Functional Analysis of ANKRD11 and FBXO31:

Two Candidate Tumour Suppressor Genes from the 16q24.3

Breast Cancer Loss of Heterozygosity Region

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This thesis is dedicated to my brother;

Although you are no longer with us, your undying determination and passion for life remains with us all.

You have always be my guiding light in times of dark, my guardian angel in the presence of evil.

I know you will always be with me, and together we will walk, hand in hand, along the road towards a cure.

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Erratum

Erratum to the doctoral thesis entitled "Functional Analysis of *ANKRD11* and *FBXO31*: Two Candidate Tumour Suppressor Genes From the 16q24.3 Breast Cancer Loss of Heterozygosity Region" by Paul Neilsen.

Abstract – Page VII – Second Paragraph – Line 3:

Replace the comma with "which"

Introduction – Page 2 – Third Paragraph – Line 7:

Change "single nucleotide polymorphism" to "tumour-restricted single nucleotide polymorphism"

Chapter 3 – Page 54 – First Paragraph – Line 4:

Insert the sentence "Transiently-expressed ANKRD11 protein accumulated in nuclear foci that were more numerous and larger in size than that of endogenous ANKRD11."

Abstract

Loss of heterozygosity (LOH) on the long arm of chromosome 16 is frequently observed during the onset of breast cancer. Our laboratory has recently identified both *ANKRD11* and *FBXO31* as candidate tumour suppressor genes in the chromosome band 16q24.3, which is the smallest region of overlap for breast cancer LOH. This thesis focuses on the functional analysis of these two novel genes and implicates a role for them as breast cancer tumour suppressors.

ANKRD11: a novel p53 coactivator involved in the rescue of mutant p53

The ability of p53 to act as a transcription factor is critical for its function as a tumour suppressor. Ankyrin repeat domain 11 (ANKRD11) was found to be a novel p53-interacting protein which enhanced the transcriptional activity of p53. ANKRD11 expression in breast cancer cell lines was shown to be down-regulated when compared to ANKRD11 expression in finite life-span HMECs and non-malignant immortalized breast epithelial cells. Restoration of ANKRD11 expression in MCF-7 (p53 wild-type) and MDA-MB-468 ($p53^{R273H}$ mutant) cells suppressed the oncogenic properties of these breast cancer cell lines through enhancement of $p21^{waf1}$ expression. ShRNA-mediated silencing of ANKRD11 reduced the ability of p53 to activate $p21^{waf1}$ expression in response to DNA damage. ANKRD11 was shown to associate with the p53 acetyltransferase, P/CAF, and exogenous ANKRD11 expression increased the levels of acetylated p53. Exogenous ANKRD11 expression enhanced the DNA-binding properties of the $p53^{R273H}$ mutant to the *CDKN1A* promoter, implicating a role for ANKRD11 in the restoration of mutant $p53^{R273H}$ function. These findings demonstrate a role for ANKRD11 as a p53 coactivator and illustrate the potential of ANKRD11 in the restoration of mutant $p53^{R273H}$ function.

ANKRD11 has roles beyond that of p53 coactivation. This thesis also presents preliminary findings to suggest that ANKRD11 may be involved in the regulation of eukaryotic cell division. Furthermore, ANKRD11 was shown to function as an estrogen receptor coactivator. Taken together, these finding suggest that ANKRD11 is a multi-functional cancer-related protein.

FBXO31: the 16q24.3 senescence gene

A BAC located in the 16q24.3 breast cancer loss of heterozygosity region was previously shown to restore cellular senescence when transferred into breast tumour cell lines. We have shown that *FBXO31*, although located just distal to this BAC, can induce cellular senescence in the breast cancer cell line MCF-7 and is the likely candidate senescence gene. Exogenous FBXO31 expression inhibited the oncogenic properties of the MCF-7 breast cancer cell line. In addition, compared to the relative expression in normal breast, levels of FBXO31 were down-regulated in breast tumour cell lines and primary tumours. FBXO31 protein levels were cell cycle regulated, with maximal expression from late G_2 to early G_1 phase. Ectopic expression of FBXO31 in the breast cancer cell line MDA-MB-468 resulted in the accumulation of cells at the G_1 phase of the cell cycle. FBXO31 was also shown to be a component of a SCF ubiquitination complex. We propose that FBXO31 functions as a tumour suppressor by generating SCF^{FBXO31} complexes that target particular substrates, critical for the normal execution of the cell cycle, for ubiquitination and subsequent degradation.

Declaration

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.

Signed: Date:

List of Publications

Kumar R, **Neilsen PM**, Crawford J, McKirdy R, Lee J, Powell JA, Saif Z, Martin JM, Lombaerts M, Cornelisse CJ, Cleton-Jansen A-M, Callen DF. (2005). FBXO31 is the chromosome 16q24.3 senescence gene, a candidate breast tumor suppressor, and a component of an SCF complex. *Cancer Res* 65:11304-11313. *Impact Factor = 7.7*

Neilsen PM, Cheney KM, Li CW, Chen JD, Cawrse JE, Schulz RB, Powell JA, Kumar R, Callen DF. (2007). Identification of ANKRD11 as a novel p53 coactivator involved in the rescue of mutant p53. *J Cell Sci.* Submitted November 2007. *Impact Factor = 6.4*

Kumar R, Cheney KM, McKirdy R, **Neilsen PM**, Schulz RB, Lee J, Cohen J, Booker GW, Callen DF (2007). CBFA2T3-ZNF652 corepressor complex regulates transcription of the E-box gene *HEB*. *J Biol Chem.* Submitted November 2007. *Impact Factor = 5.8*

Abbreviations

α-X – Anti-X antibody (e.g. α-FLAG)

ACTR – Acetyltransferase

AD – Activator domain

ADH – Atypical ductal hyperplasia

AIB1 – Amplified in breast cancer 1

AML – Acute myeloid leukaemia

ANK domain – Ankyrin repeat domain

ANOVA – Analysis of variance

AR – Androgen receptor

ASPP – Ankyrin-repeats, SH3 domain and proline-rich region containing protein

ATCC – American type culture collection

BAC – Bacterial artificial chromosome

bHLH – Basic helix-loop-helix

CBP – CREB binding protein

Cdk – Cyclin dependent kinase

cDNA – Complementary DNA

ChIP – Chromatin immunoprecipitation

CKI – Cyclin dependent kinase inhibitor

CREB – cAMP response element-binding

DAPI – 4',6-diamidino-2-phenylindole

DBD – DNA-binding domain

D-box – Destruction Box

DCIS – Ductal carcinoma *in situ*

DNA – Deoxyribonucleic acid

DSB – Double-strand breaks

DTT – Ditheothiol

E1 – Ubiquitin activating enzyme

E2 – Ubiquitin conjugating enzyme

E3 – Ubiquitin ligase

EBI – European Bioinformatics Institute

EBV – Ebstein-Barr virus

EGF – Epidermal growth factor

EGFP – Enhanced green fluorescent protein

ER – Estrogen receptor

ERα – Estrogen receptor alpha

ERβ – Estrogen receptor beta

ERE – Estrogen response element

FCS – Fetal calf serum

GFP – Green fluorescent protein

GR – Glucocorticoid receptor

GRIP-1 – Glucocorticoid receptor interacting protein 1

GST – Glutathione S-transferase

H2A – Histone 2A

HA – Hemagglutinin

HAT – Histone acetyltransferase

HDAC – Histone deacetyltransferase

HMEC – Human mammary epithelial cell

IDC – Invasive ductal carcinoma

IF – Immunofluorescence

IHC – Immunohistochemistry

ILC – Invasive lobular carcinoma

IP – Immunoprecipitation

KLH – Keyhole limpet hemocyanin

LBD – Ligand-binding domain

LCIS – Lobular carcinoma *in situ*

LOH – Loss of heterozygozity

LRES – Long-range epigenetic silencing

MAPK – Microtubule-associated protein kinase

MBP – Maltose-binding protein

MEK2 – MAPK kinase 2

miRNA – Micro RNA

MMC – Mitomycin C

MR – Mineralocorticoid receptor

mRNA – Messenger RNA

NCBI – National center for biotechnology information

NCoA – Nuclear receptor coactivator

NLS – Nuclear localisation signal

NPC – Nasopharengeal carcinoma

ONPG – *O*-nitrophenyl-β-galactopyranoside

ORC2 – Origin recognition complex subunit 2

p53-RE – p53 response element

p/CIP – p300/CBP interacting protein

PAC – P1 artificial chromosome

PAS domain – Per-Arnt-Sim domain

PCR – Polymerase chain reaction

PEST sequence – Proline, glutamic acid, serine and threonine rich sequence

PR – Progesterone receptor

RAC3 – Receptor-associated coactivator 3

RD – Repressor domain

Real-time RT-PCR – Reverse transcription real time-PCR

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RT – Room temperature

SAC – Spindle assembly checkpoint

SAHA – Suberoylanilide hydroxamic acid

SDS – Sodium dodecylsulphate

SDS-PAGE – SDS Polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SERM – Selective estrogen receptor modulator

shRNA – Short hairpin RNA

siRNA – Small interfering RNA

SNP – Single nucleotide polymorphism

SRC – Steroid receptor coactivator

SRO – Smallest region of overlap

SSCP – Single-strand conformation polymorphism

TIF2 – Transcriptional intermediary factor 2

TK – Thymidine kinase

TRAM-1 – Thyroid hormone receptor activator molecule 1

Ub – Ubiquitin

UDH – Usual ductal hyperplasia

WB – Western blot

Y2H – Yeast-2-hybrid

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Chapter 1: Introduction

1.1 – Cancer: a genetic disease

Genetic instability is the hallmark of cancer. Malignant transformation of normal breast epithelial cells represents a complex multistep process in which genetic and epigenetic alterations drive the progressive transformation of a cell into a state of uncontrolled growth (Hanahan & Weinberg, 2000). These tumorigenic alterations result in the gain of function of *oncogenes* or *proto-oncogenes*, and the inactivation of *tumour suppressor genes* (Kopnin, 2000).

1.1.1 – Oncogenes and proto-oncogenes

Oncogenes can be of cellular or viral origin, and their expression assists in the neoplastic transformation of a cell. Proto-oncogenes are normal cellular genes that have the potential to be converted into oncogenes following tumorigenic DNA alterations.

The process of tumorigenesis involves genetic alterations of these oncogenes or proto-oncogenes, rendering them constitutively active or active under conditions where the normal wild-type gene is usually not expressed (Vogelstein & Kinzler, 2004). These genetic alterations that drive the cell into malignancy include chromosomal translocations, generating oncogenic fusion proteins; gene amplification or genetic mutations that result in abnormal gene overexpression; or intragenic mutations that induce aberrant post-translational modification of their gene products (Kopnin, 2000; Vogelstein & Kinzler, 2004). Additionally, it has been recently shown that mutations in small non-protein-coding RNAs (micro RNAs - miRNAs) can result in the misregulation and subsequent over-expression of oncogenes (Esquela-Kerscher & Slack, 2006; Negrini et al., 2007). The activation of oncogenes results in uncontrolled cellular proliferation through the modulation of several

cellular pathways, including escape for apoptosis or senescence, loss of cell cycle control, abnormal cellular differentiation, genetic instability, angiogenesis and increased tissue invasion and metastasis (Hanahan & Weinberg, 2000; Kopnin, 2000).

1.1.2 – Tumour suppressor genes

The discovery of abnormally "activated" genes that contribute towards cellular transformation (oncogenes) fuelled the idea that there also exist genes whose "inactivation" increased the probability of tumorigenesis (tumour suppressor genes). To date, numerous such tumour suppressors have been identified that are involved in the suppression of several malignancies, as summarised in Table 1.1.

1.1.2.1 – Inactivation of tumour suppressor genes

During the formation of a malignant cell, tumour suppressor genes are frequently "inactivated" through mutation, resulting in either loss of expression of the encoded protein or errors in the translated protein. These mutations occur through a variety of mechanisms, including DNA replication errors during cell division (Ionov et al., 1993), ultraviolet light, ionising radiation (Jensen & Haas, 1963), chemical mutagens and oncogenic viruses (Jensen et al., 1963). These mutations can be structurally characterised as either small-scale mutations involving a tumour-restricted single nucleotide polymorphism (SNP) or large-scale structural alteration involving extensive changes to the chromosomal structure.

Table 1.1: Examples of tumour suppressor genes and the malignancy associated with their inactivation.

Tumour Suppressor Gene	***Function(s)***	***Tumour(s) associated with inactivation of tumour suppressor gene***
APC	Wnt/Wingless signaling	Familial adenomatous polyposis (Groden et al., 1991), sporadic colorectal, gastric, pancreatic, thyroid and ovarian cancers (Beroud & Soussi, 1996).
ATM	DNA damage sensor	Familial and sporadic breast cancer (Concannon, 2002), and multiple leukemias (Boultwood, 2001) and lymphomas (Savitsky et al., 1995).
BANP	Transcriptional corepressor	Infiltrating Ductal Breast Carcinomas (Rampalli et al., 2005; Singh et al., 2007)
BRCA1 *BRCA2*	DNA repair	Familial breast and ovarian cancer (Hall et al., 1990; Futreal et al., 1994; Miki et al., 1994; Wooster et al., 1994), sporadic breast (Lancaster et al., 1996; Rosen et al., 2003), ovarian (McCoy et al., 2003) and pancreatic (Beger et al., 2004) cancers.
CBFA2T3	Transcriptional corepressor	Breast carcinomas (Kochetkova et al., 2002; Bais et al., 2004).
CDH1 (E-cadherin)	Cell-cell adhesion	Lobular breast carcinoma (Berx et al., 1995; Berx et al., 1996), familial and sporadic gastric cancer (Berx et al., 1998; Guilford et al., 1998), and epithelial cancers (Hajra & Fearon, 2002).
CDKN1A ($p21^{waf1}$)	Cdk inhibitor	Familial breast (Mousses et al., 1995), lung (Chedid et al., 1994), skin, head, neck (Ralhan et al., 2000) and cervical (Harima et al., 2001) cancers and sporadic hepatocellular carcinoma (Furutani et al., 1997).

CDKN1B ($p27^{Kip1}$)	Cdk inhibitor	Frequently inactivated in multiple sporadic tumours (Philipp-Staheli et al., 2001).
CDKN2A ($p14^{ARF}$)	MDM2 antagonist	Familial melanoma and sporadic neural tumours (Foulkes et al., 1997), T-cell lymphoblastic lymphoma (Gardie et al., 1998), metastatic melanoma (Kumar et al., 1998) and many other sporadic tumours (Pollock et al., 1996).
CHK1	DNA damage response	Microsatellite unstable endometrial (Bertoni et al., 1999) and gastrointestinal (Menoyo et al., 2001) cancers.
CHK2	Protein kinase	Familial Li-Fraumeni syndrome (Wu et al., 2001b).
FBX031	Component of E3 ubiquitin ligase	Breast carcinoma (Kumar et al., 2005).
FBXW7 (FBW7)	Component of E3 ubiquitin ligase	Endometrial (Spruck et al., 2002), pancreatic (Calhoun et al., 2003) and colorectal (Rajagopalan et al., 2004) cancers.
INK4a (p16)	Cdk inhibitor	Familial melanoma (Hussussian et al., 1994) and multiple sporadic tumours (Foulkes et al., 1997).
MSH2 *MLH1*	DNA repair	Familial non-polyposis coli cancer (Bronner et al., 1994; Fishel et al., 1994), sporadic microsatellite unstable colorectal (Herfarth et al., 1997), gastric (Bevilacqua & Simpson, 2000), head and neck cancers (Liu et al., 2003), melanoma (Castiglia et al., 2003) and retinoblastoma (Choy et al., 2004).
NF1	*ras* inactivator	Familial neurofibromatosis (Gutmann et al., 1991), sporadic colon adeocarcinoma, myelodisplastic syndrome (Li et al., 1992), astrocytoma, glioblastoma, ependymoma and neuroectodermal tumours (Thiel et al., 1995).
PTCH	Hedgehog signaling	Nevoid basal cell carcinoma syndrome (Hahn et al., 1996a) and sporadic medulloblastoma (Pietsch et al., 1997).
PTEN	Lipid phosphatase	Cowden syndrome (Sulis & Parsons, 2003) and multiple sporadic tumours (Simpson & Parsons, 2001).

RB	Transcriptional corepressor	Familial retinoblastoma (Friend et al., 1986; Dunn et al., 1988), sporadic small cell lung carcinoma (Harbour et al., 1988; Yokota et al., 1988), osteosarcoma (Toguchida et al., 1989) and ductal pancreatic carcinoma (Huang et al., 1996).
SMAD4	Transcription factor	Familial juvenile polyposis (Howe et al., 1998), sporadic colon and pancreatic cancers (Hahn et al., 1996b; Schutte et al., 1996).
TP53 (p53)	Transcription factor DNA repair E3 ubiquitin ligase	Familial Li-Fraumeni syndrome (Malkin, 1994) and multiple sporadic tumours (Hernandez-Boussard et al., 1999a; Hernandez-Boussard et al., 1999b; Ikawa et al., 1999; Vogelstein et al., 2000)**.**
TSC1 *TSC2*	GTPase activating protein complex	Tuberous sclerosis (Kandt et al., 1992) and renal cell carcinoma (Urakami et al., 1997).
VHL	Component of E3 ubiquitin ligase	Von Hippel-Lindau syndrome (Richards et al., 1993), renal cell carcinoma and hemangiosarcoma (Tory et al., 1989).
WT1	Transcription factor	Familial nephroblastoma (Kaneko et al., 1991)
ZNF652	Transcription factor	Breast (Kumar et al., 2006) and vulvar (Holm et al., 2007) carcinoma.

Adapted from (Kopnin, 2000; Sherr, 2004; Payne & Kemp, 2005)

SNPs involve the exchanging of a single nucleotide for another. SNPs that occur within the protein coding region can result in the translation of the same amino acid (silent mutation), an erroneous amino acid (missense mutation) or premature termination of the transcript (nonsense mutations) (Griffiths et al., 1999). Furthermore, a nucleotide may be inserted or deleted into the coding region of a tumour suppressor gene (Read, 1999), resulting in a frameshift mutation and subsequent truncation of the protein or an absence of protein due to nonsense-mediated decay of the altered message RNA (mRNA).

Genetic instability has been frequently identified as one of the initial events during oncogenesis, perturbing the expression of tumour suppressor gene(s) through large-scale structural alterations in the integrity of chromosomal DNA. Amplifications or deletions of large chromosomal regions often lead to a concordant increase or decrease in the expression levels of the genes harboured within the affected region. In addition, chromosomal instability can also lead to the interchange of genetic regions from non-homologous chromosomes (chromosomal translocation or insertion), removal of intra-chromosomal regions (interstitial deletion) or a reverse in the orientation of a chromosomal region (chromosomal inversions). Not only can these events lead to the inactivation of tumour suppressor genes, but they may also form the basis of several malignancies through the generation of oncogenic fusion genes, such as the AML1-ETO oncoprotein derived from the frequently occurring t(8;21) translocation in acute myeloid leukaemia (AML) (Miyoshi et al., 1991).

In somatic tissue, the loss of one allele at a locus is referred to as loss of heterozygosity (LOH). LOH can be detected by observation of loss of germ-line heterozygosity in the somatic tissue. LOH arises from various mechanisms, including deletion, gene conversion, mitotic recombination, translocation, chromosome breakage and loss, chromosomal fusion, or whole chromosomal loss (Thiagalingam et al., 2002). A high frequency of chromosomal

deletions elicited as LOH is a hallmark of genetic instability during oncogenesis, and usually involves the loss of tumour suppressor gene(s) function within the affected chromosomal region (Thiagalingam et al., 2002).

The combination of mutation and LOH is a common mechanism for the inactivation of both alleles of a tumour suppressor gene during oncogenesis. For example, inactivation of the p53 tumour suppressor protein through a combination of specific point mutations within the *TP53* gene and LOH of the *TP53* gene locus on human chromosome 17p have been reported in numerous malignancies (Gleich et al., 1996; Dolan et al., 1999; Katsama et al., 2000; Forslund et al., 2002; Semczuk et al., 2005).

1.1.2.2 – Retinoblastoma as a model for Knudson's "two-hit" hypothesis

The basis principles of tumour suppressor genetics was first exemplified by Alfred Knudson and colleagues through studies involving familial retinoblastoma (Knudson, 1971). Knudson hypothesized that familial retinoblastoma was caused by two mutational events; one mutation inherited through the germ-line and therefore present in all cells in the body and the second mutation is restricted to somatic cells of the retina. This hypothesis has since evolved into what is commonly referred to as Knudson's "two-hit" model for tumorigenesis (Knudson, 1971; Comings, 1973). Subsequent cytogenetic studies revealed that the second "hit" in familial retinoblastoma was sometimes associated with an interstitial deletion of the chromosomal band 13q14 (Francke & Kung, 1976; Knudson et al., 1976; Noel et al., 1976), leading to inactivation of the remaining un-mutated allele of the *RB* tumour suppressor gene (Friend et al., 1986). In accordance with Knudson's "two-hit" hypothesis, inherited cases of retinoblastoma showed single hit kinetics and were bilateral since there was a pre-existing inherited mutation of *RB*, whilst sporadic retinoblastoma required hits to inactivate both alleles of *RB* and were predominately unilateral cases (Knudson, 1971).

1.1.2.3 – Epigenetic silencing of tumour suppressor genes

Knudson's definition of a "hit" referred to a genetic lesion involving mutation, chromosomal deletion or LOH of one allele of a tumour suppressor gene. In addition to these genetic changes, it has since been established that epigenetic changes are also frequently associated with the inactivation of tumour suppressor genes (Jones & Baylin, 2002; Laird, 2005).

In the mammalian genome, DNA methylation occurs at the C5 position within cytosines situated 5' of a guanosine in a CpG dinucleotide (Gruenbaum et al., 1981). Although this dinucleotide is under-represented throughout the genome, short regions of genomic DNA (0.5-4kb in length) have been shown to be high in CpG dinucleotides (Gardiner-Garden & Frommer, 1987; Takai & Jones, 2002). These "CpG islands" are present in approximately 40% of the 5' promoter or exonic regions of all mammalian genes and are normally unmethylated (Larsen et al., 1992). However, studies have shown that cancer cells undergo large-scale changes in 5-methylcytosine distribution, including the hypermethylation of promoter CpG islands associated with tumour suppressor genes (Holliday, 1979; Frost & Kerbel, 1983; Nyce et al., 1983; Riggs & Jones, 1983). Hypermethylation perturbs the accessibility of the transcriptional machinery to the promoter region, resulting in reduced expression of the tumour suppressor gene.

It is now realised that DNA methylation is not the only facet of integrated changes to the chromatin structure that occur during carcinogenesis. Studies have shown that cancer cells exhibit aberrant histone deacetylation (Johnstone, 2002), methylation (Kondo et al., 2003; Liang et al., 2004) and sumoylation (Shiio & Eisenman, 2003) in the promoter regions of tumour suppressor genes, leading to gene silencing and contributes to the development of cancer.

It has been recently shown that epigenetic changes in cancer are not limited to discrete individual CpG-island associated genes, but can cover multiple neighbouring CpG islands and genes (Frigola et al., 2006). This study reported wide-spread epigenetic gene silencing in colorectal cancer through DNA and histone methylation of the entire 4-Mb region of chromosome 2q14.2. This phenomenon has been termed "long-range epigenetic silencing" (LRES), and is suggested to have similar implications as LOH during oncogenesis (Frigola et al., 2006).

1.1.2.4 – Haploinsufficient tumour suppressor genes

For many tumour suppressor genes, the retention of a single, functional allele is sufficient to maintain the regulation of normal cellular growth and homeostasis. However, the function of some tumour suppressors is dependent on their gene dosage levels and the presence of only one working allele of a tumour suppressor gene is insufficient to accomplish the normal activity of that gene product in the cell (Payne & Kemp, 2005). This class of genes are referred to as haploinsufficient tumour suppressor genes, and their mode of inactivation conflicts with Knudson's classical "two hit" model of tumorigenesis (Fig 1.1).

A role for the cyclin dependent kinase inhibitor, $p27^{kip1}$ as a haploinsufficient tumour suppressor gene was elegantly shown through the use of the $p27^{+/-}$ heterozygous mouse (Fero et al., 1998). Previous studies had shown that $p27^{-/-}$ mice had increased growth rates, with adult mice 20-30% larger than wild-type littermates (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Interestingly, tissues from the $p27^{+/-}$ mouse showed approximately 50% of the total levels of normal p27 expression, and these mice showed an intermediate increase in growth rate and predisposition to tumours (Fero et al., 1998). This study clearly defined the parameters of haploinsufficient tumour suppression and has influenced our understanding of tumour suppressor genetics.

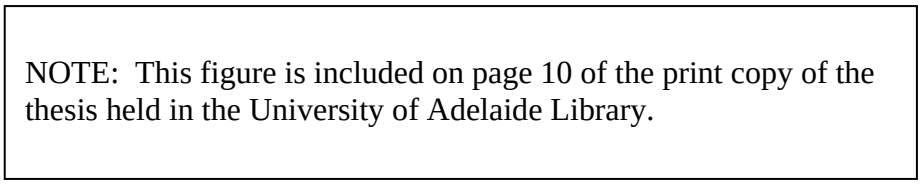


Figure 1.1. Tumour suppressor genetics

A comparative diagram depicting the classical “two hit” model for inactivation of a tumour suppressor gene versus the “one hit” inactivation of a haploinsufficient tumour suppressor gene. *Adapted from (Payne & Kemp, 2005).*

1.2 – Breast cancer

Breast cancer is the leading cause of cancer-related death in women (World Health Organisation, 2006), with one in eleven Australian women diagnosed with breast cancer before the age of 75 (Australian Institute of Health and Welfare & National Breast Cancer Centre, 2006; Breast Cancer Australia, 2006). Breast cancer is a complex disease derived from either the epilethial (carcinoma) or stromal (sarcoma) tissue, with the majority of tumours arising from the epithelium.

Although the events that occur during the onset of breast oncogenesis are poorly defined, evidence exists to suggest that breast cancer originates from pre-malignant lesions. The most common precursor lesions are derived from either the milk duct (ductal carcinoma *in situ* - DCIS) or breast lobuli (lobular carcinoma *in situ*), and develop from a proliferation of pre-malignant cells that have not invaded beyond the basement membrane (Polyak, 2001; Leonard & Swain, 2004). Other forms of non-invasive breast lesions include usual ductal hyperplasia (UDH) and atypical ductal hyperplasia (ADH) (Polyak, 2001). Women diagnosed with these lesions have an increased propensity to develop the invasive form of this disease, which includes either invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC) (Wellings & Jensen, 1973; Wheeler & Enterline, 1976; Leonard & Swain, 2004). These lesions are primarily diagnosed through mammography, followed by histopathological and immunohistochemical analysis of biopsies. Several molecular markers, such as the expression of hormone receptors or growth factors, provide valuable information pertaining to the prognosis, diagnosis and treatment of the disease.

1.2.1 – Estrogens and breast cancer

The estrogen receptor (ER) is expressed in approximately 70% of all human breast tumours, signifying the relevance of this steroid in the development and propagation of breast cancer (Dickson & Lippman, 1987; Dobrzycka et al., 2003). ER signalling has been shown to enhance both the proliferative and anti-apoptotic characteristics of breast tumour cells (Yager & Davidson, 2006). The expression of ER in breast cancer cells has proven to be an important prognostic factor that has a significant influence on the treatment options for this disease (Knight et al., 1977; Rich et al., 1978). Therefore, it is not surprising that the use of ablative endocrine therapy or selective estrogen receptor modulators (SERMs), such as tamoxifen, have proven extremely efficacious in the treatment and prevention of ER-positive breast cancer. Combined analysis of over 37,000 women with breast cancer from 55 trials showed that adjuvant tamoxifen therapy significantly reduces the recurrence and mortality rates associated with this disease (Early Breast Cancer Trialists' Collaborative Group, 1998). Tamoxifen has been widely used in the clinic for over a quarter of a century, and its success as a breast cancer therapeautic has evoked a wealth of research focused on the development of the ideal SERM with breast tissue-specific activity and minimal clinical side-effects to the patient.

1.2.2 – Molecular basis of breast tumorigenesis

The cellular and molecular heterogeneity of breast cancer arises from the vast multitude of genes potentially involved in the progression of breast tumorigenesis. This emphasizes the importance of studying genome-wide expressional alterations that occur in breast oncogenesis. The application of genomic tools such as DNA microarray analysis has furthered our understanding of the molecular basis of mammary tumorigenesis through the identification of gene signatures that are reported to predict the clinical outcome of breast cancer (van 't Veer *et al.*, 2002; van de Vijver *et al.*, 2002).

Similar studies have led to the identification of breast tumours that share distinct gene expression patterns that enable the classification of these tumours into subgroups (Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003). These subgroups include a basal epithelial-like group, a normal breast-like group, three subtypes of the luminal epithelial group and an *ERBB2*-overexpressing group (Sorlie et al., 2001). These expression-based hierarchical clustering of breast tumours were observed to segregate into clinically distinct subgroups, with the basal-like and *ERBB2*-overexpressing subgroups associated with a significantly reduced survival rate (Sorlie et al., 2001).

1.2.2.1 – Breast cancer oncogenes: ERBB2 / HER2

ERBB2 (v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog, HER2) is a breast cancer proto-oncogene that encodes a member of the epidermal growth factor (EGF) receptor family of membrane-spanning type I receptor tyrosine kinases (Harari & Yarden, 2000). *ERBB2* is amplified in approximately 20 to 30% of all invasive breast cancers, and expression of the erbB-2 oncoprotein is associated with poor prognosis (Slamon et al., 1987; Hudis, 2007). ErbB-2 associates with its receptor family members to regulate various cellular processes, including growth, survival, adhesion, migration and differentiation (Yarden, 2001; Hudis, 2007). Hyperactivation of the erbB-signalling network results in the dysregulation of homeostatic cell cycle progression, including upregulation of active cyclin D/Cdk complexes (Harari & Yarden, 2000).

The erbB-2 oncoprotein has emerged as an attractive target for breast tumour-specific therapies. Exploitation of erbB-2 as a therapeutic target has proven successful due to the extracellular accessibility of this transmembrane receptor protein. Furthermore, erbB-2 is significantly overexpressed in breast cancer cells, allowing tumour-specific targeting of erbB-

2 in breast cancer patients suffering from erbB-2-dependent tumours. (Sorlie et al., 2001). ErbB-2-targeting is achieved through the use of a humanized anti-erbB-2 monoclonal antibody, trastuzumab (marketed by Genentech as herceptin). Trastuzumab binds to the extracellular juxtamembrane domain of erbB-2 and inhibits the proliferation and survival of erbB-2-overexpressing tumours (Hudis, 2007). Although the impact of trastuzumab on the treatment of women with erbB-2-dependent breast tumours has been profound, several issues surrounding its side-effects, neo-adjuvant potential and optimal treatment duration are still under investigation (Hudis, 2007). In addition to trastuzumab, novel drugs that inhibit tyrosine kinase activity, interfere with ligand binding or block transcription of specific components of the network are expected to reach maturation within the next decade (Harari & Yarden, 2000).

1.2.2.2 – Breast cancer oncogenes: CCND1

Cyclin D1 is the product of the *CCND1* gene, located on chromosome 11q13. Cyclin D1 is overexpressed at the mRNA and protein level in over half of all invasive breast tumours, making it one of the most frequently amplified oncogenes in breast cancer (Buckley et al., 1993; Gillett et al., 1994; Ormandy et al., 2003). Cyclin D1 overexpression is particularily enriched in ebrB-2-dependent breast tumours, and data suggest that type-D cyclins are direct targets of downstream erbB-2-signalling pathways (Harari & Yarden, 2000).

Cyclin D1 is involved in the regulation of cell cycle progression of breast cancer cells and in mammary gland development (Roy & Thompson, 2006). Cyclin D1 expression shows a strong correlation with ER levels in breast tumours (Hui et al., 1996; Jares et al., 1997), and the mitogenic effects of estrogen were shown to be mediated by both cyclin D1 and c-myc breast cancer oncoproteins (Prall et al., 1998).

1.2.2.3 – Breast cancer oncogenes: MYC

The *MYC* gene is located on chromosome 8q24 and is frequently amplified in breast tumours. A meta-analysis of 97 individual studies revealed that 15.5% of breast cancer biopsies exhibit *MYC* gene amplification, and over half of these biopsies have increased levels of c-myc protein (Liao & Dickson, 2000). Studies involving MMTV-c-myc transgenic mouse models support a role for c-myc in mammary gland tumorigenesis, with over half of transgenic female mice developing breast cancer (Amundadottir et al., 1996; Rose-Hellekant & Sandgren, 2000). The c-myc oncoprotein facilitates mammary tumorigenesis by induction of cyclin E/Cdk2 activity. This is achieved by c-myc through either direct induction of cyclin E expression or through sequestration of the Cdk inhibitor, $p27^{kip1}$ (Obaya et al., 1999; Liao & Dickson, 2000).

Recent cytogenetic analysis of breast tumours has revealed that gain of the 8q24 amplicon correlates with poor patient survival (Jain et al., 2001). Furthermore, amplification of the *MYC* gene in breast tumours is associated with the presence of mutations in *TP53*, an important tumour suppressor gene. This is a likely causative for elevated levels of mutant p53 in breast tumours, as it has been previously shown that c-myc is involved in transactivation of the *TP53* promoter (Roy et al., 1994).

1.2.2.4 – Breast Cancer Tumour Suppressor Genes: TP53

The *TP53* gene is located on the short arm of chromosome 17 and encodes the p53 tumour suppressor protein. p53 was first identified in 1979 and characterised as an oncogenic tumour antigen due to the purification of over-expressed mutant p53 mRNA from tumour cell lines (DeLeo et al., 1979; Linzer & Levine, 1979). Detailed cytogenetic and mutational analysis of *TP53* in tumours later revealed that p53 was indeed a tumour suppressor gene (Baker et al., 1989; Nigro et al., 1989).

The anti-oncogenic properties of p53 arise predominately from its function as a transcription factor, regulating various target genes that control the homeostatic characteristics of normal cellular growth (Liu & Chen, 2006). In response to extracellular stress signals, p53 protein levels and activity are rapidly increased, driving the cell into a temporary arrest at G_1 phase of the cell cycle (Kastan et al., 1991). This temporary arrest allows p53 to transactivate numerous DNA repair genes to make the necessary corrections to the DNA before the cell progresses into S phase of the cell cycle and subsequent DNA replication (Hartwell & Kastan, 1994). In case the complete repair of the damaged DNA is not possible, p53 drives this potentially malignant cell into apoptosis (Lane, 1992).

The *TP53* gene maps to human chromosome 17p13.1, a region frequently associated with allelic loss in sporadic breast carcinomas (Niederacher et al., 1997; Miller et al., 2003). Inactivation of functional p53 is a crucial event during the onset of tumorigenesis, with approximately 50% of all cancers containing somatic mutations in the *TP53* gene (Vogelstein et al., 2000). In addition, germline mutations in *TP53* are associated with the familial Li-Fraumeni syndrome (Malkin, 1994). Virtually all cancer-associated mutations in *TP53* reduce the ability of the encoded protein to activate transcription, supporting the idea that the transcriptional activity of p53 is critical to its function as a tumour suppressor.

Alternatively, p53 can be inactivated in cancer cells indirectly through binding to viral proteins such as the SV40 large T-antigen, or through mutations in genes whose products interact with and modulate p53 stability or activity (Vogelstein et al., 2000). These latter proteins include the p300/CBP and P/CAF (p300/CBP-associated factor) acetyltransferases, that are multifunctional regulators of p53-mediated transcription (discussed further in Section 3.1.2, Introduction) (Lill et al., 1997). Several malignancies have been shown to harbour

genetic mutations in the genes encoding p300 and CBP, and evidence suggests that loss of p300/CBP function could disrupt p53 activation, stability and transactivation of target genes (Iyer et al., 2004). The p53 response has also been shown to be disrupted through cancer-related perturbations in the normal functioning of several other p53 regulatory proteins, including $p14^{ARF}$ (Balint & Vousden, 2001), MDM2 (a p53 ubiqitin ligase) (Momand et al., 1998) and PML (Guo et al., 2000).

1.2.2.5 – Breast Cancer Tumour Suppressor Genes: BRCA1, BRCA2 and BARD1

Family history is a significant risk factor for the development of breast cancer. Approximately 10% of all breast cancers are inherited (James et al., 2007). Hereditary breast cancer occurs through the transmission of mutations in dominately inherited breast cancer susceptibility genes through the germline. These heterozygous carriers of the inherited mutation are predisposed to breast cancer as they have already aquired one of the two "inactivational" events required for breast tumorigenesis, as proposed by Knudson (see Section 1.1.2.2). This explains why hereditary breast cancers usually occur bilaterally, and are associated with an earlier onset than the sporadic form of breast cancer (Payne & Kemp, 2005).

The two most frequently mutated breast cancer susceptibility genes are *BRCA1* and *BRCA2*, with mutations in these genes reported in up to 50% of all familial breast cancers (Berry et al., 1997; Couch et al., 1997; King et al., 2003; James et al., 2007). The *BRCA1* breast cancer susceptibility gene was mapped to human chromosome 17q21 through genetic linkage studies of families segregating early-onset of breast cancer (Hall et al., 1990). *BRCA1* mutation carriers have a 50 – 80% risk of developing breast cancer by the age of 70 (Easton et al., 1995; Struewing et al., 1997; Ford et al., 1998). In sporadic breast cancer, somatic mutations in the *BRCA1* gene are rarely observed, however the mRNA and protein levels of BRCA1 are significantly reduced in 30% of breast cancers and 70% of ovarian cancers (Yang et al.,

2001). This down-regulation of BRCA1 in sporadic cancers is believed to occur through non-mutational mechanisms, including hypermethylation of the *BRCA1* promoter or malfunctions in the upstream regulatory pathways of *BRCA1* (James et al., 2007).

The human *BRCA1* gene encodes a 220 kDa nuclear phosphoprotein that exists as a component of several different large multi-protein complexes involved in numerous cellular functions, including transcriptional regulation, protein ubiquitination, cell cycle checkpoint control, DNA repair and homologous recombination (Monteiro, 2000; Scully & Livingston, 2000; Khanna & Jackson, 2001). Both BRCA1 and BRCA1 play integral roles in the maintenance of genomic stability through the repair of double-strand breaks (DSB). This is exemplified in the observation that BRCA1-deficient tumours are associated with a high degree of anueplody and a 2 fold increase in the number of genetic alterations (Tirkkonen et al., 1997).

A role for BRCA1 in the repair of DSBs was first suggested following the report that BRCA1 nuclear foci co-localise with Rad51, a protein involved in homologous recombination and DNA damage responses (Scully et al., 1997b). BRCA1 and Rad51 have also been observed in a complex with BRCA2 (termed BRCC - **BR**CA1-BRCA2 **C**ontaining **C**omplex) and the BRCA1 heterodimeric interacting partner, BARD1 (BRCA1-associated Ring Domain 1) (Jin et al., 1997; Scully et al., 1997a; Chen et al., 1998). In addition to its function during homologous recombination, this BBRC complex has also been shown to possess ubiquitin ligase activity, regulating the protein levels of various factors involved in DNA repair (Gudmundsdottir & Ashworth, 2006).

Although the BRCA1-BARD1 heterodimer has been shown to be involved in DSB repair and ubiquitination, it is suggested that these proteins also possess independent functions as both

BRCA1 and BARD1 are not consistently coexpressed in similar tissues (Irminger-Finger et al., 1998). Indeed, BARD1 has been shown to possess a BRCA1-independent tumour suppressor function as a mediator between proapoptotic stress and p53-dependent apoptosis (Irminger-Finger et al., 2001). BARD1 was shown to interact with p53 via its ankyrin (ANK) domain, a process that is perturbed by the presence of the tumour-associated mutation, $BARD1^{Q564H}$ (Irminger-Finger et al., 2001; Feki et al., 2005). These findings signify the importance of this interaction in the prevention of breast tumorigenesis through the programmed death of pre-malignant cells.

1.3 – Identification of breast cancer tumour suppressor genes

Known tumour suppressor genes are located in chromosomal regions of frequent tumour-specific LOH. However, use of defined regions of LOH in tumours to identify novel tumour suppressor genes has been more challenging. Tumour-related allelic imbalance has assisted in the identification, mapping or cloning of numerous tumour suppressor genes, including *TP53* (Gleich et al., 1996; Dolan et al., 1999; Katsama et al., 2000; Forslund et al., 2002; Semczuk et al., 2005), *RB* (Francke & Kung, 1976; Knudson et al., 1976; Noel et al., 1976) and *PTEN* (Tokiyoshi et al., 1996; Teng et al., 1997).

1.3.1 – Identification of the smallest region of overlap for 16q LOH in breast cancer

Numerous cytogenetic studies have implicated the loss of the long arm of human chromosome 16 as an early event in breast carcinogenesis as it is frequently observed in tumours with no other genetic abnormalities (Dutrillaux et al., 1990; Hulten et al., 1993; Gong et al., 2001). Chromosome 16 has been identified as one of the most frequently altered chromosomes in breast cancer (Miller et al., 2003), with 16q LOH occuring in at least half of all breast tumours (Cleton-Jansen et al., 2001; Miller et al., 2003).

Numerous allelotyping studies have implicated loss of 16q in various tumour types including breast (Cleton-Jansen et al., 2001; Miller et al., 2003), prostate (Suzuki et al., 1996), lung (Sato et al., 1998), hepatocellular (Chou et al., 1998), gastric (Mori et al., 1999), head and neck squamous cell carcinoma (Wang et al., 1999), retinoblastoma (Gratias et al., 2007) and rhabdomyosarcoma (Visser et al., 1997). Identification of allelic imbalance at a locus in tumours has been widely reported as a means of identifying putative tumour suppressor genes (Osborne & Hamshere, 2000; Cleton-Jansen et al., 2001). Therefore, it is likely that 16q harbours several tumour suppressor genes whose products are involved in the suppression of a variety of malignancies. Therefore, attempts have been made to delineate the smallest region of overlap (SRO) for 16q LOH in breast tumours (Tsuda et al., 1994; Driouch et al., 1997; Cleton-Jansen et al., 2001; Callen et al., 2002). The largest of these studies involved the analysis of 16q LOH and used a high density of genetic markers in 712 breast tumours (Cleton-Jansen et al., 2001). From this study, three distinct regions of allelic imbalance were identified; two at 16q24.3 and one at 16q22.1.

1.3.1.1 – CDH1: the 16q22.1 breast cancer tumour suppressor gene

The epithelial cadherin gene (*CDH1*) has been identified as a tumour suppressor located within the 16q22.1 LOH region (Cleton-Jansen, 2002). The *CDH1* gene encodes a transmembrane glycoprotein involved in cell-cell adhesion, and loss of *CDH1* expression contributes to increased proliferation, invasion and metastasis of lobular breast carcinomas (Huiping et al., 1999). Truncating mutations in *CDH1* have been found in approximately 60% of ILCs (Berx et al., 1995; Berx et al., 1996; Huiping et al., 1999; Cleton-Jansen, 2002). However, no mutations in *CDH1* have been identified in invasive ductal carcinomas (IDC) (Cleton-Jansen, 2002), suggesting that a role for *CDH1* as a tumour suppressor is limited to the rarer lobular subgroup of breast carcinomas. Therefore, it is likely that other tumour suppressor genes within 16q are responsible for the suppression of IDC oncogenesis.

1.3.1.2 - Identification of the 16q24.3 breast cancer tumour suppressor gene(s)

The minimal region of breast cancer LOH at chromosome loci 16q24.3 has been delineated to an interval between the polymorphic marker *D16S498* and the 16q telomere (Cleton-Jansen et al., 2001). Prior to our research, no known breast cancer tumour suppressors were identified within this region have been identified. Therefore, our aim was to identify novel breast cancer tumour suppressor gene(s) from this 16q24.3 LOH region. As the human genome project was incomplete at this time, it was necessary for our laboratory to construct a high-resolution bacterial (BAC) and P1 artificial chromosome (PAC)-based physical map spanning over 2.4Mb of 16q24.3 from *D16S498* to the telomere (Powell et al., 2002). Following its physical mapping, our laboratory defined all the genes and transcripts located within this region. Breast cancer-specific mutation screening was conducted on seven of the genes located within this interval; *SPG7* (Settasatian et al., 1999), *BBC1* (Moerland et al., 1997), *CPNE7* (Savino et al., 1999), *CDK10* (Crawford et al., 1999), *FANCA* (Cleton-Jansen et al., 1999), *GAS11* and *C16ORF3* (Whitmore et al., 1998). None of these genes were found to harbour tumour-restricted mutations in sporadic breast cancer DNA samples.

We hypothesized that breast cancer tumour suppressor gene(s) from the 16q24.3 LOH region would be down-regulated in a proportion of breast tumours or cancer cell lines due to the presence of mutations or promoter hypermethylation. However, not all tumours would be expected to lose expression of a particular tumour suppressor gene. Therefore, our laboratory investigated the variations in expression of the 104 genes located within the 16q24.3 breast cancer LOH region in a panel of eleven breast cancer cell lines as compared to their expression profiles in normal breast tissue and cell line derived from normal breast epithelium (Powell et al., 2002). From this screen, *FBXO31*, *CBFA2T3* and *CYBA* were identified as genes that exhibited highly variable expression profiles as compared to the baseline

expressional variation of several housekeeping genes (Powell et al., 2002). Evidence for their potential role as breast cancer tumour suppressors is discussed below.

1.3.1.2.1 - FBXO31 (F-box protein 31)

Findings from our research provide significant evidence to suggest that *FBXO31* is a putative breast cancer tumour suppressor gene (Kumar et al., 2005). My contribution to the characterisation of the function of FBXO31 is discussed in further detail in Chapter 4.

1.3.1.2.2 - CBFA2T3 (Core-binding factor, runt domain, alpha subunit 2; translocated to 3)

CBFA2T3 (*MTG16, ETO2*), together with the homologues *CBFA2T1* (*MTG8, ETO, RUNX1T1*) and *CBFA2T2* (*MTGR1*), form the small "ETO" family of proteins, named after the Eight-Twenty-One chromosomal translocation, t(8;21). This translocation between *CBFA2T1* on chromosome 8, and RUNX1 (AML1) on chromosome 21 generates an oncogenic fusion protein commonly involved in the pathology of acute myeloid leukemia (AML) (Miyoshi et al., 1991; Calabi & Cilli, 1998). *CBFA2T3* is also involved in a similar leukaemia translocation, t(16;21)(q24;q22), that also generates a fusion protein with the transcription factor, *RUNX1*, that is restricted to a rarer group of therapy-related AML (Gamou et al., 1998). The ETO family members function as transcriptional repressors through the formation of high-molecular weight repressor complexes that include N-CoR, SMRT and mSin3A, leading to the recruitment of HDACs and subsequent histone deacetylation and transcriptional repression (Wang et al., 1998). Gene specificity of the ETO-based repressor complexes is achieved through the selective recruitment of DNA-binding transcription factors that bind to specific DNA response elements within the promoters of target genes. These transcription factors include BCL6 (Chevallier et al., 2004), PLZF (Melnick et al., 2000), GFI-1 (McGhee et al., 2003) and the recently identified ZNF652 (Kumar et al., 2006).

CBFA2T3 expression was shown to be significantly reduced in some primary breast tumours and breast cancer cell lines when compared with the expression of *CBFA2T3* in normal breast tissue or nontransformed human mammary epithelial cells (HMECs) (Kochetkova et al., 2002; Bais et al., 2004). Reintroduction of *CBFA2T3* into breast cancer cell lines with reduced endogenous levels of *CBFA2T3* expression resulted in a reduction of their clonogenicity on plastic and soft agar (Kochetkova et al., 2002), suggesting that CBFA2T3 displays functional properties consistent with that of a breast cancer tumour suppressor. Taken together, these findings provide evidence to support a role for *CBFA2T3* as a breast cancer tumour suppressor gene from the 16q24.3 LOH region. Despite these findings, the downstream target genes of the CBFA2T3-based corepressor complex involved in the prevention of breast oncogenesis have not as yet been determined.

1.3.1.2.3 – CYBA (Cytochrome b-245, alpha polypeptide)

CYBA was one of the three genes observed to exhibit highly variable expression in breast cancer cell lines (Powell et al., 2002). *CYBA* encodes the p22(phox) protein and has been extensively studied as an NADPH oxidase in the microbicidal function of phagocytes (Sumimoto et al., 1994). p22(phox) associates with gp91(phox) to form cytochrome b-558, which has a role as the final electron transporter during the oxidation of NADPH. Oxidation of NADPH results in the generation of reactive oxygen species, which have been widely implicated in the initiation and progression of tumorigenesis (Brown & Bicknell, 2001). Germ-line mutations in *CYBA* have been shown to cause chronic granulomatous disease, a rare inherited disorder that results in a severe defect in host defence and consequent predisposition to microbial infection (Rae et al., 2000). *CYBA* expression was also recently reported to correlate with response to neoadjuvant docetaxel treatment, suggesting a possible role for *CYBA* as a biomarker for chemosensitivity in breast cancer patients (Chang et al., 2007). Based on these findings, it is likely that CYBA is acting as a sensitive surrogate

marker for chromosome 16 LOH and is not functioning as a tumour suppressor. However, it is still possible that *CYBA* is involved in the suppression of breast cancer, but further experimentation to characterise its function during breast oncogenesis is necessary. Unfortunately, our laboratory has not had the opportunity to further investigate *CYBA* as a candidate breast cancer tumour suppressor.

1.3.1.3 - Does 16q24.3 harbour multiple breast cancer tumour suppressor genes?

Thus far, there have not been any breast cancer-specific mutations identified in either *FBXO31* or *CBFA2T3*, suggesting that these genes do not follow the classical Knudson's "two-hit" hypothesis for inactivation of a tumour suppressor gene (Knudson, 1971). Aberrant *CBFA2T3* promoter methylation was recently observed in breast cell lines and tumours (Bais et al., 2004), suggesting that the down-regulation of *CBFA2T3* expression during breast oncogenesis results from a combination of epigenetic silencing and 16q24.3 LOH. Intriguingly, both *CBFA2T3* and *FBXO31* showed a correlation in their down-regulated expression in breast tumours that was independent of 16q LOH status (Callen, unpublished), suggesting a common mechanism of gene silencing of these 16q24.3 breast cancer tumour suppressors. We hypothesize that a combination of long-range epigenetic silencing (Frigola et al., 2006) of 16q24.3 and LOH of chromosome 16q results in the simultaneous loss, or reduction in activity, of both *FBXO31* and *CBFA2T3* and this contributes to the early stages of breast tumorigenesis. This notion also raises the possibility of the existence of other additional tumour suppressor genes located within the 16q24.3 LOH region. Therefore, we hypothesize that 16q LOH associated with breast tumorigenesis is a complex process that involves loss of expression of multiple tumour suppressor genes from the 16q24.3 LOH region.

Studies in other tumour types also suggest that more than one tumour suppressor gene may reside in regions of cancer LOH; for example, the 3p LOH region in small cell lung carcinoma contains several tumour suppressor genes (Zabarovsky et al., 2002; Imreh et al., 2003). Similarily, we hypothesize that multiple breast cancer tumour suppressors are located within the 16q24.3 LOH region. This is consistent with the finding that in the majority of breast cancers with chromosome 16q LOH have extended regions of LOH over large expanses of this chromosome. In addition to the screen for 16q24.3 tumour suppressor genes based on the variablility of their expression in a panel of breast cancer cell lines (Powell et al., 2002), additional candidate tumour suppressor genes were also selected based on homologies or possible connections with genes that are associated with breast oncogenesis. From this screen, *ANKRD11* was identified as a potential candidate based on homology to a gene with a known involvement in the suppression of breast cancer (Powell, 2003).

1.3.1.3.1 - ANKRD11 (Ankyrin repeat domain 11)

ANKRD11 is located approximately 500kb from the 16q telomere and is therefore located within the 16q24.3 LOH region. *ANKRD11* was selected as a candidate breast cancer tumour suppressor as it shared a high level of homology with the ankyrin (ANK) domains of BARD1 (Powell, 2003). A major topic of this thesis is the functional characterisation of ANKRD11, and our data suggests that *ANKRD11* is a putative breast cancer tumour suppressor gene. The functional characterisation of *ANKRD11* is discussed in further detail throughout Chapter 3.

Chapter 2: General Materials and Methods

2.1 - Cell lines and transient transfections

184V and 48RS are finite life span human mammary epithelial cells (HMEC), whereas 184A1 is a non-malignant immortally transformed cell line derived from normal breast epithelium (Stampfer & Yaswen, 2003). These cell lines were kindly supplied by Dr Martha Stampfer and were grown in MCDB-170 medium (Life Technologies). GP-293 cells were purchased from BD Biosciences. The HCT116 colon carcinoma cell line and its $p53^{-/-}$ derivative were kindly supplied by Dr Bert Vogelstein (Bunz et al., 1998). MCF-10A (human non-malignant immortalised breast epithelial), MCF-7, BT-20, T47-D, MDA-MB-231, MDA-MB-468, SKBR-3 (human breast cancer), HEK293T (human embryonic kidney), Saos-2 (human osteosarcoma), HeLa (human cervical cancer), COS-7 (monkey kidney) and CHO (hamster ovary) cell lines were purchased from the American Type Culture Collection (ATCC) and were grown in the recommended media. Cells were grown at 37°C in 5% CO_2.

Transfections were performed in Opti-MEM media using Lipofectamine 2000 transfection reagent in accordance with the manufacturer's protocol (Invitrogen). Briefly, cells were seeded in a 6-well plate at 60-80% confluency in antibiotic-free medium. The indicated amount of DNA was mixed with 250 µL Opti-MEM, and 10 µL of Lipofectamine 2000 was mixed with 250 µL Opti-MEM in a second tube. The aliquots were incubated at room temperature for 5 mins, mixed and incubated for a further 20 mins at room temperature. Complexes were subsequently added drop-wise to the cells and incubated for at least 24 hours at 37°C before analysis. The medium was replaced 6-8 hours post-transfection with fresh medium.

2.2 – Antibodies

Commercial antibodies utilised throughout this thesis are as described in Table 2.1:

Table 2.1: Source and origin of commercial antibodies used throughout this research.

Antibody	**Origin**	**Source**
α-β-actin	mouse	Sigma-Aldrich
α-Cul-1 (cullin-1)	mouse	Santa Cruz Biotechnology
α-cyclin B1	mouse	Neomarkers
α-FLAG	mouse	Sigma-Aldrich
α-GFP (green fluorescent protein)	mouse	Roche
α-GST (glutathione s-transferase)	goat	Amersham Biosciences
α-HA (hemagglutinin)	rabbit	Santa Cruz Biotechnology
α-MEK (mitogen-activated protein / extracellular signal-related kinase 2)	rabbit	Neomarkers
α-myc	mouse	Neomarkers
α-nucleophosmin	mouse	Sigma-Aldrich
α-p21^{waf1}	mouse	Neomarkers
α-p53	mouse	Neomarkers
α-acetyl-p53 (Lys320)	rabbit	Upstate
α-PML (promyelocytic leukaemia)	rabbit	Santa Cruz Biotechnology
α-Skp1 (S-phase kinase-associated protein 1)	rabbit	Neomarkers
α-Roc-1	rabbit	Neomarkers
α-SUMO-1 (small ubiquitin-like modifier-1)	rabbit	Santa Cruz Biotechnology

2.3 - Immunofluorescence

Cells were seeded in Lab-Tek II Chamber Slides (Nalge Nunc), transiently-transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) or treated with mitomycin C (20 μg/mL), thymidine (2 mM) or nocodazole (1 μM). At the indicated times post-transfection or post-treatment, cells were fixed in 2% paraformaldehyde (15 mins, RT) and permeabilised in 0.4% Triton-X-100 (15 mins, RT). Cells were incubated with the indicated primary antibody in 5% donkey serum (overnight, 4°C), followed by incubation with the appropriate Alexa Fluor-, rhodamine-, fluorescein- or Cy5-conjugated antibodies (Molecular Probes) in 5% donkey serum (1 hour, RT) and mounted in VECTASHIELD mounting media with DAPI (Vector Laboratories). For staining of DNA/chromatin-rich regions, cells were initially treated with RNase A (1 μg/mL, 37°C, 30 mins) and subsequently incubated with propidium iodide (PI) solution (100 μg/mL, 37°C, 30 mins). Cells were imaged on an Olympus IX70 inverted microscope and a BioRad Radiance 2100 Confocal Microscope equipped with three lasers, Argon ion 488 nm; Green HeNe 543 nm; Red Diode 637 nm.

2.4 - Reverse transcription real time-PCR (RT-PCR)

ANKRD11, *CDKN1A* ($p21^{waf1}$) or *FBXO31* expression were determined by real-time RT-PCR using specific forward and reverse primers (as listed in Table 2.2) that spanned across intron/exon boundaries to eliminate possible genomic DNA contamination. Total RNA was isolated using RNeasy Mini Kit (QIAGEN) from pellets of cell lines grown to ~80% confluence. Complementary DNA (cDNA) was synthesized from 2 μg of RNA using the M-MLV reverse transcriptase (RNase H^-) (Promega) in accordance with the manufacturer's protocol. Briefly, 2 μg of RNA was initially incubated with 0.5 μg of oligo$(dT)_{24}$ primer at 72°C for 4 mins, and then on ice for 4 mins. The reverse transcription reaction (90 mins at 42°C, followed by 15 mins at 70°C) was performed in 0.5 μM dNTPs, 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT and 50-100 units M-MLV RT (H^-). Each

real-time RT-PCR reaction (3 min activation of the polymerase at 95°C, 45 cycles of 15 sec at 94°C, 30 sec at 57°C and 30 sec at 72°C; signal detection at 72°C) contained 0.2 µl of this cDNA and used SYBR Green (Bio-Rad IQ Supermix) on a BioRad iCycler (BioRad, Hercules, CA). Standards of known RNA concentrations were used to generate standard curves for quantitative real-time RT-PCR analysis. The housekeeping gene *cyclophilin A* was used to normalize the expression of *ANKRD11*, *CDKN1A* or *FBXO31*, as previous studies have shown minimal variation in the levels of this transcript in breast cell lines (Powell et al., 2002). On occasions, *β-actin* was used as an additional housekeeping gene to ensure the consistency of *cyclophilin A* expression in response to various treatments. Real-time PCR expression data was quantitated using the Bio-Rad iQ5 Optical System Software. Normalised expression data for the cell lines was calculated relative to the expression in the HMEC cell line 184V and presented as mean ± SEM (standard error of the mean) of triplicates. The significance of the down-regulation of average *ANKRD11* or *FBXO31* expression in breast cancer cell lines when compared with the average *ANKRD11* or *FBXO31* expression in finite life-span HMECs and non-malignant immortalized breast epithelial cells was determined using analysis of variance (ANOVA).

2.5 - Western blot analysis

Cells were lysed in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4 with 1× protease inhibitors (Roche), sonicated and then centrifuged. Clarified lysates were assayed for total protein content using the bicinchoninic acid protein assay (Pierce). Equal quantities of protein from cell lysates were resolved in 1× protein loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) on SDS-PAGE (Laemmli et al., 1970) and transferred on to Hybond-C Extra (Amersham Biosciences). Membranes were probed with the relevant primary antibodies to detect the protein of interest and hybridized with appropriate horseradish peroxidase-

conjugated secondary antibodies using an ECL detection system (Amersham Biosciences) in accordance with standard protocols (Ausubel, 2001).

2.6 – Oligo-primers and DNA constructs

Primers used during real-time RT-PCR, PCR amplification of coding regions and overlap PCR mutagenesis are listed in Table 2.2. Table 2.3 presents the generation or source of DNA constructs used throughout this research.

2.7 – DNA sequence analysis

The sequences of all constructs was confirmed through DNA sequence analysis using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in accordance with the manufacturer's protocol. Briefly, the sequencing reaction (10 μL) was performed in the presence of double stranded plasmid DNA (150 ng), primer (2.5 pmol) and BigDye Terminator v3.1 (2 μL). The sequencing reaction (1 min activation at 96°C, 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C) was isopropanol precipitated, washed and analysed using a ABI Prism 3730 DNA Analyser.

2.8 – In silico analysis

In silico analysis of the predicted ANKRD11 or FBXO31 amino acid sequences was performed to identify putative protein domains and motifs using PROSITE (ExPASy), PESTfind (EMB) and ELM (EMBL) programs. Sequence alignment was performed using the ClustalW program (EMBL-EBI). *ANKRD11* (accession NM_013275) and *FBXO31* (accession NM_024735) gene sequences were derived from Genebank, NCBI.

2.9 – Cell Culture Synchronisation

Cells were synchronized at the G_1-S phase using a double thymidine block or at G_2-M phase using a nocodazole block. For G_1-S phase synchronisation, cells were grown in the presence of 2 mM thymidine (Sigma-Aldrich) for 24 hours and then washed with warmed media and grown in fresh pre-warmed medium without thymidine for a further 10 hours. Cells were then cultured in the presence of 2 mM thymidine for a further 16 hours and finally released from the G_1-S block by washing twice with fresh medium and collected at various time-points post-release. For G_2-M phase synchronisation, cells were grown in the presence of 1 µM nocodazole for 16 hours and thoroughly washed in media to release the cells from the block. The cells were then grown in fresh media without nocodazole and immunostained at various time-points post-release.

Table 2.2: Primers used for real-time RT-PCR, PCR amplification of coding regions and overlap PCR mutagenesis.

#	Primer Name	Primer Sequence (5' → 3')	Direction	Description
1	B-act-1177-StuI-R	CACACAGGCCTCCGGACTCGTCATACTCCT GCTTG	Reverse	Real-time RT-PCR analysis of *β-actin*
2	B-act-911-BglII-F	CACACAGATCTTACCTTCAACTCCATCATGA AGTG	Forward	Real-time RT-PCR analysis of *β-actin*
3	CYC-F	GGCAAATGCTGGACCCAACACAAA	Forward	Real-time RT-PCR analysis of *cyclophilin A*
4	CYC-R	CTAGGCATGGGAGGGAACAAGGAA	Reverse	Real-time RT-PCR analysis of *cyclophilin A*
5	EGFP-T13FUS-F	CACACACACAGGATCCCGCCACCATGGTGA	Forward	For generation of pLNCX2-GFP-ANKRD11 fusion construct.
6	EGFP-T13FUS-R	CACACACACAGGATCCCTTGTACAGCTCGT	Reverse	For generation of pLNCX2-GFP-ANKRD11 fusion construct.
7	ERBP-BHI-F	CACACACACAGGATCCCCATGGTGGTTACC AGATCTGCACG	Forward	For generation of HA-ERBP construct
8	ERBP-BHI-R	CACACACACAGGATCCTTAATTGCGAAATT TCTTCTTCTTTCG	Reverse	For generation of HA-ERBP construct
9	FBXO31-NB-F	CTTCACCGATATAGACAC	Forward	For generation of FBXO31 Northern blot probe
10	FBXO31-NB-R	GGCCGTACATGCACTCCACTG	Reverse	For generation of FBXO31 Northern blot probe
11	GLprimer2-BR	CTTTATGTTTTTGGCGTCTTCCA	Reverse	Biotinylated reverse primer for pGL2 vector – used to generate the biotinylated *CDKN1A* promoter region

12	Hs7970-5-7-F1	CCGGCGGGAGGCAGGAGGAGT	Forward	Real-time RT-PCR analysis of *FBXO31*
13	Hs7970-5-7-R1	GCGGCGGTAGGTCAGGCAGTTGTCG	Reverse	Real-time RT-PCR analysis of *FBXO31*
14	myc-EcoRI-F	CACACACACAGAATTCCATGGAGCAGAAGC TGATCAGCGAG	Forward	For generation of GFP-FBXO31 and GFP-FBXO31ΔF fusion constructs
15	p14ARF-BHI-R	CACACACACAGGATCCTCAGCCAGGTCCAC GGGCAGAC	Reverse	For generation of GFP-$p14^{ARF}$ construct
16	p14ARF-ERI-F	CACACACACAGAATTCCATGGTGCGCAGGT TCTTGGTGAC	Forward	For generation of GFP-$p14^{ARF}$ construct
17	p21-F	TGGACCTGGAGACTCTCAGGGTCG	Forward	Real-time RT-PCR analysis of *CDKN1A*
18	p21-R	TTAGGGCTTCCTCTTGGAGAAGATC	Reverse	Real-time RT-PCR analysis of *CDKN1A*
19	p53-BHI-F	CACACACACACAGGATCCATGGAGGAGCC GCAGTCAGATCCTAG	Forward	For generation of pMAL-c2x-p53 fusion construct
20	p53-EcoRI-R	CACACAGAATTCTCAGTCTGAGTCAGGCCC TTCTGTCTTG	Reverse	For generation of pKS(+)-myc-p53
21	p53-N-myc-NX-F	CACACACACACAGCGGCCGCTCTAGAATGG AGCAGAAGCTGATCAGCGAGGAGGACCTG ATGGAGGAGCCGCAGTCAGATCCTAG	Forward	For generation of pKS(+)-myc-p53
22	pLNCX2-2882-F	AGCTGGTTTAGTGAACCGTCAGATC	Forward	Forward pLNCX2 primer
23	pLNCX2-3075-R	CTTAAGCTAGCTTGCCAAACCTACAG	Reverse	Reverse pLNCX2 primer
24	RVprimer3-F	CTAGCAAAATAGGCTGTCCC	Forward	Forward primer for pGL2 and pGL3 vectors - used to generate the *CDKN1A* promoter region

25	SCR-RKS-F	GATCCCCGACACGCGACTTGTACCACTTCAAGAGAGTGGTACAAGTCGCGTGTCTTTTTGGAAA	Forward	For generation of scramble shRNA
26	SCR-RKS-R	AGCTTTTCCAAAAAGACACGCGACTTGTACCACTCTCTTGAAGTGGTACAAGTCGCGTGTCGGG	Reverse	For generation of scramble shRNA
27	shT13-BBRC-F	GATCCCCGCGGAAGCTGCCCTTCACCTTCAAGAGAGGTGAAGGGCAGCTTCCGCTTTTTGGAAA	Forward	For generation of ANKRD11-specific shRNA
28	shT13-BBRC-R	AGCTTTTCCAAAAAGCGGAAGCTGCCCTTCACCTCTCTTGAAGGTGAAGGGCAGCTTCCGCGGG	Reverse	For generation of ANKRD11-specific shRNA
29	T13-1353-myc-R	CACACACACACACATTATCGATTCAGGTCCTCCTCGCTGATCAGCTTCTGTTCCTTCTGCTGCTTGGCATTAG	Reverse	For generation of ANKRD11$^{1\text{-}451aa}$-myc construct
30	T13-2446-BHI-F	CACACACAGGATCCCGGACGAATATTGTAACAAAAATCAG	Forward	For generation of FLAG-ANKRD11$^{816–1802aa}$ construct
31	T13-7507-BHI-F	CACACACACAGGATCCCCAAGGAGCTGTTCAGGCAGCAGGAG	Forward	Real-time RT-PCR analysis of *ANKRD11*
32	T13-7975-BHI-R	CACACACAGGATCCTCAGTCGTCGTTGACGTCGACCATG	Reverse	Real-time RT-PCR analysis of *ANKRD11*
33	T13-BN-ANK-F	CACACACACAGGATCCGCGGCCGCACCATGTCTACAGTGTGTCAGAAGGGAAC	Forward	For generation of FLAG-ANKRD11$^{144\text{-}313aa}$ construct
34	T13-EC-ANK-R	CACACACACAGAATTCTTAATCGATACTGGAAGGTGCGAAGGATGGTG	Reverse	For generation of FLAG-ANKRD11$^{144\text{-}313aa}$ construct
35	T13-Int1-K-F	CACACACACACAGGTACCCAAAGCCACCAGACCTCCGTTC	Forward	For cloning of the ANKRD11 intronic region encompassing the putative p53-RE

36	T13-Int1-X-R	CACACACACACACACTCGAGCAGCAGA ACCTTGCTGTGCGTGTC	Reverse	For cloning of the ANKRD11 intronic region encompassing the putative p53-RE
37	T13-p53-M4-F	TGGAGCTTGCAAAAGCTTGCAAAAACGCTT TCCTCACTAAGGATTTAA	Forward	For overlap PCR mutation of the putative p53-RE in intron 1 of *ANKRD11*.
38	T13-p53-M4-R	GCGTTTTTGCAAGCTTTTGCAAGCTCCAAAG CACTGGCACCAGTGGGCGT	Reverse	For overlap PCR mutation of the putative p53-RE in intron 1 of *ANKRD11*.
39	UR	GGAAACAGCTATGACCATG	Reverse	Universal reverse primer for pBLUESCRIPT

Table 2.3: Generation or source of constructs reported through this thesis

Construct Name	Insert and Expression Vector	Generation / Source of Construct
ANKRD11$^{1\text{-}451aa}$-myc	pLNCX2-ANKRD11$^{1\text{-}451aa}$-myc	Nucleotides 1-1353 of the ANKRD11 ORF (encoding the first 451 aa) was PCRed with primers #22 and #29 , and cloned at *Bgl*II / *Cla*I sites of pLNCX2 (generated by Dr Raman Sharma) (Kumar et al., 2005).
ANKRD11$^{2369\text{-}2663aa}$	pACT2-ANKRD11$^{2369\text{-}2663aa}$	Generated by Prof J. Don Chen's laboratory (Robert Wood Johnson Medical School, New Jersey, USA) as previously described (Zhang et al., 2004).
ANKRD11$^{2369\text{-}2663aa}$	pGBT-ANKRD11$^{2369\text{-}2663aa}$	ANKRD11 coding region from pACT2-ANKRD11$^{2369\text{-}2663aa}$ was cloned at *Bam*HI / *Xho*I sites of pGBT9 - generated by Prof J. Don Chen's laboratory.
ANKRD11-myc	pLNCX2-ANKRD11-myc	ANKRD11 ORF was cloned with a carboxyl-terminal myc epitope tag at *Bgl*II / *Cla*I sites of pLNCX2 (Powell, 2003) (generated by Dr Jason Powell).
ANKRD11-p53-RE-Luc	pGL3-Basic-ANKRD11-p53-RE-Luc	*ANKRD11* intronic region containing a putative p53-RE was PCR amplified from Hut78 genomic DNA using primers #35 and #36 and cloned at *Kpn*I / *Xho*I sites of pGL3-Basic.
ANKRD11-p53-RE-mut-Luc	pGL3-Basic-ANKRD11-p53-RE-mut-Luc	Putative p53 response element (GAACATGCCAGGTCATGTCT) in *ANKRD11* intronic region from pGL3-Basic-ANKRD11-p53-RE-Luc construct was mutated to (CTTGCAAAAGCTTGCAAAA) by overlap PCR using primers #37 and #38.
CDKN1A-pro-Luc	pGL3-Basic-CDKN1A-pro-Luc	2.3kB of genomic *CDKN1A* promoter sequence containing 2× p53Res (el-Deiry et al., 1993) cloned at *Hind*III site of pGL3-Basic - a kind gift from Prof Moshe Oren.

EBNA-LP	pSNOC-EBNA-LP	ENBA-LP ORF cloned into pSNOC – a kind gift from Dr Tom Sculley (QIMR, Queensland).
ERE-TK-Luc	ERE-TK-Luc	A TK promoter with 3× upstream consensus EREs (estrogen response elements) was cloned in pLUC – a kind gift from Prof Wayne Tilley (Dame Roma Mitchell Cancer Research Laboratories, South Australia).
ER-α	pSG5-ER-α	ER-α ORF cloned in pSG5 – a kind gift from Prof Wayne Tilley.
FLAG-ANKRD11	pCMV-Tag2-ANKRD11	ANKRD11 ORF excised from pLNCX2-ANKRD11-myc through restriction digestion using *Hind*III and *Cla*I (blunted) and cloned at *Hind*III / *Xho*I (blunted) sites of pCMV-Tag2-B (generated by Dr Raman Sharma).
FLAG-ANKRD11$^{1\text{-}817aa}$	pCMV-Tag2-ANKRD11$^{1\text{-}817aa}$	Nucleotides 1-2493 of the ANKRD11 ORF was excised from pLNCX2-ANKRD11-myc through restriction digestion using *Bgl*II / *Ssp*I and cloned at *Bam*HI / *Eco*RV sites of pCMV-Tag2-A.
FLAG-ANKRD11$^{144\text{-}313aa}$	pCMV-Tag2-ANKRD11$^{144\text{-}313aa}$	Nucleotides 429-916 of the ANKRD11 ORF (the region sharing homology with the BARD1 ANK domain) was PCR amplified from pLNCX2-ANKRD11-myc with primers #33 and #34 and cloned at *Bam*HI / *Eco*RI sites of pCMV-Tag2-C.
FLAG-ANKRD11$^{816\text{-}1802aa}$	pCMV-Tag2-ANKRD11$^{816\text{-}1802aa}$	A coding region of pLNCX2-ANKRD11-myc was PCR amplified with primers #30 and #32, restriction digested with *Bam*HI / *Eco*RI (2446bp to 5408bp) and cloned at *Bam*HI / *Eco*RI sites of pCMV-Tag2-C.
FLAG-ANKRD11$^{1803\text{-}2203aa}$	pCMV-Tag2-ANKRD11$^{1803\text{-}2203aa}$	Nucleotides 5408-6611 were excised from pLNCX2-ANKRD11-myc through restriction digestion using *Eco*RI / *Xho*I and cloned at *Eco*RI / *Xho*I sites of pCMV-Tag2-B.
FLAG-ANKRD11$^{2352\text{-}2663aa}$	pCMV-Tag2-ANKRD11$^{2352\text{-}2663aa}$	Nucleotides 7058-7992 were excised from pCMV-Tag2-ANKRD11 through restriction digestion using *Apa*I and cloned at the *Apa*I site of pCMV-Tag2-A.

FLAG-BARD1	pFLAG-CMV2-BARD1	BARD1 ORF cloned at *Not*I / *Xba*I sites in pFLAG-CMV2 – a kind gift from Dr Beric Henderson (The University of Sydney, New South Wales).
FLAG-HA-FBXO31	pQCXIN-FLAG-HA-FBXO31	Dual-tagged (FLAG and HA) FBXO31 cloned into the pQCXIN retroviral vector (generated by Dr Raman Sharma).
FLAG-Iκβα	pCMV-Iκβα	Iκβα ORF cloned into pCMV vector (Brockman et al., 1995) – a kind gift from Dr Dean Ballard (Vanderbilt University Medical Center, Tennessee, USA).
FLAG-P/CAF	pAB-FLAG-hP/CAF	Generated by Prof J. Don Chen's laboratory.
FLAG-PML I	pCI-neo-FLAG-PML I	PML isoform I – a kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).
FLAG-PML II	pCI-neo-FLAG-PML II	PML isoform II – a kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).
FLAG-PML III	pCI-neo-FLAG-PML III	PML isoform III – a kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).
FLAG-PML IV	pCI-neo-FLAG-PML IV	PML isoform IV – a kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).
FLAG-PML V	pCI-neo-FLAG-PML V	PML isoform V – a kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).
FLAG-PML VI	pCI-neo-FLAG-PML VI	PML isoform VI – a kind gift from Dr Lawrence Banks (International Centre for Genetic Engineering and Biotechnology, Trieste, Italy).

GFP-ANKRD11-myc	pLNCX2-GFP-ANKRD11-myc	GFP ORF PCR amplified from pEGFP-C1Δ*Bgl*II-*Bam*HI with primers #5 and #6, restriction digested with *Bam*HI and cloned at the *Bgl*II site of pLNCX2-ANKRD11-myc (generated by Dr Raman Sharma).
GFP-FBXO31	pEGFP-C1-FBXO31	The FBXO31 ORF was PCR amplified from pLNCX2-myc-FBXO31 using primers #14 and #23, restriction digested with *Eco*RI and cloned at *Eco*RI site of pEGFP-C1.
GFP-FBXO31ΔF	pEGFP-C1-FBXO31ΔF	The FBXO31ΔF coding region was PCR amplified from pLNCX2-myc-FBXO31ΔF using primers #14 and #23, restriction digested with *Eco*RI and cloned at *Eco*RI site of pEGFP-C1.
GFP-H2A	pEGFP-H2A	Histone H2A fused to GFP - a kind gift from Dr Michael Brandeis, Silberman Institute of Life Sciences, Israel.
GFP-p14^{ARF}	pEGFP-C1-p14^{ARF}	p14^{ARF} ORF was PCRed from MDA-MB-468 cDNA using primers #15 and #16 and cloned at *Eco*RI / *Bam*HI sites of pEGFP-C1.
GFP-p53	pEGFP-C1-p53	The p53 ORF was PCR amplified from pLNCX2-myc-p53 using primers #14 and #23, restriction digested with *Eco*RI and cloned at *Eco*RI site of pEGFP-C1.
GST-ANKRD11$^{144\text{-}313aa}$	pGEX-5X-1-ANKRD11$^{144\text{-}313aa}$	Coding region from pCMV-Tag2-ANKRD11$^{144\text{-}313aa}$ excised through restriction digestion using *Bam*HI / *Eco*RI and cloned at *Bam*HI / *Eco*RI sites of pGEX-5X-1 (generated by Jacqueline Cawrse).
GST-ANKRD11$^{2369\text{-}2663aa}$	pGEX-5X-1-ANKRD11$^{2369\text{-}2663aa}$	Generated by Prof J. Don Chen's laboratory as previously described (Zhang et al., 2004).
HA-6×His-53bp2	pCM6HK-53bp2	53bp2 ORF cloned into pCM6HK (contains an amino-terminal HA tag and 6× His tags) (Iwabuchi et al., 1998) – a kind gift from Prof Stanley Fields (Howard Hughes Medical Institute, Seattle, USA)

hADA3	pACT-hADA3	hADA3 ORF (isolated from a human placental cDNA library in yeast two-hybrid screen) cloned into pACT (generated by Prof J. Don Chen's laboratory).
hADA3f	pCMXHA-hADA3f	hADA3 ORF from pACT-hADA3 was cloned at *Asp*718 / *Nhe*I sites in pCMXHA (generated by Prof J. Don Chen's laboratory)
HA-ANKRD11	pCMXHA-ANKRD11	Generated by Prof J. Don Chen's laboratory as previously described (Zhang et al., 2004).
HA-EBNA-LP	pCMV-HA-EBNA-LP	EBNA-LP ORF was excised from pSNOC-EBNA-LP through restriction digestion using *Bam*HI and *Eco*RI (blunted) and cloned at *Bgl*II / *Not*I (blunted) sites of pCMV-HA.
HA-ERBP	pCMV-HA-ERBP	ERBP ORF was PCR amplified from kidney cDNA using primers #7 and #8 and cloned at *Bam*HI / *Bgl*II sites in pCMV-HA.
HA-Skp1	pCMV-HA-Skp1	Generated by Dr Raman Sharma (Kumar et al., 2005).
MBP-p53	pMAL-c2x-p53	PCR amplified p53 ORF from pKS(+)-myc-p53 using primers #19 and #39, restriction digested using *Bam*HI / *Hind*III and cloned at *Bam*HI / *Hind*III sites of pMAL-c2x (generated by Dr Raman Sharma).
MDM2-pro-Luc	pGL3-Basic-MDM2-pro-Luc	400bp of genomic *MDM2* promoter containing an endogenous p53-RE (Juven et al., 1993) cloned into pGL2-Basic at SmaI (blunted *Nsi*I / *Apa*I) - a kind gift from Prof Moshe Oren (Weisman Institute of Science, Rehovot, Israel).
myc-ANKRD11$^{144\text{-}313aa}$	pCMV-myc-ANKRD11$^{144\text{-}313aa}$	Coding region from pCMV-Tag2-ANKRD11$^{144\text{-}313aa}$ excised through restriction digestion using *Bam*HI / *Kpn*I and cloned at *Bgl*II / *Kpn*I sites of pCMV-myc (generated by Jacqueline Cawrse).

myc-FBXO31	pLNCX2-myc-FBXO31	Generated by Dr Raman Sharma (Kumar et al., 2005).
myc-FBXO31ΔF	pLNCX2-myc-FBXO31ΔF	Generated by Dr Raman Sharma (Kumar et al., 2005).
myc-p53	pLNCX2-myc-p53	Generated by Dr Raman Sharma (Kumar et al., 2005).
N-myc-p53	pKS(+)-myc-p53	p53 ORF was PCR amplified from breast cDNA using primers #20 and #21 and cloned at *Xba*I / *Eco*RI sites of pKS(+) (generated by Dr Raman Sharma).
p53-RE-Luc	pGL2-Promoter-p53-RE-Luc	17× tandem consensus p53-REs cloned into pGL2-Promoter - a kind gift from Prof Moshe Oren.
pCMV-HA		Clontech Laboratories
pCMV-myc		BD Biosciences
pCMV-Tag2-A		Stratagene
pCMV-Tag2-B		Stratagene
pCMV-Tag2-C		Stratagene
pEGFP-C1		BD Biosciences
pGEX-5X-1		GE Healthcare
pGL3-Basic-Luc		Promega
pKS(+)		Stratagene
pLNCX2		BD Biosciences

pMAL-c2x		New England Biolabs
pMSCV		Invitrogen
pMSCV-puro-GFP	pMSCV-puro-GFP-H1	pMSCV retroviral vector expressing puromycin and GFP under the SV40 promoter. The *Hind*III site and 3'LTR were deleted. Inserted shRNA sequences are expressed under the H1 promoter (generated by Dr Raman Sharma).
pQCXIN		Invitrogen
pRL-TK		Promega
pSG5		A kind gift from Prof Wayne Tilley
pVSV-G		BD Biosciences
$RAC3^{1\text{-}408aa}$	$pGBT\text{-}RAC3\text{-}N^{1\text{-}408aa}$	Generated by Prof J. Don Chen's laboratory as previously described (Wu et al., 2001a).
SCR	pMSCV-SCR	Scrambled shRNA using annealed primers #25 and #26 was cloned into pMSCV-puro-GFP at *Bgl*II / *Hind*III sites.
shANK	pMSCV-shANK	Previously reported ANKRD11-specific shRNA (Zhang et al., 2007a) using annealed primers #27 and #28 was cloned into pMSCV-puro-GFP at *Bgl*II / *Hind*III sites.

Chapter 3: Functional characterisation of ANKRD11

3.1 – Identification of ANKRD11 as a novel p53 coactivator involved in the rescue of mutant p53

3.1.1 – Prelude

3.1.1.1 – Selection of ANKRD11 as a candidate 16q24.3 breast cancer tumour suppressor gene

ANKRD11 (also termed ANCO-1) was initially selected as a candidate breast cancer tumour suppressor gene within the 16q24.3 breast cancer LOH region due to the high degree of homology in the ankyrin (ANK) domain between ANKRD11 and BARD1 (Powell, 2003). In addition, *in silico* analysis of the ANKRD11 sequence identified a matching EST that was reported as a nasopharyngeal carcinoma (NPC) susceptibility protein (LZ16: nucleotide accession no. AF121775). The finding that the central region of ANKRD11 protein is a tumour antigen present in childhood medulloblastoma is also further evidence for a role for *ANKRD11* as a cancer-related gene (Behrends et al., 2003).

3.1.1.2 – Characterisation of the ANKRD11 gene

The genomic structure of *ANKRD11* was established in our laboratory during the generation of the 16q24.3 physical map, and consists of 13 exons spanning across a genomic interval of approximately 263kb (Powell, 2003). The *ANKRD11* gene contains an ORF 7995 bp, encoding a large protein of approximately 299 kDa. Prior to our research, *ANKRD11* has remained uncharacterised as a consequence of long adenosine nucleotide, or poly(A), tracts present throughout the large genomic sequence, acting as primer sites for oligo-dT during reverse transcription experiments. Furthermore, the *ANKRD11* transcript exceeds the limits of

conventional reverse transcription. As a result of these limitations, the *ANKRD11* gene was only partially characterised during the initial gene expression profiling of genes within the 16q24.3 interval (Powell et al., 2002). Furthermore, the choice of primers used in this previous profiling have subsequently been shown to not efficiently detect the ANKRD11 transcript. Subsequently, our initial aim was to investigate if *ANKRD11* was aberrantly expressed in breast carcinomas (as discussed in 3.1.3.1).

In silico analysis of the ANKRD11 amino acid sequence shows the presence of an ankyrin (ANK) domain and multiple PEST and bipartite NLS (nuclear localisation signals) sequences. The ANK domain of ANKRD11 has a high degree of homology with the ANK domain of BARD1 (45% identical; 72% similar), suggesting a similarity in the functional role of the ANK domains of these two proteins. BARD1 has been previously reported to interact with p53 via its ANK domain, mediating p53-dependent apoptosis (Feki et al., 2005). This observation prompted us to investigate if the homologous ANK domain of ANKRD11 also interacted with p53.

3.1.2 – Introduction

The p53 protein is the most important tumour suppressor in the cell and is believed to be "the guardian of the genome" (Lane, 1992). The function of p53 is predominantly dependent on its activity as a sequence-specific transcription factor, controlling the expression of various target genes that mediate biological functions such as cell-cycle arrest, apoptosis, senescence, differentiation, DNA repair and inhibition of angiogenesis and metastasis (Liu & Chen, 2006). Approximately 50% of all cancers are associated with mutations in the *TP53* gene (Vogelstein et al., 2000). The six most frequently occurring tumorigenic mutations of p53 cluster within the DNA-binding surface, involving residues that either directly contact DNA ("DNA-contact" mutations – residues R248 and R273) or perturb the structural integrity of the DNA-binding surface of p53 ("structural" mutations – residues R175, G245, R249 and R282) (Bullock & Fersht, 2001).

DNA contact mutants generally retain a similar structural integrity and thermodynamic stability to that of wild-type p53, however the mutation may significantly impair or even completely abrogate its ability to interact with its cognate DNA response element (Bullock et al., 2000). Rescue of DNA contact mutants has been demonstrated through restoration of their DNA-binding affinities following treatment with a p53-derived C-terminal peptide (Selivanova et al., 1997), small p53-activating molecules (CP-257042 and CP-31398) (Foster et al., 1999) or the introduction of second site suppressor mutations (Brachmann et al., 1998). Despite these recent advances, the development of successful pharmacological therapeutics designed to rescue p53 "DNA contact" mutants has proven to be a challenging task due to the complex bio-mechanistics that occur during the restoration of mutant p53 function.

The p53 transcription factor is stabilized and activated in response to various forms of intracellular or extracellular stress. Stabilization and activation of p53 occurs through a complex pattern of post-translational modifications, including phosphorylation and acetylation. p53 is acetylated at specific lysine residues in the carboxyl terminus by two classes of histone acetyltransferases (HATs); p300/CBP (Lys373, Lys381 and Lys382) and P/CAF (Gu & Roeder, 1997; Sakaguchi et al., 1998; Liu et al., 1999). Earlier studies have shown that acetylation of p53 promotes its ability to bind its cognate DNA response element *in vitro* (Gu & Roeder, 1997; Sakaguchi et al., 1998), however, these findings are not consistent with recent *in vivo* data (Liu & Chen, 2006; Toledo & Wahl, 2006). It has been demonstrated that the critical function of p53 acetylation *in vivo* is not to enhance the DNA binding affinity of p53, but rather to promote recruitment of coactivators/HATs to the promoters of p53 responsive genes (Barlev et al., 2001).

Recent evidence suggests that acetylation of specific lysine residues in p53 may influence the selection of specific p53 target genes, resulting in induction of either growth arrest or apoptotic pathways (Di Stefano et al., 2005; Chao et al., 2006). hADA3 (Alteration/Deficiency in Activation) has been identified as a novel p53-interacting protein capable of recruiting p300/CBP and P/CAF to p53 (Wang et al., 2001). Ectopic expression of hADA3 increased the stability and transcriptional activity of p53, and was shown to have an essential role in p53 acetylation (Wang et al., 2001). Furthermore, it has been demonstrated that hADA3 is required for $p14^{ARF}$-induced senescence of human mammary epithelial cells, a process that is dependent upon p53 acetylation (Shamanin & Androphy, 2004; Sekaric et al., 2007). Nevertheless, the current understanding of the complex molecular mechanisms involved in the regulation of p53 acetylation is incomplete.

ANKRD11 was also reported to interact with and suppress the function of the p160 coactivator family, including the oncogene AIB1 (amplified in breast cancer-1) (Zhang et al., 2004). AIB1 enhances ligand-dependent transactivation of steroid nuclear receptors, including ER, and is frequently amplified and overexpressed in breast and ovarian cancers (Anzick et al., 1997). ANKRD11 was reported to recruit histone deacetylases (HDACs) through its carboxyl terminus to the AIB1/nuclear receptor complex, resulting in the inhibition of ligand-dependent transactivation (Zhang et al., 2004). Based on these findings, we investigated whether ANKRD11 has properties similar to that of a breast cancer tumour suppressor. In this study, it is established that ANKRD11 interacts with p53 and the functional consequences of this interaction are investigated.

3.1.3 – Materials and Methods

3.1.3.1 – Cell lines and antibodies

Cell lines used throughout this section are as described (General Materials and Methods; Section 2.1). Commercial antibodies used are as described in Table 2.1. A rabbit α-ANKRD11 polyclonal antibody was raised against a 16-amino acid synthetic peptide representing 326-341aa (SFAPSSSVDGNNTDSE) of the ANKRD11 sequence, conjugated to KLH and affinity-purified on sepharose beads coupled with the same peptide.

3.1.3.2 – Plasmids

The generation or source of constructs used throughout this section are described in Table 2.3. Constructs were generated using standard procedures (Ausubel, 2001), with the sequences of all constructs confirmed by DNA sequencing (General Materials and Methods; Section 2.7).

3.1.3.3 – Immunofluorescence

Immunofluorescence was performed as described (General Materials and Methods; Section 2.3).

3.1.3.4 – Glycerol Gradient Fractionation

MCF-7 cells (1×10^8) stably-expressing the GFP-ANKRD11-myc protein were lysed in buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 0.1% Triton-X-100, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4 with 1× complete protease inhibitor cocktail (Roche), sonicated and centrifuged. The clarified protein lysate was loaded on a 10-40% continuous glycerol gradient prepared in 20 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 100 mM KCl, 0.1% NP-40 and centrifuged overnight at 50,000 rpm in an MLS-50 swing out rotor (Beckman Coulter). Protein complexes from 24 gradient fractions were analysed by Western blot analysis using α-myc, α-p53 and α-PML antibodies.

3.1.3.5 – In silico analysis

In silico analysis of ANKRD11 was performed as described (General Materials and Methods; Section 2.8)

3.1.3.6 – Co-immunoprecipitation assays

Nuclei were harvested from 5×10^7 MCF-7 or HEK293T cells using methodologies described by Wysocka et al. (2001). Nuclei were resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-X-100 and 1× complete protease inhibitor cocktail, sonicated and centrifuged. Clarified lysates were pre-cleared with pre-immune serum from the rabbit used to generate the α-ANKRD11 polyclonal antibody (1 hour, 4^oC), incubated with Protein A-conjugated sepharose beads (Amersham Biosciences, Uppsala, Sweden) (1 hour, 4^oC) and immunoprecipitated using the α-ANKRD11 antibody. Co-immunoprecipitation of endogenous p53 with protein fragments of ANKRD11 was performed as described in Section 4.3.10.

3.1.3.7 – In vitro binding assays

For *in vitro* binding assays, MBP (maltose-binding protein), MBP-p53, GST (glutathione S-transferase), GST-ANKRD11$^{144\text{-}313aa}$ or GST-ANKRD11$^{2369\text{-}2663aa}$ proteins were induced in BL21pLysS bacteria (see Table 2.3 for details of constructs). GST, GST-ANKRD11$^{144\text{-}313aa}$ or GST-ANKRD11$^{2369\text{-}2663aa}$ fusion proteins were purified using glutathione sepharose 4B beads (Amersham Pharmacia Biotech) using the manufacturer's recommended protocol. MBP or MBP-p53 fusion protein were associated with the amylose resin for use as bait in the *in vitro* binding assay. ^{35}S-labeled hADA3 was synthesized from pCMXHA-hADA3f by *in vitro* transcription/translation reactions using T7-Quick reticulocyte lysate (Promega). To confirm their intactness, proteins were resolved by SDS-PAGE and visualized by colloidal Coomassie

staining. For MBP-pulldown experiments, purified GST or GST-ANKRD11$^{144\text{-}313aa}$ fusion proteins were incubated with either MBP- or MBP-p53-amylose beads in TNME buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% NP-40, 20% glycerol at 4°C), washed in TNME buffer, eluted with 10 mM maltose in 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 5% glycerol and detected by Western blot analysis. GST-pulldown assays were performed as described by Zhang et al (2004).

3.1.3.8 – Promoter binding assays

For promoter binding assays, a biotinylated p53-responsive *CDKN1A* promoter region was immobilized to streptavidin-M280 magnetic beads (Invitrogen) following the manufacturer's protocol. MDA-MB-468 (5×10^6) cells were lysed in binding buffer (20 mM Tris-HCl pH 8.0, 6 mM $MgCl_2$, 5 mM DTT, 0.1 mM EDTA, 0.01% NP-40, 10% glycerol), sonicated and centrifuged. Clarified lysates were incubated (1 hour, RT) with either *CDKN1A*-charged or uncharged beads, washed in binding buffer and eluted in 1× SDS loading buffer. Eluted proteins were detected by Western blot analysis.

3.1.3.9 – Reporter assays

HeLa or Saos-2 cells (2×10^5) were seeded in 24-well plates and transfected with 100 ng of reporter plasmid (Table 2.3) along with varying amounts of myc-p53 or ANKRD11-myc and 25 ng pRL-TK plasmid (Promega) was added as a transfection control. Empty vector was added to compensate for amounts of plasmid used in various treatments. Dual-reporter assays were performed as described previously (Kumar et al., 2006). Briefly, cells were harvested after 24 hours, lysed and assayed using the dual-luciferase reporter assay system (Promega). Firefly luciferase values were normalised to Renilla luciferase activity and expressed as relative luciferase units ± SEM of triplicates.

3.1.3.10 – Reverse transcription real time-PCR (RT-PCR)

Reverse transcription and real-time PCR analysis were performed as described (General Materials and Methods; Section 2.4).

3.1.3.11 – Cell-based assays

Amphitrophic recombinant retroviruses were generated as described (Section 4.3.6). Stable cell isolates were established by transduction of MCF-7, MDA-MB-468 (MB-468) or MDA-MB-231 (MB-231) cell lines with retroviral particles derived from either pLNCX2-GFP-ANKRD11 or pLNCX2 vector (negative vector control). Colonies arising from single cells (clonogenicity) of these stable cell isolates was determined by scoring the number of colonies initiated following the growth of low-density (1000 cells per 6 well) cultures for 10-14 days in the presence of geneticin (600 μg/ml). Cell proliferation of these stable cell isolates was assayed by plating cells at 10-20% confluence in 96 well plates and determining the number of cells at various time-points using the CellTitre-Glo Luminescent Cell Viability Assay (Promega). The shRNA-mediated silencing of ANKRD11 was performed through generation of stable cell isolates established by transduction of MCF-10A cells with retroviral particles derived from either pMSCV-shANK (Table 2.2; primers 27 and 28) of pMSCV-SCR (scramble control) (Table 2.2; primers 25 and 26).

3.1.3.12 – Yeast two-hybrid screen

The pGBT-ANKRD11$^{2369\text{-}2663aa}$ plasmid encoding a GAL4 DBD-ANKRD11 C-terminal domain (2369-2663aa) fusion protein was used as bait to screen a human placental yeast two-hybrid cDNA library. The yeast Y190 strain was sequentially transformed with the bait and library plasmids following the manufacturer's protocols (Clontech Laboratories). Yeast colonies that survived on synthetic conditional plates supplemented with 50 mM 3-aminotriazole (Sigma-Aldrich) were then tested for β-galactosidase expression. Library

plasmids from double reporter positive colonies were rescued and reconfirmed by co-transformation with the bait plasmid.

3.1.3.13 – β-Galactosidase assay

Positive yeast cultures were collected by centrifugation from an overnight culture (3 mL) in synthetic media without 3-aminotriazole, and then suspended in 500 μL Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) containing 0.27% β-mercaptoethanol. Cells were then permeabilized by vortexing in the presence of 50 μL chloroform and 50 μL 0.05% SDS. The β-galactosidase substrate ONPG (*O*-nitrophenyl-β-galactopyranoside) was added (200 μL) from a stock solution (4 mg/mL in Z-buffer). The reactions were incubated at 30^{o}C until colour development, and then stopped by the addition of 200 μL of 1M Na_2CO_3.

3.1.3.14 – Statement of work

The findings presented in Chapter 3.1 have been submitted for publication. Some data is presented that was generated by other researchers, but is presented in its entirety throughout this chapter to maintain a holistic view of the project. Contributions were as follows: Assoc Prof David Callen, generation of stably-expressing cell lines through retroviral-mediated gene transfer; Dr Raman Sharma, glycerol gradient fractionation, *in vitro* binding assays and generation of the α-ANKRD11 polyclonal antibody; Prof J Don Chen, yeast-two hybrid screen and β-galactosidase assays; Dr Kelly Cheney contributed to some of the luciferase were performed by.

3.1.4 – Results

3.1.4.1 – ANKRD11 associates with p53, p14^{ARF} and PML in extranucleolar inclusions

ANKRD11 was originally investigated as a candidate breast cancer tumour suppressor gene due to its location within the 16q24.3 breast cancer LOH region. To gain insight into the functional role of ANKRD11, we initially investigated its subcellular localisation in breast epithelial cells. GFP-ANKRD11 was retrovirally-expressed in MCF-10A, a non-malignant, immortalized human breast epithelial cell. Fluorescent and phase contrast microscopy showed that the GFP-ANKRD11 protein forms distinct foci within the nuclei of MCF-10A cells (Fig 3.1*A*). We also observed that these foci localise to visible domains within the nucleus that are distinct from nucleoli, as indicated by immunostaining of the nucleolar marker, nucleophosmin (Fig 3.1*B*).

Recent studies refer to these domains that are visible under phase contrast, yet distinct from nucleoli, as "extranucleolar inclusions" (Kashuba et al., 2003a). These extranucleolar domains have been reported to house several crucial cell cycle proteins, including p53, p14^{ARF} and PML, the promyelocytic leukemia protein. To investigate if ANKRD11 associates with these proteins within extranucleolar inclusions, we determined the localisation of endogenous ANKRD11 and p53 in MCF-10A cells in the presence or absence of mitomycin C, a DNA damaging agent that induces elevated levels of p53 protein. Our findings show that endogenous ANKRD11 and p53 co-localise within nuclear foci, and this interaction is enhanced in the presence of elevated p53 protein levels in response to mitomycin C treatment (Fig 3.1*C*).

It has also been previously reported that exogenous p14^{ARF} localises to both extranucleolar inclusions and the nucleolus (Kashuba et al., 2003a; Kashuba et al., 2003b). ANKRD11-myc

and GFP-p14^{ARF} were ectopically expressed in HEK293T cells and imaged using confocal microscopy (Fig 3.1*D*). Co-immunolocalisation results show ANKRD11-myc localises exclusively to extranucleolar p14^{ARF} foci (filled arrowheads), but not to the larger nucleolar p14^{ARF} foci (hollow arrowheads). Transiently-expressed ANKRD11 protein accumulated in nuclear foci that were more numerous and larger in size than that of endogenous ANKRD11. Endogenous PML counterstaining showed that PML bodies were also recruited to ANKRD11/p14^{ARF}-positive extranucleolar inclusions (Fig 3.1*E*) as previously reported (Kashuba et al., 2003a; Kashuba et al., 2003b). In the absence of exogenous p14^{ARF}, ANKRD11 nuclear foci were observed to exhibit partial co-localisation with PML bodies during late G_2 phase of the cell cycle (see Section 3.3.3.1). This observation suggests that the association of ANKRD11-PML complex is enhanced in the presence of p14^{ARF}. The location of nucleoli in these cells was determined through detection of nucleophosmin, and nuclei were counterstained with DAPI.

Further studies were undertaken using glycerol gradient centrifugation of lysates of MCF-7 cells stably-expressing GFP-ANKRD11-myc. Both p53 and PML co-fractionated with ANKRD11-myc (Fig 3.1*F*), providing preliminary evidence that these proteins may co-exist as a complex. Detection of p14^{ARF} in these fractions was not possible since the MCF-7 cell line does not express endogenous p14^{ARF} due to deletion of the *CDKN2A* locus (Stott et al., 1998).

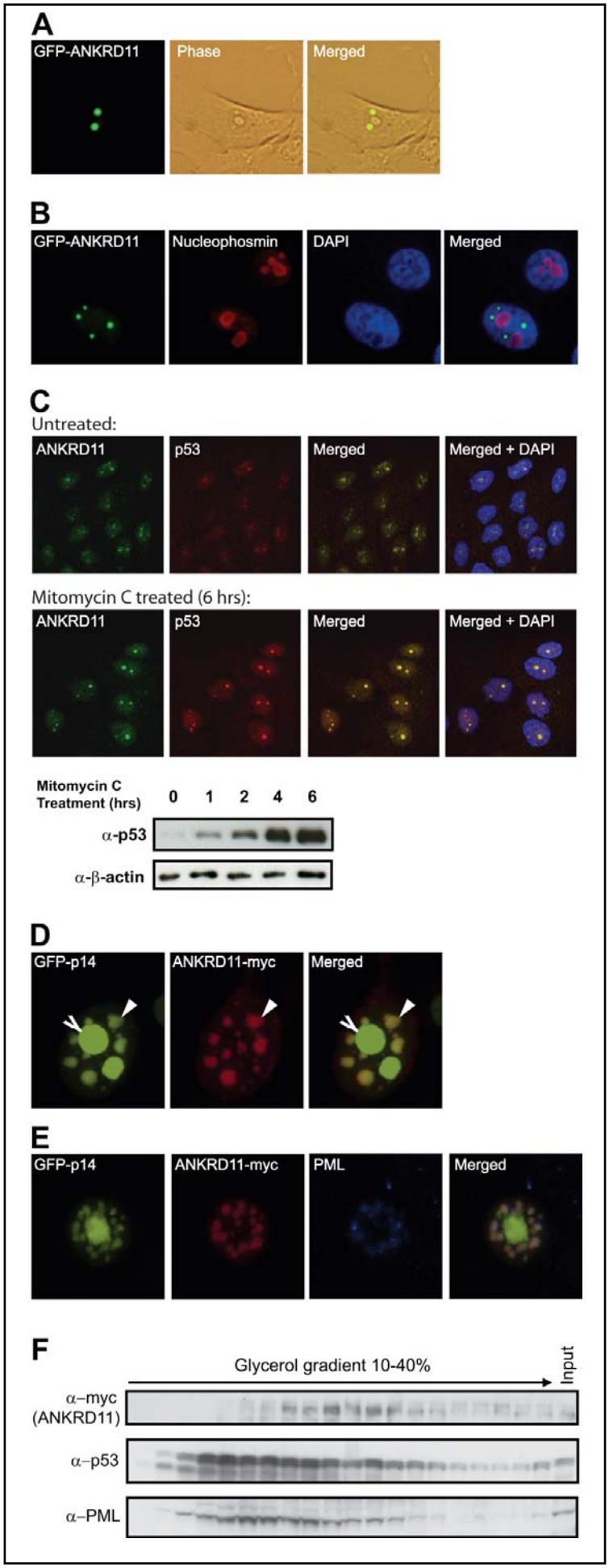

A
GFP-ANKRD11
Phase
Merged
B
GFP-ANKRD11
Nucleophosmin
DAPI
Merged
C
Untreated:
ANKRD11
p53
Merged
Merged + DAPI
Mitomycin C treated (6 hrs):
ANKRD11
p53
Merged
Merged + DAPI
Mitomycin C Treatment (hrs)
0 1 2 4 6
α-p53
α-β-actin
D
GFP-p14
ANKRD11-myc
Merged
E
GFP-p14
ANKRD11-myc
PML
Merged
F
Glycerol gradient 10-40%
Input
α–myc (ANKRD11)
α–p53
α–PML

Figure 3.1. ANKRD11 associates with $p14^{ARF}$, p53 and PML in extranucleolar inclusions.

A: GFP-ANKRD11 localises to subnuclear domains that are visible using phase contrast microscopy. MCF-10A cells were transduced with a recombinant retrovirus expressing GFP-ANKRD11, selected in geneticin and imaged using confocal microscopy.

B: GFP-ANKRD11 localises to subcellular domains that are distinct from nucleoli. MCF-10A cells expressing GFP-ANKRD11 from (*A*) were immunostained with α-nucleophosmin antibody (nucleolar marker; red) and DAPI (4',6-diamidino-2-phenylindole; DNA; blue).

C: Endogenous ANKRD11 co-localises with p53. MCF-10A cells were either untreated or treated with mitomycin C (20 µg/mL) for 6 hours and immunostained with α-ANKRD11 and α-p53 antibodies. The relative p53 protein levels in these cells with up to 6 hours of mitomycin C treatment was determined by Western blot analysis using an α-p53 antibody. β-actin was used as a loading control.

D: ANKRD11-myc localised exclusively to $p14^{ARF}$-positive extranucleolar inclusions. HEK293T cells were transiently-transfected with constructs expressing GFP-$p14^{ARF}$ and ANKRD11-myc. GFP-$p14^{ARF}$ localised to both nucleoli (hollow arrowheads) and extranucleolar inclusions (filled arrowheads). Localisation of ANKRD11-myc was determined through immunostaining with α-myc antibody.

E: Endogenous PML co-localises with $p14^{ARF}$ and ANKRD11-myc in extranucleolar inclusions. HEK293T cells from (*D*) ectopically expressing GFP-$p14^{ARF}$ and ANKRD11-myc were double-immunostained with α-myc and α-PML antibodies.

F: ANKRD11-myc co-fractionates with p53 and PML. Extracts of MCF-7 cells stably-expressing GFP-ANKRD11-myc were sedimented in a 10-40% glycerol gradient. Protein complexes from 24 gradient fractions were resolved on SDS-PAGE and Western blotted using α-myc, α-p53 and α-PML antibodies.

3.1.4.2 – ANKRD11 interacts with p53 in vivo and in vitro through the ankyrin repeat domain

Co-immunoprecipitation experiments were used to determine if ANKRD11 interacts with p53 *in vivo*. Endogenous p53 was detected in protein complexes immunoprecipitated using an α-ANKRD11 antibody from protein lysates of MCF-7 cells transduced with recombinant retroviruses expressing GFP-ANKRD11 and treated with mitomycin C for 6 hours (Fig 3.2*A*; upper panel). Similar results were observed after immunoprecipitation of endogenous ANKRD11 from HEK293T cell lysates (Fig 3.2*A*; lower panel). This interaction was specific, as p53 was not detected in complexes immunoprecipitated with pre-immune serum. To further map the region of ANKRD11 that interacts with p53, HEK293T cells were transfected with constructs expressing various FLAG-tagged regions of the ANKRD11 protein. Immunoprecipitation of these fragments using α-FLAG-agarose beads showed that endogenous p53 interacts with the amino terminal 817aa of the ANKRD11 protein (Fig 3.2*B*).

Intriguingly, this region of ANKRD11 contains an ankyrin repeat domain. *In silico* analysis of the predicted ANKRD11 amino acid sequence revealed that its ankyrin (ANK) domain (133-296aa) was highly homologous with the ANK domains of BARD1 (45% identical; 72% similar), IκBα (27% identical; 68% similar) and 53bp2 (24% identical; 57% similar). Since it has been previously reported that these proteins interact with p53 through their ANK domain (Gorina & Pavletich, 1996; Dreyfus et al., 2005; Feki et al., 2005), we investigated whether the ANK domain of ANKRD11 was indeed the minimal region of interaction with p53. For this purpose, an amino-terminal myc-tagged construct expressing a region of ANKRD11 encompassing the ANK domain (144-313aa) was used. HCT116 cells were transfected with either myc-ANKRD11$^{144\text{-}313aa}$ or empty vector and then treated with mitomycin C for 6 hours. α-myc immunoprecipitation of HCT116 cell lysates showed that ANKRD11 interacts with p53 through this 144-313aa region (Fig 3.2*C*; upper panel). Similar results were obtained after

α-FLAG immunoprecipitation of lysates from HEK293T cells expressing FLAG-ANKRD11$^{144\text{-}313aa}$ (Fig 3.2*C*; lower panel).

Subsequently, an *in vitro* MBP-pulldown experiment was used to determine if ANKRD11 directly interacts with p53. GST-ANKRD11$^{144\text{-}313aa}$ protein was precipitated after incubation with MBP-p53 amylose beads (Fig 3.2*D*). This interaction was shown to be specific, as GST-ANKRD11$^{144\text{-}313aa}$ protein did not bind MBP amylose beads alone and GST protein did not interact with the MBP-p53 amylose beads. Figure 3.2*E* depicts the ankyrin repeat domain (ANK), the two repressor domains (RD) and the activator domain (AD) of ANKRD11, summarizes these *in vivo* and *in vitro* ANKRD11-p53 interaction results and illustrates the minimal region of ANKRD11 required for p53 interaction (ANKRD11$^{144\text{-}313aa}$).

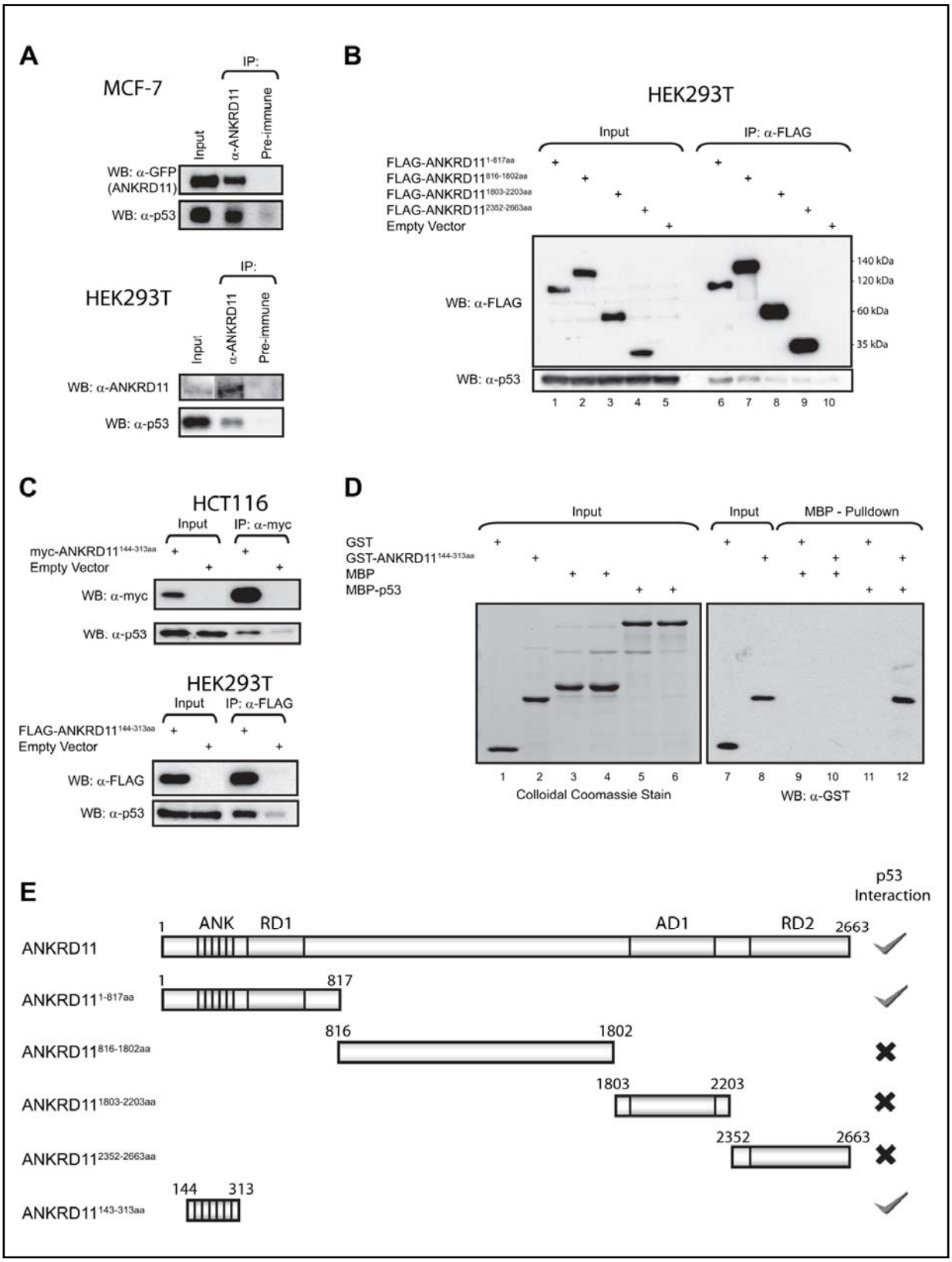

A
MCF-7
IP:
Input
α-ANKRD11
Pre-immune
WB: α-GFP (ANKRD11)
WB: α-p53
HEK293T
IP:
Input:
α-ANKRD11
Pre-immune
WB: α-ANKRD11
WB: α-p53
B
HEK293T
Input
IP: α-FLAG
FLAG-ANKRD11 1-817aa
FLAG-ANKRD11 816-1802aa
FLAG-ANKRD11 1803-2203aa
FLAG-ANKRD11 2352-2663aa
Empty Vector
140 kDa
120 kDa
60 kDa
35 kDa
WB: α-FLAG
WB: α-p53
1 2 3 4 5 6 7 8 9 10
C
HCT116
Input
IP: α-myc
myc-ANKRD11 144-313aa
Empty Vector
WB: α-myc
WB: α-p53
HEK293T
Input
IP: α-FLAG
FLAG-ANKRD11 144-313aa
Empty Vector
WB: α-FLAG
WB: α-p53
D
Input
Input
MBP - Pulldown
GST
GST-ANKRD11 144-313aa
MBP
MBP-p53
1 2 3 4 5 6 7 8 9 10 11 12
Colloidal Coomassie Stain
WB: α-GST
E
p53 Interaction
ANK
RD1
AD1
RD2
1
2663
ANKRD11
1
817
ANKRD11 1-817aa
816
1802
ANKRD11 816-1802aa
1803
2203
ANKRD11 1803-2203aa
2352
2663
ANKRD11 2352-2663aa
144
313
ANKRD11 143-313aa

Figure 3.2. ANKRD11 interacts with p53 *in vivo* and *in vitro* via the ANK domain

A: ANKRD11 co-immunoprecipitates with endogenous p53. Protein complexes from either MCF-7 (retroviral-mediated GFP-ANKRD11 expression) or HEK293T cell lysates (endogenous ANKRD11) were immunoprecipitated using a rabbit α-ANKRD11 polyclonal antibody or pre-immune serum. Immunoprecipitated protein complexes were analyzed by Western blot using α-ANKRD11 and α-p53 antibodies. WB, Western blot; IP, immunoprecipitation.

B: p53 interacts with the amino-terminal region of ANKRD11. HEK293T cells were transiently-transfected with constructs expressing one of four different FLAG-tagged regions of the ANKRD11 protein or empty pCMV-Tag2 vector as indicated. Protein complexes from total cell lysates were immunoprecipitated using α-FLAG antibody coated sepharose beads. Inputs (lanes 1-5) or immunoprecipitates (lanes 6-10) were Western blotted using α-FLAG and α-p53 antibodies. Low levels of non-specific p53 binding to the sepharose beads were also detected (lanes 7-10).

C: ANKRD11 interacts with endogenous p53 through the ankyrin repeat domain. HCT116 or HEK293T cells were transiently-transfected with constructs encoding myc- or FLAG-tagged ANKRD11$^{144\text{-}313aa}$ protein (ANK domain) or empty vector, respectively. Protein complexes were immunoprecipitated using the appropriate antibodies and Western blotted as described in (*B*).

D: ANKRD11 directly interacts with p53 through the ankyrin repeat domain. Purified recombinant GST (lanes 9 and 11) or GST-ANKRD11$^{144\text{-}313aa}$ (lanes 10 and 12) fusion proteins were incubated with either MBP (lanes 9 and 10) or MBP-p53 (lanes 11 and 12) amylose beads. GST (lane 7) and GST-ANKRD11$^{144\text{-}313aa}$ (lane 8) inputs and interacting protein complexes (lanes 9-12) were Western blotted using an α-GST antibody. To confirm their intactness, all proteins were resolved by SDS-PAGE and visualized by colloidal Coomassie staining (Lanes 1-6).

E: The minimal region of ANKRD11 required for p53 interaction. Findings from *in vivo* and *in vitro* interaction data (Fig 3.2*A*-*D*) are presented as a schematic diagram illustrating that 144-313aa is the minimal region of ANKRD11 that interacts with p53.

3.1.4.3 – ANKRD11 enhances p53-mediated transcription

Since ANKRD11 directly interacts with p53, we investigated if ANKRD11 was able to modulate the transcriptional activity of p53. Results from dual luciferase assays show that expression of ANKRD11 in Saos-2 or HeLa cells led to a significant (2.0 fold and 1.8 fold, respectively; $p < 0.05$) dose-dependent increase in p53-mediated transactivation of the pGL2-Promoter-p53-RE-Luc reporter construct containing 17 tandem p53-REs (Fig 3.3). A similar effect was observed when endogenous p53-REs from *CDKN1A* ($p21^{waf1}$) and *MDM2* promoter sequences were used in reporter assays, since expression of ANKRD11 caused a significant (maximum 3.3 fold and 4.1 fold; $p < 0.05$) dose-dependent increase in p53-mediated transactivation of the pGL3-Basic-CDKN1A-pro-Luc and pGL2-Basic-MDM2-pro-Luc reporter constructs, respectively (Fig 3.3).

3.1.4.4 – ANKRD11 expression in breast cell lines

Since ANKRD11 is located within the 16q24.3 breast cancer LOH region and is able to enhance p53 activity, it was thought possible that ANKRD11 imparts a biological function as a tumour suppressor. To further investigate this possibility, the relative expression of ANKRD11 in a panel of breast cell lines was determined by real-time RT-PCR. Results show that the average ANKRD11 expression in the six breast cancer cell lines is 6.6 fold down-regulated ($p < 0.01$) when compared to the average ANKRD11 expression in finite life-span HMECs and non-malignant immortalized breast epithelial cells (Fig 3.4*A*; dark bars).

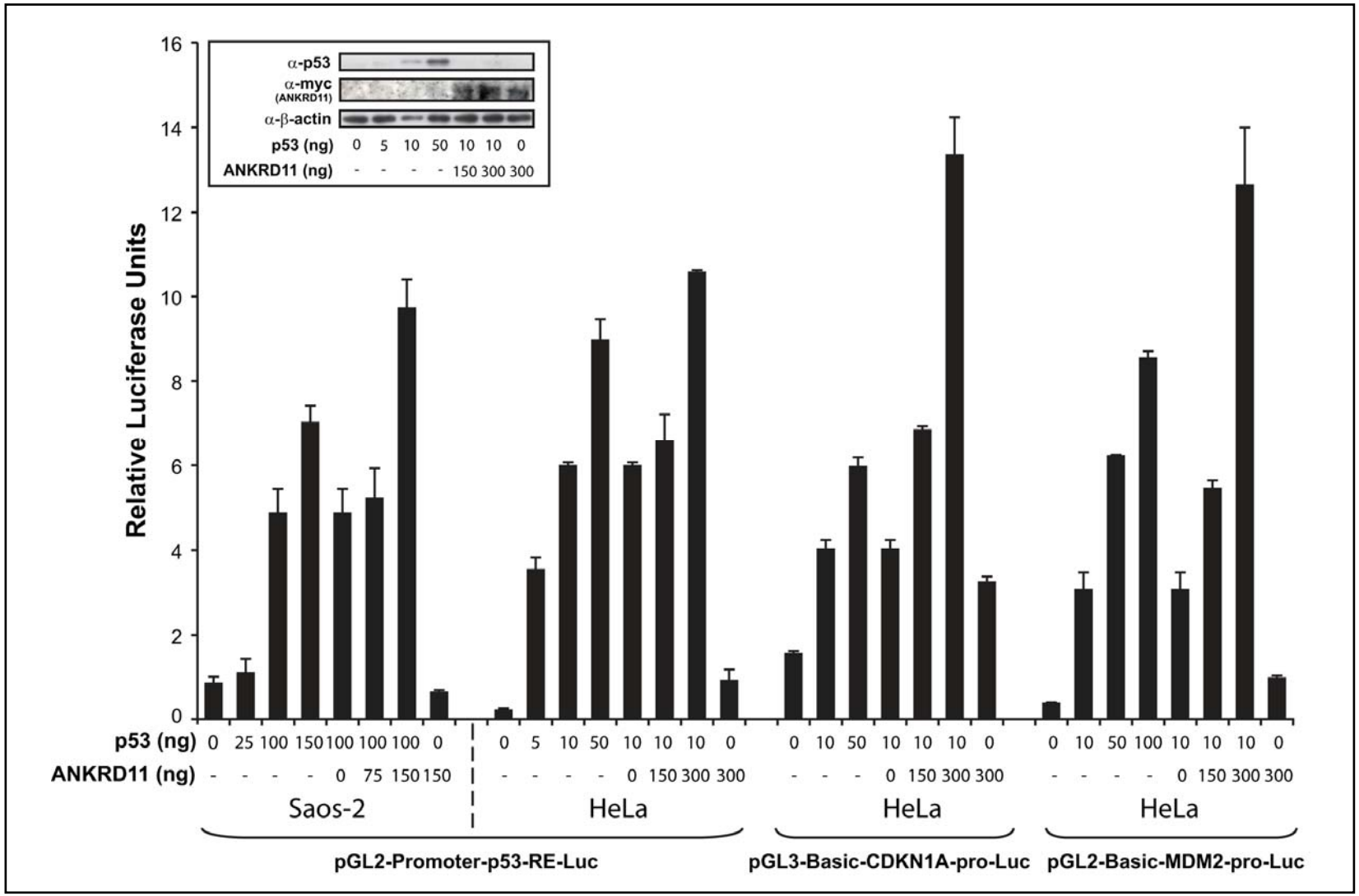

α-p53
α-myc
(ANKRD11)
α-β-actin
p53 (ng) 0 5 10 50 10 10 0
ANKRD11 (ng) - - - - 150 300 300
Relative Luciferase Units
16
14
12
10
8
6
4
2
0
p53 (ng) 0 25 100 150 100 100 100 0 0 5 10 50 10 10 10 0 0 10 50 10 10 10 0 0 10 50 100 10 10 10 0
ANKRD11 (ng) - - - - 0 75 150 150 - - - - 0 150 300 300 - - - 0 150 300 300 - - - - 0 150 300 300
Saos-2
HeLa
HeLa
HeLa
pGL2-Promoter-p53-RE-Luc
pGL3-Basic-CDKN1A-pro-Luc
pGL2-Basic-MDM2-pro-Luc

Figure 3.3. ANKRD11 expression enhances p53 transcriptional activity.

Saos-2 (left) or HeLa (right) cells were co-transfected with p53-responsive reporter constructs encoding 17 tandem p53-REs (left), the *CDKN1A* promoter region (middle) or the *MDM2* promoter region (right), together with pRL-TK and constructs expressing myc-p53 and ANKRD11-myc as indicated. Empty pLNCX2 vector was added to equalize the total amounts of plasmid used in various treatments. In HeLa cells, ANKRD11 expression resulted in a minimal increase in reporter activity in the absence of exogenous myc-p53. This is presumably due to ANKRD11-mediated activation of endogenous p53 in HeLa cells, since this phenomenon was not observed in the p53-null Saos-2 cell line. Data are represented as mean ± SEM of triplicates. The expression of protein levels from transiently-transfected constructs was determined by Western blot analysis using α-myc and α-p53 antibodies (inset).

3.1.4.5 – Restoration of ANKRD11 enhances p53-dependent expression of p21^{waf1}

Our findings suggest that increased ANKRD11 expression enhances p53-mediated transcription of a reporter gene driven by the *CDKN1A* promoter region (Fig 3.3) and ANKRD11 expression is down-regulated in breast cancer cell lines (Fig 3.4*A*; dark bars). Therefore, we hypothesized that restoring the expression of ANKRD11 in breast cancer cell lines that retain an intact p53 pathway may reduce their oncogenic phenotype by enhancing the transcriptional activity of p53. To test this hypothesis, exogenous ANKRD11 was re-introduced into the MCF-7 (wild-type p53), MB-468 (mutant p53) and MB-231 (mutant p53) cell lines. In contrast to the p53^{R273H} mutant expressed in the MB-468 cell line that possesses the ability to bind its cognate DNA response element and transactivate target genes, the MB-231 cell line expresses the p53^{R280K} mutation, completely abrogating its DNA-binding affinity and transactivation potential (Park et al., 1994; Prasad & Church, 1997).

Cultures stably-expressing GFP-ANKRD11 (MCF-7-ANK-1 to ANK-3; MB-468-ANK-1; MB-231-ANK-1 to ANK-3) or negative control cell lines (MCF-7; MB-468; MB-231) (see Materials and Methods; Cell-based assays) were established from single colonies after retroviral transduction and geneticin selection. The level of ANKRD11 re-expression in these stable cell isolates was compared to that observed in finite life span or non-malignant immortalized breast epithelial cells (Fig 3.4*A*; light bars). The relative p21^{waf1} expression level in these MCF-7, MB-468 or MB-231 cultures stably-expressing ANKRD11 was determined by real-time RT-PCR (Fig 3.4*B*). MCF-7 and MB-468 cultures stably-expressing ANKRD11 showed an average increase in p21^{waf1} expression levels by 3.3 or 2.4 fold, respectively. Restoration of ANKRD11 expression in the MB-231 cell line caused no significant change in p21^{waf1} expression levels, suggesting that the presence of an intact p53 pathway is required for ANKRD11 to enhance the transcription of downstream p53 target genes. These observations were also consistent with p21^{waf1} protein levels (Fig 3.4*B*; insets).

3.1.4.6 – Restoration of ANKRD11 expression suppresses the growth characteristics of breast cancer cell lines

The clonogenic properties of the stably-expressing MCF-7, MB-468 or MB-231 cultures were subsequently determined to investigate the effect of restoring physiologically-relevant levels of ANKRD11 expression on the growth characteristics of breast cancer cells. The average number of colonies initiated on plastic by MCF-7 or MB-468 cultures stably-expressing ANKRD11 was reduced by 76% or 92% respectively, when compared with the negative control cultures (MCF-7 or MB-468) (Fig 3.4*C*). Restoration of ANKRD11 expression in MB-231 cells resulted in a minimal inhibition (11% average reduction) of the clonogenic properties of this breast cancer cell line, suggesting that ANKRD11 suppresses the oncogenic phenotypes of these breast cancer cell lines by a p53-dependent mechanism (Fig 3.4*C*).

In addition, the effect of restoration of ANKRD11 expression in these breast cancer cell lines on their proliferative characteristics was investigated (Fig 3.4*D*). Growth curves of MCF-7, MB-468 or MB-231 cell cultures stably-expressing ANKRD11 showed an average reduction in proliferation by 36%, 47% or 30% respectively, (after 72 hours) when compared to the proliferation of negative control cultures. The findings that ANKRD11 expression is down-regulated in breast cancer cell lines and that restoration of ANKRD11 expression suppresses the oncogenic characteristics of breast cancer cells is consistent with a role of ANKRD11 as a tumour suppressor.

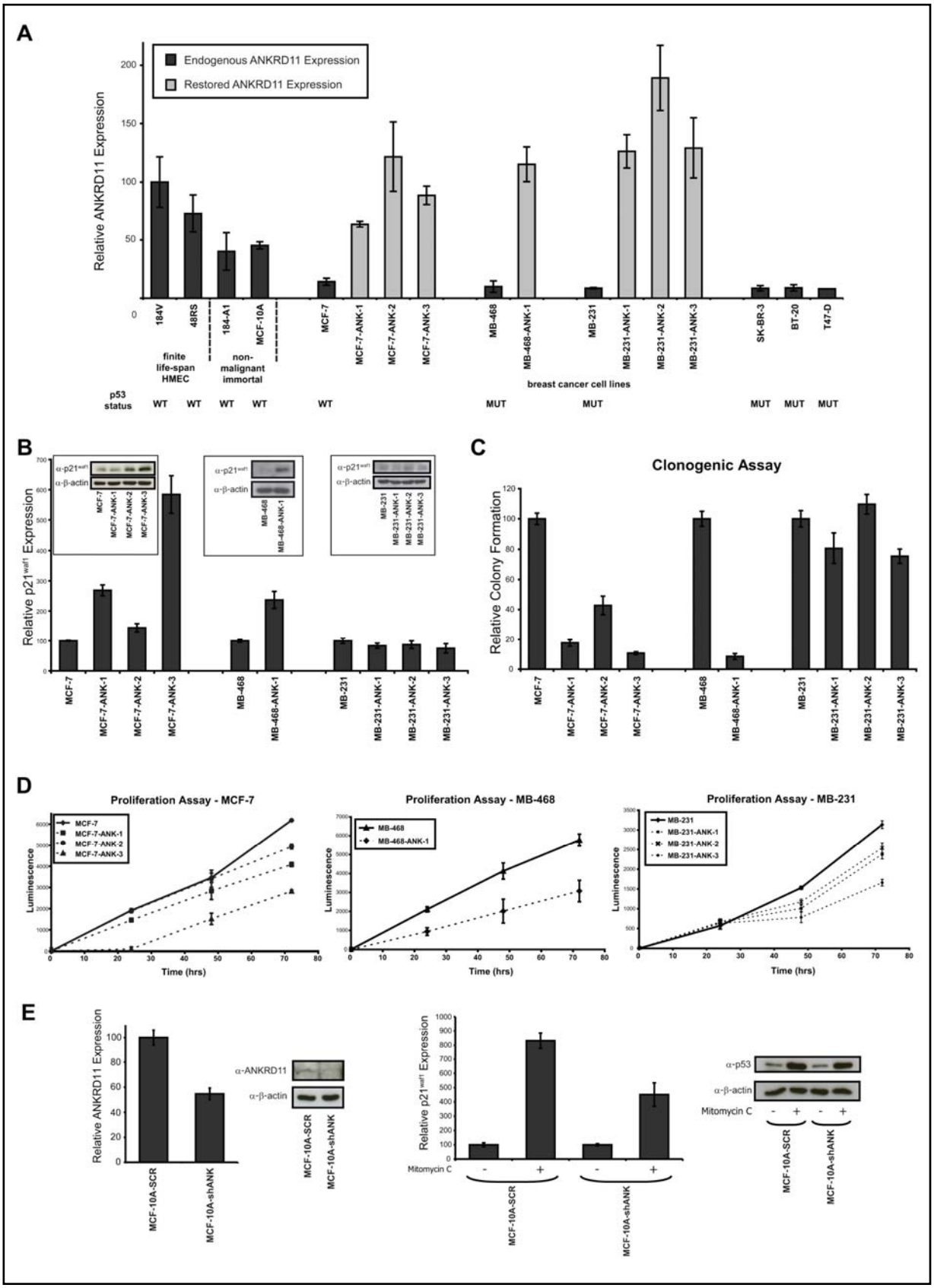

A
Endogenous ANKRD11 Expression
Restored ANKRD11 Expression
Relative ANKRD11 Expression
184V
48RS
184-A1
MCF-10A
MCF-7
MCF-7-ANK-1
MCF-7-ANK-2
MCF-7-ANK-3
MB-468
MB-468-ANK-1
MB-231
MB-231-ANK-1
MB-231-ANK-2
MB-231-ANK-3
SK-BR-3
BT-20
T47-D
finite life-span HMEC
non-malignant immortal
breast cancer cell lines
p53 status
WT
MUT
B
Relative p21waf1 Expression
α-p21waf1
α-β-actin
C
Clonogenic Assay
Relative Colony Formation
D
Proliferation Assay - MCF-7
Proliferation Assay - MB-468
Proliferation Assay - MB-231
Luminescence
Time (hrs)
E
Relative ANKRD11 Expression
α-ANKRD11
MCF-10A-SCR
MCF-10A-shANK
Relative p21waf1 Expression
Mitomycin C
α-p53

Figure 3.4. ANKRD11 regulates p53-mediated expression of $p21^{waf1}$ in breast cell lines.

A: ANKRD11 expression in the indicated breast cell lines was determined by real-time RT-PCR. The p53 status of each breast cell line is indicated. Cultures of MCF-7, MB-468 or MB-231 stably-expressing GFP-ANKRD11 (MCF-7-ANK-1 to ANK-3; MB-468-ANK-1; MB-231-ANK-1 to ANK-3) or negative control cell lines (MCF-7; MB-468; MB-231) were established from single colonies after retroviral transduction and selected in geneticin. Endogenous (dark bars) or exogenous (light bars) ANKRD11 expression levels in these stably-expressing cell lines were determined using real-time RT-PCR and compared to the levels of endogenous ANKRD11 expression in the finite life-span or non-malignant breast epithelial cell lines.

B: Restoration of ANKRD11 expression increases $p21^{waf1}$ expression only in MCF-7 and MB-468 cell lines. Both $p21^{waf1}$ mRNA and protein levels (inset) were determined in cultures stably-expressing GFP-ANKRD11 or the negative control cell cultures as described in (*B*) using real-time RT-PCR analysis and Western blot analysis.

C, D: Restoration of ANKRD11 expression reduced the growth characteristics of the MCF-7 and MB-468 breast cancer cell lines. The number of colonies formed on plastic (*C*) and proliferation rates (*D*) of the cultures stably-expressing GFP-ANKRD11 or the negative control cell cultures as described in (*A*) were determined. Data presented in (*C*) and (*D*) are represented as mean ± SEM of triplicates.

E: Silencing of ANKRD11 by shRNA reduces p53-mediated transcription. MCF-10A cultures stably-expressing ANKRD11 shRNA (MCF-10A-shANK) show both a reduction in mRNA and protein levels of ANKRD11 when compared to MCF-10A cells stably-expressing scramble shRNA (MCF-10A-SCR). MCF-10A-shANK and MCF-10A-SCR cells were either untreated or treated with mitomycin C for 12 hours and the relative $p21^{waf1}$ mRNA levels and p53 protein levels were determined using real-time RT-PCR analysis and Western blot analysis, respectively.

3.1.4.7 – Silencing of ANKRD11 reduces p53 transcriptional activity

To further investigate a role for ANKRD11 as a p53 coactivator, we silenced the expression of endogenous ANKRD11 using a previously validated short hairpin RNA (shRNA) (Zhang et al., 2007a). Stable expression of this shRNA in the MCF-10A breast epithelial cell line (MCF-10A-shANK) resulted in reduced levels of endogenous ANKRD11 mRNA and protein when compared to MCF-10A cells stably-expressing scramble shRNA (MCF-10A-SCR) (Fig 3.4*E*). Results show that shRNA-mediated silencing of ANKRD11 reduced the ability of p53 to activate $p21^{waf1}$ expression in response to DNA damage. These findings provide additional evidence to support a functional role for ANKRD11 as a p53 coactivator.

3.1.4.8 – ANKRD11 associates with hADA3 and P/CAF and increases p53 acetylation

To gain further mechanistic insight into the role of ANKRD11 as a p53 coactivator, we endeavored to identify other known p53-regulatory proteins that may be recruited by ANKRD11. hADA3 was identified from a yeast two-hybrid screen as an ANKRD11-interacting protein, as determined by the β-galactosidase reporter assay (Fig 3.5*A*). To confirm this interaction, a GST-pulldown assay was conducted using *in vitro* translated hADA3. Results show that hADA3 interacted specifically with GST-$ANKRD11^{2369\text{-}2663aa}$, but not with GST alone (Fig 3.5*B*). Recent studies have shown that hADA3 plays a key role in the regulation of p53 acetylation, stability and activity through the recruitment of HAT complexes such as p300 and P/CAF to p53 (Wang et al., 2001; Nag et al., 2007). The interaction of ANKRD11 with hADA3 suggests that ANKRD11 may also be involved in the acetylation of p53 through recruitment of HAT complexes.

To test this hypothesis, the localisation of P/CAF was determined in relation to ANKRD11 nuclear foci. Co-immunolocalisation studies demonstrated that FLAG-P/CAF protein is

recruited to HA-ANKRD11 positive nuclear foci (Fig 3.5*C*). In addition, ectopic expression of GFP-ANKRD11 increased the acetylation of p53 at Lys320 in both MCF-7 and MB-468 cell lines (Fig 3.5*D*). ANKRD11-mediated increase in Lys320-acetylated p53 levels was shown to occur solely through increased post-translation modification of p53, since exogenous ANKRD11 had no effect on total p53 protein levels in these cell lines (Fig 3.5*D*). Exogenous expression of ANKRD11 in the MB-231 cell line had no effect on Lys320-acetylated p53 levels, which is consistent with our observation that ANKRD11 did not affect $p21^{waf1}$ expression in this cell line. Taken together, these findings suggest that ANKRD11 enhances p53 transcriptional activity through increased acetylation of p53 via recruitment of acetyltransferases such as P/CAF, or cofactors such as hADA3.

3.1.4.9 – ANKRD11 enhances DNA-binding of the $p53^{R273H}$ mutant

The finding that restored ANKRD11 expression in MB-468 cells can enhance the transcriptional activity of the $p53^{R273H}$ mutant through increased Lys320 acetylation prompted us to further investigate if ANKRD11 influences the DNA-binding properties of this mutant. Results from promoter binding assays show that $p53^{R273H}$ binds the p53-responsive *CDKN1A* promoter region and that this binding is enhanced following restoration of ANKRD11 expression in the MB-468 cell line (MB-468-ANK-1) (Fig 3.5*E*). Therefore, these findings implicate a possible role for ANKRD11 in the functional rescue of the $p53^{R273H}$ mutant.

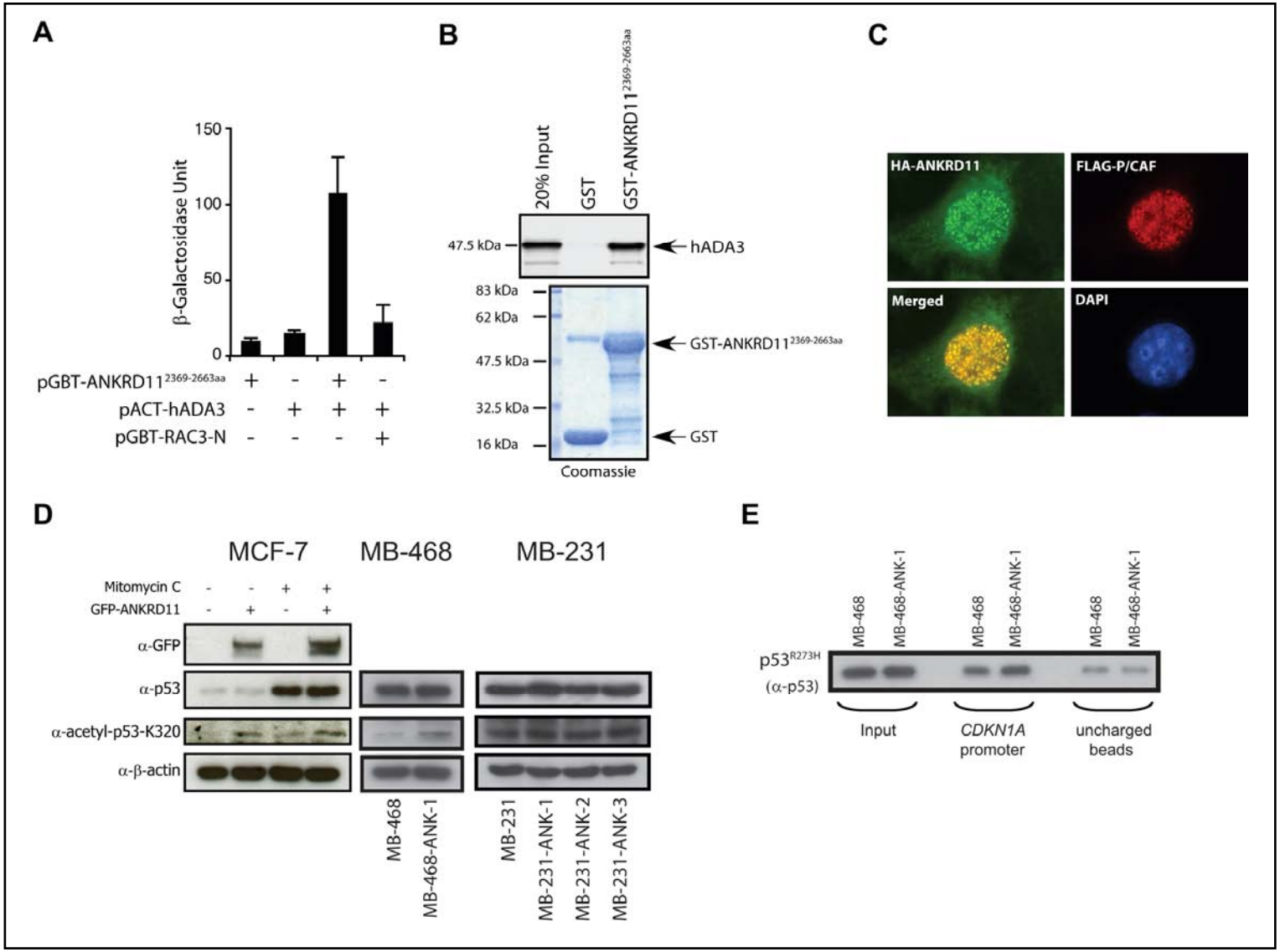

A
β-Galactosidase Unit
150
100
50
0
pGBT-ANKRD11 2369-2663aa + - + -
pACT-hADA3 - + + +
pGBT-RAC3-N - - - +
B
20% Input
GST
GST-ANKRD11 2369-2663aa
47.5 kDa
hADA3
83 kDa
62 kDa
47.5 kDa
32.5 kDa
16 kDa
GST-ANKRD11 2369-2663aa
GST
Coomassie
C
HA-ANKRD11
FLAG-P/CAF
Merged
DAPI
D
MCF-7
MB-468
MB-231
Mitomycin C - - + +
GFP-ANKRD11 - + - +
α-GFP
α-p53
α-acetyl-p53-K320
α-β-actin
MB-468
MB-468-ANK-1
MB-231
MB-231-ANK-1
MB-231-ANK-2
MB-231-ANK-3
E
MB-468
MB-468-ANK-1
MB-468
MB-468-ANK-1
MB-468
MB-468-ANK-1
p53 R273H
(α-p53)
Input
CDKN1A promoter
uncharged beads

Figure 3.5. ANKRD11 enhances P/CAF-mediated acetylation of p53 and increases the DNA-binding activity of the $p53^{R273H}$ mutant in the MB-468 cell line.

A: Yeast two-hybrid assay showing interaction between $ANKRD11^{2369\text{-}2663aa}$ and hADA3. Negative controls; yeast cells carrying pGBT-$ANKRD11^{2369\text{-}2663aa}$ plus pACT2, pACT-hADA3 plus pGBT9, and pACT-hADA3 plus pGBT-RAC3-N constructs showed no activation of the -galactosidase reporter.

B: ANKRD11 interacts with hADA3 *in vitro*. GST-pull down assay shows that GST-$ANKRD11^{2369\text{-}2663aa}$ but not GST alone pulled down significant amount of ^{35}S-labeled hADA3. Lower panel shows the Coomassie blue staining of the GST proteins.

C: ANKRD11 nuclear foci co-localise with P/CAF. COS-7 cells were transiently-transfected with FLAG-tagged P/CAF and full length HA-tagged ANKRD11. Transfected cells were immunostained with α-HA (ANKRD11; green) and α-FLAG (P/CAF; red) antibodies and DAPI (DNA; blue).

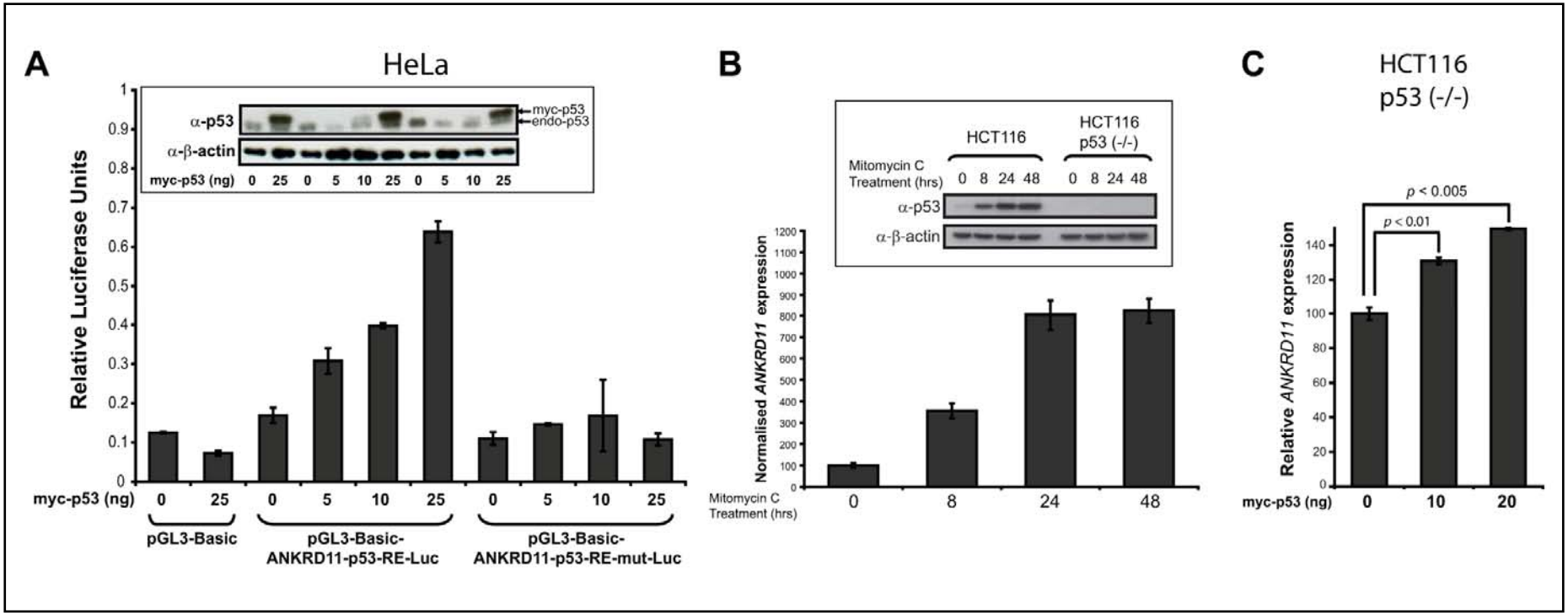
D: Restoration of expression of ANKRD11 increases p53 acetylation at Lys320. MCF-7 cells were transduced with retroviral particles derived from pLNCX2-GFP-ANKRD11 or pLNCX2 vector, selected in geneticin and treated with mitomycin C for 6 hours as indicated. Both total p53 and acetyl-lysine p53 (Lys320) protein levels were detected by Western blot analysis using the appropriate antibodies (representative of two independent experiments). Total p53 and acetyl-lysine p53 (Lys320) protein levels were also detected in MB-468 and MB-231 cultures stably-expressing GFP-ANKRD11 or the negative control cell cultures previously described (Fig 3.4*A*).

E: ANKRD11 enhances the DNA-binding affinity of the endogenous $p53^{R273H}$ mutant in MB-468 cells. Lysates from MB-468-ANK-1 cells stably-expressing GFP-ANKRD11 or the negative control MB-468 cells from (Fig 3.4*A*) were promoter precipitated using either beads charged with the *CDKN1A* promoter region or uncharged beads. Inputs or promoter precipitated $p53^{R273H}$ were detected by Western blot analysis using an α-p53 antibody.

3.1.4.10 – ANKRD11 is a p53 target gene

Two independent genome-wide ChIP-based studies have recently identified a putative p53-RE 50 kb from the 5' end of the first intron of *ANKRD11* (Hearnes et al., 2005; Wei et al., 2006). A 1.1 kb region of *ANKRD11* intronic sequence encompassing this putative p53-RE was cloned upstream of a luciferase reporter (pGL3-Basic-ANKRD11-p53-RE-Luc), and the dual-luciferase reporter system was used to investigate the transcriptional activity of this putative p53-RE located within the *ANKRD11* intronic region. Increasing expression of myc-p53 led to a dose-dependent increase in luciferase expression from this reporter construct (Fig 3.6*A*). Furthermore, p53-mediated activation of this region of *ANKRD11* intronic sequence was ablated when the putative p53-RE was mutated (pGL3-Basic-ANKRD11-p53-RE-mut-Luc) (Fig 3.6*A*). The expression level of the myc-p53 construct used in these assays was comparable with endogenous p53 levels in HeLa cells (Fig 3.6*A*; inset), suggesting that the effects observed in these experiments are mediated by physiologically relevant levels of exogenous p53.

To further investigate *ANKRD11* as a p53 target gene, we monitored endogenous ANKRD11 expression levels during the p53 response evoked through treatment with a DNA damaging agent. p53-dependent ANKRD11 transcription showed a increase by 8.2 fold at 48 hours post-treatment, and was correlated with increased p53 protein levels (Fig 3.6*B*). In addition, endogenous ANKRD11 mRNA levels increased in a dose-dependent manner ($p < 0.005$) in response to re-introduction of physiologically relevant levels of exogenous p53 in the HCT116 ($p53^{-/-}$) derivative (Fig 3.6*C*). These data, together with the previous ChIP-verified studies, suggest that *ANKRD11* is a novel p53 target gene.


A
HeLa
α-p53
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myc-p53 (ng) 0 25 0 5 10 25 0 5 10 25
Relative Luciferase Units
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pGL3-Basic
pGL3-Basic-ANKRD11-p53-RE-Luc
pGL3-Basic-ANKRD11-p53-RE-mut-Luc
B
HCT116
HCT116 p53 (-/-)
Mitomycin C Treatment (hrs) 0 8 24 48 0 8 24 48
α-p53
α-β-actin
Normalised ANKRD11 expression
Mitomycin C Treatment (hrs) 0 8 24 48
C
HCT116 p53 (-/-)
p < 0.005
p < 0.01
Relative ANKRD11 expression
myc-p53 (ng) 0 10 20

Figure 3.6. *ANKRD11* is a p53 target gene.

A: p53 transcriptionally activates a reporter gene driven by an *ANKRD11* intronic sequence carrying a p53-RE. HeLa cells were co-transfected with a construct expressing myc-p53 and the reporter constructs pGL3-Basic (left; empty vector), pGL3-Basic-ANKRD11-p53-RE-Luc (middle) or pGL3-Basic-ANKRD11-p53-RE-mut-Luc (right). The relative luciferase activity was determined in cell lysates as described in Section 3.1.3.9. Data are represented as mean ± SEM of triplicates. The expression level of the myc-p53 construct as compared to endogenous p53 levels in HeLa cells was determined by Western blot analysis using an α-p53 antibody (inset).

B: ANKRD11 expression correlates with increasing p53 protein levels during the DNA damage response. ANKRD11 expression in either HCT116 or HCT116 ($p53^{-/-}$) cells was determined at time-points 0, 8, 24 and 48 hours post-treatment with mitomycin C. The expression of ANKRD11 in HCT116 (wild-type p53) cells was normalized to that observed in the isogenic HCT116 ($p53^{-/-}$) derivative to monitor only the p53-dependent modulation of ANKRD11 expression during the DNA damage response. Induction of p53 protein levels following treatment with mitomycin C were determined by Western blot analysis using an α-p53 antibody (inset).

C: HCT116 ($p53^{-/-}$) cells were transfected in a 24-well format with the indicated amounts of myc-p53 (*A*), and the relative ANKRD11 mRNA levels in these treatments were determined using real-time RT-PCR analysis.

3.1.5 – Discussion

This study characterizes ANKRD11 as a novel p53 coactivator. Finding suggest that ANKRD11 can enhance the transactivation of p53-target genes such as $p21^{waf1}$, and that this transcriptional activation is dependent on p53 status. Furthermore, silencing of endogenous ANKRD11 expression reduces the transcriptional activity of p53. ANKRD11-mediated enhancement of p53 activity was shown to occur through recruitment of p53 acetyltransferases and cofactors such as P/CAF and hADA3, leading to increased p53 acetylation and DNA binding.

Immunofluoresence studies have demonstrated that both endogenous and exogenous ANKRD11 localises to nuclear foci, and both co-localisation and co-fractionation data indicate that ANKRD11 associates with p53, $p14^{ARF}$ and PML within these nuclear domains (Fig 3.1). Previous studies have also shown that these latter three proteins associate within similar domains, which were referred to as ‘extranucleolar inclusions’ (Kashuba et al., 2003a; Kashuba et al., 2003b). Interestingly, the association between endogenous ANKRD11 and p53 in MCF-10A cells was enhanced in the presence of a DNA damaging agent, suggesting that ANKRD11 may be involved in post-translational regulation of p53 activity during the p53 response. Our data suggests that upon cellular stress, p53 acetylation occurs within these extranucleolar inclusions through ANKRD11-mediated recruitment of hADA3 and the p53 acetyltransferases, P/CAF and p300/CBP. This notion is supported by previous findings whereby PML, another component protein recruited to extranucleolar inclusions, was also implicated in the regulation of p53 transcriptional activity (Guo et al., 2000). PML has been shown to directly interact with the DNA binding domain of p53 and enhance p300/CBP-mediated acetylation of p53 within PML nuclear bodies (Guo et al., 2000).

This study uses the MCF-7, MB-468 and MB-231 breast cancer cell lines as models to demonstrate the biological function of ANKRD11 as a p53 coactivator. Although both MB-468 and MB-231 cell lines express mutated p53, the DNA binding properties and transactivation potential of these mutants differ substantially. The $p53^{R280K}$ "DNA contact" mutant expressed in MB-231 cells is unable to bind DNA or transactivate p53 target genes (Park et al., 1994), making this an ideal negative control cell line for these studies. The observation that exogenous ANKRD11 had no significant effects on the $p21^{waf1}$ expression or clonogenic properties of the MB-231 cell line provides evidence to suggest that the coactivator function of ANKRD11 was indeed p53-dependent (Fig 3.4).

The $p53^{R273H}$ "DNA contact" mutant expressed in MB-468 cells retains the ability to interact with its cognate p53-DNA response element *in vivo* (Park et al., 1994; Prasad & Church, 1997). This was in contradiction with an *in vitro* study suggesting that $p53^{R273}$ possessed no DNA binding affinity (Bullock et al., 2000). These discrepancies between findings from *in vivo* and *in vitro* studies are a likely resultant of the use of recombinant, purified mutant p53 protein throughout these *in vitro* binding assays, suggesting that cellular post-translation modifications of p53 may influence its affinity for DNA. Our findings suggest that the restoration of mutant $p53^{R273H}$ function may be possible by enhancing the required post-translational modifications via modulation of ANKRD11 or other p53 regulatory proteins (Fig 3.5).

$p53^{R273H}$ is a potential target for restoration of mutant p53 function in cancer as it is the second most frequent cancer-associated mutation of p53, accounting for approximately 7.5% of entries in the IARC *TP53* mutation database (www.iarc.fr/p53/index.html). Crystallographic studies have demonstrated that mutation of the R273 DNA contact residue significantly impairs the affinity of p53 for DNA, but has no effect on the thermodynamic

stability or structural integrity of the DNA-binding surface of p53 (Joerger et al., 2005; Ang et al., 2006). Therefore, it is suggested that only the enhancement of the DNA binding properties of this mutant is required to restore the normal function of the $p53^{R273H}$ mutant (Bullock & Fersht, 2001). However, recent *in vitro* data suggests that the DNA-binding affinity of $p53^{R273H}$ is approximately 700 to 1000 times weaker than that of wild-type p53, making the therapeutic revival of this mutant a challenging task (Ang et al., 2006).

The transcriptional regulatory domains of ANKRD11, including two intrinsic repressor domains (RD1: 318-611aa and RD2: 2369-2663aa) and an activator domain (AD1: 2076-2145), have recently been defined (Zhang et al., 2007a). It is likely that P/CAF or other HATs are recruited to transcription factors by ANKRD11 through this AD1 domain, although this is yet to be formally demonstrated. It has previously been reported that ANKRD11 can function as a corepressor of steroid nuclear receptor transactivation through the recruitment of HDACs via the RD2 domain of ANKRD11 to the p160/nuclear receptor complex (Zhang et al., 2004; Zhang et al., 2007a). Furthermore, ANKRD11 subnuclear localisation is regulated by both nuclear import and export signals, and is essential for ANKRD11 to negatively regulate transcription of steroid nuclear receptors (Zhang et al., 2007b). It is apparent that ANKRD11 can function as either a coactivator or corepressor of transcription, although the underlying mechanisms that govern this dual-functionality are yet to be elucidated.

ANKRD11 was initially identified as a putative breast cancer tumour suppressor gene due to its location within the 16q24.3 breast cancer LOH region (Powell et al., 2002). A number of other tumour suppressors have been identified within this region, including CBFA2T3 (Kochetkova et al., 2002) and FBXO31 (as discussed in Chapter 4) (Kumar et al., 2005). It is hypothesized that LOH of chromosome 16q during the onset of breast cancer results in the simultaneous loss or reduction in activity of multiple tumour suppressor genes, and this

contributes to the early stages of tumorigenesis. A role for ANKRD11 as a breast cancer tumour suppressor was supported by the findings that ANKRD11 expression is down-regulated in breast cancer cell lines, and restoration of ANKRD11 expression in MCF-7 and MB-468 cells suppressed their oncogenic growth characteristics (Fig 3.4). A concordant increase in $p21^{waf1}$ expression was also observed in response to restored ANKRD11 expression, suggesting that the anti-oncogenic effects of ANKRD11 occur through activation of downstream p53 pathways.

An amino-terminal fragment of ANKRD11 containing the ankyrin repeat domain has been identified as a nasopharyngeal carcinoma (NPC) susceptibility protein (LZ16: nucleotide accession no. AF121775). NPC frequently exhibits 16q LOH, which correlates with a high Epstein-Barr virus (EBV) titer and contributes to the aggressive etiology of this disease (Shao et al., 2001). Intriguingly, the EBV-encoded nuclear antigen leader protein (EBNA-LP) has been shown to localise to extranucleolar inclusions, inhibit the apoptotic function of $p14^{ARF}$ and interact with p53 (Szekely et al., 1993; Kashuba et al., 2003a; Kashuba et al., 2003b). Taken together, these findings suggest a role for ANKRD11 in the suppression of EBV-mediated NPC.

ANKRD11 itself is a novel p53 target gene (Fig 3.6). A putative p53-RE was identified 50 kb from the 5' end of the first intron of *ANKRD11* by two independent ChIP-based genome wide screens mapping functional p53-REs (Hearnes et al., 2005; Wei et al., 2006). Our results have validated this p53-RE and shown that p53 does indeed modulate endogenous *ANKRD11* transcript levels. Furthermore, endogenous ANKRD11 expression directly correlates with p53 status in breast cell lines ($p < 0.05$) (Fig 3.4*A*). These findings are supported by a recent microarray study whereby induction of a non-functional $p53^{A138V}$ mutant in a human lung carcinoma cell line led to a 3.4 fold reduction of ANKRD11 expression ($p < 0.01$) when

compared with ANKRD11 expression levels in the presence of wild-type p53 (Robinson et al., 2003). Taken together, these results further support a role for *ANKRD11* as a p53 target gene. Interestingly, *P/CAF* itself was also recently identified as a p53 target gene in breast epithelial cell lines (Watts et al., 2004). The findings of both *ANKRD11* and *P/CAF* as p53 target genes suggest the existence of a positive feedback loop in the p53 acetylation pathway, although this remains to be investigated. Detailed investigations into the regulation of p53 activity by ANKRD11 or its interacting partners will significantly improve our understanding of the molecular pathways surrounding the modulation of p53 and will provide deeper insight into the development of improved pharmacological approaches to restore the function of mutant p53 in tumours.

3.2 – Identification of a putative p53-interaction motif located within the ANK domain

3.2.1 – Prelude

Findings from our co-immunoprecipitation studies show that endogenous ANKRD11 interacts with p53 via its ANK domain (Fig 3.2). Interestingly, several other proteins have also been reported to interact with p53 via their ANK domains, including BARD1 (Feki et al., 2005), 53bp2 (Gorina & Pavletich, 1996) and IκBα (Chang, 2002; Dreyfus et al., 2005). Based on this observation, it is tempting to speculate that a common ankyrin-based structure is the basis for the interaction of these proteins with p53.

3.2.2 – Introduction

3.2.2.1 – The stuctural architecture of the ANK domain

The ankyrin repeat motif is one of the most common protein-protein interaction domains found in nature (Mosavi et al., 2002). The ankyrin family of proteins exhibit a multitude of functionalities, including transcription initiation, cell cycle regulation, cytoskeletal integrity, ion transport, cell–cell signalling, endocytosis and cell fate determination (Bork, 1993; Mosavi et al., 2004).

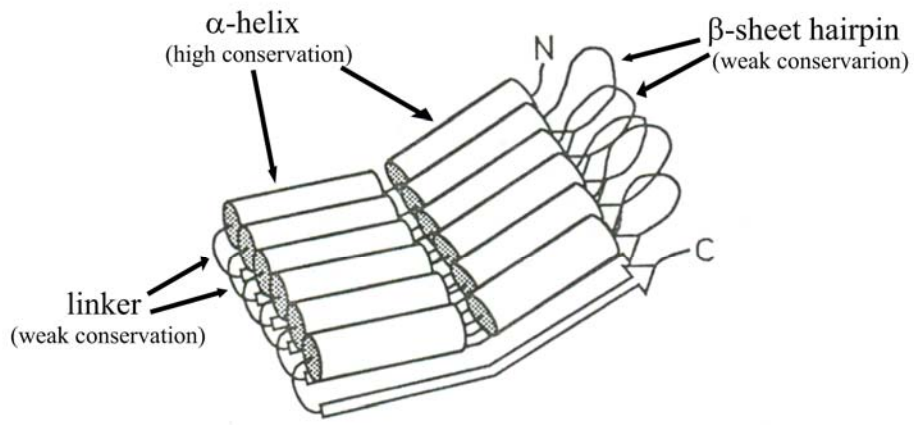
Each ankyrin repeat consists of a 33 amino acid motif, folding into a β-hairpin loop followed by two anti-parallel α-helices and a linker sequence reaching the turn of the next repeat (Fig 3.7*A*) (Sedgwick & Smerdon, 1999; Kohl et al., 2003). Studies have determined the ankyrin consensus motif derived from the combined *in silico* analysis of over 4000 ankyrin repeat proteins (Mosavi et al., 2002; Binz et al., 2003; Kohl et al., 2003). These *de novo* studies have shown that the well conserved residues within the ankyrin repeat comprise the α-helices,

whilst the β-hairpin loop and linker sequences represent less-conserved regions of the repeat (Mosavi et al., 2002 and Fig 3.7). Structural analysis has revealed that the β-hairpin loop of the ankyrin repeat is located at the ankyrin-binding interface in all ankyrin protein complex resolved thus far (Mosavi et al., 2004). The β-hairpin of the ankyrin repeat contains poorly conserved residues, consistent with its function as the recognition surface for a diverse array of macromolecular targets (Sedgwick & Smerdon, 1999; Mosavi et al., 2002).

3.2.2.2 – Ankyrin proteins: a novel family of p53 regulators?

One of the hallmarks of the p53 tumour suppressor protein is its ability to interact with a multitude of regulatory proteins (as listed in Braithwaite et al., 2006). These proteins serve to regulate both upstream and downstream pathways of p53-mediated tumour suppression through specific post-translational modification of p53. Several members of the ankyrin family of proteins have been independently reported as regulators of p53, including ANKRD11 (Section 3.1), BARD1 (Irminger-Finger et al., 2001), 53bp2 (Iwabuchi et al., 1998) and IκBα (Dreyfus et al., 2005).

A



B

NOTE: This figure is included on page 82 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.7. Structural architecture and conservation of the ANK domain.

A: Schematic diagram of a typical ANK domain. The putative p53-interaction motif is located adjacent to the α-helices in the last ankyrin repeat finger.

B: Conservation of amino acids constituting the published ankyrin repeat consensus (Mosavi et al., 2002).

3.2.2.2.1 – BARD1: a regulator of the apoptotic function of p53

The involvement of BARD1 in the apoptotic pathway was first reported following the observation that BARD1 protein and mRNA levels were elevated during apoptosis, and BARD1-deficient cells are resistant to apoptotic death following genotoxic stress (Irminger-Finger et al., 2001). Furthermore, overexpression of BARD1 induced apoptotic cell death in a BRCA1-independent manner. Further investigation revealed that BARD1 specifically interacts with p53 during apoptosis, catalyzing its phosphorylation at Ser^{15}. This post-translational modification of p53 leads to elevated cellular p53 protein levels and is a prerequisite for p53-mediated apoptosis (Irminger-Finger et al., 2001).

3.2.2.2.2 – 53bp2: a regulator of the transcriptional function of p53

The *TP53* gene is mutated in approximately half of all cancers, preventing the encoded protein from either binding its response element, folding at 37°C or interacting with its regulatory proteins (Bullock & Fersht, 2001). An *in vitro* binding study involving either wild-type p53 or two cancer-associated p53 mutants ($p53^{R175H}$ or $p53^{R273H}$) was utilized to detect regulatory proteins that show altered binding to p53 mutants (Iwabuchi et al., 1994). This screen identified 53bp2, a member of the ASPP (ankyrin-repeats, SH3 domain and proline-rich region containing protein) family of proteins, as a protein that could bind to wild-type, but not mutant p53. 53bp2 was reported to enhance p53-mediated target gene transactivation, suggesting a role of 53bp2 in the upstream p53 signal transduction pathways. Furthermore, 53bp2 was shown to co-operate with p53 to inhibit oncogene-mediated cell transformation (Iwabuchi et al., 1998). Taken together, these findings report a role for 53bp2 as a regulator of p53-mediated tumour suppression and illustrate that mutation of p53 associated with the development of oncogenesis results in loss of 53bp2-mediated p53 regulation.

3.2.2.2.3 – IκBα: a regulator of p53 localisation

IκBα has been widely reported as an inhibitor of the NFκB transcription factor through cytoplasmic sequestration (Gloire et al., 2006). However, a new role has recently emerged for IκBα in the regulation of p53 localisation and transcriptional activity. IκBα was originally shown to interact with p53 in the cytoplasm, and this cytoplasmic p53-IκBα complex dissociates in response to apoptotic stimuli such as Doxorubicin (Chang, 2002; Zhou et al., 2003). In response to genotoxic stress, IKK kinase is activated and phosphorylates the N-terminus of IκBα. Phosphorylated IκBα dissociates from cytoplasmic p53 and is subsequently ubiquitinated and degraded by the proteosome. Therefore, it is not surprising that overexpression of IκBα was reported as a negative modulator of p53-dependent transcription, presumably through its cytoplasmic sequestration of p53 (Dreyfus et al., 2005).

3.2.2.3 – The ANK domain is frequently involved in p53 interaction

Both *in vivo* and *in vitro* bindings assays support the existence of a p53-ANKRD11 complex and have mapped this interaction to the ANK domain of ANKRD11 (Section 3.1.4.2). Intriguingly, the ankyrin proteins BARD1, 53bp2 and IκBα have also been reported to interact with p53 via their respective ANK domains (Gorina & Pavletich, 1996; Chang, 2002; Dreyfus et al., 2005; Feki et al., 2005). A common ankyrin-based structure is proposed to be the basis for the interaction of these proteins with p53. It is hypothesized that these ankyrin proteins interact with and modulate p53 function through a novel p53-interaction motif located within this ANK domain.

3.2.3 – Materials and Methods

3.2.3.1 – In silico analysis

In silico analysis of the ANK domain of ANKRD11 was performed as described (General Materials and Methods; Section 2.8). ANKRD11 (accession NM_013275) BARD1 (accession NM_000465), IκBα (accession NM_020529) and 53bp2 (accession NM_001031685) sequences were derived from Genebank, NCBI.

3.2.4 – Results

3.2.4.1 – Homology between ANK domains of ANKRD11, BARD1, IκBα and 53bp2

In silico analysis of the predicted ANKRD11 amino acid sequence revealed that the five ankyrin repeats (133-296aa) constituting the ANK domain in ANKRD11 were highly homologous with the p53-interacting ANK domains in BARD1 (45% identical; 72% similar), IκBα (27% identical; 68% similar) and 53bp2 (24% identical; 57% similar).

3.2.4.2 – Identification of a putative ANK-based p53-interaction motif

Due to the high degree of homology amongst the ANK domains of these ankyrin proteins, we hypothesise that ankyrin proteins interact with and modulate p53 function through a common p53-interaction motif located within the ANK domain. This was investigated through *in silico* analysis of the aligned amino acid sequences of the p53-interacting ANK domains of ANKRD11, BARD1, 53bp2 and IκBα in an effort to identify homologous regions that were not common with the published ankyrin consensus (Mosavi et al., 2002). The precise definition of the minimal region of p53-interaction within the ANK domains of these proteins was determined from published studies (Gorina & Pavletich, 1996; Chang, 2002; Dreyfus et al., 2005; Feki et al., 2005) (Fig 3.8*A*).

These analyses identified a common motif was subsequently identified within the β-sheet hairpin of the last ankyrin repeat of these proteins (Fig 3.8*B*; dark shade) that is rich in threonine (T), tyrosine (Y), serine (S) and glutamine (Q) residues. These residues are not present in the same region of the published ankyrin repeat consensus (Fig 3.8*B*). Therefore, from this *in silico* analysis we have identified a common p53-interaction motif located within the p53-interacting ANK domains of ANKRD11, BARD1, 53bp2 and IκBα that is distinct from the published ankyrin repeat consensus.

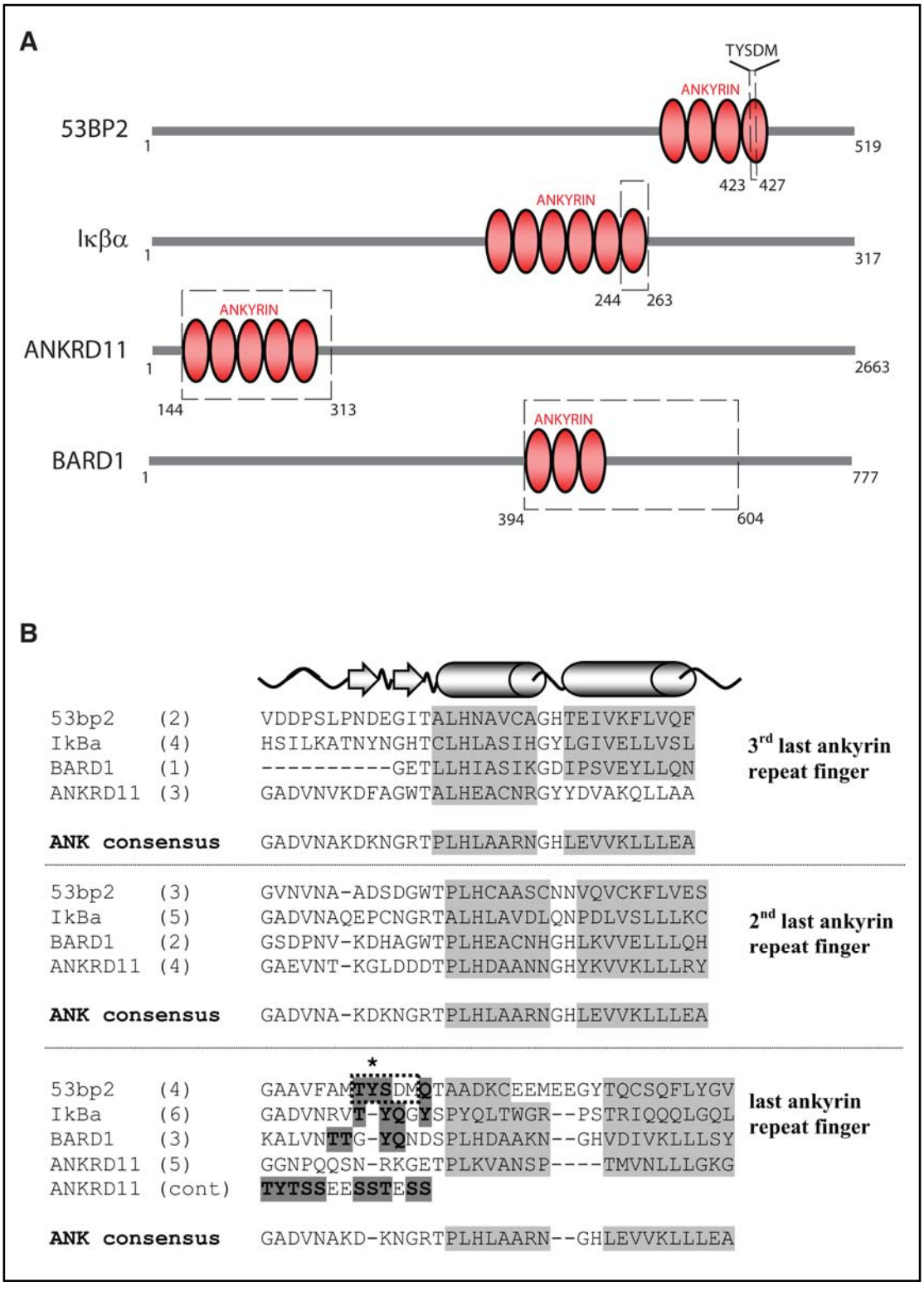

A
TYSDM
ANKYRIN
53BP2
1
519
423
427
ANKYRIN
Iκβα
1
317
244
263
ANKYRIN
ANKRD11
1
2663
144
313
ANKYRIN
BARD1
1
777
394
604
B
53bp2 (2) VDDPSLPNDEGITALHNAVCAGHTEIVKFLVQF
IkBa (4) HSILKATNYNGHTCLHLASIHGYLGIVELLVSL
BARD1 (1) ----------GETLLHIASIKGDIPSVEYLLQN
ANKRD11 (3) GADVNVKDFAGWTALHEACNRGYYDVAKQLLAA
ANK consensus GADVNAKDKNGRTPLHLAARNGHLEVVKLLLEA
3rd last ankyrin repeat finger
53bp2 (3) GVNVNA-ADSDGWTPLHCAASCNNVQVCKFLVES
IkBa (5) GADVNAQEPCNGRTALHLAVDLQNPDLVSLLLKC
BARD1 (2) GSDPNV-KDHAGWTPLHEACNHGHLKVVELLLQH
ANKRD11 (4) GAEVNT-KGLDDDTPLHDAANNGHYKVVKLLLRY
ANK consensus GADVNA-KDKNGRTPLHLAARNGHLEVVKLLLEA
2nd last ankyrin repeat finger
*
53bp2 (4) GAAVFAMTYSDMQTAADKCEEMEEGYTQCSQFLYGV
IkBa (6) GADVNRVT-YQGYSPYQLTWGR--PSTRIQQQLGQL
BARD1 (3) KALVNTTG-YQNDSPLHDAAKN--GHVDIVKLLLSY
ANKRD11 (5) GGNPQQSN-RKGETPLKVANSP----TMVNLLLGKG
ANKRD11 (cont) TYTSSEESSTESS
ANK consensus GADVNAKD-KNGRTPLHLAARN--GHLEVVKLLLEA
last ankyrin repeat finger

Figure 3.8. Identification of a putative p53 interaction motif within the ANK domain.

A: Schematic diagram depicting the minimal defined p53-interacting regions for 53bp2, IκBα, BARD1 and ANKRD11.

B: Alignment of the amino acid sequence of the last three ankyrin repeats of 53bp2, IκBα, BARD1 and ANKRD11 (repeat number indicated in brackets). The α-helices are indicated in the shaded region (light shade). The regions flanking the α-helices of the last ankyrin repeat in 53bp2, IκBα, BARD1 and ANKRD11 (continued on 2nd line) are rich in threonine (T), tyrosine (Y), serine (S) and glutamine (Q) residues (dark shade) and are suggested to be a putative p53-interacting motif. Note that these resides do not constitute part of the published ankyrin repeat consensus sequence (given below alignment; (Mosavi et al., 2002)) and are rarely seen in their respective locations within the earlier ankyrin repeats of these proteins. The region of 53bp2 reported to interact with p53, as determined through crystal structure analysis (dashed box - TYSDM - Gorina & Pavletich, 1996), is also located at the same site as this putative p53-interaction motif.

3.2.5 – Discussion

Despite multiple reports of ankyrin proteins directly interacting with p53 via their ANK domains, the notion of a common molecular mechanism that defines the interaction of ankyrin proteins with p53 has not been previously reported in the literature. We propose that p53 is recruited to the ANK domain of ankyrin proteins through a cryptic p53-interaction motif presented by the β-sheet hairpin of the final C-terminal ankyrin repeat. The existence of this p53-interaction motif is supported by several lines of evidence.

- Studies investigating the crystal structure of ankyrin repeat proteins in complex with binding partners have determined that the main recognition site for protein-protein interaction within the ANK domain occurs at the β-sheet hairpin of the final C-terminal ankyrin repeat (Mosavi et al., 2002; Mosavi et al., 2004).

- Structural analysis of the core domain of p53 bound to the ankyrin repeats of 53bp2 showed that the last β-sheet hairpin of the ankyrin repeats was responsible for physical interaction with p53 (Gorina & Pavletich, 1996). Furthermore, the proposed p53-interaction motif encompasses the precise region of 53bp2 that was previously shown to physically interact with p53, as determined by crystal structure analysis (Fig 3.8*B*; dashed box). This crystal structure analysis also revealed that "the fourth ankyrin repeat of 53bp2 contains an insertion (T**Y**SDM – Fig 3.8*B*; asterisk) compared to the ankyrin consensus, and this insertion is critical for the surface complementation at the ankyrin-p53 interface" (Gorina & Pavletich, 1996). This co-crystal study provides strong supporting evidence towards the hypothesis that a common p53-interaction motif is responsible for the interaction of ankyrin proteins with p53.

- Similarly, synthesis of data from two *in vitro* studies of the p53-IκBα complex suggests that the minimal region of IκBα required for p53 interaction also consists of the last ankyrin repeat (244-263aa) (Fig 3.8A, Chang, 2002; Dreyfus et al., 2005).

- The proposed p53-interaction motif is located in a an ANK domain region of poorly conserved residues. It is accepted in the literature that poorly conserved residues within the ANK domain are typically present at the recognition surface of ankyrin proteins (Sedgwick & Smerdon, 1999; Mosavi et al., 2002). It is unlikely that residues that are contained within the published ankyrin repeat consensus (Mosavi et al., 2002) are responsible for the interaction of the ANK domain with p53.

Future studies to test this hypothesis could involve *in vivo* and *in vitro* binding assays to precidely define the residues of ANKRD11, 53bp2, IκBα or BARD1 involved in their interaction with p53. Resolution of the crystal structure of the ANK domain of ANKRD11, IκBα or BARD1 bound to p53 would provide valuable insight into the underlying mechanistic surround the interactions of these ankyrin proteins with p53.

To date, the only ankyrin proteins known to interact with and modulate p53 function include ANKRD11, 53bp2, IκBα or BARD1. It is possible that additional ankyrin proteins may also function as p53 co-regulators and interact with p53 via the proposed p53-interaction motif. It is possible that an *in silico* screen based on this motif will identify other previously unknown p53 regulatory ankyrin proteins.

It is likely that the integrity of the ankyrin-p53 interaction is of clinical relevance, since the p53-BARD1 and p53-53bp2 complexes were disrupted by tumour-associated mutations in BARD1 and p53, respectively (Iwabuchi et al., 1994; Gorina & Pavletich, 1996; Irminger-

Finger et al., 2001; Feki et al., 2005). If ANKRD11, 53bp2, IκBα and BARD1 all regulate p53 through a common ankyrin-based structural motif, this ankyrin-p53 interaction could provide a unique therapeutic target that will allow the simultaneous modulation of multiple p53 binding partners. This could have clinical implications in the treatment of cancers that still retain wild-type p53.

The modulation or restoration of p53 pathways in cancer cells holds great promise for the development of more selective and effective cancer treatments. However, the majority of these therapeutic strategies are still in early development, and there exists an urgent need for further research in this field to clearly define the complex regulation of p53 by its co-factors.

Cancer is a heterogeneous disease, and it is likely that drugs targeting multiple proteins or pathways will be required to achieve therapeutic success. Treatments such as tyrosine kinase inhibitors (Gleevec) and HDAC inhibitors (SAHA) have already proven to be useful anti-cancer drugs (Druker et al., 1996; Richon et al., 1998). We envisage that targeting the ankyrin-p53 interaction will also prove to be beneficial in the treatment of cancer through the modulation of multiple p53-regulatory proteins and their downstream pathways.

3.3 – Characterisation of a role for ANKRD11 as a putative cell cycle checkpoint protein

3.3.1 – Introduction

3.3.1.1 – The cell cycle

Proliferation of eukaryotic cells is a tightly regulated process where cells proceed through various critical "checkpoints" of the cell cycle. The cell cycle is segregated into various phases, including G_1 phase (preparation), S phase (DNA synthesis), G_2 phase (2^{nd} preparation) and M phase (mitosis). Non-proliferative cells in multicellular eukaryotic organisms enter a state of quiescence (G_0 phase), where they remain until mitogenic stimuli initiates their re-entry into the cell cycle (Coller, 2007). In contrast, senescent cells are arrested in G_0/G_1 phase and do not re-enter the cell cycle upon mitogenic stimuli. Cellular senescence occurs in response to various types of stressful stimuli, including shortened telomeric length, aberrant oncogenic signalling, oxidative stress or DNA damage (Zhang, 2007).

3.3.1.2 – Regulation of the cell cycle

Cell cycle progression is regulated through the phosphorylation of a variety of proteins by a series of cyclin-dependent kinases (Cdks). Although Cdk protein levels remain constant throughout the cell cycle, their activity is stringently controlled by several mechanisms, including association with cyclins, phosphorylation and dephosphorylation, and interaction with Cdk inhibitors (CKIs) (Nakayama & Nakayama, 2006). Many of these cyclins and CKIs have extremely short half-lives and are rapidly degraded at critical points throughout the cell cycle through the ubiquitin proteolytic system.

3.3.1.3 – The Ubiquitin Proteolytic Pathway

The ubiquitin proteolytic pathway plays a major role in the selective degradation of a variety of short-lived proteins in eukaryotic cells. In addition to the regulation of cell cycle progression through degradation of various cyclins and CKIs (Ciechanover et al., 1984; Finley et al., 1984), the ubiquitin system has been implicated in the degradation of proteins involved in other cellular processes, including protein kinase activity (Chen et al., 1996), signal tranduction (Hicke, 1999), transcriptional regulation (Hershko & Ciechanover, 1998), receptor down-regulation and endocytosis (Hicke & Riezman, 1996; Strous et al., 1996). Protein degradation through the ubiquitin proteolytic pathway is a complex process whereby proteins are first polyubiquitinated, providing a target for subsequent degradation by the 26S proteosome (Nakayama & Nakayama, 2006).

3.3.1.3.1 – Polyubiquitination

There are four critical enzymes involved in the ubiquitin system; ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3) and the 26S proteosome (Fig 3.9). The first step in the ubiquitination pathway is the activation of ubiquitin into a high-energy, unstable intermediate by the E1 ubiquitin activating enzyme through the formation of a thioester linkage between the C-terminal glycine residue of ubiquitin and a cysteine residue on E1 (Handley et al., 1991). The ubiquitin molecule then undergoes trans-esterification onto a cysteine residue of E2 and is subsequently linked to a lysine residue on the targeted substrate. This process is facilitated by the E3 ubiquitin ligase, the enzyme that also specifies the substrates recruited (Nakayama & Nakayama, 2006).

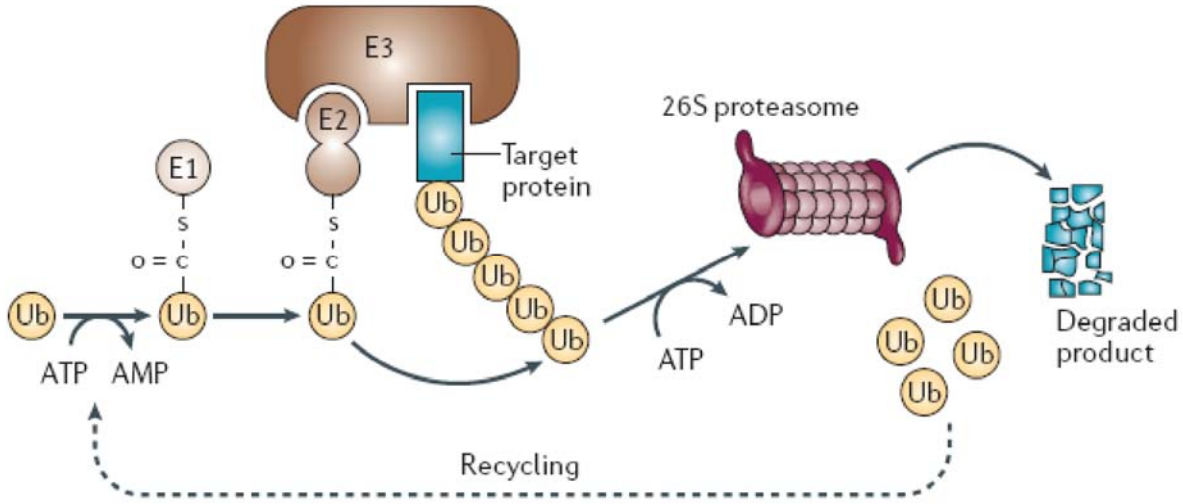


Figure 3.9. Overview of the ubiquitin-proteasome pathway. Ubiquitin (Ub) is initially activated by E1 (ubiquitin activating enzyme) and transferred to E2 (ubiquitin conjugating enzyme). Proteins are polyubiquitinated by E3 (ubiquitin ligase) through E2-mediated recruitment of the activated ubiquitin molecule target to the substrate. Finally, polyubiquitinated proteins are typically degraded by the 26S proteasome. *Adapted from (Nakayama & Nakayama, 2006).*

Ubiquitin is a small protein of 76 amino acids that can undergo covalent linkages at lysine 48 to form polyubiquitin chains on proteins. Once the polyubiquitin chain exceeds four or more molecules in length, proteins can be efficiently targeted by the 26S proteosome where they undergo ATP-dependent proteolytic degradation (Yang et al., 2004). It has been recently discovered that the functionality of the ubiquitination pathway is not limited to protein degradation. Studies have shown that monoubiquitination or polyubitination of alternative lysine residues on the substrate protein also regulates various other cellular pathways, including gene expression, endocytosis, subnuclear trafficking, Iκβ kinase complex (IKK) activation, ribosome modification and DNA repair (Pickart, 2001; Hoeller et al., 2007).

3.3.1.3.2 – E3 ubiquitin ligases

The E3 ubiquitin ligases are a highly diverse family of protein complexes responsible for the specific substrate recognition of proteins to be targeted for proteasomal-mediated degradation (Hershko, 1983). There is a vast number of proteins degraded by the ubiquitin proteolytic pathway, these E3 ligases exhibit a high degree of versatility and specificity for their substrates. Therefore, it is not surprising that 500-1000 mammalian E3 ubiquitin ligases have been identified to date (Nakayama & Nakayama, 2006). These E3 ubiquitin ligases are categorized into four distinct classes: HECT-type, RING-finger-type, U-box-type and PHD-finger-type. Two of the most common RING-finger-type E3 ubiquitin ligases include the SCF (Skp1/Cul1/F-box) and APC/C (anaphase-promoting complex/cyclosome) complexes, with their specific ubiquitin ligase activities tightly regulated throughout the various stages of the cell cycle.

3.3.1.3.2.1 – E3 ubiquitin ligases: the SCF complex

The most thoroughly characterised of these multi-component E3 ubiquitin ligases is the SCF complex, named after its major constituents, the **S**kp1, **C**ullin and **F**-box proteins (Ho et al., 2006). In the mammalian SCF complex, the Cullin protein (Cul1) acts as a molecular scaffold for the entire protein complex. Cul1 has been shown to simultaneously interact with Skp1 (Feldman et al., 1997; Skowyra et al., 1997) and the 16 kDa RING finger protein, referred to as either Roc1, Rbx1 or Hrt1 (Ohta et al., 1999; Tan et al., 1999). Roc1 plays an essential role in the SCF ligase activity through heterodimerisation with Cul1 and subsequent catalysis of the E2-dependent ubiquitin polymerisation (Ho et al., 2006). The binding of the ubiquitin-like molecule Nedd8 to Cul1 has also been shown to facilitate the association of E2 with Roc1 and increase SCF activity (Jackson & Eldridge, 2002) (Figure 3.10).

The F-box proteins constitute a large family that contain a motif referred to as an 'F-box' which is essential for association with the Skp1 adaptor protein (Nakayama & Nakayama, 2006). The F-box proteins act as selective molecular receptors and, through protein-protein interaction domains at the carboxyl-terminus, recruit specific phosphorylated substrates to the SCF complex for subsequent polyubiquitination by E2. The SCF complex is able to impart a sophisticated level of regulation of numerous crucial cell cycle regulators through the selective recruitment of numerous F-box proteins, each of which can also recognise multiple substrates. Several proteins that mediate both positive and negative imfluences on cell cycle progression are selectively targeted for degradation following the assembly of several SCF complexes, including SCF^{Skp2}, $SCF^{\beta\text{-}TRCP}$ and SCF^{Fbw7} (Table 3.1).

3.3.1.3.2.2 – E3 ubiquitin ligases: the APC/C complex

The anaphase promoting complex/cyclosome (APC/C) is the major E3 ubiquitin ligase that is active during mitosis, with its title reflecting its primary function. Similar to the SCF

complex, APC/C is structurally composed of both invariable core components and interchangeable substrate-recognition components.

The invariable core catalytic componenet of the APC/C complex is comprised by APC11 (Roc-1-related RING finger protein) and APC2 (Cul-1-related scaffolding protein) (Nakayama & Nakayama, 2006) (Fig 3.10). This two-subunit catalytic APC core can interact with the various E2 ubiquitin conjugating enzymes and ubiquitinate proteins *in vitro*, however this ubiquitin ligase reaction has limited substrate specificity and poor processivity in the absence of other APC/C constituents (Acquaviva & Pines, 2006).

The non-enzymatic APC/C component consists of four sub-units, including (Kraft et al., 2003). These APC sub-units contain multiple TPR (tetratricopeptide repeat) protein-protein interaction motifs, and are responsible for the recruitment of the APC/C cofactors, Cdc20 and Cdh1 (Acquaviva & Pines, 2006) (Fig 3.10). Cdc20 and Cdh1 confer substrate specificity to the APC/C ubiquitin ligase complex in a cell-cycle dependent manner. The recruitment of these APC/C cofactors is a tightly regulated process involving phosphorylation of either APC3, APC6, APC7 or APC8. This phosphorylation occurs at specific stages of the cell cycle, ensuring the timely degradation of key cell cycle regulators.

APC/C^{Cdc20} is active during the early stages of mitosis and has been implemented in the polyubiquitination of several cell cycle regulators, including securin, a protein involved in sister chromatid segregation, and mitotic cyclins (Liu & Fuchs, 2006) (Table 3.1). The APC/C^{Cdh1} complex is rapidly assembled during anaphase and is then active during mitotic exit and the G_1 phase of the cell cycle. APC/C^{Cdh1} is responsible for the polyubiquitination of multiple substrates, including the remaining mitotic cyclins, several G_1 regulators (including Skp2), and its predecessor, Cdc20 (Liu & Fuchs, 2006) (Table 3.1).

Recognition of substrates by the APC/C complex is typically dependent on the presence of the destruction box (D-box). The full-length consensus D-box (RxxLxxIxN) was initially identified in cyclin B and is recognised by APC/C^{Cdc20} (Glotzer et al., 1991), whereas the presence of a minimal D-box (RxxL) sequence or a KEN-box (KEN) is sufficient for APC/C^{Cdh1} recognition (Pfleger & Kirschner, 2000; Zur & Brandeis, 2002).

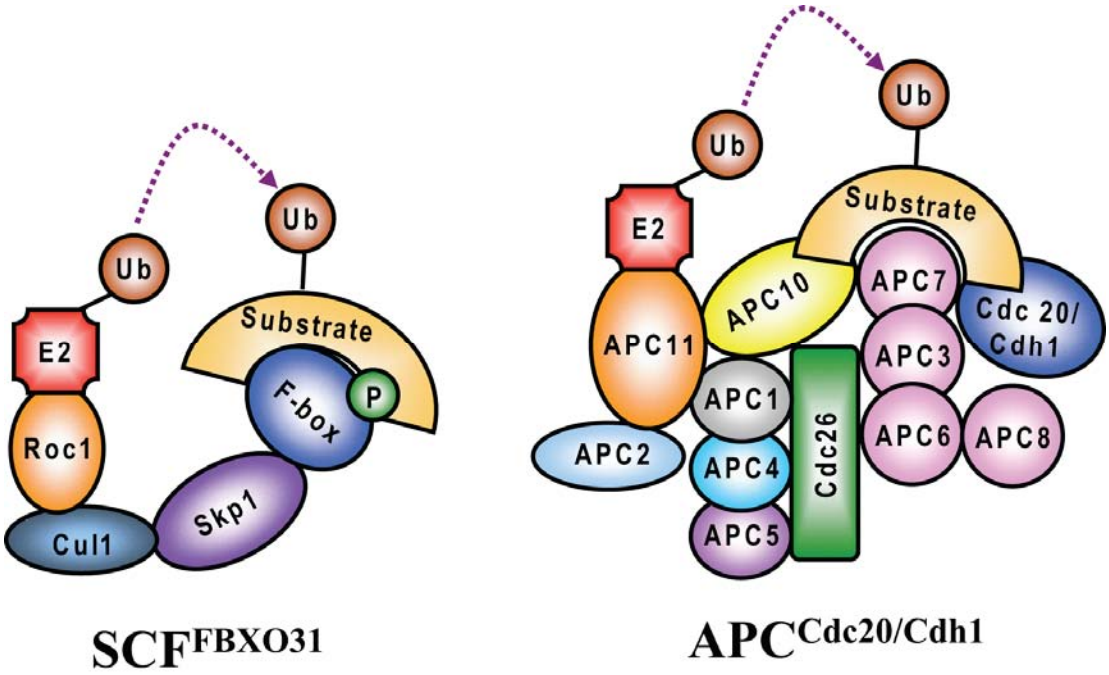


Figure 3.10. The SCF and APC/C E3 ubiquitin ligase complexes.

Table 3.1: Degradation of various cell cycle regulators through the ubiquitin proteolytic system

Cell Cycle Regulator	Cell Cycle Function	Ubiquitin Ligase	References
p21	Inhibits cyclin-Cdk2 and cyclin-Cdk4 complexes. Negative regulator of cell cycle progression during exit from the G_1 phase	SCF^{Skp2}	(Toyoshima & Hunter, 1994; Bornstein et al., 2003)
p27	Inhibits cyclin-Cdk2 and cyclin-Cdk4 complexes. Negative regulator of cell cycle progression during exit from the G_1 phase	SCF^{Skp2}	(Toyoshima & Hunter, 1994; Carrano et al., 1999; Marti et al., 1999; Tsvetkov et al., 1999)
p45 (Skp2)	Responsible for the degradation of p21, p27, p57 and free cyclin E. Positive regulator of cell cycle progression during exit from the G_1 phase.	APC/C^{Cdh1}	(Carrano et al., 1999; Marti et al., 1999; Tsvetkov et al., 1999; Nakayama et al., 2001; Bornstein et al., 2003; Kamura et al., 2003; Bashir et al., 2004a; Wei et al., 2004)
p57	Inhibits cyclin A-Cdk2, cyclin B-Cdk1, cyclin D-Cdk4 and cyclin E-Cdk2 complexes. Negative regulator of cell cycle progression during exit from the G_1 phase	SCF^{Skp2}	(Lee et al., 1995; Kamura et al., 2003)
p130	Inhibits cyclin A-Cdk2 and cyclin E-Cdk2 complexes. Negative regulator of cell cycle progression during G_0 phase and G_1 – S transition	SCF^{Skp2}	(De Luca et al., 1997; Tedesco et al., 2002; Guardavaccaro & Pagano, 2004; Ohta & Fukuda, 2004)
Cyclin A	Associates with Cdk1 and Cdk2. Positive regulator of cell cycle progression through S phase and G_2/M transition	APC/C^{Cdc20} APC/C^{Cdh1} SCF^{Skp2}	(Sherr, 1996; Philips et al., 1998; Yam et al., 2002; Nakayama & Nakayama, 2006)

Cyclin B	Associates with Cdk1. Positive regulator of cell cycle progression through G_2/M transition	APC/C^{Cdc20} APC/C^{Cdh1}	(Sherr, 1996; Nigg, 2001; Castedo et al., 2004; Nakayama & Nakayama, 2006)
Cyclin D	Associates with Cdk4 and Cdk6. Positive regulator of cell cycle during exit from the G_1 phase	SCF^{Fbx4} SCF^{Skp2}	(Sherr, 1996; Nakayama et al., 2001; Ohta & Fukuda, 2004; Lin et al., 2006)
Cyclin E	Associates with Cdk2. Positive regulator of cell cycle during exit from the G_1 phase	SCF^{Fbw7} SCF^{Skp2}	(Sherr, 1996; Nakayama et al., 2001)

3.3.1.3.3 – Cell cycle checkpoints

The process of cell division is a highly ordered system, designed for the timely execution of a variety of complex cellular processes. The term "cell cycle checkpoint" refers to mechanisms implemented by the cell to actively halt progression of the cell cycle until earlier processes, such as DNA replication or mitotis, have been successfully completed (Kastan & Bartek, 2004). Cell cycle checkpoints can also become activated in response to a variety of endogenous and exogenous stress signals, including DNA damage, defects during DNA replication or failure of the chromosomes to attach to the mitotic spindle (Giono & Manfredi, 2006). The most highly studied cell cycle checkpoints are discussed below, with a major focus on the various checkpoint proteins and signalling pathways that mediate these cell cycle checkpoints.

3.3.1.3.3.1 – The G_1/S phase checkpoint

The G_1/S checkpoint functions to prevent cells from initiating DNA replication with damaged DNA. The p53 tumour suppressor plays a prominent role throughout this checkpoint and plays a critical role in the pausing of cells at this checkpoint in response to DNA damage (Giono & Manfredi, 2006). The upstream pathways that control the transcriptional activity of p53 are key mediators of this cell cycle checkpoint. ATM/ATR and Chk1/Chk2 kinases are responsible for the phosphorylation and stabilisation of p53 during the G_1/S checkpoint and in response to DNA damage (Khanna et al., 2001; Kastan & Bartek, 2004). Induction of p53 activity leads to accumulated levels of its key transcriptional target, $p21^{waf1}$. The Cdk inhibitor, $p21^{waf1}$, is responsible for inhibiting all G_1-promoting cyclin/Cdk complexes, and prolonged expression of $p21^{waf1}$ results in permanent G_1 arrest of the affected cell (Kastan & Bartek, 2004).

In addition to the ATM-p53-$p21^{waf1}$ pathway, the G_1/S checkpoint is also regulated by the Cdk inhibitor $p27^{kip1}$ (Massague, 2004). $p27^{kip1}$ inhibits the activity of the cyclin-Cdk2 and cyclin-Cdk4 complexes, and its degradation by SCF^{Skp2} is a requirement for G_1/S phase transition (Toyoshima & Hunter, 1994; Carrano et al., 1999). Ongoing research has revealed increasingly complex cohorts of signalling pathways that are involved in the mediation of the G_1/S checkpoint.

3.3.1.3.3.2 – The intra-S phase checkpoint

The intra-S phase checkpoint is activated by DNA damage and prevents the firing of origins of DNA replication that are yet to be initiated (Kastan & Bartek, 2004). This checkpoint manifests itself as a reversible decrease in DNA synthesis, and is controlled by two distinct ATM-dependent pathways (Khanna & Chenevix-Trench, 2004). Firstly, ATM- and ATR-dependent activation of the Chk1/Chk2 kinases results in down-regulation of CDC25A and consequently, inhibition of cyclin-Cdk2 complexes. Inhibition of Cdk2 activity prevents loading of CDC45 onto chromatin, leading to the incomplete assembly of DNA replication complexes and consequently decreased DNA synthesis (Kastan & Bartek, 2004; Khanna & Chenevix-Trench, 2004).

The second pathway that mediates the intra-S phase checkpoint is imparted through ATM-mediated phosphorylation of NBS1, a component of the MRN (MRE11, Rad50, NBS1) complex involved in double strand break (DSB) repair. Phosphorylation of NBS1 is a requirement for a cell to be able to tolerate DSBs induced through exposure to ionising radiation. In addition, NBS1 phosphorylation has been shown to inhibit S-phase progression following DSBs (Khanna & Chenevix-Trench, 2004).

3.3.1.3.3.3 – The G_2/M phase checkpoint

The G_2/M checkpoint prevents DNA-damaged cells from entering mitosis and undergoing cell division (Xu et al., 2002). Following DNA damage, the G_2/M checkpoint is initiated through inhibition of the mitosis-promoting activity of the cyclin B/Cdk1 complex via an ATM/ATR-dependent mechanism (Khanna & Chenevix-Trench, 2004). The maintenance of the G_2/M checkpoint is reliant on the transcriptional activities of both BRCA1 and p53, enhancing the expression of various proteins involved in G_2 arrest, including $p21^{waf1}$, GADD45 and 14-3-3 sigma proteins (Nyberg et al., 2002; Kastan & Bartek, 2004).

3.3.1.3.3.4 – The spindle assembly checkpoint

The spindle assembly checkpoint (SAC) is active during prometaphase stage of the cell cycle and ensures that dividing cells do not undergo anaphase entry until all chromosomes are attached to the mitotic spindle and correctly aligned along the metaphase plate. The critical proteins that initiate the SAC include members of the MAD (mitotic-arrest deficient) and BUB (budding unihibited by benzimidazole) families (Musacchio & Salmon, 2007).

The major target of the SAC complex is the APC/C activator, Cdc20. The SAC complex negatively regulates the ability of APC/C^{Cdc20} to target securin and cyclin B proteins for proteasomal degradation (Musacchio & Salmon, 2007). Securin is an inhibitor of separase, the protease responsible for cleavage of the cohesion complex that holds sister chromatids together (Peters, 2006). Secondly, delayed proteolysis of cyclin B leads to prolonged activity of cyclin B/Cdk1, a complex whose inactivation is required for exit from mitosis (Musacchio & Salmon, 2007).

3.3.1.3.4 – Aberrant regulation of cell cycle proteins and checkpoints during breast tumorigenesis

Aberrations in the cell cycle regulatory mechanisms are hallmarks of malignant cells (Liu & Fuchs, 2006). Both ATM and p53 play crucial roles in the initiation and mediation of checkpoints of the cell cycle. Therefore, it is not surprising that mutations in *ATM* and *TP53* are associated with the development of familial and sporadic breast cancer, respectively (Athma et al., 1996; Inskip et al., 1999; Khanna & Chenevix-Trench, 2004; Lacroix et al., 2006).

Cyclin D1, cyclin E1, p27 and Skp2 are cell cycle proteins involved in the regulation of cell cycle progression during the G_1/S phase transition and undergo stringent regulation at various stages during the normal execution of the cell cycle (Table 3.1). Breast cancer is frequently associated with abnormal levels of these four cell cycle proteins (Ohta & Fukuda, 2004). For example, cyclin D1 is overexpressed at both the mRNA and protein levels in over half of all invasive breast tumours (Buckley et al., 1993; Gillett et al., 1994; Ormandy et al., 2003). Increased cyclin E1 protein levels are consistently associated with poor outcome for breast cancer patients (Keyomarsi et al., 2002). The absence of p27 protein expression is highly associated with poor prognosis in breast tumours (Tan et al., 1997). In breast cancers, $p27^{kip1}$ and Skp2 levels are inversely correlated, suggesting that the presence of SCF^{Skp2} is responsible for reduced $p27^{kip1}$ levels (Sonoda et al., 2006). Furthermore, cytogenetic analysis revealed that amplification of the *SKP2* gene occurs in 11% of all breast carcinomas (Forozan et al., 2000; Chen et al., 2006). These findings illustrate the importance of elucidating the mechanisms involved in post-translational regulation of these cell cycle regulators, and warrant further research into the aberrations in their regulation or degradation that contributes towards breast oncogenesis.

3.3.2 – Materials and Methods

3.3.2.1 – Cell lines and antibodies

Cell lines used throughout this section are as described (General Materials and Methods; Section 2.1). Commercial antibodies used are as described in Table 2.1.

3.3.2.2 – Plasmids

The generation or source of constructs used throughout this section are described in Table 2.3. Constructs were generated using standard procedures (Ausubel, 2001), with the sequences of all constructs confirmed by DNA sequencing (General Materials and Methods; Section 2.7).

3.3.2.3 – Immunofluroescence

Immunofluorescence was performed as described (General Materials and Methods; Section 2.3).

3.3.2.4 – Cell culture synchronization

Mitotic synchronisation was performed using a nocodazole block as described (General Materials and Methods; Section 2.9).

3.3.2.5 – Subcellular biochemical fractionation

Subcellular biochemical fractionation was performed as described in Wysocka et al (2001). Briefly, MCF-10A cells (1×10^7) were harvested, washed in PBS and resuspended in Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM DTT and protease inhibitor cocktail). Triton X-100 was added (0.1% final concentration), cells were incubated on ice for 8 minutes and nuclei were collected through centrifugation (1300 × *g*, 5 mins, 4°C). The supernatant was removed (cytosolic fraction), and

harvested nuclei were washed once in Buffer A and lysed for 30 minutes on ice in Buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitor cocktail). A chromatin-enriched fraction was collected through subsequent centrifugation (1700 × *g*, 5 mins, 4°C). The supernatant (soluble nuclear fraction) was removed and the pellet (chromatin-enriched fraction) was resuspended in lysis buffer and sonicated (General Materials and Methods; Section 2.5).

3.3.3 – Results

3.3.3.1 – ANKRD11 exhibits partial localisation with PML bodies

Our earlier findings suggest that ANKRD11 co-localised with PML in $p14^{ARF}$-positive extranucleolar inclusions (see Section 3.1.4.1). In contrast, a previous report suggested that ANKRD11 nuclear foci are distinct from PML bodies (Zhang et al., 2004). To clarify this issue, the subcellular localisation of ANKRD11 in relation to PML bodies was investigated in the absence of $p14^{ARF}$ expression. ANKRD11-myc was transiently-expressed in MCF-7, a breast cancer cell line that does not express endogenous $p14^{ARF}$ due to deletion of the *CDKN2A* locus (Stott et al., 1998). Co-immunolocalisation results show that endogenous PML is localised to the periphery of ANKRD11-myc nuclear foci (Fig 3.11*A*). A similar partial localisation was also observed by endogenous SUMO-1, confirming that these PML nuclear foci were associated with PML bodies (Fig 3.11*B*). The association of ANKRD11 with PML bodies was cell cycle-dependent, as these findings were only observed during late G_2 phase of the cell cycle.

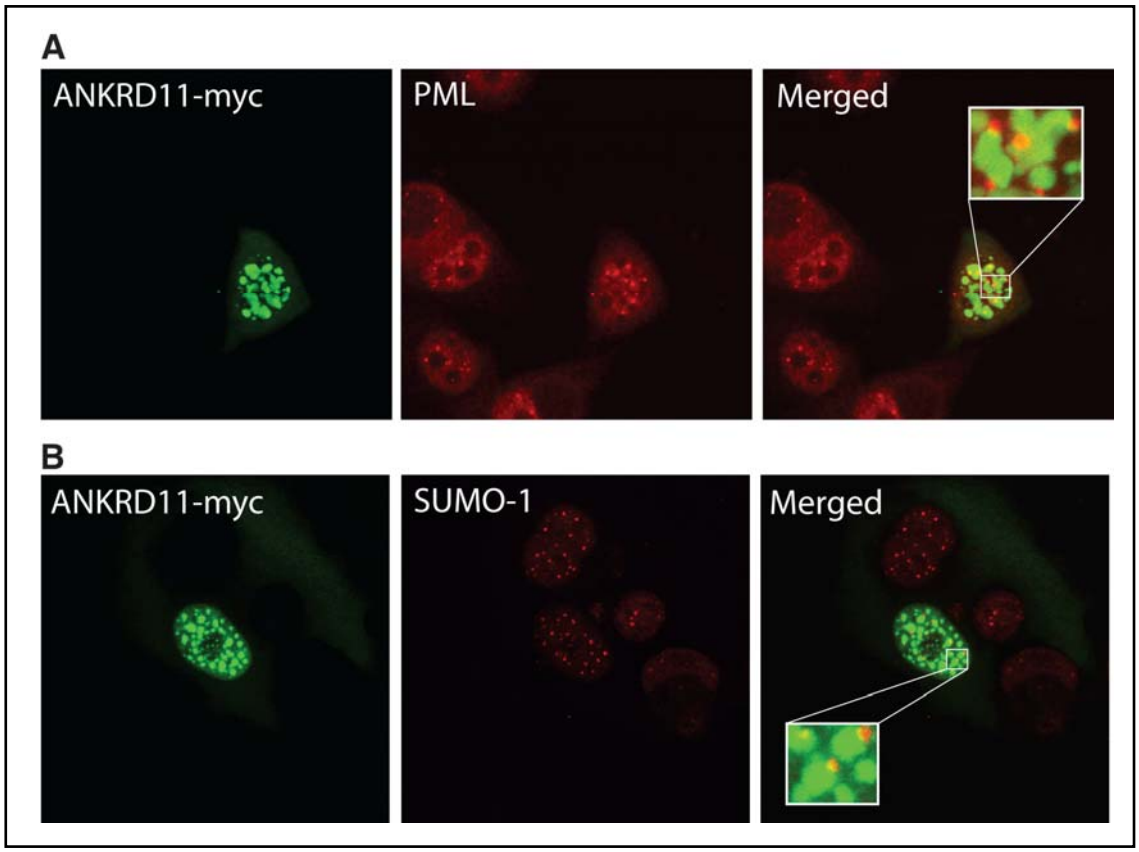


Figure 3.11. ANKRD11 partially co-localises with PML bodies.

A: ANKRD11-myc and PML partially co-localise. MCF-7 cells were transiently-transfected with a construct expressing ANKRD11-myc, blocked with nocodazole, immunostained with α-myc and α-PML antibodies and imaged using confocal microscopy.

B: ANKRD11-myc and SUMO-1 partially co-localise. MCF-7 cells were transiently-transfected and synchronized as in *(A)*. Cells were immunostained using α-myc and α-PML antibodies and imaged using confocal microscopy.

3.3.3.2 – ANKRD11 localises with PML isoforms

To gain further insight into the role or regulation of ANKRD11 by PML bodies, it was investigated if the association of ANKRD11 with PML was isoform specific. To investigate this, the localisation of ANKRD11 was determined in relation to the various isoforms of PML. GFP-ANKRD11 was retrovirally-expressed in MCF-10A cells and selected in Geneticin for two weeks. MCF-10A cells stably-expressing GFP-ANKRD11 were transfected with constructs expressing either of six FLAG-tagged isoforms of PML; PML I, PML II, PML III, PML IV, PML V or PML VI. Fluorescent microscopy showed that GFP-ANKRD11 nuclear foci co-localise with each of the PML isoforms I – VI (represented by co-localisation with PML V – Fig 3.12*A*). These findings suggest that the association between ANKRD11 and PML is not isoform specific and imply that the association between ANKRD11 and PML is mediated by the common amino-terminal region of the PML isoforms (Fig 3.12*B*).

3.3.3.3 – ANKRD11 associates with chromatin during mitosis and is rapidly degraded during mitotic progression

Following G_2/M synchronization of MCF-7 cells with nocodazole, it was observed that the morphology of exogenous ANKRD11 nuclear foci was altered (Fig 3.11). To further investigate this observation, the localisation profile of exogenous ANKRD11 was determined throughout the course of mitotic progression. ANKRD11-myc was transiently-expressed in MCF-7 cells synchronized at the G_2/M phase of the cell cycle following treatment with nocodazole. Cells were released from the nocodazole block and the subcellular localisation of ANKRD11-myc was monitored using immunoflurescence and confocal microscopy. In the presence of nocodazole, ANKRD11 nuclear inclusions became enlarged and dispersed into spherical specular foci (Figure 3.13*A*; left panels). These pre-mitotic ANKRD11 nuclear foci were shown to co-localise with DNA/chromatin rich regions, as determined through counterstaining with propidium iodide (PI), and may represent nuclear chromosomal domains.

NOTE: This figure is included on page 110 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.12. ANKRD11 co-localises with PML isoforms I – VI.

A: Co-localisation of GFP-ANKRD11 and PML V. MCF-10A cells stably-expressing GFP-ANKRD11 were transiently-transfected with a construct expressing FLAG-PML V. Cells were immunostained with an α-FLAG antibody and imaged using confocal microscopy. Similar results were observed using cells transiently-expressing FLAG-PML I, II, III, IV and VI.

B: A schematic diagram representing the PML isoforms I – VI. The various PML isoforms are depicted as the alternative mRNA splice-variants cloned downstream of the FLAG coding sequence. *Adapted from (Guccione et al., 2004)*.

Chromosomal condensation was observed approximately 1 hour post-release from the nocodazole block, as determined by propidium iodide counterstaining. The level of exogenous ANKRD11-myc expression was significantly reduced during this stage of mitosis, with the remaining ANKRD11 nuclear foci exported to the periphery of the cell and not associated with chromosomal DNA (Figure 3.13*A*; right panels). Exogenous ANKRD11-myc expression was almost undetectable at 3 hours post-release.

The specific stages of mitotis were determined by counterstaining cells with the mitotic spindle protein, β-tubulin. Specular ANKRD11 nuclear foci were observed during the prometaphase stage of mitosis (30 mins post-release), as indicated by the absence of mitotic spindle formation or chromosomal condensation (Fig 3.13*B*). The expression of ANKRD11-myc was not observed in cells with polar mitotic spindle formation or alignment of the chromosomal metaphase plate, suggesting that degradation of ANKRD11 occurs prior to metaphase entry.

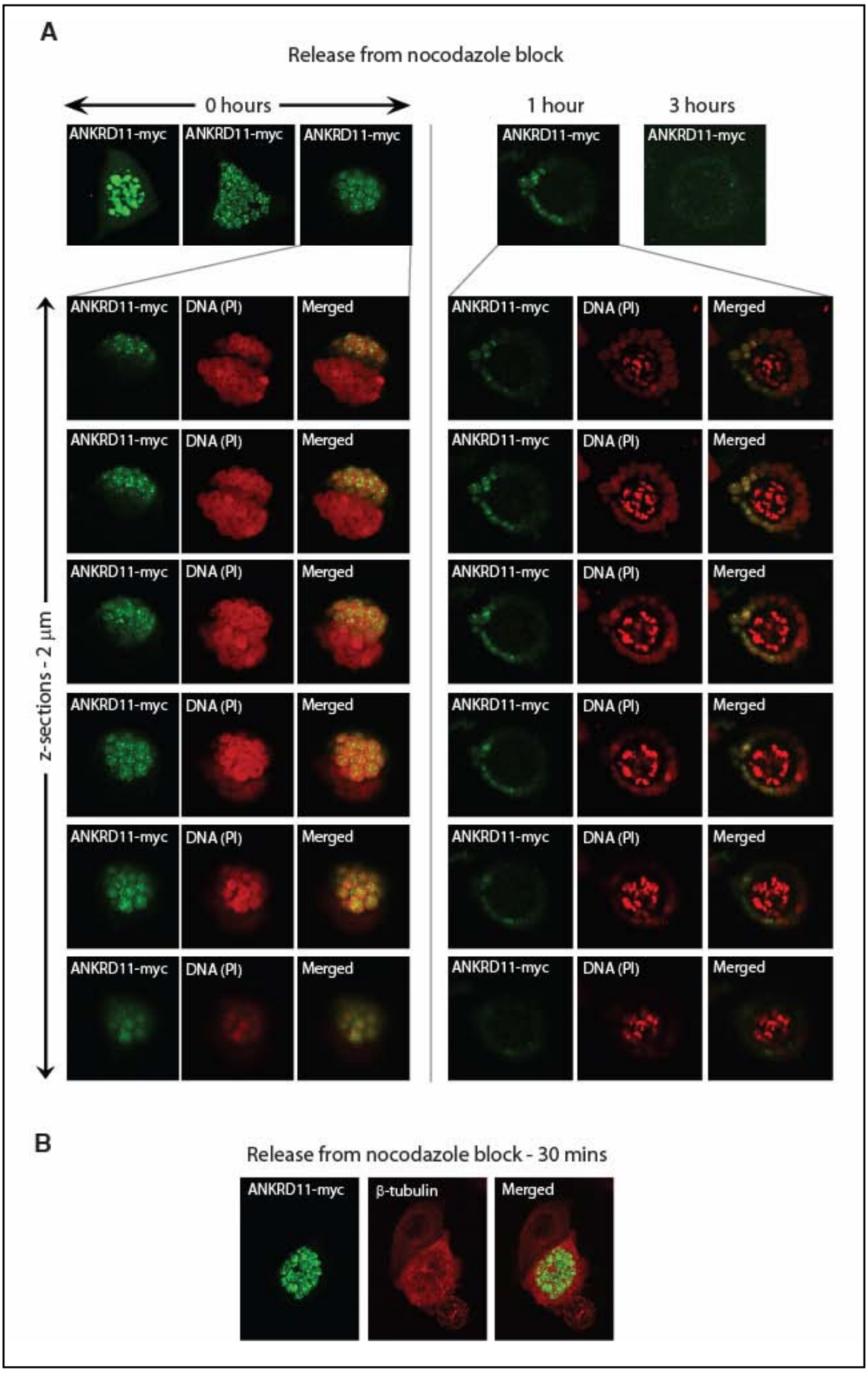

A
Release from nocodazole block
0 hours
1 hour
3 hours
ANKRD11-myc
DNA (PI)
Merged
z-sections - 2 µm
B
Release from nocodazole block - 30 mins
ANKRD11-myc
β-tubulin
Merged

Figure 3.13. ANKRD11 localisation during mitotic progression

A: ANKRD11 localises to chromatin-rich regions during prometaphase. MCF-7 cells transiently-expressing ANKRD11-myc were blocked in nocodoazole (left 3 panels) or released for the indicated times (right 2 panels). Cells were immunostained with an α-myc antibody, DNA/chromatin-rich regions were counterstained with propidium iodide (PI) and imaged using confocal microscopy. Z-section images of 2 μm thickness were collected for the indicated cells.

B: ANKRD11 localises to chromatin-rich regions prior to mitotic spindle formation. MCF-7 cells transiently-expressing ANKRD11-myc were released from nocodazole block for 30 minutes, immunostained with α-myc and α-β-tubulin antibodies and imaged using confocal microscopy.

Findings from immunoflurescence studies suggest that ANKRD11 associates with chromatin. To further investigate this association, biochemical fractionation was performed to determine the subcellular compartmentalization of endogenous ANKRD11 protein. MCF-10A cells were fractionated into either cytosolic, soluble nuclear or chromatin-enriched fractions and subjected to Western blot analysis. In accordance with previous immunoflurescence findings, the majority of endogenous ANKRD11 protein was detected in the chromatin-enriched fraction (Fig 3.14). The efficiency of subcellular fractionation was determined through the detection of the cytosolic (MEK2) and chromatin-bound (ORC2) markers.

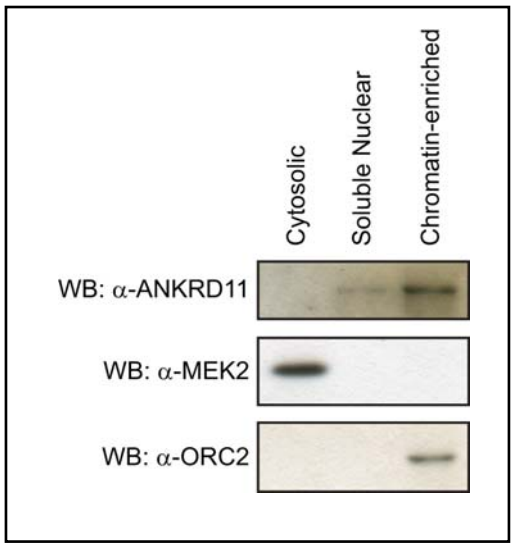


Figure 3.14. Endogenous ANKRD11 is compartmentalized in the chromatin-enriched subcellular fraction.

MCF-10A were fractionated into cytosolic, soluble nuclear or chromatin-enriched subcellular fractions. Endogenous ANKRD11, MEK2 or ORC2 protein were detected in the fractionated lysates analysis following Western blot analysis with either α-ANKRD11, α-MEK2 or α-ORC2 antibodies, respectively.

3.3.3.4 – ANKRD11 associates with histone H2A

ANKRD11 was recently shown to possess intrinsic activator (AD) and repressor (RD) domains (Zhang et al., 2007a), suggesting a role for ANKRD11 in the remodelling of chromatin structure through the recruitment of various acetyltransferases or deacetylases to the nucleosomal histones. It is tempting to hypothesize that the localisation of ANKRD11 to chromatin-rich regions resulted from its association with histone modification. Therefore, we determined the localisation of ANKRD11 in relation to histone H2A. ANKRD11-myc and GFP-histone H2A (GFP-H2A) were transiently-expressed in MCF-7 cells. Results show that ANKRD11-myc and GFP-H2A co-localise within the nucleus of MCF-7 cells, with both proteins exhibiting a diffuse nuclear localisation pattern (Fig 3.15*A*). Co-expression of GFP-H2A resulted in a morphological change in the localisation pattern of ANKRD11-myc (Fig 3.15*A*) from its usual nuclear focal arrangement, as observed in cells transiently-expressing ANKRD11-myc alone (Fig 3.15*B*). Taken together, these findings suggest the existence of a dynamic association between ANKRD11 and nucleosomal histones.

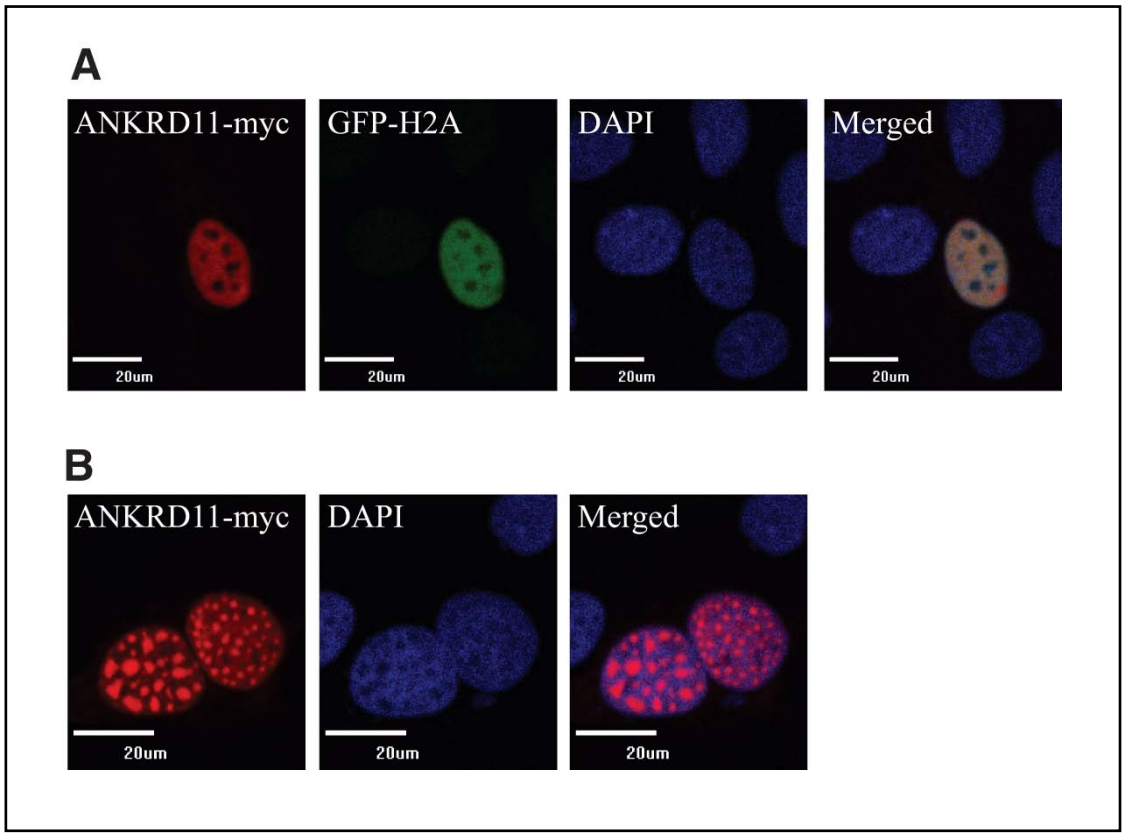


Figure 3.15. ANKRD11 co-localises with histone H2A.

A: ANKRD11 co-localises with histone H2A in a diffuse nuclear localisation pattern. MCF-7 cells were transiently-transfected with constructs expressing ANKRD11-myc and GFP-H2A were immunostained with an α-myc antibody and imaged using confocal microscopy.

B: ANKRD11 focal localisation in the absence of exogenous histone H2A. MCF-7 cells were transiently-transfected with a construct expressing ANKRD11-myc and were immunostained and imaged as described in *(A).*

3.3.3.5 – ANKRD11 is a cell cycle regulated protein

As exogenous ANKRD11 protein in the cell appeared to be rapidly degraded upon metaphase entry (Fig 3.13), we investigated if endogenous ANKRD11 protein was also cell cycle regulated. Endogenous ANKRD11 protein levels at different stages of the cell cycle were determined in the MCF-10A cell line by collecting samples at various time points following release from a double thymidine block (G_1/S phase synchronization). Endogenous ANKRD11 was detected in cell lysates following Western blot analysis using an α-ANKRD11 polyclonal antibody (Section 3.1.3.1). Variation in levels of ANKRD11 at different times after release of the block indicates that endogenous ANKRD11 protein is indeed cell cycle regulated (Fig 3.16). The cell cycle synchronization was confirmed by change in the levels of cyclin B1, which has been reported to be rapidly degraded prior to mitotic exit (Sigrist et al., 1995). Endogenous ANKRD11 protein levels were at a maximum at 10 hours post-release. Comparison with the cyclin B1 cell cycle profile indicates that levels of endogenous ANKRD11 protein were at a maximum during the early stages of mitosis and were subsequently degraded during mitotic progression and early G_1 phase of the cell cycle. These results are consistent with the observed regulation of ectopically-expressed ANKRD11 and suggest that machinery required for degradation of ANKRD11 is active during the later stages of mitosis and early G_1 phase of the cell cycle.

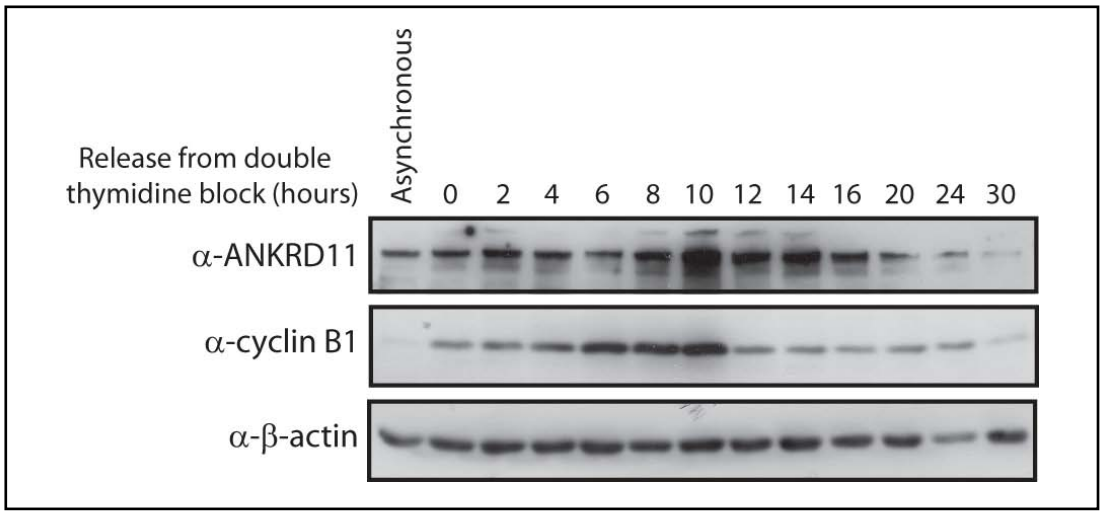


Figure 3.16. ANKRD11 is a cell cycle regulated protein.

MCF-10A cells were synchronized using a double thymidine block, released into fresh media without thymidine and harvested at the indicated timepoints. ANKRD11, cyclin B1 and β-actin protein levels were detected using Western blot analysis.

3.3.3.6 – ANKRD11 translocates to the nucleolus following proteasomal inhibition

Nucleolar accumulation of proteins that are degraded by the ubiquitin-proteasomal pathway have previously been observed in response to proteasomal inhibition (Klibanov et al., 2001; Mattsson et al., 2001; Pokrovskaja et al., 2001; Arabi et al., 2003). To investigate the involvement of the ubiquitin-proteasomal pathway in the degradation of ANKRD11, the subcellular localisation of exogenous ANKRD11 in MCF-7 cells was determined following treatment with MG132, an inhibitor of proteasomal-mediated degradation. Findings show nucleolar translocation of GFP-ANKRD11 protein in the presence of MG132 (Fig 3.17). Previous studies have also reported nucleolar accumulation of several components of PML bodies following proteasomal inhibition (Mattsson et al., 2001). Consistent with these findings, our co-immunolocalisation data shows that PML co-localises with ANKRD11 in nucleoli following treatment with MG132 (Fig 3.17).

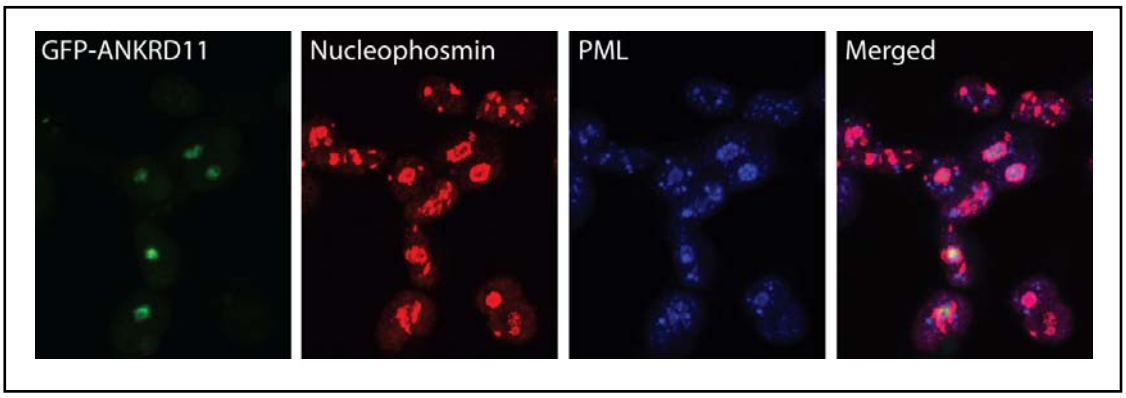


Figure 3.17. ANKRD11 and PML translocate to the nucleolus following proteasomal inhibition.

MCF-7 cells were transiently-transfected with a construct expressing GFP-ANKRD11 and treated with MG132 (1 μM) for 16 hours. Cells were immunostained using α-nucleophosmin and α-PML antibodies and imaged using confocal microscopy.

3.3.3.7 – ANKRD11 contains a consensus destruction box and multiple PEST sequences

In silico analysis of the ANKRD11 amino acid sequence was performed to determine the location of putative motifs involved in proteasomal-mediated degradation. In addition to the ANK domain and bipartite nuclear localisation signals (NLS), ANKRD11 also contains twelve minimal (RxxL) destruction box (D-box) motifs, including one full-length consensus D-box (RxxLxxIxN) (Fig 3.18; light blue shade). D-boxes are the hallmark of proteins degraded via the anaphase-promoting complex/cyclosome (APC/C) (Zur & Brandeis, 2002; Bashir et al., 2004b). ANKRD11 also contains multiple PEST sequences (Fig 3.18; blue text), which are peptide signals for proteasomal degradation and usually contained by proteins with short intracellular half-lives (Rogers et al., 1986).

ANKRD11 amino acid sequence (NM_013275)

ANKYRIN REPEAT DOMAIN
PEST SEQUENCE
NUCLEAR LOCALISATION SIGNAL
DESTRUCTION BOX

```
   1 MPKGGCPKAP QQEELPLSSD MVEKQTGKKD KDKVSLTKTP KLERGDGGKE VRERASKRKL
  61 PFTAGANGEQ KDSDTEKQGP ERKRIKKEPV TRKAGLLFGM GLSGIRAGYP LSERQQVALL
 121 MQMTAEESAN SP***VDTTPKHP SQSTVCQKGT PNSASKTKDK VNKRNERGET RLHRAAIRGD***
 181 ***ARRIKELISE GADVNVKDFA GWTALHEACN RGYYDVAKQL LAAGAEVNTK GLDDDTPLHD***
 241 ***AANNGHYKVV KLLLRYGGNP QQSNRKGETP LKVANSPTMV NLLLGKGTYT SSEES***STESS
 301 EEEDAPSFAP SSSVDGNNTD SEFEKGLKHK AKNPEPQKAT APVKDEYEFD EDDEQDRVPP
 361 VDDKHLLKKD YRKETKSNSF ISIPKMEVKS YTKNNTIAPK KASHRILSDT SDEEDASVTV
 421 GTGEKLRLSA HTILPGSKTR EPSNAKQQKE KNKVKKKRKK ETKGREVRFG KRSDKFCSSE
 481 SESESSESGE DDRDSLGSSG CLKGSPLVLK DPSLFSSLSA SSTSSHGSSA AQKQNPSHTD
 541 QHTKHWRTDN WKTISSPAWS EVSSLSDSTR TRLTSESDYS SEGSSVESLK PVRKRQEHRK
 601 RASLSEKKSP FLSSAEGAVP KLDKEGKVVK KHKTKHKHKN KEKGQCSISQ ELKLKSFTYE
 661 YEDSKQKSDK AILLENDLST ENKLKVLKHD RDHFKKEEKL SKMKLEEKEW LFKDEKSLKR
 721 IKDTNKDISR SFREEKDRSN KAEKERSLKE KSPKEEKLRL YKEERKKKSK DRPSKLEKKN
 781 DLKEDKISKE KEKIFKEDKE KLKKEKVYRE DSAFDEYCNK NQFLENEDTK FSLSDDQRDR
 841 WFSDLSDSSF DFKGEDSWDS PVTDYRDMKS DSVAKLILET VKEDSKERRR DSRAREKRDY
 901 REPFFRKKDR DYLDKNSEKR KEQTEKHKSV PGYLSEKDKK RRESAEAGRD RKDALESCKE
 961 RRDGRAKPEE AHREELKECG CESGFKDKSD GDFGKGLEPW ERHHPAREKE KKDGPDKERK
1021 EKTKPERYKE KSSDKDKSEK SILEKCQKDK EFDKCFKEKK DTKEKHKDTH GKDKERKASL
1081 DQGKEKKEKA FPGIISEDFS EKKDDKKGKE KSWYIADIFT DESEDDRDSC MGSGFKMGEA
1141 SDLPRTDGLQ EKEEGREAYA SDRHRKSSDK QHPERQKDKE PRDRRKDRGA ADAGRDKKEK
1201 VFEKHKEKKD KESTEKYKDR KDRASVDSTQ DKKNKQKLPE KAEKKHAAED KAKSKHKEKS
1261 DKEHSKERKS SRSADAEKSL LEKLEEEALH EYREDSNDKI SEVSSDSFTD RGQEPGLTAF
1321 LEVSFTEPPG DDKPRESACL PEKLKEKERH RHSSSSSKKS HDRERAKKEK AEKKEKGEDY
1381 KEGGSRKDSG QYEKDFLEAD AYGVSYNMKA DIEDELDKTI ELFSTEKKDK NDSEREPSKK
1441 IEKELKPYGS SAINILKEKK KREKHREKWR DEKERHRDRH ADGLLRHHRD ELLRHHRDEQ
1501 KPATRDKDSP PRVLKDKSRD EGPRLGDAKL KEKFKDGAEK EKGDPVKMSN GNDKVAPSKD
1561 PGKKDARPRE KLLGDGDLMM TSFERMLSQK DLEIEERHKR HKERMKQMEK LRHRSGDPKL
1621 KEKAKPADDG RKKGLDIPAK KPPGLDPPFK DKKLKESTPI PPAAENKLHP ASGADSKDWL
1681 AGPHMKEVLP ASPRPDQSRP TGVPTPTSVL SCPSYEEVMH TPRTPSCSAD DYADLVFDCA
1741 DSQHSTPVPT APTSACSPSF FDRFSVASSG LSENASQAPA RPLSTNLYRS VSVDIRRTPE
1801 EEFSVGDKLF RQQSVPAASS YDSPMPPSME DRAPLPPVPA EKFACLSPGY YSPDYGLPSP
1861 KVDALHCPPA AVVTVTPSPE GVFSSLQAKP SPSPRAELLV PSLEGALPPD LDTSEDQQAT
1921 AAIIPPEPSY LEPLDEGPFS AVITEEPVEW AHPSEQALAS SLIGGTSENP VSWPVGSDLL
1981 LKSPQRFPES PKRFCPADPL HSAAPGPFSA SEAPYPAPPA SPAPYALPVA EPGLEDVKDG
2041 VDAVPAAIST SEAAPYAPPS GLESFFSNCK SLPEAPLDVA PEPACVAAVA QVEALGPLEN
2101 SFLDGSRGLS HLGQVEPVPW ADAFAGPEDD LDLGPFSLPE LPLQTKDAAD GEAEPVEESL
2161 APPEEMPPGA PGVINGGDVS TVVAEEPPAL PPDQASTRLP AELEPEPSGE PKLDVALEAA
2221 VEAETVPEER ARGDPDSSVE PAPVPPEQRP LGSGDQGAEA EGPPAASLCA PDGPAPNTVA
2281 QAQAADGAGP EDDTEASRAA APAEGPPGGI QPEAAEPKPT AEAPKAPRVE EIPQRMTRNR
2341 AQMLANQSKQ GPPPSEKECA PTPAPVTRAK ARGSEDDDAQ AQHPRKRRFQ RSTQQLQQQL
2401 NTSTQQTREV IQQTLAAIVD AIKLDAIEPY HSDRANPYFE YLQIRKKIEE KRKILCCITP
2461 QAPQCYAEYV TYTGSYLLDG KPLSKLHIPV IAPPPSLAEP LKELFRQQEA VRGKLRLQHS
2521 IEREKLIVSC EQEILRVHCR AARTIANQAV PFSACTMLLD SEVYNMPLES QGDENKSVRD
2581 RFNARQFISW LQDVDDKYDR MKTCLLMRQQ HEAAALNAVQ RMEWQLKVQE LDPAGHKSLC
2641 VNEVPSFYVP MVDVNDDFVL LPA
```

Figure 3.18. ANKRD11 amino acid sequence contains a consensus destruction box and multiple PEST sequences.

In silico analysis of the ANKRD11 amino acid sequence, depicting the ANK domain (bold and italics), nuclear localisation signals (red), PEST sequences (blue) and a carboxyl-terminal consensus destruction box motif (blue shade).

3.3.4 – Discussion

This study provides preliminary data to suggest a role for ANKRD11 as a putative mitotic checkpoint protein. PML bodies were recruited to the periphery of ANKRD11 nuclear foci in an isoform-independent manner. Exogenous ANKRD11 was observed to associate with chromatin-rich regions during pro-metaphase and was rapidly degraded upon metaphase entry, presumably via the ubiquitin-proteasomal pathway. Endogenous ANKRD11 protein was also shown to be cell cycle regulated, following a similar cell cycle profile to that of cyclin B1. Proteasomal inhibition resulted in translocation of ANKRD11 to the nucleolus, suggesting that ANKRD11 is degraded via the ubiquitin-proteolytic pathway.

3.3.4.1 – The dynamic regulation of ANKRD11 during mitotis

Immunofluroescence studies show that ANKRD11 has a dynamic role during the initial stages of mitosis (Fig 3.13). Exogenous ANKRD11 was completely degraded upon progression into metaphase, suggesting that ANKRD11 may function as a cell cycle control mechanism to block or arrest cells prior to cell division until the cell is ready to enter mitosis. This was further supported by the cell cycle profile of endogenous ANKRD11, suggesting that ANKRD11 protein levels reach a maximum during mitosis and is subsequently degraded during mitotic exit (Fig 3.16). A similar mechanism of rapid protein degradation during mitosis has been reported for cyclin B1 and is a prerequisite for mitotic exit (Harper et al., 2002). Cyclin B is degraded by both APC/C^{Cdc20} and APC/C^{Cdh1}, and its degradation during anaphase is required for sister chromatid separation and subsequent division into daughter cells (Harper et al., 2002; Nakayama & Nakayama, 2006). Furthermore, endogenous ANKRD11 protein levels follow a similar oscillating pattern throughout the cell cycle to that of cyclin B1 (Fig 3.16), suggesting that these two proteins are degraded during the same phase of the cell cycle.

Although this study has shown that ANKRD11 associates with chromatin during pro-metaphase, the specific functional role that ANKRD11 exerts during mitotic progression is yet to be determined. It is unlikely that this will be achieved through experiments involving ANKRD11 over-expression, as this study shows that the endogenous intracellular proteasomal machinery is capable of degrading high levels of transiently-expressed ANKRD11 protein during mitosis. Therefore, RNA interference experiments must be used to determine if ablation of endogenous ANKRD11 expression perturbed the normal execution of mitosis. Similar studies have been previously described for various checkpoint proteins, including ATM (Zhang et al., 1998), p53 (Arora & Iversen, 2000), Chk1, Wee1 and Myt1 (Wang et al., 2004).

These studies are yet to be performed in multiple cell lines using alternative synchronization methods to ensure that these findings are not an artifactual occurance in the MCF-7 cell line. Furthermore, it would be ideal to monitor the cell cycle regulation of GFP-ANKRD11 through live-cell imaging of stably-expressing cell isolates. These studies will conclusively determine the cell cycle profile of ANKRD11 and provide valuable insight into the complex function and dynamic regulation of ANKRD11 during mitosis.

3.3.4.2 – Association of ANKRD11 with chromatin-rich regions

3.3.4.2.1 – Is ANKRD11 recruited to chromatin via interaction with nucleosomes?

Findings from this study indicate that ANKRD11 is associated within chromatin-rich regions. Of particular interest was the observation that exogenous histone H2A expression disrupted the typical focal nuclear localisation pattern of ANKRD11 (Fig 3.15), suggesting that both histone H2A and ANKRD11 exist in a subcellular protein complex. Histones comprise the major protein portion of chromatin, and form nucleosome cores through the interaction of two

H2A-H2B dimers with a H3-H4 tetramer (Luger et al., 1997; Bernstein & Hake, 2006). Therefore, we hypothesize that ANKRD11 is recruited to chromatin through its interaction with nucleosomal histones.

During mitosis, the activity of HATs and HDACs are down-regulated and general hypoacetylation of histones takes place, leading to the displacement of transcription factors from the mitotic chromosomes (Nishiyama et al., 2006). Since ANKRD11 has been previously been reported to recruit HDACs (Zhang et al., 2004) and presumably HATs (Fig 3.5*C*), it is possible that the rapid degradation of ANKRD11 prior to metaphase entry is a cellular process designed to prevent chromatin remodelling and histone modification during cell division. Further studies regarding the role of ANKRD11 during mitosis are certainly warranted, and may provide insight into the complex mechanisms that initiate and regulate the progression of a eukaryotic cell through mitosis.

3.3.4.2.2 – ANKRD11-histone association: direct or indirect?

It is possible that the above mentioned association between ANKRD11 and histone H2A is not direct. ANKRD11 was previously reported to possess both intrinsic activator and repressor domains, functioning as a negative regulator of liganded nuclear receptor transcriptional activity through the recruitment of HDACs by the carboxyl-terminal RD2 domain (Zhang et al., 2004; Zhang et al., 2007a). It is possible that the observed localisation between ANKRD11 and histone H2A is mediated through the HDAC contingent of ANKRD11, rather than ANKRD itself. Histone H2A is frequently associated with heterochromatin, representing regions of tightly-packed DNA where transcription is limited (Dillon, 2004). Heterochromatin regions stain darkly in the presence of propidium iodide and are typically located at the periphery of the nucleus (as observed in Fig 3.13). Taken together, one cannot rule out the possibility that the observed localisation of ANKRD11 with histone

H2A is an artifactual resultant of its ability to recruit HATs and HDACs for its function as a nuclear receptor corepressor via chromatin remodelling and histone modification.

3.3.4.3 – Proteasomal-mediated degradation of ANKRD11

To provide preliminary evidence that ANKRD11 is degraded via the ubiquitin-proteasomal pathway, the localisation of exogenous ANKRD11 was investigated following treatment with the proteasomal inhibitor, MG132 (Fig 3.17). Proteasomes accumulate in euchromatic regions of the nuclei and avoid peripheral and perinucleolar regions rich in heterochromatin (Mattsson et al., 2001). Therefore, nucleolar translocation is a common mechanism used by proteins to evade the proteasome in the presence of MG132. Nucleolar translocation in response to proteasomal inhibition has been reported for several other proteins, including PML bodies (PML, SUMO-1 and Sp100 - Mattsson et al., 2001), p53 (Klibanov et al., 2001), c-myc (Arabi et al., 2003) and EBNA-5 (Pokrovskaja et al., 2001). Intriguingly, several of these proteins have been shown to co-localise with ANKRD11 in untreated cells (Figs 3.1*C,* 3.1*E,* 3.11), suggesting that these proteins translocate to the nucleolus in response to proteasomal inhibition through a common mechanism.

It was recently shown that PRIMA-1, a low molecular weight compound that restores the transcriptional and apoptotic activity of mutant $p53^{R175H}$, a frequently observed cancer-assocaited mutation (Rokaeus et al., 2007). This was achieved through PRIMA-1-mediated nucleolar translocation of mutant $p53^{R175H}$, along with p53-regulatory proteins PML, CBP and Hsp70. PRIMA-Dead, a compound structurally related to PRIMA-1 but unable to induce mutant $p53^{R175H}$-dependent apoptosis, failed to induce nucleolar translocation of mutant $p53^{R175H}$. Taken together, it is possible that redistribution of mutant $p53^{R175H}$ to nucleoli plays a role in the restoration of function for this p53 DNA-contact mutant. Since ANKRD11 can restore the transactivation potential of the $p53^{R273H}$ mutant, and both p53 and ANKRD11

translocate to nucleoli upon MG132 treatment, it would be tempting to investigate if ANKRD11 restores mutant p53 function through a similar localisation-dependent manner as PRIMA-1.

3.4 – Investigation into a role for ANKRD11 in the modulation of estrogen receptor activity

3.4.1 – Prelude

The estrogen receptor (ER) is expressed in approximately 70% of all human breast cancer and, in the presence of estrogen, promotes the development and propagation of the tumour (see Section 1.2.1). Intriguingly, ANKRD11 was reported to repress the transcriptional activity of several steroid nuclear receptors, including progesterone (PR), androgen (AR), mineralocorticoid (MR) and glucocorticoid (GR) receptors (Zhang et al., 2004). However, the effects of ANKRD11 on the transcriptional activity of ER are yet to be determined. The aim of this section is to investigate if ANKRD11 also functions as a co-repressor of ER, and to determine if this process is associated with ANKRD11-mediated suppression of breast tumorigenesis.

3.4.2 – Introduction

3.4.2.1 – Steroid nuclear receptors and transcriptional regulation

Steroid nuclear receptors are key regulators of growth, differentiation, development and metabolism in a wide range of tissues. They belong to superfamily of ligand-dependent transcriptional activators that regulate gene expression in response to various steroid hormones. Steroid nuclear receptors have a highly-conserved DNA-binding domain (DBD) that recognizes sequence specific response elements in the promoter regions of target genes. In contrast to other families of transcription factors, the steroid nuclear receptor family possess a carboxyl-terminal ligand binding domain (LBD) (Edwards, 2000). The LBD functions as a molecular switch, activating the transcription of the target gene following the conformational change in the steroid nuclear receptor in the presence of its hormonal ligand.

The steroid nuclear receptor family members include the estrogen receptors (ERα and ERβ), progesterone receptors (PR-A and PR-B), androgen receptor (AR), glucocorticoid receptor (GR) and minerlocorticoid receptor (MR) (Robyr et al., 2000).

3.4.2.2 – The p160 family of steroid recetor coactivators

Upon binding of the hormonal ligand to the LBD, these steroid nuclear receptors recruit the p160 steroid receptor coactivator (SRC) protein complex to drive the transcription of target genes (Edwards, 2000). Three homologous members of the SRC family have been identified in humans; SRC-1 (NCoA-1), SRC-2 (GRIP-1, TIF-2 or NCoA-2) and SRC-3 (p/CIP, RAC3, ACTR, AIB1 or TRAM-1) (Xu & Li, 2003).

3.4.2.2.1 – Structural and function domains of the SRC family – the bHLH-PAS domain

The p160 coactivator family is highly conserved with an overall sequence homology of 43 to 48% among the three classes of SRCs. The SRC family of coactivators also share a common domain structure, with the most highly conserved region containing the amino-terminal bHLH (basic helix-loop-helix) and PAS A/B (Per-Arnt-Sim) domains (Xu & Li, 2003). Previous studies have shown that bHLH is a DNA binding motif present in several eukaryotic transcription factors (Murre et al., 1989), and the PAS domain has been reported to be involved in protein dimerization (Huang et al., 1993). Despite the high degree of sequence homology within this domain, it is neither required for p160 interaction with steroid nuclear receptors nor does it contribute towards the coactivator function of the protein, suggesting that this region has a role that is yet to be appreciated (Edwards, 2000).

3.4.2.2.2 – Structural and function domains of the SRC family – the LXXLL motif

The SRC family members directly interact with steroid nuclear receptors in a ligand-dependent fashion via their receptor interaction domain. This receptor interaction domain

contains three highly conserved LXXLL motifs, termed nuclear receptor boxes (Heery et al., 1997). Studies involving peptide competition, or site-directed mutatgenesis of these conserved lysine residues, provide strong evidence to suggest that this motif mediates SRC-binding to the liganded nuclear receptor complex (Heery et al., 1997; Torchia et al., 1997; Ding et al., 1998).

3.4.2.2.3 – Structural and function domains of the SRC family – the AD domain

Two autonomous transcriptional activator domains (AD1 and AD2) are located within the carboxyl-terminus of all SRC family members. Interestingly, AD1 also contains three additional nuclear receptor boxes that are involved in the recruitment of the p300/CBP and P/CAF histone acetyltransferases (HATs) (McInerney et al., 1998; Voegel et al., 1998). It has been comprehensively demonstrated that histone hyperacetylation results in transcriptional activation through unpacking of the condensed chromatin structure, thereby facilitating access of transcription factors to the promoters of target genes (Chan & La Thangue, 2001). Recruitment of p300/CBP to the AD1 was shown to be required for SRC-mediated coactivation of steroid nuclear receptors (McInerney et al., 1998).

A protein termed coactivator associated arginine methyltransferase (CARM1) has been shown to be recruited by the AD2 of all three classes of SRCs. CARM1 can methylate histone H3, resulting in transcriptional activation through a chromatin-remodelling mechanism similar to that of histone hyperacetylation (Chen et al., 1999). SRC-1 and SRC-3 have also been shown to possess intrinsic transcriptional activation function within their carboxyl terminus, however this intrinsic HAT activity is much weaker than that imposed by p300/CBP or P/CAF and therefore does not contribute to SRC coactivation (Leo & Chen, 2000; Xu & Li, 2003).

3.4.2.3 – AIB1 and breast cancer

Several studies have suggested a specific role for AIB1 (Amplified in Breast Cancer 1; SRC-3 family member) in mammary gland growth and development. AIB1 was shown to be highly expressed in mammary gland epithelial cells, oocytes, vaginal epithelial layer, hepatocytes, and in the smooth muscle cells of many tissues such as blood vessels, intestines, and oviducts (Xu et al., 2000; Yuan et al., 2002). Severe abnormalities were observed in the development and functionality of reproductive system in AIB1$^{-/-}$ female mice (Xu et al., 2000). These mice exhibited a significantly decreased development of mammary gland alveolar following combined stimulation by estrogen and progesterone. Taken together, these observations suggest that AIB1 has a major physiological role as a coactivator for ER and PR-mediated transcription.

Since SRC-3 was shown to specifically enhance ER and PR, it was suggested that this coactivator may play a critical role in hormonal-dependent malignancies such as breast cancer. Indeed, AIB1 mRNA was over-expressed in 31-64% of breast tumours (Anzick et al., 1997; Bouras et al., 2001). In breast cancer cells, AIB1 was shown to be recruited to the estrogen-responsive *CCND1* promoter to enhance cyclin D1 expression (Planas-Silva et al., 2001). Furthermore, mammary gland tumorigenesis induced by the *MMTV-v-HA-RAS* transgene was significantly suppressed in AIB1$^{-/-}$ mice, suggesting that this coactivator may have a permissive role in breast oncogenesis (Liao et al., 2002). These findings indicate that the selective inhibition of AIB1 function in the mammary gland may prove to be a useful approach for the prevention and treatment of breast cancer (Xu & Li, 2003).

3.4.2.4 – ANKRD11: a novel antagonist of the p160 coactivators

ANKRD11 (also termed ANCO-1; **An**kyrin Repeats Containing **Co**factor-**1**) was recently identified as a novel AIB1-interacting protein (Zhang et al., 2004). ANKRD11 was reported

to recruit HDACs to the AIB1-nuclear receptor complex, functioning as a negative regulator of ligand-dependent transcriptional activity of several steroid nuclear receptors, including PR, AR, MR and GR. ANKRD11 was shown to inhibit SRC-2 coactivation of PR-mediated transcription, suggesting that ANKRD11 may function as an antagonist for the p160 coactivator family (Zhang et al., 2004).

Surprisingly, this study did not determine the effects of ANKRD11 on the transcriptional activity of ER. Over half of all breast tumours have abnormally high expression of ER, and negative regulators of ER activity have proven to be extremely useful treatments in the fight against breast cancer. The existing findings warrant further investigation into the regulation of ER transcriptional activity by ANKRD11. Furthermore, the antagonistic effects of ANKRD11 on the AIB1-ER complex may provide the foundation for the development of future breast cancer therapeutics.

3.4.3 – Materials and Methods

3.4.3.1 – Cell lines and antibodies

Cell lines used throughout this section are as described (General Materials and Methods; Section 2.1). Commercial antibodies used are as described in Table 2.1.

3.4.3.2 – Plasmids

The generation or source of constructs used throughout this section are described in Table 2.3. Constructs were generated using standard procedures (Ausubel, 2001), with the sequences of all constructs confirmed by DNA sequencing (General Materials and Methods; Section 2.7).

3.4.3.3 – Estrogen receptor transactivation assays

HEK293T, HeLa or MCF-7 cells (2×10^5) were seeded in 24-well plates in steroid-free media (Phenol Red-free media with 10% charcoal-stripped FCS) and transfected with 100 ng of the ERE-TK-Luc reporter construct (see Table 2.3) together with varying amounts of ERα and ANKRD11-myc constructs and 25 ng of pRL-TK plasmid as a transfection control. Varying amounts of empty vector was added to compensate to give a total quantity of transfect DNA in each treatment of 800 ng. Four hours post-transfection, cells were treated with 17β-estradiol (1 nM) for 20 hours. Dual-reporter luciferase assays were performed as described previously (Section 3.1.3.9). Briefly, cells were harvested, lysed and assayed using the dual-luciferase reporter assay system (Promega). Firefly luciferase values were normalised to Renilla luciferase activity and expressed as relative luciferase units ± SEM of duplicates.

3.4.3.4 – Co-immunoprecipitation assays

Co-immunoprecipitation of HA-ERBP with either myc-ANKRD11 or ANKRD11$^{1\text{-}451aa}$-myc was performed in HEK293T cells as described (Section 4.3.10).

3.4.4 – Results

3.4.4.1 – ANKRD11 enhances estrogen receptor-mediated transcriptional activity

As ANKRD11 was previously shown to function as a corepressor for AR, PR, MR and GR-mediated transactivation (Zhang et al., 2004), it was determined if ANKRD11 also modulates the activity of ERα. Initially, ERα-transactivation assays were optimised using a reporter construct containing 3 tandem EREs cloned upstream from the luciferase gene driven by a thymidine kinase (TK) promoter. Results from a dose-response curve (0, 0.1, 1 or 10 nM) of estradiol in the HEK293T cell line shows that an intermediate transactivation potential was observed following treatment with 1 nM estradiol and 100 ng of exogenous ERα (Fig 3.19). Similar results were obtained in HeLa cells and therefore these standardised conditions were used in subsequent ERα transactivation assays.

Results from ERα transactivation assays showed that transiently-expressed ANKRD11 in HeLa cells led to a significant (12.7 fold; $p < 0.01$) dose-dependent increase in ligand-dependent ERα-mediated transcriptional activity (Fig 3.20). However, a minimal effect (3.2 fold increase; $p < 0.01$) was observed in the absence of estradiol, suggesting a partial ligand-independent enhancement of ERα activity by ANKRD11.

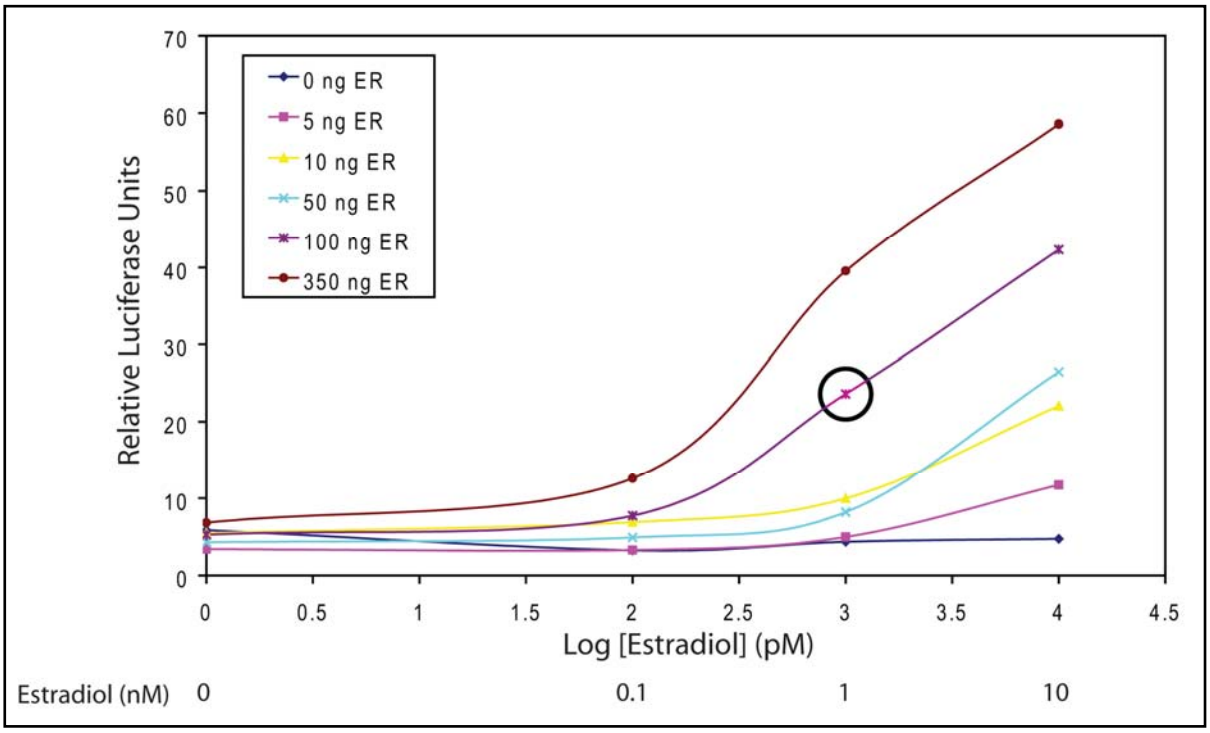


Figure 3.19. Optimisation of ERα and estradiol treatment for ERα transactivation assays

HEK293T cells were co-transfected with a reporter constructs encoding 3 tandem EREs, together with pRL-TK and increasing amounts of ERα as indicated. Empty pLNCX2 vector was added to equalize the total amounts of plasmid used in various treatments. Cells were either untreated or treated with 0, 0.1, 1 or 10 nM estradiol as indicated. Dose-response curves of ERα vs estradiol were plotted, and the optimal concentrations of ERα and estradiol were subsequently identified at the mid-point of these curves. Data are represented as mean ± SEM of duplicates.

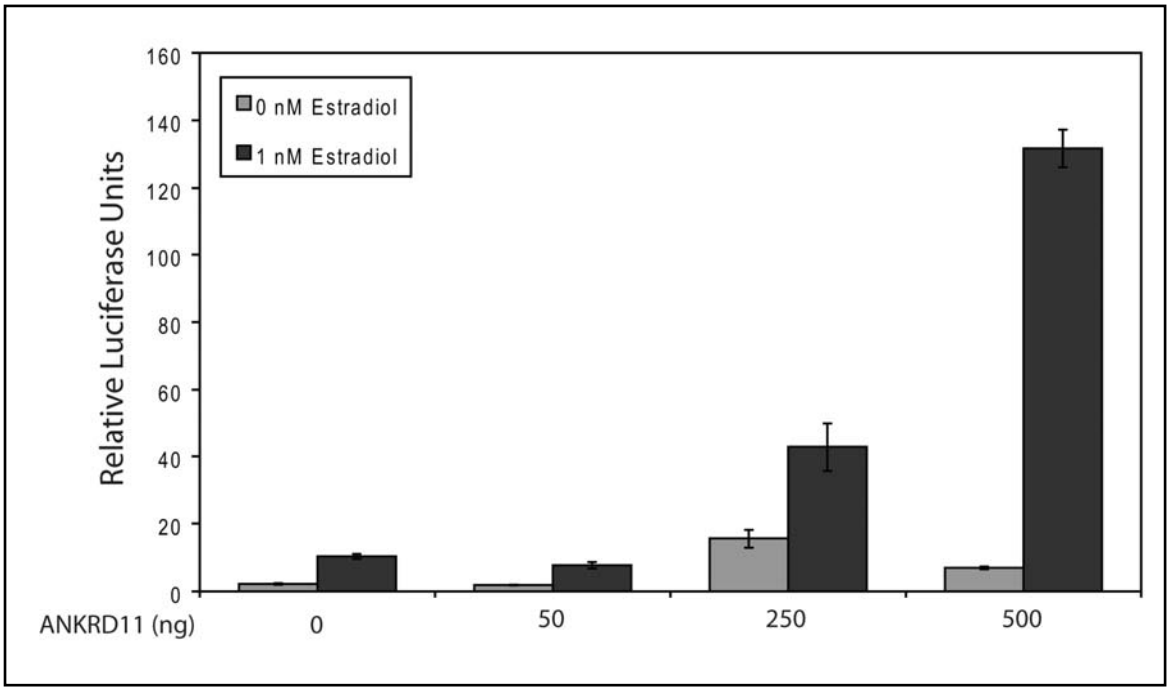


Figure 3.20. ANKRD11 expression enhances ERα-mediated transcriptional activity.

HeLa cells were co-transfected with a reporter constructs encoding 3 tandem EREs, together with ERα (100 ng), pRL-TK and increasing amounts of ANKRD11-myc as indicated. Empty pLNCX2 vector was added to equalize the total amounts of plasmid used in various treatments. Cells were either untreated or treated with estradiol (1 nM). Data are represented as mean ± SEM of duplicates.

To further investigate a role for ANKRD11 as an activator of ER-mediated transactivation, the effect of ANKRD11 expression on endogenous ERα activity in the ER-positive MCF-7 breast cancer cell line was determined. Ectopic-expression of GFP-ANKRD11 in MCF-7 cells significantly enhanced the ligand-dependent transcriptional activity of ERα (2.3 fold; $p < 0.01$) (Fig 3.21). Consistently, ectopic ANKRD11 expression in MCF-7 cells was also associated with a concordant increase in the ligand-independent activity of ERα (1.7 fold; $p < 0.17$). Taken together, these findings suggest that ANKRD11 can enhance the transcriptional activity of ERα using both a ligand-dependent and independent mechanism.

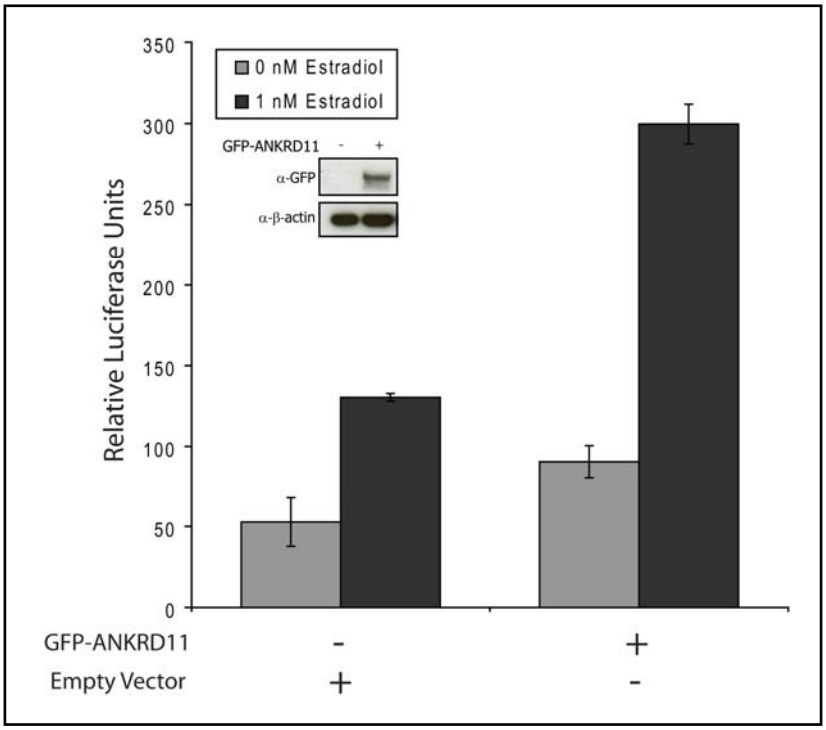


Figure 3.21. ANKRD11 expression enhances the activity of endogenous ERα.

MCF-7 cells were transduced with retroviral particles derived from either pLNCX2-GFP-ANKRD11 or pLNCX2 vector and selected in geneticin for two weeks. Expression of GFP-ANKRD11 was determined by Western blot analysis (Insert). Cells were then co-transfected with a reporter construct encoding three tandem EREs, pRL-TK and either untreated or treated with estradiol (1 nM). Data are represented as mean ± SEM of duplicates.

3.4.4.2 – ANKRD11 interacts with ERBP in vivo via the amino-terminal ankyrin region

To gain further mechanistic insight into the role of ANKRD11 as a positive regulator of ERα activity, we endeavoured to identify other known ER-regulatory proteins that may be recruited by ANKRD11. ERBP, an ERα coactivator, was previously identified from a yeast two-hybrid screen as an ANKRD11-interacting protein (Powell, 2003). Co-immunoprecipitation experiments were performed to confirm the existence of an ANKRD11-ERBP complex *in vivo.* ERBP-HA was detected in protein complexes following anti-myc immunoprecipitation of cell lysates from HEK293T cells transiently co-expressing myc-ANKRD11 and HA-ERBP (Fig 3.22*A*). These experiments were subsequently repeated using the amino-terminus of ANKRD11 (1-451aa), since this was the region used as bait in the yeast two-hybrid screen. Consistently, ERBP-HA was detected in a protein complex with ANKRD11$^{1\text{-}451aa}$-myc, confirming this interaction *in vivo* (Fig 3.22*B*). These findings validate the previously reported identification of ERBP as a putative ANKRD11-binding protein and present evidence to suggest that ANKRD11 may participate with ERBP to facilitate the positive regulation of ERα activity.

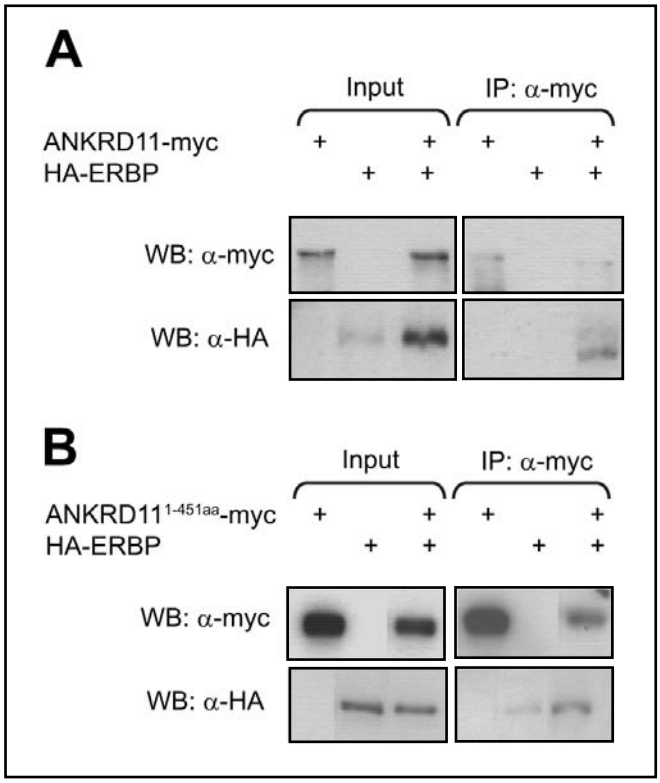


Figure 3.22. ANKRD11 interacts with ERBP *in vivo*.

A: ANKRD11 co-immunoprecipitates with ERBP. HEK293T cells were transiently-transfected with constructs expressing HA-ERBP and/or myc-ANKRD11 as indicated. Protein complexes from total cell lysates were immunoprecipitated using α-myc antibody coated sepharose beads. Inputs (lanes 1-3) or immunoprecipitates (lanes 4-6) were Western blotted using α-myc and α-HA antibodies.

B: ERBP interacts with the amino-terminal region of ANKRD11. HEK293T cells were transiently-transfected with constructs encoding HA-ERBP and/or myc-ANKRD11$^{1-451aa}$ protein (region used as bait in yeast two-hybrid screen). Protein complexes were immunoprecipitated using the appropriate antibodies and Western blotted as described in (*A*).

3.4.5 – Discussion

In this study, we have provided evidence to suggest a role for ANKRD11 as a positive modulator of ERα transcriptional activity. It is possible that this coactivator functionality of ANKRD11 is mediated through its association with ERBP via its amino-terminal ankyrin region.

Results from dual luciferase assays show that ANKRD11 enhances the transcriptional activity of ERα. These findings are in contrast with previous reports characterizing ANKRD11 as a corepressor for AR, PR, MR and GR-mediated transactivation (Zhang et al., 2004; Zhang et al., 2007a; Zhang et al., 2007b). These findings suggest the existence of a rather enigmatic modulation of nuclear receptors by ANKRD11, functioning in a receptor-specific context as either a coactivator or corepressor. This dual-functionality of nuclear receptor modulation is reflected by the multiple transcriptional regulatory domains in the ANKRD11 protein. ANKRD11 was shown to possess both intrinsic repressor and activation domains (Zhang et al., 2007a). ANKRD11 has also been shown to interact with both negative, HDACs (Zhang et al., 2004) or positive, p160 family (Zhang et al., 2004), P/CAF (Fig 3.5*D*) and ERBP (Fig 3.22) regulators of nuclear receptor transcriptional activity (as summarised in Fig 3.23). Together, these results indicate that transcriptional potential of ANKRD11-mediated nuclear receptor modulation occurs through a complex process involving mechanisms that drive either transcriptional activation or repression.

This hypothesis describes ANKRD11 as a dual-functional co-regulator of nuclear receptor activity, however, some fundamental aspects are yet to be addressed. To date, studies performed by our laboratory (Chapter 3.4) and others (Zhang et al., 2004; Zhang et al., 2007a; Zhang et al., 2007b) involve the use of transient nuclear receptor transactivation assays to investigate the transcriptional modulatory effects imparted by ANKRD11. Although the

ligands used throughout these experiments are administered at physiologically-relevant concentrations (e.g. 1 nM estradiol), the findings from these artificial reporter assays are yet to be confirmed by the use of endogenous nuclear receptor target genes. In addition, the underlying mechanisms involved in the transcriptional modulation of nuclear receptors by ANKRD11 are yet to be clearly defined. As ANKRD11 does not directly intact with nuclear receptors, it is possible that ANKRD11 could modulate nuclear receptor activity by either the direct recruitment of regulatory proteins to the genomic nuclear receptor response elements or by the sequestration of these regulatory proteins away from nuclear receptor transcriptional complexes.

Co-immunoprecipitation data suggests that ANKRD11 interacts with ERBP via the amino-terminal 451aa region. This region contains the ANK domain, a known protein-protein interaction domain, and therefore it is logical to suggest that the ANK domain of ANKRD11 is responsible for its interaction with ERBP. *In vitro* binding assays have shown that ERBP directly associates with ERα (Bu et al., 2004). However, ERBP itself was not reported to possess intrinsic transcriptional activity, suggesting that other transcriptional modulators are required to mediate its function as an ERα coactivator. Our findings provide evidence to suggest that ANKRD11 is one such coactivator recruited by ERBP to modulate the transcriptional potential of ERα. This has similarities to the model proposed for ANKRD11-mediated co-repression of nuclear receptor activity, where ANKRD11 is recruited to the nuclear receptor transcriptional complex by the p160 family (Zhang et al., 2004).

A recent report suggests that the function of ANKRD11 as a co-repressor of nuclear receptor activity is required for normal bone development and homeostasis (Barbaric et al., 2007). Mice heterozygous for an *N*-ethyl-*N*-nitrosourea (ENU)-induced mutation in the *ANKRD11* gene (encoding a ANKRD11^{E2604K} mutant protein) had a phenotype of abnormal cranial

morphology, reduced body size and reduced mineral density. This mutation was mapped to the RD2 domain of ANKRD11 within the minimal region required for HDAC-interaction (Fig 3.23). It is possible that this mutation affects the ability of ANKRD11 to inhibit hormone-receptor activity by perturbing the recruitment of HDACs to the transcriptional complex. Pertubations in the normal functioning of hormone receptors have been previously implicated in the pathogenesis of bone loss, leading to osteoporosis (Gennari et al., 2002). Embryonic lethality also occurred in mice that are homozygous for this mutation, suggesting that ANKRD11 is involved in basic processes critical for the normal function of the cellular development (Barbaric et al., 2007).

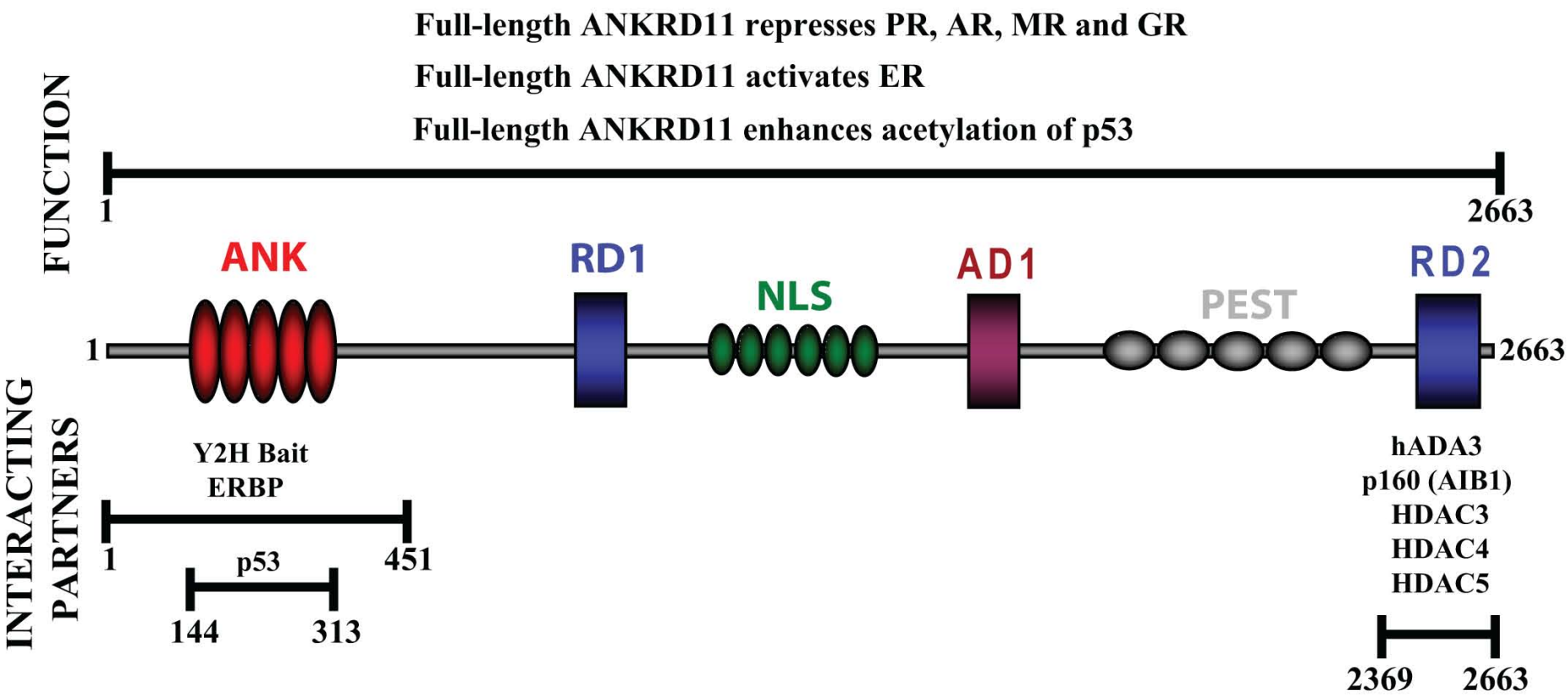


Figure 3.23. The ANKRD11 protein: location of protein motifs, interacting partners and known functions.

A collective schematic diagram indicating the various domains, functional regions and interacting partners (as determined by *in vitro* or *in vivo* binding assays) of ANKRD11 as identified from both our laboratory (Chapter 3) and others (Zhang et al., 2004; Zhang et al., 2007a). AD: activation domain, RD: repressor domain, ANK: ankyrin domain.

Chapter 4: FBXO31 is the Chromosome 16q24.3 Senescence Gene, a Candidate Breast Tumour Suppressor, and a Component of an SCF Complex

4.1 - Prelude

FBXO31 was initially identified as a candidate breast cancer tumour suppressor within the 16q24.3 breast cancer LOH region (Powell et al., 2002 - see Section 1.3.1.2). The subsequent functional characterisation of FBXO31 was published (Kumar et al., 2005) and is presented in Chapter 4.

4.2 – Introduction

Previous studies showed that the microcell-mediated transfer of human chromosome 16q fragments caused senescence in human- and mouse-immortalized cell lines (Reddy et al., 1999). This senescence-associated region was subsequently localised to a 360-kb yeast artificial chromosome (YAC) d792t2 mapping between the markers D16S498 and D16S476 (Reddy et al., 2000) and more recently to an 85-kb BAC clone 346J21 (Kaur et al., 2005). Transfer of this BAC into several cell lines, including the human breast cancer line MCF-7, was shown to restore cellular senescence. These findings are consistent with the location of a senescence gene within the BAC 346J21, and this gene may also function as a tumour suppressor gene. Physical mapping of the two previously identified potential tumour suppressor genes located *FBXO31* within the YAC d792t2, whereas *CBFA2T3* is 1.7 Mb distal to this YAC (Callen et al, unpublished data).

Herein, we report mapping of the BAC 346J21 with respect to the *FBXO31* gene and investigate if the properties of *FBXO31* are consistent with a senescence and tumour

suppressor gene. In addition, because *FBXO31* encodes an F-box protein and other proteins with F-box domains have been shown to be variant components of SCF complexes (Kipreos & Pagano, 2000), we have investigated whether FBXO31 mediates its function as part of an SCF^{FBXO31} E3 ubiquitin ligase complex.

4.3 – Materials and Methods

4.3.1 - Cell lines and antibodies

Cell lines used throughout this section are as described (General Materials and Methods; Section 2.1). Stable cell lines expressing FLAG-HA-FBXO31 were generated by geneticin selection of 293T cells transduced with a pQCXIN-based retroviral construct. Commercial antibodies used are described in Table 2.1. A rabbit polyclonal antibody was raised against a 15-amino-acid synthetic peptide representing the COOH terminus (amino acids 525-539; QAFDEMLKNIQSLTS) of the FBXO31 protein conjugated to keyhole limpet hemocyanin. The polyclonal antibody was then affinity purified on Sepharose beads coupled with the same peptide.

4.3.2 – Plasmids

The generation or source of constructs used throughout this section are described in Table 2.3. Constructs were generated using standard procedures (Ausubel, 2001), with the sequences of all constructs confirmed by DNA sequencing (General Materials and Methods; Section 2.7).

4.3.3 – Human tissues

Primary breast tumours with known histology, stage, and differentiation grade had 16q LOH status determined as reported previously (Cleton-Jansen et al., 2001). Patient material was obtained on approval of local medical ethics committees.

4.3.4 – Northern blot

Multiple Tissue Northern Blot (BD Biosciences) carrying polyadenylated RNA from various human tissues was probed with a ^{32}P-labeled *FBXO31* DNA fragment. The 224-bp DNA probe was generated by PCR amplification from the full-length clone using the above

mentioned primers (Table 2.2) and radiolabelled with α-^{32}P-dCTP using the Megaprime DNA labelling system (Amersham Biosciences).

4.3.5 – Real-time reverse transcription-PCR

Reverse transcription and real-time PCR analysis were performed as described (General Materials and Methods; Section 2.4). The geometric mean expression of three housekeeping genes (*HNRPM, CPSF6,* and *TBP*) was used to normalize the expression of *FBXO31* in breast tumour cDNA according to published methods (Vandesompele et al., 2002).

4.3.6 – Cell-based assays

To generate amphotropic recombinant retroviruses, GP2-293 cells were transfected with pVSV-G (BD Biosciences) and various gene constructs cloned into the pLNCX2 vector using Lipofectamine 2000 transfection agent (Invitrogen) (General Materials and Methods; Section 2.1). Forty-eight hours after transfection, the culture medium containing the retrovirus was collected, filtered, and used to transduce the appropriate cell line at ~50% confluence. Transduction was enhanced by adding 8 µg/mL polybrene and centrifuging plates at 1,250 rpm for 30 minutes. To determine the frequency of colony formation, after 24 hours, the cells from each treatment were plated in six-well plates at concentrations of 4 to 10 $\times$ 10^3 cells/mL in selective media containing geneticin, and colonies were counted in triplicate wells after growth for a further 2 to 3 weeks. The empty vector was used as a negative control. The transduction efficiency was monitored by immunofluorescence detection of the myc-tag. Cellular senescence was assayed using a kit from Cell Signaling Technologies based on senescence-specific acidic ß-galactosidase activity (Dimri et al., 1995). Senescent cells stained intense blue after overnight incubation at 37°C. Colonies of >30 cells containing >10 blue staining cells were scored as senescent. Proliferation assays were determined on transduced cells selected for 10 to 14 days in the presence of geneticin. These cells were

plated at 10% to 20% confluence in 96-well plates and at various times samples (n = 6-8) assayed by incubating cells for 2 hours with the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega) and measuring absorbance at 490 nm.

4.3.7 – Cell synchronization

Cell culture synchronisation using a double thymidine block and release was performed as described (General Materials and Methods; Section 2.9).

4.3.8 – Cell cycle analysis

MCF-7 or MDA-MB-468 cells were transfected with pEGFP-FBXO31, pEGFP-FBXO31ΔF, pEGFP-p53, or pEGFP-C1 using Lipofectamine 2000 (Invitrogen). Cells were collected 24 and 48 hours after transfection and treated as described (Schmid & Sakamoto, 2003). Briefly, cells were pelleted (300 × g for 5 minutes at 4°C), washed twice with cold PBS, resuspended in 500 µL of cold PBS, and then fixed for 1 hour at 4°C by adding 500 µL fixation solution (2% w/v paraformaldehyde in PBS, pH 7.2). The fixed cells were pelleted, washed with cold PBS, resuspended in 1 mL of 70% ethanol added dropwise to the pellet while vortexing, and then incubated overnight at 4°C. The next day, the cells were pelleted and resuspended in 1 mL propidium iodide solution (40 µg/mL with 100 µg/mL RNase A) for 30 minutes at 37°C in the dark and analyzed on a FACScan flow cytometer (BD Biosciences), and the multivariate data were collected using CellQuest software (BDIS). Cell cycle analysis of DNA histograms was done using ModFit LT V2.0 (Verity) software.

4.3.9 – Western blot analysis

Western blot analysis was performed as described (General Materials and Methods; Section 2.5).

4.3.10 – Coimmunoprecipitation and affinity purification

Plasmids (2 μg each) expressing either myc-FBXO31 or HA-Skp1 or both were transfected into 5×10^5 HEK293T cells in 35-mm wells. Twenty-four hours after transfection, cells were harvested and lysed as for Western blot analysis. Clarified lysates were incubated with either anti-myc or anti-HA-conjugated agarose beads (Sigma-Aldrich). Beads were washed twice with 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8), then 20 mM Tris-HCl (pH 7.5), and the protein complexes were eluted with 1× protein-loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol) (Laemmli, 1970). Input and immunoprecipitated proteins were resolved by Western blot analysis, and membranes were immunoblotted with rat anti-HA, mouse anti-myc, mouse anti-Cullin-1, and rabbit anti-Roc-1 antibodies. HEK293T and HEK293T cell lines stably expressing FLAG-HA-FBXO31 protein were used to affinity purify the tagged FBXO31. Cells were lysed in 20 mM Tris-HCl (pH 8), 200 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% Tween 20, 0.2 mM phenylmethylsulfonyl fluoride, 1× protease inhibitors (Roche) on ice for 15 minutes. Clarified lysates were incubated for 2 hours with anti-FLAG M2 agarose (Sigma-Aldich). The beads were then washed with the lysis buffer, and the protein complexes were eluted with the same buffer containing 250 μg/mL FLAG peptide (Roche). The inputs and eluates were analyzed by Western blotting with rat anti-HA, rabbit anti-Roc-1, mouse anti-Cullin-1, and mouse anti-Skp1 antibodies.

4.3.11 – Statement of work

The findings presented in Chapter 4 have been published in the journal *Cancer Reseach* (Cancer Res 2005; 65(24): 11304-13). Some data is presented that was generated by other researchers, but is presented in its entirety throughout this chapter to maintain a holistic view of the project. Contributions were as follows: Assoc Prof David Callen, retroviral-mediated gene transfer and cell-based assays; Dr Raman Sharma, generation of the α-FBXO31

polyclonal antibody and immunoprecipitation experiments; Jo Crawford, northern blot analysis; Dr Anne-Marie Cleton-Jansen, expression of FBXO31 in tumours.

4.4 – Results

4.4.1 – Analysis of 16q24.3 senescence region

The relative positions of the *FBXO31* gene together with the YAC d792t2, the BAC 346J21, and the various sequence-tagged sites (STS) used previously to define the location of the BAC (Kaur et al., 2005) are given in Fig 4.1*A*. The sequenced extremities of the BAC 346J21 are defined from the available end sequences with accession nos. B15982 and B15983 available at the National Center for Biotechnology Information (NCBI). In BAC 346J21, the marker D16S3063 was reported to be distal to D16S3048 as determined from the mapping of STSs and overlapping BAC clones (Kaur et al., 2005). However, the order of markers as defined by the NCBI genomic sequence places D16S3063 proximal to D16S3048 and some 14 kb proximal to the STS 344A17.T7. The STS 346J21.T7, together with WI15838, WI12410, and the 3' end of *FBXO31* were all located within 200 bp.

The YAC d792t2 was purchased and shown by PCR with STS primers derived from the cDNA sequence BX119888 to possess an interstitial deletion extending at least 1.2 kb (Fig 4.1*A*). These results were confirmed by multiplex PCR with additional STSs present on the YAC (data not shown). The YAC d792t2 is a chimera because it also possesses a segment of chromosome 11q13 (Reddy et al., 2000).

The BAC 346J21 only contains 290 bp of the 3' end of *FBXO31* with the majority of the gene positioned telomeric to this BAC. The genomic region encompassed by the BAC does not contain any predicted CpG islands and contains 44% low copy repeats as determined by RepeatMasker. The NCBI build 35.1 of the human genome sequence predicts three possible transcripts completely contained within the genomic region of the BAC 346J21 (as defined by the location of the end sequences B15982 and B15983 from chromosome 16 base 85,835,220

to 85,920,731): *LOC388305*, *LOC400552*, and *LOC440391*. Based on the absence of introns for *LOC400552* and *LOC440391*, the lack of significant representation of any of the three transcripts in expressed sequence tag (EST) libraries, and the absence of any motifs or species conservation of the predicted proteins, these are unlikely to represent functionally transcribed genes. Because well-defined genes within the confines of this BAC were absent, it was decided to further characterize the *FBXO31* gene, located within the YAC d792t2 and immediately adjacent to the BAC 346J21.

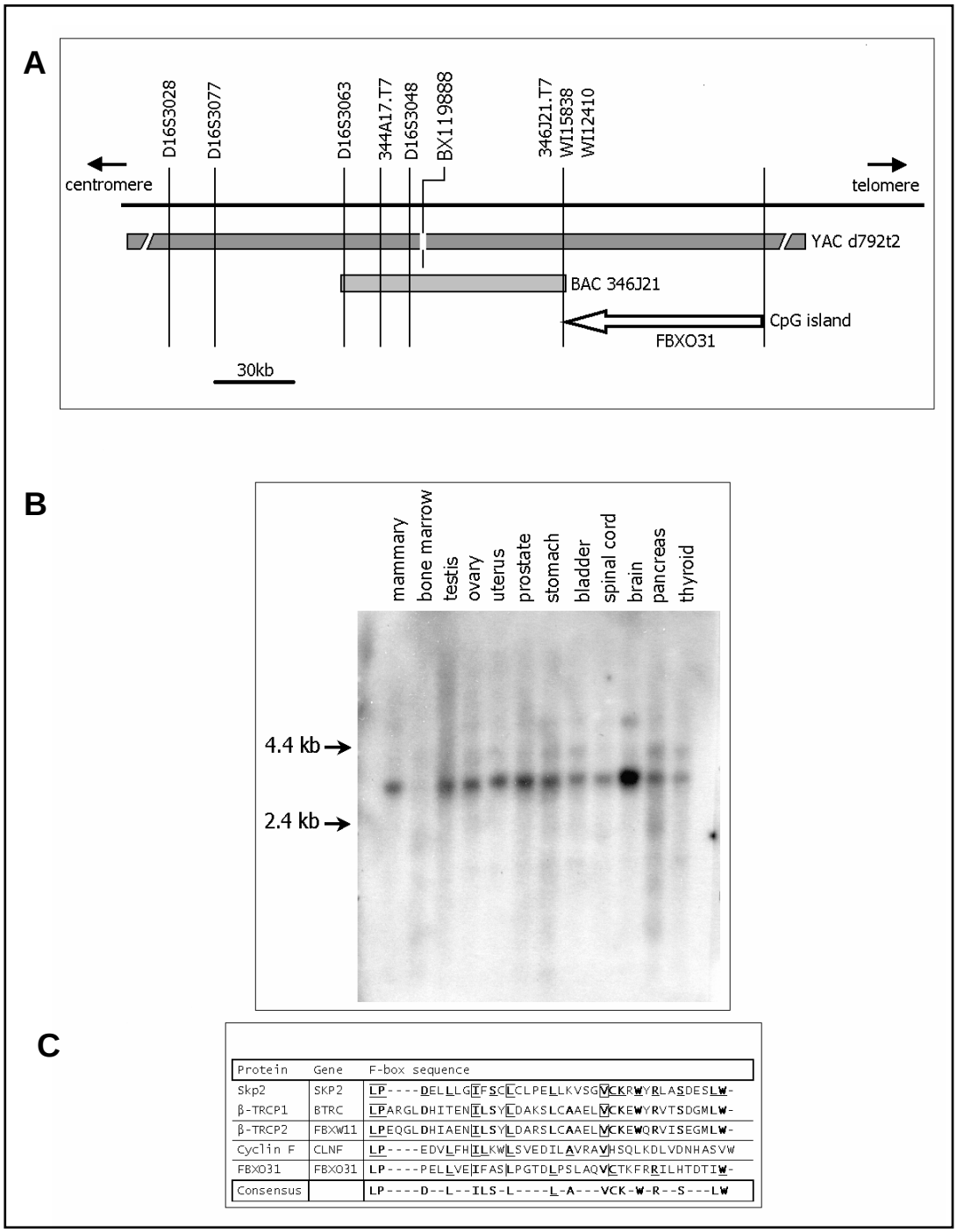

A
D16S3028
D16S3077
D16S3063
344A17.T7
D16S3048
BX119888
346J21.T7
WI15838
WI12410
centromere
telomere
YAC d792t2
BAC 346J21
CpG island
FBXO31
30kb
B
mammary
bone marrow
testis
ovary
uterus
prostate
stomach
bladder
spinal cord
brain
pancreas
thyroid
4.4 kb
2.4 kb
C
Protein Gene F-box sequence
Skp2 SKP2 LP----DELLLGIFSCLCLPELLKVSGVCKRWYRLASDESLW-
β-TRCP1 BTRC LPARGLDHITENILSYLDAKSLCAAELVCKEWYRVTSDGMLW-
β-TRCP2 FBXW11 LPEQGLDHIAENILSYLDARSLCAAELVCKEWQRVISEGMLW-
Cyclin F CLNF LP----EDVLFHILKWLSVEDILAVRAVHSQLKDLVDNHASVW
FBXO31 FBXO31 LP----PELLVEIFASLPGTDLPSLAQVCTKFRRILHTDTIW-
Consensus LP----D--L--ILS-L----L-A---VCK-W-R--S---LW

Figure 4.1. Location and tissue expression of the *FBXO31* gene and presence of an F-box domain in the encoded protein.

A: location of the *FBXO31* gene. Positions of various markers are determined by analysis of the genomic sequence of this region of chromosome 16. Positions of the YAC d792t2 and the BAC 346J21 are shown (Reddy et al., 2000; Kaur et al., 2005).

B: Northern blot of polyadenylated RNAs from various human tissues showing a 3.6-kb *FBXO31* transcript. *Arrows,* location of molecular weight markers. *C,* alignment of F-box domains from FBXO31 and other selected F-box proteins. *Boxed,* individual amino acids identical among all five F-box sequences; *underlined,* when present in three to four sequences. Nucleotide sequences are derived from Genbank, NCBI.

4.4.2 – Northern blot and in silico analysis

The presence of a 3.6-kb RNA band on a Northern blot shows that *FBXO31* expression is negligible in bone marrow, highly expressed in brain, and expressed at similar levels in the other 10 human tissues, including breast (Fig 4.1*B*). *FBXO31* mRNA contains a 1,620-bp ORF that is transcribed from the genomic DNA with nine exons spanning ~52 kb. The *FBXO31* transcript has an exceptionally short 23-base 5'-untranslated region. *In silico* analysis of the EST database and 5' rapid amplification of cDNA ends (data not shown) failed to identify any additional 5' transcript sequence. In addition, the presence of a 2.48-kb CpG island (66% G + C and with 9% CpG) encompassing the first 350-bp exon of *FBXO31* also supports the 5' start of the transcript.

FBXO31 encodes a 539-amino-acid protein with a predicted molecular mass of 61 kDa. The predicted amino acid sequence of *FBXO31* does not have significant homology to known proteins, except for a consensus F-box domain at the amino terminus (Fig 4.1*C*). The 40-amino-acid F-box domain is involved in protein-protein interactions and is present in a large family of proteins (Jin et al., 2004). FBXO31 also contains six minimal (RxxL) destruction box (D-box) motifs that are hallmark of proteins degraded via the anaphase-promoting complex/cyclosome (APC/C) (Zur & Brandeis, 2002; Bashir et al., 2004b).

4.4.3 – Ectopic expression of FBXO31 induces cellular senescence in MCF-7

Colonies of MCF-7 growing in the presence of geneticin after retroviral transduction were stained for the presence of the senescence-specific acidic ß-galactosidase activity (Dimri et al., 1995). Senescent cells had intense blue staining with a large flattened morphology. The MCF-7 cell line has a spontaneous background of senescent cells usually evident as occasional blue staining cells (Fig 4.2*A*). Following several weeks of growth, 35% to 40% of colonies ectopically expressing FBXO31 showed senescence compared with 5% to 7% of senescent

colonies with vector alone (Fig 4.2*B-C*; Table 4.1). Colonies from cells transduced with FBXO31ΔF were similar to the vector alone, suggesting that the F-box motif of FBXO31 is critical for this senescent function. In subsequent experiments, MCF-7 cells ectopically expressing a GFP-tagged FBXO31 showed that the presence of cell senescence was correlated to the expression of FBXO31 (Fig 4.2*D-I*). In MCF-7 colonies, senescent cells were frequent in regions with GFP fluorescence, whereas there were few senescent cells in regions without GFP.

Table 4.1. MCF-7 senescent colonies ectopically expressing FBXO31

	2 weeks growth			3 weeks growth		
	Number colonies			Number colonies		
	Senescent	Total	% Senescent	Senescent	Total	% Senescent
FBXO31	27	78	34.6	26	65	40.0
FBXO31ΔF	2	80	2.5	5	38	13.2
Vector	5	93	5.4	4	56	7.1

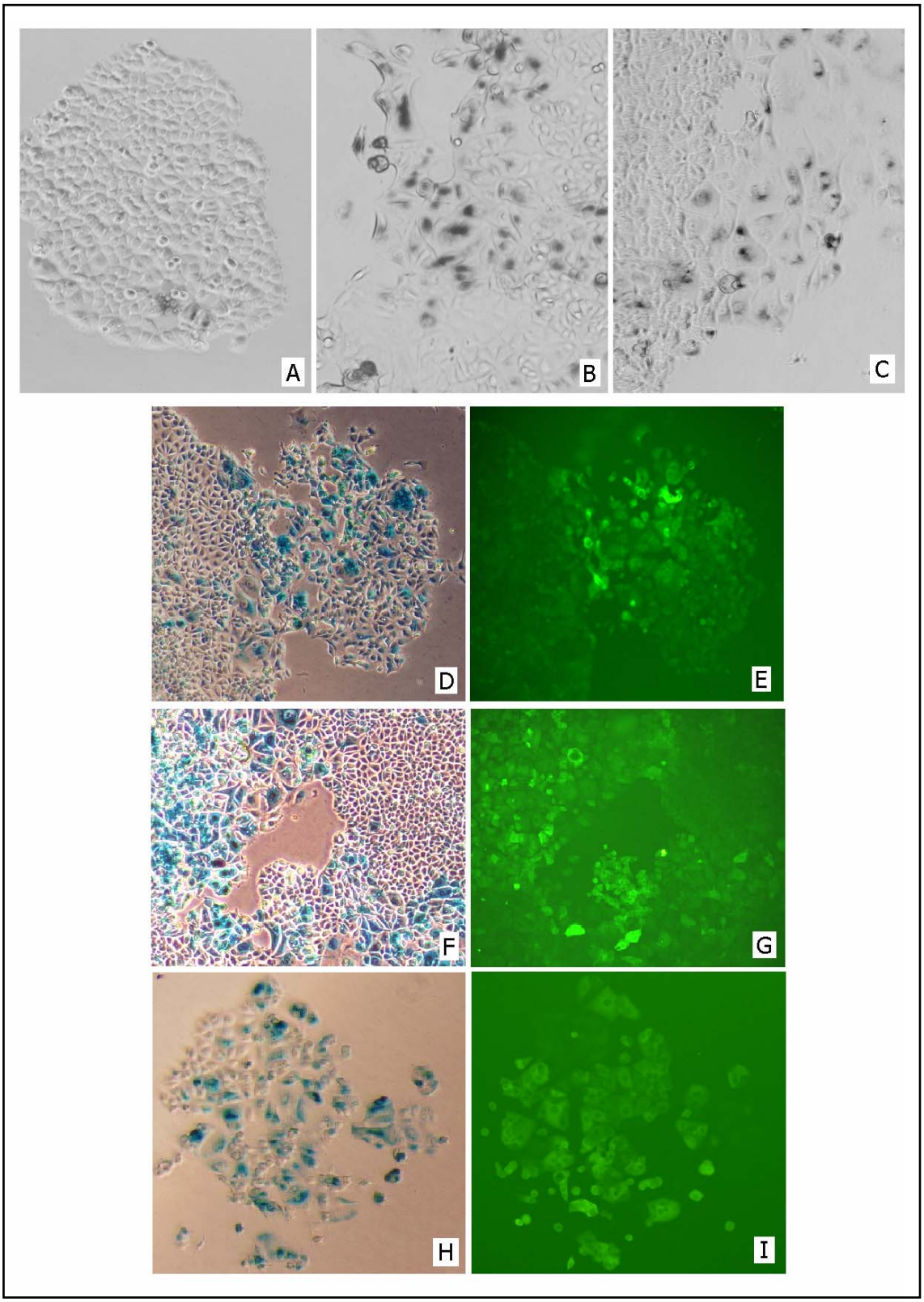


Figure 4.2. FBXO31 ectopic expression induces cellular senescence in MCF-7 cells.

Staining of senescent cells in colonies of MCF-7 ectopically expressing empty vector (*A*) and FBXO31 (*B* and *C*). Colonies of MCF-7 stained for senescence (*D, F*, and *H*) and fluorescent images showing the ectopically expressing GFP-FBXO31 (*E, G*, and *I*).

4.4.4 – Alteration of FBXO31 expression affects growth of breast cell lines

Because it was determined that ectopic expression of FBXO31 could induce senescence in the breast cell line MCF-7, we investigated if this gene could function more generally as a tumour suppressor. Ectopic expression of a tumour suppressor in a cancer cell line would be expected to inhibit the ability of cells to initiate colonies on plastic and inhibit cell proliferation.

FBXO31 was ectopically expressed in the breast cancer cell lines MCF-7 and MDA-MB-468 by retroviral transduction and colony formation on plastic assessed. Both cell lines showed a reduction in colony formation, compared with the vector control, when transduced with retroviruses expressing FBXO31 (Fig 4.3*A*-*B*). This reduction in colony formation averaged 50% for MCF-7 from five independent experiments and 77% for MDA-MB-468 from six independent experiments (Fig 4.3*C*). In all experiments, an expected severe inhibition in colony formation (generally 95% compared with the vector control) was observed in cells transduced with retrovirus expressing the p53 protein. Ectopic expression of FBXO31ΔF, a construct with a deletion of the F-box domain, showed an intermediate reduction in colony formation (MCF-7) or no significant reduction (MDA-MB-468) compared with the vector control (Fig 4.3*A*-*B*).

The effect of FBXO31 ectopic expression on the proliferation of MCF-7 cells was also determined by generating cultures of geneticin-resistant MCF-7 cells following retroviral transduction. Growth curves of these cell lines showed that cells expressing FBXO31 or p53 had a similar reduction in their proliferation (after 72 hours, 34% and 38%, respectively) compared with the control (Fig 4.3*D*). Cells expressing FBXO31ΔF had an intermediate 20% reduction of proliferation compared with the control.

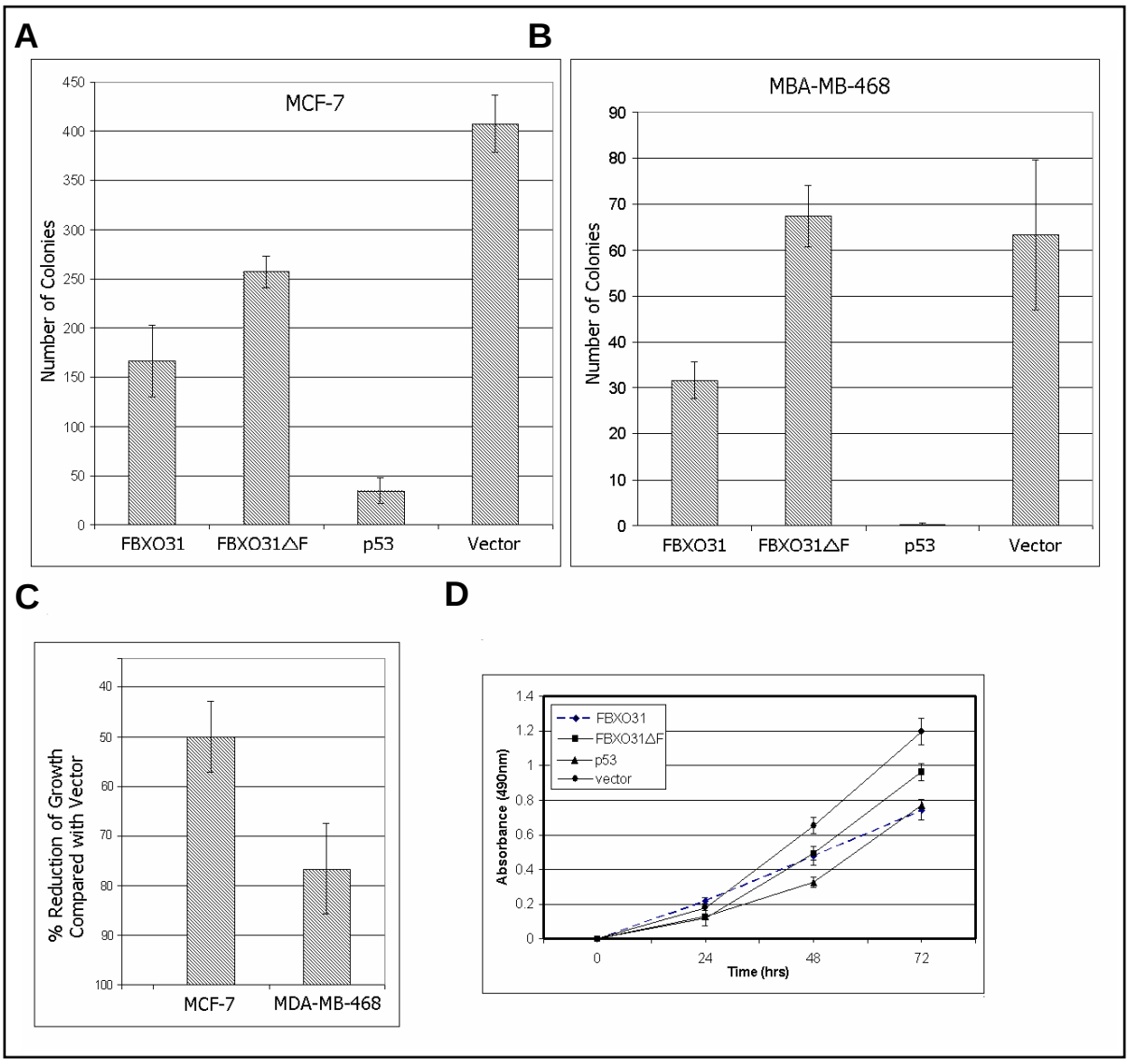

A
MCF-7
Number of Colonies
450
400
350
300
250
200
150
100
50
0
FBXO31
FBXO31ΔF
p53
Vector
B
MBA-MB-468
Number of Colonies
90
80
70
60
50
40
30
20
10
0
FBXO31
FBXO31ΔF
p53
Vector
C
% Reduction of Growth Compared with Vector
40
50
60
70
80
90
100
MCF-7
MDA-MB-468
D
Absorbance (490nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FBXO31
FBXO31ΔF
p53
vector
0
24
48
72
Time (hrs)

Figure 4.3. Ectopic expression of FBXO31 reduced the growth characteristics of breast cancer cell lines MCF-7 and MDA-MB-468.

A, B: The MCF-7 (*A*) and MDA-MB-468 (*B*) breast cancer cell lines were transduced with recombinant retroviruses expressing FBXO31, FBXO31ΔF, p53 (positive control), and empty vector (negative control). Number of colonies growing on plastic dishes in the presence of geneticin, 2 weeks after transduction.

C: Average effect of ectopic expression of FBXO31 on colony growth, compared with vector alone, from five independent experiments with MCF-7 and six experiments with MDA-MB-468.

D: FBXO31 ectopic expression inhibited MCF-7 cell growth. Growth assays were initiated from cultures transduced with retroviruses expressing FBXO31, FBXO31ΔF, p53, and empty vector and selected for 14 days in media containing geneticin.

4.4.5 – FBXO31 expression and mutation screening

To further investigate the possible role of FBXO31 as a tumour suppressor, the relative expression of *FBXO31* was determined by real-time RT-PCR in a panel of breast cell lines (Fig 4.4*A*). Results showed levels of expression in finite life span HMEC and nonmalignant immortalized cells that averaged 7.9 times greater than the average expression in breast cancer cell lines. The chromosome 16q LOH of the breast cancer cell lines has been determined previously (Callen et al., 2002). The average relative expression of *FBXO31* in the cell lines MCF-7 and BT20, without chromosome 16q LOH, was significantly higher than the cell lines T47-D, MDA-MB-231, MDA-MB-468, and SKBR3 with chromosome 16 LOH (1.27 versus 0.58, $p < 0.01$).

Based on the findings of reduced expression in breast cancer cell lines, expression was then assessed by real-time RT-PCR in a panel of primary breast tumours selected to have at least 50% tumour cells as estimated from H&E-stained sections. Presented in Fig 4.4*B* is the relative *FBXO31* expression in tumours classified by analysis of polymorphic markers (Cleton-Jansen et al., 2001) into those without chromosome 16q LOH and those with LOH for the entire long arm of chromosome 16. There was a trend for lower *FBXO31* expression in tumours with 16q LOH (10 of 15 tumours had expression of <50% of the average expression in normal breast) compared with tumours without LOH of 16q (6 of 11 tumours).

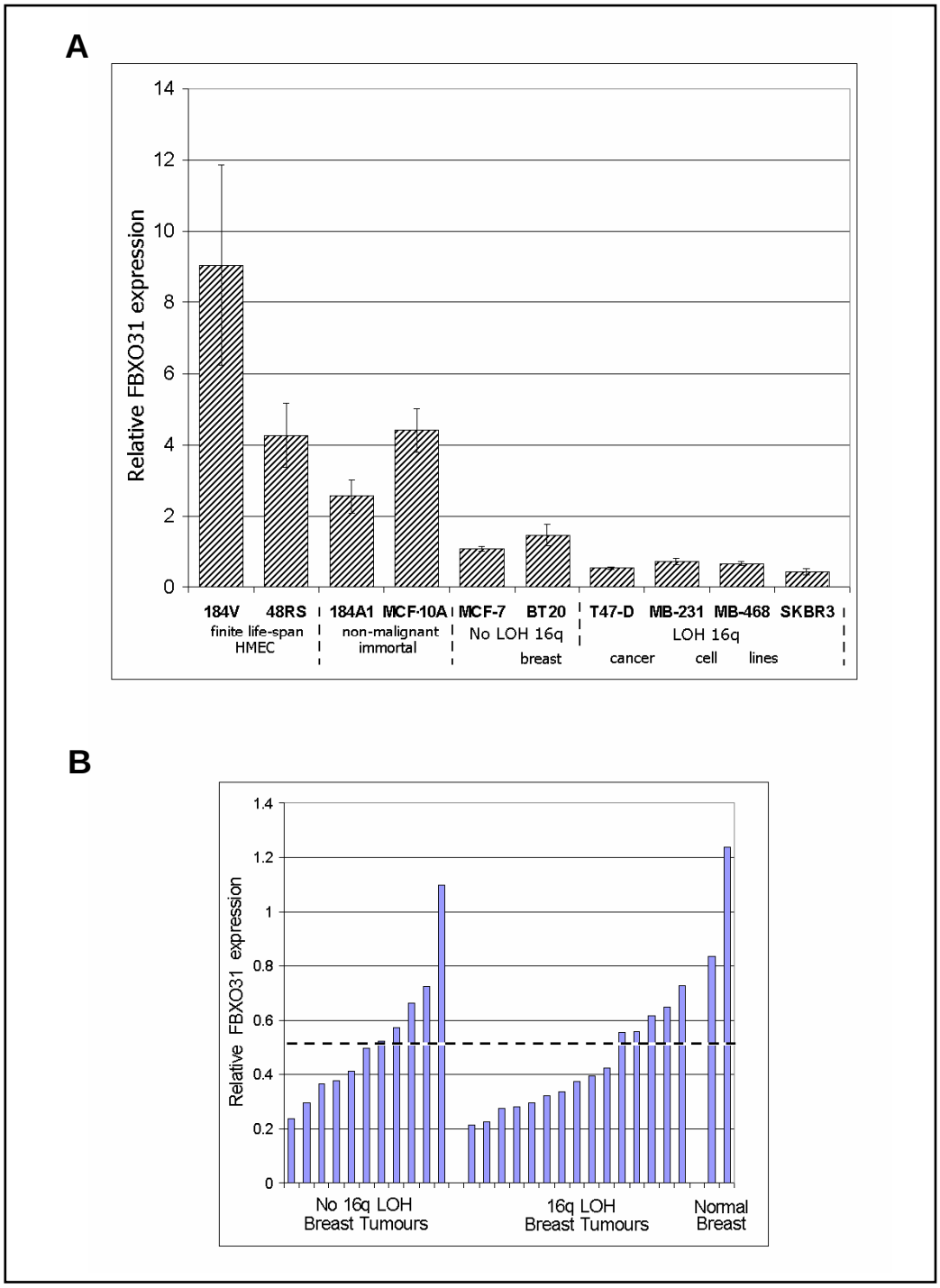


Figure 4.4. *FBXO31* expression as measured by real-time RT-PCR is reduced in breast cell lines and tumours.

A: *FBXO31* expression in various breast cell lines.

B: *FBXO31* expression in breast tumours with and without 16q LOH. *Horizontal dashed line,* at 50% of the average expression in the normal breast samples.

Based on the observation of reduced *FBXO31* expression in some tumours, it was considered possible that the *FBXO31* gene may function as a tumour suppressor. Tumour-restricted mutations can provide definitive evidence for a tumour suppressor gene (Paige, 2003). Accordingly, screening for the presence of mutations in *FBXO31* was undertaken on DNA isolated from a panel of 24 breast cancer cell lines by single strand conformational analysis (SSCA). In addition, DNA from 44 breast tumours with known LOH of the long arm of chromosome 16, and the DNA from the normal peripheral blood lymphocytes of the same patients were also screened. Primers for SSCA were designed to bind within the intronic sequences such that coding and flanking intronic regions could be analyzed (data available upon request). No tumour specific mutations were detected for *FBXO31* both in breast cancer cell lines and tumour DNA.

4.4.6 – FBXO31 is cell cycle regulated

Following the findings that ectopic expression of *FBXO31* suppresses growth, a fluorescence-activated cell sorting–based cell cycle analysis was used to determine if the mechanism of this growth suppression is due to a specific effect on the cell cycle. Cell cycle analysis was done on the breast cancer cell line MDA-MB-468 transiently expressing the GFP-tagged proteins for 24 and 48 hours (Fig 4.5). Analysis of the asynchronous cell populations showed a 13% increase in the proportion of GFP-FBXO31-expressing G_1 cells from 24 to 48 hours (Fig 4.5*A*-*B*) compared with the 3% increase observed in only GFP-expressing G_1 cells in the same time period (Fig 4.5*G*-*H*). Ectopic expression of GFP-FBXO31ΔF resulted in profiles similar to the control with a 3% increase in G_1 cells from 24 to 48 hours (Fig 4.5*C*-*D*). However, when GFP-p53 was ectopically expressed, there was a 17% increase in G_1 cells from 24 to 48 hours (Fig 4.5*E*-*F*). Similar results were observed in independent experiments and in the MCF-7 cell line (data not shown). These observations suggest that a block in the cell cycle at

G_1 is the probable cause of the observed negative effect of FBXO31 ectopic expression on growth of breast cancer cell lines.

The cell cycle regulation of FBXO31 levels was then investigated. The endogenous FBXO31 levels were determined using an affinity-purified rabbit polyclonal anti-FBXO31 antibody. Specificity of the anti-FBXO31 antibody was confirmed by Western blot analysis on protein lysates of MCF-10A and HEK293T cells transiently expressing myc-FBXO31. The protein samples showed a band at 78 kDa, suggesting that this antibody could specifically detect endogenous and ectopically expressed FBXO31 (data not shown). FBXO31 levels at different stages of the cell cycle were investigated in MCF-10A and the breast cancer cell line SKBR3 by collecting samples at various time points following release from a double thymidine block. For both cell lines, the variation in levels of FBXO31 at different times after release of the block shows that this protein is cell cycle regulated (Fig 4.6*A*). The cell cycle synchronization was confirmed by change in the levels of cyclin B1. These levels were at a maximum at 6 to 10 hours for MCF10A and 8 to 12 hours for SKBR3. Cell progression from mitosis is associated with decreased levels of cyclin B1. For both cell lines, the levels of FBXO31 were a maximum at similar times, from 8 to 16 hours after release (Fig 4.6*A*). Comparison with the cyclin B1 cell cycle profile indicate that levels of FBXO31 protein were at a maximum at late G_2 to early G_1 phase.

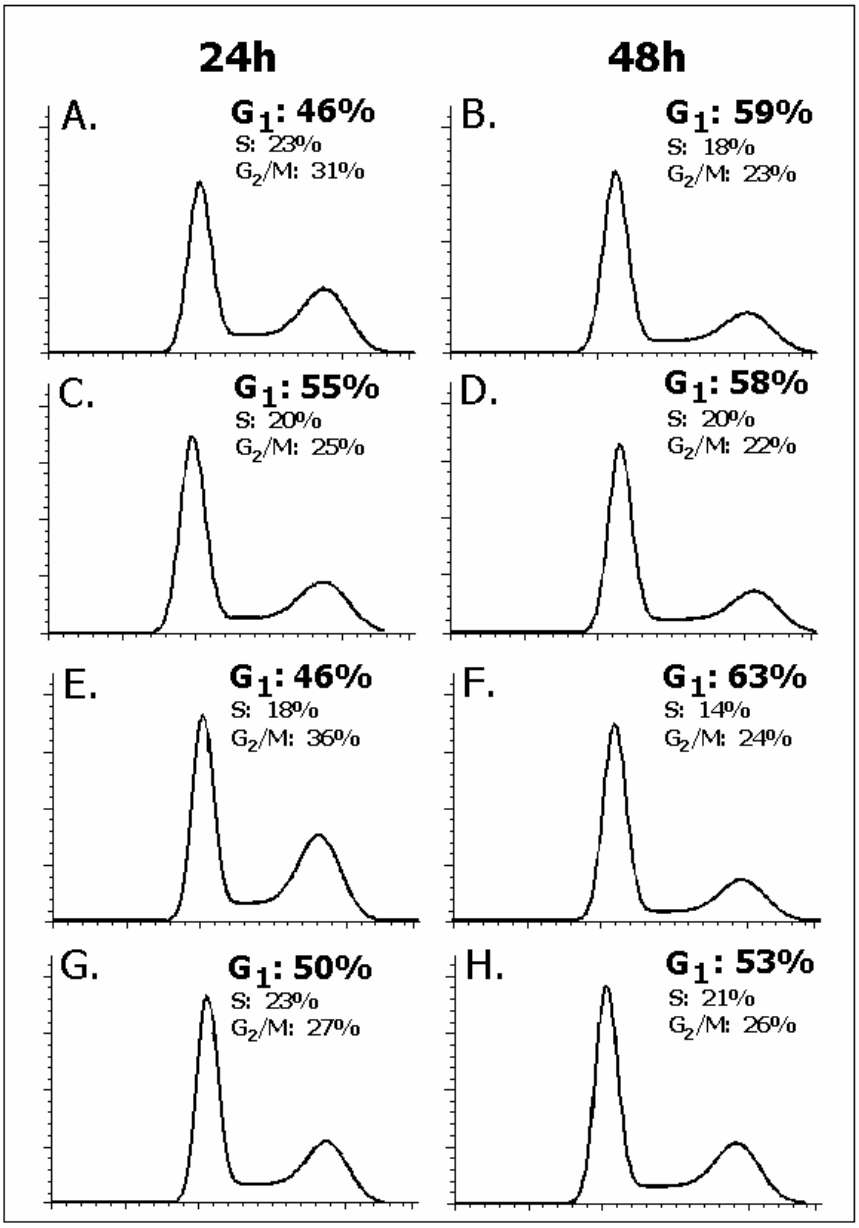


Figure 4.5. FBXO31 ectopic expression blocks cells at G_0-G_1 of the cell cycle. Cell cycle curves of GFP-positive asynchronous MDA-MB-468 cultures 24 and 48 hours after transfection with GFP fusion constructs of FBXO31 (*A-B*), FBXO31ΔF (*C-D*), p53 (*E-F*), and empty vector (*G-H*).

4.4.7 – FBXO31 exists as a component of SCF^{FBXO31} complex

Immunoprecipitation was used to determine if FBXO31 exists as a component of the SCF ubiquitination complex. Reciprocal experiments on HEK293T lysates prepared from cells transfected with constructs either expressing myc-FBXO31, myc-FBXO31ΔF, HA-Skp1, both myc-FBXO31 and HA-Skp1, or both myc-FBXO31ΔF and HA-Skp1 proteins showed that FBXO31 specifically interacts with the Skp1 protein (Fig 4.6*B*). The FBXO31-Skp1 interaction was effectively eliminated when the F-box was deleted in cells transfected with the myc-FBXO31ΔF construct. Endogenous Cullin-1 and Roc-1 were also present in the immunoprecipitations from cells transfected with constructs expressing myc-FBXO31/HA-Skp1. The trace amounts of Cullin-1 and Roc-1 detected in anti-myc immunoprecipitations from cells transfected with myc-FBXO31ΔF and/or HA-Skp1 were considered to originate from nonspecific interactions, because Cullin-1 and Roc-1 were not detected in the reciprocal anti-HA immunoprecipitations done on the same lysates.

To provide additional evidence for FBXO31 as a component of an SCF^{FBXO31} complex, stable HEK-293T-based cell lines were generated by retroviral transfer of FLAG-HA-FBXO31. The expression of the tagged FBXO31 protein was at near endogenous levels (data not shown). Cell lysates from these stable cell lines were affinity purified using anti-FLAG antibody, and the presence of endogenous Skp1, Cullin-1, and Roc-1 proteins was confirmed by Western blot analysis (Fig 4.6*C*). Because Skp1, Cullin-1, and Roc-1 constitute invariant components of the SCF complexes (Cenciarelli et al., 1999; Winston et al., 1999), it is concluded that FBXO31, either ectopically expressed or expressed in stable clones at near physiologic levels, exists as part of an SCF^{FBXO31} complex.

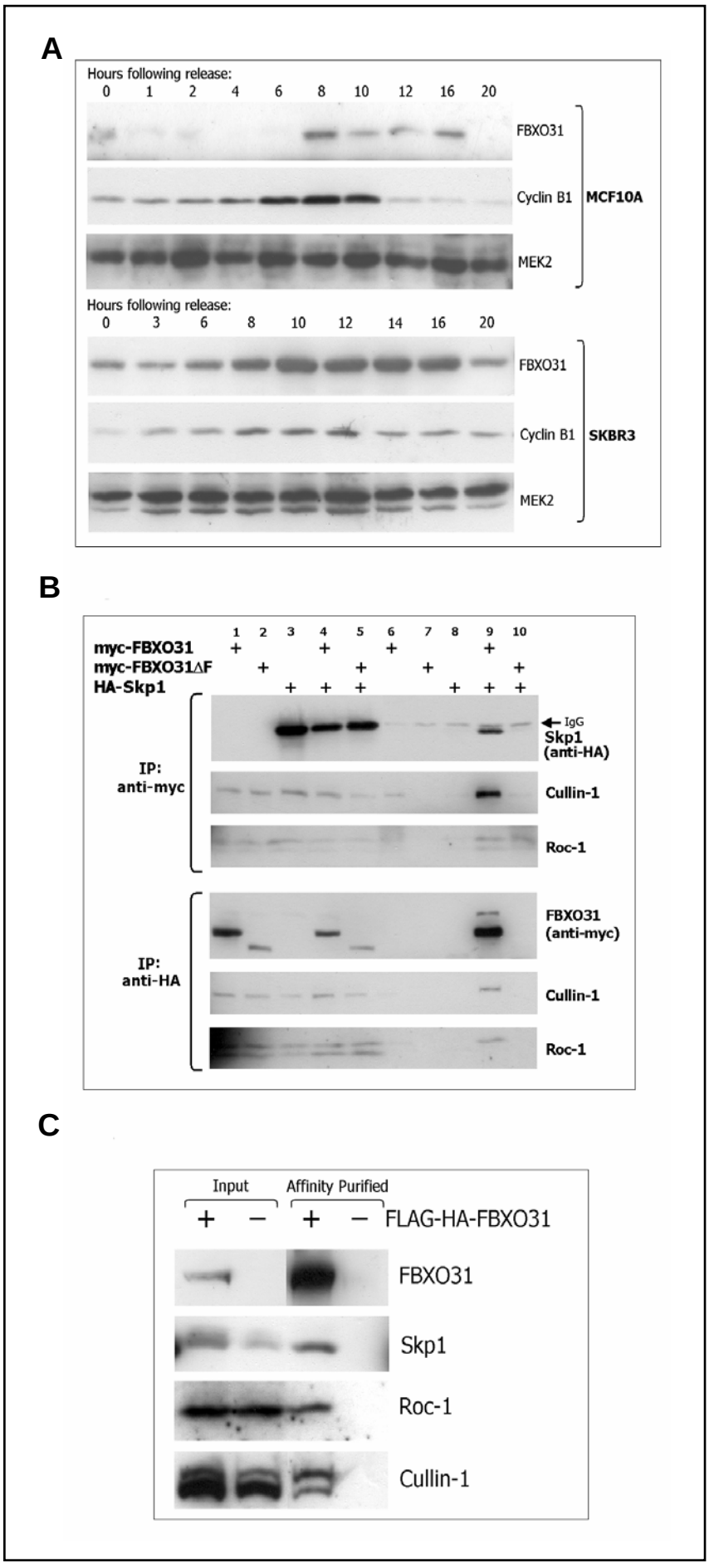

A
Hours following release:
0 1 2 4 6 8 10 12 16 20
FBXO31
Cyclin B1
MCF10A
MEK2
Hours following release:
0 3 6 8 10 12 14 16 20
FBXO31
Cyclin B1
SKBR3
MEK2
B
1 2 3 4 5 6 7 8 9 10
myc-FBXO31
myc-FBXO31ΔF
HA-Skp1
IP: anti-myc
IgG
Skp1 (anti-HA)
Cullin-1
Roc-1
IP: anti-HA
FBXO31 (anti-myc)
Cullin-1
Roc-1
C
Input
Affinity Purified
+ − + − FLAG-HA-FBXO31
FBXO31
Skp1
Roc-1
Cullin-1

Figure 4.6. FBXO31 is cell cycle regulated and is a component of the SCF complex.

A: MCF-10A and SKBR3 cells were synchronized with a double thymidine block, harvested at the indicated time points after release from the block, and analyzed by Western blot analysis. Blots were probed with anti-FBXO31, anti-cyclin B1, and anti-MEK2 antibodies.

B: HEK293T cells were transiently transfected with constructs expressing individually or combinations of myc-FBX031, myc-FBX031ΔF, or HA-Skp1 proteins as shown. Total cell lysates (*lanes 1-5*) were immunoprecipitated (*lanes 6-10*) with either anti-*myc* (*top three blots*) or anti-HA (*bottom three blots*) antibodies. Input (*lanes 1-5*) and immunoprecipitated (*lanes 6-10*) proteins were resolved by SDS-PAGE and probed with appropriate antibodies to detect FBXO31, FBXO31ΔF, Skp1, Cullin-1, and Roc-1 proteins.

C: Lysates from HEK293T and a stable HEK293T clone expressing FLAG-HA-FBXO31 were affinity purified using anti-FLAG M2-coated agarose beads. Input and affinity purified proteins were resolved by SDS-PAGE and probed with appropriate antibodies to detect FBXO31, Skp1, Cullin-1, and Roc-1 proteins.

4.5 – Discussion

Previous studies determined that the YAC d792t2, and subsequently the transfection of a retrofitted BAC 346J21, caused cellular senescence when transferred into cell lines, and this was inferred to be caused by a gene termed *SEN16* (Reddy et al., 2000; Kaur et al., 2005). Our analysis of the YAC d792t2 showed an interstitial deletion was present in the region encompassed by the BAC, whereas analysis of the genomic sequence contained within the BAC 346J21 did not identify any potential candidate senescence genes. We chose to characterize the gene *FBXO31*, as it was located within the YAC d792t2. *FBXO31* possessed properties consistent with the *SEN16* senescence gene, because ectopic expression of FBXO31 in the breast cell line MCF-7 (one of the cell lines used in the *SEN16* studies - (Kaur et al., 2005)) resulted in an increase in the number of senescent colonies. In addition, colonies of transduced MCF-7 showed the expression of FBXO31 was correlated with the presence of regions of cellular senescence. *In vitro* systems using ectopic expression driven by highly active promoters can lead to nonphysiologic consequences. However, it is unlikely that the observed functional consequences of FBXO31 ectopic expression reflect nonphysiologic expression, because the senescence phenotype observed in MCF-7 cells (Fig 4.2*B*-*C*) was similar to that previously reported (Kaur et al., 2005). In addition, we have observed that geneticin-selected cells with retrovirally induced expression of FBXO31 have near physiologic levels of protein.

Although the data presented here are consistent with *FBXO31* being the previously identified cellular senescence gene *SEN16* (Kaur et al., 2005), the location of *FBXO31* is immediately distal to the BAC 346J21. There are several possible explanations for the apparent absence of the ORF of the *FBXO31* gene in the BAC 346J21. First, the BAC may be rearranged and actually contain the *FBXO31* gene. The possibility of a rearranged clone is suggested by the

reversal of the order of the markers D16S3063 and D16S3048 in the reported physical mapping of BAC 346J21 (Kaur et al., 2005). The second possibility is that uncharacterized DNA rearrangements in the clones containing the BAC result in changes in endogenous *FBXO31* expression. MCF-7 transfected with the retrofitted BAC 346J21 were identified as rare geneticin-resistant colonies, but these could not be further characterized due to the absence of polymorphic markers unique to the introduced BAC. Therefore, there is no information regarding whether the introduced DNA is integrated, the sites of integration and the copy number. Studies suggest that it is extremely difficult to obtain stable cell lines with intact introduced DNA when transformed with large fragments of DNA, and alternative vector systems have been specifically designed to alleviate this problem (Wade-Martins et al., 1999).

Previously, based on breast cancer LOH studies, the chromosome band 16q24.3 was delineated as the smallest region of overlap and therefore the likely location of one or more breast cancer tumour suppressor genes (Cleton-Jansen et al., 2001). Therefore, the possibility was investigated that FBXO31 has a broader role as a breast tumour suppressor. The data presented suggest that this may be the case. The expression of the *FBXO31* gene was reduced in both breast tumour cell lines and sporadic primary breast tumours compared with nonmalignant breast epithelium (Fig 4.4*A* and *B*). In addition, there was a significantly reduced expression in breast cancer cell lines with 16q LOH compared with those without 16q LOH. Similarly, in primary tumour samples, there was a trend for a higher proportion of tumours showing reduced expression when LOH of 16q was present. However, complete elimination of *FBXO31* expression was not observed in breast tumours, because the maximum reduction of the relative *FBXO31* expression determined by RT-PCR was about 20% of the expression in normal breast. This suggests that down-regulation of *FBXO31* is a likely scenario for function as a tumour suppressor but that residual levels of expression are retained.

Additional studies were then undertaken to further investigate the potential function of FBXO31 as a tumour suppressor. Ectopic expression of FBXO31 in the breast cancer cell lines MCF-7 and MDA-MB-468 resulted in a moderate inhibition of colony growth on plastic and reduced the proliferation of MCF-7 cells. Cell cycle analysis of the breast cancer cell lines ectopically expressing FBXO31 was consistent with a block in the cell cycle at G_1. Investigation of endogenous FBXO31 expression in synchronized MCF-10A and SKBR3 cells show FBXO31 protein levels are at a maximum from late G_2 to early G_1 phase (Fig 4.6*A*). The timing of FBXO31 destruction is consistent with APC-mediated degradation (Zur & Brandeis, 2002). Cell cycle analysis showed that inhibition of growth of breast cancer cell lines by ectopic FBXO31 expression is likely to be caused by cells not progressing normally past G_1 phase of the cell cycle (Fig 4.5). These results are consistent with the analysis of replicative senescence in primary cells where entry into senescence is associated with the accumulation of cells at G_1 phase (Shelton et al., 1999). In summary, the expression and functional studies and the role of FBXO31 in the cell cycle are consistent with a tumour suppressor role of FBXO31. Tumour haploinsufficiency for FBXO31 as a consequence of being in a region of frequent LOH in breast cancer is likely because tumours possess a low level of expression, and it is predicted that the protein is still subject to cell cycle variation. It is suggested that FBXO31 haploinsufficiency contributes to the acquisition by tumour cells of escape from senescence and the normal controls of cell proliferation. These conclusions are further supported by a breast cancer gene expression microarray study, which predicted the clinical outcome in breast cancer based on the combined relative expression profiles of 70 genes (van 't Veer et al., 2002). Represented in these 70 genes is *FBX031* (denoted as contig 51464).

The predicted amino acid sequence of the FBXO31 protein does not have significant homology to known proteins except for the presence of a 40-amino-acid F-box domain at the amino acid terminus. F-box proteins can exist as part of SCF ubiquitin ligase complexes that are involved in diverse cellular functions, including signal transduction, control of G_1-S progression, and orderly execution of the cell cycle (DeSalle & Pagano, 2001; Reed, 2003). Skp1, Cullin-1, and Roc-1 are invariant proteins of the SCF complex, whereas the F-box proteins that bind to Skp1 are the components that impart functional specificity. For example, Skp2 specifically binds phosphorylated p27 resulting in a critical role in the degradation of p27 and therefore a control of S phase entry of the cell cycle (Carrano et al., 1999).

Our results support the existence of a SCF^{FBXO31} complex. First, FBXO31 coimmunoprecipitated with Skp1, Cullin-1, and Roc1 (Fig 4.6*B*) and copurified with FBXO31 in stable cell lines expressing tagged FBXO31 at near physiologic levels (Fig 4.6*C*). Second, analysis of the nonmalignant breast cell line MCF-10A and the breast tumour cell line SKBR3 showed that FBXO31 is cell cycle regulated (Fig 4.6*A*). Third, *in silico* analysis showed that FBXO31 protein has six minimal D-box (RxxL) motifs that are the hallmark of proteins degraded via the APC/C (Zur & Brandeis, 2002). FBXO31 shares all these features with the Skp2 protein present in the $SCF^{Skp2\text{-}Cks1}$ complex (Bashir et al., 2004b), suggesting a functional similarity.

It is therefore proposed that FBXO31 is likely to form a functional SCF^{FBXO31} complex that would recruit and ubiquitinate specific proteins for subsequent degradation. Most F-box proteins bind to their substrates through regions located COOH-terminal to the F-box motif (Cardozo & Pagano, 2004). F-box proteins notated as "FBXO" do not have recognizable substrate binding domains; however, as analysis of FBXO proteins progresses, new substrate binding domains are being recognized. For example, Fbx7 has been shown to recruit a

substrate; the region responsible was shown to be a proline-rich region and was found in two other FBXO proteins (Hsu et al., 2004). The COOH-terminal part of FBXO31 contains a 175-amino-acid region that is unusually rich (25%) in glycine and arginine residues that may represent the substrate binding region. Regions rich in glycine and arginine have been implicated as protein binding domains, although such reported domains have a more defined glycine/arginine repeat structure than present in FBXO31 (Friesen & Dreyfuss, 2000; Hsu et al., 2004). It is proposed that the substrates of the SCF^{FBXO31} complex that are ubiquitinated and subsequently degraded are critical cell cycle proteins, and we are presently working towards their identification.

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