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Investigating the Role of Retinoblastoma Protein and Transforming Growth Factor-Beta in Growth Inhibition

Mehdi Amiri, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biochemistry

INVESTIGATING THE ROLE OF RETINOBLASTOMA PROTEIN AND TRANSFORMING GROWTH FACTOR-BETA IN GROWTH INHIBITION

(Thesis format: Monograph)

by

Mehdi Amiri

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The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

The Retinoblastoma protein (pRB) is a key regulator of cell proliferation in the G1 phase of the cell cycle. The LxCxE binding cleft is a highly conserved region of pRB. Using a knock-in mouse model, called *Rb1*$^{\Delta L}$, with disrupted pRB and LxCxE interactions, our lab has shown that epithelial cells from *Rb1*$^{\Delta L/\Delta L}$ mice do not respond to TGF-β1 mediated growth arrest. Using shRNAs to deplete the expression of components of LxCxE motif containing complexes, data showed that SAP18 is not involved in TGF-β1 mediated growth arrest. However, depletion of SAP30 and MTA2 compromised TGF-β1 mediated growth arrest. Furthermore, depletion of MTA2 resulted in derepression of E2F target genes in response to TGF-β while depletion of SAP30 repressed the expression of E2F target genes.

Keywords

pRB, TGF-β, LxCxE binding cleft, MTA2, SAP30, SAP18, transcriptional repression

Co-Authorship Statement

All chapters were written by Mehdi Amiri and edited by Dr. Fred Dick.

All experiments were performed by Mehdi Amiri.

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Abbreviations

2-ME	2-Mercaptoethanol
AMH	Anti-Müllerian hormone
APC	Anaphase-promoting complex
ARF	Alternate open reading frame
ASAP	Apoptosis-and splicing-associated protein
BAH	Bromo-adjacent homology
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine
BRG1	Brahma related gene 1
CAP-D3	Chromosome associated protein-D3
CCNE1	Cyclin E1
CCNA2	Cyclin A2
CDH1	Cadherin-1
cDNA	complementary DNA
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
Co-Smad	Common-partner Smad
CRF	Chromatin regulatory factor
CSC	Cancer stem cell
CtBP	C-terminal binding protein
CtIP	C terminal Interacting Protein
DMBA	7-12-Dimethylbenz (a) anthracene
DMEM-F12	Dulbecco's modified eagle medium: nutrient mixture F-12
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferases 1
DP	Dimerization partner
DREAM	DP, pRB, E2F and MuvB
DYRKA1	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
E2F	E2 promoter binding factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELM	Egl-27 and MTA1 homology
EMT	Epithelial to mesenchymal transition
Erbin	ErbB2/Her2-interacting protein
Erk	Extracellular signal regulated kinases
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FKBP12	FK506 binding protein 12
FoxH1	Forkhead box H1
GDF	Growth and differentiation factor
GFP	Green fluorescent protein
GS domain	Glycine-serine-rich domain
GSE	Gel shift extract
GST	Glutathione S-transferase
GTPase	Guanosine-5'-triphosphatase
HDAC 1/2	Histone deacetylase 1 or 2

HID	Histone deacetylase interacting domain
HLB	Hypotonic lysis buffer
HPV	Human papillomavirus
HRP	Horseradish peroxidase
I-Smad	Inhibitory Smad
JNK	c-Jun N-terminal kinases
LAP	Latency associated peptide
LATS2	Large tumor suppressor kinase 2
LSD1	Lysine specific demethylase 1
MAD2	Mitotic arrest deficient 2
MAPK	Mitogen activated protein kinase
MBD	Methyl-CpG binding domain
MCM	Mini-chromosome maintenance
MEC	Mouse epithelial cell
MEF	Mouse embryonic fibroblast
MH1/2	Mad homology 1 or 2
miRNAs	microRNAs
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MTA	Metastasis associated 1 family
NET1	Neuroepithelial cell transforming 1
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NuRD	Nucleosome remodeling and histone deacetylase
PAH	Paired amphipathic helix
PAI-1	Plasminogen activator inhibitor-1
PBF	Papillomavirus binding factor
PBS	Phosphate buffered saline
P/CAF	p300/CBP-associated factor
PcG	Polycomb-group
pCMV	Cytomegalovirus promoter
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI	Propidium iodide
PI3K	Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PML	Promyelocytic leukemia
PPM1A	Protein phosphatase, Mg2+/Mn2+ dependent, 1A
pRB	Human retinoblastoma tumor suppressor protein
pRb	Mouse retinoblastoma protein
R-Smad	Receptor-regulated Smad
RB1	Human retinoblastoma susceptibility gene
Rb1	Mouse retinoblastoma susceptibility gene
RbAp46/48	Retinoblastoma associated protein 46/48
RNAi	RNA interference
Rho	Ras homolog gene family
RNPS1	RNA-binding protein
RNA	Ribonucleic acid
Runx	Runt-related transcription factor
SANT	SWI3, ADA2, N-CoR, TFIIB

SAP30	Sin3 associated polypeptide- 30 kDa
SARA	Smad anchor for receptor activation
SBE	Smad-binding element
SDS3	Suppressor of sefective silencing 3
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ShLuc	Short hairpin luciferase
ShRNA	Short hairpin RNA
Ski	Sloan-Kettering institute oncogene
Smad	SMA- and MAD-related
SnoN	SKI-like oncogene
SV40	Simian virus 40
SWI/SNF	Switching defective/sucrose non-fermenting
TAK1	TGF beta-activated kinase 1
TBS	Tris-buffered saline
TβIR	TGF-β type I receptor
TβIIR	TGF-β type II receptor
TFA	Acetonitrile/trifluoroacetic acid
TFIID	Transcription factor II D
TGF-β	Transforming growth factor-β
TIEG2	Transforming growth factor-beta-inducible early gene 2
TIMP	Tissue inhibitors of metalloproteinase
TNBC	Triple-negative breast cancer
TNF	Tumor necrosis factor
TRAP	Triiodothyronine receptor auxiliary protein
UTR	Untranslated region
WAP	Whey acidic protein
YY1	Yin yang 1

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1 Introduction

1.1 Retinoblastoma; the first tumor suppressor to be identified

1.1.1 Retinoblastoma and *RB1* Discovery

Before the "two hit" hypothesis proposed by Knudson, it was believed that a large number of mutations are required for tumorigenesis. Using the retinoblastoma cancer model, Knudson proposed that this disease could arise from as few as two mutations. He proposed that in familial form of retinoblastoma, in which a germline mutation is present, a second somatic mutation is required for tumorigenesis whereas in sporadic retinoblastoma, both somatic mutations are required for tumorigenesis (1). This notion was supported by the fact that sporadic cases have a later onset (1). A decade later, several studies cloned a locus containing the product of retinoblastoma susceptibility gene (pRB) and mapped the gene on chromosome 13 (2-5). Studies conducted after cloning of the *RB1* gene showed that pRB is a nuclear phospho-protein with the ability to bind to DNA (6-8). In addition, the germline mutation in a single allele of *RB1* promoted a loss of heterozygosity, a chromosomal event that results in loss of the entire gene, confirming Knudson's two hit hypothesis (9).

With the emergence of transgenic mouse models, three independent studies reported that heterozygous mice for *RB1* did not develop retinoblastoma (10-12). More importantly, these animals survived and did not show any overt phenotype except low-

penetrance pituitary tumour formation reported by Jacks and colleagues (10). The RB1 null mice died in the early embryonic stages due to apoptosis in the nervous system (10-12). These findings led to the discovery of other members of pocket protein family and the suggestion that pRb function can be compensated by other pocket protein family (p107 and p130) in mice. Development of the first animal model with Rb1/p107 null cells revealed evidence for the compensation of the pocket proteins family as these mice developed retinoblastoma (13). Since the discovery of *RB1*, many studies have examined different roles for pRB in cancer progression and have expanded our knowledge of different pRB functions.

1.1.2 Structure of pRB

pRB contains 928 amino acids, and consists of three domains, the N- terminal domain, the C-terminal domain and a "small pocket" linker domain. This pocket domain contains two major domains, A and B, which are linked by a spacer region. Many proteins such as LxCxE (Leu-X-Cys-X-Glu; X= any amino acid) partners, E2 promoter binding factor (E2F) and cyclin dependent kinase (CDK) complexes bind to the small pocket domain (14). The small pocket together with the C-terminal domain creates the "large pocket" which is required and sufficient for the ability of pRB to induce cell cycle arrest (15, 16).

The LxCxE binding cleft in the small pocket region is one of the most highly conserved regions of pRB (17). This cleft was initially identified as a contact site for LxCxE motifs in viral oncoproteins such as simian virus 40 (SV40) large T antigen, adenovirus E1A and human papillomavirus E7 (HPV E7) (18, 19). This cleft interacts

with over 30 cellular proteins. Many of these proteins have the ability to modify chromatin, including Histone deacetylase 1/2 (HDAC1/2), DNA methyltransferases 1 (DNMT1), Brahma Related Gene 1 (BRG1) (20) (Figure 1-3). The majority of research in the pRB field has focused on the role of E2F transcription factors in pRB`s tumor suppressor function with little focus on how proteins with LxCxE motif contribute to this function. This thesis focuses on an aspect of how these proteins with LxCxE motif contribute to pRB`s ability to act as a tumor suppressor.

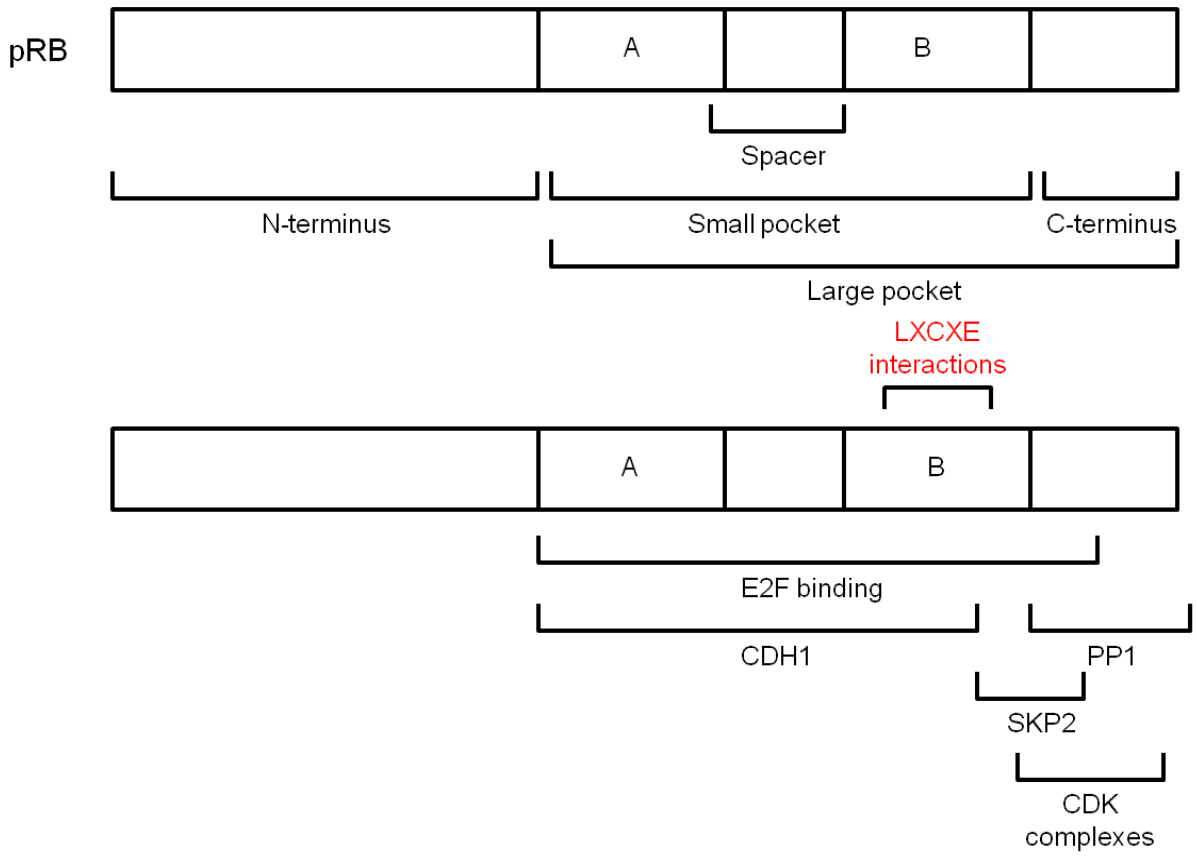


Figure 1-1 Domain structure of pRB

The domain structure of pRB is shown with the large pocket, small pocket and C-terminal regions. pRB binds to over one hundred protein partners and mediates transcriptional regulation of hundreds of target genes.

1.1.3 E2F transcription factors

1.1.3.1 Structural features of E2Fs

The protein-protein interactions between pRb and E2F transcription factors are well characterized. E2Fs are a family of transcription factors with many common features. They are divided into two categories based on their transcriptional activity: activator E2Fs and repressor E2Fs. Activator E2Fs consist of E2F1, E2F2 and E2F3a while E2F3b, E2F4, E2F5, E2F6, E2F7 and E2F8 are classified as repressor E2Fs (21).

All E2Fs except E2F7 and E2F8 interact with dimerization partner (DP) proteins through the dimerization domain. DP proteins are required to enhance DNA binding activity of E2Fs (22-24). There are three different DP proteins (DP1, DP2/3, DP4) but not much is known about their activity (25). E2F subunits determine the specificity of E2F/DP complex rather than DP subunits (25). E2F-DP interactions are necessary for E2Fs function, as E2F1-6 are not capable of interacting with deoxyribonucleic acid (DNA) without dimerization. E2F7 and E2F8 contain two DNA binding domains allowing them to interact with DNA independently of DP proteins (26, 27). E2F1, E2F2 and E2F3a are structurally similar and share many domains such as the transactivation domain. This domain is responsible for E2F target genes` activation by recruiting the transcription machinery such as transcription factor II D (TFIID), p300 and triiodothyronine receptor auxiliary protein (TRAP) to E2F target genes promoters (28-30). Pocket proteins also interact with this domain to block its ability to drive transcription of E2F target genes (31, 32). E2F 6-8 do not contain a transactivation domain and act as a constitutive repressor independently of pocket proteins (21).

1.1.3.2 E2F binding sites of pRB

pRB contains two distinct binding sites to interact with E2F1: The large pocket and the C-terminal region of pRB (33). The large pocket region, also called the "general site" of pRB, interacts with the transactivation domain of E2F1-4. The C-terminal region of pRB, also known as the "specific site", uniquely binds to the marked box domain of E2F1 (34). The 'general site' functions in proliferative control while the 'specific site' functions in E2F1 induced apoptosis (33-35). However, the exact mechanism by which specific site regulates E2F1 induced apoptosis remains to be investigated. Moreover, E2F1-pRB complex through specific site showed low affinity for the canonical E2F recognition sequence (33) and was resistant to disruption by E1A infection and CDK phosphorylation (36). These data suggest that this complex is regulated through distinct mechanisms and functions in nontraditional pathways compared to other E2F-pRB complexes.

1.1.3.3 Roles of E2F transcription factors

Multiple gene targeted mouse models have been developed to investigate the role of each individual E2F. The data showed that all the single knockout mice are viable suggesting that none of the E2Fs is required in development. However, there are some defects in different tissue for each knockout suggesting that E2Fs may have tissue specific roles (21). For instance, $E2F4^{-/-}$ mice show maturation defects in hematopoietic lineages and $E2F2^{-/-}$ mice show a higher activity of T-Cell receptor signaling leading to development of autoimmune diseases (37, 38).

Although activator E2Fs appear to have redundant roles in proliferative control, E2F1 shows a unique connection to apoptosis. This unique function was discovered by the fact that E2F1 null mice showed a defective apoptosis in their lymphocytes (45). More importantly, none of the other E2F knockout mice showed any defect in apoptosis suggesting that this is a unique function of E2F1(38-44). During DNA damage, E2F1 effectively activates an apoptotic program through the activation of p53 or its homologue p73 (46-48). It has been shown that E2F1 activates a series of apoptotic targets such as the p19-alternate open reading frame (ARF), which is responsible for inhibiting the degradation of p53 (49). E2F1 also acts to induce apoptosis through the direct activation of p73 (50).

1.1.4 Mechanisms of pRB-mediated gene repression

Briefly, pRB binds to E2Fs and inhibits their transcriptional activity to induce G1 arrest. In the G1 phase of the cell cycle, pRB is phosphorylated and bound to E2Fs and co-repressors, which results in transcriptional repression of S phase entry genes. During late G1, the phosphorylation of pRB by CDK4/6/Cyclin D complexes alleviates repression of E2F. Further phosphorylation of pRB by CDK2/Cyclin E drives the cell into S-phase (64) (Figure 1-4).

In addition to pRB and E2Fs interactions, which masks the transactivation domain of E2F and inhibits E2F transcriptional activation, pRB and other members of the pocket protein family recruit multiple co-factors that change the activation state of target genes (52). The best-characterized interactions occur between pRB and HDACs. HDACs are responsible for removing acetyl groups from activated histones to induce transcriptional

repression (53). The pRB-HDAC complex strengthens pRB-E2F mediated repression during the G1 phase of the cell cycle (54, 55, 51 and 56). pRB also interacts with other proteins to regulate transcription and affect chromatin dynamics. One such example is DNMT1, which interacts with pRB to promote DNA methylation and repress transcription (57). RBP2 is another pRB binding protein involved in chromatin regulation. This protein is a demethylase, which removes methyl groups of an active transcription mark, named trimethylated H3K4 (58-61). Further experiments showed that Rbp2 knockdown in Rb1 null MEFs opposes proliferation of these cells while promoting differentiation (62).

As mentioned here, some LxCxE binding proteins can change the activation state of target genes. However, the exact mechanisms by which these proteins change the transcriptional program remains to be discovered.

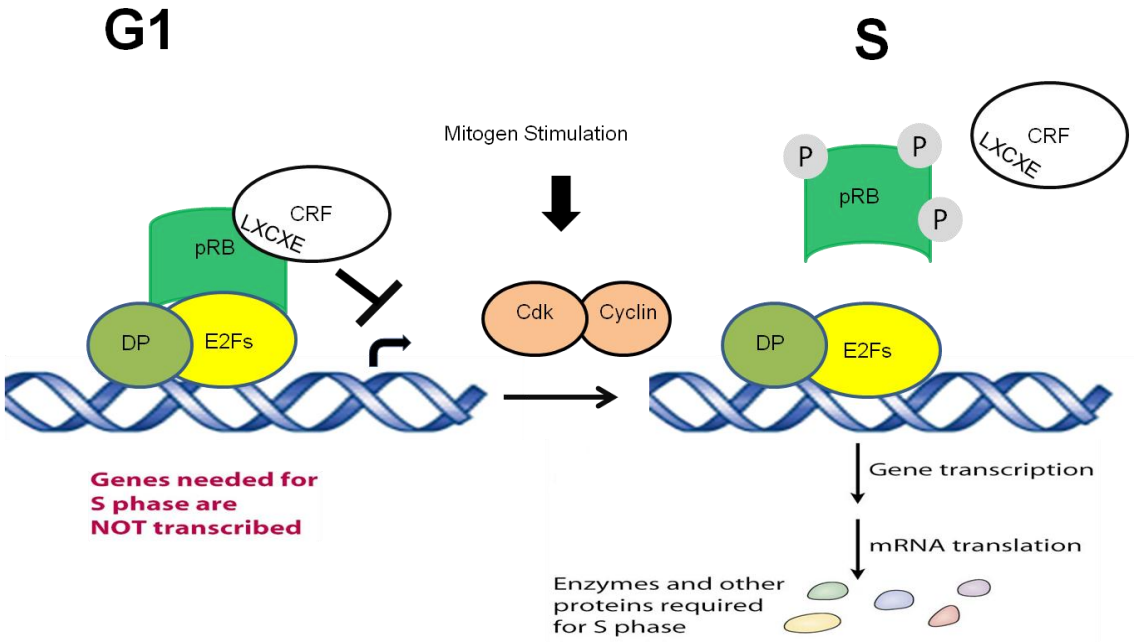


Figure 1-2 The proposed model of pRB function

In the G1 phase of the cell cycle, hypophosphorylated pRB acts as a tumor suppressor by binding to E2Fs and inhibiting transcriptional activation of genes required for S phase entry. When cells progress to the S phase, CDK-cyclins complexes phosphorylate pRB and inhibit its activity. The phosphorylation of pRB releases E2F-DP, allowing them to activate the transcription of genes, which are required for S phase entry.

1.1.5 Biological functions of pRB

1.1.5.1 Regulation of cell cycle

In the G1 phase of the cell cycle, hypophosphorylated pRB masks the transactivation domain of E2Fs bound to their DP protein partners, which blocks the activation of E2F target genes such as *CCNE1*, *CCNA2* and *E2F1* (63). pRB-E2F-DP complexes are also able to recruit chromatin regulatory factors (CRFs) to further repress the transcription of these genes (64). As cells progress into the S phase, CDK/Cyclin complexes phosphorylate pRB, leading to release of E2F/DP complexes. Free E2Fs drive the transcription of E2F target genes required for the S phase entry.

1.1.5.2 pRB and controlling cell death

It is believed that pRB has a dual role in apoptosis through multiple mechanisms (65, 66). The first evidence for the anti-apoptotic role of pRb rose from an experiment where Rb null mice showed an increased level of apoptosis (10). However, recent studies revealed that this phenotype is due to the deregulation of cell cycle regulators and overproliferation of the placenta which result in hypoxia in embryonic tissues and is not cause by E2Fs (67-69). In contrast, recent studies have shown that pRB has an apoptotic role in highly proliferative cells (65, 70 and 71). In these studies, the hyperphosphorylated pRB forms a complex with E2F1 and histone acetyltransferase p300/CBP-associated factor (P/CAF) at the promoter of proapoptotic genes such as caspase7 and p73 driving their expression to induce apoptosis (65). Moreover, a recent study revealed another mechanism for apoptotic activity of pRB. This study showed that

pRB expression increases tumor necrosis factor-α (TNF-α)-induced apoptosis, which depends on its localization to the mitochondrial outer membrane and acts in a Bax-dependent manner (72).

1.1.5.3 pRB and the maintenance of genome stability

pRB is also able to both prevent and respond to genotoxic stress. A recent study showed that disruption of pRB function by oncogenes could cause replicative stress during the S phase of the cell cycle (73). This study showed that pRB inactivation by oncoproteins results in an uncoordinated S phase entry. This leads to increased replication stress which ultimately causes DNA damage and genome instability (73).

Many of E2F target genes involved in chromatin condensation, spindle checkpoint and chromosome segregation have been identified (74, 75). Mitotic arrest deficient 2 (Mad2) protein, which is responsible for proper chromosome segregation, is an example of direct E2F target gene. pRB inactivation deregulates the expression of Mad2, which leads to mitotic defects and aneuploidy (76). These data suggest that pRB has essential roles in maintaining genome stability beyond its G1/S function.

pRB is also essential for maintaining chromosome stability and preventing tumor growth in mice. Using an LxCxE mutant mouse model, our lab showed that the LxCxE motif of pRB recruits the chromosome associated protein D3 (CAP-D3) protein to the centromeric heterochromatin. We also showed that recruitment of CAP-D3 to this region is required for condensin II complex formation and maintaining chromosome stability (77). Furthermore, these results were supported by findings from other research groups using fruit flies and human retinal cells (78, 79).

1.1.5.4 The role of pRB in senescence

pRB plays an essential role in senescence through the stable repression of E2F target genes and heterochromatin formation (80, 81). Studies over several years have revealed how pRB contributes to senescence. It has been shown that the activator E2Fs are recruited to promyelocytic leukemia (PML) nuclear bodies by the tumor suppressor PML. This leads to transcriptional repression of E2F target genes, which causes stable cell cycle arrest in a pRB-dependent manner (82). These findings provide an explanation for how pRB mediates the stable repression of E2F target genes in oncogene-induced senescence, but how pRB interacts with other co-factors to perform its role in inducing stable cell cycle exit remains to be addressed.

Recently, a study showed that large tumor suppressor kinase 2 (LATS2 kinase) mediates the formation of p130/DP, pRB, E2F and MuvB (DREAM) complexes at E2F target gene promoters. The formation of this complex represses transcription of E2F target genes and induces senescence (83). In addition, LATS2 kinase phosphorylates dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRK1A) and in turn phosphorylates LIN52, which is required for DREAM assembly (84). It is interesting to mention that p130 and p107 alone are not sufficient for the oncogenic Ras induced senescence (85). However, p130 and p107, but not pRB, were found in the DREAM complex (86). These findings provide a mechanism for oncogene induced senescence. pRB could be responsible in recruiting p107 and p130 to the DREAM complex in oncogene induced senescence; however, how pRB contributes to this mechanism needs to be investigated.

1.1.6 pRB in breast cancer

pRB is the central regulator of the cell cycle that is inactivated in the majority of human cancers. Primary tumors analyses have shown that 20-35% of tumors lose pRB expression and 7-37% of tumors show loss of heterozygosity or other alterations of the *RB1* locus (87-90).

In breast cancer, pRB function is abrogated via multiple mechanisms such as loss of p16ink4a, which promotes cell growth (91, 92). Microarray analyses have revealed that pRB/E2F complex regulates approximately 150 target genes involved in cell cycle control (93). Furthermore, it has been shown that *RB1* deficiency results in deregulation of several E2F target gene such as proliferating cell nuclear antigen gene (*PCNA*), *CCNE1* (Cyclin E1 gene) and *CCNA2* (Cyclin A2 gene), all of which are required for cell cycle progression (94). It is logical that elevated expression levels of these target genes are responsible for the accelerated proliferation rates observed in *RB1*-deficient breast cancer cells and tumors.

In addition, the influence of *RB1* status on disease severity has been investigated in a broad range of tumors. In the context of breast cancer, these analyses have revealed that *RB1* loss correlates with advanced disease and often estrogen receptor (ER)-negative disease (95, 96). Although there are many data showing E2F-pRB mediated gene expression changes in breast cancer cells and tumors, the main question of whether direct *RB1* loss or various upstream effects on the pRB pathway are responsible for these changes in gene expression remains to be addressed. Answering this question could lead to improved treatment for breast cancer patients.

1.1.7 Importance of LxCxE interactions in pRB function

The majority of research in the pRB field has focused on its role in binding and inhibiting E2F transcription factors, with little focus on the role of LxCxE-pRB interactions in pRB`s tumor suppressor function. The LxCxE binding motif interacts with many factors (20). Initial *in vitro* studies have showed that the mutation of the LxCxE binding cleft prevents pRB induced growth arrest in fibroblasts. However, the LxCxE mutant was sufficient to maintain growth arrest in terminally differentiated muscle tissue (97). Later study by La Thangue group called into question the effect of LxCxE interactions in growth arrest, as LxCxE mutants were dispensable for growth arrest (98). Thus, the development of mouse models that disrupt the interaction between pRB and LxCxE binding cleft were necessary to study the function of this domain in multiple cellular contexts *in vivo*.

In order to understand the importance of the LxCxE binding cleft in pRb, our lab created a gene-targeted mouse model. This mouse, called $Rb1^{\Delta L}$, carries three substitutions which disrupt the interaction between pRb and LxCxE containing proteins, whereas interactions with other molecules, such as E2F transcription factors, are intact (99) (Figure 1-5). In accordance with La Thangue group study, MEFs isolated from $Rb1^{\Delta L/\Delta L}$ mice have the ability to maintain proliferative control in asynchronous growing cultures, in response to serum starvation and confluence arrest (99). Furthermore, these mice are viable, fertile and do not develop spontaneous tumor.

In the context of tumor initiation, $Rb1^{\Delta L/\Delta L}$ showed several mitotic defects leading to chromosomal instability, which resulted in tumour formation in conjunction with the

p53 null model (77, 99). It has been demonstrated that CAP-D3, the condensin II complex subunit, which is essential for proper chromosome segregation during mitosis, interacts with pRB through the LxCxE binding cleft (78). This provides a mechanistic explanation for genomic instability seen in the study by our lab (85).

Finally, in the context of cellular senescence, pRB recruits different co-factors from normal physiological G1 arrest (208, 213). Using the *Rb1*$^{\Delta L/\Delta L}$ mouse model, our lab showed that LxCxE mutants prevent Ras-induced senescence (100). Although no specific LxCxE partners have been identified, Jarid1a (Rbbp2) was shown to regulate senescence by interaction with pRB. Even though it has not been determined whether Jarid1a interacts with pRB through the LxCxE binding cleft, this presents a possible mechanism of regulation (101).

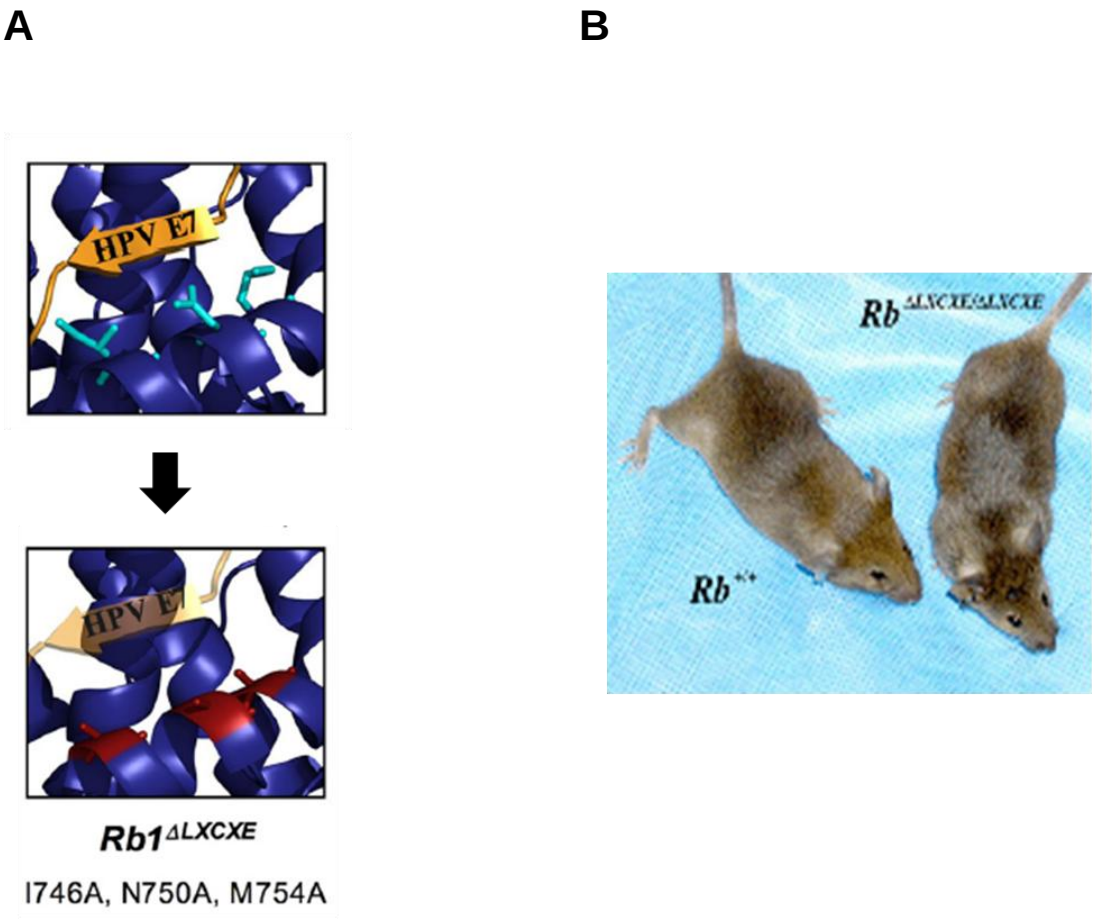


Figure 1-3 The knock-in mouse strain with disrupted LxCxE interactions

A) pRB interaction with the human papillomavirus (HPV) E7 through the LxCxE binding cleft. Amino acids mediating the interaction are shown in turquoise. The *Rb1*$^{\Delta L}$ mutation changes these residues to alanines (red) which results in disrupting the pRB-LxCxE interaction
B) ΔLxCxE mice are viable and develop relatively normally. (Adopted from Francis, S., MCB 29: 4455-66)

1.1.8 LxCxE binding partners

As mentioned above, the LxCxE binding motif interacts with many factors (20). This introduction focuses on the complexes relevant to this thesis (Table 1-1).

1.1.8.1 Sin3 complex

Sin3 is a nuclear protein, which consists of Sin3A and Sin3B isoforms in human (102). In addition to these isoforms, splice variants occur in these genes, adding more complexity to these isoforms (103). Structural analyses of Sin3 have revealed that there are four conserved regions within this protein called paired amphipathic helix (PAH) domains which are responsible for protein-protein interactions (104). Another conserved region in Sin3 is the HDAC interacting domain (HID) located between PAH3 and PAH4 domains. Both HID and PAH domains are essential for transcriptional repression activity of Sin3 (105, 106).

The Sin3 complex consists of several components including retinoblastoma associated protein 46 (RbAp46), RbAp48, HDAC1, HDAC2, Sin3 Associated Polypeptide-30 kDa (SAP30) and Sin3 associated polypeptide-18 kDa (SAP18) (107). RbAp46 and RbAp48 are highly similar in their sequence (90% homology) (108-110). Furthermore, they have been identified using immobilized pRB (108, 109). Interestingly, these proteins are capable of interacting with histone H4 and H2A suggesting that they may be involved in Sin3 complex interaction with histones (111).

SAP30 is one of the unique components of Sin3 complex. SAP30 is a highly conserved protein from yeast to human. There are several biochemical evidences

suggesting that this protein is a component of the Sin3/HDAC complex in yeast and mammalian cells (112). In mutational analysis of yeast, disruption of the SAP30 shows the similar phenotypes as the strains with Sin3 disruption, suggesting that SAP30 plays an essential role in the Sin3 complex (112). SAP30 directly binds to multiple subunits of the Sin complex, including RBBP4/7 and HDAC1, suggesting that SAP30 may play a role in stabilizing the complex (112). In addition to serving a role a stabilizing molecule in Sin3 complex, several studies indicate that SAP30 may serve as a bridging protein between the Sin3 complex and other transcription factors and corepressors such as Papillomavirus binding factor (PBF) and Yin Yang 1 (YY1). PBF acts as a repressor of HPV transcription through the recruitment of the Sin3/HDAC complex to the promoters of certain HPV proteins via direct interaction with SAP30 (113). SAP30 also interacts with YY1 and enhances YY1 induced repression through direct recruitment of HDAC1 (114, 115).

SAP18 is another core protein in the Sin3 complex, which directly interacts with HDAC1 (116). SAP18 has been identified to be a part of other complexes such as apoptosis-and splicing-associated protein *(*ASAP*)*.This complex also contains an RNA-binding protein (RNPS1) and a caspase (Acinus) (117). In Drosophila melanogaster, SAP18 plays an important role in mRNA splicing, by interacting with the homolog of Pinin (dPnn) (117). More recently, another component of the Sin3 complex has been identified in yeast and mouse, called the suppressor of defective silencing 3 (SDS3) (118-120). SDS3 is an integral component of the Sin3 complex, which interacts with HID domain of Sin3 and regulates the catalytic activity of the Sin3 complex (121, 122).

Furthermore, depletion of SDS3 compromises deacetylation of pericentric heterochromatin, leading to aneuploidy and defective karyokinesis (123).

1.1.8.2 Mi-2/NuRD (nucleosome remodeling and deacetylation) complex

Mi-2/NuRD and Sin3 complexes share several components including RbAp46, RbAp48, HDAC1 and HDAC2. In addition, Mi-2/NuRD complex contains other subunits such as Mi-2α, Mi-2β, p66α, p66β and metastasis associated 1 family member 2 (MTA2) (124-126). Mi-2 proteins contain chromodomain and switching defective/sucrose non-fermenting (SWI2/SNF2)-type helicase/ATPase domains with chromatin remodeling activity (127, 128). p66α and p66β directly interact with the methyl CpG binding proteins, MBD2b and MBD3. However, their exact role in humans remains to be investigated (129).

The MTA protein family consists of three proteins including MTA1, MTA2 and MTA3 (130). MTA proteins localize to the nucleus except MTA1, which localizes to both the cytoplasm and nucleus (130-132). This family contains several common domains including the SANT (SWI, ADA2, N-CoR and TFIIIB-B) domain which is involved in DNA binding, bromo-adjacent homology (BAH) domain which is essential for protein-protein interactions and the egl-27 and MTA1 homology (ELM) domain (131, 132). These common domains suggest that MTA proteins may function in signal transduction and transcriptional regulation.

MTA2 is a component of the NuRD complex, which represses transcription and exerts HDAC activity (124). MTA2 is the first member of MTA family to be found in the NuRD complex. This protein contains putative zinc fingers and leucine zipper domains (133). MTA2 has been identified in association with HDAC and p53, which results in deacetylation of p53 and repression of p53 dependent transcription (134).

1.1.8.3 C-terminal binding protein (CtBP) complex

It has been shown that pRB recruits histone deacetylase to mediate transcriptional repression of E2F target genes. However, many genes subjected to E2F-pRB mediated repression are not activated following “trichostatin A” treatment, a histone deacetylase inhibitor, suggesting additional factor may contribute to the repression.

CtBP isoforms one, two and three are cellular proteins that bind to the C-terminal region of the human adenovirus E1A proteins (135-137). CtBP proteins are highly conserved among invertebrates and vertebrates (137). CtBP proteins play an important role in development and oncogenesis (137). CtBP protein is recruited by a protein with PLDLS motif to the promoter of genes such as *Knirps* and *Snail* to repress transcription of these genes in fruit flies (136, 138). In addition, it has been shown that mammalian CtBP protein is recruited to E2F target genes promoters through an interaction with the C-terminal interacting protein (CtIP) (135). Since CtIP does not directly bind to DNA, it appeared that CtIP bridges CtBP to target promoters by interaction with pRB or p130 and then E2F. However, the exact mechanism by which CtBP represses transcription of E2F targets needs to be investigated.

Table 1-1 pRB-LxCxE chromatin regulating complexes

Complex	**Subunits**							
Sin3	SAP18	RBP1	Sin3	HDAC 1/2	RBBP 4/7	SDS3	ING1/2	SAP30
NuRD	LSD1	MTA2	Mi-2a/b	HDAC 1/2	RBBP 4/7	p66a/b	MBD3b	MBD3a
CtBP	LSD1	CtIP	CDYL	HDAC 1/2	CtBP	GLP	CoREST	LCoR

1.2 TGF-β, a highly pleiotropic cytokine: An overview

Transforming growth factor-β (TGF-β) isoforms one, two and three are members of a large family of cytokines with similar structure. TGF-β regulates different biological functions such as apoptosis, cell growth, T-cell activation and differentiation (139). TGF-β isoforms act as tumor suppressors whose growth suppressive function is overcome during cellular transformation (140, 141). In contrast, TGF-β also regulates processes such as cell motility, which can stimulate metastatic dissemination of cancer cells (142). From this perspective, understanding the mechanism of action of TGF-β signaling holds great promise for developing new cancer therapies.

1.2.1 Overview of signal transduction by TGF-β

TGF-β initiates signaling cascade by inducing two serine/threonine kinase receptors. Upon ligand binding, the TGF-β type II receptor (TβIIR) forms a complex with the TGF-β type I receptor (TβIR) and phosphorylates TβIR at its glycine-serine-rich domain (GS domain). Activated TβIR in turn phosphorylates and activates receptor-regulated Smad (R-Smad) proteins (143-145). Phosphorylated R-Smads then bind to Smad4 through their mad homology 2 (MH2) domains, and translocate into the nucleus (146-149). Once in the nucleus, these complexes mediate the expression of different genes such as plasminogen-activator inhibitor-1 (PAI-1), p15, p21 and collagen by interacting with various transcription factors and co-factors (150-154). (Figure 1-1)

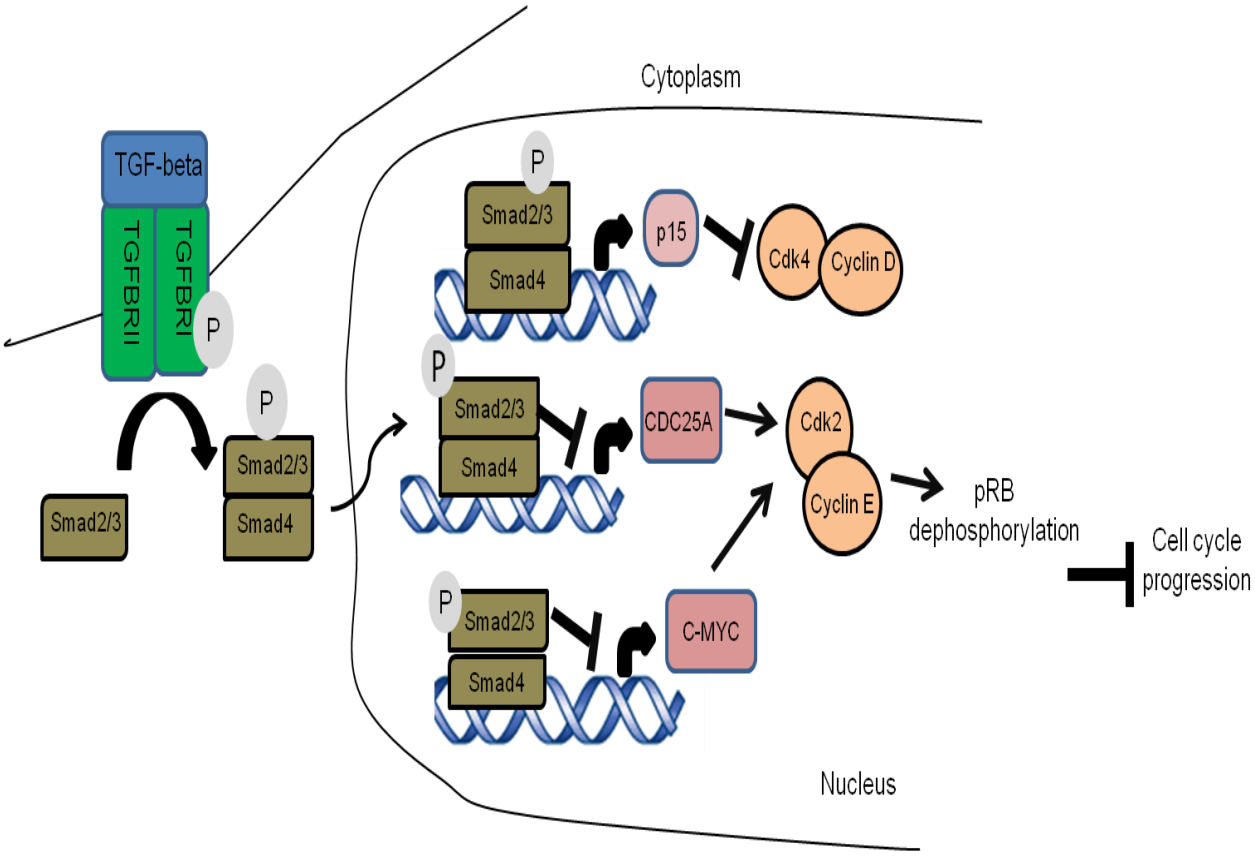


Figure 1-4 The current model of TGF-β signaling in the cell cycle

In the presence of TGF-β ligand, TβIR is phosphorylated which allows it to phosphorylate and activate R-Smads. Activated R-Smad then binds to co-Smad and translocates into the nucleus. Once in the nucleus, Smad4 recruits other cofactors, which results in regulation of many genes and dephosphorylation and activation of pRB in the G1 phase of the cell cycle.

1.2.2 Components of TGF-β signaling pathways

1.2.2.1 TGF-β ligands and receptors

The TGF-β superfamily includes over 30 polypeptides. They are divided into two subfamilies: the TGF-β subfamily which includes TGF-βs (1-3), activins (A, B), nodal and myostatins, and the bone morphogenetic protein (BMP) subfamily which includes BMPs (1-9), Anti-Müllerian hormone (AMH) and growth and differentiation factors (GDFs) (155, 156). These ligands are synthesized as the C-terminal domain of a precursor molecule. The signal peptide region is cleaved before secretion and these cleaved cytokines remain inactive due to the function of the latency-associated peptide (LAP) (157-159). Once in the extracellular matrix (ECM), TGF-β is activated by proteases that remove LAP, resulting in the stable and active dimeric form of the TGF-β cytokine (146).

TGF-β triggers signaling events by inducing heterodimerization of TβIR and TβIIR. Both receptors contain a single transmembrane domain, an N-terminal domain and a C-terminal domain (160). A complex of TβIR and TβIIR homodimers is formed after ligand binding creating a tetrameric structure. Once assembled, the constitutively active TβIIR phosphorylates TβIR at its cytoplasmic GS domain. Phosphorylation of the serine residues within the GS domain creates a docking site for R-Smads (161, 162).

1.2.2.2 Smad proteins

1.2.2.2.1 Structure and diversity

Smad proteins were originally identified in *Drosophila melanogaster* and *Caenorhabditis elegans* (163, 164). There are eight Smad proteins in mammals that are divide into three subtypes: R-Smads including Smad2 and Smad3 for TGF-β signalling pathways, Smad1, Smad5 and Smad8 for BMP signaling pathways, common-partner Smads (Co-Smads) such as Smad4 and inhibitory Smads (I-Smads) such as Smad6 and Smad7 (165-167).

R-Smad and Smad4 proteins contain two conserved domains: The MH1 domain in the N-terminal region and the MH2 domain in the C-terminal region bridged by a linker region (168, 146). The MH2 domain in R-Smads contains a 'SSXS' motif phosphorylated by TβIR for Smad activation (143, 170 and 171). Smad4 does not contain the 'SSXS' sequence in its MH2 domain, Therefore, TβIR is not able to phosphorylate Smad4 (169, 172). The linker region contains a 'PPXY' motif, a regulatory site for Smad activation, which promotes Smad degradation via proteasomal machinery (146). I-Smads (Smad6 and Smad7) lack the MH1 domain and the 'SSXS' motif (173) (Figure 1-2).

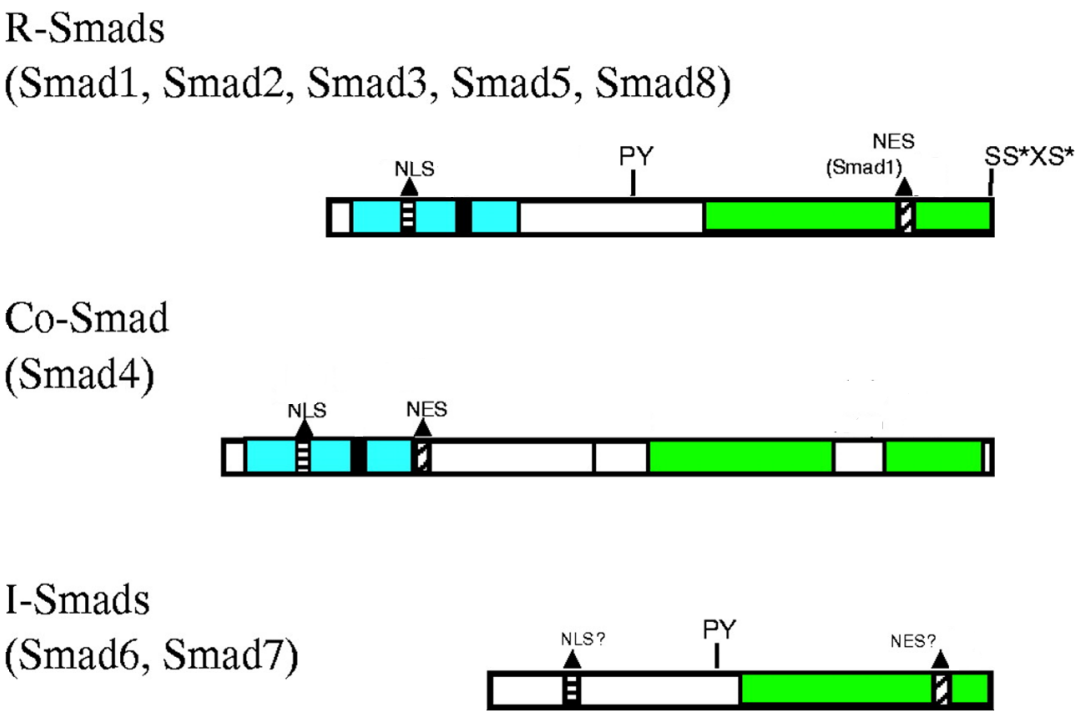


Figure 1-5 Diagrammatic representation of Smads

The MH1 domain is shown in blue and the MH2 domain is shown in green. Phosphorylated serine residues in the SSXS motif of R-Smads are shown with asterisks. NLS: nuclear localization signal, NES: nuclear export signal, PY: PPXY motif (Adopted from Moustakas, A. et al. J Cell Sci (2001) 114: 4359-4369)

1.2.2.2.2 Mechanism of transcriptional regulation by Smads

In resting cells, R-Smads are located in the cytoplasm and they interact with different proteins such as the Smad anchor for receptor activation (SARA). SARA binds to R-Smads to inhibit receptor interaction. This protein also prevents nuclear entry of unphosphorlated Smads by interacting with R-Smads (174, 175). Upon TGF-β stimulation, activated receptor complexes phosphorylate R-Smads at their 'SSXS' motif. In this stage, SARA mediates and facilitates the interactions between R-Smads and TGF-β receptors. Once R-Smads are phosphorylated, they bind to Smad4 for nuclear entry and complex formation. There are several studies showing that Smad4 is essential for mediating of TGF-β signaling (149, 172, 176 and 177). For instance, TGF-β responsiveness is rescued by ectopic expression of Smad4 in a Smad4 null cell line, suggesting that Smad4 is required for TGF-β signaling (178).

Once the complex is formed, R-Smads bind to the major groove of DNA. However, Smad2 and Smad4 do not bind to DNA (168, 179). A specific DNA sequence (5'- CAGAC-3') termed as the Smad-binding element (SBE), is an essential factor in recognition of DNA by Smad complexes (150, 180 and 181). Many Smad target genes have SBEs in their promoters (182-184). Since Smads interact with SBEs with low affinity, DNA binding partners are required to make specific high affinity interactions of Smads and SBEs and drive transcriptional responses of Smads. Examples of these DNA binding partners are forkhead box H1 (FoxH1) family (e.g., Fast1), Rel/ nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family (e.g., NF-κB2), runt-related transcription factor (Runx), E-box, p300, HDAC, Sloan-Kettering Institute

oncogene (Ski), SKI-like oncogene (SnoN) and E2Fs which regulate gene expression (185-193).

1.2.3 Regulation of TGF-β signaling

As a critical and important signaling pathway, the TGF-β superfamily signaling is tightly regulated to ensure a proper physiological response. Thus, there are several regulatory mechanisms for this pathway at multiple levels.

1.2.3.1 Regulation of ligand

TGF-β remains in an inactive form after secretion by binding to the LAP. TGF-β must cut off LAP to obtain its function (194). Many extracellular proteases such as thrombospondin-1, cathepsin D and matrix metalloproteinase 2 (MMP2) are responsible for this cleavage (195-197).

1.2.3.2 Regulation of receptor activation

There are many mechanisms involved in regulation of receptor activation. For instance, FK506-binding protein 12 (FKBP12) is able to terminate signal transmission by blocking the phosphorylation site of TβIR (198, 199). The internalization of receptors is another mechanism to regulate TGF-β signaling at the receptor level. This process occurs through two pathways: clathrin-mediated endocytosis of the receptors and lipid raft or caveolin-mediated endocytosis. In clathrin-coated endocytosis, vesicles bring the receptors to the cell surface in the absence of TGF-β. These vesicles also bring the activated receptor in close proximity to their R-Smad to facilitate their phosphorylation

(200-202). However, lipid rafts negatively regulate the receptors (200, 203). Upon ligand stimulation, the Smad7-WW-HECT domain E3 ligases complex interacts with the TGF-β receptors. This interaction induces lipid raft dependent endocytosis, causing receptor degradation by proteasomal machinery.

1.2.3.3 Regulation of Smad activity and levels

Smad activity and levels are tightly regulated by a variety of different mechanisms and complexes. For instance, phosphorylated R-Smads compete with the ErbB2/Her2-interacting protein (Erbin) to bind to Smad4 (204). Additionally, protein phosphatase, $Mg^{2+/}Mn^{2+}$ dependent, 1A (PPM1A) removes the phosphate group from nuclear R-Smads to inactivate and bring them back to the cytoplasm (205). Furthermore, I-Smads, Smad6 and Smad7, regulate TGF-β signaling. Smad6 is an inhibitory protein for the BMP signaling pathway whereas Smad7 functions in both the BMP and TGF-β signaling pathways (206, 207). Smad6 and Smad7 recruit E3 ligases for degradation of BMP and TGF-β receptors, respectively (208, 209). In addition, Smad6 and Smad7 interfere with R-Smads for binding to the TβIR, inhibiting the phosphorylation of R-Smads (206, 210). Interestingly, TGF-β activates the expression of Smad6 and Smad7 providing a negative feedback loop (211, 206).

1.2.4 Smad-independent signaling

In addition to Smad-dependent signaling, there are several Smad-independent TGF-β signaling pathways through the crosstalk with other pathways. One example of these pathways is mitogen activated protein kinase (MAPK) family signaling pathways

(212). C-Jun N-terminal Kinase (JNK), extracellular substrate-regulated kinase (Erk) and p38 MAPK kinase pathways are examples of this family signaling pathway, which are activated by TGF-β. These signaling pathways can cause a variety of cellular responses including cell proliferation, apoptosis and differentiation (213). It has been demonstrated that TGF-β can activate p38 MAPK signaling independent of Smads (214). Although the exact mechanism of JNK, Erk or p38 MAPK activation by TGF-β is not well understood, one possible mechanism for JNK and p38 MAPK activation is through the TGF-β-activated kinase 1 (TAK1) (215). TAK1 is one of the members of the MAPK kinase kinases (MAPKKKs) family which are responsible for activation of JNK and p38 MAPK in respond to a variety of stimuli such as TGF-β. It is also possible that TGF-β activates the NF-kB signaling pathway because TAK1 is able to phosphorylate and activate IkB kinase, which stimulates the NF-kB signaling. Further characterization of this network will provide more details and insights into the MAPK activation by TGF-β.

TGF-β is also capable of activating Ras homolog gene-like (Rho-like) GTPases including Rac, RhoA, RhoB, NET1 (RhoA-specific guanine exchange factor) and Cdc42 (216-219). These GTPases regulate many cellular events such as maintaining focal contacts, contractile stress fibers and cell motility. It has been shown that RhoA and its downstream signaling molecules such as p160ROCK have a critical role in the epithelial to mesenchymal transition (EMT) induced by TGF-β (217).

1.2.5 Biological functions of TGF-β

TGF-β isoforms play important roles in many cellular processes. Most importantly, TGF-β inhibits cell proliferation and regulates differentiation in different

cell types (220). TGF-β inhibits cell proliferation by Smad2 and Smad3 transcriptional program. Smads activate the expression of key cell cycle regulators such as CDK inhibitors, p15 and p21 (221-224). Additionally, TGF-β downregulates the expression of c-Myc. Downregulation of c-Myc results in induction of p15 and p21 expression (225, 226). The result of all these transcriptional changes is blocking the activities of CDKs, which are required for the G1-S phase transition (Figure 1-1).

TGF-β is also responsible for mediating the expression of genes responsible for ECM formation such as fibronectin, tissue inhibitors of metalloproteinases (TIMPs), PAI-1, type I and type VII collagen (184, 227-230).

TGF-β is also a key player in EMT (231). EMT is a complex process in which epithelial cells lose their cell-cell contacts, and begin to leave their community and spread into surrounding tissues (232). TGF-β mediates EMT by regulating transcriptional program of genes involved in this process such as Snail1, Twist1 and Cadherin-1(CDH1) (233, 234). These transcriptional changes are coupled by the phosphorylation of PAR6 mediated by TβIIR to dissolve tight junctions and promote EMT (235).

Lastly, TGF-β1 null mice showed an excessive inflammatory response and increased levels pro inflammatory cytokine such as TNF-α and interferon-γ. These data suggest that TGF-β plays an important role in immune system by blocking pro inflammatory chemokine synthesis (236, 237).

1.2.6 Dual role of TGF-β in tumorigenesis

In the early stages of cancer initiation, TGF-β acts as a tumor suppressor similar to its role in normal cells. However, in the later stages of cancer progression where genetic interruptions of the TGF-β signaling pathway occur, cancer cells lose their sensitivity to TGF-β mediated growth arrest. In this scenario, mutations of TGF-β signaling components, which disturb TGF-β mediated growth arrest role is thought to, cause a loss of proliferative control in cancer (142). However, it has been demonstrated that many tumor cells, which lack any mutations in TGF-β signaling pathway components also become refractory to TGF-β induced growth arrest and even, show severe phenotype changes seen in EMT (238,239).

During cancer progression, crosstalk interactions of TGF-β pathway components with altered oncogenic signalling affect transcriptional responses to TGF-β (240). For instance, in pancreatic cancer cells with mutated Ras, the Smad3/TGF-β inducible early gene two (TIEG2) complex loses its ability to downregulate the expression of c-Myc resulting in loss of TGF-β growth inhibition (240). Further investigations have revealed that the phosphorylation of TIEG2 by Erk prevents the binding of the Sin3A corepressor to the c-Myc promoter, which results in loss of growth control by TGF-β (241, 242). These studies suggested that the loss of Smad transcriptional responses is a key event by which TGF-β loses its growth inhibitory role in cancer (141). Additionally, TGF-β can promote tumor progression by inducing EMT through other signaling pathways such as phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) signaling, Notch signaling, RhoA, Rac1 and p38 MAPK (243, 216, 244).

In summary, lose of TGF-β`s function as a tumor suppressor at later stages of cancer progression through interactions with other oncogenic signaling pathways, leads to changes in the transcriptional regulation of Smads and their interacting proteins and induction of EMT. Since TGF-β plays a dual role in cancer, understanding of the exact mechanisms by which TGF-β acts as a tumor suppressor or tumor promoters will provide a great promise to develop new therapies for cancer patients with minimized unwanted side effects. This thesis will focus on understanding of TGF-β growth controlling mechanism to find better therapy for cancer patients.

1.2.7 Mouse models of TGF-β in development and cancer

To study the role and mechanism of TGF-β in development and cancer, a large number of mouse models have been developed. This introduction briefly focuses on gene-targeted disruptions of Smads and the gain and loss of function models of TGF-β receptors and ligands.

To study the function of different TGF-β ligands and Smads, several knockout mouse models were developed. These studies uncovered that TGF-β2 null mice have several developmental defects such as cardiac, spinal and pulmonary defects (245). These mice were also defective in processes such as ECM production, EMT and cell proliferation (245). TGF-β3 null mice also showed defects in EMT and abnormal lung development (246). Several knockout models of Smad proteins also have been developed which provide more insights into the function of these proteins. Smad2 and Smad4 null mice died early in the embryonic stage due to several developmental defects in gastrulation and anterior-posterior axis formation (247-250). Surprisingly, Smad3

knockout mice survived between 1-10 months after birth and then died due to the impaired mucosal immune response, wound healing, and skeletal development (251-253). Smad5 null mice also showed defects in angiogenesis and died at embryonic stage (254).

Multiple mouse models also have been developed to study mammary gland development and cancer. Transgenic mice expressing an active form of TGF-β in mammary epithelium, mouse mammary tumor virus (MMTV)-Tgf-$\beta1^{223/225}$, showed a hypoplastic mammary gland (255). In another study, mice expressing a dominant-negative form of TβIIR also showed increased ductal extension through the end bud (256). These data suggest that TGF-β inhibits ductal growth and side branching during mammary gland development.

Several studies have confirmed that TGF-β acts as a tumor suppressor in the mammary gland. For instance, in mice expressing MMTV-Tgfβ$1^{223/225}$, this active form of TGF-β inhibited mammary tumor formation in 7-12-dimethylbenz (a) anthracene (DMBA)-treated mice compared to DMBA-treated wild type controls (257). In contrast, the dominant negative form of TβIIR in mouse mammary epithelium (MMTV-DNIIR) increased the rate and number of tumor formation after treatment with the DMBA (258). Furthermore, Mice expressing the active form of TGF-β under control of whey acidic protein promoter (WAP-Tgfβ$1^{223/225}$) introduced with MMTV during their pregnancy, showed a decrease in the rate of tumor formation compared to control mice (256).

In addition to studies supporting the role of TGF-β as a tumor suppressor, there is evidence that TGF-β also acts as a cancer promoter. One such evidence was seen in transgenic mice expressing an activated TGF-β receptor in mammary gland (MMTV-

TβRI^{AAV}). These modifications inhibited Neu-induced tumorigenesis but increased lung metastasis (259). Conversely, mice with the activated ligand under control of MMTV promoter (MMTV-Tgfβ1$^{223/225}$) did not affect tumor latency but enhanced tumor invasiveness and metastasis to lungs (260).

1.3 Unique connection between LxCxE binding cleft and TGF-β

As mentioned earlier, MEFs isolated from *Rb1*$^{\Delta L/\Delta L}$ mice have the ability to maintain proliferative control in asynchronous growing cultures, in response to serum starvation and confluence arrest (99). Furthermore, these mice are viable, fertile and able to develop relatively normally but they show defects in mammary gland development characterized by hyperplasia (261). The pups from female *Rb1*$^{\Delta L/\Delta L}$ animals were not nursed regularly resulting in the neonatal lethality of animals raised by *Rb1*$^{\Delta L/\Delta L}$ mothers. There was no defect in milk production, but milk was not ejected properly (261). Interestingly, the mice hemizygous for *tgf-β1* or mice expressing a dominant-negative TβIIR show excessive ductal proliferation (262, 263). Furthermore, dominant-negative TβIIR mice also display a nursing defect (264).

Since *Rb1*$^{\Delta L}$ mice and mice defective for TGF-β1 signaling within the mammary epithelium have similar phenotype, this encouraged our lab to look at the ability of *Rb1*$^{\Delta L/\Delta L}$ cells to respond to a TGF-β1 mediated growth inhibition. Our lab showed that epithelial cells from *Rb1*$^{\Delta L/\Delta L}$ mice do not respond to TGF-β1 mediated growth arrest, suggesting that the pRB-LxCxE interaction is necessary for TGF-β1-induced growth arrest. Our lab also showed that TGF-β1 is able to induce the dephosphorylation and

activation of mutant pRB and the defect in growth inhibition is downstream of pRB activation (261). Our lab also showed that *Rb1*$^{\Delta L/\Delta L}$ mice are not able to repress E2F target genes in response to TGF-β1 (261). This work suggested that the pRB-LxCxE interaction plays a unique role in TGF-β1 growth inhibition.

In order to investigate the role of E2F transcription in TGF-β induced growth arrest, our lab generated another knock-in mouse, called *Rb1*$^{\Delta G}$. This mouse carries a mutation that disrupts the interaction between pRB and E2F but its ability to bind to LxCxE proteins is intact. *Rb1*$^{\Delta G}$ mice showed the same hyperplasic phenotype as the *Rb1*$^{\Delta L}$ mutants and they were defective in response to TGF-β1 mediated growth arrest. This confirms that E2F regulation by pRB is a requirement in TGF-β induced growth arrest**.** Taken together, these data show that a repressor complex including pRB, one or more LxCxE motif containing co-repressor(s) and an E2F transcription factor is involved in TGF-β growth inhibition. Since the exact LxCxE interacting protein(s) that cooperate with pRB in TGF-β growth arrest paradigm is unclear, it is logical to search for LxCxE motif containing proteins that cooperate with pRB in E2F transcriptional repression in response to TGF-β to understand how TGF-β inhibits cell proliferation.

To identify candidates that bind to pRB through the LxCxE binding cleft, our lab performed a Glutathione S-transferase (GST) pull down screen. In order to perform GST pull-downs, nuclear extracts from HeLa cells were mixed with GST-RB^{WT} and GST-RB$^{\Delta L}$ proteins. Bound proteins were resolved by SDS-PAGE and silver stained. The data showed that numerous proteins were missing from GST-RB$^{\Delta L}$. These bands were subjected to Mass Spectrometry and western blotting analysis identified some of these proteins. Taken together, pRB could interact with four major complexes including

anaphase-promoting complex (APC), Sin3, NuRD and CtBP through the LxCxE binding cleft (99, 265). Since APC regulates proliferation independently of E2F, we did not include this complex in our research (265). All components of three potential complexes are shown in Table 1-1 (266-268, 135).

1.4 Thesis hypothesis and objectives

The majority of proteins with LxCxE motif have been reported to bind to pRB before RNAi technology and as a result, there is no evidence of the requirement of LxCxE motif containing proteins in cell cycle arrest under physiological conditions. Thus, we are searching for proteins reported to bind to pRB through the LxCxE binding cleft but lack verification in a physiological setting. It is interesting to mention that whether these proteins directly or indirectly bind to the LxCxE binding cleft is unclear.

Based on the evidence from literature and experiments done in our lab, **the hypothesis of this study is that pRB interacts with specific repressor complexes through the LxCxE binding cleft to repress E2F dependent transcription in response to TGF-β and this function is important for cancer suppression.**

Objective: Identification of proteins cooperating with pRB in E2F transcriptional repression in response to TGF-β

To identify proteins cooperating with pRB in response to TGF-β, lentiviral vectors carrying short hairpin RNA (shRNA) were used. Using these shRNAs, we depleted the expression of each component and examined if depletion of these components compromises TGF-β mediated growth arrest. This experimental approach

will identify complexes such as Sin3, NuRD and CtBP, which cooperate with pRB in E2F transcriptional repression in response to TGF-β.

2 Materials and Methods

2.1 Cell lines and cultures

MCF-10A cells were a generous gift from Gabriel Dimattia lab (Western University). The original MCF-10 cell line was isolated from mastectomy performed on a 36-year-old woman (269). From the original diploid mortal cell, MCF-10A cells, a spontaneously immortal subline of MCF-10, have been derived after extending trypsin passages in the normal calcium levels (1.05 mM). MCF-10A cells have characteristic features of normal mammary epithelial cells and are sensitive to TGF-β mediated growth arrest (269).

MCF-10A cells were grown in Dulbecco's modified Eagle's Medium-Ham`s F12 (DMEM-F12) (Invitrogen, Cat # 11320-033). DMEM-F12 was supplemented with 20 ng/ml Epidermal Growth Factor (EGF) (Sigma, Cat # E9644), 5% horse serum (Gibco, Cat# 26050-088), 0.5 mg/ml hydrocortisone (Sigma, Cat # H4001), 10 g/ml insulin (Sigma, Cat # I6634), 100 ng/ml cholera toxin (Sigma, Cat# C8052) and 1% penicillin/streptomycin (Gibco, Cat #15070-063) at 37°C in a 5% CO_2 humidified incubator.

Routine passaging of these cells involved aspirating the growth medium and washing the cells with Phosphate Buffered Saline (PBS) (Sigma, Cat # P3813), adding trypsin solution and incubating them in a 5% CO_2 humidified incubator at 37°C. Once cells were dislodged by gently tapping and trypsin incubation, growth medium was added

to neutralize the trypsin. The cells were then spun down in a 15 ml conical tube and were split in 1:5 ratios.

2.2 ShRNA transfection

HEK 293T cells were a generous gift form Joe Mymryk lab (Western University). HEK 293T cells were plated at a density of 7×10^5 cells in 5 ml of DMEM + 10% FBS without antibiotics (no penicillin/streptomycin) media in a 6 cm plate the day before the transfection. The following morning, the media was changed to a fresh media without antibiotics and in the late afternoon, cells were transfected with lentiviral shRNA plasmids. To transfect the cells, 6 µg shRNA plasmid (Open Biosystems), 3 µg psPAX2 packaging plasmid, 3 µg pMD2.G envelope plasmid (Addgene #12259 and 12260) and up to 20 µl serum-free OPTI-MEM media (Gibco, Cat # 31985-062) was added to a microcentrifuge tube. In another tube, 74 µl serum-free OPTI-MEM and 6 µl FuGENE transfection reagent (Roche Applied Biosciences) was added, mixed and incubated at room temperature. After 5 minutes of incubation, 80 µl FuGENE master mix from the second tube was added to the first tube to make a total 100 µl master mix and then incubated at room temperature. After 15 minutes, the mix was added dropwise to the plate and the cells were incubated at 37°C in a 5% CO_2 incubator for 15 hours. The following morning, the media was replaced with 3 ml fresh DMEM + 10% FBS + penicillin/streptomycin and incubated for 48 hours to produce lentiviral particles.

Table 2-1 List of the shRNAs used in knockdown experiments (Open Biosystems)

Target gene	Accessions	Clone ID	Antisense Sequence	Vector
RB1 (shRB#1)	NM_000321	TRCN0000040163	TTTGGACTAGAAATAATGTGG	pLKO.1
RB1 (shRB#2)	NM_000321	TRCN0000040164	TTGCAGTAGAATTTACACGCG	pLKO.1
RB1 (shRB#3)	NM_000321	TRCN0000040165	TTCACAAAGTGTATTTAGCCG	pLKO.1
SAP30	NM_003864	TRCN0000021687	AACACCACTATCAACCTTGAG	pLKO.1
MTA2	NM_004739	TRCN0000013374	TATCTGTCTCATTCAAGAGGG	pLKO.1
SAP18	NM_005870	TRCN0000021660	AAACCCAGGGCTGCCTTGGAAAAG	pLKO.1

2.3 Lentiviral Infection

To infect MCF-10A cells with lentiviral particles, the media from HEK 293T cells transfected with lentiviral shRNA was filtered through a 0.45 μm filter after 48 hours transfection and supplemented with 8 μg/mL of Polybrene (Sigma, Cat # H9268) to increase the efficiency of viral infection. The filtered media, which contains lentiviral particles, was directly added to MCF-10A cells that had been plated the previous day at density of 3×10^5 cells in a 6 cm dish. MCF-10A cells were incubated for 24 hours in the incubator. Next morning, the media was replaced with a fresh media. After 24 hours, the media from MCF-10A cells was removed and puromycin was added to the cells at a final concentration of 1.6 μg/ml. The culture was replaced with media containing puromycin every other day. One uninfected plate of cells in parallel was treated with puromycin to serve as a positive control for the puromycin selection. Infected MCF-10A cells were then expanded for further experiments.

2.4 TGF-β1 preparation and treatment

To make TGF-β1 (R&D systems, Cat # 240-B-010) stock solution, 30% acetonitrile/0.1% trifluoroacetic acid (TFA) solution was made and filtered through a 0.22 μm filter. TGF-β1 powder was diluted with the above solution to a 0.1 μM stock concentration and aliquoted to several microcentrifuge tubes. Filled tubes were placed at -20°C overnight, and then transferred to -80°C for long-term storage.

TGF-β1 was used at a final concentration of 100 pM. The stock was then stored at 4°C after usage and used within a few weeks.

2.5 5-bromo-2-deoxyuridine (BrdU)-propidium iodide (PI) staining

MCF-10A cells were labeled with BrdU and PI as previously described (270). 1 µl of Cell Proliferation Labeling Reagent (BrdU) (GE Healthcare, Cat # RPN201) was added for each ml of cell culture medium (1 to 1000 dilutions) and incubated at 37°C. After 4 hours of incubation, the cells were washed with PBS and trypsin was added to dislodge the cells. Once MCF-10A cells dislodged, they were transferred to a 15 ml conical tube and spun down at 500 g for 5 minutes. The cells were washed with PBS one more time and then resuspended in 100 µl PBS. Then, 1 ml of 95% EtOH was added dropwise while vortexing to fix the cells and incubated for 30 minutes in 4°C. After fixing the cells, EtOH was removed and 1 ml of 2N HCl/0.5% Tx-100 was added in a dropwise fashion while vortexing and the cells were incubated at room temperature for 30 minutes. To neutralize HCl, 1 ml of 0.1 M NaB_4O_7 (pH 8.5) was gently added after removing HCl from cell pellet. After 30 minutes incubation at room temperature, the cell pellet was resuspended in 0.5 ml of antibody solution (PBS containing 0.2% Tween-20 and 1% BSA) with mouse anti-BrdU antibodies (BD Biosciences, 347580) diluted 1 to 25 and incubated at room temperature for 30 minutes in dark. The cells were pelleted and resuspended in 50 µl of antibody solution containing rabbit anti-mouse secondary antibodies conjugated to fluorescein isothiocyanate (FITC) (Vector Laboratories, Cat # FI-2000) diluted 1 to 10 and incubated for 30 minutes at room temperature in dark.

Finally, cells were resuspended in 0.5 ml of PI and RNase A solution (PBS with 1% BSA, 1 mg/ml PI, 0.25 mg/ml RNase A) and incubated in dark at 37°C for 30

minutes. The solution was passed through a cell strainer to remove cell clumps before running the samples on flow cytometer.

2.6 Flow cytometry analysis

After staining MCF-10A cells with PI and BrdU, cell populations were analyzed by flow cytometry on a Beckman-Coulter EPICS XL-MCL instrument, which is capable of detecting PI and FITC. The sensitivity of photomultiplier tubes for PI and FITC was adjusted such that 2N and 4N peaks are centered at 200 and 400 (arbitrary units) on the X-axis for PI plot and BrdU positive cells are approximately 10 times brighter for FITC plot. Finally, 5000 to 10000 single cell events were collected for each sample in order to ensure that results obtained are representing the cells cultured in each plate.

2.7 Preparation of nuclear extract from cells

To generate nuclear extracts, MCF-10A cells were washed twice with PBS and collected into 1 ml of PBS with cell scrapers. Collected cells were centrifuged at 200 g for 5 minutes at 4°C and supernatant was removed. The pellet was resuspended in 200 µl of Hypotonic Lysis Buffer (HLB) (10 mM Tris pH 7.5, 10 mM KCl, 3 mM $MgCl_2$ and 1 mM EDTA). HLB was supplemented with the protease inhibitors cocktail (1 mM DTT, 1 mM PMSF, 5 mM NaF, 0.1 mM Na_3VO_4, 5 µg/ml Leupeptin and 5 µg/ml Aprotinin) and incubated on ice for 5 minutes. NP-40 was added to each sample for a final concentration of 0.05% and incubated for 5 minutes on ice. The lysate was centrifuged at 1800 g for 10 minutes, supernatant was then removed and 200 µl HLB supplemented with protease inhibitors cocktail and 0.05% NP-40 was added to pellet and resuspended lysate was

incubated for 10 minutes on ice. This was repeated three more times. Then, the pellet was resuspended in 100 µl Gel Shift Extract (GSE) buffer (20 mM Tris pH 7.5, 420 mM NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA and 25% Glycerol) supplemented with the protease inhibitors cocktail (25 mM DTT, 0.5 mM NaF, 0.1 mM Na_3VO_4, 0.1% NP-40, 5 µg/ml Leupeptin and 5 µg/ml Aprotinin). Extracts were frozen and kept at -80°C. Finally, extracts were thawed and cellular debris was removed by centrifugation at 21000 g for 20 minutes when they were used for protein concentration measurement.

2.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

SDS-polyacrylamide gels were prepared in mini gel. Resolving gel mix was prepared according to the volume required for making 10% SDS-polyacrylamide gels, added in the gel apparatus and EtOH was poured to the top. Clear distinction between EtOH and gel indicates the gel has dried. EtOH was poured out, and immediately stacking gel was poured in, and the comb was inserted and allowed to solidify at room temperature. 30-50 µg protein samples were mixed with 5× sample buffer (0.225 M Tris-HCl pH 6.8, 5% SDS, 50% Glycerol, 0.05% bromophenol blue and 0.25 M DTT) and denatured by heating at 95°C for 5 minutes, glass plates (with solidified gel) were locked into the gel electrophoresis cassette and the electrophoresis cassette was placed into SDS-PAGE apparatus. The inner chamber was filled with 1× SDS-PAGE running buffer and the outer chamber was filled half way with the same buffer. Protein samples were loaded into the wells and electrophoresed at constant current at 120 V for 1.5 hours.

SDS-PAGE gel was transferred onto nitrocellulose membrane at 500 mA for 60 minutes at 4°C. The transferred membrane was incubated in a blocking solution (3% fat free milk in 1×TBS-Tween (200 mM Tris pH 7.5, 1.5 M NaCl, 3% Tween-20)) for one hour at room temperature while shaking. Membrane was directly incubated in primary antibodies overnight at 4°C while shaking. After incubation, the membrane was washed in 1X TBS-Tween for 10 minutes for three times. Then, the membrane was incubated in horseradish peroxidase (HRP) conjugated secondary antibodies against mouse or rabbit antibody for one hour at 25°C and then washed in 1 X Tris-buffered saline (TBS)-Tween for two times and 5 minutes each. Proteins were visualized with chemiluminescent detection method.

Table 2-2 List of the antibodies used for western blotting and staining

Antibody Name	**Protein Recognized**	**Host Species**	**Company**	**Dilution ratio**
sc-6200	Smad2	Goat	Santa Cruz	1:500
AB3849	Phospho-specific Smad2 (Ser465/467)	Rabbit	Chemicon	1:700
347580	BrdU	Mouse	BD-Bioscience	1:25

2.9 RNA isolation

Total RNA was isolated using GenElute^{TM} Mammalian total RNA miniprep kit (Sigma, Cat # RTN70-1KT). Before beginning the procedure, the lysis solution/2-mercaptoethanol (2-ME) mixture was prepared by adding 10 µl of 2-ME to each ml of lysis solution that was sufficient for the same day use. In addition, the concentrate wash solution 2 was diluted with 100% EtOH in ratio 1:4 (1 part concentrate wash solution 2: 4 parts 100% EtOH).

To extract RNA from MCF-10A cells plated in a 6 cm plate, cells were washed with PBS twice and then PBS was removed completely. To lyse cells, 250 µl lysis solution/ 2-ME mixture was directly added to the plate and incubated for 3 minutes at room temperature. Then, the plate was titled to collect the lysate. The lysed cells were transferred into a GenElute Filtration Column (blue insert with a 2ml receiving tube) and centrifuged at maximum 12000 g for 2 minutes to remove cellular debris and shears DNA. Then, the filtration column was discarded and the receiving tube was kept. An equal volume of 70% EtOH was added and mixed with the filtered lysate by vortexing. The lysate/EtOH mixture was loaded into GenElute Binding Column (colorless insert with a red o-ring seated in a 2 ml receiving tube) and centrifuged at 12000g for 15 seconds. The flow through liquid was discarded but the collection tube was retained. Then, 500 µl of wash solution 1 was added into the column and centrifuged at 12000 g for 15 seconds. The binding column was transferred into a fresh 2 ml collection tube and flow-through liquid and the original collection tube were discarded. 500 µl of diluted wash solution 2 was loaded into the column and centrifuged at 12000 g for 15 seconds.

The flow-through liquid was discarded but the collection tube was retained. This step was repeated but the column was centrifuged for three more minutes to dry. Finally, the binding column was transferred to a fresh 2 ml collection tube and 50 µl elution solution was then loaded into the binding column and centrifuged at 12000 g for one minute. The quality and quantity of RNA were evaluated by measuring OD 260/280 and 260/230 ratio. Purified RNA in the collection tube was stored at -80°C.

2.10 Generation of total cDNA

The total complementary DNA (cDNA) was generated using SuperScript® III Reverse Transcriptase kit (Invitrogen, Cat # 18080-044). 1 µg RNA and up to a total volume of 10 µl RNase free H_2O was added to an RNase free PCR tube. 1 µl DNase I and 1 µl 10× DNase I reaction buffer was added to the PCR tube and the sample was incubated at room temperature for 15 minutes to degrade all genomic DNA. The reaction was stopped by adding 1 µl 25 mM EDTA, 1 µl of 10 mM dNTP mixture and incubating at $70^{\circ C}$ for 5 minutes. Then, the reaction mixture was prepared in a total volume of 9 µl by adding 4 µl of 5× First Strand buffer, 2 µl of 100 mM dithiothreitol (DTT), 1 µl 50µM oligo $(dT)_{20}$, 1 µl RNase OUT™ (40 U/µl) and 1 µl of SuperScript® III Reverse Transcriptase (250 U/µl). In addition to this mixture, one mixture without SuperScript® III Reverse Transcriptase was made to serve as a control. The reaction mixture was gently vortexed and added to the sample. The final mixture was incubated for the following times: 5 minutes at 25°C, 40 minutes at 42°C, 30 min at 50°C, 40 minutes at 55°C and finally 15 minutes at 70°C. The generated cDNA was diluted in RNase free H_2O for downstream application.

2.11 Real-Time PCR

Real-time PCR was performed by the fluorescent dye SYBR Green methodology using the iQ™ SYBR® Green Supermix (Bio-Rad, Cat # 170-8880). To perform Real-Time PCR, the following master mix was prepared for each reaction: 5 µl iQ™ SYBR® Green Supermix, 1 µl forward primer, 1 µl reverse primer and up to 8 µl H_2O. 2µl of cDNA prepared by SuperScript® III Reverse Transcriptase system was added to each master mix. Finally, the samples were run on a CFX96 Touch™ real-time PCR detection system (Bio-Rad). Each reaction included GAPDH as a reference gene for normalization, and reactions lacking cDNA served as negative controls. The primers used in Real-Time PCR and RT-PCR are in the following table. (Table 2-3)

To calculate the gene expression, C_t values for all genes were acquired in all samples. To normalize the expression of each target genes, the difference between GAPDH and target gene C_t values was calculated for each sample. Then, the relative expression of each target gene was calculated using the following formula in each sample: $2^{(C_t \text{GAPDH} - C_t \text{target gene})}$. Finally, to determine the fold change expression of the target gene, the relative expression of the target gene in each sample was divided by its relative expression in the calibrator sample (Untreated shLuc in our case).

Table 2-3 List of the primers used for Real-Time PCR

Gene	Forward Primer	Reverse Primer
RB1	GCATGGCTCTCAGATTCACCT	CTTCTGGGTCTGGAAGGCTG
SAP30	AAGAGCGCAAGGCATCTTTA	GTCCTGGTCTGGTTGGTAGC
MTA2	TGGTTAGACGGATTGAGGAGC	GCGCCGGAAAAGACAGACA
SMAD7	TTCCTCCGCTGAAACAGGG	CCTCCCAGTATGCCACCAC
GAPDH	GCCGCATCTTCTTTTGCGTC	GATCTCGCTCCTGGAAGATGG
PCNA	TCCTGTGCAAAAGACGGAGT	TCTACAACAAGGGGTACATCTGC
CCNE1	GCCAGCCTTGGGACAATAATG	AGTTTGGGTAAACCCGGTCAT
CCNA2	CCTGGACCCAGAAAACCATTG	ATTTAACCTCCATTTCCCTAAGGT
SAP18	CCTCGCGAGAGACTTAGTGC	AAGACCCGTAGCAACAGTGG

2.12 Statistical analysis

Each experiment was repeated three times. Values are shown as the mean ± standard deviation (SD) of triplicate measurements. Student's paired *t*-test was used to analyze differences between the sample of interest and its control. A *p* value of less than 0.05 was considered statistically significant.

3 Results

3.1 Identification of proteins cooperating with pRB in E2F transcriptional repression in response to TGF-β

3.1.1 Rationale

As mentioned earlier, our experiments using the *Rb1*$^{\Delta L}$ mouse model uncovered that the LxCxE-pRB interaction plays an essential role in TGF-β growth inhibition. The exact LxCxE interacting protein(s) that cooperate with pRB in TGF-β growth arrest paradigm is unclear. Therefore, it is logical to search for LxCxE motif containing proteins that cooperate with pRB in E2F transcriptional repression in response to TGF-β to understand how TGF-β inhibits cell proliferation.

Previous experiments in our lab showed that four complexes including APC, Sin3, NuRD and CtBP interact with pRB in an LxCxE dependent manner (99, 265). Lentiviral vectors carrying short hairpin RNA (shRNA) was used to deplete the expression of components of each complex in order to identify components cooperating with pRB in E2F transcriptional repression in response to TGF-β.

A difficulty in this screen is that some components have different isoforms such as Sin3A/B, Mi2α/β that share functional and structural similarities, so their function in cooperation with pRB may be redundant. In addition, some components are common between different complexes such as HDAC1/2, RbAp46/78 and lysine specific

demethylase 1 (LSD1). This makes them less desirable to start the screening. Therefore, the screening was started with some unique components of these complexes.

3.1.2 TGF-β growth arrest is pRB dependent in MCF-10A cells

Previous experiments in our lab showed that MEFs from LxCxE mutant mice were unresponsive to TGF-β mediated growth arrest. Our lab also analyzed TGF-β growth control in primary mouse epithelial cells (MECs) and keratinocytes, which are more sensitive to TGF-β mediated cell cycle arrest. Both cell types isolated from LxCxE mutant mice showed the same defect in response to TGF-β mediated growth inhibition (261). Isolation of MECs or keratinocytes requires many mice, therefore, MCF-10A cells, a normal human mammary epithelial cell, were used for the growth arrest experiments.

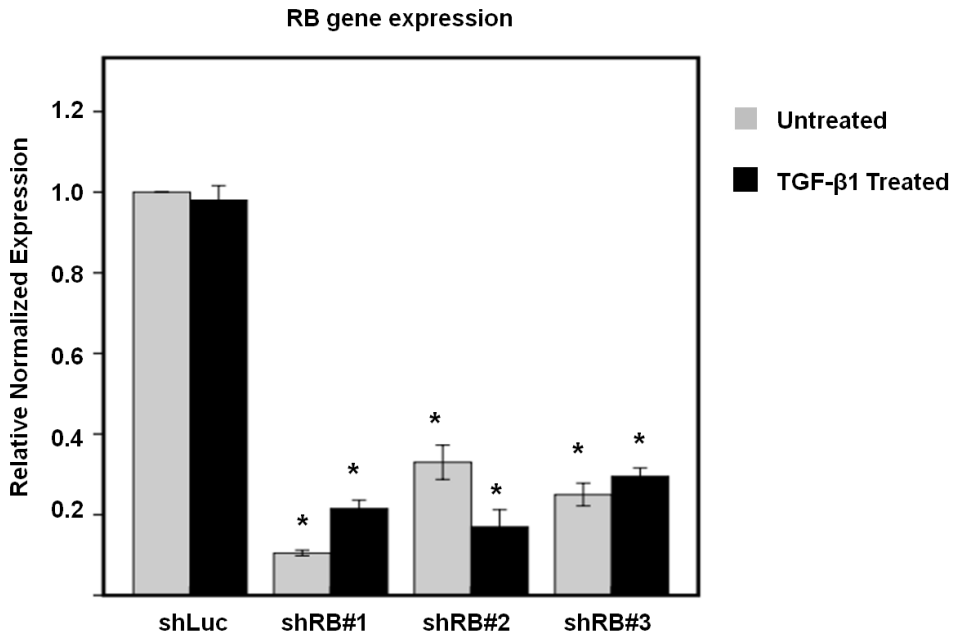
TGF-β1 mediated growth inhibition in mouse fibroblast requires pRB (271); therefore, we wanted to ensure that TGF-β1-induced growth arrest is pRB dependent in MCF-10A cells. To do this, 3×10^5 MCF-10A cells were plated in 6 cm plates in duplicate. Then, these cells were infected with lentiviral vector carrying shRNA to deplete the expression of pRB along with shRNA expressing luciferase (shLuc) as a control. After infection and three days of selection with puromycin, the infected cells were expanded for further experiments.

To confirm knockdown efficiency, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for each shRNA and treated one plate of each shRNA with TGF-β1 (100 pM) for 24 hours. After treatment with TGF-β1, RNA was isolated and converted to cDNA and Real-Time PCR was performed for pRB to confirm knockdown

efficiency. As shown in Figure 3-1A, the level of pRB transcript was significantly reduced in MCF-10A cells.

To perform growth arrest assays, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for each shRNA. TGF-β1 (100 pM) was added to one plate of each shRNA. After 24 hours TGF-β1 treatment, the cells were pulse-labeled with BrdU for 4 hours. Then, the percentage of cells with incorporated BrdU was quantified by flow cytometry. As shown in Figure 3-1B, MCF-10A cells expressing shLuc showed a significant decrease in BrdU incorporation in response to TGF-β1 whereas pRB depleted cells did not respond to TGF-β1-induced growth arrest. This suggests that depletion of pRB compromises TGF-β-induced growth arrest. To avoid off-target effects, the same experiment was repeated with two more shRNAs targeting pRB and they all showed the same results. In conclusion, these results show that TGF-β1-induced growth arrest is pRB dependent in MCF-10A cells, because multiple shRNAs against pRB can interrupt TGF-β1-induced growth arrest in these cells.

A


RB gene expression
Relative Normalized Expression
1.2
1.0
0.8
0.6
0.4
0.2
shLuc
shRB#1
shRB#2
shRB#3
Untreated
TGF-β1 Treated

B

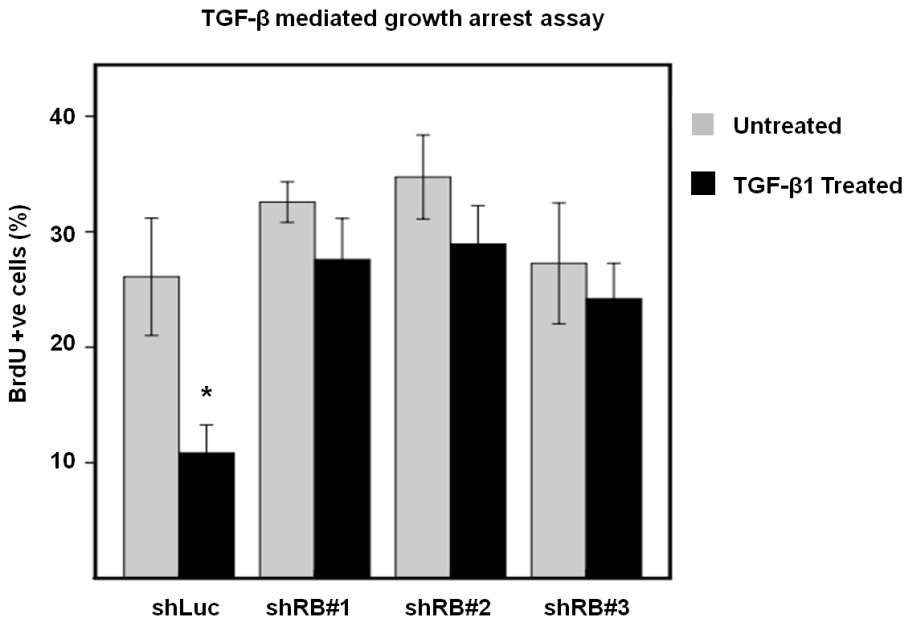

TGF-β mediated growth arrest assay
BrdU +ve cells (%)
40
30
20
10
shLuc
shRB#1
shRB#2
shRB#3
Untreated
TGF-β1 Treated

Figure 3-1 TGF-β1-induced growth arrest is pRB dependent in MCF-10A cells

(A) MCF-10A cells were infected with shRNA to deplete the expression of pRB. After infection and three days selection, the infected cells were treated with TGF-β1 for 24 hours. The mRNA level of pRB was measured by Real-Time PCR to verify knockdown efficiency. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance. (B) The depleted cells were treated with TGF-β1 for 24 hours and pulse-labeled with BrdU for 4 hours. The percentage of cells incorporating BrdU was measured by flow cytometry. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference from untreated shLuc (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean. (+ve, positive)

3.1.3 E2F target genes are deregulated upon pRB depletion in response to TGF-β

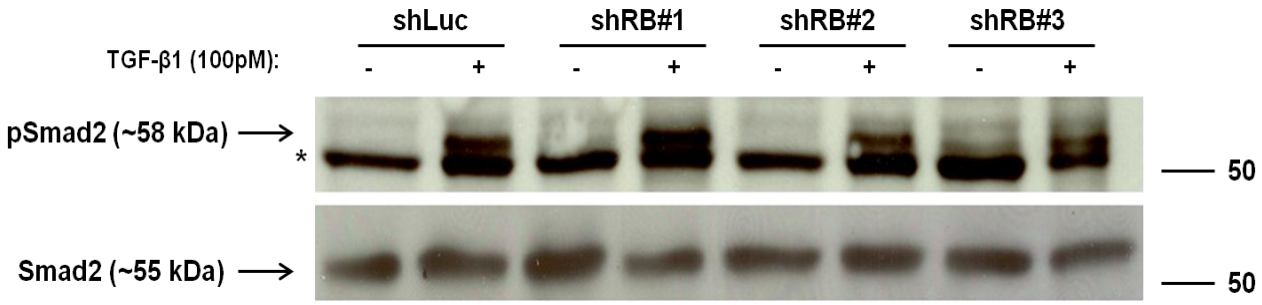
A recent study has shown that the TGF-β signaling pathway is regulated by multiple microRNAs (miRNAs) (272). A large-scale RNAi screen was performed to identify novel components and modulators of the TGF-β pathway by looking at the nuclear translocation of a green fluorescent protein (GFP)-Smad2 fusion protein. It was identified that 176 siRNAs inhibit nuclear localization of Smad2 in response to TGF-β. However, after finding genes correlating to each siRNA hit, they were not able to group these genes in any relevant biological process nor were any of these genes relevant to any genes involved in the TGF-β pathway. Further analysis revealed that these selected hits would significantly reduce mRNA levels of TGF-β receptors (TβRs), particularly TβIIR through miRNA off-target effects. It has been demonstrated that complementarity between a heptamer or hexamer 'seed' match of siRNA and 3' untranslated region (UTR) of an off-target gene mediates the off-target effects observed in siRNA screens (273, 274). This was confirmed by sequence analysis of siRNA and 3' UTR of TβIIR in their study. Thus, the risk of obtaining misleading results using shRNA in single-assay readout is substantial. Therefore, control experiments are essential in the interpretation of such results.

To ensure that the TGF-β1-induced growth arrest in our knockdown experiments is not a result of TβIIR down regulation or its disability to act on Smads, western blotting was performed to look at phosphorylation of Smad2. To do this, 2.8×10^7 pRB depleted MCF-10A cells were plated in 15 cm plates in duplicate along with shLuc MCF-10A

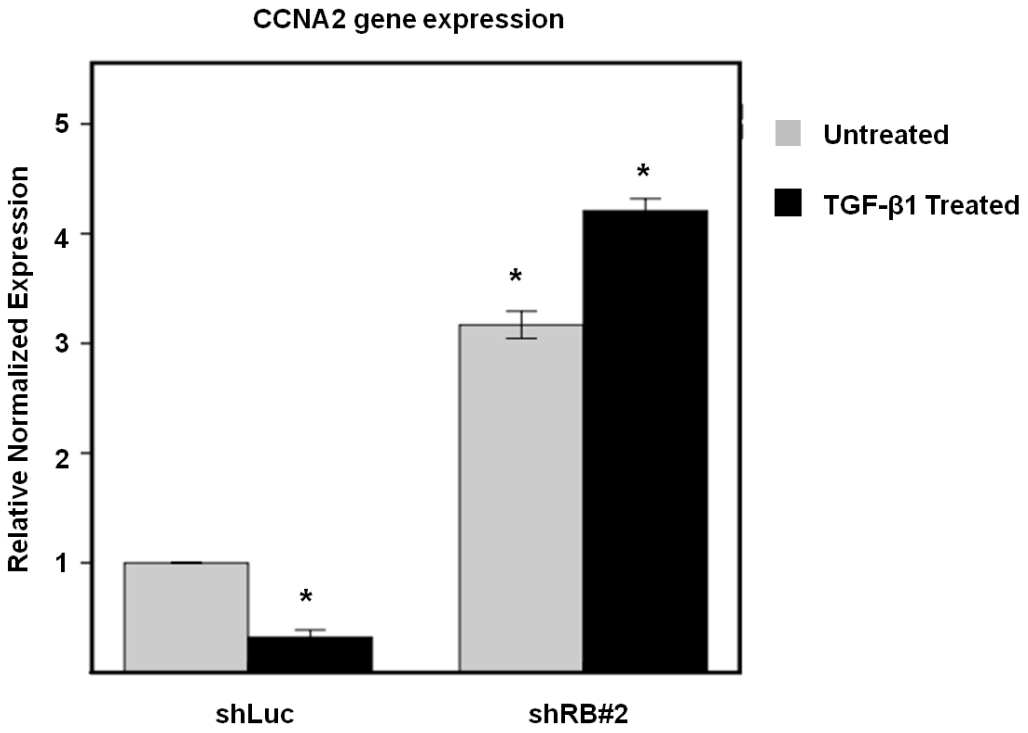
cells and then TGF-β1 (100 pM) was added to one plate of each shRNA for 24 hours. After treatment with TGF-β1, the nuclear extract was isolated and was blotted for phospho-Smad2. As shown in Figure 3-2A, TGF-β1 stimulation of MCF-10A cells resulted in phosphorylation of Smad2 in all pRB knockdown cells similar to control cells suggesting that the expression and activity of TGF-β1 receptor is intact after pRB knockdown by shRNAs.

It has been shown that pRB binds to E2F transcription factors and represses the transcription of E2F responsive genes and loss of pRB results in a deregulation of E2F target genes expression (275). Thus, the transcript levels of E2F targets were examined to investigate if they are repressed in TGF-β1 mediated growth arrest and if this effect is disrupted by pRB depletion. To do this, pRB depleted and shLuc MCF-10A cells were treated with TGF-β1 for 24 hours, RNA was then isolated and converted to cDNA and Real-Time PCR was performed for two E2F responsive genes. As shown in Figure 3-2B, E2F target genes, *CCNE1* and *CCNA2*, are repressed in response to TGF-β1 in shLuc MCF-10A cells and this repression is disrupted after depletion of pRB. Based on these analyses, further experiments were performed to knockdown the expression of components of complexes, which can bind to pRB through the LxCxE binding cleft.

A


shLuc
shRB#1
shRB#2
shRB#3
TGF-β1 (100pM):
- + - + - + - +
pSmad2 (~58 kDa)
*
50
Smad2 (~55 kDa)
50

B


CCNA2 gene expression
Relative Normalized Expression
1
2
3
4
5
*
*
*
Untreated
TGF-β1 Treated
shLuc
shRB#2

C

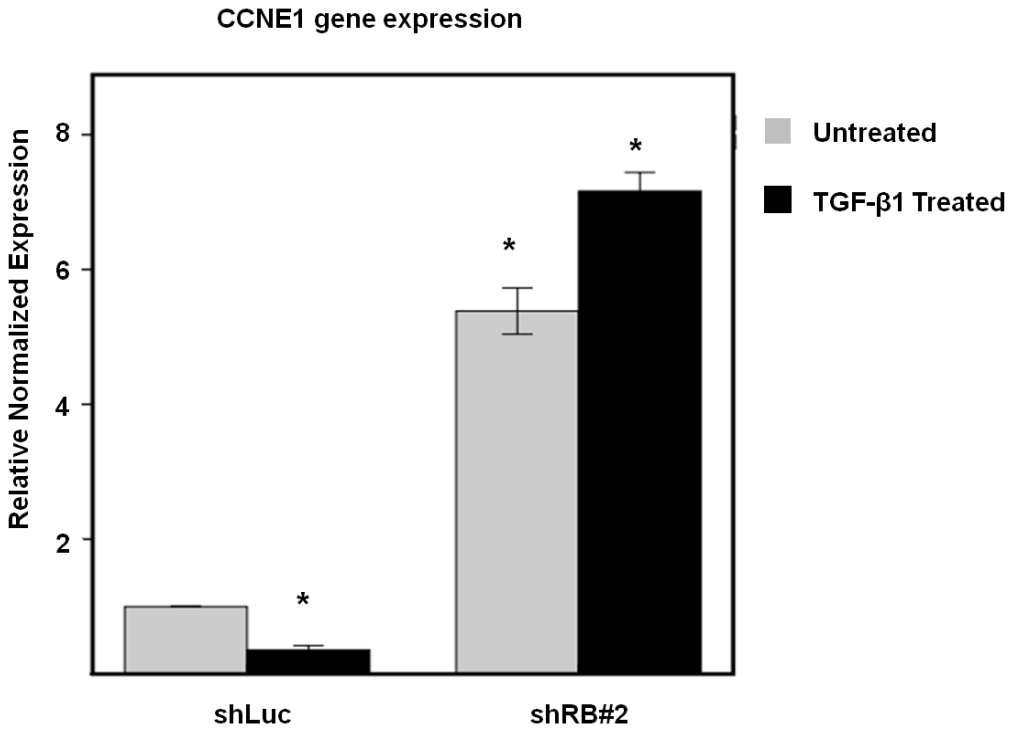


Figure 3-2 The TGF-β signaling is intact and E2F target genes repression is disrupted by pRB depletion

(A) Total phospho-Smad2 expression levels were measured in pRB depleted and shLuc MCF-10A cells by western blot analysis. The upper band is phospho-Smad2 and the asterisk indicates the non-specific lower band. (B-C) Total RNA from MCF-10A cells infected by shLuc and shRB #2 was isolated using GenElute Mammalian Total RNA Purification Kit. To eliminate DNA genomic contamination, RNA was digested by DNAse1 and then cDNA was generated using Superscript III. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference from untreated shLuc (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance.

3.1.4 SAP18, a unique component of the Sin3 complex

SAP18 is another core protein in the Sin3 complex. SAP18 protein directly interacts with both mammalian Sin3 and HDAC1 (116). It has been suggested that SAP18 may have a potential role in stabilizing the HDAC1-Sin3 interaction and enhancing HDAC1 enzymatic activity (116). Furthermore, SAP18 can interact with other proteins of various transcriptional regulatory circuits (116). It has been shown that one of the mechanisms by which E2F-pRB mediates repression of E2F target genes is through the recruitment of HDAC (38). Therefore, SAP18 may have a role in TGF-β1 mediated growth arrest. This motivated us to examine the role of SAP18 in TGF-β1 mediated growth arrest.

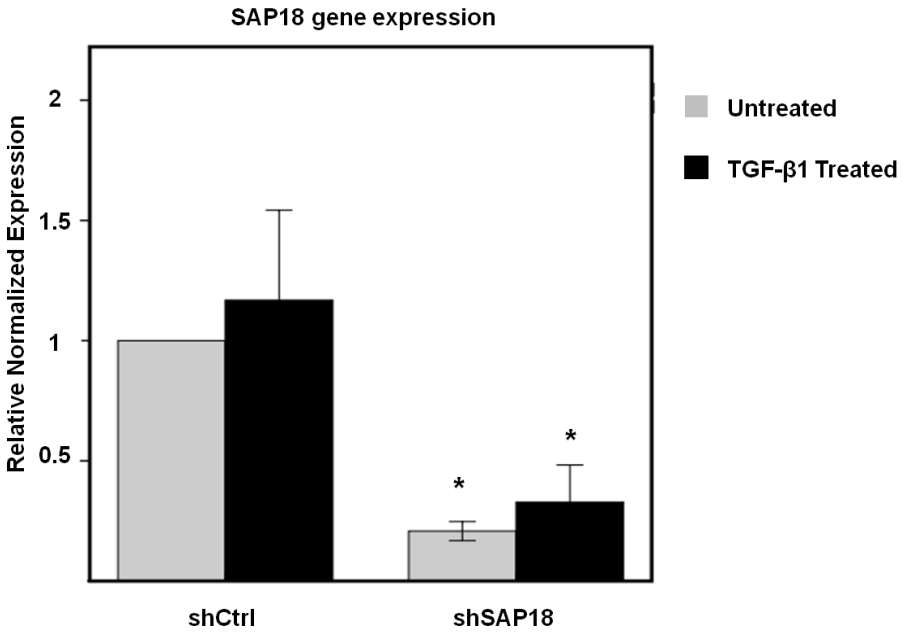
3.1.4.1 SAP18 is not involved in TGF-β1 mediated growth arrest

To examine whether SAP18 is involved in TGF-β1-induced growth arrest, 3×10^5 MCF-10A cells were plated in 6 cm plates in duplicate. Then, these cells were infected with lentiviral vector carrying SAP18 shRNA along with shCtrl as a control. After lentiviral infection and selection with puromycin, the infected cells were expanded for further experiments.

To confirm knockdown efficiency, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for each shRNA. TGF-β1 (100 pM) was added to one plate of each shRNA for 24 hours. RNA was isolated and converted to cDNA and Real-Time PCR was performed for SAP18. As shown in Figure 3-6A, the level of SAP18 transcript was significantly reduced in MCF-10A cells.

To do TGF-β1 growth arrest assay, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for each shRNA and TGF-β1 (100 pM) was added to one plate of each shRNA. After 24 hours TGF-β1 treatment, cells were pulse-labeled with BrdU for 4 hours. Then, flow cytometry was performed to quantify the number of cells incorporating BrdU. As shown in Figure 3-3B, both MCF-10A cells expressing shCtrl and shSAP18 showed a significant decrease in BrdU incorporation in response to TGF-β1. This suggests SAP18 is not involved in TGF-β1-induced growth arrest.

A


SAP18 gene expression
Relative Normalized Expression
2
1.5
1
0.5
*
*
shCtrl
shSAP18
Untreated
TGF-β1 Treated

B

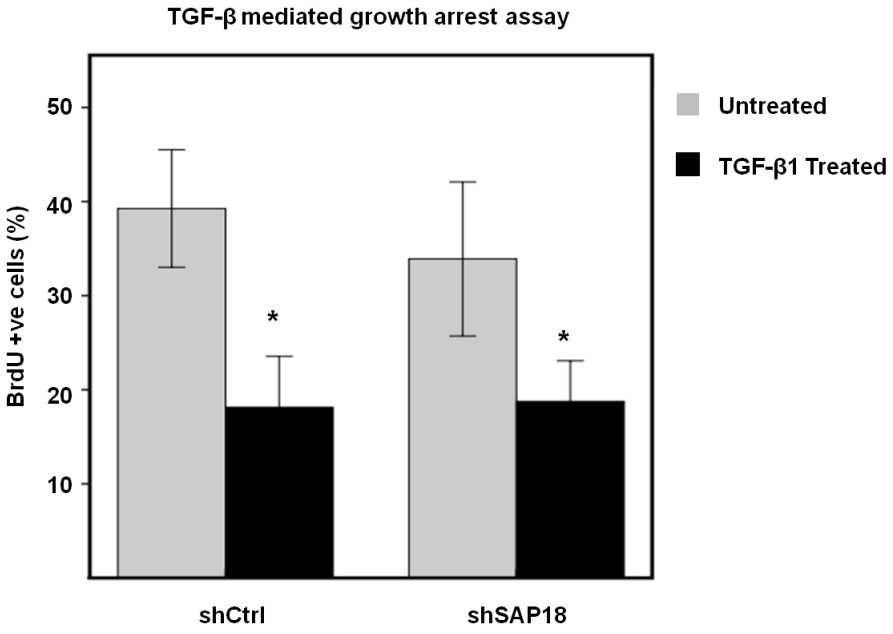

TGF-β mediated growth arrest assay
BrdU +ve cells (%)
50
40
30
20
10
*
*
shCtrl
shSAP18
Untreated
TGF-β1 Treated

Figure 3-3 Depletion of SAP18 does not compromise TGF-β1 mediated growth arrest

(A) MCF-10A cells were infected with shRNA to deplete the expression of SAP18. After infection and three days selection, the infected cells were treated with TGF-β1 for 24 hours, the mRNA level of SAP18 was measured by Real-Time PCR to verify knockdown efficiency. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance. The asterisks indicate a statistically significant difference from untreated shCtrl (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean. (B) The depleted cells were treated with TGF-β1 for 24 hours and pulse-labeled with BrdU for 4 hours. The percentage of cells incorporating BrdU was measured by flow cytometry. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference from untreated samples (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean. (+ve, positive)

3.1.5 SAP30, a potential component involved in TGF-β growth arrest

SAP30 is one of the unique components of Sin3 complex (112). SAP30 directly interacts with HDAC1 and multiple subunits of the Sin3 complex suggesting that SAP30 may play a role in stabilizing the complex (112). It has been demonstrated that pRB can repress transcription of E2F target genes through the recruitment of HDAC, which removes the acetyl groups of positive charge residues from histones on the promoter, thereby promoting the formation of closed nucleosomes that inhibit transcription (51). Therefore, it is logical to look for unique components, which associate with HDACs in potential complexes. Since SAP30 interacts with HDAC1, this motivated us to examine the ability of MCF-10A cells to respond to TGF-β in absence of SAP30.

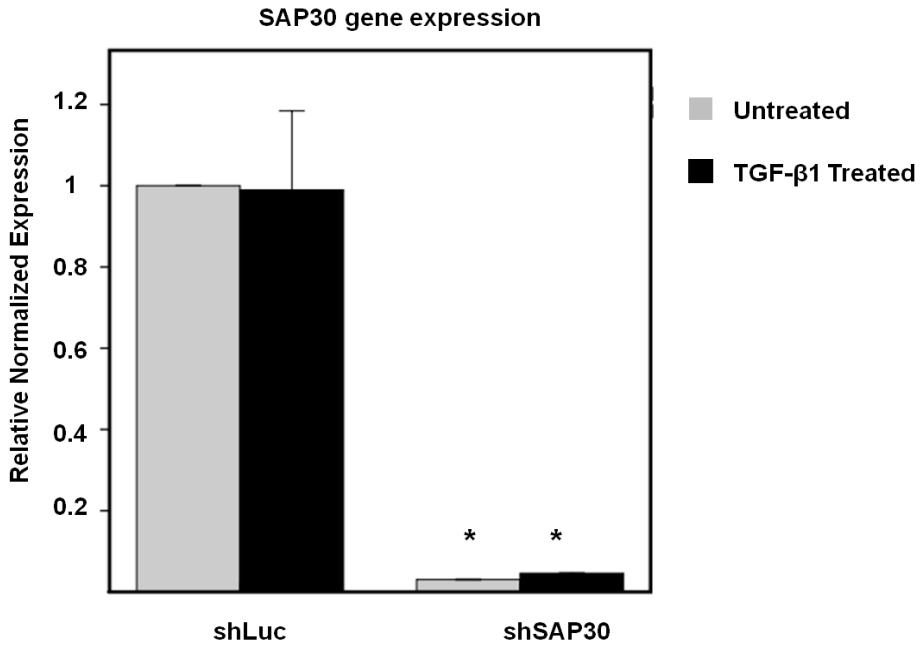
3.1.5.1 Depletion of SAP30 compromises TGF-β growth arrest

To examine whether SAP30 is involved in TGF-β growth inhibition, 3×10^5 MCF-10A cells were plated in 6 cm plates in duplicate. Then, these cells were infected with lentiviral vector carrying shRNA to deplete the expression of SAP30 along with shLuc as a negative control. After infection and three days of selection with puromycin, the infected cells were expanded for TGF-β1-induced growth arrest assays and knockdown efficiency.

To examine knockdown efficiency, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for each shRNA and one plate was treated with TGF-β1 (100 pM) for 24 hours. After treatment with TGF-β1, RNA was isolated and converted to cDNA and Real-Time PCR was performed for SAP30. As shown in Figure 3-4A, the level of SAP30 transcript was significantly reduced in MCF-10A cells.

To examine the role of SAP30 in TGF-β growth inhibition, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for each shRNA and TGF-β1 (100 pM) was added to one plate of each shRNA. After 24 hours TGF-β1 treatment, the cells were pulse-labeled with BrdU for 4 hours and then the percentage of cells incorporating BrdU was quantified by flow cytometry. As shown in Figure 3-4B, MCF-10A cells expressing shLuc showed a significant decrease in BrdU incorporation in response to TGF-β1 whereas the ability of SAP30 depleted cells to induce TGF-β1 growth arrest was reduced. This suggests that depletion of SAP30 compromises TGF-β1-induced growth arrest.

A


SAP30 gene expression
Relative Normalized Expression
1.2
1
0.8
0.6
0.4
0.2
*
*
shLuc
shSAP30
Untreated
TGF-β1 Treated

B

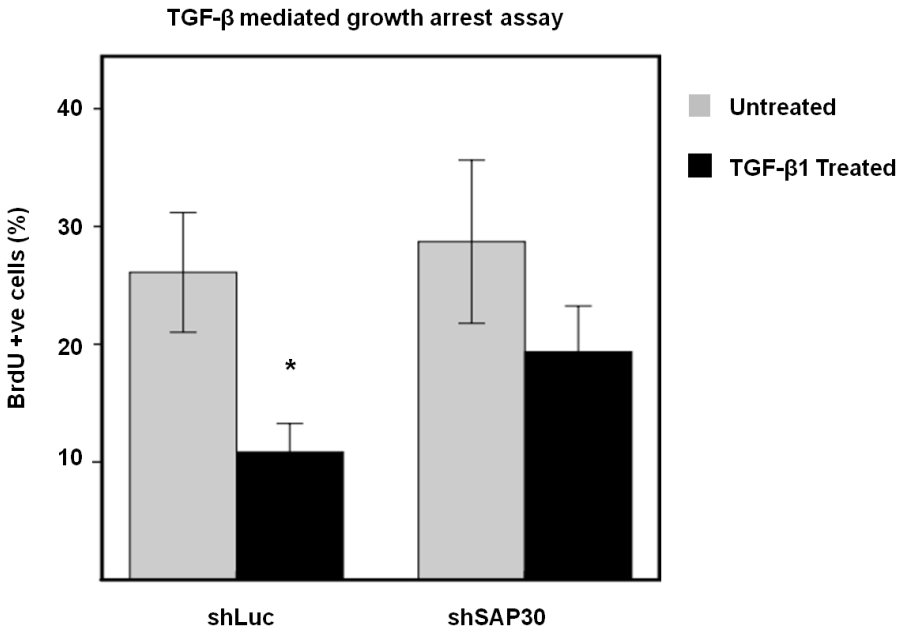

TGF-β mediated growth arrest assay
BrdU +ve cells (%)
40
30
20
10
*
shLuc
shSAP30
Untreated
TGF-β1 Treated

Figure 3-4 Depletion of SAP30 compromises TGF-β1 mediated growth arrest

(A) MCF-10A cells were infected with shRNA to deplete the expression of SAP30. After infection and three days selection, the infected cells were treated with TGF-β1 for 24 hours; the mRNA level of SAP30 was measured by Real-Time PCR to verify knockdown efficiency. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance. (B) The depleted cells were treated with TGF-β1 for 24 hours and pulse-labeled with BrdU for 4 hours. The percentage of cells incorporating BrdU was measured by flow cytometry. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference from untreated shLuc (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean (+ve, positive).

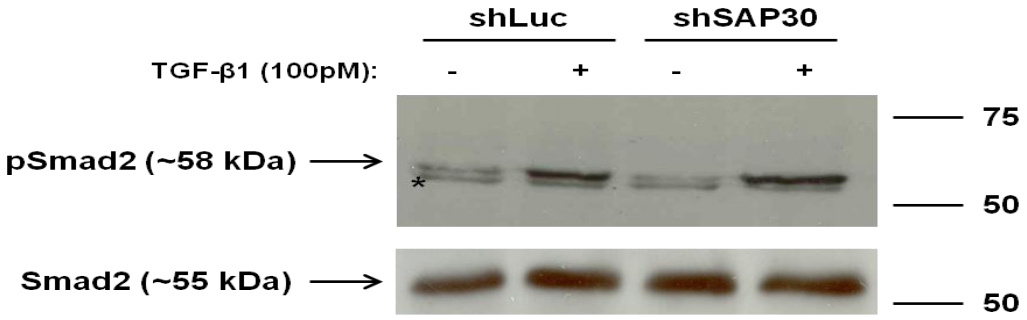
3.1.5.2 TGF-β signaling is intact in absence of SAP30

The mechanism by which SAP30 depletion disrupts TGF-β1-induced growth inhibition was then investigated. In the presence of the TGF-β ligand, the TβIIR receptor phosphorylates the TβIR. This phosphorylation allows TβIR to phosphorylate and activate Smad2/3 proteins. After Smad2/3 activation, they bind to Smad4 proteins and then this complex translocates to the nucleus. Once in the nucleus, this complex binds to specific regions of the genome along with other co-repressors or co-activators to regulate gene transcription, which ultimately leads to G1 growth arrest (146). To determine whether SAP30 is involved in TGF-β1-induced growth inhibition and to ensure that shRNA targeting SAP30 transcript is not downregulating TβIIR level leading to growth arrest observed in knockdown experiment, the TGF-β signaling pathway in SAP30 depleted cells was analyzed. To do this, 2.8×10^7 SAP30 depleted MCF-10A cells were plated in 15 cm plates in duplicate along with MCF-10A cells infected by shLuc as control and treated one plate of each with TGF-β1 (100 pM) for 24 hours. After treatment with TGF-β1, nuclear extract was isolated and was blotted for phospho-Smad2. As shown in Figure 3-5A, Smad2 proteins are phosphorylated in response to TGF-β1 in SAP30 depleted MCF-10A cells in the same manner as shLuc MCF-10A cells. This suggests that depletion of SAP30 does not block TGF-β1 receptor expression and function.

Phosphorylated Smads along with other cofactors can activate transcription of several genes such as Smad7 (211). To investigate the effect of SAP30 depletion on Smad-dependent transcription, the ability of SAP30 depleted MCF-10A cells were

examined in transcriptional activation of Smad7 in response to TGF-β1. To do this, 4×10^5 SAP30 depleted MCF-10A cells were plated in 6 cm plates in duplicate along with shLuc MCF-10A cells and treated one plate of each shRNA with TGF-β1 (100 pM) for 24 hours. After treatment with TGF-β1, RNA was isolated and converted to cDNA and Real-Time PCR was performed for Smad7. As shown in Figure 3-5B, Smad complexes are able to activate transcription of Smad7 in the absence of SAP30 suggesting that Smad-dependent transcription is intact in the absence of SAP30.

A


shLuc
shSAP30
TGF-β1 (100pM):
- + - +
pSmad2 (~58 kDa)
*
75
50
Smad2 (~55 kDa)
50

B

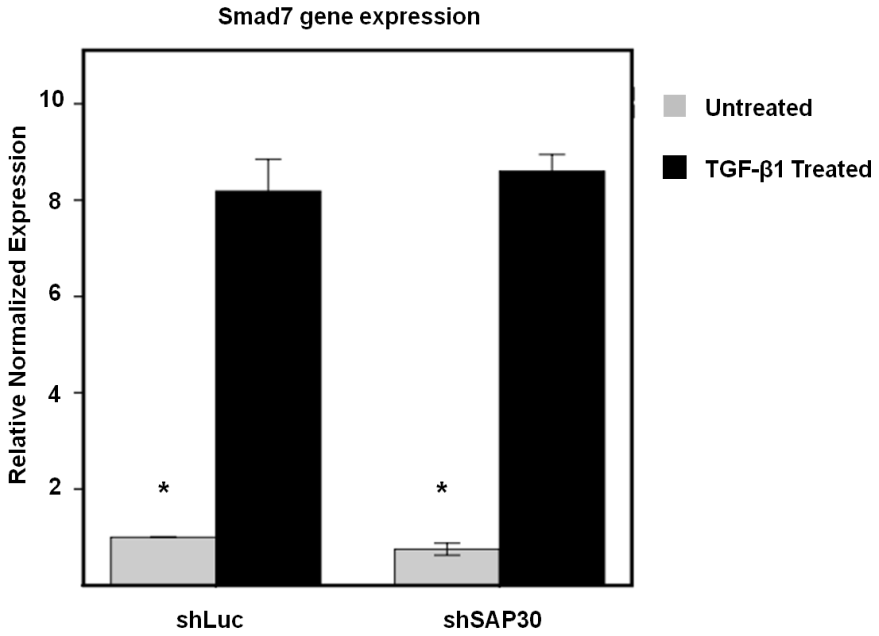

Smad7 gene expression
Relative Normalized Expression
10
8
6
4
2
*
*
shLuc
shSAP30
Untreated
TGF-β1 Treated

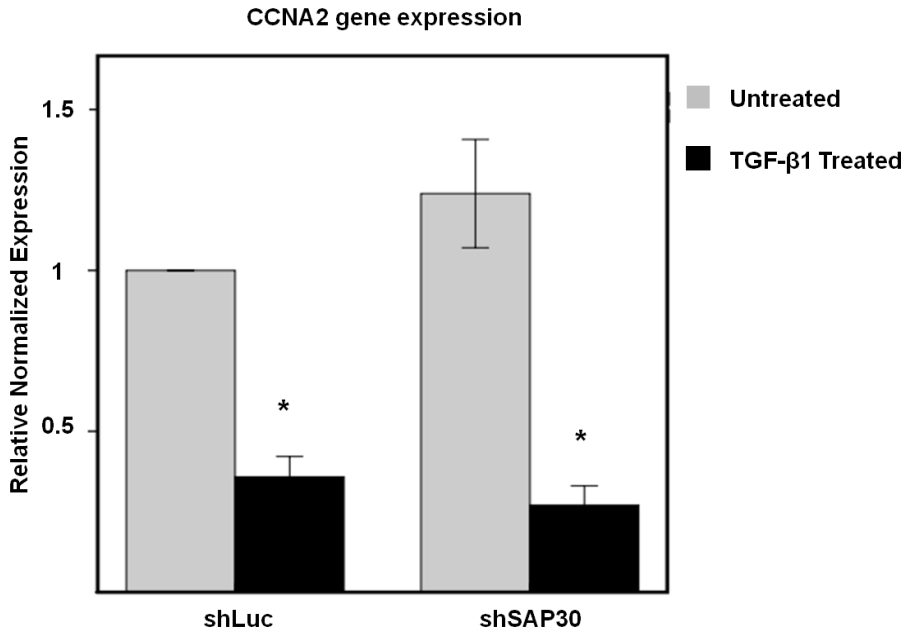
Figure 3-5 TGF-β signaling is intact in absence of SAP30

(A) The total phospho-Smad2 expression level was measured in TGF-β1-treated and untreated SAP30 depleted MCF-10A and shLuc MCF-10A by western blot analysis. The upper band is phospho-Smad2 and the asterisk indicates the lower non-specific band. (B) The mRNA level of Smad7 was measured in TGF-β1-treated and untreated SAP30 depleted MCF-10A and shLuc MCF-10A by Real-Time PCR. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference of treated samples from untreated samples (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance.

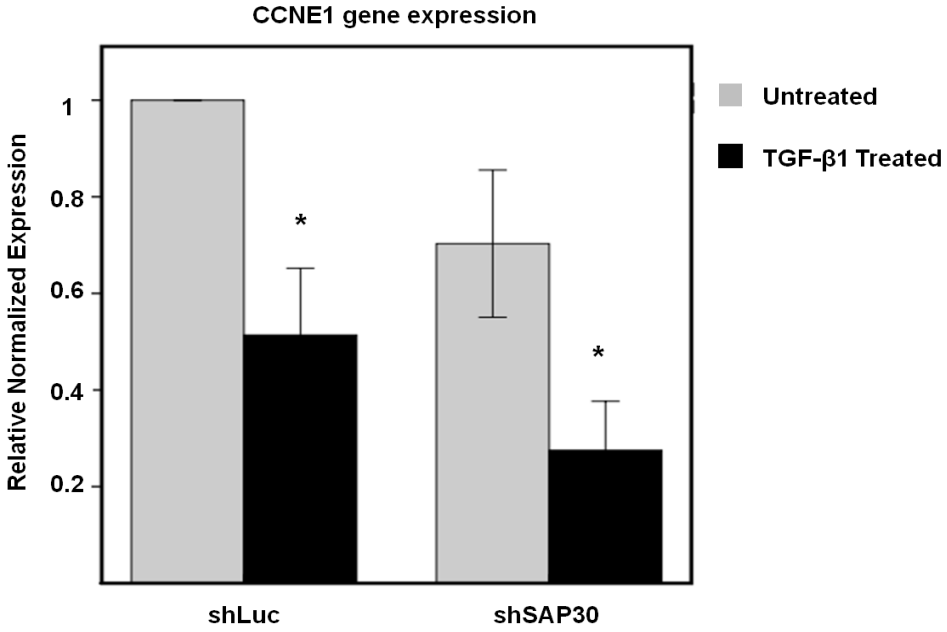
3.1.5.3 E2F target genes are repressed by TGF-β in absence of SAP30

In many cell types, TGFβ activates the transcription of CDK inhibitors such as p15 and p21 and downregulates the expression of proliferative genes including Cdc25A, c-Myc. The net result of these transcriptional changes is CDK activity inhibition, which results in dephosphorylation of pRB and repression of E2F target genes necessary for the G1/S phase transition (221-226). To determine the status of E2F target genes expression in SAP30 depleted cells in response to TGF-β, Real-Time PCR was performed to measure the mRNA level of three E2F-responsive genes in response to TGF-β. As shown in Figure 3-6, levels of *PCNA*, *CCNE1* and *CCNA2* transcripts decreased in both SAP30 depleted and shLuc MCF-10A cells in response to TGF-β. Our previous experiments using $Rb1^{\Delta L}$ MEFs showed that pRb`s ability to repress transcription of E2F responsive genes in response to TGF-β1 is lost. This suggests that pRB also needs LxCxE binding partner to repress transcription of E2F target genes. As this result shows, depletion of SAP30 compromises TGF-β growth arrest; however, SAP30 serves this role in an E2F independent manner, because E2F target genes are repressed in absence of SAP30 in response to TGF-β. Therefore, SAP30 is not our desired component.

A


CCNA2 gene expression
Relative Normalized Expression
1.5
1
0.5
*
*
shLuc
shSAP30
Untreated
TGF-β1 Treated

B


CCNE1 gene expression
Relative Normalized Expression
1
0.8
0.6
0.4
0.2
*
*
shLuc
shSAP30
Untreated
TGF-β1 Treated

C

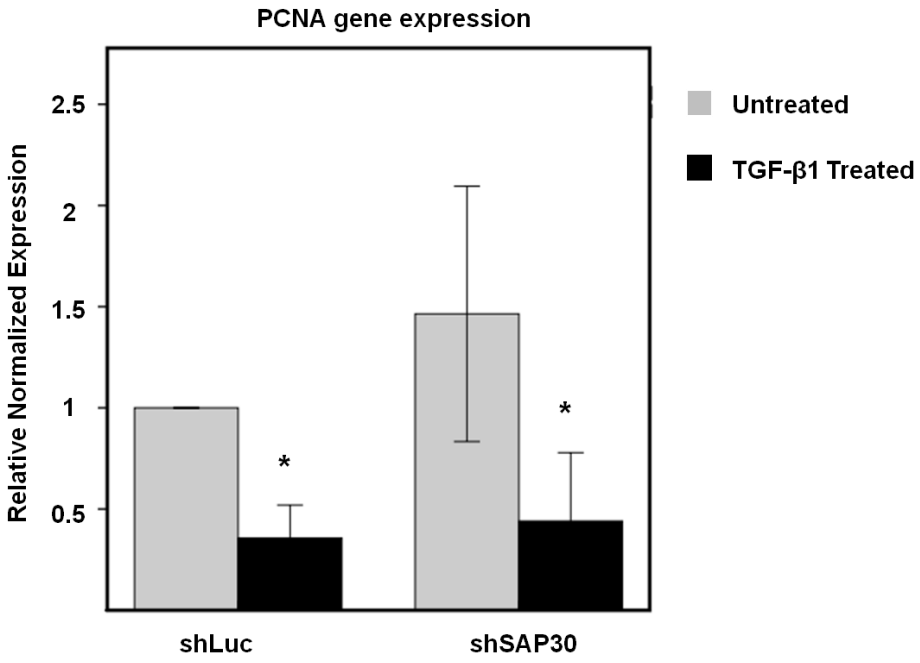


Figure 3-6 E2F target genes are repressed in response to TGF-β in absence of SAP30

(A-C) The mRNA level of *CCNE1*, *CCNA2* and *PCNA* was measured in TGF-β1 treated and untreated SAP30 depleted MCF-10A and shLuc MCF-10A by Real-Time PCR. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference of treated samples from untreated samples (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean.

3.1.6 MTA2, a unique component of NuRD complex involved in E2F transcriptional repression

MTA2 is essential in the assembly of an active histone HDAC complex and the association of MTA2 with the core HDAC complex requires MBD3 (276). It has also been shown that one of the mechanisms by which E2F-pRB mediates repression of E2F target genes is through recruitment of HDAC (51); Therefore, MTA2 may have a role in E2F transcriptional repression in response to TGF-β1. This motivated us to examine the effect of MTA2 in TGF-β1 mediated growth arrest.

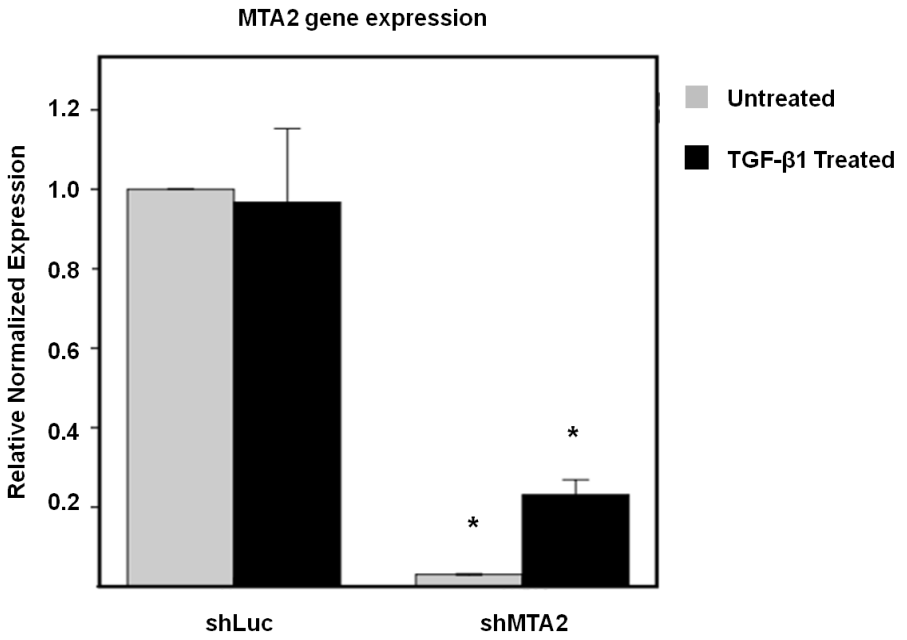
3.1.6.1 Depletion of MTA2 compromises TGF-β growth arrest

To examine whether MTA2 is involved in TGF-β1-induced growth arrest, 3×10^5 MCF-10A cells were plated in 6 cm plates in duplicate. Then, these cells were infected with lentiviral vector carrying MTA2 shRNA along with shLuc as a control. After lentiviral infection and selection with puromycin, the infected cells were expanded for further experiments.

To confirm knockdown efficiency, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for MTA2 shRNA and shLuc. TGF-β1 (100 pM) was added to one plate of each shRNA for 24 hours. After TGF-β1 treatment, RNA was isolated and converted to cDNA and Real-Time PCR was performed for MTA2. As shown in Figure 3-7A, the level of MTA2 transcript was significantly reduced in MCF-10A cells.

To do TGF-β1 growth arrest assay, 4×10^5 infected MCF-10A cells were plated in 6 cm plates in duplicate for each shRNA. TGF-β1 (100 pM) was added to one plate of each shRNA. After 24 hours TGF-β1 treatment, the cells were pulse-labeled with BrdU for 4 hours. Then, flow cytometry was performed to quantify the number of cells incorporating BrdU. As shown in Figure 3-7B, MCF-10A cells expressing shLuc showed a significant decrease in BrdU incorporation in response to TGF-β1 whereas the ability of MTA2 depleted cells to induce to TGF-β1 growth arrest was reduced. This suggests that depletion of MTA2 compromises TGF-β1-induced growth arrest.

A


MTA2 gene expression
Relative Normalized Expression
1.2
1.0
0.8
0.6
0.4
0.2
*
*
shLuc
shMTA2
Untreated
TGF-β1 Treated

B

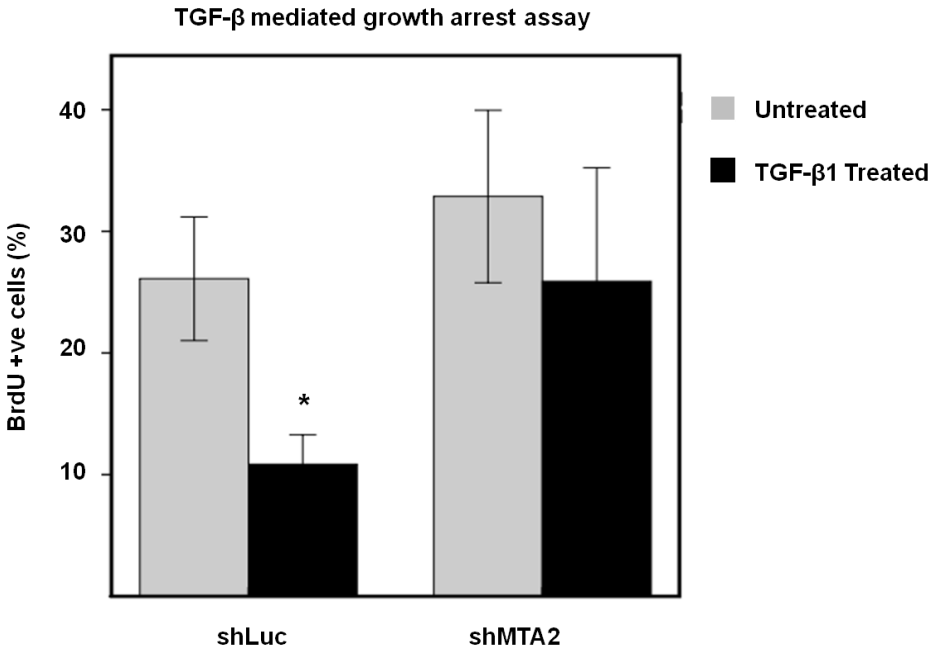

TGF-β mediated growth arrest assay
BrdU +ve cells (%)
40
30
20
10
*
shLuc
shMTA2
Untreated
TGF-β1 Treated

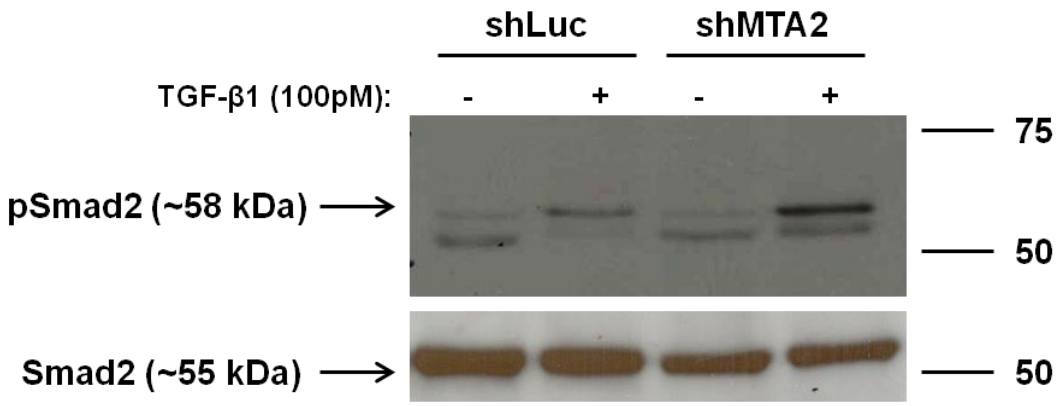
Figure 3-7 Depletion of MTA2 compromises TGF-β1 mediated growth arrest

(A) MCF-10A cells were infected with shRNA to deplete the expression of MTA2. After infection and three days selection, the infected cells were treated with TGF-β1 for 24 hours; the mRNA level of MTA2 was measured by Real-Time PCR to verify knockdown efficiency. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance. (B) MTA2 depleted cells were treated with TGF-β1 for 24 hours, pulse-labeled with BrdU for 4 hours. The percentage of cells incorporating BrdU was measured by flow cytometry. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference from untreated shLuc (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean. (+ve, positive)

3.1.6.2 TGF-β signaling is intact in absence of MTA2

In order to investigate whether depletion of MTA2 affects TGF-β1 signaling pathway upstream of pRB dephosphorylation and activation, the ability of MTA2 depleted MCF-10A cells to phosphorylate Smad2 in response to TGF-β1 was examined. To do this, 2.8×10^7 MTA2 depleted MCF-10A cells were plated in 15 cm plates in duplicate along with MCF-10A cells infected by shLuc as a control and treated one plate of each with TGF-β1 (100 pM) for 24 hours. After treatment with TGF-β1, the nuclear extract was isolated and was blotted for phospho-Smad2. As shown in Figure 3-8A, Smad2 is phosphorylated in response to TGF-β1 in absence of MTA2. This suggests that depletion of MTA2 does not block TGF-β1 receptor expression and function. In addition, the Smad-dependent transcription of MTA2 depleted MCF-10A cells was examined; To do this, Real-Time PCR was performed to look at the transcription level of Smad7. As shown in Figure 3-8B, Smad complexes are able to activate transcription of Smad7 in the absence of MTA2 suggesting that Smad-dependent transcription is intact in absence of MTA2.

A


shLuc
shMTA2
TGF-β1 (100pM):
-
+
-
+
pSmad2 (~58 kDa)
Smad2 (~55 kDa)
75
50
50

B

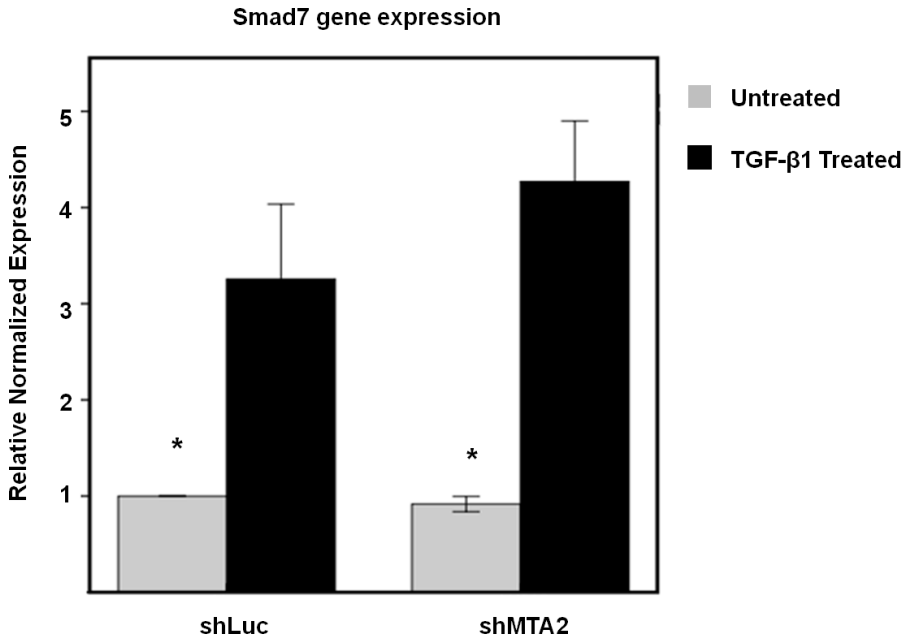

Smad7 gene expression
Relative Normalized Expression
1
2
3
4
5
*
*
shLuc
shMTA2
Untreated
TGF-β1 Treated

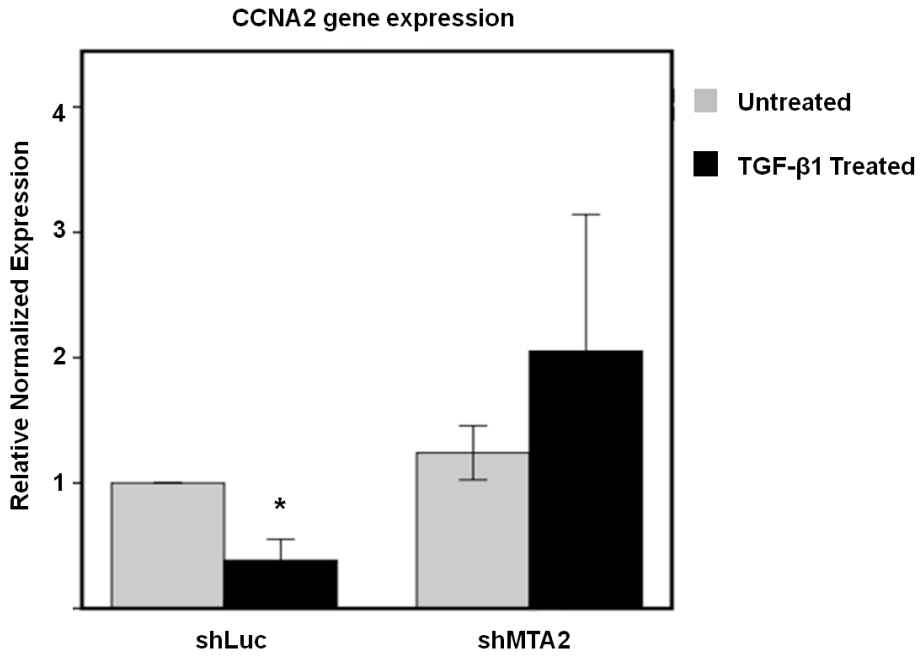
Figure 3-8 TGF-β signaling is intact in absence of MTA2

(A) Total phospho-Smad2 expression levels were measured in TGF-β1-treated and untreated MTA2 depleted MCF-10A and shLuc MCF-10A by Western blot analysis. (B) The mRNA level of Smad7 was measured in TGF-β1-treated and untreated MTA2 depleted MCF-10A and shLuc MCF-10A by Real-Time PCR. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference from treated samples (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean.

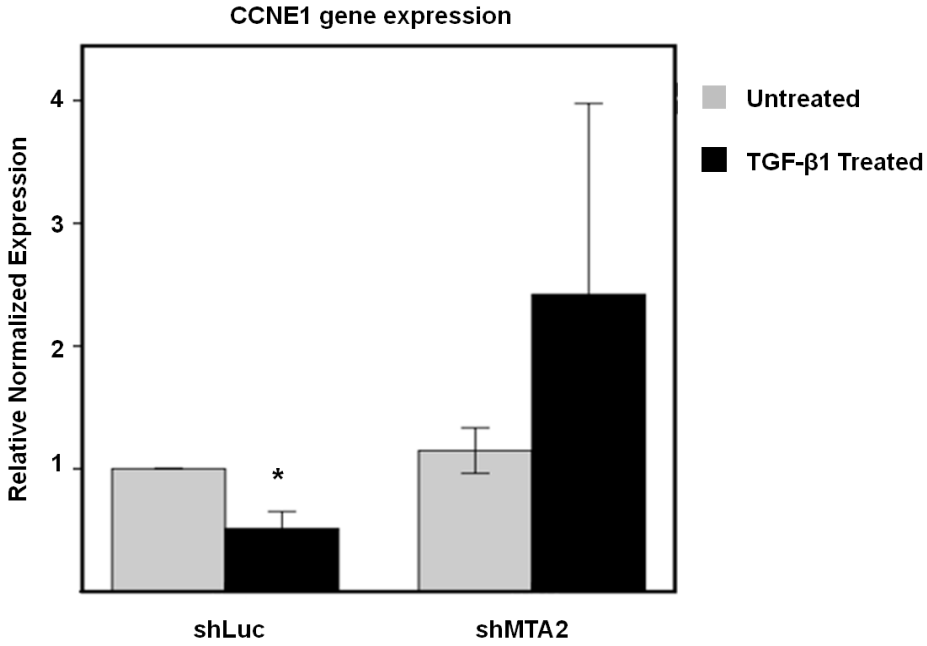
3.1.6.3 E2F target genes are deregulated in response to TGF-β in absence of MTA2

To determine the status of E2F target genes expression in the absence of MTA2 and in response to TGF, Real-Time PCR was performed to measure the mRNA level of three E2F-responsive genes including *PCNA*, *CCNE1* and *CCNA2* in response to TGF-β. As shown in Figure 3-9, these E2F target are repressed in response to TGF-β1 in shLuc MCF-10A cells. However, MTA2 depleted MCF-10A cells show a slight increase in the expression of E2F target genes. This suggests that E2F target genes are derepressed in MTA2 depleted cells. This finding is consistent with previous experiment in our lab suggesting that E2F target genes are derepressed in $Rb1^{\Delta L}$ MEFs in response to TGF-β1.

A


CCNA2 gene expression
Relative Normalized Expression
4
3
2
1
*
shLuc
shMTA2
Untreated
TGF-β1 Treated

B


CCNE1 gene expression
Relative Normalized Expression
4
3
2
1
*
shLuc
shMTA2
Untreated
TGF-β1 Treated

C

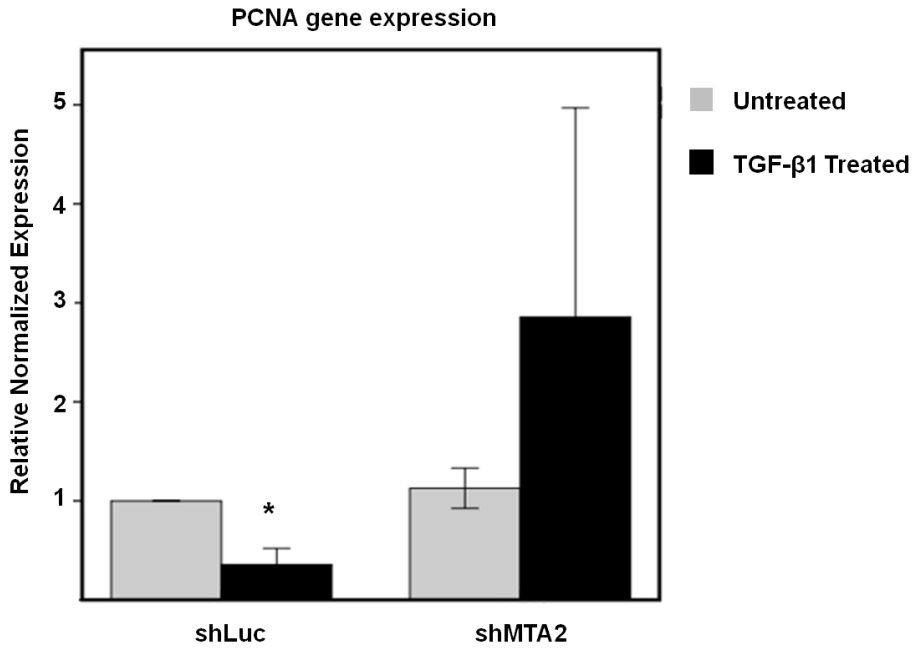


Figure 3-9 E2F target genes are deregulated in response to TGF-β in absence of MTA2

(A-C) The mRNA level of *CCNA2*, *CCNE1* and *PCNA* was measured in TGF-β1-treated and untreated MTA2 depleted MCF-10A and shLuc MCF-10A by Real-Time PCR. The average of three independent experiments is shown. The asterisks indicate a statistically significant difference from untreated shLuc (Student's *t* test; $P < 0.05$). The error bars indicate one standard deviation from the mean. All values for Real-Time PCR experiments were normalized to the level of GAPDH mRNA abundance.

4 Discussion

4.1 Summary

pRB is a key regulator of cell proliferation in the G1 phase of the cell cycle. Previously, it was thought that pRB`s function as a tumor suppressor was only due to its ability to inhibit E2F target gene transcription in the G1 phase. Now, it is believed that there are many proteins, which cooperate with pRB to act as a tumor suppressor. TGF-β induces G1 growth arrest by inhibiting CDK`s activity which leads to dephosphorylation and activation of pRB (63, 64). While many scientists did not consider TGF-β as a part of the pRB pathway; our lab, using a knock-in mouse that carries a three amino acid substitution mutant to disrupt LxCxE cleft in pRB (called $Rb1^{\Delta L}$), showed that mammary epithelial cells from $Rb1^{\Delta L/\Delta L}$ mice do not respond to TGF-β-induced growth arrest (261). We found that TGF-β stimulation of epithelial cells results in dephosphorylation of pRB and the defect in growth inhibition is downstream of pRB's activation. Furthermore, repression of E2F responsive cell cycle genes is defective in the $Rb1^{\Delta L/\Delta L}$ cells in response to TGF-β (261). This shows that the interactions mediated by the LxCxE binding cleft of pRB are necessary for TGF-β mediated growth arrest. Using shRNAs to deplete the expression of chromatin regulating complexes, we tried to identify specific proteins, which interact with pRB through the LxCxE binding cleft in mediating E2F target gene repression in response to TGF-β. First, data showed that TGF-β mediated growth arrest is pRB dependent in MCF-10A cells and pRB depletion deregulates E2F target gene expression in these cells confirming that these cells are a suitable system for our study. Then, our results showed that the depletion of MTA2 and SAP30 compromises TGF-β

mediated growth arrest. In addition, depletion of MTA2 and SAP30 did not block TGF-β1 signaling upstream of pRB as demonstrated by phosphorylation of Smad2 and upregulation of Smad7 level. Furthermore, depletion of MTA2 resulted in derepression of E2F target genes in response to TGF-β while depletion of SAP30 repressed the expression of E2F target genes. These data suggest that SAP30 induces TGF-β1 mediated arrest in an E2F independent manner. It has been demonstrated that TGF-β enhances the interaction of pRB with the MCM complex at the G1 phase of the cell cycle, which prevents the activation of replication origin and the G1- S phase transition (277). Although we currently do not know how pRB controls MCM function molecularly. One possible explanation is that pRB recruits other regulatory factors to MCM complexes to mediate this interaction. Since our data suggests that SAP30 mediates TGF-β growth arrest independent of E2F transcription, it is possible that SAP30 plays a role in pRB`s interaction with replication origins to induce growth inhibition. In addition, our data suggests that MTA2 is involved in an E2F dependent TGF-β1 mediated growth arrest. In summary, SAP30 and MTA2 are involved in TGF-β1 mediated growth arrest. However, they will use different mechanisms to induce growth inhibition.

4.2 Plausible mechanisms of MTA2 mediated TGF-β growth arrest

These findings suggest that MTA2 is one of the components involved in E2F transcriptional repression in response to TGF-β. There are several possibilities for how MTA2 represses the transcription of E2F target genes and how this protein induces TGF-β mediated growth arrest (Figure 4-1).

One possibility is that MTA2 in the NuRD complex is recruited independent of LxCxE interaction and through the direct interaction with chromatin, because the C-terminal domain of MTA proteins in the NuRD complex directly interacts with histone H3 tails (278). Furthermore, it has been shown that the NuRD complex can be recruited to chromatin through the interaction with other proteins (278). One example is that the NuRD complex can associate with chromatin by interacting with different co-factors such as HP1. In this case, NuRD complex is recruited to E2F target genes through the direct interaction of MTA2 with chromatin. Once recruited, the NuRD complex uses its ability to remodel chromatin structure and repress the transcription of E2F target genes (Figure 4-1A).

Another possibility is that MTA2 in the NuRD complex is recruited through HDAC1, because HDAC1 can bind to pRB through the LxCxE binding cleft and MTA2 is required for the assembly of HDAC complex (51, 276). In this scenario, pRB recruits the NuRD complex through the HDAC1 interaction to E2F target genes promoters. Once recruited, the NuRD complex changes chromatin dynamics and represses E2F target genes transcription (Figure 4-1B). Since MTA2 directs the assembly of the complex, its` depletion makes the complex incapable of repressing E2F target genes transcription in response to TGF-β. This will lead to defective TGF-β mediated growth arrest in absence of MTA2.

Lastly, the NuRD complex may interact with chromatin through the MBD2 subunit, which has the ability to bind to the methylated DNA (279). In this scenario, pRB interacts with DNMT1, an enzyme responsible for catalyzing DNA methylation of promoters, through the LxCxE binding cleft (280, 57). Once E2F target genes are

methylated, MBD2 subunit in the NuRD complex binds to the methylated DNA and recruits the NuRD complex to E2F target genes. The NuRD complex has the HDAC activity and once it is recruited, can repress transcription of E2F target genes by deacetylating histones on E2F target genes` promoter (Figure 4-1C). The experiments proposed in the next section will examine these models.

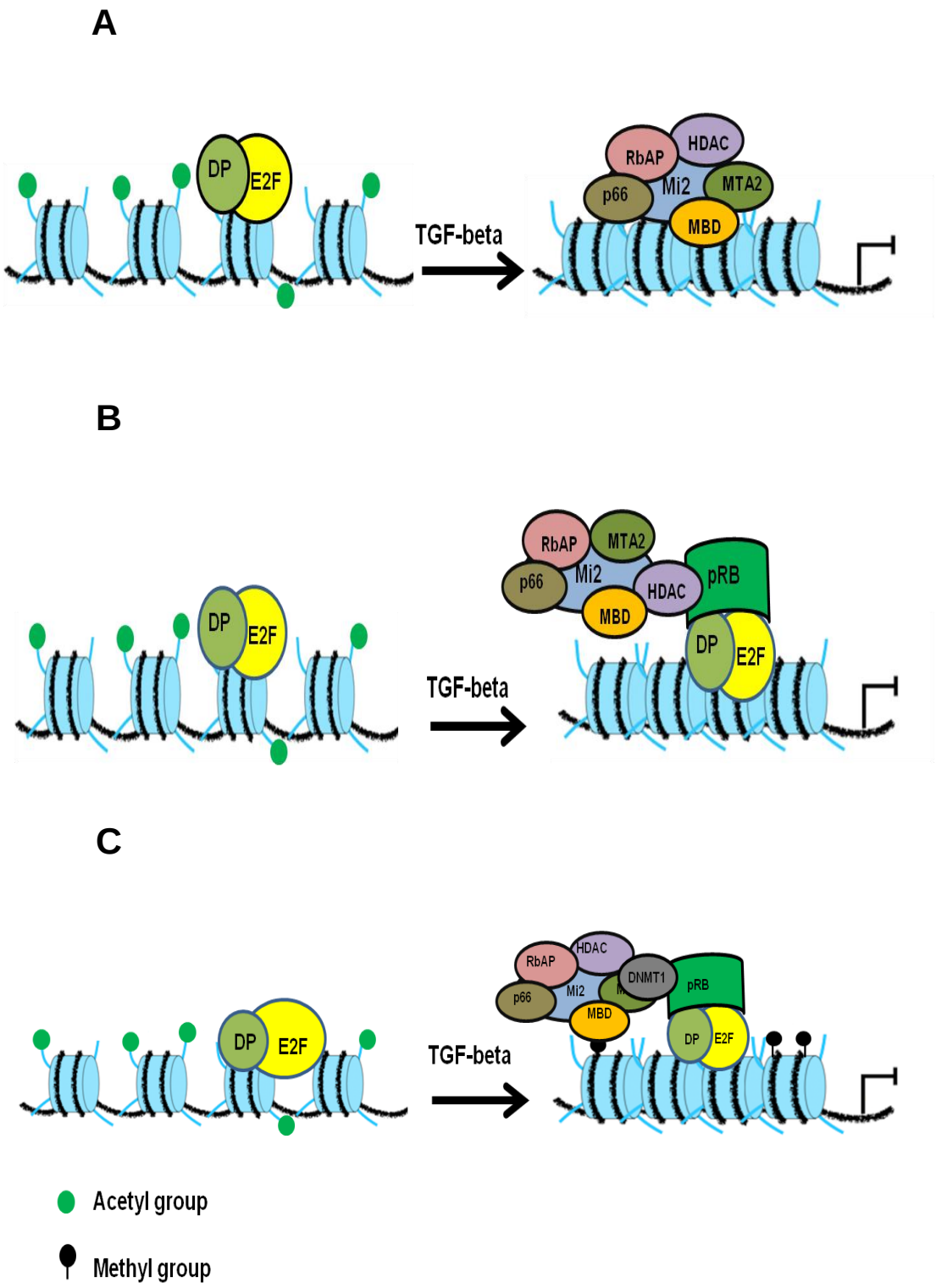

A
DP
E2F
TGF-beta
RbAP
HDAC
p66
Mi2
MTA2
MBD
B
DP
E2F
TGF-beta
RbAP
MTA2
p66
Mi2
HDAC
pRB
MBD
DP
E2F
C
DP
E2F
TGF-beta
HDAC
RbAP
DNMT1
pRB
p66
Mi2
MBD
DP
E2F
Acetyl group
Methyl group

Figure 4-1 Plausible mechanisms of MTA2 mediated TGF-β growth arrest

(A) NuRD complex is recruited to E2F target genes through the direct interaction of MTA2 with chromatin. Once recruited, the NuRD complex uses its ability to remodel chromatin structure and repress the transcription of E2F target genes. (B) pRB recruits the NuRD complex through HDAC1 interaction with E2F target genes promoters. Once recruited, the NuRD complex changes chromatin dynamics and represses E2F target genes transcription. (C) pRB interacts with DNMT1 through the LxCxE binding cleft. Once E2F target genes are methylated, MBD2 subunit in the NuRD complex binds to the methylated DNA and recruits the NuRD complex to E2F target genes. The NuRD complex has the HDAC activity and once it is recruited, can repress transcription of E2F target genes by deacetylating histones on E2F target genes promoter.

4.3 Further investigating of the mechanism of MTA2 mediated TGF-β growth arrest

To examine our proposed models, we can use shRNAs against DNMT1 and MBD2 to knockdown their expression and examine whether depletion of these proteins compromise TGF-β mediated growth arrest. In addition, we can use methylation specific PCR for E2F target genes after TGF-β treatment to investigate if they become methylated in response to TGF-β and if their methylation pattern will change after DNMT1 and MBD2 knockdown. If DNA methylation is a mechanism by which the NuRD complex serves to induce the growth arrest, we expect that depletion of DNMT1 and MBD2 compromise the growth arrest. Furthermore, since the NuRD complex has the HDAC activity and MTA2 can direct the assembly of an active HDAC complex, it is logical to examine the acetylation changes at E2F target genes in the absence of MTA2 in response to TGF-β1. To this end, MTA2-depleted MCF-10A would be treated with TGF-β1. Then chromatin immunoprecipitation (ChIP) assays will be performed to look at histone tail modifications using pan acetyl-Histone H3 antibody and pan acetyl-Histone H4 antibody. To do this, DNA released from precipitated complexes will be amplified by PCR using primers specific to the promoter regions of E2F target genes such as *PCNA, CCNE1, CCNA2*. The same ChIP assays also can be performed to determine whether MTA2 is recruited to E2F target genes promoter in a TGF-β1 dependent manner.

Additionally, immunoprecipitation (IP) assays for other components of NuRD complex in absence of MTA2 can be performed to ensure that the complex integrity is intact in absence of MTA2 and the complex does not fall apart upon depletion of MTA2. This experiment would tell us whether MTA2 has a very specific role in TGF-β induced

arrest and its loss leaves the rest of the complex alone or the NuRD complex is completely disrupted by MTA2 depletion and that would explain why this would disrupts its ability to arrest proliferation.

In summary, these experiments identify how the NuRD complex cooperates with pRB in response to TGF-β1 and determine how these downstream targets of the pRB-LxCxE interaction function in response to TGF-β1 in this critical cancer-suppressing pathway.

4.4 Other potential LxCxE partners involved in TGF-β growth arrest

In addition to MTA2 and SAP30, there are other components in both Sin3 and NuRD complexes, which associate with HDAC. Therefore, they may have a role in transcriptional repression of E2F target genes in response to TGF-β. One such example is SDS3. SDS3 mutants showed very similar phenotypes previously observed in Sin3, suggesting that SDS3 plays an essential role in the Sin3 complex (281). Depletion of SDS3 results in a dramatic loss of HDAC activity and a significant reduction in Sin3-mediated repression (119). SDS3-deficient cells fail to deacetylate pericentric heterochromatin histones, resulting in a general failure of cytokinesis and aneuploidy. These studies indicate that SDS3 is a core subunit in the Sin3 complex and augments HDAC activity. Therefore, it may have a role in transcriptional repression of E2F target genes in response to TGF-β.

In addition to Sin3 and NuRD complexes, CtBP binds to pRB through the LxCxE binding cleft. It appeared that CtBP mediates transcriptional repression in a HDAC-dependent or HDAC-independent manner (135, 282). It has been suggested that CtBP mediates HDAC-independent repression through the recruitment of the Polycomb-group (PcG) complex and pRB by CtIP (283). Since CtBP serves its role as a transcriptional repressor through the recruitment of HDAC as well as PcG, it may have a role in transcriptional repression of E2F target genes in response to TGF-β.

The complexes mentioned above (Sin3, NuRD and CtBP) contain subunits which are able to modify chromatin structure resulting in the loss of pRB proliferative control. For example, LSD1 is a component of the NuRD, which is capable of demethylating H3K4me2 from nucleosomes (284). Furthermore, it has been demonstrated that trimethylation of histone 3 lysine 9 (H3K9Me3), which is mediated by Suv39h1 enzyme, is reduced at the E2F target genes at the senescent $Rb1^{\Delta L}$ cells (100) and also long term exposure to TGF-β1 can induce cellular senescence (285). To complement shRNA approach and identify the other potential LxCxE partners, we can further investigate the chromatin modifications at E2F target genes after TGF-β1 treatment. To this end, $Rb1^{\Delta L}$ MECs will be treated with TGF-β1. Then, ChIP assays will be performed to look at histone tail modifications using methyl/acetyl-Histone H3 antibody and methyl/acetyl-Histone H4 antibody. DNA released from precipitated complexes will be amplified by PCR using primers specific to the promoter regions of E2F target genes such as *Pcna, Ccne1, Mcm3* and *Mcm5.*

As a result, these experiments will determine chromatin structure changes at E2F target genes promoters in response to TGF-β1. This way, the change in chromatin

structure after TGF-β1 treatment will lead us to pick suitable components to continue forward in the experiments. These experiments will also determine the requirement of LxCxE interacting proteins in TGF-β1 mediated growth arrest.

4.5 An unbiased approach to identify LxCxE partners involved in TGF-β growth arrest

Many potential candidates have been identified that may cooperate with pRB in an LxCxE dependent manner to induce TGF-β mediated growth arrest. With such a diverse list, alternative approaches may be required to determine the mechanism behind of pRB-LxCxE dependent TGF-β mediated growth arrest. One unbiased approach would utilize shRNA library screening to identify proteins cooperating with pRB in response to TGF-β1 by knocking down proteins at random. To do this, NMuMG-Fucci cell line, a subline of the NMuMG cell line expressing a cell cycle marker, will be used. Fucci technology allows dual-color imaging, which can distinguish between live cells in the G1 (red) and the S/G2/M phases (green) (286). After knocking down with shRNA library, the cells would be tested for TGF-β1-induced growth arrest and those proteins, which cooperate with pRB in TGF-β1-induced growth arrest, can be identified. As a result, these experiments will identify the identity of any remaining LxCxE interacting proteins in TGF-β1 mediated growth arrest.

4.6 Therapeutic potential of uncovering the exact mechanism of TGF-β mediated growth arrest

TGF-β has a tumor promoting effect in tumor development, when carcinoma cells become insensitive to TGF-β induced growth inhibition (142). Furthermore, the majority of triple-negative breast cancers (TNBCs) recur after chemotherapy. A subpopulation of cancer stem cells (CSCs) drives recurrences following treatment with anticancer chemotherapy (287, 288). Chemotherapy induces TGF-β activity, which has been shown to induce these tumor stem-like properties (289). Thus, TGF inhibitors are being developed by pharmaceuticals as anti-metastatic therapies in patients with this cancer to decrease the CSC population to prevent TNBC recurrences. However, TGF-β is essential for normal development and plays crucial roles in wound healing, inflammation and tissue repair; therefore, using these TGF-β inhibitors may create life-threatening side effects in other tissues later in life.

Since TGF-β has a dual role in tumorigenesis, the detailed understanding of TGF-β signaling pathways is required in order to differentiate between tumor suppressor and promoting effect of TGF-β to prevent unwanted side effects in other tissues. In order to develop new therapeutics that targets the TGF-β signaling pathway in tumor progression, it is important to determine the unique components that mediate the tumor promoting or tumor suppressor properties of TGF-β. My project provides an excellent opportunity to elucidate the exact growth-controlling mechanism and determine the unique components of tumor suppressor properties of TGF-β. My project provides a better understanding of the TGF-β growth inhibitory pathway in order to distinguish it from tumor promoting

role of TGF-β. This will lead to find better therapy for breast cancer patients, which minimizes unwanted off-target side effects in these patients.

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Curriculum Vitae

Name: Mehdi Amiri

Education

Western University, London, Ontario — Jan 2011- present
M.Sc Candidate in Biochemistry

University of Tehran, Tehran, Iran — Sep 2005- June 2010
B.Sc in Cell & Molecular Biology

Honors and Awards

CIHR Strategic Training Program in Cancer Research and Technology Transfer (CaRTT) award (Sep 2011-Aug 2012)

Western Graduate Research Scholarship (Jan 2011-April 2014)

Tuition fee exemption for undergraduate study Award (Sep 2005-July 2010)

Admitted to the University of Tehran (Sep 2005); after passing Iranian Nationwide University Entrance Examination ranking in the first 400 of more than one million applicants

One of the selected students in the final stage of 7th National Biology Olympiad, Tehran, Iran (2004)

Presentations

1) Amiri, M., and Dick, F. A. Investigating the role of Retinoblastoma protein (pRB) and Transforming Growth Factor-β (TGF-β) in cancer suppression, The 9th Annual Oncology Research & Education Day, June 22nd, 2012. *(Poster Presentation)*

2) Amiri, M., and Dick, F. A. Investigating the role of Retinoblastoma protein (pRB) and Transforming Growth Factor-β (TGF-β) in cancer suppression, Cancer Research Laboratory Program Seminar Series, London Regional Cancer Program, ON, Canada, March 8th, 2012. *(Oral Presentation)*

Publication

1) Cecchini, M.J., **Amiri, M**., and Dick, F.A. (2012) Analysis of cell cycle position in mammalian cells. *J. Vis. Exp. 21 ;(59). pii: 3491. doi: 10.3791/3491.*

Research Experience

Graduate research assistant in Victoria Research Labs, London Regional Cancer Program, Victoria Hospital, London, ON (Jan 2011- April 2014)
Supervisor: Dr. Fred Dick
Worked on the role of negative growth regulation by the Retinoblastoma protein (pRB) and Transforming Growth Factor-β (TGF-β) in suppression of mammary tumorigenesis

Undergraduate research assistant in Cellular and Developmental Biology Lab, School of Biology, University of Tehran (Oct 2008 - Sep 2009)
Supervisor: Dr.Nasrin Motamed
Worked on the effect of silibinin on viability, migration and adhesion of the MDA-MB 231 cell line and expression of genes associated with metastasis suppressor gene, D4GDI, in the MDA-MB 231 cell line

Teaching Experience

Teaching General Biology in Biology Olympiad preparatory classes held by Ministry of Education for talented high school students in different regions of Iran.(2007- 2008)

Teaching Biology, Chemistry and English courses to high school students, Private Tutor, Tehran, Iran (2007- 2009)