REGULATION MECHANISMS OF DROSOPHILA RETINOBLASTOMA PROTEIN STABILITY AND REPRESSION ACTIVITY

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ABSTRACT

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The retinoblastoma (RB) family proteins are pivotal transcriptional corepressors involved in diverse cellular events. As a potent tumor suppressor, RB plays a variety of important roles including regulating cell cycle, promoting cellular differentiation, inducing cell senescence and balancing apoptotic death, many of which are controlled by specific post-translational modifications of RB. We identified an evolutionarily conserved instability element (IE) in the C-terminus of *Drosophila* RB-related protein Rbf1 that simultaneously regulates degradation and repression activity. Paradoxically, when the IE is deleted, increased protein levels do not cause enhanced repression activity. Rather, these mutations diminish repression activity of Rbf1, indicating a linkage between Rbf1 activity and instability. I found that the IE is an independent module which may serve as an interaction domain for multiple cofactors linking protein turnover and transcriptional repression. I showed that loss of the IE promotes cell growth, a disastrous consequence favorable to cancer progression. To better understand the control of this unique bifunctional element of Rbf1, I investigated the effects of phosphorylation by Cyclin-Cdk complexes. I show that protein stability and activity governed by the IE are cleanly separable, possibly by coordinated interactions with E2F transcription factors and E3 ubiquitin ligases. A highly conserved lysine residue K774 in the IE, frequently mutated in RB family protein p130 in lung cancers, is critical for Cyc-Cdk-mediated phosphorylation. My data suggest

that the IE governs distinct functional outputs of Rbf1 through post-translational modifications and potentially cofactor binding. The similarities with mammalian systems suggest that parallel processes may regulate RB family proteins in human cancers.

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KEY TO ABBREVIATIONS

c-Abl	Cellular Abelson murine leukemia
c-Myc	Cellular Myelocytomatosis viral oncogene C
CBP	CREB-Binding Protein
cdc2	cell division cycle 2
Cdk	Cyclin dependent kinase
CNS	Central Nervous System
COP9	Constitutive Photomorphogenic 9
CSN	COP9 Signalosome
dCAP-D3	Chromosome Associated Protein D3
DNA	Deoxynucleic Acid
DP	E2F Dimerization Partner
E3	Ubiquitin ligase enzyme 3
E1A	Early region 1A
E2F	E2 promoter binding Factor
EID-1	EP300-interacting Inhibitor of Differentiation 1
H3K9	Histone 3 Lysine 9
H3K18	Histone 3 Lysine 18
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase Complex
HP1	Heterochromatin Protein 1
HPV E7	Human Papilloma Virus Early 7

JAK/STAT	Janus Kinase and Signal Transducer and Activator of Transcription
JNK	c-Jun N-terminal Kinase
L3MBTL1	Lethal 3 Malignant Brain Tumor-Like 1
MAP	Mitogen-Activated Protein
Mdm2	Murine double minute 2
PCNA	*Proliferating Cell Nuclear Antigen*
PP1	Protein Phosphatases type 1
RB	Retinoblastoma protein
Rbf1	Retinoblastoma family protein 1
Rbf2	Retinoblastoma family protein 2
RING	Really Interesting New Gene
RNR2	Ribonucleotide Reductase 2
S2 cells	Schneider 2 cells
SCF	Skp/Cullin/F-box complex
Skp	S-phase kinase-associated protein
SMYD2	SET and MYND domain containing 2
SUV39H1	Suppressor of Variegation 3-9 Homolog 1
SV40	Simian Virus 40
SWI/SNF	Switch/Sucrose Nonfermenting complex
Tip60	TAT interacting protein 60
VP-16	Virion Protein 16

CHAPTER 1

Introduction

The most important lesson scientists have learned in the field of cancer research over the last decades is that a diverse spectrum of genetic and epigenetic changes occur and accumulate to promote cancer in different ways. This heterogeneity hinders the development of efficient therapeutic strategies. To better understand underlying properties of the disease state, one essential task is to use model systems to understand the basic properties of regulatory molecules that are affected in cancer, to identify general principles that can be applied to systems of higher complexity.

In 1971, Knudson proposed a “two-hit hypothesis” to explain occurrences of sporadic and inherited forms of retinoblastoma, a juvenile retinal cancer. He suggested that this type of cancer was initiated by separately occurring lesions in each allele of the same gene, either inherited from a parent or acquired during somatic development (Knudson, 1971). This rather simple hypothesis, supported by clinical observations, ignited extensive research on tumor suppressor genes and cancer development. The retinoblastoma protein (RB) was identified as the first tumor suppressor due to its loss of function being associated with tumorigenesis.

After decades of biochemical and physiological studies on the retinoblastoma family proteins, RB and the structurally related proteins p107 and p130, scientists have arrived at an understanding of their tumor suppressor functions, which include controlling cell cycle arrest, inducing cellular differentiation, maintaining genomic stability and inducing cell senescence (Burkhart and Sage, 2008). Consistent with these important cellular roles of RB, the protein is frequently inactivated in a broad range of human cancers other than retinoblastoma, including lung cancer, melanoma, prostate cancer, breast cancer, bladder cancer, leukemia,

brain cancer, oesophageal cancer and liver cancer (Burkhart and Sage, 2008). Moreover, upstream regulators of RB family proteins are also frequently mutated, attesting the functional importance of RB and its tight regulation (Knudsen and Knudsen, 2008).

Despite diverse roles of RB in many cellular events, a common molecular mechanism is thought to be involved; RB serves as a recruiter to assemble protein-DNA complexes or protein-protein complexes in transcriptional-dependent or -independent manners. Numerous partner proteins have been found to associate with RB (Morris and Dyson, 2001). Understanding these physical and physiological interactions in different pathways can illuminate the functional importance of RB in tumor suppression.

Due to its diverse regulatory roles, RB is subject to tight spatial and temporal control by diverse post-translational modifications. Phosphorylation of RB is a well-studied mechanism of manipulating RB structure and activity in response to cellular and environmental stimuli (Rubin, 2013). Other post-translational modifications, including methylation, acetylation and ubiquitination, are involved in different regulation pathways that control RB functional outputs in diverse physiological conditions (Munro et al., 2012).

In this introduction, I will focus on mammalian RB family proteins and their *Drosophila* counterparts. I discuss their functions, binding partners and regulatory mechanisms revealed in diverse studies over the decades. I will also discuss the identification of physical and functional targets of RB on a genome-wide scale, which points to exciting newly discovered roles of RB that will be the focus of future research.

Retinoblastoma family proteins

General properties of retinoblastoma family proteins

After identification of a genetic locus on human chromosome 13 that was associated with

retinoblastoma, the retinoblastoma susceptibility gene was successfully cloned and found to encode a nuclear phosphoprotein that is bound to chromatin and may regulate other genes (Friend et al., 1986; Lee et al., 1987a; Lee et al., 1987b). The first cellular function identified for RB is its negative regulation of the cell cycle, which provides a simple explanation for RB tumor suppression (Goodrich et al., 1991). RB was reported as an important target of viral oncoproteins such as adenovirus E1A, SV40 T antigen and human papilloma virus E7 protein; mutant viral oncoproteins incapable of RB binding lose their ability to transform host cells into tumor cells (Helin and Ed, 1993). Thus, a clear correlation between DNA tumor viruses and RB regulation of cell proliferation has been established.

Further experimentation identified a key player in RB biology: the E2F1 transcription factor, which was the first cellular target of RB to be identified (Helin et al., 1992; Kaelin et al., 1992). E2F family proteins bind to and activate a variety of genes important for progression through the cell cycle, including Cyclin A, Cyclin E, Cdc2, c-Myc and DNA polymerase α (Helin, 1998). The current model suggests that RB cell cycle regulation is mediated by repressive interactions with E2F family proteins, in which histone modifiers and chromatin remodelers are recruited to promoters to inhibit E2F-dependent transcription of cell cycle genes (Brehm and Kouzarides, 1999). RB proteins are also thought to directly antagonize the activation domain of certain E2F proteins.

RB-mediated cell cycle regulation is controlled by Cyclin-dependent kinases (Cdks). Phosphorylation of RB disrupts association between RB and E2F and releases transcription of E2F-dependent genes (Buchkovich et al., 1989). Therefore, RB itself is subject to a cell cycle-controlled regulatory network. Genetic lesions in these upstream regulators cause a broad range of tumors, indicating that disruption of the RB pathway is a common hotspot in tumorigenesis.

Further research has demonstrated that the cellular functions of RB as a potent tumor

suppressor also include genes in many categories other than cell cycle regulation, including DNA repair, DNA replication and apoptosis, some of which involve E2F-independent functions (Burkhart and Sage, 2008). It is widely considered that the diverse roles of RB all contribute to its tumor suppression and may govern cell type-specific and tumor stage-specific functions.

Identification of homologous genes containing a conserved region similar to the RB "pocket", the portion of the RB binding domain targeted by viral oncoproteins, revealed two RB-related proteins, p107 and p130. Cloning of these two genes suggest that RB, p107 and p130 share sequence homology especially in the central pocket domain that mediates interactions with viral oncoproteins. These proteins are collectively called the pocket proteins. RB family proteins share not only sequence similarities but also biochemical similarities. Like RB, p107 and p130 also associate with E2F family proteins to repress E2F-dependent transcription by recruiting common cofactors such as HDAC. Their cell cycle activities are also regulated by Cdks (Classon and Dyson, 2001).

The highly conserved central pocket domain shared by RB family proteins contains two well-ordered subdomains separated by an unstructured spacer (Fig. 1-1). Each subdomain bears cyclin box folds which are well-characterized structures for protein-protein interactions (Kim and Cho, 1997). The N-terminal domain of RB also contains cyclin fold-like structures (Hassler et al., 2007). The less structured C-terminal domain is not well conserved in RB family proteins but may account for some functional differences in pocket proteins.

Despite sequence and functional similarities, significant differences exist between p107, p130 and RB. Expression levels of the pocket proteins and their association with E2F proteins are clearly different depending on cell types. RB interacts with E2F1-4 in both quiescent and dividing cells, whereas p107 and p130 primarily interact with E2F4 and 5. p107 binds to E2F in the S phase of cell cycle and p130 interacts with E2F in quiescent cells

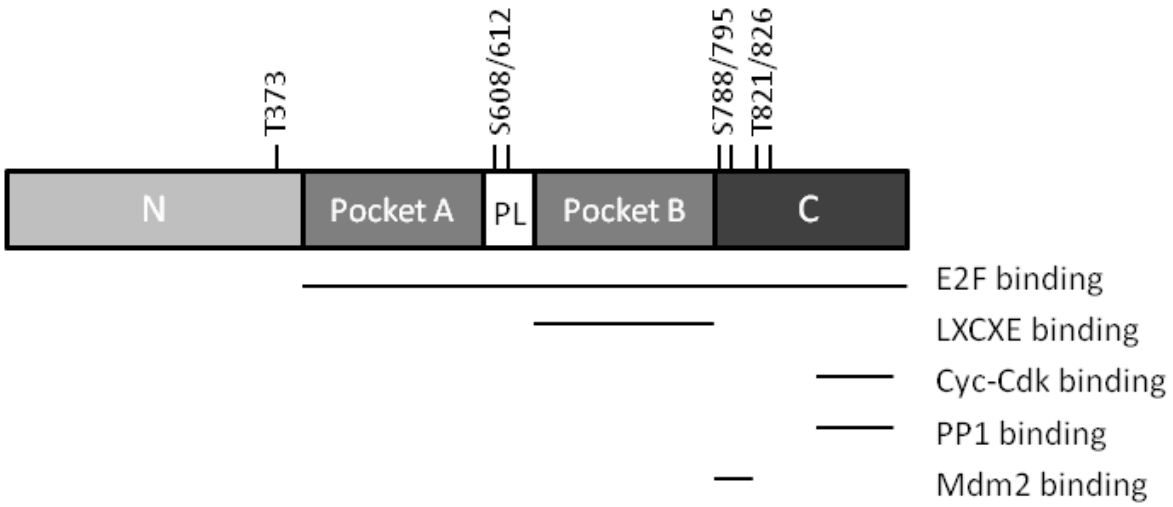
(Classon and Dyson, 2001). These biochemical differences indicate that the pocket proteins may govern differential functional outputs at different stages of the cell cycle and in different cell types.

Drosophila retinoblastoma family proteins

The pocket proteins are highly conserved in vertebrates, invertebrates and plants. The RB homologues have been extensively studied in *Drosophila melanogaster* due to its simpler RB-E2F network. Studies of RB family proteins in this model organism can provide insights into control of diverse biological functions, such as cell cycle regulation, differentiation and apoptosis, in different organisms.

Drosophila contains two retinoblastoma family proteins, Rbf1 and Rbf2, two E2F family proteins E2F1 and E2F2, and one DP partner for E2F binding (van den Heuvel and Dyson, 2008). Rbf1 interacts with E2F1, a potent transcriptional activator, and E2F2, a transcriptional repressor, while Rbf2 exclusively interacts with E2F2 (Stevaux et al., 2002). Rbf1-knockout embryos show constitutive expression of *PCNA* and *RNR2*, two E2F-regulated genes for DNA replication, and ectopic S-phase entry, indicating the importance of Rbf1 for regulating E2F-dependent transcription and cell cycle progression during embryogenesis (Du and Dyson, 1999). Rbf1 is expressed relatively uniformly at all stages of embryonic development, while Rbf2 is dynamically expressed during development and peaks during early embryogenesis, indicating that these two factors may have redundant and unique roles (Keller et al., 2005; Stevaux et al., 2002). *rbf1* is essential for *Drosophila* development, whereas *rbf2* nulls are viable, although fertility is impaired. Rbf2 has also been found to govern tissue-specific regulation of E2F2 target genes (Stevaux et al., 2005). As with mammalian RB proteins, *Drosophila* Rbf proteins are subject to phosphorylation by Cyclin-Cdk complexes (Xin et al., 2002).

Figure 1-1. Schematic diagram of RB structure.



RB contains three domains: the N-terminal domain, the pocket domain and the C-terminal domain. Two subdomains in the pocket are separated by the pocket linker (PL). Five phosphorylation sites important for controlling conformational change of RB are indicated. Regions in RB for protein binding are indicated by lines.

These studies in *Drosophila* do not directly address cancer-related questions, but they illuminate essential biological functions of the well-conserved RB pathway and help us better understand potential roles and regulation of RB in human development and cancer progression.

Functions of RB proteins

The fact that RB is functionally inactivated in a broad range of human cancers supports the general notion that the protein is a tumor suppressor. It was originally believed that RB tumor suppression function was largely due to its cell cycle regulation during G1-S transition by inhibiting E2F-dependent transcription of cell cycle genes. Now it is generally considered that RB has many functional roles in addition to serving as a G1 checkpoint. RB might suppress tumorigenesis by inducing cellular differentiation, promoting cell senescence and preserving genomic stability in different cell types. RB is also associated with controlling angiogenesis and metastasis, which are not well understood. A further complexity of RB regulation is the dual context-dependent pro-apoptotic and anti-apoptotic functions (Burkhart and Sage, 2008).

Cell cycle regulation

The most thoroughly studied function of RB is its interaction with sequence-specific DNA binding E2F transcription factors in the regulation of cell proliferation. The pocket proteins negatively regulate E2F-dependent transcription through at least two mechanisms. First, the transactivation domain of E2F1 initiates transcription by recruiting general transcription factors and histone acetyltransferases (Emili and Ingles, 1995; Hagemeier et al., 1993; Lang et al., 2001; Pearson and Greenblatt, 1997). RB binding with E2F is primarily through the

interaction between the pocket domain of RB and the transactivation domain of E2F, interfering with the transactivation domain's ability to engage the transcriptional machinery (Rubin, 2013). Second, when bound to E2F, RB also recruits a large set of histone modifiers and chromatin remodeling complexes including histone deacetylases, histone methyltransferases, histone demethylases, DNA methyltransferases and SWI/SNF complexes (Frolov and Dyson, 2004). However, the molecular details of these two mechanisms for gene expression control in cell cycle are not well understood.

Histone acetylation and deacetylation seem to be a key mechanism for regulation of E2F-dependent transcription. E2F recruits several histone acetyltransferseas (HAT) as coactivators such as CBP, p300 and Tip60 to E2F-regulated promoters. Histone deacetylases (HDAC) recruited by RB family proteins reverse the histone marks placed by E2F activation complexes. RB also interacts with the histone methyltransferase SUV39H1, which methylates H3K9 and recruits heterochromatin protein HP1. RB serves as an adaptor protein to tether histone modifiers to the promoter and revamp the chromatin status (Munro et al., 2012). H3K9 methylation and HP1 binding are considered to be heterochromatin markers, indicating that RB-mediated repression may drive some E2F-regulated genes into a permanently repressed status. In *Drosophila*, some genes are stably repressed in proliferating cells by E2F2-Rbf complexes, which are resistant to cell cycle regulation (Dimova et al., 2003). These RB-bound genes are likely involved in cellular events other than cell cycle progression.

Cell division and genomic stability

Loss of RB leads to upregulation of E2F, which drives cells into ectopic S phase followed by apoptosis due to genomic instability, suggesting that RB plays an essential role in maintaining mitotic fidelity (Chau and Wang, 2003). This specific role of RB is appealing

because it might explain why inactivation of RB contributes to both cancer initiation and progression.

Development of aneuploidy has been long considered as a key transition in metastatic progression (Hartwell, 1992). Genomic instability, including deletions, inversions and chromosomal rearrangements, caused by inappropriate mitotic chromosome segregation may introduce mutations in tumor suppressor genes and oncogenes, which is conducive to cancer development. This notion is well supported by identification of chromosomal alterations in many human cancers (Albertson et al., 2003).

Inactivation of RB increases mitotic defects and genomic instability, leading to tumorigenesis. Loss of RB function results in supernumerary centrosomes, centromeric defects and merotelic kinetochore attachments during mitosis, which is associated with chromosome mis-segregation (Manning and Dyson, 2011). However, there is no evidence supporting a direct relationship between RB and mitosis. It is more likely that loss of RB causes defects in early preparation for mitosis.

An early defect includes misregulated E2F-dependent transcription of genes required for chromosome segregation during mitosis. One example is that expression of Mad2, an E2F target, is upregulated by inactivation of RB. The overexpression of Mad2 is sufficient to induce chromosome mis-segregation (Hernando et al., 2004). E2F-independent functions of RB are also important for maintaining genomic stability. Loss of RB causes defects in recruitment of cohesin and condensin to the centromere of chromosomes, which promotes merotelic kinetochore attachments and chromosome mis-segregation. In *Drosophila*, Rbf1 also colocalizes with condensin II protein dCAP-D3 on polytene chromosomes in an E2F-independent manner (Longworth et al., 2008).

Chromosomal instability drives tumor evolution by gradual acquisition of deleterious mutations in tumor suppressor genes and oncogenes. Based on the mitotic defects observed

following inactivation of RB, it is likely that the normal tumor suppressive role of RB involves the preservation of genomic stability.

Apoptosis

RB is involved in apparently mutually exclusive cellular events: cell proliferation and apoptotic death. This raises one important question of how cells coordinate growth and death. Many cancers inactivate RB by phosphorylation, rather than destabilizing or eliminating the protein entirely, possibly because of the inhibitory function of RB on apoptosis during cancer initiation (Knudsen and Knudsen, 2008).

One model to explain the anti-apoptotic function of RB is that the increased apoptosis in RB-null cells is an indirect outcome caused by defects in ectopic S phase entry correlated with loss of RB. However, some studies suggest that E2F1 is a pro-apoptotic transcription factor and that RB represses E2F1-regulated promoters to play a negative role in apoptosis (Field et al., 1996; Leone et al., 2001). Loss of RB in mice sensitizes cells to apoptosis in the nervous systems, lens and skeletal muscles, which requires the activity of E2F1 and p53 (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). For example, apoptosis protease activating factor-1 (Apaf1), a common target for transcriptional regulation by E2F1 and p53, is deregulated in RB-null CNS neurons (Moroni et al., 2001). Therefore, RB has a direct role in inhibiting apoptosis.

E2F1 is not the only mediator for the anti-apoptotic function of RB. RB can directly bind to c-Abl tyrosine kinase and inhibit its activity, which promotes apoptosis (Wang, 2000; Welch and Wang, 1993). The JNK kinase is also involved in stress-induced apoptosis and it seems to be inhibited by RB (Shim et al., 2000). Taken together, RB is able to inhibit apoptosis through interactions with other cellular proteins in an E2F1-independent manner.

However, the role of RB in apoptosis is rather complicated as an RB-dependent

proapoptotic function has also been reported. In response to DNA damage and oncogenic stress, the RB-E2F1 complex binds to apoptotic genes that are transcriptionally active and RB is required to achieve maximal apoptotic response (Ianari et al., 2009; Knudsen et al., 1999). Taken together, RB is able to either suppress or promote apoptosis, depending on the cellular context.

Tumor suppression functions of p107 and p130

Viral oncoproteins do not only bind to RB but also interact with the other two family members, p107 and p130, suggesting potential functional roles of these two RB-related proteins in tumor suppression (Dyson et al., 1989; Harlow et al., 1986). However, due to the functional overlap within the RB family, it is difficult to distinguish the individual contribution of RB family members in diverse cellular events.

Genetic lesions in the upstream regulators, such as Cyclin, Cdk and Cdk inhibitors, of RB family proteins functionally inactivate all three family members by hyperphosphorylation, suggesting that all RB family members contribute to tumor suppression (Canepa et al., 2007; Pei and Xiong, 2005). It is known that p107 and p130 are weak tumor suppressors, since mutations in them are rarely found in human tumors (Beroukhim et al., 2010; Burkhart and Sage, 2008). But in the context of RB loss, p107 and p130 contribute some tumor suppression functions. For example, mice with mutations in both RB and p107, but not mutation in RB only, develop papillomatous lesions and squamous cell carcinomas in the epidermis (Lara et al., 2008). RB/p107 and RB/p130-deficient mice develop many types of tumors, a larger spectrum than tumorigenesis in RB-deficient mice, suggesting p107 and p130 contribute to tissue-specific functional compensation in response to the loss of RB (Dannenberg et al., 2004).

Binding partners of RB proteins

Consistent with the diverse cellular roles of RB, the protein has been found to associate with numerous factors involved in different cellular events(Morris and Dyson, 2001). Nevertheless, despite the ability of RB to associate with different partners, it is thought that RB has a common activity as an adaptor protein to assemble protein-protein complexes and protein-DNA complexes (Fig. 1-1). Its major binding partner E2F is involved in transcriptional regulation of many types of genes by RB. RB exerts full repression potency by recruiting numerous cofactors including histone modifiers and chromatin remodeling complexes to the promoter. Moreover, because this protein has pleiotropic activities, RB itself is tightly controlled by upstream regulators through different pathways. RB is subject to phosphorylation and dephosphorylation mediated by Cyclin-Cdk and phosphatase. Ubiquitination of RB targeted by Mdm2 and Skp2 is also a key mechanism for proper depletion of RB in a timely fashion. Understanding how RB is involved in different cellular events and is regulated through different pathways requires thorough investigation of protein-protein interactions on a structural basis.

Binding of E2F family proteins

The best characterized interactions of RB are with E2F family proteins. E2F transactivation domain (TD) binds to the cleft between two subdomains of the pocket (Lee et al., 2002; Xiao et al., 2003). The RB C-terminus also makes an important second interaction with the Marked Box (MB) domain of E2F and its heterodimer binding partner DP. Two separate regions in the C-terminus are involved in the second interaction (Rubin, 2013).

Phosphorylation induces global conformational changes in RB to disrupt E2F association involving at least three mechanisms. Phosphorylation of S608/S612 in the pocket linker (PL) induces PL binding to the pocket domain to compete with E2F TD binding. Phosphorylation

of T373 in the N-terminus creates a hydrophobic surface to promote RBN-pocket binding (Burke et al., 2012). Phosphorylation of S788/S795 in the C-terminus inhibits that domain from binding to the E2F MB, while phosphorylation of T821/T826 induces RBC binding to the pocket domain (Rubin et al., 2005).

Binding of transcription cofactors

A binding motif LXCXE is found in viral oncoproteins such as adenovirus E1A, SV40 large T-antigen and HPV E7 (Moran, 1993). Structural studies show that this motif binds to a shallow groove in the B subdomain of the pocket domain (Lee et al., 1998). Interestingly, histone modifiers, chromatin remodeling complexes, and the condensin II complex all bind to RB in an LXCXE-dependent manner (Brehm and Kouzarides, 1999; Longworth et al., 2008; Morris and Dyson, 2001). At least some of the components in these complexes have the LXCXE sequence. For example, HDAC contains an LXCXE motif that is required for RB binding (Brehm et al., 1998; Kouzarides, 1999; Luo et al., 1998; Magnaghi-Jaulin et al., 1998).

Binding of E3 ubiquitin ligases

The Mdm2 E3 ubiquitin ligase physically interacts with RB and inhibits RB growth regulatory function, and as an oncoprotein, Mdm2 is frequently overexpressed in a variety of human cancers (Xiao et al., 1995). Mdm2 contains a central acidic region that interacts with the C-terminus of RB (Sdek et al., 2004). The C-terminal RING finger domain of Mdm2 functions as a ubiquitin ligase to target substrate proteins, including p53 and RB. Interestingly, Mdm2 binds preferentially to hypophosphorylated RB, suggesting a potential phosphorylation-mediated regulation of Mdm2-RB interaction (Sdek et al., 2004). In contrast, p130 is regulated by a distinct E3 ubiquitin ligase complex, Skp1/Cul1/Skp2, which binds

and promotes p130 degradation, a process which requires prior phosphorylation by Cdks (Bhattacharya et al., 2003).

Binding of Cyclin-Cdk and phosphatase

The C-terminus of RB contains a docking site for CycA/E-Cdk2 and CycD-Cdk4 (Adams et al., 1999; Pan et al., 2001). Interestingly, it overlaps with the binding site for protein phosphatase 1 (PP1). Competition by PP1 with Cyc-Cdk for RB binding is sufficient to block inactivation of RB and cell cycle progression (Hirschi et al., 2010; Tamrakar and Ludlow, 2000).

Unlike RB, p107 and p130 share a conserved spacer region before A and B subdomains in the pocket. Importantly, this region provides a high-affinity binding site for CycA/E-Cdk2 (Hannon et al., 1993; Lees et al., 1992; Li et al., 1993). This alternative mode of Cyc-Cdk binding may provide a discriminatory regulation of RB family proteins in response to different stimuli. Additionally, the N-terminal region of p107 and p130 seems to inhibit the activities of Cyc-Cdk (Woo et al., 1997).

Regulation of RB proteins

Viral oncoprotein binding and inactivation

DNA tumor viruses transform host cells into proliferating cancer cells by inactivating tumor suppressor proteins such as p53 and RB (DeCaprio, 2009). Specifically, viral oncoproteins, such as adenovirus E1A, SV40 large T-antigen and HPV E7, sequester RB from E2F and disrupt its function of cell cycle regulation (Helin and Ed, 1993). In addition, RB can be phosphorylated by viral proteins mimicking cyclin-CDK function (Hume et al., 2008).

Phosphorylation

The best-studied regulation mechanism of RB is phosphorylation mediated by Cyclin-Cdk family kinases. A simple model is that during G1-S transition, RB is phosphorylated by Cyc-Cdk and dissociates from E2F, resulting in progression of the cell into S phase (Brehm and Kouzarides, 1999). However, structural and functional studies have provided evidence to support that RB physically interacts with numerous proteins and that it is progressively phosphorylated and inactivated by multiple Cyc-Cdk complexes (Mittnacht, 1998).

Several Cyc-Cdk complexes have been reported to regulate RB, namely CycD-Cdk4, CycE-Cdk2 and CyCA-Cdk2, each of which is responsible for RB phosphorylation at a specific time point in the cell cycle. CycD-Cdk4 is active in mid to late G1, CycE-Cdk2 in late G1 and CycA-Cdk2 in S phase (Munro et al., 2012). The relative contribution of different Cyc-Cdk complexes is not fully understood, however.

One indication of differential regulation functions by different Cyc-Cdk complexes comes from the finding that they phosphorylate different sites on RB. Different Cyc-Cdk kinases have different preferred target sites *in vitro*. For example, CycD-Cdk4 preferentially phosphorylates S780 (Kitagawa et al., 1996). T826 is also specifically targeted by CycD-Cdk4 (Zarkowska and Mittnacht, 1997). Interestingly, these two sites are different from the typical Cdk concensus S/TPXK/R, suggesting that site selection by different Cyc-Cdk might be determined by chemical specificity.

Differential phosphorylation patterns have distinct effects on RB functions. As described above, several key residues in the N-terminus, the pocket and the C-terminus are phosphorylated to induce conformational changes for the release of RB from E2F (Rubin, 2013). Moreover, phosphorylation of T821 and T826 in the C-terminus of RB is required for dissociation of LXCXE binding proteins whereas phosphorylation of S807 and S811 is required to disrupt c-Abl binding (Knudsen and Wang, 1996).

The phosphorylation mechanism is utilized for the regulation of RB not only in the context of cell cycle progression but also during other cellular events. Under conditions of DNA damage, RB is phosphorylated by p38 MAP kinase, which is activated by a signaling cascade in response to stress stimuli (Delston et al., 2011). Additionally, the checkpoint kinases Chk1 and Chk2 regulate RB function on apoptotic genes during DNA damage response (Inoue et al., 2007). These examples demonstrate that phosphorylation mediated by multiple kinases modulates RB functions involved in different pathways, generating distinct molecular outputs.

Ubiquitination and degradation

In addition to phosphorylation control, RB protein levels are tightly controlled by proteolysis, which is less understood. A variety of viral oncoproteins, such as HPV E7, human cytomegalovirus pp71 and hepatitis C virus NS5B, can bind to RB and target it for degradation through a proteasome-dependent pathway (Boyer et al., 1996; Kalejta and Shenk, 2003; Munakata et al., 2005). As mentioned above, Mdm2, a RING finger protein, serves as a ubiquitin E3 ligase to promote proteasome-dependent degradation of RB, and in cancers overexpressing Mdm2, RB levels are diminished (Sdek et al., 2005; Uchida et al., 2005). Moreover, Mdm2 mediates both ubiquitin-dependent and ubiquitin-independent pathways, as has also been described for the degradation of p53. Mdm2-mediated degradation of RB is an important mechanism to block RB-E2F formation and facilitate E2F-dependent transcription.

Unlike RB and p107, p130 protein levels are dramatically reduced when the protein is hyperphosphorylated during G1-S transition in cell cycle (Tedesco et al., 2002). In this process, Skp1-Cul1-Skp2 SCF E3 ubiquitin ligase mediates ubiquitination and degradation of hyperphosphorylated p130 through a proteasome-dependent pathway, suggesting an important mechanism for negative regulation of p130 transcriptional activity (Bhattacharya et

al., 2003).

Other post-translational modifications

Acetylation of lysine residues is also a well-studied regulation mechanism of RB. The p300/CBP histone acetyltransferase acetylates RB on K873 and K874 during cell cycle progression and differentiation. This acetylation hinders Cdk-mediated phosphorylation of RB and enhances the binding of Mdm2, suggesting that acetylation may be involved in regulating protein-protein interactions (Chan et al., 2001a). Consistent with this, acetylation of RB during myogenesis is associated with RB hypophosphorylation. Acetylated RB recruits Mdm2 to target EID-1, a differentiation inhibitor, for degradation to promote terminal differentiation (Nguyen et al., 2004).

Interestingly, K873 and K874 fall within the binding motif for Cdks, providing a possible explanation of antagonistic effects of acetylation to Cdk-mediated phosphorylation and inactivation. Moreover, in response to DNA damage, acetylation of K873/K874 releases the E2F1 Marked Box domain from the C-terminal interaction domain of RB, indicating that acetylation of RB C-terminus has gene-specific regulation of RB activity (Markham et al., 2006).

Unlike RB, acetylation of K1079 in p130 enhances Cdk4-mediated phosphorylation *in vitro*, suggesting that interplay between acetylation and phosphorylation may contribute to differential functions of the pocket proteins (Saeed et al., 2012).

The C-terminal lysine residues are also subject to methylation control. Methylation at K873 in RB by the methyltransferase Set7/9 creates a binding site for the heterochromatin protein HP1 involved in RB-dependent cell cycle arrest and transcriptional repression (Munro et al., 2010). Similarly, methylation of K860 by SMYD2 provides a binding site for the methyl-binding domain of the transcriptional repressor L3MBTL1 (Saddic et al., 2010). In

response to DNA damage, K810 in the C-terminus of RB can be methylated to block the interaction between RB and Cdk4, inhibiting phosphorylation of the protein, suggesting an interplay between methylation and phosphorylation (Carr et al., 2011).

Genome-wide regulation by RB proteins

By performing genome-wide analysis of the RB binding profile and RB-regulated transcription in recent years, researchers have started to reveal previously less understood functions of RB family proteins, especially for nonoverlapping cellular roles between different RB proteins.

Genome-wide binding and functional targets of RB

Adenovirus oncoprotein E1A binding to RB family proteins is an essential step in the transformation process of the host cells. Upon transformation, E1A induces large-scale epigenetic and transcriptional reprogramming through relocalization of RB, p107, p130 and the p300/CBP histone acetyltransferase, driving cells into replication mode and inhibiting antiviral responses and cellular differentiation. This whole process is in a temporal order. E1A first depletes RB from cell cycle genes and recruits p300/CBP to the promoters, causing enrichment of H3K18Ac and transcriptional activation. Subsequently, RB and p130 bind to and repress antiviral genes, induced by E1A binding. E1A also associates with p107 on development and differentiation genes to inhibit these target genes (Ferrari et al., 2008; Ferrari et al., 2012). These data reveal distinct roles of RB family proteins involved in transformation-induced transcriptional reprogramming associated with epigenetic activities, which is also possibly used in nonviral mechanisms. This idea is supported by the study of the unique role of RB during senescence. RB plays a nonredundant role of targeting

E2F-dependent cell cycle genes in oncogene-induced senescence (Chicas et al., 2010).

Genome-wide binding and functional targets of Rbf1

In our recent study, we performed a genome-wide survey of Rbf1 targets in *Drosophila* embryos. We found that in addition to cell cycle genes Rbf1 also targets many components of the insulin, Hippo, JAK/STAT, Notch and other signaling pathways. Interestingly, many Rbf1 target genes identified in our study lack canonical E2F sites, suggesting that Rbf1 might be recruited by other transcription factors involved in different regulatory programs (Acharya et al., 2012a). However, Rbf1 binding on E2F-regulated promoters and promoters lacking E2F sites requires DP, the binding partner of E2F, suggesting that the binding of E2F-DP complexes to the promoter is an essential step in the recruitment of Rbf1 and that they may bind to weak E2F sites or bind through other DNA binding proteins (Korenjak et al., 2012b).

Consistent with genome-wide binding profiles of Rbf1, both cell cycle-dependent and cell cycle-independent functional targets are found in the genome-wide gene expression analysis in S2 cells. Rbf1-E2F2 and Rbf2-E2F2 complexes repress a subset of genes in proliferating cells, indicating possible functions of Rbf proteins beyond cell cycle control (Dimova et al., 2003).

Dissertation overview

Chapter 2 follows our initial observations that Rbf1 is specifically destabilized when the COP9 signalosome is inactivated. In this study, we characterized mutant forms of the Rbf1 protein in *Drosophila* and identified an instability element (IE) in the C-terminal region that is responsible for the instability of Rbf1. Paradoxically, when the IE is deleted, increased protein levels do not cause enhanced repression activity. Rather, these mutations diminish repression activity of Rbf1, indicating a linkage between Rbf1 activity and instability.

In Chapter 3, I focus on determining how the instability element (IE) influences Rbf1 stability and activity. By assaying the IE in the context of chimeric GFP proteins, I find that the IE is a modular degron that is able to direct the turnover of a heterologous protein through the ubiquitination pathway. More importantly, the IE itself is a repression domain when directly tethered to the promoter, suggesting that the IE may serve as an interaction domain for multiple cofactors linking protein turnover and transcriptional repression. Ubiquitination of Rbf1 not only enhances protein turnover, but also promote the repression activity of Rbf1 in a gene-specific manner. Interestingly, the IE domain of Rbf1 is not required for transcriptional repression of certain promoters, indicating that the IE is not the sole repression domain and that Rbf1 exerts functions through distinct mechanisms possibly by interacting with different transcription factors.

In Chapter 4, I show that COP9 may play a dual role in regulation of E2F1, through cullin-based turnover of E2F1, as well as control of Rbf1 levels that influence E2F1 stability. Transcriptionally inactive Rbf1 proteins that lack the IE can still stabilize E2F1, supporting a model that disruption of the functions of the IE may promote activity of E2F1. By performing BrdU incorporation assays in S2 cells, I show that expression of Rbf1ΔIE drives cells into ectopic S phase while the wild-type Rbf1 causes G1 arrest, suggesting the mutant protein confers an uncontrolled growth advantage, which provides a possible explanation to human cancer development upon loss of RB functions.

In Chapter 5, I focus on the regulation of the Rbf1 IE by Cyclin-Cdk complexes, and demonstrate that protein stability and activity are separable, suggesting that the multifunctional IE acts in parallel regulatory roles involving interactions with E2F transcription factors and E3 ubiquitin ligases. An N-terminal threonine contributes independently to control of turnover and activity. Disruption of IE-mediated regulation of Rbf1 induces dramatic developmental phenotypes in the *Drosophila* eye. Our data show

evidence for a complex phosphorylation code that may have gene- or stage-specific implications for RB protein function.

In Chapter 6, I describe my current understanding of the regulation mechanisms of Rbf1 and propose research directions for our future study. We will look for potential proteins which interact with the IE and identify IE-dependent and IE-independent functional targets. We will further characterize the physical interaction between Rbf1 IE and E2F and investigate the functional consequences of loss of the IE.

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CHAPTER 2

Paradoxical instability-activity relationship defines a novel regulatory pathway for Retinoblastoma proteins

Abstract

The Retinoblastoma (RB) transcriptional corepressor and related family of pocket proteins play central roles in cell cycle control and development, and the regulatory networks governed by these factors are frequently inactivated during tumorigenesis. During normal growth, these proteins are subject to tight control through at least two mechanisms. First, during cell cycle progression, repressor potential is downregulated by Cdk-dependent phosphorylation, resulting in repressor dissociation from E2F family transcription factors. Second, RB proteins are subject to proteasome-mediated destruction during development. To better understand the mechanism for RB family protein instability, we characterized Rbf1 turnover in *Drosophila*, and the protein motifs required for its destabilization. We show that specific point mutations in a conserved C-terminal instability element strongly stabilize Rbf1, but strikingly, these mutations also cripple repression activity. Rbf1 is destabilized specifically in actively proliferating tissues of the larva, indicating that controlled degradation of Rbf1 is linked to developmental signals. The positive linkage between Rbf1 activity and its destruction indicates that repressor function is governed in a fashion similar to that described by the degron theory of transcriptional activation. Analogous mutations in the mammalian RB family member p107 similarly induce abnormal accumulation, indicating substantial conservation of this regulatory pathway.

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My contribution to this study was the demonstration that destabilization of Rbf1 occurs specifically in proliferating tissues of the larva, indicating that Rbf1 degradation is tightly regulated and is likely triggered by developmental signals.

Introduction

Originally identified as an important player in juvenile retinal cancer, and the first example of a tumor suppressor protein, the retinoblastoma (RB) gene product has been recognized as a key regulator of the eukaryotic cell cycle. RB is also inactivated in a significant proportion of adult onset human cancers (Classon and Harlow, 2002; Knudson, 1978) attesting to the centrally important role for RB in proliferation control. Further analyses in mammals have revealed that other RB related proteins, p130 and p107, contribute to cell cycle governance, but the partitioning of cell cycle duties among family members is not well defined. Nonetheless, the RB family and their cognate regulatory networks are well conserved among metazoans, substantiating the physiological significance of RB family function (van den Heuvel and Dyson, 2008).

As potent regulators of cellular proliferation, the activities of RB family proteins are tightly regulated. The canonical pathway for RB family regulation is mediated by cyclin/Cdk complexes that phosphorylate pocket proteins at key points during the cell cycle. In response, phospho-RB dissociates from E2F binding partners, and transcription of cell cycle related genes such as *PCNA* can initiate at the G1/S phase transition (Dyson, 1998). In addition to phosphorylation control, RB protein activities are also regulated by proteolysis. During in vitro differentiation of 3T3-L1 adipocytes, p130 levels are transiently decreased relative to p107 by a proteasome-mediated pathway, and this switch is associated with successful differentiation (Prince *et al.*, 2002). RB levels can be regulated by the Mdm2 ubiquitin ligase, better known for its control of levels of the p53 tumor suppressor, and in cancers overexpressing Mdm2, RB levels are diminished (Sdek *et al.*, 2005; Uchida *et al.*, 2005). The idea that altered RB protein levels contribute to disease etiology is further highlighted during infection by certain oncogenic viruses that hijack the proteolytic process and induce RB family member turnover to relieve host control of cellular proliferation (Boyer *et al.*, 1996; Stubdal *et al.*, 1997). Together,

these examples demonstrate that regulation of RB family protein levels are important for normal cellular growth, but that these processes are often deregulated in disease.

In *Drosophila*, the RB family (Rbf) is comprised of two members, Rbf1 and Rbf2, and like their mammalian counterparts, these proteins function as transcriptional corepressors that interact with the E2F family of transcription factors (Sutcliffe *et al.*, 2003). The *Drosophila* Rbf proteins provide canonical cell cycle control functions, and they are similarly regulated by phosphorylation involving cyclin/cdk complexes (Frolov et al., 2005; Swanhart et al., 2007; Xin et al., 2002). Rbf proteins are further subjected to influence of their turnover rates. Our recent studies indicated that proteasome-mediated turnover of both Rbf1 and Rbf2 is prevented through an association with the COP9 signalosome (Ullah *et al.*, 2007). This linkage may contribute to COP9 control of cell cycle and development in plants and animals (Wei *et al.*, 2008). The COP9 signalosome consists of 8 subunits (CSN1-8), many of which exhibit limited similarity to subunits of the 19S regulatory lid of the proteasome, suggesting that the COP9 signalosome may play a direct role in modulating protein stability, possibly via interactions with the catalytic 20S core proteasome (Chang and Schwechheimer, 2004; Su et al., 2003). The COP9 signalosome may also control protein degradation through interactions with and subsequent deneddylation of the cullin subunits of SCF ubiquitin E3 ligase complexes (Wei *et al.*, 2008). Multiple subunits of the COP9 signalosome were found to physically associate with Rbf proteins, and the depletion of any of these subunits lead to destabilization of both Rbf1 and Rbf2 in cultured cells and embryos (Ullah *et al.*, 2007), suggesting that the entire complex is involved in stabilizing Rbf proteins. However, it is not known whether the COP9 regulation of Rbf proteins is a constitutive process, or whether this control is regulated during development. The CSN4 subunit of the COP9 signalosome co-occupies cell cycle regulated genes simultaneously with Rbf proteins, suggesting that processes affecting repressor stability are spatially and temporally linked to repressor function during gene regulation (Ullah *et al.*,

2007).

While proteasome-mediated destruction of cellular proteins is clearly linked to downregulation of factor activity, the converse relationship has also been described, notably, that the potency of transcriptional regulatory proteins is directly linked to processes that mediate their destruction. This somewhat paradoxical relationship has been described for a variety of eukaryotic transcriptional activator proteins, including c-Jun, c-Fos, Myc, E2F1, and Gal4, all of which harbor degradation signals in regions closely overlapping with their activation domains (Salghetti et al., 2001; Salghetti et al., 1999; Salghetti et al., 2000). Synthetic constructs with multiple degradation domains exhibit higher levels of transcriptional activation, suggesting that the correspondence is not just coincidental (Salghetti *et al.*, 1999; Salghetti *et al.*, 2000). One proposed explanation for the tight correlation between protein lability and increased transcriptional potency, posits that the proteasome, which is essential for turnover of ubiquitylated substrates, also mediates transcriptional activation functions directly (Ferdous et al., 2007; Gonzalez et al., 2002). A second mechanism suggests that activator ubiquitylation serves to recruit co-activator proteins, such as P-TEFb, to increase RNA polymerase elongation while simultaneously increasing the susceptibility of the activator to proteasome-mediated destruction (Collins and Tansey, 2006; Daulny et al., 2008; Lee et al., 2005; Muratani and Tansey, 2003). Although this effect has been observed for transcriptional activator proteins, no transcriptional repressor has been reported as potentiated by proteolytic susceptibility. In this study, we provide evidence that the lability of the *Drosophila* RB-related factor Rbf1 is tightly linked to its function as a transcriptional repressor, and that this evolutionarily conserved feature may provide an additional level of developmental control of the cell cycle.

Results

The Rbf1 C-terminal region encodes an instability element

Our previous studies demonstrated that endogenous Rbf1 and Rbf2 proteins are dependent on the presence of the COP9 signalosome for stability; depletion of COP9 subunits resulted in a loss of Rbf protein, which was prevented by the addition of proteasome inhibitors, indicating the involvement of the 26S proteasome pathway (Ullah *et al.*, 2007). To identify regions involved in Rbf turnover as first step towards understanding the process of Rbf stabilization, we examined the stability of epitope-tagged, transfected Rbf1 proteins in S2 cells. We focused on Rbf1 because this protein represents the predominant functional RB family member in *Drosophila*; *rbf1* null mutations are lethal, while *rbf2* null mutants have only very modest phenotypes (Stevaux *et al.*, 2005). Furthermore, previous data suggested that endogenous Rbf1 levels fluctuate during embryogenesis (Keller *et al.*, 2005; Stevaux *et al.*, 2005). We initially examined the importance of the conserved central pocket domain, as well as the less-conserved N- and C-terminal regions (Fig. 2-1A; Table 2-1). In this process, we identified a region in the C-terminus of the protein as an instability element (IE); proteins lacking residues 728-786 accumulated to high levels, and these levels were not further increased by treatment with the proteasome inhibitor MG132 (Fig. 2-1B). In contrast, Rbf1 proteins containing the IE were expressed at lower levels, and these levels were enhanced by proteasome inhibition. Rbf1 stability was sensitive to growth conditions; Rbf1 ΔIE proteins were expressed at higher levels than proteins containing this domain under conditions of higher cell density, longer periods of cell culture, or with low amounts of transfected DNA (Fig. 2-1C). This last observation suggested that the system for Rbf1 turnover can be saturated, and indeed we observed greater differences between the wild-type and mutant Rbf1 ΔIE proteins in cells expressing lower levels of each protein (not shown). We conclude that

the C-terminal region encompassing amino acids 728-786 harbors element(s) that contribute to Rbf1 instability and proteasome responsiveness.

Critical roles of lysine residues within instability element

The striking accumulation of wild-type Rbf1 protein in cells treated with the proteasome inhibitor MG132 indicated that this protein, but not the mutant forms lacking the IE, is subject to active degradation. We hypothesized that the Rbf1 IE may serve as a target for protein ubiquitylation as one mechanism explaining the contribution of this region to proteasome-mediated turnover. Protein ubiquitylation of lysine residues often directs processing by the 26S proteasome, therefore we tested whether the lysine residues in the IE are involved in the stability of Rbf1 (Fig. 2-2; Table 2-1). Mutant Rbf1 in which three, four, or all of the six lysines were converted to alanine (K to A) were assessed for expression. All three of these mutant forms accumulated to significantly higher levels than the wild-type protein. In contrast, mutant Rbf1 proteins harboring charge-conserving lysine-to-arginine substitutions in the same residues did not over accumulate, suggesting that the positive charge of the side chain, rather than its ability to be ubiquitylated, is important for low steady state levels (Fig. 2-2A). To determine whether the change in steady state levels is due to altered stability, we next tested whether the half-life of wild-type and mutant (4KA) Rbf1 proteins differed by treating S2 cells with the translational inhibitor cycloheximide. Three days after transfection at a point when our previous data indicated that Rbf1 (4KA) mutant protein was expressed at higher levels than wild type Rbf1, S2 cells were treated with cycloheximide and Rbf1 protein levels subsequently measured at 0, 6, and 12 hours (Fig. 2-2B, 2-2C). By 6 hours, levels of the wild-type Rbf1 protein, but not the mutant Rbf1 (4KA), were significantly decreased, confirming that the heightened accumulation of Rbf1 proteins lacking the IE is caused by reduced rate of Rbf1 degradation (Fig. 2-2D).

Figure 2-1. Identification of an instability element (IE) in Rbf1.

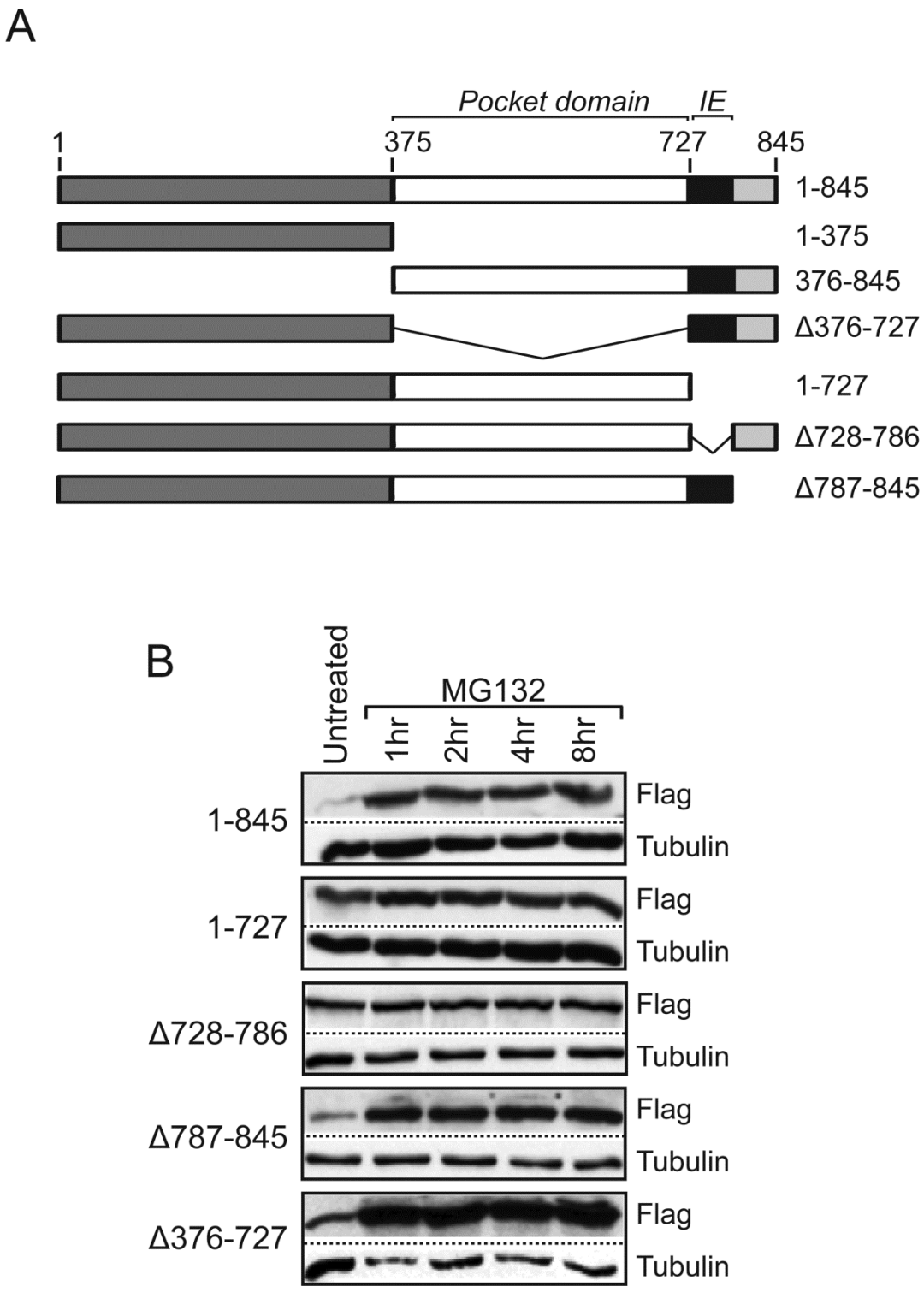

Figure 2-1 (cont'd)

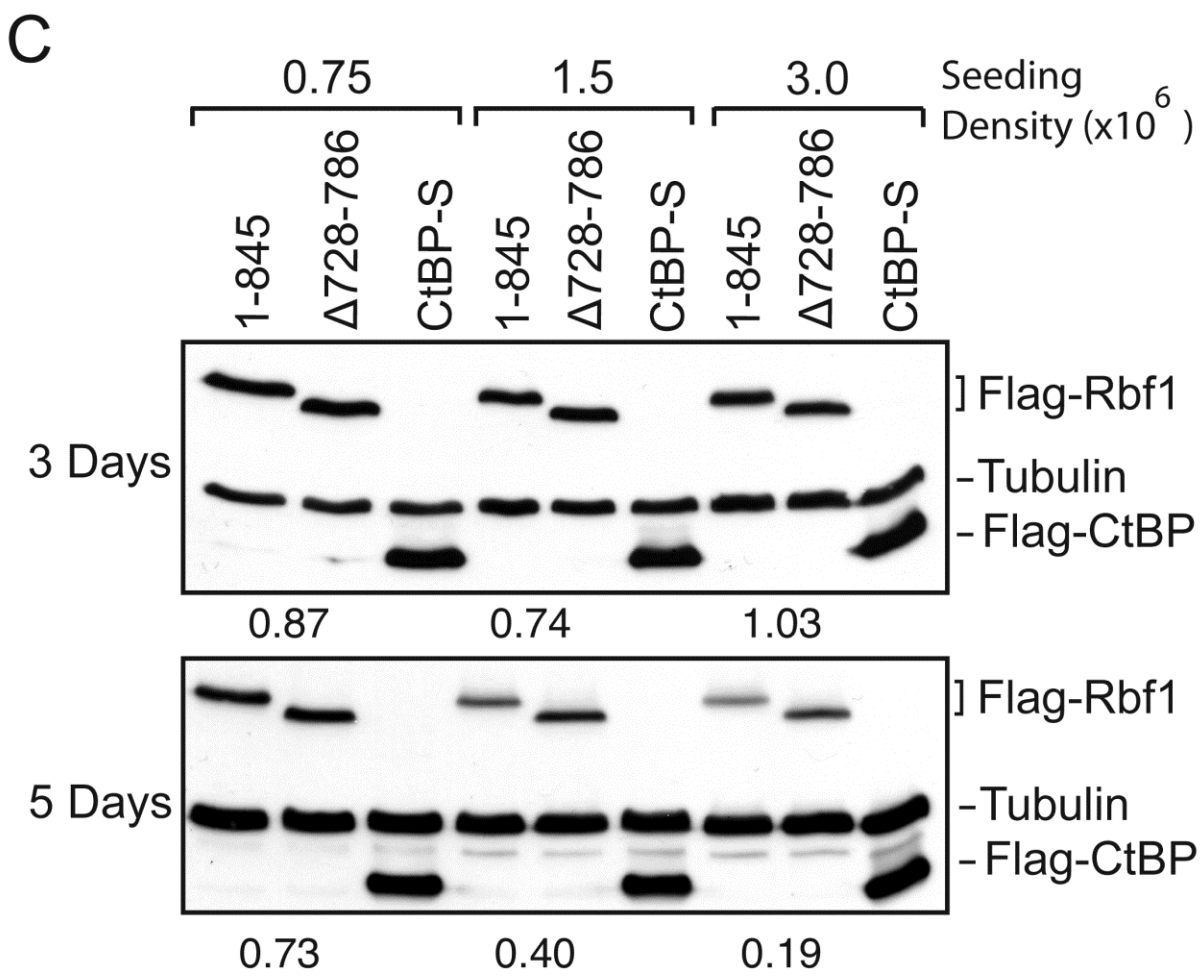

Figure 2-1 (cont'd)

(A) Schematic diagram of Rbf1 proteins expressed in *Drosophila* S2 cells. The N and C termini are indicated in dark and light gray respectively; the black box represents the instability element; the E2f-binding pocket domain is in white. (B) Effect of proteasome inhibitor MG132 on Rbf1 protein levels. Cells were transfected to express the indicated proteins and treated for 1-8 hours with MG132, and protein levels assayed by Western blot using antibodies to C-terminal Flag epitope tag. The wild-type 1-845 and mutants lacking the extreme C terminus (Δ787-845) or the pocket domain deletion mutant (Δ376-727) were expressed at lower levels, and were strongly stabilized by this drug, while the mutants lacking the IE (Δ728-786 and 1-727) were expressed at higher levels and were not much further stabilized by MG132 treatment. (C) Effects of cell density and culture time on differential expression of wild-type Rbf1 and IE mutant. 400 ng of Rbf1 expression plasmid was transfected into S2 cells. At lower initial cell densities (0.75×10^6/ml) and shorter growth times (3 days), expression of wild-type Rbf1 (1-845) and a deletion mutant lacking the IE (Δ728-786) accumulate to similar levels. Normalized protein levels are shown below the lanes containing Rbf1. Cells at higher initial densities ($1.5\text{-}3 \times 10^6$/ml) grown for longer times (5 days) show higher levels of the mutant protein relative to the wild-type form. Levels of transfected CtBP protein, and endogenous tubulin protein, are shown as controls.

Table 2-1. Rbf1 repression, stability, and localization.

Rbf1 construct	Repression activity ± stdev	Protein stability	Nuclear localization
1-845	100 ± 9		+
1-375	12 ± 1		-
376-845	42 ± 3		+
1-727	16 ± 2	+	-
Δ728-786	16 ± 4	+	+
Δ787-845	107 ± 14		-
K754A	65 ± 6		+
K754R	81 ± 9		+
K774A	151 ± 15		+
K774R	125 ± 22		+
3K-A.1	35 ± 11	+	+
3K-R.1	105 ± 26		+
4K-A.1	22 ± 5	+	+
4K-R.1	86 ± 7		+
6K-A.1	36 ± 9	+	+
6K-R.1	110 ± 9		+

Constructs marked (-) for nuclear localization were not exclusively nuclear.

Figure 2-2. Conserved lysine residues in IE play critical roles in accumulation and stability of Rbf1.

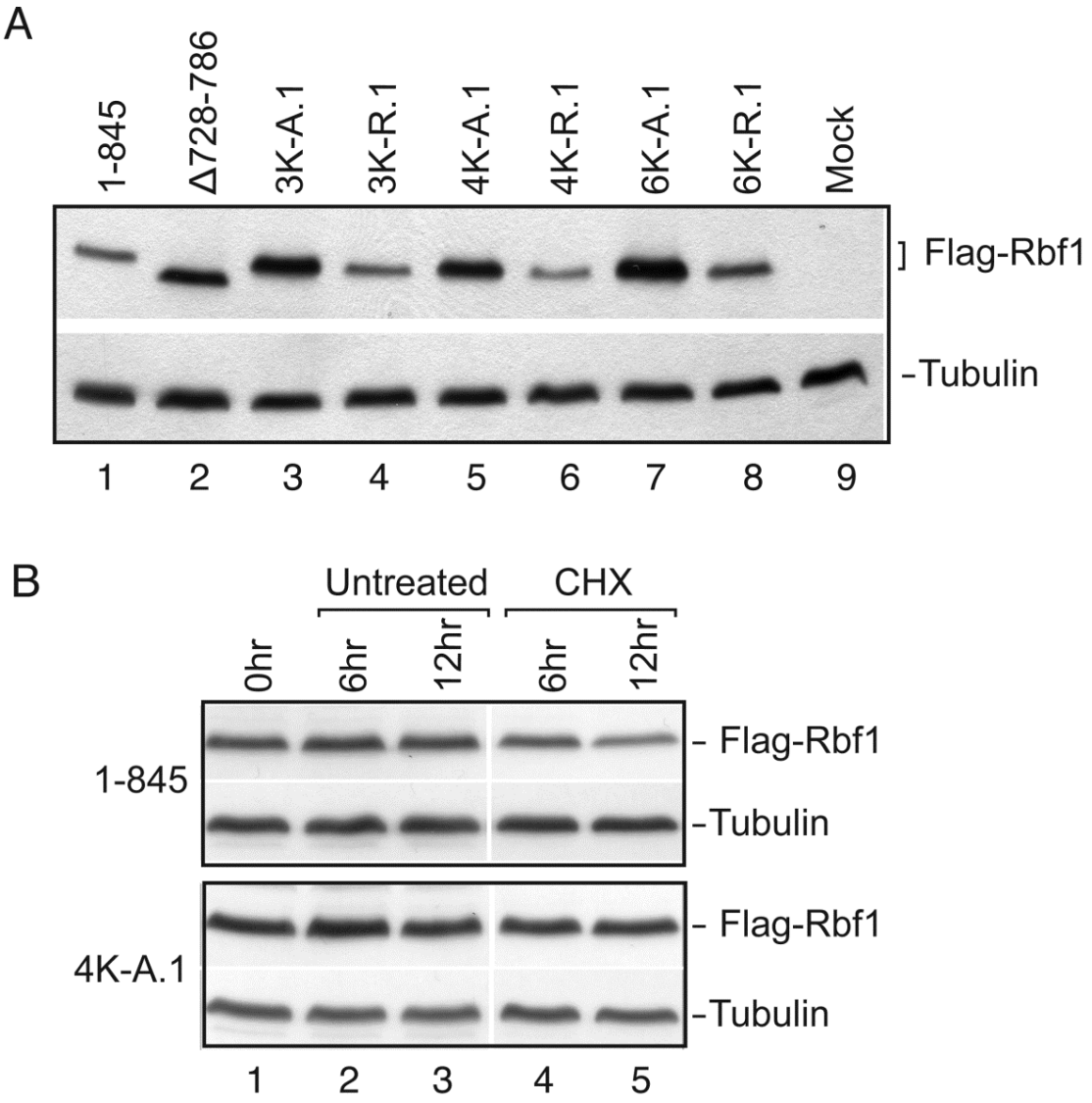

Figure 2-2 (Cont'd)

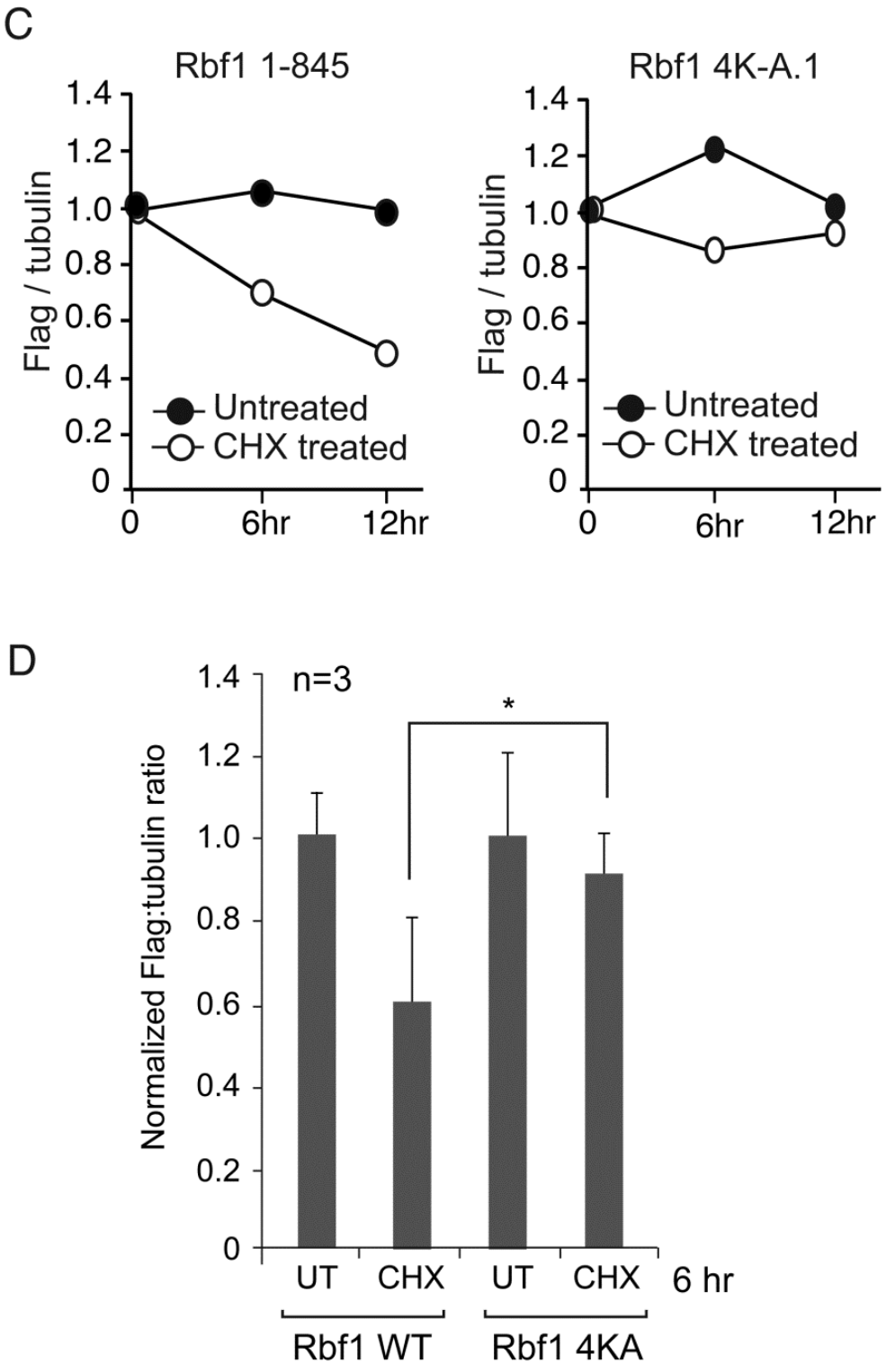

Figure 2-2 (Cont'd)

(A) Mutation of multiple lysine residues within the IE leads to increased protein accumulation. Lysine residues were changed to alanine (K732A, K739A, K740A for 3K-A; also K754A for 4K-A; also K774A and K782A for 6K-A) or to arginine. Rbf1 over-accumulation is not observed with the lysine to arginine substitution. 1.5 x 10^6 S2 cells were transfected with 100 ng of Rbf1 expression plasmid and grown for five days. The data shown is representative of three biological experiments. (B, C) Half-life measurements of unstable wild-type and stable IE mutant Rbf1 proteins. Three days after transfection, cells were treated with cycloheximide and harvested at the indicated times. Rbf1 protein levels were quantitated by photon-capture analysis with a Fuji LAS-3000 Imager and normalized to tubulin levels. (D) Bar graphs showing averaged normalized flag:tubulin ratios for the Rbf1 wild-type and 4K-A mutant proteins at the 6 hour time point from three biological replicates. At this time point, the difference between the wild-type and the 4K-A mutant protein levels was statistically significant ($p=0.05$).

To assess whether the Rbf1 IE functions as an instability element in the context of normal *Drosophila* development, we devised a rescue construct that expresses epitope-tagged Rbf1 under the control of the endogenous *rbf1* regulatory sequences. Developmental expression of the wild-type Rbf1 and Rbf1 ΔIE (Δ728-786) proteins was then assessed by Western blotting. As shown in Fig 2-3A (left panel), the overall levels of both proteins were similar in third-instar larval extracts, suggesting that the deletion mutant accumulated to wild-type levels. However, a very different picture emerged when we measured protein expression in imaginal disc tissue from third-instar larvae as shown through Western blots in Fig. 2-3A (right panel) and imaginal disc staining in Fig. 2-3B-J. Steady state levels of wild-type Rbf1 were far lower than Rbf1 ΔIE in eye, wing and leg imaginal discs; this observation was consistent for three independent lines of each construct (Fig. 2-3B-J). The relationship between this effect and previously characterized Rbf1 function is especially evident in the eye imaginal disc. The terminally differentiating cells of the posterior eye disc normally have no transcription of *rbf1* and low or nonexistent levels of Rbf1 (Keller *et al.*, 2005), but the Rbf1 ΔIE mutant also shows staining in these posterior cells, suggesting an abnormal perdurance of the protein (Fig. 2-3C, D). The marked difference between the steady-state levels of the two proteins in these contexts indicates that the wild-type Rbf1 protein is specifically destabilized in the proliferating and differentiating tissue of the imaginal discs. The tissue-specific stability of the Rbf1 wild-type and mutant proteins suggests that turnover of Rbf1 is a regulated event, and is likely triggered by developmental signals. The cell-density-dependent difference in protein accumulation for wild-type and IE-deleted Rbf1 proteins as described in Fig. 2-1C also supports this hypothesis.

The Rbf1 instability element contributes to repression potency

In the previous experiment, the *rbf1-Flag* transgene rescued an rbf^{14} null mutant,

substituting for both zygotic and maternal Rbf1 protein as demonstrated by its ability to support viable flies for generations (Table 2-2 and data not shown). In contrast, the similar construct expressing Rbf1 (ΔIE)–Flag protein was not capable of rescuing the mutation, despite robust expression in imaginal discs and wild type expression at the third-instar larval stage. We therefore hypothesized that the IE is required for Rbf1's role in regulating activity. To test this hypothesis, S2 cells were co-transfected with expression plasmids encoding wild type or mutant Rbf1 proteins and the effect on repression potency was determined using *PCNA*-luciferase reporter construct, which sensitive to repression by Rbf1 (Stevaux *et al.*, 2002). As expected, proteins lacking the central pocket domain were inactive; this region of the protein is required for interaction with the E2F transcription factors that recruit Rbf1 to the promoter (Fig. 2-4A). Removal of the N-terminal portion of the protein had only a mildly deleterious effect on repression, consistent with previous studies that suggested it is not required for transcriptional activity in vivo and in vitro (Hiebert *et al.*, 1992). In contrast, removal of portions of the entire C-terminus revealed multiple effects. First, deletion of the IE region alone had a strong inhibitory effect on transcriptional repression, and this effect was just as severe as removal of the critical pocket domain. The Rbf1 ΔIE and pocket deletion mutant proteins did not exhibit aberrant localization, but remained in the nucleus (Fig. 2-4B). Second, loss of the adjacent C-terminal 59 amino acids (Δ787-845) did not abolish repression, but did change its sub-cellular localization so that the protein was no longer strictly nuclear. This data indicates that this region harbors a nuclear targeting element governing Rbf1 cytoplasmic/nuclear distribution. As observed for deletion of the entire IE (Δ728-786), removal of portions of this fifty-nine amino acid region in blocks of twenty was sufficient to inhibit repression activity, suggesting that the function of the IE is distributed over numerous residues throughout this region (data not shown).

Our previous data indicated that multiple lysine residues within the Rbf1 IE contributed to

Rbf1 stability, thus we tested whether these same residues were involved in the transcriptional repression mediated by Rbf1. Indeed, as shown in Fig. 2-4C, Rbf1 proteins bearing multiple lysine to alanine substitutions were less effective repressors, even though these proteins were more stable than the wild-type Rbf1. This effect was most notable for the Rbf1 4KA mutant whose repression capability was similar to that mediated by Rbf1 lacking the IE. Surprisingly, alanine substitution of two additional lysine resides (6KA) reproducibly improved the function of Rbf1 in repression. This observation raised the possibility that this region harbors elements that throttle Rbf1 repressor potency, as discussed further below. In contrast to alanine substitution, Rbf1 proteins harboring multiple lysine to arginine substitutions did not over-accumulate, and significantly, were just as potent as wild type Rbf1 for transcriptional repression. Based on these data, we conclude that these residues contribute both to Rbf1 instability and to repressor function. These data further indicate that modification of these residues is not essential to either process. To test whether the effects on transcriptional repression of these Rbf1 mutations were evident in other contexts, we compared transcriptional repression of wild-type and mutant Rbf1 proteins on the *Pol*α promoter, which has somewhat different requirements for E2F and DP activation compared to the *PCNA* promoter (Fig. 2-4D) (Dimova *et al.*, 2003). Deletion of the IE or point mutations within this region similarly reduced the repression activity on this promoter as well, indicating that the relationship between protein activity and instability is independent of promoter context. Taken together, these data strongly indicate that the ability of the Rbf1 protein to act as a transcriptional repressor is tightly associated with its instability, and that the IE in the Rbf1 C terminus is multifunctional, linking these two features.

The Rbf1 IE is not essential for E2F interactions and promoter binding

Previous studies have shown that both the pocket domain as well as the carboxy-terminus

of the human RB protein can make molecular contacts with E2F1 (Lee et al., 2002; Rubin et al., 2005; Xiao et al., 2003). We reasoned that the reduced activity of the Rbf1 instability element mutants might be a direct result of their inability to physically associate with the E2F transcription factors. Therefore, we performed GST pull-down and co-immunoprecipitation (Co-IP) assays to test for interactions between Rbf1 and E2f proteins. In the GST pull-down assays, both GST-Rbf1 1-845 and the IE mutant (Δ728-786) displayed similar binding ability to in vitro translated E2f1 and E2f2 proteins (Fig. 2-5A, lanes 5 and 6). No interaction was observed with beads alone or GST protein (Fig. 2-5A, lanes 3 and 4). Similarly in Co-IP assays from *Drosophila* S2 cells, Myc-tagged E2f1 co-precipitated with Rbf1 1-845 and two IE mutants (Δ728-786 and 4K-A.1) but not with the pocket domain deletion mutant (Δ376-727) (Fig. 2-5B; top panel, lanes 3-6). These results show that the IE mutants retain a capacity to interact with both E2f1 and E2f2 proteins.

To assess whether the IE plays a role in Rbf1 promoter occupancy we performed chromatin immunoprecipitation (ChIP) assays using embryos expressing the Flag-tagged Rbf1 wild-type or ΔIE mutant to test for promoter binding of these proteins at the *DNA primase* promoter (Fig. 2-5C). Binding at an intergenic locus and a non-target gene (*sloppy paired 1*) promoter was assessed as negative controls. Interestingly, the *DNA primase* promoter was found to be enriched in immunoprecipitates from chromatin derived from embryos expressing both the wild-type Rbf1 as well as the Rbf1 IE mutant proteins indicating that the Rbf1 IE mutant can still occupy promoters (Fig. 2-5C; top panel). Binding of the IE mutant at this locus was slightly reduced compared to the wild-type Rbf1 although the association was significantly above background as no enrichment was observed at an intergenic locus (middle panel) or the non-target *sloppy paired 1* promoter (bottom panel). It appears that, unlike the Rbf1 pocket deletion mutant, the reduced activity of the Rbf1 IE mutants cannot be attributed simply to their inability to interact with E2F proteins or target gene promoters.

Figure 2-3. Expression of wild-type and IE mutant forms of Rbf1 in the *Drosophila* larva.

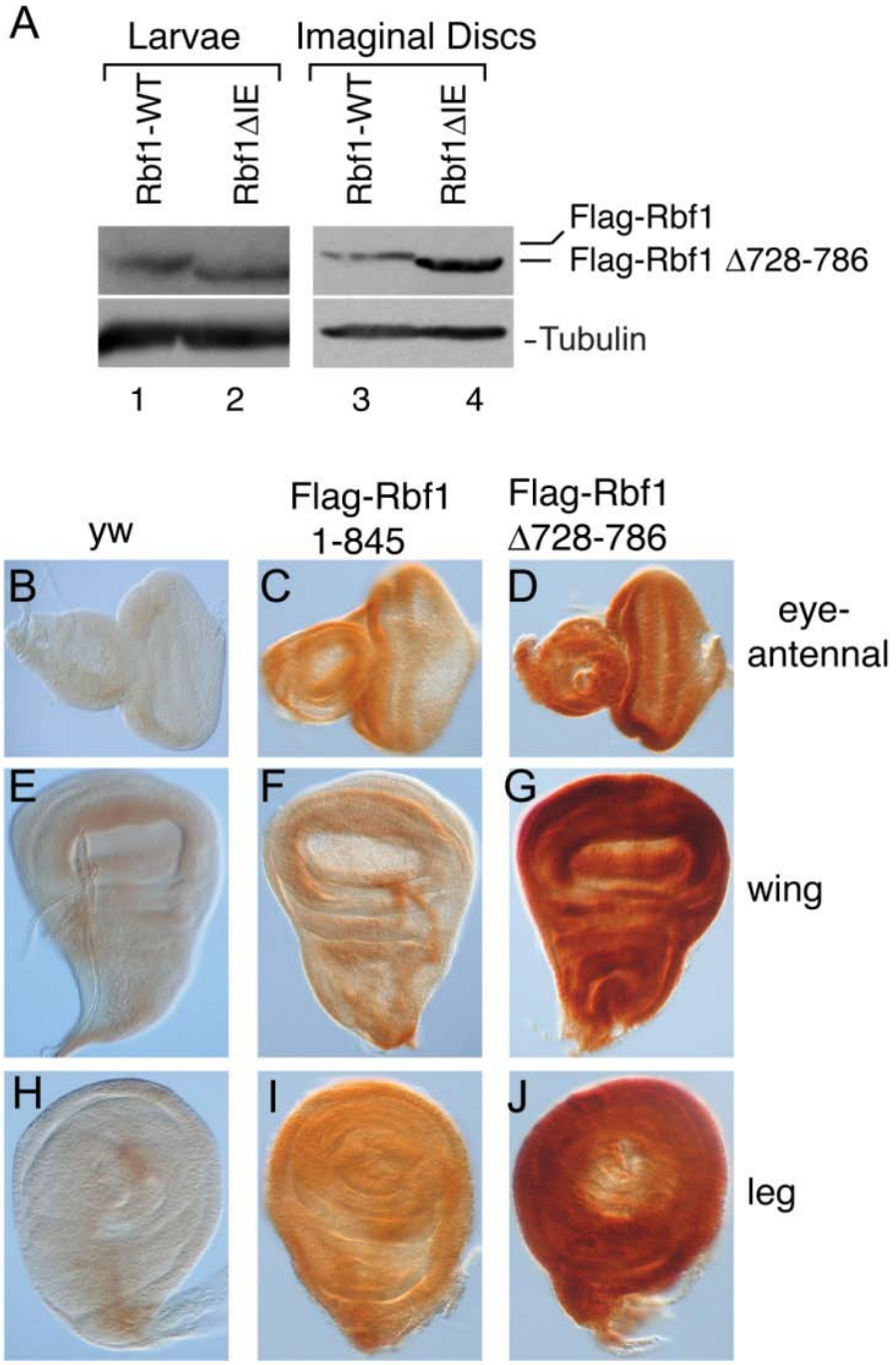

Figure 2-3 (cont'd)

Indicated proteins were expressed from the endogenous *rbf1* promoter, and expression levels were assayed in total larval extracts as well as in imaginal discs. (A) Western blot showing expression of Flag-tagged Rbf1 from third-instar larvae (left panel) and pooled imaginal discs (right panel) carrying homozygous copies of *rbf1* genomic constructs. Equivalent levels of proteins were noted in whole larval extracts whereas the mutant protein was found to accumulate to about 4-fold of the wild-type protein in the imaginal discs. The Western blot of whole larval extracts is representative of four biological replicates for the two lines shown in C, F, I, and D, G, J; the average difference in protein levels in total larval extracts was 13% +/- 2%. (B-J) Rbf1 expression in third-instar larval imaginal discs. (B-D) eye discs, (E-G) wing discs, and (H-J) leg discs. Weak background staining was observed in non-transgenic *yw* flies (B, E, and H), and specific, but weak staining was evident in discs expressing wild-type Rbf1 protein (C, F, and I). Strong expression was noted in flies expressing the inactive Rbf1Δ 728 -786 IE mutant protein (D, G, and J). The imaginal disc staining is representative of stainings of three different lines for each construct; in all cases, the IE mutant protein was expressed at higher levels. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Table 2-2. *rbf*^{14} rescued by transgenic Rbf1.

rbf^{14} mutant male flies rescued by *rbf1* transgene

Strain	Genotype (%)				n
	rbf^{14}/Y	FM7/Y	*rbf*^{14}/+	FM7/+	
Rbf1 L1	3.7	19.1	41.2	36.0	1116
Rbf1 L2	3.6	22.6	39.8	34.0	1163
Rbf1Δ728-786	0	30.0	37.4	32.6	697

rbf^{14} mutant female flies rescued by *rbf1* transgene

Strain	Genotype (%)				n
	rbf^{14}/Y	FM7/Y	*rbf*^{14}/*rbf*^{14}	*rbf*^{14}/FM7	
Rbf1 L1	6.1	39.6	9.8	44.5	164
Rbf1 L2	1.1	36.7	8.5	53.7	188

L1 and L2 are two independent transgenic lines expressing wild-type Rbf1 protein. Rbf1Δ728-786 expresses a nonfunctional, proteolytically stabilized form of Rbf1. *rbf*^{14} is a complete deletion mutant of Rbf1. FM7 represents an X-chromosome balancer. *rbf*^{14}/Y represents rescued males; *rbf*^{14}/*rbf*^{14} represents rescued females. The larger percentage of flies carrying the wild-type (+) or balancer (FM7) X-Chromosome indicates that some flies are not rescued.

Figure 2-4. Rbf1 requires the IE for transcriptional repression.

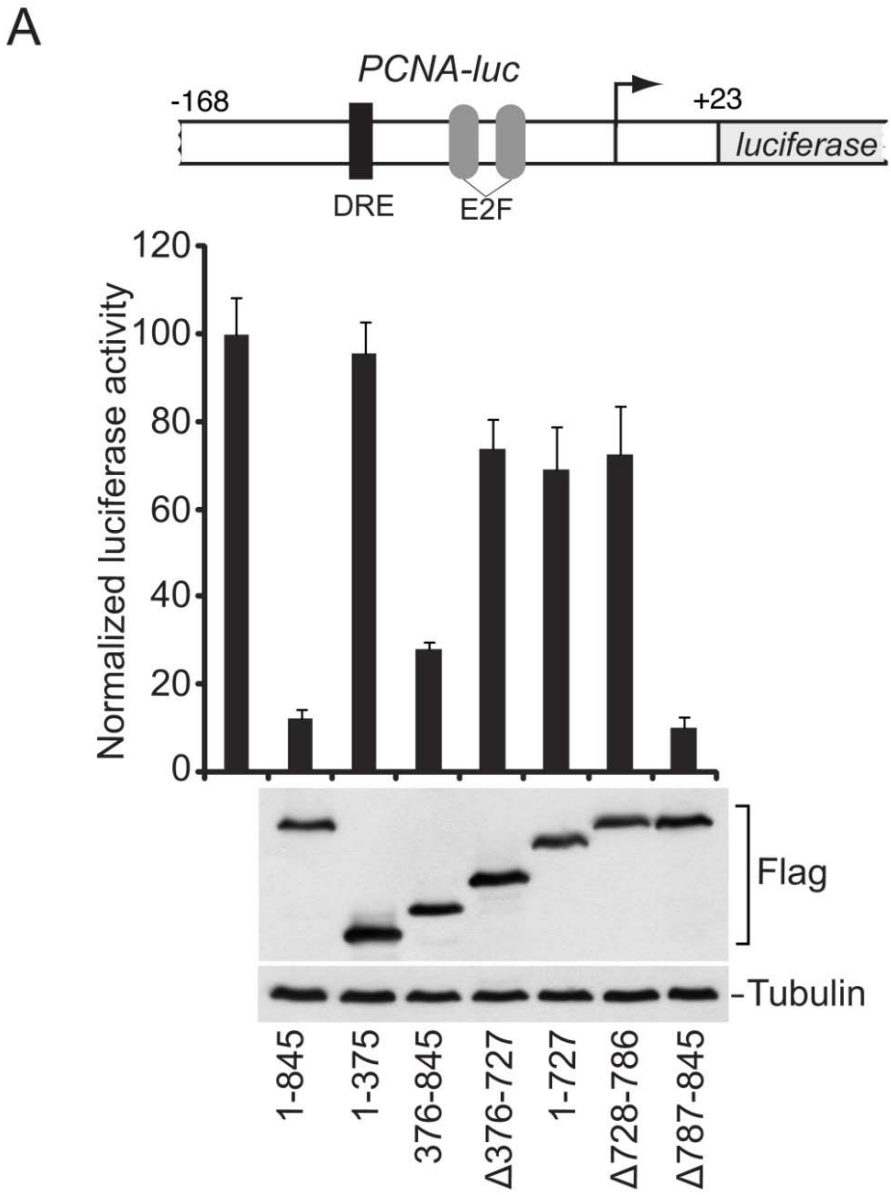

Figure 2-4 (cont'd)

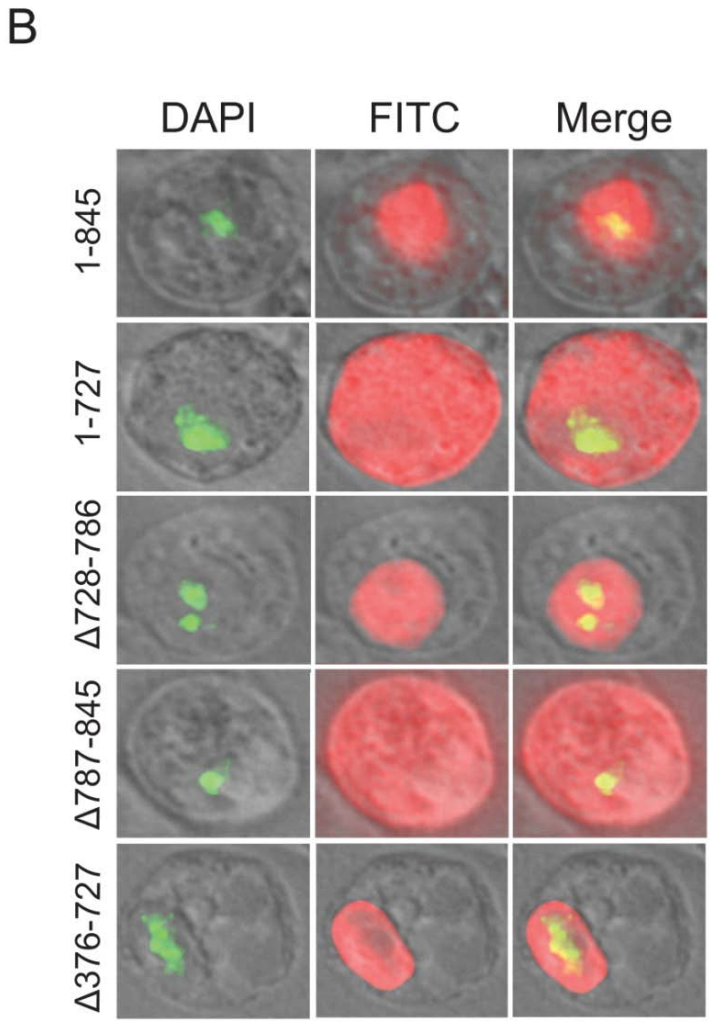

Figure 2-4 (cont'd)

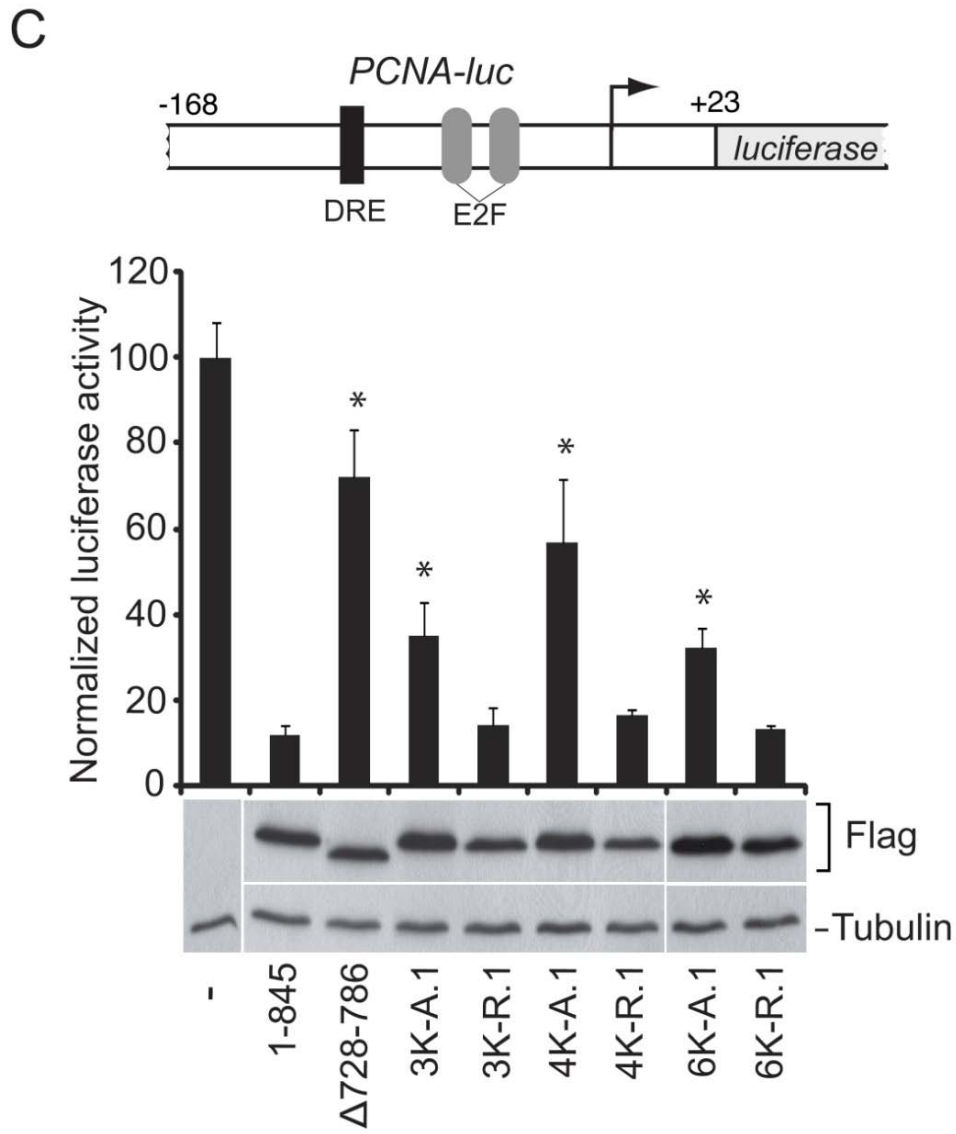

Figure 2-4 (cont'd)

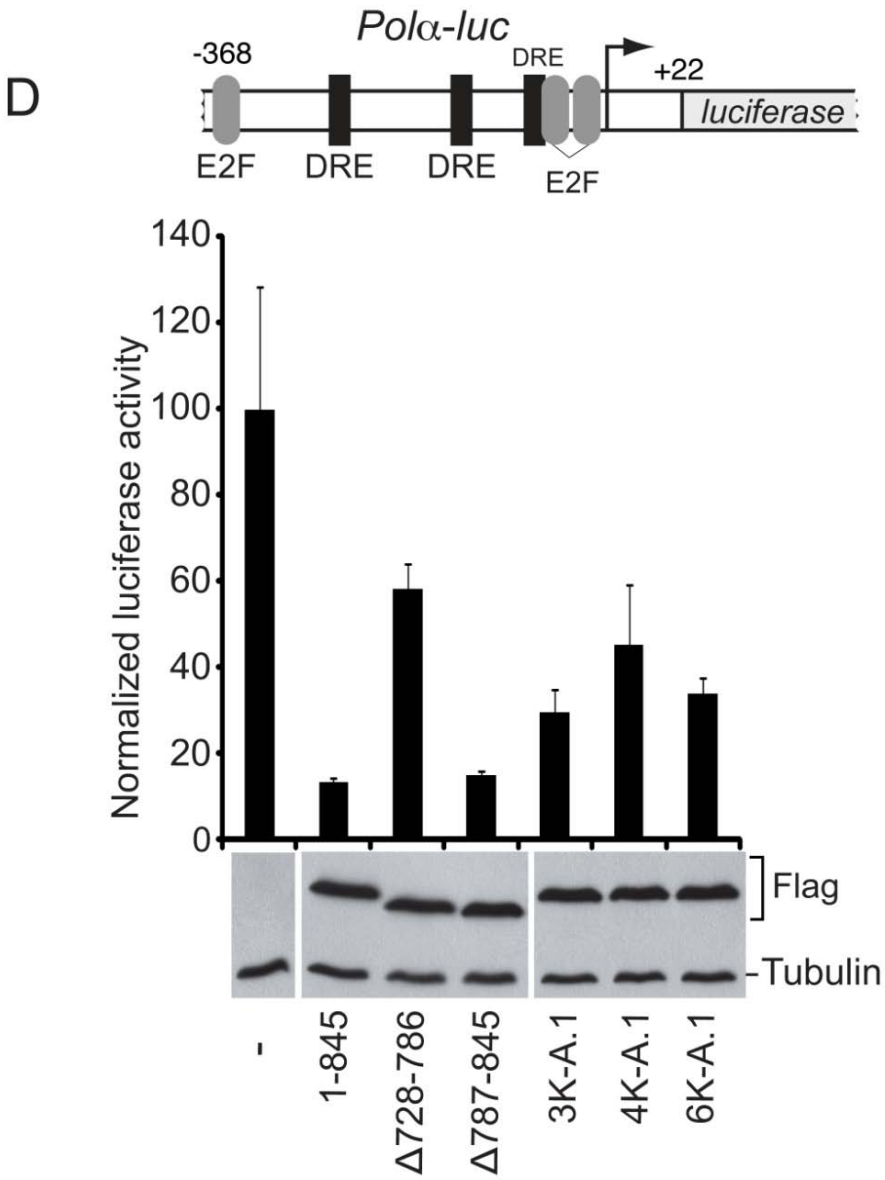

Figure 2-4 (cont'd)

(A) Deletion of the IE (Δ728-786) or E2F binding pocket (Δ376-727) compromises transcriptional repression activity of Rbf1 proteins measured on the *PCNA-luciferase* reporter gene (bar graph). Under these transfection conditions, proteins were expressed at similar levels (Western blot). (B) Subcellular localization of wild-type (1-845) and deletion mutants. DAPI staining indicates DNA in nucleus, and FITC staining the Rbf1 proteins. Proteins lacking residues 787-845, which include the presumptive nuclear localization signal, are found predominantly in the cytoplasm. (C) Transcriptional activity of Rbf1 IE deletion and point mutant proteins assayed on *PCNA-luciferase* reporter. Mutant proteins lacking the IE, or with multiple lysine to alanine mutations, were compromised for transcriptional repression activity. Lysine to arginine mutant proteins exhibited wild-type repression activity. Error bars indicate standard deviation, and asterisks indicate $p < 0.05$. (D) Rbf1 repression of *Drosophila Polα-luciferase* reporter. Deletion of the IE largely inactivates the protein for transcriptional repression (top panel). Data in 4A represent two biological replicates, each with three technical replicates, except for 1-845 and Δ 728 -786, which represent 16 and 9 biological replicates. Other transfections include data from at least three biological replicates. Firefly luciferase activity is expressed relative to Renilla luciferase control.

Figure 2-5. Rbf1 IE is not essential for E2F interactions and promoter binding.

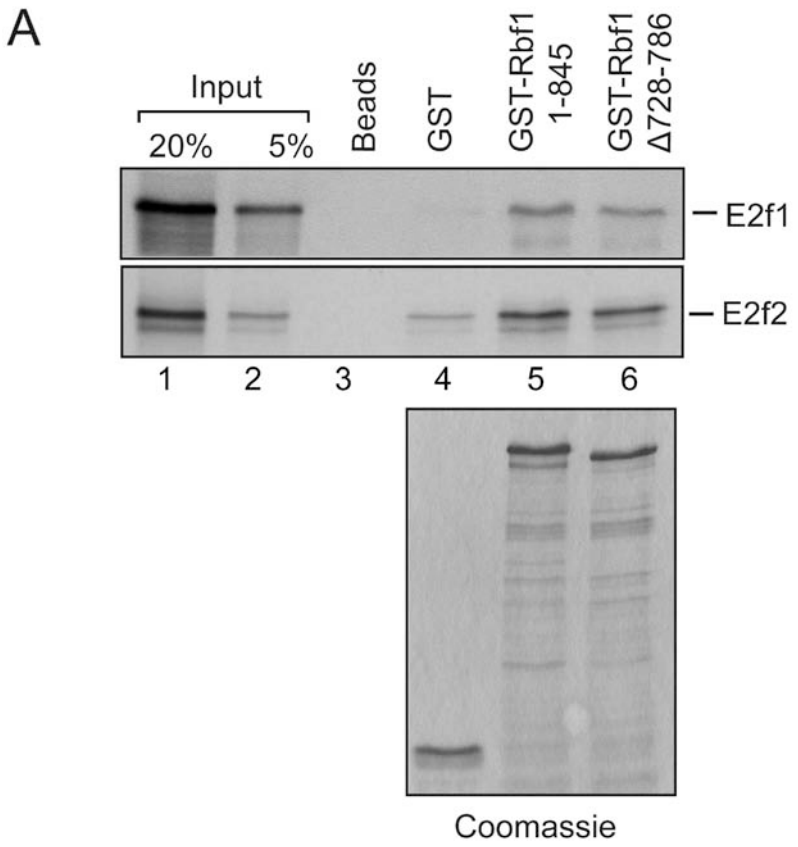

Figure 2-5 (cont'd)

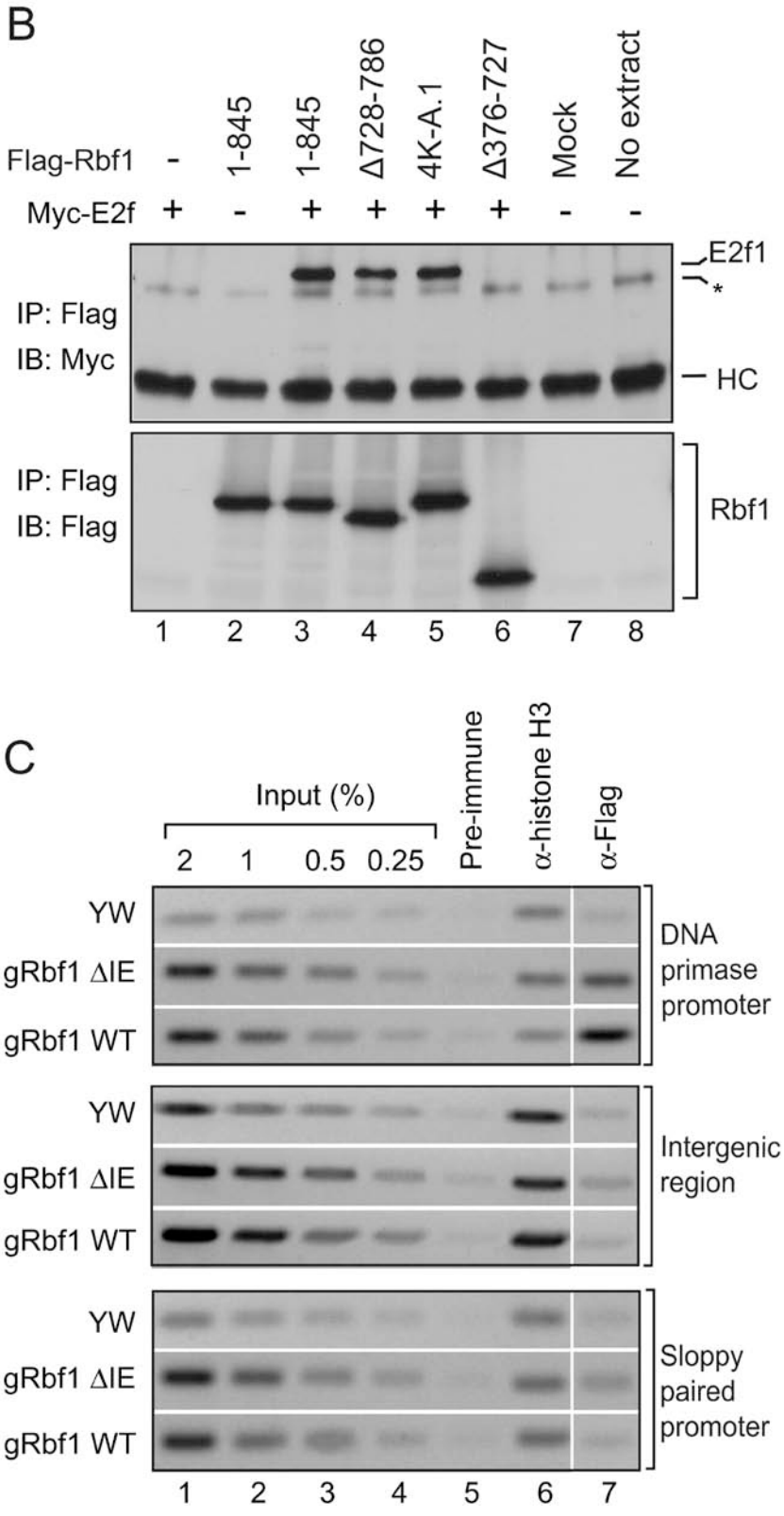

Figure 2-5 (cont'd)

(A) GST-Rbf1 and E2f interaction assay. Indicated GST fusion proteins were bound to radio-labeled E2f proteins and bound proteins were analysed by SDS-PAGE and autoradiography. GST-Rbf1 1-845 and ΔIE mutant displayed similar binding ability to both in vitro translated E2f1 and E2f2 proteins (compare lanes 5 and 6). No interaction was observed with beads alone and GST protein (lanes 3 and 4). Coomassie stained gel showing equal amounts of GST fusion proteins used in binding assays (bottom panel). The data shown is representative of three biological replicates. (B) Co-immunoprecipitation assay. Rbf1/E2f1 interactions in co-transfected S2 cells. Cells were co-transfected with Myc-tagged E2f1 and Flag-tagged Rbf1 expression constructs. Whole cell lysates were used for Flag immunoprecipitations (IP) and the samples were assayed using Western blots with anti-Myc antibody (top panel). Myc-tagged E2f1 co-precipitated with Rbf1 1-845 and two IE mutants (Δ728-786 and 4K-A.1) but not with the pocket domain deletion mutant (Δ376-727) (top panel, lanes 3-6). Mock is IP performed using cell lysate from untransfected cells (lane 7). The asterisk indicates a non-specific band that is contributed by the Flag M2 beads since it appeared in the no extract control where IP was performed in the absence of any cell lysate (lane 8). Equivalent levels of the heavy chain IgG (marked as HC) were seen in all samples indicating the use of equal amount of antibody for each IP reaction. The IP samples were also blotted with the anti-Flag antibody (bottom panel) to verify the amount of Flag-tagged protein that was captured in each assay. The data shown is representative of two biological replicates. (C) Promoter occupancy by Flag-tagged Rbf1 wild-type and Rbf1 IE mutant proteins measured by chromatin immunoprecipitation. Formaldehyde cross-linked chromatin was prepared from 0 to 20 hour embryos expressing the wild-type or mutant Rbf1 protein and immunoprecipitated using the indicated antibodies. Enrichment of the Rbf-regulated promoter (*DNA primase*) was observed by anti-Flag antibody immunoprecipitation reactions with both wild-type and IE

Figure 2-5 (cont'd)

mutant fly embryos but not in reactions using pre-immune IgG (top panel) or at an intergenic locus (middle panel) and a non-target gene promoter (*sloppy paired 1*) (bottom panel).

The Rbf1 IE is a dual-function regulator of repressor potency

Our data indicates that the Rbf1 IE region influences Rbf1 instability and contributes to Rbf1 repression potency, providing a link between these two activities. However, during these analyses we additionally observed that Rbf1 (6KA), harboring substitutions of all lysine residues within the IE was reproducibly a more potent repressor than Rbf1 (4KA), harboring substitutions of only the four most N-terminal lysine residues within the IE. This observation raised the possibility that while most of the lysines play a positive role in Rbf1 repression, one or both of the C-terminal-most lysine residues (K774, K782) play a negative role, restricting Rbf1 activity. Therefore, to determine whether the lysine residues within the IE contribute to both positive and negative regulation of Rbf1 function, we tested the repression activities of Rbf1 proteins with individual alanine substitutions of each lysine residue within the IE. A subset of these results is shown in Fig. 2-6A, revealing three outcomes. In one case (K732), alanine substitution did not affect repressor potency, and was indistinguishable from wild type Rbf1. The second class of mutants were hypomorphic (K739, K740, K754) exhibiting modest but reproducible inhibitory effects on repression, consistent with these residues contributing a positive influence on repressor potency (Fig. 2-6A, B). In contrast, three mutants, K774A, K774R, and K782A exhibited hypermorphic phenotypes with modest but reproducibly higher repression activity than the wild-type Rbf1 protein, suggesting that these residues are involved in a negative control of repressor activity (Fig. 2-6A, B). In cases where lysine to arginine substitution did not moderate activity to wild type levels, such as with K754 and K774, it is possible that the lysine in question is a target of modification, as a positive charge is not the sole important feature. However, for mutants with only single point mutations, we did not observe the robust stabilization of mutant proteins compared to the wild-type protein (not shown). Together, these data also indicate that the IE exerts both positive and negative influences on transcriptional activity. Those mutant forms of Rbf1

lacking all lysines exhibited intermediate repression phenotypes because of two distinct and opposite effects, with decreased activity caused by mutations in K739, 740, and 754 partially offset by increased activity mediated by the mutation of K774 and K782.

To test the physiological importance of these positively and negatively-acting residues for repressor regulation in *Drosophila*, we expressed Rbf1 isoforms in the developing eye imaginal disc using an *eyeless-Gal4* driver system (Fig. 2-7A-H). As noted in previous studies, misexpression of the wild-type Rbf1 protein induced rough eyes in a large percentage of offspring. The mutant form of Rbf1 (Δ728-786) lacking the IE was completely inert, despite robust expression of the protein in the fly (not shown), consistent with a role for the IE in repression. Individual point mutations that had modest effects on repression in cell culture assays similarly showed modest effects on eye development, exhibiting milder phenotypes, and lower penetrance than the wild-type Rbf1. In contrast, the hypermorphic K774A mutant, which exhibited elevated repression activity in cell culture assays, induced dramatic phenotypes (Fig. 2-7E-H). A large percentage of offspring expressing this protein exhibited very severe eye defects, including complete loss of the eye or developmental abnormalities including antennal outgrowths and fewer transgenic individuals were recovered relative to non-expressing controls, suggesting lethality (Fig. 2-7I, J). Thus, the effects of the mutant forms of Rbf1 on eye development mirror exactly the relative potencies of these proteins as measured in cell-based repression assays indicating that Rbf1 is subjected to both positive and negative regulation of repressor potency via the C-terminal IE *in vivo*. This result additionally demonstrates the importance of limiting Rbf1 repression activity during development.

Conserved instability domain of mammalian p107

The correlation between Rbf1 activity and instability in *Drosophila* prompted us to

examine whether similar regulation affects mammalian RB proteins. The overall level of amino acid conservation is highest between the “pocket” domains of RB family members, but there are clearly conserved blocks of residues in the C-terminal region. The primary structure of the C-terminus of Rbf1 most closely resembles that of p107, including the amino acids residues located in the instability element of Rbf1 (Fig. 2-8A). To directly compare Rbf1 and p107, we transfected S2 cells with wild-type p107 and mutant forms in which conserved lysine and arginine residues were replaced with alanine, as well as a deletion of the region most similar to the Rbf1 IE (amino acids 964-1024). Similar to the stabilization effects noted with Rbf1, mutant p107 exhibited increased accumulation compared to the wild-type protein (Fig. 2-8B), suggesting that the C-terminal region of p107 harbors an instability element that funnels p107 into similar turnover pathways even in this heterologous system.

Discussion

During *Drosophila* development, cell-cycle regulation deviates considerably from the classical four-stage G1/S/G2/M pattern, exhibiting rapid direct S-M cycling early in development, stepwise acquisition of G2 and G1 phases, and endoreplication. These alternative cycles involve a variety of regulatory features, including constitutive inactivation of Rbf proteins by phosphorylation, transcriptional regulation of the *rbf1* and *rbf2* genes, and regulated degradation of the E2F1 protein. Here, we provide evidence that this regulatory richness also includes a novel developmentally-triggered degradation of Rbf1 that paradoxically appears to be required for repression activity. Our study indicates that Rbf1 lability is tightly linked to repression activity, both in a cellular as well as a whole organismal context. The IE identified in the C-terminus of this protein appears to be a complex domain with dual functions, so that even a few lysine to alanine mutations can dramatically enhance

protein stability while inhibiting transcriptional activity, while other lesions enhance the protein's activity (Figs. 2-1, 2-3, and 2-4).

Not only is the turnover of Rbf1 required for effective gene regulation, but it appears that this turnover can be developmentally cued, presumably to be coordinated with the engagement of Rbf1 with regulation of the cell cycle (Fig. 2-3). Highly proliferative imaginal disc tissue appears to provide one such context, where levels of wild-type, but not an instability element mutant, Rbf1 protein decrease sharply, presumably in response to the engagement of this protein during cell cycling. In the eye imaginal disc, the Rbf1 protein levels drop sharply in the posterior, where cells are becoming terminally differentiated. Presumably, Rbf1 is activated and consumed in the coordinated cell divisions that occur in the two stripes flanking the morphogenetic furrow; the absence of any further transcription leads to global depletion of Rbf1. The Rbf1 protein lacking the IE accumulates inappropriately in differentiating cells.

How might the repression activity of Rbf1 be linked to protein turnover? Protein lability has previously been found to underlie the action of some eukaryotic transcriptional activators (Kim et al., 2003; Salghetti et al., 2001). The activation domain of the VP16 protein was found to be subject to modification by ubiquitylation, enhancing the transcriptional potency of this factor as well as destabilizing it. This process is thought to affect other transcriptional activators as well (Salghetti *et al.*, 2000). The exact mechanism by which ubiquitylation enhances transcriptional activation is poorly understood. The ubiquitin tag may serve a dual purpose of facilitating interactions with the transcriptional machinery as well as attracting the 26S proteasome. Alternatively, the proteasome itself, or portions of this multi-protein complex, may directly enhance transcription; chromatin immunoprecipitation experiments have placed the "lid" of the proteasome on specific genomic locations (Ferdous et al., 2007; Gonzalez et al., 2002).

Figure 2-6. Rbf1 IE harbors positive and negative regulatory elements.

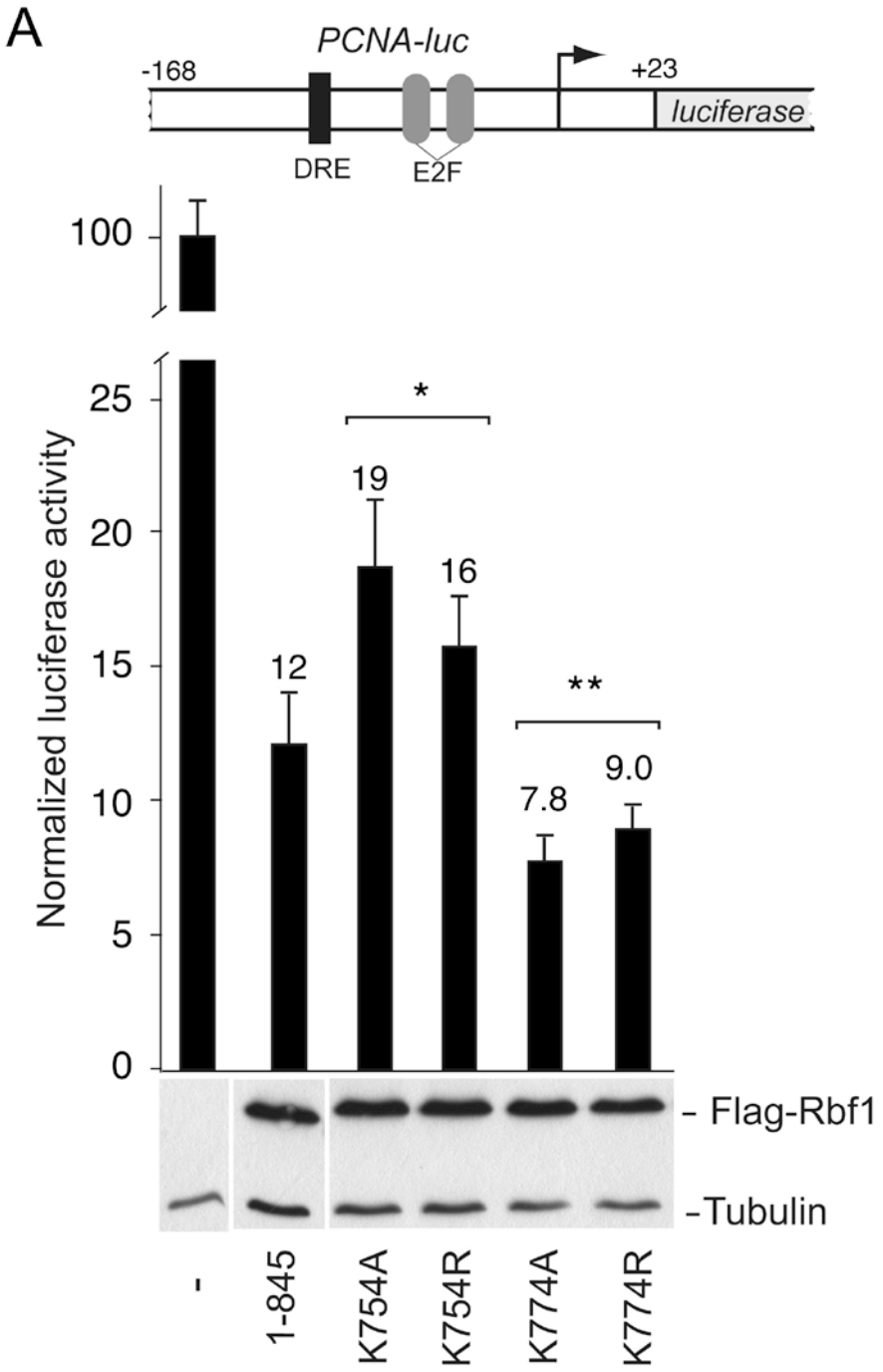

Figure 2-6 (cont'd)

B

Category	Mutation	*p* value
Neutral	K732A	0.76
Hypomorph	K739A	2.8E-05
	K740A	1.0E-03
	K754A	4.9E-16
	K754R	1.3E-05
Hypermorph	K774A	3.5E-22
	K774R	2.6E-08
	K782A	5.8E-05

C

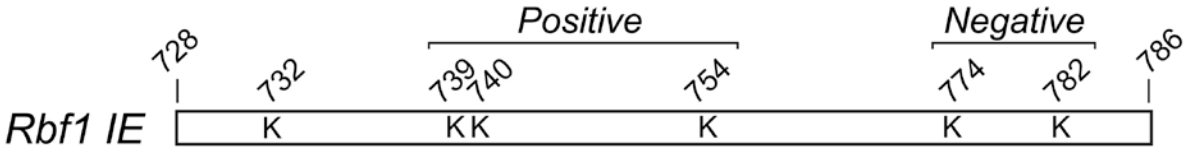

Figure 2-6 (cont'd)

(A) Transcriptional repression activity of Rbf1 lysine point mutant proteins. Examples of mutant proteins that show either enhanced or reduced repression activity. Mutation of K754 to alanine or arginine attenuates repression activity while K774 to alanine mutant exhibited enhanced repression activity with respect to the wild-type protein (top panel). Under these transfection conditions, proteins were expressed at similar levels (lower panel). Error bars indicate standard deviations, and asterisks indicate $p < 0.05$ compared to wild-type Rbf1. (B) The lysine point mutants were classified as neutral, hypo- or hypermorphic based on the indicated t-test results. (C) Schematic representation of the Rbf1 IE indicating the location of lysine residues that play a positive or negative role in Rbf1-mediated repression.

Figure 2-7. Severe developmental consequences of expression of hyperactive Rbf1.

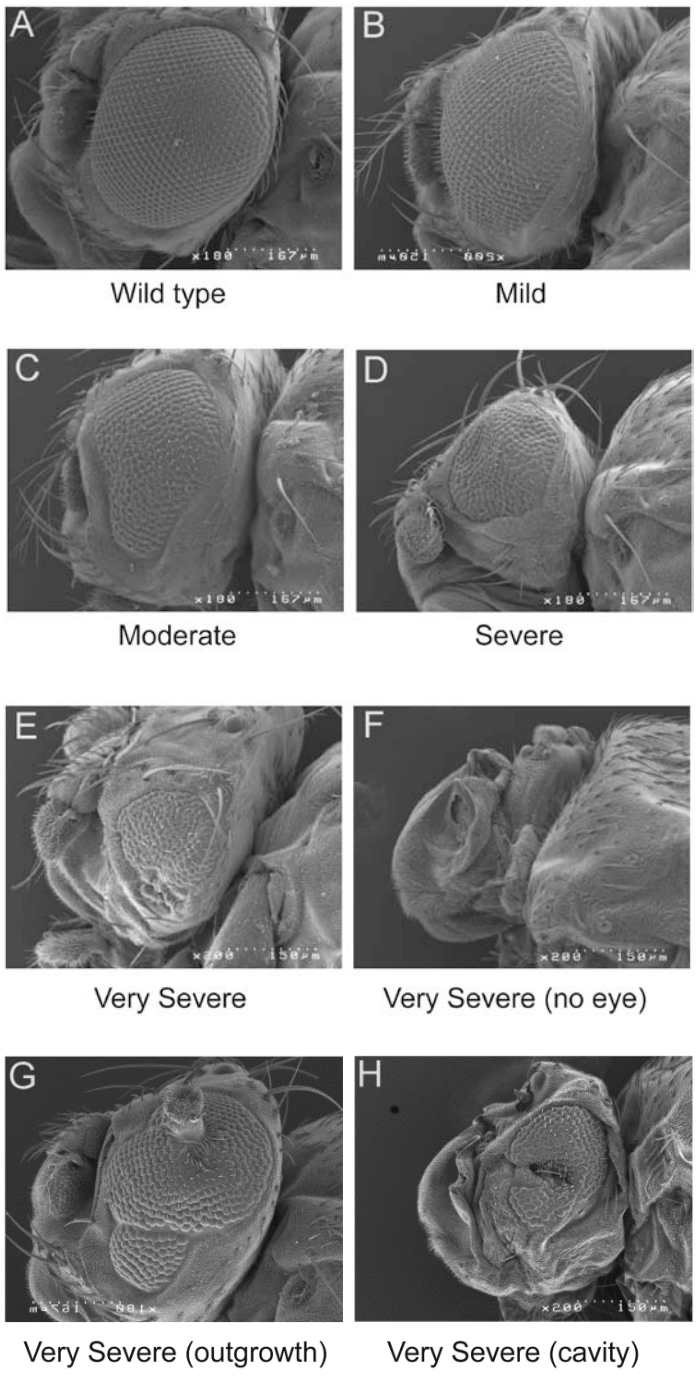

Figure 2-7 (cont'd)

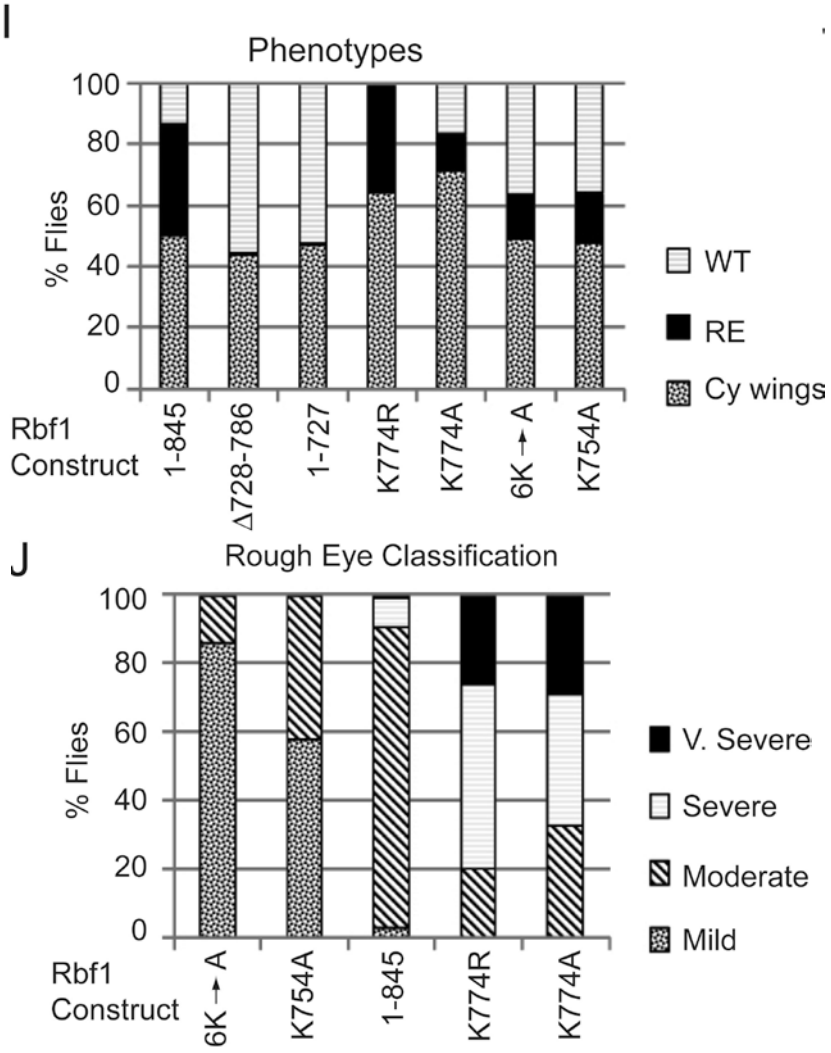

Figure 2-7 (cont'd)

cDNAs of *rbf1* wild-type and IE hypermorphic and hypomorphic mutants were misexpressed in the eye imaginal disc using the *eye-Gal4* driver. (A-H) representative eyes exhibiting wild-type, mild, moderate, severe, and four very severe phenotypes. (I) Bar graphs representing frequency with which flies carrying the *eye-Gal4* driver and UAS-*rbf1* gene were recovered, as well as frequency with which these latter flies exhibited a phenotype ("WT" normal eye, "RE" rough eye of any degree of severity, "Cy wings" indicates flies that lacked the *Gal4* driver, did not express the *rbf1* transgene, and had wild-type eyes). Note that Δ728-786 and 1-727, which lack the IE and were inactive in cell culture, never showed a phenotype, and that the hyperactive K774 mutants exhibited a partially lethal phenotype, as judged by lower recovery of flies containing the *eye-Gal4* driver. (J) Severity of eye phenotype in flies exhibiting rough eyes. Mutants are shown in order of increasing severity; point mutations in the IE that decreased function in cell culture assays also exhibited weaker eye phenotypes, and hypermorphic K774 alleles exhibited much stronger phenotypes.

Figure 2-8. Mutations in the conserved IE of p107 enhance expression.

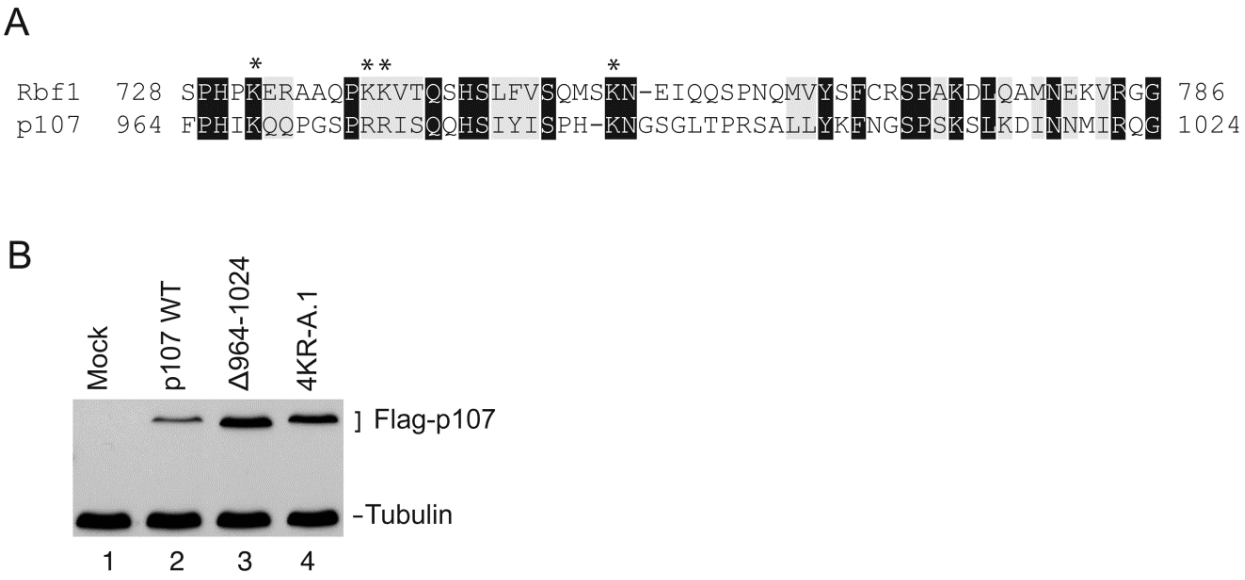

Figure 2-8 (cont'd)

(A) Similarities between Rbf1 IE and homologous region of p107, which is most similar to Rbf1. Asterisks mark basic residues mutated in each protein to stabilize expression. (B) Genes for Flag-tagged wild-type p107 or IE mutants were transfected into S2 cells and expression quantitated by Western blot. The 60 amino acid region deleted from p107 in Δ964 -1024 is similar to the Rbf1 IE. Endogenous tubulin levels are shown as controls.

Until now, there have been no examples of a connection between transcriptional repression and turnover. If it is the modification of the protein with ubiquitin that potentiates Rbf1's repressor activity, this moiety may allow efficient interaction with the transcriptional machinery, similar to the manner in which SUMOylation of PPAR-γ enhances interaction with NCoR corepressors to silence inflammatory genes (Pascual *et al.*, 2005). Ubiquitylation would in this case attract the 26S proteasome in a competing, parallel reaction that enables Rbf1 turnover. Alternatively, Rbf1 recruitment of the proteasome may allow this complex to directly mediate repression, in a way opposite to that produced by activation domains.

The C-terminus of Rbf1 appears to represent a regulatory nexus for this protein; in addition to the instability/repression activity described here, key residues appear to provide a damper to modulate its overall activity (Fig. 2-6), and phosphorylation within this region by cyclin kinases can inactivate the protein (Xin *et al.*, 2002). The deep conservation of residues within the Rbf1 IE argues strongly for similar activities in mammalian pocket proteins; indeed, mutations of key residues in p107, the closest homolog to Rbf1, strongly stabilize the levels of this protein (Fig. 2-8). In addition, the spectrum of mutations associated with the human retinoblastoma gene indicates that the C-terminal region correlating to the Rbf1 IE may similarly contain critical functions for the mammalian RB protein. One common class of genetic lesion associated with retinoblastomas are nonsense mutations that cause a truncation of the C-terminus of the RB protein, and several cancer-associated missense mutations have similarly been mapped to the region corresponding to the Rbf1 IE (Lohmann, 1999).

Previous studies have shown that the RB C-terminus interacts with the E3 ligase Skp2 and the anaphase promoting complex (APC/C) to regulate turnover of the p27 cyclin kinase inhibitor (Binne et al., 2007; Ji et al., 2004). This pathway has been suggested to represent a transcription-independent mechanism by which RB controls the cell cycle, and indeed RB was shown not to be subject to APC/C degradation (Binne *et al.*, 2007). Our results indicate

that a clean separation of transcription and proteolytic control in the context of RB proteins may be oversimplified; here we see evidence for a separate route of proteolytic regulation that modulates transcriptional regulatory potential and protein stability of Rbf1, and possibly related mammalian pocket proteins. Interestingly, the regulation of this pathway may involve the evolutionarily conserved COP9 signalosome. Our previous biochemical studies indicated that the COP9 signalosome regulatory complex is physically associated with Rbf proteins and limits turnover of these repressors (Ullah *et al.*, 2007). From the results of the current study, we postulate that COP9 antagonizes the function of the Rbf1 IE, perhaps by blocking the access of ubiquitin-modifying E3 ligases that would otherwise potentiate Rbf1 activity and turnover. Alternatively, inhibition of E3 ligases may involve the enzymatic activity of COP9, whereby this complex downregulates E3 ligases by deneddylation of their cullin subunits (Wei *et al.*, 2008). How the instability of pocket proteins potentiates their activities, and how these processes relate to developmental control of retinoblastoma family proteins and cancer, will be an area of active investigation.

Materials and Methods

Expression Constructs and Transgenic Lines

To express Rbf1 proteins under control of the endogenous regulatory sequences, an 8.8 kbp genomic locus of Rbf1 was cloned, extending from 2.4 kb upstream of first exon to 2.4 kb downstream stop (2.1 kb downstream end of last exon) into pCaSpeR (Schejter and Shilo, 1989) between *Kpn*I and *Xho*I sites, in three steps using PCR amplification of genomic DNA. Two Flag epitope tags were inserted immediately 5' of the *rbf1* stop codon into an *Xba*I site. The genomic construct of Rbf1 Δ728-786 was made by site-directed mutagenesis. For genes used in S2 cell culture transfection, *rbf1* cDNA was PCR amplified and various mutants produced by

site-directed mutagenesis were cloned from pLD02906 (Keller *et al.*, 2005) into *Kpn*I and *Xba*I sites of pAX vector (Ryu and Arnosti, 2003). Two Flag epitope tags were inserted 5' of the stop codon. For misexpression in the fly, the constructs were cloned into *Kpn*I and *Xba*I sites of pUAST (Brand and Perrimon, 1993). For bacterial expression of GST fusion proteins, the pRSF Duet-1 vector (Novagen) was modified to introduce a GST ORF followed by a ligation independent cloning (LIC) site into its multiple cloning site I (MCS I) to generate the pRSF GST-Tb/LIC vector. *rbf1* cDNA was PCR amplified and cloned into this LIC site to generate the pRSF GST-Rbf1 1-845 construct. The pRSF GST-Rbf1 Δ728-786 construct was generated by site-directed mutagenesis. For expression of human p107 in S2 cells, the cDNA and various mutants produced by site-directed mutagenesis were cloned into the pAX vector and modified with a C-terminal double Flag epitope. The pCaSpeR and pUAST plasmids were used to generate transgenic flies by *P*-element mediated germline transformation of *yw* flies. The transgenic flies were then balanced with SM2 CyO or TM3 Sb balancers.

Luciferase Reporter Assay

Drosophila S2 cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Typically, 1.5 million cells were transfected with 1 µg of *PCNA*-Luciferase reporter, 0.25 µg of pRL-CMV Renilla luciferase reporter (Promega) and 0.2 µg of one of pAX-*rbf1* constructs. Cells were harvested 72 hours after transfection and luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega) and quantified using the Veritas microplate luminometer (Turner Biosystems). Firefly luciferase activity was normalized to renilla luciferase activity.

Immunocytochemistry

Drosophila S2 cells were transfected with 400ng of each *rbf* mutant using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were grown directly on cover slips pre-treated with 0.01% poly-L-Lysine (Sigma). Three days after

transfection, cells were washed once in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) and fixed in 4% paraformaldehyde **(**in PBS) for 30 min. at room temperature. Cells were then washed four times in PBS, permeabilized in PBS+Triton-X-100 (0.4%v/v) for 10 min at room temperature, and blocked with 1% bovine serum albumin (in PBS)**.** Cells were then incubated with M2 anti-Flag antibody (Sigma**;** final concentration 20 g/ml) in 1% w/v BSA in PBS buffer, washed three times in TBST (10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween-20) for 5 min at room temperature and incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G (1:500 dilution) (Boehringer Mannheim and Invitrogen). Cells were then washed three times in TBST and mounted in Vectashield mounting medium (Vector laboratories) containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) and incubated overnight at room temperature. Cells were visualized using an Olympus BX51 fluorescent microscope.

Western Blot Analysis

To measure protein expression in larval tissue, third-instar larvae were collected from transgenic lines expressing Flag-tagged Rbf1 and Rbf1 Δ728-786, mashed with a plastic pestle and sonicated (3 cycles of 12 pulses each) in lysis buffer (50 mM HEPES, pH 7.9, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA, 12.5 mM $MgCl_2$, Complete mini-EDTA free protease inhibitor cocktail, Roche). Imaginal discs were dissected out from ten third-instar larvae and extracts were prepared in lysis buffer. Extracts were run on 10% SDS-PAGE gels and analysed by Western blotting using M2 anti-Flag (mouse monoclonal, 1:10,000, 5 mg/ml Sigma; F3165). Antibody incubation was performed in TBST (20 mM Tris-Cl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) with 5% non-fat dry milk. Blots were developed using HRP-conjugated secondary antibodies (Pierce) and SuperSignal West Pico chemiluminescent substrate (Pierce). To measure protein expression in cell culture, 50 µg S2 cell lysates were resolved by SDS-PAGE, transferred to a PVDF membrane and probed with M2 anti-Flag mouse monoclonal at 1:10,000

dilution, mouse monoclonal anti-tubulin (Iowa Hybridoma Bank) at 1:20,000 dilution.

Treatments with MG132 Proteasome Inhibitor and Cycloheximide

For proteasome inhibitor treatments, S2 cells were transfected with 0.5 µg of pAX*rbf1* constructs using the calcium phosphate transfection method. The cells were grown for 5 days, then treated with 50 µg/ml MG132 (Sigma-Aldrich) or the vehicle DMSO for the indicated times. For determination of Rbf1 protein half-life, 1.5 million S2 cells were transfected using Effectene transfection reagent (Qiagen) with 10 ng of pAX*rbf1* 1-845 or 4K-A.1 genes. 72 hrs post-transfection the cells were treated with 100 µM cycloheximide for the indicated times.

Protein-protein Interaction Studies

For the expression of GST fusion proteins, the appropriate expression constructs were transformed into Rosetta2 (DE3) *E.coli* cells (Novagen). Protein expression was induced by 0.5 mM IPTG for 3 hours at 37 °C. The proteins were purified on Glutathione sepharose beads (GE Healthcare). The [^{35}S]-Met labeled E2f proteins were generated using the TNT T7 Quick for PCR DNA Kit (Promega). In vitro translated proteins were bound to ~1 µg of preincubated immobilized GST fusion proteins for 3 hours at room temperature. The beads were washed three times with HEMGT-150 buffer (25 mM HEPES, 0.1 mM EDTA, 12.5 mM $MgCl_2$, 10% Glycerol, 0.1% Tween-20, 150 mM KCl). Bound proteins were eluted by boiling in 1X Laemmli sample buffer and analysed by SDS-PAGE and autoradiography. For the co-immunoprecipitation assays, 200 ng Myc-tagged E2f1 and 200 ng of various Flag-tagged Rbf1 constructs were co-transfected into S2 cells using Effectene transfection reagent (Qiagen). Cells were grown for 3 days after which whole cell extracts were prepared and Flag immunoprecipitation reactions were performed (Anti-Flag M2 affinity gel, Sigma) followed by anti-Myc Western blotting (mouse monoclonal, 1:3000 dilution, 5 mg/ml, Roche).

Chromatin Immunoprecipitation

Chromatin was prepared and analysed from 0-20 hour old embryos as described previously

(Martinez and Arnosti, 2008) except that the chromatin (1 ml) was incubated with 5 µl (5 µg) of Flag antibody (Sigma; F7425) or 2 µl H3 antibody (Abcam; 0.4 µg/µl) overnight at 4 °C. The recovered DNA was dissolved in 40 µl water. 2 µl of each ChIP sample was used for 28 cycles of PCR. The oligos used for PCR were 5'- CCGCAAGCATCGATAATGAGCAGA-3' and 5'-AGTTGTGCGGGTACTTGGTTTC C-3' for the *DNA primase* promoter; 5'-TGTGGGCTCTCTTCGTGTAGACTT-3' and 5'-TGGTTTCTGATTCTCACACACGAC-3' for the *sloppy paired 1* promoter and 5'-GTTGAGAATGTGAGAAAGCGG-3' and 5'-CGAAAAAGGAGAAGGCACAAAG-3' for an intergenic region.

Fly Assays

Flies harboring the wild-type or mutant *rbf1* forms in the pUAST vector were crossed with flies containing an *eyeless-Gal4* / CyO driver (Gilbert *et al.*, 2006) and the offspring were screened for eye phenotypes. The rbf^{14} mutant (stock number 7435) was obtained from the Bloomington Stock Center.

Immunohistochemical Staining of Imaginal Discs

Imaginal discs were dissected in chilled PBS from third-instar larvae of *rbf1* and *rbf1*Δ728-786 flies and fixed in 3.7% formaldehyde in 10 mM potassium phosphate, pH 6.8; 15 mM NaCl; 45 mM KCl; 2 mM $MgCl_2$ for 30 minutes at room temperature. Antibody detection was performed by diaminobenzadine staining using the Vectastain kit (Vector Labs). Primary M2 α-Flag dilution was 1:1500. Following the horseradish peroxidase reaction, discs were mounted in 70% glycerol.

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CHAPTER 3

Ubiquitination of Retinoblastoma family protein 1 potentiates gene-specific repression function

Abstract

The Retinoblastoma (RB) tumor suppressor family functions as a regulatory node governing cell cycle progression, differentiation and apoptosis. Post-translational modifications play a critical role in modulating RB activity, but additional levels of control, including protein turnover, are also essential for proper function. The Drosophila RB homolog Rbf1 is subjected to developmentally cued proteolysis mediated by an instability element (IE) present in this protein's C-terminus. Paradoxically, instability mediated by the IE is also linked to Rbf1 repression potency, suggesting that proteolytic machinery may also be directly involved in transcriptional repression. We show that the Rbf1 IE is an autonomous degron that stimulates both Rbf1 ubiquitination and repression potency. Importantly, Rbf1 IE function is promoter-specific, contributing to repression of cell cycle responsive genes but not to repression of cell signaling genes. The multifunctional IE domain thus provides Rbf1 flexibility for discrimination between target genes embedded in divergent cellular processes.

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Introduction

The RB tumor suppressor protein functions as a crucial regulator of the G1/S transition during cell cycle progression, and thus plays a central role in restricting cellular proliferation (Burkhart and Sage, 2008). Consistent with this property, the RB1 gene is inactivated in a broad range of human cancers, often as a seminal event contributing to both cancer initiation and progression (Hanahan and Weinberg, 2011). RB has been further implicated in the governance of diverse physiological processes, including differentiation and apoptosis, and as a central hub connecting these processes, RB activity is subjected to strict control by post-translational modification during normal growth and development (Nguyen and McCance, 2005; Skapek et al., 2006). Indeed, in many tumor types, upstream regulatory pathways governing RB are inactivated with similar frequencies as inactivation of RB itself, attesting to the importance of close supervision over RB function (Wikenheiser-Brokamp, 2006a).

In an intricate network of gene control, RB and its related family members, p107 and p130, function as transcriptional repressors of diverse gene sets through interactions with members of the E2F family of transcriptional activator proteins (Genovese et al., 2006; Morris and Dyson, 2001). RB family members govern apparently mutually exclusive physiological processes, notably cell cycle progression and apoptosis, thus distinct regulatory mechanisms must ensure that RB-mediated induction of apoptosis does not ensue, even as RB proteins are periodically activated on cell cycle genes during normal proliferation (Delston and Harbour, 2006). Canonical regulation of RB activity is governed by cyclin/cdk regulatory kinases (Chen et al., 1989; Hinds et al., 1992; Kato et al., 1993; Lin and Wang, 1992). Timely phosphorylation blocks RB/E2F association, and unleashes waves of E2F-mediated transcription that contribute to cell cycle progression (Knudsen et al., 1998). However, RB continues to reside at a number of genomic sites after cyclin/cdk-mediated deactivation

(Wells et al., 2000; Wells et al., 2003), revealing that cyclin/cdk activity does not universally de-repress all RB target genes. Indeed, RB phosphorylation by p38MAPK at a site that is not a target for cyclin/cdks can modulate RB-mediated repression of apoptotic response genes (Delston and Harbour, 2006; Delston et al., 2011). This model suggests that RB is subjected to a protein-modification code that enables gene specific outcomes, namely cyclin/cdk kinases regulate cell cycle-responsive promoters and stress responsive kinases regulate apoptosis-responsive promoters.

In Drosophila, RB family proteins Rbf1 and Rbf2 interact with E2F transcription factors as corepressors, similar to their mammalian counterparts. Drosophila Rbf proteins are also controlled by a canonical phosphorylation mechanism through cyclin-cdk complexes (Du et al., 1996a; Du et al., 1996b). Mutant rbf1 embryos show constitutive expression of PCNA and RNR2, two E2F1-regulated genes for DNA replication, and ectopic S-phase entry, indicating the importance of Rbf1 for arresting cells in G1 phase (Du and Dyson, 1999). Rbf1 associates at numerous canonical E2F cell cycle-regulated genes in the early embryo (Acharya et al., 2010; Stevaux and Dyson, 2002), indicating that key components of the RB regulatory pathway are evolutionarily conserved. However, in the embryo, Rbf1 also associates with numerous E2F1-independent target genes beyond the canonical cadre of E2F1-dependent target genes (Acharya et al., 2012b; Korenjak et al., 2012a). Many of these candidate E2F1-independent target genes encode components of signaling pathways, exemplified by the insulin receptor (InR), and whose expression is regulated independently of the cell cycle. Thus, Drosophila Rbf regulatory influence during development appears to extend beyond cell cycle progression and apoptosis to include cellular signaling, although in a mechanism likely independent of E2F1.

In addition to regulation by phosphorylation, Rbf proteins are subject to developmental regulation of their proteolytic turnover. Developmental regulation occurs in imaginal disc

tissue (Acharya et al., 2010) with stability controlled by the COP9 signalosome (Ullah et al., 2007), a developmentally regulated complex that controls proteasome-mediated protein degradation via modulation of E3 ubiquitin ligase activity (Bech-Otschir et al., 2002; Chamovitz and Glickman, 2002). The COP9 signalosome is physically associated with Rbf1 and Rbf2, and depletion of COP9 subunits stimulates Rbf1 turnover (Ullah et al., 2007). Rbf1 stability is influenced by a C-terminal instability element (IE) that positively contributes to both repressor destruction and potency (Acharya et al., 2010). The conservation of the IE in mammalian RB family proteins suggests that these pathways operate in higher eukaryotes, however the function of the IE in integrating protein turnover and transcriptional control is poorly understood. Here, we show that the Rbf1 IE is sufficient to facilitate ubiquitination and turnover, and directly mediates transcriptional repression. Strikingly, Rbf1 ubiquitination enhances E2F1-dependent PCNA repression but not E2F1-independent repression of InR transcription. Thus, the IE is a key protein motif directing promoter-specific activity of Rbf1. These studies reveal a novel level of regulatory discrimination within the RB protein modification code that enables gene-specific repression during development.

Results

A modular degron influences Rbf1 ubiquitination and stability

Drosophila Rbf proteins are subjected to developmentally regulated turnover, exhibiting tissue-specific modulation in both the developing embryo and larvae (Acharya et al., 2010; Keller et al., 2005). To understand the mechanism underlying this regulation, we tested whether the Rbf1-IE can autonomously control protein stability by fusing the IE region (728-786) to GFP (Fig. 3-1A), and measuring the half-lives of GFP and GFP-Rbf1-IE chimeras in S2 cells after cycloheximide treatment. Steady state levels of GFP-Rbf1-IE, but

not GFP, were substantially decreased by 12 hours after cyclohexamide challenge, indicating that the IE directly enhanced GFP turnover (Fig. 3-1B). Thus, the IE region can function autonomously as a degron, and independently of other domains within Rbf1. This ability is consistent with the previously discovered role of the IE in control of full-length Rbf1 stability during development (Acharya et al., 2010).

Previous models of degron function indicate that subcellular location of substrate proteins influences turnover (Hammond-Martel et al., 2012). Therefore, to examine the effect of substrate localization on Rbf1-degron function, the Rbf1 nuclear localization signal (NLS, Fig. 3-S1) was appended to GFP-Rbf1-IE, largely confining the chimera protein to the nucleus (Fig. 3-1C). Accumulation of the GFP chimera proteins was then measured; testing lysine-to-alanine substitutions within the IE that were previously shown to both inactivate and stabilize wild type Rbf1 (Acharya et al., 2010). In all experiments, both GFP-Rbf1-IE (-NLS) and GFP-Rbf1-C (+NLS) behaved similarly, with K to A mutants accumulating to levels approximately three fold higher than those of their wild-type counterparts. Consistent with these observations, the GFP-Rbf1-IE 4K-A mutant displayed a significantly longer half-life compared to GFP-Rbf-IE (Fig. 3-S2). The steady state levels of both GFP-Rbf1-IE and GFP-Rbf1-C were unaffected by lysine-to-arginine substitution of the same amino acids, indicating that the positive charges of the side chains are important for IE substrate destabilization and that these lysine residues are unlikely targets for ubiquitination (Fig. 3-1D). These data indicate that the function of the IE as a modular degron is unaffected by its preferential nuclear localization, and is consistent with a model wherein some components of the Rbf1 degradation pathway occur in the nucleus.

Regulated protein turnover often involves the activity of the 26S proteasome, which interacts with substrates that have been modified with ubiquitin, but also in some cases proteins that are not ubiquitinated. In mammals, RB and p107 are substrates of E3 ubiquitin

ligases and are turned over in a proteasome-dependent manner (Barbash et al., 2007; Sdek et al., 2004; Uchida et al., 2005; Ying and Xiao, 2006). Rbf1 is likewise dependent on the proteasome pathway, but there are no reports of ubiquitination of this protein. To test whether Rbf1 is ubiquitinated in vivo, we expressed Flag-tagged Rbf1 and HA-tagged ubiquitin proteins in S2 cells, and immunoprecipitated the Rbf1 proteins. As shown in Fig. 3-2A, poly-ubiquitinated Rbf1 species were detected in heat denatured extracts prepared from cells co-expressing both Flag-Rbf1 and HA-ubiquitin. Ubiquitinated species were not observed in mock-transfected samples, in samples containing only one of the two proteins, or in extracts containing Rbf1 and HA-ubiquitin from denatured extracts containing individually expressed HA-Ub or Flag-Rbf1 proteins that were mixed together prior to immunoprecipitation. In the presence of the MG132 proteasome inhibitor, higher levels of polyubiquitinated Rbf1 were observed (Fig. 3-2B). We conclude that the Rbf1 protein was ubiquitinated in vivo, and is targeted for proteasome-mediated turnover, an outcome that is consistent with previous observations linking the COP9 signalosome to protection of Rbf1 from destruction by the proteasome (Ullah et al., 2007). Interestingly, Rbf1 lacking the IE region (Rbf1-ΔIE) exhibited a substantial reduction, but not complete loss, of Rbf1 ubiquitination (Fig. 3-3A), a result that was also observed for Rbf1-4KA (Fig. 3-S3), suggesting that the IE enhances ubiquitination, but is not essential for all modification events. We tested whether the IE is sufficient to independently drive ubiquitination by co-expressing HA-tagged ubiquitin and the GFP-IE chimera. Indeed, as shown in Fig. 3-3B, levels of poly-ubiquitinated GFP were substantially increased by appending the Rbf1-IE region as compared to levels observed for untagged GFP. GFP-Rbf1 IE ubiquitination was reduced by the introduction of the 4K-A substitutions (Fig. 3-S4). Together, these data show that one function of the Rbf1 IE is to facilitate substrate ubiquitination.

Figure 3-1. The instability element (IE) of Rbf1 is a modular degron.

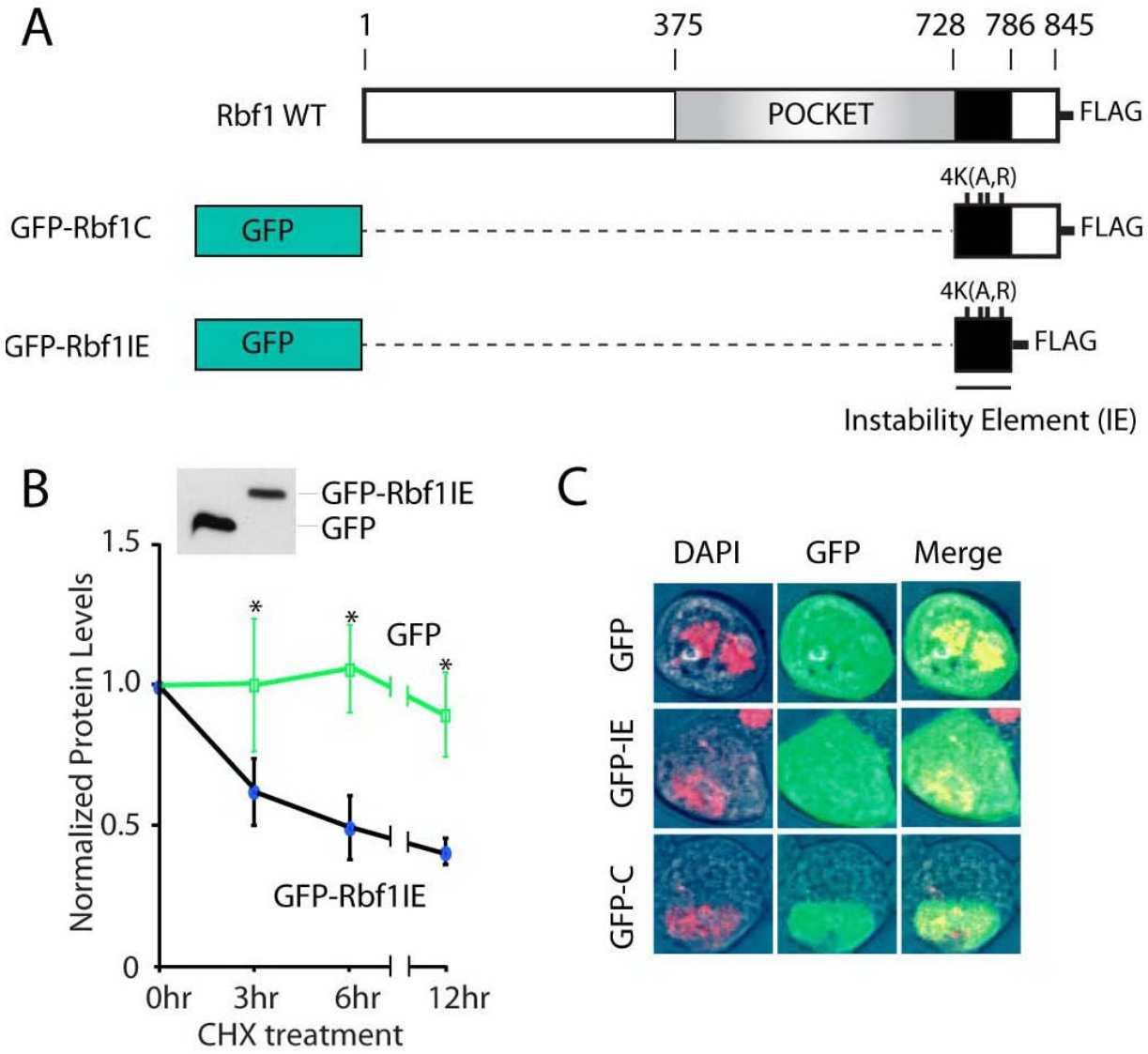

Figure 3-1 (cont'd)

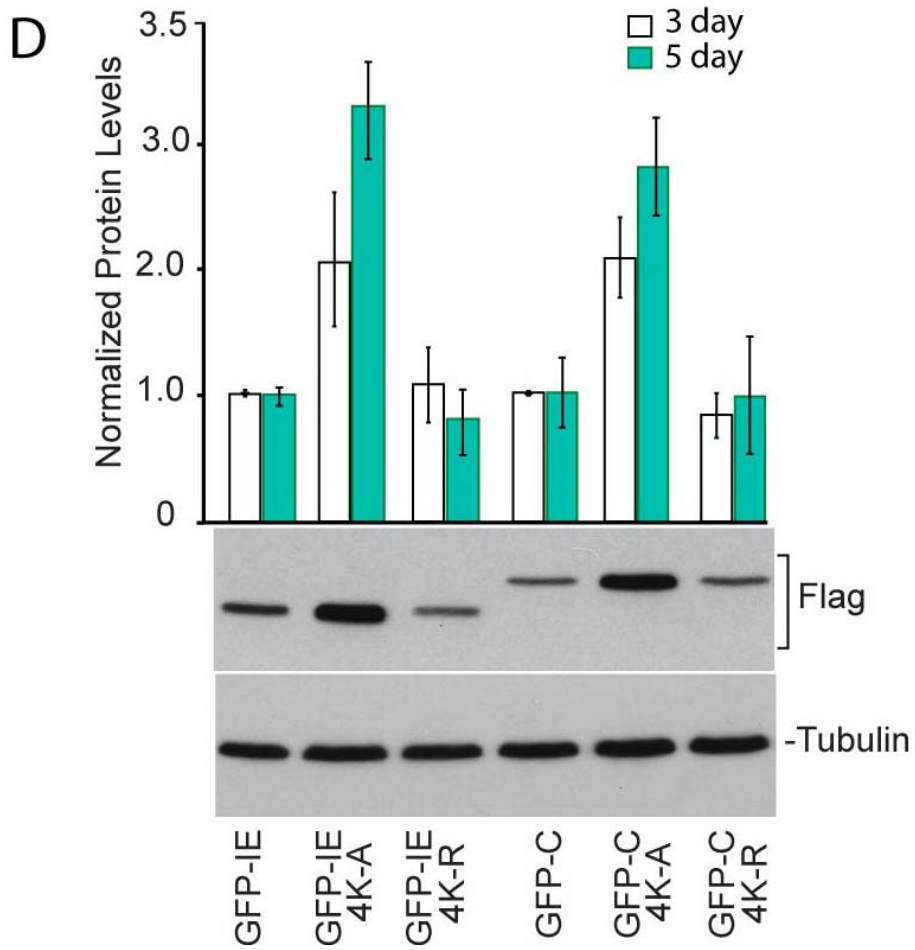

Figure 3-1 (cont'd)

(A) Schematic diagram of GFP-fusion proteins expressed in Drosophila S2 cells. (B) Presence of the IE increases protein turnover. Half-lives of GFP-fusion proteins were measured by Western blot after cycloheximide (CHX) treatment (error bars are standard deviation, $p<0.01$). Inset Western blot shows the steady-state levels of GFP and GFP IE fusion protein before CHX treatment. (C) Subcellular localization of GFP and GFP-fusion proteins as measured by confocal microscopy. (D) IE function modulates GFP stability. Indicated GFP-fusion proteins were expressed in S2 cells for 3 or 5 days and measured by Western blot with antibodies against the Flag epitope. Lysine residues (K732, K739, K740 and K754) were changed to alanine or to arginine. Protein levels were quantitated by photon-capture analysis with a Fuji LAS-3000 Imager and normalized to tubulin levels. Error bars indicate standard deviation, and asterisks indicate $p < 0.01$. Western blot data is a representative from the 5-day set of experiments.

Figure 3-2. Rbf1 is degraded via an ubiquitin-proteasome dependent pathway.

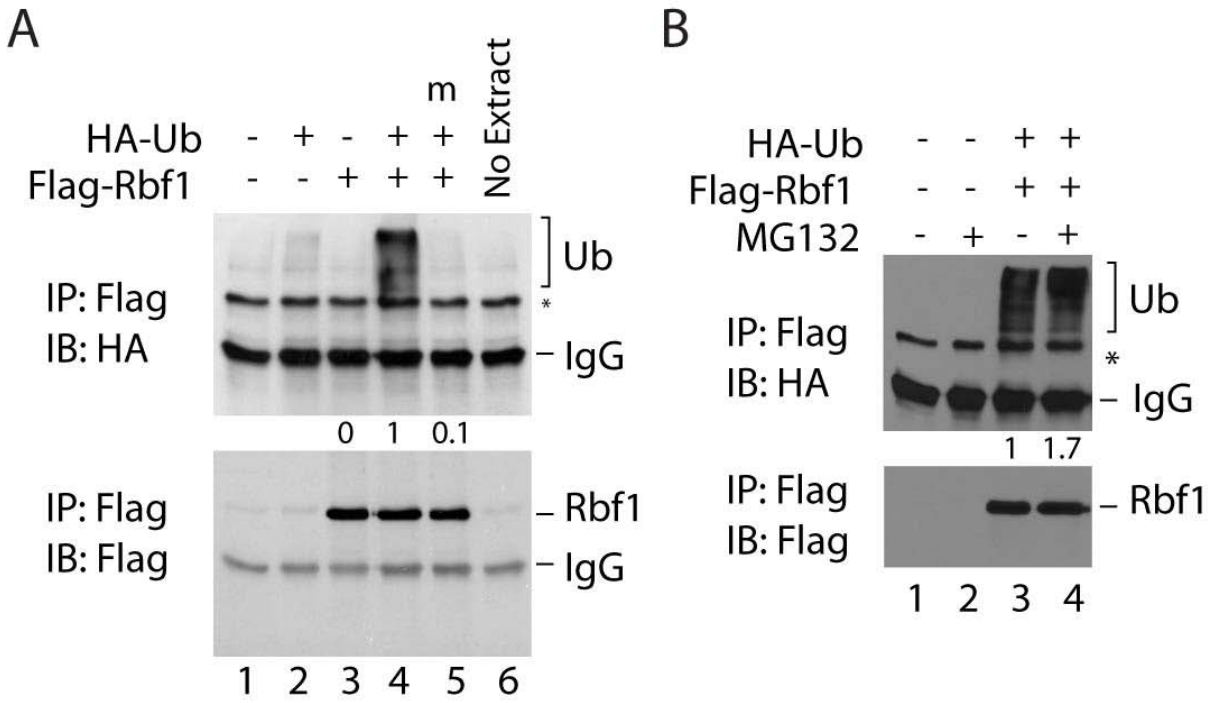

Figure 3-2 (cont'd)

(A) Rbf1 is ubiquitinated in vivo. S2 cells were transfected with Flag-tagged Rbf1 and HA-tagged ubiquitin expression constructs. Denatured protein extracts were used for Flag immunoprecipitation (IP) and recovered samples were assayed by anti-HA Western blot analysis (top panel). The asterisk indicates a non-specific band and "m" indicates reaction performed using mixed samples from those in lanes 2 and 3. The IP samples were also blotted with anti-Flag antibody (bottom panel) to verify equivalent Rbf1 recovery (lanes 3-5). The numbers underneath the HA Western blot panel represent the ratios of HA/Flag signals. The data shown are representative of three biological replicates. (B) Rbf1 ubiquitination is sensitive to proteasome inhibition. Samples were treated as in (A) except that they were treated with MG132, a proteasome inhibitor.

Figure 3-3. The Rbf1 instability element enhances protein ubiquitination.

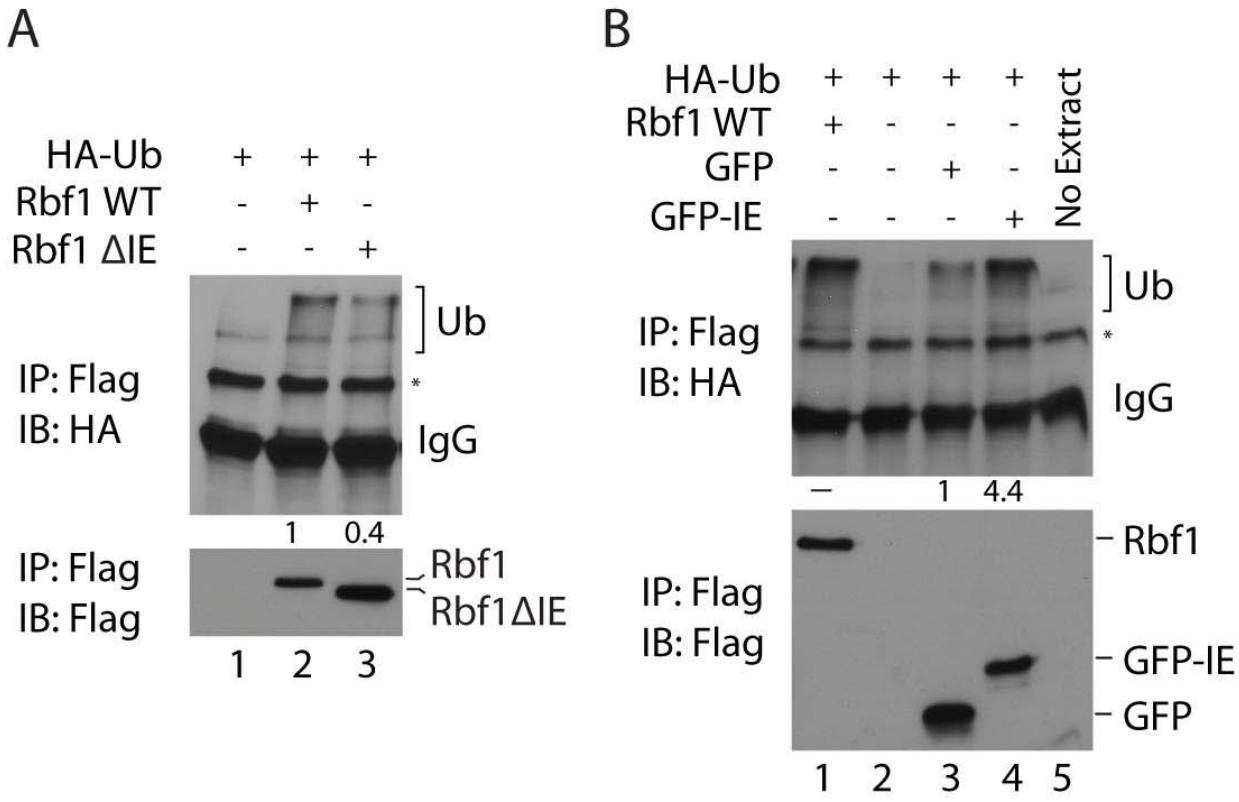

Figure 3-3 (cont'd)

(A) The Rbf1 IE enhances ubiquitination. Wild type and mutant Rbf1 lacking the IE (Rbf1-ΔIE) were compared for ubiquitination as performed in Figure 2. (B) The Rbf1 IE is sufficient to drive the ubiquitination of a heterologous protein, GFP. Fusion of the Rbf1-IE to GFP led to a substantial increase in the levels of its ubiquitination as compared to the levels observed for GFP as measured by co-transfection and CO-IP/Western analysis.

The Rbf1-IE can function independently in transcriptional repression

We showed previously that in addition to influencing protein stability, the IE region is critical for Rbf1 repressor activity on E2F1-dependent promoters, such as *PCNA* and *Polα* (Acharya et al., 2010). We therefore hypothesized that the Rbf1 degron functions as a bona fide transcriptional repression domain. To test this hypothesis, the Rbf1 degron alone or degron plus NLS was fused to the Tet repressor, and the activity of these proteins was assayed on an Actin5C reporter harboring two Tet binding sites (Fig. 3-4A). Indeed, when directly tethered to its target promoter in the absence of doxycycline, both Tet-Rbf1-IE and Tet-Rbf1-C showed strong repression activity at levels approaching that observed with Tet-Knirps, a potent short-range repressor that was included as a positive control on this reporter (Fig. 3-4B). As expected, treatment with doxycycline to inhibit DNA binding also diminished repression (not show). The Tet repressor DNA binding domain alone lacked notable repression activity. These data are consistent with a direct role for the IE in transcriptional repression. Interestingly, both Tet-Rbf1-C and Tet-Rbf1-IE harboring the K-A substitutions repressed transcription to similar levels as observed for the wild type Tet-Rbf1-IE chimera. Thus, these lysine residues that influence repression in the context of full-length Rbf1 are not essential in this context (Acharya et al., 2010).

The ability of the IE to independently repress transcription next prompted us to examine whether the IE is an essential element within full-length Rbf1 when targeted to a promoter independently of E2F1. Strikingly, the Tet-Rbf1 chimera lacking the IE (Tet-Rbf1-ΔIE) was not compromised for activity; the protein repressed transcription from the Actin5C-Tet reporter as effectively as did the wild type Tet-Rbf1 chimera, indicating that the IE is not essential in this context (Fig. 3-4C). When assayed on the PCNA reporter that lacks Tet binding sites but utilizes E2F1 to recruit Rbf1, the Tet-Rbf1-ΔIE chimera was compromised for repression, consistent with previous observations that the IE is important for Rbf1

repression of cell cycle genes (Acharya et al., 2010). Therefore, this outcome suggests that the mechanism of promoter targeting does influence whether the IE region functions in repression. Interestingly, both Tet-Rbf1-C (4KA) and Tet-Rbf1-IE (4KA) were expressed at similar levels as their wild type counterparts, and under conditions wherein the same alanine substitutions increased Tet-full-length Rbf1 steady state levels (Fig. 3-4D). These observations suggest that the function of these IE-lysine residues is context dependent for both repression and stability.

Context-dependent repression by Rbf1-IE regulatory domain

The substantial repression exhibited by the Rbf1-ΔIE mutant protein when directly recruited to the Tet promoter demonstrated that this protein is not inherently defective. This observation also raised the interesting possibility that the IE provides gene specific repression capability. To examine the possibility that the IE provides repression capability specifically in the context of E2F1-regulated promoters, the repression potency of wild type Rbf1 was compared to Rbf1-ΔIE on E2F1-regulated promoters (*PCNA*, *Polα,* and *Mcm7*) (Fig. 3-5A) and non-canonical E2F1-independent promoters (*InR*, *wts*, *Pi3K68D*) (Fig. 3-5B). The *InR*, *wts*, and *Pi3K68D* gene promoters are devoid of recognizable E2F1 binding sites and were refractory to activation by E2F1, but are directly bound by Rbf1 in the embryo (Acharya et al., 2012b). On the canonical target genes, Rbf1-ΔIE was much weaker than wild-type Rbf1 for E2F1-dependent gene repression, but both repressors exhibited similar potency on the non-canonical Rbf1 reporter genes. As previous data showed that Rbf1-ΔIE can interact with E2F1 and associate with endogenous E2F1 target genes (Acharya et al., 2012b), the IE may provide post-recruitment functions that are dispensable when Rbf1 is recruited independently of E2F1.

Figure 3-4. Rbf1 IE functions as a transcriptional repression domain.

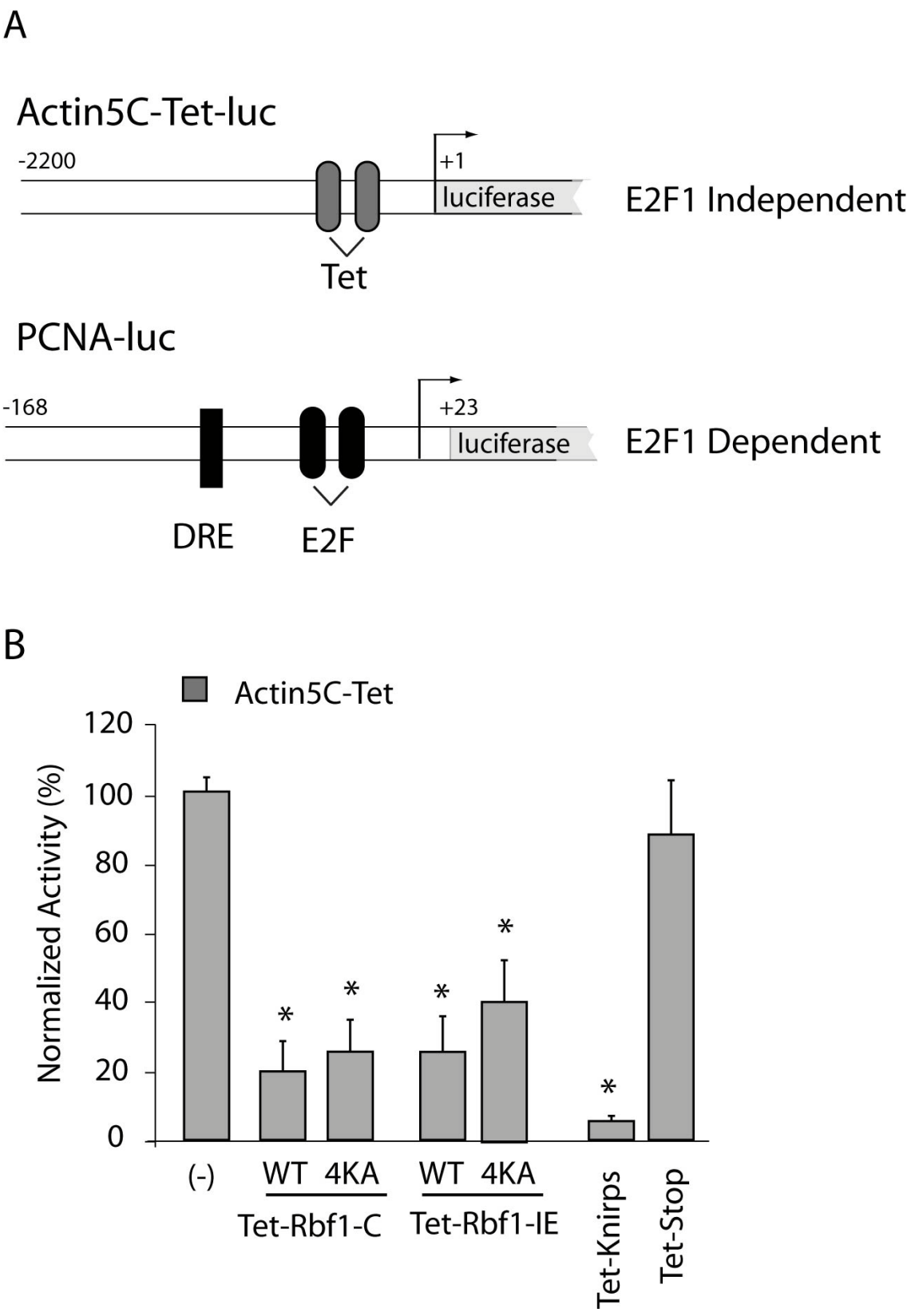

Figure 3-4 (cont'd)

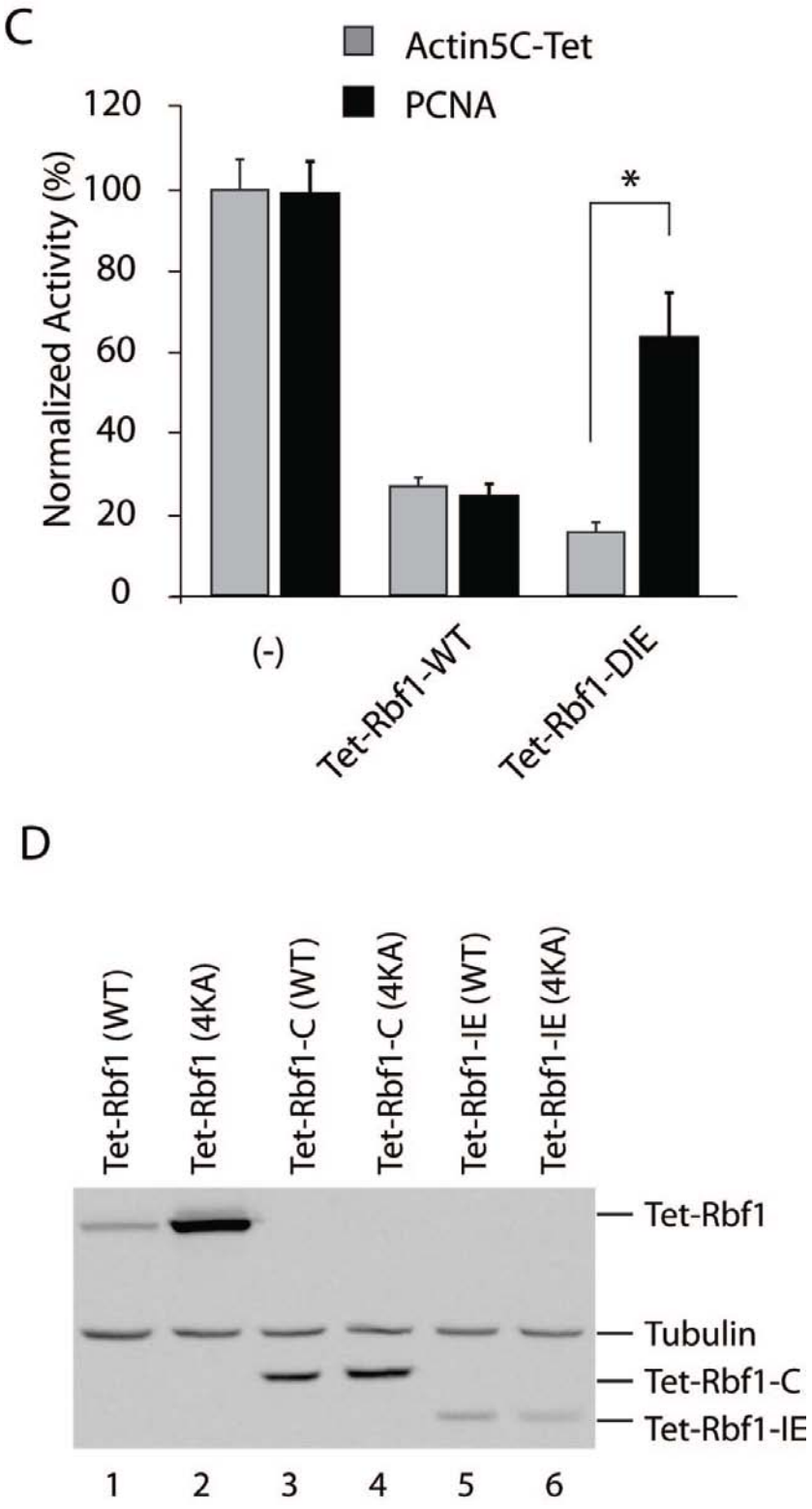

Figure 3-4 (cont'd)

(A) Schematic representation of the E2F1-independent and E2F1-dependent reporter genes used in this study (B) Transcriptional activities of Tet-fusion proteins were assayed on the *Actin5C*-Tet-luc reporter. The IE with or without the NLS repressed the target gene when directly tethered to the promoter compared to reactions lacking Rbf1 fusion proteins (*, $p<0.05$). Both the WT and 4KA mutant versions repressed transcription equivalently. A Knirps fusion protein (Tet-Knirps) and Tet protein alone (Tet-Stop) served as positive and negative controls, respectively. (C) Transcriptional activities of the Tet-Rbf1 WT and Tet-Rbf1 ΔIE chimeras were compared on the *Actin5C*-Tet-luc and *PCNA*-luc reporters. Data are from at least three biological replicates. (D) Levels of the indicated Tet-Rbf1 fusion proteins were determined by anti-Flag Western blot analysis 3 days after transfection. Lysine to alanine substitution did not affect steady state levels of the Tet-Rbf1-IE and Tet-Rbf1-C proteins under conditions wherein Tet-Rbf1 levels were increased. Tubulin levels are shown as a loading control.

Figure 3-5. Context dependence of the Rbf1-IE for transcriptional repression.

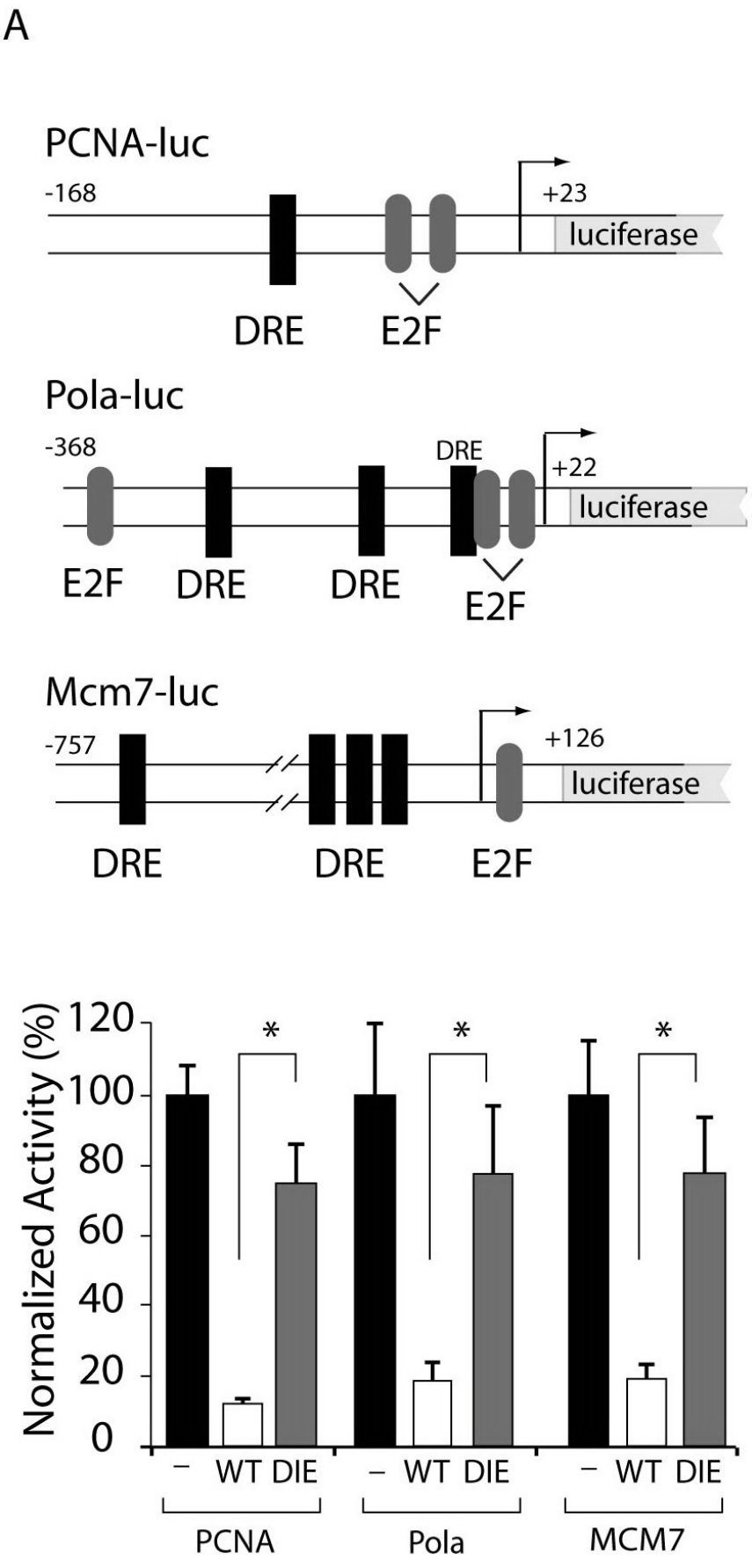

Figure 3-5 (cont'd)

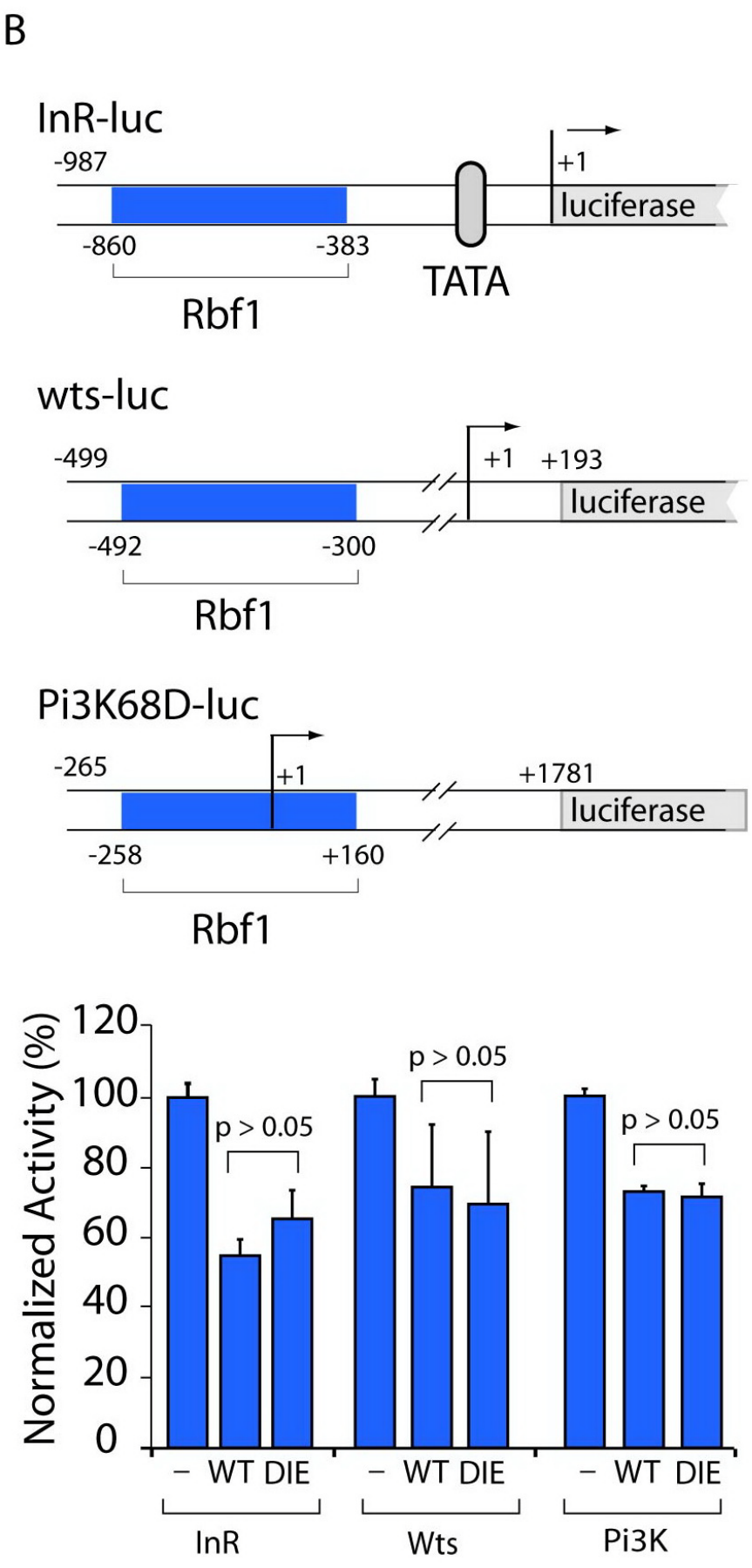

Figure 3-5 (cont'd)

(A, B) Rbf1 WT and Rbf1 ΔIE showed dissimilar repression activities on the E2F1 dependent reporters as compared to the E2F1 independent promoters. Transcriptional activity was measured as described in Figure 4. Data are from at least three biological replicates.

Rbf1 ubiquitination stimulates repressor potency

The function of the instability element as both a repression domain and a degron that stimulates Rbf1 ubiquitination, suggested that ubiquitin might function directly in Rbf1-mediated repression. We showed above that MG132 treatment substantially increases the levels of ubiquitinated Rbf1. Therefore, we measured Rbf1-mediated repression of the PCNA reporter in the presence or absence of MG132 (Fig. 3-6A). A modest but reproducible enhancement in repression potency of wild type Rbf1 was observed within 2 hours of drug treatment, an effect that was not observed with the Rbf1-ΔIE mutant. This data is consistent with IE-directed ubiquitination influencing repression activity. Although MG132 affected only the wild type Rbf1, a general concern remained that global proteasome inhibition may induce pleiotropic effects (Deroo and Archer, 2002). Therefore, to directly assess the effect of ubiquitin on Rbf1 function, repression assays were performed using chimera proteins containing ubiquitin fused to the N-terminus of full length Rbf1. As ubiquitin attachment markedly destabilized full-length Rbf1 (see also Fig. 3-6C) consistent with this modification directing Rbf1 for proteasome destruction, repression assays were performed using differing amounts of expression plasmids to equalize repressor concentration. Under conditions wherein both Rbf1 and Ub-Rbf1 were expressed at comparable levels, the presence of ubiquitin markedly improved Rbf1 repression activity on the PCNA promoter on average 4-5 fold (Fig. 3-6B). This outcome supports the hypothesis that ubiquitin can contribute directly to target gene repression.

The potent role of ubiquitin in Rbf1 target gene repression noted above allowed the possibility to examine whether poly-ubiquitination at this site is essential for enhanced repressor potency. To test this possibility, K48R and K63R substitutions were incorporated within the N-terminal ubiquitin at positions expected to impede poly-ubiquitination. Indeed, as shown in Fig. 3-6C, Rbf1 appended with mutant ubiquitin (K48R, K63R) was maintained

at higher steady state levels than Rbf1 fused to wild type ubiquitin when expressed using comparable amounts of expression plasmid. Thus, the N-terminal ubiquitin was functional in the proteasome-mediated turnover of Rbf1. When compared to wild type Rbf1 lacking ubiquitin, Rbf1 harboring mutant ubiquitin remained a more potent repressor of PCNA transcription. This result suggests that while ubiquitination at the Rbf1 N-terminus can contribute to repression potency, poly-ubiquitination at this site is not essential for this enhancement. Nonetheless, in all experiments, Rbf1 containing wild type ubiquitin did exhibit improved specific activity, suggesting that higher order ubiquitination can contribute to repression.

Based on the observation that Rbf1-ΔIE is defective for repression on E2F1 target genes, whether the forced ubiquitination of Rbf1-ΔIE could stimulate repression potency was tested. In this experiment, higher levels of Rbf1-ΔIE were tested to ensure that active proteins were being compared. Under these conditions, and despite substantially lower steady state protein levels associated with forced ubiquitination, Rbf1-ΔIE harboring the appended wild type ubiquitin exhibited increased repression ability of PCNA transcription (Fig. 3-6D). However, ubiquitin did not enhance Rbf1-ΔIE repression of the InR reporter, suggesting that the effect of this modification is restricted to certain types of target genes. These observations imply that insufficient ubiquitination observed with IE deletion underlies the loss of repression activity at cell cycle regulated genes.

Figure 3-6. Rbf1 ubiquitination enhances gene specific repression activity.

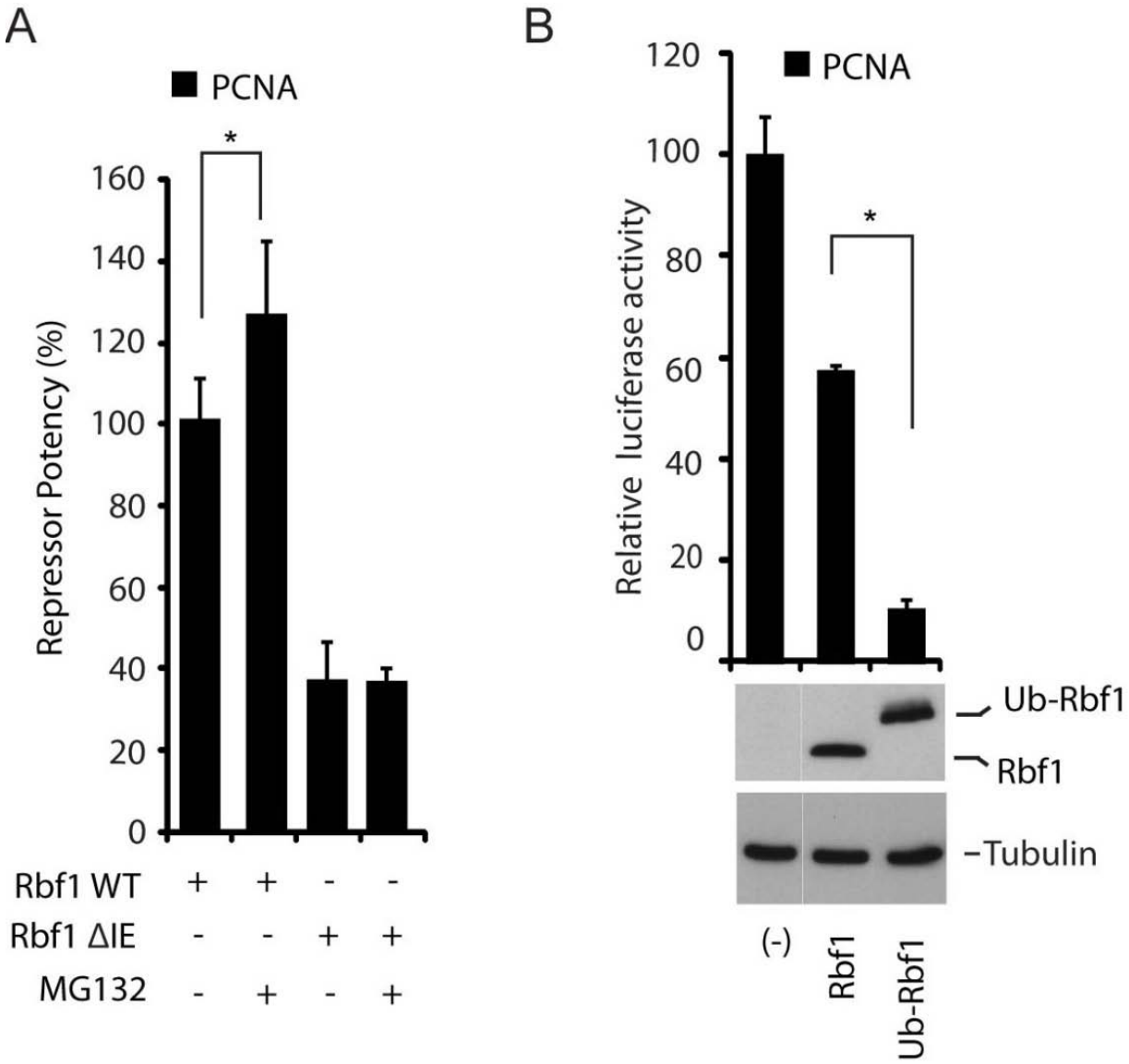

Figure 3-6 (cont'd)

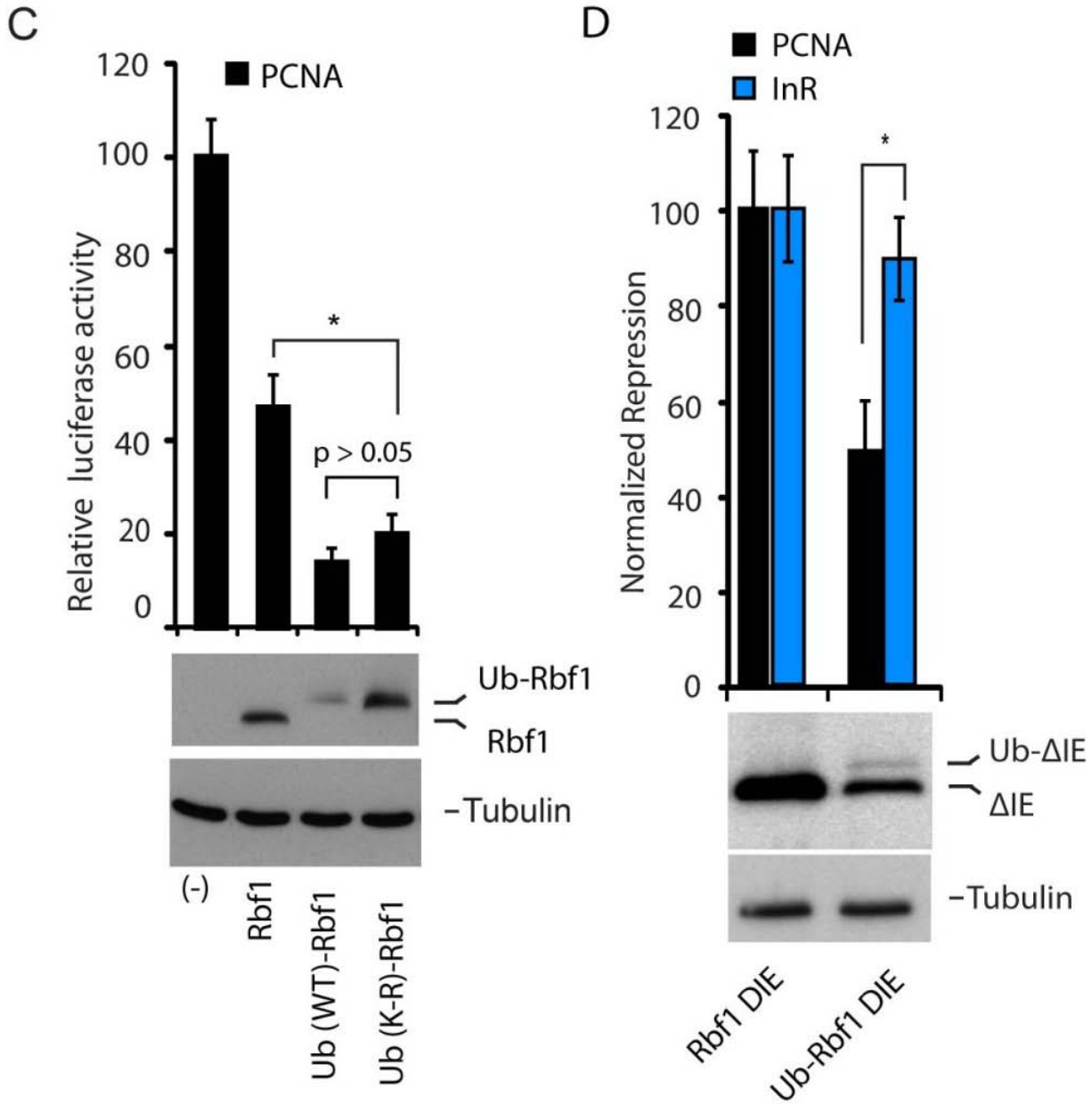

Figure 3-6 (cont'd)

(A) Proteasome inhibition by MG132 influences transcriptional repression activity of Rbf1 on the PCNA-luc reporter. Repression potency of WT Rbf1 on the *PCNA*-luc reporter (set to 100%), but not the ΔIE mutant was significantly enhanced after MG132 treatment (*, $p<0.01$) (B) Ubiquitin enhances Rbf1 repression potency. In this experiment, wild type Rbf1 expression was adjusted to match that of the unstable Ub-Rbf1 chimera (3 ng pAX-Rbf1 WT vs. 1000 ng pAX-Ub-Rbf1 WT) for testing using the *PCNA*-luc reporter (upper panel). At comparable levels of repressor, as detected by Flag Western analysis (lower panel), ubiquitin improved Rbf1 specific activity 3-4 fold. Tubulin levels are shown as a loading control. (C) Poly-ubiquitination of the N-terminal ubiquitin is not essential for enhanced repression. K to R substitutions at positions 48 and 63 within the N-terminal ubiquitin tag increased Rbf1 steady state levels as compared to wild type ubiquitin-Rbf1 chimeras in transfection experiments using equal amounts of DNA (lower panel). At comparable protein levels, the mutant Ub-Rbf1 chimera repressed transcription better than Rbf1 lacking the ubiquitin tag (*, $n=3$, $p<0.05$) and to levels similar as observed for the Rbf1 chimera harboring the wild type ubiquitin tag. (D) Ubiquitin fusion partially restores transcriptional repression activity to Rbf1-ΔIE on the *PCNA*-luc reporter ($p<0.05$) but not on the *InR*-luc reporter using equal amounts of DNA during transfection. In these experiments, the faster migrating protein observed with the Ub-Rbf1-ΔIE fusion protein (lower panel) is likely due to substantial cleavage of the ubiquitin tag.

Discussion

The RB family of proteins governs diverse physiological processes including cell cycle, apoptosis, and differentiation. An important question remains how these factors maintain differential influence over mutually exclusive pathways. Previous studies demonstrated that mammalian RB phosphorylation by cell cycle dependent kinases or stress responsive kinases can distinguish between cell cycle arrest or apoptotic responses (Delston et al., 2011). In this study of the Drosophila Rbf1 protein, we uncovered a direct role for ubiquitination in differential gene regulation. In particular, the C-terminal regulatory domain of Rbf1 was found to harbor an independently acting degron that directs Rbf1 ubiquitination. Post-translational modification by ubiquitin improved Rbf1 transcriptional repression, directly linking repressor potency to ubiquitin-mediated turnover pathways. Furthermore, Rbf1 lacking the degron was also debilitated for repression of cell cycle regulated *PCNA*, *Polα*, and *Mcm7* promoters, but not for regulation of non-canonical Rbf1 target genes, thus highlighting a role for ubiquitination in differential regulation of Rbf target genes. These findings point to distinct modes of transcriptional repression depending upon the promoters targeted. Recent genomic studies have shown that Rbf1 association at many non-canonical promoters, including the *InR* locus, is independent of E2F1 but is dependent upon the general E2F partner, DP1 (Acharya et al., 2012b; Korenjak et al., 2012a). Thus, it remains possible that the Rbf1 degron functions primarily when recruited by E2F1/DP1 and not when recruited by E2F2/DP1. This concept is consistent with structural studies of human RB that show the corresponding region located within the RB C-terminus is important for interactions with E2F1/DP1 complexes (Rubin et al., 2005). As the Rbf1 degron sequence is highly conserved within the mammalian RB homologs p107 and p130, degron function in differential gene repression may be evolutionarily conserved.

While ubiquitin clearly enhanced Rbf1 activity towards the *PCNA* promoter, the molecular

mechanism by which ubiquitination is associated with transcriptional repression is unknown. In one model, repression is enhanced by direct proteasome recruitment to a promoter through interactions mediated by ubiquitin. In a second model, ubiquitination serves two roles, recruiting essential cofactors to a promoter, and separately interacting with the protein degradation machinery. Aspects of this mechanism are analogous to the degron theory of gene activation previously described for the c-Myc proto-oncoprotein (Geng et al., 2012; Salghetti et al., 2001; Salghetti et al., 1999; Salghetti et al., 2000). During activation, ubiquitin can function for co-factor recruitment, such as described for recruitment of p-TEFb by the viral activator VP16 (Kurosu and Peterlin, 2004), and thus ubiquitin may similarly contribute to RB co-repressor recruitment. As our studies demonstrate that the C-terminal degron may recruit an E3 ligase, a direct role for these enzymes in Rbf1 gene regulation is possible. Such a direct role for E3 ligases in repression was observed for BRCA1-mediated transcriptional regulation (Horwitz et al., 2007); however, in that example, ubiquitin interfered with assembly of the preinitiation complex. Alternatively, Rbf1-mediated E3 recruitment could promote E2F1 ubiquitination. However, the IE region does not appear to influence Rbf1-mediated E2F1 stabilization (42). Whether E3 ligases participate directly in Rbf1-mediated repression is unknown, nonetheless, observations that the COP9 signalosome, an evolutionarily conserved complex that functions to inhibit E3 ligase activity, was directly found at Rbf1 target genes simultaneously with the Rbf1 repressor (Ullah et al., 2007) suggests that a complex network of feedback regulation is proximally available at Rbf1 target gene promoters.

Materials and Methods

Expression Constructs

Generation of Rbf1 WT and mutant expression constructs was described previously (Acharya et al., 2010). To generate GFP fusion proteins, eGFP cDNA was PCR-amplified from phs-eGFP and cloned into KpnI site of pAX vector. Two Flag epitope tags were inserted 5' of the stop codon. The C-terminus and the IE of Rbf1 were made by site-directed mutagenesis. To minimize the differences among mRNAs transcribed from GFP fusion protein constructs, the first two amino acids of the IE were mutated into stop codons to generate GFP alone constructs. Tet fusion protein expression constructs were generated as described previously (Ryu and Arnosti, 2003). Rbf1 WT and mutants were digested from pAX-rbf1 vector and ligated into KpnI and XbaI sites of pAX-Tet vector. The C-terminus and the IE were amplified with KpnI and XbaI on the ends and inserted into pAX-Tet vector. To generate ubiquitin fusion proteins, the ubiquitin coding sequence was amplified using oligonucleotides with KpnI sites on both ends, and the amplicon was inserted into the KpnI site of the pAX vector. The C-terminal glycine residues at the junction were initially mutated to alanine to prevent ubiquitin removal by isopeptidases (Ub-Rbf1-ΔIE, Fig. 3-6D) and then to isoleucine (Ub-Rbf1, Figs. 3-6B, 3-6C) to provide a more complete block to cleavage.

Luciferase Reporter Assay

Drosophila S2 cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Typically, 1.5 million cells were transfected with 100 ng of Ac5C2T50-Luciferase reporter, 0.25 μg of pRL-CMV Renilla luciferase reporter (Promega) and 20 ng of one of pAX-Tet-rbf1 constructs. For PCNA-luciferase assay, 1.5 million cells were transfected with 1 μg of PCNA-Luciferase reporter, 250 ng of pRL-CMV Renilla luciferase reporter (Promega) and 200 ng of pAX Rbf1-WT, pAX Rbf1-ΔIE, or pAX-Ub-Rbf1-ΔIE constructs. 1000 ng of pAX-Ub-Rbf1-WT and 3 ng of pAX Rbf1-WT was used in Fig. 3-6B. Cells were harvested 3 days after transfection and luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega) and quantified using

the Veritas microplate luminometer (Turner Biosystems). Firefly luciferase activity was normalized to Renilla luciferase activity except when analyzing Rbf1 activity on the InR promoter. For doxycycline treatment (1µg/ml), the drug was added to the media immediately after transfection.

Western Blot Analysis

To measure protein levels in S2 cell culture, cells were harvested 3 or 5 days after transfection and lysed by freeze-and-thaw cycles three times in lysis buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 1% Triton X-100). Typically, 50 µg S2 cell lysates were separated by 12.5% SDS-PAGE, transferred to PVDF membrane for analysis using M2 anti-Flag (mouse monoclonal, 1:10,000, Sigma, F3165), anti-GFP (mouse monoclonal, 1:1,000, Santa Cruz Biotechnology, sc-9996) and anti-tubulin (mouse monoclonal, 1:20,000, Iowa Hybridoma Bank). Antibody incubation was performed in TBST (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) with 5% non-fat dry milk. Blots were developed using HRP-conjugated secondary antibodies (Pierce) and SuperSignal West Pico chemiluminescent substrate (Pierce).

Stability Assays

For determination of GFP fusion protein half-life, 1.5 million S2 cells were transfected with 200 ng of pAX-GFP-Rbf1-IE or 400ng of pAX-GFP. After 3-day incubation, cells were treated with 100 µM cycloheximide for the indicated times. For proteasome inhibitor treatments in Figs. 3-2B and 3-6A, seventy-two hours post-transfection, cells were treated with DMSO or DMSO containing 50 µg/ml MG132 (Sigma-Aldrich) for 2 hours.

In vivo Ubiquitination Assay

In experiments shown in Fig. 3-2A and 3-2B, S2 cells were co-transfected with 250 ng of pAX Rbf1-WT, 250 ng of pAcGal4 and 250 ng of UAS-Ub constructs using Effectene transfection reagent (Qiagen, Valencia, CA). In Fig. 3-3A, cells were transfected with 50 ng

pAX Rbf1-WT or pAX Rbf1-ΔIE, 50 ng of pAcGal4 and 50 ng of UAS-Ub constructs. In Fig. 3-3B, cells were transfected with 200 ng of Rbf1 WT, 400 ng of pAX-GFP-flag and 200 ng of pAX GFP-Rbf1-IE constructs. In all cases, cells were grown for 3 days after which extracts were prepared using SDS lysis buffer (2% SDS, 150mM NaCl, 10mM Tris-HCl, pH 8.0). The extracts were heat denatured and sonicated followed by a 10-fold dilution using dilution buffer (10mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100). Flag immunoprecipitation reactions were performed (Anti-Flag M2 affinity gel, Sigma) followed by anti-HA Western blotting (mouse polyclonal, 1:5000 dilution).

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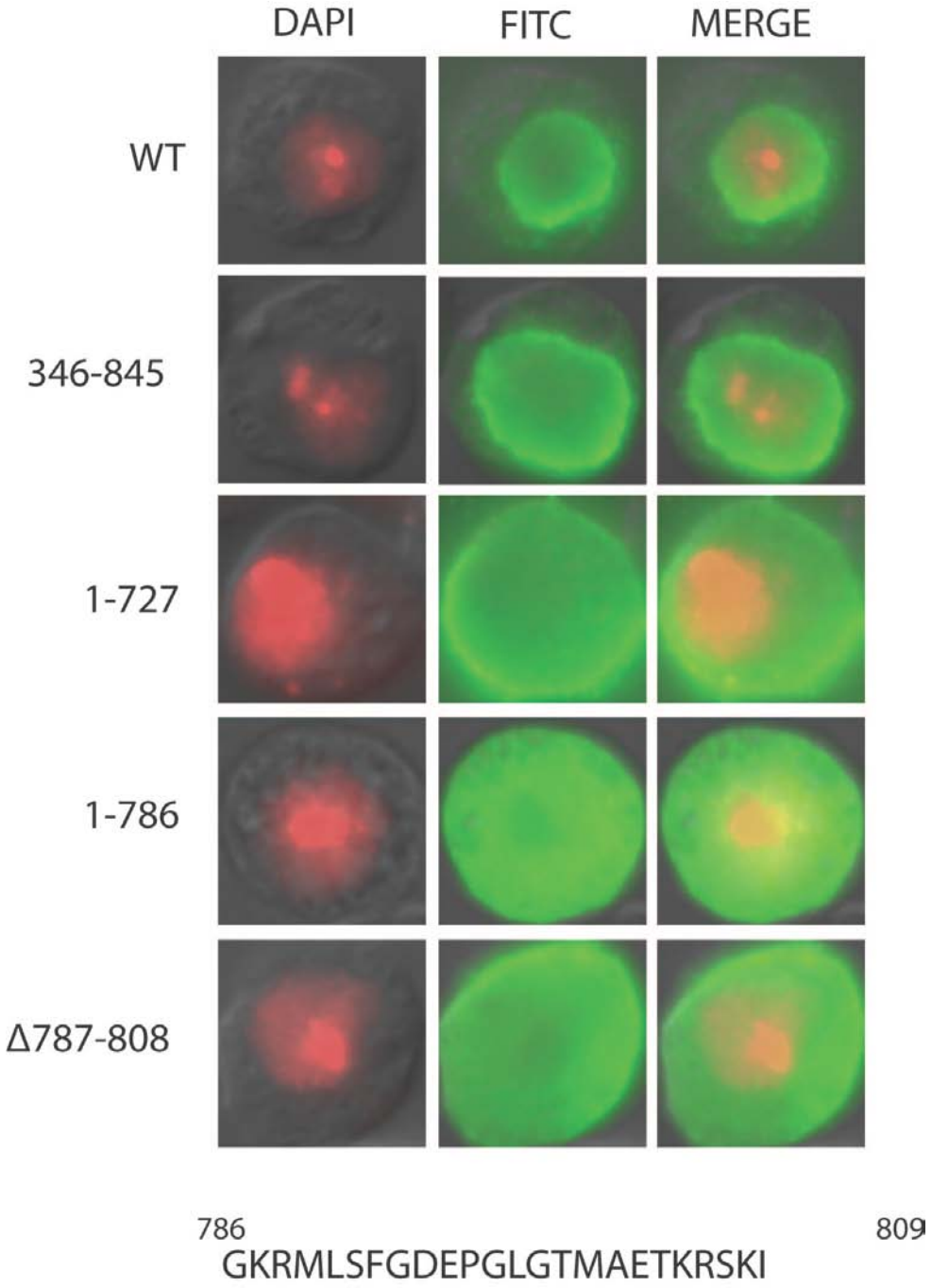
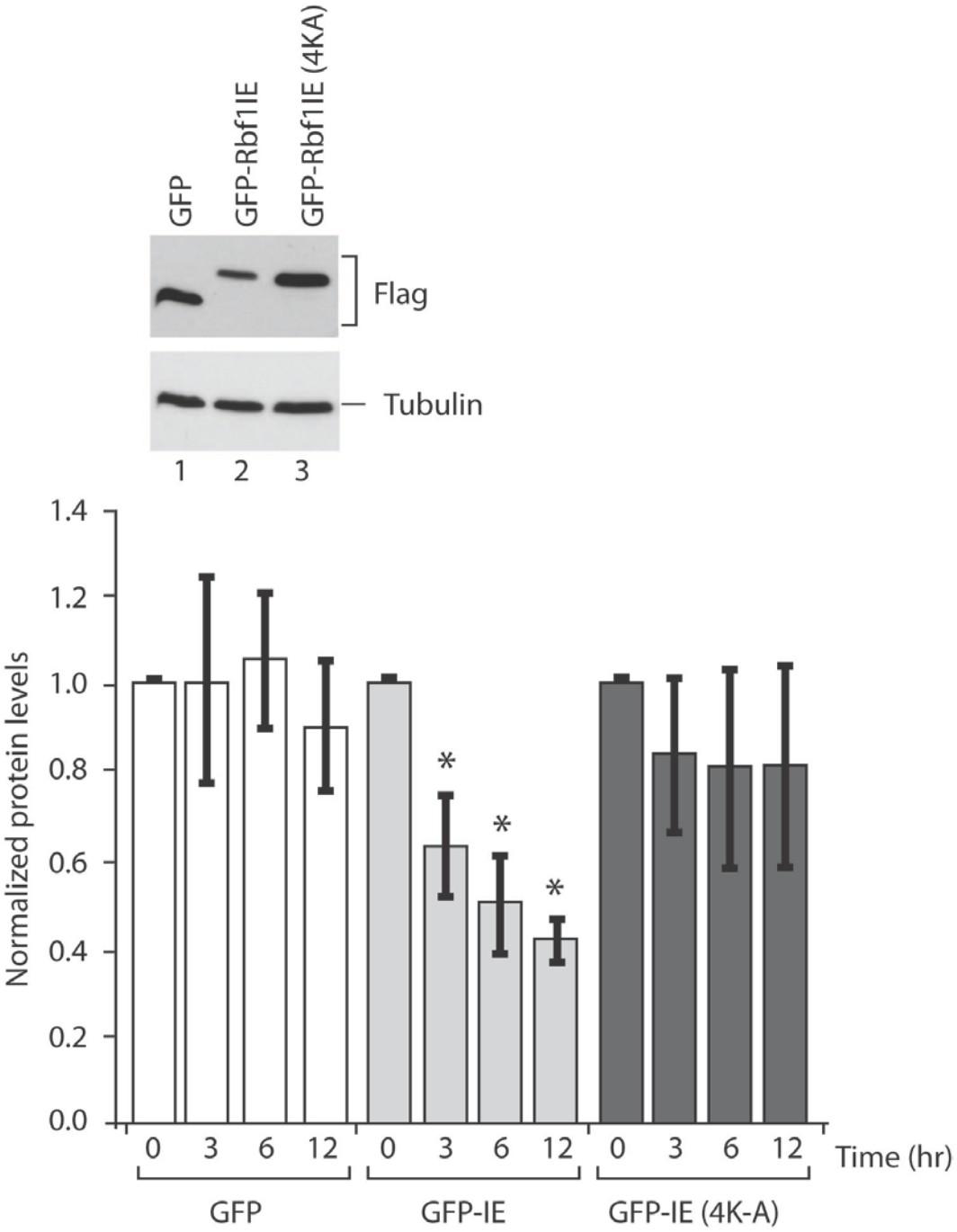
APPENDIX

Figure 3-S1. Identification of the Rbf1 nuclear localization sequence (NLS).



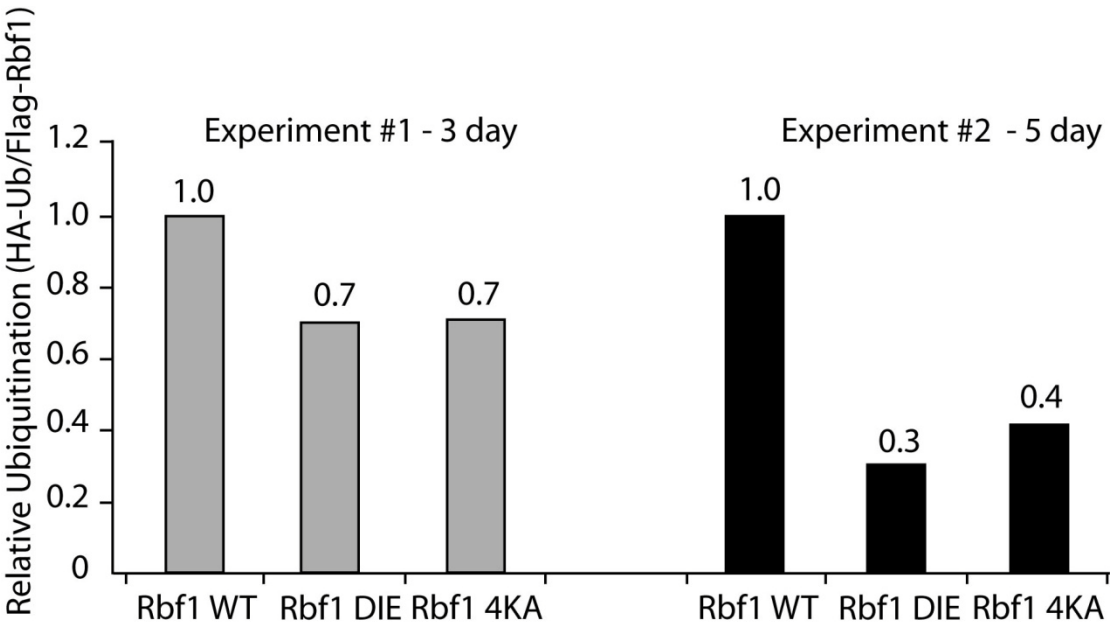
The indicated Rbf1 proteins were expressed in *Drosophila* S2 cells for subcellular localization assessment by immunostaining (FITC). DNA within the nucleus was measured by DAPI staining. The amino acids required for nuclear localization are contained within 787-808 of the C-terminus of Rbf1 - key residues are indicated in bold (bottom).

Figure 3-S2. The Rbf1 C-terminal lysines (K732, K739, K740 and K754) contribute to degron function in GFP degradation.



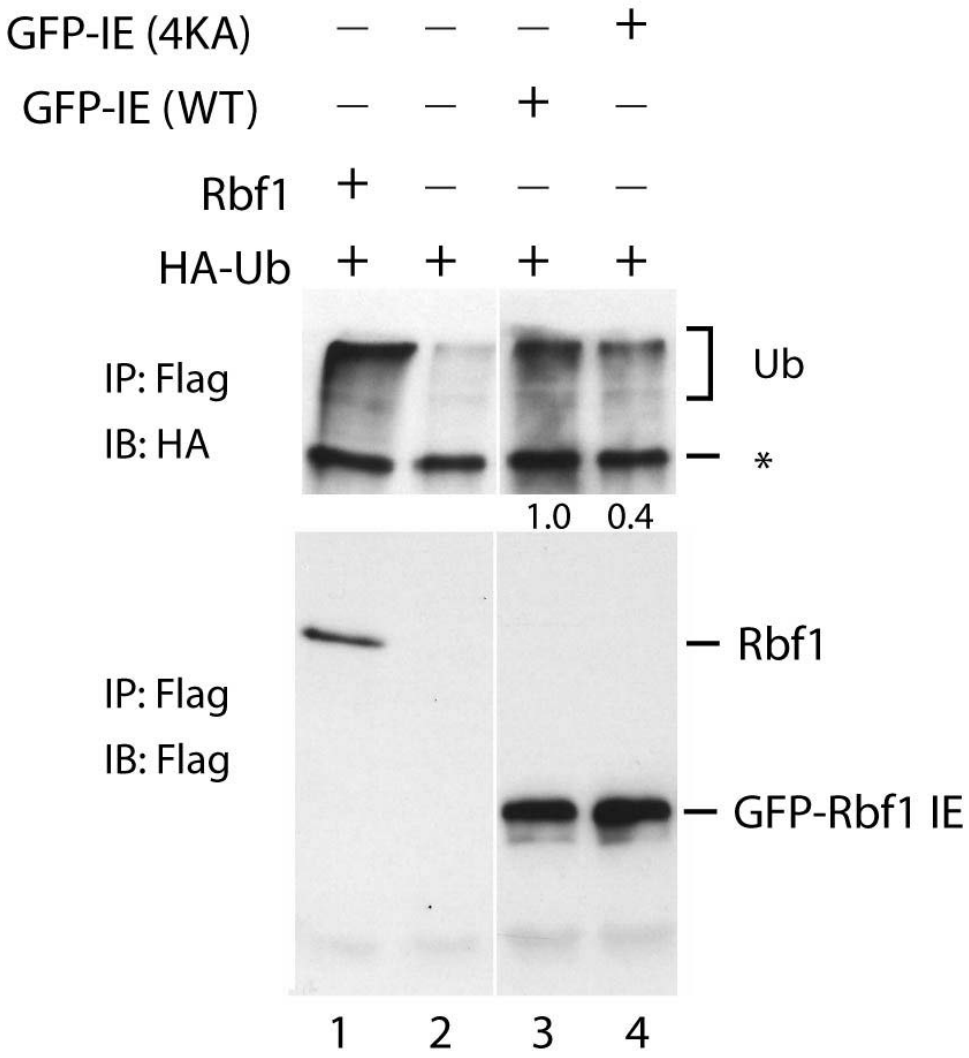
Steady state levels of GFP fusion proteins were measured after cycloheximide treatment for the indicated times. Lysine to alanine substitutions (GFP-IE 4K-A) in the IE resulted in a significant extension of protein half-life compared to the GFP-IE protein. Error bars indicate standard deviation, and asterisks indicate $p<0.05$.

Figure 3-S3. Lysine residues within the Rbf1 C-terminal degron influence Rbf1 ubiquitination.



S2 cells were transfected with HA-tagged ubiquitin and the indicated Flag-tagged Rbf1 expression constructs. After 3 or 5 days, denatured protein extracts were prepared for Flag immunoprecipitation (IP). Recovered samples were assayed by anti-HA Western blot analysis to detect ubiquitinated species and anti-Flag analysis to estimate recovery of Rbf1 proteins. The amounts of each species were quantified and the ratio of ubiquitin to Rbf1 was calculated. In each experiment, the ratio for wild type Rbf1 was set to 1.0.

Figure 3-S4. Lysine residues within the Rbf1 C-terminal degron participate in enhanced GFP ubiquitination.



GFP-IE and GFP-IE 4K-A were compared for ubiquitination as performed in Figure 3B. Under conditions wherein expression levels of GFP-IE and GFP-IE (4KA) were comparable, the presence of the 4K-A substitutions decreased ubiquitination as compared to the ubiquitination levels observed for GFP-IE, as measured by co-transfection and Co-IP/Western analysis.

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CHAPTER 4

Rbf1 degron dysfunction enhances cellular DNA replication

Abstract

The E2F family of transcription factors contributes to oncogenesis through activation of multiple genes involved in cellular proliferation, a process that is opposed by the Retinoblastoma tumor suppressor protein (RB). RB also increases E2F1 stability by inhibiting its proteasome-mediated degradation, but the consequences of this post-translational regulation of E2F1 remain unknown. To better understand the mechanism of E2F stabilization and its physiological relevance, we examined the streamlined Rbf1-dE2F1 network in *Drosophila*. During embryonic development, Rbf1 is insulated from ubiquitin-mediated turnover by the COP9 signalosome, a multi-protein complex that modulates E3 ubiquitin ligase activity. Here, we report that the COP9 signalosome also protects the Cullin4-E3 ligase that is responsible for dE2F1 proteasome-mediated destruction. This dual role of the COP9 signalosome may serve to buffer E2F levels, enhancing its turnover via Cul4 protection and its stabilization through protection of Rbf1. We further show that Rbf1-mediated stabilization of dE2F1 and repression of dE2F1 cell-cycle target genes are distinct properties. Removal of an evolutionarily conserved Rbf1 C-terminal degron disabled Rbf1 repression without affecting dE2F1 stabilization. This mutant form of Rbf1 also enhanced G1-to-S phase progression when expressed in Rbf1-containing S2 embryonic cells, suggesting that such mutations may generate gain-of-function properties relevant to cellular transformation. Consistent with this idea,

several studies have identified mutations in the homologous C-terminal domains of RB and p130 in human cancer.

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Introduction

The RB/E2F regulatory nexus

The Retinoblastoma family of proteins consists of the RB, p107, and p130 members that control multiple processes associated with cellular proliferation, including cell cycle, differentiation, apoptosis, and cellular biosynthetic potential (Henley and Dick, 2012). Consistent with their regulatory governance of these processes, RB family members are frequently inactivated in human cancers (Lohmann, 2010; Lohmann and Gallie, 2004). In some diseases, such as retinoblastoma and small cell lung carcinoma, mutations in the RB1 gene itself are potentially causative for disease. In other cancer types, deregulation is accomplished through altered function of upstream regulatory factors, including the cyclin-dependent kinases (cdk) and cyclin/cdk inhibitors, with effects encompassing all RB family members (Wikenheiser-Brokamp, 2006b). Together, these genetic changes are so pervasive as to be recognized as a hallmark of cancer (Hanahan and Weinberg, 2000, 2011).

One important target for RB family members in gene regulation is the E2F family of transcription factors that like RB are tightly linked to growth control. In humans, at least eight different E2F species (E2F1-E2F8) have been identified, and are classified as either transcriptional activators (E2F1-3) or repressors (E2F4-8) based on their sequence homology and functional properties (Dimova and Dyson, 2005; van den Heuvel and Dyson, 2008). In *Drosophila*, these pathways are streamlined with two RB family proteins, Rbf1 and Rbf2, contributing to regulation of two E2F proteins, dE2F1 and dE2F2 (Stevaux et al., 2002). During G0 and early G1 of cell-cycle progression, RB family members directly bind

to different sets of E2F factors (Goodrich et al., 1991; Takahashi et al., 2000), and at least for E2F1, RB association reverses regulatory polarity from activation to repression (Flemington et al., 1993; Helin et al., 1993; Hiebert et al., 1992; Takahashi et al., 2000; Weintraub et al., 1995; Weintraub et al., 1992). Cyclin-cdk kinase mediated phosphorylation of RB in late G1 causes RB/E2F1 dissociation, allowing E2F to activate numerous proliferation genes that drive entry into S phase (Dynlacht et al., 1994; Ewen et al., 1993; Hinds et al., 1992; Kato et al., 1993; Nevins et al., 1991). In human cancer, increased E2F activity is frequently observed (Chen et al., 2009; Eymin et al., 2001; Imai et al., 2004; Saberwal et al., 2004), and is associated with poor prognosis, particularly in melanoma and breast cancer (Alla et al., 2010; Baldini et al., 2006; Hallett and Hassell, 2011; Vuaroqueaux et al., 2007), highlighting the importance of imposing regulatory curbs on E2F1 expression and activity.

The ubiquitin-proteasome system and RB/E2F regulation

In addition to limitation through cyclin/cdk-mediated phosphorylation, the RB/E2F axis is governed by the ubiquitin-proteasome system. Indeed, inappropriate RB turnover contributes to disease as demonstrated during cellular immortalization by viral proteins leading to enhanced RB ubiquitination (Boyer et al., 1996). Although RB levels often appear stable in actively proliferating cells (Classon and Harlow, 2002; Haberichter et al., 2007), steady state fluctuations have been correlated with phosphorylation changes during cellular stress (Tedesco et al., 2002), suggesting that a negative correlation exists between RB levels and its activity in certain contexts. For example, in response to nocodazole

blockade, U2OS osteosarcoma cells exhibit marked elevation of RB levels in the G2/M phase of the cell cycle, and upon release into early G1, RB destabilization reestablishes lower baseline steady state levels (not shown). In seminal experiments linking RB to cell cycle control, microinjected RB induced cellular G1 arrest only when introduced during the window of time immediately after nocodazole release and not when injected in asynchronously proliferating cells (Goodrich et al., 1991), suggesting that RB function is correlated with conditions in early G1 amenable to its diminishing steady state levels. An inverse relationship between steady state levels and repressor potency was also observed for the *Drosophila* Retinoblastoma family member Rbf1 wherein unstable Rbf1 proteins were potent for target gene repression while stable mutant proteins were impotent (Acharya et al., 2010). A tight activity-instability linkage may ensure that RB repression programs remain dynamic and sensitive to growth conditions, such as previously suggested for dynamic p53 fluctuation in response to DNA damage (Batchelor et al., 2011; Batchelor et al., 2008). Similar to RB, both p107 and p130 exhibit differential expression during the cell cycle with p107 levels peaking in S phase and p130 levels highest in G0 (Beijersbergen et al., 1995; Classon and Harlow, 2002; Tedesco et al., 2002), and thus multiple mechanisms likely influence turnover of these different RB family members. Interestingly, cyclin/cdk kinase activity is correlated with changes in RB family member levels (Beijersbergen et al., 1995; Tedesco et al., 2002), suggesting that the cyclin/cdk and ubiquitin/proteasome regulatory arms crosstalk to govern both RB family activity and stability.

In previous studies of the *Drosophila* embryo, we observed that the Rbf1 and Rbf2 proteins associate with the COP9 signalosome (Ullah et al., 2007), a developmentally

regulated complex that controls proteasome-mediated degradation of many proteins through interactions with SCF (SKP1/cullin/F-box) E3 ubiquitin ligase complexes (Wei et al., 2008). CSN5, the catalytic core of COP9, contains a metalloprotease motif termed JAMM (Jab1/MPN domain-associated metalloisopeptidase) that removes Nedd8 from the cullin subunits (Cope et al., 2002). As cullin neddylation activates E3 ligase activity, the COP9 signalosome thus serves to protect substrates from turnover. Indeed, both Rbf1 and Rbf2 are destabilized in the absence of COP9 function (Ullah et al., 2007), connecting the regulation of Rbf protein turnover to the ubiquitin proteasome system. The involvement of a specific ubiquitin ligase remains unknown, although in mammals, RB and p130 turnover has been linked to MDM2 (Sdek et al., 2005; Xiao et al., 1995) and SCF^{Skp2} (Tedesco et al., 2002), respectively. As with the RB family, E2F family members are degraded through ubiquitin-mediated turnover, both at defined points during the cell cycle (Peart et al., 2010) and in response to DNA damage (Blattner et al., 1999; Hofferer et al., 1999) with E2F1 subjected to ubiquitination via the S-phase specific F-box protein SCF^{Skp2} and degradation in the S/G2 phases of the cell cycle (Marti et al., 1999; Zhang et al., 2005). Other ubiquitin ligases including APC/C (Peart et al., 2010) and ROC-Cullin ligases (Ohta and Xiong, 2001) likely contribute to E2F1 degradation in these contexts. In contrast, a protective role is suggested for the MDM2 ubiquitin ligase (Zhang et al., 2005), and consistently, $p19^{ARF}$-mediated inhibition of MDM2 encourages E2F1 turnover (Chang et al., 2007; Martelli et al., 2001). Interestingly, a key determinant of E2F1 degradation turns out to be RB itself (Hateboer et al., 1996; Hofmann et al., 1996; Ikeda et al., 1996; Martelli and Livingston, 1999). RB can bind to a carboxy-terminal instability element in E2F1, and may

stabilize E2F1 by occluding the cellular ubiquitination machinery (Campanero and Flemington, 1997). In *Drosophila*, E2F1 destruction is mediated by the $Cul4^{Cdt2}$ E3-Ubiquitin ligase (Shibutani et al., 2008; Zielke et al., 2011), suggesting that both Rbf1 and COP9 may coordinately influence E2F1 stability. Herein, we show that dE2F1 levels are indeed influenced by Rbf1 and COP9, but through distinct mechanisms. While Rbf1 can stabilize dE2F1 through pocket-domain dependent protein-protein interactions, the COP9 signalosome complex down regulates dE2F1 levels through modulation of the Cul4 E3 ligase. Rbf1-mediated dE2F1 stabilization and repression activity are separate properties as select mutant Rbf1 forms lacking dE2F1 repression capability retained their capacity to stabilize dE2F1. We further show that this class of repression-inactive Rbf1 mutants enhanced the rate of S-phase entry perhaps through their inappropriate stimulation of dE2F1 levels.

Results & Discussion

The COP9 signalosome regulates the Rbf1/E2F1 pathway

To test whether loss of COP9 function is associated with destabilized E2F1, endogenous dE2F1 steady-state levels were examined in S2 embryonic cells that were depleted of the largest subunit of the COP9 complex, CSN1, using dsRNA. This treatment strongly reduced levels of transfected flag-tagged RBF1 and endogenous RBF2, as expected, while E2F1 levels were increased significantly (Fig. 4-1A). This result shows that the COP9 signalosome stabilizes Rbf proteins, as previously noted (Ullah et al., 2007), but instead of

protecting E2F1, the COP9 complex contributes to its turnover. Similar results were obtained during CSN4 and CSN5 knockdowns (not shown), suggesting that the COP9 complex rather than individual COP9 subunits contribute to E2F modulation. Previous studies showed that the COP9 signalosome can stabilize cullin E3 ligase-containing SCF complexes (Wu et al., 2005), and a Cul4-containing SCF complex contributes to dE2F1 ubiquitination in S2 cells(Shibutani et al., 2008). Therefore, Cul4 levels were examined in Csn1 knockdown cells, ascertaining that Cul4 was indeed diminished during COP9 knockdown. Direct knockdown of Cul4 but not Cul5 also led to increased levels of E2F1, consistent with previous reports (Shibutani et al., 2008). Thus, a pathway emerges wherein the Cul4 E3 ligase responsible for E2F1 turnover is stabilized by the COP9 signalosome (Fig. 4-1B). Direct modulation of Cul4 and Cul5 levels had no discernable effect on Rbf levels, indicating that Rbf1 and E2F1 are ubiquitinated through distinct pathways. We conclude that COP9 signalosome is associated with opposing roles for Rbf1 and E2F1, contributing to Rbf1 stabilization but E2F1 destabilization.

Drosophila Rbf1 enhances dE2F1 levels

The data presented above highlights that a complicated network governs E2F stability with the COP9 signalosome contributing to low E2F1 levels during normal function. Previous studies have shown that in humans, RB can stabilize E2F1 (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). We therefore examined dE2F1 levels using *Drosophila* S2 cells that harbor wild type COP9 function in the absence or presence of increased Rbf1 expression (Fig. 4-2A). Three days post-transfection, steady-state protein

levels of Myc-tagged dE2F1 were measured in the presence or absence of the MG132 proteasome inhibitor. Consistent with previous studies on mammalian E2F1, increased levels of *Drosophila* dE2F1 were observed during Rbf1 expression and at levels comparable to those observed with MG132 proteasome inhibition (Fig. 4-2A). Under the conditions selected for this experiment, Rbf1 was relatively stable and its levels largely unaffected by MG132 treatment, although under different growth conditions Rbf1 is proteasome sensitive (Acharya et al., 2010). These data indicate that dE2F1 is targeted by the ubiquitin-proteasome pathway and is responsive to the steady-state levels of Rbf1.

We next tested whether the direct binding of Rbf1 is required for dE2F1 stabilization. The conserved RB-family pocket domain is the primary site for E2F1 interaction (Lee et al., 2002; Rubin et al., 2005), therefore a deletion mutant of Rbf1 lacking this domain was tested. Unlike with wild type Rbf1, dE2F1 levels were unaffected by this mutant form of RBF1, while retaining responsiveness to proteasome inhibition. The pocket domain alone was sufficient to confer at least partial stabilization on dE2F1, suggesting that this domain is necessary but not sufficient for complete stabilization (Fig. 4-2B). These data are consistent with a model wherein dE2F1 is stabilized by direct contacts with Rbf1. Combined with our previous analysis of COP9 function, we conclude that the COP9 signalosome complex influences dE2F1 levels through two separate pathways, positively by stabilization of Rbf1, which binds dE2F1 to enhance cellular levels, and negatively by stabilizing the E3 ligase Cul4.

Our previous studies of Rbf1 indicated that an evolutionarily conserved C-terminal instability element (IE) functions as an autonomous degron that stimulates both Rbf1

ubiquitination and repression potency (Acharya et al., 2010; Raj et al., 2012b). RB family proteins require multiple domains to mediate gene repression, including the pocket domain that facilitates E2F interaction and co-factor recruitment (Chan et al., 2001b; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998), and the C-terminal region that harbors the IE (Acharya et al., 2010), and may provide additional dE2F1 contacts, as was shown for human RB and p107 (Rubin et al., 2005). Interestingly, Rbf1 degron deletion mutants retained their capacity to physically associate with dE2F1 at target gene promoters (Acharya et al., 2010). To determine whether Rbf1 repression activity and dE2F1 stabilization are biochemically separable, we generated a series of Rbf1 deletion constructs that were tested for both properties (Fig. 4-3A). Consistent with previous studies, transcription from the *PCNA*-promoter was activated by dE2F1, but was repressed upon co-expression with wild type Rbf1 (Fig. 4-3B). Also consistent, wild type Rbf1 (1-845) robustly stabilized dE2F1. Mutant forms of Rbf1 lacking the pocket domain (1-375 and 376-727) were inactive for both repression and dE2F1 stabilization, attesting to the importance of this domain for both these properties. Significantly, three different mutant forms of Rbf1 that lacked the IE entirely or had mutations in four key lysines were defective for repression, but continued to stabilize E2F1. Together, these data identify one class of Rbf1 mutations that disable repression without affecting dE2F1 stabilization, and another class that disables both repression and stabilization.

Figure 4-1. Dual roles of the COP9 signalosome in regulation of the Rbf1-dE2F1 network.

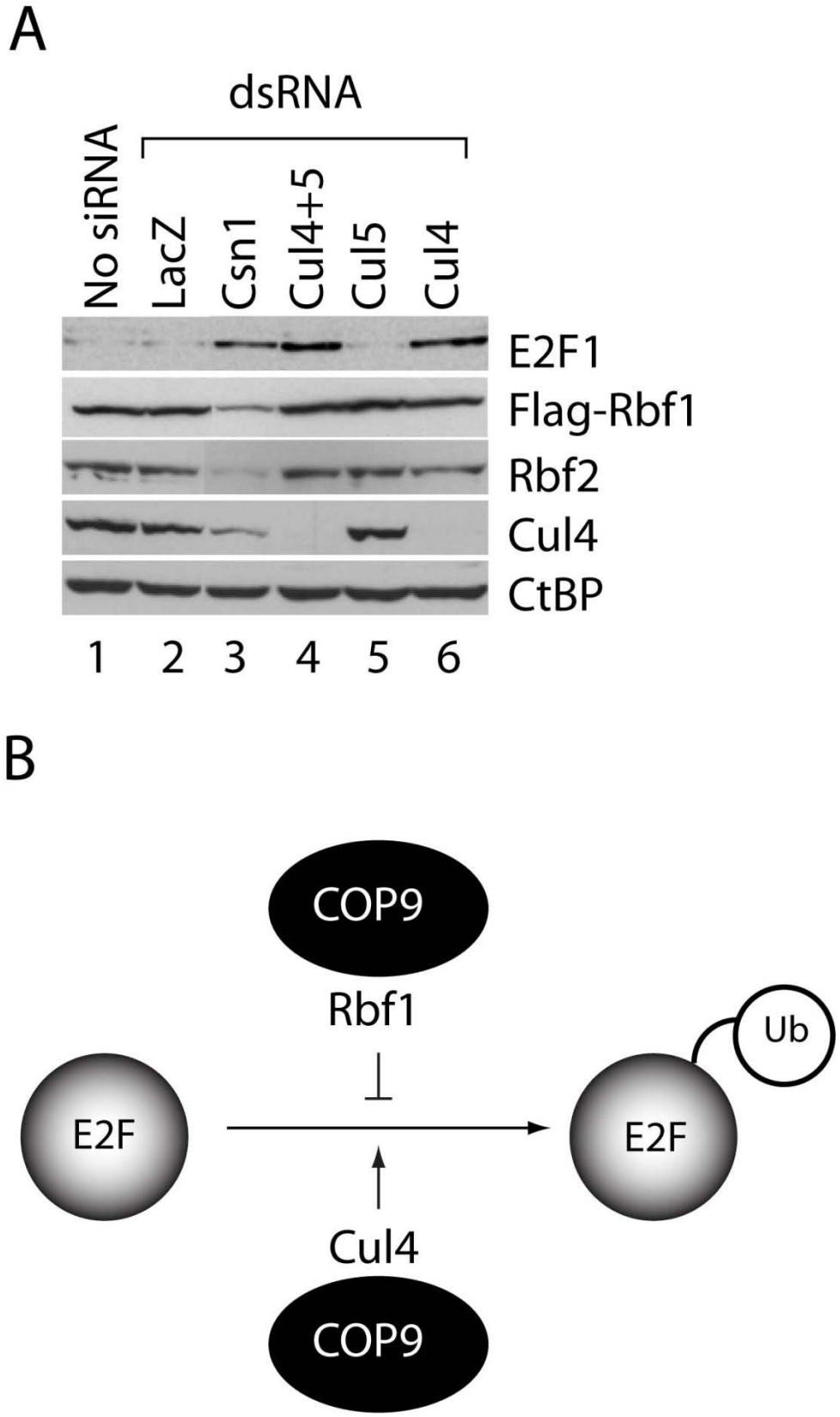

Figure 4-1 (cont'd)

(A) The COP9 signalosome complex governs both Rbf and E2F1 stability. S2 cells were treated with indicated dsRNA and proteins were measured by western blot analyses. Endogenous dE2F1 levels were dramatically increased due to reduced Cul4 levels when Cul4 (lane 4 and 6) or its upstream regulator COP9 (CSN1 subunit, lane 3) were depleted. Flag-Rbf1 and endogenous Rbf2 levels were substantially decreased by the CSN1 knockdown, but were not affected by Cullin knockdowns. (B) COP9 is a dual-functional regulator of dE2F1 stability. First, COP9 plays a protective role on Rbf1, which in turn stabilizes dE2F1. Second, COP9 restrains dE2F1 level by maintaining a Cul4-based E3 ligase, which targets dE2F1 for degradation.

Figure 4-2. The Rbf1 pocket domain contributes to dE2F1 protection from proteasome-mediated degradation.

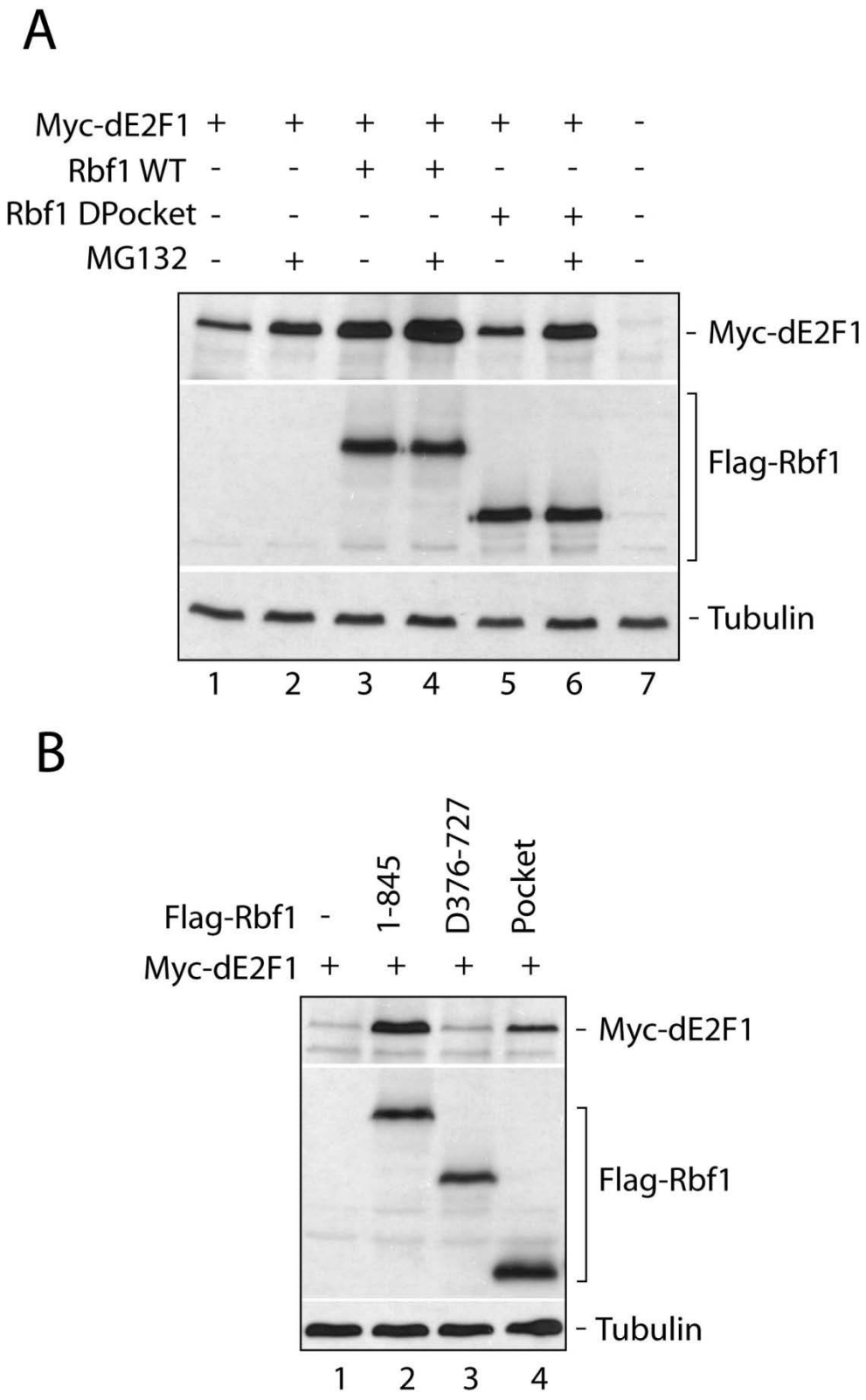

Figure 4-2 (cont'd)

(A) dE2F1 is sensitive to proteasome inhibition and is robustly stabilized by Rbf1 WT protein but not by forms of Rbf1 lacking the central pocket domain. Under these experimental conditions, Rbf1 WT and ΔPocket forms were expressed at equivalent levels and both are insensitive to proteasome inhibition. Endogenous tubulin levels are shown as loading controls. The experiment shown is representative of three biological replicates. (B) The Rbf1 pocket domain is insufficient for robust dE2F1 stabilization. The Rbf1 WT protein stabilizes dE2F1 protein, whereas at equivalent levels of expression, the Rbf1 pocket deletion mutant is incapable of stabilizing dE2F1 protein, while the Rbf1 pocket-only mutant provides only partial stabilization. Endogenous tubulin levels are shown as loading controls.

Figure 4-3. Mutant Rbf1 lacking IE function stabilizes dE2F1 but cannot fully repress its transcriptional activity.

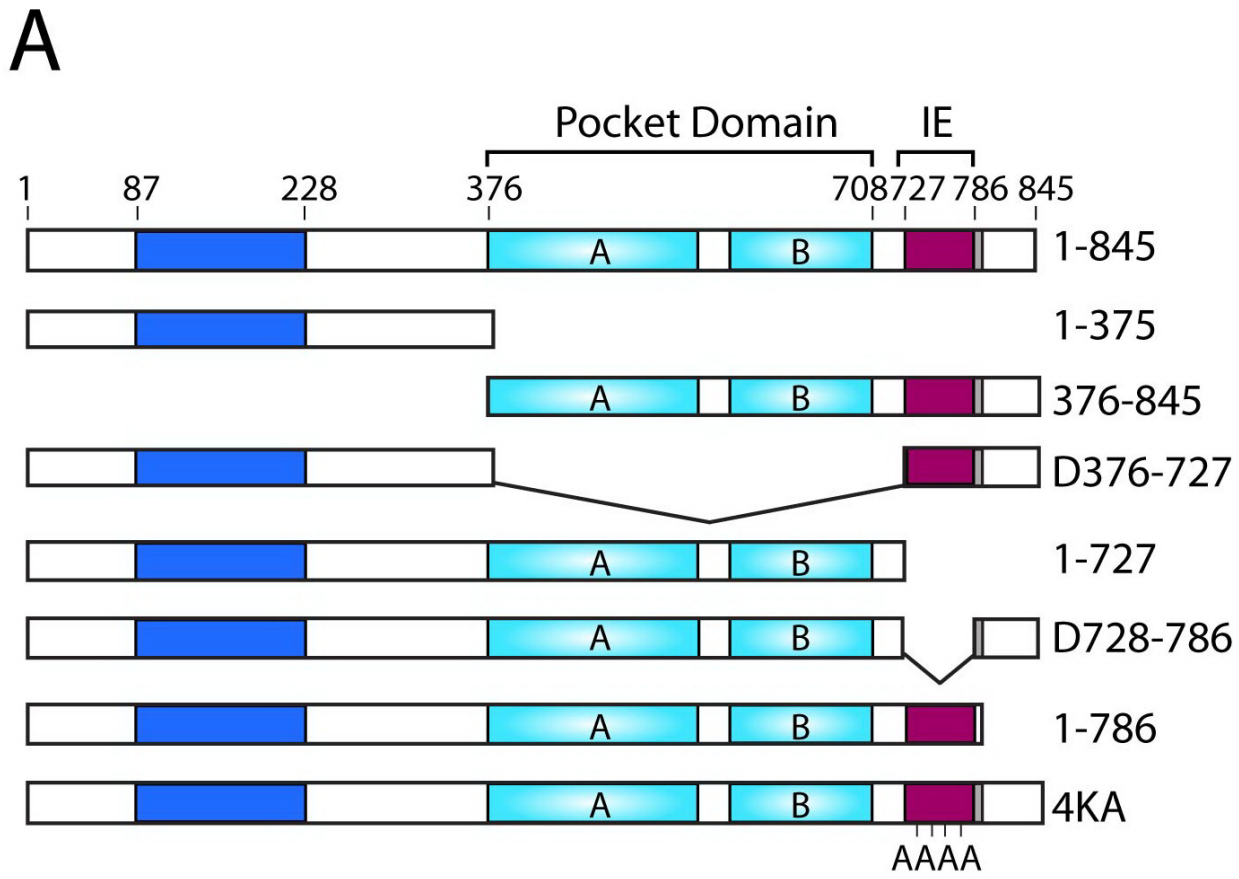

Figure 4-3 (cont'd)

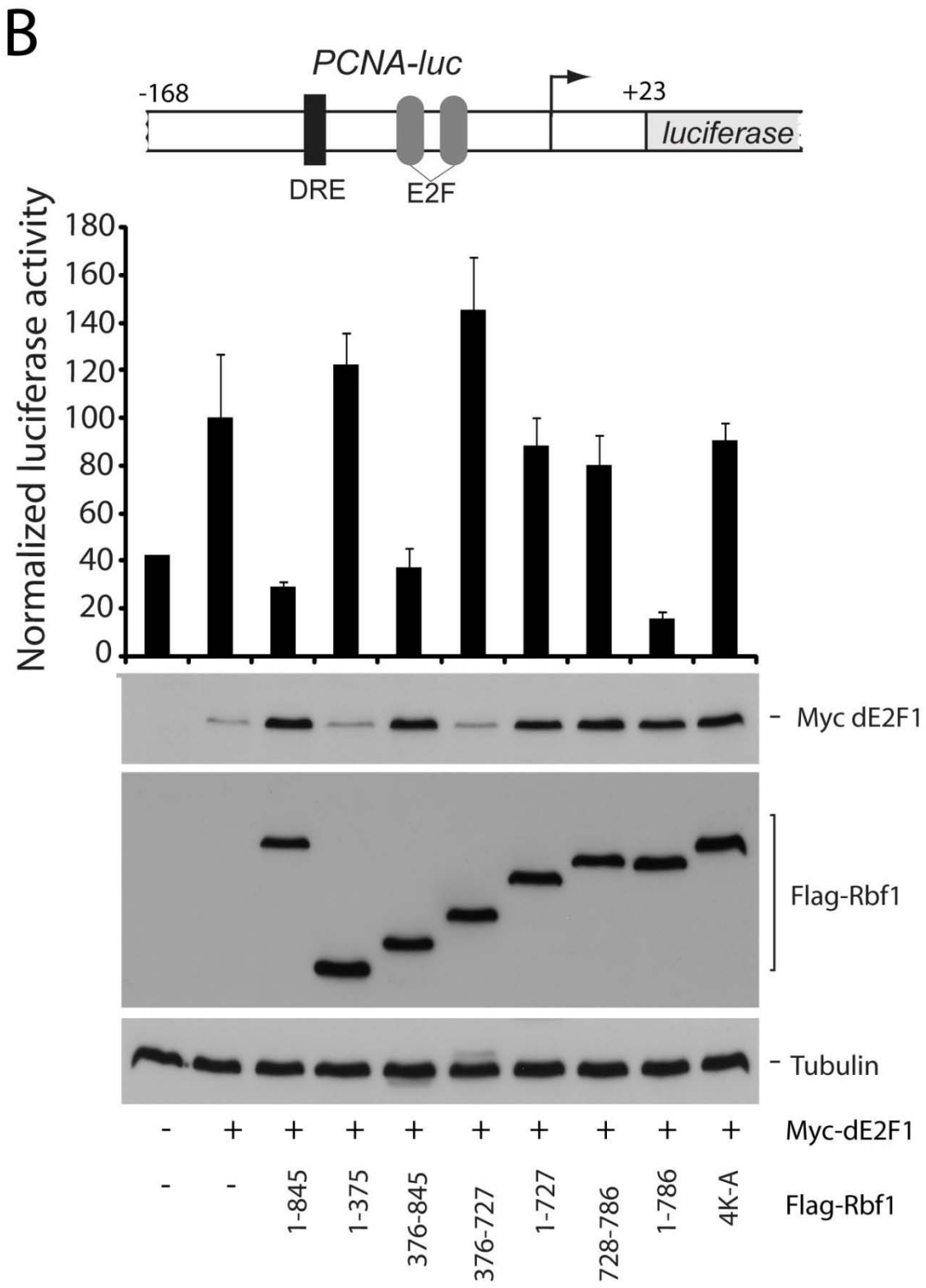

Figure 4-3 (cont'd)

(A) Schematic representation of Rbf1 proteins used for functional testing showing the relative positions of the pocket domain and the IE region. (B) Functional characterization of Rbf1. Mutations in the IE (728-786 and 4K-A) compromise transcriptional repression activities of Rbf1 proteins measured on the *PCNA*-luciferase reporter gene (bar graph) but do not affect dE2F1 stabilization property (anti-Myc Western blot). Under these transfection conditions, Rbf proteins were expressed at similar levels (anti-Flag Western blot). Data represents at least three biological replicates.

Rbf degron mutations enhance cellular S-phase entry

Previous studies have shown that elevated E2F levels are associated with increased cellular proliferation, and therefore we hypothesized that enhanced dE2F1 stabilization enabled by repression-incompetent Rbf1 would facilitate ectopic S phase and contribute to deregulated cell growth. To test this possibility, GFP-Rbf1 WT and the Rbf1 ΔIE mutant were expressed in S2 cells and the effect on cell cycle progression was examined by FACS analysis (Fig. 4-4). Consistent with the established role of Rbf1 in G1-to-S phase transition, GFP-Rbf1 WT induced a strong G1 arrest in the transfected cells (GFP positive) that was not observed in untransfected cells (GFP negative) from the same culture (not shown) or in cells expressing the GFP-Rbf1 ΔIE mutant. The lack of G1 arrest by GFP-Rbf1 ΔIE is consistent with a parallel lack of dE2F1 repression potency associated with IE loss. Interestingly, GFP-Rbf1 ΔIE-expressing cells also displayed a modest increase in their S-phase percentage, as estimated by Modfit analysis (Fig. 4-4, inset). Therefore, as a direct measure of the ability of this mutant form of Rbf1 to stimulate S-phase entry, we performed BrdU incorporation assays. In this assay, cells expressing wild type or mutant Rbf1 were visualized by anti-Flag epitope immunofluorescence, and cells undergoing *de novo* DNA synthesis were identified by BrdU staining (Fig. 4-5A). To assess the effect of transfected proteins on cell cycle, we calculated a proliferation index comparing the percentage of BrdU-positive cells in the transfected Rbf1-expressing population to the total population of cells. If a transfected protein exhibits no effect on cell cycle entry, the index should be equal to one, whereas the index should be less than one should the transfected protein cause cell cycle arrest. A protein that induces ectopic S phase entry should result in an index

greater than one. As shown in Fig. 4-5B, expression of Rbf1 lacking the pocket domain had no effect on DNA synthesis (P.I. = 1.03), consistent with observations that this protein is defective for repression and is unable to interact with and stabilize dE2F1. Therefore, in the context of S2 cells that express endogenous Rbf1, this defective Rbf1 protein had no effect. Cells expressing wild type Rbf1 experienced substantial cell cycle arrest (P.I. = 0.63), suggesting that repression-competent Rbf1 down regulated key genes required for S phase entry. Importantly, expression of the repression-defective Rbf1 ΔIE protein resulted in a substantial increase in the percentage of cells undergoing DNA replication (P.I. = 1.24), presumably through effects on dE2F1 stabilization. We conclude that expression of mutant Rbf1 harboring alterations to IE function confers a distinct growth advantage due to increased rate of S phase entry. In the model proposed in Fig. 4-5C, dE2F1 activity increases during early G1 concomitant with increases in its steady state levels mediated, in part, by either wild type or mutant Rbf1. However, loss of Rbf1-mediated repression associated with IE mutation permits premature S phase entry, a process that would normally be delayed in the presence of wild type Rbf1 until licensing by cyclin/cdk phosphorylation. Based on these observations, we hypothesized that mutations in the IE domain of human RB family members may be selected for in human cancers. Indeed, in one study of non-small lung carcinoma, a substantial percentage of patients (84%) were found to harbor mutations in the p130 IE region (Claudio et al., 2000). We previously showed that mutant Rbf1 harboring alanine substitutions of lysine residues within the IE diminish repressor potency but do not eliminate Rbf1-dE2F1 protein-protein interactions (Acharya et al., 2010). It is interesting to note that many of the p130 IE cancer-associated

mutations were observed in these conserved basic residues. Together, these studies suggest an unexpected role for RB family turnover in cellular proliferation and cancer.

Figure 4-4. The IE region contributes to Rbf1-mediated G1 arrest.

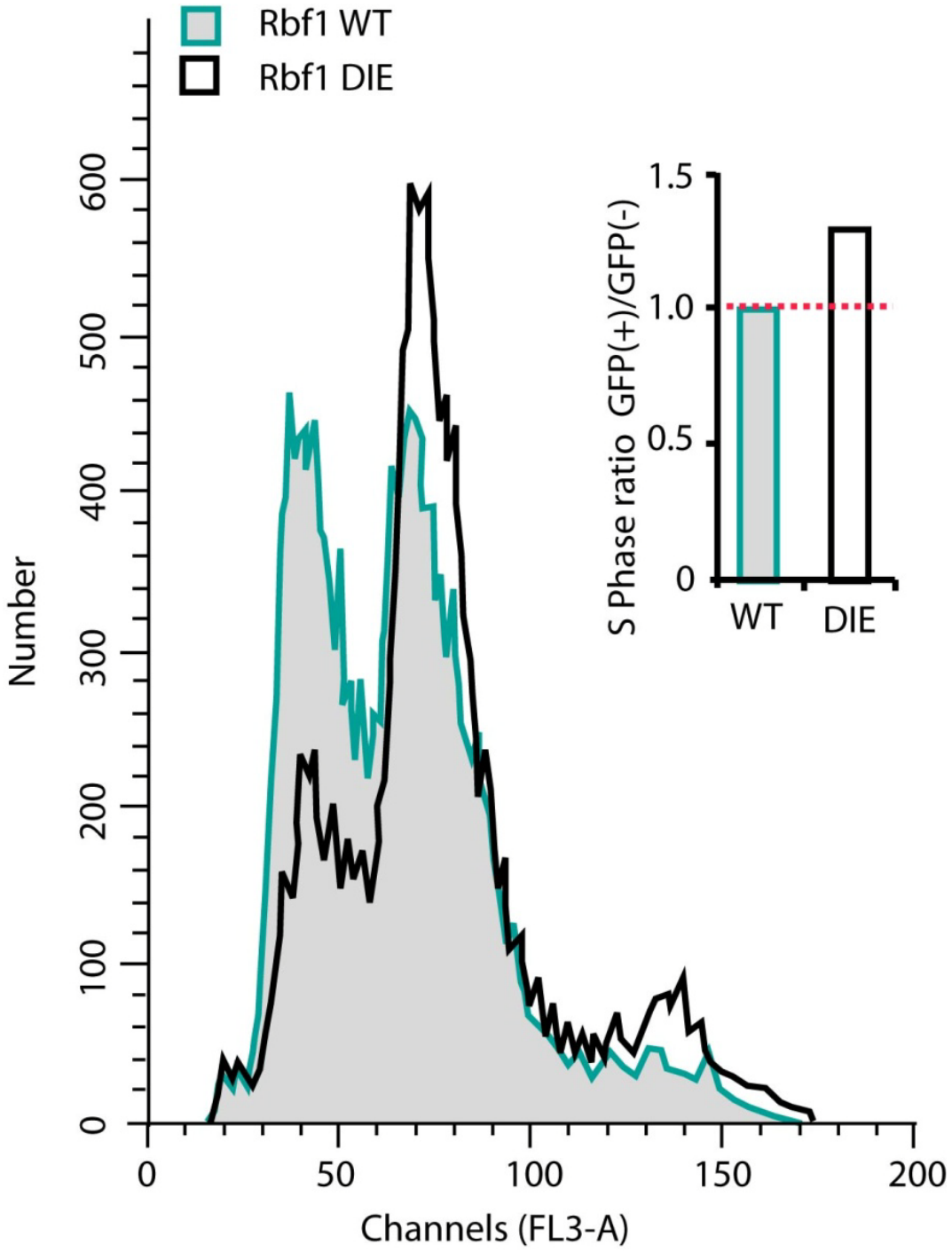

Figure 4-4 (cont'd)

Wild type or mutant GFP-Rbf1ΔIE proteins were expressed in Drosophila S2 cells and the effect on cell cycle was determined by propidium iodide staining and FACS analyses. An overlay of the DNA content histograms for wild type GFP-Rbf1 (grey) and mutant GFP-Rbf1ΔIE-expressing cells (solid unfilled) shows that the loss of IE function is correlated with a diminished proportion of cells in the G1 phase. (Inset) Bar graph shows the ratios of total S-phase percentages for GFP positive versus GFP negative populations for GFP-Rbf1 WT and GFP-Rbf1ΔIE transfected samples. In two separate experiments, GFP-Rbf1ΔIE expressing cells exhibited an increased proportion of cells in S phase, as estimated using Modfit analysis.

Figure 4-5. Mutation of the RB-family degron positively influences DNA replication frequency.

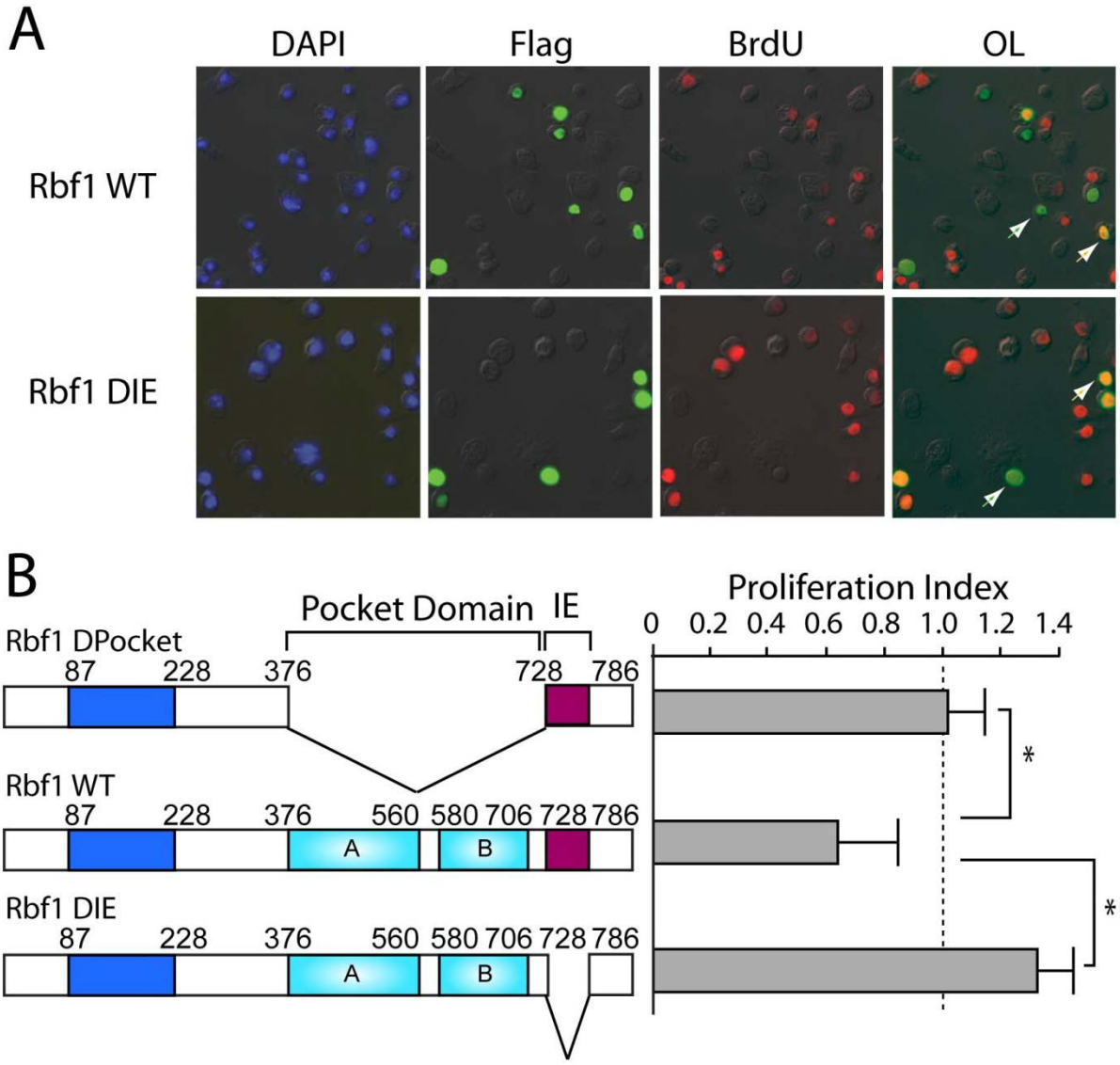

Figure 4-5 (cont'd)

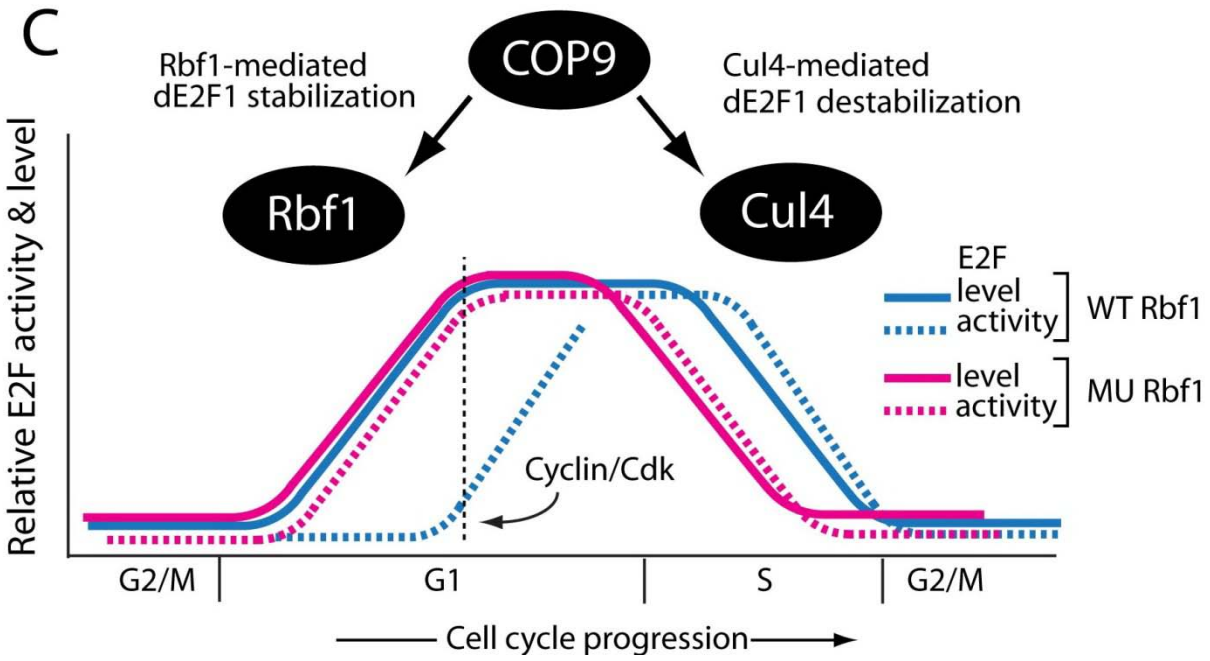

Figure 4-5 (cont'd)

(A) Rbf1 ΔIE enhances S-phase entry. S2 cells were transfected with the indicated Rbf1 proteins and the effect on DNA synthesis was monitored by BrdU incorporation. Transfected cells and BrdU-positive cells were visualized by immunofluorescent staining with anti-Flag and anti-BrdU antibodies. Arrows indicate representative cells that are transfected/BrdU negative (green) or transfected/BrdU positive (yellow). Rbf1 ΔIE expression was associated with increased BrdU positive staining compared to Rbf1 WT expressing cells. (B) The effect of Rbf1 proteins on DNA replication was indicated as a proliferation index calculated as a ratio of the percentage of BrdU-positive cells in transfected cells to that in the total population. The Rbf1ΔPocket mutant that is unable to both stabilize and repress dE2F1 also showed no effect on BrdU incorporation. Rbf1 WT expressing cells exhibited diminished BrdU incorporation consistent with increased G1 arrest, whereas Rbf1ΔIE expressing cells exhibited enhanced S phase. Data from three biological replicates were analyzed. Error bars indicate standard deviation, and asterisks indicate $p < 0.05$. (C) A model for the regulation of E2F function and stability during cell cycle progression. In this model, E2F levels are stabilized during early G1 by both wild type and mutant Rbf1 proteins. The COP9 signalosome contributes to enhanced dE2F1 steady state levels during this stage by stabilization of Rbf1. However, dE2F1 activity is not restrained by mutant Rbf1 leading to premature S phase entry. After Rbf1-dE2F1 estrangement mediated by cyclin cdk phosphorylation, the COP9 signalosome contributes to dE2F1 destruction via protection of Cul4.

Materials and Methods

Expression Constructs

Generation of Rbf1 WT and mutant expression constructs was described previously (Acharya et al., 2010). To generate GFP-Rbf1 fusion proteins, eGFP cDNA was amplified from phs-eGFP and inserted into *KpnI* site of pAX-Rbf1 WT and pAX-Rbf1 □728-786 vectors. The Myc-E2F1 expression construct was a gift from Dr. Maxim Frolov, Univ. of Chicago).

E2F1 Stabilization Assay

1.5 million *Drosophila* S2 cells were transfected with 0.2 μg of pAXRbf1 WT or mutants and 0.2 μg of pIE-E2F1 constructs using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. The cells were grown for 3 days after which protein levels were analyzed through Western blotting. For proteasome inhibition in Fig. 4-2, the cells were treated with 50 μg/ml MG132 (Sigma-Aldrich) or the vehicle DMSO for 2 hours.

Western Blot Analysis

To measure protein levels in *Drosophila* S2 cell culture, cells were harvested 3 days post-transfection and lysed by three freeze-thaw cycles in lysis buffer (50mM Tris-HCl pH8.0, 150mM NaCl, 1% Triton X-100, protease inhibitors). 50 μg of S2 cell lysates were run on 12.5% SDS-PAGE gels, transferred to a PVDF membrane, and probed with M2 anti-Flag (mouse monoclonal, 1:10,000, Sigma; F3165), anti-Myc (mouse monoclonal,

1:3000, Roche; 9E10), anti-tubulin (mouse monoclonal, 1:20,000, Iowa Hybridoma Bank), anti-Cul4 (1:1000, a gift from Dr. Robert Duronio), anti-E2f1 (1:1000, gift from Dr. Maki Asano), anti-Rbf2 (rabbit polyclonal, 1:5000, R2C1) and anti-CtBP (rabbit polyclonal, 1:5000, DNA208).

Luciferase Reporter Assay

Drosophila S2 cells were transfected using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Typically, 1.5 million cells were transfected with 1 µg of *PCNA*-Luciferase reporter, 0.25 µg of pRL-CMV Renilla luciferase reporter (Promega), 200 ng of pIE-E2F1 and 200 ng of one of pAXRbf1 constructs. Cells were harvested 72 hours after transfection and luciferase activity was measured using the Dual-Glo luciferase assay system (Promega) and quantified using the Veritas microplate luminometer (Turner Biosystems). Firefly luciferase activity was normalized to renilla luciferase activity.

Fluorescence-activated Cell Sorting and Cell Cycle Analysis

To analyze the effects of Rbf1 proteins on *Drosophila* S2 cell cycle, FACS analyses were performed using cells expressing GFP-tagged Rbf1 proteins. 1.5 million cells were transfected with 1 µg of pAXGFP-Rbf1 WT or pAXGFP-Rbf1 ΔIE constructs using the Effectene transfection reagent (Qiagen). Cells were harvested four days post-transfection and analyzed by flow cytometry to separate the GFP positive and GFP negative populations. Sorted cells were fixed with 70% ethanol and stained with propidium iodide (PI) for DNA content measurements using a BD Bioscience Vantage SE flow cytometer. The cell cycle data was analyzed through ModFit LT v3.3 (Verity Software House).

BrdU Incorporation Assay

Cell proliferation was assessed by examining bromodeoxyuridine (BrdU) incorporation 20 h after the addition of BrdU to the S2 cell cultures that were transiently transfected with the indicated pAXRbf1 expression constructs. 2 million S2 cells were plated on polylysine coated glass coverslips and transfected with 400ng pAX vector expressing Flag-tagged Rbf1 WT or mutants. Cells were incubated at 25 °C for one day and then incubated in medium with 100uM BrdU for 20 hours. Cells were washed once with PBS and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were permeabilized with 0.4% Triton X-100 for 10 min and blocked with 1% BSA for 1 hour. Cells were then incubated with rabbit polyclonal anti-Flag (1:250, F7425, Sigma-Aldrich) for 1 hour. After two washes with TBST (20 mM Tris pH 7.5, 120 mM NaCl, 0.1% Tween 20), cells were incubated with Alexa488-cojugated chicken anti-rabbit (1:500). For detection of BrdU, cells were fixed again in 4% paraformaldehyde for 30 min, treated with 2M HCl for 30 min and blocked with 1% BSA for 30 min. cells were then incubated with mouse monoclonal anti-BrdU (1:250, BD Pharmingen) for 1 hour. After two washes with TBST, cells were incubated with Alexa555-cojugated goat anti-mouse (1:500). Cover slips were mounted in Vectashield mounting medium containing 1.5ug/ml DAPI.

RNA Interference

Double-stranded RNAs were transcribed with MEGAscript T7 High Yield Transcription Kit (Ambion). S2 cells were maintained in Sf-900 II serum free medium (GIBCO) supplemented with 0.5% Penicillin-Streptomycin. 1.5 million cells were incubated with fresh medium containing 15 µg dsRNA for 30 min and then transfected with 200 ng

pAX-Rbf1. Cells were grown for 5 days at 25 °C.

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CHAPTER 5

Differential phosphorylation events govern stability-activity linkage of *Drosophila* retinoblastoma protein Rbf1

Abstract

The retinoblastoma (RB) family proteins are transcriptional corepressors that play diverse roles in many cellular events to exert potent tumor suppression functions. Precise regulation of activity and turnover of these proteins is effected through a variety of post-translational modifications. We identified an evolutionarily conserved C-terminal instability element (IE) in the *Drosophila* RB-related protein Rbf1 that simultaneously regulates degradation and repression activity. Surprisingly, stabilizing mutations in the IE are less, not more, active in repression, suggesting that instability is tightly linked to Rbf1 function. To better understand the control of this unique bifunctional element of Rbf1, we investigated the effects of phosphorylation by Cyclin-Cdk complexes. We show by directed *in vivo* phosphorylation of Rbf1 and mutagenesis of target sites that protein stability and activity are cleanly separable, suggesting that the multifunctional IE domain can act in parallel regulatory roles, possibly by coordinated interactions with E2F transcription factors and E3 ubiquitin ligases. A separate phosphorylation event in the N-terminus of the protein contributes independently to overall turnover control. Dramatic developmental phenotypes are observed in *Drosophila* eyes for all Rbf1 mutations that affect protein activity, independent of protein lability. Included in this class of mutants are those that affect K774, a conserved residue in RB family proteins mutated in human tumors. Our data suggests that

a phosphorylation code governs distinct functional outputs, which may have gene- or stage-specific implications in disease-related RB family mutations.

Introduction

The retinoblastoma protein RB, recognized as a key tumor suppressor, has a plethora of cellular roles in diverse pathways, including cell cycle control, induction of cellular differentiation, regulation of apoptosis and maintenance of genomic stability (Burkhart and Sage, 2008). Functional inactivation of RB in a broad range of human cancers contributes to both cancer initiation and progression, suggesting cell type-specific and tumor stage-specific functions of RB as a potent tumor suppressor. Consistent with diverse regulatory roles, RB is subject to tight controls through multiple levels of regulation mechanisms which are often severely disrupted under disease conditions (Chau and Wang, 2003).

Phosphorylation of RB, and related family members p107 and p130, is recognized as a pivotal mechanism which significantly influences RB functions under different cellular conditions including cell cycle regulation, apoptosis and DNA repair (Classon and Dyson, 2001; Munro et al., 2012). A canonical phosphorylation pathway of RB involves activity of cyclin-dependent kinases (Cdk) during cell cycle progression. Inactivation of RB during G1-S transition requires sequential phosphorylation events by different Cyclin-Cdk complexes (Mittnacht, 1998). Under conditions of DNA damage, RB is phosphorylated by p38 MAP kinase which is activated by a signaling cascade in response to stress stimuli (Delston et al., 2011). Additionally, phosphorylation of RB mediated by checkpoint kinases, Chk1 and Chk2, regulates RB function on apoptotic genes during DNA damage response (Inoue et al., 2007). These examples demonstrate that phosphorylation mediated by multiple kinases modulates RB functions involved in different pathways, generating

distinct molecular outputs.

Numerous phosphorylation sites have been mapped to the RB protein, supporting the idea that phosphorylation events throughout the protein participate in the regulation of RB functions (Adams, 2001). More importantly, differentially phosphorylated forms of RB controlled by different kinases have distinct regulatory functions. For example, Cyclin-Cdk complexes target overlapping as well as unique phosphorylation sites involved in different cell cycle stages (Zarkowska and Mittnacht, 1997). p38 phosphorylates RB at a specific site S567 to modulate RB repression function on apoptotic genes (Delston et al., 2011). S612 of RB is phosphorylated by checkpoint kinases in response to DNA damage to strengthen rather than alleviate RB anti-apoptotic activity (Inoue et al., 2007). Therefore, phosphorylation is not a unimodal modification for the diverse cellular functions of RB on its target genes. It has been proposed that the phosphorylation code in RB translates regulatory inputs into signals for recruitment of cofactors and modulation of gene activity (Munro et al., 2012).

A mechanism by which differential phosphorylation affects RB is that modification of specific sites causes intramolecular conformational changes, affecting interactions with RB binding partners. At least three key phosphorylation events induce structural changes in different domains of RB to inhibit RB binding to E2F family proteins (Rubin, 2013). The association between the pocket domain in RB and the transactivation domain (TD) in E2F is inhibited by phosphorylation of S608/S612 in the pocket domain (Burke et al., 2012). T373 phosphorylation induces the binding of RB N-terminus to the pocket domain to disrupt E2F binding (Burke et al., 2012). Phosphorylation of the C-terminal region in RB

has two major structural functions: inhibiting C-terminus binding to the marked box (MB) domain of E2F and inducing RB C-terminus binding to the pocket domain (Rubin et al., 2005). Additionally, phosphorylation also regulates association between RB and cofactors such as HDAC (Rubin et al., 2005). Conformational changes in RB caused by phosphorylation provide a platform on which different phosphorylation events can be translated into different functional consequences.

In addition to phosphorylation control of activity, RB is also subject to proteolysis through a proteasome-dependent pathway in the context of normal cellular growth. RB can be regulated by Mdm2, a well known E3 ubiquitin ligase for its regulation of the p53 tumor suppressor (Sdek et al., 2005; Uchida et al., 2005). Additionally, certain viral proteins can induce RB protein turnover to promote host cell proliferation, supporting the idea that control of RB stability is a key process in normal cell growth (Boyer et al., 1996; Stubdal et al., 1997). Interestingly, several studies of cell cycle regulation by RB family proteins show that RB protein levels fluctuate in corresponding to phosphorylation status, suggesting an intimately connected network between phosphorylation control of RB activity and stability (Buchkovich et al., 1989; Tedesco et al., 2002).

In *Drosophila,* the RB-E2F network and its upstream regulation pathways are well conserved in cellular events including cell cycle regulation and apoptosis, attesting the physiological importance of phosphorylation and proteolysis controls (van den Heuvel and Dyson, 2008). The *Drosophila* RB family protein Rbf1 is subject to the canonical phosphorylation mechanism mediated by Cyclin-Cdk (Xin et al., 2002). Additionally, Rbf1 protein level is controlled by a developmentally regulated turnover process which involves

the function of the COP9 signalosome, a conserved complex that controls E3 ligase activity and proteasome-dependent protein degradation (Ullah et al., 2007). Our previous studies indicate that a C-terminal instability element (IE) of Rbf1 influences its turnover rate and contributes to Rbf1 repression activity, suggesting a tight correlation between Rbf1 stability and activity (Acharya et al., 2010; Raj et al., 2012b). Post-translational modifications including ubiquitination and phosphorylation are likely to be involved to bridge potential crosstalk between different regulation pathways. However, the molecular details of the IE as a regulatory module in integrating protein turnover and transcriptional control are not well understood.

In this study, we show that N-terminal and C-terminal phosphorylation sites contribute to Cyclin-Cdk-mediated regulation of Rbf1 stability and activity, but functional importance of phosphorylation in different regulation pathways is not equally distributed among these phosphorylation sites, supporting the idea of the phosphorylation code translated into functional consequences. Interestingly, a key conserved lysine residue in the Rbf1 IE appears to influence phosphorylation effects; the homologous residue is found to be mutated in p130 in lung cancers. These studies demonstrate the functions of the IE as a nexus connecting regulatory power of phosphorylation involved in multiple pathways.

Results

The instability element (IE) harbors phosphorylation sites critical for controlling Rbf1 stability and activity

Drosophila Rbf1 protein is subject to a phosphorylation control by Cyclin-Cyclin-dependent kinase (Cyc-Cdk) during cell cycle progression, leading to cell cycle-dependent modulation of its transcriptional repression activity (Xin et al., 2002). Studies of mammalian RB family proteins suggest a tight correlation between their phosphorylation status, activity and stability (Tedesco et al., 2002). To understand the phosphorylation mechanism underlying the activity-stability connection, we first asked whether the IE mediates phosphorylation-induced stabilization. We first examined the function of exogenous Cyclin-Cdk complexes on wild-type Rbf1 in *Drosophila* S2 cells by cotransfecting CycD-Cdk4 or CycE-Cdk2 pairs with Flag-epitope tagged Rbf1 (Fig. 5-1A). The activity of Rbf1 under these conditions was assayed on a *PCNA*-luciferase reporter. As expected, Rbf1 was inactivated substantially by both of the Cyc-Cdk complexes, consistent with the known function of Cyc-Cdk-mediated phosphorylation on Rbf1. Intriguingly, the steady-state level of Rbf1 was increased under the condition of Cyc-Cdk overexpression (Fig. 5-1B). In addition to higher levels of the original Rbf1 protein, slower-migrating band was enhanced in the presence of overexpressed Cyc-Cdk, indicating a hyperphosphorylated species. The IE functions as an autonomous degron, destabilizing the levels of a heterologous GFP protein to which it is fused (Raj et al., 2012b); we did not observe stabilization of this chimera upon expression of Cyc-Cdk kinases, suggesting that the IE

alone is insufficient to mediate interaction of these enzymes (Fig. 5-S1).

The common effects on protein stability and activity suggested that the C-terminal IE, which is important for both of these effects, may be directly involved in the regulation by phosphorylation (Raj et al., 2012b). Structural studies of RB demonstrate that multiple phosphorylation events in the C-terminus can disrupt RB binding to E2F1, as well as induce a conformational change in RB (Rubin et al., 2005). We compared the sequence of Rbf1 IE with human RB family proteins and noted three highly conserved SP sites (S728, S760 and S771), which are potential phosphorylation targets by Cyc-Cdk (Fig. 5-2A). Previous studies have indicated that the sites in Rbf1 or RB family members can be phosphorylated *in vivo* (Canhoto et al., 2000; Cantin et al., 2008; Dephoure et al., 2008; Gauci et al., 2009; Leng et al., 2002; Mayya et al., 2009; Zhai et al., 2008). We examined whether these three serine residues are critical for Rbf1 stability by mutating them to either aspartates or alanines. The mutant Rbf1 harboring phosphomimetic Ser-to-Asp mutations accumulated to about twofold of the wild-type Rbf1 protein, whereas the mutant with unphosphorylatable Ser-to-Ala mutations was expressed at a lower level than the wild-type protein (Fig. 5-2B). These results indicate that the serine residues influence Rbf1 stability, possibly as targets of phosphorylation, providing a regulatable switch to turn up or down Rbf1 protein levels. Our previous study of the IE domain indicated that there was a close positive association between Rbf1 lability and activity, and earlier work has indicated that phosphorylation of RB family proteins downregulates their transcriptional activity (Acharya et al., 2010; Classon and Dyson, 2001; Mittnacht, 1998). We therefore hypothesized that the phosphomimetic Ser-to-Asp mutations in the Rbf1 IE would

inactivate Rbf1. We assayed the activity of the stabilized Rbf1 3SD and destabilized Rbf1 3SA mutants on the *PCNA*-luciferase reporter gene (Fig. 5-2C). Surprisingly, both mutants showed strong repression activity at levels similar to the wild-type Rbf1. As expected, the stabilized Rbf1 4KA, included as a negative control, was a much weaker repressor in this assay. Thus, the C-terminal phosphorylation sites that influence Rbf1 stability are not essential for repression activity in this context. One possibility is that other sites are important for phosphorylation-mediated regulation of Rbf1 activity, and these sites only affect stability. We tested this notion by overexpression of Cyc-Cdk, and noted that Rbf1 3SD and 3SA were less effectively inactivated than Rbf1 WT, suggesting that the serine residues within the IE are indeed important for the phosphorylation control of activity (Fig. 5-2D). The partial responsiveness of the Rbf1 mutants does indicate that additional phosphorylation sites may be involved.

The comparison between Rbf1 4KA and Rbf1 3SD enabled us to revisit our previous model that Rbf1 exhibits strong repression potency when it becomes labile and ready for degradation (Raj et al., 2012b). Both sets of mutations stabilize Rbf1, but they lead to distinct outcomes in repression activity; Lys-to-Ala mutations abolish Rbf1 activity whereas Ser-to-Asp mutations show little effect in the same context. This inconsistency with the model suggests that at least two distinct mechanisms for Rbf1 activity are utilized to govern regulations by different post-translational modifications.

Figure 5-1. *Drosophila* Rbf1 is subject to Cyc-Cdk-mediated inactivation and stabilization.

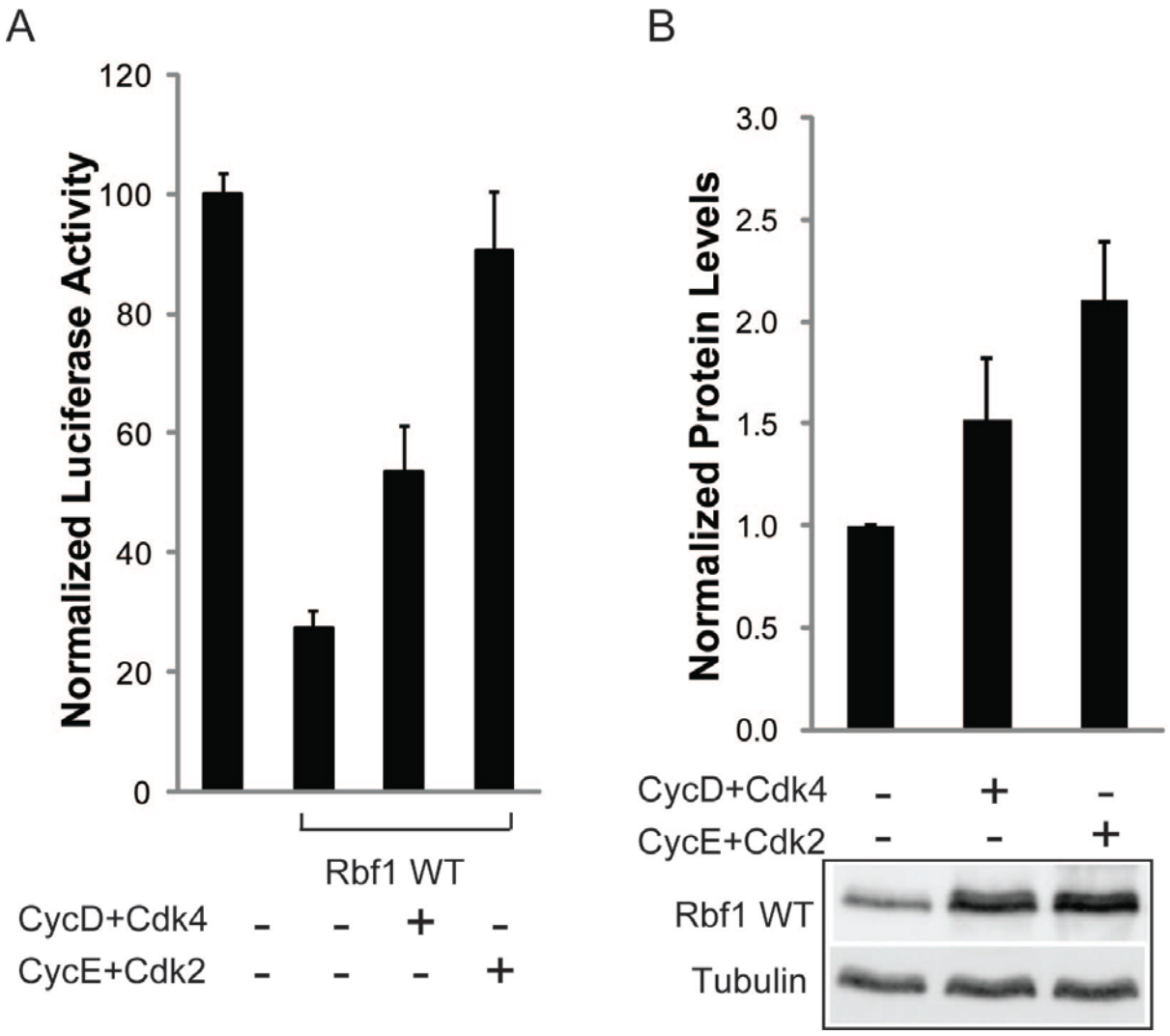

Figure 5-1 (cont'd)

(A) The wild-type Rbf1 was coexpressed with CycD-Cdk4 or CycE-Cdk2 in S2 cells and its repression activity was assayed on the *PCNA-luciferase* reporter gene. Rbf1-mediated repression is reduced by Cyc-Cdk. Bar graph shows average of four biological replicates. (B) The wild-type Rbf1 protein level with or without coexpression of Cyc-Cdk was assayed by western blotting using anti-Flag antibody in this and subsequent experiments. Rbf1 levels are increased in the presence of overexpressed Cyc-Cdk. Bar graph represents average of eight biological replicates. Protein levels were quantitated by photon-capture analysis with a Fuji LAS-3000 Imager and normalized to tubulin levels. In subsequent figures, data represent at least three biological replicates in all luciferase activity and western blot assays. Error bars indicate standard deviation.

Figure 5-2. Three serine residues in the IE are critical for Rbf1 protein stability and repression activity.

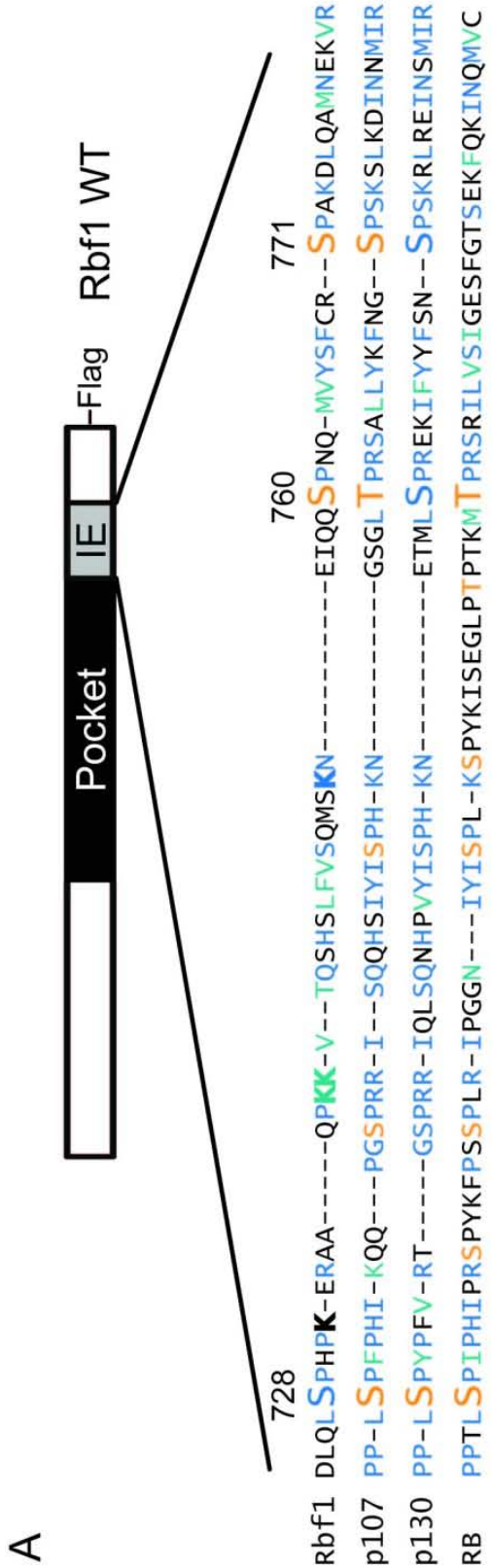

Figure 5-2 (cont'd)

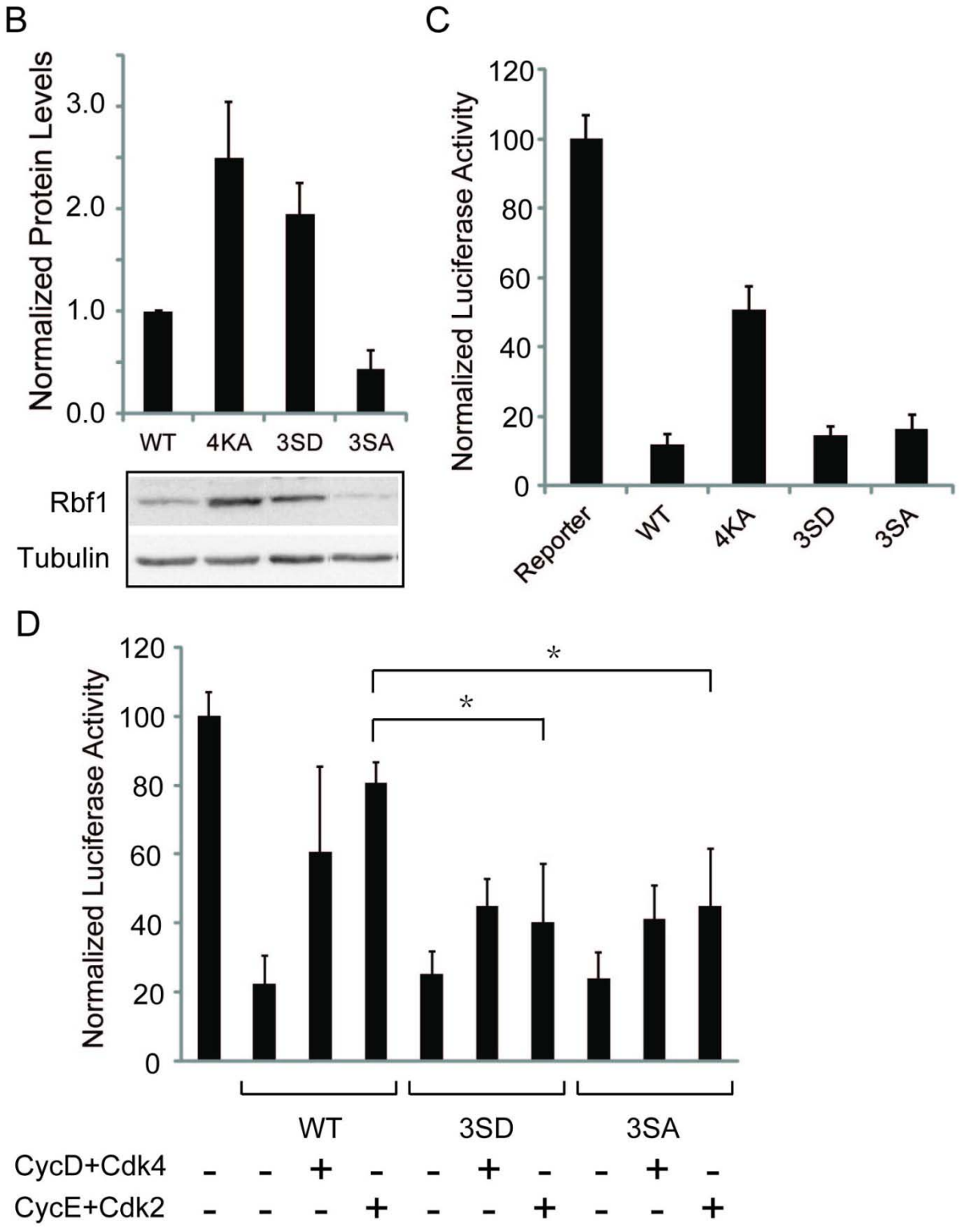

Figure 5-2 (cont'd)

(A) Schematic diagram of the wild-type Rbf1 and sequence alignment of the IE in Rbf1 and human RB family proteins. The three serine residues subject to mutagenesis are indicated by numbers (728, 760 and 771). Identical residues are colored in blue and similar residues are in aquamarine. Known phosphorylation sites are indicated in orange. The four lysine resides in the IE critical for protein stability are shown in boldface. (B) Western blot measuring expression levels of Rbf1 proteins, comparing wild-type (WT), a stabilized 4KA mutant, and Ser-to-Asp or Ser-to-Ala mutants. Phosphomimetic S-D mutations stabilize Rbf1, whereas unphosphorylatable S-A mutations lead to lower expression levels. (C) Rbf1 3SD and 3SA activities were assayed on the *PCNA-luciferase* reporter gene. Both mutants retain wild-type repression activity. The 4KA mutant exhibits low activity, as previously shown (Acharya et al., 2010). (D) Responsiveness of 3SD and 3SA to Cyc-Cdk overexpression was assayed on the *PCNA-luciferase* reporter gene. Rbf1 WT is inactivated by Cyc-Cdk, while 3SD and 3SA are partially resistant to Cyc-Cdk-mediated inactivation. Asterisks indicate $p < 0.01$.

The N-terminal T356 plays a dominant role in Cyc-Cdk-mediated stabilization of Rbf1

Studies of the N-terminus of RB show that phosphorylation of T373 induces a conformational change that allows the N-terminus to bind to the pocket region, blocking E2F association and relieving RB repression (Burke et al., 2012). In Rbf1, T356 is located in a conserved region homologous to that in which the RB T373 is found (Fig. 5-3A). We first assessed the role of T356 in Rbf1 stability by mutating this residue to either aspartate or alanine (Fig. 5-3B). Unlike the distinct effects of Ser-Ala and Ser-Asp mutations in the IE, both Thr-to-Asp and Thr-to-Ala destabilized Rbf1, suggesting that T356 also affects Rbf1 stability, and that both mutations may block phosphorylation to affect the steady-state levels. (T356D is apparently not phosphomimetic in this context). To test whether enhanced phosphorylation would further affect Rbf1 proteins in which T356 is mutated, we assayed Rbf1 response to overexpressed Cyc-Cdk with T356D alone or in combination with the three Ser-to-Asp mutations in the IE (Fig. 5-3C). Strikingly, mutants with T356D alone or all four mutations were resistant to Cyc-Cdk-mediated stabilization, consistent with a model that phosphorylation of T356 in Rbf1 stabilization plays a key step that may involve facilitating phosphorylation of other targets on Rbf1 (Fig. 5-3D). The weak residual response of Rbf1 T356D to Cyc-Cdk overexpression suggests that phosphorylation of additional sites may contribute to Rbf1 stabilization. We conclude that Cyc-Cdk stabilizes Rbf1 through two distinct regions containing phosphorylation sites; phosphorylation of T356 is predominant and may facilitate the downstream phosphorylation events.

Figure 5-3. N-terminal T356 is a key residue for the regulation of Rbf1 protein levels.

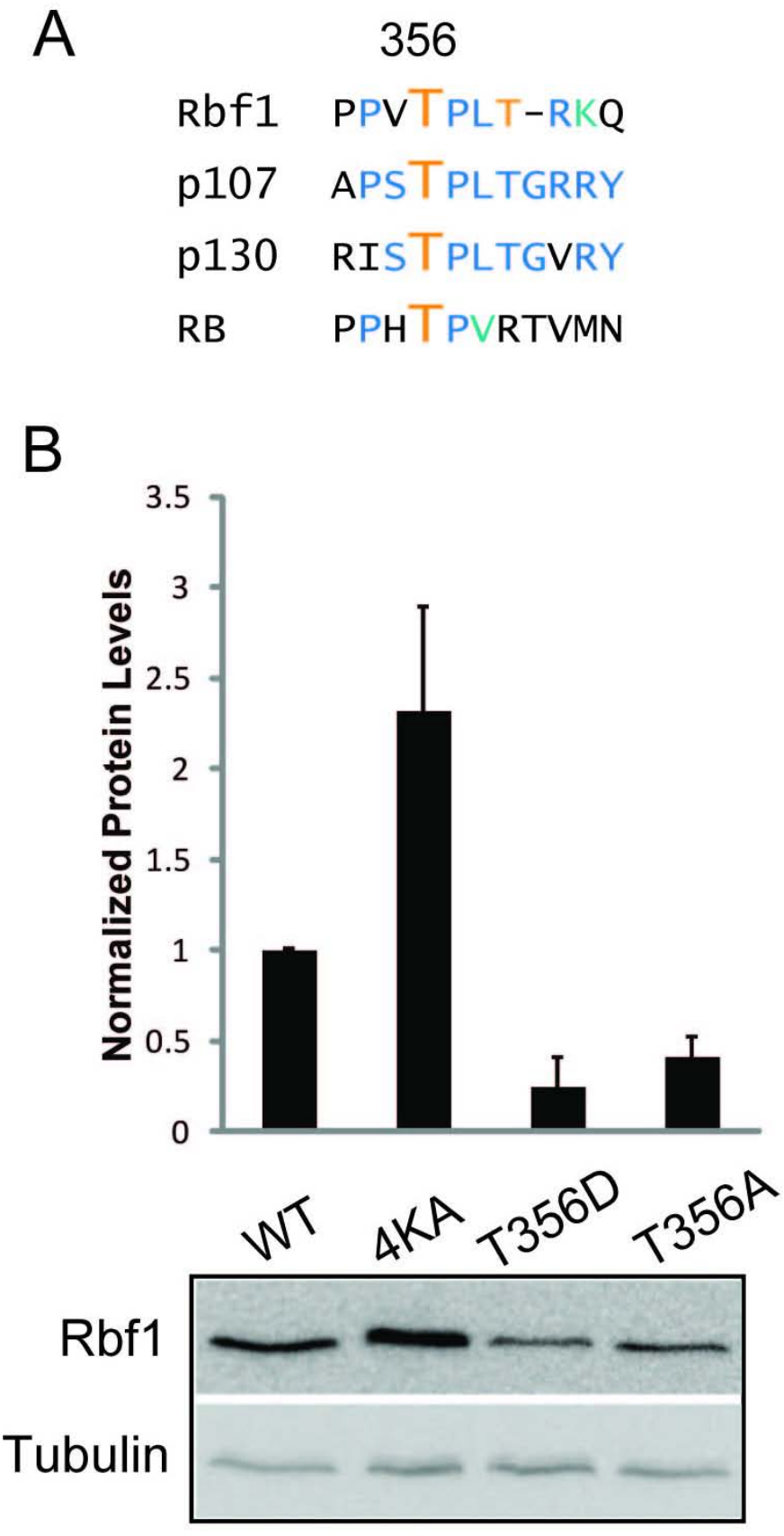

Figure 5-3 (cont'd)

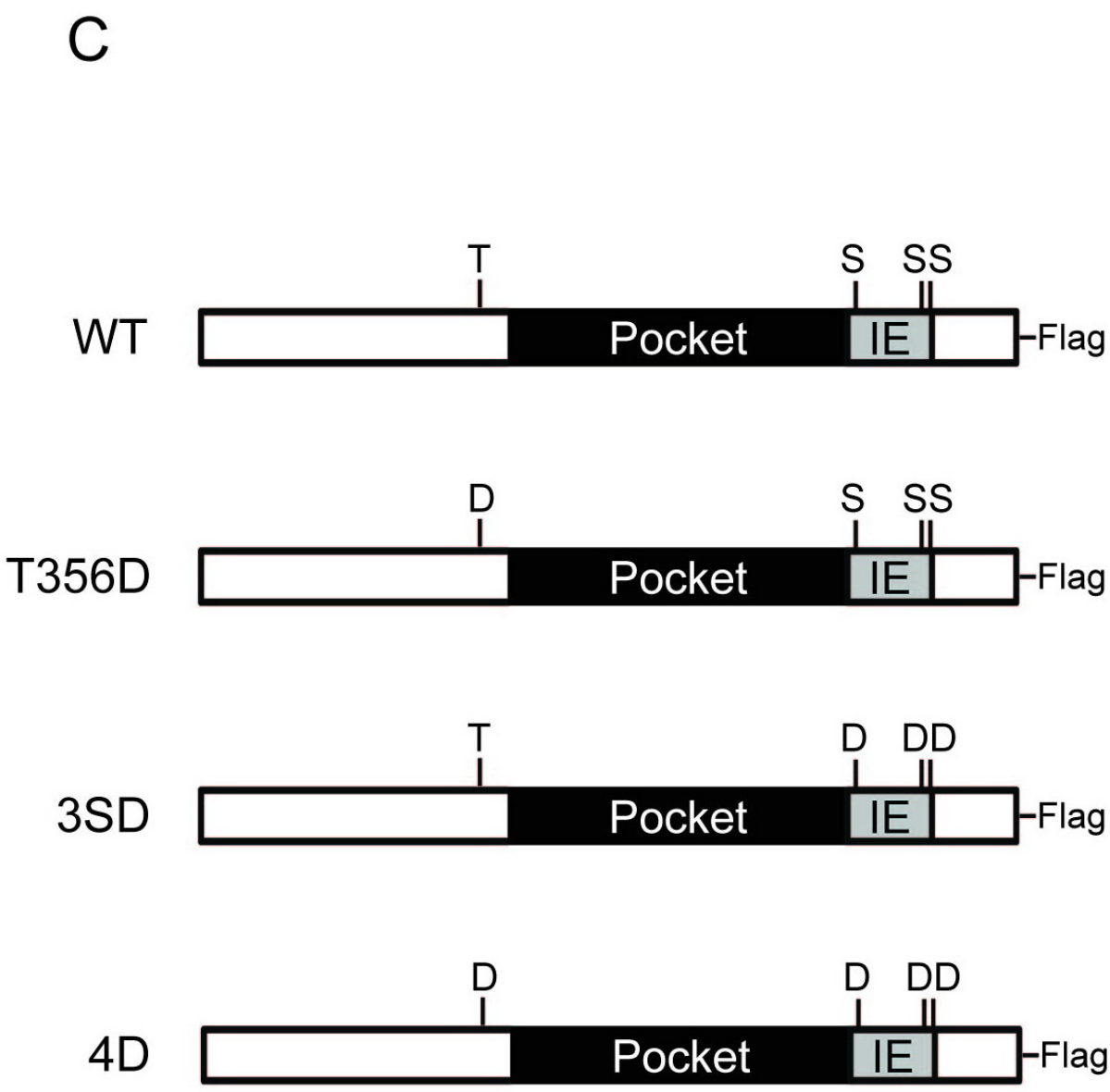

Figure 5-3 (cont'd)

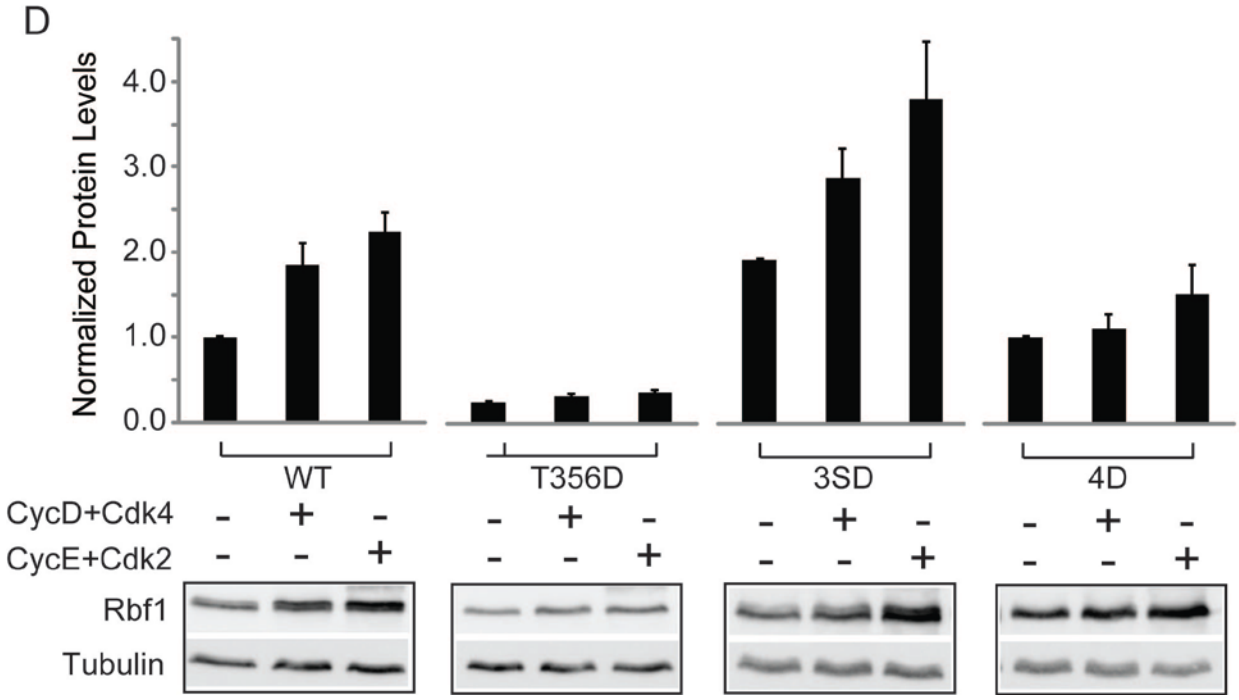

Figure 5-3 (cont'd)

(A) Protein sequence alignment of the N-terminal region harboring T356 in Rbf1 and human RB family proteins. The threonine residue is indicated by number 356. Identical residues are colored in blue and similar residues are in aquamarine. Known phosphorylation sites are indicated in orange. (B) Both mutants with Thr-to-Asp or Thr-to-Ala are destabilized in S2 cells, shown in western blot assays. (C) Schematic diagram of Rbf1 mutants with the N-terminal Thr-to-Asp mutation and the C-terminal Ser-to-Asp mutations. (D) Rbf1 WT and 3SD levels are increased by expression of Cyc-Cdk, whereas Rbf1 mutants with a Thr-to-Asp mutation (T356D and 4D) show much less responses. The difference between the fold increase of WT and T356D or WT and 4D under the condition of CycE-Cdk2 overexpression is significant ($p = 0.01$).

T356 is critical for Cyc-Cdk-mediated inactivation of Rbf1

Our ability to separate control of turnover and transcriptional activity with respect to the IE led us to ask whether the regulation mediated by T356 had similar or disparate effects on Rbf1 repression activity. We measured the transcriptional activity of Rbf1 T356D and Rbf1 4D in response to Cyc-Cdk overexpression (Fig. 5-4A). Strikingly, like the wild-type protein, the T356D was still highly responsive to Cyc-Cdk inactivation, whereas the Rbf1 mutant bearing all four mutations of phosphorylation sites was completely resistant to Cyc-Cdk inactivation. In this assay of transcriptional activity, the nature of the mutation at T356 was critical; unlike the similar effects of Thr-Ala and Thr-Asp on stability, Rbf1 T356A responsiveness to Cyc-Cdk was significantly reduced, while Rbf1 4A was completely resistant under these conditions (Fig. 5-4B). These results suggest that phosphorylation of T356 is critical to induce inactivation of Rbf1 and that all four phosphorylation sites are necessary for this process. In this case, the T356D mutation does appear to be phosphomimetic with respect to inactivation, although not with respect to stabilization. Phosphorylation of T356 plays distinct roles in the stability and activity pathways: it is a key event in phosphorylation-mediated stabilization but only contributes partially to phosphorylation-mediated inactivation, reflecting separable pathways.

Phosphorylation mediates Rbf1 stabilization independently of the lysine residues in the IE

Mutations of Ser-to-Asp and Lys-to-Ala within the IE both stabilize Rbf1, therefore we asked whether the two sets of residues contribute in an additive fashion to regulate Rbf1

degradation. We compared steady-state levels of proteins with 4KA (stabilizing) mutations to those with 4KA and 3SA (destabilizing) mutations. Both were overexpressed to a similar extent, raising the possibility that the lysines of the IE may regulate modifications of the adjacent serines (Fig. 5-5A, B). However, when *in vivo* phosphorylation was stimulated by overexpression of Cyc/Cdk kinases, the 4KA mutant was expressed at higher levels, but not the 4KA3SA mutant, indicating that modification of these serines is still possible, and can contribute to protein lability independently, (Fig. 5-5C). Together, these data suggest that lysines and serines are involved in distinct regulation mechanisms both of which influence Rbf1 stability in a nonredundant but cumulative manner.

Rbf1 responsiveness to Cyc-Cdk-mediated inactivation determines Rbf1 potency in Drosophila eye development

To assess the physiological importance of the phosphorylation sites under developmental settings, we expressed these mutant forms of Rbf1 in eye imaginal discs using an *eyeless*-Gal4 driver system (Fig. 5-6A). As shown in our previous studies, the wild-type Rbf1 induced mild and moderate eye phenotypes, and the mutant form of Rbf1 lacking the IE had no effect on eye development (Fig. 5-6B). The T356D mutant, which like the wild-type protein was inactivated by Cyc/Cdk in luciferase assays, induced slightly more severe phenotypes than wild-type Rbf1. In contrast, Rbf1 3SD and 4D mutants exhibited very severe phenotypes, including complete loss of the eye. These proteins were noted to be resistant to Cyc-Cdk inactivation in cell culture assays. Similarly, the T356A mutant caused severe and very severe phenotypes. Further mutation of serines in the IE did not appreciably alter this strong phenotype. Overall, the spectrum of eye phenotypes correlates

well with the responsiveness of each mutant protein to Cyc-Cdk-mediated inactivation, supporting the idea that Cyc-Cdk-mediated phosphorylation is a key mechanism to restrain Rbf1 activity under physiological conditions. In contrast, Rbf1 mutants that either showed enhanced stabilization (3SD) or destabilization (T356A) were equally active in this overexpression assay.

K774 in the IE is critical for response to Cyc-Cdk-mediated phosphorylation

The hypermorphic 3SD and 4D mutants phenocopy a previous identified mutant K774A, suggesting a potential link between the K774A mutation and effects of phosphorylation (Fig. 5-6B). Therefore, we tested whether K774 is important for response of Rbf1 to Cyc-Cdk overexpression. To test this hypothesis, we examined K774A mutant protein level and activity in response to Cyc-Cdk overexpression (Fig. 5-7A, B). Whereas the wild-type Rbf1 was stabilized and inactivated by Cyc-Cdk overexpression, the K774A mutant showed a muted response, suggesting that the K774A mutation reduces susceptibility of Rbf1 to Cyc-Cdk control. As shown in Figure 4 and Figure 5, N-terminal and IE phosphorylation sites play different roles in Cyc-Cdk mediated modulation of Rbf1 stability and activity. The phenotype exhibited by the K774A mutation indicates K774 might be critical for effects mediated by both N-terminal and IE residues.

Figure 5-4. T356 is critical for Cyc-Cdk-mediated inactivation of transcriptional repression activity.

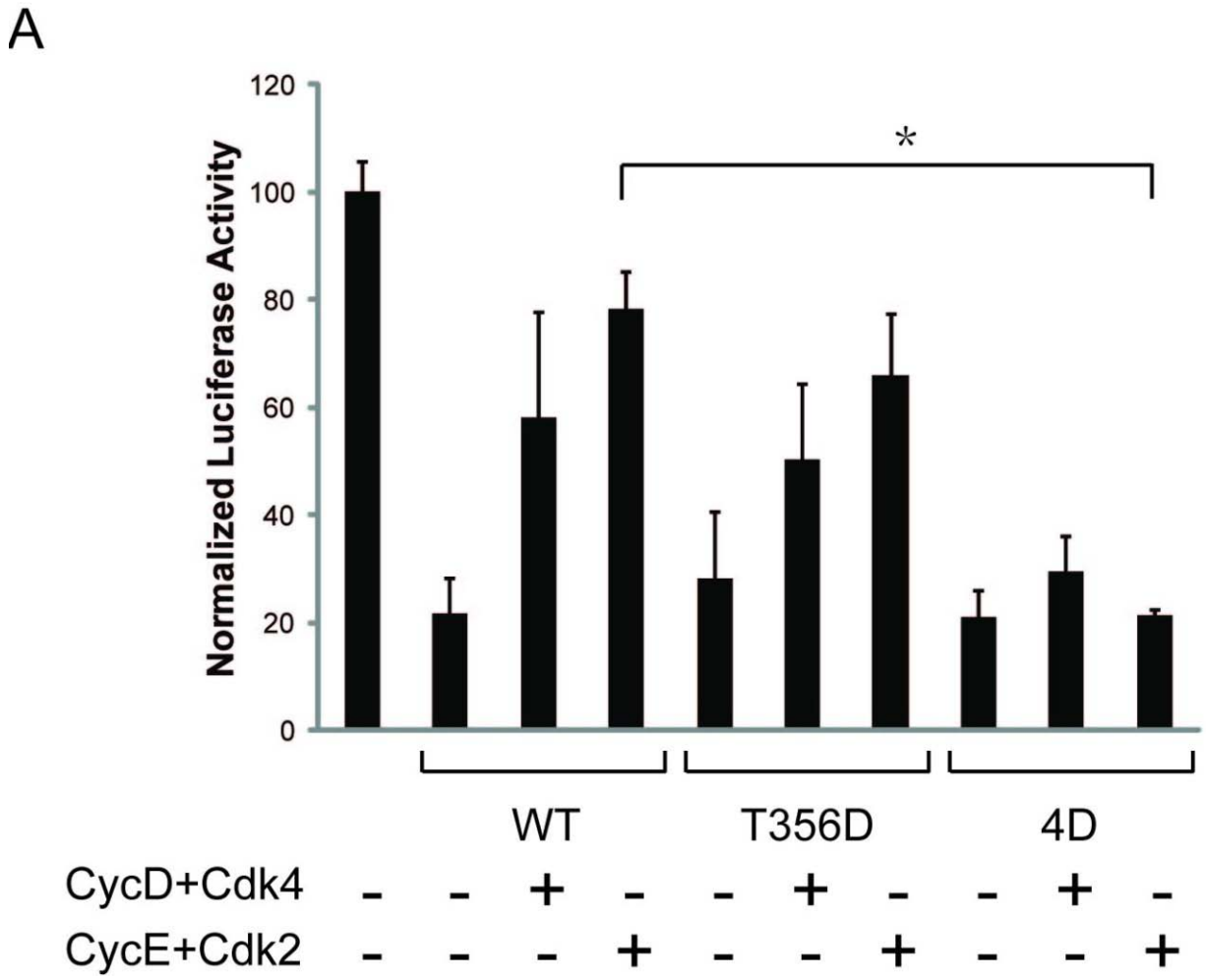

Figure 5-4 (cont'd)

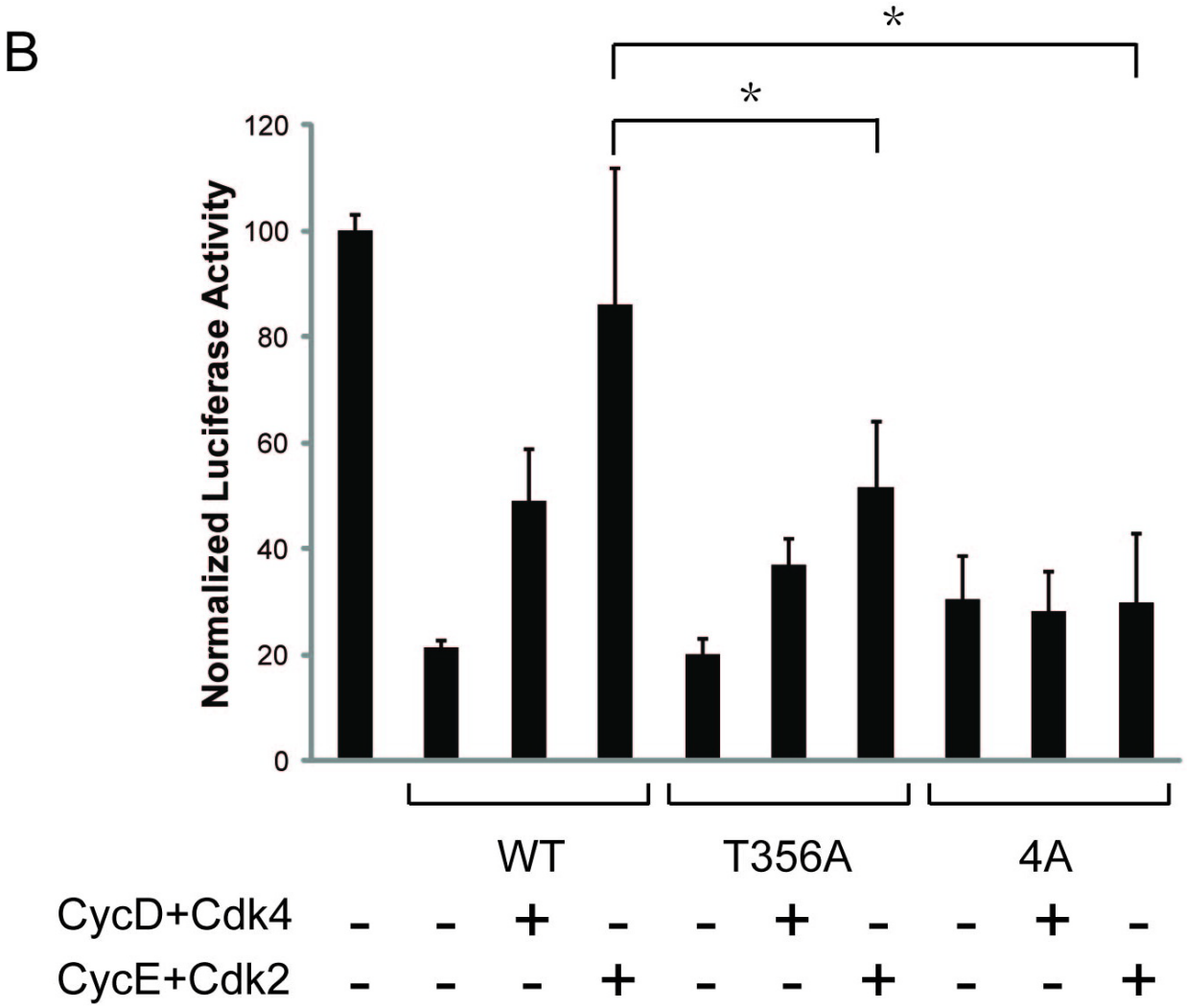

Figure 5-4 (cont'd)

(A) *PCNA*-luciferase assays were used to measure Rbf1 repression activity. Rbf1 WT and Rbf1 T356D are inactivated by Cyc-Cdk, whereas Rbf1 4D shows no response to Cyc-Cdk overexpression. (B) Rbf1 T356A is partially resistant to Cyc-Cdk inactivation, whereas Rbf1 4A is completely resistant. Asterisks indicate $p < 0.01$.

Figure 5-5. Conserved serine residues within the IE influence Rbf1 stability independently of the lysine residues in the IE.

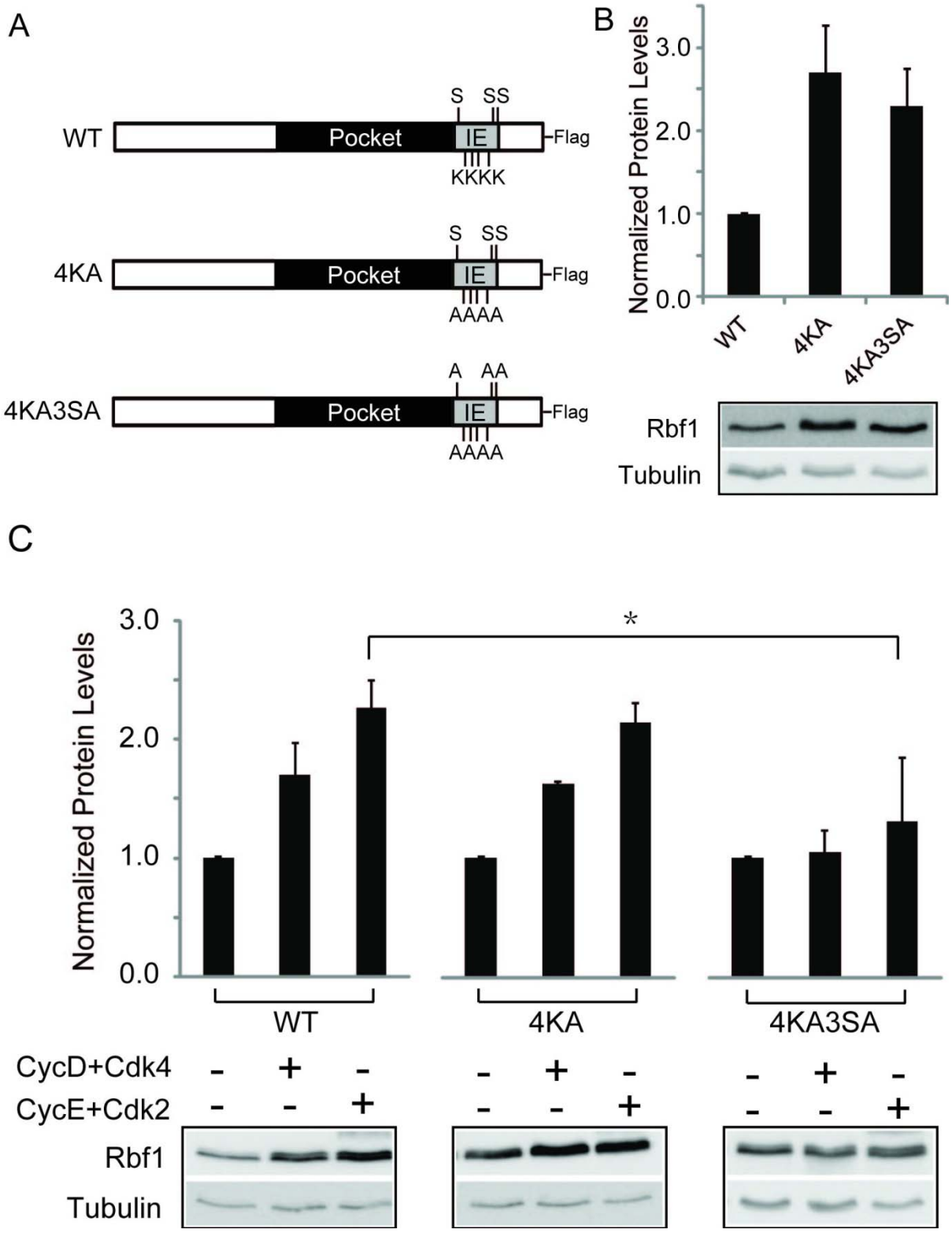

Figure 5-5 (cont'd)

(A) Schematic diagram of Rbf1 mutants with Lys-to-Ala and Ser-to-Ala mutations in the IE. (B) Western blot of Rbf1 proteins expressed in S2 cells. S-A mutations do not further significantly change level of stabilized Rbf1 4KA mutant. (C) Protein levels of stabilized Rbf1 4KA are further increased by Cyc-Cdk overexpression. However, mutant with combined K-A and S-A mutations is unresponsive to Cyc-Cdk overexpression. Asterisks indicate $p < 0.05$.

Figure 5-6. Dramatic developmental defects induced by Cyc-Cdk-resistant Rbf1 proteins.

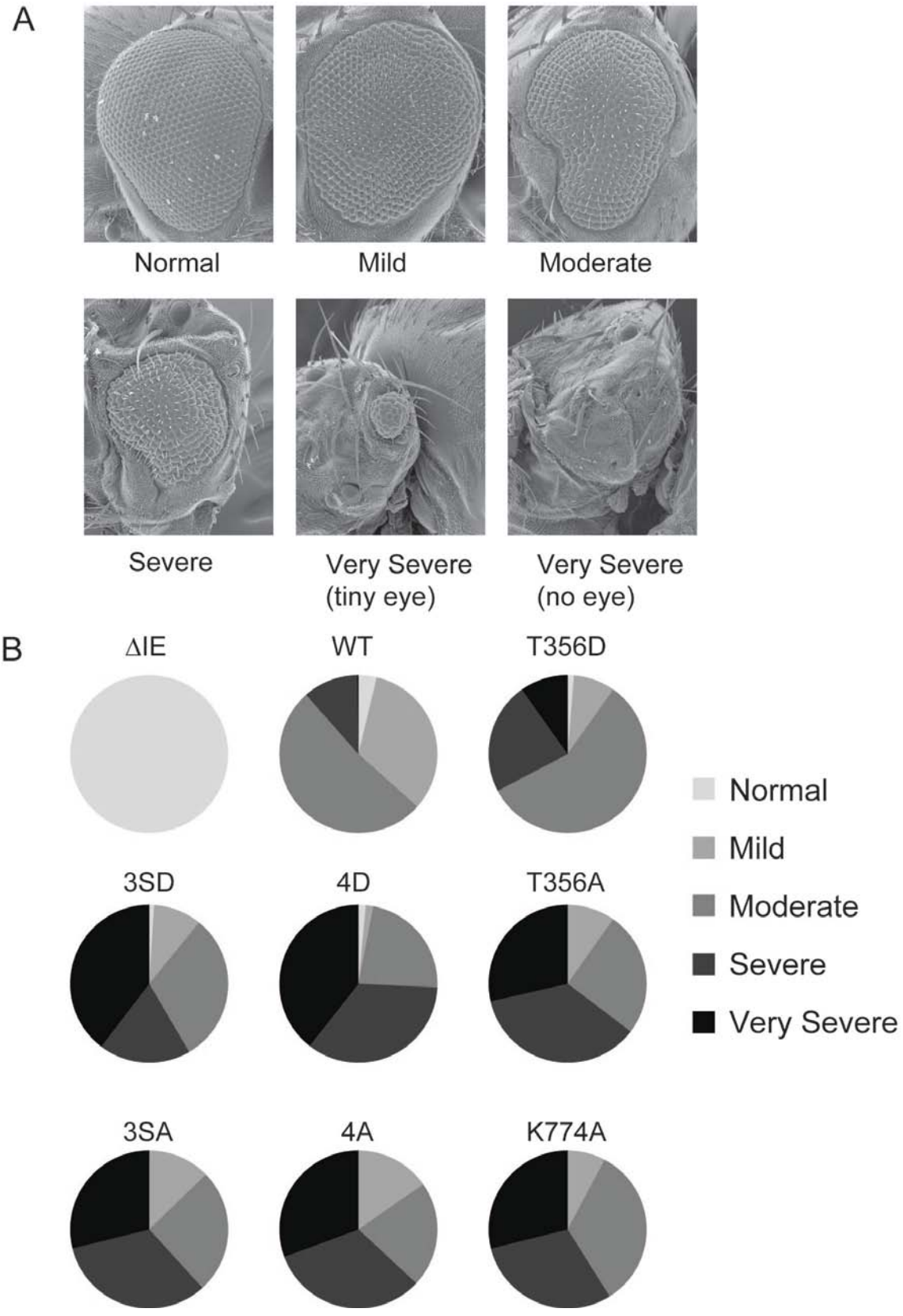

Figure 5-6 (cont'd)

(A) Representative *Drosophila* eyes exhibiting wild-type, mild, moderate, severe and very severe phenotypes induced by expression of *rbf1* genes in the eye imaginal disc. (B) Bar graphs represent percentage of flies exhibiting different eye phenotypes caused by Rbf1 overexpression. A mutant lacking the IE (ΔIE) has no effect on eye development, while the wild-type Rbf1 (WT) causes a high percentage of mild and moderate phenotypes. The T356 mutant exhibited a slightly stronger phenotype than Rbf1 WT, and other mutants had much more pronounced effects. 70-400 flies were scored for each of these lines.

Figure 5-7. K774 influences Cyc-Cdk control of both Rbf1 protein levels and transcriptional activity.

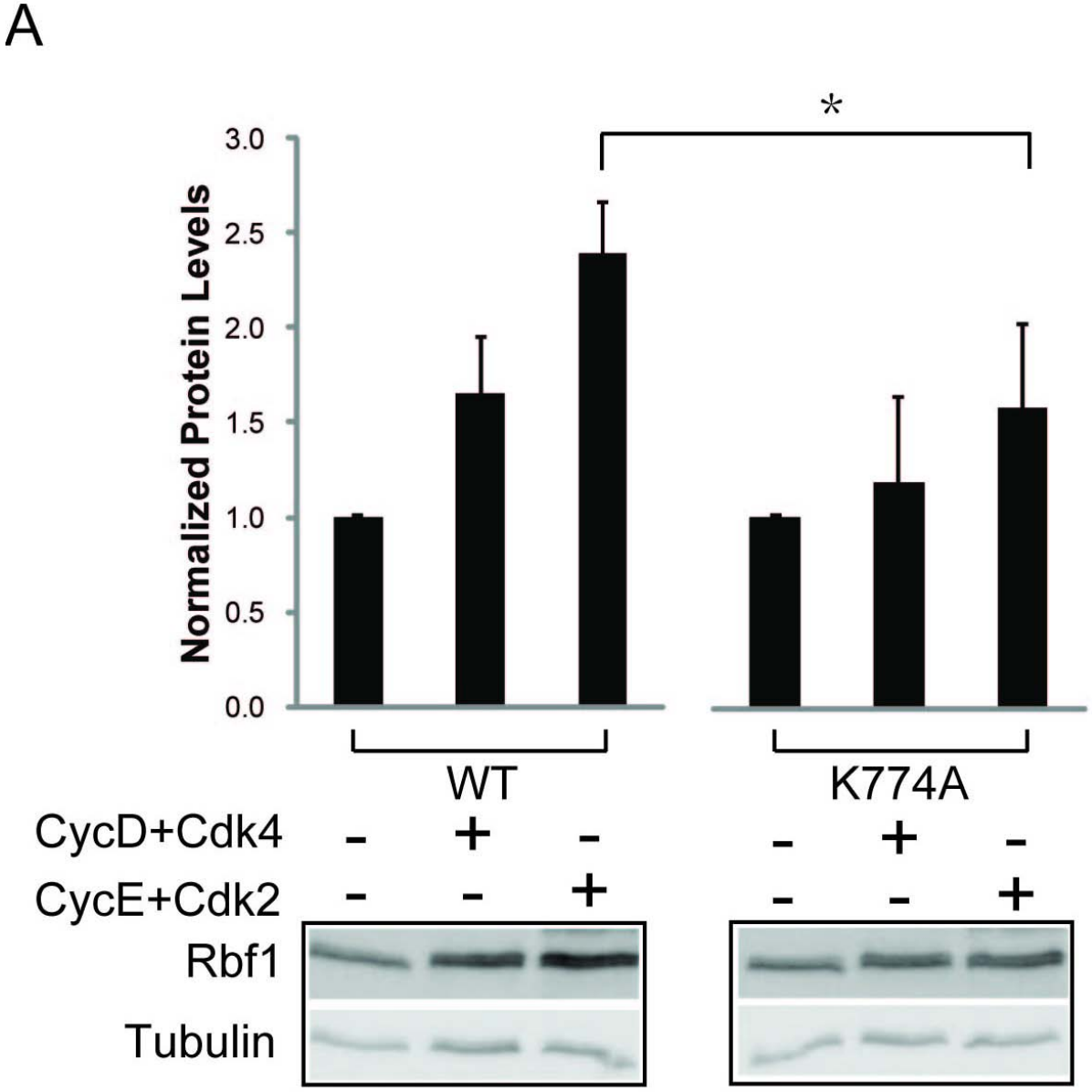

Figure 5-7 (cont'd)

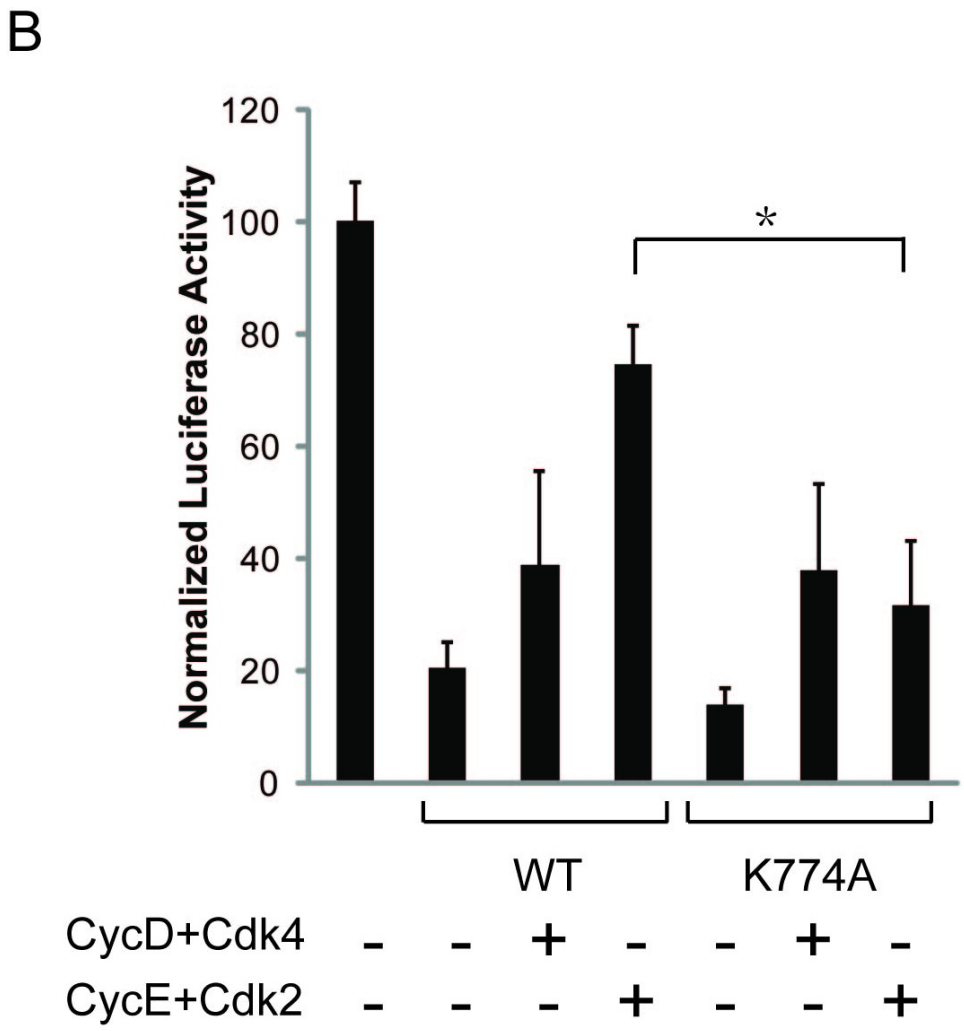

Figure 5-7 (cont'd)

(A) Western blot analysis of Rbf1 WT and K774A mutant. The latter is partially resistant to Cyc-Cdk-mediated stabilization. The Rbf1 WT and K774A have similar levels of expression without Cyc-Cdk overexpression. (B) *PCNA*-luciferase assay to measure Rbf1 WT and K774A mutant. Repression activity of K774A is partially resistant to Cyc-Cdk overexpression. Asterisks indicate $p < 0.05$.

Discussion

RB family proteins play significant roles in diverse cellular events including cell cycle progression, cellular differentiation and apoptotic death (Burkhart and Sage, 2008). As described for posttranslational modifications of histone proteins and transcription factors such as p53, a biochemical regulatory code of conduct has been proposed for RB based on extensive research on RB phosphorylation and other modifications (Munro et al., 2012). In particular, phosphorylation serves as a key mechanism of transmitting upstream regulatory signals into functional outputs, which allows the channeling of RB activity into diverse pathways. However, the roles of specific phosphorylation patterns are not yet fully understood. In this study, we provide evidence for essential phosphorylation sites in two regions involved in regulation of Drosophila Rbf1 stability and activity, two key features frequently disrupted in human diseases.

The N-terminal T356 phosphorylation site functions as a critical switch for Rbf1 protein stability and repression activity, presumably by serving as a priming site for other phosphorylation events, for instance, in the IE. This highly conserved threonine residue (T373) in RB, when phosphorylated, induces a global conformational change to facilitate the N-terminus binding to the pocket domain, which blocks association of the pocket domain with E2F transactivation domain (TD) (Burke et al., 2012). Consistent with the structural importance of T373, its phosphorylation is sufficient to inactivate RB in human and rodent cells (Lents et al., 2006). This similarity between the involvements of this single phosphorylation site in the inactivation mechanism suggests that phosphorylation of T356 in Rbf1 may also cause a conformational change. This level of regulation may facilitate

Cdk targeting of additional phosphorylation sites in the pocket domain or the IE, disrupting protein-protein interactions important for function and stability (Fig. 5-8).

Our previous studies demonstrate that the C-terminal IE in Rbf1 is crucial for both protein turnover and repression activity (Acharya et al., 2010; Raj et al., 2012b). This multifunctional region may serve as a surface for protein-protein interactions that regulate contacts with E2F factors and E3 ligases. Mutations of the lysines would both weaken E3 ligase binding to block the degradation pathway and impair E2F binding, attenuating transcriptional repression. In this study of phosphorylation control, we uncovered a second set of residues involved in stability-activity linkage through the IE. Phosphorylation of the serine residues in the IE may block E3 and E2F associations to stabilize and inactivate the protein respectively (Fig. 5-8). The IE of Rbf1 is highly conserved with RB family members, p107 and p130, implying a similar IE-mediated control of stability and activity. Indeed, a p107 mutant lacking the IE, when expressed in S2 cells, accumulates to a much higher level than the wild-type p107 (Acharya et al., 2010), supporting one aspect of the regulation mechanisms mediated by the IE. The conservation of the three serines in the IE indicates that the phosphorylation control through the IE may also be a conserved mechanism. In mammalian RB, the C-terminus is required for RB activity due to its essential association with the Marked Box (MB) of E2F. Structural studies of RB C-terminus reveal that phosphorylation in the conserved IE region destabilizes association of C-terminus with the E2F MB and induces an intramolecular interaction between the RB C-terminus and the pocket (Rubin et al., 2005). A similar mechanism may apply to Rbf1 inactivation, and additionally the conformational change caused by these phosphorylation

events may also influence E3 binding (Fig. 5-8).

Our previous model of paradoxical instability-activity relationship suggests that these two processes are tightly linked, and that ubiquitination driven by the IE contributes to both protein degradation and repression activity (Raj et al., 2012b). However, in this study, we identified a way to separate these two pathways that converge on the IE. Ser-to-Asp mutations only affect the Rbf1 degradation pathway, but do not disrupt repression activity. We propose that these mutations specifically block E3 ligase binding to Rbf1 to reduce the level of ubiquitination, but are not disruptive to E2F interactions. Phosphorylation control of E3 ligase binding is also observed for RB: the Mdm2 E3 ligase binds preferentially to the C-terminal region of hypophosphorylated RB. The ability to disengage turnover and repression activity suggests that high levels of ubiquitination are not essential for Rbf1 activity in every context. In rapidly dividing cells, Rbf1 activity may be only controlled by phosphorylation and dephosphorylation so that it can be recycled and reused efficiently. In contrast, in differentiating cells Rbf1 may be ubiquitinated and degraded to ensure a rapid depletion when it is no longer needed. In the mammalian system, Mdm2 targets RB for degradation to release E2F-dependent transcription, suggesting a conserved degradation pathway. However, there may be specific contexts in which the linkage between ubiquitination-mediated degradation and activity of Rbf1 is important. We previously showed that a single ubiquitin tag is able to enhance Rbf1 activity, therefore this modification may allow Rbf1 to exert different levels of activity on the same genes depending on the cellular context. The ubiquitin tag may help recruit transcription cofactors to achieve the maximal repression activity of Rbf1, and even some components of the

degradation machinery, such as the proteasome, may be directly involved in transcriptional repression.

Within the Rbf1 IE, lysine and serine residues appear to each contribute to the regulation of the IE, however, it is possible that there is functional interplay between the two sets of residues. Of particular interest are recent findings that in response to DNA damage, K810 in the C-terminus of RB can be methylated to block phosphorylation of serine sites by impeding the interaction between RB and Cdk4 (Carr et al., 2011). In addition, a recent study shows that acetylation of K1079 (equivalent to Rbf1 K774) in p130 enhances Cdk4-mediated phosphorylation *in vitro* (Saeed et al., 2012). This particular residue of p130 is found to be mutated in lung cancers, which suggests that there may be specific growth advantages to disruption of physiological controls of stability and activity (Claudio et al., 2000).

Figure 5-8. Model for the phosphorylation-mediated functional inactivation and stabilization of Rbf1.

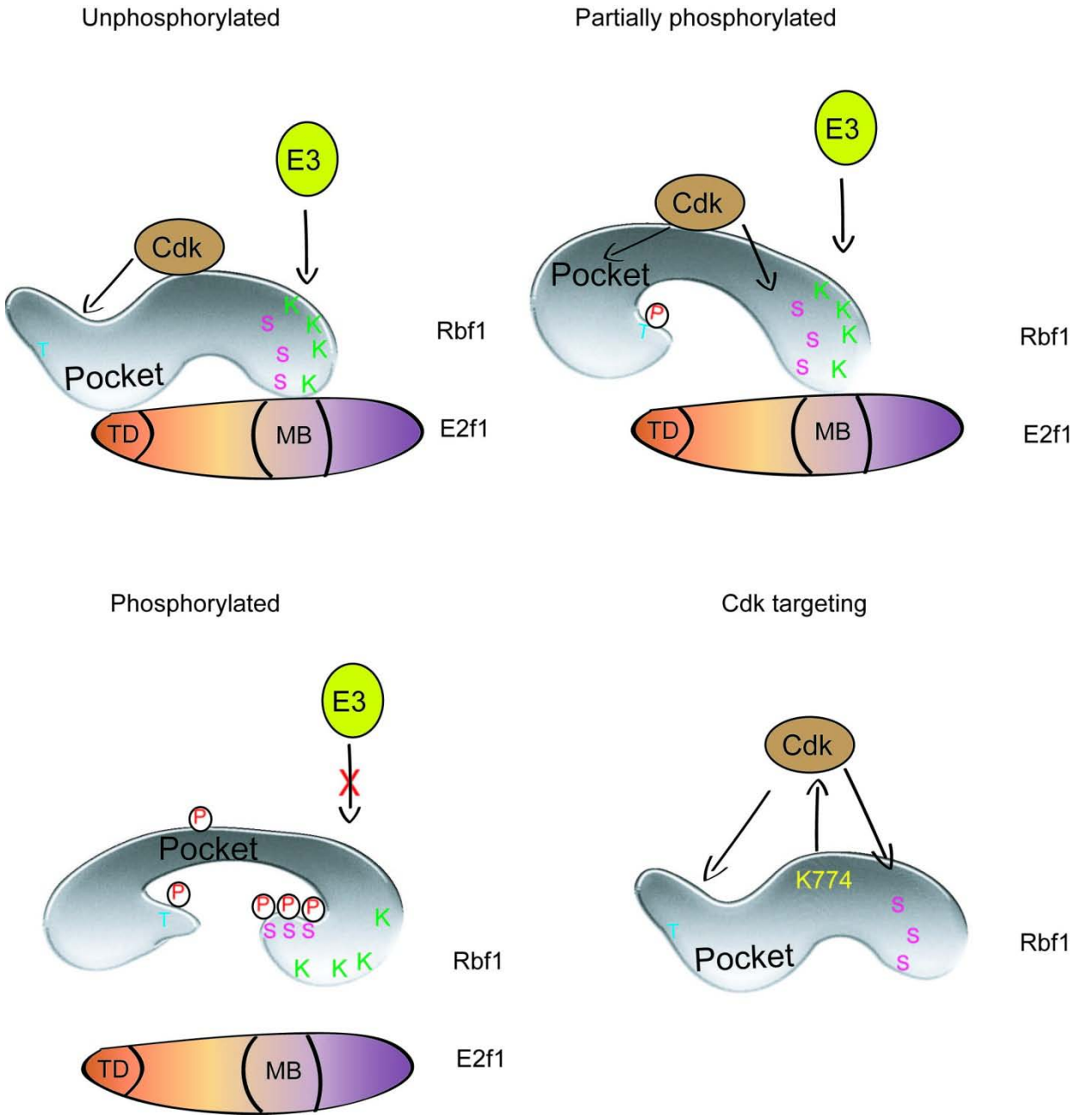

Figure 5-8 (cont'd)

Unphosphorylated Rbf1 binds to E2f1 through Pocket-Transactivation domain (TD) and C domain-Marked Box (MB) interactions. Phosphorylation of T356 by Cdk induces a partial conformational change which may serve as a priming event for other phosphorylation events in the pocket or in the IE. When the C-terminus is phosphorylated, Rbf1 dissociates from E2f1 and loses its repression activity and repels E3 binding to be stabilized. A key lysine K774 in the IE is critical for Cdk targeting on phosphorylation sites to affect Rbf1 sensitivity toward Cyc-Cdk for stability and activity.

Materials and Methods

Expression Constructs

Generation of Rbf1 WT and mutant expression constructs was described previously (Acharya *et al.*, 2010). The mutations of phosphorylation sites in Rbf1 were made by site-directed mutagenesis using a Quick-Change TM strategy (Stratagene). To generate Cyclin and Cdk expression constructs, *Cyclin D, Cyclin E, Cdk2 and Cdk4* cDNA were PCR-amplified from respective pOT construct (DGRC) and cloned into the *KpnI* and *NotI* or *NotI* and *XbaI* sites of pAX vector (Ryu and Arnosti, 2003). Two Flag epitope tags were inserted 5' of the stop codon. To generate Rbf1 expression constructs used in the fly eye assays, Rbf1 WT and mutants were cloned into pUASTattB (Bischof et al., 2007). The plasmids were then injected by Rainbow Transgenics into the 51D site of *yw* flies to generate transgenic lines.

Western Blot Analysis

Drosophila S2 cells were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. 1.0 million cells were transfected with 100ng of pAX-Rbf1. For the Cyc-Cdk overexpression assays, 100ng of pAX-Rbf1 was cotransfected with 200ng of pAX-CyclinE or D and 400ng of pAX-Cdk2 or 4. Cells were harvested 5 days after transfection and lysed by freeze-and-thaw cycles three times in lysis buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 1% Triton X-100). Total protein levels were measured by Bradford assays. To measure Rbf1 protein levels, 50 µg S2 cell lysates were run on 12.5% SDS-PAGE gels, transferred to PVDF membranes and probed with M2 anti-Flag antibody (mouse monoclonal, 1:10,000, Sigma, F3165) and anti-tubulin (mouse monoclonal, 1:10,000,

Iowa Hybridoma Bank). Antibody incubation was performed in TBST (20 mM Tris-Cl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) with 5% non-fat dry milk. Blots were developed using HRP-conjugated secondary antibodies (Pierce) and SuperSignal West Pico chemiluminescent substrate (Pierce).

Luciferase Reporter Assays

For *PCNA*-luciferase assays, 1.5 million Schneider S2 cells were transfected with 600ng of *PCNA*-luciferase reporter (Frolov et al., 2003), 200ng of pAX-Rbf1 WT, 200ng of pAX-CyclinE or D and 400ng of pAX-Cdk2 or 4. Cells were harvested 3 days after transfection and luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega) and quantified using the Veritas microplate luminometer (Turner Biosystems).

Fly Assays

Heterozygous lines harboring the wild-type or mutant *rbf1* forms in the pUAST vector were crossed with flies containing an *eyeless-Gal4* / CyO driver (Gilbert *et al.*, 2006) and the offspring containing both driver and UAS-*rbf1* transgenes (70-400 flies per construct) were screened for eye phenotypes.

Acknowledgments

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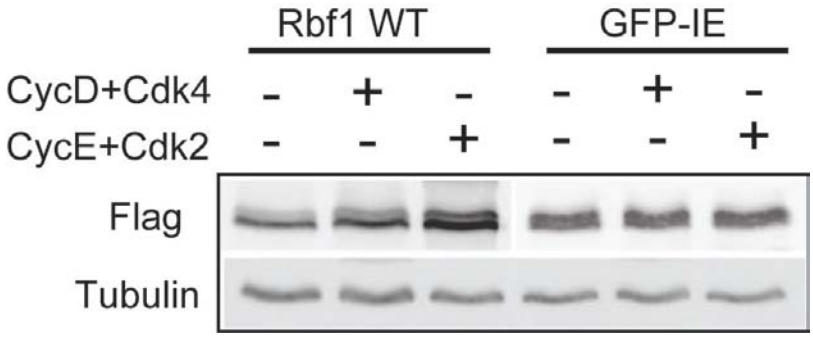
APPENDIX

Figure 5-S1. The IE alone is insufficient for Cyc-Cdk-mediated stabilization.



GFP-IE protein level is not affected by overexpression of Cyc-Cdk when the wild-type Rbf1 is stabilized under these conditions.

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CHAPTER 6

Future directions

Here I discuss how my work on the *Drosophila* retinoblastoma protein Rbf1 has changed our understanding of RB biology, leading to future perspectives and suggested research directions in this area.

My studies have demonstrated that the C-terminal instability element (IE) of Rbf1 has key functions in the regulation of Rbf1 stability and activity through post-translational modifications (Acharya et al., 2010; Raj et al., 2012b). One plausible model to explain the central role of the IE is that it serves as an interaction domain for proteins and cofactors involved in diverse regulation pathways (Chapter 5). To further investigate the roles of the IE, one essential step will be to identify potential binding proteins to the IE region. I have generated a chimeric protein with the minimal IE fused to a heterologous GFP, and showed that in this context, the IE is sufficient to direct destabilization of the chimera. This epitope-tagged chimeric protein can be used in immunoprecipitation and mass spectrometry assays to identify physically associated proteins in S2 cells and flies. A similar assay to identify Rbf2 interaction proteins was performed in the Henry and Arnosti laboratories previously (Ullah et al., 2007).

One class of proteins that we will be looking for is E3 ubiquitin ligases that may bind to the IE and target the protein for ubiquitination and degradation. Although my preliminary RNAi experiments failed to reveal any potential E3 ligases among the Cullin family that influences Rbf1 protein levels, I am confident that such activity exists. For example,

studies of mammalian RB demonstrate that the E3 ligase Mdm2 binds to the conserved C-terminus and promotes RB degradation (Sdek et al., 2004). Therefore, the question about candidate E3 ligases remains open. Another class of proteins of particular interest is transcription cofactors that interact with the IE and contribute to the repression activity of Rbf1 at the target promoter. The interaction domain for many RB binding proteins, especially LXCXE-containing proteins, has been mapped to the pocket domain, but the C-terminus may still be important for certain aspects of protein binding. For example, HDAC1 binds to the C-terminus of p130 and enhances its repression on the E2F-dependent Cyclin A promoter (Stiegler et al., 1998). More importantly, the C-terminus may provide a control of gene-specific activities of RB. C-terminal binding of RB with c-Jun promotes c-Jun-dependent transcription, showing a role of RB as a transcriptional activator in early G1 during keratinocyte differentiation (Nead et al., 1998). Consistent with this, in our studies, we show that the IE is required for Rbf1 activity on cell cycle genes, but not on other classes of genes, such as the ones in signaling pathways, suggesting functional importance of the IE as a module to discriminate distinct roles of RB in different pathways.

Related to the above goal, another interesting and essential direction is to identify IE-dependent and IE-independent functional targets of Rbf1. Our lab has generated stable S2 cell lines to express the wild-type Rbf1 and the ΔIE mutant under an inducible promoter. We will first test a select group of previously identified Rbf1 target genes to validate this assay and then perform a genome-wide survey of functional targets by microarrays. We anticipate that IE-dependent and IE-independent genes may fall into different gene ontology categories, showing distinct requirements of the IE for different Rbf1 regulation

mechanisms. In this case, regulation of Rbf1 by modification of the IE may impact certain functional classes of target genes preferentially, permitting alternative Rbf1 regulation of a subset of the regulon.

A third long-term goal worth pursuing is to characterize physical protein-protein interactions between the IE and E2f and some of the binding partners identified in the above assays. My work indicates that the IE may have critical contacts with E2F, which are subject to Cyc-Cdk-mediated phosphorylation (Chapter 5). The structure and the phosphorylation control of the RB C-terminus have been well studied, but how these structural features contribute to RB functions remains elusive (Rubin, 2013). I have generated a cohort of Rbf1 mutants with mutations on key lysine and serine residues important for Rbf1 activity and stability. A structural study of these mutants with E2F binding would elucidate the importance of post-translational modifications in the IE for E2F interaction and possible functional outputs. In particular, K1079 (equivalent to Rbf1 K774) is frequently mutated in lung cancers and it governs an interplay between its acetylation and cdk4-mediated phosphorylation (Claudio et al., 2000; Saeed et al., 2012). This class of mutations may greatly affect RB-E2F interactions and therefore the transcriptional regulation of genes involved in cancer progression. Structural studies may provide insights into the basis by which such mutations are beneficial to transformed cells.

Our cell culture-based assays show that overexpression of the Rbf1ΔIE mutant promotes DNA replication, a phenotype favorable to cancer growth (Raj et al., 2012a). It will be interesting to investigate if this mutant is capable of disrupting normal cell growth and driving excess DNA replication under developmental settings. However, overexpression of

Rbf1ΔIE in the fly eye imaginal disc does not cause any observable abnormal eye phenotype, suggesting that additional cofactors may be required to mediate the effect of Rbf1ΔIE in regulating the cell cycle (Acharya et al., 2010). Consistent with this idea, a previous study showed that overexpression of E2F1 alone has a modest eye phenotype, whereas when it is overexpressed with DP, a more pronounced rough eye phenotype is observed (Du et al., 1996b). Therefore, we will overexpress Rbf1ΔIE with DP in the eye imaginal disc to look for eye morphology defects and monitor DNA replication by BrdU staining. We expect to observe similar phenotypes as with E2F1 and DP overexpression. This assay would provide insights into the importance of the IE for controlling cell cycle arrest and gain of function in cell proliferation when it is mutated in human cancers.

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