REGULATION OF THE MAMMALIAN RETINOBLASTOMA PATHWAY BY THE UBIQUITIN-PROTEASOME SYSTEM

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ABSTRACT

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The Retinoblastoma (RB) family of proteins play critical roles in normal development through their governance of genes involved in cell fate. During normal growth RB family activity is tightly regulated through Cdk-dependent phosphorylation, resulting in their dissociation from E2F family transcription factors. In addition, the RB pathway is also governed through the ubiquitin-proteasome system, with deregulated degradation of RB proteins frequently associated with human cancer. Recent studies from our labs have shown in *D*rosophila that the Retinoblastoma family (Rbf) proteins are subjected to proteasome mediated turnover during embryonic development and this process enhances Rbf engagement in transcriptional repression. This positive link between Rbf1 activity and its destruction indicates that repressor function is governed in a manner similar to that described by the degron theory of transcriptional activation.

To understand the relationship between RB family stability and their repressor function during early mammalian development, we initiated studies in mouse embryonic stem (ES) cells. Our studies suggest that differentiation of mouse ES cells is associated with the establishment of a functional RB pathway and simultaneous changes in stability of RB family members. As pluripotent ES cells are characterized by unrestrained cdk activity which plummets at the onset of differentiation, we speculated that the observed changes in protein stability upon ES cell differentiation reflects an intimate relationship between RB phosphorylation and stability. Indeed, we show that phosphorylation dependent turnover of RB, p107 and p130 is mediated by an evolutionarily conserved and autonomous instability element (IE) located in their C-terminal

regulatory domain. Moreover, stabilizing mutations within the IE elements also debilitate them for transcriptional repression. We conclude that the overlap of degron sequences and repression modules is a conserved feature shared among the RB homologues, and represents a novel mode of transcriptional repression. Together, these findings implicate the Retinoblastoma family IE region as a regulatory nexus linking repressor potency to the ubiquitin-proteasome system in development and disease.

I dedicate this thesis to my parents, Mr. Lokendra Sankar Sengupta and Mrs. Soma Sengupta for teaching me the values of education, and for their unconditional love and support.

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TABLE OF CONTENTS

LIST OF FIGURES

KEY TO ABBREVIATIONS

APC/C- Anaphase Promoting Complex/Cyclosome

ATP- Adenosine Triphosphate

BRG1- BRM-related Gene product 1

CC-MB- Coiled-Coil-Marked-Box

CCNA2- Cyclin A2

CDK- Cyclin Dependent Kinase

ChIP- Chromatin Immunoprecipitation

CMV- Cytomegalovirus

COP9- Constitutive Photomorphogenic 9

CSN- COP9 Signalosome

CtBP- C-terminal Binding Protein

DP- E2F Dimerization Partner

DREAM- DP, RB-like, E2F and MuvB complex

E1A- Early region 1A

E3- Ubiquitin activating enzyme 3

E2F- Adenovirus E2 promoter binding factor

GFP- Green Fluorescent Protein

HCV- Hepatitis C Virus

HDAC1/2- Histone Deacetylase 1/2

HGP- Hutchison-Gilford-Progeria

HPV E7- Human Papilloma Virus Early 7

IE-Instability Element

LAP2α- Lamin Associated Protein 2α

LIF- Leukemia Inhibitory Factor

LOH- Loss of Heterozygosity

MCM10- Mini Chromosome Maintenance 10

MDM2- Murine double minute 2

MEFs- Mouse Embryonic Fibroblasts

Nedd4/8- Neural precursor cell-expressed developmentally down regulated gene 4/8

NSCLC- Non Small Cell Lung Cancer

PCNA- Proliferating Cell Nuclear Antigen

PIP- PCNA Interacting Protein

RB- Retinoblastoma Protein

RBL1-Retinoblastoma Like 1

RBL2-Retinoblastoma Like 2

RBP1- retinoblastoma Binding Protein 1

Rbf1/2- Retinoblastoma Factor 1/2

SCF- Skp/Cullin/F-box complex

SUMO- Small Ubiquitin Like Modification

SWI/SNF-Switch/Sucrose nonfermenting complex

TAD- Transactivation Domain

TKO Triple Knock Out

TSS Transcriptional Start Site

CHAPTER 1: INTRODUCTION[1]

[1]Part of the work described in this chapter is used in the following manuscript: Satyaki Sengupta and R. William Henry (2015) Regulation of the RB-E2F pathway by the ubiquitin-proteasome system, Invited review from *Biochemical Biophysical Acta. (BBA) Gene Regulatory Mechanisms*

Abstract

The Retinoblastoma tumor suppressor (RB) and its related family members p107 and p130 regulate cell proliferation through the transcriptional repression of genes involved in cellular G1 to S phase transition. However, RB proteins are functionally versatile, and numerous genetic and biochemical studies point to expansive roles in cellular growth control, pluripotency, apoptotic response, genomic stability, metastasis, and senescence. For the vast majority of genes, RB family members target the E2F family of transcriptional activators as an integral component of its gene regulatory mechanism. These interactions are regulated via reversible phosphorylation by Cyclins/Cyclin-dependent kinase (Cdk) complexes, a major molecular mechanism that regulates transcriptional output of RB/E2F target genes. Recent studies indicate an additional level of regulation involving the ubiquitin-proteasome system that renders pervasive control over each component of RB pathway. Disruption of the genetic circuitry for proteasome-mediated targeting of the RB pathway has serious consequences on development and cellular transformation during cancer progression, and is associated with several forms of human cancer. In this review, I discuss the role of the ubiquitin-proteasome system in proteolytic control of RB-E2F pathway components, and recent data that points to surprising non-proteolytic roles for the ubiquitin-proteasome system in novel transcriptional repression mechanisms.

Retinoblastoma tumor suppressor protein

Precise regulation of cell cycle is key to normal development and maintenance of physiological homeostasis. Disruption of these regulatory mechanisms leads to deregulated cellular proliferation associated with human cancers (1, 2). Retinoblastoma (RB) protein actively engages in transcriptional repression of cell cycle genes and thereby coordinates from G1-to-S phase transition during cell cycle to limit aberrant cellular proliferation (3). Historically, RB was attributed as a tumor suppressor protein through studies that implicated mutation in the RB1 gene as a causal event in the development of Retinoblastoma, a devastating cancer of the juvenile retina (4). We now know that mutations in RB *"per se"* or it's inactivation through upstream mechanisms is a common theme in cancer initiation (3). Besides its anti-proliferative function, RB employs a repertoire of others tumor suppressive mechanisms pertaining to regulation of apoptosis, genomic stability senescence, pluripotency, tumor metabolism, angiogenesis and metastasis (3).

In mammals, the RB family is composed of RB, and the RB-like proteins p107 (RBL1) and p130 (RBL2). These transcriptional repressor proteins are primarily related through the conservation of the cyclin fold pocket domains that provides a docking interface for co-regulatory factors required for target gene regulation (5). The pocket domain is evolutionarily conserved and can be easily recognized in RB proteins from diverse metazoan species, including two members, Rbf1 and Rbf2, found in Drosophila species (6). In the canonical repression mechanism, RB interacts with and actively blocks the function of a heterodimeric transcription factor complex composed of E2F and DP proteins bound to gene promoters (7, 8). The E2F family is composed of multiple members, but only some of these (E2F1-3) function as transcriptional activators in humans, while others (E2F4-8) lack activator capacity per se, but instead can function as co-repressors when tested in gene expression assays (7, 8). RB associates preferentially with E2F1-

3 complexes, while p107 and p130 tend to associate with the co-repressor class of E2F complexes (7, 8). Similar E2F specialization has been observed in Drosophila with dE2F1 providing activator functionality while the only other member, dE2F2, functions as transcriptional repressor (6).

RB interacts with E2F1 in a highly modular fashion to influence differential gene regulation. RB is organized into three structured regions that includes the N-terminus (RB-N), the Pocket domain (RB-pocket) and the C-terminus (RB-C) (5). RB-pocket binds to E2F1-transactivation domain (E2F1-TAD) to regulate transcription of cell cycle genes (9, 10, 11, 12) whereas RB-C interacts with the E2F1-Coiled-Coil-Marked Box domain (E2F1-CC-MB) to regulate transcription of apoptotic genes (11, 13, 14). Each interaction is regulated by distinct phosphorylation events that induce significant allosteric changes to impede the function of one domain while potentially leaving other functionality intact (15). *In vivo*, targeted disruption of the interaction between RB-pocket and E2F-TAD leads to severe impairment of RB-mediated repression of cell cycle genes, although surprisingly these animals undergo normal development and show no evidence of tumor formation (12). Together these studies suggest that RB-pocket dependent interaction with E2F-TAD is necessary for RB mediated transcriptional repression, but is dispensable for its tumor suppressor function.

Another interesting and conserved feature of the RB family pocket domain is the presence of a conserved hydrophobic cleft that provides a binding surface for Leu-X-Cys-X-Glu (LXCXE) containing peptides. This is particularly intriguing because LXCXE serves as a signature motif in several RB-interacting proteins such as chromatin modifying enzymes and chromatin remodelers (16). *In vivo* an allele of RB that lacks functional LXCXE binding cleft ($RB^{\Delta L/\Delta L}$) is impaired for interaction with several chromatin modifying enzymes such as RBP1, Sin3, CtBP, HDAC1, HDAC2 while retaining intact binding with E2F1(17) . Consistent with the role of repressive

chromatin in RB mediated gene regulation, several cell cycle genes are derepressed in $RB1^{\Delta L/\Delta L}$ cells during quiescence and oncogenic senescence, in part through deficiencies in establishing heterochromatin marks on target genes (17, 18). Together these studies suggest that L-X-C-X-E dependent interactions with the transcriptional machinery is critical for establishing an epigenetic landscape suited for RB mediated transcriptional control (16, 17, 18).

Regulation of RB-E2F pathway: general mechanisms

Attesting to its central significance in growth control, the RB/E2F pathway is subjected to tight regulation. In the canonical pathway, sequential RB phosphorylation by the Cyclin-D/Cdk4 and Cyclin-E/Cdk2 complexes during G1-S phase progression, mitigates the two general sets of interactions that are essential for RB-mediated repression, namely E2F/DP association and co-factor binding (Figure 1-1) (19-24). An emerging, but surprising model of RB regulatory tactics suggests that RB is phosphorylated at only one of its many potential sites by Cyclin-D/Cdk4 during early G1, whereas RB becomes hyper-phosphorylated by Cyclin-E/Cdk2 in late G1 allowing wholesale activation of E2F driven transcription of cell cycle genes required for S phase function (25). Rather than blocking global RB function, early but limited phosphorylation may create a suite of RB molecules with specialized functions depending upon particular phosphorylation events. Such restrained phosphorylation may provide for more refined regulation of target genes with selective association of some co-regulatory factors, such as histone deacetylases or ATP-dependent chromatin remodeling factors at some target genes. In addition to control by reversible phosphorylation, output of the RB pathway can also be influenced by modulating the abundance of RB through transcriptional and post-transcriptional mechanisms (26). *In vivo* RB transcription is cell cycle independent, however steady state abundance of RB-mRNA is governed through autoregulatory feedback circuits in a highly cell and tissue specific manner (27).

Figure 1-1. Regulation of RB-E2F pathway during cell cycle progression

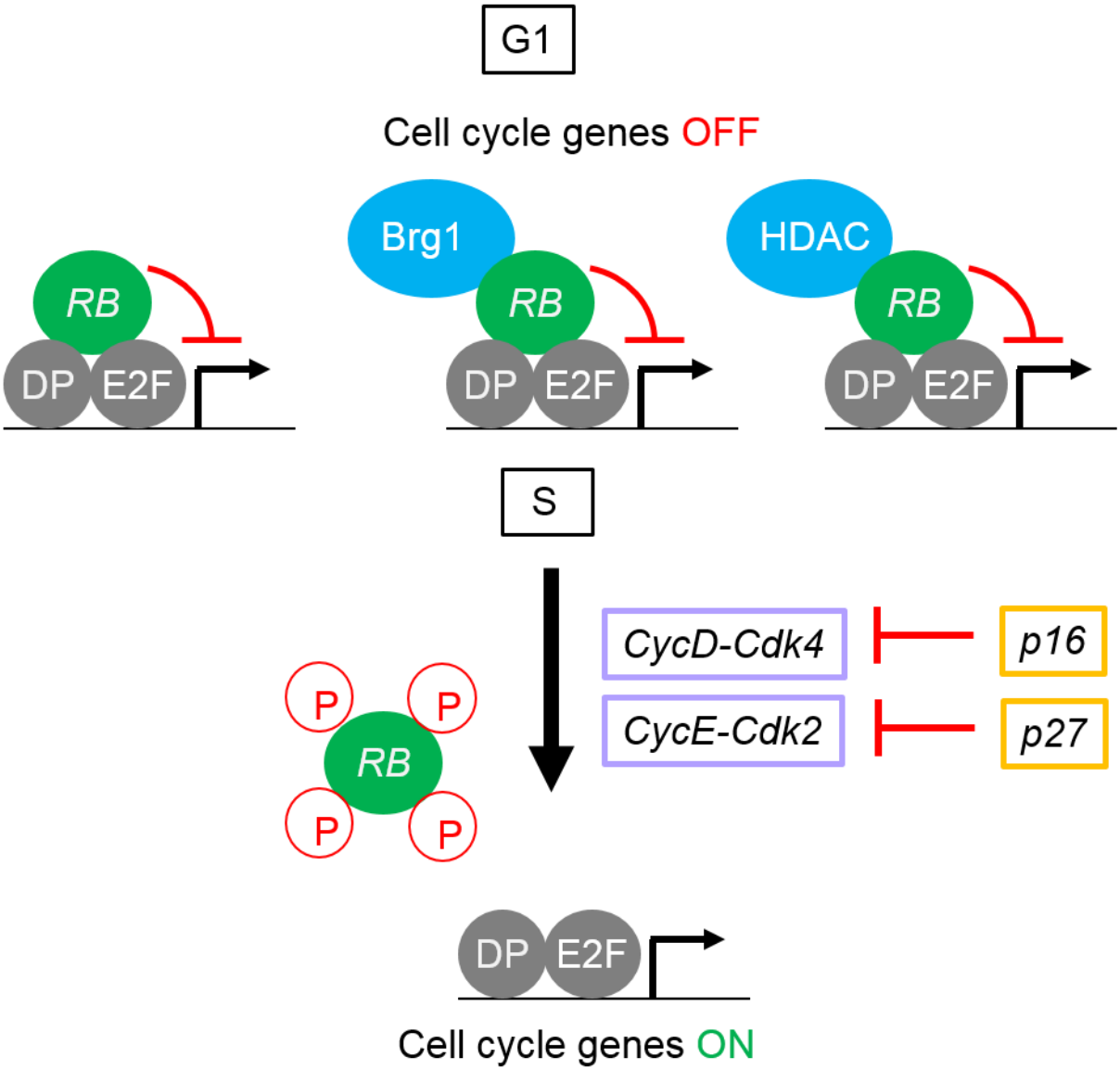

Figure 1-1 (cont'd)

During G1, RB is actively engaged in repression of cell cycle genes (OFF) by binding to, and inhibiting E2F-DP heterodimer. RB mediated transcriptional repression also depends upon interaction with chromatin remodelers (e.g., Brg1) and chromatin modifying enzymes (e.g., HDAC). During S-phase, multisite phosphorylation mediated by Cyclin D/Cdk4 and Cyclin E/Cdk2 disengages RB from E2F-DP and promotes transcription of cell cycle genes (ON). Cyclin D/Cdk4 and Cyclin E/Cdk2 activity is inhibited by Cyclin-dependent-Kinase Inhibitors (CKIs) p16 and p27 respectively.

In contrast, p107 transcription is cell cycle dependent and also subjected to cell type specific regulatory networks that dictates transcriptional output (28, 29). Oncogenic mutations resulting in diminished RB transcription have been found in many cancer cell types (30, 31). RB family abundance is also dictated post-transcriptionally through regulation of protein stability by the ubiquitin-proteasome system and contributes to cell cycle coupled fluctuations RB levels (32). Inappropriate degradation of RB proteins through the proteasome is also associated with cellular transformation driven by viral oncogenes (33).

Ubiquitin-Proteasome mediated regulation of RB family proteins

Polyubiquitination of intracellular proteins directs them for proteasome mediated degradation (126). Unhindered progression through cell cycle relies on timely degradation of mitotic and G1 cyclins by the ubiquitin-proteasome system (35). In addition, the proteasome is also implicated in degradation of RB and E2F family members. While unified by their ability to repress E2F dependent transcription, RB, p107 and p130 exhibit distinct expression patterns during cell cycle progression indicating that these factors are regulated through additional mechanisms governing their steady state levels. Data from cell cycle block and release studies demonstrated that p130 is abundantly expressed in quiescent cells, while both RB and p107 are maintained at lower steady state levels (32, 35, 36). As cells progress through G1 to S phase, p107 and RB are expressed at higher levels, as p130 steady state levels plummet. The best experimental support for the ubiquitin-proteasome system in these processes was initially noted for p130 turnover involving the SCFSkp2 E3 ubiquitin ligase complex (32, 36). In this process, p130 regulation is sensitive to at least two conditions. First, early in cell cycle progression Skp2 activity is rate-limiting for p130 degradation, but upon serum stimulation and progression into S phase, Skp2 levels accumulate (37-39). Secondly, Cdk4 phosphorylates p130 to trigger subsequent ubiquitination and

proteasome-mediated destruction (32). The timely destruction of p130 during G1 and S phase has important consequences for activation of gene expression programs during cell cycle progression. Firstly, destabilization of p130 during G1-S transition ensures transcriptional activation of activator E2Fs (E2F1-E2F3), whose expression is repressed by p130-E2F4 complexes (40, 41). Secondly, as p130 binds to and inactivates Cdk2 (42), its degradation also ensures activation of Cdk2 that allows progression to S phase. It is interesting that during G1-S transition p130 turnover is mediated by the same E3 ligase complex employed for turnover of the p27 Cdk2 inhibitor, reinforcing the timely activation of Cdk2 for coordinated cell cycle progression (43). As noted for p130, p107 also exhibits a strong linkage between cell cycle progression and turnover, but in a pattern that was inversely correlated with p130 behavior (32, 35, 36). In this case, p107 turnover occurs during the point wherein it is engaged in gene repression, suggesting that p107 potency is linked to its modification and destruction by the ubiquitin-proteasome system. Consistent with this notion, studies using the Cdk4-specific inhibitor PD-0332991, a cytostatic drug that causes a robust G1 arrest demonstrated that the hypo-phosphorylated and active form of p107 is degraded by the proteasome (44). Earlier studies had reported that p107 levels are unaffected by treatment with proteasome inhibitors. However, in those studies, the effect of proteasome inhibition may have been obscured because MG132 treatment was carried out in cells that have entered into S-phase, a period wherein p107 is phosphorylated and refractory to proteasome-mediated turnover (45). Our studies suggest that dual control of p107 stability and activity is rendered by a C-terminal instability motif which functions as a phosphorylation sensitive degron to influence protein turnover and a repression domain that mediates molecular contacts with E2F to mediate gene repression (46). The instability element is evolutionarily conserved and related sequences are clearly found in Drosophila Rbf1 and human p130 (46, 47). While divergent at the amino acid

level, functionally related features linking activity and proteasome-mediated turnover are also observed in regions of the human RB C-terminal domain previously demonstrated to mediate E2F/DP contacts (46). This correlation led us to propose a model for the RB family in which signaling pathways converge on common motifs to simultaneously regulate protein longevity and activity.

In contrast with both p107 and p130, which exhibit robust changes in steady state levels during cell cycle progression, RB fluctuations are muted, and the connection between RB degradation during cell cycle control is less well understood (32). Nonetheless, in some disease contexts, RB stability is intimately connected to p16 mediated cell cycle arrest, but in somewhat unexpected ways. RB is anchored to the nuclear matrix by a trimeric complex formed by Lamin-A/C and LAP2α (48). Disruptions in RB anchoring either in cells lacking Lamin-A function or via LAP2α depletion are associated with a marked reduction in endogenous RB levels (49, 50). Moreover, Lamin-A deficient cells are also insensitive to $p14^{arf}$ and $p16^{arf}$ mediated arrest potentially due to inappropriate RB destruction (50). It has been speculated that diminution of RB may also have implications for progressive muscular dystrophy in a mouse models and in patients with laminopathies. Deletion mutations in the Lamin-A gene are widely implicated in Hutchison-Gilford-Progeria (HGP) syndrome (51). Recent study suggests that levels of RB protein decrease in fibroblasts from HGP patients, but whether this is due altered stability remains to be determined (52).

Additional evidence supports a role for the ubiquitin-proteasome system in RB control in response to DNA damage. Firstly, several lines of evidence suggest that the Mdm2 E3-ligase physically interacts with RB to promote its degradation (53, 54, 55). Human Mdm2 is frequently amplified in many cancers (56), suggesting that deregulated RB turnover may contribute to

tumorigenesis. In support of Mdm2-mediated RB degradation as a putative oncogenic mechanism, an inverse correlation between Mdm2 and RB protein expression has been found in non-small cell lung cancer (NSCLC) tumors that do not exhibit loss-of-heterozygosity (LOH) for RB (55). The physiological significance of this regulation is further underscored by the observation that Mdm2 overexpression disrupts the formation of RB-E2F complexes (53). Secondly, in cells that harbor genomic amplification of Mdm2, depletion of Mdm2 inhibits DNA replication in a RB-dependent manner (54). This is consistent with previous studies showing that oncogenicity of Mdm2 relies in part on its S-phase promoting function that is independent of p53. Evidence to date further suggests that RB degradation may involve two distinct mechanisms. In one model, Mdm2 interacts with endogenous RB, and promotes its ubiquitination in a process that is dependent on the E3 ligase activity of the Ring finger domain of Mdm2 (55). In this study, RB-Mdm2 interactions and RB ubiquitination were also dependent on the C terminal domain of RB harboring the instability element (46). A second model suggests Mdm2-mediated degradation of RB involves ubiquitin independent degradation by the 20S proteasome (54). In this scenario, Mdm2 bound to RB and promoted an interaction between RB and C8-α subunit of the 20S proteasome, which allows for efficient tethering of RB to the 20S complex thereby facilitating its degradation. Even though all three pocket domain proteins can interact with this E3 ligase, Mdm2 driven ubiquitination appears specific for RB (55). Nonetheless, Mdm2 mediated control of RB family stability and function may also extend to p107 as Mdm2 overexpression enables p53 null cells to overcome p107 mediated G1 arrest (57), although the molecular details are currently unknown. Recent studies suggest that RB stability is also regulated by MDMX, a structural homolog of MDM2 that is deficient for E3 ligase activity (58). MdmX enhances RB-Mdm2 interaction to efficiently target RB for proteasome mediated degradation and inhibits RB mediated transcriptional repression (59).

In vivo ablation of MdmX in highly tumorigenic p53-null cells results in an RB-dependent reduction of tumor growth in mouse xenograft assays, suggesting that MdmX mediated RB degradation is potentially oncogenic (59). Dual regulation by MdmX and Mdm2 have also been implicated in regulation of p53 stability and transactivation (60). Collectively these studies suggest that the Mdm2-MdmX axis functions as negative regulators of RB and p53 tumor suppressor pathways.

Regulation of RB protein stability by viral oncogenes

Inactivation of tumor suppressor pathways is a hallmark of viral oncogene induced transformation. Replication of viral genome is heavily reliant on sustained availability of host cell replication factors which are abundant only during S phase, and remain transcriptionally repressed by RB family proteins during the remainder of the cell cycle. Thus disruption of RB function by viral oncogenes provides a milieu amenable for viral replication. Indeed, early works from the Harlow (61) and Nevins lab (62) showed that oncoproteins derived from DNA tumor viruses such as Adenoviral E1A and Human Papilloma virus E7 (HPV-E7) binds to RB, sequestering it away from E2F and thereby unleashing E2F transactivation potential needed for cellular transformation. Interestingly, several E7 mutants deficient for RB sequestration retained transformation potential, suggesting that an additional E7 function is needed to harness oncogenic potential (63-66). This additional function of E7 required for cellular transformation was defined as its ability to trigger ubiquitin-mediated degradation of RB (67). Structural (9) and biochemical studies (33) suggest that the LXCXE motif in E7 interacts with the RB-pocket, and these contacts are crucial for E7 directed RB degradation. Mechanistically, RB bound E7 forms a complex with Cullin2-Rbx1-Elongin B/C to constitute an active E3 ligase that directs proteasome dependent RB degradation (68). ZER1, a Cullin-2 substrate specificity factor mediates the interaction between E7 and the

Cullin-2 E3-complex, and this bridging function of ZER1 is necessary for HPV-E7 mediated RB destabilization (69).

In addition to RB, p107 and p130 are also targeted by E7 for proteasome mediated degradation (33), with consequent effects on cell proliferation. The maintenance of cellular quiescence during G0 is achieved by repression of cell cycle genes by the DREAM complex, comprised of p130, E2F4, DP1 and the MuvB core (70, 71). E7 mediated degradation of p130 prevents the formation of DREAM repressor, leading to sustained activation of S phase genes that allows cell to escape growth arrest and sustain proliferation (72). A similar of strategy of degrading cellular RB in order to gain control over host cell machinery is employed by RNA viruses such as hepatitis C virus (HCV). In HCV infected cells, a LXCXE-containing viral RNA-polymerase binds to RB in the cytoplasm and recruits E6-associated-protein (E6AP) E3-ligase to promote RB degradation (73, 74). Together these studies suggest that invoking proteasome mediated degradation of RB family proteins is a commonly employed mechanism for oncogenesis.

Regulation of E2F1 stability by the ubiquitin-proteasome system

Similar to RB family proteins, the levels of E2F transcription factors are also tightly regulated through transcription dependent (41, 75, 76) and independent mechanisms (77, 78). Post-transcriptional regulation of E2F abundance through proteasome mediated turnover is an essential feature of eukaryotic cell cycle control during development and adulthood (79, 80, 81). Most of our understanding pertaining to E2F turnover is derived from studies involving E2F1, the major activator E2F which serves as a dual regulator of cell proliferation and apoptosis (7, 82). E2F1 protein is most abundant during late-G1 consistent with its role in activation of S phase genes, however at the onset of S/G2 phase E2F1 levels decline rapidly, as it gets richly ubiquitinated and targeted to the proteasome (79, 83, 84). E2F1 protein is extremely unstable due to the presence of

an autonomously acting C-terminal degron (85, 86). Interestingly the E2F1 degron sequence overlaps with the RB binding motif located within its transactivation domain, suggesting that RB binding could potentially occlude the usage of the degron, thereby preventing E2F1 degradation (85, 86, 87). Indeed overexpression of RB stabilizes E2F1 by preventing its proteosomal degradation, and mutant forms of RB that are unable to bind E2F1 also fail to stabilize E2F1. Together these studies suggest that during G1, target gene repression is achieved by stable RB-E2F1 complexes, whereas during S-phase as RB-E2F complexes disengage, free E2F molecules are rapidly degraded. Cell cycle coupled E2F1 turnover is achieved through temporal recruitment of distinct E3 ligases. For example, during S phase, E2F1 is degraded by the SCF^{skp2} E3-ligase complex (83) whereas during early mitosis its degradation is mediated by Anaphase Promoting Complex/C (APC/C^{cdc20}) E3-ligase (84).

Insight into the physiological significance of cell-cycle associated changes in E2F1 stability is obtained from genetic analysis of S-phase coupled degradation of Drosophila E2F1 (dE2F1). dE2F1 harbors a degron sequence known as PCNA Interacting Protein (PIP) box, which in replicating cells enables E2F1 degradation through recruitment of $Cul4^{Cdt2}$-E3 ligase (88). Transgenic overexpression of a stable allele of *d*E2F1 that is refractory to degradation during S-phase, results in accelerated progression through cell cycle and induces severe apoptosis through transcription dependent and independent mechanisms (88, 89). Interestingly, in a genetic background muted for apoptotic response, stabilized E2F1 results in hyperproliferation, suggesting that apoptotic clearance of cells expressing aberrant levels of E2F1 enables a check on inappropriate cellular proliferation (89). Together, these observations suggests that *in vivo*, S-phase coupled E2F1 degradation limits inappropriate gene expression and is critical for maintenance of tissue homeostasis. It is important to note that regulation of E2F1 stability is highly

context specific. Whereas E2F1 is degraded during S-phase, its levels rapidly increase following DNA damage as a result of altered protein stability (82, 90, 91). In this context stabilized E2F1 is critical for efficient induction of proapoptotic genes.

Regulation of the ubiquitin-proteasome system by RB proteins

As discussed in the previous sections, components of the RB axis are highly regulated by the ubiquitin-proteasome system. Interestingly, RB family members in turn regulate components of the ubiquitin machinery through transcriptional and posttranscriptional mechanisms to affect cellular proliferation and tumor suppression. In particular, most studies have focused on RB-dependent regulation of Skp2, the F-box component of the SCF^{Skp2}-E3 ligase complex that regulates the stability of p130 (32) and the Cdk-inhibitor p27 (43). Skp2 abundance is cell cycle dependent (37-39). Growth arrest leads to diminished Skp2 levels, whereas its expression is induced upon active proliferation, enabling the formation of catalytically active SCF^{Skp2} complexes. Consistently, p27 is stabilized during quiescence, resulting in RB dephosphorylation and growth arrest, whereas during S-phase p27 is destabilized through SCF^{Skp2} (92), resulting in RB inactivation and cellular proliferation. RB binds to the N-terminus of Skp2 and disrupts Skp2-p27 interaction, resulting in p27 degradation and G1 arrest (93). In addition, RB also interacts with the catalytically active Anaphase promoting complex/cyclosome (APC/C), a multisubunit E3 ligase involved in targeted destruction of proteins at the G1/S boundary and mitosis (94). Interestingly, RB-APC/C association doesn't affect RB stability, but rather provides a scaffold to recruit Skp2 and facilitate its degradation. RB interacts with Skp2 and APC through distinct surfaces and it is highly likely that a trimeric complex formed by RB-Skp2-APC is required for effective stabilization of p27 during cell cycle arrest. In addition to regulating Skp2 stability and activity, RB is also involved in direct repression of Skp2 transcription in growth arrested cells (95,

96). RB heterozygous (RB +/-) mice develops pituitary tumors with elevated Skp2 and diminished p27 levels (97). However, pituitary specific loss of Skp2, or introduction a stable p27 allele, completely abolishes pituitary tumors in RB+/- mice, thereby attesting to the notion that RB mediated regulation of Skp2 abundance and activity serves a tumor suppressive function (97-99). Furthermore, RB depleted human cone precursor cells and retinoblastoma cell lines exhibits similar reliance on RB-Skp2-p27 axis for survival and proliferation (97, 100). Like RB, p107 promotes Skp2 turnover resulting in p27 stabilization and cell cycle arrest, however using mechanisms that are distinct from RB induced Skp2 destabilization (101). The p107-Skp2-p27 axis is also tumor suppressive, as loss of p107 in RB null mice increases Skp2 abundance, resulting in diminished p27 and heightened CDK2 activity that leads to the formation of retinoblastoma (102). Together these studies suggest that RB mediated regulation of SCF^{Skp2}-E3 ligase complex is an important regulatory feature of mammalian cell cycle control and supports the idea of crosstalk between these diverse regulatory modules.

Roles for ubiquitin-proteasome in RB-E2F mediated gene regulation

In addition to its function in controlling timely degradation of several transcription factors including RB and E2F, the ubiquitin-proteasome system is also directly involved in transcriptional regulation (103). Ubiquitination of several transcriptional activators promotes their activation potency (104, 105). Interestingly, these activator proteins show an intimate sequence overlap between domains involved in transactivation and degradation (104). Together these observations suggests a model where ubiquitination within this overlapping region may additionally function in transactivation by promoting recruitment of transcriptional cofactors (103, 106).

The following lines of evidence suggest that the ubiquitin-proteasome system may similarly be involved in regulating transcriptional output from the RB-E2F pathway. *Firstly,* in a

series of recent work we showed that domains involved in regulating RB family stability through ubiquitin-proteasome, are also involved in mediating transcriptional repression (46, 47). Consistently, deletion of this region both stabilizes the repressor (RB) and renders it ineffective for transcriptional repression, suggesting that ubiquitin mediated proteolysis may positively contribute towards gene repression. In support of this idea, ubiquitination of *D*rosophila Rbf1 potentiates repression of target genes (Figure 1-2 B), although the exact role for ubiquitin in this process remains unknown (107). *Secondly,* the RB-E2F pathway is subjected to regulation by COP9 signalosome (CSN), an evolutionarily conserved octameric protein complex that regulates the assembly of Skp1-Cullin1-Fbox (SCF)-E3 ligase (108). During embryonic development *D*rosophila RB homologs Rbf1 and Rbf2 are stabilized through interaction with COP9 signalosome (CSN) (109). Interestingly, Rbf1-CSN interaction is evident on chromatin and suggests that CSN may function as a corepressor by stabilizing chromatin-bound Rbf1 (Figure 1-2 A). Attesting to CSN corepressor function, there is a substantial overlap between genomic regions bound by Rbf1 and CSN7 subunit of the signalosome (110). Furthermore, CSN regulates transcription of E2F dependent cell cycle genes (110, 111) probably by facilitating E2F1 degradation by the Culin4-E3 ligase (112). Indeed in the absence of CSN, E2F1 is stabilized (112) but surprisingly rendered inert for transcriptional activation of its target genes (110, 111), suggesting that ubiquitin mediated proteolysis may positively contribute to transactivation. Consistent with this notion, CSN5 mediated ubiquitination and proteolysis of Myc activator protein positively regulates its transcriptional potency (113). Yet another involvement of CSN in gene regulation is reflected in its role as a specificity factor for E2F dependent transcription of apoptotic genes (114-117).

Figure 1-2. Regulation of RB-E2F dependent transcription by the ubiquitin-proteasome system.

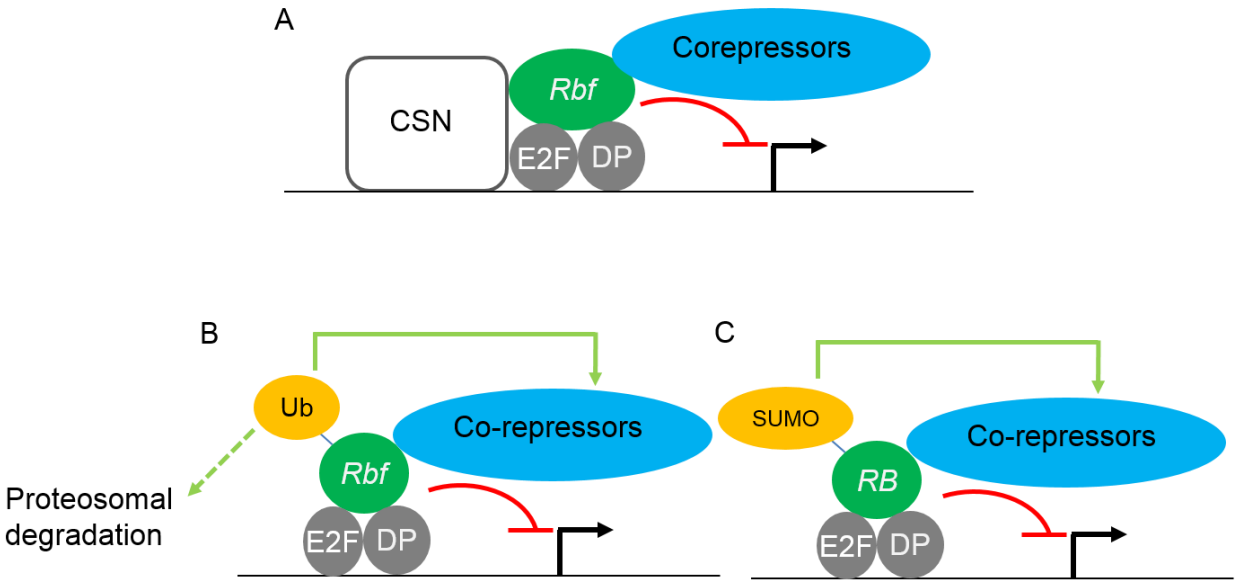

Figure 1-2 (cont'd)

(A) Chromatin bound *D*rosophila Rbf1 is stabilized by COP9 signalosome (CSN) and may enable appropriate gene expression program during embryonic development. **(B)** A proposed model depicting that ubiquitin (Ub) attached to *D*rosophila Rbf1 may engage in both targeting Rbf1 for proteosomal degradation (broken green arrow), and in enhancing Rbf1 repression by promoting Rbf1 interaction with co-repressors (solid green arrow). **(C)** Hypophosphorylated RB is SUMOylated and the SUMO tag may promote RB interaction with transcriptional co-repressors to facilitate repression.

Consistently, CSN5 binds to E2F1 target genes involved in apoptosis and is absent at promoters of replication genes (117). Intriguingly, in this context the transcriptional function of CSN5 is independent of its deneddylase activity, suggesting that CSN contributes to transcriptional activation through non-proteolytic mechanisms (115). One such possible mechanism may involve CSN5 mediated disruption of the interaction between RB-C terminus and E2F1 marked box domain that specifically inhibits transcription of apoptotic genes (11, 14). Alternatively CSN5 may be involved in recruiting coactivators needed for transcriptional activation. *Thirdly*, RB-E2F mediated gene regulation is reliant on chromatin remodelers that affect histone H2B ubiquitination, a chromatin mark associated with active transcription (121). RB and E2F interact with two distinct remodelling complexes, Brg-BAF250a containing SWI-SNF-A, and Brm-BAF250b containing SWI-SNF-B (118, 119). During quiescence, both complexes are required to repress E2F target genes, whereas only BAF250b containing complex remain bound during proliferation, suggesting an activator function for this complex (120). BAF250b via its BC-box interacts with ElonginC-Cullin2-Roc1 E3-ligase to promote H2B ubiquitination and activate transcription. Consistently, cells devoid of BAF250b exhibit delayed induction of cell cycle genes upon exit from quiescence (120). Together, these studies suggest that appropriate induction of E2F target genes may rely on chromatin modifications driven by E3-ubiquitin ligases.

Finally, in addition to ubiquitination, RB is also posttranslationally modified by conjugation of Small-Ubiquitin-Like-Modification (SUMOylation) catalyzed by various classes of SUMO-E3 ligases (122, 123). Hypophosphorylated RB is SUMOylated in the pocket domain that enhances its potency for transcriptional repression (122). In this context, the SUMO tag on RB may promote its interaction with transcriptional co-repressors to facilitate repression (Figure 1-2 C) (125). Moreover, overexpression of SUMO-E3 ligases induce a senescent phenotype that

is reliant on a functional RB pathway (123, 124). Taken together, these studies suggest that the ubiquitin-proteasome system plays diverse roles in regulation RB-E2F dependent transcriptional control.

Summary

In addition to regulation by reversible phosphorylation, RB-E2F pathway is governed by the ubiquitin-proteasome system, and this mode of governance significantly contributes towards RB-E2F functions during development and disease. RB regulation through both of these mechanisms involve hierarchical control by upstream regulators, and there is substantial crosstalk between these regulatory schemes. Continuing work from our lab has characterized the molecular pathway involved in ubiquitin-proteasome mediated regulation of *D*rosophila Rbf1, and its implication for Rbf function in development and gene regulation. However such detailed understanding of mammalian RB regulation by the ubiquitin-proteasome system is missing. In chapter II I present and discuss my findings pertaining to molecular mechanisms involved in regulating mammalian RB family stability and their functional significance. In chapter III I discuss the implications of this work, and summarize ongoing and future experiments that aim at better understanding of RB regulation by the ubiquitin-proteasome system.

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CHAPTER 2: THE EVOLUTIONARILY CONSERVED C-TERMINAL DOMAINS IN THE MAMMALIAN RETINOBLASTOMA FAMILY SERVE AS DUAL REGULATORS OF PROTEIN STABILITY AND TRANSCRIPTIONAL POTENCY[1]

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Abstract

The Retinoblastoma (RB) tumor suppressor and related family of proteins play critical roles in development through their regulation of genes involved in cell fate. Multiple regulatory pathways impact RB function, including the ubiquitin-proteasome system with deregulated RB destruction frequently associated with pathogenesis. With the current study, we explored the mechanisms connecting proteasome-mediated turnover of the RB family to the regulation of repressor activity. We find that steady state levels of all RB family members, RB, p107, and p130 were diminished during embryonic stem (ES) cell differentiation concomitant with their target gene acquisition. Proteasome-dependent turnover of the RB family is mediated by distinct and autonomously acting instability elements (IE) located in their C-terminal regulatory domains in a process that is sensitive to Cyclin-dependent kinase (CDK4) perturbation. The IE regions include motifs that contribute to E2F/DP transcription factor interaction, and consistently, p107 and p130 repressor potency was reduced by IE deletion. The juxtaposition of degron sequences and E2F interaction motifs appears to be a conserved feature across the RB family, suggesting the potential for repressor ubiquitination and specific target gene regulation. These findings establish a mechanistic link between regulation of RB family repressor potency and the ubiquitin-proteasome system.

Introduction

The Retinoblastoma (RB) tumor suppressor regulates cell fate through its governance of distinct gene sets that promote cell division, differentiation, and apoptosis (1,2). RB is related to two other family members, p107 and p130 that share substantial structural conservation along with some overlapping function in target gene regulation (3-6). While not as tightly linked to tumor suppression as RB, tumor suppressive capacity has been assigned to these other family members in some contexts. In mouse studies, RB deficient mice were prone to pituitary tumor formation (7), whereas mice deficient for RB and p107 or p130 developed retinoblastoma (8,9), suggesting that p107 and p130 can influence tissue specific predisposition towards tumor development. Similarly, conditional loss of p130 in adult lung epithelial cells in a $RB^{-/-}/p53^{-/-}$ null background enhanced development of small cell lung carcinoma (10). Thus, all three RB family members can act as tumor suppressors in specific contexts.

In their roles as tumor suppressors, RB family members predominately function as transcriptional repressors of target genes through their antagonism of the E2F/DP family of transcription factors (11,12). Recent evidence suggests that RB also plays a positive role in transcriptional activation of some pro-apoptotic response genes, again in a mechanism requiring E2F activity (13), although RB may also induce apoptosis through a mechanism that is independent of transcription (14). In this scenario, RB-mediated tumor suppression is enabled through blockade of gene products necessary for cell growth with concomitant invocation of cell death pathways. As key regulators of cell fate, the RB family is tightly controlled by CDK-mediated phosphorylation in response to environmental conditions (15-17). Hypo-phosphorylated RB interacts with E2F1/DP1 (18), blocking activated transcription of cell cycle genes involved in DNA replication and S-phase progression (11,12). In response to mitogenic signals, serial

phosphorylation via cyclin D-CDK 4/6 and cyclin E-CDK2 renders RB family members inactive by disengaging their association with E2F complexes (19-22). Cyclin-CDK activity is also critically regulated during early steps in normal embryonic development (23). Rapidly dividing pluripotent embryonic stem (ES) cells of the early developing embryo employ constitutive CDK-mediated inhibition of RB proteins as a mechanism to maintain rapid cell division during blastocyst formation (24-26). As ES cells differentiate, CDK activity plummets, allowing RB family proteins to regulate E2F activity in a cell cycle dependent manner (26,27). Despite this unifying model for cyclin-CDK regulation, there are significant differences in the coordination of RB family member activities and steady state levels. For example, RB and p107 are active in cycling cells, while p130 functions predominately in quiescent cells that have exited from the cell cycle. Experiments performed with staged cells showed that steady state p130 levels peak in G0 in contrast to RB and p107 that increase as cells progress through G1 (28,29). Consistently, CDK4 activity has opposite effects on p107 and p130 steady state levels; inhibition of the enzyme leads to diminished levels of p107 and higher levels of p130 (30). Thus, RB family member activity and stability clearly respond differently to cyclin-CDK signaling during cell cycle progression. However, the mechanisms that link regulation of RB family activity to their turnover are poorly understood.

Previous studies from our lab showed that the Drosophila melanogaster RB homologue Rbf1 is subjected to proteasome mediated turnover during embryonic development (31,32). We further demonstrated that Rbf1 turnover is influenced by an "instability element" (IE) located within its C-terminal regulatory domain. Importantly, the IE region is also critical for full repressor potency for some cell cycle regulated genes, but not for non-canonical targets whose expression is not usually integrated with the cell cycle (31,33). Interestingly, Rbf1 ubiquitination also enhanced

specific activity at select cell cycle target genes (33), suggesting that the potency of the repressor at specific genes and overall Rbf1 stability are coordinated. The IE region is well conserved within the mammalian p107 and p130 factors, and we hypothesized that the activity of mammalian RB family members may also be coordinated via integration of the cyclin-CDK signaling pathway with the ubiquitin-proteasome system. We demonstrate here that this regulatory mechanism is indeed shared among the human RB family proteins. The IE regions within the RB, p107, and p130 C-terminal domains negatively regulate repressor stability through a cyclin-CDK responsive proteasome dependent pathway and contribute to effective gene repression. These findings indicate that an evolutionarily conserved regulatory pathway links stability and potency for the mammalian RB family.

Materials and Methods

Expression Constructs **-** Expression plasmids encoding mutant forms of human RB, p107 and p130 were obtained by site-directed mutagenesis of the pCMV-GFP-RB, pCMV-GFP-p107, pCMV-GFP-p130 parental plasmids (34). To generate GFP fusion proteins, PCR amplified instability elements from RB (residues 786-864), p107 (residues 964-1024), and p130 (residues 1035-1095) were fused in frame between the HindIII and KpnI sites of pEGFP-C3 (Clontech). All plasmids were verified by sequencing for the desired mutation.

ES cell culture, differentiation, and immunofluorescence **-** Mouse R1 ES cells were obtained from American Type Culture Collection (ATCC, Manassas, VA), and were cultured on mitomycin-treated mouse embryonic fibroblasts (MEFs) in medium containing high-glucose DMEM supplemented with fetal calf serum (FCS), LIF, L-glutamine, nonessential amino acids, and β-mercaptoethanol. J1-ES cells and the RB-/-, p107-/-, p130-/- triple knock out (TKO) ES cells were a kind gift from Julien Sage (35). For ES cell differentiation, cells were plated on

gelatin-coated plates to eliminate contaminating MEFs. Differentiation was induced by growing cells in presence of 10 µM retinoic acid (R2625, Sigma) for 72h. Control cells were treated with DMSO for similar time. For immunofluorescence analysis, ES cells were grown on lab-tek II chamber slides (Nalge Nunc International, Naperville, IL) under similar conditions and differentiation was induced as discussed above. Cells were fixed in 3.7% freshly made paraformaldehyde for 20 min, washed three times in wash buffer (phosphate buffered saline (PBS) pH 7.4, 0.1% BSA, and 0.01% Tween-20). Cells were permeabilized in PBS containing 0.1% Triton X-100 for 15 min, washed, and blocked for one hour at RT in blocking solution (PBS pH 7.4, 1% BSA and 0.01% Tween 20). Cells were incubated in primary antibody against anti-RB (G3245, mouse monoclonal, 1:100, BD Pharmingen), anti-p107 (SC-318, rabbit polyclonal, 1:100, Santa Cruz Biotechnology), or anti-p130 (SC-317, rabbit polyclonal, 1:100, Santa Cruz Biotechnology) in blocking buffer either overnight at 4^{o}C (Fig 1A-C) or for 2 h at room temperature (Fig 2-1 D-F). Following three washes in wash buffer, cells were incubated in secondary antibody (Alexa Fluor 488 Goat anti-rabbit-A11008, Alexa Fluor 488 Goat anti-mouse-A11001, Life technologies, Carlsbad, CA) for one hour. Following three washes in wash buffer, slides were mounted with Vectashield mounting media containing DAPI (Vector Laboratories Inc., Burlingame, CA). Images were obtained using an Olympus Fluoview 1000 Filter-based laser scanning confocal microscope.

Chromatin Immunoprecipitation **-** ES cells were grown on T-125 flasks and treated with either DMSO or 10 µM RA (R2625, Sigma) for 3 days. Cells were washed in PBS, trypsinized, suspended in DMEM and cross-linked with 1% formaldehyde for 18h at 4°C. Cells were then pelleted, washed with PBS and flash frozen in liquid nitrogen. Soluble chromatin was prepared as previously described (36). Chromatin bound protein complexes were immunoprecipitated in low

salt buffer (20mM Tris HCl, pH 8.1, 2mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X 100) using 5µg of anti-RB (G3245, BD) or anti-p107 (SC-318) or anti-p130 (SC-317) or 5 µg of rabbit non-specific IgG (Millipore). Chromatin-antibody complexes were isolated using 50 µl protein G Dynabeads (Life technologies, Carlsbad, CA). Beads were washed once each in low salt buffer, high salt buffer (20 mM Tris HCl, pH 8.1, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X 100), LiCl buffer (10 mM Tris HCl pH 8.1, 1 mM EDTA, 250 mM LiCl, 1% deoxycholate, 1% IGEPAL630), and twice with TE buffer (10mM Tris HCl, pH 8.1, 1mM EDTA). Chromatin-antibody complexes were eluted from the beads in 200 µl of elution buffer (100 mM $NaHCO_3$, 1% SDS) at 65°C for 30 min with occasional vortexing. Crosslinking was reversed by addition of 8 µl of 5 M NaCl and incubation overnight at 65°C. Extracts were then treated with 1 µl of RNase A (10 mg/ml) for 1h. Subsequently, 13 µl of Proteinase K buffer (8 µl 1M Tris pH 6.8, 4 µl 0.5M EDTA and 1 µl Proteinase K (10 mg/ml) was added and samples were incubated for an additional 1.5 h at 45°C. Associated DNA was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). Quantitative real-time PCR was performed on input DNA, and antibody specific ChIP DNA using SYBR Green Master Mix reagents with an ABI Step one plus thermocycler (Applied Biosystems, Foster City, CA) detection system. Enrichment of RB family members at target gene promoters was examined using primers spanning known E2F binding sites at the murine *CCNA2* and *MCM10* loci. An intergenic region on mouse chromosome 6 was used as a negative control. Primer sequences are as follows: *CCNA2-F:* AATAG TCGCGGGCTACTTGA; *CCNA2-R:* GAGCG TAGAGCCCAGGAG; *MCM10-F:* AGCGTC CTCCACAAATGAAC; *MCM10-R:* ACCCCG TGACGCTTACCTA; *Intergenic mouse chr6F:* TTTTCAGTT CACACATATAAAGCAGA; *Intergenic mouse chr6R:* TGTT GTTGTTGTT GCTTCACTG.

RNA extraction and gene expression analysis using quantitative real time PCR – Pluripotent or differentiated ES cells were harvested, snap frozen and stored at -80°C. RNA was extracted using RNeasy Mini kit (Qiagen, Valencia, CA) according to manufacturer's instruction. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Quantitative real-time PCR for RB, p107, and p130 was performed using gene specific primers (10)) and SYBR Green Master Mix reagents with an ABI Step one plus thermocycler (Applied Biosystems, Foster City, CA) detection system. Primers sequences are as follows: *RB-F*: GCTTGGCTAACTTGGGAG; *RB-R*: CAACTGCTGCGATAAAGATG; *p107-F*: CCGAAGCCCTGGATGACTT; *p107-R*: GCATGCCAGCCAGTGTATAACTT; *p130-F*: AAGGCACATGCTAACCAATGAA; *p130-R*: GAGCAGTTACCGCAGCATGA. Transcript levels were measured using Taqman probes (Applied Biosystems, Foster City, CA) for Pou5f1 (Mm03053917_g1), Nanog (Mm02019550_s1), and the Eukaryotic elongation factor 1a1 (Mm01966122_u1) as an endogenous control. Relative gene expression was measured by the $2^{-\Delta\Delta C_T}$ method (37).

Human cell culture, transfection, and drug treatments - To determine the effect of CDK4 inhibition on endogenous RB family stability, approximately 5 x 10^5 U2OS cells were grown for 48h in DMEM containing 10% fetal bovine serum and penicillin-streptomycin. Cells were then treated with 1 μM PD0332991 (Selleck Chemicals, Houston, TX), and were cultured for an additional 24 hours with or without 1 μM MG132 for the last 6h. Cells were harvested and the pellet was snap frozen in liquid nitrogen. Cell extracts were prepared in lysis buffer (50mM Tris HCl pH 8.0, 150mM NaCl, 1% Triton X-100) and the total protein concentration determined using Bradford assay. Equal amounts of whole cell extracts (50 μg) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes for Western analyses. Endogenous proteins

were detected by using the following antibodies: anti-RB (G3245, mouse monoclonal, 1:1,000, BD Pharmingen), anti-p107 (SC-318, rabbit polyclonal, 1:1,000, Santa Cruz Biotechnology), anti-p130 (610261, mouse monoclonal, 1:1000, BD Bioscience), anti-tubulin (mouse monoclonal, 1:20,000, Iowa Hybridoma Bank), and anti-actin (A5441, mouse monoclonal, 1:10,000, Sigma). In Figure 2-2B, p130 was detected using the rabbit polyclonal antibody (SC-317, rabbit polyclonal, Santa Cruz Biotechnology). All antibody incubations were performed in 5% milk in TBST (20 mM Tris HCl, pH 7.5, 120 mM NaCl, 0.1% Tween-20). Blots were developed using peroxidase conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, as appropriate (1:5000, Thermo Scientific, Waltham, MA) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA). To measure the effect of CDK4 inhibition on recombinant RB family proteins, approximately 5 x 10^5 U2OS cells were cultured for 24 h prior to transfection with GFP-tagged full-length or ΔIE mutant constructs using Nanojuice transfection reagent (Novagen, EMD Chemicals, San Diego, CA). 24 hours after transfection, cells were treated with 1 μM PD0332991 for an additional 24 hours. Western analysis was performed, as above, using anti-GFP antibodies (SC-9996, mouse monoclonal, 1:1,000, Santa Cruz Biotechnology).

Stability assays – The steady state abundance of GFP-tagged full length, ΔIE, and 4KRA proteins (Figure 3) was determined by Western blot analyses, as described above. To determine the relative stability of GFP and GFP fused to the RB family instability elements, transfected cells were treated with 100 μM cycloheximide 40 hour after transfection, and samples were harvested at 3h, 6h, and 9h post treatment. For proteasome inhibitor treatments in Figures 4B and 5A, cells were treated with DMSO or 1 μM MG132 for 24h.

Luciferase Reporter Assay - *U2OS* cells were transfected using with Nanojuice transfection reagent, as described above. Typically 5 x 10^5 cells were transfected with 100 ng of a human cyclin A promoter-driven luciferase reporter (human Cyclin A promoter (-89 to +11, (38)), 50 ng of pRL-CMV Renilla luciferase reporter (Promega), and 500 ng of plasmid expressing the GFP-tagged effector proteins. After 48 h, cells were harvested and luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega) and Veritas microplate luminometer (Turner Biosystems). Firefly luciferase activity was normalized to Renilla luciferase reading. Luciferase measurements were made in triplicate and at least three biological experiments were performed.

Structural Homology Modeling - Structure homology modeling of the p130 IE in complex with the E2F4/DP1 was performed using SWISS-MODEL (39). The crystal structure of the RB C-terminal domain bound to an E2F1-DP1 heterodimer (PDB code: 2AZE) (40) was used to generate the homology model.

Results

Regulation of RB, p107, and p130 localization and stability in mouse ES cells –Previous studies in Drosophila suggested that Rbf1 turnover and function are linked during embryonic development (31,32). We therefore examined the behavior of mammalian RB family members in pluripotent self-renewing mouse embryonic stem (ES) cells before and after differentiation. RB function is limited in ES cells due to elevated cyclin-CDK activity, but is established at the onset of differentiation, in part due to down regulation of cyclin-CDK kinase complexes (23,27). Thus, these cells offer a useful system to examine regulation of the RB family as members are mobilized to gene promoters in response to dynamic CDK activity during development. In these

experiments, ES cells were cultured on mitotically inactive embryonic fibroblasts in the presence of leukemia inhibitory factor (LIF) to sustain their self-renewing potential or in the presence of retinoic acid (RA) to induce differentiation, and the effect on RB, p107, and p130 was first examined by immunofluorescence analyses. In undifferentiated R1-ES cells, RB and p107 exhibited predominate cytoplasmic staining, which shifted to a stronger nuclear pattern after RA treatment (Figure 2-1 A, B). In contrast, p130 was detected in the nuclear compartment both before and after RA-induced differentiation (Figure 2-1 C). To control for antibody specificity, RB family staining in WT J1-ES were compared to triple knock out (TKO) ES cells under identical imaging conditions, showing that RB and p107 were preferentially detected in the WT-ES cells (Figures 2-1, D-E). The pattern for RB subcellular localization was similar for both R1-ES and J1-ES cells with increased nuclear retention observed after RA challenge (see also Figure 2-1, H). However, the strong cytoplasmic staining for p107 was muted in J1-ES cells, and nuclear staining was observed both before and after RA treatment. Unexpectedly, p130 was equivalently detected in the nuclei of both WT and TKO ES cells (Figure 2-1, F). In Western blot analyses, we also detected anti-p130 reactive species in both WT and TKO cells (Figure 2-1, G) and using multiple antibodies that recognize distinct epitopes (not shown), whereas RB and p107 were detected only in WT but not TKO ES cells, confirming specificity for these antibodies. Based on these findings, we conclude that RB and p107 can exhibit differential subcellular localization in response to induced differentiation. During the execution of these experiments, we frequently observed that the staining intensity of endogenous RB family members was reduced after RA treatment. These differences are not obvious in Figures 2-1, A-C because the relative luminosity of the images before and after differentiation was normalized.

Figure 2-1. Cellular localization of RB, p107, and p130 during mouse embryonic stem (ES) cell differentiation.

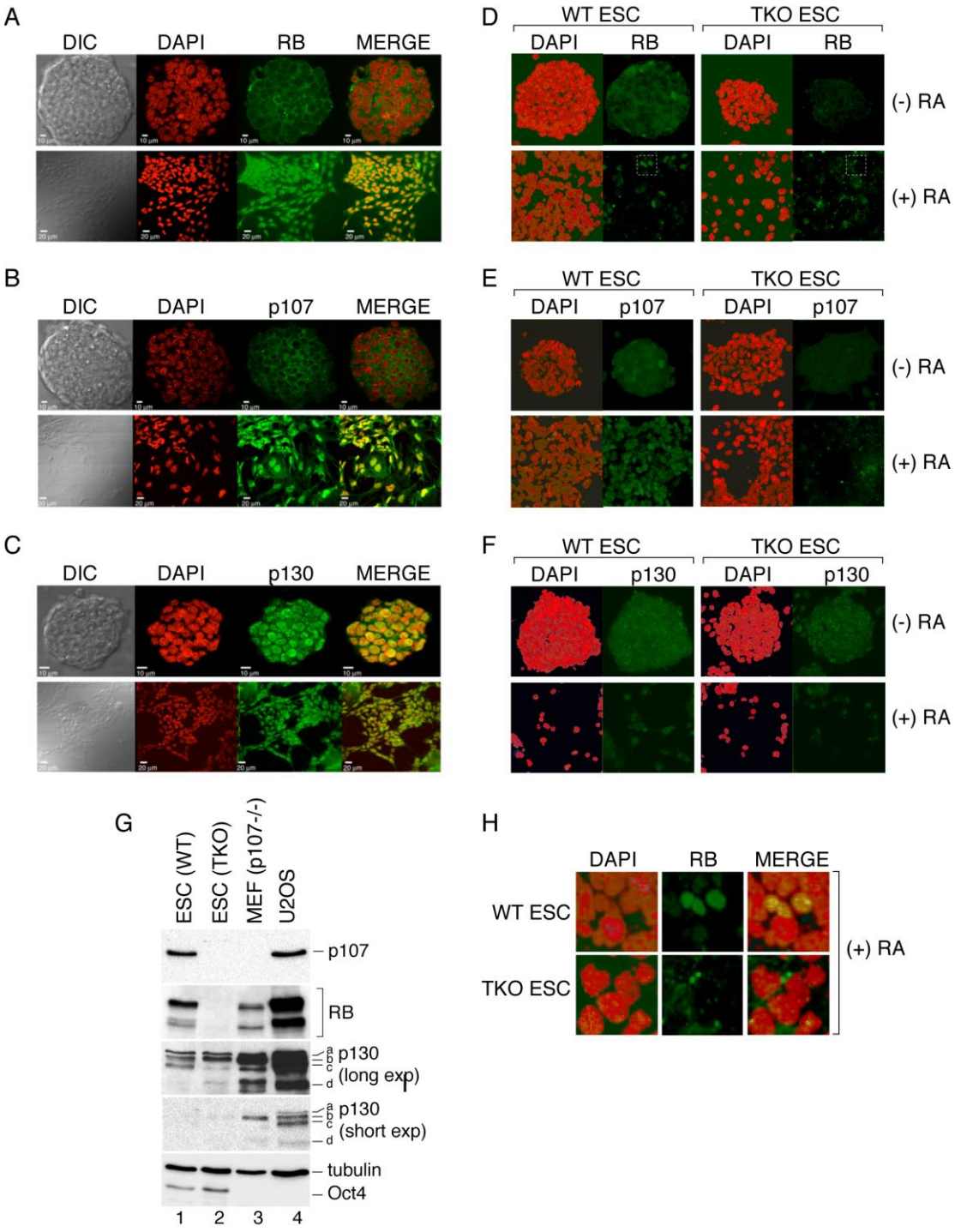

Figure 2-1 (cont'd)

(A-C) Confocal imaging showing localization of RB, p107, and p130 in a single section of pluripotent mouse R1-ES cell colonies before and after differentiation with retinoic acid (RA), as indicated. Cells were counterstained with DAPI to detect nuclear DNA. Three biological replicates were performed and at least ten ES cell colonies were imaged for each experiment. Representative examples are presented. **(D-F)** Maximal intensity projection confocal images comparing WT J1-ES and TKO cells before and after RA treatment. Samples were processed in parallel and data collected using identical imaging parameters **(G)** Western blots were performed on whole cell extracts prepared from wild type (lane 1) and triple knockout (TKO) ES (lane 2) cells using the indicated antibodies. Comparable amounts of p107-/- MEFs (lane 3) and U2OS (lane 4) extracts were included for comparison. Different exposures are shown for the p130 Western blot to permit visualization of the differently migrating species (labeled a-d). **(H)** RB exhibits nuclear localization in response to RA treatment. Sections of the data presented in panel D (white boxes) were enlarged to demonstrate the predominately nuclear staining pattern for RB in WT ES cells, which was not observed for the TKO cells. Total RB staining was significantly reduced after RA treatment with a broad range of intensity noted among cells.

However, reduced RB expression after RA treatment is noticeable for the experiment presented in Figure 2-1, D wherein the images were acquired using identical parameters, suggesting that ES cell differentiation is correlated with reduced steady state expression. Variation in RB family abundance has been observed during cell cycle progression (24,28,29,41), therefore, we considered that RB family levels during differentiation might be associated with the differing cell cycle profiles for pluripotent ES cells compared to cells undergoing differentiation. As shown in Figure 2-2 A, the proportion of cells in G1 indeed increased concomitant with a diminished S phase population in differentiated ES cells, consistent with tight relationship between RB family levels and cell cycle progression.

Our previous studies in Drosophila further demonstrated that Rbf1 is less stable in its active state (31,42), and we surmised that mammalian RB family proteins likewise become destabilized as they engage target genes in differentiating cells during G1 phase. To test this hypothesis, the levels of RB family proteins before and after RA treatment were compared to their genomic binding at endogenous target genes. Levels in ES cells were also compared to those in MCF-7 breast adenocarcinoma cells, as a positive control for RB family members and as a negative control for the stem cell marker Oct-4. As shown in Figure 2-2 B, Oct-4 expression was significantly reduced by RA treatment, in line with the expected changes for this molecular marker of pluripotency. Consistent with the reduced RB family staining that we previously noted using confocal microscopy, the steady state abundances of all three family members were clearly reduced after RA-induced differentiation as observed by direct Western blot assay. In two replicates, RB levels were reduced by 69 percent while levels of p107 and p130 were reduced by 64 and 46 percent, respectively. The decrease in RB family protein levels were not due to change in transcription or

Figure 2-2. RB family protein abundance decreases during differentiation concomitant with increased engagement at target gene promoters.

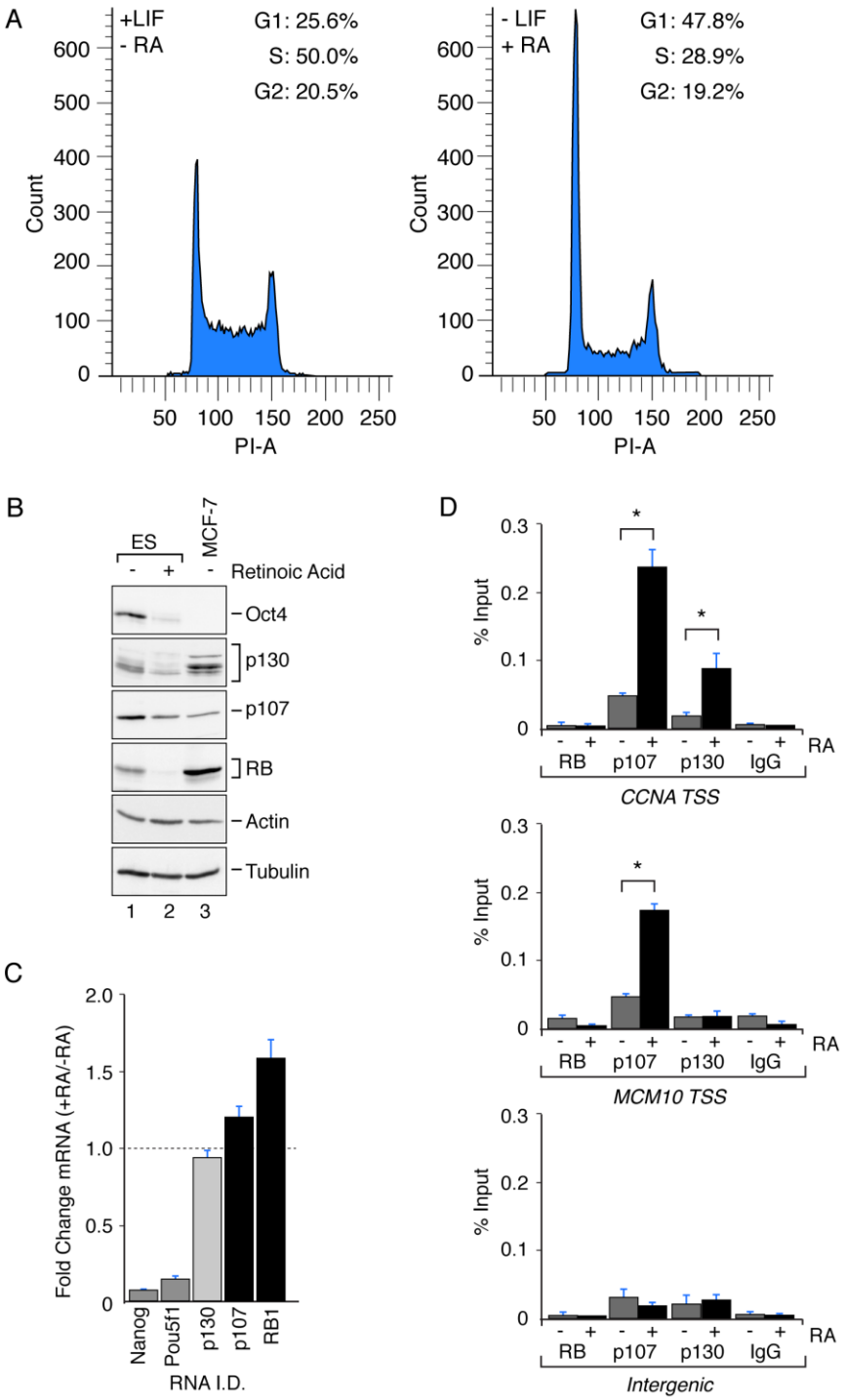

Figure 2-2 (cont'd)

(A) FACS analysis showing increased G1 and reduced S phase population in differentiated ES cells (-LIF, +RA) as compared to pluripotent ES cells (+LIF, -RA). **(B)** Western blot analysis of RB, p107 and p130 in whole cell extract derived from pluripotent (-RA, lane 1) and differentiated (+RA, lane 2) mouse ES cells. RB, p107, and p130 levels in differentiated ES cells were decreased by 69, 64, and 46 percent, respectively, as compared to pluripotent ES cells (n=2). Oct4 was analyzed as a positive control of differentiation and was substantially diminished in RA treated ES cells (lane 2). Actin and tubulin were analyzed as loading controls. Whole cell extracts from MCF7 breast adenocarcinoma cells (lane 3) were analyzed as a negative control for Oct4 and as a positive control for RB, p107, and p130 detection. **(C)** Quantitative real time PCR showing relative changes in abundance of RB, p107, and p130 mRNA transcripts upon differentiation (+RA/-RA). RB and p107 mRNA levels increased modestly after differentiation whereas p130 levels were modestly reduced. Transcript levels of Nanog and Pou5f1 (Oct4) were reduced after RA treatment, as expected. **(D)** p107 and p130 association at target promoters is stimulated during RA-induced differentiation. Chromatin immunoprecipitation assays were performed with the indicated antibodies to determine enrichment of RB, p107, and p130 on the *CCNA2* and *MCM10* promoters before and after RA treatment. After differentiation, CCNA2 start site (TSS) DNA was significantly enriched in both the p107 and p130 immunoprecipitated samples ($n=6$, $p<0.05$), whereas enrichment of the MCM10 promoter was observed only during p107 immunoprecipitation ($n=6$, $p<0.05$). Under these conditions, no significant enrichment of any loci was observed for the anti-RB immunoprecipitated samples, nor was enrichment observed with species matched IgG control antibodies. Amplification of an intergenic region on mouse chromosome 6 was performed as an additional negative control.

or RNA stability because steady state mRNA levels were either unaffected by RA treatment, such as for p130, or were modestly stimulated, such as for p107 and RB (Figure 2-2 C). *Nanog* and *Pou5f1* (Oct-4) expression were significantly reduced during differentiation, as expected. These results point to a post-translational mechanism for RB family regulation, such as through proteasome mediated turnover pathway. To examine this possibility, we treated pluripotent ES cells and RA-differentiated cells with the proteasome inhibitor MG132. However, proteasome inhibition induced substantial cell death for both pluripotent and differentiated ES cells (not shown), precluding direct assessment of proteasome involvement for RB family turnover in these cells.

Next, we determined whether the observed changes in cell cycle arrest and RB family localization during differentiation could be correlated with repressor binding at target gene promoters as one measure of function. To this end, we measured RB family occupancy on a set of well-characterized E2F-dependent promoters that were demonstrated to be RB family target genes (5,6) and whose expression was affected by RB family loss in ES cells (43). As shown in Figure 2-2 D increased promoter binding by p107 and p130 was correlated with RA-induced differentiation and cell cycle arrest. Interestingly, p107 and p130 exhibited distinct gene association, as both were bound to the CCNA2, TK1, and E2F1 loci (Figure 2-2 D and data not shown), while only p107 but not p130 was associated with the MCM10 locus. Finally, our data provide some evidence for differential binding by RB family members depending upon cell type. Specifically, p130 was not associated at the MCM10 locus in ES cells, even though it had been detected at this locus in other cell types (6). We also did not observe RB association at any of these target genes, although we have routinely used this antibody to detect RB binding in other cell types (44), and we cannot conclude whether the lack of RB binding in these experiments is biologically

relevant. The lack of RB signal at these cell cycle loci, otherwise bound by p107 and/or p130 is consistent with data previously published using T98G cells (5). Interestingly, the current observations indicate that p107 levels drop during differentiation even as there is increased residency at target genes. Unlike p107, increased p130 presence at target promoters and its diminished expression upon differentiation are independent of any changes in subcellular localization, suggesting that turnover regulation is probably not coupled to nuclear transport processes.

The RB family C-terminal regulatory domains influence repressor stability – We next considered a model that signaling mechanisms governing mammalian RB family activity are involved in regulation of repressor turnover. In Drosophila, the Rbf1 homolog harbors an instability element (IE) within its C-terminal domain that contributes to both repressor activity and destruction (31,33). As indicated by the alignments shown in Figures 2-3 A and 2-3 B, just such a sequence is clearly identifiable within the C-terminal regions of both p107 and p130. RB exhibits substantial sequence differences throughout this region. Nonetheless, previous studies demonstrated that the RB C-terminal region is structurally related to p107 and thus, this region might likewise participate in both repression and turnover. Within RB, the IE can be subdivided into two regions previously called the RBC^{NTer} and RBC^{Core}, which are important for specific interactions with the marked box domains of the E2F1/DP1 complex (see Figure 2-3 A, and (40)). The RBC^{NTer} region can also interact with the MDM2 E3 ubiquitin ligase (45), suggesting that corresponding IE region within RB similarly coordinates repressor stability. To test whether the IE regions are important for regulation of RB family turnover, we deleted these regions from RB, p107, and p130 and examined the effect on steady state expression during transient transfection in U2OS cells. These cells were chosen because we could achieve more efficient and reliable transfection in this system than in ES

cells. Moreover, U2OS cells do not express the p16 CDK inhibitor (46,47); hence these cells exhibit unrestrained cyclin-CDK activity, analogously to ES cells (25). As shown in Figure 2-3C, deletion of the RBC^{NTer} region (Δ786-800) alone, harboring the putative MDM2 binding site, did not affect RB steady state levels. In contrast, mutant RB lacking both the RBC^{NTer} plus RBC^{Core} regions (RBΔ786-864) exhibited a significant elevation in steady state abundance. Thus, the IE region within RB negatively influences repressor abundance. The C-terminal regulatory regions from p107 and p130 are less well characterized than for RB, and yet these proteins clearly exhibit the highest homology to the Drosophila Rbf1 IE region, as noted previously. Therefore, a more detailed analysis of these family members was undertaken. As shown in Figure 2-3 D, IE deletion from GFP-tagged p107 increased steady state expression. Similarly, GFP-p107 abundance was increased by alanine substitution of four conserved lysine and arginine residues within the p107 IE region (GFP-$p107^{4KRA}$) that were previously shown to influence Rbf1 half-life. As observed for p107, deletion of the IE region in GFP-p130 resulted in an even more profound fold-increase in abundance, a result that was mirrored by the corresponding 4KR to A substitution (Figure 2-3 E). Ablating E2F/DP interaction by deletion of the entire p130 A/B pocket domain did not affect steady state expression, suggesting that E2F/DP association *per se* does not modulate p130 abundance. Moreover, IE deletion did not affect the nuclear accumulation of either p107 or p130 as observed by immunofluorescence assay (data not shown). The quantification of the effects of IE deletion on RB family abundance is summarized in Figure 2-3 F. The effects of IE deletion were similar in magnitude for RB and p107 showing an increase of 2.4 fold ($n=5$, $p<0.05$) and 2.8 fold ($n=7$, $p<0.05$), respectively, while p130 abundance was increased 8.8 fold ($n=3$, $p<0.05$). Together, these data demonstrate that all three mammalian RB family members harbor C-terminal regulatory domains that contribute to their reduced steady state expression.

Figure 2-3. The C-terminal instability element is conserved within the mammalian RB family.

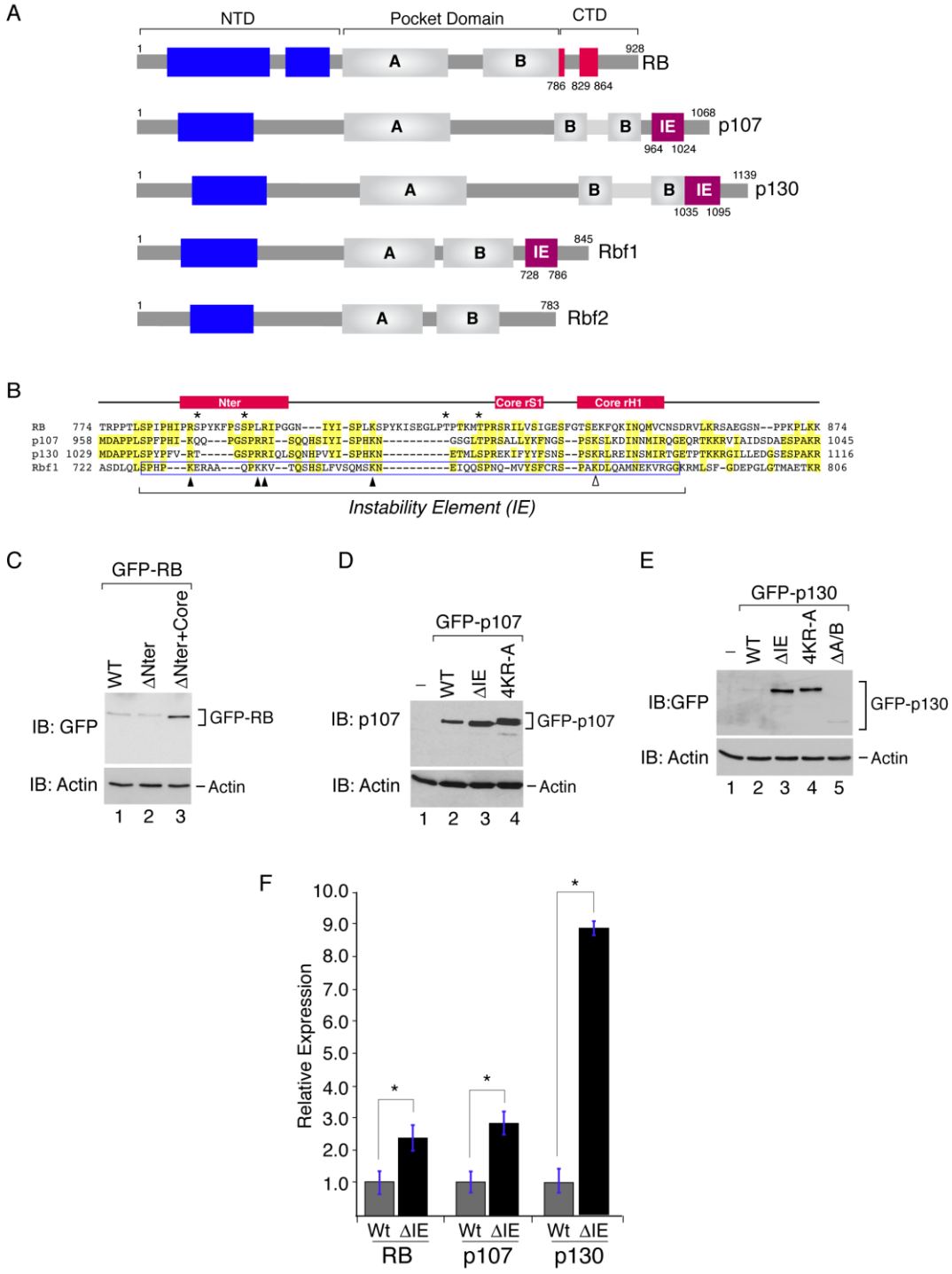

Figure 2-3 (cont'd)

(A) Schematic representation of the human and Drosophila RB family. The canonical instability element (magenta box) first discovered in Rbf1 (residues 728-786) is also present in the C-terminus of human p107 (residues 964-1024) and p130 (residues 1035-1095). The corresponding C-terminal region in human RB that functions in E2F1/DP1 interactions, shown in red, contains two discrete regions called RBC^{NTer} (residues 786-800) and RBC^{Core} (residues 829-864) (40). Drosophila Rbf2 does not appear to harbor a C-terminal IE. The A and B cyclin fold domains within the central pocket domains are shown as grey boxes. Potential cyclin fold domains within the N-terminal regions are shown as blue boxes. **(B)** Sequence alignment of the C-terminal regions from RB, p107, p130, and Rbf1. Residues that are identical in at least two members are highlighted in yellow. The position of the experimentally determined instability element within Rbf1 is boxed in blue. The RBC^{NTer} and RBC^{Core} regions are schematically represented above the alignment. Asterisks indicate the position of CDK phosphorylation sites within RB that modulate intermolecular interactions with E2F1/DP1 and intramolecular interactions with the B domain (40). The positions of positively charged lysine and arginine residues that increase Rbf1 stability when mutated are indicated as black triangles. The position of a lysine residue within Rbf1 (K774) that induces profound developmental phenotypes when mutated (31) is indicated as an open triangle. **(C)** Western blot analysis of whole cell extracts derived from U2OS osteosarcoma cells transfected with GFP-RB^{WT} (lane 1), GFP-$RB^{\Delta NTer}$ (lane 2) and GFP-$RB^{\Delta NTer+Core}$ (lane 3). **(D)** Western blot analysis of whole cell extracts derived from U2OS osteosarcoma cells transfected with wild type GFP-p107 (lane 2) or mutant GFP-107 harboring a deletion of the C-terminal instability element (GFP-$p107^{\Delta IE}$, lane 3) or alanine substitutions at four conserved positively charged residues (K970, R977, R978 and K991) within the instability element (GFP-$p107^{4KR-A}$,

Figure 2-3 (cont'd)

lane 4). **(E)** Western blot analysis of mutant p130 harboring a deletion of the C-terminal instability element (GFP-p130$^{\Delta IE}$, lane 2) or bearing alanine substitutions at four conserved positively charged residues (R1041, R1046, R1047, and K1062) within the instability element (GFP-p130^{4KR-A}, lane 4). Total p130 levels were unaffected by deletion of the A/B pocket domain GFP-p130ΔA/B (lane 5). Actin was used as a loading control for all experiments. **(F)** Quantification of the Western data presented in 3C-E for IE-deletion mutants. Deletion of the IE within RB (n=5), p107 (n=7), and p130 (n=3) resulted in 2.4-fold, 2.8-fold, and 8.8-fold increase in abundance, respectively (*, $p<0.05$).

RB family members lacking their IE regions exhibited increased steady state abundance; therefore, we hypothesized that the IE-containing regions function as degradation signals or degrons to direct destruction of their cognate proteins. To test whether the Rbf1-related IE regions within p107 and p130 are sufficient for autonomous recognition and target protein degradation by the ubiquitin proteasome system, these regions were appended to GFP, and the effect on chimera protein abundance was examined. As shown by the immunofluorescence data presented in Figure 2-4 A, the GFP-IE^{p107} chimera was expressed at reduced levels in most, but not all cells when directly compared to GFP. The total numbers of transfected cells were similar for both constructs, as assessed by scoring the number of GFP-positive cells independently of fluorescence intensity. These analyses indicate that the major difference in steady state abundance is likely due to reduced accumulation rather than differential transfection efficiency. The GFP-IE chimeras containing either the p107 or p130 IE regions were also markedly diminished compared to GFP alone in Western blot analyses (Figure 2-4 B). MG132 treatment increased the steady state abundance of both GFP-IE^{p107} and GFP-IE^{p130}, whereas GFP levels were unaffected, consistent with the model that the p107 and p130 IE regions facilitate degradation by the proteasome (Figure 2-4 B). Significantly, alanine substitution of key lysine and arginine residues that affected p107 and p130 expression in the context of the full-length proteins also lead to elevated steady state levels of the GFP-IE chimeras (Figure 2-4 C). These data also indicate that the cellular degradation machinery does not require additional interactions with other domains for turnover activity. Nonetheless, neither proteasome inhibition by MG132 nor IE mutation in this context restored expression to levels observed for GFP alone, suggesting that other unidentified features contribute to regulation of degron activity. We next treated cells with cycloheximide to test whether the reduced levels of GFP-IE fusion proteins were indeed a function of accelerated turnover. The measured stabilities

Figure 2-4. The canonical instability elements in human p107 and p130 function as autonomously acting degrons.

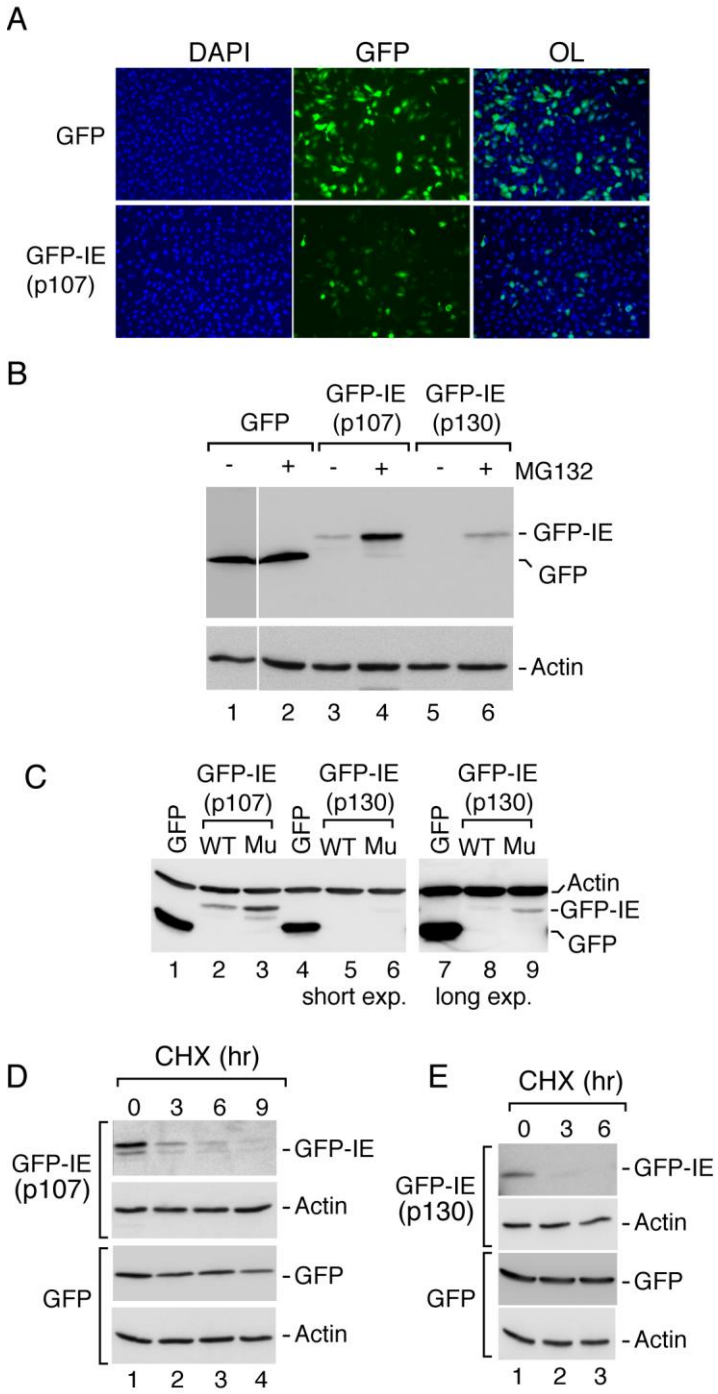

Figure 2-4 (cont'd)

(A) The p107 IE contributes to reduced steady expression of GFP. GFP fluorescence was measured in U2OS cells transfected with GFP alone or the GFP-IE^{p107} chimera containing GFP fused to residues 964-1024, corresponding to the p107 instability element. Cells were counterstained with DAPI to detect cellular DNA, and overlays (OL) of the GFP and DAPI signals are shown. GFP-IE^{p107} showed much reduced expression as compared to GFP in most, but not all cells. **(B)** GFP-IE^{p107} and GFP-IE^{p130} steady state expression is enhanced by proteasome inhibition. Anti-GFP western blot analysis was performed on whole cell extracts derived from U2OS cells transfected with GFP (lanes 1, 2), GFP-IE^{p107} (lane 3, 4), or GFP-IE^{p130} containing GFP fused to residues 1035-1095 corresponding to the p130 instability element (lane 5, 6), in the absence or presence of MG132, as indicated. Whereas GFP remained insensitive to the proteasome inhibition, the GFP-IE fusion proteins accumulated to higher levels during MG132 treatment, suggesting the involvement of the instability element in proteasome mediated turnover. Actin was detected as a loading control and was refractory to proteasome inhibition. **(C)** Conserved positively charged amino acids contribute to autonomous IE function. Anti-GFP western blot analysis was performed on cells extracts transfected with either wild type GFP-IE^{p107} (lane 2) or mutant GFP-IE^{p107} containing alanine substitutions of four positively charged resides within the IE (lane 3). Similar analysis was performed for wild type GFP-IE^{p130} (lanes 5, 8) and mutant GFP-IE^{p130} (lanes 6, 9). In all cases, the GFP-chimeras containing the wild type IE sequences were expressed at lower levels than GFP alone (lanes 1, 4, 7), while alanine substitution within the IE resulted in increased steady state expression. The effect of alanine substitutions on GFP-IE^{p130} was more evident at a higher exposure of the same blot (lane 8 vs. lane 9). Actin was detected as a loading control. **(D, E)** The p107 and p130 instability elements contribute to enhanced protein turnover. Anti-GFP

Figure 2-4 (cont'd)

Western analyses was performed on cells expressing either GFP or the wild type GFP-IE chimeras incubated in the presence of the translation inhibitor cycloheximide for 0, 3, 6, or 9 h, as indicated. GFP exhibited a half-life greater than 9 hours, whereas the half-lives of the GFP-IE chimeras were less than 3 h. Actin was detected as a loading control and its levels remained unperturbed by cycloheximide treatment.

of the GFP-IE chimeras were substantially lower, demonstrating that these effects are directed towards protein turnover (Figures 2-4 D, 2-4 E). Together, these studies define these canonical IE regions as independently acting degrons that are capable of directing substrate degradation by the proteasome.

The canonical IE regions from p107 and p130 differ from RB at the primary sequence level. However, structure prediction analysis suggested that these regions may be conserved at the tertiary level, and thus we next tested the ability of the non-canonical RB-IE region to function as a degron. As shown in Figure 2-5 A, GFP appended with the RB-IE was expressed at a substantially lower level than GFP, and levels were increased by MG132 treatment, suggesting that this region is a bona fide degron. Cycloheximide treatment demonstrated that the RB-derived IE was also destabilizing (Figure 2-5 B), as previously noted for the canonical p107 and p130 IE constructs. We conclude that the RBC^{NTer} and RBC^{Core} regions together constitute a functional degron.

RB family members are differentially expressed during cell cycle progression with low levels of RB and p107 during G0 or early G1, and increasing levels as cells progress towards late G1/S (29,48). In contrast, p130 is typically expressed at its highest levels during G0 but at reduced levels at other stages. These differences suggest that cyclin/CDK activity may be key for regulation of RB family protein levels. At least for p130, proteasome-mediated turnover is known to contribute to cell cycle associated changes (28,29). To assess the potential role of the IE in this process, we first measured the effect of CDK4 inhibition by PD0332991 on endogenous RB family members in asynchronously dividing U2OS cells. As shown in Figure 2-6 A, p107, and RB levels

Figure 2-5. The non-canonical instability element in human RB functions as an autonomous degron.

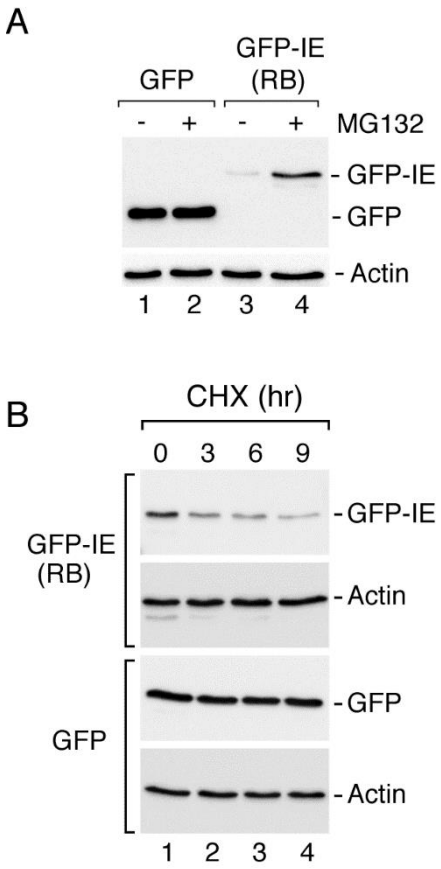

Figure 2-5 (cont'd)

(A) The RB IE contributes to reduced steady expression. Anti-GFP Western blot analysis was performed on whole cell extracts derived from U2OS cells that were transfected with GFP (lanes 1, 2) or GFP-IE^{RB} containing GFP fused to residues 786-864 from human RB (lanes 3, 4) in the absence or presence of the proteasome inhibitor MG132, as indicated. Actin was detected as a loading control. In these experiments, the GFP-IE^{RB} chimera was expressed at much lower levels than GFP alone. GFP-IE^{RB} expression was also enhanced during proteasome inhibition. **(B)** The RB IE region contributes to enhanced substrate turnover. Cycloheximide experiments were performed as described previously, demonstrating that the GFP-IE^{RB} chimera exhibited diminished stability ($t^{1/2}$ < 3hr), as compared to GFP ($t^{1/2}$ > 9hr). Actin was used as a loading control.

were markedly diminished after CDK4 inhibition, while endogenous p130 levels were significantly increased. These data are consistent with the divergent regulation of the RB family members during cell cycle progression in staged cells (28, 29), with experiments testing the effect of PD0332991 in asynchronously dividing hepatocellular carcinoma cells (30). Moreover, levels of p107 and p130 were significantly increased during a short duration (6 h) of MG132 treatment, while RB levels were modestly diminished. These data indicate that endogenous p107 and p130 are subjected to proteasome-mediated turnover under these growth conditions. Secondly, RB is either not subjected to proteasome turnover or MG132 induced RB turnover via a proteasome-independent pathway. Interestingly, levels of p107, but not RB, were rescued by proteasome blockade during Cdk4 inhibition (PD+MG132), suggesting that Cdk4-mediated phosphorylation of p107 prevents its proteasome-mediated turnover. In contrast, concomitant proteasome and Cdk4 inhibition did not affect p130 levels compared to that observed for PD alone, suggesting that hypo-phosphorylated p130 is not a substrate for ubiquitin mediated degradation, as described previously (29).

We next tested whether the IE is essential for the observed destabilization of p107 by examining the effect of CDK4 inhibition on wild type and mutant p107 lacking the IE in transiently transfected cells. Indeed, PD0332991 treatment destabilized the wild type but not the p107ΔIE protein, indicating that the IE is required for CDK-sensitive proteasome-mediated turnover of p107 (Figure 2-6 B). Wild type RB was also destabilized during Cdk4 inhibition, while RB lacking both the RBC^{NTer} plus RBC^{Core} (GFP-$RB^{\Delta IE}$) regions was refractory to PD0332991 influence (Figure 2-6 C), suggesting that the IE plays a similar role for CDK4 regulation of RB. In contrast to endogenous p130, recombinant GFP-p130 was destabilized, not stabilized during CDK4 inhibition, and this destabilization was observed regardless of IE status (Figure 2-6 D). This

Figure 2-6. RB and p107 destabilization during CDK4 inhibition is dependent upon instability element function.

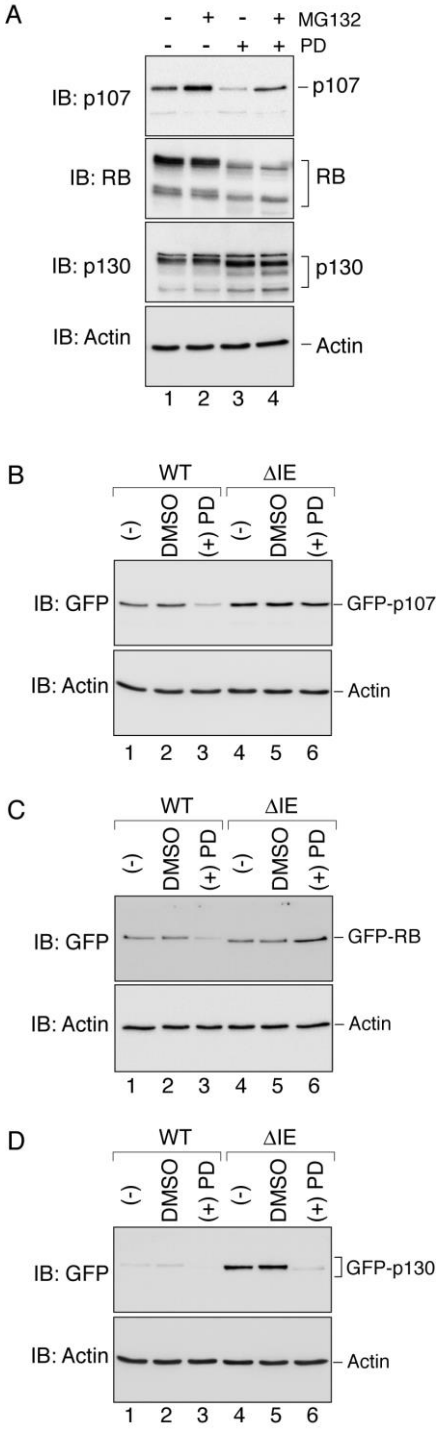

Figure 2-6 (cont'd)

(A) CDK4 inhibition differentially affects steady state expression of endogenous RB, p107, and p130. Western blot analysis was performed to detect endogenous RB, p107 and p130 in whole cell extracts derived from U2OS cells treated with DMSO (lanes 1), MG132 (lane 2), the CDK4-specific inhibitor PD0332991 (lane 3), or PD0332991 plus MG132 (lane 4). Proteasome inhibition by MG132 (6h) increased steady state abundance of p107 and p130 by 90% and 30%, respectively as compared to the DMSO treated samples ($n=3$, $p<0.05$), whereas RB abundance decreased by 15% ($n=2$, $p<0.05$). The steady state expression of both RB and p107 were reduced by 80% during PD treatment as compared to the DMSO treated samples ($n=5$, $p<0.05$), whereas p130 expression was increased by 49% ($n=7$, $p<0.05$). The PD-induced down-regulation of p107, but not RB, could be restored by MG132 to levels comparable to the DMSO control. Actin as used as a loading control and its levels remained unperturbed by PD or MG132 treatment. **(B)** p107 destabilization during CDK4 inhibition requires IE function. Western blots analysis was performed on U2OS cells that expressed wild type GFP-p107 (lanes 1-3) or mutant GFP-p107ΔIE (lanes 4-6) in the absence or presence of PD0332991, as indicated. In response to CDK4 inhibition, wild-type GFP-p107 levels were reduced by 68% as compared to the DMSO control ($n=3$, $p<0.05$), whereas the levels of p107 lacking the instability element were modestly reduced (18%) as compared to the DMSO control ($n=3$, $p<0.05$). **(C)** RB destabilization during CDK4 inhibition requires IE function. Western blot analyses were performed as above, showing that wild-type GFP-RB levels were reduced by 66% in response to PD as compared to the DMSO control ($n=3$, $p<0.05$), whereas steady state expression of GFP-RBΔIE was increased by 82% compared to the DMSO control samples ($n=3$, $p<0.05$). **(D)** p130 destabilization during CDK4 inhibition does not require IE function. Western blot analyses were performed as above, showing that both wild type GFP-p130

Figure 2-6 (cont'd)

and GFP-p130ΔIE were destabilized to similar extents during PD0332991 treatment. On average, the levels of both proteins were decreased by 92% in response to PD as compared to DMSO control ($n=3$, $p<0.05$). Actin was used as a loading control in panels B-D.

outcome suggests that an additional IE-independent pathway can contribute to p130 turnover. The differences in response to CDK4 inhibition for the endogenous and overexpressed p130 proteins also suggests that some turnover pathways are active only in one setting, perhaps dictated by additional regulatory phosphorylation events that affected endogenous p130 in this context.

Previous biochemical and structural studies of the C-terminal domains of the human RB family showed that these regions are important for interactions with the marked box domains of E2F/DP complexes (40,49-51). A model of the p130 IE region in a complex with the marked box domains of E2F4 and DP1 was generated by homology modeling using the RB C-terminal domain bound to a heterodimer of E2F1 and DP1 (Figure 2-7 A). In this model, the C-terminal portion of the IE harbors a sheet-turn-helix motif, which contacts the E2F4/DP1 complex, consistent with a potential role for the IE in repression. Interestingly, we noted that p107 (*RBL1*) and p130 (*RBL2*) harbor low frequency somatic mutations in cancer patients that map within the IE regions, as documented in the TCGA and COSMIC databases (http://cancergenome.nih.gov/) (52). These missense and nonsense mutations are found in carcinomas of the ovary, large intestine, endometrium, and pancreas. An additional independent study focusing on *RBL2* found that mutations within the p130 IE were frequently observed in a cohort of lung adenocarcinomas (53), including missense mutations affecting lysine 1083. It is notable that comparable lesions have strong biological effects in Drosophila wherein Rbf1 bearing a homologous substitution at K1083 (K774R) caused severe developmental defects (31,54), suggesting that some mutations may play important roles in vivo. As also shown in Figure 2-7 A, the locations of cancer-associated *RBL2* mutations map to different regions of the p130 structure, suggesting that these mutations may generate different effects, including modulation of E2F/DP interactions or potential E3 ligase association. We first tested whether cancer-associated point mutations can affect p130 stability by

expression of the proteins in U2OS cells. Substitutions within the C-terminal portion of the alpha helix (S1090I, I1092M) significantly enhanced p130 levels, comparable to the effect of mutating four conserved lysine/arginine residues in the adjacent but unstructured region of the IE. Other point mutations tested, including R1070G, N1079F, K1083R, and K1083T that map more proximal to the E2F4/DP1 dimer interface had no effect on p130 steady state levels (Figure 2-7 B). Thus, some IE-associated cancer mutations result in enhanced expression of p130 potentially due to disrupted E3 ligase binding.

We next tested whether deletion of the entire IE or amino acid substitutions within the IE regions from p107 and p130 can impact repression potency (Figures 2-7 C, 2-7 D). Both wild type p107 and p130 repressed transcription driven by the *CCNA2* reporter to levels approximately 50-63% of that observed for the control. This magnitude of repression is consistent with previously reported activities for p107 and p130 (34), but is not as profound as that reported for Drosophila Rbf1 (31,33). Removal of the IE, or substitution of four conserved lysines/arginines with alanine significantly impaired repression activity for both p107 and p130, indicating that the IE in these mammalian homologs contribute to full repression potential. Consistently, truncation of the entire C-terminus (p130-ΔC) significantly impaired repression, consistent with the role of the C-terminus in nuclear localization and in mediating contacts with E2F4/DP (55). In contrast, none of the missense mutations within the p130-IE, as reported in human cancer samples, were significantly altered for repression of either the cell cycle regulated *CCNA2* gene or the apoptotic *TP73* reporter (not shown). We conclude that these particular point mutations are unlikely to critically affect transcriptional regulation of canonical E2F/DP target genes.

Figure 2-7. p130 IE activity in regulating repressor potency and stability are biochemically separable functions.

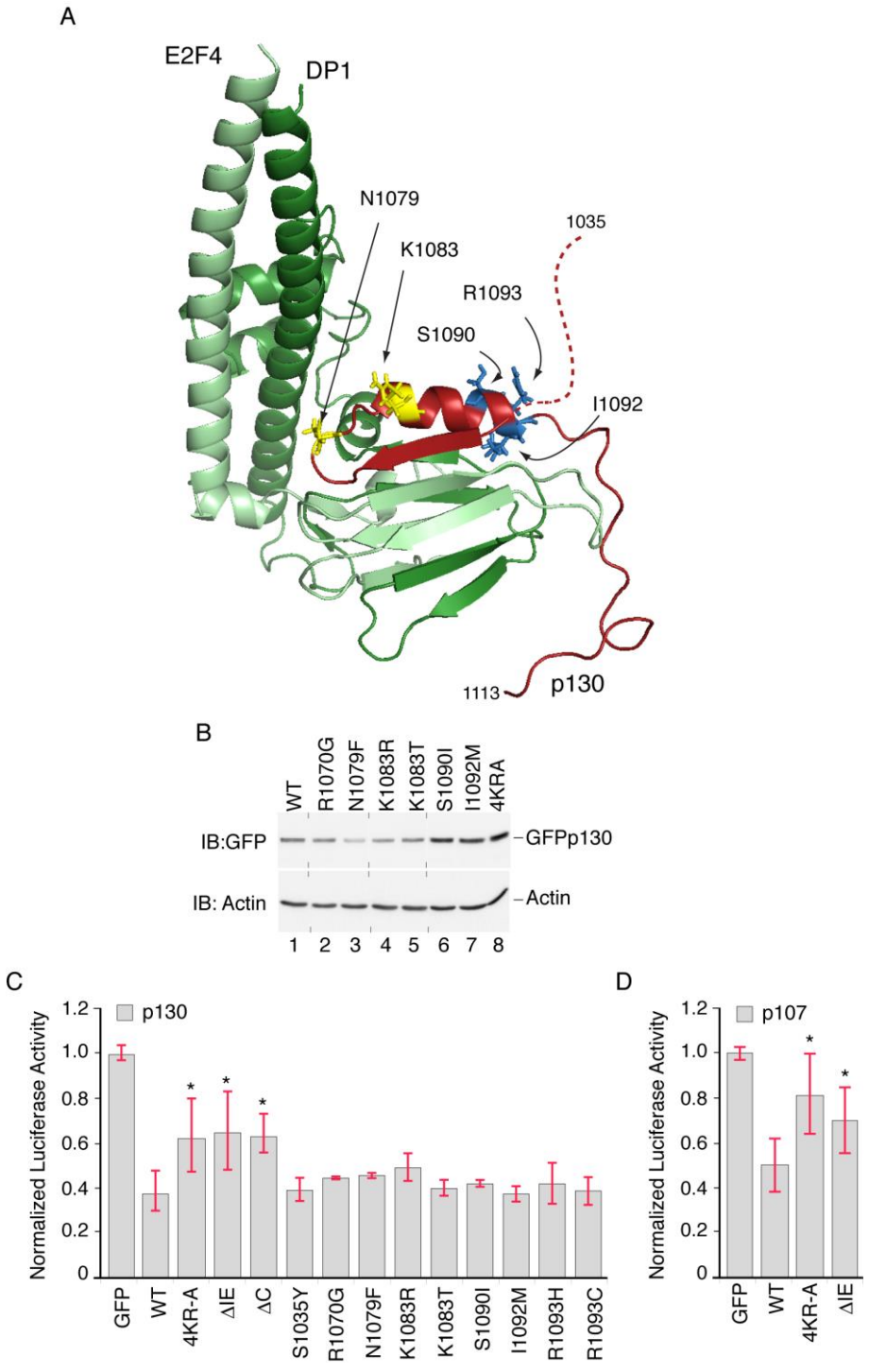

Figure 2-7 (cont'd)

(A) Model of the human p130 IE in a complex with E2F4/DP1. Homology model of the p130 C-terminus (residues 1035-1113, red) in complex with the coiled coil-marked box domains from E2F4 (residues 94-198, light green) and DP1 (residues 199-350, dark green) was generated using the crystal structure of the RB C-terminal domain bound to an E2F1-DP1 heterodimer as an template (PDB code: 2AZE, (40)). The N-terminal portion of the p130 IE is unstructured in this model (dashed red line), whereas the C-terminal portion of the IE harbors a sheet (residues 1071-1077)-turn-helix (1083-1093) motif. The positions of some amino acid residues altered in human cancer patients are indicated (documented in COSMIC - S1090I: TCGA-23-1118-01; I1092M: TCGA-AA-3864-01; R1093H: TCGA-AA-A01Q-01; R1093C: TCGA-D1-A15W-01A-11D-A122-09). In all cases, these mutations are rare occurring at a frequency $\leq 0.3\%$. Additional mutants (R1070G, 2/14 cases; N1079F, 2/14 cases; K1083R, 4/14 cases; K1083T, 1/14 cases) are based on the study presented in reference 53. Residues highlighted in yellow (N1079, K1083) are located towards the N-terminal region of the α-helix while residues highlighted in blue (S1090, I1092, R1093) are located within the C-terminal region of this helix. **(B)** Cancer associated IE mutations have variable effects on p130 levels. Anti-GFP Western blot analysis was performed on U2OS cells transfected with plasmids expressing either wild type GFP-p130 or mutant versions harboring single point substitutions, as indicated. In two replicates, GFP-130 containing the S1090I and I1092M substitutions was expressed 2.2- and 1.7-fold greater than the wild type (n=2). Vertical dashed lines indicate positions where image had been cut to rearrange the order of lanes. **(C)** The p130 IE is required for full repression potency. Luciferase reporter assays were performed in the presence of wild type or mutant versions of GFP-130, as indicated, testing repression of the human CCNA2 luciferase reporter. Wild type GFP-p130 repressed transcription by 63%, as

Figure 2-7 (cont'd)

compared to GFP alone. GFP-p130^{4KR-A}, GFP-p130$^{\Delta IE}$, and GFP-p130$^{\Delta C}$ were significantly less effective than wild type GFP-p130 (n=8, $p<0.05$, *). None of the IE mutations reported in human cancers statistically altered the repression of CCNA2 reporter by GFP-p130. **(D)** The p107 IE is required for full repression potency. Luciferase reporter assays were performed in the presence of wild type or mutant versions of GFP-107, as indicated, testing repression of the human CCNA2 luciferase reporter. Wild GFP-p107 repressed transcription by 50%, as compared to GFP alone. Both GFP-p107^{4KR-A} and GFP-p107$^{\Delta IE}$ were significantly less effective than wild type GFP-p107 (n=5, $p<0.05$, *).

Discussion

The RB tumor suppressor family governs key steps in cellular proliferation through the transcriptional regulation of distinct genes associated with growth control (3,4). Phosphorylation of RB proteins by cyclin/CDK complexes in mouse and human ES cells was previously demonstrated to inhibit RB/E2F interaction with consequent effects on gene expression and cell proliferation (25,26,43,56). In this study, we report that early steps during developmental regulation of the RB family in ES cells are additionally rendered through governance of subcellular localization. Specifically, RB and p107 accumulated in the nucleus during mouse ES cell differentiation mediated by concomitant LIF withdrawal and retinoic acid addition. This transition was concurrent with enhanced p107 and p130 association at select target genes. Promoter association by p107 and p130, but not RB in this developmental context is similar to that observed in tissue culture experiments performed using T98G glioblastoma cells (5). It is also interesting that p107 and p130 exhibited different behavior for promoter binding with some genes harboring only p107 and others harboring both p107 and p130. However, the functional significance of differential promoter association during ES differentiation remains to be determined. It should be noted that ES cells can undergo differentiation independently of RB family control (43), and thus consequences for promoter-specific binding may depend upon additional cues, such as cell type and developmental context. These data point to two mechanisms governing activity of some RB family members during early development, one involving post-translational regulation by the cyclin/CDK system and another involving control of subcellular localization.

Our data further indicate that RB family function in transcriptional repression is linked to increased repressor turnover. This connection was first suggested by observations that steady state levels of RB family members in ES cells were diminished during RA-induced differentiation and

cell cycle attenuation. Our observations are also consistent with previous studies in mouse ES cells where total RB levels dropped in early G1 after release from nocodazole blockade (24). Taken together these observations suggest an intimate connection between cell cycle progression and RB protein levels in development. In response to RA, cellular mRNA levels encoding RB, p107, and p130 were either unaffected or were slightly increased, suggesting that changes in protein abundance during differentiation are influenced at a post-transcriptional level, potentially involving regulated protein turnover. To understand the mechanism underlying RB family abundance, we tested the effect of proteasome inhibition on repressor levels in ES cells. However, these cells were extremely sensitive to MG132 treatment precluding direct assessment of RB family member half-lives. We therefore performed a biochemical structure-function study in a human osteosarcoma cell line that also maintains elevated CDK activity, analogously to that observed in ES cells. In this context, p107, and p130 turnover were indeed directed via a proteasome-mediated pathway that involved the evolutionarily conserved instability elements located within their C-terminal regulatory domains. The primary sequences of the mammalian p107 and p130 IE regions are most similar, and both of these are clearly related to the prototypical IE initially identified within Drosophila Rbf1 (31). However, RB differs substantially in primary sequence throughout the IE. Nonetheless, structural studies have indicated that pocket proteins maintain secondary and tertiary conservation throughout this region (40). We show herein that the corresponding region within RB also functions as an autonomously acting degron, suggesting that regulation of repressor stability via these C-terminal degrons represents an important and evolutionarily conserved component of global RB family control.

Mammalian RB family members are differentially expressed during cell cycle progression (28,29,48) with rapid degradation of hyper-phosphorylated p130 correlated with G0 exit and cell

cycle reentry. In contrast, p107 and RB levels tend to increase as cells progress towards S phase. Our studies showed that both wild type p107 and RB but not mutant versions lacking the IE are diminished by CDK4 inhibition, and are consistent with a role of IE-mediated degron function in these cell cycle fluctuations. These findings are consistent with earlier observations wherein mouse ES cells ablated for the CDK2 inhibitor Cdk2AP1 exhibited enhanced RB phosphorylation concomitant with increased RB abundance (57). Together, these studies demonstrate a clear linkage between the onset of CDK regulatory activity in ES cells and inversely correlated changes in RB family activity and abundance.

Although the IE regions of RB, p107, and p130 share similar function in turnover control, the notable primary sequence divergence within these regions suggests that different E3 ligases participate in RB family turnover. We propose that these distinct IE regions provide regulatory flexibility for RB family responses to distinct cell signaling events. Such events may include differential responses to DNA damage or during regulated cell cycle progression. For example, the RBC^{NTer} region can bind to the Mdm2 E3 ligase for targeted destruction of hypo-phosphorylated RB (45,58,59). As noted, corresponding regions in p107 and p130 are only minimally conserved with RB, and these proteins are refractory to Mdm2 expression (59). In contrast, proteolysis of p130, but not that of RB or p107 is dependent on the ubiquitin ligase activity of SCF^{Skp2} (28,29), which is minimally expressed during G0 but peaks during S phase (60-62), suggesting that E3 availability also plays a significant role in turnover of specific family members. Although SCF^{Skp2} and Mdm2 have been suggested as E3 ligases for cell cycle and DNA damage-associated degradation of RB family proteins, the involvement of these ligases in turnover during ES cell differentiation remains to be established. It is interesting that the RB C-terminal domain is also sufficient for F-box protein Skp2 association, but in this case, the RB-Skp2

interaction is mainly implicated in the regulation of p27 turnover (63), suggesting a role for the RB-IE in non-autonomous protein turnover. We consider it likely that multiple E3 ligases participate in RB family regulation through differential contacts with the IE regions of the different RB family members.

Our study has additionally uncovered an intriguing aspect of mammalian RB regulation, namely that the sequences guiding repressor instability physically overlap with regions that are important for transcriptional repression. The inability of mutant p107 and p130 lacking the IE to fully engage in transcriptional repression is consistent with biochemical studies that have demonstrated a role for the IE in intermolecular contacts with the coiled coil-marked box (CC-MB) regions of E2F1-DP1 complexes ((40), see also Figure 7). In this regard, our observations with the RB family of repressor proteins are similar to the intimate association of degrons within the activation domains of potent trans-activator proteins, such as E2F1 and c-Myc (64), regulatory factors that control critical steps in cellular proliferation. A common theme emerges from these studies that key activators and repressors governing cell fate outcomes are inherently engineered with limitations on their life span through turnover by the ubiquitin-proteasome system. Depending upon how the RB family interacts with distinct E2F/DP complexes, the interesting possibility arises that IE-E2F/DP engagement may reciprocally influence interactions with E3 ubiquitin ligases. In one model, E2F/DP complexes compete with E3 ligase for access to IE surfaces. In an alternative cooperative model, IE interactions with E2F complexes may portend engagement with E3 ubiquitin ligases. This latter model is supported, in part, by our data showing that the steady state levels of p107 and RB are diminished by Cyclin D/Cdk4 inhibition, a process that also licenses these pocket proteins for E2F/DP interactions and target gene engagement. Moreover, some cancer-associated p130 mutants tested in the current study showed increased

steady state expression without significant effects on *CCNA2* repression in vitro, suggesting that E3 binding and E2F/DP engagement are biochemically separable. While ineffectual for perturbation of p130-mediated repression in this context, it remains possible that *in vivo*, these mutations are associated with deregulation of other, as yet uncharacterized, classes of target genes with significant effects on cellular physiology (6,65). We note that the in the developing Drosophila embryo, Rbf1 associates with many genes involved in cell signaling and metabolism (66) and similar categories of genes may likewise become deregulated during human cancer progression in cells lacking IE function. In flies, expression of Rbf1 lacking the IE enhanced DNA replication *in vitro* (67) and drove increased organ size when expressed in a tissue specific manner during development (54), suggesting a critical role for IE function in developmental and proliferative pathways.

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CHAPTER 3: SUMMARY

The Retinoblastoma family proteins regulate cellular proliferation through transcriptional repression of genes involved in transition from G1-S phase (1). The underlying mechanism of gene repression by RB proteins involve inhibition of E2F transcriptional activators. Multisite phosphorylation on RB by Cyclins/Cyclin-dependent kinase (Cdk) complexes, abolishes interaction with E2Fs and increases transcriptional output of RB/E2F target genes (2, 3). As discussed in Chapter 1, an additional level of regulation on RB and E2F is implemented by the ubiquitin-proteasome system. Regulation of RB function through both these mechanisms involve hierarchical control by upstream regulators, and there is a substantial crosstalk between these regulons (Figure 3-1). Disruption of the genetic circuitry involved in phosphorylation and proteasome-mediated targeting of the RB and E2F molecules results in severe deficiencies in embryonic development (4-9), and is also an early event in cellular transformation during cancer progression (10).

Recent studies from our labs have shown that in *D*rosophila the Retinoblastoma family (Rbf) proteins are subject to proteasome mediated turnover during embryonic development, and this process enhances Rbf engagement in transcriptional repression (4,5,6,11). This positive linkage between Rbf1 activity and its destruction indicates that repressor function is governed in a manner similar to that described by the degron theory of transcriptional activation (12). To understand the relationship between RB family stability and their repressor function during early mammalian development, I initiated studies in mouse embryonic stem (ES) cells. These studies revealed that differentiation of mouse ES cells is associated with the establishment of a functional RB pathway and simultaneous destabilization of RB family members (13).

Figure 3-1. Parallel regulation of the RB-E2F pathway through reversible phosphorylation and the ubiquitin-proteasome system.

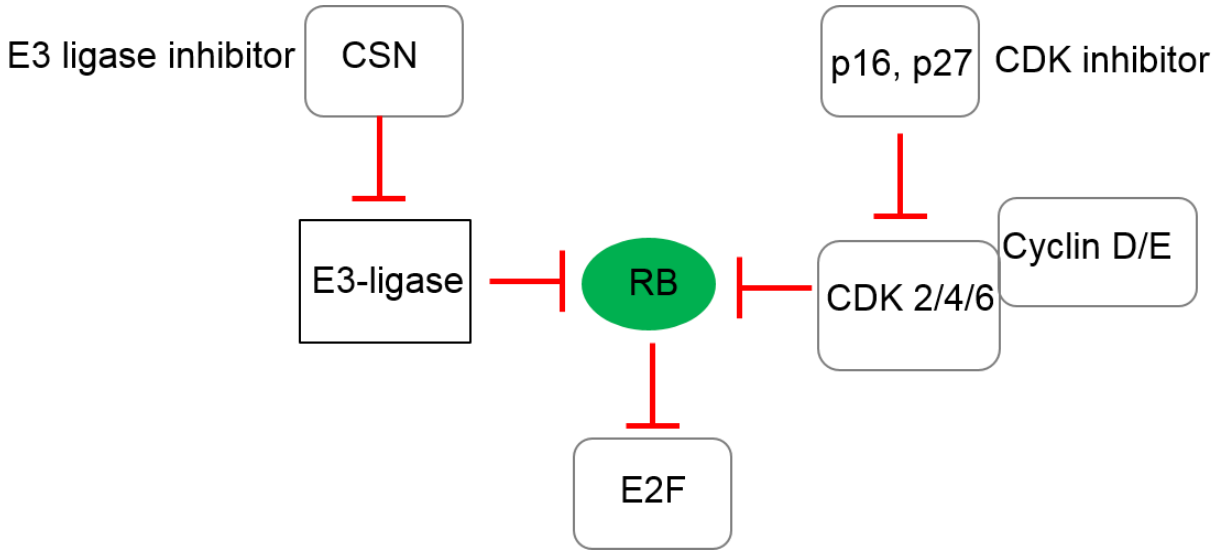

Figure 3-1 (cont'd)

RB is inactivated by upstream Cyclin/Cdk complexes, which in turn are negatively regulated by CDK inhibitors. In parallel RB is inactivated by E3-ligases whose activity is modulated by upstream E3-ligase inhibitors. Regulation of RB function through both these mechanisms involve hierarchical control by upstream regulators, and there is substantial crosstalk between these regulons.

As pluripotent ES cells are characterized by unrestrained Cdk activity which plummets at the onset of differentiation (14), we speculated that the observed changes in protein stability upon ES cell differentiation reflects an intimate relationship between RB phosphorylation and stability. Indeed, a C-terminal instability element (IE) in RB, p107 and p130 mediates their proteasome dependent turnover in response to changes in phosphorylation status (13). The IE sequence is evolutionarily conserved and functions autonomous to direct degradation of heterologous proteins (13). This study has additionally uncovered another intriguing aspect of mammalian RB regulation, namely that the sequences involved in regulating repressor instability physically overlaps with regions that are important for transcriptional repression. This idea is supported by the observation that mutant p107 and p130 lacking the IE although very stable, were unable to fully engage in transcriptional repression. This dependence on IE for transcriptional repression is consistent with its role in mediating intermolecular contacts with the coiled coil-marked box (CC-MB) regions of E2F1-DP1 and E2F4-DP1 (Figure 3-2A) (13, 15). To this end, it is intriguing to speculate that IE-E2F/DP interaction may reciprocally influence interactions with E3 ubiquitin ligases. The observation that the steady state levels of RB and p107 were diminished by Cyclin D/Cdk4 inhibition, a process that also licenses these pocket proteins for E2F/DP interaction and gene regulation, supports a model where IE interactions with E2F complexes may allow interaction association with E3-ubiquitin ligases (Figure 3-2B). This overlap of degron sequences and repression domains is a conserved feature shared among the RB homologues (5, 13), and represents a novel mode of regulated transcriptional repression, whereby repressors governing critical cell fate outcomes are inherently engineered with limitations on their longevity through turnover by the ubiquitin-proteasome system. Together, these findings implicate Retinoblastoma family IE region as a regulatory nexus linking repressor potency to the ubiquitin-proteasome system in development and

disease. The instability element (IE) mediated regulation of RB family abundance and transcriptional potency is evolutionarily conserved (5, 13). As part of ongoing and future experiments, I am most keen on understanding the differential use of RB family degron for protein turnover in response to diverse physiological perturbations, and the mechanisms of cross-talk between CDK-directed phosphorylation control of RB proteins and the cellular degradation machinery. *Firstly,* as deregulated proteosomal degradation of RB, p107 and p130 contributes to cellular transformation (10), it will be very intriguing to determine whether the instability element is involved in viral oncogene (HPV-E7) induced RB degradation through the proteasome. *Secondly*, based on our recent observation that UV induced DNA damage results in proteosomal degradation of p107 and p130 (Figure 3-3A, B), it will be interesting to study the role of IE in DNA damage induced regulation of p107 and p130 stability. *Thirdly,* we also found that the Mdm2 inhibitor Nutlin-3 stabilizes Mdm2, and induces proteasome mediated degradation of RB and p107 (Figure 3-3C). Given the involvement of Mdm2 in RB turnover, we hypothesize that Nutlin-3 promotes IE-MDM2 interaction that ultimately leads to RB/p107 degradation. Based on our previous findings that the instability element (IE) binds to E2F and directs protein turnover in an ubiquitin dependent manner, we propose a model where E2F and Mdm2 cooperatively binds to the hypo-phosphorylated p107 via the instability element, thereby ensuring degradation of active p107. *Finally,* our studies suggested that Cdk4 inhibition by PD0332991 leads to destabilization of wild-type RB and p107 via proteasome dependent degradation, and mutant form of these proteins lacking the instability element were refractory to PD mediated destabilization. This led us to propose a model whereby, dephosphorylation of specific Cdk4 S-T/P sites within the IE may lead to direct binding of E3 ligases, resulting in protein turnover. We are currently testing this model using a battery of phospho-resistant and phospho mimetic mutations in RB and p107.

Figure 3-2. Participation of the RB family C-terminal instability element (IE) in dual regulation of protein stability and transcriptional potency.

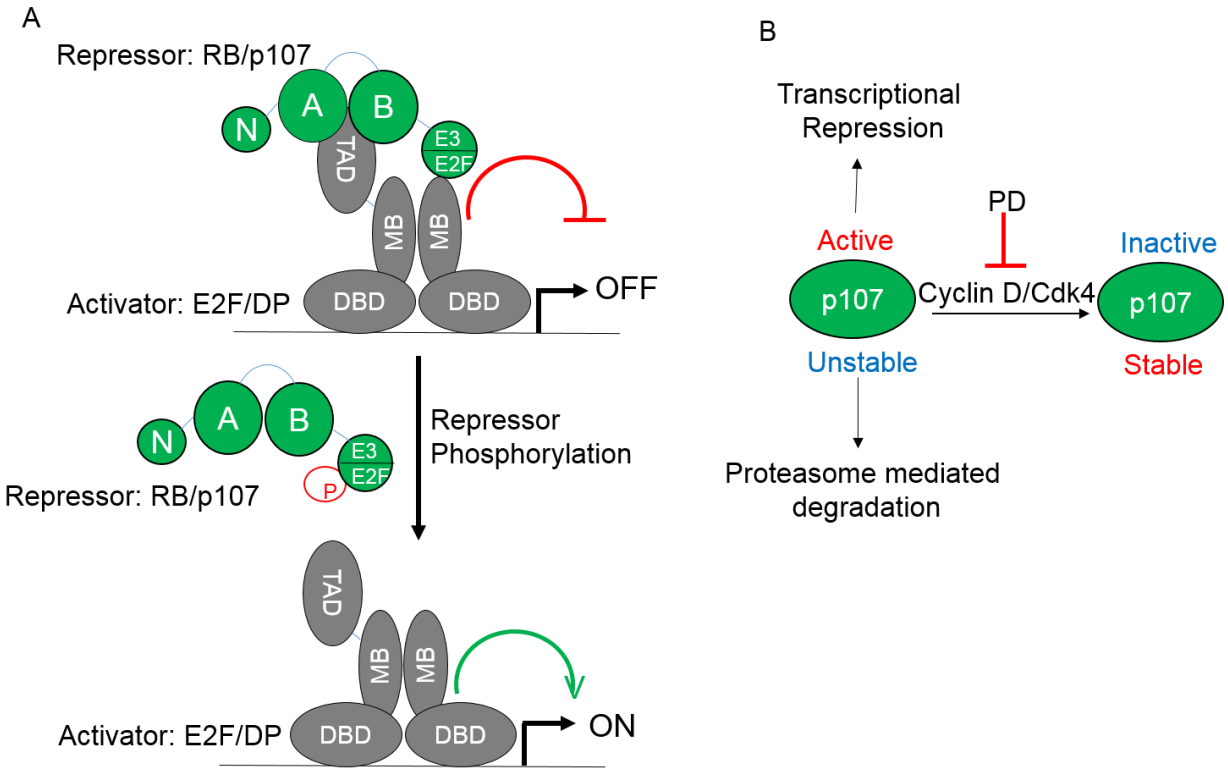

Figure 3-2 (cont'd)

(A) IE mediates intermolecular contacts with the coiled coil-marked box (CC-MB) regions of E2F1-DP1 and E2F4-DP1, and thereby contributes to transcriptional repression. **(B)** RB and p107 is destabilized upon Cyclin D/Cdk4 inhibition, a process that also licenses these pocket proteins for E2F/DP interaction and gene regulation. This supports a model whereby IE interactions with E2F complexes may allow simultaneous interaction with E3-ubiquitin ligases to limit the life span of active repressor molecules.

Figure 3-3. Regulation of RB family stability by DNA damage and Nutlin-3.

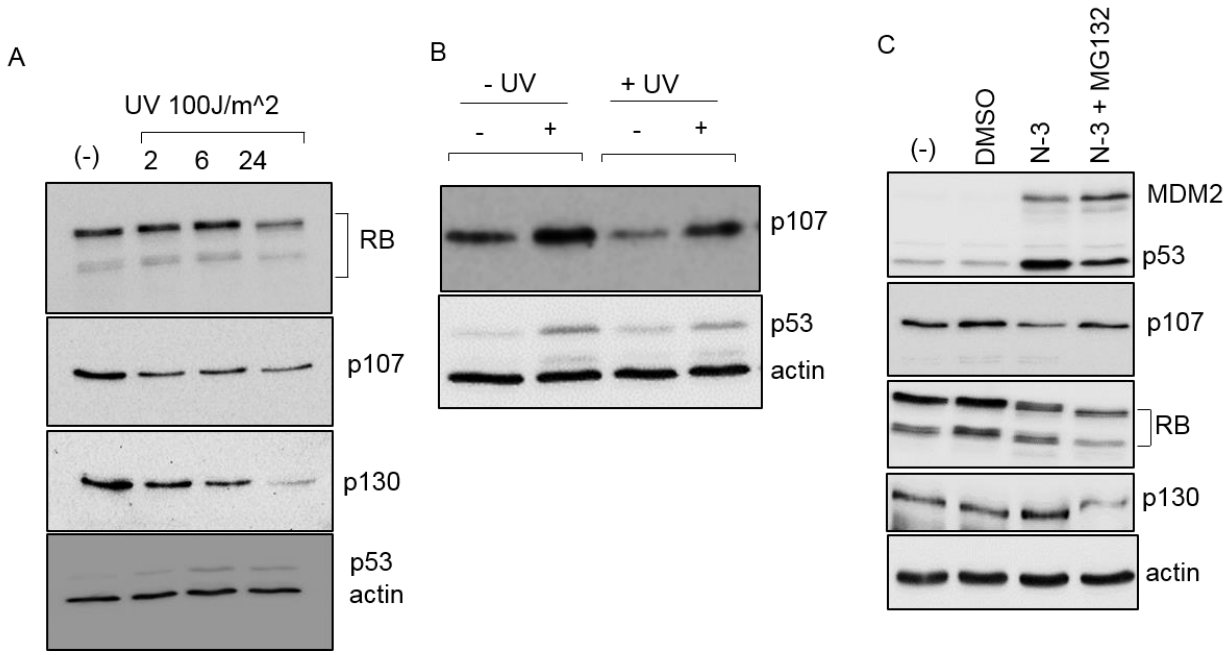

Figure 3-3 (cont'd)

(A) Western blot analysis of whole cell extracts from U2OS cells treated with 100J/m^2 UV and collected after indicated times. Levels of p107 and p130 rapidly declined within 2-6 h of UV treatment, whereas RB levels remained constant during this time period. **(B)** Reduced levels of p107 upon UV induced damage is a result of proteasome dependent turnover, as p107 destabilization during this window is rescued by concomitant MG132 treatment. **(C)** Nutlin-3 (N3) treatment results in altered abundance of Rb family proteins. Reduced levels of p107 upon Nutlin-3 (N3) treatment is a result of proteasome dependent turnover, as p107 destabilization during this window is rescued by concomitant MG132 treatment.

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APPENDIX

COP9 regulation of RB stability in mammalian cells

COP9 signalosome (CSN) is an evolutionarily conserved protein complex comprised of eight subunits (CSN1-CSN8) that regulates the assembly of active Skp1-Cullin1-Fbox (SCF) E3-ligase complex, and thereby controls ubiquitin mediated protein turnover (reviewed in 1-3). The SCF core, comprised of Cullin1 and the Ring-finger protein Roc1, provides a molecular scaffold that via the adaptor protein Skp1 allows modular recruitment of distinct F-box receptors, which in turn recruits specific substrates, thereby generating a repertoire of substrate specific E3 ligases. Covalent conjugation of Nedd8 to Culin1 (*Nedd*ylation) promotes the assembly of active SCF by recruitment of the adaptor and substrate specific F-box proteins to the SCF core. Similarly, removal of Nedd8 from Cul1 catalyzed by CSN5 (de*Nedd*ylation), disassembles and inactivates the SCF complex. This dynamic neddylation and deneddylation of Culin1 mediated by COP9 is the major regulatory control that ensures optimal function of SCF E3-ligases. Studies from our lab found that during embryogenesis, *D*rosophila RB homologs Rbf1 and Rbf2 are stabilized through interaction with COP9 signalosome (CSN) (4). Interestingly, Rbf1-CSN interaction was evident on chromatin and suggested that CSN may function as a corepressor by stabilizing chromatin-bound Rbf1 (4). Both RB and COP9 being evolutionarily conserved proteins, we hypothesized that a similar mechanism governing RB stability should exist in human cells. To test this hypothesis, we knocked down CSN subunits in MCF7 breast adenocarcinoma cell lines, and examined the steady state abundance of RB, p107 and p130 through western blot analysis. In parallel, we also studied the effect of CSN knockdown on cell cycle progression, because CSN has been implicated in transcriptional control of cell cycle genes (5).

As shown in Figures AP-1.1A and AP-1.1B, siRNAs against CSN1 and CSN5 lead to significant decrease in CSN1 and CSN5 protein levels, without any significant effect on the steady

state abundance of RB family proteins. Similar experiments were also performed in three other RB-positive cell lines namely mammary MDA-MB-231, mammary 184B5, and colorectal HCT116 cells, and no significant changes in RB protein levels were observed (data not shown) in any of the cell types. In addition, we also examined the effect of CSN5 knockdown on cell cycle distribution (8-11) and in regulating p53 stability (7) as positive controls for CSN5 deficiency. As shown in Figure AP-1.1D, CSN5 knockdown resulted in a G1 arrest, consistent with previous reports that CSN5 deficiency influences cellular proliferation as a result of reduced CDK2 activity and impaired RB phosphorylation (6). Moreover, CSN5 knockdown resulted in increased p53 abundance upon Doxorubicin induced DNA damage (AP-1.2), consistent with the notion that CSN5 plays a role in MDM2-mediated p53 ubiquitination and degradation (7). In agreement with our observation, two independent groups recently reported that unlike Drosophila Rbf1, mammalian RB proteins do not interact with CSN and, its knockdown is ineffectual for mammalian RB family abundance (5, 6). Taken together, these studies suggests that in mammals, CSN is not directly involved in regulation of RB family stability in differentiated cells, but affects the transcriptional output of the RB-E2F pathway by modulating RB phosphorylation by CDKs. Based on our studies with *D*rosophila Rbf1, an alternative possibility is that COP9 mediated protection of RB occurs only in the context of early embryonic development. Interestingly, knockdown of CSN2 in mouse embryonic stem (ES) cells caused a modest reduction in RB levels (data not shown). Future studies should aim at understanding context dependent function of CSN in regulating RB biology.

Figure AP-1. COP9 signalosome is not involved in regulation of RB family protein stability in differentiated human cells.

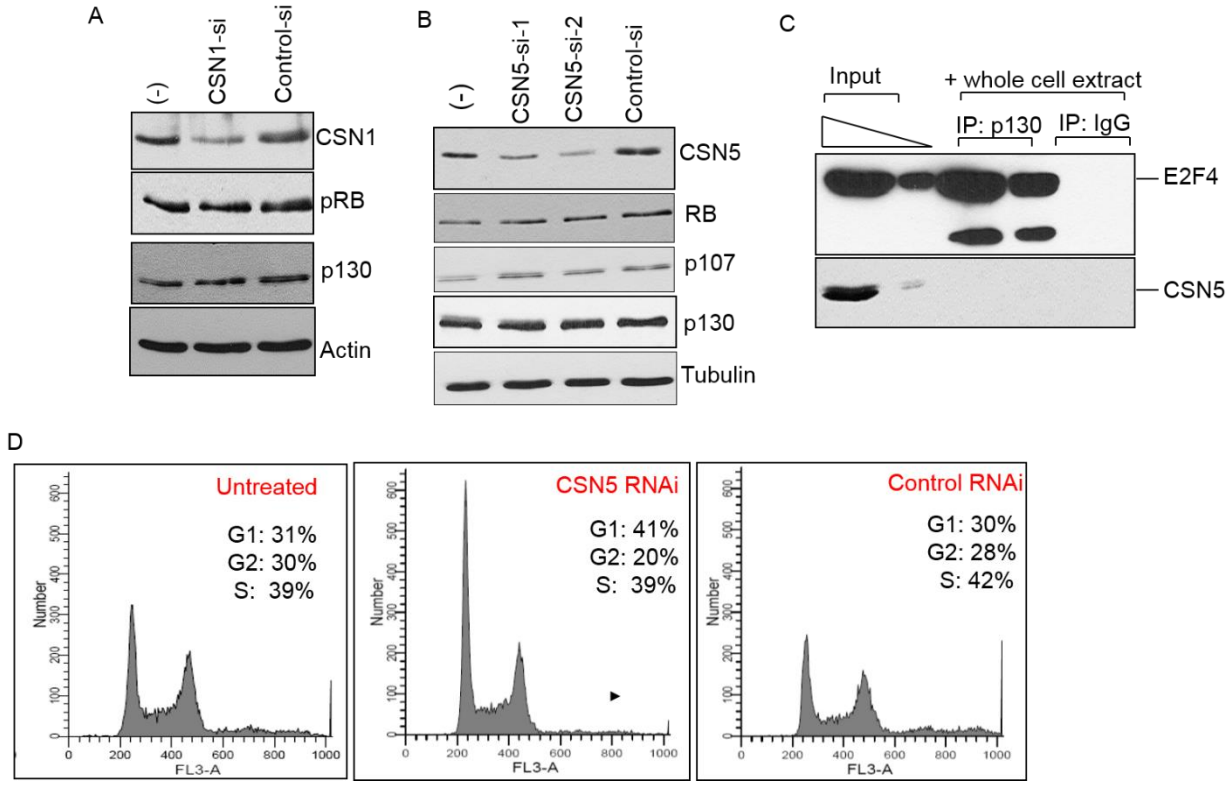

Figure AP-1 (cont'd)

(A, B) Western blot analysis of MCF-7 cell extracts for RB, p107 and p130 after siRNA mediated knockdown of CSN1 and CSN5. Two specific siRNAs against CSN5 were used. CSN1 and CSN5 levels were substantially reduced using respective siRNAs, but none of the siRNAs affected RB, p107, and p130 levels. Actin and tubulin were used as loading controls. **(C)** Endogenous p130 interacts with E2F4, but not CSN5. **(D)** CSN5 knockdown causes a G1 cell cycle arrest in MCF7 cells.

Figure AP-2. COP9 signalosome regulates p53 abundance upon doxorubicin induced DNA damage

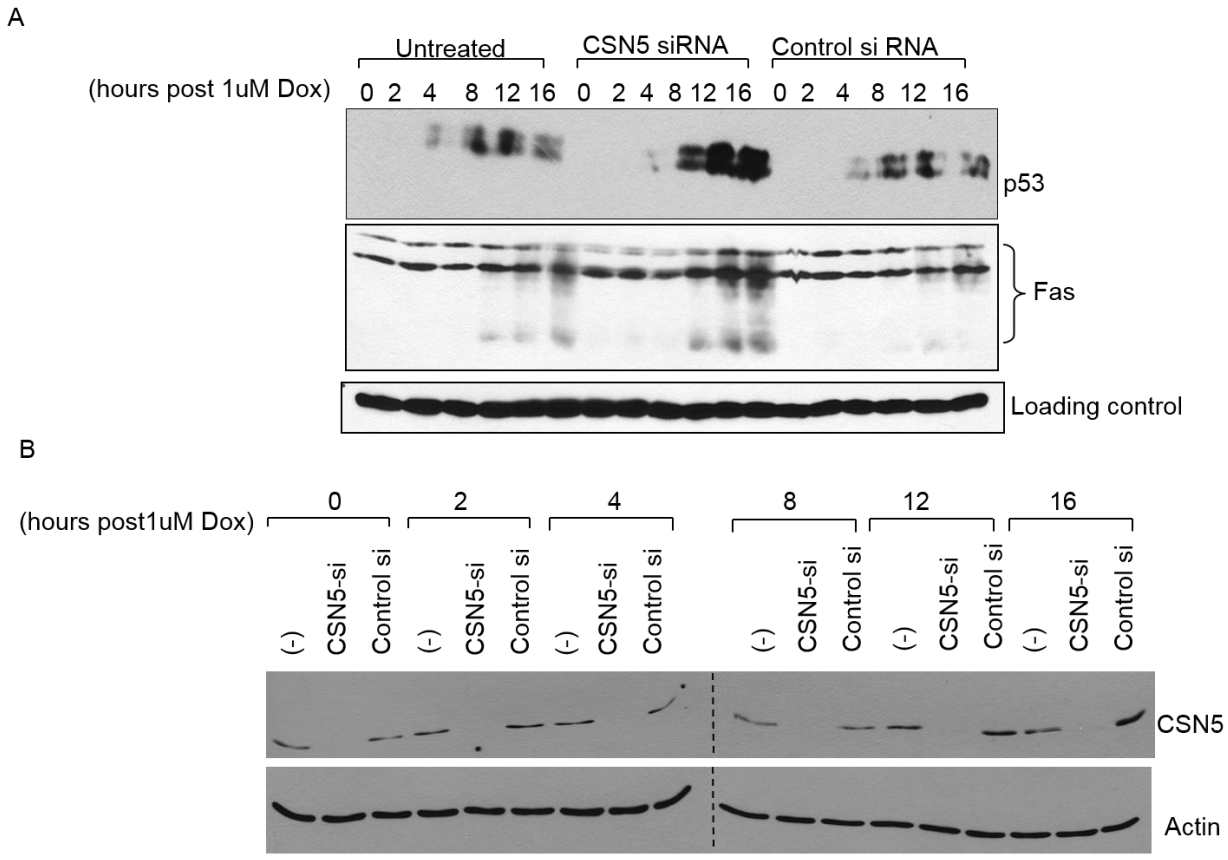

Figure AP-2 (cont'd)

(A) Western blot analysis of MCF-7 cell extracts for p53, after doxorubicin induced DNA damage in cells that were transfected with either control-siRNA or CSN5-siRNA **(B)** Validation of CSN5 knockdown in these samples using western blot analysis.

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