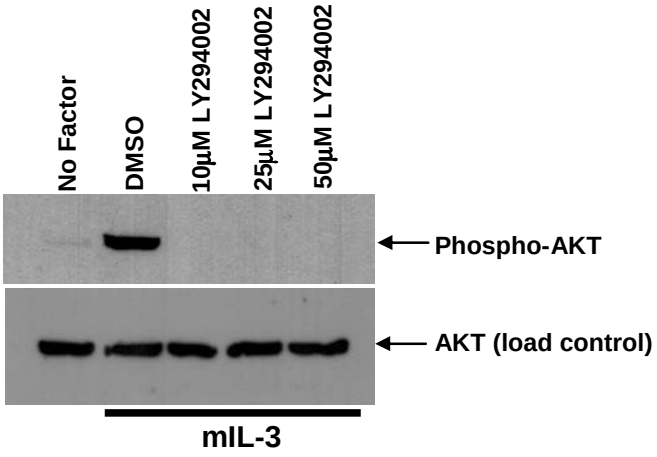
3.



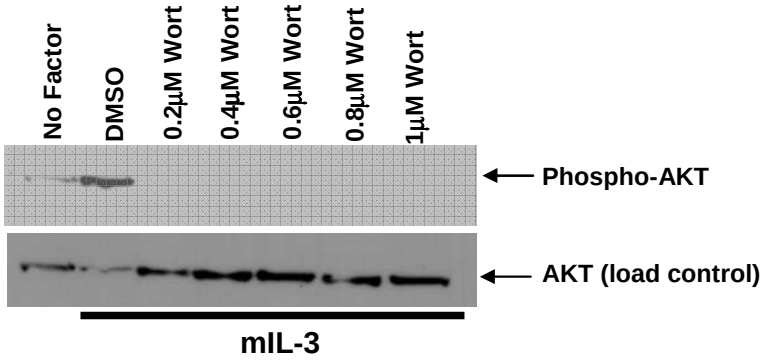
Validation of AG490, JAK2 Inhibitor II, PD98059 and U0126 Inhibitors. FDB1 cells were withdrawn from mIL-3 and varying concentrations of AG490 **(1)**, JAK2 Inhibitor II **(2)**, PD98059 **(3)** and U0126 **(3)** were added to the cultures for 16 hours. The control inhibitor U0124 (50mM) was used for comparison with U0126 and PD98059 inhibitors. Cells were then stimulated with mIL-3 where indicated. Phosphorylated JAK2, STAT5A/B and p44/42 MAPK were detected in total cell lysates following immunoblot with phospho-specific antibodies.

Appendix E

1.



2.



Validation of LY294002 and Wortmannin Inhibitors. FDB1 cells were withdrawn from mIL-3 and varying concentrations of LY294002 **(1)** or Wortmannin **(2)** were added to the cultures for 16 hours. Cells were then stimulated with mIL-3 where indicated Phosphorylated AKT was detected in total cell lysates following immunoblot with phospho-specific antibodies.