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CHARACTERIZING pRB'S ROLE IN CELL CYCLE CONTROL, MAMMARY GLAND DEVELOPMENT, AND TUMORIGENESIS: A UNIQUE CONNECTION TO TGF-p SIGNALLING

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CHARACTERIZING pRB'S ROLE IN CELL CYCLE CONTROL, MAMMARY GLAND DEVELOPMENT, AND TUMORIGENESIS: A UNIQUE CONNECTION TO TGF-β SIGNALLING

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Abstract

The ability to respond to anti-growth signals is critical to maintain tissue homeostasis and loss of this proliferative control mechanism is considered a hallmark of cancer. Negative growth regulation generally occurs during the G0/G1 phase of the cell cycle, yet the redundancy and complexity among components of this regulatory network have made it difficult to discern how negative growth cues protect cells from aberrant proliferation.

Transforming growth factor β (TGF-β) is a crucial mediator of mammary epithelial morphogenesis and can negatively regulate cell cycle progression. TGF-β has been shown to inhibit cyclin dependent kinase activity, which leads to activation of the retinoblastoma protein (pRB) and growth arrest. However, unlike other components of TGF-β cytostatic signalling, pRB is thought to be dispensable for mammary development. Using gene-targeted mice where the LXCXE binding cleft on pRB has been disrupted ($Rb1^{\Delta L}$ and $Rb1^{NF}$), we have discovered that pRB plays a crucial role in mammary gland development. In particular, *Rb1* and $Rb1^{NF}$ mutant female mice have hyperplastic mammary epithelium due to insensitivity to TGF-β growth inhibition. In contrast with previous studies that highlight the inhibition of cyclin/CDK activity by TGF-β signalling, these experiments reveal that active transcriptional repression of E2F target genes by pRB is also a key component of TGF-β cytostatic signalling. However, loss of pRB-LXCXE interactions does not cause overt defects in other TGF-β signalling pathways such as apoptosis and differentiation. Taken together, this work demonstrates a unique functional connection between pRB and TGF-β in growth control and mammary development.

These findings were extended to explore the importance of the pRB anti-proliferative response during tumour formation and progression. Cytostatic control is considered a key tumour suppressive mechanism in the mammary gland. Here I show that LXCXE-dependent growth control by pRB blocks formation of mammary tumours in *Wap-p53*^{R172H} transgenic mice. In contrast, the same growth control mechanism is unnecessary to protect against *Neu* or *7,12*-dimethylbenz[a]anthracene-induced mammary tumorigenesis. Taken together, this work demonstrates that anti-proliferative control by pRB can act as a barrier against oncogenic transformation. Strikingly, these data also reveals that this tumour suppressive effect is context-dependent.

Key Words

Retinoblastoma, TGF-β, mammary gland, proliferation, LXCXE, cell cycle, breast cancer, metastasis

Co-authorship

This thesis contains material from previously published and submitted manuscripts. The original manuscripts used in Chapter 2 and 4 of this thesis were written entirely by Sarah Francis and Fred Dick. A copyright release for Chapter 2 is provided in Appendix III.

All of the experimental work presented in this thesis was performed by Sarah Francis, with the following exceptions that were included for continuity:

Chapter 2:

Jacqueline Bergsied created the $Rb1^{N750F}$ mouse used in the nursing and developmental studies. Christian Isaac performed the TGF-β growth inhibition assay on murine embryonic fibroblasts in Figure 2.4 and the cyclin-dependent kinase inhibitor overexpression assays in Figure 2.6. Courtney Coschi performed the nursing study for $Rb1^{N750F}$ mice in Table 2.1. Alison Martens and Carlo Hojilla aided with mammary gland transplant experiments. Subrata Chakrabarti aided in histological analysis for data presented in Figures 2 and 3.

Chapter 4:

Fred Dick performed the soft agar colony formation assay in Figure 4.1. Subrata Chakrabarti aided in the histological analysis.

In loving memory of

Maureen Kenney (1953-1994)

and Caitlin Kenney (1984-2003).

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And to Owen, Emily, and Tessa: for making me smile and filling me with pure joy. I love you very much.

This thesis has been brought to you by the letters "R", "B" and the number "1".

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List of Abbreviations

A	Alanine
aa	Amino acid
ABS	Adult bovine serum
ALK5	Activin receptor-like kinase 5 (aka TβRI)
APC/C	Anaphase promoting complex/cyclosome
ArppP0	Acidic ribosomal phosphoprotein
Atf3, ATF3	Activating transcription factor gene, protein
BMP	Bone morphogenic protein
BrdU	5-bromo-2-deoxyuridine
BRG1	Brahma-related gene 1
Cap-D3	Chromosome-associated protein D3
Ccna2	Gene encoding Cyclin A2
Ccne1	Gene encoding Cyclin E1
CIP/KIP	CDK inhibitory protein/kinase inhibitory protein
CDK	Cyclin-dependent kinase
Cdkn1a	Cyclin dependent kinase inhibitor 1a gene – encodes $p21^{CIP1}$
Cdkn1b	Cyclin dependent kinase inhibitor 1b gene – encodes $p27^{KIP1}$
Cdkn1c	Cyclin dependent kinase inhibitor 1c gene – encodes $p57^{KIP2}$
Cdkn2a	Cyclin dependent kinase inhibitor 2a gene – encodes $p16^{INK4a}$
Cdkn2b	Cyclin dependent kinase inhibitor 2b gene – encodes $p15^{INK4b}$
Cdkn2c	Cyclin dependent kinase inhibitor 2c gene – encodes $p18^{INK4c}$
Cdkn2d	Cyclin dependent kinase inhibitor 2d gene – encodes $p19^{INK4d}$
CKI	Cyclin dependent kinase inhibitor
CMV	Cytomegalovirus
co-SMAD	Common (partner) SMAD
CRF	Chromatin remodelling factor
DAPI	4',6-diamidino-2-phenylidole
DAPK	Death-associated protein kinase gene
DMBA	7,12-dimethylbenz[z]anthracene
DMEM:F12	Dulbecco's Modified Eagle's Medium Nutrient Mixture F12 Ham
dn	Dominant negative
dnIIR	Dominant negative TGF-β type II receptor
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dpc	Day post-coital
dREAM	*Drosophila melanogaster* RBF, E2F, and MYB complex
DREAM	DP, RB-like, E2F, And MuvB complex (mammalian cells)
DRM	DP, RB, and MuvB complex (*C. elegans*)
E1A	Adenovirus early-region 1A gene
E2F	Early-region 2 transcription factor
EGF	Epidermal growth factor
EMEM	Eagle's Minimum Essential Medium
EMT	Epithelial-to-mesenchymal transition
ER	Estrogen receptor

F	Phenylalanine
FBS	Fetal bovine serum
G0	Quiescence
G1	Gap 1 phase of the cell cycle
GDF	Growth and differentiation factor
GST	Glutathione S-transferase
H	Histidine
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
His-E2F3	Histidine-tagged-E2F3
HMGA2	High mobility group A2
hp	Hyperplastic
HPV	Human papilloma virus
I	Isoleucine
I0, I2, I16	Involution day 0, day 2, day 16
ID	Inhibitor of differentiation
INK4	Inhibitor of cyclin dependent kinase 4
JNK	Jun N-terminal kinase
kb	Kilobase
kDa	Kilodalton
LA	Lobuloalveolar
LAP	Latency associated protein
LG	*β-Lactoglobulin* promoter
LSM	Lung surface metastases
LXCXE	Leucine, any amino acid, cysteine, any amino acid, glutamic acid
M	Methionine
MAPK	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
MEF	Murine embryonic fibroblast
MMTV	Murine mammary tumour virus
N	Asparagine
NuRD	Mi2/nucleosome remodelling and deacetylase complex
ORC	Origin replication complex
P0, P2	Day 0 post-parturition, Day 2 post-parturition
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pcna	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3 kinase
PP1	Protein phosphatase 1
pRB	Retinoblastoma protein
PyVmT	Polyomavirus middle T antigen gene
qRT-PCR	Quantitative real time reverse transcriptase PCR
R	Arginine
RB1	Human retinoblastoma gene
Rb1	Murine retinoblastoma gene

Rbl1	Retinoblastoma like gene 1 – encodes murine p107
R-SMAD	Receptor-activated SMAD
RT-PCR	Reverse transcriptase PCR
S	DNA synthesis phase of the cell cycle
SA	Surface area
SARA	Smad anchor for receptor activation
SCLC	Small cell lung carcinoma
SDS	Sodium dodecyl sulfate
Ser	Serine
shRNA	Short hairpin RNA
SKP2	S phase kinase-associated protein 2
Suv39h1	Suppressor of variegation 3-9 homolog 1
SV40 TAg	Simian virus large T antigen
SWI/SNF	Switch/Sucrose non-fermentable
SynMuv	Synthetic multivulva
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
TEB	Terminal end bud
TGF-α	Transforming growth factor α
TGF-β	Transforming growth factor β
Tgfbr2	Gene encoding the TGF-β type II receptor
TIEG1	TGF-β-inducible early response gene 1
Tyms	Gene encoding thymidylate synthase
U/mL	Units per millilitre
V	Valine
Wap	Whey acid protein promoter

Chapter 1: Introduction

1.1 General Introduction

The ability to control growth is essential during development and maintenance of homeostasis; loss of this control is considered one of the hallmarks of cancer (76). The mammalian cell has developed a network of pathways to safeguard the cell against aberrant proliferative cues (137). The retinoblastoma protein (pRB) and transforming growth factor beta (TGF-β) are key components in this web of cellular growth control, so it is not surprising that their activities are subverted in many forms of cancer, including breast cancer (20, 138). Despite the wealth of knowledge that has been gathered since the discovery of these two proteins (6, 40, 61, 62, 123), many questions remain about how they function both during development and tumorigenesis. It is becoming increasingly clear that cellular context plays an important role in how cells respond to both tumour suppressive and oncogenic signals. For that reason, this thesis examines the role of pRB-dependent proliferative control in mammary gland development and tumorigenesis, and demonstrates a unique connection with the TGF-β pathway.

1.2 Identification and Cloning of the Retinoblastoma Tumour Suppressor Protein

The first tumour suppressor system identified in humans was that governing the formation of the childhood eye cancer, retinoblastoma. This cancer occured sporadically in some patients, but appeared to be inherited in others (114). Based on the genetic data, Knudson proposed a "two-hit" hypothesis, suggesting that retinoblastoma was caused by two mutational events. In familial cases, one mutation conferring susceptibility to

retinoblastoma was inherited through the germ line, while the second, rate-limiting step occurred in somatic cells. In sporadic cases, both mutations occurred in somatic cells. This initial observation led to the mapping of the putative retinoblastoma susceptibility gene to chromosome 13q14, and the subsequent cloning of the retinoblastoma tumour suppressor gene (*RB1*) (58, 61, 62, 123, 201). As predicted by Knudson's hypothesis, both alleles of the *RB1* gene were frequently deleted or mutated in cases of both sporadic and familial retinoblastoma (61, 62, 123). Consistently, there was little to no expression of the mRNA transcript in retinoblastoma cells, while transcripts were readily detected in human retinal and placental tissue (61, 62, 123). This strongly implicated the loss of RB1 as causative in the development of retinoblastoma tumours. Mutations in *RB1* were also identified in osteosarcoma cell lines, linking loss of pRB to other human cancers (61, 62). Inactivation of the *RB1* gene is now known to occur with variable incidence in other tumours, including 90% of small cell lung carcinomas (SCLC) and 20-30% of breast cancers (14, 20). In other cancers, upstream components of the pRB pathway are disrupted, leading to loss of pRB function (14, 20, 54, 137). Thus, pRB appears to play a tumour suppressive role in many forms of human cancers. Nearly 25 years after the initial cloning of *RB1*, many cellular functions and interacting partners of pRB have been determined. However, it remains unclear which of these functions are physiologically relevant and are involved in suppression of tumorigenesis.

1.3 pRB Structure

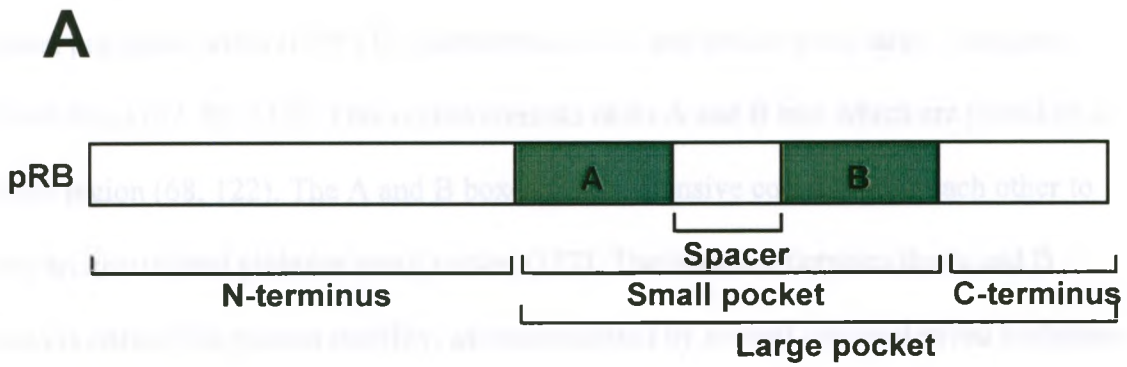
1.3.1 The Pocket Protein Family

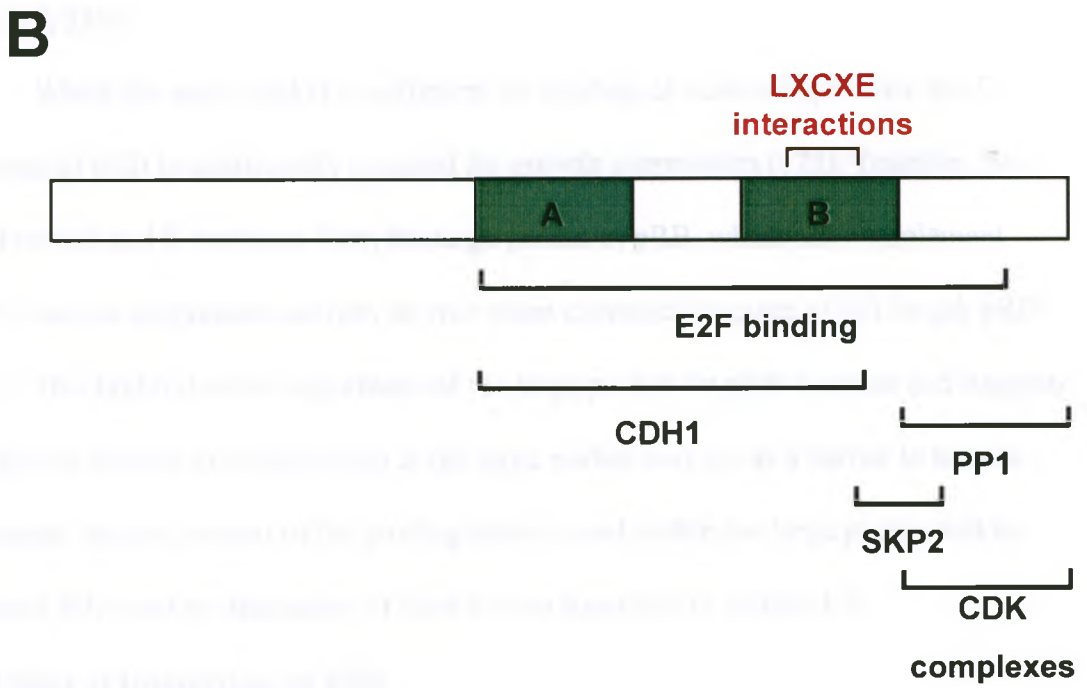
pRB is a member of the pocket protein family which also includes p107 and p130. All three pocket proteins can induce similar phenotypes when overexpressed in culture (31, 232) and in some cancers, upstream regulators of all three pocket proteins are altered (14, 54, 137). The pocket proteins have also been shown to compensate for the loss of one another in at least some cell types (149), so it is possible that in some cellular contexts, all three pocket proteins need to be disrupted to allow tumour progression. However, there are also several important differences between the members of the pocket protein family. For instance, sequence alignment has shown that p107 and p130 are more closely related to one another than to pRB (29). Furthermore, their cellular expression varies. p107 is mainly controlled at the transcriptional level and is expressed in cycling cells. p130 is most abundant in quiescent and differentiated cells, and its levels drop as quiescent cells re-enter the cell cycle (29). In contrast, pRB is expressed in both cycling and non-cycling cells, suggesting that it is ubiquitously required while p107 and p130 are used in more specialized situations. Importantly, pRB is unique among the pocket proteins in that it is the only one that is commonly mutated in human cancers (20). For these reasons, this thesis will focus on the functions and physiological roles of pRB.

1.3.2 General Structure

pRB shares the same general structure as all pocket proteins and is conserved across many different species (29, 122, 148). *RB1* encodes a 928 amino acid long nuclear phosphoprotein (124) which can be divided into N-terminal, "small pocket", and C-terminal regions (Fig. 1.1a) (29). The small pocket was originally identified as the

Figure 1.1 pRB structure and binding partners. (A) The general structure of pRB is outlined. (B) The large pocket of pRB can interact with many cellular proteins. Details of many of these cellular interactions are discussed in section 1.3.3 of the text. (Figure adapted from Classon and Dyson, Exp. Cell Research, 2001 and Dick, Cell Division, 2007)


A
pRB
A
B
Spacer
N-terminus
Small pocket
C-terminus
Large pocket


B
LXCXE
interactions
A
B
E2F binding
CDH1
PP1
SKP2
CDK
complexes

minimal region of pRB that is necessary for interactions with viral oncoproteins such as human papilloma virus (HPV) E7, adenovirus E1A, and simian virus large T antigen (SV40 TAg) (92, 93, 110). This region consists of an A and B box which are joined by a spacer region (68, 122). The A and B boxes make extensive contacts with each other to form an intertwined globular small pocket (122). The interface between the A and B boxes is critical for protein stability, as demonstrated by several cancer-derived mutations that disrupt multiple interaction sites found within the small pocket (88, 113, 159, 160, 187, 219, 229).

While the small pocket is sufficient for binding of viral oncoproteins, the C-terminus of pRB is additionally required for growth suppression (173). Together, the small pocket and C-terminus form the large pocket of pRB, which can complement pRB's tumour suppressive activity *in vivo* when expressed in place of full length pRB (230). This highlights the importance of the large pocket for pRB function and suggests that growth control via interactions at the large pocket may act as a barrier to tumour formation. Below, several of the binding sites located within the large pocket will be outlined, followed by discussion of their known functions in section 1.4.

1.3.3 Sites of Interaction on pRB

(A) Cyclin Dependent Kinase and Protein Phosphatase 1 Binding Domains

While pRB contains no obvious protein interaction domains, it can interact with many cellular proteins (44). Phosphorylation of pRB is mediated by cyclin dependent kinase (CDK) complexes. Both cyclin D-CDK4/6 and cyclin E-CDK2 complexes can bind to and phosphorylate pRB (1). Interactions of cyclin E-CDK2 complexes with their substrates depend on contacts between an RXL motif on the substrate and a hydrophobic

patch on the cyclin (3). Several RXL related motifs are present within the last 100 residues of pRB, and deletion of the RXL motif starting at residue 870 diminishes phosphorylation (2). Under certain conditions, phosphorylation of pRB by cyclin D1-CDK4 also depends on an intact pRB RXL motif. However, cyclin D-CDK4 appears to recognize pRB by a mechanism that is distinct from that used by cyclin E-CDK2 complexes (1). CDK4 recognizes a 19 amino acid long sequence at the C-terminus of pRB that is necessary for binding and CDK4-dependent phosphorylation of pRB (217). Several studies suggest that not only do these two cyclin-CDK complexes interact with different regions of pRB, but that they can act in a non-redundant manner to phosphorylate pRB (52, 134). Taken together, these data identify regions on pRB that are necessary for interactions with multiple cyclin-CDK complexes and their catalytic activities.

Intriguingly, Protein Phosphatase 1 (PP1) can also bind to the C-terminus of pRB. Amino acids 792 to 928 are both necessary and sufficient for binding to PP1α (206). Surprisingly, PP1 appears to bind both hyper and hypophosphorylated forms of pRB (207), suggesting the possibility that it may regulate the phosphorylation status of pRB both by catalytic removal of phosphate groups and by competition with CDK complexes for access to the C-terminus. Further work to define the PP1 interaction site and mechanism of action should shed light on this possibility.

(B) E2F Interactions

pRB does not possess any recognizable DNA binding domains (44). Instead, pRB appears to be recruited to DNA through its interactions with cellular proteins, such as E2F transcription factors, which regulate expression of genes necessary for cell cycle

progression. E2F1 was one of the first identified cellular interacting partners for pRB (82, 109). Further work has identified interactions between pRB and the first four members of the E2F family of transcription factors. Amino acids throughout the large pocket are necessary for interactions with E2Fs (Fig. 1.1). Interestingly, the pRB-binding site on E2Fs appears to overlap with their transactivation domain (44, 120, 226). This suggests that pRB may interact with E2Fs in order to regulate their activity.

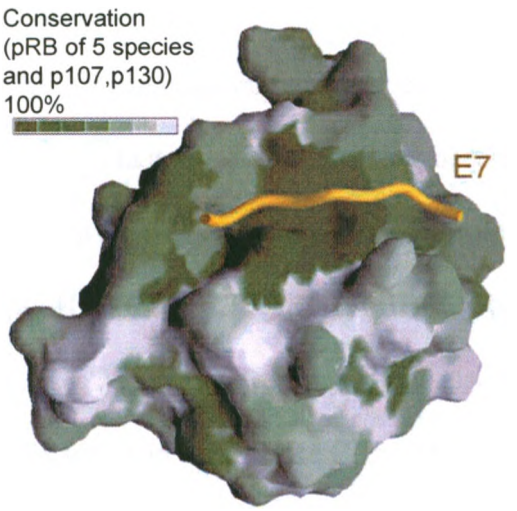
(C) Skp2 Interactions

pRB can also bind to the S-phase kinase-associated protein 2 (SKP2). SKP2 is a subunit for the SKP1-CUL1-F-box protein (SCF) E3 ubiquitin ligase complex. The SCF^{SKP2} complex targets many cell cycle regulatory proteins, such as $p27^{KIP1}$, $p21^{CIP1}$, p57, cyclin E, and p130 for degradation during the G1 to S phase transition. (21, 59, 203, 214). As an F-box protein, SKP2 provides substrate specificity to the SCF complex. However, SKP2 does not contact pRB using its substrate-recognition motif (59). Instead, it interacts with the C-terminus of pRB using amino acids in its N-terminus (104). This suggests that SKP2 may not target pRB for degradation, but that pRB may regulate its activity in some way.

(D) LXCXE Binding Cleft

As mentioned above, diverse classes of viral oncoproteins can interact with the small pocket of pRB. These viral oncoproteins use a conserved LXCXE motif that facilitates binding to pRB (48, 74, 151). This interaction maps to a shallow hydrophobic groove on the B box of pRB, termed the LXCXE binding cleft, which is one of the most highly conserved regions of the protein (Fig. 1.2) (122). Four amino acids within the LXCXE binding cleft make direct contact with the HPV E7 LXCXE peptide: Y709,

Figure 1.2 The LXCXE binding cleft on pRB is highly conserved. The crystal structure of pRB bound to the HPV-E7 LXCXE peptide. Based on this crystal structure, LXCXE-containing proteins are predicted to sit within a shallow groove on the surface of the B box on pRB. This is one of the most highly conserved regions on pRB, as denoted by the dark green shading in the pocket region. Below, the sequence alignment from multiple species is shown. Residue numbering corresponds to the murine amino acid sequence and residues that were mutated in the mutant mice used in this thesis are highlighted in red. (Figure adapted from Lee, et al., Nature, 1998)



Amino acid #:		745	757	
Homologue:	pRB	I**I**VFY**N**SVF**M**QRL	*Homo sapiens*
	p107	L**I**KFY**N**TIY**V**GRV	*Homo sapiens*
	p130	L**I**QFY**N**NIY**I**KQI	*Homo sapiens*
	RB1	I**I**TFY**N**EVF**V**PAA	*Mus musculus*
	RBF	I**I**HFY**N**HTY**V**PLM	*Drosophila malanogaster*
	LIN-35	I**I**KYY**N**IEF**R**DRI	*Caenorhabditis elegans*
	Mat3p	I**I**GFY**N**AVF**V**PAM	*Chlamydomonas reinhardti*

ΔLXCXE
I746A, N750A, M754A

NF
N750F

K713, Y756, and N757 (Y702, K706, Y749, and N750 in mice). N757 also adds to the distinct curvature of the binding cleft. This asparagine residue is located within a helix that forms one side of this shallow pocket, and its presence within the helix creates a kink that gives the cleft its unique shape (122).

Both the convergent evolution of the viral oncoproteins to contact this site and the high conservation of the LXCXE binding cleft across species suggests that it must have important roles within the cell. In fact, the cleft region has been shown to interact with over 30 cellular proteins (reviewed in (44)). Many of these proteins have the ability to modify chromatin, including HDAC1 and 2 , BRG1, hBRM, DNMT1, and Suv39h1 (16, 46, 55, 135, 136, 157, 178, 200, 231). However, this site is also used for binding to a diverse array of proteins: transcription factors like CtIP, HBP1, and ELF1 (119, 142, 208, 218); the anaphase promoting complex through interactions with the CDH1 subunit (13); and Cap-D3, a condensin subunit involved in chromosome condensation during mitosis (129).

While some of these interacting proteins contain an LXCXE-like motif, work is still needed to validate these sites as the region on cellular proteins that make contact with the LXCXE binding cleft. It is also possible that cellular proteins do not use an LXCXE-like motif at all or that it is part of a larger binding motif needed to interact with the LXCXE binding cleft. This is plausible given the fact that some of the interacting partner sequences do not contain a classic LXCXE domain, yet require the binding cleft to interact with pRB (13, 129, 157).

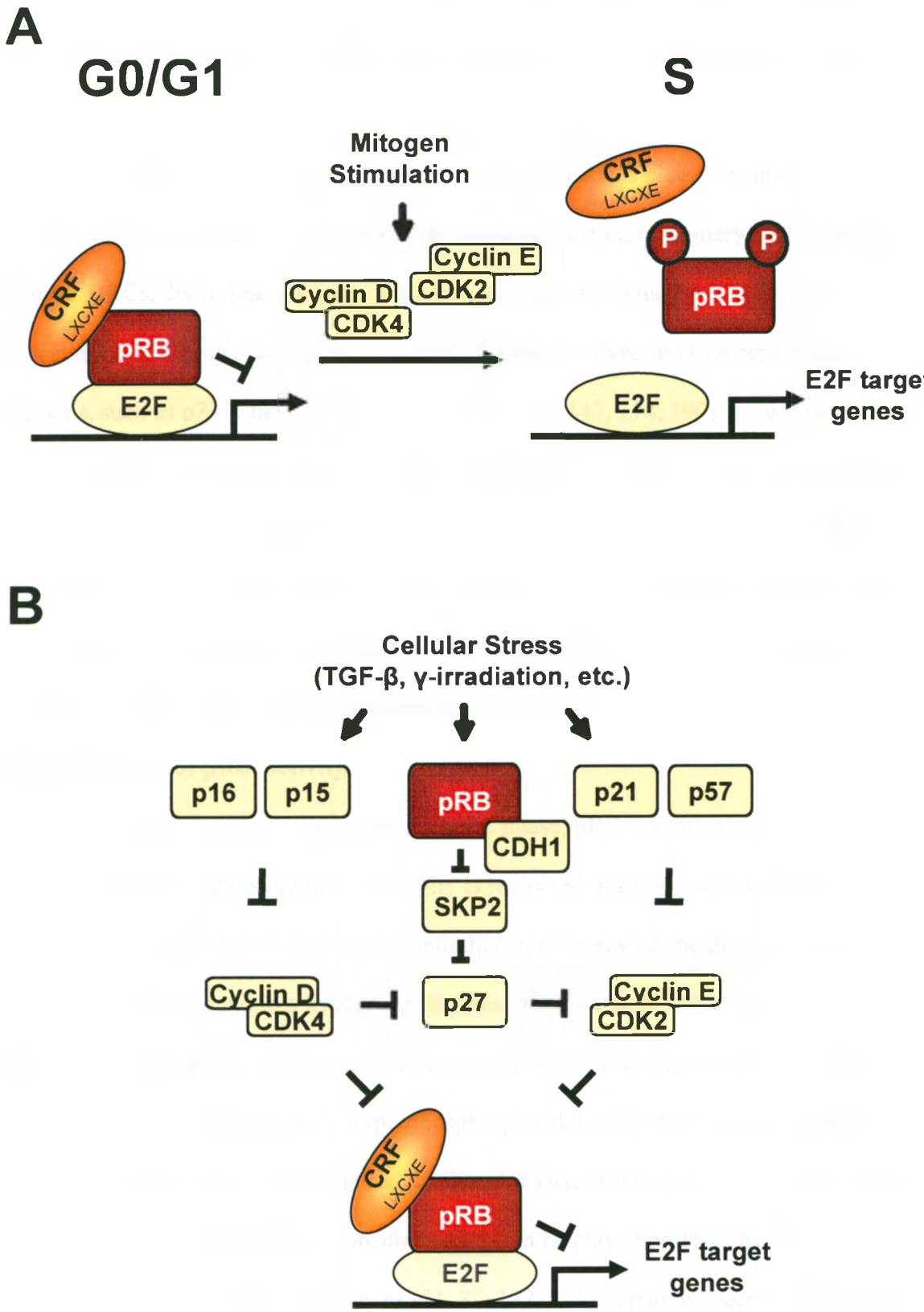
1.4 pRB and Cell Cycle Control

The interaction between pRB and viral oncoproteins also provided some of the first indications about how pRB functions. Viral oncoproteins such as HPV E7 hijack the cellular replication machinery in order to drive viral DNA synthesis. By binding to pRB, it was thought that they were preventing pRB from regulating cell proliferation (37, 151, 220). Direct evidence that pRB is involved in cell cycle regulation came from single cell microinjection experiments (70). Injection of hypophosphorylated pRB into *RB1*$^{-/-}$ Saos-2 cells early in G1 resulted in cell cycle arrest. However, when added in late G1 or early S phase cells, pRB was unable to inhibit progression. Similar experiments using transfection of *RB1* into Saos-2 cells also resulted in a G1 arrest (87). Conversely, pRB depletion accelerated progression into S phase (70, 85). This gave rise to the concept of pRB as an important negative regulator of cell cycle progression.

1.4.1 Transcriptional Control of the Cell Cycle

If viral oncoproteins could regulate pRB activity, it was likely that cellular proteins could play a similar role during the normal cell cycle. In the most basic sense, this role is filled by E2Fs, and pRB acts as a switch turning on and off E2F-dependent transcription (32). During quiescence (G0) or G1, pRB can bind to activator E2Fs, masking their transactivation domain to inhibit the transcription of genes that are necessary for cell cycle advancement (Fig. 1.3a) (56, 81, 86). In support of this, overexpression of E2Fs can drive transcription of target genes and aberrant entry into S phase (reviewed in (45)). pRB can also bind to E2F4, which is a repressor E2F. E2F4 can recruit pRB to E2F target genes to actively repress their transcription (45). Additionally, pRB can recruit chromatin remodelling factors to these repressor complexes through

Figure 1.3 Model of pRB proliferative control. (A) In early to mid-G1 of the cell cycle, pRB can bind E2F transcription factors, preventing the transcription of genes necessary for progression into S phase. Chromatin remodelling factors (CRFs) can also interact with pRB at its LXCXE binding cleft to further repress transcription of these genes. Upon mitogenic stimulation, cyclin dependent kinase (CDK) complexes phosphorylate pRB, releasing E2Fs and allowing the transcription of genes that are necessary for cell cycle progression. (B) In response to cellular stresses such as DNA damage or TGF-β signalling, cells in the G1 phase of the cell cycle can arrest in a pRB-dependent manner. This requires the inhibition of CDK complexes by cyclin dependent kinase inhibitors (CKIs). pRB can also complex with the anaphase promoting complex/cyclosome (APC/C) through the CDH1 subunit to mediate the degradation of SKP2. This prevents the SCF^{SKP2} E3 ubiquitin ligase from degrading $p27^{KIP1}$. The coordinated action of these different pathways results in hypophosphorylation of pRB, which can then recruit CRFs to form repressive complexes at E2F target genes.


A
G0/G1
S
Mitogen
Stimulation
CRF
LXCXE
pRB
E2F
Cyclin D
CDK4
Cyclin E
CDK2
P
P
pRB
E2F
E2F target
genes
B
Cellular Stress
(TGF-β, γ-irradiation, etc.)
p16
p15
pRB
CDH1
p21
p57
SKP2
Cyclin D
CDK4
p27
Cyclin E
CDK2
CRF
LXCXE
pRB
E2F
E2F target
genes

interactions at the LXCXE binding cleft. These chromatin remodelling factors are then thought to modify the chromatin structure to further silence E2F transcription (16, 46, 117, 136, 157, 178, 215, 231).

E2F targets include genes directly involved in the G1/S phase transition such as cyclin E, CDK2, and E2F1, and components of the replication machinery such as MCM proteins, ORCs, thymidine synthase, and DNA polymerase alpha (65, 85, 90, 198). Interestingly, E2Fs can also regulate a subset of genes involved in DNA repair and apoptosis, such as p73, Chk1, Apaf1, and p53 (97, 128, 147, 174, 199), as well as genes involved in mitosis, such as cyclin B1 and B2, Bub1, Cdk1, Cdc20, Smc2 and Smc4, and Mad2 (99, 147). Of note, both pRB and p107 are E2F target genes. This may account for the increase in p107 during S phase, as well as the ability of p107 to compensate for the loss of pRB, which results in deregulated E2F transcription. This may also create a negative feedback loop to prevent aberrant proliferation.

1.4.2 Regulation of pRB Activity

While pRB appears to be active in the G1 phase, pRB is synthesized throughout the cell cycle (19), suggesting that its activity is regulated in some manner. Again the viral oncoproteins provided early insight into this regulation with the discovery that SV40 TAg preferentially bound to a hypophosphorylated form of pRB (130). The demonstration that pRB appeared to be hypophosphorylated during G0/G1 in multiple cell types, and was predominantly hyperphosphorylated during other phases, helped to solidify that pRB activity was related to its phosphorylation status (19, 24, 38, 144). pRB contains 16 putative phosphorylation sites which can be phosphorylated by cyclin D-CDK4/6 and cyclin E-CDK2 complexes (51, 87, 112, 125). Upon mitogenic signalling at

early and mid G1, cyclin D-CDK4/6 complexes phosphorylate pRB at a number of sites. This is thought to allow low level transcription of E2F target genes, including cyclin E. Along with its catalytic subunit, CDK2, cyclin E can then phosphorylate additional sites on pRB at the G1/S phase boundary (Fig. 1.3a) (30). This hyperphosphorylation inactivates pRB, releasing it from E2Fs, and allowing cell cycle advancement.

The half life of pRB ranges from 10 to 12 hours (144, 177) and pulse chase experiments have shown that pRB from a preceding cell cycle is carried over into the next G1 phase (132, 133).Thus, in order for the cell to advance through a subsequent cell cycle, pRB must be returned to its hypophosphorylated state. This dephosphorylation occurs between anaphase and G1 and is mediated by interactions between PP1 and the C-terminus of pRB (4, 47, 131). Protein phosphatase 2A (PP2A) can also dephosphorylate pRB, though this appears to occur in response to growth inhibitory signals such as irradiation and oxidative stress (7, 27). Thus, PP1 and PP2 can dephosphorylate pRB to modulate various aspects of proliferative control. Together, the coordination of phosphatases and CDK complexes regulate the phosphorylation and activity of pRB in a cell cycle-dependent manner.

1.4.3 pRB-Dependent Cell Cycle Arrest

G1 progression and proliferation are limited by extracellular factors that maintain cytostasis. Just as the cell cycle advances by mitogenic stimulation, it can be arrested during G1 by growth-inhibiting cytokines, DNA damage, and other cellular stresses (Fig. 1.3b) (137). Experiments using *Rb1*$^{-/-}$ fibroblasts demonstrated that pRB is essential for the cell cycle arrest response induced by DNA damage or TGF-β1 (17, 78, 84). Fundamentally, these processes work by stimulating members of the CIP/KIP and/or

INK4 families of cyclin dependent kinase inhibitors (CKIs) (141). The INK4 family includes $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$ (encoded by *Cdkn2b, Cdkn2a, Cdkn2c,* and *Cdkn2d*, respectively). These CKIs bind to cyclin D-CDK4 complexes and inhibit their kinase activity (191). The CIP/KIP family is comprised of $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ (encoded by *Cdkn1a, Cdkn1b*, and *Cdkn1c* respectively) which interact with and inhibit cyclin E-CDK2 complexes (190). The cell has evolved complex mechanisms of regulation of CDKs and CKIs in order to tightly control proliferation. For instance, during cell cycle advancement, $p27^{KIP1}$ is sequestered by cyclin D-CDK4/6 complexes, preventing inhibition of cyclin E-CDK2 activity (191). In response to cellular stress, $p16^{INK4a}$ is induced, which has two effects. First, $p16^{INK4a}$ can bind to and inhibit the catalytic activity of its target, cyclin D-CDK4/6. Second, binding of $p16^{INK4a}$ disrupts the cyclin D-CDK4/6- $p27^{KIP1}$ interaction, freeing $p27^{KIP1}$ to inhibit CDK2 activity (191). The specific CKIs used to induce cell cycle arrest vary depending on the stress signal and cell type in question. Regardless of which CKIs are used, the net result is the same: CKIs block CDK phosphorylation of their substrates, which include pRB. Thus, pRB remains in an active state, allowing it to block E2F transcription, and preventing cell cycle progression (Fig. 1.3b).

Several LXCXE interacting proteins have also been implicated in the induction of cell cycle arrest. The general model suggests that pRB recruits chromatin remodelling factors to its LXCXE binding cleft and create a closed chromatin structure at E2F target genes. While many of these interacting partners have been shown to repress E2F-mediated transcription in a pRB-dependent manner, their role in pRB growth arrest has yet to be validated (16, 46, 117, 136, 157, 178, 215). This type of validation is required

given one group demonstrated that while pRB can recruit HDAC activity to repress transcription of E2F target genes, the human SWI-SNF ATP-dependent nucleosome remodelling complex was required for full cell cycle arrest (231). Thus, while some of the known LXCXE-interacting partners are necessary for pRB proliferative control, more work is required to understand the necessity of other interacting proteins in this process. It is possible that pRB can interact with multiple chromatin remodelling complexes, based on the cellular availability of the different complexes and external cell signals. This could allow for some of the cell-type and context specificity of pRB activities.

Interplay between pRB, SKP2, and CDH1 provides an alternative mechanism to induce cell cycle arrest. pRB can inhibit CDK activity and G1 to S progression by increasing the expression of $p27^{KIP1}$ (5, 104). Work in *RB1*$^{-/-}$ Saos-2 cells demonstrated that the increase in $p27^{KIP1}$ levels preceded, and was required for, cell cycle arrest (104). Several elegant studies outlined the mechanism for this growth arrest paradigm. As mentioned earlier, $p27^{KIP1}$ is targeted for degradation by the SCF^{SKP2} E3 ubiquitin ligase. pRB can block this degradation by simultaneously binding SKP2 at the C-terminus, and CDH1 in an LXCXE-dependent manner. This allows APC/C-mediated degradation of SKP2, preventing SCF^{SKP2} from ubiquitinating $p27^{KIP1}$ (13, 104). The accumulated $p27^{KIP1}$ is then free to inhibit CDK activity and block cell cycle progression.

Collectively, this work illustrates the coordinated mechanisms used by pRB and its interacting proteins to induce cell cycle arrest. First, pRB can bind to E2Fs, blocking transactivation of cell cycle genes. Second, it can recruit chromatin remodelling factors to E2F repressor complexes. These chromatin modifiers can then alter the chromatin architecture to further repress E2F-dependent transcription. Finally, pRB can act as a

scaffold to bring an E3 ubiquitin ligase into contact with its substrate to increase the levels of CKIs upstream of E2F-mediated transcription. Together, these studies reveal the complexity of pRB-mediated growth arrest. However, the relative contribution of each of these interactions in a given growth arrest paradigm have been unclear. Work in chapter two of this thesis will address the necessity of pRB-LXCXE interactions in proliferative control in mammary epithelial cells.

1.5 Physiological Roles for pRB in the Mammary Gland

pRB interacts with a myriad of proteins in the cell in order to elicit cell cycle control. While the scope of this thesis prevents an in depth review of all the different functions of pRB, it is becoming abundantly clear that pRB also has roles in a wide variety of cellular processes, including DNA replication, differentiation, apoptosis, and mitotic control (reviewed in (20)). Despite this knowledge, it is still unclear which, if any, of these functions are part of pRB's tumour suppressive actions. This has been difficult to discern because of the high number of interacting proteins and the lack of a clear functional relevance for many of these interactions. Furthermore, most tumour-derived mutations of pRB result in a truncated or unstable protein, making it difficult to determine which specific interactions are most important for its tumour suppressive function (30). While the work of many labs has led to an extensive knowledge of the biochemical functions of pRB, in particular its role in growth control, the development of transgenic and gene-targeted mouse models has allowed the field to address which of these biochemical functions are sufficient and necessary, both during development and tumorigenesis. Genetic studies have provided a plethora of information about pRB

function, however, for the purposes of this thesis, our knowledge of pRB in mammary gland development and cancer will be emphasized.

1.5.1 Roles for Cell Cycle Proteins in Mammary Development

Transgenic mouse models that either overexpress or disrupt expression of key components of the cell cycle machinery have highlighted the importance of the pRB pathway in mammary gland development. Complete loss of cyclin D1 results in viable mice with surprisingly few defects. However, they do have defects in mammary gland development (193, 194). In the absence of cyclin D1, ductal development occurs normally but lobuloalveolar development is impaired and females fail to lactate. This phenotype is recapitulated when the epithelium is transplanted into cleared wild-type mammary glands, demonstrating the epithelial-specific necessity for cyclin D (53, 66). Sufficient mammary-specific overexpression of $p16^{INK4a}$, which inhibits cyclin D-CDK4 activity, results in a similar phenotype (63). This demonstrates that cyclin D1 activity must be carefully regulated for proper mammary gland development. A lack of more dramatic defects at multiple stages of mammary gland development may reflect the ability of other cyclins to compensate for loss of cyclin D1 at certain stages of mammary gland development.

Cyclin D can also sequester $p27^{KIP1}$ to prevent it from inhibiting cyclin E-CDK2 activity. Interestingly, loss of $p27^{KIP1}$ restores normal mammary development in the cyclin $D1^{-/-}$ background (67, 210), demonstrating the need for $p27^{KIP1}$ in mammary gland development. Consequently, several groups developed mammary-specific *Cdkn1b*$^{-/-}$ models. These studies have yielded conflicting results, from increased proliferation and apoptosis to decreased proliferation and impaired development (reviewed in (155)).

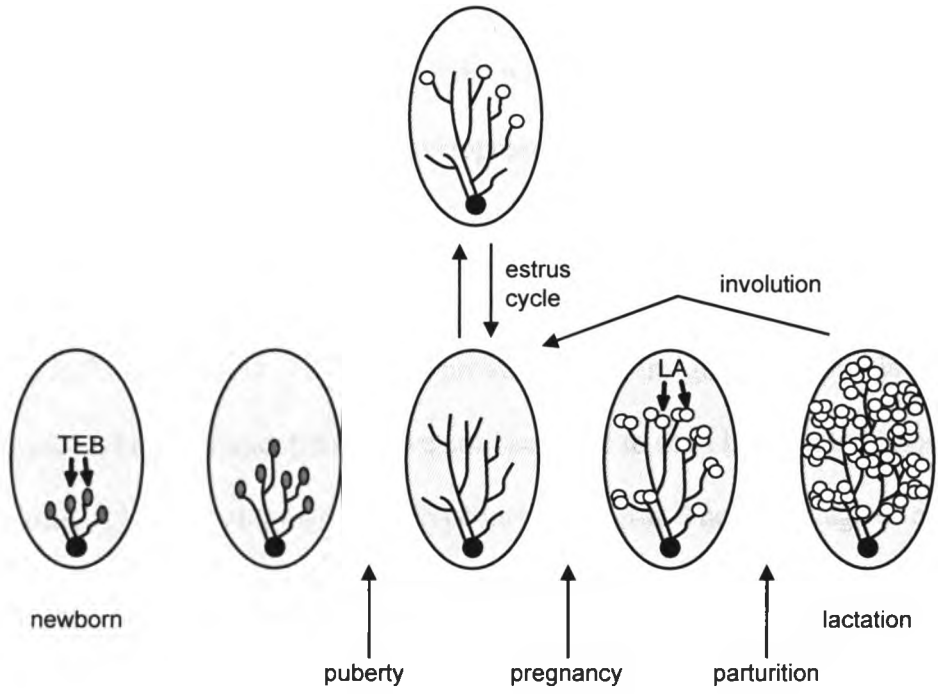
However, even in the studies where loss of $p27^{KIP1}$ resulted in decreased proliferation, $p27^{KIP1}$ heterozygosity was associated with increased proliferation (36, 154). While more work is required to resolve these differences, the data demonstrate that $p27^{KIP1}$ plays a role during mammary gland development.

All of the cell cycle regulators discussed above lie genetically and biochemically upstream of pRB. The cyclin D-CDK4/6 complex phosphorylates pRB in order to inactivate it, while $p16^{INK4a}$ and $p27^{KIP1}$ counter CDK acitivity via several different mechanisms (Fig. 1.3). As the point of convergence among these pathways, it would seem logical that pRB would play an important role in mammary gland development. Studies that address this possibility are discussed below.

1.5.2 Complete loss of pRB

The initial examination of the physiological roles for pRB began with complete ablation of the *Rb1* gene. Germ line loss of *Rb1* is embryonic lethal between day 13.5 and 15.5 of gestation (28, 100, 121). During embryogenesis, pRB is most highly expressed in the nervous system, blood cells, skeletal muscle, and lens (105, 107). Not surprisingly, $Rb1^{-/-}$ mice have pronounced defects in the development of these tissues. Strikingly, $Rb1^{-/-}$ embryos provided with normal placentae can survive to birth (224). Many of the $Rb1^{-/-}$ defects are rescued in these animals, identifying key function for pRB in extraembryonic cell lineages that are required for embryonic development and viability. However, the skeletal muscle defect persists and the mice die shortly after birth (224). This precludes study of the role of pRB in mammary gland development, which occurs almost entirely post-natally (Fig. 1.4).

Figure 1.4 Stages of murine mammary gland development. Mammary gland development occurs almost entirely post-natally. The newborn mouse has only a rudimentary mammary anlage, which sits within the mammary fat pad. At the onset of puberty, hormonal stimulation results in ductal extension from large clusters of highly proliferative epithelial cells, known as terminal end buds (TEBs). In the adult virgin female, the fat pad is filled with ducts. With the influx of hormones and growth factors during pregnancy, there is extensive side-branching, proliferation, and differentiation of the epithelial population to form lobuloalveolar (LA) structures, which become the milk-producing units during lactation. After pups are weaned, the mammary gland reverts back to a virgin-like state through a process of apoptosis and tissue remodelling, known as involution. (Figure adapted from Hennighausen and Robinson, Genes Dev., 1998)



Adapted from Hennighausen and Robinson,
Genes Dev. 12;449-455

Study of heterozygous mice has yielded little more insight into the importance of pRB during mammary gland development and cancer. *Rb1*$^{+/-}$ mice are viable and display very few phenotypic abnormalities (91, 101). These mice develop primarily pituitary tumours by 11 months of age, with almost 100% penetrance. The formation of these pituitary tumours is associated with loss of the wild type *Rb1* allele. However, the onset of pituitary tumours may preclude the development of slower growing mammary tumours in these mice.

1.5.3 *Rb1*$^{-/-}$ Transplant Studies

While *Rb1*$^{-/-}$ and *Rb1*$^{+/-}$ mice have provided some insights into the role of pRB in the cell cycle and development, they have not been able to shed light on the importance of pRB during breast development, which occurs after birth. The only stage of mammary gland development that occurs *in utero* is the formation of the anlage within the surrounding fat pad (Fig. 1.4). However, this structure contains only a few rudimentary ducts near the nipple, and it is not until the onset of puberty that hormonal signals induce elongation and branching of the mammary ducts from terminal end buds (TEBs) into the fat pad (83). This precludes the study of mammary gland development in the embryonic lethal *Rb1* null mice.

It seems likely that pRB would have a role in mammary gland development, given that many other members of the G1 cell cycle machinery are necessary for the formation and function of the mammary gland. Furthermore, pRB, p107, and p130 are expressed in both ductal and alveolar epithelial cells, which is consistent with pocket proteins playing a functional role there (106). To gain further insight into the role of pRB during mammary gland development, one group took advantage of the fact that development of

the primitive mammary anlage begins between day post-coital (dpc) 10 and 11, before the death of *Rb1*$^{-/-}$ embryos (176). They transplanted anlagen from knockout embryos into the cleared fat pads of wild type females (179). Surprisingly, they did not find any defects in mammary gland development or a predisposition to cancer. However, caution must be taken when interpreting these results. First, transplanted mammary glands are not able to make a functional connection to the host nipple, and therefore, the group was unable to assess the ability of these mice to nurse their young. Second, complete loss of pRB can result in the upregulation of other members of the pocket protein family, which can functionally compensate for the loss of pRB (182, 183). Since p107 and p130 are also expressed in the mammary gland, it is possible that they serve to protect this highly proliferative tissue from loss of a single pocket protein. In support of this idea, overexpression of a form of SV40 TAg that specifically disrupts pRB interactions in the mammary gland, which disrupts all three pocket proteins, results in hyperplasia of mammary epithelial tissue and tumorigenesis (73). Therefore, the role of pRB during mammary gland development and tumorigenesis remains an open question. New techniques and models are necessary to address the role of pRB in the function of the mammary gland without compensation by other pocket proteins. This thesis will outline the use of two gene-targeted mouse models that have provided new insight into the importance of pRB, both during development of the mammary gland and during tumour formation.

1.6 The TGF-β Family

1.6.1 The TGF-β Superfamily

TGF-β is the prototypic member of a superfamily of over 30 cytokines that includes the TGF-βs, activins, inhibins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) (79). This superfamily emerged with the evolution of multicellular organisms, potentially as a way to establish communication and order within the organism (225). These proteins are regulated both spatially and temporally to control a diverse number of cellular processes including proliferation, differentiation, migration, immune responses, and cell death. Because of their roles in mammary gland development and cell cycle arrest, this thesis will focus on the function of the TGF-β subfamily of proteins.

1.6.2 The TGF-β Isoforms

The TGF-β subfamily is made up of three related isoforms, TGF-β1, TGF-β2, and TGF-β3. These proteins are highly conserved between species, which suggests they play important roles within the cell. This is further supported by the fact that loss of any of the isoforms in the mouse results in peri-natal death from a variety of defects including inflammatory, cardiovascular, pulmonary, and neural crest abnormalities (108, 116, 172, 185, 192). The differences in phenotype are likely the result of the distinct, though sometimes overlapping, spatial and temporal patterns of expression for each isoform in the developing embryo and adult tissue (145, 164, 186). While each isoform may have unique properties and functions, acting in culture, all three isoforms display similar activity (139, 196). Therefore, for the purposes of this thesis, the term TGF-β will be used to refer to the isoforms in a general sense, unless specified.

1.7 The TGF-β Signalling Cascade

1.7.1 Secretion and Extracellular Regulation of TGF-β

TGF-β is a secreted protein that consists of two identical chains of 112 amino acids linked by disulfide bonds. Each chain is synthesized as the C-terminal region of a 390 amino acid precursor protein. Upon secretion, the pro-region of this precursor, termed the latency associated protein (LAP), is cleaved, yet remains associated with the bioactive dimer, forming a biologically inactive complex (139). LAP can interact non-covalently with latent TGF-β binding partners that facilitate the storage, secretion, and localization of the latent complex (140).

Physiological activation of the latent LAP-TGF-β complex is only partially understood, although many different pathways have been implicated in the process (140). The strongest evidence implicates thrombospondin and the αvβ6 integrin. Thrombospondin appears to activate TGF-β through a conformational modification of LAP, activating a large proportion of TGF-β *in vivo* (33). LAP-TGF-β is also a ligand for the αvβ6 integrin, which appears to induce spatially restricted expression of TGF-β (150). It remains to be determined if there are other mechanisms of TGF-β activation, and what triggers this reaction within the extracellular matrix. It is clear, however, that release of the active ligand initiates a chain of signalling events in the recipient cell that results in a diverse range of biological outcomes in the mammary gland.

1.7.2 Sensing TGF-β Signals – Receptor Binding and Activation

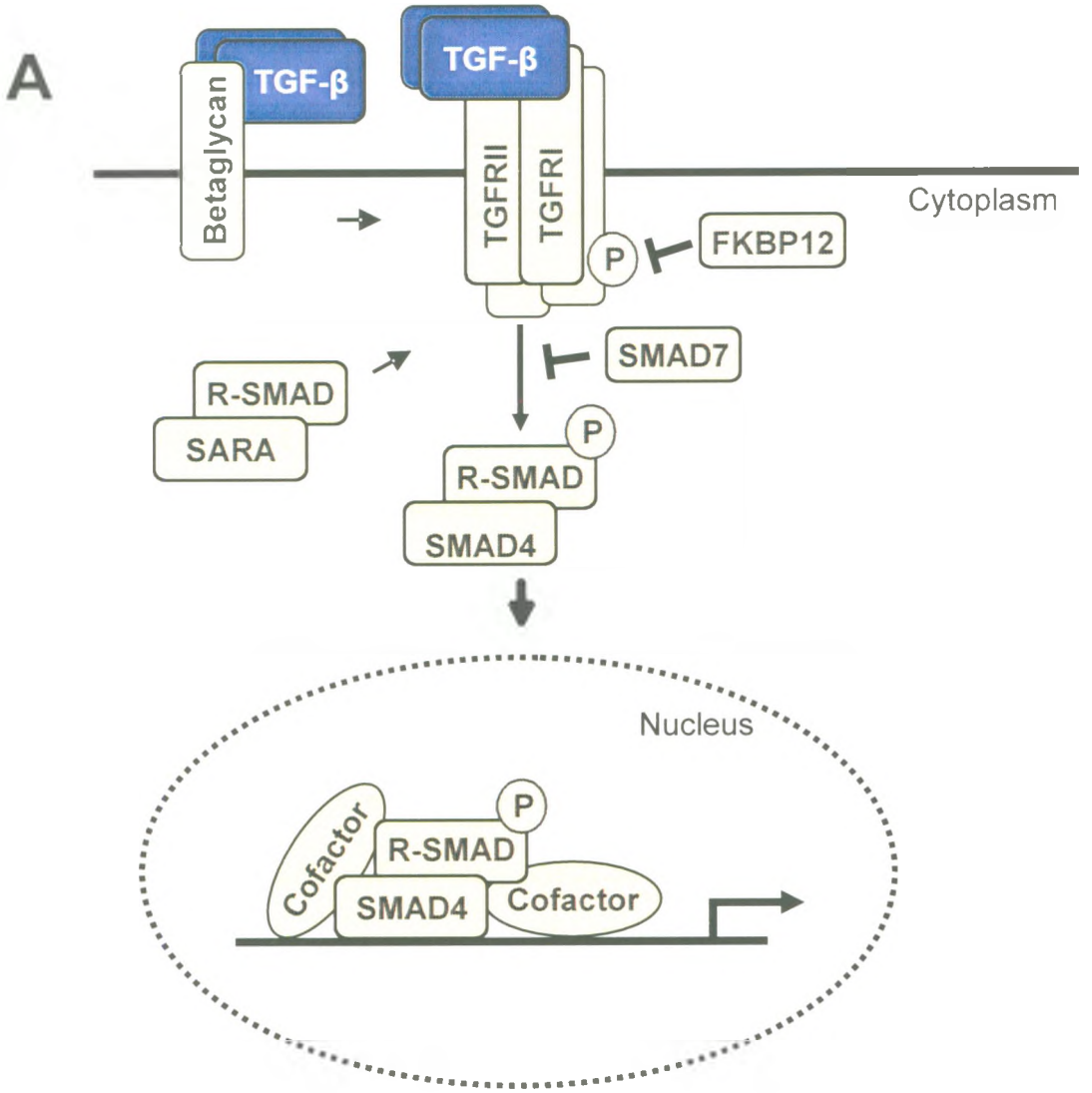
Liberated TGF-β initiates a signalling cascade by binding to a complex of two pairs of receptor subunits, the TGF-β type II (TβRII) and TGF-β type I receptors (TβRI,

also known as ALK5) (Fig. 1.5) (22, 222). A membrane-bound proteoglycan, betaglycan, can aid in the presentation of TGF-β to its receptors (141). Both the TβRII and TβRI are receptor threonine/serine kinases (204, 212), and when complexed to TGF-β, the TβRII phosphorylates threonine and serine residues in the Glycine/Serine (GS) region of the TβRI (223).This phosphorylation event switches this region from acting as a binding site for an inhibitor, FKBP12, to a docking site for its substrates (94, 95).

1.7.3 Propagating TGF-β Signals – Smad Signalling

SMAD proteins are the main substrate of the TGF-β receptor complex. There are at least nine members of the SMAD family, which can be subdivided into three groups: receptor-activated SMADs (R-SMADs), partner SMADs (or co-SMADs), and inhibitor SMADs. SMAD2 and 3 act as the R-SMADs for the three TGF-β isoforms (80). SMAD7, an inhibitory SMAD, can compete for binding at the TβRI receptor, thus blocking recruitment and phosphorylation of the R-SMADs (41). In the basal state, SMAD2 and 3 can also be retained in the cytoplasm through interaction with the Smad Anchor for Receptor Activation (SARA) (213). Interactions with SARA block the exposure of the nuclear import signal on SMAD2 and 3 (227). Receptor-mediated phosphorylation at C-terminal serine residues on SMAD2 or 3 destabilizes SMAD interactions with SARA, exposing the nuclear import signal. The phosphorylation of R-SMADs also augments their affinity for the co-SMAD, SMAD4 (227). The combined effect is rapid accumulation of activated SMADs in the nucleus, where they can interact with a variety of DNA-binding cofactors, co-activators, and co-repressors to regulate target gene transcription.

Figure 1.5 Model of TGF-β signalling to the nucleus. Activated TGF-β is presented to the TGF-β type II and I receptors (TβRII and TβRI) by betaglycan. In the presence of their ligand, two copies of each receptor subunit come together, allowing the phosphorylation of TβRI by TβRII. This frees TβRI to phosphorylate receptor SMADs (R-SMADs). Phosphorylation of R-SMADS allows them to interact with SMAD4 and translocate to the nucleus, where they recruit co-factors to regulate target gene expression. SMADs can be sequestered in the cytoplasm by SARA, while FKBP12 and SMAD7 inhibit receptor and SMAD phosphorylation. These functions are described in sections 1.7.2 and 1.7.3 of the text.


A
TGF-β
Betaglycan
TGF-β
TGFRII
TGFRI
Cytoplasm
P
FKBP12
SMAD7
R-SMAD
SARA
P
R-SMAD
SMAD4
Nucleus
P
Cofactor
R-SMAD
SMAD4
Cofactor

1.7.4 The Transcriptional Response to TGF-β

The relative simplicity of the TGF-β signalling cascade is in apparent contradiction to its ability to regulate multiple cellular processes. The key to the diversity of TGF-β signalling lies in the cellular context and the coordination of a large set of transcriptional regulators that can interact with SMAD proteins (138). TGF-β signalling is context-dependent. Cells of different types or exposed to different conditions express different SMAD-interacting proteins. Thus, distinct groups of cofactors can interact with SMAD proteins in various cellular contexts to either up-regulate or down-regulate transcription (Fig. 1.5). This allows TGF-β to elicit responses from proliferative control to epithelial-to-mesenchymal transition (EMT) to apoptosis. Common co-factors for SMADs include FoxO transcription factors and C/EBPβ (69, 141). Specific combinations of these and other factors are recruited along with SMADs to induce or repress gene transcription. This large number of combinations of SMADs and cofactors that form complexes based on cellular availability allow for the diversity in TGF-β signalling. This point will be illustrated further in the context of the TGF-β cytostatic response (see section 1.8.1A).

1.7.5 Non-canonical TGF-β Signalling Pathways

There are variant branches of TGF-β signalling that do not involve all of the components of the canonical pathway. For instance, TGF-β can activate SMAD2/3 to bind p68, a component of the microRNA processing complex DROSHA, in a SMAD4-independent manner (35). This targets production of miR-21 in vascular smooth muscle cells. The net result is induction of a contractile cell phenotype by downregulation of the suppressor PDCD4. Like many of the non-canonical TGF-β pathways, this phenomenon

has been demonstrated in one specific cell type, so it remains unclear if TGF-β elicits similar responses in other cell lineages.

SMADs mediate most, but not all, TGF-β gene responses. TGF-β has been shown to activate other mediators such as mitogen-activated protein kinases (MAPKs), ERK, Jun N-terminal kinase (JNK), phosphatidylinositol-3 kinase (PI3K), p38, members of the Rho family, and PP2A phosphatases (41, 141). Again, many of these interactions appear to be cell type-dependent. An exception of note is the phosphorylation of PAR6 by TβRII, which frees PAR6 from a preformed PAR6-TβRI complex in many epithelial cell types. This allows PAR6 to dissolve tight junctions in the context of EMT (161). Studies using breast cancer cell lines have demonstrated that TGF-β can lead to the rapid activation of ERK2 and sustained activation of JNK (60, 146). Activation of the MAPK pathway appears to be linked to the ability of cells to respond to TGF-β-dependent growth arrest signals, suggesting that this signalling pathway may be intact in primary mammary epithelial cells. However, little work has been done in primary cells to formally prove this. Thus, the relative impact of these alternative pathways versus SMAD signalling in MECs remains unclear.

1.8 TGF-β Signalling in the Mammary Gland

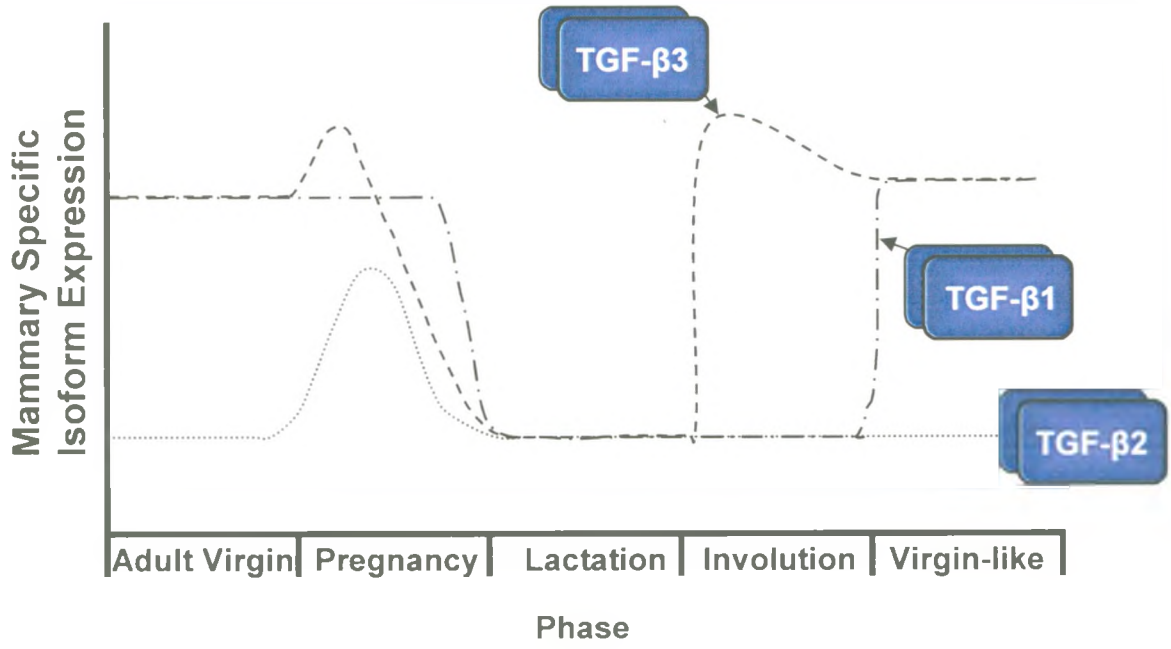
As mentioned above, the specific combination of available nuclear co-factors within the cell determines which genes SMADs will regulate and this allows TGF-β to regulate a wide array of cellular processes within a given tissue. This emphasizes the importance of studying TGF-β function within a given cell of interest, since there is a

high degree of variation from cell type to cell type. For the purposes of this thesis, data relevant to mammary epithelial cells will be discussed.

All three TGF-β isoforms are expressed in the mammary epithelium, although they are expressed differentially throughout mammary gland development and display unique expression patterns (Fig. 1.6) (181). TGF-β1 and TGF-β3 transcripts are strongly expressed in the mammary gland throughout all stages of mammary gland development, with the notable exception of lactation. TGF-β2, on the other hand, is expressed at very low levels except during pregnancy. During ductal outgrowth, both TGF-β1 and TGF-β3 are expressed in epithelial cells of the TEBs, with TGF-β1 expression concentrated at the tips of the end buds and TGF-β3 at the flank region. All three isoforms show overlapping epithelial expression in quiescent ducts. TGF-β1 mRNA expression is fairly consistent throughout pregnancy, while TGF-β2 and TGF-β3 increase throughout pregnancy and peak at dpc 15. However, all three isoforms show a dramatic post-natal reduction in expression (180, 181). In the case of TGF-β1, this correlates with a decrease in overall TGF-β1 protein levels in the ductal epithelium (50). Only TGF-β3 is upregulated during involution, a period of marked apoptosis and tissue remodelling that returns the post-weaned mammary gland to a virgin-like state (Fig. 1.4) (181).

This expression data must be interpreted carefully, since several of the experiments used RNA antisense labelling or used antibodies that do not reliably distinguish between latent and active protein. However, the expression of the TGF-β family transcripts suggests that they play important roles in the various stages of mammary gland development; the temporal and spatial differences in expression patterns suggests

Figure 1.6 Differential expression of TGF-β isoforms in the mammary gland. In the virgin mammary gland, TGF-β1 and TGF-β3 are expressed at high levels, while TGF-β2 is expressed at very low levels. TGF-β1 expression remains fairly constant throughout pregnancy, while TGF-β2 and TGF-β3 peak during mid-pregnancy. Upon parturition, expression of all three isoforms drops to low levels. Expression of TGF-β3 peaks again during involution, while TGF-β1 levels rise again in the quiescent virgin-like mammary gland.


TGF-β3
TGF-β1
TGF-β2
Mammary Specific
Isoform Expression
Adult Virgin
Pregnancy
Lactation
Involution
Virgin-like
Phase

that the various isoforms may have both overlapping and unique signalling roles within the mammary gland.

1.8.1 Mouse Models of TGF-β in the Mammary Gland

Our current understanding of TGF-β signalling in the mammary gland is the culmination of many biochemical, molecular, and biological studies. The overwhelming evidence suggests that TGF-β is crucial for mammary gland development. Cell culture studies have indicated a role for TGF-β in many processes, including growth control, differentiation, EMT, and apoptosis (138). Mouse models have revealed a need for TGF-β during all stages of mammary development: morphogenesis, quiescence, pregnancy, lactation, and involution (11). However, it has been difficult to correlate the various biochemical and molecular data with specific biological outcomes. The fact that multiple cell types secrete and respond to TGF-β signalling in the breast have added a further layer of complexity to this puzzle. Below, the various mouse models that have been used to elucidate the role of TGF-β in the mammary epithelial compartment are briefly outlined, followed by dissection of the various molecular and biological roles associated with TGF-β signalling in mammary epithelial cells.

(A) Gain-of-Function Models

Two basic strategies have been employed to determine the role of TGF-β in the mammary gland: systems where TGF-β signalling is stimulated, and systems where the pathway is disrupted (Table 1.1). Gain-of-function models come in two different varieties. The first is overexpression of a TGF-β isoform, using transgenic models where expression is driven by mammary-specific promoters such as the *mouse mammary tumour virus* (*MMTV*) promoter or the *whey acidic protein* (*Wap*) promoter. *MMTV*

Table 1.1 Known physiological roles for TGF-β in the mammary gland

Genotype	Developmental phenotypes	Tumour phenotypes	Ref
Wap-TGF-β1	• aberrant LA apoptosis during pregnancy	ND	(103, 115)
MMTV-TGF-β1$^{S223/225}$	• reduced ductal outgrowth	• no spontaneous tumours -DMBA • increased latency -*Neu* cross • decreased tumour proliferation • increased lung metastasis	(153, 166, 167)
β-LG-TGF-β3	• aberrant apoptosis of LA cells	ND	(156)
MMTV-TβRI(AAD)	• decreased proliferation • increased apoptosis, especially during late pregnancy	• no spontaneous tumours -*Activated Neu* cross • increased latency • increased metastatic extravasation	(195)
MMTV-Alk5^{T204D}	• delayed ductal outgrowth • reduced apoptosis in TEBs and during involution • nursing defect	• no spontaneous tumours -*Neu* cross • increased lung metastasis	(152)
Tgf-β1$^{+/-}$	• increased proliferation/accelerated ductal outgrowth • accelerated LA differentiation during pregnancy	ND	(50)
MMTV-dnIIR	• lobuloalveolar formation in virgins	• no spontaneous tumours -DMBA • decreased latency	(15)
MMTV-dnIIR	• LA formation in virgins • impaired development during late pregnancy • delayed involution • nursing defect	• spontaneous tumours (median latency 27.5 months) *MMTV-TGF-α* cross • reduced tumour cell invasion	(71, 72)
Wap- dnIIR	• delay in second phase of involution	ND	(12)
Tgfbr2^{MGKO}	• increased proliferation of LA cells • increased apoptosis	• no spontaneous tumours -*MMTV-PyVmT* cross • decreased latency • increased lung metastasis	(57)
Tgf-β3$^{-/-}$ (transplant)	• decreased apoptosis during involution	ND	(156)
MMTV-TβRII(ΔCyt)		• no spontaneous tumours -*Activated Neu* crosses • decreased latency • decreased extravasation of lung metastases	(195)

ND – no data
LA – lobuloalveolar
LG - lactoglobulin
DMBA – 7,12-dimethylbenz[a]anthracene
PyVmT – polyomavirus middle T antigen

targets expression in the mammary gland, but is also expressed in the salivary gland and other tissues. *MMTV* has the benefit of being expressed in virgin as well as lactating mammary glands. *Wap*, on the other hand, is very specific for the mammary gland, but is only expressed during pregnancy and lactation (216). Thus, use of each model results in different spatial and temporal expression of TGF-β in the mammary gland. Since TGF-β is secreted as a latent protein, groups have used a constitutively active form of TGF-β (TGF-$\beta1^{S223/225}$) in order to ensure activity of the transgene (18). However, phenotypic outcomes must be weighed carefully, since TGF-β is no longer subject to all of the normal regulatory processes. To date, *Wap* and *MMTV* transgenic models overexpressing active TGF-β1 have been developed (103, 115, 166). Overexpression of wild typeTGF-β3 has also been achieved using the promoter for β-lactoglobulin, another milk protein (156).

The second approach involves activating downstream components of the TGF-β pathway. Two groups have used this type of strategy to create mice with a constitutively active form of the TβRI (ALK5). Both groups used a mutant form of the TβRI where threonine 204 is substituted for an aspartic acid residue, resulting in constitutive activation of the receptor kinase (221). The first group drove expression of the transgene ($Alk5^{T204D}$) using the *MMTV* promoter (152). The second group introduced two secondary point mutations to prevent binding of the inhibitor FKBP12 (195). Expression of this mutant form of TβRI, termed TβRI(AAD) was also driven by the *MMTV* promoter.

(B) Loss-of-Function Models

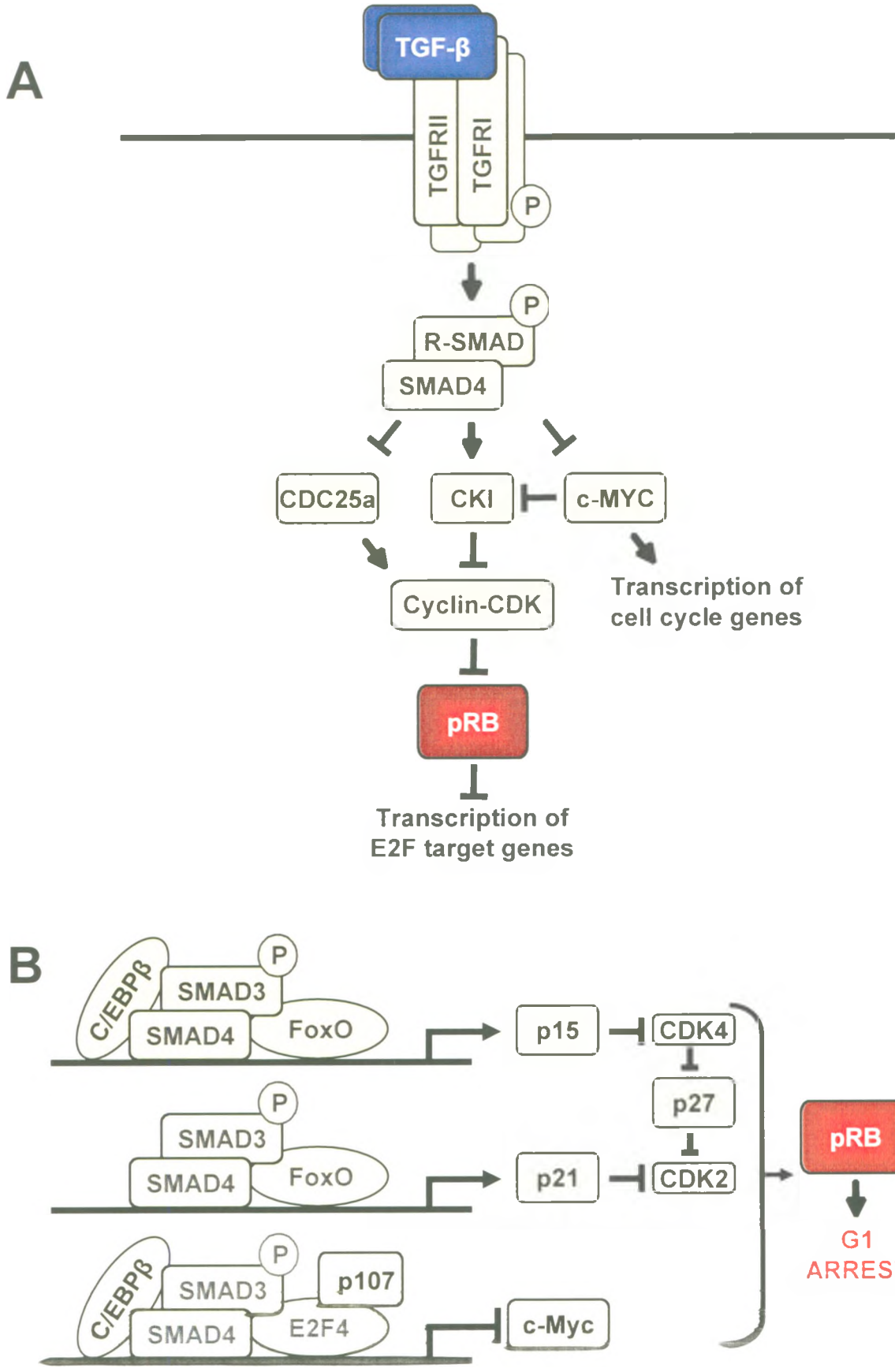
Since loss of any of the TGF-β isoforms results in neonatal death, knockout studies have provided little information about the requirement for these proteins in the developing mammary gland. Nonetheless, studies using *Tgf-β1*$^{+/-}$ females have yielded some clues, since they express only 10% of the TGF-β1 found in wild type mice (50). Two groups have driven expression of a dominant negative type II receptor (dnIIR) using the *MMTV* promoter in order to block TGF-β activity (15, 72). These dnIIRs can bind TGF-β and TβRI, but lack the kinase domain, preventing phosphorylation of TβRI and SMAD proteins (15, 25). Recently, TβRII has also been conditionally knocked out in the mammary epithelium (termed *Tgfbr2*^{MGKO}) (57).

Collectively, this group of mouse models has provided insight into the roles that TGF-β plays in the mammary gland. One of the main challenges remaining is to connect the molecular understanding of TGF-β functions with the phenotypic data that has emerged. Below, the evidence for various TGF-β functions that influence the different stages of mammary development is discussed.

1.8.2 TGF-β-Mediated Growth Arrest

One of the most studied functions of TGF-β is its control of epithelial proliferation through regulation of a cytostatic gene response. TGF-β arrests many cells, including breast epithelial cells, in the G1 phase of the cell cycle (89, 118, 168). This response is elicited by the coordinated upregulation of cell cycle inhibitors and repression of cell cycle promoters (Fig. 1.7) (141). Treatment with TGF-β results in an increase in CKIs, but the choice of CKI appears to be cell type-dependent. For instance, both p15^{INK4b} and p21^{CIP1} are induced in keratinocytes, while only p15^{INK4b} is induced in

Figure 1.7 Model of TGF-β proliferative control. (A) In order to induce a TGF-β cytostatic response, SMAD complexes upregulate the expression of CKIs while repressing c-MYC. Repression of c-MYC has two purposes: first, it prevents transcription of pro-proliferative genes, and second, it releases c-MYC repressive complexes from CKI promoters. CKIs can then inhibit the CDK-dependent phosphorylation of pRB, activating pRB, and inducing a cell cycle arrest. (B) The differential availability of cellular cofactors allows specificity of TGF-β signalling. In the case of TGF-β growth control, activation of both $p21^{CIP1}$ and $p15^{INK4b}$ requires FoxO binding, while $p15^{INK4b}$ induction requires additional binding of C/EBPβ. Repression of c-MYC also requires C/EBPβ recruitment, as well as binding of a p107-E2F4 complex.


A
TGF-β
TGFRII
TGFRI
P
P
R-SMAD
SMAD4
CDC25a
CKI
c-MYC
Transcription of
cell cycle genes
Cyclin-CDK
pRB
Transcription of
E2F target genes
B
C/EBPβ
P
SMAD3
SMAD4
FoxO
p15
CDK4
p27
p21
CDK2
pRB
G1
ARREST
p107
E2F4
c-Myc

human breast epithelial cells (34, 77, 175, 184). $p27^{KIP1}$ also plays an important, though non-transcriptional, role in this process (77, 170, 171, 211). Upon induction of $p15^{INK4b}$ by TGF-β, $p15^{INK4b}$ displaces $p27^{KIP1}$ from CDK4/6, freeing $p27^{KIP1}$ to inhibit CDK2 (175).

Concomitant with the upregulation of CKIs, TGF-β induces repression of c-MYC, a transcription factor that drives proliferation (168, 169). Not only does this prevent transcription of positive regulators of the cell cycle, but also relieves c-MYC-mediated repression of *Cdkn2a* and *Cdkn1a* promoters (encoding $p15^{INK4a}$ and $p21^{CIP1}$), rendering them competent for activation (189, 197). TGF-β has also been shown to repress transcription of the phosphatase Cdc25a in MCF10a mammary epithelial cells (96). This prevents the removal of inhibitory phosphorylation marks on CDK4/6. Together, this coordinated induction of CKIs and inhibition of cell cycle promoters provides an elegant mechanism for TGF-β-mediated growth suppression.

Transcriptional regulation of these genes is SMAD-dependent and involves the recruitment of specific cofactors to each promoter region (Fig. 1.7b). A SMAD-FoxO complex mediates $p21^{CIP1}$ induction, while induction of $p15^{INK4b}$ additionally requires C/EBPβ (69, 188). Repression of c-MYC, on the other hand, involves transcriptional repression from a Smad3/4-p107-E2F4-C/EBPβ complex (23). The use of different co-factor complexes provides an elegant system to initiate a cell cycle arrest: SMAD3/4-p107-E2F4-C/EBPβ repression of c-MYC inhibits the induction of pro-proliferative genes and relieves c-MYC-dependent repression of $p15^{INK4b}$ and $p21^{CIP1}$ while SMAD-FoxO and SMAD-FoxO-C/EBPβ complexes further stimulate CKI expression. CKIs can

then inhibit CDK phosphorylation of their target substrates. Thus, the cellular availability of co-factors allows for a careful orchestration of events that results in cell cycle arrest.

pRB is one of the main targets for CDKs, and as the final barrier to cell cycle progression in G1, it stands to reason that it should play a large part in the TGF-β cytostatic response. Treatment with TGF-β does correlate with the expression of hypophosphorylated pRB (118) and work in *Rb1*$^{-/-}$ MEFs also demonstrated that pRB is required for TGF-β growth inhibition (84). It is presumed that pRB induces growth arrest by an E2F-dependent mechanism, however, at the outset of this work, little had been done to prove or disprove this theory. In chapter 2, I examine how pRB elicits the TGF-β cytostatic response.

Regardless of the specific mechanism, the physiological data demonstrates that TGF-β controls proliferation *in vivo*. Overexpression of *MMTV-TGF-β1* inhibits ductal outgrowth in the virgin mouse (166). Conversely, *Tgf-β1*$^{+/-}$, *Tgfbr2*$^{MGKO/MGKO}$ and *MMTV-dnIIR* mice display increased proliferation of the ductal epithelium (49, 50, 57, 71, 72). Thus, evidence from both the gain-of-function and loss-of-function models supports an important role for TGF-β in growth control of the mammary epithelial population.

1.8.3 TGF-β Signalling in Differentiation

Members of the TGF-β family have been implicated in the differentiation of many vertebrate tissues, including the immune, haematopoietic, neuronal, and epithelial compartments (39). At the molecular level, TGF-β has been shown to repress members of the Inhibitor of Differentiation (ID) family of proteins in multiple cell lines, including the immortalized MCF10a breast cell line (111). ID proteins act in a conserved manner to

negatively control cell cycle arrest and commitment to differentiation in many cell lineages (43). In the case of ID1, TGF-β controls expression both directly and indirectly. Initial treatment with TGF-β results in the upregulation of the transcriptional repressor, ATF3, which then acts as a cofactor for SMAD3-dependent repression of *ID1* (111). Consistently, ID1 expression decreases in SCp2 mammary epithelial cells stimulated to differentiate by lactogenic hormone treatment (42), suggesting that TGF-β inhibits ID1 in order to induce differentiation.

Physiological evidence also suggests that TGF-β is important for differentiation in the mammary gland, although it is somewhat at odds with the biochemical data. Roles in differentiation are most clearly seen during pregnancy and lactation in the mammary gland, when epithelial cells undergo differentiation into lobuloalveolar structures that become the milk producing units following parturition (Fig. 1.4) (176). Ectopic expression of a constitutively active form of TGF-β1 leads to stunted alveolar development during pregnancy (115) while *MMTV-dnIIR* virgin females develop lobuloalveolar structures and express milk protein prematurely (15, 72). Similarly, TGF-β1 suppresses the expression of the milk protein β-casein in mammary explants and murine mammary epithelial cell lines stimulated with lactogenic hormones (143, 180, 202). The concept that TGF-β suppresses differentiation correlates well with the changes in expression levels of the three isoforms during pregnancy and lactation (Fig. 1.6). Together, these data suggest that members of the TGF-β family prevent full differentiation of epithelia into milk producing units, and downregulation of the isoforms at birth allows lactation to commence.

The biochemical and mouse model data present an apparent paradox. How can TGF-β both allow differentiation of MCF10a cells while preventing differentiation in the mammary gland? The ability of TGF-β to inhibit or stimulate differentiation of mammary epithelial cells likely depends on the cellular environment. TGF-β1 can restrain proliferation of ER-α-positive cells and in turn, ovarian hormones can regulate ductal and alveolar proliferation (49, 50). Furthermore, work on mammary explants demonstrates that TGF-β inhibits casein secretion in differentiating mammary gland explants, but not ones isolated from lactating females (202). Therefore, TGF-β's role in differentiation is likely to be influenced by the combinations of hormones and growth factors that are present in the microenvironment at each stage of development.

1.8.4 TGF-β Signalling in Apoptosis

TGF-β can also trigger apoptosis, although the environmental stimuli that induce this process have not been identified. NMuMG mammary epithelial cells can be induced to apoptose after several days of TGF-β1 exposure (64), via undefined mechanisms. Work with other cell types has implicated both SMAD-dependent and -independent pro-apoptotic mechanisms. SMADs can regulate expression of genes implicated in the apoptotic response such as *TIEG1*, *GADD45*, *BIM*, *DAPK*, and *SHIP* (163). In lymphocytes and hepatocytes, the TβRII can also directly associate with the FAS receptor adaptor protein, DAXX, in response to TGF-β and during apoptosis (165). DAXX then mediates activation of the JNK-p38-MAPK kinase pathway, resulting in the expression of genes involved in the apoptotic response (165). Which, if any, of these pathways are used to induce apoptosis in the mammary epithelial compartment have yet to be determined.

While the exact pro-apoptotic mechanism remains elusive, there is strong physiological evidence that TGF-β plays an important pro-apoptotic role in the ductal epithelia. This is most evident during involution of the mammary gland, the process of programmed cell death and remodelling of the mammary epithelium to a virgin-like state after weaning. TGF-β3 is rapidly induced upon weaning and precedes apoptosis (156). Expression of *β-lactoglobulin-TGF-β3* in the alveolar epithelium induces apoptosis of these cells, but not tissue remodelling. Furthermore, transplantation experiments demonstrated that loss of TGF-β3 in the mammary tissue leads to prolonged involution (156). Studies driving TGF-β3 expression with the *Wap* promoter demonstrated that it also plays a pro-apoptotic role in the later stages of involution (12). Conversely, blocking TGF-β signalling using the *MMTV-dnIIR* results in delayed involution of the mammary gland (71). Interestingly, in *Wap-TGF-β1* mice, alveolar development was compromised because of an increase in apoptosis during pregnancy (103, 115), implying that other isoforms may play a role in mediating apoptosis. However, this may result from the inappropriate expression of TGF-β1 in the mammary gland rather than a true physiological role in apoptosis. Further work will be required to ascertain this. While it is possible that multiple TGF-β isoforms can induce apoptosis within the mammary gland, the expression patterns strongly implicate TGF-β3 as the isoform responsible for apoptosis during involution.

There is also evidence that TGF-β can act as a pro-survival factor in the mammary epithelial compartment. NMuMG cells become resistant to apoptosis after prolonged exposure to TGF-β, in contrast to the pro-apoptotic response induced by shorter-term exposure to TGF-β (64). The reason behind this shift is not immediately clear, since

downstream components of each pathway are expressed at low levels in these cells. However, there is some indication from mouse models that TGF-β can also activate survival pathways. For instance, in mammary glands of females from *MMTV-Alk5*^{T204D} females, there are decreased levels of apoptosis both in the TEBs and during involution (152). MECs derived from these mammary glands have increased PI3K and AKT activities. In contrast, in the *MMTV-TβRI(AAD)* model, there was increased apoptosis of the mammary epithelium (195). While this discrepancy has not been reconciled in full, it is apparent that TGF-β can induce or suppress apoptosis. However, much more work is needed to clarify the cellular and environmental contexts where TGF-β induces each pathway and the mechanisms behind them.

1.8.5 TGF-β Signalling in EMT, Invasion, and Motility

TGF-β is also a potent inducer of the transdifferentiation of epithelial cells to mesenchymal cells. EMT occurs naturally during vertebrate development as well as during disease states such as fibrosis and cancer (39). TGF-β promotes EMT through Smad-dependent and –independent mechanisms. First, SMAD-mediated expression of high-mobility group A2 (HMGA2) can induce expression of SNAIL, SLUG, ZEB1 and 2, and TWIST, which are potent repressors of the cell adhesion receptor, E-Cadherin (79, 209). As outlined above, TβRII can also phosphorylate PAR6, which leads to the dissolution of cell junction complexes (161). Recently, the E3 ubiquitin ligase TRAF6 has also been shown to interact with the TGF-β receptor complex and treatment with TGF-β1 induces EMT in NMuMG cells in a TRAF6-dependent manner (228). Finally, activation of the RAS pathway in conjunction with TGF-β appears to be required for

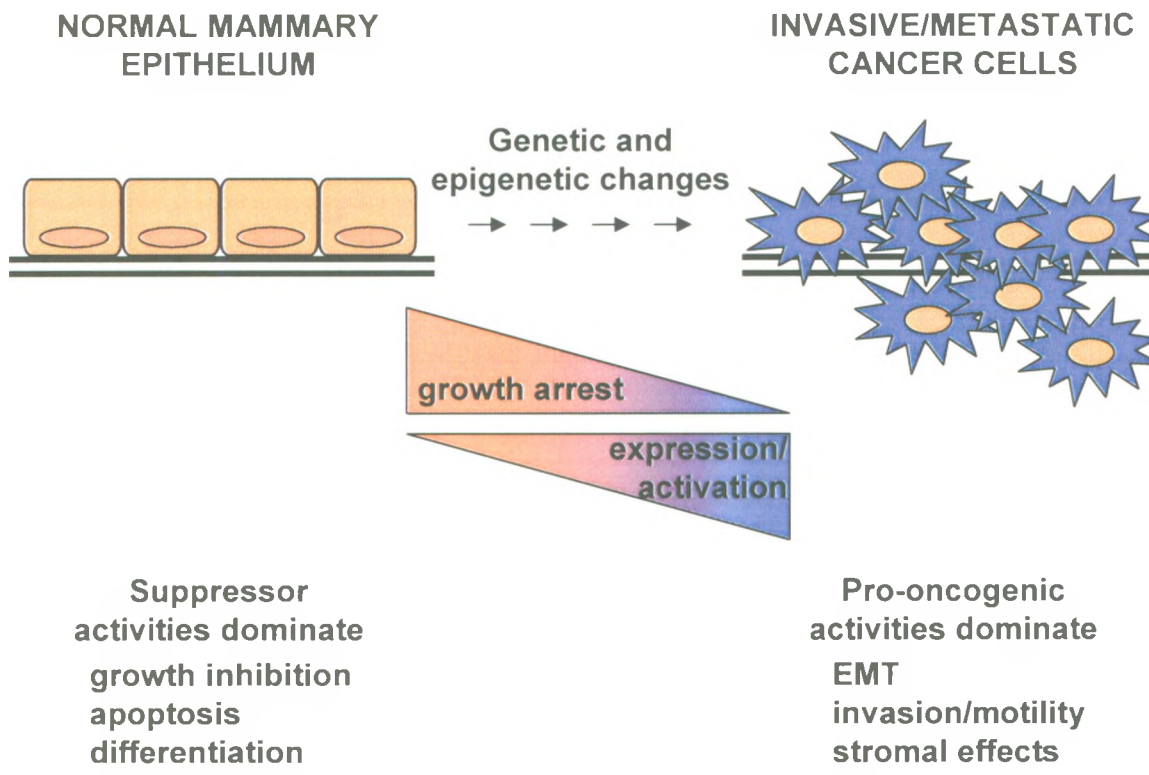
induction of complete EMT and invasion in the EpH4 murine mammary epithelial cell line (102, 158).

The net result of these as well as TGF-β-independent effects is the production of a more mesenchymal type cell with acquired motility and invasive properties. In the most extreme case, TGF-β can further promote the differentiation of these fibroblastic-like cells into myofibroblasts, which are contractile and can produce pro-metastatic factors, such as matrix metalloproteinases, vascular endothelial growth factor, and chemokine receptors (39). While there are no clear examples of TGF-β-mediated EMT during normal mammary gland development, it appears to play a role in invasion and metastasis of cancer cells (see section 1.9.2 and 1.9.3). In the future, it will be important to determine if signalling pathways are differentially required for EMT during development and pathogenesis. It was also previously unknown if primary mammary cells induce an EMT response in a similar manner to immortalized cell lines. This concept was addressed in this thesis.

1.9 TGF-β and Breast Cancer

As outlined in the preceding sections, careful orchestration and balancing of pro- and anti-growth TGF-β signals is critical for mammary gland development and homeostasis (Fig. 1.8). However, in many cancers, including breast cancer, this balance is disrupted. In some cases genetic mutations within the core TGF-β machinery result in disruption of all TGF-β signalling, and are associated with increased risk of breast cancer (reviewed in (10). More commonly, core signalling is retained, and in many cases elevated, suggesting there is a lack of selective pressure to lose TGF-β signalling

Figure 1.8 Duality of TGF-β signalling. TGF-β is involved in many signalling pathways, both during development and disease states. In normal mammary epithelial cells, homeostasis is maintained by a balance of tumour suppressive TGF-β functions and those considered pro-oncogenic. In contrast, the pro-oncogenic activities of TGF-β seem to dominate in cancer cells. At the same time, many breast cancer cell lines become insensitive to TGF-β growth arrest, leading to the hypothesis that TGF-β growth control is an important tumour suppressive mechanism, and that specific loss of the cytostatic response allows expression of TGF-β to drive breast cancer progression and metastasis. The different functions of TGF-β and their roles in cancer prevention and progression are discussed in sections 1.8 and 1.9 of the text. (Figure adapted from Roberts and Wakefield, Proc. Natl. Acad. Sci. USA, 2003)


NORMAL MAMMARY EPITHELIUM
INVASIVE/METASTATIC CANCER CELLS
Genetic and epigenetic changes
growth arrest
expression/ activation
Suppressor activities dominate
growth inhibition
apoptosis
differentiation
Pro-oncogenic activities dominate
EMT
invasion/motility
stromal effects

completely. In fact, a growing body of evidence suggests that in breast cancer, TGF-β tumour suppressive functions are lost, while the majority of TGF-β signalling is retained and subverted to drive tumorigenesis and metastasis.

1.9.1 TGF-β as a Tumour Suppressor

Early in breast cancer progression, TGF-β appears to protect against tumour formation and growth. While *MMTV-dnIIR* mice either do not develop spontaneous tumours (15) or do so after a very long latency (71), *MMTV-dnIIR* females have an increased rate of tumorigenesis when treated with 7,12-dimethylbenz[a]anthracene (DMBA) (Table 1.1) (15). *Tgfbr2*$^{MGKO/\,MGKO}$ females do not undergo spontaneous tumorigenesis either, but develop tumours faster than wild type animals when crossed with the mouse mammary tumour virus-polyomavirus middle T antigen (*PyVmT*) transgenic strain (57). Finally, crossing mice with a truncated form of TβRII to mice expressing an active form of the *Neu* proto-oncogene in the mammary gland leads to earlier tumour onset (195). Conversely, when crossed into the *Neu* oncogenic background, overexpression of active TGF-β results in decreased proliferation of tumours (153). Similarly, active TGF-β protects against mammary tumour formation in mice treated with DMBA (167). Constitutive activation of TβRI led to delayed tumour formation in one study (195), although it did not affect primary tumour latency in another (152). This discordance potentially reflects the fact that the *MMTV-Alk5*^{T204D} mutant can still be bound by the inhibitor FKBP12, while *MMTV- TβRI(AAD)* cannot (Table 1.1). Further, Muroaka-Cook, *et al.* crossed the *MMTV-Alk5*^{T204D} mutant into the *Neu* background, which overexpresses wild type *Neu*, while Siegel, *et al.* used constitutively active forms of Neu in their crosses with *MMTV- TβRI(AAD)* mice, which may have

influenced the results. Nevertheless, combined with the loss-of-function models, the preponderance of data suggests that TGF-β can suppress primary mammary tumour progression.

1.9.2 Pro-tumorigenic roles for TGF-β

While the majority of tumour studies suggest that TGF-β has tumour suppressive properties, the same studies suggest that overexpression of TGF-β signalling can drive tumorigenesis and metastasis (Table 1.1). Activation of TβRI or overexpression of active TGF-β1 promotes the formation and extravasation of pulmonary metastases driven by overexpression of wild type Neu or constitutively active forms of Neu (152, 153, 195). In contrast, expression of the truncated TβRII protein protects against the invasion of *Neu*-induced lung metastases into the lung parenchyma (195). A truncated version of TβRII also reduces tumour cell invasion when crossed to mice expressing transforming growth factor α (TGF-α) (71). Collectively, these studies demonstrate that TGF-β can behave as an oncogene as well as a tumour suppressor.

1.9.3 Duality of TGF-β Functions in Breast Cancer

The wealth of information from both cell culture and mouse models demonstrates that TGF-β can protect against tumour formation in the mammary gland during the initial stages of breast cancer, but can also result in increased metastasis. How does this happen? The current dogma in the field says that the TGF-β cytostatic response protects cells against tumour formation, and that in later stages of progression, this cytostatic response is lost, leaving other aspects of TGF-β signalling like EMT, invasion, motility, as well as paracrine responses, to drive metastasis (Fig. 1.8) (10, 138, 162). Many tumour cells, including breast cancer cells, do become resistant to TGF-β-mediated growth inhibition

over time in culture. Furthermore, short term culture of breast cancer cells taken from pleural effusions of patients with metastatic disease demonstrated a partial or complete loss of TGF-β growth suppression (69). These samples lacked $p15^{INK4b}$ induction and c-MYC repression despite retaining other TGF-β gene responses. This correlates with many studies demonstrating that members of the G1 cell cycle arrest machinery are aberrantly expressed in breast cancers (54). This suggests that the cytostatic response is specifically severed during breast cancer progression. Furthermore, tumour cells often have increased production of one or more of the TGF-β isoforms, which renders these cells more invasive and metastatic (138). Certainly, there is a wealth of data implying that TGF-β proliferative control is an essential aspect of TGF-β tumour suppression. That is not to say, however, that the less well studied roles of apoptosis and differentiation may not also protect against tumour formation and progression. Since all mouse models to date have relied on manipulation of the entire TGF-β pathway, it has been challenging to conclusively determine the role of each TGF-β response during tumour formation, progression, and metastasis. Thus, the TGF-β mechanisms of tumour suppression have remained unknown. In this thesis, the importance of TGF-β growth control as a tumour suppressive mechanism in the mammary epithelial compartment was examined.

1.10 Gene-targeted Strategies to Study the Function of the pRB LXCXE Binding Cleft

The primary interest of our lab is to understand how pRB functions. As stated above, it is still not clear which of pRB's functions are necessary for its tumour suppressor activities. This has been complicated by the large number of proteins that bind

pRB at multiple binding sites. In order to understand how pRB functions, our lab has taken a structure function approach, disrupting specific binding sites on pRB, allowing the study of their significance in isolation. Of particular interest to this thesis, previous work has yielded mutant forms of pRB where the LXCXE binding cleft are disrupted (*Rb1*$^{\Delta L}$ and *Rb1*^{NF}). Initial characterization of these proteins demonstrated that RB$^{\Delta L}$ and RB^{NF} cannot bind to viral oncoproteins or to known LXCXE binding partners, but still interact with E2F proteins (26, 98). Asynchronously dividing MEFs derived from *Rb1*$^{\Delta L}$ mice express normal levels of E2F target genes, suggesting that LXCXE interactions may be disepensable for cell cycle control in proliferating cells. Interestingly, *Rb1*$^{\Delta L/\Delta L}$ MEFs are unable to repress E2F-dependent transcription in response to serum starvation, although this treatment does induce a G1 arrest (98, 205). Similarly, pRB^{NF} only partially inhibits E2F transcription in luciferase-based assays, but is able to induce growth arrest when transfected into *RB*$^{-/-}$ Saos-2 cells (26). In contrast, both E2F target repression and negative proliferative control are lost in *Rb1*$^{\Delta L}$ MEFs treated with γ-irradiation or in response to oncogenic stress (205). This suggests several intriguing possibilities. First, this suggests that pRB-LXCXE interactions are dispensable in normal cycling cells, but are necessary for E2F transcriptional control in response to growth arrest signals. This could explain why *Rb1*$^{\Delta L/\Delta L}$ mice are viable, but display more subtle defects (8, 9, 98, 205). Second, it implies that the contribution of LXCXE interactions to a growth arrest signal may be context-dependent, and more globally, that there may be multiple means by which pRB induces growth arrest. Finally, it suggests that pRB proliferative control mediated by its LXCXE binding cleft may be an important tumour suppressive

mechanism. In this thesis, these issues will be explored within the context of the mammary gland in mice.

1.11 Objectives

As discussed in this introductory chapter, both pRB and TGF-β interact with many cellular proteins to elicit a variety of cellular responses and have tumour suppressive properties. However, relatively little is known about which of these responses are needed to exert these tumour suppressive effects (20, 162). In particular, because of the multitude of binding partners that can affect pRB activity, it has been difficult to discern which of these interactions are important to prevent tumour formation. Furthermore, while most components of the G1 regulatory pathway are involved in mammary gland development, the importance of pRB in this process remains unclear. The aim of this thesis was to use knock-in mouse models where the LXCXE binding cleft on pRB is disrupted, to study the role of LXCXE interactions in mammary gland development and tumorigenesis.

First, I examined the effects of loss of pRB-LXCXE interactions on mammary gland development. I reasoned that $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mice would allow an opportunity to examine both mammary development and function because they are viable and do not overexpress the other pocket proteins. I hypothesized that, like other members of the cell cycle machinery, pRB is necessary for mammary gland development. Since pRB plays a role in TGF-β proliferative control, and TGF-β is necessary for proper mammary gland development, I also endeavoured to determine any functional

connections between the two pathways in mammary gland development. These results are discussed in detail in chapter 2.

The findings in chapter 2 demonstrated that pRB is critical for proper TGF-β growth control and mammary gland development. pRB has been implicated in TGF-β growth control, but not other aspects of TGF-β signalling. However, it is possible that pRB has unidentified roles in other TGF-β pathways or can influence them indirectly. The next objective was to use a combination of molecular and biological techniques to examine other TGF-β pathways in the mammary gland to determine if they were affected by loss of pRB-LXCXE interactions. The results of this work are discussed in chapter 3.

Finally, I explored the necessity of interactions at the LXCXE binding cleft for tumour suppression by pRB. The findings in chapter 2 and chapter 3 suggested that pRB is necessary for TGF-β proliferative control but not other aspects of TGF-β signalling, like TGF-β-dependent apoptosis and differentiation. To my knowledge, this is the first time that TGF-β growth inhibitions has been disrupted in isolation. This allowed me to address whether TGF-β growth inhibition is necessary to protect mammary cells from cancer progression. Three different approaches were used to address this question. First, mice were treated with DMBA, since TGF-β is known to protect against tumour formation in this model. Two genetic crosses were also used to explore the role of pRB proliferative control in tumour suppression: *Wap-p53*^{R172H}, which results in genomic instability (126, 127) and *Neu,* which has been used to define roles for TGF-β in mammary tumour development and lung metastases (75). This has allowed me to address pRB's role in proliferative control in both primary tumour formation as well as metastasis. These results are discussed in detail in chapters 3 and 4.

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Chapter 2: A functional connection between pRB and TGF-β in growth inhibition and mammary gland development

2.1 Introduction

TGF-β is a potent inhibitor of mammary epithelial cell proliferation and plays a key role in mammary gland development (55). Specific loss of its ability to arrest proliferation is considered an essential step in the development of breast cancer, while its ability to induce other cellular changes is maintained and used to drive oncogenesis (30). However, selective loss of TGF-β growth inhibition responses rarely occur at the level of the TGF-β receptor or SMAD proteins, which are common to many aspects of TGF-β signalling (55). Instead, disruption of the TGF-β cytostatic response often occurs at the level of CDK regulation, leaving other pro-tumorigenic aspects of TGF-β signalling intact (56). This underscores the importance of understanding all cell cycle regulatory targets of TGF-β, as they are candidates for mutation in breast cancer (16).

TGF-β suppresses proliferation by inducing a growth arrest in the G1 phase of the cell cycle (54, 55). TGF-β signalling results in transcriptional repression of pro-proliferative genes such as c-Myc (65) and CDC25A (38), and concomitantly, transcriptional induction of the CDK inhibitors $p21^{CIP1}$ (12) and $p15^{INK4b}$ (31), as well as stabilization of the $p27^{KIP1}$ protein (66). This creates a global inhibition of CDK activity that leads to dephosphorylation and activation of the retinoblastoma protein (pRB) in G1 (56). Despite pRB's requirement in TGF-β induced cell cycle arrest (34), it is rarely considered a component of this signalling pathway (55). Because pRB controls the final regulatory step before commitment to DNA replication (87), activation of any pathway

that results in G1 arrest regulates pRB function, suggesting that pRB uses the same G1 arrest mechanism independently of the initial stimulus that causes it. However, most experiments investigating pRB's growth arrest mechanism have relied on its re-expression in the *RB1* deficient Saos-2 cell line as the arrest stimulus (6, 7, 15, 35, 68, 76). The artificial nature of these experiments leaves open the possibility that pRB may have unique activities that are invoked depending on the growth arrest signal.

Mice deficient for TGF-β1, -2, or -3 die as embryos or neonates due to extensive defects in development (42, 45, 67, 74, 79). Strikingly, disruption of TGF-β signalling specifically in the mammary gland causes defects such as hyperplastic ductal epithelium and defective nursing (20, 21, 28, 29, 41). The relative importance of TGF-β growth inhibition compared to its other morphogenic signals in mammary gland development is unclear (22, 77). However, many targets and components of TGF-β's cytostatic signalling cascade, such as cyclin D1 and $p27^{KIP1}$, also participate in controlling mammary epithelial proliferation during development (25, 27, 48, 59, 80, 81). Surprisingly, it has been suggested that pRB may be dispensable for this process (73). Complete loss of pRB function in mice results in embryonic lethality shortly after the formation of the mammary anlagen (8, 40, 49). To study postnatal mammary development, Robinson, *et al.* transplanted $Rb1^{-/-}$ anlagen into clarified fat pads of wild type females (73). They found no differences in mammary gland development or tumour formation. However, transplant experiments have a number of shortcomings. For example, transplanted anlagen do not form a connection with the nipple, preventing a complete study of mammary function. Furthermore, complete loss of pRB results in upregulation of the related protein p107, which can compensate for some aspects of pRB

function (37, 71). This highlights our limited knowledge of pRB function in mammary gland development and emphasizes the need for more sophisticated approaches to study its potential role in this tissue.

To exert control over proliferation, pRB interacts with E2F transcription factors and co-repressor proteins to block expression of genes that are involved in cell cycle progression (5, 18, 82, 83). Most co-repressors contact pRB using an LXCXE peptide motif. This allows pRB-E2F complexes to recruit chromatin remodelling factors such as DNA methyltransferases, histone methyltransferases, histone deacetylases, and helicases, among others, to actively repress transcription (4, 17, 46, 53, 61, 72, 84). The binding cleft on pRB that contacts the LXCXE motif is a highly conserved region of the growth suppressing 'pocket' domain (50). This hydrophobic cleft was first identified as the site of contact for LXCXE motifs in viral oncoproteins such as Adenovirus E1A, Simian Virus 40 large T antigen, and Human Papilloma Virus E7 (13, 19, 58, 85). The fact that so many cellular proteins can use an LXCXE motif to bind to pRB suggests that this cleft serves an important physiological purpose. However, few LXCXE motif-containing proteins are known to be required for a pRB-dependent cell cycle arrest (2, 88). Thus, it remains unclear whether LXCXE-dependent interactions are broadly required for pRB action, or for a subset of its growth inhibitory activities.

In an effort to understand the importance of the LXCXE binding cleft in pRB growth arrest during development, I have used two knock-in mutant mouse strains termed $Rb1^{\Delta L}$ and $Rb1^{NF}$, in which the LXCXE binding site on pRB has been disrupted by mutagenesis (39). Contrary to previous reports, we demonstrate that pRB has a critical role in mammary gland development. Loss of pRB-LXCXE interactions leads to defects

in nursing and epithelial growth control. These phenotypes are linked to a disruption in TGF-β growth inhibition in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mammary glands. The inability of TGF-β to block proliferation occurs despite inhibition of CDKs, and appears to be dependent on the ability of pRB to actively repress expression of E2F target genes. This suggests that pRB has a more intimate role in the TGF-β growth arrest pathway because TGF-β requires LXCXE-dependent interactions where other pRB-dependent arrest mechanisms do not. Furthermore, this study reveals an unappreciated role for pRB in mammary gland development.

2.2 Materials and Methods

Mouse strains

The *Rb1*$^{\Delta L}$ mouse strain containing three amino acid substitutions in the *Rb1* locus has been previously described (39). Analysis of *Rb1*$^{\Delta L/\Delta L}$ mice was performed on a mixed 129/B6 background. To generate the *Rb1*^{NF} strain, correctly targeted TC1 ES cells were identified by Southern blotting as shown in Figure 2.1b and c and injected into blastocysts to generate chimeric mice. Male chimeras were bred to C57/BL6 females, and agouti progeny were bred to 129 Sv/Ev/Tac mice that contained the Cre recombinase gene driven by the protamine promoter (PrmCre)(62). Males that carried *Rb1*$^{NF(Neo)}$ and PrmCre expressed Cre recombinase during spermatogenesis, which led to excision of the Neo cassette in sperm. These mice were then bred to generate *Rb1*$^{NF/NF}$ progeny and were subsequently studied in a mixed 129/B6 background. Genotyping methods and primer sequences can be found in Appendix I. *MMTV-TGF-β1*$^{223/225}$ mice express simian TGF-β1 carrying serine mutations at cysteines 223 and 225 of the pro region of the TGF-β1 precursor, resulting in the production of a constitutively active form of the mature protein (64). These mice were obtained from the Jackson Labs on a C57/B6 background and were bred to the *Rb1*$^{\Delta L}$ mutation creating a mixed 129/B6 genetic background.

Nursing data was collected from birth (P0) to weaning. Females were considered unable to nurse if all pups died within the first two days post-parturition, and considered able to partially nurse if some, but not all, pups survived past P2. Both multiparous and uniparous females were used in the study. All animals were housed and handled as approved by the Canadian Council on Animal Care.

Histology and Mammary Whole Mounts

The second and third thoracic mammary glands were dissected at 8 weeks of age or the second day post-parturition and fixed in neutral buffered 10% formalin. Fixed tissues were embedded in paraffin, cut into 5μm thick sections, and stained with H&E. To determine the extent of hyperplasia, the ductal cross sections present per mouse were counted and the fraction of hyperplastic ducts per genotype was calculated. Cross sections from three to nine females per genotype were quantified. Ductal cross sections with more than three layers of epithelial cells were scored as hyperplastic. For whole mount experiments, the fourth inguinal mammary gland was removed, mounted on a glass slide, and stained with Carmine Red using standard methods.

Detection of cytokeratin 18 and cytokeratin 14 was performed on paraffin sections that had been deparaffinized and rehydrated using a series of xylene and ethanol washes. Sections were brought to a boil in sodium citrate buffer and then maintained at 95˚C for 10 minutes. Cooled sections were rinsed in water three times for five minutes each, then rinsed in PBS for five minutes. Sections were blocked in 2.5% horse serum/2.5% goat serum in PBS-0.3% Triton-X for one hour. Sections were incubated with anti-cytokeratin 18 (KS18.04; Fitzgerald) and anti-cytokeratin 14 (AF64; Covance) overnight at 4˚C and then rinsed in PBS three times for five minutes each. Slides were incubated with horse anti-mouse IgG-FITC and goat anti-rabbit IgG-Texas Red secondary antibodies (FI-2000, TI- FI-1000; Vector) for 1.5 hours and then rinsed in PBS as above. Slides were mounted with Vectashield plus DAPI (H-1200; Vector) and sealed with nail polish. Fluorescent images were captured on a Zeiss Axioskop40 microscope and Spot Flex camera and coloured using EyeImage software (Empix Imaging, Mississauga, Ontario).

Mammary transplants

Mammary transplants were performed as described by Moorehead (57). All transplants were performed using the fourth inguinal glands. The epithelial portion of 3 week old $Rb1^{\Delta L/\Delta L}$ mammary glands was removed by harvesting the tissue between the lymph node and nipple. A 2x2 mm section of this tissue was placed into the cleared fat pad of Fox Chase SCID mice, and the epithelial tissue from $Rb1^{+/+}$ females was placed in the contralateral fat pad. SCID females were euthanized at 8 weeks of age and the fraction of hyperplastic ducts was determined as outlined above.

Cell culture

Primary mammary epithelial cells (MECs) were harvested as described by Hojilla (36). Each MEC preparation consisted of the mammary glands of four female mice. $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ mammary glands were minced and dissociated in 2 mg/mL collagenase IV in DMEM:F12, supplemented with 100 μg/mL gentamycin, 60 U/mL nystatin and 100U/mL penicillin/streptomycin for 2 hours at 120 rpm at 37˚C. Cells were then washed with PBS supplemented with 5% adult bovine serum (ABS) and plated onto collagen-coated dishes. MEC cultures were maintained with DMEM:F12 media supplemented with 1% ABS, 10 μg/mL insulin, 5 ng/mL epidermal growth factor (EGF), 50μg/mL gentamycin, 20 U/mL nystatin, and 100 U/mL penicillin/streptomycin. Finally, the cultures were passaged and purified using a differential dispase treatment.

Keratinocytes were harvested as previously described (10). P0-P2 animals were euthanized and immersed in 70% ethanol for 25 minutes at 4˚C to sterilize. Limbs, tails, and heads were removed before the dermis and epidermis were isolated from the mice,

dermis side down, and rinsed in PBS to remove blood. One millilitre of 0.25% trypsin was added to each skin, prior to incubating at 4°C overnight. Skins were then placed in 2 mL of fresh trypsin and the skins were incubated at 37°C for 30 minutes to 1 hour. The epidermis was then separated from the dermis, and minced finely with scissors in 15 mL conical tubes. 15 mL of keratinocyte growth medium (No-calcium EMEM, 8% chelex-treated fetal bovine serum (FBS), 74 ng/mL hydrocortisone, 6.7 ng/mL T3, 5 μg/mL insulin, 10^{-10} M cholera toxin, 5 ng/mL epidermal growth factor, and 0.1% penicillin/streptomycin) were added to each tube and the tubes were rocked gently at 37°C for 10 to 15 minutes. The suspension was filtered through a 70 μm nylon filter and plated at 400 000 cells per well onto collagen and poly-L-lysine coated coverslips in 24 well plates. The day after plating the cells were rinsed with PBS and fresh medium was added. Medium was changed every other day to maintain proliferation.

Rb1$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ primary murine embryonic fibroblast (MEF) cultures were derived as previously described (39). Cell culture experiments were carried out using passage 2 MECs, passage 4 MEFs, and passage 1 keratinocytes.

TGF-β growth arrest assays

Asynchronously proliferating *Rb1*$^{+/+}$, *Rb1*$^{\Delta L/\Delta L}$, and *Rb1*$^{NF/NF}$ MEFs were treated with 100 pM TGF-β1 (R&D Systems) for 24 hours. Cells were then pulse-labelled with 5-bromo-2-deoxyurdine (BrdU) (RPN201V1, Amersham Biosciences) according to the manufacturer's instructions for 1.5 hours. BrdU incorporation was quantified using flow cytometry as previously described (9). Flow cytometry was carried out on a Beckman-

Coulter EPICS XL-MCL instrument. Data analysis was carried out using CXP version 2 software.

Rb1$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MECs and keratinocytes were treated with TGF-β1 as outlined above and BrdU incorporation was measured using immunofluorescence microscopy. Cells were fixed and stained with an antibody against BrdU (1:500)(347580, BD Biosciences) using methodologies outlined by Foster, *et al.* (23). The percentage of BrdU-positive cells was determined from 10 fields of view per treatment group, and the average fold decrease in proliferation was calculated relative to untreated controls cultured in parallel.

Retroviral infections

Retroviral infections were performed as previously described (63). BOSC packaging cells were plated at 10^7 cells per 15 cm dish in 25 to 30 mL of media 24 hours prior to transfection. Each dish was transfected by calcium phosphate with 60 μg of pBabe plasmid containing $p16^{INK4a}$, $p21^{CIP1}$, or vector alone. BOSC media was replaced with 10-15 mL of fresh media the next morning. Two days later, the viral supernatant was filtered and supplemented with 4 μg/mL of polybrene before being placed directly on passage 3 MEFs that had been plated at 8 x10^5 cells per 10 cm dish a day earlier. BOSC cells were given fresh media and this was used for a second round of infection 12 hours later. After another 12 hours of incubation with viral supernatant, MEFs were given fresh media for 8 to 12 hours, at which point infected MEFs were selected for 4 days with media containing 5 μg/mL puromycin. After drug selection, MEFs were replated at low

density in drug containing media for BrdU labelling and subsequent flow cytometry analysis.

Protein and RNA quantification

To isolate milk, female mice were injected with 4.5 U oxytocin (Sigma) four hours after removal of their offspring. Thirty minutes later milk was extracted manually. Equal volumes of milk and 2X SDS-PAGE buffer were mixed, denatured, and resolved by SDS-PAGE. Gels were stained with coomassie blue.

To examine levels of phospho-SMAD2 and phospho-pRB, $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MEFs were treated with 100 pM TGF-β1 (R&D Systems) for 2 or 24 hours respectively. Total cellular extracts were isolated in radio-immunoprecipitation assay buffer. Equal amounts of total cellular proteins were resolved in each lane by SDS-PAGE, transferred to membranes, and probed using standard methods. Proteins were detected using the following antibodies: SMAD2 (sc-6200; Santa Cruz), phospho-SMAD2 S465/467 (AB3849; Chemicon), pRB (G3-245; BD-Pharmingen), phospho-pRB S807/811 (9308; Cell Signalling).

Messenger RNA levels of E2F target genes were detected using the Quantigene Plex 2.0 reagent system (Panomics, Freemont, CA) and measured using a BioPlex200 multiplex analysis system according to the manufacturer's instructions. To measure expression of *MMTV-TGF-β1* mRNA, RNA was extracted from mammary glands of 3 week old and 8 week old females using Trizol reagent (Invitrogen). RNA was then converted to cDNA using the Reverse Transcription System (Promega) as per the

manufacturers' instructions. RT-PCR was performed using primers against the simian TGF-β1 transcript (see Appendix I).

Luciferase reporter assays

Luciferase reporter assays were performed as described by Sarker (75). MEFs were seeded at 75 000 cells/well in a 6 well plate 24 hours prior to transfection. Cells were then cotransfected with 3TP-lux (250 ng/well) and cytomegaolovirus (CMV)-β-galactosidase vector (50 ng/well) using Fugene 6 (Roche) according to the manufacturer's directions. Twenty-four hours post-transfection, the cells were treated with 100 pM TGF-β1 (R&D Systems) for 20 hours at 37°C. Extracts were prepared using Luciferase Assay Buffer (Promega) and luciferase activity was measured on a Wallac Victor2 1420 multilabel reader. β-galactosidase activity was measured colorimetrically using 2-nitrophenyl-β-D-galactopyranoside as the substrate. Luciferase activity (measured in relative light units) was normalized to β-galactosidase measurements.

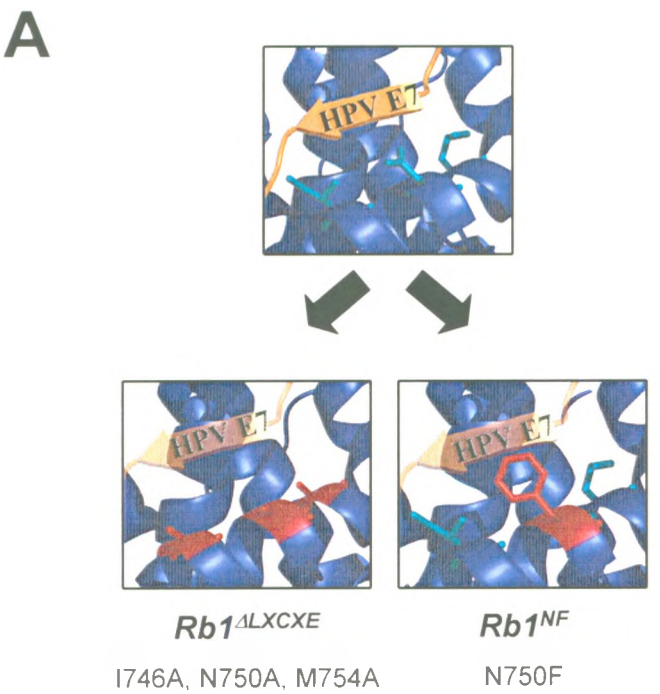
2.3 Results

Two distinct strategies to eliminate pRB-LXCXE interactions

The LXCXE binding cleft is one of the most highly conserved regions of the retinoblastoma protein (50), and is the contact site for many proteins involved in chromatin regulation (5). However, it is noteworthy that proteins like Suv39h1, Cdh1, and the condensin subunit CAP-D3 do not contain a classic LXCXE motif, yet require the LXCXE binding cleft for interaction with pRB (2, 51, 61). To understand the importance of interactions between pRB and cellular partners that use this interaction surface, we generated two knock-in mouse models that use distinct mutation strategies to disrupt interactions with this region of pRB. The $Rb1^{\Delta LXCXE}$ (herein referred to as $Rb1^{\Delta L}$) mutant replaces three well conserved amino acids (I746, N750, and M754) with alanines and has been previously reported (39) (Fig. 2.1a). These substitutions are predicted to make the leucine and cysteine residues of the LXCXE motif a loose fit. A different gene targeting strategy was utilized to block access to the LXCXE binding cleft in the $Rb1^{N750F}$ ($Rb1^{NF}$) mouse. The $Rb1^{NF}$ mutant substitutes a bulky phenylalanine for asparagine at amino acid 750, which is predicted to sterically block access to the LXCXE binding cleft (Fig. 2.1a). The targeting strategy used to create this mouse is shown in Figure 2.1B and a representative Southern blot shows targeting by homologous recombination (Fig. 2.1c). The selectable marker was removed by breeding Cre transgenic and chimeric mice. F1 offspring were subsequently intercrossed to eliminate the transgene and produce homozygous $Rb1^{NF/NF}$ animals.

Previous cell culture based studies showed that $pRB^{\Delta L}$ and pRB^{NF} are unable to bind LXCXE-containing proteins including Adenovirus E1A, Human Papilloma Virus

Figure 2.1 Two knock-in mouse strains with disrupted LXCXE interactions. (A) Structural depiction of pRB interacting with the LXCXE motif of HPV E7. Side chains from amino acids 746, 750, and 754 on pRB mediate the interaction with the LXCXE peptide and are coloured turquoise. The *Rb1*$^{\Delta LXCXE}$ mutation changes these amino acids to alanines (red), removing one side of the LXCXE binding cleft, while the *Rb1*^{NF} mutation adds a bulky phenylalanine instead of asparagine at amino acid 750 (red). This is predicted to occupy more space and block access to the LXCXE binding cleft. (B) The genomic structure of *Rb1* is shown. The targeting vector containing a LoxP-flanked PGK-neo cassette inserted into intron 23 and the mutation of N750F in exon 22 are indicated. A new XbaI site was introduced into intron 21. Homologous recombination resulted in the *Rb1*$^{NF\text{-}neo}$ allele. Location of the 5' probe used for Southern blotting is also shown. Following germ line transmission, the correctly recombined allele was generated by crossing chimeric males to a Cre-expressing transgenic strain. The structure of the *Rb1*^{NF} allele in which Neo has been correctly excised is shown at the bottom. (C) A Southern blot of representative ES clones digested with XbaI and probed with the 5' probe is shown. (D) The ability of GST-E1A and GST-DP1/His-E2F3 to interact with pRB$^{\Delta L}$ and pRB^{NF} was tested in GST-pulldown assays, and bound pRB protein was detected by western blot analysis.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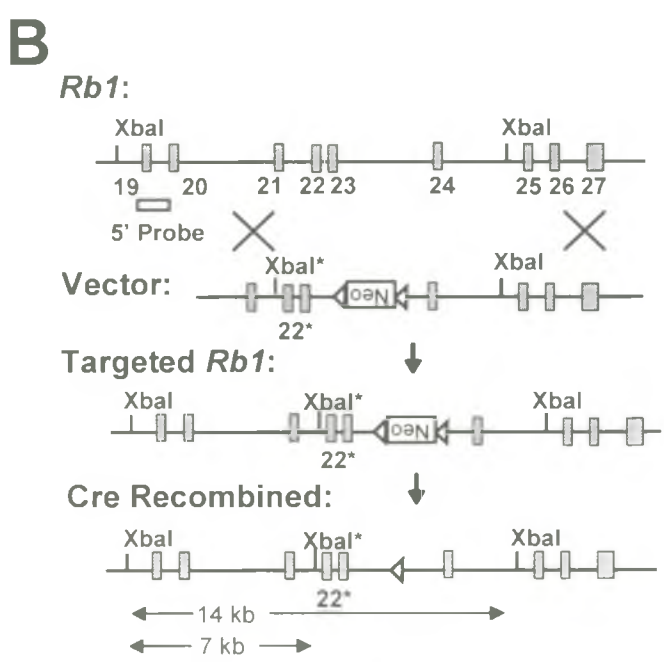
HPV E7
HPV E7
HPV E7
Rb1ΔLXCXE
I746A, N750A, M754A
Rb1NF
N750F


B
Rb1:
XbaI
XbaI
19 20 21 22 23 24 25 26 27
5' Probe
Vector:
XbaI*
XbaI
Neo
22*
Targeted Rb1:
XbaI
XbaI*
XbaI
Neo
22*
Cre Recombined:
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XbaI*
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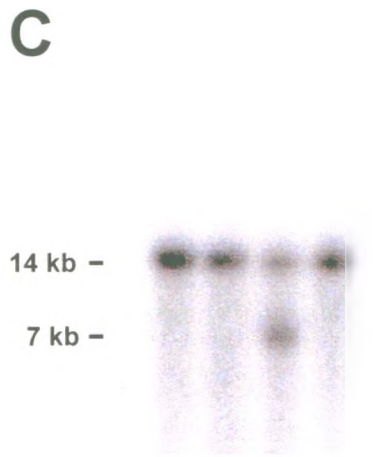
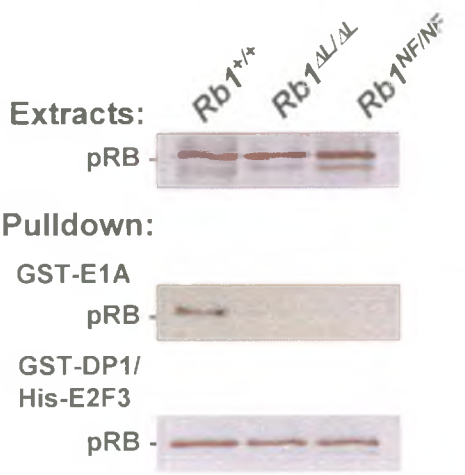

C
14 kb –
7 kb –


D
Rb1+/+
Rb1ΔL/ΔL
Rb1NF/NF
Extracts:
pRB
Pulldown:
GST-E1A
pRB
GST-DP1/
His-E2F3
pRB

E7, histone deacetylase 1, Retinoblastoma Binding Partner 1, Sin3, and C-terminal binding protein 1, but these pRB mutants retain normal interactions with E2F transcription factors (7, 39). GST-pulldown experiments further confirm that $pRB^{\Delta L}$ and pRB^{NF} mutant proteins derived from $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ cells are defective for binding to proteins containing a classic LXCXE motif like E1A (Fig. 2.1d). In addition, both mutant forms of pRB interact with recombinant E2F3-DP1 equivalently to wild type pRB. These experiments demonstrate that together the two mouse strains have the necessary properties to define the physiological contexts where pRB-LXCXE interactions are required, regardless of the nature in which interacting proteins contact this binding site on pRB.

Nursing defects in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ female mice

Mice homozygous for LXCXE binding cleft mutations are viable and indistinguishable from wild type littermates, however, mutant females display a distinct defect in mammary gland function. When bred, pups from $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mothers frequently did not survive past day 2 post-parturition (P2) (Table 2.1). Furthermore, many pups that did survive had very small white spots in their abdomens (Fig. 2.2a), indicating that they were not being nursed regularly.

In the majority of cases, $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ females built nests, and after delivery, offspring were cleaned and present in the nest. Mothers quickly retrieved offspring that we removed from the nests, and pups were routinely observed attempting to suckle. Thus, despite ostensibly normal maternal and offspring behaviour, little or no

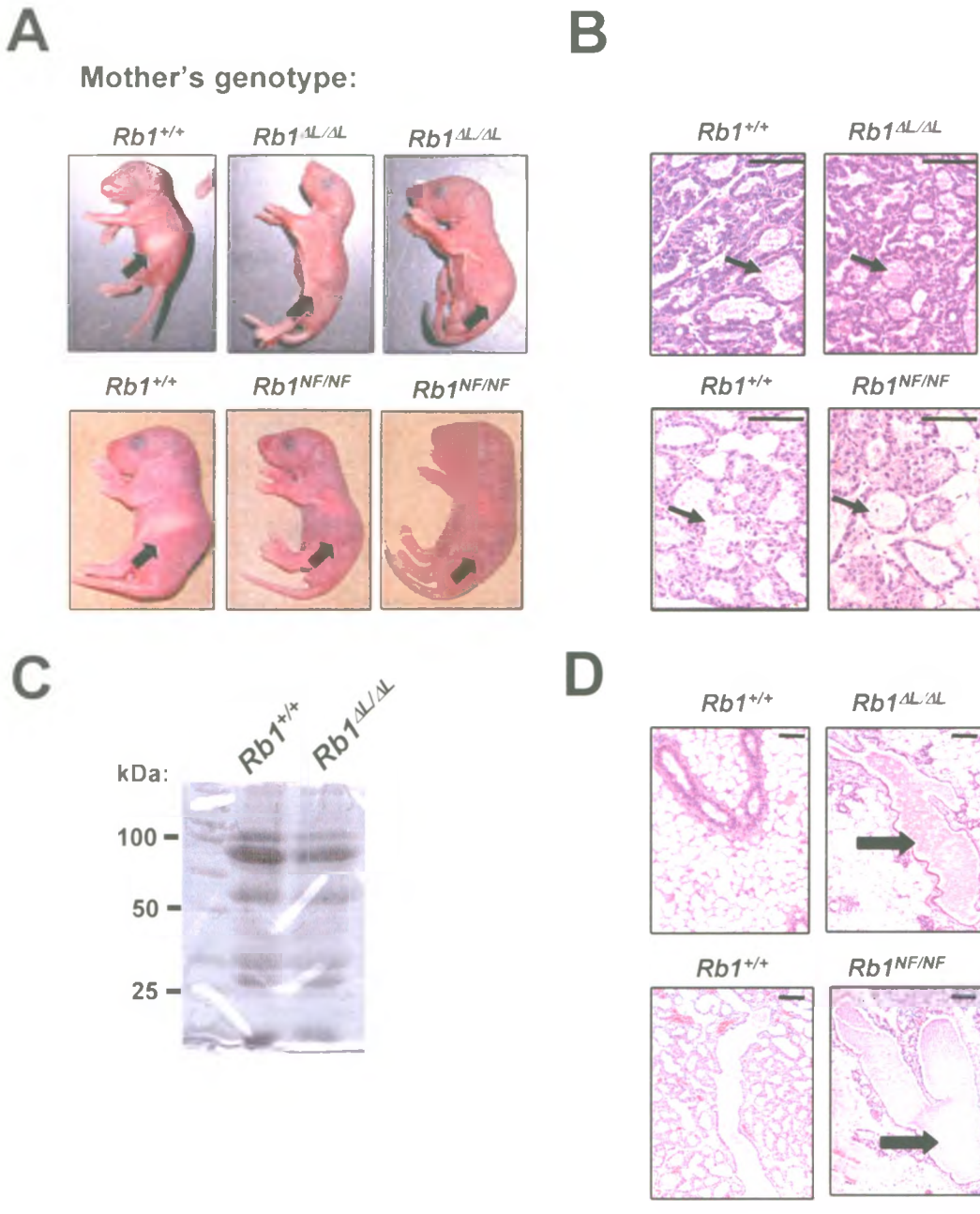
Table 2.1 The effect of pRB LXCXE cleft mutations on the ability of female mice to nurse. Mothers were considered unable to nurse if their pups died within the first two days post-parturition (P2). Females that lost at least one pup and had at least one pup survive past P2 were considered to have partially nursed. Proportions were compared between relevant groups using a chi-square test.

	Proportion Unable to Nurse		Proportion Partially Nursed		Proportion Nursed Completely		Number of Litters	
ES line	$Rb1^{+/+}$	$Rb1^{\Delta L/\Delta L}$	$Rb1^{+/+}$	$Rb1^{\Delta L/\Delta L}$	$Rb1^{+/+}$	$Rb1^{\Delta L/\Delta L}$	$Rb1^{+/+}$	$Rb1^{\Delta L/\Delta L}$
27C4	0.05	0.44***	0.03	0.23	0.92	0.32	40	34
94	0	0.45*	0.08	0.09	0.91	0.45	12	22
TOTAL	**0.04**	**0.44****	**0.04**	**0.17**	**0.92**	**0.38**	**52**	**56**

	Proportion Unable to Nurse		Proportion Partially Nursed		Proportion Nursed Completely		Number of Litters	
ES line	$Rb1^{+/+}$	$Rb1^{NF/NF}$	$Rb1^{+/+}$	$Rb1^{NF/NF}$	$Rb1^{+/+}$	$Rb1^{NF/NF}$	$Rb1^{+/+}$	$Rb1^{NF/NF}$
5F11	0.03	0.33**	0.43	0.29	0.54	0.37	37	27

* $P < 0.05$
** $P < 0.01$
*** $P < 0.005$

Figure 2.2 Defective nursing in *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{NF/NF}$ females. (A) Representative offspring from *Rb1*$^{+/+}$, *Rb1*$^{\Delta L/\Delta L}$, and *Rb1*$^{NF/NF}$ mothers at day two post-parturition (P2). Arrows indicate the stomachs of the offspring. (B) Paraffin sections of *Rb1*$^{+/+}$, *Rb1*$^{\Delta L/\Delta L}$, and *Rb1*$^{NF/NF}$ mammary glands from post-partum females (P2) were stained with H&E to verify the presence of milk. Arrows indicate milk-filled alveoli. Scale: 50 μm. (C) SDS-PAGE and coomassie staining of milk obtained from *Rb1*$^{+/+}$and *Rb1*$^{\Delta L/\Delta L}$ post-partum females. (D) H&E staining of sections at P2 also indicated dilation of the ducts in *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{NF/NF}$ females. Arrows indicate dilated ducts. Scale bar: 200 μm.


A
Mother's genotype:
Rb1+/+
Rb1ΔL/ΔL
Rb1ΔL/ΔL
Rb1+/+
Rb1NF/NF
Rb1NF/NF
B
Rb1+/+
Rb1ΔL/ΔL
Rb1+/+
Rb1NF/NF
C
Rb1+/+
Rb1ΔL/ΔL
kDa:
100
50
25
D
Rb1+/+
Rb1ΔL/ΔL
Rb1+/+
Rb1NF/NF

milk was observed in the stomachs of newborns from $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mothers, indicating that impaired milk intake caused the neonatal lethality (Fig. 2.2a).

To confirm that there were no defects in milk production, we performed histological analysis of postpartum mammary tissue from $Rb1^{+/+}$, $Rb1^{\Delta L/\Delta L}$, and $Rb1^{NF/NF}$ females. All had undergone a similar degree of lobuloalveolar formation and alveoli contained milk at P2 (Fig. 2.2b). SDS-PAGE and coomassie staining of milk obtained from $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ mammary glands revealed no differences in milk protein content between genotypes, suggesting that neonatal morbidity was not due to poor milk quality from *Rb1* mutant mothers (Fig. 2.2c). However, histological analysis of mammary glands from lactating and multiparous mutant females revealed large, dilated ducts containing milk (Fig. 2.2d), a phenotype consistent with an inability to secrete milk (43).

These experiments indicate that while $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ females are able to produce milk, they have difficulty excreting it from their mammary glands, frequently resulting in neonatal lethality. The prevalence of this nursing defect in mouse lines from two separate ES clones of the $Rb1^{\Delta L}$ mutation as well as the $Rb1^{NF/NF}$ mutant indicates that pRB-LXCXE interactions are critical for mammary gland function. By extension, we conclude that pRB has an essential function in mammary gland development.

$Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ females develop hyperplasia of the mammary ductal epithelium

The disruption in milk expulsion exhibited by mutant *Rb1* mammary glands prompted us to examine mammary gland development in these mice. Mammary gland histology revealed hyperplastic growth in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mammary glands

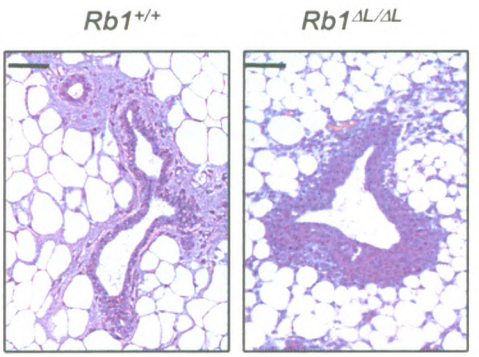
throughout development (Fig. 2.3a and c, and data not shown). Hyperplasia was characterized by increased luminal epithelial cell layers (Fig. 2.3f), as well as invagination of the epithelium into the lumen of the duct. The tables associated with Figure 2.3A and C show a significantly elevated frequency of hyperplastic ducts in *Rb1* mutant mice compared with controls ($P<0.0001$). This data suggests that pRB-LXCXE interactions are required for proliferative control of mammary ductal epithelium during development. Conversely, ductal infiltration of the fat pad was similar between wild type and mutant genotypes as revealed by Carmine Red staining of mammary gland whole mounts (Fig. 2.3b and d). In addition, branching frequency and overall ductal morphogenesis appeared normal, suggesting that hyperplasia that is visible at a microscopic level throughout development does not manifest in more severe developmental problems.

Both epithelial and stromal factors influence ductal development. To determine whether disruption of LXCXE interactions within the mammary epithelium was sufficient to enhance ductal growth, we transplanted mammary epithelial tissue from wild type and $Rb1^{\Delta L/\Delta L}$ mutants into cleared fat pads of Fox Chase SCID recipients prior to puberty. H&E staining revealed that hyperplastic epithelia were evident in $Rb1^{\Delta L/\Delta L}$ glands, even in the presence of wild type stroma and endocrine factors (Fig. 2.3e). This demonstrates that overproliferation of the mammary ductal epithelium in *Rb1* mutant mice is not a secondary consequence of altered endocrine signalling, nor signalling from the surrounding stroma, but rather, is epithelial cell autonomous.

This analysis reveals a striking defect in mammary ductal development in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ virgin mice. This defect is specific to the epithelial compartment,

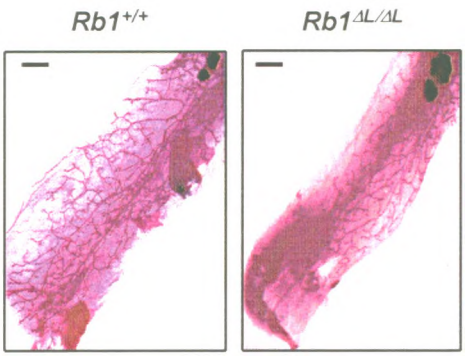
Figure 2.3 *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{NF/NF}$ females develop hyperplasia of mammary ductal epithelia. (A) H&E staining of *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mammary tissue sections from 8 week old mice. Each image displays a representative cross section of ducts used to count epithelial layers. Ducts three or more cells thick were scored as hyperplastic. The accompanying table displays the proportion of hyperplastic (hp) ducts found in wild type and *Rb1*$^{\Delta L/\Delta L}$ mammary glands. (B) Carmine Red-stained mammary whole mounts are shown from 12 week old mice for the indicated genotypes. (C) An identical analysis to that performed in A is shown for *Rb1*$^{+/+}$ and *Rb1*$^{NF/NF}$ mice. (D) Whole mount analysis was also performed on matched wild type and *Rb1*$^{NF/NF}$ mice. (E) Mammary epithelial tissue from *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mice was transplanted into clarified fat pads of Fox Chase SCID hosts. Tissue sections from transplanted mammary glands were stained and analyzed as in A. The proportion of hyperplastic ducts for each genotype was compared using a chi-square test. (F) Paraffin sections from 8 wk old mice were stained for the luminal epithelial and basal/myoepithelial markers, cytokeratin 18 (green) cytokeratin 14 (red). A, C, E scale bar: 200 μm, B, D scale bar: 2 mm, F scale bar: 50 μm.

A

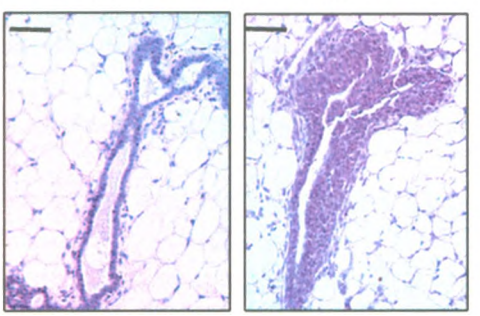


	# of mice	# of ducts	# of hp ducts	Proportion of hp ducts	*P* value
Rb1$^{+/+}$	6	455	11	0.024	
Rb1$^{\Delta L/\Delta L}$	9	390	62	0.160	<0.0001

B

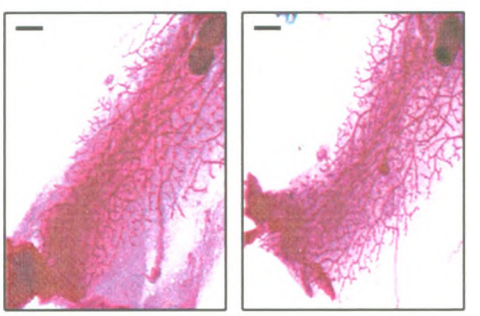


C

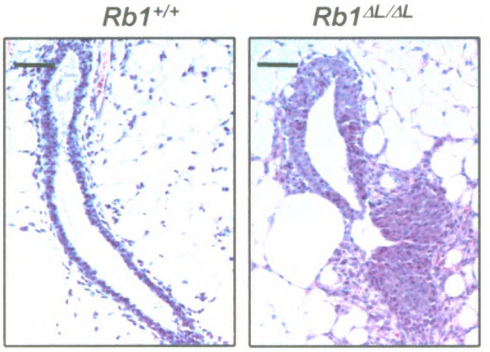


	# of mice	# of ducts	# of hp ducts	Proportion of hp ducts	*P* value
Rb1$^{+/+}$	5	712	67	0.097	
Rb1$^{NF/NF}$	5	151	31	0.205	<0.0001

D

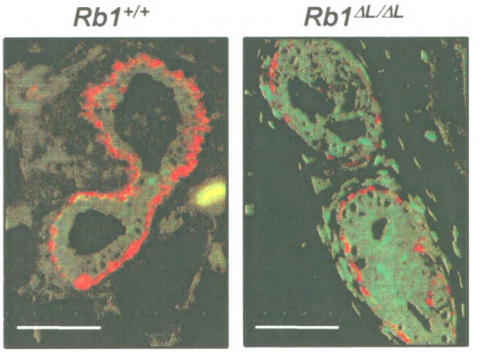


E



	# of mice	# of ducts	# of hp ducts	Proportion of hp ducts	*P* value
Rb1$^{+/+}$	8	950	60	0.063	
Rb1$^{\Delta L/\Delta L}$	8	641	128	0.200	<0.0001

F

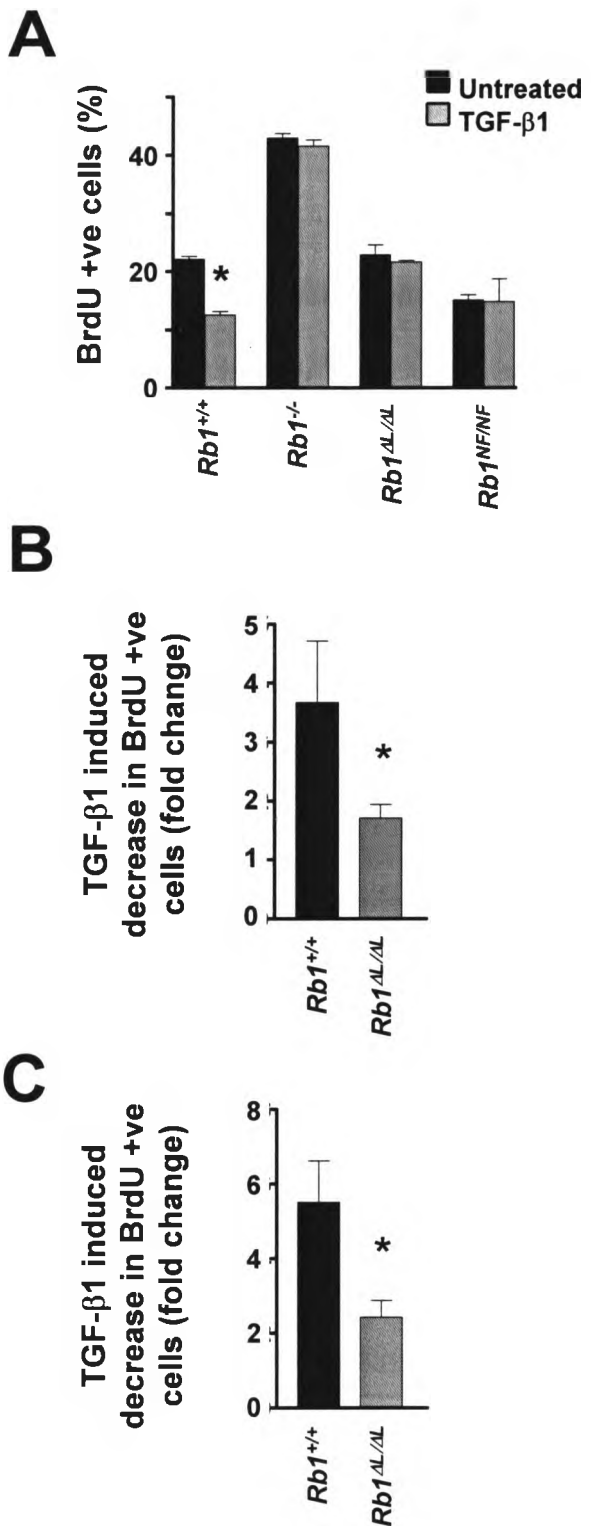


as ductal branching, which relies on stromal signalling (41), is intact, and the transplants reveal that the hyperplasia persists even in the presence of wild type stroma. Transplantation experiments further demonstrate that the hyperplasia is phenotypically distinct from the apparently normal development that takes place with transplanted *Rb1*$^{-/-}$ mammary anlagen (73). Consequently, these *Rb1* mutant strains have revealed a key role for pRB in mammary epithelial proliferation and function.

Defective TGF-β growth inhibition in *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{NF/NF}$ cells contributes to hyperplasia

TGF-β is essential for growth control and development of the mammary gland (22, 55). Interestingly, excessive ductal proliferation is seen in mice hemizygous for *Tgf-β1* or expressing a dominant negative TGF-β type II receptor (20, 21, 28, 29, 41). Furthermore, dominant negative TGF-β type II receptor mice display a nursing defect (29). The similarity of phenotypes between mice defective for pRB-LXCXE interactions and mice defective for TGF-β signalling within the mammary epithelium prompted us to examine the ability of *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{NF/NF}$ cells to respond to a TGF-β1 growth arrest signal. We treated primary MEFs from *Rb1*$^{+/+}$, *Rb1*$^{\Delta L/\Delta L}$, and *Rb1*$^{NF/NF}$ mice with TGF-β1 for 24 hours, pulse labelled with BrdU, and then quantified the percentage of cells incorporating BrdU by flow cytometry (Fig. 2.4a). *Rb1*$^{-/-}$ cultures serve as an important control because they are known to be refractory to TGF-β1 growth arrest (34). In this experiment, *Rb1*$^{+/+}$ MEFs showed reduced BrdU incorporation in response to TGF-β1, while *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{NF/NF}$ fibroblasts were unresponsive, indicating that pRB-LXCXE interactions are necessary for TGF-β–mediated growth arrest.

Figure 2.4 Defective TGF-β growth inhibition in *Rb1*$^{\Delta L/\Delta L}$ and *Rb1*$^{NF/NF}$ cells. (A) *Rb1*$^{+/+}$, *Rb1*$^{-/-}$, *Rb1*$^{\Delta L/\Delta L}$, and *Rb1*$^{NF/NF}$ murine embryonic fibroblasts (MEFs) were treated with TGF-β1 and pulse labelled with BrdU 24 hrs later. BrdU incorporation was measured by flow cytometry and the percent incorporation is shown for each genotype. (B) Mammary epithelial cells (MECs), and (C) keratinocytes were treated with TGF-β1 for 24 hours and pulse labelled with BrdU as described above. The percentage of cells incorporating BrdU was measured by immunofluorescence microscopy. The fold decrease in proliferation between treated and untreated cultures was determined. The average of three independent experiments is shown. *Indicates a statistically significant difference (Student's t test; $P<0.05$). Error bars indicate one standard deviation from the mean.

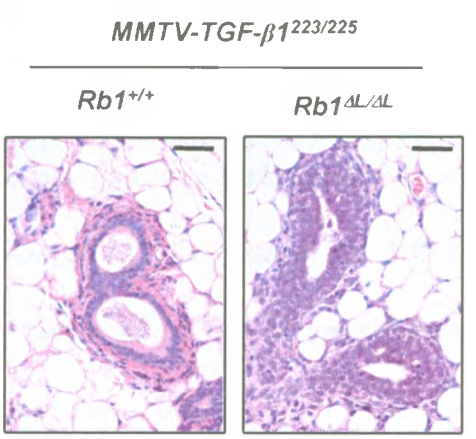

A
Untreated
TGF-β1
BrdU +ve cells (%)
0
20
40
*
Rb1+/+
Rb1-/-
Rb1ΔL/ΔL
Rb1NF/NF
B
TGF-β1 induced decrease in BrdU +ve cells (fold change)
0
1
2
3
4
5
*
Rb1+/+
Rb1ΔL/ΔL
C
TGF-β1 induced decrease in BrdU +ve cells (fold change)
0
2
4
6
8
*
Rb1+/+
Rb1ΔL/ΔL

This analysis of TGF-β growth control was expanded to include other cell types that are more sensitive to TGF-β–induced cell cycle arrest. We prepared primary MECs, plated them in duplicate, and TGF-β1 was added to one of each pair. The percentage of BrdU positive cells was determined by immunofluorescence microscopy and the fold decrease of incorporation was calculated using the untreated control as a reference (Fig. 2.4b). We found that the ability to induce a TGF-β1 growth arrest was drastically reduced in $Rb1^{\Delta L/\Delta L}$ mammary epithelial cells. $Rb1^{+/+}$ MECs had almost a four-fold decrease in cell proliferation, while $Rb1^{\Delta L/\Delta L}$ MECs showed less than two-fold reduction in BrdU incorporation (P=0.03). We also performed this experiment with $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ primary keratinocytes (Fig. 2.4c). $Rb1^{+/+}$ keratinocytes displayed a large decrease in BrdU incorporation, while $Rb1^{\Delta L/\Delta L}$ cells demonstrated only a 2.4-fold reduction in proliferation (P=0.0113). From these experiments we conclude that pRB-LXCXE interactions are critical for TGF-β growth control in multiple cell types.

To validate that resistance to TGF-β growth inhibition contributes to the developmental defects seen in the mammary glands of mice lacking LXCXE interactions, we combined the $Rb1^{\Delta L}$ mutation with an *MMTV-TGF-β1* transgene to determine whether hyperplastic ductal growth of $Rb1^{\Delta L/\Delta L}$ epithelia could be suppressed in the presence of excess TGF- β1. Figure 2.5 shows our analysis of ductal hyperplasia in 8 week old $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ mice overexpressing a constitutively active form of TGF-β1. H&E staining of ductal cross sections shows a persistent hyperplastic phenotype that is indistinguishable from $Rb1^{\Delta L/\Delta L}$ alone (compare Fig. 2.5a with Fig. 2.3a). Furthermore, the frequency of hyperplastic ducts in $Rb1^{\Delta L/\Delta L}$ mice overexpressing active TGF-β1 is also similar to $Rb1^{\Delta L/\Delta L}$ alone (compare Fig. 2.5b with Fig. 2.3a). We also investigated

Figure 2.5 Mammary ductal hyperplasia is caused by defective TGF-β growth inhibition in *Rb1*$^{\Delta L/\Delta L}$ mice. *Rb1*$^{\Delta L/\Delta L}$ mice were crossed into the *MMTV-TGF-β1*$^{223/225}$ background. (A) H&E staining of paraffin sections from mammary glands isolated from 8 week old mice. Cross sections of individual ducts are shown. Ducts that contained three or more epithelial layers were scored as hyperplastic. Scale bar: 200 μm. (B) The proportion of hyperplastic (hp) ducts in *MMTV-TGF-β1*$^{223/225}$; *Rb1*$^{+/+}$ and *MMTV-TGF-β1*$^{223/225}$; *Rb1*$^{\Delta L/\Delta L}$ mammary glands was determined and compared using a chi-square test. (C) Reverse transcriptase-PCR was used to detect the constitutively active, simian-derived TGF-β1 transcript expressed by the MMTV promoter in *MMTV-TGF-β1*$^{223/225}$, *Rb1*$^{\Delta L/\Delta L}$ mammary glands.

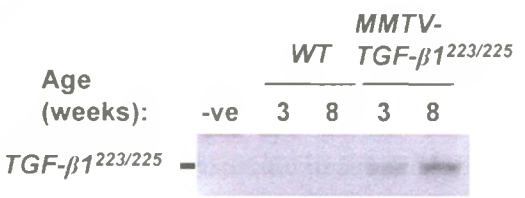
A



B

	# of mice	# of ducts	# of hp ducts	Proportion of hp ducts	*P* value
Rb1$^{+/+}$	4	1403	76	0.054	
Rb1$^{\Delta L/\Delta L}$	3	1432	348	0.243	<0.0001

C



the expression pattern of the *MMTV* transgene using RT-PCR to detect the simian TGF-β1 transcript (Fig. 2.5c). This shows that expression of the transgene is evident as early as 3 weeks of age. Thus, even after 5 weeks of persistent expression of a constitutively active form of TGF-β1, the mammary ductal epithelium still overproliferates. This reveals that resistance to TGF-β growth inhibition is an important component of the ductal hyperplasia phenotype.

These data link the hyperplastic phenotypes observed in mammary epithelium in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mice with an inability to respond to TGF-β growth inhibition. In addition, a small increase in BrdU positive basal keratinocytes has been observed in $Rb1^{\Delta L/\Delta L}$ mice compared to controls (1), suggesting that defective TGF-β growth arrest in $Rb1^{\Delta L/\Delta L}$ keratinocytes may have a mild effect on the epidermis. Our experiments have identified a previously unappreciated role for pRB in mediating TGF-β growth control in mammary epithelium that is necessary for mammary development and function.

$Rb1^{\Delta L/\Delta L}$ cells transduce TGF-β1-dependent signals

We next wanted to address the mechanism by which mutations in the LXCXE binding cleft of *Rb1* disrupt TGF-β growth inhibition. TGF-β stimulates its receptors to phosphorylate SMAD proteins, which translocate to the nucleus, and along with co-regulators, activate or repress gene transcription of a diverse number of genes. Targets for activation include plasminogen activator inhibitor 1 (11, 14), and the CDK inhibitors $p15^{INK4b}$ and $p21^{CIP1}$(12, 70), among others. To determine where pRB-LXCXE interactions are required in TGF-β-mediated growth arrest, we analyzed the TGF-β signalling pathway in $Rb1^{\Delta L/\Delta L}$ MEFs. Phospho-specific western blots showed that TGF-

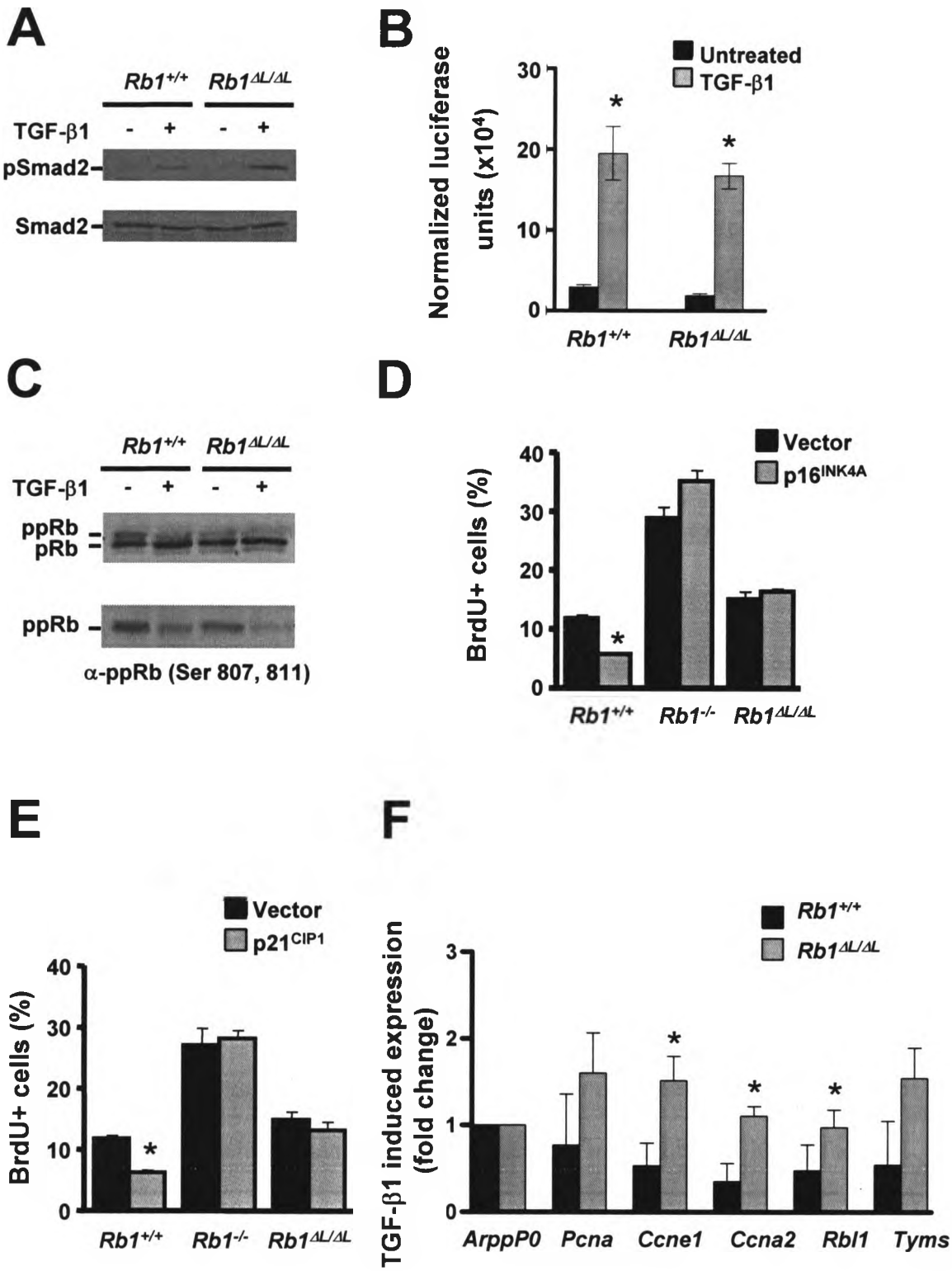
β1 treatment of $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MEFs resulted in phosphorylation of SMAD2 (Fig. 2.6a). This suggests that TGF-β receptor expression and function are not significantly altered in $Rb1^{\Delta L/\Delta L}$ cells.

To examine SMAD-dependent transcription, we utilized the 3TP-lux reporter, which contains TGF-β responsive elements from the promoter of the *plasminogen activator inhibitor 1* gene driving the expression of firefly luciferase (86). Transfected $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MEFs had comparable levels of luciferase activity when stimulated with TGF-β1 (Fig. 2.6b). Importantly, luciferase expression was increased to the same extent when $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ cells were treated with TGF-β1. Together with the phospho-specific western blot analysis, the luciferase assay data indicates that SMAD-dependent signal transduction functions normally in $Rb1^{\Delta L/\Delta L}$ cells. From these experiments it is clear that the $Rb1^{\Delta L}$ mutation disrupts growth control, but does not cause pleiotropic defects in TGF-β signalling.

$Rb1^{\Delta L/\Delta L}$ cells are unable to repress E2F target genes in response to TGF-β

Growth inhibition by TGF-β is thought to be the result of multiple, overlapping means of inhibiting cyclin dependent kinase activity (54, 55). In G1 this leads to the accumulation of hypophosphorylated pRB and cell cycle arrest (24, 26, 47). To investigate this aspect of TGF-β growth inhibition, we performed phosphospecific western blot analysis on MEFs treated with TGF-β1. $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MEFs had comparable levels of dephosphorylated pRB when treated with TGF-β1 (Fig. 2.6c), yet $Rb1^{\Delta L/\Delta L}$ cell proliferation was not reduced under these conditions (Fig. 2.4a). This

Figure 2.6 TGF-β1 signalling in *Rb1*$^{\Delta L/\Delta L}$ cells does not repress E2F target genes. (A) Phospho-SMAD2 levels were measured in TGF-β1 treated *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MEFs by western blot analysis. (B) *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MEFs were transfected with the 3TP-luciferase reporter and β-galactosidase plasmids. MEFs were then treated with TGF-β1 for 24 hrs. The luciferase activity was normalized to β-galactosidase expression and is shown as arbitrary units. (C) Total pRB expression levels as well as phospho-pRB levels were measured in TGF-β1-treated *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MEFs by western blot analysis. (D, E) *Rb1*$^{+/+}$, *Rb1*$^{-/-}$, and *Rb1*$^{\Delta L/\Delta L}$ MEFs were infected with retroviruses expressing either (D) p16^{INK4a} or (E) p21^{CIP1}. Following drug selection, cells were pulse labelled with BrdU. BrdU incorporation was measured by flow cytometry and the percent incorporation is shown. (F) The fold change in mRNA levels in response to TGF-β1 treatment is shown for E2F responsive genes as well as the non-E2F responsive control, Acidic ribosomal phosphoprotein (*ArppP$_0$*). Error bars indicate one standard deviation from the mean. *Indicates a statistically significant difference (Student's t test; $P<0.05$).


A
Rb1+/+
Rb1ΔL/ΔL
TGF-β1 - + - +
pSmad2
Smad2
B
Untreated
TGF-β1
Normalized luciferase units (x10⁴)
30
20
10
0
*
*
Rb1+/+
Rb1ΔL/ΔL
C
Rb1+/+
Rb1ΔL/ΔL
TGF-β1 - + - +
ppRb
pRb
ppRb
α-ppRb (Ser 807, 811)
D
Vector
p16INK4A
BrdU+ cells (%)
40
30
20
10
0
*
Rb1+/+
Rb1-/-
Rb1ΔL/ΔL
E
Vector
p21CIP1
BrdU+ cells (%)
40
30
20
10
0
*
Rb1+/+
Rb1-/-
Rb1ΔL/ΔL
F
Rb1+/+
Rb1ΔL/ΔL
TGF-β1 induced expression (fold change)
3
2
1
0
*
*
*
ArppP0
Pcna
Ccne1
Ccna2
Rbl1
Tyms

indicates that mutant pRB is activated by TGF-β1 signalling and suggests that the defect in growth inhibition is downstream of CDK regulation.

To further confirm that *Rb1*$^{\Delta L/\Delta L}$ cells are unable to arrest despite the inhibition of cyclin/CDK activity, we sought to inhibit CDK activity directly. Hypophosphorylation of pRB and G1 arrest can be induced by ectopic expression of INK4 and CIP/KIP family proteins, and this arrest is known to be lost in cells deficient for pRB (44, 52, 60, 78). We used retroviral infection to express either p16^{INK4a} or p21^{CIP1} in *Rb1*$^{+/+}$, *Rb1*$^{-/-}$, and *Rb1*$^{\Delta L/\Delta L}$ MEFs to study the effects of representative members of the INK4 or CIP/KIP protein families on cell cycle arrest. *Rb1*$^{+/+}$ cells had decreased BrdU incorporation after infection with either p16^{INK4a}- or p21^{CIP1}- expressing viruses, while *Rb1*$^{\Delta L/\Delta L}$ MEFs behaved like *Rb1*$^{-/-}$ MEFs, with no reduction in BrdU incorporation (Fig. 2.6d and e). Thus, even when inhibitor expression blocked CDK activity, *Rb1*$^{\Delta L/\Delta L}$ MEFs were unable to arrest growth. Based on this analysis we conclude that TGF-β growth arrest requires a unique aspect of pRB function beyond becoming dephosphorylated and binding to E2Fs.

To understand the nature of the pRB-LXCXE-dependent function that is required for TGF-β induced growth arrest, we determined whether mutant pRB still represses transcription of E2F target genes. We measured the mRNA levels of five E2F responsive genes under conditions where TGF-β1 stimulation inhibits proliferation of *Rb1*$^{+/+}$ MEFs. While the levels of *Pcna*, *Ccne1*, *Rbl1*, *Ccna2*, and *Tyms* decreased in wild type TGF-β1-treated cells, there was little change in transcript levels for a number of these genes in *Rb1*$^{\Delta L/\Delta L}$ cells (Fig. 2.6f). In some cases expression appeared to increase slightly. Given that both wild type and mutant pRB became hypophosphorylated under these TGF-β1

treatment conditions (Fig. 2.6c), we interpret this to mean that mutant pRB is active but unable to repress transcription.

This indicates that pRB functions as part of an active repressor complex in TGF-β growth inhibition. Presumably this complex contains pRB, an LXCXE motif-containing co-repressor, and an E2F transcription factor. Since the most obvious defect in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mice lies in proliferative control during mammary gland development, this reveals a novel requirement for pRB-LXCXE interactions in the TGF-β cytostatic response that is uniquely important for mammary development and function.

2.4 Discussion

This study reveals a number of unexpected findings about TGF-β signalling and pRB in regulating cell proliferation. First, our work highlights a previously unrecognized role for pRB in mammary gland development. Additionally, mutation of the highly conserved LXCXE binding region of pRB creates a very discrete functional defect in the mammary glands of otherwise normal mice. Because TGF-β signalling underlies the mammary defects in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mice, our work argues that pRB-LXCXE interactions have a unique functional role in TGF-β-induced growth inhibition.

Our work appears to contradict the report by Robinson, *et al.*, which showed that complete ablation of pRB in transplanted epithelium results in normal mammary development (73). However, these apparently paradoxical results may be explained by differences in experimental approaches. First, we discovered hyperplasia in early development of virgin animals, a defect that we were unable to detect in densely packed lactating mammary glands. Since these authors examined only the structure of lactating $Rb1^{-/-}$ mammary glands, it is perhaps not surprising that they did not detect hyperplastic growth. Similar to Robinson, *et al.*, we investigated the density and morphology of alveoli between genotypes in lactating females and did not detect differences. The inability of transplanted mammary glands to form a functional connection with the nipple precludes further assessment of a phenotype in *Rb1* null glands. However, our intact mouse models clearly showed a defect in expelling milk, indicating that fully functional pRB is necessary for lactation. To ascertain the importance of pRB in TGF-β proliferative control, Robinson *et al.* transplanted *WAP-TGF-β1*; $Rb1^{-/-}$ epithelium into wild type recipients. These mice express TGF-β1 in alveolar cells during pregnancy and lactation.

Again, these alveoli were indistinguishable from wild type controls. In contrast, the *MMTV-TGF-β1* transgene used in our experiments reveals *in vivo* resistance to TGF-β1-induced growth arrest during early development. The challenges presented by transplanting embryonic $Rb1^{-/-}$ anlagen limits the range of developmental events that can be investigated, and likely explains why pRB's role in mammary development and function has gone undetected until now.

Most breast cancers originate from ductal epithelium and nearly all cell lines derived from breast cancer patients are unresponsive to the growth inhibiting effects of TGF-β1 in culture (16, 55). Similar to the transplant experiments in Robinson *et al.,* we have not detected spontaneous mammary tumours in $Rb1^{\Delta L/\Delta L}$ or $Rb1^{NF/NF}$ mice (73). However, it is noteworthy that transgenic mice expressing dominant negative TGF-β type II receptors have similar defects in their mammary glands and either did not develop spontaneous tumours (3), or developed tumours only after a very long latency (28). Future studies using transgenic induction of mammary tumorigenesis in our *Rb1* mutant mice will allow TGF-β's cell cycle control function in cancer development and metastasis to be studied in isolation.

$Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ cells are largely refractory to TGF-β1 growth inhibition in cell culture and our genetic cross to *MMTV-TGF-β1* mice suggests that loss of this proliferative control mechanism results in hyperplasia. We speculate that TGF-β signalling defects also lead to the nursing defect in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ females, given that mice expressing a dominant negative TGF-β type II receptor are also reported to have nursing defects (29). We envision a number of scenarios that could explain this defect. One possibility is that overproliferation of the ductal epithelium causes physical

blockage of the lumen, preventing milk letdown, and ultimately leading to dilated ducts. Another possibility is that the nursing defect is not proliferation-related. Since TGF-β signalling is necessary for contraction of smooth muscle cells (32, 33, 69), the distended milk filled ducts could result from reduced tension in myoepithelial cells. We did observe some ducts that lacked a complete ring of basal/myoepithelial cells in $Rb1^{\Delta L/\Delta L}$ sections (Fig. 3f), suggesting that there may be disruption of the myoepithelial layer. Therefore, it is possible that TGF-β confers a more contractile phenotype on the myoepithelium during lactation and this is lost in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ mammary glands.

We have demonstrated that pRB has a much more intimate role in TGF-β-mediated growth arrest than previously anticipated. This interpretation is based on the fact that TGF-β regulated growth control requires LXCXE interactions. Since $Rb1^{-/-}$ mice are not viable and exhibit numerous proliferative control defects (8, 40, 49) that are complemented in viable $Rb1^{\Delta L/\Delta L}$ and $Rb1^{NF/NF}$ animals, this indicates that pRB-LXCXE interactions are uniquely needed for a TGF-β cell cycle arrest in a very specific tissue. We interpret defective repression of E2F responsive genes to be the cause of the TGF-β arrest defect because pRB is hypophosphorylated after TGF-β stimulation, but transcript levels of E2F targets remain elevated as the cell cycle continues to advance. The identity of the exact LXCXE interacting protein(s) that pRB needs to contact in this growth arrest paradigm is unclear as numerous binding partners have been implicated in chromatin regulation during transcriptional repression (4, 17, 46, 53, 61, 72, 84). Identifying and characterizing the co-repressor(s) that cooperate with pRB in response to TGF-β will be critical to fully understanding how TGF-β inhibits cell proliferation.

We have demonstrated that pRB has an essential role in growth control of the mammary gland during development. This study also reveals that pRB is a key component of TGF-β induced growth arrest because it functions differently in this growth arrest pathway compared to other pRB-dependent growth suppressing functions in development. The $Rb1^{\Delta L}$ and $Rb1^{NF}$ mouse strains will be ideal to further advance our understanding of the mechanism of TGF-β growth arrest in the future.

2.5 References

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Chapter 3: Loss of pRB-LXCXE interactions specifically disrupts TGF-β proliferative control in mammary epithelial cells

3.1 Introduction

Transforming growth factor-β (TGF-β) induces broad cellular effects in vertebrate development and disease. In epithelial cells, TGF-β signalling on any one target cell is pleiotropic and potentially regulates many functions, including proliferation, apoptosis, differentiation, migration, and invasion (28, 38). To date, it has not been possible to separate the various functions of TGF-β, so the contribution of each of these pathways during development and disease pathologies remains unclear.

TGF-β can act as a potent inhibitor of cell proliferation *in vitro*, and loss of this type of negative growth regulation is thought to be a hallmark of human cancers (14). In some cancers, such as head and neck, gastric, pancreatic, ovarian, and colorectal carcinomas, components of the canonical pathway such as the TGF-β receptors or SMAD proteins are disrupted, resulting in loss of all TGF-β signalling (28). However, in other tumour types, including breast cancer, downstream targets of the growth regulatory pathway, such as cyclin dependent kinase inhibitors (CKIs) are deregulated (8, 27, 28). This is thought to result in the loss of TGF-β growth control, while other morphogenic aspects of its signalling can drive cancer progression and metastasis (15, 28, 38, 45). Support for this theory comes from several animal models. In mice expressing a dominant negative type II TGF-β receptor (dnIIR) in the mammary gland, the entire canonical TGF-β pathway is disrupted, and when treated with 7,12-dimethylbenz[a]anthracene (DMBA) or crossed to mice expressing an active form of the *Neu* proto-oncogene, tumour latency was

significantly reduced (5, 43). However, the apparent frequency of extravasation to form lung metastases was also reduced with loss of TGF-β signalling in the *Neu* model. Conversely, overexpression of TGF-β1 protects against primary tumour formation induced by DMBA, overexpression of wild type *Neu*, or overexpression of active forms of *Neu* but drives metastasis in these *Neu* models of breast cancer (32, 36, 43). The same holds true in mice expressing constitutively active forms of the transforming growth factor type I receptor (TβRI) (31, 43). Taken together, these models indicate that some aspects of TGF-β signalling protect against tumorigenesis. However, these models rely on manipulating all aspects of TGF-β signalling, and thus cannot conclusively demonstrate that negative growth regulation by TGF-β acts to protect against tumour formation and progression. Therefore, there remains a need to validate TGF-β-dependent proliferative control as a tumour suppressive mechanism.

Previous attempts to address this theory have focused on modulating cyclin dependent kinase inhibitors (CKIs) such as $p15^{INK4b}$ and $p21^{CIP1}$, which are SMAD-dependent targets. However, disruption of either CKI alone does not disrupt TGF-β growth control or result in mammary tumorigenesis *in vivo*, likely because of functional overlap among the different CKIs (7, 9, 10, 18, 23, 24, 26, 30, 33). Despite the extensive array of correlative data suggesting that TGF-β growth inhibition suppresses tumorigenesis, this model has been difficult to substantiate and the work in this chapter will address this question.

The activities of these different CKI networks converge upon activation of the retinoblastoma protein (pRB) (27). I have previously shown that mice with disruption of the LXCXE binding cleft of pRB ($Rb1^{\Delta L}$) display defective TGF-β growth control in the

mammary epithelium (11). In contrast, I demonstrate here that other TGF-β-mediated processes such as mammary alveolar differentiation and induction of apoptosis during involution appear unchanged in the mutant mammary gland. Surprisingly, I also demonstrate that pRB proliferative control does not protect against DMBA-induced tumorigenesis in $Rb1^{\Delta L/\Delta L}$ mice. Since this proliferative control relies in part on TGF-β-mediated anti-growth signals, this work raises questions about the importance of TGF-β growth inhibition as a tumour suppressive mechanism. Taken together, this work indicates that TGF-β growth regulation can be separated from other aspects of TGF-β signalling. Furthermore, despite its necessity during mammary gland development, TGF-β proliferative control appears to be largely dispensable for protection against carcinogen-induced tumorigenesis.

3.2 Materials and methods

Mouse strains and chemical induction of tumours

The *Rb1*$^{\Delta L}$ mouse strain has been described previously (19). Analyses of *Rb1*$^{\Delta L/\Delta L}$ mice were performed on a mixed 129/B6 background. Genotyping methods and PCR primers were provided by the suppliers or are as outlined by Isaac, *et al.* (See Appendix I) (19). All animals were housed and handled as approved by the Canadian Council on Animal Care.

To induce carcinogenesis, mice were treated by oral gavage with 1mg/mL of 7,12-dimethylbenz[a]anthracene (DMBA) in canola oil weekly for 4 weeks. Full necropsies were performed on animals with mammary tumours larger than 2 cm^2 or who displayed signs of distress, such as weight loss, piloerection, or lethargy.

Immunohistochemistry and immunofluorescence

The second and third thoracic mammary glands were dissected at day post-coital (dpc) 13.5, the second day of lactation (P2), the second day after pups were weaned (I2), or day 16 after involution (I16) (11) and fixed in neutral buffered 10% formalin. Fixed tissues were embedded in paraffin, cut into 5μm thick sections, and stained with hematoxylin and eosin (H&E).

To examine apoptosis, pups were removed from lactating females at P2. Two days post-weaning, females were sacrificed and the second and third thoracic mammary glands were formalin-fixed. Detection of cleaved caspase-3 was performed as per the manufacturer's instructions (9604; Cell Signaling). The total number of cleaved caspase-3-positive cells from 10 random fields of view was quantified for each of three mammary

glands per genotype, and the average number of apoptotic cells per field of view was calculated for each genotype. Images were captured on a Zeiss Axioskop40 microscope and Spot Flex camera using EyeImage software (Empix Imaging, Mississauga, Ontario).

Primary cell culture assays

Mammary epithelial cells (MECs) were harvested and cultured as previously described (11, 16). Cell culture experiments were carried out on passage one MECs. TGF-β1 growth inhibition assays were performed as previously described (11).

To examine differentiation in the mammary epithelial compartment, MECs were harvested from females at dpc 10.5-13.5. Cells were grown to confluence in normal MEC media (11), and then serum starved in MEC media containing 0.01% adult bovine serum (ABS) and lacking epidermal growth factor (EGF) for 48 hours. Cells were then induced to differentiate using MEC media containing 0.01% adult bovine serum (ABS), 1μg/mL hydrocortisone, 5μg/mL prolactin, and lacking epidermal growth factor (EGF), and supplemented with or without 500 pM TGF-β1 for 24 hours. Cells were then harvested and total RNA isolated using Trizol reagent (Invitrogen). The RNA was converted to cDNA using the Reverse Transcription System (Promega) as per the manufacturers' instructions. Quantitative real time PCR (qRT-PCR) was then performed using iQ SYBR Green Supermix (Bio-Rad) on a PTC-200 Thermal Cycler equipped with a Chromo 4 Continuous Fluorescence Detector, and the data was analyzed using Opticon Monitor 3.1 software (Bio-Rad). qRT-PCR was performed using primers against mouse β-casein and normalized to *Gapdh* expression (see Appendix I).

Migration and invasion assays

Collagen assays were performed in a similar manner as described by Muraoka, *et al.* (32). Briefly, MECs were plated onto a collagen bed and grown in MEC media with 0.01% serum and lacking epidermal growth factor (EGF) or insulin for 96 hours. Cells were then grown in this serum- and growth factor-reduced MEC media supplemented with or without various concentrations of TGF-β1 for 10 days. Cells were photographed on an Olympus IX70 microscope.

Scratch wound assays were performed using a protocol modified from Lamouille and Derynck (25). MECs were grown to confluence in a 24 well plate and then left in the serum- and growth factor-reduced media outlined above for 96 hours prior to wounding. Monolayer cells were scratched to create a wound with a p20 or p1000 pipette tip, and rinsed twice with serum- and growth factor-reduced media to remove debris. Cells were then treated with serum- and growth factor-reduced media supplemented with or without various concentrations of TGF-β1 for 24 hours. Cells were photographed at 0, 6, 12, and 24 hours on an Olympus IX70 microscope. Percent of initial wound area was calculated by measuring the wound area at each time point using Volocity 4 software (Improvision) and dividing by the initial wound area for three replicates from each treatment group.

Protein and mRNA quantification

pRB expression levels were measured by western blot analysis on nuclear extracts from $Rb1^{+/+}$, and $Rb1^{\Delta L/\Delta L}$ mammary glands using antibodies against pRB (G3-245; BD-Pharmingen), and Lamin A/C (MAB3211, Chemicon).

To measure messenger RNA levels, total RNA was extracted from asynchronous or TGF-β1 treated (100pM of TGF-β1 for 2 or 24 hours, for *Atf3* and *Cdkn2b* expression, respectively) MECs as outlined above. qRT-PCR was performed as described above and levels of mRNA were normalized to those of *Actb* (encodes β-actin) (see Appendix I).

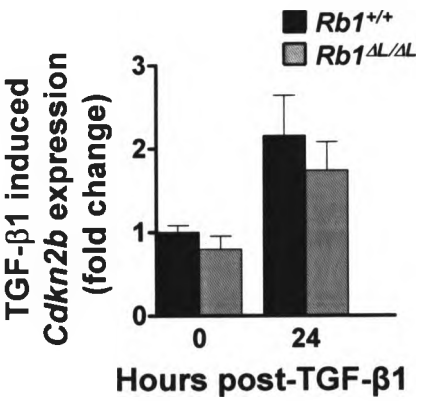
3.3 Results

Loss of LXCXE interactions disrupts TGF-β proliferative control in mammary epithelial cells

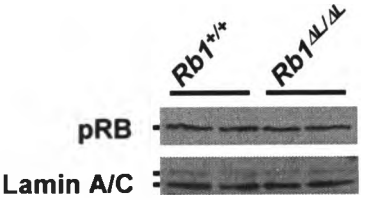
Our lab has previously used the $Rb1^{\Delta L}$ knock-in mouse model to disrupt the LXCXE binding cleft on the retinoblastoma protein (pRB). Disruption of LXCXE-mediated interactions results in defective TGF-β growth arrest of several cell types, including mammary epithelial cells (MECs). I have previously demonstrated that this defect occurs downstream of pRB in mutant murine embryonic fibroblasts (11). However, the mechanisms of TGF-β growth inhibition are tissue-specific, so it was essential to first confirm that the TGF-β anti-proliferative pathway was maintained upstream of pRB within the mammary epithelial compartment. In MECs, *Cdk2nb* (encodes $p15^{INK4b}$) is induced by SMAD-dependent transcription, leading to inhibition of CDKs, hypophosphorylation of pRB, and cell cycle arrest (42). I confirmed that *Cdk2nb* expression is induced to equal levels in $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MECs (Fig. 3.1a), suggesting that SMAD-dependent transcription of cell cycle regulatory proteins is functional in $Rb1^{\Delta L/\Delta L}$ MECs. As has been shown previously in MEFs, loss of the TGF-β proliferative response is not due to altered expression of pRB in MECs, since pRB levels were not different in the mammary glands of mice from both genotypes (Fig. 3.1b). These results complement the previous finding that TGF-β growth arrest is disrupted in mutant MECs (11). I have now demonstrated that SMAD-dependent transcription of one of the key proteins involved in maintaining pRB in a hypophosphorylated, active state in mammary epithelial cells is functional in $Rb1^{\Delta L/\Delta L}$ MECs. Together with our previous study, this work demonstrates the importance of pRB for TGF-β growth control and suggests that

Figure 3.1 Defective TGF-β proliferative control in *Rb1*$^{\Delta L/\Delta L}$ mammary epithelial cells. (A) RNA was extracted from *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MECs that had been treated with 100 pM TGF-β1 for 24 hours. The levels of *Cdkn2b*, which encodes the cyclin dependent kinase inhibitor p15^{INK4b}, were then measured by qRT-PCR. Error bars indicate one standard deviation from the mean for three MEC pairs. (B) pRB expression levels were examined by western blot analysis for *Rb1*$^{+/+}$, *Rb1*$^{\Delta L/+}$, and *Rb1*$^{\Delta L/\Delta L}$ mammary nuclear extracts.

A


Rb1+/+
Rb1ΔL/ΔL
TGF-β1 induced
Cdkn2b expression
(fold change)
3
2
1
0
0
24
Hours post-TGF-β1

B


Rb1+/+
Rb1ΔL/ΔL
pRB
Lamin A/C

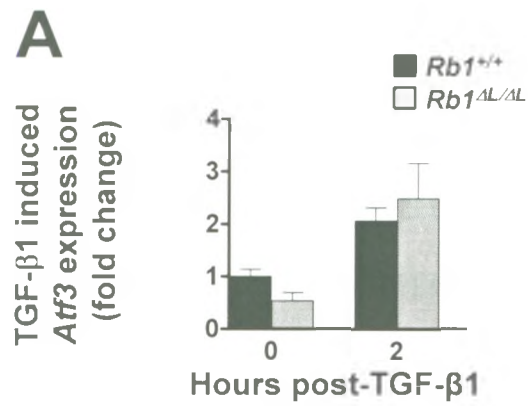
LXCXE interactions are critical for the TGF-β cytostatic response in the mammary epithelial compartment.

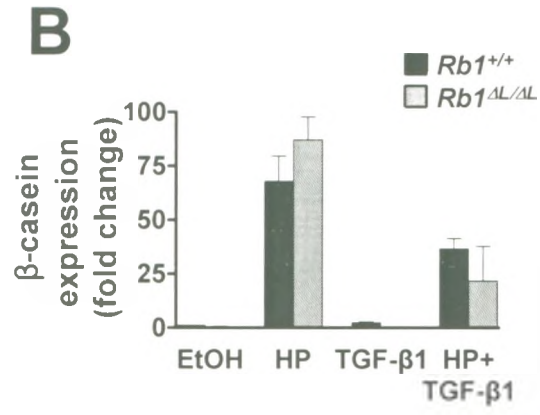
TGF-β-dependent differentiation and apoptosis are intact in mutant mice

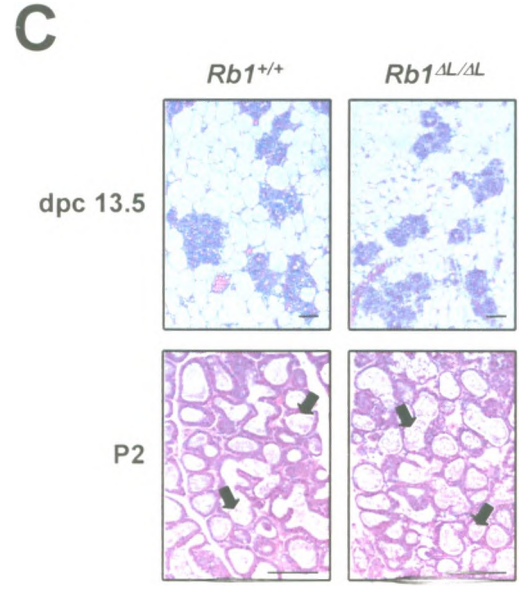
The growth arrest response is only one aspect of the complex TGF-β signalling network. While the only known role for pRB is the induction of the TGF-β cytostatic response, it was possible that pRB disruptions to the LXCXE binding cleft could affect other TGF-β-dependent pathways. I next determined if other arms of TGF-β signalling were disrupted by loss of LXCXE interactions in the mammary gland of *Rb1* mutant mice. At the molecular level, TGF-β has been implicated in the differentiation of MECs through activation of ATF3, which, in conjunction with SMAD3, can repress the Inhibitor of Differentiation 1 (ID1) (22). *Atf3* message levels were measured by qRT-PCR in MECs treated with TGF-β1. Levels of *Atf3* increased to a similar extent in $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MECs, suggesting that this aspect of TGF-β-mediated differentiation is intact (Fig. 3.2a).

Within the mammary gland, one of the most pronounced periods of differentiation occurs during pregnancy, when the epithelial cells differentiate into milk-producing lobuloalveolar structures. Functionally, TGF-β has been shown to inhibit this process. Expression of TGF-β family members is downregulated at the end of pregnancy (40), and mice with constitutive expression of TGF-β1 in the pregnant mammary gland exhibit significantly reduced lobuloalveolar formation (21). Conversely, inhibition of TGF-β signalling in the mammary gland using *MMTV-dnIIR* results in formation of alveolar structures in virgin females and the production of β–casein (13). Cell culture studies have also confirmed that TGF-β inhibits expression of β–casein (6, 29, 39, 44). Using MECs

Figure 3.2 TGF-β-dependent differentiation remains intact in *Rb1*$^{\Delta L/\Delta L}$ mammary epithelial cells. (A) *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MECs were treated with 100 pM TGF-β1 for two hours. RNA was then extracted and levels of *Atf3* were measured by qRT-PCR. Error bars indicate one standard deviation from the mean for three MEC pairs. (B) *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ MECs were serum starved for 48 hours and then treated with ethanol (EtOH), hydrocortisone (1 μM) and prolactin (5 μg/mL) (HP), 500 pM TGF-β1, or HP + TGF-β1 for 72 hours. RNA was then extracted and levels of *Csn2* (encodes β-casein) were measured by qRT-PCR. This trend was confirmed in a second MEC pair. (C) Paraffin sections of *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mammary glands from pregnant (dpc 13.5) and post-partum females (P2) were stained with H&E to verify differentiation of the mammary epithelium. Arrows indicate milk-filled alveoli. Scale bar: 100 μm.


A
Rb1+/+
Rb1ΔL/ΔL
TGF-β1 induced
Atf3 expression
(fold change)
4
3
2
1
0
0
2
Hours post-TGF-β1


B
Rb1+/+
Rb1ΔL/ΔL
β-casein
expression
(fold change)
100
75
50
25
0
EtOH
HP
TGF-β1
HP+
TGF-β1


C
Rb1+/+
Rb1ΔL/ΔL
dpc 13.5
P2

derived from mid-pregnant females, differentiation was stimulated and the effect that TGF-β had on this process was examined. Stimulation with differentiation media induced expression of *Csn2* (encodes β-casein) in both wild type and $Rb1^{\Delta L/\Delta L}$ MECs (Fig. 3.2b). Importantly, *Csn2* expression was inhibited in cells from both genotypes when TGF-β was added to the media (Fig. 3.2b). This demonstrates that the role of TGF-β during hormone-dependent differentiation of MECs is not affected by disruption of the LCXCE binding cleft. While this is in stark contrast to the pro-differentiation signal induced in the previous assay, the two experiments recapitulate two known roles for TGF-β (6, 22, 29, 39, 44).

It is possible that the opposing cellular outcomes in the above experiments (pro- or anti-differentiation) result from differences in microenvironment and extracellular signals in the two assays. Therefore, in order to understand the physiological relevance of TGF-β differentiation control, I examined the mammary glands of wild type and mutant females during pregnancy and lactation. Mice from both genotypes underwent a similar degree of lobuloalveolar formation during pregnancy and had milk-filled alveoli during lactation (Fig. 3.2c). This is consistent with previous data showing that $Rb1^{\Delta L/\Delta L}$ mice are able to produce milk with levels of milk protein comparable to wild type females (11). Together, these data suggest that the TGF-β-dependent effects on mammary epithelial differentiation are not affected by loss of LXCXE interactions.

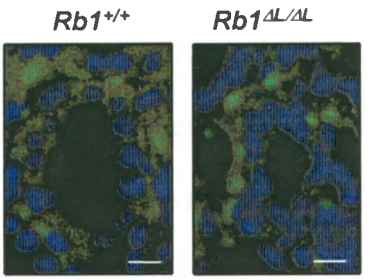
TGF-β can also control cell survival in epithelia (15, 28). In the mammary gland, TGF-β-dependent apoptosis occurs during involution of the post-lactational epithelia (1, 12, 34). To determine if TGF-β-dependent cell death was disrupted in mice lacking LXCXE interactions, I examined the levels of cleaved caspase-3 in paraffin sections from

Rb1$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ I2 mammary glands, a period of extensive TGF-β-mediated apoptosis. Immunofluorescence staining revealed no difference in cell death between genotypes (Fig. 3.3a and b). Consistently, histological analysis of paraffin sections from involuting mammary glands (I2) showed the presence of cells being shed into the lumens of the alveoli during this period, a common characteristic of apoptosis during involution (Fig. 3.3c) (37). Furthermore, mammary glands from mice of both genotypes had reverted back to a virgin-like state by 16 days post-weaning (Fig. 3.3c). This is in contrast to studies where overexpression of TGF-β3 in the mammary epithelium caused aberrant apoptosis and loss of *Tgf-β3* or expression of a dominant negative form of the type II receptor led to decreased apoptosis during involution (4, 12, 34). These experiments suggest that apoptosis during involution is unaffected by loss of pRB-LXCXE interactions. Since this apoptosis is dependent, in part, on TGF-β signalling, it suggests that TGF-β apoptosis is intact in the *Rb1*$^{\Delta L/\Delta L}$ mammary epithelial compartment.

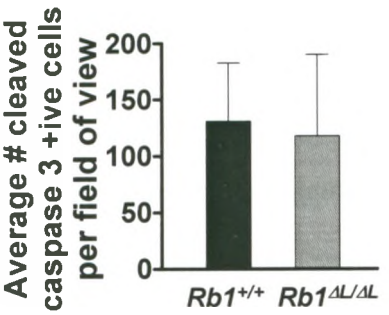
The biological data demonstrates that apoptosis and differentiation occur in an overtly normal manner in the *Rb1*$^{\Delta L/\Delta L}$ mammary gland. This was complemented by molecular studies demonstrating that known roles for TGF-β in differentiation and apoptosis in mammary cell lines are intact in mutant mammary epithelial cells. In contrast to the TGF-β cytostatic response, these experiments suggest that TGF-β-dependent differentiation and apoptosis are unaffected by the loss of pRB-LXCXE interactions in the mammary gland.

Figure 3.3 TGF- β-dependent apoptosis is similar in wild type and *Rb1*$^{\Delta L/\Delta L}$ mammary glands. (A) Apoptosis in *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mammary glands from females two days post-weaning (I2) was examined by cleaved caspase-3 staining (green) and counterstaining with DAPI (blue). Scale bar: 10 μm. (B) The average number of cells stained positive for cleaved caspase-3 per field of view was quantified for each genotype. Error bars represent one standard deviation from the mean of 30 fields of view for each genotype. (C) Paraffin sections of *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ mammary glands during involution (I2 and I16). Arrows indicate cells shed into the lumen of the ducts, which is characteristic of apoptosis. Scale bar: 50 μm.

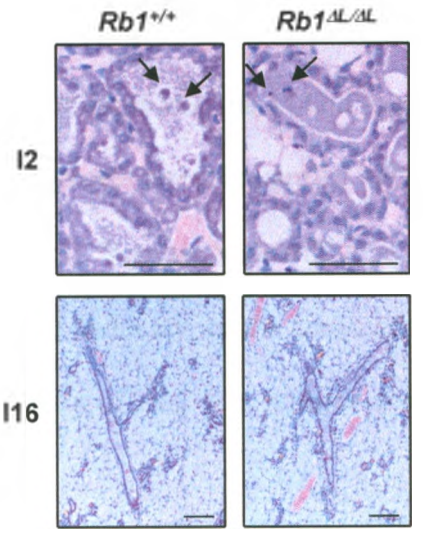
A


Rb1+/+
Rb1ΔL/ΔL

B


Average # cleaved caspase 3 +ive cells per field of view
200
150
100
50
0
Rb1+/+
Rb1ΔL/ΔL

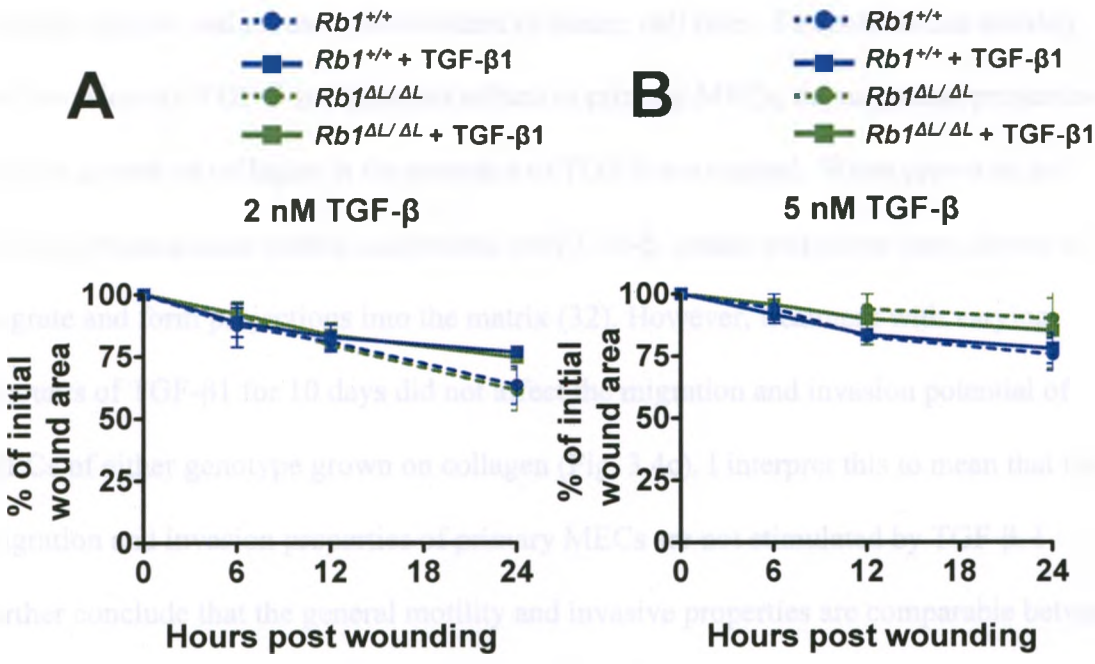
C

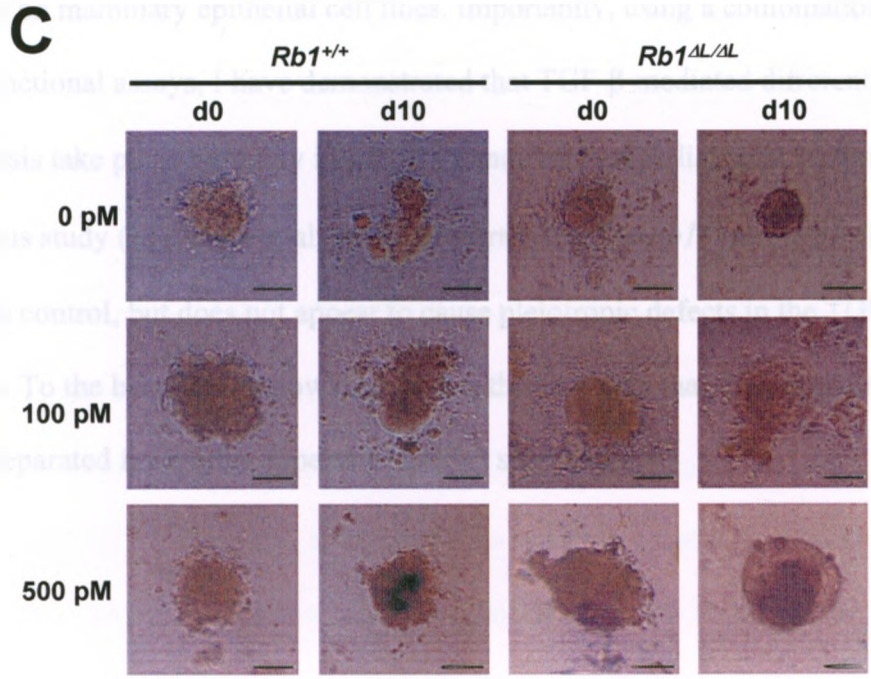

Rb1+/+
Rb1ΔL/ΔL
I2
I16

Invasion and motility occur independently of TGF-β signalling in primary murine mammary epithelial cells

Finally, the ability of TGF-β to control motility and invasion in mutant mammary epithelial cells was examined. Expression of TGF-β has been shown to enhance motility and invasion of murine mammary epithelial cell lines (3, 25), however this has not been investigated in primary MECs. To determine the effect of TGF-β on motility of cells derived from the ductal epithelium, I performed scratch wound assays on wild type and mutant MECs. These cultures were pre-treated with serum- and growth factor-reduced media for 4 days prior to wounding and TGF-β1 treatment so that proliferative effects would be minimized. Serum starved NMuMG cells normally migrate slowly to fill in a scratch wound, and the rate of wound healing is increased upon treatment with TGF-β1 (25). However, a significant difference in the rate of wound closure was not observed between TGF-β1 treated and untreated primary MECs of either genotype (Fig. 3.4a). The assay was repeated with increased amounts of TGF-β1, however, this did not significantly alter the primary observation on wound healing (Fig. 3.4b). The wound size and time of serum starvation were also varied and differences were not found between the treatment groups or genotypes (data not shown). Pre-treatment with TGF-β1 was also unable to stimulate the cells to migrate into the wound more rapidly (data not shown). Importantly, TGF-β1 induced growth arrest in parallel cultures of the TGF-β-sensitive HaCaT cell line (data not shown). Because both wild type and $Rb1^{\Delta L/\Delta L}$ cells could not be stimulated to migrate by TGF-β under any of the above conditions, it appears that motility may occur independently of TGF-β in primary mammary epithelia.

Figure 3.4 Migration and invasion occur independently of TGF-β in primary mammary epithelia. The percent of initial wound area was calculated for $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MECs that were untreated or treated with (A) 2 nM or (B) 5 nM TGF-β over a 24 hour time course. Error bars represent one standard deviation from the mean. (C) $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MECs were grown in serum- and growth factor-reduced medium on a collagen matrix supplemented with the given concentrations of TGF-β1. Representative micrographs are shown. Scale bar: 100 μm.


A
Rb1+/+
Rb1+/+ + TGF-β1
Rb1ΔL/ΔL
Rb1ΔL/ΔL + TGF-β1
2 nM TGF-β
% of initial wound area
100
75
50
25
0
0
6
12
18
24
Hours post wounding
B
Rb1+/+
Rb1+/+ + TGF-β1
Rb1ΔL/ΔL
Rb1ΔL/ΔL + TGF-β1
5 nM TGF-β
% of initial wound area
100
75
50
25
0
0
6
12
18
24
Hours post wounding


C
Rb1+/+
Rb1ΔL/ΔL
d0
d10
d0
d10
0 pM
100 pM
500 pM

This result is counter to the known TGF-β-dependent motility demonstrated in multiple human and mouse immortalized or cancer cell lines. To confirm that motility and invasion are TGF-β-independent effects in primary MECs, the migration properties of cells grown on collagen in the presence of TGF-β were tested. When grown on an artificial extracellular matrix and treated with TGF-β, cancer cells have been shown to migrate and form projections into the matrix (32). However, treatment with varying amounts of TGF-β1 for 10 days did not affect the migration and invasion potential of MECs of either genotype grown on collagen (Fig. 3.4c). I interpret this to mean that the migration and invasion properties of primary MECs are not stimulated by TGF-β. I further conclude that the general motility and invasive properties are comparable between wildtype and $Rb1^{\Delta L/\Delta L}$ mammary epithelial cells.

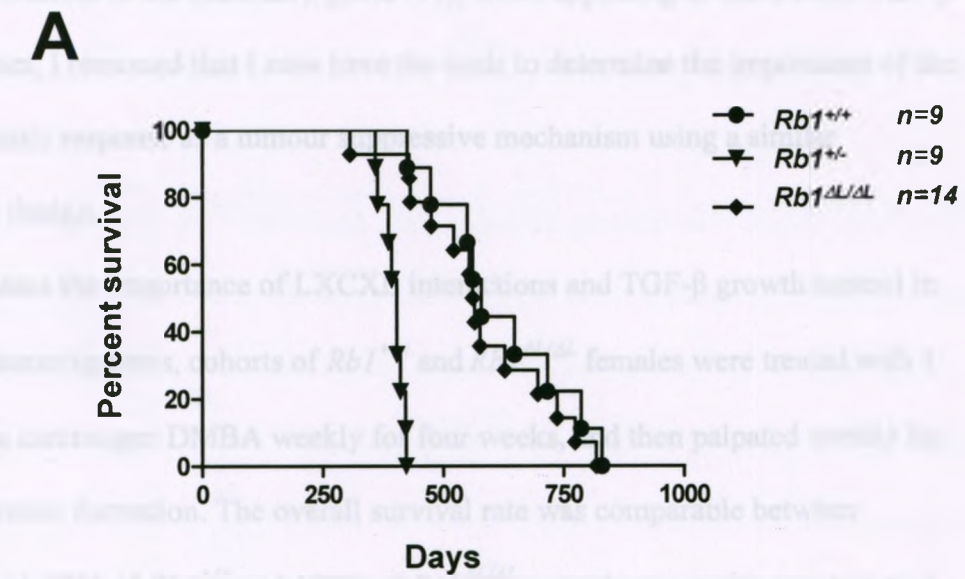
This work suggests that invasion and motility of primary murine MECs is not dependent on TGF-β signalling. In contrast, primary MECs appear to respond to TGF-β-dependent apoptotic and differentiation signals in a manner consistent with previous reports on mammary epithelial cell lines. Importantly, using a combination of biological and functional assays, I have demonstrated that TGF-β-mediated differentiation and apoptosis take place normally in $Rb1^{\Delta L/\Delta L}$ mammary epithelial cells. Together with our previous study (11), these analyses demonstrate that the $Rb1^{\Delta L}$ mutation disrupts TGF-β growth control, but does not appear to cause pleiotropic defects in the TGF-β pathway in MECs. To the best of my knowledge this is the first time that TGF-β growth control has been separated from other aspects of TGF-β signalling.

TGF-β growth inhibition does not protect against DMBA-induced tumorigenesis

Disruption of LXCXE interactions results in defective TGF-β proliferative control, while leaving other TGF-β signalling events intact. A similar phenomenon is thought to occur during breast cancer progression, where the TGF-β cytostatic response is severed, leaving pro-tumorigenic functions such as motility, invasion, and epithelial-to-mesenchymal transition to drive cancer progression (2, 28, 35). Thus, I next wanted to address the role of LXCXE interactions during mammary tumorigenesis. Defects caused by the $Rb^{\Delta L}$ mutation were not sufficient to cause spontaneous tumour formation in the mammary gland during the natural lifespan of the mice (Fig. 3.5a). Importantly, $Rb^{\Delta L/\Delta L}$ mice did not develop pituitary or thyroid tumours, which are associated with loss of a single allele of pRB (Fig. 3.5b) (17, 20). In contrast, $Rb1^{\Delta L/\Delta L}$ mice have a life expectancy similar to wild type controls and do not display any distinct pathology at time of euthanasia. However, this does not exclude the possibility that pRB-LXCXE interactions can protect against mammary tumour formation, since spontaneous mammary tumorigenesis does not occur in many mouse models of disrupted TGF-β signalling (see Table 1.1). Instead, a tumour suppressive role for TGF-β in the mammary gland was established by exposing those mice to carcinogens or using transgenic induction of mammary tumorigenesis.

To determine if pRB-LXCXE interactions play a tumour suppressive role in the mammary gland, wild type and $Rb^{\Delta L/\Delta L}$ females were treated with DMBA. Two previous reports have examined the role of TGF-β in DMBA-induced carcinogenesis. In the *MMTV-TGF-β1*$^{S223/225}$ model, mammary cells were protected from DMBA-mediated tumour formation, while loss of TGF-β signalling using the dominant negative type II

Figure 3.5 *Rb1*$^{\Delta L/\Delta L}$ mice do not develop spontaneous tumours. (A) Kaplan-Meier survival curves are shown for *Rb1*$^{+/+}$, *Rb1*$^{+/-}$, and *Rb1*$^{\Delta L/\Delta L}$ mice. (B) Photographs of normal *Rb1*$^{+/+}$ and *Rb1*$^{\Delta L/\Delta L}$ pituitaries at the time of necropsy. A pituitary tumour from an *Rb1*$^{+/-}$ control is shown for comparison. Scale bar: 2 mm.


A
Percent survival
100
80
60
40
20
0
0
250
500
750
1000
Days
Rb1+/+ n=9
Rb1+/- n=9
Rb1ΔL/ΔL n=14

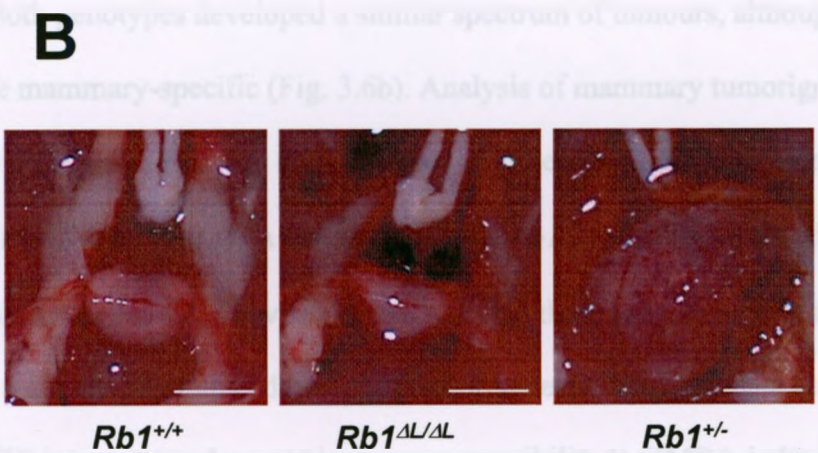
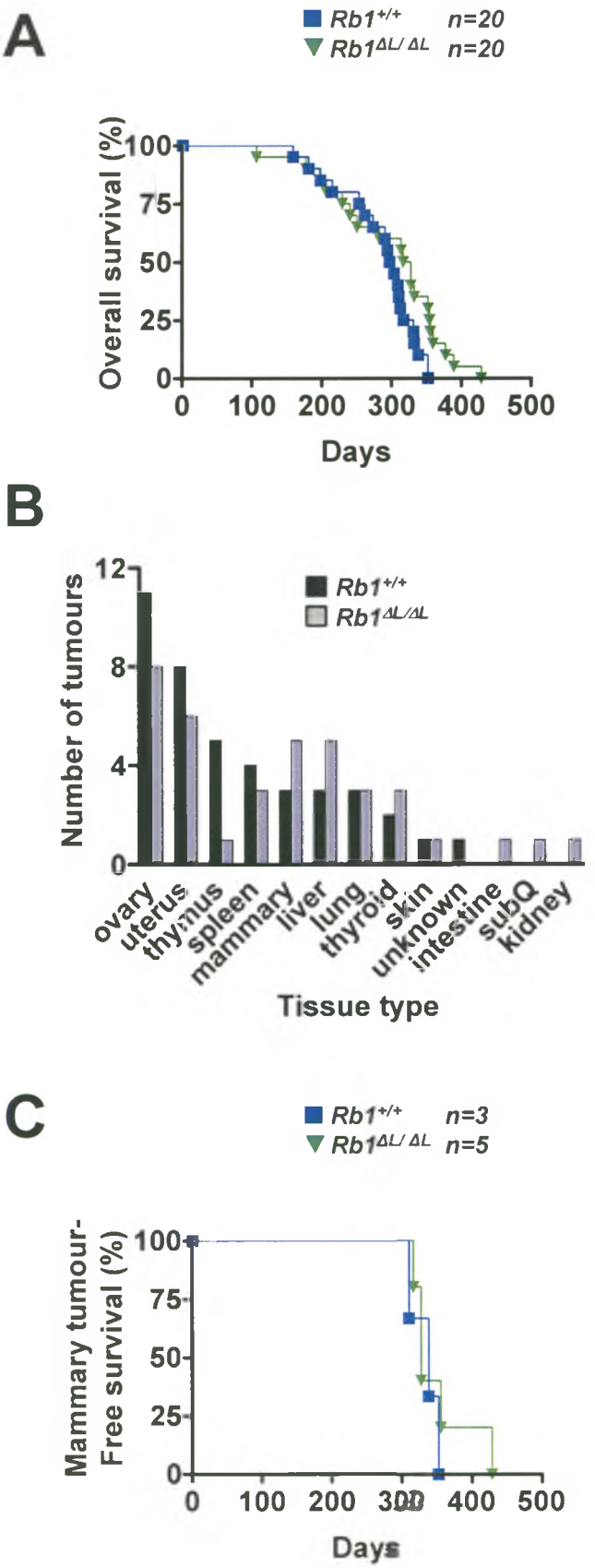

B
Rb1+/+
Rb1ΔL/ΔL
Rb1+/-

Figure 3.6 LXCXE interactions do not protect against DMBA-induced carcinogenesis. (A) Overall survival for all tumour types is shown for both genotypes (log rank test; *P=0.0534*). (B) The tumour spectrum from $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ females treated with DMBA. SubQ, subcutaneous. (C) Survival of the subset of mice that developed mammary tumours is shown for each genotype (log rank test; *P=0.4558*).


A
Rb1+/+ n=20
Rb1ΔL/ΔL n=20
Overall survival (%)
100
75
50
25
0
0
100
200
300
400
500
Days
B
Rb1+/+
Rb1ΔL/ΔL
Number of tumours
12
8
4
0
ovary
uterus
thymus
spleen
mammary
liver
lung
thyroid
skin
unknown
intestine
subQ
kidney
Tissue type
C
Rb1+/+ n=3
Rb1ΔL/ΔL n=5
Mammary tumour-Free survival (%)
100
75
50
25
0
0
100
200
300
400
500
Days

3.4 Discussion

Using the $Rb1^{\Delta L}$ knock-in mouse, I have revealed several novel findings about TGF-β signalling. First, by disrupting interactions at pRB's LXCXE binding cleft, I provide evidence that TGF-β growth control can be separated from other aspects of its signalling such as apoptosis and differentiation in mammary epithelial cells. I also demonstrate that migration of primary mammary epithelia in culture occurs via TGF-β-independent mechanisms. Finally, I report the striking finding that LXCXE interactions are not required for suppression of DMBA-induced carcinogenesis. By extension, because TGF-β proliferative regulation is disrupted in this model, this work argues that other aspects of TGF-β signalling may be more important than negative growth control for TGF-β-mediated tumour suppression for DMBA-driven tumorigenesis.

TGF-β can act as a strong inducer of motility and invasion in a variety of cell types. To determine if this aspect of TGF-β signalling was intact in the mutant mammary gland, I performed scratch wound and collagen assays on $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ MECs. Surprisingly, primary wild type mammary epithelial cells treated with TGF-β1 did not show an increase in motility in either the scratch wound or collagen assays compared to untreated controls. It is possible that the TGF-β1 used in the assay had lost its biological activity, and that is why no effect was seen. I do not favour this possibility because different aliquots of TGF-β1 from the same lot were able to induce a growth arrest signal in HaCaT cells. Furthermore, TGF-β1 did stimulate expression of *Atf3* in both wild type and $Rb1^{\Delta L/\Delta L}$ cells, suggesting that the TGF-β1 used in these assays were biologically active. It was also possible that more TGF-β1 is required to induce TGF-β-dependent motility than a growth arrest response. To test this theory, cells were treated with varying

concentrations of TGF-β in both experiments, yet this did not elicit a response in wild type cells, even at the highest concentrations. Instead, I hypothesize that primary cells respond differently to TGF-β invasion and motility cues than cells from established immortalized cell lines. A comparison of primary mammary epithelial cells versus NMuMG cells in motility and invasion assays may clarify this situation. However, using a combination of functional and biological assays, I have shown that differentiation and apoptosis, which are regulated in part by TGF-β, are intact within the $Rb1^{\Delta L/\Delta L}$ mammary gland. I cannot entirely rule out the possibility that some morphogenic signals transmitted by TGF-β family members are lost or reduced in the $Rb1^{\Delta L/\Delta L}$ mutant mice. However, I interpret the phenotypes of $Rb1^{\Delta L/\Delta L}$ mice as the first proof of principle that TGF-β's cell cycle arrest signals and other morphogenic signals are separable.

To examine the role of LXCXE interactions in tumorigenesis, I used a chemical model of tumour induction. DMBA has been used extensively to induce mammary tumorigenesis in mice and rats. Surprisingly, DMBA-treated mice developed a wide array of tumours but only a small proportion was mammary in origin. It is possible that differences in mouse strain or dosage schemes resulted in the low number of mammary tumours in this study. It was also surprising that the overall tumour latency was unchanged in the mutant mice compared to wild type controls. One interpretation of this result is that $Rb^{\Delta L}$ confers protection against DMBA. Saenz-Robles, *et al.* have shown that epithelial cells expressing the SV40 Large T antigen (TAg) contain significantly lower amounts of mRNA for several drug metabolizing/detoxifying enzymes (41). This effect depends on an intact LXCXE motif in TAg, suggesting that pRB plays a role in drug metabolism in an LXCXE-dependent manner. Therefore, it is possible that loss of

LXCXE interactions disrupts expression of cytochrome P450 enzymes that metabolize DMBA into its more carcinogenic form. This could counter the loss of proliferative control in $Rb1^{\Delta L/\Delta L}$ mice and mask a tumour suppressive role for the LXCXE binding cleft. Future studies examining the levels of various drug metabolizing enzymes in wild type and $Rb1^{\Delta L/\Delta L}$ animals may shed light on this possibility.

An alternative interpretation is that TGF-β growth control is dispensable for tumour suppression in the DMBA model. Overexpression of TGF-β1 in the mammary gland has been shown to protect against DMBA tumour formation (36). Conversely, DMBA has been shown to induce mammary tumours with a shorter latency in mice expressing a dnIIR in the mammary gland (5). In that study, all aspects of the TGF-β signalling pathway were lost, but the more aggressive phenotype was attributed to loss of TGF-β's ability to induce a cell cycle arrest. In contrast, TGF-β growth control was the only aspect of TGF-β signalling disrupted in the mammary gland of the $Rb1^{\Delta L/\Delta L}$ mice, yet tumorigenesis was not enhanced. This suggests that other aspects of TGF-β signalling, such as apoptosis and differentiation may play stronger tumour suppressive roles than the cytostatic response. Future studies looking at the role of these aspects of TGF-β signalling in isolation will help to clarify their specific roles in tumour suppression and/or progression.

3.5 References

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Chapter 4: The role of proliferative control by the retinoblastoma protein in mammary cancer and metastasis

4.1 Introduction

Cellular proliferation is a tightly regulated process and the loss of responsiveness to negative growth signals is a hallmark of cancer cells (16). This is especially evident in the breast, where epithelial cells undergo constant fluctuations in proliferation, differentiation, and apoptosis during the menstrual cycle and as a result of pregnancy (17). Tight growth control of the mammary epithelial compartment is crucial, and disruptions to the balance of mitogenic and anti-growth signals can leave this highly proliferative tissue susceptible to the formation of cancer (27, 39). Therefore, delineating how proliferative control of breast epithelial cells is lost during tumour formation is essential to understand the pathogenesis of breast cancer.

The decision for a somatic cell to remain quiescent or re-enter the cell cycle is coordinated by the retinoblastoma protein (pRB) pathway (27, 39). In response to mitogenic signals, pRB is inactivated by cyclin dependent kinase (CDK) complexes by phosphorylation. Simultaneously, growth inhibiting signals can activate pRB by blocking cyclin dependent kinase activity through the actions of cyclin dependent kinase inhibitors (CKIs) such as $p15^{INK4b}$, $p16^{INK4a}$, $p21^{CIP1}$, and $p27^{KIP1}$ (27, 40). In cell culture, disruption of an individual CKI, such as $p21^{CIP1}$, can result in deregulated proliferation despite signals from negative growth regulatory stimuli such as DNA damage (4, 5). However, this does not cause a complete loss of responsiveness to double stranded DNA breaks, and residual growth inhibition in the absence of $p21^{CIP1}$ suggests that there is

redundancy amongst the Ink4 and CIP/KIP protein families (5). Indeed, negative growth regulators such as transforming growth factor β (TGF-β) are capable of inducing a cell cycle arrest in the absence of any one of these CKI proteins (13, 19, 29, 31). Furthermore, individual ablation of these genes in mice has no effect on viability and is accompanied by surprisingly few developmental abnormalities. Interestingly, each knockout mouse strain develops tumours in a specific subset of tissues, but none of these mice develop mammary tumours (9, 12, 21-24, 31, 38). This is surprising because pRB pathway components such as cyclin D are commonly amplified and pRB itself is sometimes lost in human breast cancers and these are direct targets of CKI regulation (2, 3, 11, 32, 37). Thus, even though the current body of literature suggests that anti-proliferative effects greatly influence mammary epithelium, mouse models reveal layers of complexity and variation that make it challenging to understand how negative growth responses protect mammary epithelial cells from aberrant proliferation.

Activation of pRB is the last opportunity to arrest proliferation in G1 and avoid inappropriate cell cycle entry (27). Breast epithelial cells frequently respond to anti-growth signals from DNA damage, exogenous growth factors like TGF-β, and other cellular stresses. All of these serve to activate pRB and induce cell cycle arrest in the G1 phase (43). Using a knock-in mouse model ($Rb1^{\Delta L}$) with a discrete defect in its growth suppressive pocket domain that eliminates interactions with LXCXE-motif containing proteins, we have demonstrated the importance of pRB in proliferative control during mammary gland development (14). Loss of LXCXE interactions prevents pRB from recruiting co-repressors, such as histone deacetylases, to E2F responsive promoters. This results in an inability to silence transcription and in turn causes defects in proliferative

control, including insensitivity to TGF-β and senescence cues (14, 42). Furthermore, cells from these mice fail to arrest proliferation in response to ectopic expression of the CKI proteins $p16^{INK4a}$ and $p21^{CIP1}$, suggesting that there are likely additional negative growth signals to which cells from these mice are resistant (14). In the mammary gland, this results in hyperplasia of the mammary ductal epithelium (14), a known risk factor for human breast cancer (10, 35). Since pRB-dependent negative growth control is reduced in the mammary glands of $Rb1^{\Delta L}$ mice, it provides us with a unique opportunity to examine the function of proliferative control in mammary cancer and metastasis.

In order to understand the role of pRB LXCXE-dependent proliferative control during mammary tumour formation and progression, I have crossed our mice with a *Wap-*$p53^{R172H}$ transgenic strain. *Wap-* $p53^{R172H}$; $Rb1^{\Delta L/\Delta L}$ females developed mammary tumours more frequently and at a faster rate than control mice. In many cases these animals also developed metastases. In contrast, co-expression of *Neu* and $Rb1^{\Delta L}$ did not accelerate mammary tumour progression or metastasis. Our data indicate that pRB LXCXE-dependent proliferative control forms a barrier to primary tumour formation. Surprisingly, the contrasting data between the two transgenic mammary tumour models indicates that the response to negative growth signals by pRB is context-dependent.

4.2 Materials and Methods

Mouse strains

The *Rb1*$^{\Delta L}$ mouse strain has been described previously (20). Analyses of *Rb1*$^{\Delta L/\Delta L}$ mice were performed on a mixed 129/B6 background. *Wap-p53*^{R172H} mice were obtained from the Mouse Models of Human Cancer Consortium on an FVB background. These mice express the p53^{R172H} mutation driven by the *whey acidic protein (Wap)* promoter (25). These mice were bred to the *Rb1*$^{\Delta L}$ mutation, creating a mixed 129/B6/FVB genetic background. *Wap-p53*^{R172H}; *Rb1*$^{+/+}$ and *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ females were bred through five or six rounds of pregnancy to induce expression of p53^{R172H}. Live pups were removed at P2 to allow equivalent timing of transgene expression between genotypes. *Tg(MMTVneu)202Mul* mice express the wild type form of the rat *Neu* oncogene driven by the *murine mammary tumour virus* promoter (*MMTV*) (15). These mice were obtained from Jackson Labs on an FVB background and were bred to the *Rb1*$^{\Delta L}$ mutation, creating a mixed 129/B6/FVB genetic background. Genotyping methods and PCR primers were provided by the suppliers or are as outlined by Isaac, *et al.* (20)(Appendix I). All animals were housed and handled as approved by the Canadian Council on Animal Care.

Histology and mammary whole mounts

Full necropsies were performed on tumour-bearing animals after 60 days or at the time of euthanasia. Mammary tumours, lung tissues, and any other tissues that appeared abnormal were fixed in formalin and sectioned as previously described (14). The mitotic indices were manually counted in 5 high-power fields of view (400x) for mammary tumours from each genotype. Lung metastases were identified by gross morphological analysis (surface metastases) and microscopic analysis (micro metastases). Percent

metastatic surface area (SA) was calculated by measuring the total two dimensional area occupied by lung metastases in five hematoxylin and eosin (H&E) stained lung sections, divided by the total area of the lung in these sections using Volocity 4 software (Perkin Elmer, Waltham, MA). Analysis of hyperplasia in H&E stained sections of $Rb1^{\Delta L/+}$ mammary glands, as well as *Neu* expressing mammary glands, was performed as described previously (14). For whole-mount analysis, unaffected mammary glands from tumour-burdened mice were removed, mounted on glass slides, and stained with Carmine Red using standard techniques.

Primary cell culture assays

Mammary epithelial cells (MECs) were harvested and cultured as previously described (14, 18). Cell culture experiments were carried out on passage 1 or 2 MECs. TGF-β1 growth inhibition assays were performed as previously described (14).

Soft Agar Colony Formation Assay

$Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ mouse embryonic fibroblasts (MEFs) were retrovirally transfected with the pLXSN dominant negative (dn) p53/Ras^{V12} virus as previously described (33, 36). Infected cells were then grown in soft agar according to standard protocols (8). Cells were allowed to grow for 2 weeks, at which time colonies were photographed and counted. The cut-off for scoring a colony as transformed was that its size needed to correspond with the volume of at least 5 cells (as judged by neighbouring single cells). In this way we were confident that these colonies represented multiple cell divisions.

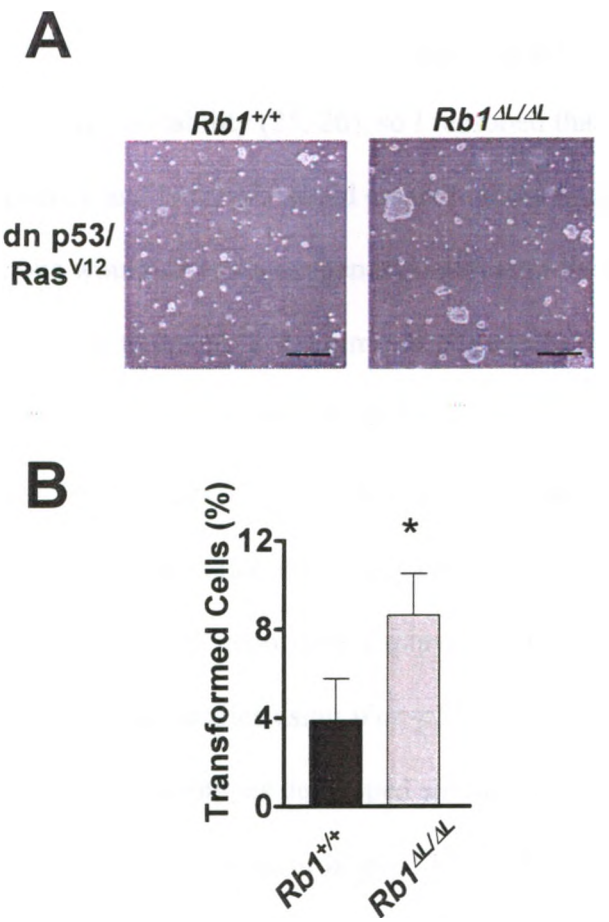
4.3 Results

pRB-LXCXE interactions act as an initial barrier to tumour formation

Our lab has previously generated a knock-in mouse model ($Rb1^{\Delta L}$) to disrupt the LXCXE binding cleft on the retinoblastoma tumour suppressor protein (pRB) (20). Loss of LXCXE-dependent interactions disrupts proliferative control in the mammary gland during development, leading to hyperplasia of the ductal epithelium that is ubiquitously detectable in virgin animals between four and 16 weeks of age (14). Surprisingly, the $Rb1^{\Delta L}$ mutation alone does not predispose mice to mammary cancer (14). Since hyperplasia of ductal epithelia is considered a risk factor for human breast cancer (10, 35), I postulated that LXCXE interactions play a tumour suppressive role in the mammary gland when combined with other oncogenic changes. To explore this possibility, a soft agar colony assay was performed using $Rb1^{+/+}$ and $Rb1^{\Delta L/\Delta L}$ mouse embryonic fibroblasts infected with a dominant negative form of p53 and an oncogenic allele of Ras (pLXSN dn p53/Ras^{V12}) (33). This dominant negative form of p53 has been shown to cooperate with Ras to form colonies in soft agar, and loss of LXCXE interactions led to an increase in the number of colonies formed (Fig. 4.1a, b). $Rb1^{\Delta L/\Delta L}$ colonies were also larger than control colonies (Fig. 4.1a), suggesting that cells lacking LXCXE interactions were able to transform earlier. This provided preliminary evidence that LXCXE-dependent anti-proliferative effects can protect cells from oncogenic transformation.

To validate that LXCXE interactions can play a tumour suppressive role in the mammary gland, I crossed our mice into the *Wap-p53*^{R172H} background. *Wap-p53*^{R172H} is a dominant negative form of p53 driven by the *whey acidic protein* promoter, which is

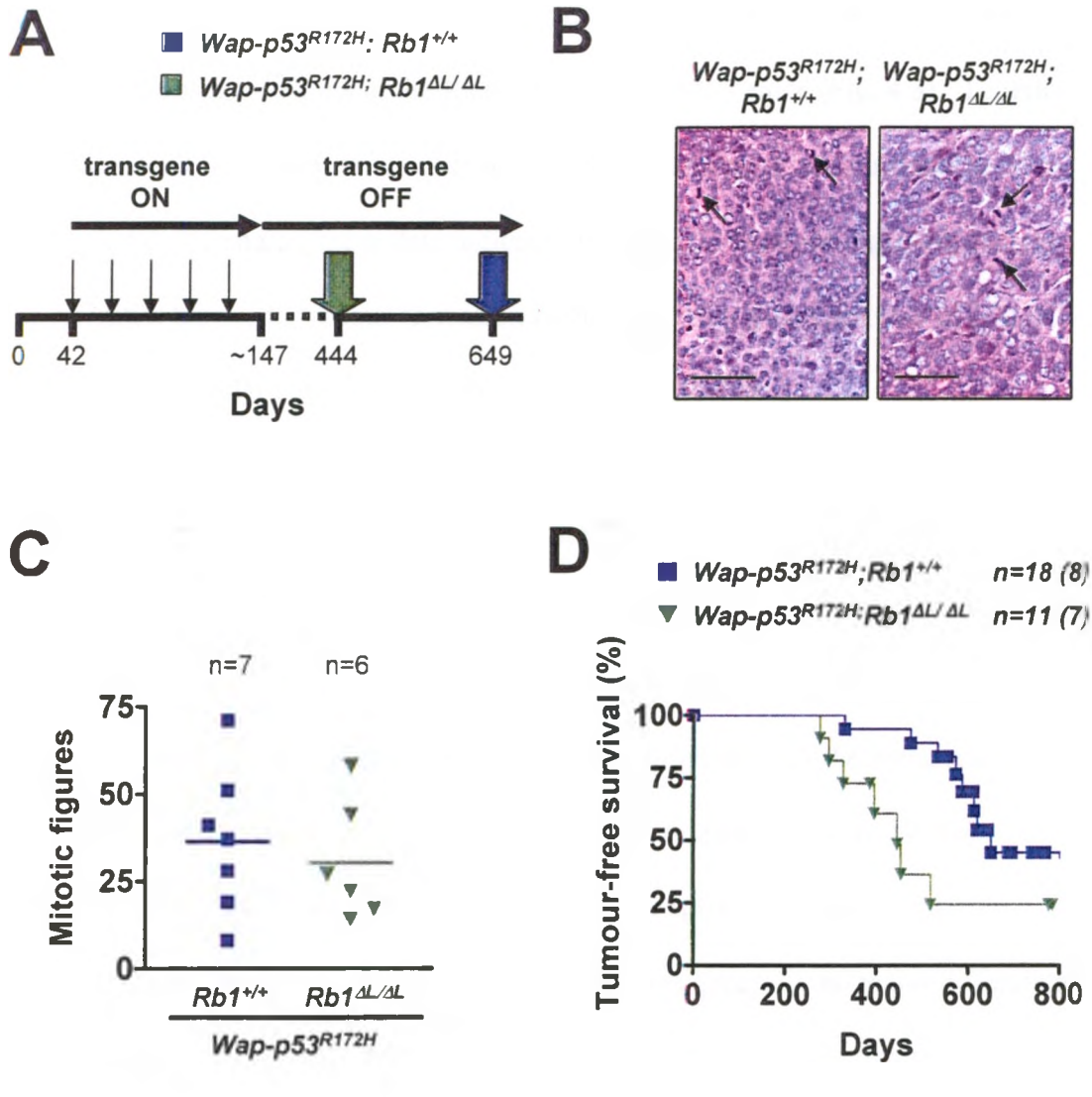
Figure 4.1 The *Rb1*$^{\Delta L}$ mutation confers sensitivity to oncogenic transformation. (A) MEF cells corresponding to the indicated genotypes were transduced with retroviruses expressing dominant negative (dn) p53 and Ras^{V12} and plated in soft agar to allow colonies to form. Photomicrographs were taken after a two-week growth period. Scale bar: 200 μm. (B) The percentage of wild type and mutant cells that transformed and grew into a colony was calculated from five randomly photographed microscopic images. A cell was counted as transformed if it formed a colony whose size appeared to be at least 5 cells. * indicates a statistically significant difference (Student's t test; $P<0.005$). Error bars indicate one standard deviation from the mean for at least three replicates.


A
Rb1+/+
Rb1ΔL/ΔL
dn p53/
RasV12
B
Transformed Cells (%)
12
8
4
0
*
Rb1+/+
Rb1ΔL/ΔL

expressed in the mammary gland during pregnancy and lactation. Cohorts of *Wap-p53^{R172H}; Rb1$^{+/+}$* and *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* females were bred through five rounds of pregnancy in order to induce transgene expression (Fig. 4.2a). Since *Rb1$^{\Delta L/\Delta L}$* females are frequently unable to nurse their pups (14), all live pups were removed two days after birth to ensure consistent timing of transgene expression between genotypes. Wap-p53^{R172H} expression leads to genomic instability (25, 26), so I reasoned that expression of Wap-p53^{R172H} during pregnancy and lactation would create random mutations. I expected that some of these mutations would drive tumorigenesis later, after the transgene was turned off, and this would allow us to assess how a diminished response to negative growth regulators affects mammary tumorigenesis. Both *Wap-p53^{R172H}; Rb1$^{+/+}$* and *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* females developed high grade mammary adenocarcinomas, characterized by high cytological variability. Many cells exhibited large cellular and nuclear size and tumours from both genotypes displayed high rates of mitosis (Fig. 4.2b, c), which is comparable to other studies using *Wap-p53^{R172H}* mice (25, 26).

While mice from both genotypes developed similar types of tumours, I did find an increased frequency of tumour formation in *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* females. 63.6% of *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* females compared to 44.4% of *Wap-p53^{R172H}; Rb1$^{+/+}$* females developed tumours over the course of the study. Importantly, loss of LXCXE interactions in the *Wap-p53^{R172H}* background resulted in a statistically significant decrease in tumour latency (Fig. 4.2d) (Log rank test, P=0.0238). Like the data from our soft agar colony assay, this suggests that LXCXE-dependent proliferative control can act as a barrier to tumour initiation. To explore this concept further, I examined tumour-free mammary glands from our tumour-burdened mice. Some mammary glands had extensive

Figure 4.2 pRB-LXCXE interactions protect against tumour formation caused by the *Wap-p53^{R172H}* transgene. (A) Experimental outline for the *Wap-p53^{R172H}* tumour study. Mice were bred through five rounds of pregnancy (thin arrows) to transiently induce p53^{R172H} expression within the mammary gland. After the fifth pregnancy, males were removed and females were palpated weekly to monitor tumour formation. Median tumour-free survival for each genotype is marked with the colored arrows. (B) Representative H&E stained paraffin sections from tumours harvested from *Wap-p53^{R172H}; Rb1$^{+/+}$* and *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* mice. Arrows indicate mitotic figures. Scale bar: 50 μm. (C) The mitotic index for *Wap-p53^{R172H}; Rb1$^{+/+}$* and *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* mice is indicated, along with the average mitotic index for each genotype. Values were derived by quantifying the number of mitotic figures in five random fields of view for each mouse. (D) Kaplan-Meier graph of mammary tumorigenesis is shown for *Wap-p53^{R172H}; Rb1$^{+/+}$* and *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* females (log rank test; *P=0.0238*). Values in brackets indicate the number of mice that developed tumours.


A
Wap-p53R172H; Rb1+/+
Wap-p53R172H; Rb1ΔL/ΔL
transgene ON
transgene OFF
0
42
~147
444
649
Days
B
Wap-p53R172H; Rb1+/+
Wap-p53R172H; Rb1ΔL/ΔL
C
n=7
n=6
Mitotic figures
75
50
25
0
Rb1+/+
Rb1ΔL/ΔL
Wap-p53R172H
D
Wap-p53R172H;Rb1+/+ n=18 (8)
Wap-p53R172H;Rb1ΔL/ΔL n=11 (7)
Tumour-free survival (%)
100
75
50
25
0
0
200
400
600
800
Days

lobuloalveolar development, preventing an assessment of abnormal proliferation by whole mount staining. However, among the remaining necropsied animals, I examined glands where tumours were not palpable, and discovered that there was an increase in the number of hyperplastic lesions in the *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ mice (Fig. 4.3). Together with the increased frequency of tumours and shortened timing of tumour onset in *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ females, these data indicate that LXCXE-dependent growth control acts as an initial barrier to tumour formation in the mammary gland and loss of LXCXE interactions leaves cells vulnerable to oncogenic transformation.

The data from our *Wap-p53*^{R172H} cross revealed that LXCXE interactions can act as an important barrier to tumour formation. I next asked whether loss of LXCXE proliferative control could also affect the formation of metastases at secondary sites. Both *Wap-p53*^{R172H}; *Rb1*$^{+/+}$ and *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ tumours were able to metastasize to the lungs, and there were no major differences in the appearance of the metastases, or the number, or size of these metastases (Fig. 4.4). Of note, one female from the *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ cohort developed a metastasis in the spleen and had extensive colonization of the lungs (Fig. 4.4). However, there were generally no major differences in metastatic potential between the two genotypes. Coupled with the primary tumour data, I conclude that reduced responsiveness to negative growth signals likely has a limited role in tumour progression and metastasis, but functions as a barrier to tumour initiation at a very early stage in cancer pathogenesis.

LXCXE interaction-dependent anti-tumorigenic effects are context-dependent

The advantage of the *Wap-p53*^{R172H} model is that it introduces random mutations into the genome and this creates a selection for mutations that can cooperate with defects

Figure 4.3 Hyperplastic nodule formation in the *Wap-p53*^{R172H} background. Carmine Red–stained mammary whole mounts from tumor-free glands in mice that had mammary tumours are shown for both genotypes in the *Wap-p53*^{R172H} background. Arrows indicate hyperplastic nodules and LN indicates lymph nodes. Scale bar: 2 mm. The number of hyperplastic nodules in each whole mount section was also quantified for each genotype along with the average number of hyperplastic nodules.

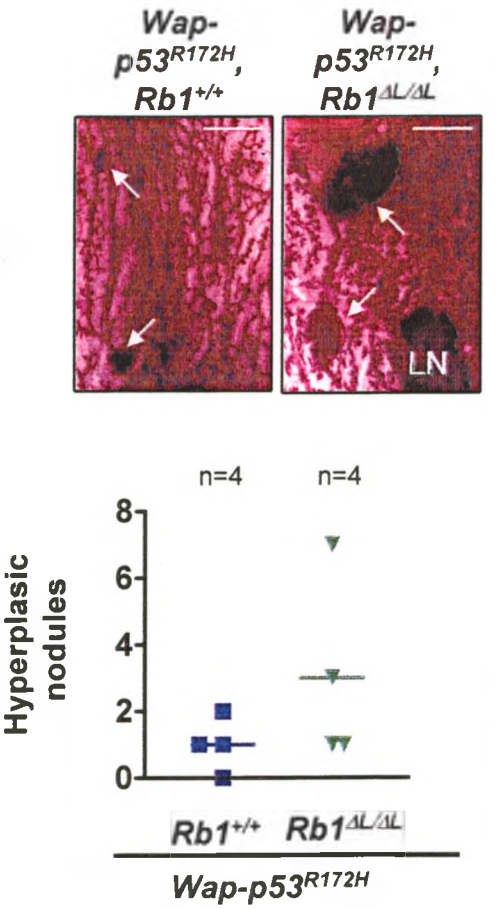
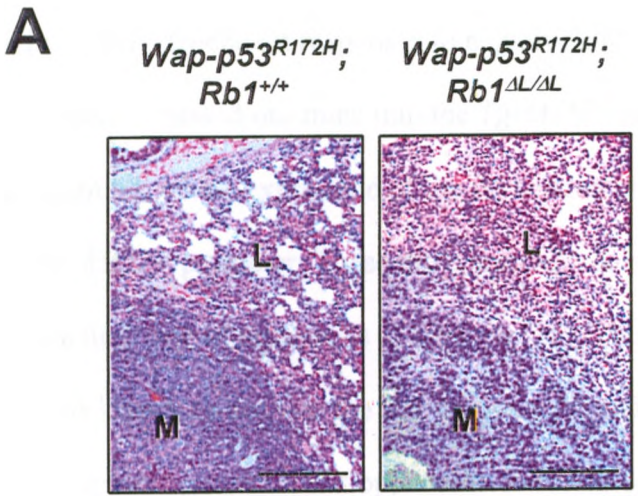
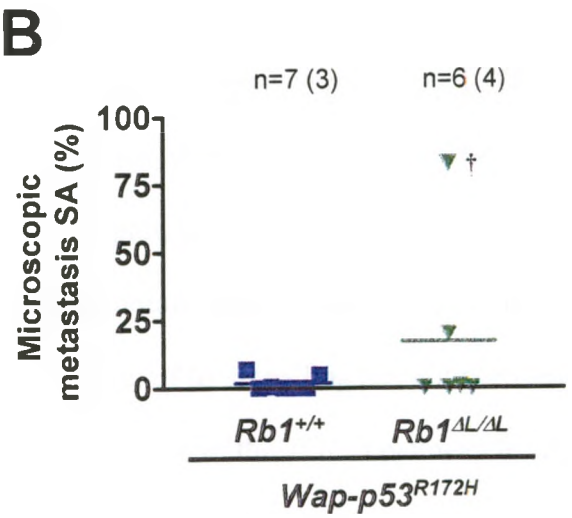

Wap-p53R172H, Rb1+/+
Wap-p53R172H, Rb1ΔL/ΔL
LN
n=4
n=4
Hyperplasic nodules
8
6
4
2
0
Rb1+/+
Rb1ΔL/ΔL
Wap-p53R172H

Figure 4.4 Metastases form in the *Wap-p53^{R172H}; Rb1$^{+/+}$* and *Wap-p53^{R172H}; Rb1$^{\Delta L/\Delta L}$* mice. (A) Representative H&E stained paraffin sections of lungs harvested from tumour-burdened mice of each genotype. M denotes metastasis, and L denotes neighbouring lung tissue. Scale bar: 100μm. (B) The surface area (SA) occupied by lung metastases relative to the total lung area in tissue sections was quantified for mice from each genotype along with the average SA. Values in brackets indicate the number of mice that developed metastases and † indicates a female that developed metastases to both the lung and spleen.


A
Wap-p53R172H; Rb1+/+
Wap-p53R172H; Rb1ΔL/ΔL
L
M
L
M


B
n=7 (3)
n=6 (4)
Microscopic metastasis SA (%)
100
75
50
25
0
†
Rb1+/+
Rb1ΔL/ΔL
Wap-p53R172H

found in $Rb1^{\Delta L/\Delta L}$ mice. However, this also prevents us from knowing what the initiating oncogenic mutations were and how they engaged negative growth responses that activate a pRB-LXCXE-dependent arrest. For this reason I also used a transgenic line that expresses a dominantly acting oncogene so that the origin of oncogenesis would be known. To determine how pRB-dependent responses to negative signals affect mammary tumorigenesis in this context, I crossed our mice into the *Tg(MMTVneu)202Mul* (herein referred to as *Neu*) background, where expression of the rat version of the *Neu* proto-oncogene is driven by the *MMTV* promoter. These mice normally develop focal mammary tumours with a high rate of metastasis to the lung (15). This transgenic line was chosen because *Neu* is known to activate the Ras pathway (7) and our data in Figure 4.1 indicates that pRB-dependent growth arrest opposes it. Furthermore, the *Neu* mouse has been used extensively to examine the influence of TGF-β on tumorigenesis. Expression of active forms of *Neu* in mice with disrupted TGF-β signalling results in reduced tumour latency (41). Conversely, when crossed to mice that overexpress TGF-β or a constitutively active receptor, primary tumour formation is delayed or tumour growth is slowed (30, 41). This anti-tumour effect is commonly attributed to TGF-β-induced cell cycle arrest although this aspect of its signalling has not been testable in isolation before now. In these same mice, activation of the TGF-β pathway leads to increased metastasis to the lungs, presumably because pro-tumorigenic aspects of TGF-β signalling such as epithelial-to-mesenchymal transition, migration, and invasion drive the metastatic cascade (28, 30, 34, 41).

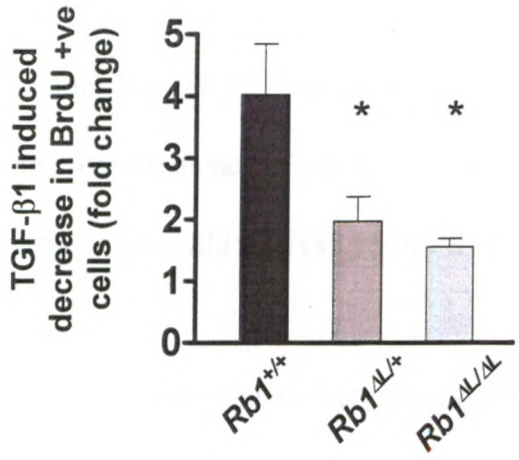
Since TGF-β is a key regulator of proliferation in the mammary epithelial compartment and induces a G1 arrest in a pRB-dependent manner, I next wanted to

characterize the *Neu; Rb1*$^{\Delta L/\Delta L}$ genotype to ensure that this experimental system would allow us to address the role of negative growth responses during tumorigenesis as I expected. To this end I tested *Rb1*$^{+/+}$, *Rb1*$^{\Delta L/\Delta L}$, and *Rb1*$^{\Delta L/+}$ mammary epithelial cells (MECs) for their ability to respond to TGF-β–induced G1 arrest (Fig. 4.5a). This confirmed our previous results that TGF-β's cytostatic response is vastly diminished in *Rb1*$^{\Delta L/\Delta L}$ cells. Surprisingly, *Rb1*$^{\Delta L/+}$ MECs have a similar defect in TGF-β growth control, indicating that mutation of only one copy of *Rb1* is sufficient to abrogate its arrest mechanism. Consistent with this observation, examination of mammary epithelia in *Rb1*$^{\Delta L/+}$ virgin female mice revealed they have a similar degree of hyperplasia as we have previously reported for *Rb1*$^{\Delta L/\Delta L}$ mice (Fig. 4.5b). I also examined the mammary glands of 8 week old *Neu* mice combined with each *Rb1* genotype in the 129/B6/FVB background (Fig. 4.5c). Expression of *Neu* raised the basal level of hyperplasia in these mice. However, the complete loss of LXCXE interactions in *Rb1*$^{\Delta L/\Delta L}$ mice still exacerbated this phenotype. This confirmed that the proliferative control defects caused by the *Rb1*$^{\Delta L}$ mutation, which led to hyperplasia of mammary epithelia, are present in these experimental animals too.

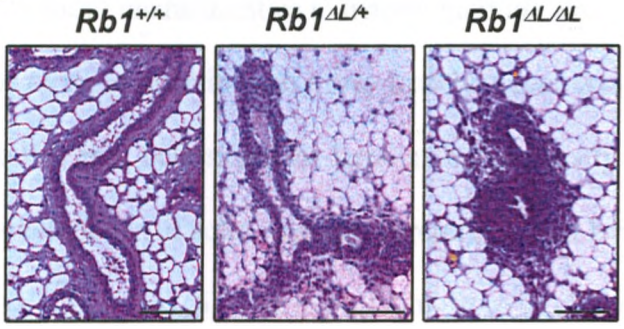
To assess the importance of LXCXE-dependent negative growth control in suppression of primary tumour formation and growth, I followed cohorts of *Neu; Rb1*$^{+/+}$, *Neu; Rb1*$^{\Delta L/+}$ and *Neu; Rb1*$^{\Delta L/\Delta L}$ females throughout their natural lives and palpated them weekly to determine the onset of mammary tumour formation. Unfortunately, the long latency before tumour formation resulted in excessive grooming in many of our mice and the need to euthanize them before palpable tumours formed. This was particularly true of the *Neu; Rb1*$^{\Delta L/\Delta L}$ mice. However, the *Neu; Rb1*$^{\Delta L/+}$ animals

Figure 4.5 Defective TGF-β proliferative control in $Rb1^{\Delta L/\Delta L}$ and $Rb1^{\Delta L/+}$ mice. (A) $Rb1^{+/+}$, $Rb1^{\Delta L/+}$, and $Rb1^{\Delta L/\Delta L}$ mammary epithelial cells were treated with TGF-β1 and pulse-labelled with BrdU 24 hrs later. The percentage of cells incorporating BrdU was measured by immunofluorescence microscopy. The fold decrease in proliferation between treated and untreated parallel cultures was determined and the average of three independent experiments is shown. * indicates a statistically significant difference from wild type (Student's t test; $P<0.05$). Error bars indicate one standard deviation from the mean. (B, C) H&E staining of paraffin sections of (B) $Rb1^{+/+}$, $Rb1^{\Delta L/+}$, and $Rb1^{\Delta L/\Delta L}$ and (C) *Neu;* $Rb1^{+/+}$, *Neu;* $Rb1^{\Delta L/+}$, and *Neu;* $Rb1^{\Delta L/\Delta L}$ mammary tissue from 8 week old mice. Each image displays a representative cross section of ducts. The table below displays the proportion of hyperplastic (hp) ducts found in $Rb1^{+/+}$, $Rb1^{\Delta L/+}$, and $Rb1^{\Delta L/\Delta L}$ mammary glands. Proportions were compared between genotypes using a chi-square test. Scale bar: 100 μm. † denotes previously published data that has been provided for comparison purposes.

A

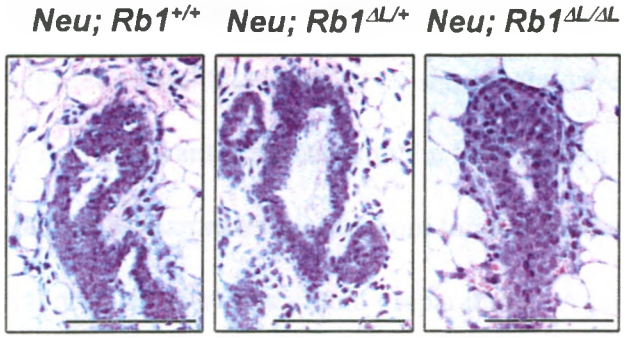


B



Genotype	# of ducts	# of hp ducts	Proportion of hp ducts	# of mice	*P* value
Rb1$^{+/+}$ †	455	11	0.024	6	
Rb1$^{\Delta L/+}$	456	53	0.116	3	<0.0001
Rb1$^{\Delta L/\Delta L}$ †	390	62	0.160	9	<0.0001

C

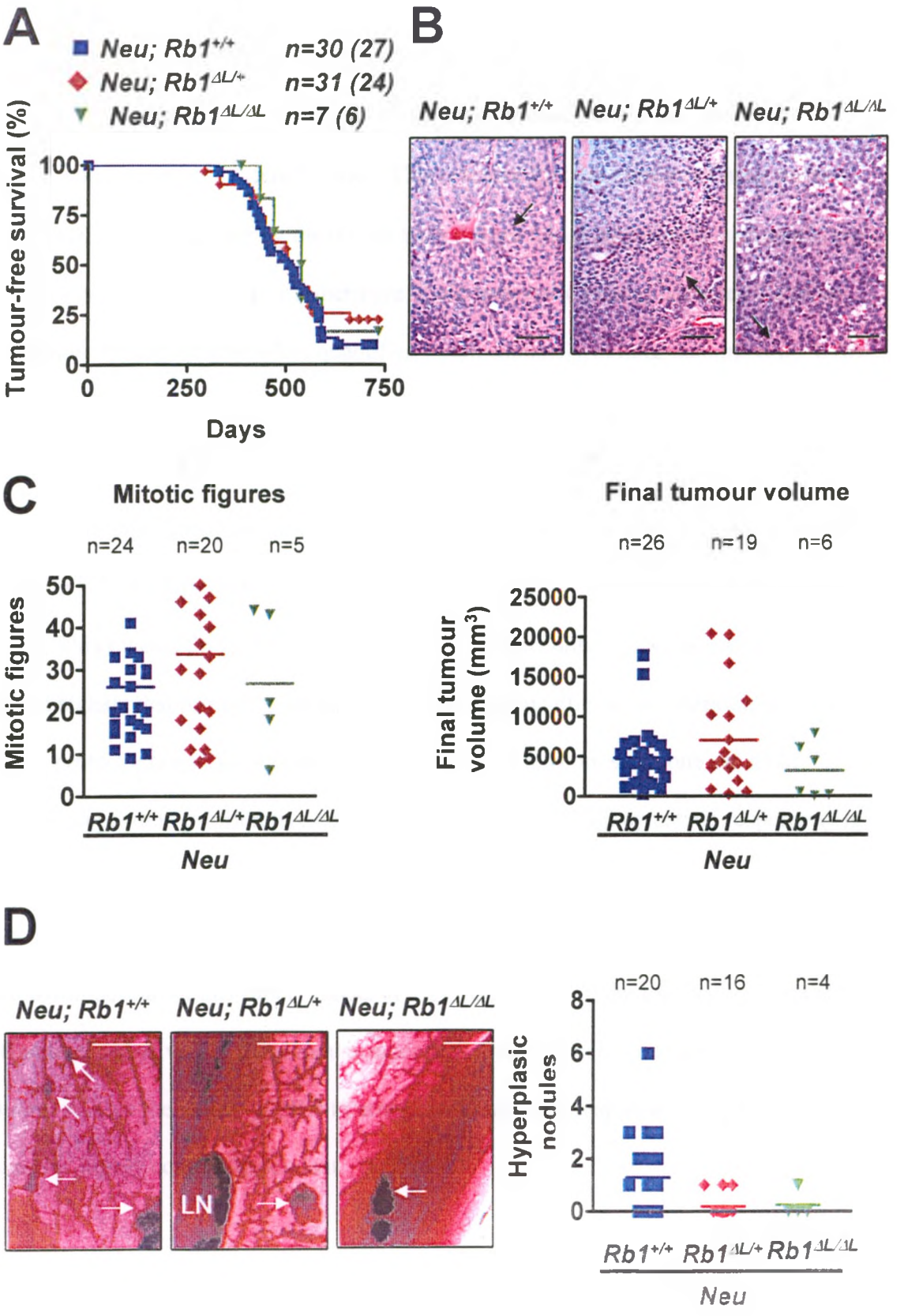


Genotype	# of ducts	# of hp ducts	Proportion of hp ducts	# of mice	*P* value
Rb1$^{+/+}$	612	69	0.113	3	
Rb1$^{\Delta L/+}$	761	87	0.114	3	
Rb1$^{\Delta L/\Delta L}$	473	71	0.149	3	0.08

have a similar response to TGF-β, indicating that they offer an equally valid means to address how negative growth signals impact tumorigenesis in this transgenic model. In stark contrast to the *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ mice, there was no difference in tumour latency between the remaining *Neu; Rb1*$^{\Delta L/\Delta L}$ females or the *Neu; Rb1*$^{+/+}$ and *Neu; Rb1*$^{\Delta L/+}$ females (Fig. 4.6a). The frequency of tumorigenesis in the *Rb1* mutant genotypes was also relatively unchanged from wild type (85.7% for *Rb1*$^{\Delta L/\Delta L}$ and 77.4% for *Rb1*$^{\Delta L/+}$ vs. 90% for wild type).

This result suggests that negative growth regulatory signals do not significantly influence cancer pathogenesis in *Neu* transgenic mice. Because this was unexpected, I also investigated other tumour characteristics to determine if the *Rb1* mutant genotypes altered the tumour type of these mice in such a way that the direct comparison in Figure 4.6a is misleading. To this end, I classified the tumours histologically and discovered that they all fit the characteristics of solid or acinar carcinomas that have been reported previously for *Neu* mice (Fig. 4.6b) (6). Our expectation from the *Wap-p53*^{R172H} cross is that negative growth responses are most important at the initiation step. However, Muraoka *et al.* found that overexpression of TGF-β did not affect tumour latency of *Neu* mice, but instead reduced tumour proliferation (30). For this reason I measured the number of mitotic figures in five randomly selected microscopic fields for each tumour as a means to compare proliferation and this revealed no significant differences (Fig. 4.6c). Furthermore, there were no significant differences in the final tumour volume (Fig. 4.6c). Lastly, I investigated unaffected mammary glands from tumour burdened animals for evidence of premalignant nodules by whole mount preparations. Again, there were no

Figure 4.6 Loss of pRB-LXCXE interactions does not affect *Neu*-driven tumorigenesis. (A) Onset of mammary tumorigenesis is shown for the indicated genotypes (log rank test; *P=0.6788*). Values in brackets indicate the number of mice that developed tumours. (B) Representative H&E stained paraffin sections from tumours harvested from *Neu; Rb1*$^{+/+}$, *Neu; Rb1*$^{\Delta L/+}$, and *Neu; Rb1*$^{\Delta L/\Delta L}$ mice 60 days after initial tumour palpation. Arrows indicate mitotic figures. Scale bar: 50 μm. (C) The mitotic index and final tumour volumes for *Neu; Rb1*$^{+/+}$, *Neu; Rb1*$^{\Delta L/+}$, and *Neu; Rb1*$^{\Delta L/\Delta L}$ mice are indicated, along with the average values for each genotype. Mitotic indices were derived by quantifying the number of mitotic figures in five random fields of view for each mouse. Final tumour volume was calculated using the formula $V=0.52xW^2xL$. (D) Carmine Red–stained mammary whole mounts from tumour-free glands in mice that had mammary tumours are shown for the given genotypes. Arrows indicate hyperplastic nodules and LN indicates lymph nodes. Scale bar: 2 mm. The number of hyperplastic nodules in each whole mount section was also quantified for each genotype along with the average number of hyperplastic nodules.

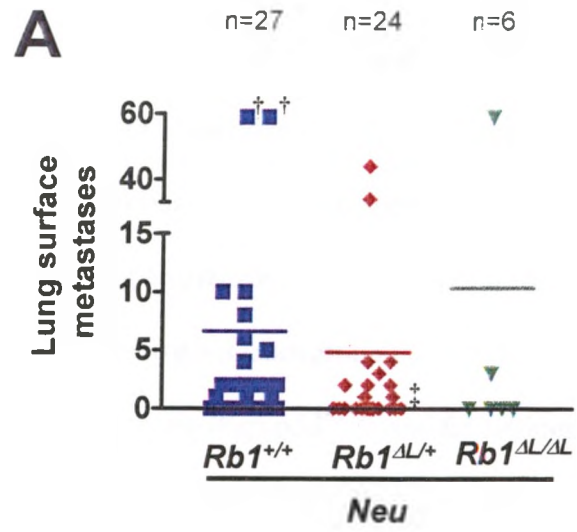

A
Neu; Rb1+/+ n=30 (27)
Neu; Rb1ΔL/+ n=31 (24)
Neu; Rb1ΔL/ΔL n=7 (6)
Tumour-free survival (%)
100
75
50
25
0
0
250
500
750
Days
B
Neu; Rb1+/+
Neu; Rb1ΔL/+
Neu; Rb1ΔL/ΔL
C
Mitotic figures
n=24
n=20
n=5
Mitotic figures
50
40
30
20
10
0
Rb1+/+ Rb1ΔL/+ Rb1ΔL/ΔL
Neu
Final tumour volume
n=26
n=19
n=6
Final tumour volume (mm3)
25000
20000
15000
10000
5000
0
Rb1+/+ Rb1ΔL/+ Rb1ΔL/ΔL
Neu
D
Neu; Rb1+/+
Neu; Rb1ΔL/+
Neu; Rb1ΔL/ΔL
LN
n=20
n=16
n=4
Hyperplasic nodules
8
6
4
2
0
Rb1+/+ Rb1ΔL/+ Rb1ΔL/ΔL
Neu

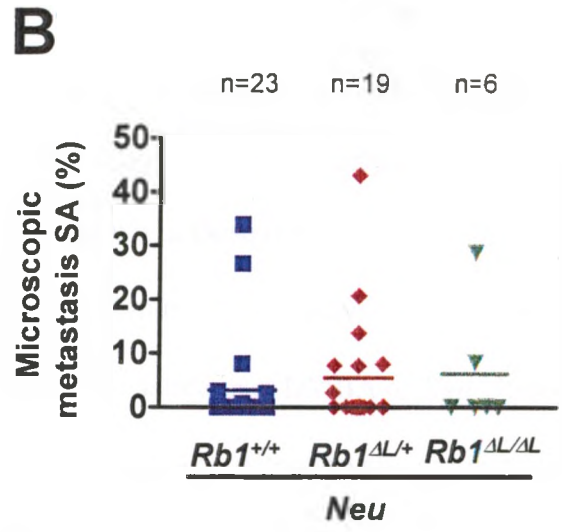
statistically significant differences between the three *Rb1* genotypes and, if anything, there was a trend toward fewer nodules in mice bearing the $Rb1^{\Delta L}$ mutation (Fig. 4.6d).

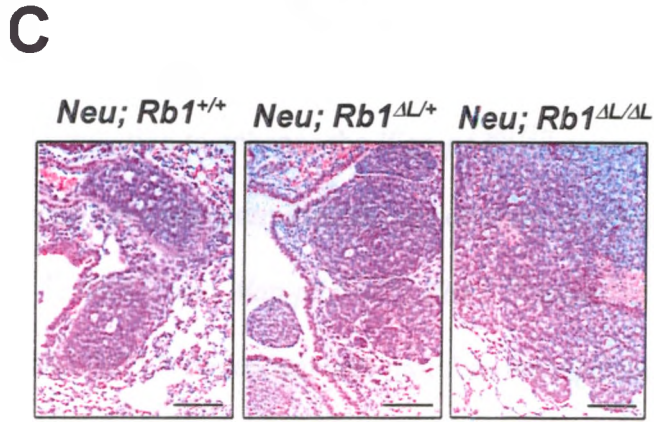
In an effort to better relate the combination of the $Wap\text{-}p53^{R172H}$ and *Neu* transgenes with our *Rb1* mutant, I also investigated metastasis in *Neu;* $Rb1^{+/+}$, *Neu;* $Rb1^{\Delta L/+}$ and *Neu;* $Rb1^{\Delta L/\Delta L}$ female mice. This revealed that the number of lung surface metastases that formed during the 60 day period from initial palpation to euthanasia were relatively similar (Fig. 4.7a). Furthermore, these metastatic lesions occupied a similar proportion of lung volume when quantified microscopically in lung sections (Fig. 4.7b). Lastly, there were no differences in histology between metastases from the respective genotypes (Fig. 4.7c). From these experiments it is clear that the $Rb1^{\Delta L}$ allele does not enhance the metastatic potential of mammary tumours whether they form in the *Neu* or $Wap\text{-}p53^{R172H}$ backgrounds.

This reveals that pRB-LXCXE interactions can confer responsiveness to negative growth signals that protect against $p53^{R172H}$ tumorigenic effects, but surprisingly, they cannot protect against *Neu*-driven oncogenesis. By extension, since one aspect of LXCXE-dependent proliferative control is linked to the TGF-β cytostatic response (Fig. 4.5) (14), and TGF-β has been shown to protect against *Neu*-driven mammary oncogenesis (30, 41), this suggests that TGF-β's other tumour suppressive functions may be most important in this cancer model (1, 28). Regardless of the explanation for the differential sensitivity of these mouse models of breast cancer to our *Rb1* mutation, these data reveal that the ability of pRB to arrest proliferation in response to negative growth signals is highly context-dependent.

Figure 4.7 Loss of pRB-LXCXE interactions does not affect metastatic potential in *Neu* mice. (A) The number of lung surface metastases (LSM) was quantified for individual mice and plotted along with the average number of LSM per genotype. † indicates lungs with >59 LSM and ‡ indicates a lung where each of the two metastases encompassed an entire lobe. (B) The SA occupied by lung metastases relative to the total lung area in tissue sections was quantified for mice from each genotype along with the average SA for each genotype. (C) Representative H&E stained paraffin sections of lungs from tumour-burdened mice are shown for each genotype. Scale bar: 100 μm.


A
n=27
n=24
n=6
Lung surface metastases
60
40
15
10
5
0
Rb1+/+
Rb1ΔL/+
Rb1ΔL/ΔL
Neu


B
n=23
n=19
n=6
Microscopic metastasis SA (%)
50
40
30
20
10
0
Rb1+/+
Rb1ΔL/+
Rb1ΔL/ΔL
Neu


C
Neu; Rb1+/+
Neu; Rb1ΔL/+
Neu; Rb1ΔL/ΔL

4.4 Discussion

Using two transgenic mouse models of breast cancer, I have examined the importance of pRB-LXCXE interactions during cancer formation and progression. Our work has revealed that LXCXE-dependent proliferative control can act as a barrier to tumour formation in the mammary gland. Surprisingly, this anti-oncogenic effect is context-dependent, protecting against *Wap-p53*^{R172H}- induced tumour formation, while having no effect in the *Neu* transgenic background.

Our experiments utilize the *Wap-p53*^{R172H} model differently than in previously published studies. Specifically, these mice display very low levels of spontaneous tumorigenesis during the first year of life (25). As a result, previous investigators have coupled expression of the transgene with other oncogenic stimuli such as DMBA or the *Neu* oncogene (25, 26), to rapidly induce tumorigenesis. In contrast, I did not experience the robust enhancement shown in these reports. I envision two possibilities to explain these differences that are not mutually exclusive. First, the *Rb1*$^{\Delta L}$ mutation and its effects on proliferation may be more subtle than DMBA or *Neu*. Indeed, the *Rb1*$^{\Delta L}$ mutation alone does not cause cancer (14, 42). Alternatively, our experiments were performed in a mixed 129/B6/FVB background and this may have delayed tumour induction. However, our experimental design, in which tumour onset was relatively late, created an opportunity for our *Rb1* mutation to enhance the *Wap-p53*^{R172H} cancer phenotype. This longer latency period may have also permitted the opportunity to compare metastatic disease in *Wap-p53*^{R172H}; *Rb1*$^{+/+}$ and *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ mice.

The precise pRB-LXCXE-dependent anti-proliferative mechanism that protects against *Wap-p53*^{R172H} driven tumours is unclear since mutant p53 acts as a random

generator of mutations that drive tumorigenesis almost a year after the transgene has been silenced. Loss of TGF-β growth regulation is an attractive candidate since it is a potent inhibitor of cell proliferation in the mammary gland and it induces cell cycle arrest in an LXCXE-dependent manner (14). However, tumour incidence in the *Neu* background suggests that the TGF-β cytostatic response may not be the reason that TGF-β is tumour suppressive in mouse models of mammary cancer. For this reason, loss of TGF-β-dependent growth arrest may not be the defect that allows more rapid mammary tumour formation in *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ mice. Other possibilities for why the *Rb1*$^{\Delta L}$ mutation cooperates with the *Wap-p53*^{R172H} transgene in tumour initiation are that *Rb1*$^{\Delta L/\Delta L}$ cells also fail to arrest in response to cellular stresses like DNA damage and oncogene induced senescence (42). Future experiments to better elucidate this question will include crosses to transgenic strains that challenge these specific anti-proliferative pathways to ascertain their importance in pRB's tumour suppressor function.

Regardless of the exact mechanism of anti-proliferative control that is responsible for the enhanced cancer phenotype in *Wap-p53*^{R172H}; *Rb1*$^{\Delta L/\Delta L}$ mice, I have demonstrated that pRB plays a critical role in protecting mammary epithelial cells from oncogenic transformation. This tumour suppressive role depends on the ability of pRB to make contacts at its LXCXE binding cleft, suggesting that recruitment of co-repressors to silence E2F target gene expression is key to blocking tumour formation. Interestingly, our work suggests that the role of proliferative control in cancer pathogenesis may be more complicated than previously thought. Unresponsiveness to negative growth signals is described as a hallmark of cancer, implying a ubiquitous need for it to be eliminated during tumorigenesis (16). But how extensive a defect in responding to external cues is

required? For example, some cancer derived cell lines that are pRB deficient reliably growth arrest as monolayers in culture. This suggests that in the absence of pRB, some growth arrest signals can be retained and are compatible with tumorigenesis. The molecular context that dictates the requirement for pRB-dependent growth arrest, versus other proliferative control mechanisms, in preventing cancer initiation is unknown. Coming to an understanding of what these factors are *in vivo* will greatly influence our understanding of proliferative control in cancer, and will undoubtedly impact the classification and treatment of cancer.

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Chapter 5: Summary and Perspectives

5.1 Summary of Findings

As highlighted in this thesis, pRB proliferative control is essential for TGF-β growth inhibition and mammary gland development. Furthermore, pRB plays a context-dependent role in tumour suppression. In chapter 2, I demonstrated that loss of LXCXE interactions results in a nursing defect and hyperplasia of the mammary ductal epithelium. Because these phenotypes are similar to those described for mice lacking a functional TGF-β pathway, I next examined if disrupting LXCXE interactions affected TGF-β growth inhibition. Mutant forms of pRB were unable to induce a proper TGF-β growth arrest, and this contributed, in part, to the hyperplastic phenotype *in vivo*. Finally, I demonstrated that this defect occurs downstream of pRB in the TGF-β signalling pathway, and results in the disruption of E2F target gene regulation.

These data suggested that the cytostatic arm of TGF-β signalling is disrupted with loss of pRB-LXCXE interactions. However, I next wanted to know if other aspects of TGF-β signalling were intact in the $Rb1^{\Delta L/\Delta L}$ mice. In chapter 3, a combination of molecular and biological assays was used to demonstrate that TGF-β-mediated apoptosis and differentiation are intact in the $Rb1^{\Delta L/\Delta L}$ mammary gland. Interestingly, invasion and motility of primary murine mammary epithelial cells appear to occur independently of TGF-β signalling. Together, these data suggest that only TGF-β growth control is affected by loss of pRB-LXCXE interactions.

Finally, knowing that loss of LXCXE interactions disrupts proliferative control in the mutant mice, I sought to understand if this was an important aspect of pRB tumour

suppression. Because the pRB and TGF-β cytostatic responses are linked, this also allowed me to address the importance of TGF-β proliferative control as a tumour suppressive mechanism. Using three mouse models of breast cancer, I demonstrated that pRB proliferative control can block tumour initiation in the mammary gland. However, this effect is context-dependent, because loss of pRB-LXCXE interactions did not change the tumour latency in *Neu* mice or mice treated with DMBA. Furthermore, since TGF-β has been shown to protect against tumorigenesis in these two contexts (39, 43, 48), these data suggest that TGF-β growth inhibition may not be required for suppression of mammary tumour formation and progression.

Overall, this work identifies a unique functional connection between pRB and TGF-β in mammary gland development and proliferative control. Surprisingly, these studies demonstrate that pRB-LXCXE interactions can suppress tumorigenesis in the mammary gland, but only in specific circumstances. This highlights the complexity and context-dependence of pRB growth control as a tumour suppressive mechanism. The implications of these findings are discussed in the following sections.

5.2 Mediators of pRB Proliferative Control

In Chapter 2, I demonstrated that pRB-LXCXE interactions are critical for pRB proliferative control in the mammary epithelial population. This is due, in part, to an inability of $pRB^{\Delta L}$ to induce a full growth arrest in response to TGF-β. While these pRB-LXCXE interactions are necessary for full suppression of E2F target genes upon treatment with TGF-β, the underlying mechanism remains unclear. Although greater than 30 LXCXE-dependent interacting partners of pRB have been identified, very few have

been demonstrated to play a role during G1 arrest (3, 60). Thus, it is difficult to predict which LXCXE-interacting proteins are necessary for pRB to induce the TGF-β cytostatic response. Below, the candidate proteins are described, along with the evidence for and against their potential for mediating pRB-LXCXE-dependent proliferative control.

5.2.1 CDH1 and the Anaphase Promoting Complex/Cyclosome

An interaction between pRB and CDH1 was recently identified using a combination of GST pulldowns and mass spectrometry (3). CDH1 is a component of the anaphase promoting complex/cyclosome (APC/C), which targets proteins for degradation in a cell cycle-dependent manner. One such target is SKP2, a component of the SCF^{SKP2} E3 ubiquitin ligase. The SCF^{SKP2} complex targets the CDK inhibitor, $p27^{KIP1}$, for degradation to allow progression through the cell cycle (7, 51, 54). Intriguingly, during cell cycle arrest, pRB can bind SKP2 at the C-terminus and CDH1 at the LXCXE binding cleft. The APC/C then targets SKP2 for degradation, and $p27^{KIP1}$accumulates in the cell, inhibiting CDK phosphorylation of target proteins, including pRB. In its active state, pRB can then block cell cycle advancement (Fig. 1.3) (3, 17). APC/C-mediated stabilization of $p27^{KIP1}$ has been demonstrated in a TGF-β growth arrest paradigm, highlighting CDH1 interactions at the LXCXE binding cleft as a candidate mechanism for cell cycle exit in response to TGF-β (30). However, several lines of evidence indicate that this interaction may be dispensable for mediating this pRB anti-growth signal. First, overexpression of two different CKIs, $p16^{INK4a}$ and $p21^{CIP1}$, was unable to induce arrest in $Rb1^{\Delta L/\Delta L}$ MEFs, suggesting that the defect is independent of CKI activity (Fig. 2.6). Furthermore, pRB became hypophosphorylated in $Rb1^{\Delta L/\Delta L}$ cells when treated with TGF-β1, suggesting that CKIs were active in those cells (Fig. 2.6). Together, these data

demonstrate that the *Rb1*$^{\Delta L/\Delta L}$ defect occurs downstream of CKI-dependent activation of pRB, suggesting that CDH1 may not be the LXCXE-interacting protein that cooperates with pRB to induce proliferative control in the context of TGF-β signalling.

5.2.2 HDAC, BRG1, and hBRM interactions

Several chromatin remodelling factors can interact with pRB in an LXCXE-dependent manner and are thought to create a closed chromatin structure at E2F target genes to repress transcription. Interestingly, the coordinated expression of HDAC, BRG1, hBRM, and pRB, has been shown to control cell cycle exit (60). All three of these chromatin remodellers can interact with pRB in an LXCXE-dependent manner and act as co-repressors of E2F target gene expression (6, 9, 31, 32, 49, 53). Zhang, *et al.*, demonstrated that overexpression of pRB and HDAC could repress E2F-dependent transcription, while the hSWI-SNF complex was additionally required to induce cell cycle arrest (60). However, the authors did not demonstrate that all four proteins interact simultaneously, so it is unclear if these proteins function as a complex or independently to exert this growth arrest. Several studies have shown that BRG1 can control cell cycle arrest indirectly by upregulating expression of p21^{CIP1} and this induction does not require interactions between BRG1 and pRB (14, 18). Therefore, it is possible that HDAC, BRG1, and hBRM using both pRB-interaction-dependent and -independent mechanisms to induce a full growth arrest. The artificial nature of the overexpression experiments also makes it difficult to interpret the biological context of these results. For instance, it is unclear if these proteins can induce cell cycle exit in response to various stimuli in physiological settings. Therefore, whether an HDAC-BRG1-hBRM-pRB complex mediates the TGF-β cytostatic response remains to be determined.

5.2.3 The Sin3 Complex

HDAC is a constituent of two other cellular complexes: the Sin3 and Mi2/nucleosome remodelling and deacetylase (NuRD) complexes. The core sin3 complex in mammals contains mSin3A and mSin3B, SAP30, SAP18, HDAC1 and 2, and the histone binding proteins RbAp46/48 (21). The core complex can also recruit other chromatin modifiers and transcriptional repressors, such as MeCP2, Ski, and Ikaros, to elicit specific effects depending on the cellular context (21). The Sin3-SAP30-HDAC complex is recruited to pRB by another LXCXE-interacting partner, RBP1 (25). In terms of a functional connection to pRB growth control, the Sin3 complex co-localizes with pRB-E2F4 repressor complexes in quiescent human cells at sites that may represent the initial origins of DNA replication following growth stimulation. Therefore it is possible that pRB-induced cell cycle arrest is mediated in part by recruiting this repressor complex to alter the chromatin structure at origins of replication (25). However, using chromatin immunoprecipitation (ChIP), another group demonstrated that recruitment of the Sin3 complex to E2F target gene promoters in quiescent cells required p107 or p130 activity, while an anti-pRB antibody did not pull down E2F target promoter sequences (45). Since E2F-dependent transcription is deregulated in $Rb1^{\Delta L/\Delta L}$ cells, this implies that the Sin3 repressor complex is not a strong candidate mediator of pRB growth control in the context of TGF-β signalling. However, it is possible that pRB-Sin3 interactions have a different role during quiescence than p130-Sin3 or that pRB plays a more transient role of recruiting Sin3 to E2F target genes while p130 is required for maintenance of this complex. Finally, different pocket proteins may interact with the Sin3 complex depending on the arrest signal. Therefore it is possible that components of the Sin3 complex interact

with pRB during a TGF-β-induced growth arrest to alter chromatin structure and repress E2F target gene transcription.

5.2.4 The NuRD Complex

HDAC-RbAp46/48 can also interact with the NuRD complex (21). This complex contains Mi-2β, which has a DNA helicase/ATPase domain, MTA2, methyl-DNA-binding protein 3 (MBD3), and the core HDAC complex. Lysine specific demethylase 1 (LSD1) also interacts with the NuRD complex in human cell lines (58). Thus, NuRD allows the coordinated activity of multiple chromatin remodelling factors in one repressor complex. Interestingly, the *Caenorhabditis elegans RB*-related gene, *lin-35*, as well as many of the worm homologues for NuRD components, fall into a similar class of genes involved in vulval development (55). In *C. elegans*, vulval formation is triggered by an EGF-like (LIN-3) signal to specific vulval precursor cells, while synthetic multivulva (synMuv) proteins repress expression of LIN-3 in the surrounding epidermis. SynMuv genes fall into three classes, designated A, B, and C. Mutants from different classes develop a Muv phenotype when combined with mutants from either of the other two classes, suggesting that redundant mechanisms block LIN-3 expression (55). Interestingly, LIN-35 and LIN-53, HDA-1 and LET-418 (*C. elegans* homologues of RB, RbAp48, HDAC, and Mi2 respectively) are all class B synMuv proteins (55). This suggests that either members of this NuRD-like complex are candidate *in vivo* partners of pRB or that they contribute independently to a common process in worm vulval development. The HDAC subunit common to both Sin3 and NuRD interacts with the pRB LXCXE binding cleft in human cells (6, 26, 32), implying that pRB may interact with the NuRD repressor complex in certain cellular contexts. Interestingly, the same

GST pulldown/mass spectrometry screen that identified the interaction between CDH1 and pRB also found that pRB interacted with the Sin3 and NuRD complexes in an LXCXE-dependent manner (Dr. Fred Dick, personal communication). Thus, components of both the Sin3 and NuRD complexes are candidate pRB LXCXE binding cleft-interacting proteins that cooperate with pRB to induce TGF-β growth arrest.

5.2.5 The DREAM complex

Recent work in *Drosophila* identified a multi-subunit complex consisting of the fly pocket protein RBF, *drosophila* E2F2, and dMyb-interacting proteins (dREAM) that repressed transcription of numerous developmentally regulated E2F target genes (23). This complex is conserved in flies (dREAM), worms, (DRM) and mammals (DREAM) (13, 23, 27, 29, 44, 55). Of note, components of the worm DRM complex also fall within the B class of synMuv genes, but they form a complex that is distinct from NuRD and interact with LIN-35 (13). Intriguingly, *in vitro* binding experiments have demonstrated that this complex contains subunits that bind to pRB in an LXCXE binding cleft-dependent manner (23). However, if the dREAM and DRM complexes regulate an extensive array of developmentally regulated genes, how is the $Rb1^{\Delta L/\Delta L}$ mouse viable? A recent study provided some insight, demonstrating that p130 is the main pocket protein that interacts with the mammalian DREAM complex during quiescence (29). This does not exclude the possibility that subunits of the DREAM complex can bind the LXCXE binding cleft on pRB to repress transcription of a subset of target genes or in specific cellular contexts. Therefore, while p130 interacts with the DREAM complex during serum starvation, it is possible that pRB can interact with the complex during other growth arrest paradigms, such as TGF-β-dependent negative growth control.

5.2.6 Histone methyltransferases

Many of the complexes outlined above contain subunits capable of modifying the chromatin environment and disruption of epigenetic modifications at E2F target genes could result in the loss of pRB proliferative control. This has been demonstrated in the case of cellular senescence (52) where the trimethylation of histone 3 lysine 9 (H3K9Me3) is reduced at E2F target genes in senescent $Rb1^{\Delta L/\Delta L}$ cells. Loss of this repressive mark is associated with deregulation of target gene expression as well as re-entry into the cell cycle. The methyltransferases responsible for the placement of this marker, Suv39h1 and 2, interact with pRB through the LXCXE binding cleft (40, 56). It is also noteworthy that long term exposure to TGF-β can induce cellular senescence (28). Therefore, it is possible that heterochromatization of E2F target genes may be necessary for TGF-β-dependent growth inhibition in much the same way it is required for senescence. This places the Suv39h methyltransferases as potential LXCXE-interacting proteins involved in TGF-β growth control.

5.2.7 An Unbiased Approach to Identify Mediators of pRB-dependent TGF-β Growth Control

The studies outlined above have identified many potential candidates that may interact with pRB at the LXCXE binding cleft in order to induce the TGF-β cytostatic response. This includes several chromatin modifiers that form large complexes to repress transcription during development and cell cycle exit. However, it also includes other classes of proteins, such as CDH1, that mediate cell cycle arrest via degradation of proteins that drive cell cycle progression. Many other proteins also interact with pRB at the LXCXE binding cleft, although the biological outcomes of these interactions are not

known (reviewed in (8)). With such a diverse list of candidates, an unbiased approach is necessary to determine the mechanism of pRB-LXCXE-dependent TGF-β G1 arrest. One such approach would involve GST pulldown/mass spectrometry on GST-tagged wild type pRB or $pRB^{\Delta L}$ mixed with TGF-β1-treated or untreated extracts. Alternatively, candidates could be identified by knocking down proteins at random using an shRNA library. Knock down cells would then be tested for TGF-β-induced cell cycle arrest. These approaches could be used alone or in tandem to identify target proteins that interact with pRB during TGF-β-induced growth arrest. They could also be repeated in the context of DNA damage and senescence cues, which are also disrupted in $Rb1^{\Delta L/\Delta L}$ MEFs (52). Together, these experiments would provide new insight into how pRB coordinates various protein interactions to assert proliferative control in different cell cycle arrest paradigms.

5.3 TGF-β-Mediated Tumour Suppression

The current dogma in the TGF-β field suggests that TGF-β proliferative control suppresses tumorigenesis early in breast cancer development, but is selectively lost during cancer progression, leaving other pro-tumorigenic aspects of TGF-β signalling to drive metastasis (reviewed in (2, 33, 35, 41, 57)). However, attempts to separate various aspects of TGF-β signalling have proven complicated, leaving open the question of which of TGF-β's many functions suppress tumorigenesis *in vivo*. Here, I have shown that TGF-β cytostatic control is disrupted in mutant mammary epithelial cells. In contrast, this work demonstrates that TGF-β-dependent apoptosis and differentiation occur in an overtly normal manner in $Rb1^{\Delta L/\Delta L}$ mammary glands. Furthermore, motility and invasion appear

to occur independently from TGF-β signalling in both wild type and mutant primary MECs. To the best of my knowledge this is the first model selectively disrupting TGF-β proliferative control, while leaving other aspects of TGF-β signalling intact. This allowed the role of TGF-β growth inhibition as a protective mechanism during mammary tumour progression to be addressed.

In both the DMBA study and the cross into the *Neu* background, there was no increase in tumorigenesis with loss of pRB-LXCXE interactions and the TGF-β cytostatic response. This is in striking contrast to previous studies where constitutive expression of TGF-β signalling protected against primary mammary tumour formation while driving lung metastases (39, 43, 48). If TGF-β growth control normally protects against breast cancers, I would have anticipated that loss of TGF-β proliferative control in these models would lead to a decrease in tumour latency or more aggressive tumours. Instead, disruption of pRB-LXCXE interactions had no effect on primary tumours in these models. If that is the case, how does this work fit with the protective effect associated with TGF-β overexpression in the mouse models outlined above? I envision two possible explanations for this outcome. The first is that there is a threshold of TGF-β growth suppression necessary for effective tumour suppression. Loss of LXCXE interactions in MECs reduced TGF-β growth arrest, but did not abrogate the activity entirely (Fig. 2.4). Therefore, I cannot rule out the possibility that residual TGF-β growth control was sufficient to suppress tumorigenesis.

An alternative explanation for the protective effect found in previous models exploring the role of TGF-β during tumorigenesis is that other TGF-β-dependent mechanisms such as apoptosis or differentiation mediate tumour suppression. These other

TGF-β-dependent mechanisms could work in conjunction with, or instead of, the cytostatic response to protect cells from tumour formation. For example, the use of patient-derived metastatic breast cancer cells also showed defects in TGF-β-induced differentiation. ID1 is normally downregulated in response to TGF-β signalling, but in metastatic breast cancer cells, ID1 is actually induced by TGF-β (42). It is not clear how this pathway is disrupted in metastatic cells, but ID1 expression has been shown to correlate with relapse in ER- patients and metastatic potential in xenograft models (12, 37). This places TGF-β-mediated differentiation and ID1 repression as another potential tumour suppressive mechanism that may contribute to the phenotypes displayed in the various mouse models presented in this thesis and in the literature.

TGF-β-mediated apoptosis may also be lost during breast cancer progression. As outlined in the introduction, depending on unknown factors and environmental cues, TGF-β can either induce or suppress apoptosis. One possibility is that TGF-β triggers growth arrest or apoptosis depending on the intensity of proliferative signals in the environment (33). In the adult virgin mammary gland, TGF-β proliferative control appears to balance out local mitogen signals. However, in the case of intense mitogenic stimulation that occurs during late pregnancy, constitutive TGF-β signalling induces apoptosis (16, 48). Similar phenomena have also been described in the transition epithelia of the anogenital region (11, 33). It is possible that TGF-β-dependent apoptosis is induced early during tumour progression to protect against aberrant mitogenic signalling in premalignant cells as well, and disruption of this mechanism contributes to more aggressive metastatic tumours.

There are still many gaps in our knowledge of how TGF-β differentiation and apoptosis are mediated in the mammary gland. TGF-β can block ID1 expression in breast cancer cells (19), which should induce differentiation, but other studies show that TGF-β can block full differentiation and milk production of alveolar cells (5, 10, 22, 36, 46, 50). In a similar manner, TGF-β can have both pro- and anti-apoptotic effects in the cell. Clearly, the molecular mechanisms underlying these TGF-β responses, as well as the cellular contexts where they are relevant need to be discerned before their importance as tumour suppressors can be addressed in full. This will require the development of new model systems where these pathways can be disrupted in isolation. These types of studies will be critical to gain a better understanding of how TGF-β acts as a tumour suppressor.

5.4 TGF-β as a Therapeutic Target

One of the biggest debates in the TGF-β field is whether drugs should be developed against this signalling pathway. As outlined earlier, a wealth of data suggests that constitutive activation of TGF-β can drive invasion and metastasis in tumour cells, and high amounts of TGF-β are often produced by the tumour cell (33). TGF-β is also involved in mediating paracrine effects in the mammary gland, which modulate the tumour microenvironment and the host immune system to enhance tumour growth (reviewed in (33)). This highlights TGF-β as a prime target for anticancer therapies. On the other hand, many TGF-β responses, like proliferative control, apoptosis, and the induction of differentiation are anti-tumorigenic. Thus, in mouse models, constitutive expression of TGF-β signalling protects against tumour formation while driving

metastasis (39, 48). Because of the dual roles that TGF-β can play during tumorigenesis, the risks of such a therapeutic strategy must be determined.

TGF-βs and their receptors are expressed in many mammalian tissues (35). This poses a potential problem because long-term exposure to TGF-β antagonists is likely to have adverse side effects. A major perceived risk of blocking TGF-β signalling is the potential for chronic inflammation or autoimmune effects. TGF-β1, in particular has a clear role in the immune system. Mice lacking TGF-β1 die shortly after birth due to systemic inflammation (24, 47) and mice lacking Smad3 also have an impaired inflammatory response (1). In humans, loss of normal TGF-β function has been implicated in the pathogenesis not only of cancer, but also autoimmune and inflammatory diseases, while excess TGF-β has been implicated in immunosuppression and fibrosis, as well as metastasis (4, 34). However, lifetime exposure to a soluble TGF-β antagonist did protect *Neu* mice from lung metastasis without significant adverse immune effects (59). This raises optimism for the use of TGF-β antagonists in cancer therapies.

Since TGF-β is a potent inhibitor of proliferation in epithelial cells, perhaps the greatest risk is potential acceleration of preneoplastic lesions or cancers where TGF-β still exerts growth restraint. This was found when a gene encoding a soluble truncated form of TβRII was transfected into a hepatoma cell line (20). However, in two models of breast cancer, similar antagonists did not have tumour-promoting effects (38, 59). At the same time, treatment reduced metastases in these mice. The data from this thesis lends further support to the potential efficacy of blocking TGF-β during cancer therapy. Loss of TGF-β growth inhibition did not affect the rate of tumorigenesis in either the DMBA or *Neu* studies. Together with the previous work with soluble antagonists (59), the data

presented here suggests that TGF-β antagonists should have minimal effects on TGF-β growth control. The developmental studies also reveal that it is dispensable for the formation and homeostasis of most tissues in the mouse. However, to test whether TGF-β growth inhibition or other TGF-β-dependent mechanisms protect against tumorigenesis in this model system, the *Neu; Rb1*$^{\Delta L/\Delta L}$ experiment should be repeated in the *MMTV-TGF-β1* background. If excess TGF-β1 suppressed tumorigenesis in both *Neu; Rb1*$^{+/+}$ and *Neu; Rb1*$^{\Delta L/\Delta L}$ mice, it would suggest that TGF-β-dependent apoptosis, differentiation, or other mechanisms may block tumour formation and progression. These types of studies will be critical to gain a better understanding of how TGF-β acts as a tumour suppressor. If this hypothesis holds true, the *Rb1*$^{\Delta L/\Delta L}$ mouse should prove a valuable tool to test new TGF-β therapies, not only for cancer but also other diseases affected by changes in TGF-β levels, as they are developed.

In a clinical setting, one would want to selectively neutralize the TGF-β pathway that is involved in disease pathogenesis without affecting the normal protective and homeostatic roles of TGF-β in unaffected tissues. Since the work presented here suggests that TGF-β growth inhibition is not important for most development and tumour suppression, it is of even greater importance to understand when and how TGF-β mediates apoptosis and differentiation, so that more selective TGF-β drug targets can be developed that do not disrupt these aspects of TGF-β signalling.

5.5 Perspectives

The work in this thesis has extended our knowledge about the roles of pRB and TGF-β in mammary gland development and cancer. First, using knock-in mouse models

where pRB LXCXE interactions are disrupted, I showed that pRB is necessary to maintaining proliferative control within the developing mammary gland. This growth regulation is intimately tied to the ability of pRB to induce TGF-β growth inhibition. In the TGF-β field, pRB represents the end point in the cytostatic response, and little attention has been paid to how it exerts this effect. The work presented here demonstrates that pRB-LXCXE interactions are critical for inducing a full arrest. With the use of affinity purification, mass spectrometry, and shRNA technologies, further expansion of the downstream effectors and mechanisms involved in TGF- β growth control can be expected. Similar techniques should also help to identify the LXCXE binding partners that mediate a variety of cellular processes that pRB has recently been implicated in, such as senescence control, DNA damage, and genomic stability (15, 52).

Second, I examined the importance of pRB-dependent negative growth regulation for blocking tumour formation. This work has identified pRB proliferative control as a tumour suppressive function of pRB in the mammary gland. However, since treatment with DMBA and the cross into the *Neu* background did not yield changes in tumour latency or aggressiveness, it reveals that pRB proliferative control is activated in a context-dependent manner. As already outlined, this data also suggests that in the *Wap-p53*^{R172H} model, TGF-β growth suppression is not the mechanism by which pRB-LXCXE interactions block tumorigenesis. A major challenge in the future will be identifying the upstream signals that induce pRB proliferative control. The use of transgenic mouse models that disrupt other pathways mediated by LXCXE interactions as outlined above should give rise to a fuller understanding of how pRB proliferative control protects against tumour formation.

5.6 References

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Appendix I: PCR Methods and Primers

Reagents for Genotyping PCR

Component	*Rb1 (Intron 20LoxP)*	*MMTV-Neu*	*MMTV-TGF-β1$^{S223/225}$*	*Wap-p53^{R172H}*
10X PCR buffer	2 µL	2 µL	2µL	2 µL
dNTPs (2 mM)	2 µL	2 µL	2 µL	2 µL
$MgCl_2$ (50 mM)	0.5 µL	0.5 µL	0.5 µL	0.5 µL
Primers (20 µM)	0.25 µL of: FD134, FD135	0.25 µL of: IMR042, IMR043, IMR386, IMR387	0.25 µL of: IMR042, IMR043, IMR086, IMR087	0.25 µL of: T022 T023
H_2O	12.8 µL	12.3 µL	12.3 µL	12.8 µL
Taq (5U/µL)	0.2 µL	0.2 µL	0.2 µL	0.2 µL
DNA*	2 µL	2 µL	2 µL	2 µL
TOTAL	20 µL	20 µL	20 µL	20 µL

*DNA for *Intron20LoxP* and *Wap-p53R172H* genotyping may be isolated by the Hotshot method. DNA for the *MMTV-Neu* and *MMTV-TGF-β1$^{S223/225}$* must be isolated by Proteinase K digestion and ethanol precipitation

Thermal Cycling Conditions for Genotyping (PTC-100 or -200 Thermal Cycler)

	Rb1 (Intron 20LoxP)	*MMTV-Neu*	*MMTV-TGF-β1$^{S223/225}$*	*Wap-p53^{R172H}*
Program	SLO1	SLO1	SLO1	SLO1
Annealing Temperature	60°C	60°C	60°C	60°C
Number of cycles	30	30	30	30

RT-PCR and qRT-PCR

RNA was extracted from tissues or cells using Trizol reagent (Invitrogen) and then converted to cDNA using the Reverse Transcription System (Promega) as per the manufacturers' instructions. RT-PCR was performed on a PTC-100 or -200 Thermal Cycler. Quantitative real time PCR (qRT-PCR) was then performed using iQ SYBR Green Supermix (Bio-Rad) on a PTC-200 Thermal Cycler equipped with a Chromo 4 Continuous Fluorescence Detector, and the data was analyzed using Opticon Monitor 3.1 software (Bio-Rad).

Reagents for cDNA Synthesis and RT-PCR

Component (Promega kit)	cDNA synthesis	Component (standard reagents)	RT-PCR
$MgCl_2$ (25 mM)	4 µL	10X PCR buffer	2 µL
RT 10X Buffer	2 µL	dNTPs (2 mM)	2 µL
dNTPs (10 mM)	2 µL	$MgCl_2$ (50 mM)	0.2 µL
Ribonuclease Inhibitor (40 U/µL)	0.5 µL	Primers (10 µM)	0.8 µL each
Random primers (0.5 mg/mL)	1 µL	DEPC-H_2O	11.7 µL
AMV Reverse Transcriptase (25 U/ µL)	0.68 µL	Taq (5U/µL)	0.5 µL
RNA*	1 µg up to 9.82 µL in DEPC-H_2O	cDNA**	2 µL
TOTAL	20 µL	**TOTAL**	20 µL

*heat RNA at 70°C for 10 minutes and then place on ice prior to cDNA synthesis
**After cDNA synthesis, dilute cDNA to 100 µL in DEPC-H_2O prior to RT-PCR or qRT-PCR

Thermal Cycling Conditions for RT-PCR (PTC-100 or -200 Thermal Cycler)

	MMTV-TGF-$\beta1^{S223/225}$	***Cdkn2b***	***Atf3***	***Csn2***	***Actb***	***Gapdh***
Program	TGFRT	TGFRT	TGFRT	TGFRT	TGFRT	TGFRT
Primers	SF01and SF02	p15 for and p15 rev or p15 for2 and p15 rev	ATF for and ATF rev	cas m for and cas m rev	act for2 and act rev2	GAP for and GAP rev
Annealing Temperature	60°C	60°C	60°C	60°C	55-60°C	60°C
Number of cycles	35	35	35	35	30-35	30-35

Reagents for qRT-PCR

Component (Bio-Rad)	qRT-PCR
DEPC-H_2O	7 µL
iQ	10 µL
Primers (10 µM)	1 µL each
cDNA**	1 µL
TOTAL	20 µL

** After cDNA synthesis, dilute cDNA to 100 µL in DEPC-H_2O prior to qRT-PCR

Thermal Cycling Conditions for qRT-PCR (PTC-200 Thermal Cycler with Chromo 4 Continuous Fluorescence Detector)

	All reactions
Program	Opticon 3.1
Protocol	Sarah F, 63
Annealing Temperature	63°C
Number of cycles	35

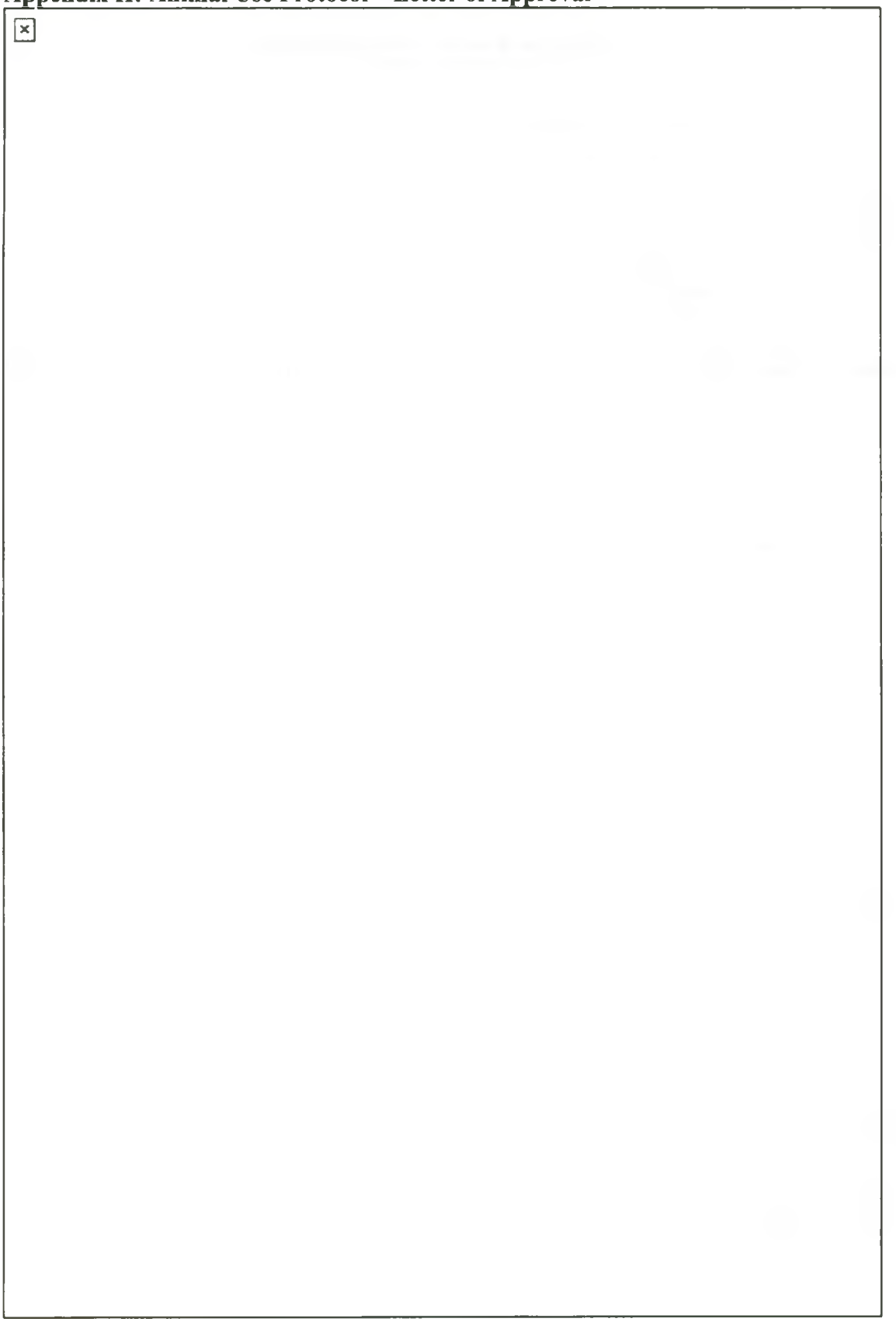
Primer Sequence Information

Gene/locus/ transgene	Application	Primer name	Primer sequence	Amplicon size	Comments
MMTV-TGF-β1$^{S223/225}$	genotyping	IMR0186	TCA CTC CTC AGG TGC AGG CTG CCT	502 bp (with IMR0187)	Provided by JL
MMTV-TGF-β1$^{S223/225}$	genotyping	IMR0187	ACA GCT ATG ACT GGG AGT AGT CAG	502 bp (with IMR0186)	Provided by JL
MMTV-Neu	genotyping	IMR386	TTT CCT GCA GCA GCC TAC GC	500 bp (with IMR387)	Provided by JL
MMTV-Neu	genotyping	IMR387	CGG AAC CCA CAT CAG GCC	500 bp (with IMR386)	Provided by JL
Il2	genotyping	IMR0042	CTA GGC CAC AGA ATT GAA AGA TCT	324 bp (with IMR0043)	Provided by JL; internal ctl for *MMTV-TGF-β1$^{S223/225}$* and *MMTV-Neu PCR*
Il2	genotyping	IMR0043	GTA GGT GGA AAT TCT AGC ATC ATC C	324 bp (with IMR0042)	Provided by JL; internal ctl for *MMTV-TGF-β1$^{S223/225}$* and *MMTV-Neu PCR*
Wap-53^{R172H}	genotyping	T022	CCG TCG ACG GCC ACA GTG AAG ACC TCC GGC CAG	1300 bp (with TO23)	Provided by MMHCC
Wap-53^{R172H}	genotyping	T023	GCC TGA AAA TGT CTC CTG GCT CAG AGG G	1300 bp (with TO22)	Provided by MMHCC
Rb1	genotyping	FD134	AGC TTC ATA CAG ATA GTT GGG	*Rb1* - 136 bp; *Rb1$^{\Delta L}$* – 274 bp (with FD135)	Amplifies intron 20 for *Rb1*, intron 20 + LoxP for *Rb1$^{\Delta L}$*
Rb1	genotyping	FD135	CAC ACA AAT CCC CAT ACC TAT G	*Rb1* - 136 bp; *Rb1$^{\Delta L}$* – 274 bp (with FD134)	Amplifies intron 20 for *Rb1*, intron 20 + LoxP for *Rb1$^{\Delta L}$*
MMTV-TGF-β1$^{S223/225}$	RT-PCR	SF01	AAG GAC CTC GGC TGG AAG T	196 bp (with SF02)	Amplifies simian *TGF-β1$^{S223/225}$*; does not align with mouse *TGF-β1*
MMTV-TGF-β1$^{S223/225}$	RT-PCR	SF02	TAG TAC ACG ATG GGC AGT GGC T	196 bp (with SF01)	Amplifies simian *TGF-β1$^{S223/225}$*; does not align with mouse *TGF-β1*
Cdkn2b (encodes p15)	qRT-PCR	p15 for	TGC CAC CCT TAC CAG ACC TGT G	167 bp (with p15 rev)	Within exon 2
Cdkn2b (encodes p15)	qRT-PCR	p15 for2	CAA GTG GAG ACG GTG CGG CAG C	322 bp (with p15 rev)	Within exon 1; used to confirmed amplification

					of cDNA from p15 for
Cdkn2b (encodes p15)	qRT-PCR	p15 rev	GCA GAT AC C TCG CAA TGT CAC G	167 bp with p15 for1); 322 bp (with p15 for2)	Within exon 2
Atf3	qRT-PCR	ATF for	CCT CTC ACC TCC TGG GTC ACT G	214 bp (with ATF rev)	Within exon 2
Atf3	qRT-PCR	ATF rev	ATT TCT TTC TCG CCG CCT CC	214 bp (with ATF for)	Within exon 3
Csn2 (encodes β-casein)	qRT-PCR	cas m for	TAT CAA TGA GCA GAA ACT TCA GAA GGT	130 bp (with casein m rev)	Spans intron-exon boundary (between exon 5/6)
Csn2 (encodes β-casein)	qRT-PCR	cas m rev	GGT TTG AGC CTG AGC ATA TGG	130 bp (with casein m for)	Within exon 6
Actb (encodes β-actin)	qRT-PCR	act for	ATGGAGAAGATCTGG CAC	616 bp (with act for)	Obtained from Berubé lab; within exon 3; qRT-PCR ctl
Actb (encodes β-actin)	qRT-PCR	act rev	CGTCACACTTCATGA TGG	616 bp (with act rev)	Obtained from Berubé lab; unknown origin; qRT-PCR ctl
Actb (encodes β-actin)	qRT-PCR	act for2	CTG TCG AGT CGC GTC CAC CC	128 bp (with act rev2)	Within exon 1; qRT-PCR ctl
Actb (encodes β-actin)	qRT-PCR	act rev2	ACA TGC CGG AGC CGT TGT CG	128 bp (with act for2)	Within exon 2; qRT-PCR ctl
Gapdh	qRT-PCR	GAP for	CAA CGA CCC CTT CAT TGA CCT	634 bp (with GAP rev)	Within exon 4; qRT-PCR ctl
Gapdh	qRT-PCR	GAP rev	ATC CAC GAC CGA CAC ATT GG	634 bp (with GAP for)	Within exon 6 qRT-PCR ctl

JL – Jackson Laboratories
MMHCC - Mouse Models of Human Cancers Consortium
ctl - control

Appendix II: Animal Use Protocol – Letter of Approval



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