REGULATION OF THE *DROSOPHILA* RETINOBLASTOMA NETWORK BY THE UBIQUITIN-PROTEASOME PATHWAY

By

Nitin Raj

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics

2012

ABSTRACT

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Studies of the Retinoblastoma tumor suppressor protein (RB) have been at the forefront of cancer research as its loss of function has been implicated in a diverse profile of deadly human cancers. In this study, we describe research that has contributed to our understanding of the regulation of the RB network by the ubiquitin-proteasome system. Through studies on the *Drosophila melanogaster* RB family homolog proteins, Rbf1 and Rbf2, we uncovered a novel regulatory pathway that governs their function as transcriptional repressors of diverse gene sets. First, we showed that a C-terminal autonomous degron, which we termed the Instability Element (IE), directs Rbf1 ubiquitination and mediates its gene-specific repression functions. The Rbf1 degron-mediated ubiquitination was paradoxically found to be a critical component for enhancing proteasome-mediated Rbf1 degradation as well as potentiating Rbf1-mediated repression of a subset of its target genes. Thus, this study uncovered a direct role for Rbf1 ubiquitination in Rbf1 transcriptional repression and adds a key piece to the long unresolved puzzle as to how RB proteins simultaneously regulate mutually exclusive cellular processes such as cell-cycle progression and apoptosis.

Interestingly, previous studies have linked gene activation to protein degradation via the promoter-associated proteasome. Hence, our findings suggest that Rbf repression similarly involves the proteasome and this intricate instability-activity relationship potentially provides regulatory responsiveness to changes in environmental conditions. These finding are also

relevant to the mammalian RB family proteins as the Rbf1 degron was found to be evolutionarily conserved both in terms of its structure and roles in protein turnover and repression.

Second, we show that in case of Rbf2, the evolutionarily conserved pocket domain enhances its ubiquitin-proteasome-mediated degradation. Additionally, unlike Rbf1, the Rbf2 N-terminus which harbors a conserved 'Domain of unknown function (DUF)', as well as the pocket domain are required for Rbf2-mediated repression of cell-cycle promoters. Thus, the two *Drosophila* RB family proteins utilize distinct protein domains to enact their roles in the regulation of cell cycle.

Furthermore, we show evidence that mutation of the Rbf1 degron, unexpectedly, leads to an enhanced rate of cellular DNA replication due to its unique ability to stabilize but not inhibit the activator protein, dE2F1. Thus, through a gain of function, the Rbf1 degron dysfunction has the potential to convert the tumor suppressor into an oncoprotein. This important observation is especially relevant in the context of cancer cells where certain mutations in RB family degron may provide a selective growth advantage. In such cancers, an additional therapeutic intervention may be required to counteract the effects of the rogue RB family alleles.

I dedicate this thesis to my mother, Mrs. Kumkum Saxena, my father, Mr. Santosh Kumar and to my wife, Akanksha, for their unconditional love and support.

ACKNOWLEDGEMENTS

From the formative stages of this thesis, to the final draft, I owe an immense debt of gratitude to my supervisor, Dr. R. William Henry. He has been the ideal thesis supervisor and mentor who taught me innumerable scientific and life lessons. His energetic demonstrations of scientific ideas and infectious enthusiasm towards science have been a huge source of inspiration to me. His sound advice, insightful criticisms and careful guidance were invaluable during the writing of this thesis.

I would also like to thank Dr. David Arnosti whose constant support and guidance was greatly needed and deeply appreciated. I want to express my gratitude to my committee members, Dr. Laura McCabe and Dr. Steven J. Triezenberg and my graduate program director, Dr. Barb Sears, for their support, guidance and helpful suggestions.

I have had the great fortune of having the ceaseless support of my wonderful labmates and friends at MSU. I would especially like to thank Tejas, Soumya, Satyaki, Rupinder, Pankaj, Gaurav, Ashwini and Sunetra for providing the necessary ‘distractions’ without which this work would not have been possible.

Last but not the least, this work would not have been successful without the unconditional love and support of my family. I am deeply indebted to my beloved parents (Mrs. Kumkum Saxena and Mr. Santosh Kumar), brother (Atin), little sister (Shruti), sister-in-law (Meghna), my adorable nephew (Ansh), parent-in-laws (Mrs. Rashi Khare and Mr. S.C. Khare), brother-in-law (Akash) and above all, my darling wife, Akanksha whose sweet love and affection makes it all worth it.

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LIST OF ABBREVIATIONS

AR	Androgen Receptor
ATP	Adenosine Triphosphate
BRG1	BRM-related Gene product 1
BRM	Brahma
CDK	Cyclin Dependent Kinase
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A
ChIP	Chromatin Immunoprecipitation
CMV	Cytomegalovirus
COP9	Constitutive Photomorphogenic 9
CSN	COP9 Signalosome
DNA	Deoxyribonucleic Acid
DNMT1	DNA Methyltransferase 1
DNMT3a	DNA Methyltransferase 3a
DNMT3b	DNA Methyltransferase 3b
DP	E2F Dimerization Partner
dREAM	DP, RB-like, E2F and MuvB complex
E1A	Early region 1A
E1	Ubiquitin activating enzyme 1
E2	Ubiquitin activating enzyme 2
E3	Ubiquitin activating enzyme 3
E2F	Adenovirus E2 promoter binding factor
HCC	Hepatocellular carcinoma

HDAC	Histone Deacetylase
HPV E7	Human Papilloma Virus Early 7
IE	Instability Element
InR	Insulin Receptor
ISWI	Imitation Switch
LOH	Loss of Heterozygosity
MDM2	Murine double minute 2
MEFs	Mouse Embryonic Fibroblasts
Nedd4	Neural precursor cell-expressed developmentally down regulated gene 4
Nedd8	Neural precursor cell-expressed developmentally down regulated gene 8
NURF	Nucleosome Remodeling Factor
P/CAF	p300/CBP Associated Factor
PCNA	Proliferating Cell Nuclear Antigen
PIC	Pre-Initiation Complex
PP1	Protein Phosphatase type 1
PP2A	Protein Phosphatase type IIA
RB	Retinoblastoma Protein
Rbf1	Retinoblastoma Factor 1
Rbf2	Retinoblastoma Factor 2
RNA	Ribonucleic Acid
S2 cells	Schneider 2 cells
SCF	Skp/Cullin/F-box complex
SIRT1	Sirtuin 1

SUV39H1	Suppressor of variegation 3-9 homolog 1
Suv4-20h1	Suppressor of variegation 4-20 homolog 1
SV40	Simian Virus 40
SWI/SNF	Switch/Sucrose nonfermenting complex
TKO	Triple Knock Out
TSS	Transcriptional Start Site
VP16	Virion Protein 16

CHAPTER ONE

INTRODUCTION

Preface

Retinoblastoma is a rare pediatric cancer of the human retina that usually occurs in children under the age of 15 years. It is caused by mutations in the Retinoblastoma susceptibility gene (*RB1*), which encodes the classic tumor suppressor protein (RB) (*1*). Retinoblastoma tumors can be either inheritable and associated with germline *RB1* mutations or non-inheritable. Through statistical analysis on inherited and sporadic cases of retinoblastoma, Dr. Alfred G. Knudson proposed the "two-hit hypothesis" which states that tumor formation is initiated by biallelic loss of tumor suppressor gene (*2*). Following his seminal work, *RB1* was the first identified and cloned tumor suppressor gene in the human genome (*1, 3*). Biallelic loss of RB results in juvenile eye tumors and osteosarcomas in adults (*4, 5*). Mutational inactivation of RB protein is associated with cancer initiation and progression in a diverse profile of deadly human cancers, in particular lung cancer, breast cancer, and sarcomas (*6, 7*).

Since its initial identification as a classic tumor suppressor protein, molecular studies have revealed a role for RB in the negative regulation of cell proliferation (*6*). The RB protein functions as a transcriptional repressor that inhibits cell cycle progression at the G1 to S phase transition through the regulation of important cell-cycle genes. Although RB is transiently inactivated by various mechanisms as cells progress through the cell cycle in normal cells, these control mechanisms are perturbed in many human cancers. Inactivation of RB protein through various mechanisms results in deregulated cellular growth and tumorigenesis. Similar to their mammalian counterparts, the *Drosophila* retinoblastoma family homolog proteins, Rbf1 and Rbf2, play key roles in cell cycle control and fly development (*8, 9*).

We have utilized the streamlined Rbf network in *Drosophila melanogaster* to understand the molecular mechanisms of regulation of the RB and E2F family of proteins. The reduced

complexity combined with the biochemical and genetic tractability of *Drosophila* makes it a premier model system to study RB biology. Here we describe how the ubiquitin-proteasome pathway regulates *Drosophila* Rbf1 and Rbf2 protein degradation and, counter intuitively, stimulates their activity as transcriptional repressors of cell cycle genes. First, we show that an evolutionarily conserved RB family degron, which we named Rbf1 IE (instability element), is present in the C-terminal region of Rbf1, and that Rbf1 IE mediated ubiquitination serves to potentiate gene-specific repression of a subset of Rbf1 target gene promoters. Second, we show that dysfunction of Rbf1 degron results in enhanced cellular DNA replication, a property that has the potential to convert the RB tumor suppressor into an oncoprotein. Lastly, I show that Rbf2 is a two-component repressor which, unlike Rbf1, utilizes both its N-terminal and pocket domains in the regulation of cell cycle genes and that the pocket domain in Rbf2 governs its ubiquitin-proteasome mediated degradation.

The retinoblastoma protein family

RB governs the G1/S transition of the cell cycle, facilitates differentiation and restrains apoptosis (*10-14*). RB functions as a transcriptional repressor that regulates the expression of several key genes involved in cell cycle progression and other cellular pathways (*15, 16*). The mammalian retinoblastoma family consists of three proteins, namely, RB, p107 and p130, that have redundant as well as antagonistic functions (*17, 18*). Together, these proteins are referred to as ‘pocket proteins’ due to the presence of a characteristic ‘pocket domain’ which is essential for their tumor suppressing activity. The RB family pocket consists of two domains, termed the pocket region A and B, and both domains exhibit structural similarities with cyclin box fold of TFIIB and cyclin proteins (*19, 20*). Two protein-binding sites are located within the RB pocket:

one for E2F transcription factors and another one for “Leu-X-Cys-X-Glu” or “LxCxE” motif containing proteins (*19, 21, 22*). Several viruses produce proteins targeting the RB “LxCxE” motif to inactivate the tumor suppressor, thereby potentiating transformation. These include viral oncoproteins such as the adenovirus E1A, SV40 Large T antigen and human papilloma virus E7 (HPV E7) that bind and inactivate RB (*21, 23*).

Retinoblastoma family members are conserved in all metazoans, plants and protists (*18*). Most lower organisms have only one RB-related gene while higher organisms tend to possess two or three family members reflecting the increasing complexity of growth control in these species. In mammals, the RB family members p107 and p130, display overlapping and redundant functions. The homozygous germline deletion of *RB1* results in embryonic lethality in mice (*24*), however, *p107* or *p130* mutant mice exhibit normal development (*25*). Interestingly, the mammalian RB family members are differentially expressed in tissues and during different stages of the cell cycle suggesting that they may have evolved unique functions in addition to their overlapping functions (reviewed in (*18, 26*)).

RB family proteins lack DNA binding domains but are capable of negatively regulating transcription of various target genes through their interactions with the heterodimeric transcription factor E2F/DP (*27*). The growth inhibitory effects of RB family proteins are in part dependent on its negative regulation of the E2F family of transcription factors which regulate the expression of genes involved in cell-cycle progression and DNA replication. The RB-E2F interaction, mediated by the ‘pocket domain’, is dependent on the phosphorylation status of RB. During G0 or G1 phases of the cell-cycle, unphosphorylated RB associates with members of E2F family of transcription factors and various chromatin remodeling factors resulting in silencing of E2F regulated promoters. In late G1 phase of the cell-cycle, RB phosphorylation mediated by

cyclin D and cyclin E associated cyclin dependent kinases (cdk) leads to release of E2F proteins resulting in S-phase entry and DNA synthesis (Figure 1-1).

The role of activator E2Fs in cellular proliferation and human cancer

In mammals, the G1 to S transition of the cell cycle is controlled by the activity of the E2 factor (E2F) family of transcription factors. E2F was originally identified as the cellular factor that is required for the activation of the E2 gene promoters during adenovirus infection (*28*). So far, nine different E2F species (E2F1, 2, 3a, 3b, 4, 5, 6, 7 and 8) have been identified and characterized. They are classified as transcriptional activators (E2F1-3a) or repressors (E2F3b-8) based on their sequence homology and functional properties. RB preferentially interacts with E2F1-4, while p107 and p130 interact with E2F4 and E2F5 (*29*). Together the E2F family functions as oncogenes or tumor suppressors in a tissue-specific manner (*30*). The E2F family plays crucial roles in the regulation of cellular proliferation, differentiation and apoptosis (*31, 32*). The activator E2Fs drive cell cycle progression at the G1/S phase, as they are necessary for the expression of DNA replication factors as well as cell cycle regulators. Absence of E2F3 results in compromised proliferation of mouse embryonic fibroblasts (MEFs) and the loss of all three activator E2Fs completely blocks proliferation (*33, 34*). Conversely, overexpression of exogenous E2F1 promotes premature S-phase entry and subsequently leads to apoptosis (*35, 36*). Therefore, the deregulated expression of E2F1 is considered an oncogenic event due to its ability to mediate uncontrolled cellular proliferation that induces tumor formation.

A strong correlation exists between increased E2F1 expression and many human cancers such as lung, breast, ovarian, colon, liver and skin cancers (*37-41*). In melanomas and breast cancers in particular, elevated E2F1 levels are indicative of poor disease prognosis, highlighting

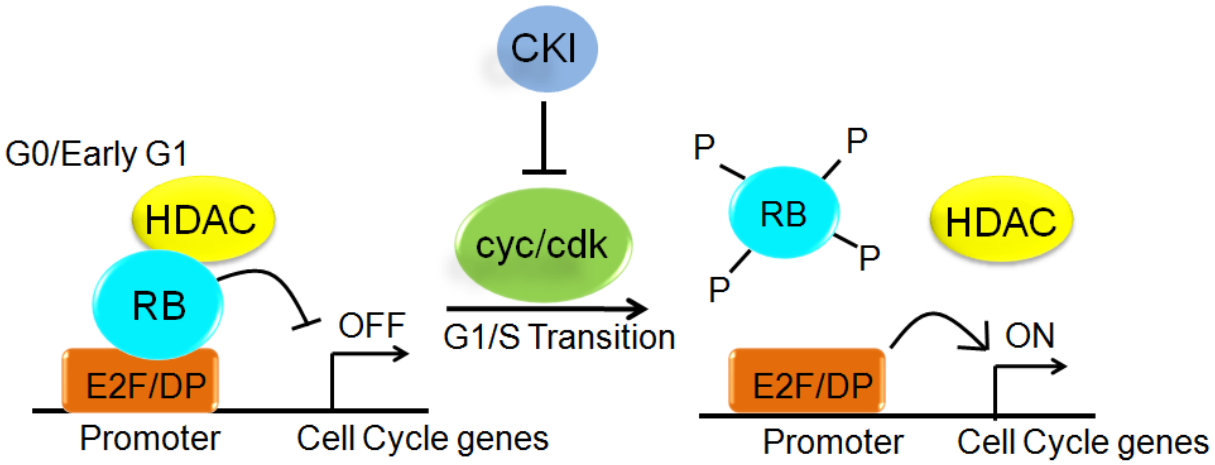

Figure 1-1. Regulation of E2F transcription factor activity during cell cycle. The RB-E2F interaction, mediated by the 'pocket domain', is dependent on the phosphorylation status of RB. In G0 or early G1 phase, RB protein is hypophosphorylated (left), and it can bind the E2F transcription factor as well as recruit cofactors such as HDACs to repress target gene expression. In late G1 phase, RB phosphorylation mediated by cyclin D1-cdk4/6 and cyclin E-cdk2 complexes (right) leads to dissociation of RB-E2F complexes and the transcription of genes that promote S-phase entry and cell cycle progression. The kinase activity of CDKs is inhibited by cyclin kinase inhibitors (CKI) such as p16, p21 and p27. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

the importance of imposing strong regulatory curbs on E2F1 expression and activity (*42-45*). Amplification of the E2F3 locus has also been reported in retinoblastomas and small cell lung carcinomas (SCLCs), and in the basal-like subtype of human breast cancer (*38, 46-48*). Hence, the deregulated expression of the activator E2Fs is a common occurrence in many human cancers, but whether this contributes to the initiation of these cancers has not been established.

Due to the dire consequences of the deregulated expression of the activator E2Fs, it comes as no surprise that E2F transcriptional activity is subject to tight regulation by multiple mechanisms, particularly during the cell-cycle. First, during different phases of the cell cycle and during differentiation, the RB family members directly bind and inactivate the transactivation domain of the activator E2Fs, converting them from activators to repressors (*27, 49*). Phosphorylation of RB family proteins mediated by cyclin-cdk kinases leads to the dissociation of RB/E2F complexes and this allows the protein level and transcriptional activity of E2Fs to peak. Second, in the mid to late S phase of the cell cycle, the cyclin A/cdk2 complex directly binds and phosphorylates E2F1 inhibiting the DNA binding affinity of the E2F1/DP1 heterodimer (*50*). Third, E2F activity is also regulated by the ubiquitin-proteasome pathway. This mode of E2F regulation is discussed in detail later in this chapter.

A streamlined RB/E2F network in Drosophila

The *Drosophila* RB/E2F pathway is much less complex compared to that in mammals due to the involvement of fewer components. This property therefore, presents an opportunity to study RB biology in a simpler and genetically tractable model system. *Drosophila* has two retinoblastoma family homolog proteins (Rbf1 and Rbf2) and two E2F proteins (dE2F1 and dE2F2) (*51-53*). *Drosophila* Rbf proteins display several of the structural features of the

mammalian RB family proteins, suggesting that these may have evolved from a common ancestor. The sequence homology between Rbf and human RB proteins extends throughout the entirety of the proteins but is most significant in the 'pocket domain'. Interestingly, both Rbf1 and Rbf2 show higher sequence similarities to p107 and p130 than to RB although the Rbf1 structural organization resembles that of RB. Most notably, Rbf1 lacks the spacer domain that is highly conserved between p107 and p130 and mediates their interactions with cdks (*54*) (Figure 1-2). Like the human RB family, Rbf1 contains a cluster of potential cdk phosphorylation sites immediately C-terminal of the pocket domain, suggesting that the fly Rbf proteins are similarly subject to phosphorylation regulation by cyclin/cdk kinases.

Rbf1 represents the dominant functional form of RB family proteins in *Drosophila,* as *rbf1* null mutants are larval lethal and never reach the late pupal stages of development (*55*). On the other hand, *rbf2* null mutants are viable and fertile. However, interestingly, the double mutant of *rbf1* null and *rbf2* null exhibits poorer viability and developmental delays, than either single mutant, suggesting that Rbf2 contributes significantly to fly development. Rbf1 plays an essential role in the introduction of G1 control during development (*55*). Similar to their mammalian counterparts, both Rbf proteins associate with dE2F/dDP proteins and repress dE2F-dependent transcription. The transcriptional activities of the *Drosophila* Rbf proteins are subject to control by cyclin-cdk mediated phosphorylation in a cell cycle dependent manner (*56*).

dE2F1 is characterized as a transcriptional activator and dE2F2 as a repressor (*57*). Similar to the mammalian E2F1, dE2F1 is essential for G1 to S phase progression in *Drosophila* embryos (*58*). Coexpression of dE2F1 and dDP in the *Drosophila* eye lead to ectopic S-phase entry in the regions of the eye disc that are normally post-mitotic (*58, 59*). Overexpression of dE2F1 and dDP in the developing eye results in a rough eye phenotype that is suppressed by coexpression of

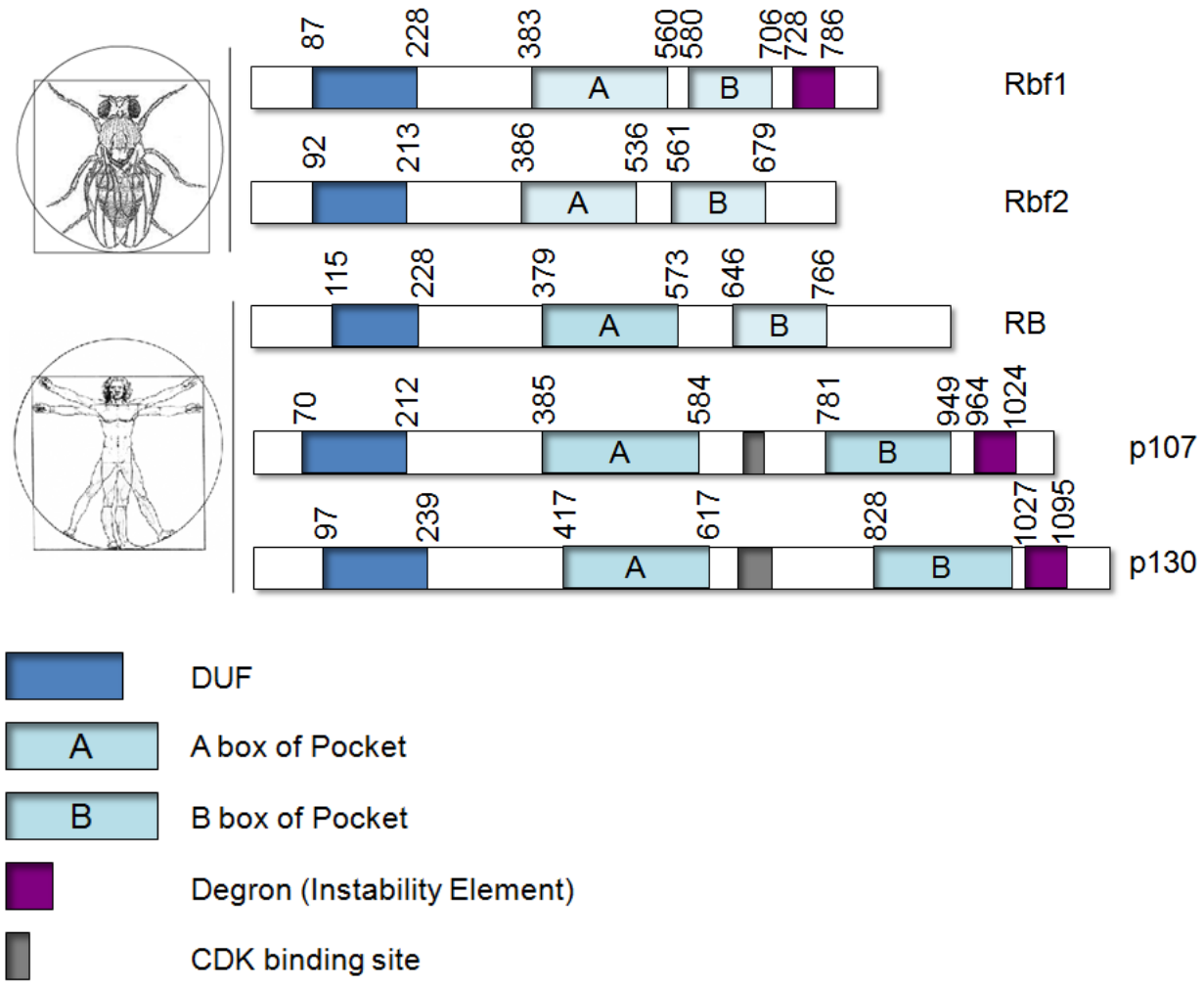

Figure 1-2. Schematic diagram of *Drosophila* and human retinoblastoma family proteins. Comparison of the structure of fly and human RB family proteins is presented here. The conserved 'Domain of Unknown Function' (DUF) is shown in dark blue. The A and B boxes of the pocket region are shown in light blue. A putative degron that is present in Rbf1, p107 and p130 is indicated in pink. A cyclin/cdk binding site found only in p107 and p130 is shown in gray. The numbers represent amino acid residues demarcating the different conserved domains. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Rbf1 (*54*). Global gene expression profiles of *Drosophila* S2 cells depleted of either of the two dE2F factors suggested that dE2F1 primarily regulates cell-cycle genes whereas dE2F2 regulates genes involved in differentiation (*60*).

Unlike the mammalian RB-E2F network where a highly complex pattern of interactions between the various RB and E2F members emerged, the *Drosophila* RB-E2F network displayed a relatively simpler pattern of interactions. Whereas Rbf1 interacts with both activator and repressor E2Fs, Rbf2 exclusively regulates the repressor dE2F2, suggesting that Rbf1 and Rbf2 regulate E2F-dependent transcription in a distinct manner (*61*). Rbf1 and Rbf2 are both able to repress transcription from E2F-regulated promoters and these proteins are normally found at these promoters in vivo (*61*). The presence of both *Drosophila* pocket proteins at dE2F promoters suggests that Rbf1 and Rbf2 may have overlapping functions in the regulation of E2F targets genes. However, the recruitment of Rbf2 to E2F-regulated promoters, and its ability to repress transcription, requires dE2F2 (*53, 61*). In support of the idea that Rbf2 acts in a stable complex with dE2F2, these proteins act synergistically when overexpressed in S2 cells or in transgenic animals, and Rbf2 levels are strongly reduced in *de2f2* mutant larvae. Rbf2 was shown to function together with E2f2 in vivo to repress the expression of differentiation markers in ovaries and in embryos where Rbf2 is highly expressed. Through RNAi knock-down of Rbf and E2F proteins in S2 cells, it was shown that these proteins repress transcription of both cell-cycle and developmentally regulated genes. Interestingly, of all the genes examined, only one was found to be elevated in Rbf2 depleted cells but not in Rbf1 depleted cells, indicating that there may be very few genes solely regulated by Rbf2.

Drosophila Rbf1 and Rbf2 are coexpressed at several stages of development but spatio-temporal differences are also observed particularly in the embryo CNS. Rbf1 is expressed at

fairly uniform levels throughout embryogenesis (0-20h) whereas Rbf2 expression is observed between 4 and 10 hours followed by a gradual decline. Rbf1 protein expression is also detected in larvae and adults whereas Rbf2 is undetectable in these stages (*62*). This differential expression pattern suggested that Rbf1 and Rbf2 may have different functions during embryogenesis.

Mechanisms of retinoblastoma transcriptional regulation

The ability of RB to repress E2F-mediated transcription is central to its function as a cell cycle regulator and tumor suppressor. Biochemical studies revealed that RB can repress E2F target genes using three distinct non-exclusive models. First, RB occludes the E2F transactivation domain by directly binding to it thereby blocking its ability to stimulate transcription (*63*). Second, through recruitment of cofactors that act as corepressors, RB can actively modulate the chromatin landscape of its target gene promoters into a more repressive state (*64, 65*). These cofactors include enzymes such as histone acetyl transferases (HATs), histone deacetylases (HDACs), histone methyl transferases (HMTs) and ATP-dependent chromatin remodeling complexes that repress transcription by modifying histones and changing chromatin structure. Third, RB protein may directly interfere with the basal transcriptional machinery as its recruitment to a promoter blocks the assembly of pre-initiation complexes. At present, it is not clear which of these mechanisms of repression is most important for RB mediated cell cycle control.

A significant body of literature supports the hypothesis that the pocket proteins utilize cofactors to repress gene activity. All three pocket proteins have been shown to associate with the histone modifying enzymes, HDACs 1-3 (*64-69*). Histone deacetylation is correlated with

chromatin compaction which is associated with repressed transcription due to the limited access of transcription factors to DNA. RB recruits HDACs to gene promoters and causes gene repression (*70*). Consistent with this observation, treatment of cells with Trichostatin A, an HDAC inhibitor, results in misregulation of various RB target genes (*66*). Interestingly, RB mutants that are defective for binding HDAC 1/2 are still capable of causing G1 arrest, suggesting that RB may also recruit other cofactors in some contexts (*71*).

The pocket proteins are also known to interact with histone methyl transferases such as SUV39H1, which specifically methylates H3K9 (*72*). SUV39H1 helps recruit HP1 (*73*), a mark of silenced chromatin. RB associates with SUV39H1 and HP1 on target gene promoters (*74*). Cell lacking all three RB family proteins show increased acetylation of histone H3 and decrease in the trimethylation of H4K20 resulting in a more permissive chromatin state (*75*). RB family proteins also recruit polycomb complex proteins, which are involved in histone methylation. RB is known to repress the transcription of *p16* gene promoter by recruitment of polycomb complex proteins BMI1 and EZH2 (*76*).

Besides covalent modifications of histones, chromatin structure can be modulated by nucleosome-remodeling complexes. Chromatin remodeling complexes hydrolyze ATP to move, exchange or remove nucleosomes along DNA (*77*). RB family proteins are also known to interact with SWI/SNF chromatin remodeling complexes which play extensive roles in the regulation of gene expression. RB family proteins physically associate with SWI/SNF complexes containing either Brahma (BRM) or BRM-related gene product (BRG1) ATPases, which repress E2F-responsive genes and induce cell cycle arrest (*78-82*). In summary, these studies suggest that the RB family proteins use diverse chromatin modifying enzymes to repress transcription.

Apart from its ability to modulate histone methylation, RB is also involved in DNA methylation (*83*). Methylation of cytosine at CpG dinucleotide is carried out by DNA methyl transferase enzymes; DNMT1, DNMT3a and DNMT3b (*84-86*). Methylated DNA provides a binding surface for proteins possessing the methyl-CpG-binding domain (MBD). These MBD domain containing proteins interact with HDACs, histone methyl transferases, and corepressors such as Sin3A to repress genes (*87*). E2F target genes are known to be silenced via promoter hypermethylation in tumors and during terminal differentiation (*88-94*). Interestingly, DNMT1 forms a complex with RB, E2F1 and HDAC1, and these proteins can synergistically repress transcription of E2F-responsive promoters (*95*).

Some of the above mentioned RB interactions that enable it to modulate gene expression have been found to be conserved in *Drosophila*. The *Drosophila* BRM complex physically interacts with Rbf1 (*96*) and interestingly, components of this complex such as brahma, moira and osa are also known to genetically interact with dE2F1 (*97*). Through biochemical purification of native dE2F-Rbf complexes from *Drosophila* embryo extracts, two recent studies showed that Rbf and dE2F proteins are a part of large multi-protein complexes called dREAM (*Drosophila* RBF, E2F, and Myb-interacting proteins) and MMB (Myb-MuvB) (*98*, *99*). The Rbf containing dREAM complex is shown to be associated with nucleosome remodelers such as ISWI and NURF301 (*99*) , another ATP dependent chromatin remodeler (*100*). Both complexes contain either Rbf1 or Rbf2 together with E2F and its dimerizing partner, dDP proteins. In addition, they also involve MYB and its various interacting proteins such as MIP120, MIP130 and MIP140. These complexes have no known enzymatic activity and currently it is not clear how they repress transcription. ChIP-on-chip experiments revealed that these complexes bind to

several thousand chromosomal sites in the *Drosophila* genome performing both repression and activation of genes.

The complete profile of the *Drosophila* Rbf associated factors is not clear. A previous study from our lab identified several factors associated with Rbf2 during embryogenesis. The recovered proteins included some expected RB cofactors such as histone acetyl transferases (TRRAP), ATP-dependent chromatin remodeling complexes such as Moira, Pontin, Reptin, BAP111, BAF53, dREAM complex components. This study additionally revealed a novel association between Rbf2 and the evolutionarily conserved COP9 signalosome complex (CSN). Several subunits of the COP9 complex (CSN) including CSN1, CSN3, CSN4, CSN5, CSN6 and CSN7 were recovered during Rbf2 immunoprecipitation, suggesting that Rbf2 might be interacting with the whole complex (*101*). Further analysis showed that COP9 protects both Rbf proteins from their proteasome-mediated degradation. Also CSN4, a subunit that is essential for the integrity of the complex, was shown to co-occupy Rbf target gene promoters with Rbf1 and Rbf2 (*101*). These observations led us to hypothesize that by protecting Rbf proteins at their target promoters, COP9 may allow stable repression of Rbf target genes.

Another mechanism through which RB can cause gene repression is through direct interactions with general transcription factors. Consistent with this model, it has been shown previously that RB is capable of repressing transcription in reconstituted *in vitro* transcription systems lacking histones (*102*). By utilizing such chromatin-free systems, it was shown that RB represses transcription during early stages of pre-initiation complex (PIC) formation by inhibiting the formation of E2F/TFIID/TFIIA activator complex (*63*). Additional, *in vitro* studies suggested that RB may directly interact with the TATA-binding-protein associated factor $TAF_{II}250$ subunit of the human TFIID complex although this result has remained controversial

as some other studies showed that RB does not interact with native TFIID (*103, 104*). RB has been shown to repress transcription by RNA Polymerase I (PolI) and RNA polymerase III (PolIII) as well (reviewed in (*105, 106*)). In case of type 1 and type 2 Pol III genes, Rb represses their transcription by inhibition of PIC formation and subsequent Pol III recruitment through direct contacts with Brf1-TFIIIB (*106, 107*).

In summary, RB family proteins repress their target genes using diverse mechanisms, including direct inhibition of E2F transactivation domain, recruitment of cofactors such histone modifying proteins and nucleosome remodelers, and preventing formation of the PIC through interference with the basal transcription machinery.

Regulation of the RB/E2F network by the ubiquitin-proteasome system

In addition to their regulation by cyc/cdk complex mediated phosphorylation, the RB family proteins are also subject to regulation by the ubiquitin-proteasome pathway (Figure 1-3). This aspect of RB biology is not well appreciated and is less well characterized than RB regulation through its phosphorylation. Similar to several proteins involved in cell cycle regulation, the RB protein is also targeted for ubiquitin-dependent or ubiquitin-independent proteasome mediated turnover by several cellular and viral proteins (*108*). MDM2 is a cellular oncoprotein and E3 ubiquitin ligase for p53 that is overexpressed in many human cancers (*109*), leading to p53 degradation through the ubiquitin-proteasome pathway. Interestingly, it has been reported that MDM2 also interacts with RB (*110*). Another study showed that MDM2, like viral oncoproteins, binds to hypophosphorylated RB and this interaction blocks RB-E2F binding, thereby suppressing the repression activity of RB (*111*). MDM2 interacts with the C8 subunit of 20S proteasome and promotes RB-C8 interaction and thus enhances RB degradation (*112*). Two

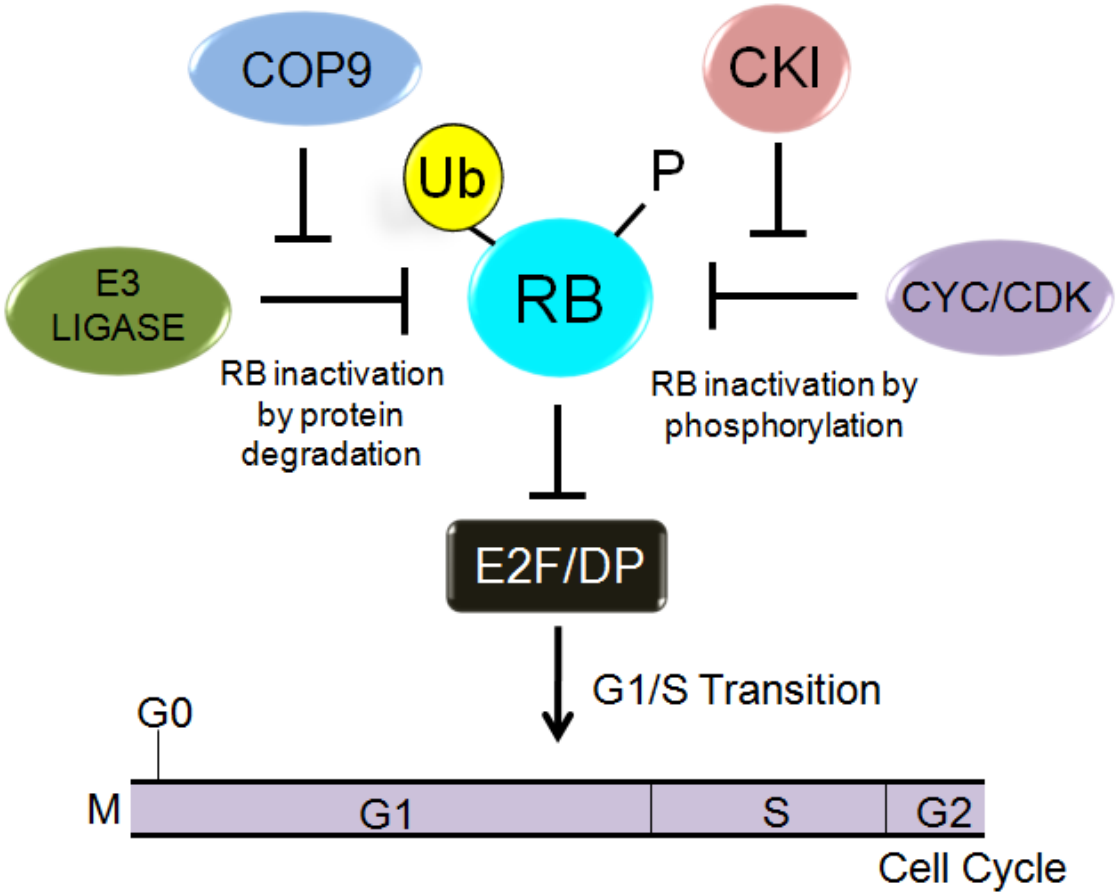

Figure 1-3. Regulation of RB by cyc/cdk phosphorylation and the ubiquitin-proteasome pathway. RB family proteins are subject to regulation through two distinct pathways. First, CYC/CDK kinases inactivate the RB protein by phosphorylation, which inhibits its ability to repress E2F-dependent transcription of genes that are required for cell-cycle progression at the G1/S phase transition. This activity of CYC/CDKs is inhibited by enzymes called Cyclin Kinase Inhibitors (CKI). In an analogous situation, the UPS is also throught to governs RB function by regulating its protein levels. The E3 ubiquitin ligase enzyme MDM2 targets RB for its ubiquitination, resulting in the downregulation of its protein levels. This activity of E3 ligase may be inhibited by the COP9 Signalosome complex that is known to protect the *Drosophila* RB family proteins from their proteasome mediated degradation.

different groups have demonstrated that MDM2 targets RB to ubiquitin-dependent (*113*) and ubiquitin-independent (*112*) proteasome mediated turnover. Another cellular oncoprotein, gankyrin, was shown to interact with RB and promote proteasome mediated turnover (*114*).

Interestingly, RB stability is also affected by certain viral oncoproteins that bind and inactivate RB leading to cellular transformation. The E7 protein of HPV binds to the LxCxE binding motif of RB (*113*) targeting the protein to ubiquitin-proteasome dependent degradation (*114, 115*). The human cytomegalovirus (CMV) pp71, was shown to promote degradation of all three mammalian RB proteins via a ubiquitin-independent but proteasome-dependent pathway (*116*). Epstein-Barr virus, that causes lymphoid malignancies in humans, produces an Epstein-Barr virus nuclear antigen 3C (EBNA3C) oncoprotein that interacts with and destabilizes RB by forming a complex with SCF^{Skp2} E3 ubiquitin ligase (*117*). Hepatitis C virus NS5B also interacts with and down-regulates RB levels (*118*). Similar to RB, both p107 and p130 are inactivated through proteasome mediated degradation by SV40 Large T antigen (*119*). These studies demonstrate that the elevated degradation of RB family proteins is one of the key mechanisms in promoting tumorigenesis by tumor viruses.

Similar to the RB family proteins, the E2F protein family is also subject to regulation by the ubiquitin-proteasome pathway through targeted degradation. E2F1 is fundamentally unstable and undergoes temporally controlled elimination at defined points during the cell cycle. Several studies have pointed towards an ubiquitin-proteasome dependent pathway for E2F1 degradation. A S-phase specific F-box protein $p45^{Skp2}$ containing SCF E3 ligase targets E2F1 for ubiquitination and subsequent degradation in the S/G2 phases of the cell-cycle (*120*). Consistent with its critical role in human cancer, a variety of perturbations in the E2F1 degradation pathway, leading to elevated E2F1 protein levels, have been reported in various human cancers.

A key cellular protein, MDM2, in contrast to its known E3 ligase activity, was shown to prolong E2F1 half-life by inhibiting its ubiquitination by displacing the SCF^{Skp2} complex and in the process augmenting its transcriptional activity (*121*). It is interesting to note that MDM2 is frequently overexpressed in many human tumors and this is correlated with a more aggressive tumor phenotype (*122*). Interestingly, another regulatory factor in E2F1 degradation turned out to be the RB protein itself. Overexpression studies revealed that RB stabilizes E2F1 by binding to a carboxy-terminal instability element in E2F1 (*123*). One study demonstrated that RB inhibits E2F1 ubiquitination and degradation, perhaps by preventing cellular ubiquitination machinery from recognizing E2F1 (*124*). These observations point towards an interesting possibility that mutations in upstream regulators of E2F1 turnover such as MDM2 and RB may result in elevated E2F1 levels, with concomitant increase in E2F1-driven cellular proliferation. They also highlight the importance of identifying more such regulators of E2F1 turnover such that they could be used as potential prognostic markers in human cancers.

In Chapter IV, we show that similar to its mammalian counterpart, the degradation of the *Drosophila* homolog of E2F1 (dE2F1) occurs in an ubiquitin-proteasome dependent manner and that this is governed by one of the two *Drosophila* Retinoblastoma-family proteins, Rbf1, and an evolutionarily conserved protein complex, COP9 signalosome. We additionally demonstrate the potential ramifications of a stabilized and activated dE2F1 on cellular proliferation. A specific class of inactivating mutations in the C-terminal domain of Rbf1 tumor suppressor retains the capacity to stabilize dE2F1 in vivo and this may lead to enhanced S-phase progression in *Drosophila* S2 cells.

Ubiquitin-proteasome system and transcription

Recent evidence suggests that deep mechanistic connections exist between transcription and the ubiquitin-proteasome systems (UPS) (*125*). Just about every step of transcription, from initiation through to mRNA export, is influenced by the UPS. The UPS pathway is one of two major intracellular proteolytic pathways in the cells (*126*). Ubiquitin-mediated protein degradation is a multistep process that typically involves a three reaction cascade (*127*). In the first reaction, the ubiquitin activating enzyme, E1, hydrolyses ATP and forms a high-energy thioester linkage with ubiquitin. The 'activated' ubiquitin is then transferred to the active-site cysteine of a Ub-conjugating enzyme, E2. The E2-Ubiquitin then functions in concert with a E3-Ubiquitin ligase to attach ubiquitin to an amino group on the substrate, typically at a lysine residue. There are hundreds of E3 ligases that provide specificity through direct protein-protein interactions with their substrates. A protein could be simply monoubiquitinated or may undergo additional rounds of ubiquitination leaving them in a polyubiquitinated state. The polyubiquitinated substrate is then targeted to the 26S proteasome, where it is deubiquitylated, unfolded by chaperone proteins and finally degraded through proteolysis (*127*).

The cellular levels of several key transcription factors that regulate cell growth, such as p53, Myc, Jun, Fos and E2Fs are governed by the UPS (*123, 128-133*). Intriguingly, recent studies have shown that ubiquitin-mediated proteolysis can counterintuitively promote the activity of several transcriptional activators such as p53, β-Catenin, Rpn4, glucocorticoid receptor (GR), c-Jun, Hif1α, VP16, Myc, Gcn4, Ste12, androgen receptor (AR), estrogen receptor, Gal4 and the Notch intracellular domain (*133-140*). Curiously, it has been noted that in case of many of these unstable activators, their transcriptional activation domains (TADs) often overlap with their degrons (*134*). Although the mechanism of how degradation and

transcriptional activation are linked is not clear, a model proposed by Bill Tansey's group suggests that, when activators interact with general transcription machinery, they recruit ubiquitin ligases to the site of transcription. These ubiquitin ligases then ubiquitinate several factors such as the activators, RNA polymerase II and histones, which in turn recruit the 26S proteasome. This model proposes the possibility of ubiquitin modification on activators serving as a dual tag that mediates the proteasome-mediated destruction of the activator as well as licences the protein to perform transcriptional activation possibly through the recruitment of co-repressors which promote transcriptional elongation (*134*). One study showed that the ubiquitination of VP16 activator increased its interaction with P-TEFb that augments rates of elongation of transcription (*141*). Recently, it has also been proposed that the 26S proteasome components play a direct role in regulation of transcription (*134, 142*).

Although established for activators, this instability-activity link has not been established for any repressors. In chapters II and III, I discuss my findings that suggest that there is an instability element (IE) in the C-terminus of Rbf1 that functions as an independent degron and is critical for Rbf1 function as a transcriptional repressor. I further present evidence that the instability mechanism is conserved in the p107 human homolog. These findings suggest that the turnover of RB family proteins is required for their activity and that the instability-activity relationship holds for repressors as well as activators, possibly representing a general property of these proteins in multicellular organisms. In chapter III, I discuss my findings that suggest that Rbf1 IE directed ubiquitination contributes to Rbf1 repression of E2F-dependent target genes but not E2F-independent target genes. Thus, this work uncovers a potential mechanism of regulatory discrimination of the diverse RB targets that participate in seemingly divergent cellular processes. In chapter V, I discuss my findings that Rbf2 harbors instability-elements within its

pocket domain that direct Rbf2 ubiquitination and subsequent degradation. I further present evidence that Rbf2 N-terminus is required for the repression of E2F-dependent target genes. Overall, my studies have brought to light several crucial aspects of RB biology that were previously unappreciated.

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CHAPTER TWO

PARADOXICAL INSTABILITY-ACTIVITY RELATIONSHIP DEFINES A NOVEL REGULATORY PATHWAY FOR RETINOBLASTOMA PROTEINS[1]

[1]The work described in this chapter was published as the following manuscript: Pankaj Acharya*, Nitin Raj*, Martin S. Buckley*, Liang Zhang, Stephanie Duperon, Geoffrey Williams, R. William Henry, David N. Arnosti, Paradoxical instability-activity relationship defines a novel regulatory pathway for Retinoblastoma proteins. *Molecular biology of the cell* **21**, 3890 (Nov 15, 2010) (* co-first authors)

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 especially 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.

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 of human cancers (*1, 2*) 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 (*3*).

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 (*4*). 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 (*5*). 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 (*6, 7*). 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 (*8, 9*). 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 (*10*). The *Drosophila* Rbf proteins provide canonical cell cycle control functions, and they are similarly regulated by phosphorylation involving cyclin/cdk complexes (*11-13*). 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 (*14*). This linkage may contribute to COP9 control of cell cycle and development in plants and animals (*15*). 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 (*16, 17*). The COP9 signalosome may also control protein degradation through interactions with and subsequent deneddylation of the cullin subunits of SCF ubiquitin E3 ligase complexes (*15*). 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 (*14*), 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 (*14*).

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 (*18-20*). Synthetic constructs with multiple degradation domains exhibit higher levels of transcriptional activation, suggesting that the correspondence is not just coincidental (*19, 20*). 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 (*21, 22*). 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 (*23-26*). 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.

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 the last exon) into pCaSpeR (*27*) 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 (*28*) into *Kpn*I and *Xba*I sites of pAX vector (*29*). 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 (*30*). For bacterial expression of GST fusion proteins, the pRSF Duet-1 vector (Novagen, Narmstadt, Germany) was modified to introduce a GST ORF followed by a ligation independent cloning (LIC) site into its multiple cloning site (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 Reproter Assay

Drosophila S2 cells were transfected using Effectene transfection reagent (Qiagen, Valencia, CA) 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, Madison, WI) and 0.2 µg of one of pAX-*rbf1* constructs. Cells were harvested 72 h after transfection, and luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega) and quantified using the Veritas microplate luminometer (Turner Biosystems, Synnyvale, CA). Firefly luciferase activity was normalized to renilla luciferase activity.

Immunocytochemistry

Drosophila S2 cells were transfected with 400 ng of each *rbf* mutant using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Cells were grown directly on cover slips pretreated with 0.01% poly-L-Lysine (Sigma, St. Louis, MO). 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, permealized in PBS + Triton-X-100 (0.4% vol/vol) 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% wt/vol 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, Mannheim, Germany, and Invitrogen, Carlsbad, CA). Cells were then washed three times in TBST and mounted in Vectashield mounting medium

(Vector Laboratories, Burlingame, CA) 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 MgCl2, Complete mini-EDTA free protease inhibitor cocktail, Roche, Indianapolis, IN). 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 analyzed 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% nonfat dry milk. Blots were developed using HRP-congjugated secondary antibodies (Pierce, Rockford, IL) and SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) 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, anti-Groucho (mouse mAb obtained from Developmental Studies Hybirdoma Bank and used at 1:50 dilution) and anti-Rbf1 antibody as described previously (*28*).

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 d then

treated with 50 µg/ml MG132 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. Seventy-two hours 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 h at 37˚C. The proteins were purified on Glutathione sepharose beads (GE Healthcare, Piscataway, NJ). 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 h 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 analyzed by SDS-PAGE and autoradiography. For the coimmunoprecipitation assays, 200 ng Myc-tagged E2F1 and 200 ng of various Flag-tagged Rbf1 constructs were cotransfected into S2 cells using Effectene transfection reagent (Qiagen). Cells were grown for 3 d 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 analyzed from 0- to 20-h-old embryos as described previously (*31*), except that the chromatin (1 ml) was incubated with 5 µl (5 µg) of Flag antibody (Sigma; F7425) or 2 µl H3 antibody (Abcam, Cambridge, MA; 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'-AGTTGTGCGGGTACTTGGTTTCC-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 (*32*), 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 min 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.

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 (*14*). To identify regions involved in Rbf turnover as first step toward 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 (*33*). Furthermore, previous data suggested that endogenous Rbf1 levels fluctuate during embryogenesis (*28, 33*). We initially examined the importance of the conserved central pocket domain, as well as the less-conserved N- and C-terminal regions (Figure 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 (Figure 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 (Figure 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.

Figure 2-1

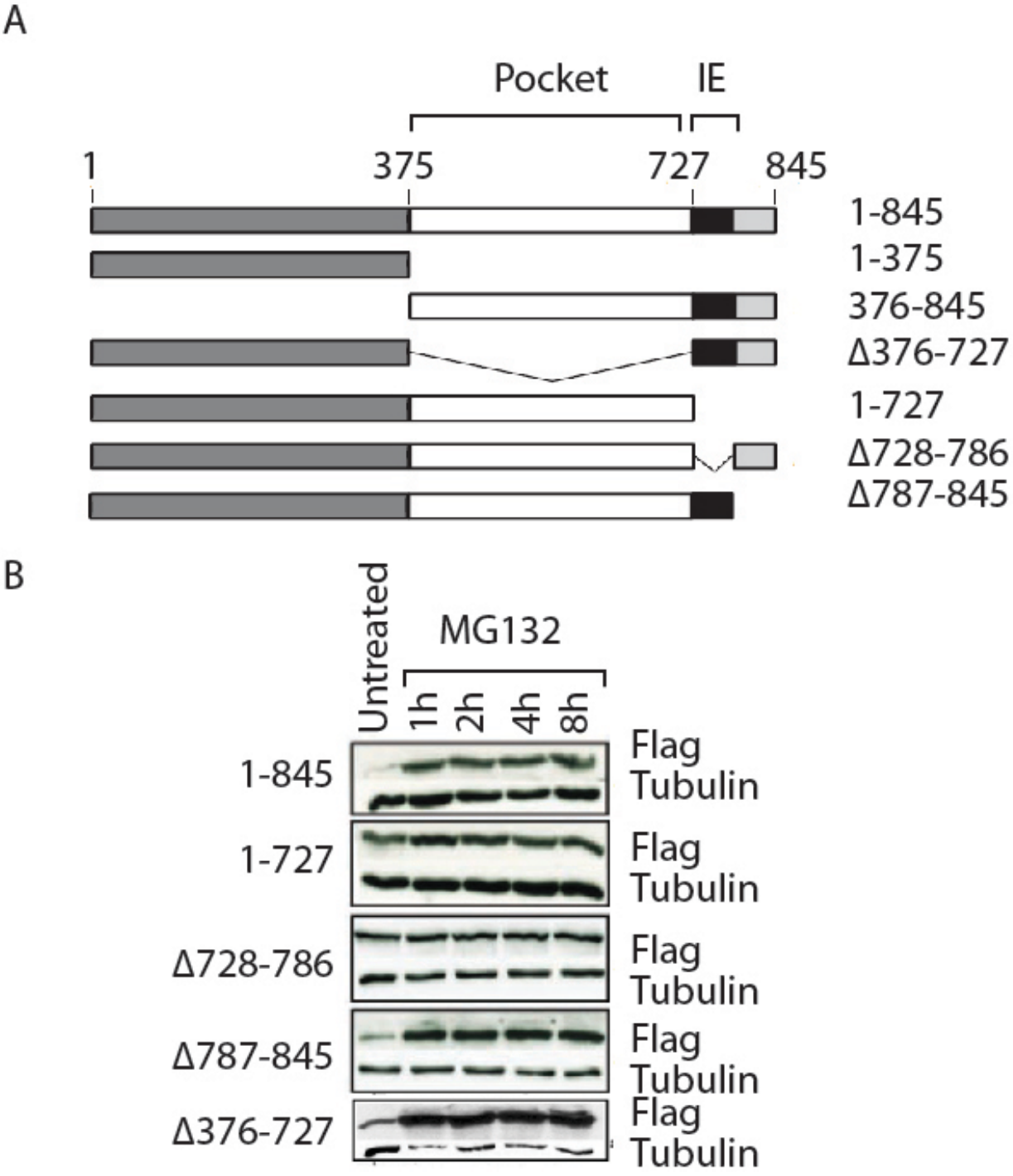

A
Pocket
IE
1
375
727
845
1-845
1-375
376-845
Δ376-727
1-727
Δ728-786
Δ787-845
B
Untreated
MG132
1h
2h
4h
8h
1-845
1-727
Δ728-786
Δ787-845
Δ376-727
Flag
Tubulin

Figure 2-1 cont'd.

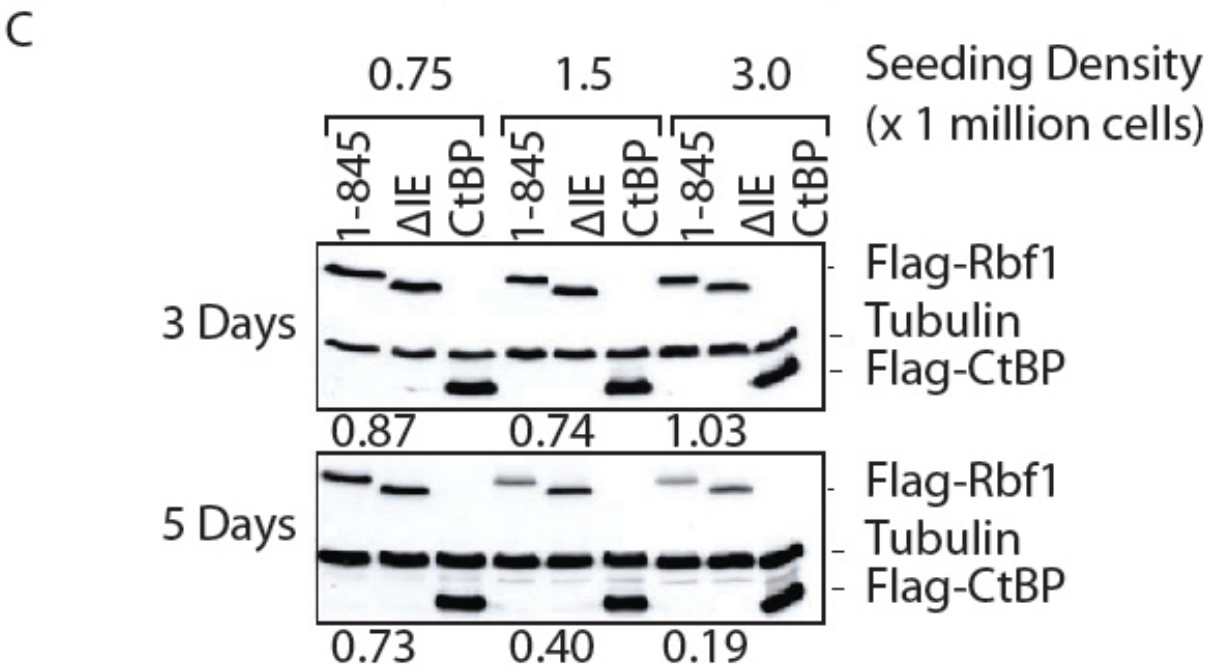


Figure 2-1 cont'd.

Figure 2-1. Identification of an instability element (IE) in Rbfl. (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 h 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 tranfected into S2 cells. At lower initial cell densities (0.75 X 10^6 / ml) and shorter growth times (3 d), 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 (1.5-3 X 10^6 / ml) grown for longer times (5 d) 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. The experiments in Figure 2-1B were performed by Dr. Martin S. Buckley, a former graduate student in the Arnosti lab.

Figure 2-2

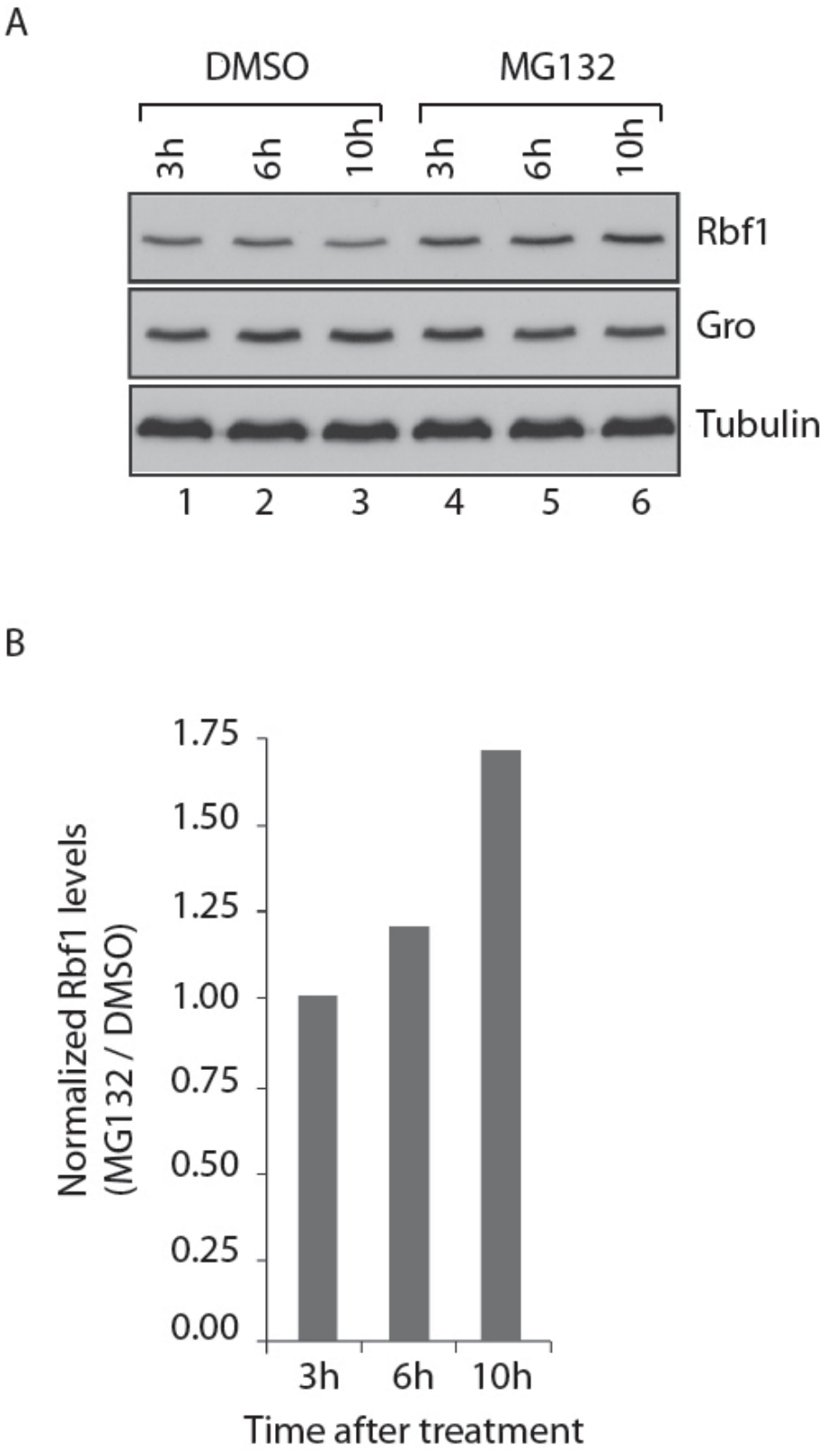

A
DMSO
MG132
3h
6h
10h
3h
6h
10h
Rbf1
Gro
Tubulin
1
2
3
4
5
6
B
Normalized Rbf1 levels
(MG132 / DMSO)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
3h
6h
10h
Time after treatment

Figure 2-2 cont'd.

Figure 2-2. (A) Effect of proteasome inhibitor MG132 on endogenous Rbf1 and Groucho corepressor protein levels. S2 cells were plated at a density of 1.5 million/ml, grown for 5 days, then treated with proteasome inhibitor MG132 or vehicle (DMSO) as indicated. The protein levels were assayed by Western blot and quantitated by photon-capture analysis with a Fuji LAS-3000 Imager. Under these cell culture conditions, the Rbf1 protein but not Groucho was found to be specifically stabilized upon MG132 treatment. (B) Quantitative assessment of Rbf1 levels shown in (A). Bars indicate ratios of Rbf1 from MG132 treated / DMSO treated cells. All values were first normalized to tubulin levels.

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.

of each protein (not shown). Under these cell culture conditions we also observed that the endogenous Rbf1 protein was stabilized by addition of MG132 (Figure 2-2). 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 (Figure 2-3; 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 (Figure 2-3A). 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 h (Figure 2-3B, 2-3C). By 6 h, 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 (Figure 2-4D).

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 Figure 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 Figure 2-4A (right panel) and imaginal disc staining in Figure 2-4, B-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 (*28*), but the Rbf1 ΔIE mutant also shows staining in these posterior cells, suggesting an abnormal perdurance of the protein (Figure 2-4C, 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 Figure 2-1C also supports this hypothesis.

Figure 2-3

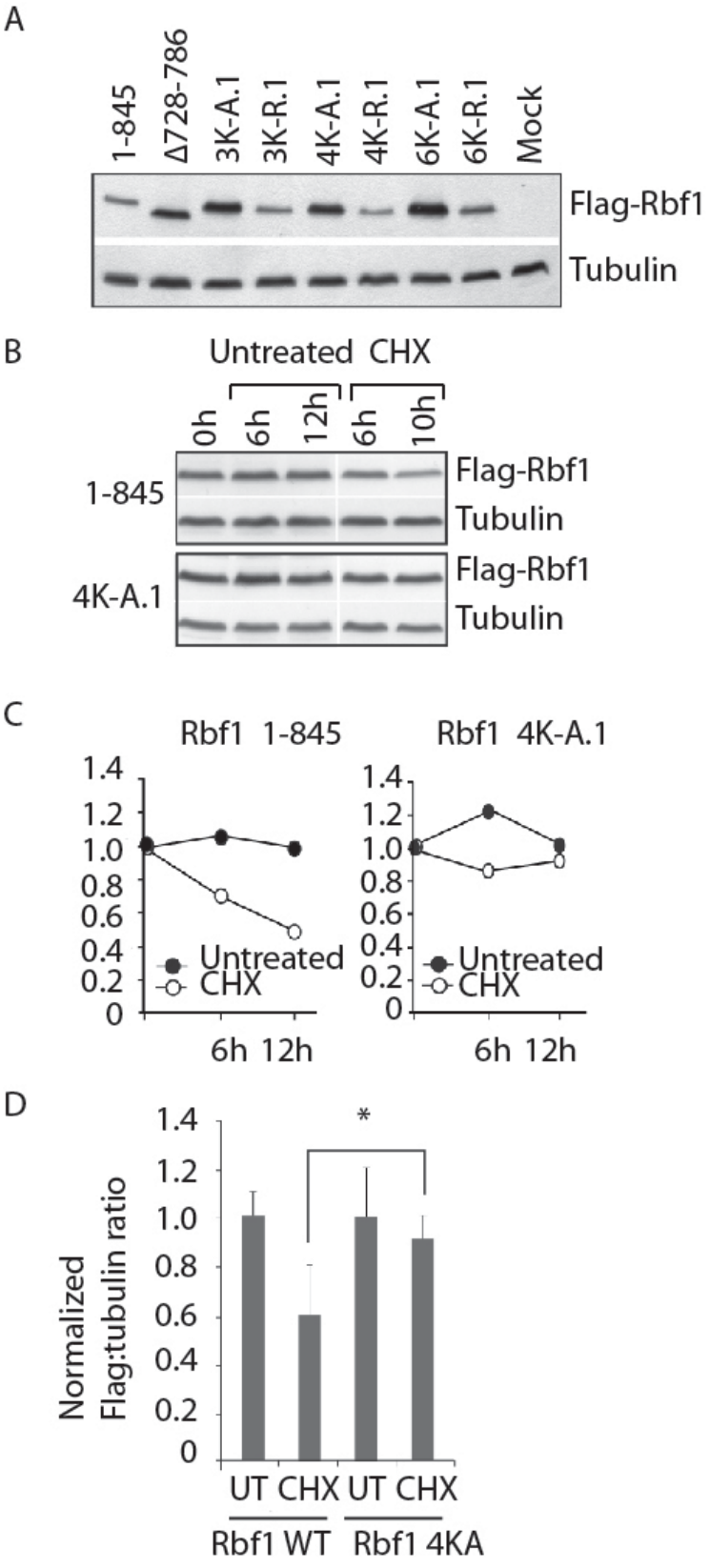

A
1-845
Δ728-786
3K-A.1
3K-R.1
4K-A.1
4K-R.1
6K-A.1
6K-R.1
Mock
Flag-Rbf1
Tubulin
B
Untreated
CHX
0h
6h
12h
6h
10h
1-845
Flag-Rbf1
Tubulin
4K-A.1
Flag-Rbf1
Tubulin
C
Rbf1 1-845
Rbf1 4K-A.1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Untreated
CHX
6h 12h
D
Normalized Flag:tubulin ratio
*
UT CHX UT CHX
Rbf1 WT
Rbf1 4KA

Figure 2-3 cont'd.

Figure 2-3. Conserved lysine residues in IE play critical roles in accumulation and stability of Rbf1. (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 overaccumulation 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 are representative of three biological experiments. (B and C) Half-life measurements of unstable wild-type and stable IE mutant proteins. Three days after transfection, cells were treated with cycloheximide and harvested at the indicated times. Rbf1 protein levels were quantified 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-h 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 ($p < 0.05$).

Figure 2-4

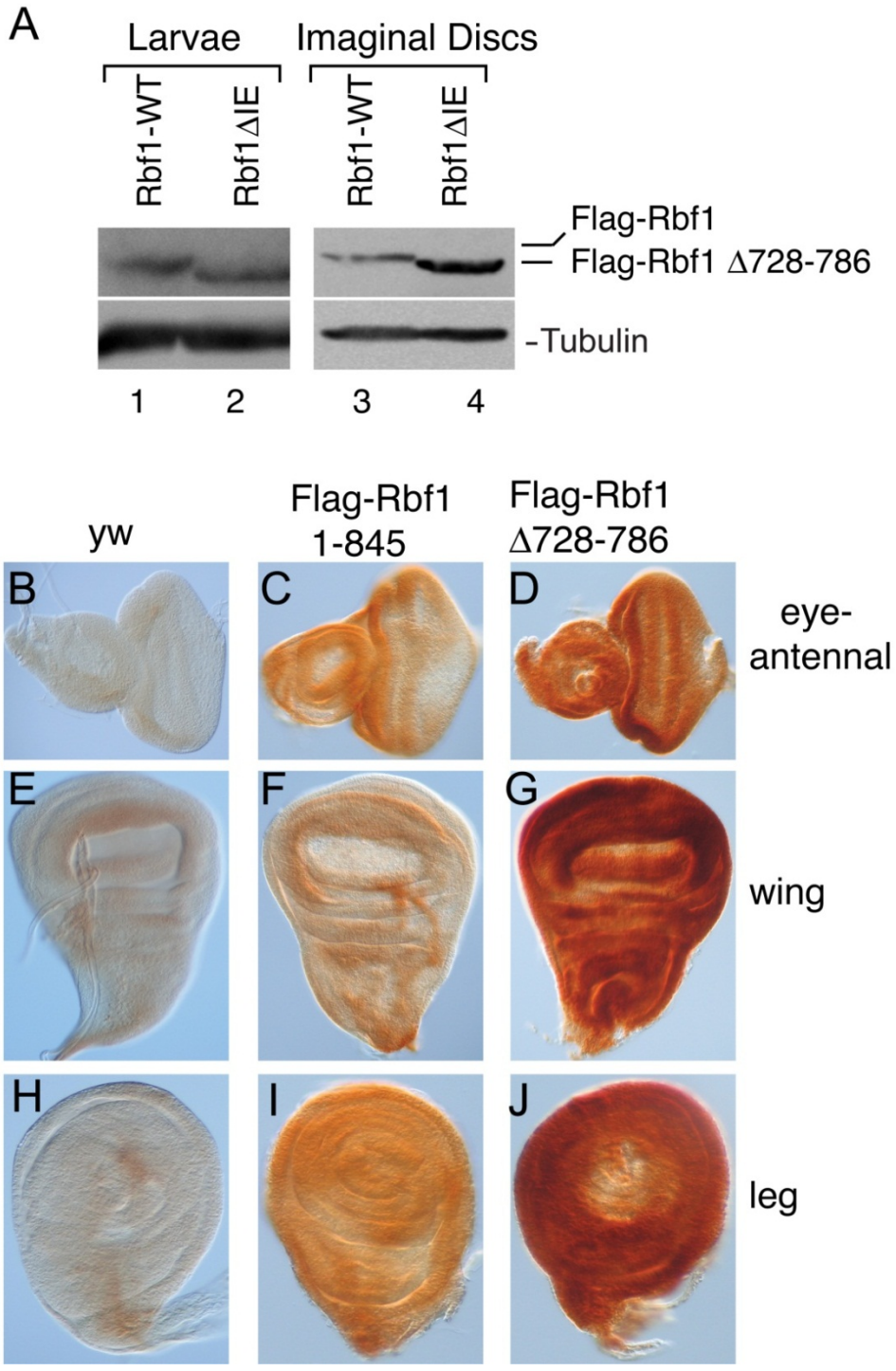

A
Larvae
Imaginal Discs
Rbf1-WT
Rbf1ΔIE
Rbf1-WT
Rbf1ΔIE
Flag-Rbf1
Flag-Rbf1 Δ728-786
-Tubulin
1
2
3
4
yw
Flag-Rbf1 1-845
Flag-Rbf1 Δ728-786
B
C
D
eye-antennal
E
F
G
wing
H
I
J
leg

Figure 2-4 cont'd

Figure 2-4. Expression of wild-type and IE mutant forms of Rbf1 in the *Drosophila* larva. 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 ~fourfold 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 nontransgenic *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 Rbf1 Δ728-786 IE mutant (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. All experiments in this figure were performed by Liang Zhang, a graduate student in the Arnosti lab. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

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 cotransfected with expression plasmids encoding wild-type or mutant Rbf1 proteins and the effect on repression potency was determined using *PCNA*-luciferase reporter construct, which is sensitive to repression by Rbf1 (*34*). 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 (Figure 2-5A). 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 (*35*). 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 (Figure 2-5B). Second, loss of the adjacent C-terminal 59 amino acids (Δ787-845) did not abolish repression but did change its subcellular localization so that the protein was no longer strictly nuclear. These data indicate that this region harbors a nuclear targeting element governing Rbf1 cytoplasmic/nuclear distribution.

Figure 2-5

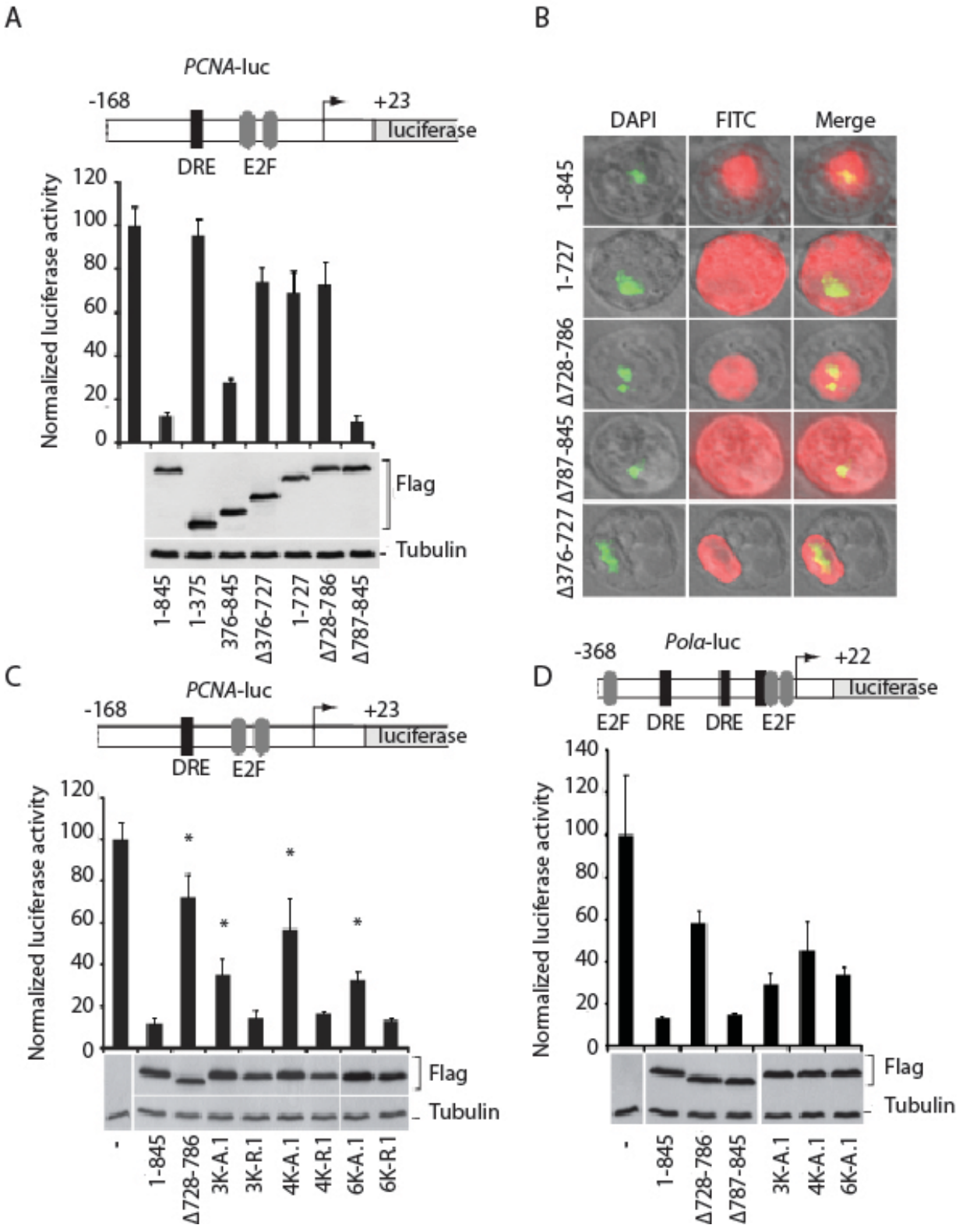

A
PCNA-luc
-168
+23
luciferase
DRE
E2F
Normalized luciferase activity
120
100
80
60
40
20
0
Flag
Tubulin
1-845
1-375
376-845
Δ376-727
1-727
Δ728-786
Δ787-845
B
DAPI
FITC
Merge
1-845
1-727
Δ728-786
Δ787-845
Δ376-727
C
PCNA-luc
-168
+23
luciferase
DRE
E2F
Normalized luciferase activity
120
100
80
60
40
20
0
*
*
*
*
Flag
Tubulin
-
1-845
Δ728-786
3K-A.1
3K-R.1
4K-A.1
4K-R.1
6K-A.1
6K-R.1
D
Pola-luc
-368
+22
luciferase
E2F
DRE
DRE
E2F
Normalized luciferase activity
140
120
100
80
60
40
20
0
Flag
Tubulin
-
1-845
Δ728-786
Δ787-845
3K-A.1
4K-A.1
6K-A.1

Figure 2-5 cont'd

Figure 2-5. Rbf1 requires the IE for transcriptional repression. (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 SD, 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 2-5A represents two biological replicates, each with three technical replicates, except for 1-845 and Δ728-786, which represent 16 and 9 biological replicates respectively. Other transfections include data from at least three biological replicates. Firefly luciferase activity is expressed relative to Renilla luciferase control. 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.

As observed for deletion of the entire IE (Δ728-786), removal of portions of this 59-aa region in blocks of 20 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 Figure 2-5C, 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 residues (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 overaccumulate, and significantly, were just as potent as wild-type Rbf1 for transcriptional repression. Based on these data, we conclude that these residues contribute both 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 *Polα* promoter, which has somewhat different requirements for E2F and DP activation compared with the *PCNA* promoter (Figure 2-5D) (*36*). 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 domains as well as the carboxy terminus of the human RB protein can make molecular contacts with E2F1 (*37-39*). 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 coimmunoprecipitation (CoIP) 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 (Figure 2-6A, lanes 5 and 6). No interaction was observed with beads alone or GST protein (Figure 2-6A, lanes 3 and 4). Similarly in CoIP assays from *Drosophila* S2 cells, Myc-tagged E2F1 coprecipitated with Rbf1 1-845 and two IE mutants (Δ728-786 and 4K-A.1) but not with the pocket domain deletion mutant (Δ376-727) (Figure 2-6B; 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 (Figure 2-6C). Binding at the intergenic locus and a nontarget gene (*sloppy paired 1*) promoter was assesses 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

Figure 2-6

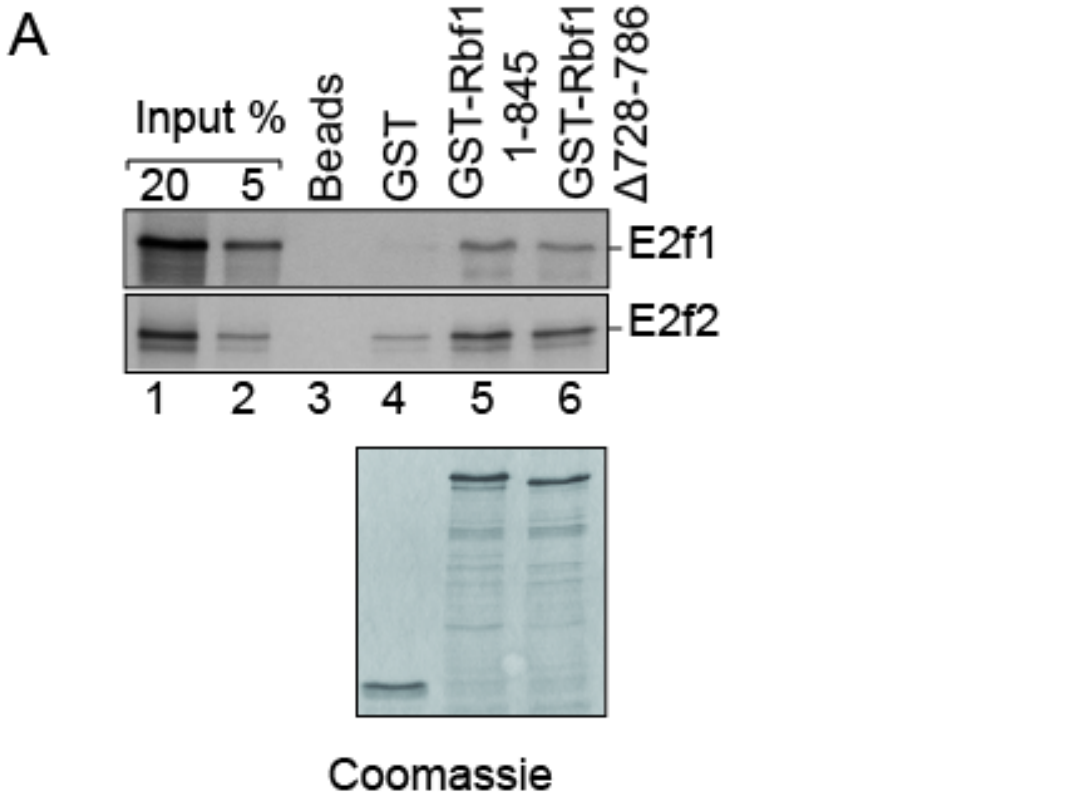

A
Input %
20
5
Beads
GST
GST-Rbf1 1-845
GST-Rbf1 Δ728-786
E2f1
E2f2
1
2
3
4
5
6
Coomassie
B
Flag-Rbf1
Myc-E2f1
1-845
1-845
Δ728-786
4K-A.1
Δ376-727
Mock
No extract
Myc-E2f1
Heavy Chain
Flag
1
2
3
4
5
6
7
8

Figure 2-6 cont'd.

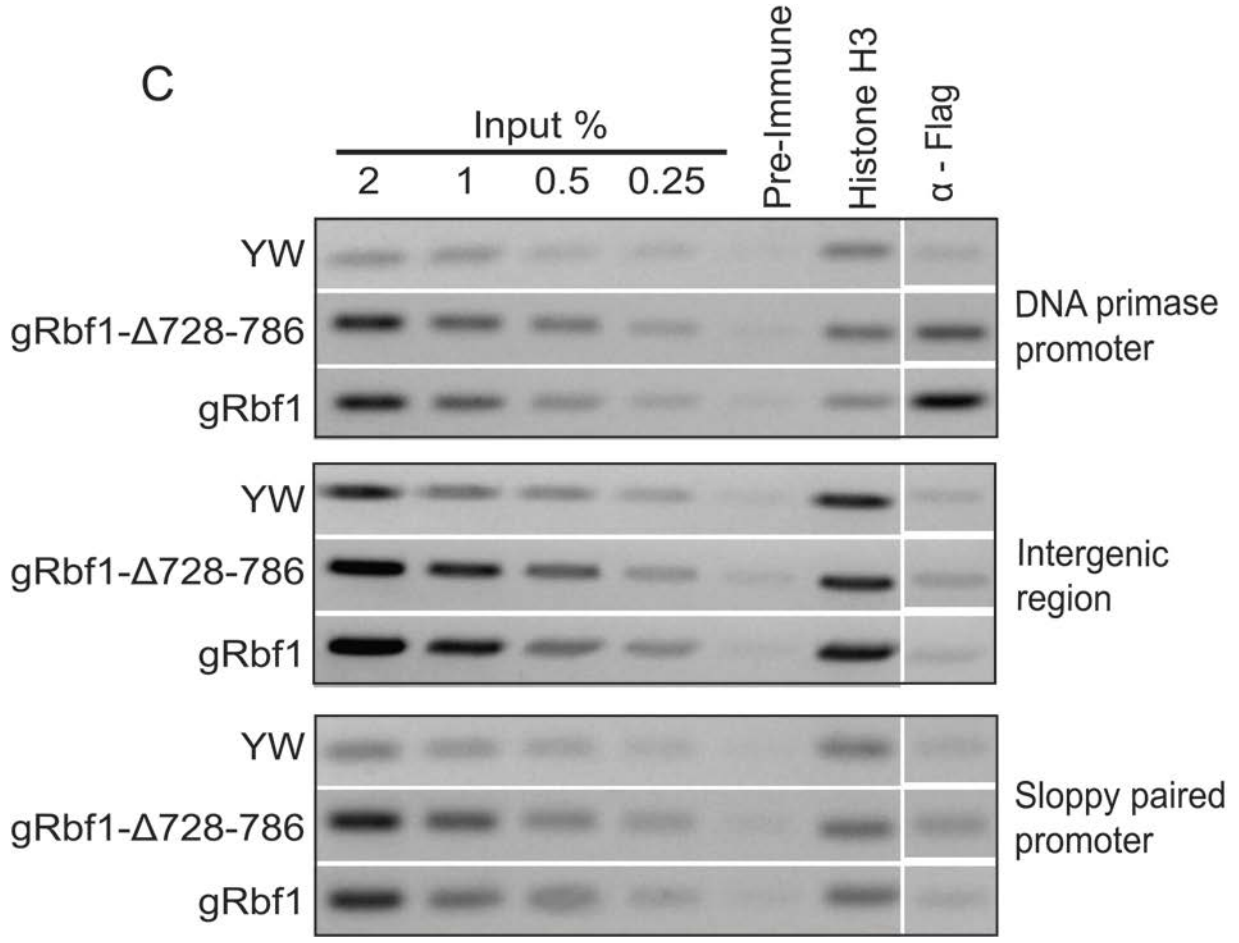


Figure 2-6. Rbf1 IE is not essential for E2F interactions and promoter binding. (A and B) Physical association between Rbf1 IE mutants and E2F proteins. (A) GST-Rbf1 and E2F interaction assay. Indicated GST fusion proteins were bound to radio-labeled E2F proteins and bound proteins were analyzed by SDS-PAGE 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 are representative of three biological replicates.

Figure 2-6 cont'd.

(B) Coimmunoprecipitation assay. Rbf1/E2F1 interactions in cotransfected S2 cells. Cells were cotransfected 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 coprecipitated with Rbf1 1-845 and two IE mutants (Δ728-786 and 4K-A.1) but not with the pocket domain deletion mutant (Δ376-786) (top panel, lanes 3-6). Mock is IP performed using cell lysate from untransfected cells (lane 7). The asterisk indicates a nonspecific 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 are 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 h 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 mutant fly embryos but not in reactions using pre-immune IgG (top panel) or at an intergenic locus (middle panel) and a nontarget gene promoter (*sloppy paired 1*) (bottom panel). The experiments in Figure 2-6C were performed by Dr. Pankaj Acharya, a former graduate student in the Arnosti lab.

occupy promoters (Figure 2-6C; top panel). Binding of the IE mutant at this locus was slightly reduced compared with the wild-type Rbf1 although the association was significantly above background as no enrichment was observed at an intergenic locus (middle panel) or the nontarget *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.

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 with 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 Figure 2-7A, 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 (Figure 2-7, A and 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 (Figure 2-7, A and 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, for mutants with only single point mutations, we did not observe the robust stabilization of mutant proteins compared with 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.

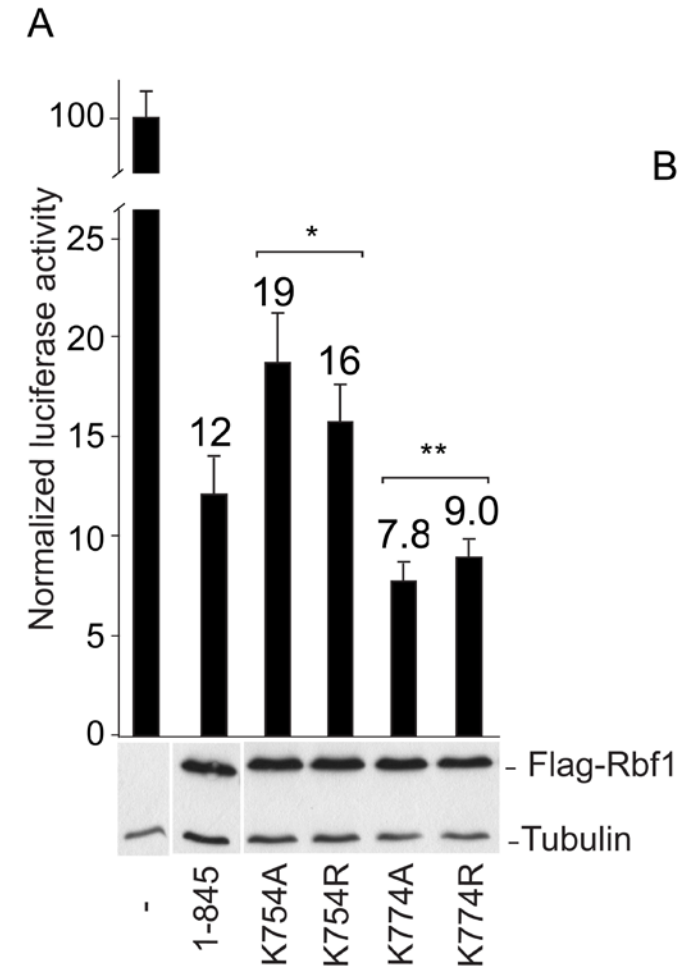
To test the physiological importance of these positively and negatively-acting residues for repressor regulation in *Drosophila*, we expressed Rbf1 isoforms in the developing eye imaginal disc using an *eyeless-Gal4* driver system (Figure 2-8, A-H). As noted in previous studies, misexpression of the 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 that the wild-type Rbf1. In contrast, the hypermorphic K774A mutant, which exhibited elevated repression activity in cell culture assays, induced dramatic phenotypes (Figure 2-8, E-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 nonexpressing controls, suggesting lethality (Figure 2-8, I and 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 (Figure 2-9A). 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 with the wild-type protein (Figure 2-9B), suggesting that the C-terminal region of p107 harbors an instability element that funnels p107 into similar turnover pathways even in this heterologous system.

Figure 2-7



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

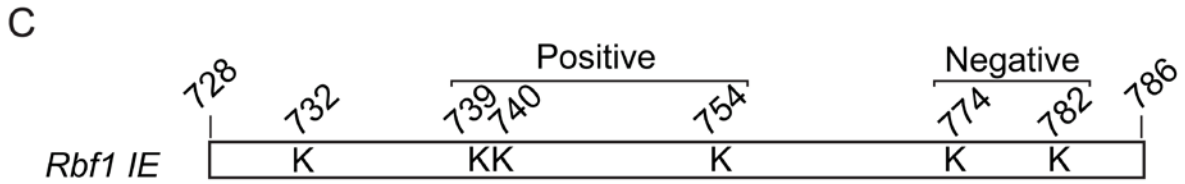
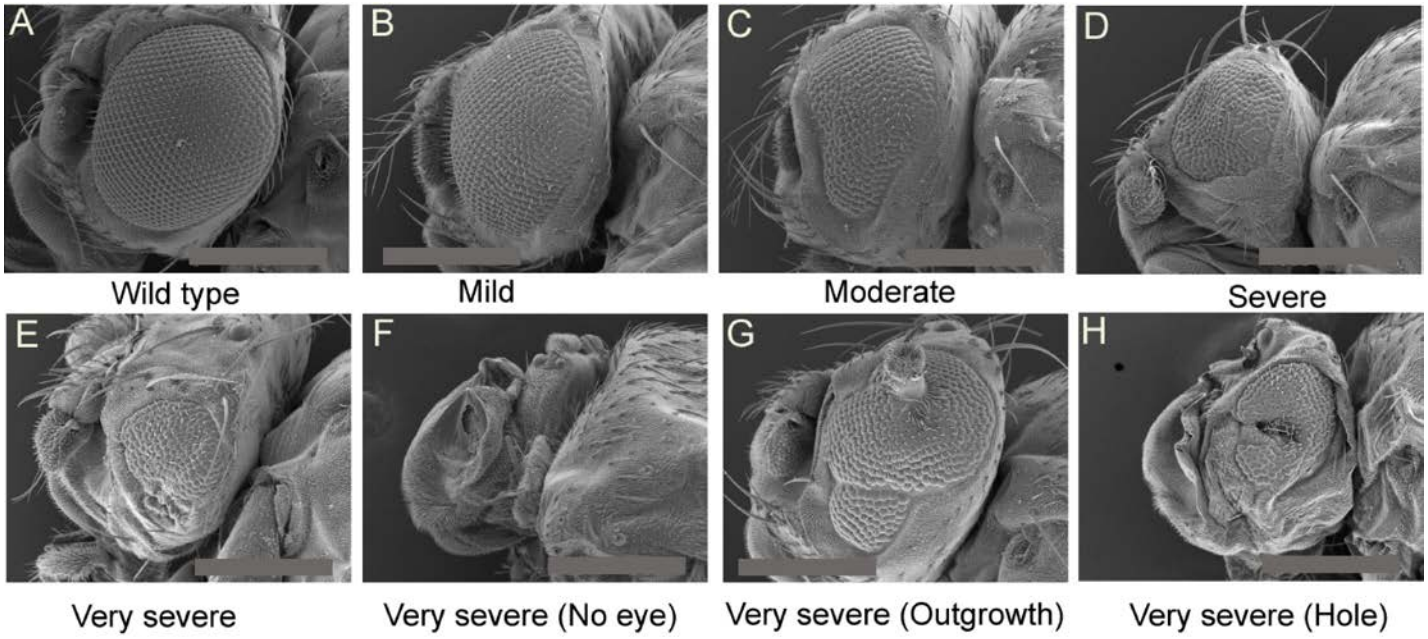
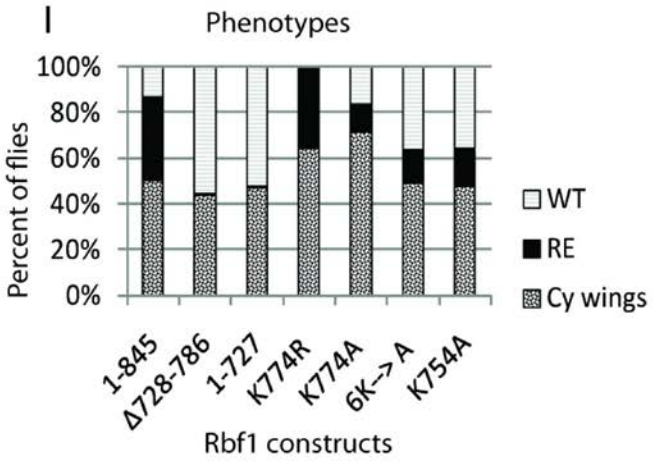


Figure 2-7 cont'd.

Figure 2-7. Rbf1 IE harbors positive and negative regulatory elements. (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 with 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 2-8


A
B
C
D
Wild type
Mild
Moderate
Severe
E
F
G
H
Very severe
Very severe (No eye)
Very severe (Outgrowth)
Very severe (Hole)


I
Phenotypes
100%
80%
60%
40%
20%
0%
Percent of flies
WT
RE
Cy wings
1-845
Δ728-786
1-727
K774R
K774A
6K--> A
K754A
Rbf1 constructs

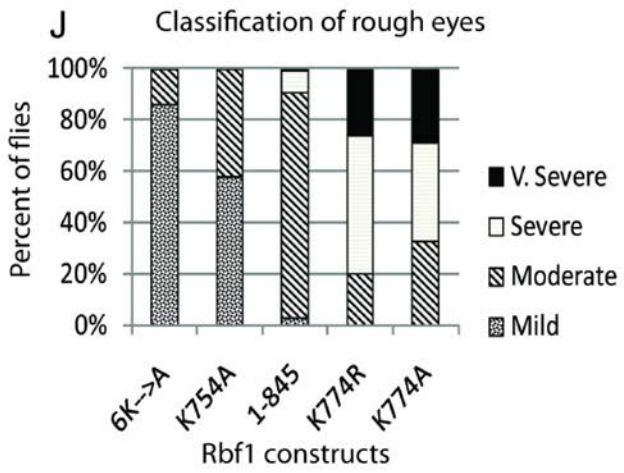

J
Classification of rough eyes
100%
80%
60%
40%
20%
0%
Percent of flies
V. Severe
Severe
Moderate
Mild
6K-->A
K754A
1-845
K774R
K774A
Rbf1 constructs

Figure 2-8 cont'd.

Figure 2-8. Severe developmental consequences of expression of hyperactive Rbf1. 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 mutants in the IE that decreased function in cell culture assays also exhibited weaker eye phenotypes, and hypermorphic K774 alleles exhibited much stronger phenotypes. The experiments in this figure were performed by Dr. Pankaj Acharya and Ms Stephanie Duperon.

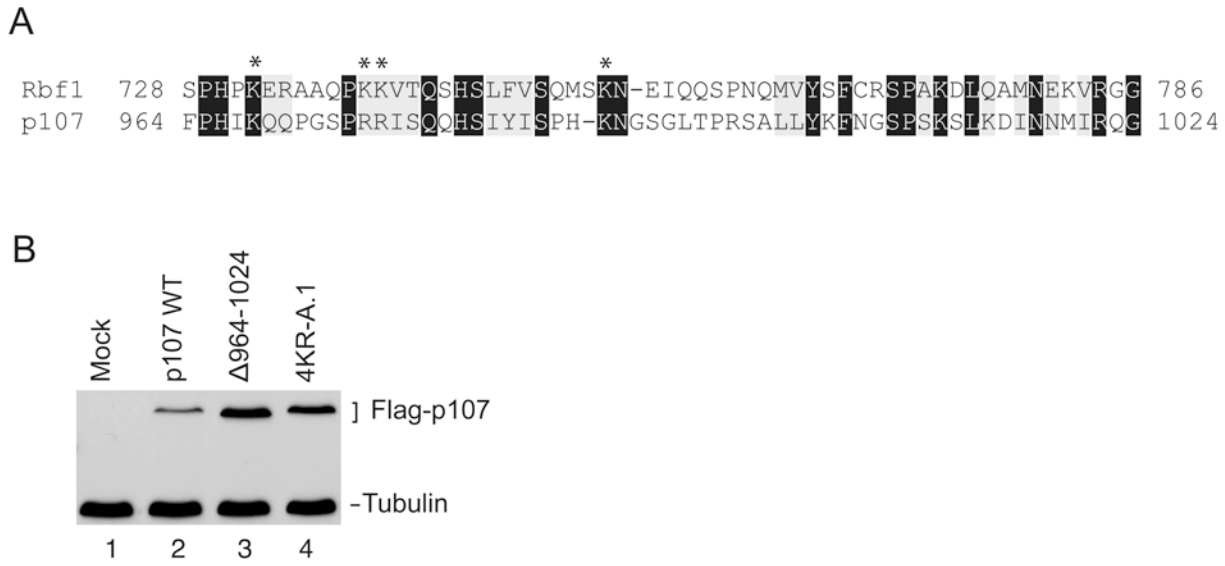


Figure 2-9. Mutations in the conserved IE of p107 enhance expression. (A) Similarities between Rbf1 IE and homologous region of p107, which is most similar to Rbf1. Asterisks mark basic residues mutated in each protein to stabilize expression. (B) Genes for Flag-tagged wild-type p107 or IE mutants were transfected into S2 cells and expression quantitated by Western blot. The 60-aa region deleted from p107 in Δ964-1024 is similar to the Rbf1 IE. Endogenous tubulin levels are shown as controls.

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 (Figures 2-1, 2-4, and 2-5).

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 (Figure 2-4). 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 cells 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 (*18*, *40*). 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 (*20*). 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 (*21*, *22*).

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 (*41*). 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 (Figure 2-7), and phosphorylation within this region by cyclin kinases can inactivate the protein (*11*). 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 of Rbf1, strongly stabilize the levels of this protein (Figure 2-9). 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 (*42*).

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 (*43, 44*). 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 (*44*). 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 (*14*). 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 (*15*). 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.

Acknowledgements

We thank Nick Dyson for sharing the *PCNA* and *Polα* luciferase reporter genes, Maxim Frolov for providing the *Myc-E2F1* construct, Stefan Gaubatz for providing the *p107* expression construct, Min-Hao Kuo and Jianjun Luo for aid with fluorescence microscopy, Jaclyn Peraino for generating Rbf1 single lysine point mutants, and John Wang for reagents and friendly advice on protein localization assays. We also appreciate the assistance from Satyaki Sengupta for his helpful discussions. This work was supported by an undergraduate summer training grant from the Michigan State University Genetics Program (to S.D.), the Michigan State University Gene Expression in Development and Disease Group, and a grant from the National Institute of Health (to D.N.A. and R.W.H.).

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CHAPTER THREE

UBIQUITINATION OF RETINOBLASTOMA FAMILY PROTEIN 1 POTENTIATES GENE-SPECIFIC REPRESSION FUNCTION[1]

[1]The work described in this chapter was published as the following manuscript: Nitin Raj*, Liang Zhang*, Yiliang Wei, David N. Arnosti, R. William Henry, Ubiquitination of Retinoblastoma family protein 1 potentiates gene-specific repression function. *The Journal of biological chemistry* [published online ahead of print Oct 18, 2012] (* co-first authors)

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.

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 (*1*). 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 (*2*). 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 (*3, 4*). 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 (*5*).

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 (*6, 7*). 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 (*8*). Canonical regulation of RB activity is governed by cyclin/cdk regulatory kinases (*9-12*). Timely phosphorylation blocks RB/E2F association, and unleashes waves of E2F-mediated transcription that contribute to cell cycle progression (*13*). However, RB continues to reside at a number of genomic sites after cyclin/cdk-mediated deactivation (*14, 15*), 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 (*8, 16*). 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 (*17, 18*). 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 (*19*). Rbf1 associates at numerous canonical E2F cell cycle-regulated genes in the early embryo (*20, 21*), 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 (*22*, *23*). 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 (*20*) with stability controlled by the COP9 signalosome (*24*), a developmentally regulated complex that controls proteasome-mediated protein degradation via modulation of E3 ubiquitin ligase activity (*25, 26*). The COP9 signalosome is physically associated with Rbf1 and Rbf2, and depletion of COP9 subunits stimulates Rbf1 turnover (*24*). Rbf1 stability is influenced by a C-terminal instability element (IE) that positively contributes to both repressor destruction and potency (*20*). 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.

Materials and Methods

Expression Constructs

Generation of Rbf1 WT and mutant expression constructs was described previously (*20*). 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 (*27*). 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 were mutated to alanine or isoleucine to prevent removal of ubiquitin by isopeptidases.

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 Figure 3-9B. 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 (50 mM Tris-HCl pH8.0, 150 mM 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 Figures 3-4B and 3-9A, 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 Figure 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 Figure 3-5A, 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 Figure 3-5B, 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, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0). The extracts were heat denatured and sonicated followed by a 10-fold dilution using dilution buffer (10 mM 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).

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 (*20, 28*). 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 (Figure 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

cycloheximide challenge, indicating that the IE directly enhanced GFP turnover (Figure 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 (*20*).

Previous models of degron function indicate that sub-cellular location of substrate proteins influences turnover (*29*). Therefore, to examine the effect of substrate localization on Rbf1-degron function, the Rbf1 nuclear localization signal (NLS, Figure 3-2) was appended to GFP-Rbf1-IE, largely confining the chimera protein to the nucleus (Figure 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 (*20*). 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 (Figure 3-3). 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 (Figure 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

Figure 3-1

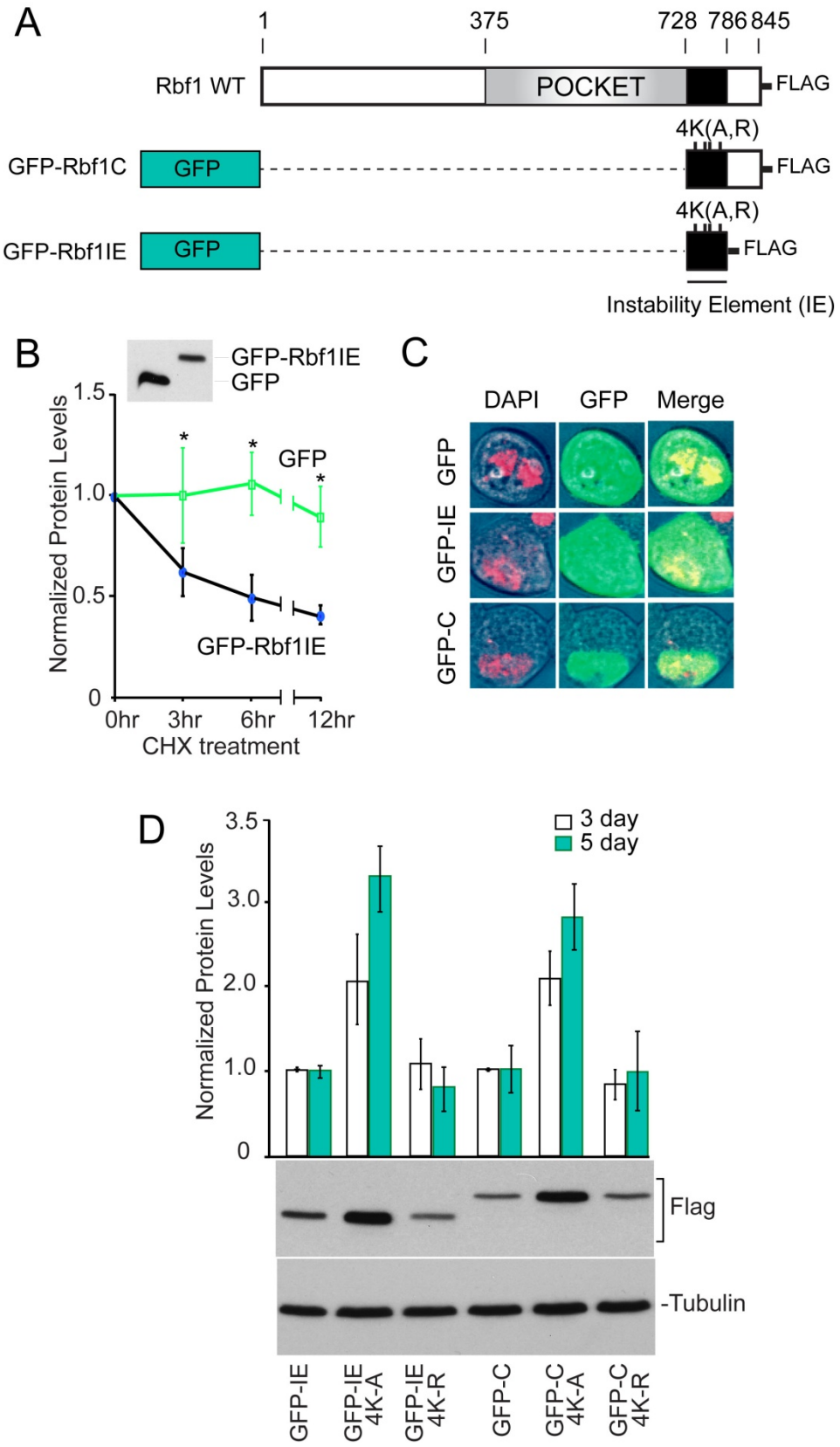
A
1
375
728
786
845
Rbf1 WT
POCKET
FLAG
4K(A,R)
GFP-Rbf1C
GFP
FLAG
4K(A,R)
GFP-Rbf1IE
GFP
FLAG
Instability Element (IE)
B
GFP-Rbf1IE
GFP
Normalized Protein Levels
1.5
1.0
0.5
0
GFP
GFP-Rbf1IE
0hr
3hr
6hr
12hr
CHX treatment
C
DAPI
GFP
Merge
GFP
GFP-IE
GFP-C
D
3 day
5 day
Normalized Protein Levels
3.5
3.0
2.0
1.0
0
Flag
-Tubulin
GFP-IE
GFP-IE 4K-A
GFP-IE 4K-R
GFP-C
GFP-C 4K-A
GFP-C 4K-R

Figure 3-1 cont'd

Figure 3-1. The instability element (IE) of Rbf1 is a modular degron. (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, $p<0.01$, $n=3$). 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. Western blot data is a representative from the 5-day set of experiments (n=2). Experiments in 3-1B, 3-1C and 3-1D (3 day) were performed by Liang Zhang, a graduate student in Arnosti lab. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 3-2

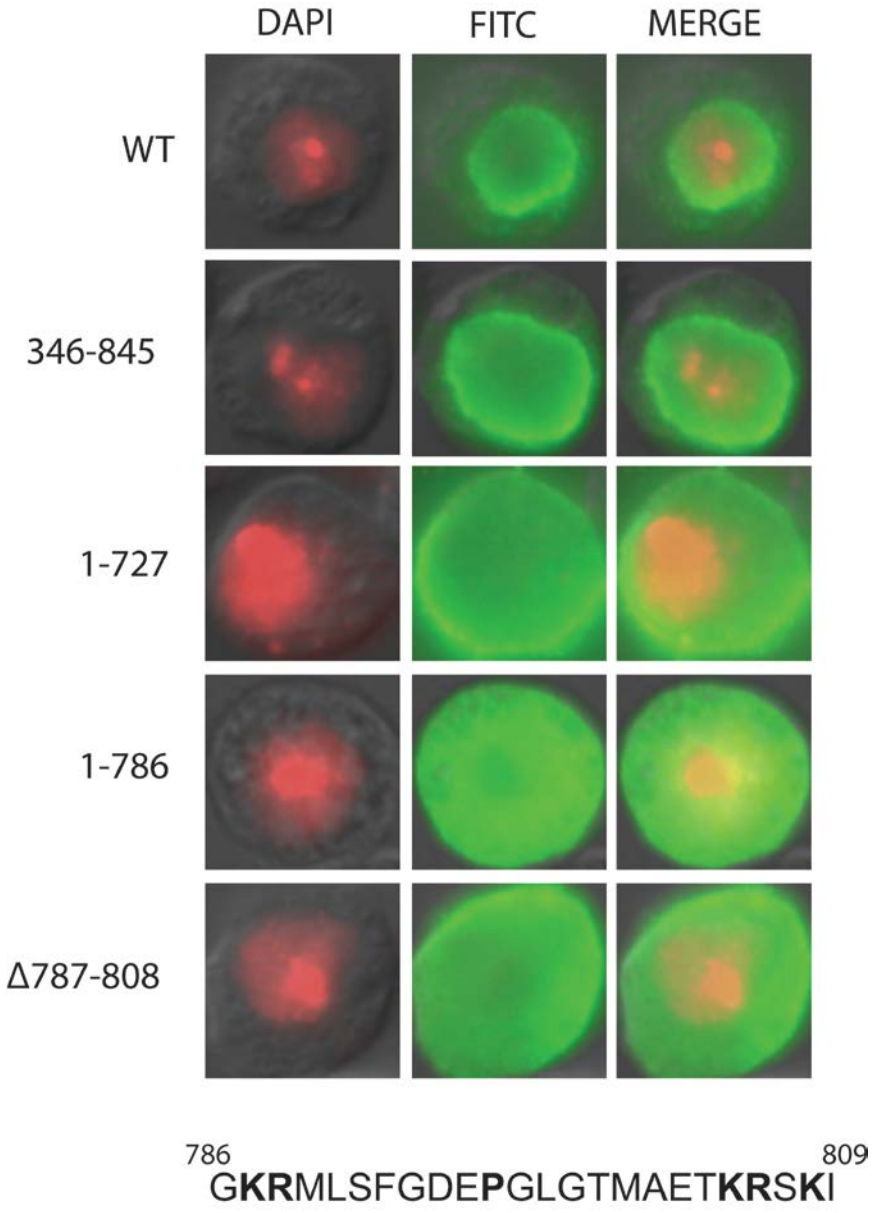
DAPI
FITC
MERGE
WT
346-845
1-727
1-786
Δ787-808
786
809
GKRMLSFGDEPGLGTMAETKRSKI

Figure 3-2 cont’d

Figure 3-2. 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). These experiments were performed by Liang Zhang. . For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

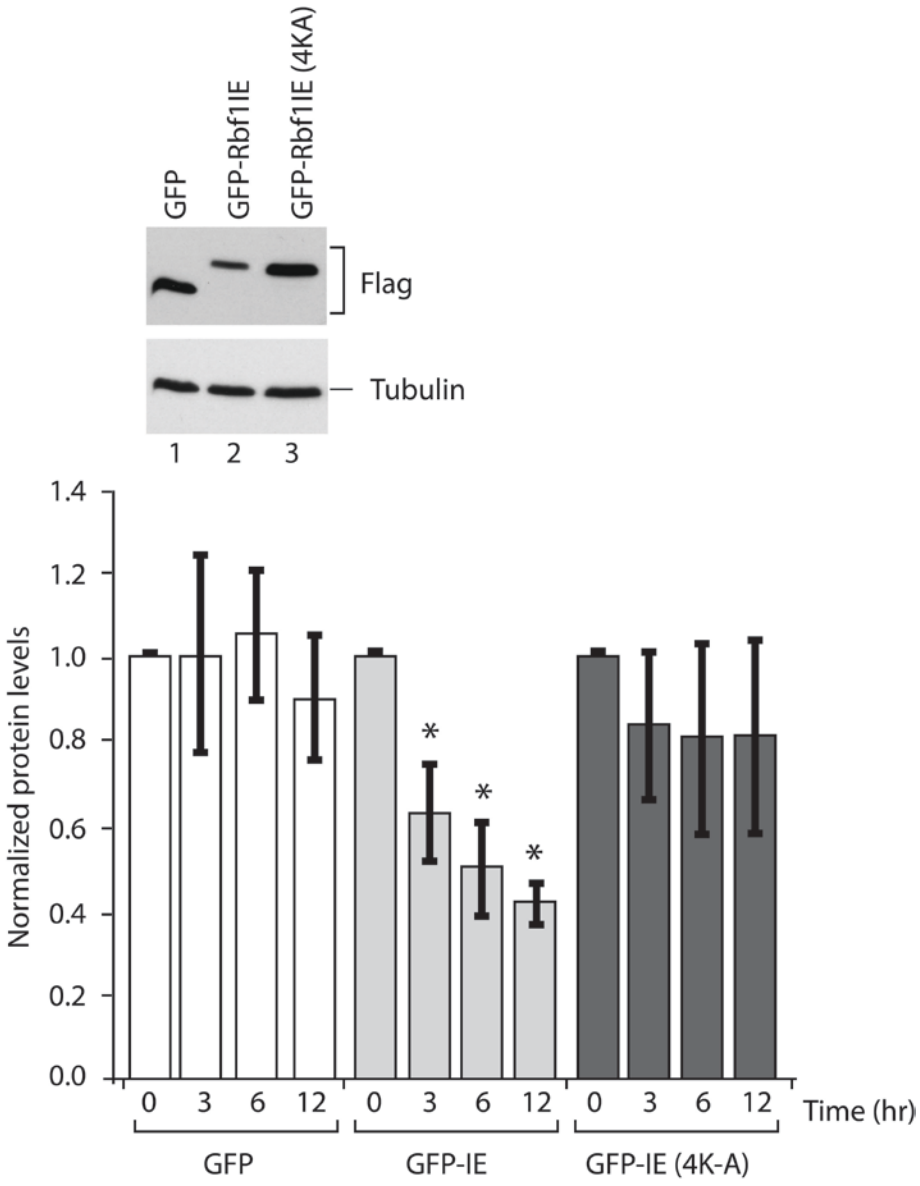

Figure 3-3. The Rbf1 C-terminal lysines (K732, K739, K740 and K754) contribute to degron function in GFP degradation. Steady state levels of GFP fusion proteins were measured after cycloheximide treatment for the indicated times. Lysine to alanine substitutions (GFP-IE 4K-A) in the IE resulted in a significant extension of protein half-life compared to the GFP-IE protein. Error bars indicate standard deviation, and asterisks indicate $p<0.05$. These experiments were performed by Liang Zhang (except inset western blot).

that are not ubiquitinated. In mammals, RB and p107 are substrates of E3 ubiquitin ligases and are turned over in a proteasome-dependent manner (*30*-*33*). 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 Figure 3-4A, 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 (Figure 3-4B). 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 (*24*). Interestingly, Rbf1 lacking the IE region (Rbf1-ΔIE) exhibited a substantial reduction, but not complete loss, of Rbf1 ubiquitination (Figure 3-7A), 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 Figure 3-5B, levels of poly-ubiquitinated GFP were substantially increased by appending the Rbf1-IE region as compared to those levels observed for untagged GFP. GFP-Rbf1 IE ubiquitination was reduced by the presence of the 4K-A substitutions (Figure 3-6). Together, these data show that one function of the Rbf1 IE is to facilitate substrate ubiquitination.

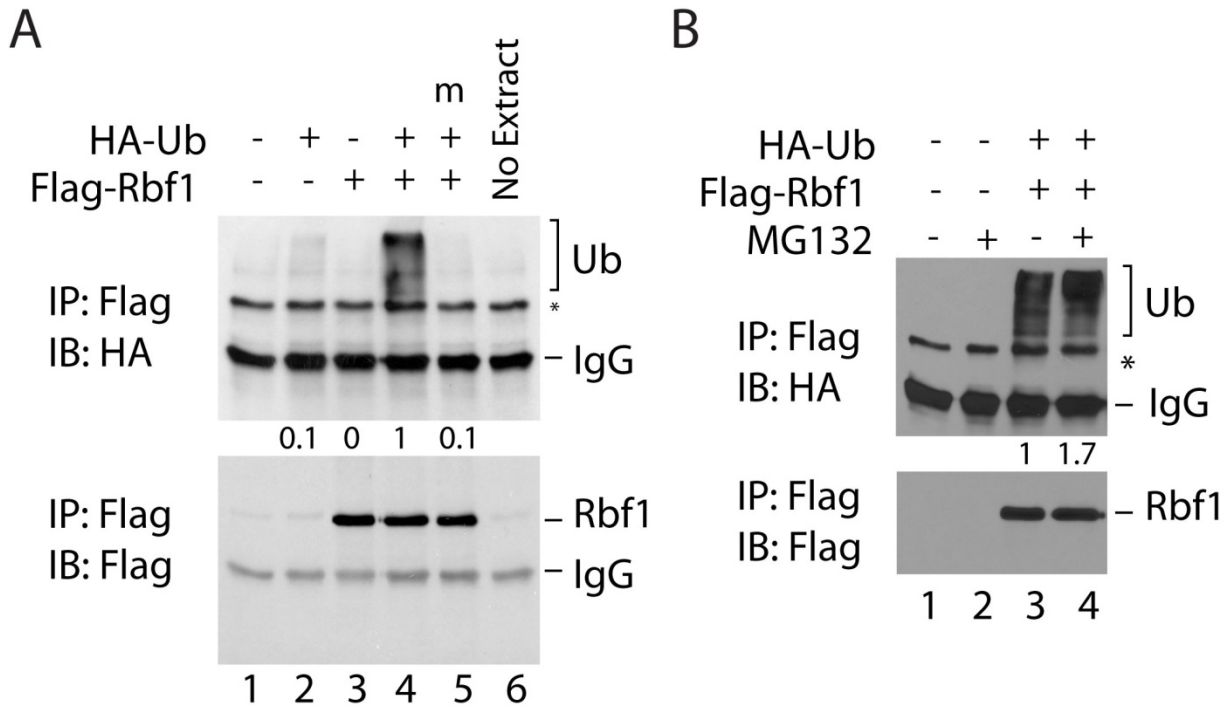

Figure 3-4. Rbf1 is degraded via an ubiquitin-proteasome dependent pathway. (A) Rbf1 is ubiquitinated in vivo. S2 cells were transfected with Flag-tagged Rbf1 and HA-tagged ubiquitin expression constructs. Denatured protein extracts were used for Flag immunoprecipitation (IP) and recovered samples were 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.

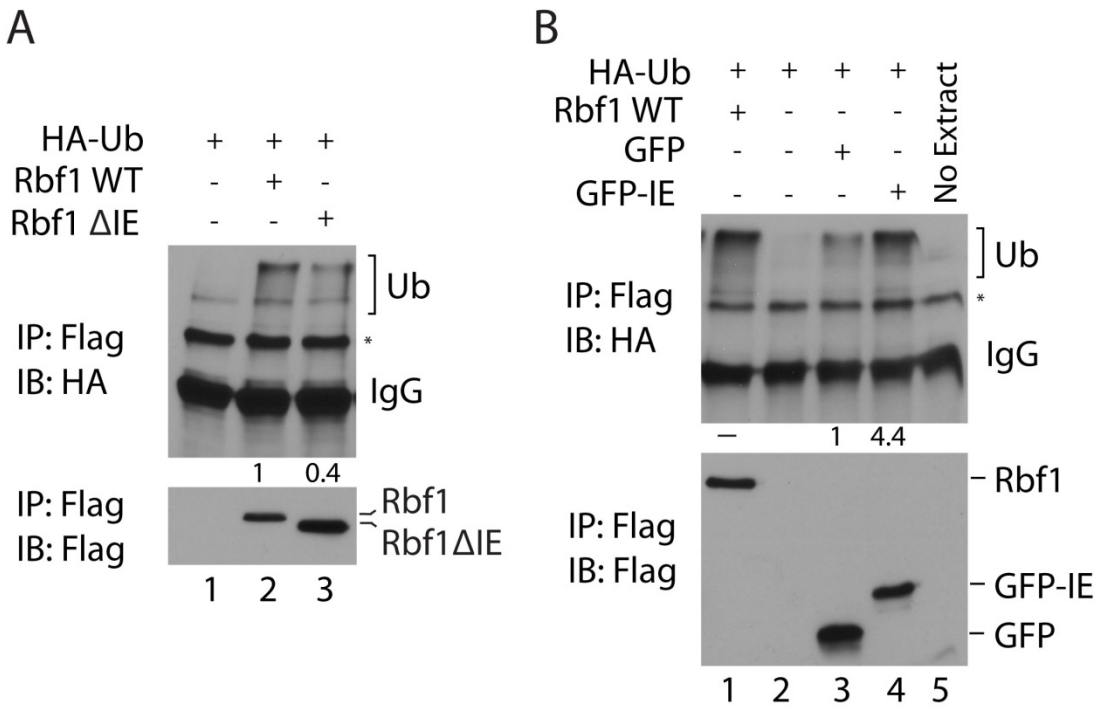

Figure 3-5. The Rbf1 instability element enhances protein ubiquitination. (A) The Rbf1 IE enhances ubiquitination. Wild type and mutant Rbf1 lacking the IE (Rbf1-ΔIE) were compared for ubiquitination as performed in Figure 3-4. (B) The Rbf1 IE is sufficient to drive the ubiquitination of a heterologous 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.

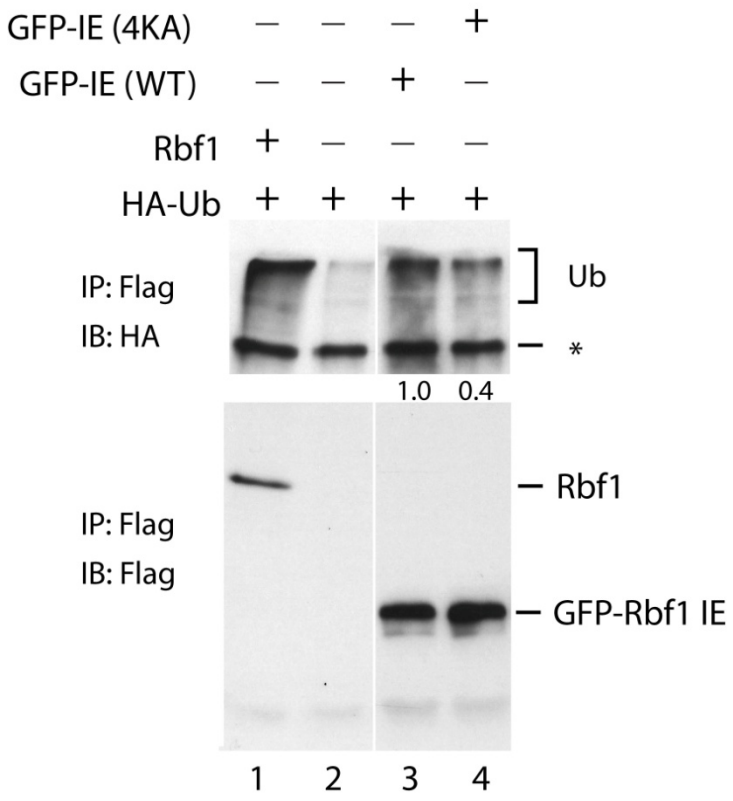

Figure 3-6. **Lysine residues within the Rbf1 C-terminal degron participate in enhanced GFP ubiquitination.** GFP-IE and GFP-IE 4K-A were compared for ubiquitination as performed in Figure 3-5B. Under conditions wherein expression levels of GFP-IE and GFP-IE (4KA) were comparable, the presence of the 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.

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* (*20*). 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 (Figure 3-7A). 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 (Figure 3-7B). As expected, treatment with doxycycline to inhibit DNA binding also diminished repression (not shown). 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 (*20*).

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 (Figure 3-7C). 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,

Figure 3-7

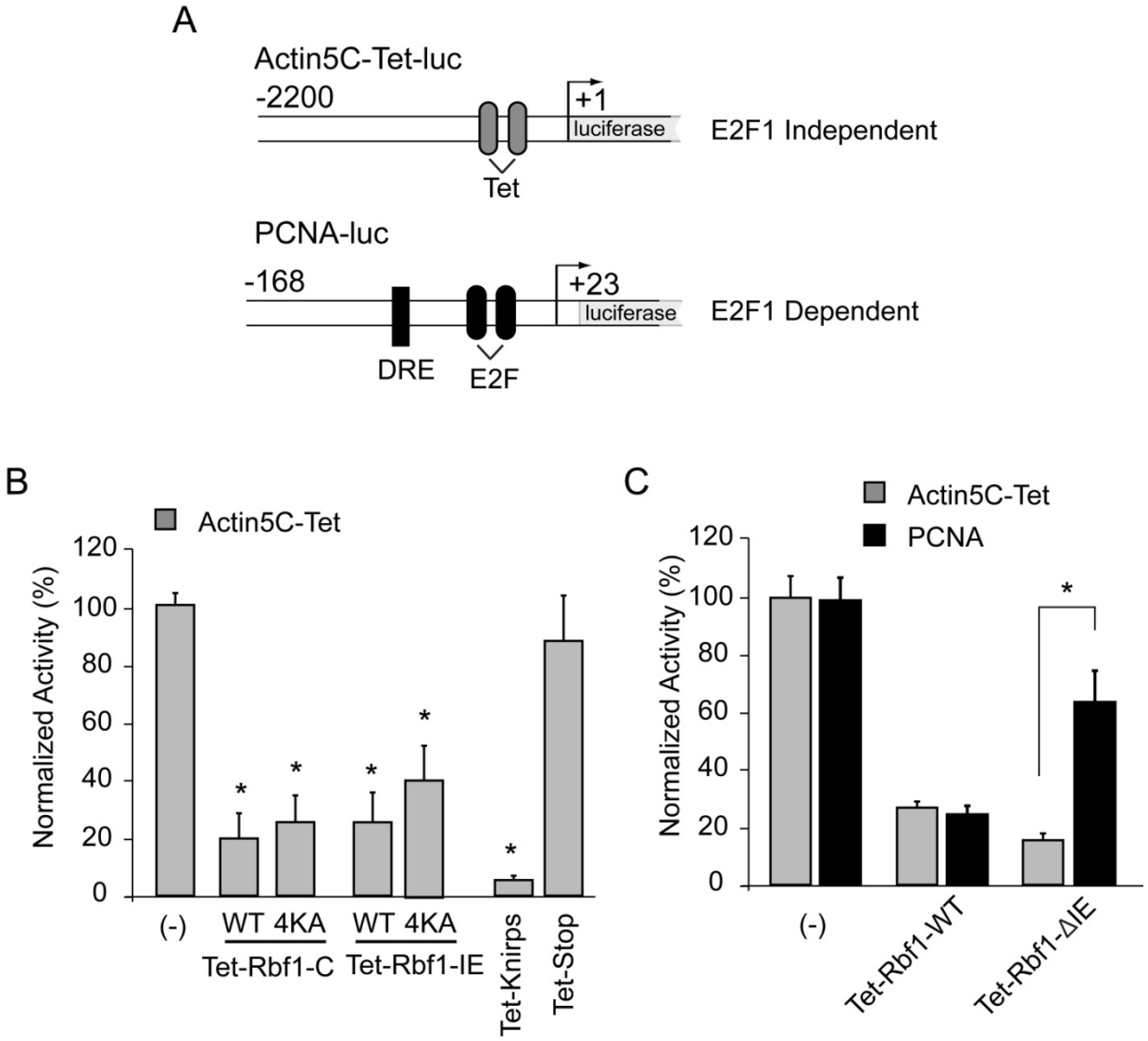
A
Actin5C-Tet-luc
-2200
+1
luciferase
E2F1 Independent
Tet
PCNA-luc
-168
+23
luciferase
E2F1 Dependent
DRE
E2F
B
Actin5C-Tet
Normalized Activity (%)
120
100
80
60
40
20
0
(-)
WT
4KA
WT
4KA
Tet-Rbf1-C
Tet-Rbf1-IE
Tet-Knirps
Tet-Stop
C
Actin5C-Tet
PCNA
Normalized Activity (%)
120
100
80
60
40
20
0
(-)
Tet-Rbf1-WT
Tet-Rbf1-ΔIE

Figure 3-7 cont’d

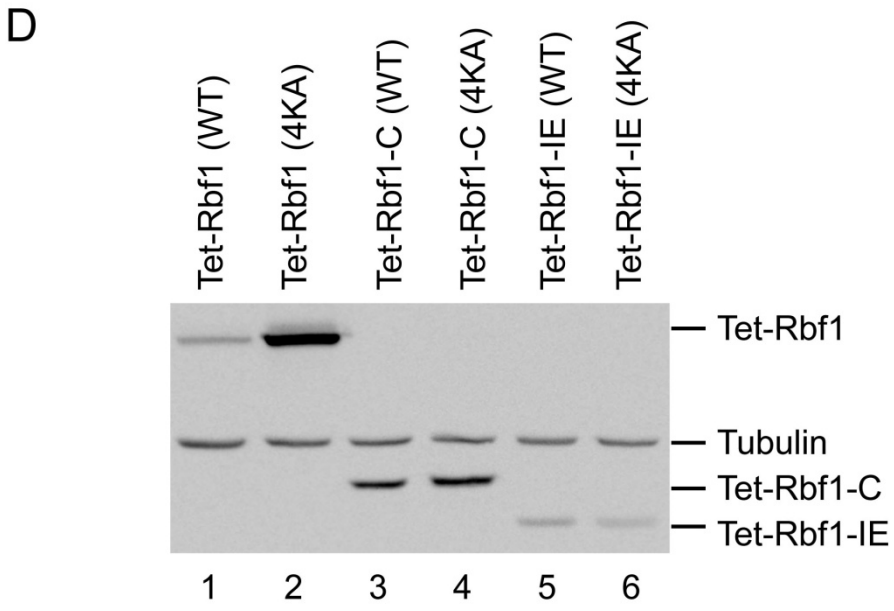

Figure 3-7 cont'd

Figure 3-7. Rbf1 IE functions as a transcriptional repression domain. (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. These experiments were performed by Liang Zhang.

consistent with previous observations that the IE is important for Rbf1 repression of cell cycle genes (*20*). 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 (Figure 3-7D). 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 activity exhibited by the Rbf1-ΔIE mutant protein when directly recruited to the Tet promoter demonstrated that this protein is not inherently defective for repression. 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*) (Figure 3-8A) and non-canonical E2F1-independent promoters (*InR*, *wts*, *Pi3K68D*) (Figure 3-8B). 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 (*22*). 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 (*22*), the IE may provide post-recruitment functions that are dispensable when Rbf1 is recruited independently of E2F1.

Figure 3-8

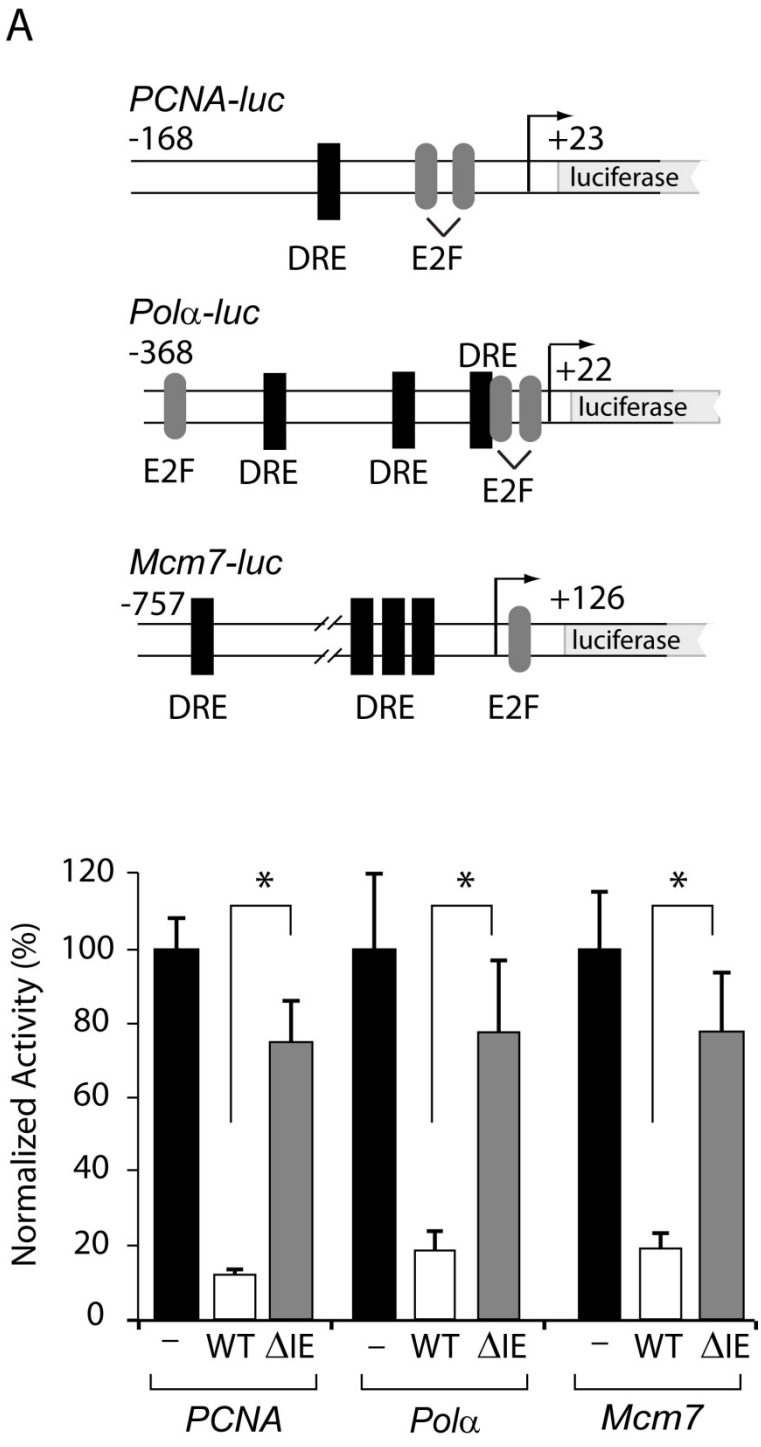
A
PCNA-luc
-168
+23
luciferase
DRE
E2F
Polα-luc
-368
DRE
+22
luciferase
E2F
DRE
DRE
E2F
Mcm7-luc
-757
+126
luciferase
DRE
DRE
E2F
Normalized Activity (%)
120
100
80
60
40
20
0
*
*
*
– WT ΔIE – WT ΔIE – WT ΔIE
PCNA
Polα
Mcm7

Figure 3-8 cont'd

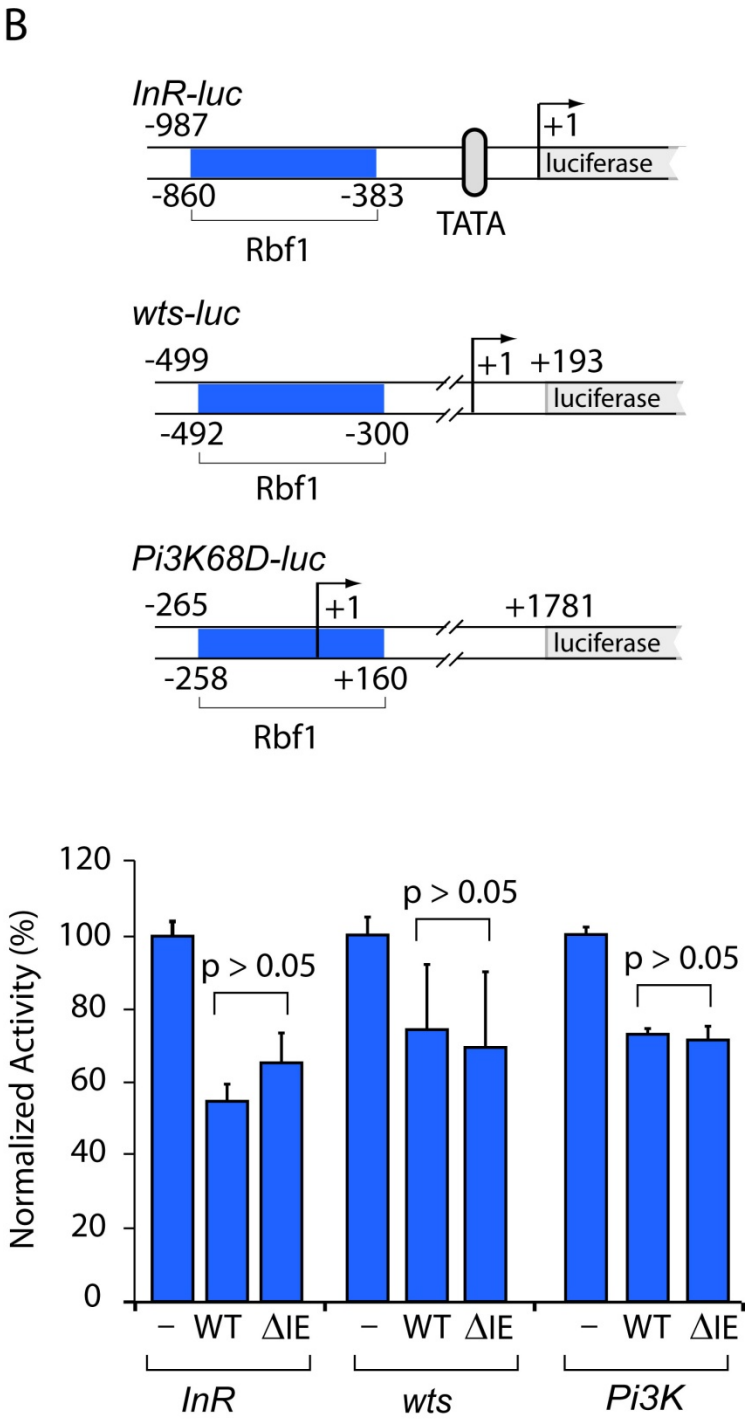

Figure 3-8 cont'd

Figure 3-8. Context dependence of the Rbf1-IE for transcriptional repression. (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 3-7. Data are from at least three biological replicates. Experiments in 3-8B were performed by Yiliang Wei, a graduate student in the Arnosti lab.

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 (Figure 3-9A). 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 (*34*). 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 Figure 3-9C) 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 (Figure 3-9B). 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

Figure 3-9C, 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 contributes 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. Despite substantially lower steady state protein levels associated with forced ubiquitination, Rbf1-ΔIE harboring the appended wild type ubiquitin exhibited more increased repression ability (Figure 3-9D). 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.

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

Figure 3-9

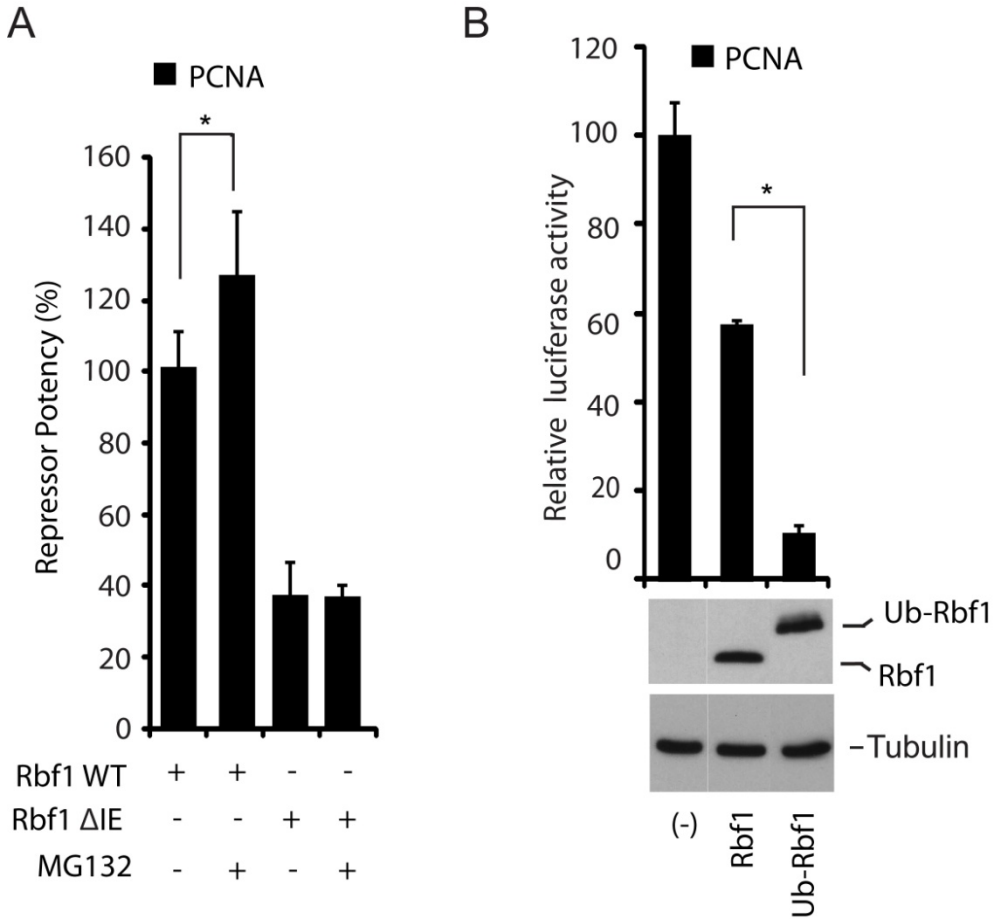
A
PCNA
*
Repressor Potency (%)
160
140
120
100
80
60
40
20
0
Rbf1 WT + + - -
Rbf1 ΔIE - - + +
MG132 - + - +
B
PCNA
120
100
80
60
40
20
0
Relative luciferase activity
*
Ub-Rbf1
Rbf1
Tubulin
(-)
Rbf1
Ub-Rbf1

Figure 3-9 cont'd

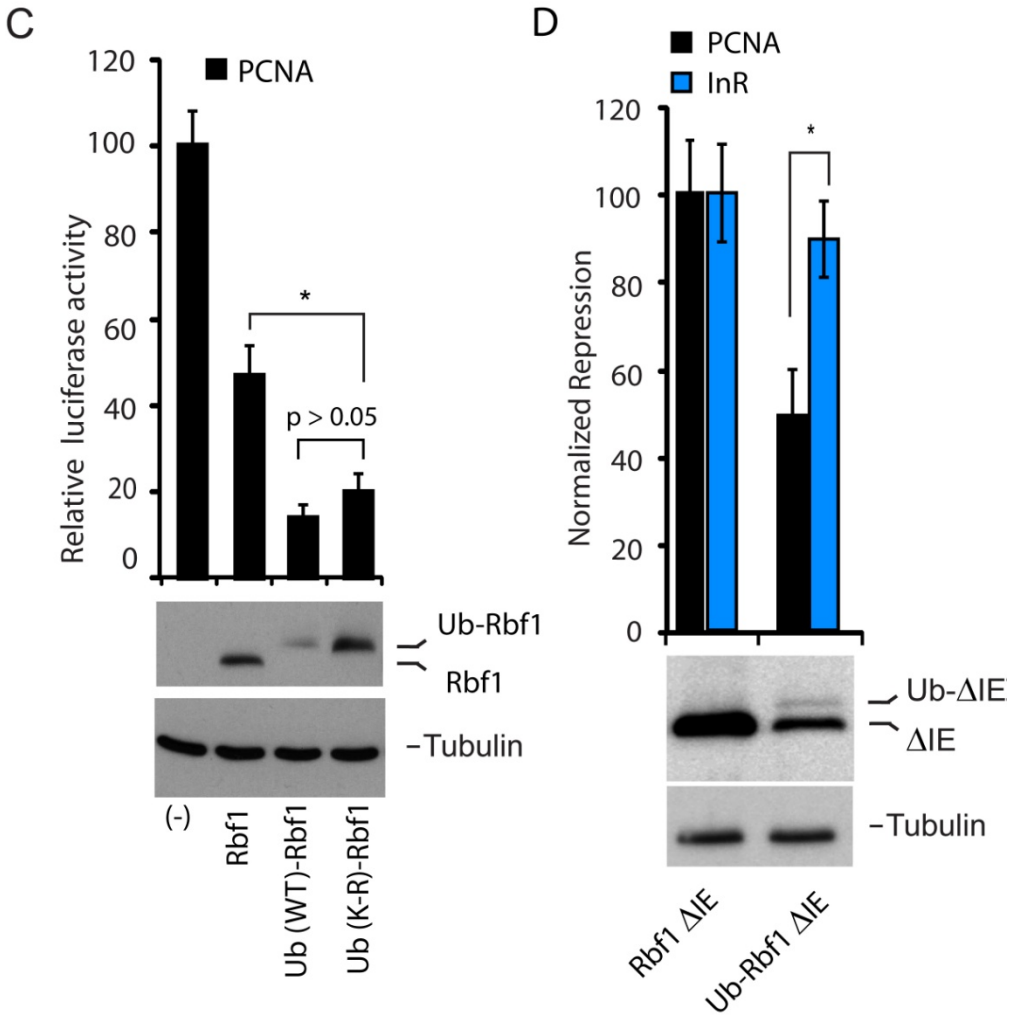

Figure 3-9 cont'd

Figure 3-9. Rbf1 ubiquitination enhances gene specific repression activity. (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. 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 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 (*, $p<0.05$) and to levels similar as observed for the Rbf1 chimera harboring the wild type ubiquitin tag ($p>0.05$). (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 Ub-Rbf1-ΔIE fusion protein was observed only upon longer exposure of the Western blot (lower panel) due to substantial cleavage of the ubiquitin tag. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

mammalian RB phosphorylation by cell cycle dependent kinases or stress responsive kinases can distinguish between cell cycle arrest or apoptotic responses (*16*). 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 (*22*, *23*). 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 (*35*). 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 (*36-39*). During activation, ubiquitin can function for co-factor recruitment, such as described for recruitment of p-TEFb by the viral activator VP16 (*40*), 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 (*41*); however, in that example, ubiquitin interfered with assembly of the preinitiation complex. 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 (*24*) suggests that a complex network of feedback regulation is proximally available at Rbf1 target gene promoters.

Acknowledgments

We thank Nick Dyson for sharing the PCNA-luciferase reporter gene, Cathie M. Pfleger for the HA-Ubiquitin construct, and Rupinder Sayal for aid with confocal microscopy. This work was supported by the MSU Gene Expression in Development and Disease Group, and the National Institutes of Health (1R01GM079098, 1R01CA142644).

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CHAPTER FOUR

RBF1 DEGRON DYSFUNCTION ENHANCES CELLULAR DNA REPLICATION[1]

[1]The work described in this chapter was published as the following manuscript: **Nitin Raj***, Liang Zhang*, Yiliang Wei, R. William Henry, David N. Arnosti, Rbf1 degron dysfunction enhances cellular DNA replication. *Cell Cycle*, **11**, 3731 (Oct 15 2012) (* co-first authors)

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.

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 (reviewed in ref. (*1*)). Consistent with their regulatory governance of these processes, RB family members are frequently inactivated in human cancers (*2, 3*). 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 (*4*). Together, these genetic changes are so pervasive as to be recognized as a hallmark of cancer (*5, 6*).

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 (*7, 8*). In *Drosophila*, these pathways are streamlined with two RB family proteins, Rbf1 and Rbf2, contributing to regulation of two E2F proteins, dE2F1 and dE2F2 (*9*). During G0 and early G1 of cell-cycle progression, RB family members directly bind to different sets of E2F factors (*10, 11*), and at least for E2F1, RB family members directly bind to different sets of E2F factors (*11-16*). Cyclin-cdk kinase mediated phosphorylation of RB in late G1 causes RB/E2F1 dissociation, allowing E2F to activate numerous proliferation genes drive entry into S phase (*17-21*). In human cancer, increased E2F activity is frequently observed (*22-25*), and is associated with poor prognosis, particularly in melanoma and breast cancer (*26-29*), highlighting the importance of imposing regulatory curbs on E2F1 expression and activity.

The ubiquitin-proteasome system and RB/E2F regulation

In addition to limitation though 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 (*30*). Although RB levels often appear stable in actively proliferating cells (*31, 32*), steady state fluctuations have been correlated with phosphorylation changes during cellular stress (*33*), 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 (*10*), 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 (*34*). 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 (*35, 36*). 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 (*31, 33, 37*), 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

(*33*, *37*), 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 (*38*), a developmentally regulated complex that controls proteasome-mediated degradation of many proteins through interactions with SCF (SKP1/cullin/F-box) E3 ubiquitin ligase complexes (*39*). CSN5, the catalytic core of COP9, contains a metalloprotease motif termed JAMM (Jab1/MPN domain-associated metalloisopeptidase) that removes Nedd8 from the cullin subunits (*40*). 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 (*38*), 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 (*41*, *42*) and SCF^{Skp2} (*33*), respectively. As with the RB family, E2F family members are degraded through ubiquitin-mediated turnover, both at defined points during the cell cycle (*43*) and in response to DNA damage (*44*, *45*) 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 (*46, 47*). Other ubiquitin ligases including APC/C (*43*) and ROC-Cullin ligases (*48*) likely contribute to E2F1 degradation in these contexts. In contrast, a protective role is suggested for the MDM2 ubiquitin ligase (*47*), and consistently, $p19^{ARF}$-mediated inhibition of MDM2 encourages E2F1 turnover (*49*, *50*). Interestingly, a key determinant of E2F1 degradation turns out to be RB itself (*51-54*). RB can bind to a carboxy-terminal instability element in E2F1, and may stabilize E2F1 by occluding the cellular ubiquitination machinery (*55*). In *Drosophila*, E2F1 destruction is mediated by the $Cul4^{Cdt2}$ E3-Ubiquitin ligase (*56, 57*), 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; 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.

Materials and Methods

Expression constructs

Generation of Rbf1 WT and mutant expression constructs was described previously (*34*). 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 Δ728-786 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 Figure 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 (50 mM Tris-HCl pH 8.0, 150 mM 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 1.5 ug/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.

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 (Figure 4-1A). This result shows that the COP9 signalosome stabilizes Rbf proteins, as previously noted (*38*), 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 (*58*), and a Cul4-containing SCF complex contributes to dE2F1 ubiquitination in S2 cells (*56*). 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 (*56*). Thus, a pathway emerges wherein the Cul4 E3 ligase responsible for E2F1 turnover is stabilized by the COP9 signalosome (Figure 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.

Figure 4-1

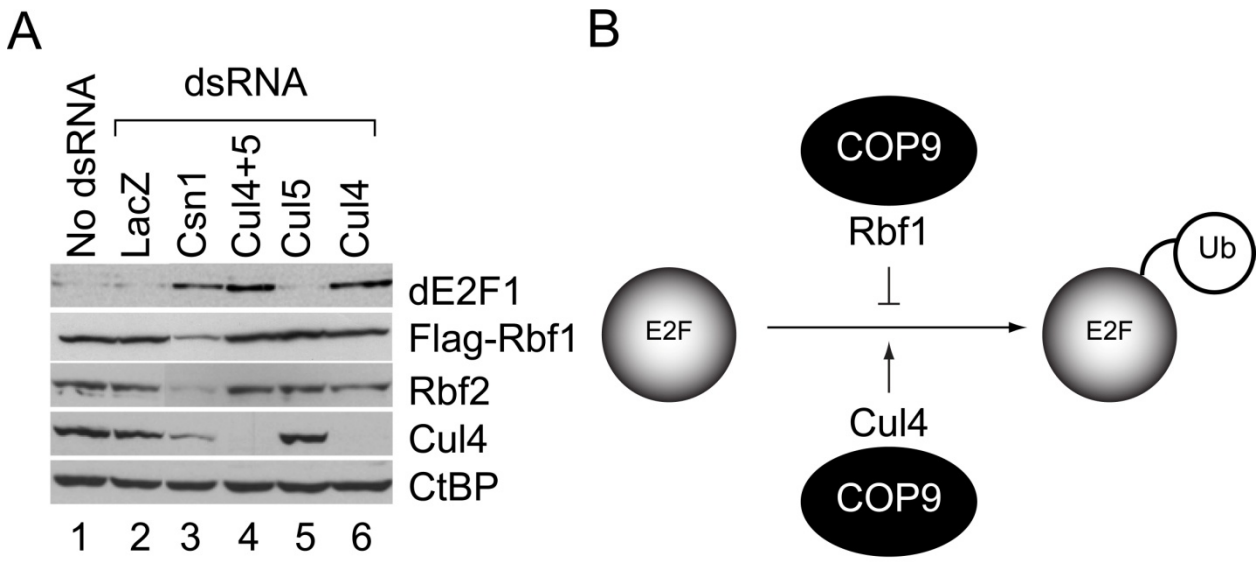

Figure 4-1. Dual roles of the COP9 signalosome in regulation of the Rbf1-dE2F1 network. (A) The COP9 signalosome complex governs both Rbf and E2F1 stability. S2 cells were treated with indicated dsRNA and proteins were measured by western blot analyses. Endogenous dE2F1 levels were dramatically increased 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.

Drosophila RBF1 enhances dE2F1 levels

The data presented above indicates 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 (*51, 52, 55*). We therefore examined dE2F1 levels using *Drosophila* S2 cells that harbor wild type COP9 function in the absence or presence of increased Rbf1 expression (Figure 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 (Figure 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 (*34*). 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 (*59, 60*). 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 (Figure 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

Figure 4-2

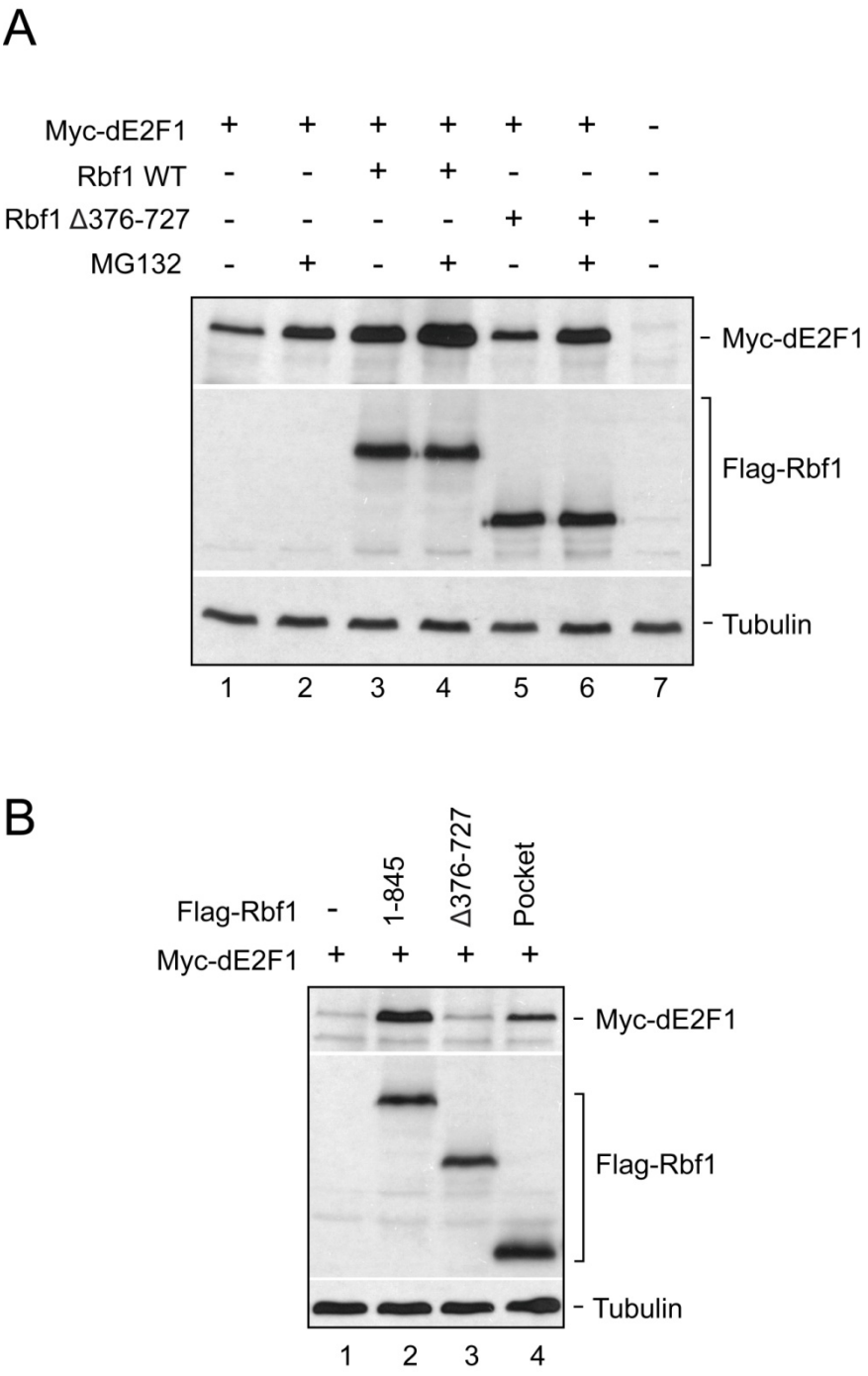
A
Myc-dE2F1 + + + + + + -
Rbf1 WT - - + + - - -
Rbf1 Δ376-727 - - - - + + -
MG132 - + - + - + -
Myc-dE2F1
Flag-Rbf1
Tubulin
1 2 3 4 5 6 7
B
Flag-Rbf1 - 1-845 Δ376-727 Pocket
Myc-dE2F1 + + + +
Myc-dE2F1
Flag-Rbf1
Tubulin
1 2 3 4

Figure 4-2 cont'd.

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

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 (*34*, *61*). RB family proteins require multiple domains to mediate gene repression, including the pocket domain that facilitates E2F interaction and co-factor recruitment (*62-65*), and the C-terminal region that harbors the IE (*34*), and may provide additional dE2F1 contacts, as was shown for human RB and p107 (*60*). Interestingly, Rbf1 degron deletion mutants retained their capacity to physically associate with dE2F1 at target gene promoters (*34*). 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 (Figure 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 (Figure 4-3B). Also consistent, wild type Rbf1 (1-845) robustly stabilized dE2F1. Mutant forms of Rbf1 lacking the pocket domain (1-375 and Δ376-727) 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 4-3

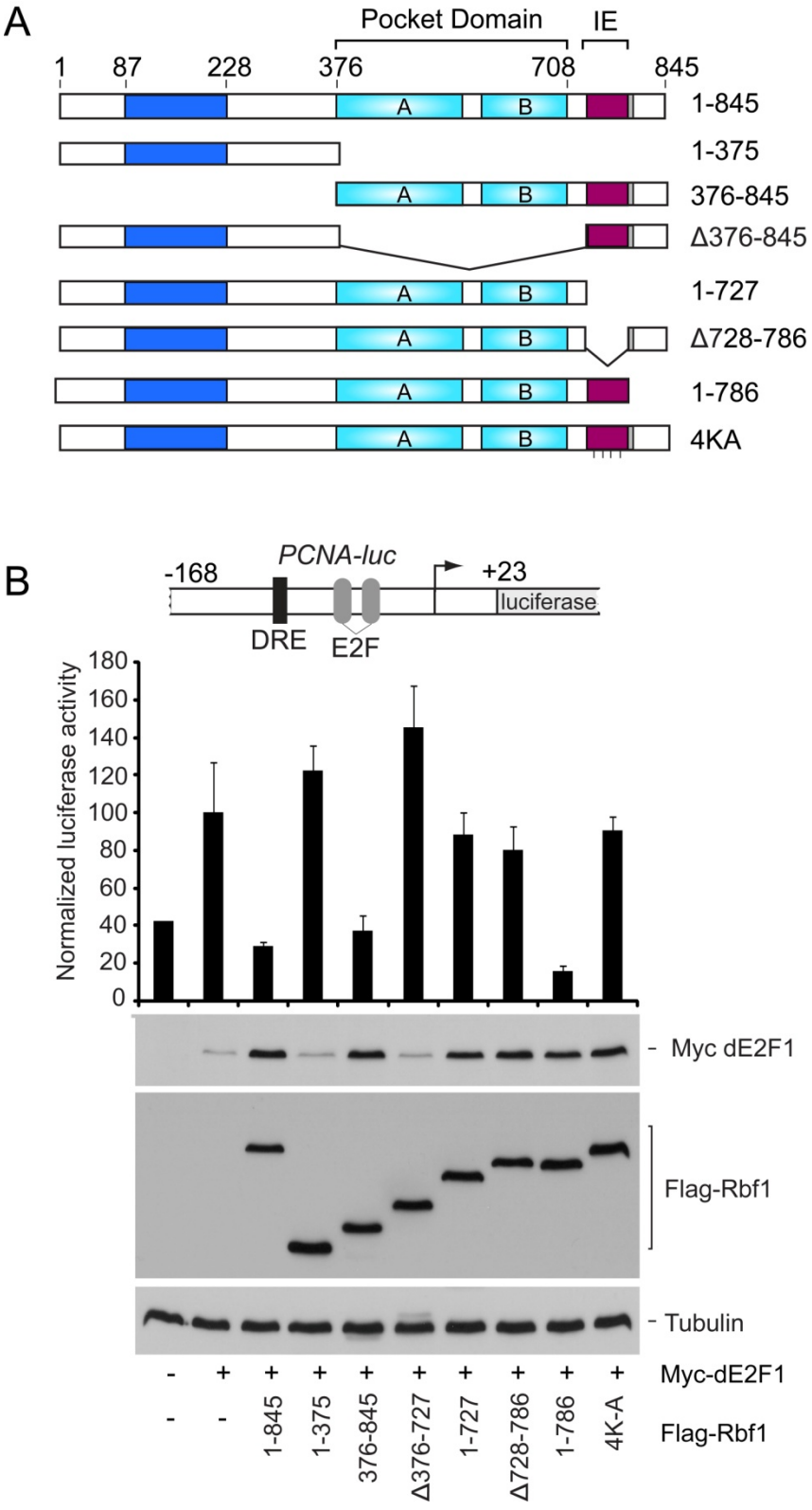
A
Pocket Domain
IE
1
87
228
376
708
845
A
B
1-845
1-375
376-845
Δ376-845
1-727
Δ728-786
1-786
4KA
B
PCNA-luc
-168
+23
luciferase
DRE
E2F
Normalized luciferase activity
180
160
140
120
100
80
60
40
20
0
Myc dE2F1
Flag-Rbf1
Tubulin
Myc-dE2F1
Flag-Rbf1
- + + + + + + + + +
- - 1-845 1-375 376-845 Δ376-727 1-727 Δ728-786 1-786 4K-A

Figure 4-3 cont'd.

Figure 4-3. Mutant Rbf1 lacking IE function stabilizes dE2F1 but cannot fully repress its transcriptional activity. (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 (Δ728-786 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 (Figure 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 (Figure 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 (Figure 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 Figure 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

Figure 4-4

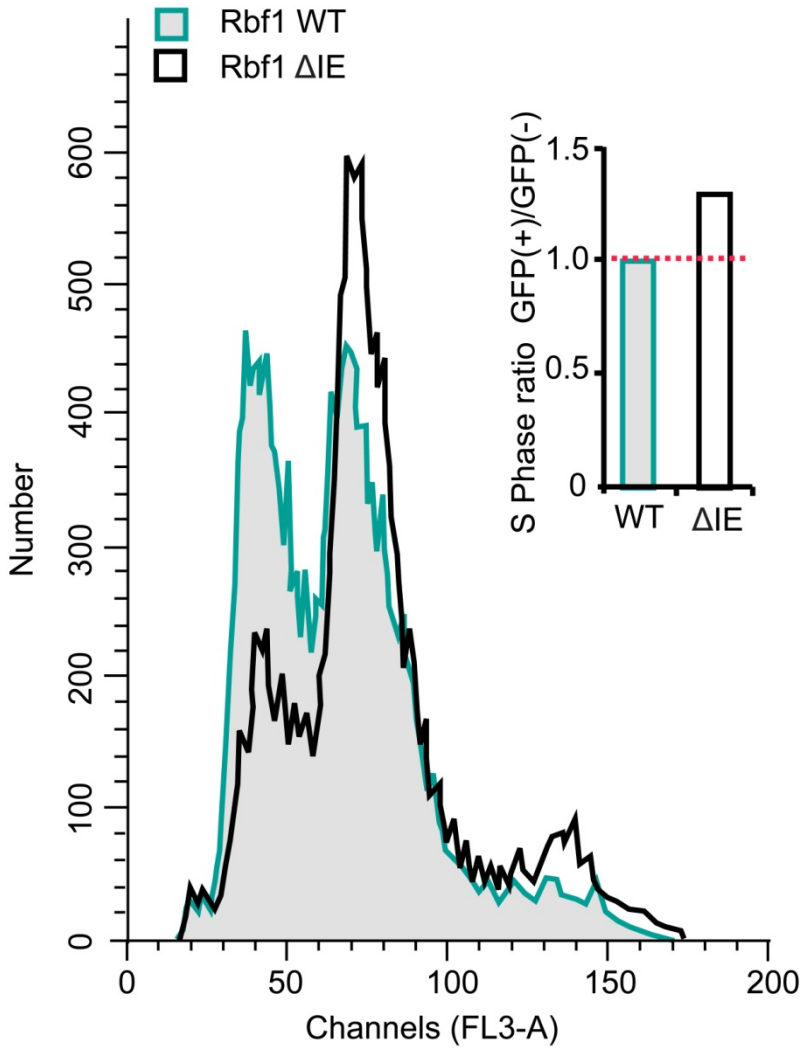
Rbf1 WT
Rbf1 ΔIE
Number
0
100
200
300
400
500
600
0
50
100
150
200
Channels (FL3-A)
S Phase ratio GFP(+)/GFP(-)
1.5
1.0
0.5
0
WT
ΔIE

Figure 4-4 cont'd.

Figure 4-4. The IE region contributes to Rbf1-mediated G1 arrest. Wild type or mutant GFP-Rbf1 ΔIE 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-Rbf1 ΔIE-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-Rbf1 ΔIE transfected samples. In two separate experiments, GFP-Rbf1 ΔIE expressing cells exhibited an increased proportion of cells in S phase, as estimated using Modfit analysis. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 4-5

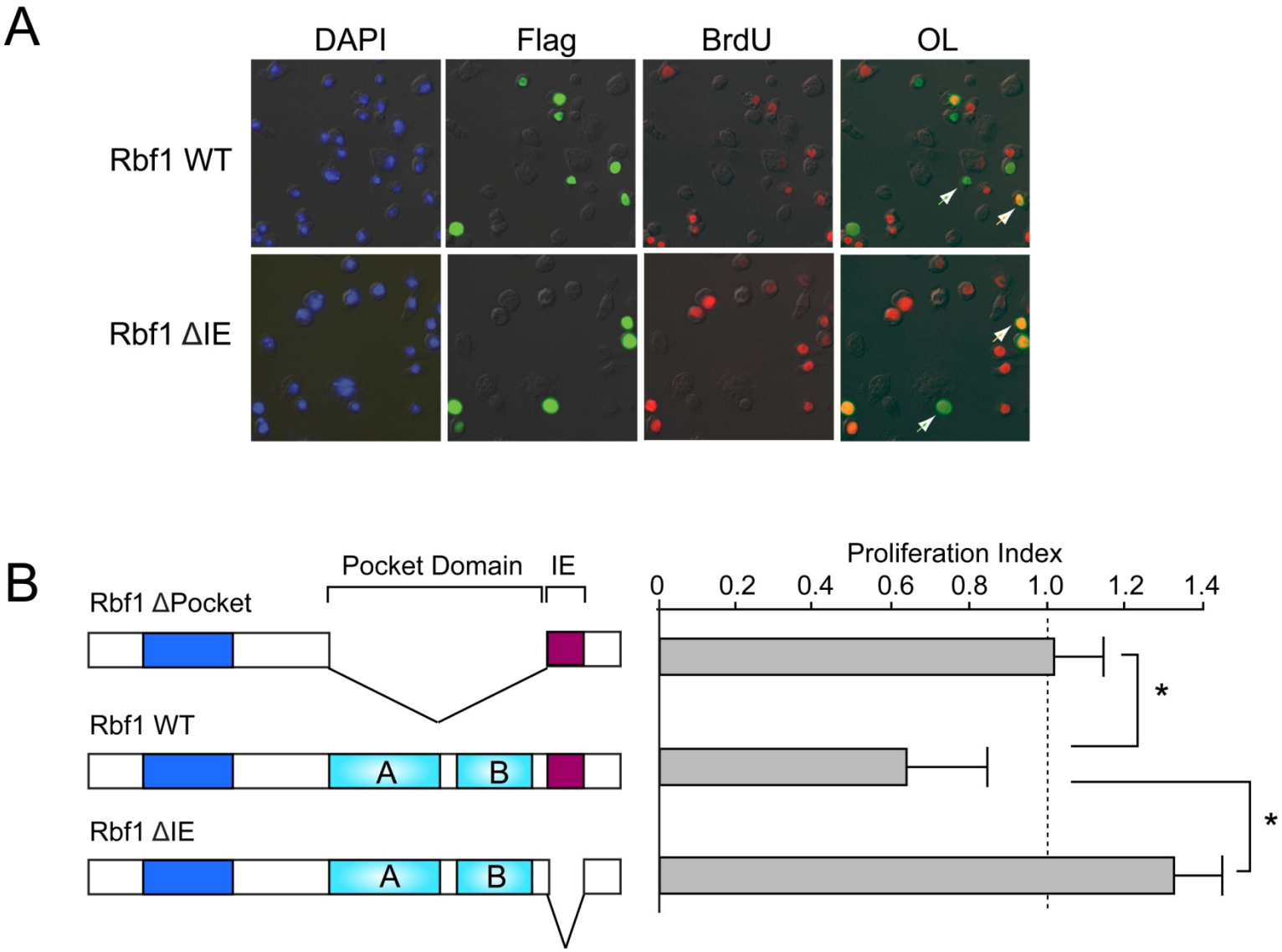
A
DAPI
Flag
BrdU
OL
Rbf1 WT
Rbf1 ΔIE
B
Pocket Domain
IE
Rbf1 ΔPocket
Rbf1 WT
A
B
Rbf1 ΔIE
A
B
Proliferation Index
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
*
*

Figure 4-5 cont'd.

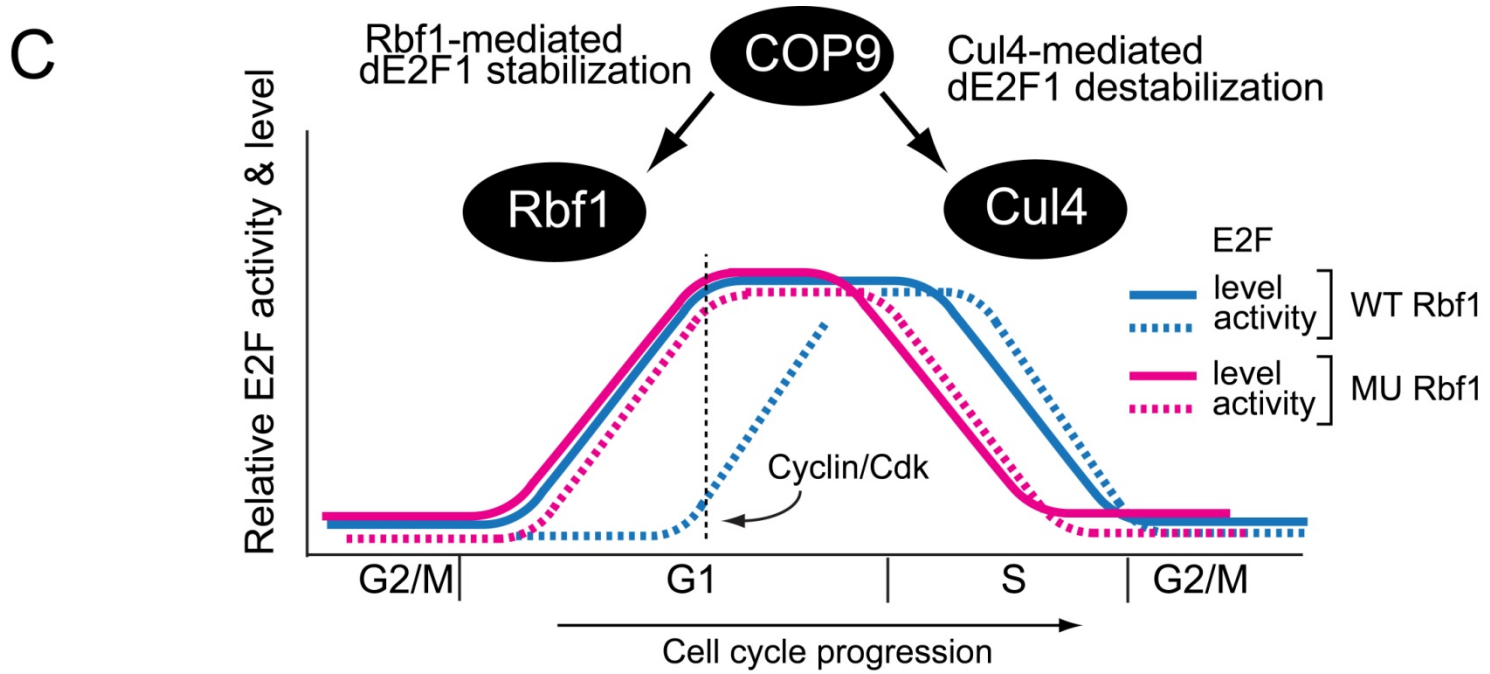

Figure 4-5 cont'd.

Figure 4-5. Mutation of the RB-family degron positively influences DNA replication frequency. (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). Rbf1ΔIE 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. At least 200 BrdU positive cells per preparation were scored manually. 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. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

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 Figure 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 (*66*). 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. 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.

Acknowledgements

We thank Nick Dyson for sharing the *PCNA-luciferase* reporter gene, Maxim Frolov for the pIE-E2F1 construct, Robert Duronio for the anti-Cul4 antibody, Maki Asano for the anti-E2F1 antibody, Louis King for expert assistance with flow cytometry, and Satyaki Sengupta for his thoughtful comments. This work was supported by the MSU Gene Expression in Development and Disease Group, and a grant from the NIH to DNA and RWH (1R01GM079098).

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CHAPTER FIVE

THE *DROSOPHILA* RBF2 POCKET DOMAIN COORDINATES REPRESSOR DESTABILIZATION AND TARGET GENE REPRESSION

Abstract

The Retinoblastoma (RB) family of proteins regulates cell cycle, differentiation, and apoptosis through the transcriptional repression of distinct gene sets that are involved in these processes. *Drosophila melanogaster* has two RB homologues, Rbf1 and Rbf2 that are characterized by an evolutionarily conserved RB-family pocket domain, and like their mammalian counterparts, provide differential gene regulation during development. We showed previously that the Rbf1 carboxy-terminal region harbors an autonomously acting degron that participates in gene-specific repression of cell cycle-regulated genes but also facilitates its proteasome-mediated destruction. This linkage between repression and repressor turnover could provide dynamic regulatory responsiveness to changing environmental conditions, and may be a widespread property of key cell fate regulators. Here we show that the Rbf2 C-terminal domain does not confer repressor instability. Rather, this region acts in conjunction with the conserved A/B pocket domain to direct Rbf2 modification. In this relationship, the Rbf2 pocket domain is the preeminent feature that links repressor ubiquitination to potency. Interestingly, the Rbf2 pocket domain is structurally related to its N-terminal region, which is predicted to harbor dual cyclin fold domains. Like the Rbf2 pocket, the evolutionarily conserved N-terminal region is required for cell cycle target gene repression, but this domain does not directly participate in Rbf2 turnover. This two-component repression model may ensure repressor instability and function at select target genes while retaining stable association at others, permitting gene specific repression capability.

Introduction

The RB tumor suppressor is a key regulator of cellular proliferation, and it functions at multiple levels to control tumorigenesis through intimate roles in both cell cycle progression and apoptosis. RB function in tumor suppression has been linked to repression of the E2F family of transcription factors (*1-3*), which regulate extensive networks of genes involved in cell growth (*4, 5*). RB interaction with E2F proteins and co-repressor proteins are mediated via the A/B pocket domain (*6, 7*), a feature that is conserved in two other mammalian RB family members, p107 and p130 (*8, 9*). These pocket proteins provide some redundant functions with RB, but genetic studies in mice clearly show that p107 and p130 provide additional unique functions during development (*10*) and in some contexts may provide tumor suppressive functions (*11-13*). An understanding of the RB family in cellular proliferation and apoptosis is complicated by richness of the mammalian E2F family that contains at least eight E2F proteins with only three members functioning as transcriptional activators, and the rest as repressors (*14*). The different RB family members interact with distinct sets of E2F family members consistent with specialized developmental roles (*15*). In *Drosophila*, two retinoblastoma family proteins, Rbf1 and Rbf2, are required for regulation of two E2F proteins, E2F1, an activator, and E2F2, a repressor, by a mechanism similar to the human RB protein (*16*). Thus, the *Drosophila* RB pathway is less complicated due to the involvement of fewer components, and presents a genetically tractable system to study the molecular mechanism of RB family function.

While the RB family functions in cell cycle regulation and apoptosis, how it differentially regulates genes involved in mutually exclusive cell fates is unknown. One possibility is that different RB family members provide gene specific regulation, a notion partially supported by analysis of Rbf1 and Rbf2 in SL2 cells, wherein cell cycle regulated genes are primarily

governed by dE2F1 activation and Rbf1 repression (*17*). A second possibility arose from observations that different sets of co-repressors contribute to RB-mediated repression at select target genes. This mechanism was hypothesized as important for cell cycle governance by RB wherein both SWI/SNF and histone deacetylase (HDAC) activity were required for repression of some target genes and as a component of checkpoint control, phosphorylation-mediated HDAC dissociation induced de-repression at some genes and not others (*18-21*). Differential cofactor usage has also been observed in actively proliferating *Drosophila* S2 cells wherein an Rbf/E2F cofactor called the dREAM complex (*22, 23*) could contribute to repression of differentiation specific genes but not cell cycle regulated genes (*17, 24*).

An important underlying mechanism for differential target gene regulation may also be related to the responses of individual RB family members to specific cell signaling cascades. In response to mitogenic signals, RB repression is disrupted late in G1 phase by serial phosphorylation by the cyclinD/cdk4 and cyclinE/cdk2 kinase complexes liberating E2F proteins for activation of cell cycle regulated genes (*25-29*). However, some RB family/E2F family complexes remain stably associated at target gene promoters during S phase and after activation of G1 cyclins (*30-35*). Hence, cyclin/cdk-mediated phosphorylation is insufficient to universally perturb RB family function at all genes. RB is also a substrate for phosphorylation by p38MAPK in response to cell stress (*36*). Interestingly, MAPK activation disrupted RB function only at apoptotic response genes, consistent with a model wherein distinct RB post-translational modification induced by mitogenic or stress activated events contributing to cell fate choices. Interestingly, RB family member phosphorylation during G1 is also correlated with divergent effects on their steady state levels. In cell cycle synchronization experiments, the levels of both RB and p107 were increased in late G1 concomitant with their cyclin/cdk-mediated

phosphorylation. In contrast, p130 levels were diminished during this period (*37*), suggesting that RB family member phosphorylation influences both activity and turnover.

Our initial studies for Rbf1 and Rbf2 during embryogenesis showed spatio-temporal differences in their steady state levels particularly in the embryonic central nervous system (*38*). Substantial discrepancies between absolute repressor levels and that of their cognate mRNAs further suggested that Rbf functions are governed by post-transcriptional mechanisms. We found that the COP9 signalosome associates with Rbf2 in the developing embryo, and could bind to some target gene promoters associated with cell cycle functions. The COP9 signalosome is a developmentally regulated multi-protein complex (*39, 40*) that inhibits ubiquitin E3 ligases through deneddylation of their cullin subunits (*41, 42*). Both Rbf1 and Rbf2 were destabilized in the absence of COP9 function, consistent with the hypothesis that the COP9 complex protects Rbf proteins from ubiquitination. We further discovered that a C-terminal instability element in Rbf1 acts as a degron to direct repressor ubiquitination, but this region also functions directly in repression (*43, 44*). *In vitro*, the Rbf1 degron was important for repression for cell cycle target genes, but was dispensable for repression of other non-canonical target genes that function in cell signaling pathways (*44*). Appending ubiquitin directly to Rbf1 diminished its stability but markedly increased its specific activity in cell cycle gene repression, thus linking repressor potency at some target genes to its ubiquitination and proteasome-mediated destruction. In the current study, we examined the connection between Rbf2-mediated repression and its turnover for regulation of cell cycle target genes. As was previously observed for Rbf1, Rbf2 ubiquitination and repression are indeed linked but unlike Rbf1, these processes are mediated through the conserved A/B pocket domain rather than a dedicated C-terminal degron, as observed for Rbf1. Rbf2 also harbors a novel N-terminal domain that can complement the A/B

pocket domain for full repression potency. Unlike the A/B pocket domain, the Rbf2 N-terminal region does not affect Rbf2 stability or ubiquitination. These observations indicate that Rbf2 utilizes multiple repression domains with both ubiquitin-dependent and ubiquitin-independent modes to enact cell cycle target gene repression.

Materials and Methods

Expression constructs

The full length *Drosophila rbf2* cDNA was PCR amplified and cloned into *Kpn*I and *Xba*I sites of pAX vector. Two Flag epitope tags were inserted 5' of the stop codon.Various mutants were produced by site-directed mutagenesis.

Luciferase Reporter Assay

Drosophila S2 cells were transfected using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. 1.5 million cells were transfected with 1 µg of *PCNA*-Luciferase reporter, 0.25 µg of pRL-CMV Renilla luciferase reporter (Promega), 120 ng of Myc-E2f2 and 0.5 µg of one of pAXRbf2 constructs. Cells were harvested 72 hours after transfection and luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega) and quantitated using the Veritas microplate luminometer (Turner Biosystems). Firefly luciferase activity was normalized to renilla luciferase activity.

Western Blot analysis

Western blots were performed utilizing 1:10,000 Flag antibody (Sigma), 1:1000 Myc antibody (Roche), 1:20,000 Tubulin antibody, 1:50 Groucho antibody (Iowa hybridoma bank), 1:5000 rabbit polyclonal R2N1, R2M1, R2C1 antibodies (Henry lab) and 1:1000 anti-ubiquitin mouse monoclonal antibody (Roche).

Treatments with proteasome inhibitor and cycloheximide

Drosophila S2 cells were transfected with 0.5 µg of pAXRbf2 constructs using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. The cells were grown for 3 days after which they were treated with 50 µg/ml MG132 (Sigma-Aldrich) or the vehicle DMSO for the indicated times. For experiment determining Rbf2 protein half-life, S2 cells were transfected using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. 1.5 million cells were transfected with 10 ng of pAX Rbf2 constructs. 72 hrs post-transfection the cells were treated with 100 µM CHX for the times indicated.

Protein-Protein Interaction Studies

For the coimmunoprecipitation assays, 200 ng Myc-tagged E2F2 and 500 ng of various Flag-tagged Rbf2 constructs were cotransfected into S2 cells using Effectene transfection reagent (Qiagen). Cells were grown for 3 d 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).

In vivo ubiquitination assay

Drosophila S2 cells were transfected with 500 ng of pAX Rbf2 WT, 362-783 (ΔN), ΔPocket and 1-702 (ΔC) constructs using Effectene transfection reagent. Cells were grown for 3 days after which extracts were prepared using 2% SDS lysis buffer. The extracts were immediately heat denatured for 10 minutes in a boiling water bath followed by a 10 fold dilution to bring down the SDS concentration down to range whereas immunoprecipitation reactions can be performed. Flag immunoprecipitations were performed (Anti-Flag affinity gel, Sigma) followed by anti-Ubiquitin Western blotting.

Results

The evolutionarily conserved pocket domain of Rbf2 confers protein instability

Previous studies from our lab uncovered a distinct developmental expression pattern for *Drosophila* Rbf proteins. Rbf1 was expressed at uniform levels throughout *Drosophila* embryogenesis (0-20 hour) whereas Rbf2 expression was seen between 4 and 10 hours followed by a gradual decline (*38*). This observation suggested that during embryogenesis Rbf2 activity could be regulated by its turnover. We also showed that endogenous Rbf2 is protected by the COP9 signalosome complex from proteasome-mediated turnover (*45*). Based on these observations we proposed that Rbf2 harbors instability element(s) that drives its turnover through the ubiquitin-proteasome pathway. To test this hypothesis, we analyzed the stability of Flag-epitope tagged Rbf2 proteins in *Drosophila* S2 cell cultures. To determine whether ectopically expressed Rbf2 proteins were sensitive to proteasome-mediated turnover, S2 cells were transiently transfected with an Rbf2 expression vector and treated with MG132, a proteasome inhibitor. As expected, the ectopically expressed Rbf2 wild-type protein (Rbf2 1-783) was greatly stabilized by MG132 treatment but not by the vehicle DMSO (Figure 5-1A). DIAP1, a protein previously known to be turned over by the ubiquitin-proteasome system, served as a positive control for MG132 treatment (*46*). In order to identify the protein instability elements in Rbf2, various deletion mutants were constructed (Figure 5-1B) and their steady-state expression levels were examined (Figure 5-1C). Interestingly, mutants lacking the conserved central pocket domain spanning residues 362-702 accumulated to significantly higher levels compared to the wild-type protein, indicating that this domain is necessary for Rbf2 degradation. However, the deletion of other portions of Rbf2 such as the amino-terminal 361 residues (Rbf2 362-783) or the carboxy-terminal 81 residues (Rbf2 1-702) or both (Rbf2 362-702) did not seem to affect the

steady-state levels of Rbf2, suggesting that the pocket domain is sufficient for the normal turnover of the protein. Consistent with this, Rbf2 pocket domain (Rbf2 362-702) accumulated to levels similar to the wild-type protein indicating that elements within this domain lead to Rbf2 destabilization.

The striking difference in the steady-state levels of the wild-type Rbf2 and the ΔPocket protein indicated that the latter is either expressed at higher levels or more stable than the former. In order to differentiate between these two possibilities, we determined the turnover rates of these proteins by treating S2 cells, which were transiently transfected with constructs that express these proteins, with a protein translation inhibitor cycloheximide (CHX). Three days after transfection, cells were treated with 100 µM of cycloheximide drug and protein levels were measured at 0, 3, 6 or 12 hours (Figure 5-1D). Protein levels were quantitated by photon-capture imaging with a Fuji LAS-3000 imager (data not shown). By 6 hours, protein levels of the wild-type Rbf2 protein, but not the mutant, were significantly decreased, confirming that the increased accumulation of the mutant proteins observed in previous experiments was caused by a reduced rate of degradation. We conclude that the pocket domain encompassing amino acids 362-702 harbors instability element(s) that are required for Rbf2 turnover.

Rbf2 N-terminus and pocket domain are required for repression of cell cycle genes

Recent work from our lab uncovered a paradoxical relationship between the instability and activity of Rbf1 protein (*47*). This observation raised a question whether such a correlation existed for Rbf2 and if so then through which domain of the protein. The identification of an instability element in the pocket domain of Rbf2 led us to hypothesize that this region may also play a critical role in Rbf2 mediated repression activity.

Figure 5-1

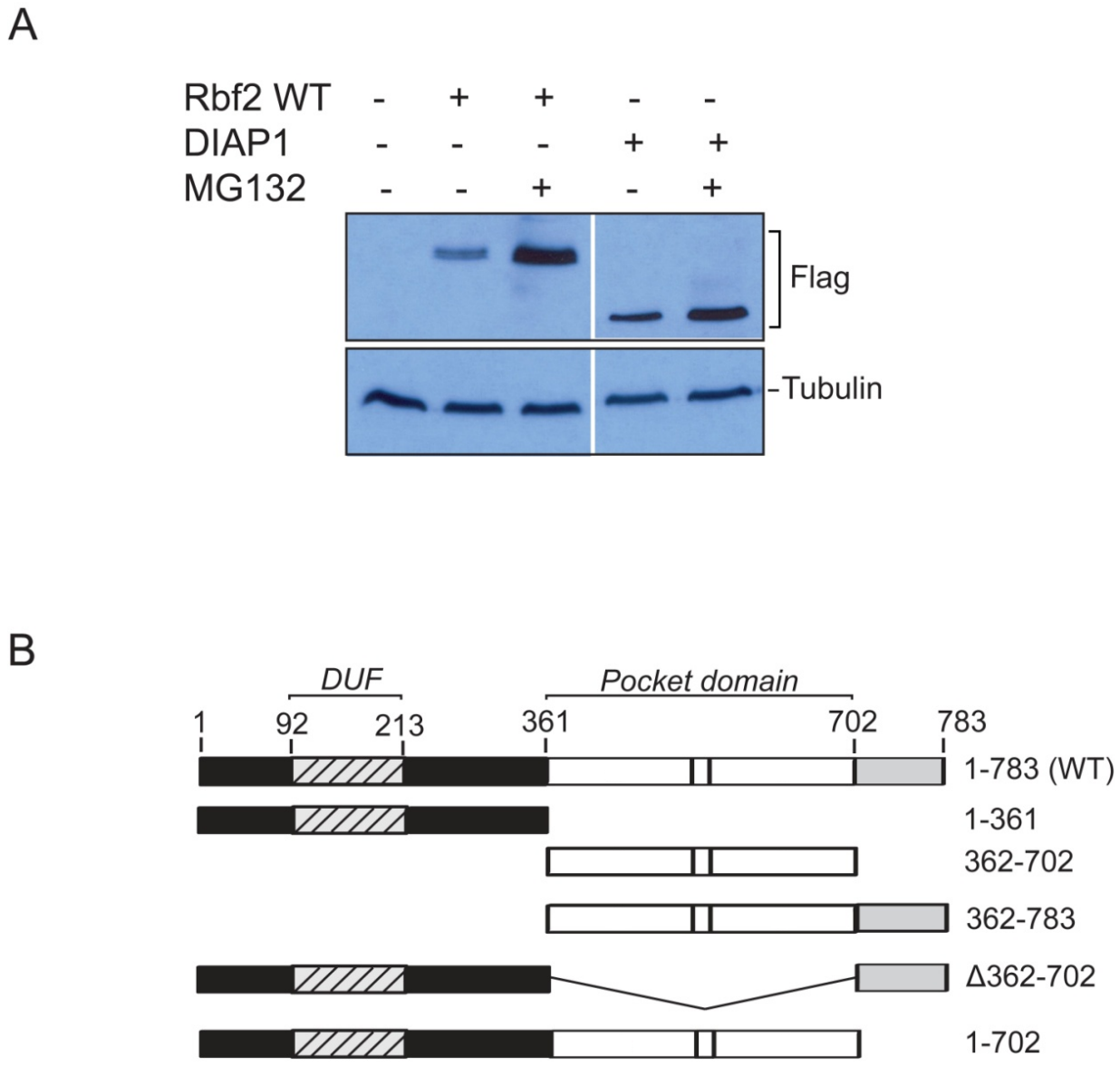
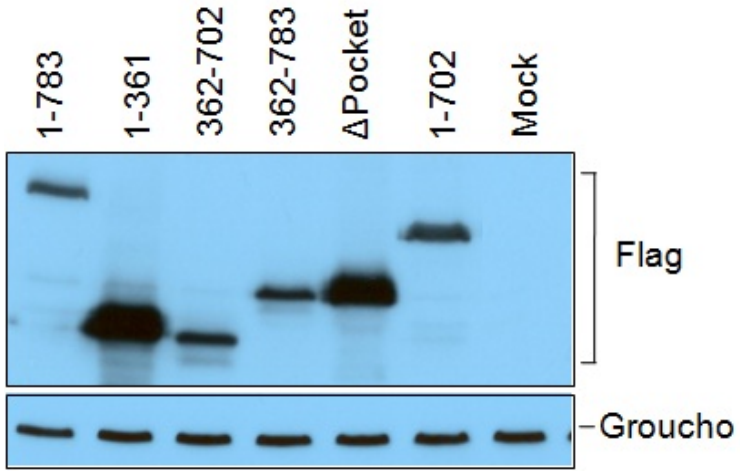

A
Rbf2 WT - + + - -
DIAP1 - - - + +
MG132 - - + - +
Flag
Tubulin
B
DUF
Pocket domain
1
92
213
361
702
783
1-783 (WT)
1-361
362-702
362-783
Δ362-702
1-702

Figure 5-1 cont'd

C



D

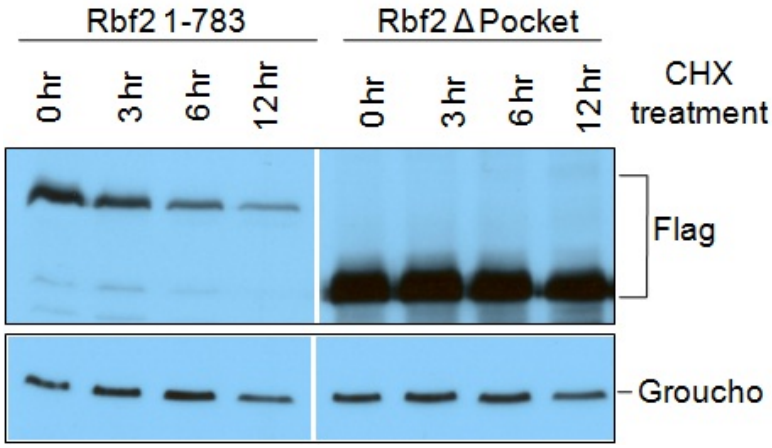


Figure 5-1 cont'd

Figure 5-1. Rbf2 pocket domain governs protein stability. (A) Effect of proteasome inhibitor MG132 on Rbf2 protein levels. Cells were transfected with construct expressing Flag-epitope tagged Rbf2 or DIAP1 proteins and treated for 2 hours with the proteasome inhibitor drug, MG132 or the vehicle DMSO. Both Rbf2 and DIAP1 were stabilized by MG132 treatment but not by DMSO. (B) Schematic diagram of Rbf2 proteins expressed in *Drosophila* S2 cells. The N and C termini are indicated in black and grey boxes respectively; the pocket domain is in white; the 'Domain of unknown function' (DUF) is denoted by slashed lines. (B) Steady-state levels of various Rbf2 forms. 500 ng of Rbf2 wild-type or mutants was transfected into S2 cells. Cells were grown for 3 days after which the proteins levels were analyzed through Flag western blotting. Groucho levels are shown as loading controls. (C) Half-life measurements of wild type and pocket domain deletion mutant Rbf2 proteins. Three days after transfection, cells were treated with 100 µg cycloheximide and harvested at the indicated times. The half-life of the wild-type Rbf2 protein was found to be ~ 6 hours while that of the mutant was >12 hours.

To test this hypothesis, we performed luciferase reporter assays to assess Rbf2 repression potential on two of its cell cycle target genes, *PCNA* and *Polα*. In this assay we scrutinized the repression potency of the various forms of Rbf2 whose steady-state levels were previously analyzed. Previous studies have shown that Rbf2-mediated repression of these target genes requires the corepressor dE2f2 (*18, 48, 49*). Hence, we tested the activity of the different Rbf2 forms in the presence of ectopically expressed Myc-epitope tagged dE2f2. As expected, cells expressing both Rbf2 and E2f2 displayed more effective repression (~3 fold) on both promoters than either one of these proteins by themselves (Figure 5-2A). Next, we examined the repression potency of various forms of Rbf2 lacking its different domains on *PCNA-luc* and *Polα-luc* (Figure 5-2A,B). The deletion of the Rbf2 pocket domain resulted in a complete loss of repression activity, a finding similar to what was observed for Rbf1 (*43*). Interestingly, unlike Rbf1, the deletion of the C-terminal region had no significant effect on its repression activity, indicating that this region is not essential for function. In contrast, deletion of the N-terminal domain impaired Rbf2-mediated repression, a result not observed for Rbf1. We conclude that Rbf1 and Rbf2 share a common requirement for pocket domain for full activity, but rely on different adjacent domains to complement the pocket domain for function. Interestingly, consistent with our previous observations, the deletion of the pocket domain but not the N-terminus resulted in a strongly stabilized protein. We conclude that Rbf2 is a two-component repressor that requires both its N-terminus and pocket domains to enact full repression of its cell-cycle target genes. To test whether either of these domains is sufficient for repression, we analyzed the activities of Rbf2 N- terminus and pocket domains alone (Rbf2 1-361 and Rbf2 362-702). Both these mutants failed to show any significant repression of the *PCNA* promoter suggesting that neither of these two domains is sufficient for Rbf2 repression activity.

Figure 5-2

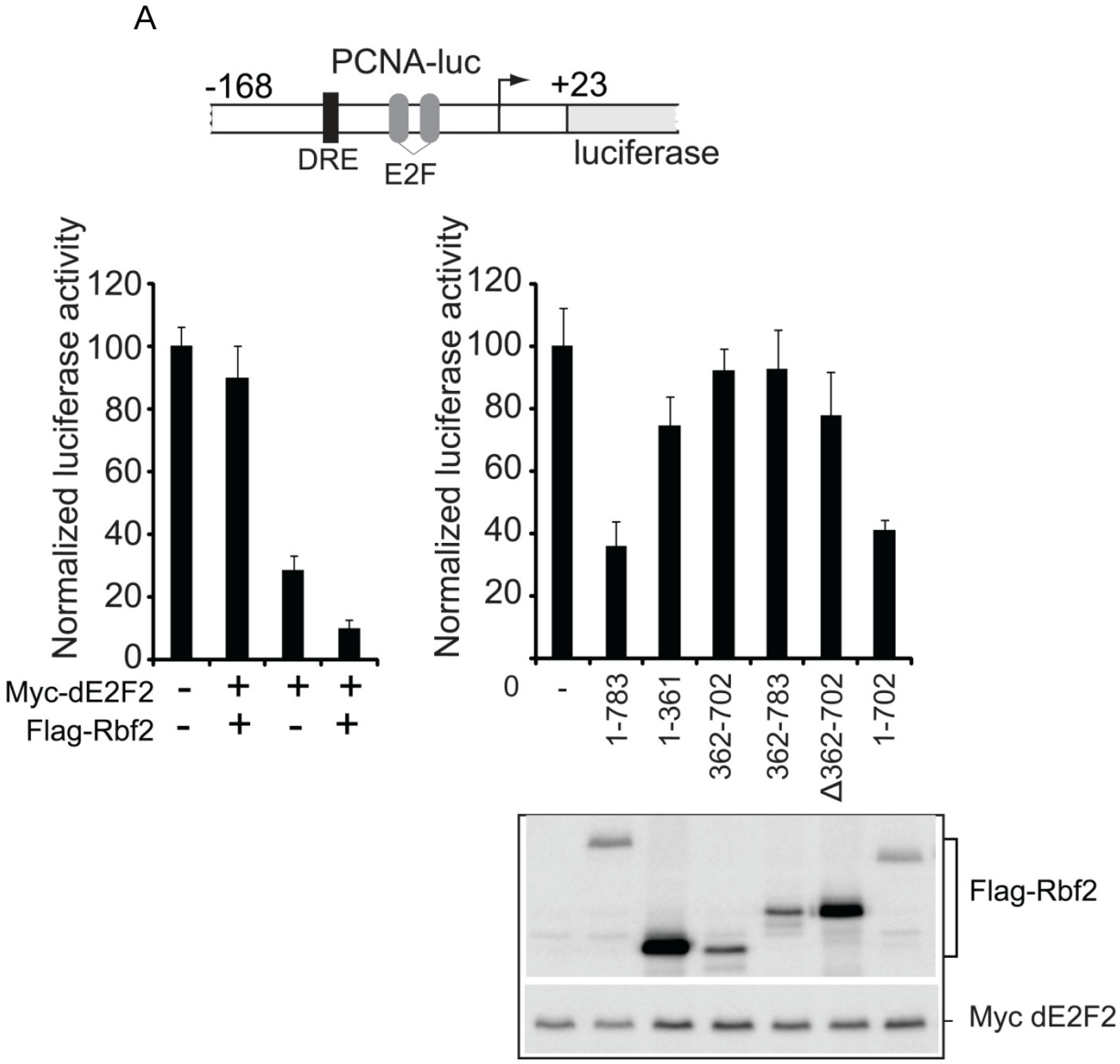

A
PCNA-luc
-168
+23
DRE
E2F
luciferase
Normalized luciferase activity
120
100
80
60
40
20
0
Myc-dE2F2 - + + +
Flag-Rbf2 - + - +
Normalized luciferase activity
120
100
80
60
40
20
0
-
1-783
1-361
362-702
362-783
Δ362-702
1-702
Flag-Rbf2
Myc dE2F2

Figure 5-2 cont'd

B

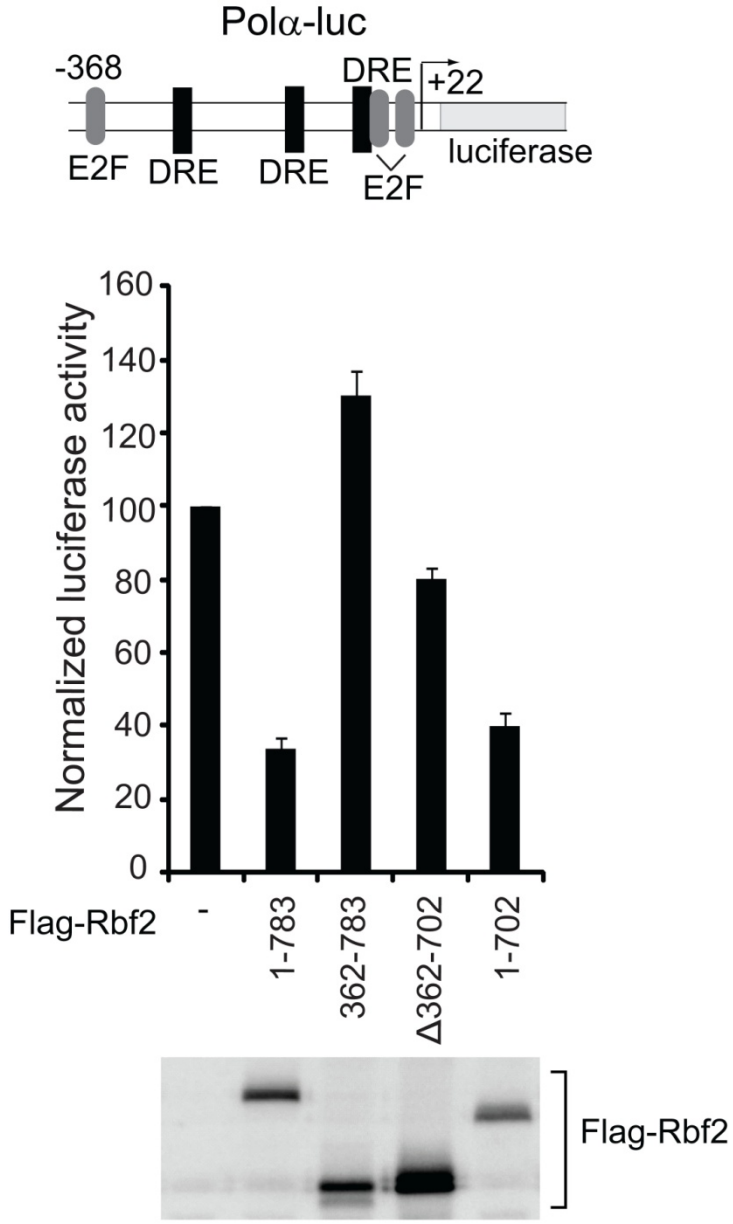


Figure 5-2 cont'd

Figure 5-2. Rbf2 requires its N-terminus and pocket domain for full repression activity. (A) Deletion of the N-terminus (362-702 and 362-783) or pocket domain (1-361 and ΔPocket) severely compromises transcriptional repression activity of Rbf2 on *PCNA-luciferase* reporter (bar graph). The pocket deletion mutants accumulated to higher levels than the wild-type protein (Western blot). (B) Repression activity of Rbf2 wild-type and mutants on *Drosophila Polα-luciferase* reporter. Deletion of the N-terminus or the pocket domain inactivates the protein for transcriptional repression. Data in 5-2A represents four biological replicates, each with three technical replicates. Data in 5-2B is from a single experiment. Firefly luciferase activity is expressed relative to Renilla luciferase control.

In order to further characterize the contributions of the two cyclin fold boxes within the pocket domain, we generated smaller deletions within this domain and tested these mutants for their stability and repression activity. RB family pocket domains are characterized by the presence of two conserved cyclin folds that are separated by a spacer region. Hence, we generated deletions in these three regions. Deletion of either Rbf2 cyclin fold A or B resulted in a significant loss of repression activity but did not alter protein stability suggesting that the Rbf2 pocket domain harbors redundant elements for regulating protein turnover but not repression activity (Figure 5-3). Deletion of the short spacer between the two cyclin boxes did not perturb protein stability or activity.

Since the N-terminus of the human RB has been predicted to be similar in architecture to the RB family pocket domain, we analyzed whether the Rbf2 N-terminal region contributes to E2F interaction. This was examined using co-immunoprecipitation assays using S2 cells expressing various forms of Flag-Rbf2 and Myc-tagged dE2F2. As shown in Figure 5-4, robust dE2F2 association with Rbf2 was observed for samples expressing both dE2F2 plus Rbf2, a result that was not observed in the absence of Rbf2. Thus, specific E2F2 recovery depended upon Rbf2 expression in this system. Interestingly, dE2F2 was co-immunoprecipitated with the Rbf2 N-terminal region alone, and at levels comparable to that observed with Rbf2 lacking the N-terminal region or with the Rbf2 A/B pocket domain alone, a region that is known to mediate E2F interaction. E2F2 recovery was not observed with CtBP, a short-range co-repressor that does not interact with E2F2, again attesting to the dependence on Rbf2 for specific E2F2 recovery. Based on these results, we conclude that Rbf2 harbors at least two regions capable of E2F2 interaction, the A/B pocket domain and the N-terminal region.

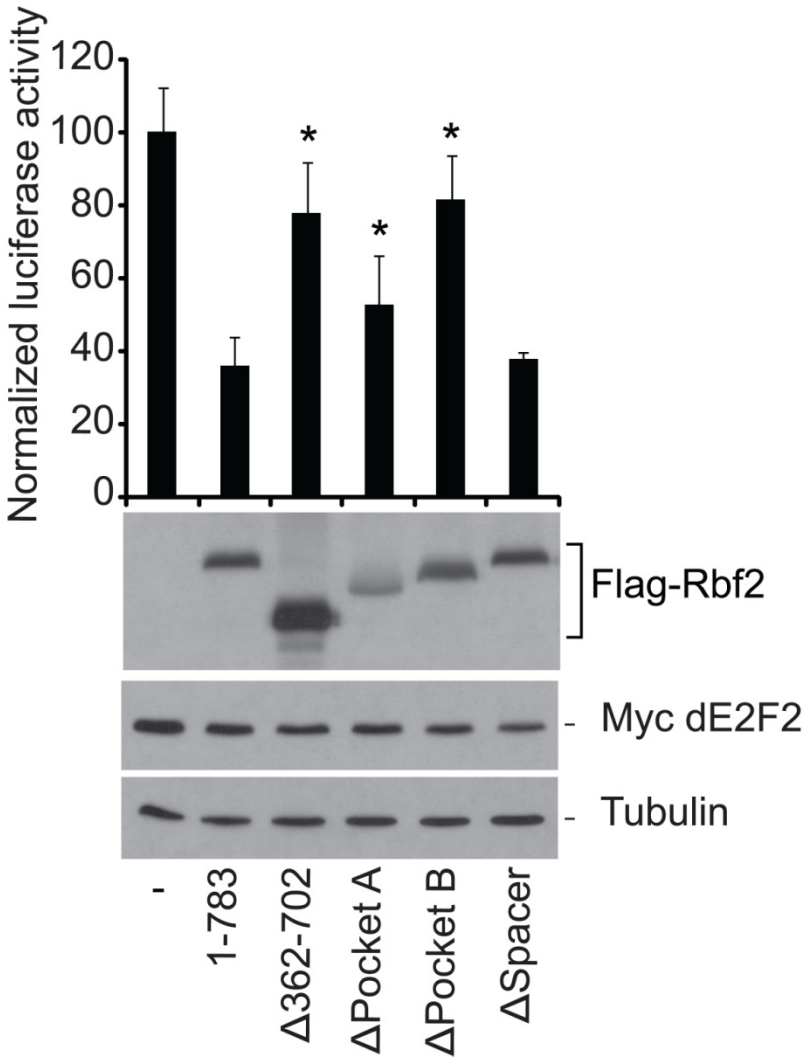


Figure 5-3. Rbf2 pocket domain harbors redundant elements for regulating protein turnover but not repression activity. Deletion of either Rbf2 pocket domain cyclin box A or B resulted in loss of repression activity (bar graphs) but does not alter protein stability (Western blot). Deletion of the short spacer between the two cyclin boxes doesn't perturb protein stability or activity. Tubulin levels are shown as loading control.

Figure 5-4

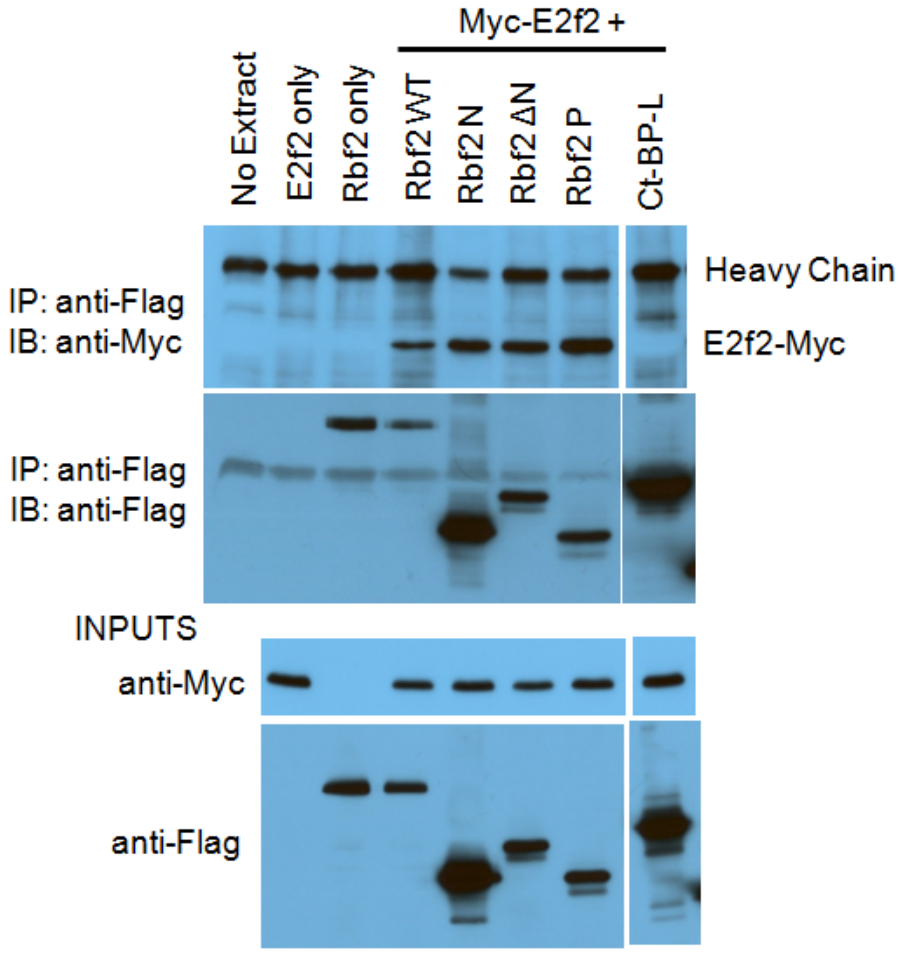

Myc-E2f2 +
No Extract
E2f2 only
Rbf2 only
Rbf2 WT
Rbf2 N
Rbf2 ΔN
Rbf2 P
Ct-BP-L
IP: anti-Flag
IB: anti-Myc
Heavy Chain
E2f2-Myc
IP: anti-Flag
IB: anti-Flag
INPUTS
anti-Myc
anti-Flag

Figure 5-4 cont'd

Figure 5-4. Rbf2 N and Pocket domains are sufficient but not necessary for dE2F2 interactions. Rbf2/E2F2 interactions were analyzed by Coimmunoprecipitation assays. S2 cells were cotransfected with Myc-tagged E2F2 and Flag-tagged Rbf2 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 E2F2 coprecipitated with Rbf2 WT (1-783), N (1-361), ΔN (Rbf2 362-783) and Rbf2 P (Rbf2 362-702) mutants but not with the CtBP-L protein (top panel, lanes 4-8). Equivalent levels of the heavy chain IgG (marked as Heavy Chain) 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 are representative of two biological replicates.

The conserved 'Domain of unknown function' (DUF) within Rbf2 N-terminus contributes to transcriptional repression activity

Our data indicates that the Rbf2 N-terminus is required for Rbf2-mediated repression. To dissect out the N-terminal elements that contribute to Rbf2 repression potency, we identified the conserved domains in this region of the protein. Analysis of Rbf2 N-terminal 361 amino-acid residues using the NCBI Conserved Domain Database (CDD) search tool revealed the existence of a conserved domain which resembles the domain of unknown function DUF3452 superfamily (hereby referred to as DUF). This domain encompasses residues 92 to 216 in the middle of Rbf2 N-terminus. This presumed domain is found in both bacteria and eukaryotes and is functionally uncharacterized. Typically, this domain is 124-150 amino acids in length and contains a single completely conserved tryptophan (W) residue that may be functionally important.

Based on the high sequence conservation of DUF amongst *Drosophila* and mammalian RB family homologs p107 and p130, as revealed by a Clustal W2 analysis (Figure 5-5A), we proposed that this domain may be important for Rbf2 function as a repressor. To test this hypothesis, we analyzed the activity of a Rbf2 mutant that lacks this domain. Interestingly, the deletion of Rbf2 DUF resulted in a significant decrease in its repression activity although not as severe as that observed with deletion of the entire N-terminus (Figure 5-5B). In contrast, deletion of the first 91 amino acids of Rbf2, just N-terminal to the DUF, had no impact on repression activity (not shown). Deletion of the DUF domain or the entire N-terminus did not affect Rbf2 steady-state levels. Hence, unlike the pocket domain, the Rbf2 DUF does not participate in Rbf2 degradation but contributes to its repression activity.

Figure 5-5

A

```
Rbf2  1 NVSLTRLLRSFKMNVSQFLRRMEHWNWLTQNENTFQLEVEELRCRLGITSTLLRHYKHIF 60
p107  2 CVSLTRILRSAKLSLIQFFSKMKKWMDMSNLPQEFRERIERLERNFEVSTVIFKKYEPIF 60
p130  3 YVSLTRILKCSEQSLIEFFNKMKKWEDMANLPPHFRERTERLERNFTVSAVIFKKYEPIF 60
         *****:*:. : .: :*: :*::*  :::    *: . *.*. .: :::.::::*: **

Rbf2  1 RSLFVHPGKGADPGAAN--------HYQALYEFGWLLFLVIRNELPGFAITNLINGCQVL 112
p107  2 LDIFQNPYEEPPKLPRSRKQRRIPCSVKDLFNFCWTLFVYTKGNFR-MIGDDLVNSYHLL 119
p130  3 QDIFKYPQEEQPRQQRGRKQRRQPCTVSEIFHFCWVLFIYAKGNFP-MISDDLVNSYHLL 119
         .:*  * :       .          . ::.* * **:  :.::  :   :*:*. ::*

Rbf2  1 VCTMDLLFVN 122
p107  2 LCCLDLIFAN 129
p130  3 LCALDLVYGN 129
        :* :**:: *
```

Figure 5-5 cont'd

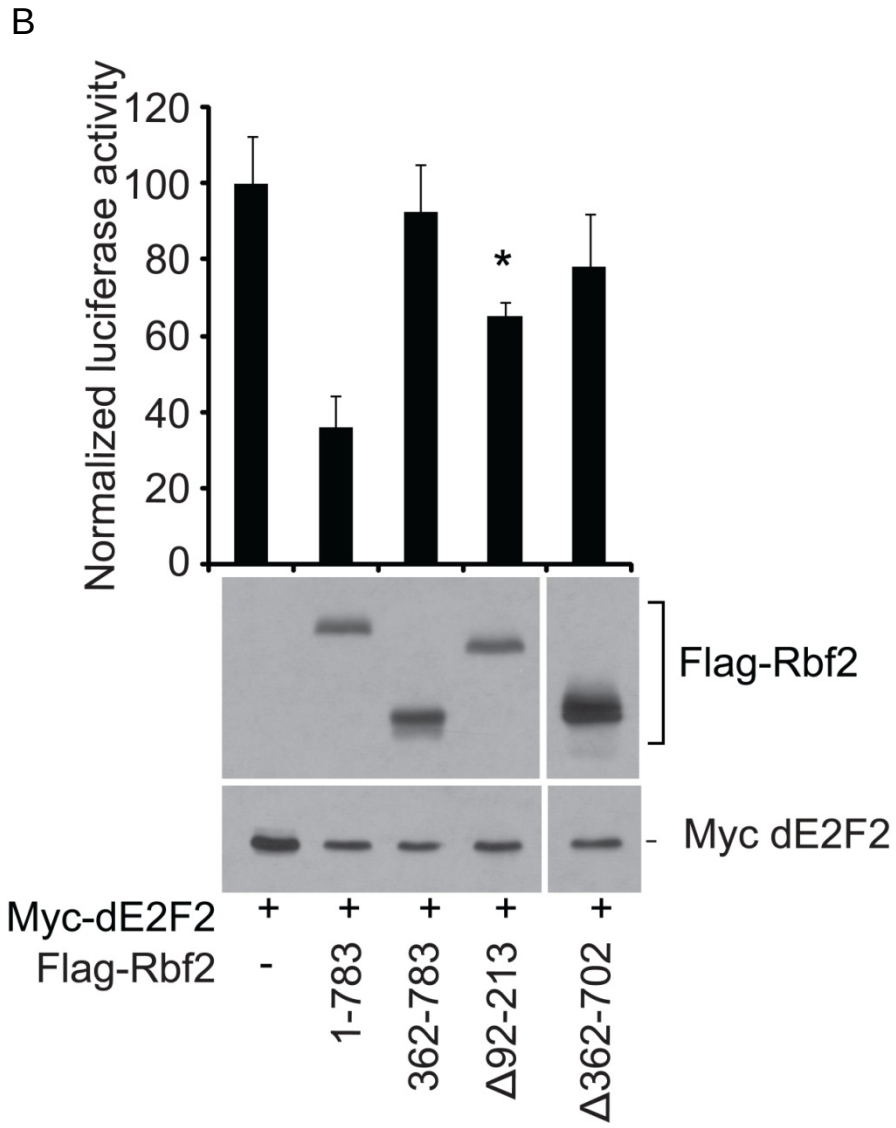


Figure 5-5 cont'd

Figure 5-5. The evolutionarily conserved Rbf2 DUF contributes to its transcriptional repression activity. (A) Clustal W2 alignment of DUF domains of *Drosophila* Rbf2 and human RB family homologs p107 and p130. Asterisk (*) and bold letters indicate identical residues, Colon (**:**) indicates different but highly similar amino acids and dot (**.**) indicates different and somewhat similar residues. (B) Transcriptional activity of Rbf2 deletion mutant proteins assayed on *PCNA-luciferase* reporter. The activity of Rbf2 ΔDUF was analyzed on the *PCNA-luc* reporter and compared to that of Rbf2 WT and Rbf2 ΔN (362-783). Rbf2 ΔPocket served as a negative control. Firefly luciferase activity is expressed relative to Renilla luciferase control. Data in 5-5B represents two biological replicates, each with three technical replicates.

The pocket domain of Rbf2 promotes its ubiquitination in vivo

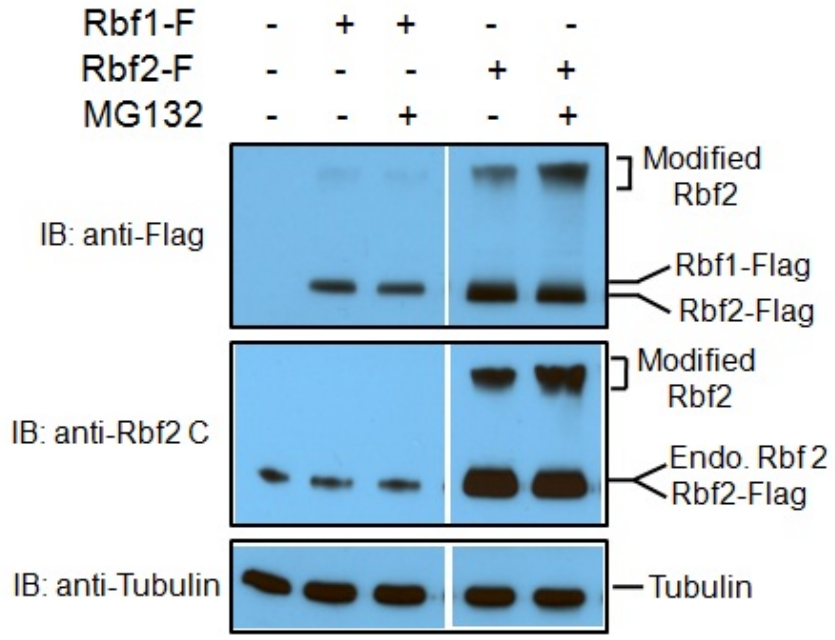
Previous studies on transcriptional activators such as VP16 and Myc have shown that ubiquitin modification on these proteins plays a direct role in transcriptional activation possibly through the recruitment of co-activators (*50-52*). Hence the ubiquitin tag provides a link between instability and transcriptional activity for activators. Based on these studies and our own observation that the pocket domain of Rbf2 plays a dual role in regulating protein stability and activity, we hypothesized that the pocket domain is modified by ubiquitination.

During the course of our studies on the turnover of Rbf2 by the ubiquitin-proteasome pathway, we indirectly observed the presence of potentially modified forms of Rbf2. S2 cells transfected with Flag-Rbf1 or Flag-Rbf2 constructs and treated with MG132 were analyzed by Western blots using anti-Flag or anti-Rbf2 C terminal epitope antibodies (Figure 5-6A). In extracts containing Flag-Rbf2 but not Flag-Rbf1, a proteasome-responsive slower migrating species was observed indicating that Rbf2 protein may be post-translationally modified. To test the contribution of different domains of Rbf2 to its modified form, we analyzed the status of the modified form in two mutants, 1-702 and ΔPocket, and compared it to the wild-type. Whereas the relative amounts of the Rbf2 modified form were similar for the wild-type and ΔPocket mutant, its levels were greatly enhanced in the 1-702 mutant suggesting that the C-terminus of Rbf2 downregulates this modification (Figure 5-6B).

The typical candidate for this type of modification that results in high molecular weight species is poly-ubiquitin. To test whether Rbf2 interacts with ubiquitin in vivo, S2 cells were transiently transfected with Flag-Rbf1 or Flag-Rbf2 expression constructs and subjected to Flag immunoprecipitation (IP) under non-denaturing conditions. IP with IgG antibody served as a negative control. The samples were then analyzed by anti-ubiquitin Western blotting to detect

Figure 5-6

A


Rbf1-F - + + - -
Rbf2-F - - - + +
MG132 - - + - +
IB: anti-Flag
Modified Rbf2
Rbf1-Flag
Rbf2-Flag
IB: anti-Rbf2 C
Modified Rbf2
Endo. Rbf 2
Rbf2-Flag
IB: anti-Tubulin
Tubulin

B

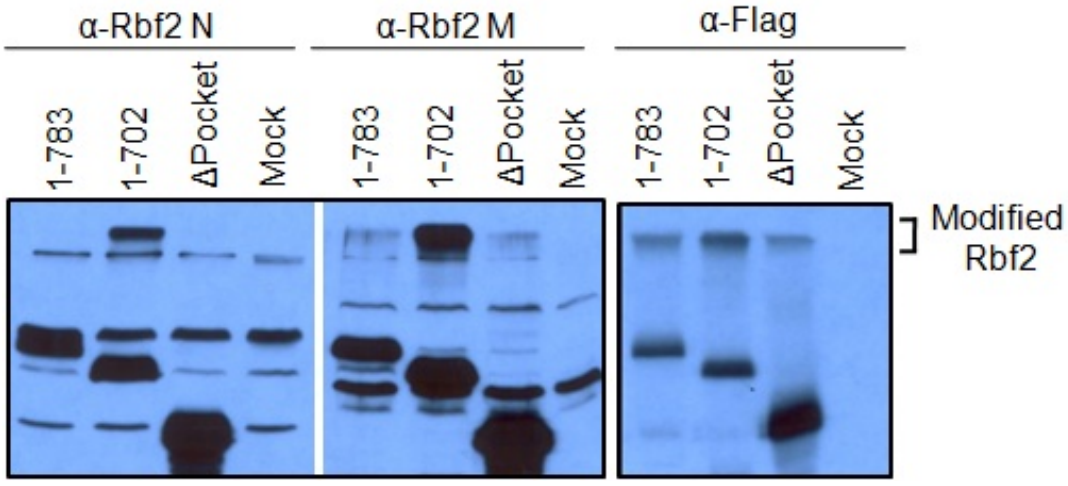

α-Rbf2 N
α-Rbf2 M
α-Flag
1-783
1-702
ΔPocket
Mock
Modified Rbf2

Figure 5-6 cont'd

Figure 5-6. Rbf2 C-terminus downregulates a proteasome-responsive post-translational modification. (A) Post-translational modification of Rbf2 that is enhanced by proteasome inhibition. Western blot analysis of extracts from S2 cells transiently transfected with Flag-epitope tagged Rbf1 or Rbf1 constructs revealed a potentially modified form of Rbf2 migrating well above the unmodified form. These higher migrating species can be detected by Flag antibody (top panel) as well as rabbit polyclonal antibodies against Rbf2 C-terminal epitope (middle panel). Tubulin levels are shown as loading controls (bottom panel). (B) Rbf2 C-terminus downregulates the abundance of modified Rbf2. S2 cells were transfected with Rbf2 WT, 1-702 or ΔPocket expression constructs and the extracts were subjected to Western blot analysis using anti-Rbf2 N-terminal epitope (left panel), anti-Rbf2 Middle epitope (middle panel) or anti-Flag antibodies (right panel). All three antibodies revealed a greatly enhanced signal for potentially modified Rbf2 in extracts containing Rbf2 1-702 mutant protein.

association of Rbf2 with endogenous ubiquitin. Indeed, the ubiquitin antibody detected a low mobility species in both Flag-Rbf1 and Flag-Rbf2 containing extracts that were immunoprecipitated with Flag antibody but not with IgG antibody (Figure 5-7A). Mock transfected cells did not show this signal. Since the protein extracts were prepared under non-denaturing condition, one possibility was that the ubiquitin signal emerged from co-factors of Rbf2 that were co-immunoprecipitated. Therefore, as a direct test of Rbf2 ubiquitination, we performed the above assay using extracts that were prepared under highly denaturing conditions to disrupt Rbf2 protein-protein interactions. Under the denaturing conditions, Rbf2 WT retained a robust ubiquitin signal in the form of a low mobility smear suggesting that the protein is indeed directly modified by poly-ubiquitination (Figure 5-7B). Based on our previous observation that the pocket domain destabilizes Rbf2, we hypothesized that the ubiquitination directed by this domain is responsible for Rbf2 degradation. To test this hypothesis, we screened three deletion mutants of Rbf2 in the above described ubiquitination assay. Consistent with the significantly enhanced stability of the pocket deletion mutants, we found that the pocket domain is indeed critical for Rbf2 ubiquitination as its deletion resulted in almost complete loss of Rbf2 poly-ubiquitinated species. The deletion of the Rbf2 N-terminus or the C-terminus, however, did not have any significant effects on ubiquitination as both mutants displayed poly-ubiquitin levels similar to the wild-type protein (Figure 5-7B). A similar result was observed when this assay was performed using the Rbf2 C epitope antibody except that in this case the Flag-Rbf2 1-702 protein was not immunoprecipitated due to the absence of the epitope (Figure 5-7C). Together, these data indicate that Rbf2 degradation is ubiquitin-proteasome dependent and that this is governed by its evolutionarily conserved pocket domain.

Figure 5-7

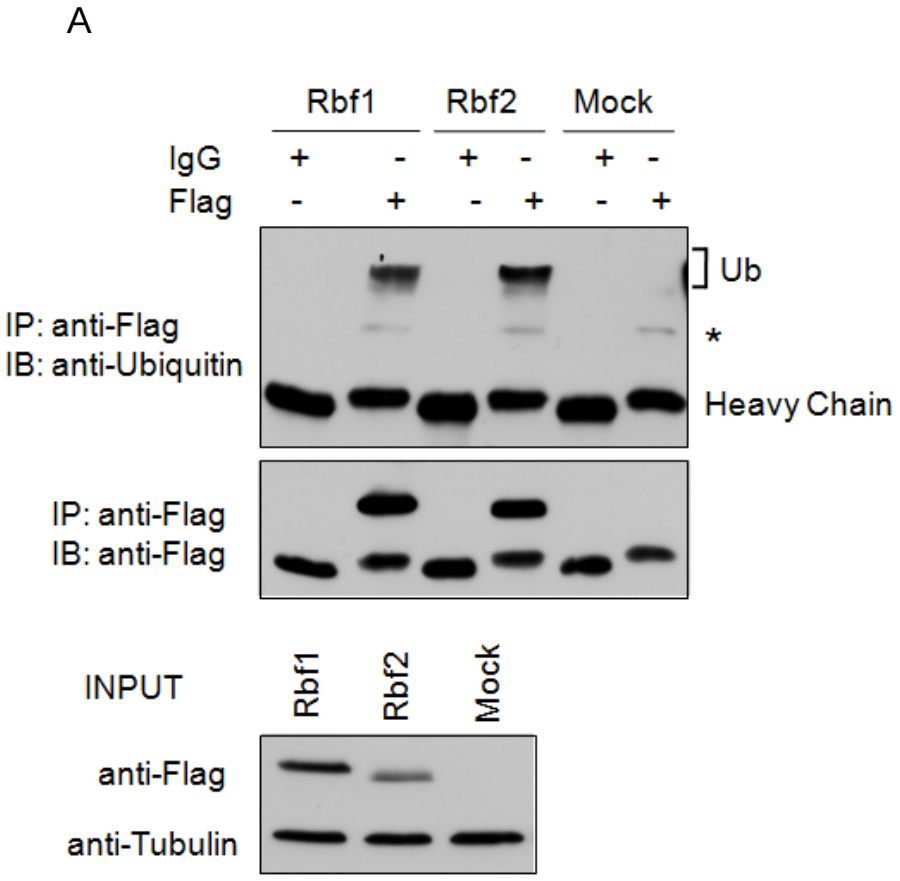

A
Rbf1
Rbf2
Mock
IgG
Flag
IP: anti-Flag
IB: anti-Ubiquitin
Ub
*
Heavy Chain
IP: anti-Flag
IB: anti-Flag
INPUT
Rbf1
Rbf2
Mock
anti-Flag
anti-Tubulin

Figure 5-7 cont'd

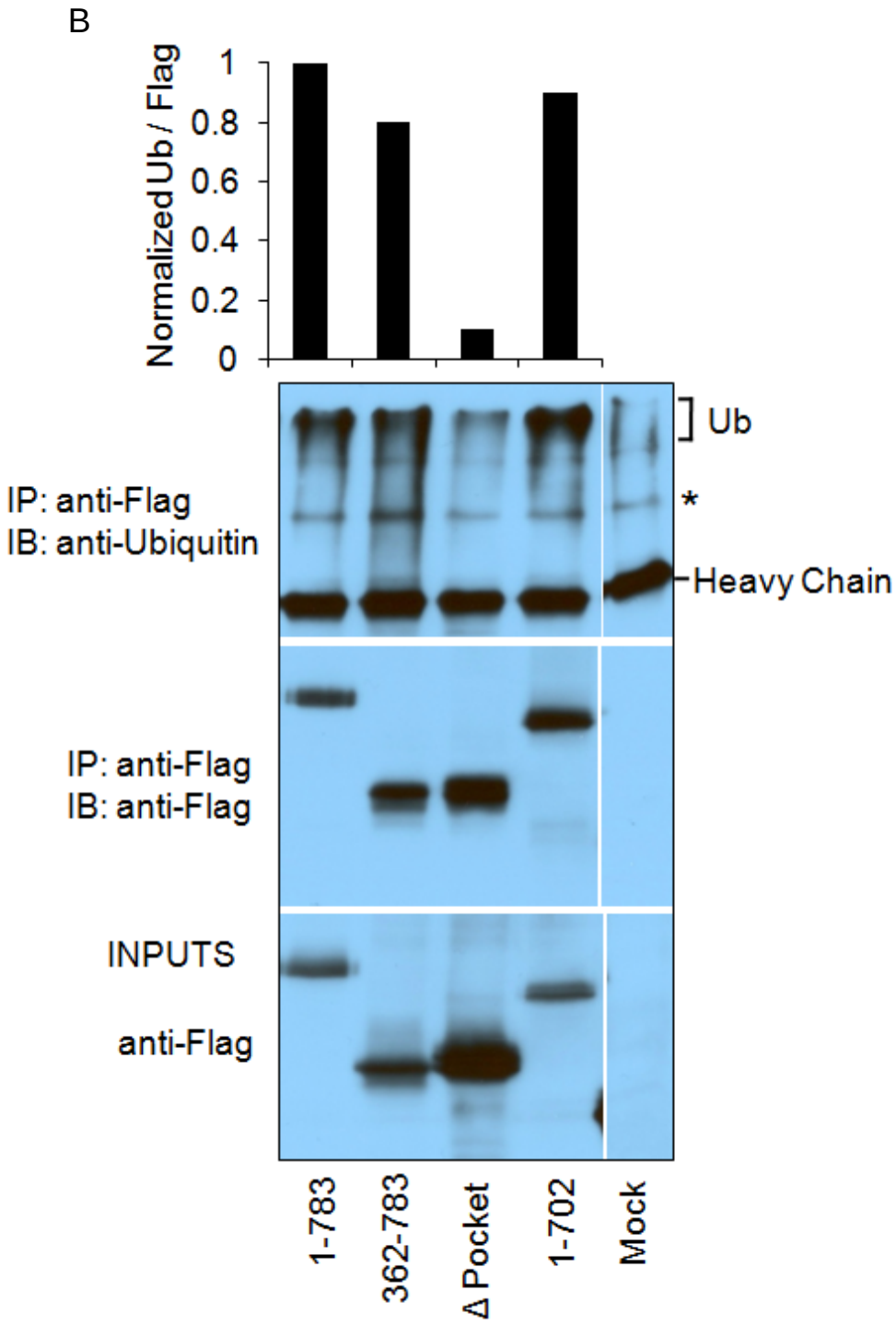


Figure 5-7 cont’d

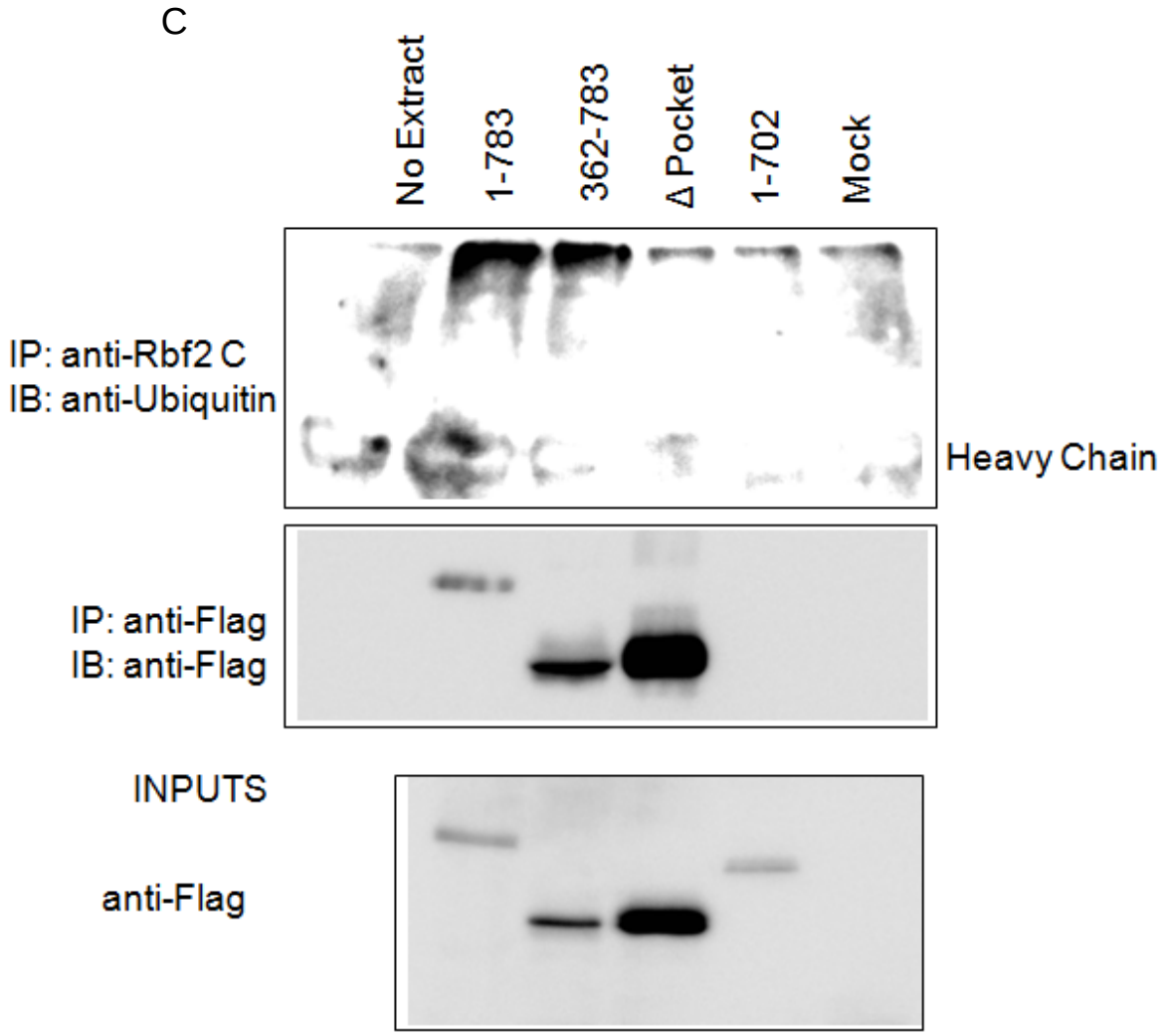


Figure 5-7 cont'd

Figure 5-7. Rbf2 pocket domain promotes its ubiquitination. (A) Rbf proteins interact with ubiquitin. S2 cells transfected with Flag-Rbf1 or Flag-Rbf2 were lysed under non-denaturing conditions and subjected to Flag immunoprecipitation (IP). IP with mouse IgG antibody served as a negative control. The samples were assayed using Western blots with anti-Ubiquitin antibody (top panel). Samples containing either Rbf1 or Rbf2 showed a low mobility species only when the IP was done with the Flag antibody and not with the non-specific antibody. Mock transfected cells did not show this signal. (B & C) Pocket domain is required for Rbf2 ubiquitination. (B) S2 cells transfected with various Flag-Rbf2 forms were lysed under highly denaturing conditions (2% SDS). These extracts were heat denatured and then subjected to Flag IP. The samples were analyzed using Western blots with anti-Ubiquitin antibody (top panel). Equivalent levels of heavy-chain IgG were seen in all samples indicating the use of equal amount of antibody for each IP reaction. Bar graphs represent the ratios of Ubiquitin/Flag signals as measured with photon-capture imaging using the Fuji LAS-3000 imager. Asterisk indicates a non-specific band that was contributed by the Flag beads. The data shown are representative of three biological replicates. (C) Rbf2 ubiquitination assay was performed as in (B) except that Flag-Rbf2 proteins were immunoprecipitated using Rbf2 C epitope (Bleed 4.7) antibody. The data shown are representative of two biological replicates.

Discussion

Similar to their mammalian counterparts, the transcriptional activities of the *Drosophila* Rbf proteins are subject to tight control by cyclin-cdk mediated phosphorylation in a cell cycle dependent manner (*53*). Our previous studies uncovered a novel mode of regulation of Rbf1 repressor function by its proteasome-mediated degradation. In this pathway, a C-terminal instability element (IE) was found to potentiate Rbf1 degradation; a property which paradoxically correlated with enhanced repressor function (*43, 44*). These observations led us to explore whether such an instability element that governs repression function existed in Rbf2. Consistent with this possibility, we had earlier demonstrated that protein levels of the endogenous Rbf2 fluctuate during embryogenesis and that its degradation is proteasome-dependent (*38, 45*). This observation indicated towards the possibility that Rbf2 degradation could be one possible mechanism of regulation of its transcriptional activity. Therefore, in this study we set out to test the contribution of the various domains of Rbf2 to its turnover and repression activity. We show that the evolutionarily conserved 'pocket domain' of Rbf2 stimulates its ubiquitin-dependent proteasome mediated degradation and is required for the repression of dE2F2 target genes. Thus, we present first evidence that the RB family pocket domain harbors elements that invoke ubiquitin-proteasome-mediated degradation in addition to operating as a repression domain. We also demonstrate that Rbf2 is a two-component repressor which, unlike Rbf1, utilizes both its N-terminal and pocket domains for mediating repression of its target genes. Additionally, we present first evidence that the previously uncharacterized Rbf2 N-terminal 'domain of unknown function' (DUF) contributes to its repression activity.

First, to test whether Rbf2 levels affect its transcriptional activity, we set out to determine the parts of Rbf2 that contribute to its degradation. Interestingly, the conserved Rbf2 pocket

domain was found to be necessary and sufficient for its degradation. The deletion of the pocket domain resulted in a significant increase in Rbf2 protein stability. This effect was not observed upon deletion of the N or C terminal regions suggesting that the Rbf2 pocket domain contains all elements that are necessary for Rbf2 degradation. We also show that unlike Rbf1, the C-terminus of Rbf2 is dispensable for transcriptional repression of dE2F target genes. Thus, in stark contrast to Rbf1, the C-terminal domain of Rbf2 is dispensable for both protein turnover and repression activity. The minimal sequence conservation between the C-terminal domains of Rbf1 and Rbf2 suggests that this instability element may be unique to Rbf1. In future, it will be interesting to test whether the Rbf2 C-terminus contributes to properties other than protein degradation and transcriptional repression.

Next, we further probed the mechanism of Rbf2 proteasome-mediated degradation by testing whether it involves poly-ubiquitination of Rbf2. Here, we present first evidence of Rbf2 poly-ubiquitination that is governed by its pocket domain. Since the pocket domain was found to be necessary and sufficient for Rbf2 degradation, it appears that it contains all elements that are involved in targeting Rbf2 to the proteasome. These may include E3 ligase binding sites and poly-ubiquitination target sites. Consistent with this possibility, the deletion of the Rbf2 pocket domain resulted in a significant loss of Rbf2 poly-ubiquitination levels. Future work will focus on determining if in fact an Rbf2 specific E3 ligase binds within the pocket domain and targets specific pocket domain lysines for ubiquitination. Interestingly, the smaller deletions within the Rbf2 pocket domain such as in ΔPocket A, ΔPocket B and ΔSpacer did not result in any significant change in Rbf2 steady-state or ubiquitination levels. Thus, these data suggest that the entire pocket domain, and not a discrete element within it, is required for Rbf2 degradation. It

may also be possible that the pocket domain harbors multiple redundantly acting instability elements interspersed within it.

Another main finding of this study was that unlike Rbf1, the N-terminus of Rbf2 is critical for its repression activity. We further show that the Rbf2 N domain provides contact site for interactions with the corepressor dE2F2. Consistent with this observation, the crystal structure of the human RB N domain revealed that this domain contains cyclin fold boxes similar to those found within the pocket domain (*54*) suggesting that the RB family N terminus may participate in E2F interactions and thereby RB mediated repression. We further show that an N terminal domain, that was previously dubbed 'Domain of Unknown Function' (DUF), contributes to Rbf2 repression of dE2F2 targets. Future work will focus on determining the mechanism by which DUF participates in Rbf2 repression. It is interesting to note that unlike the Rbf2 pocket domain, the N-terminal region does not participate in protein turnover and is therefore unlikely to utilize the UPS for potentiating repression. One possibility is that the DUF simply facilitates Rbf2-dE2F2 interactions at gene promoters. It may also be involved in the recruitment of Rbf2 cofactors that may be essential for Rbf2 repression activity. Overall, this study highlights that the two *Drosophila* retinoblastoma family proteins, Rbf1 and Rbf2, utilize distinct protein domains to repress target gene expression.

Acknowledgements

We thank Nick Dyson for sharing the *PCNA* and *Polα* luciferase reporter genes, Maxim Frolov for providing the *Myc-E2F2* construct, Ryan Blower for generating Rbf2 deletion mutants, and Tepe lab for sharing the anti-ubiquitin antibody. We also appreciate the assistance

from Satyaki Sengupta for his helpful discussions. This work was supported by the Michigan State University Gene Expression in Development and Disease Group, and a grant from the National Institute of Health (to D.N.A. and R.W.H.).

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CHAPTER SIX

CONCLUSIONS AND FUTURE PERSPECTIVES

Abstract

The RB protein is a prototypical tumor suppressor that plays a crucial role in the negative regulation of cell cycle progression. Here I describe how my studies of the *Drosophila* Retinoblastoma family proteins, Rbf1 and Rbf2, have brought to light several previously unappreciated aspects of RB biology. First, I will discuss the importance of our finding that Rbf1 repression activity is paradoxically linked to its proteasome-mediated degradation. Second, I will discuss the significance of our finding that Rbf1 ubiquitination may be a mechanism for the regulatory discrimination of diverse Rbf1 gene targets. Thirdly, I will discuss our results that suggest that certain gain-of-function mutations within the conserved RB family degron may have the potential to convert the RB tumor suppressor into an oncoprotein.

Rbf1 C-terminal IE regulates protein stability and repression activity

Previous studies have shown that the ubiquitin-proteasome system actively participates in almost all steps of DNA transcription—from initiation to export of RNA from the nucleus (*1*). Also, interestingly, the transcriptional activity of many activator proteins such as Myc, p53, Hif1α, VP16, β-catenin, Rpn4, glucocorticoid receptor (GR), c-Jun, Gcn4, Ste12, androgen receptor (AR), estrogen receptor, and Gal4 is intricately tied with their proteolytic destruction (*2-4*). These studies further pointed out that the transcriptional activation domains and degrons overlap in most of these transcriptional activators (*5*). Based on these observations, the so called 'Degron hypothesis of gene activation' was proposed wherein labile activators interact with general transcriptional machinery and the ubiquitin-proteasome system simultaneously at their target promoters. The activators are thought to recruit ubiquitin ligases to the site of transcription. These ligases then ubiquitinate the activators as well as other factors such as RNA polymerase II and histones, which serves as a molecular tag in the recruitment of the 26S

proteasome. The activator ubiquitination serves the dual purpose of protein degradation as well as for licensing the protein to perform its activation functions (*5*). The exact mechanism by which ubiquitination stimulates transcriptional activation is not clear. One study showed that the ubiquitination of VP16 activator increase its interaction with P-TEFb that augments rates of elongation of transcription (*6*). In Chapter II, we present a first report of a repressor protein, Rbf1, whose lability is tightly linked to its repression activity (*7*). We showed that a conserved instability element (IE) in the C-terminal region of Rbf1 is responsible for the 26S proteasome mediated Rbf1 degradation as well as, paradoxically, the repression of two of its cell cycle target genes, *PCNA* and *Polα*. We showed that this region is responsible for the accumulation of Rbf1 in actively proliferating tissues and not in the other tissues, suggesting that it has a role in developmental regulation of Rbf1. Interestingly, we also showed that the orthologous region in human p107 is similarly responsible for its degradation even in the heterologous S2 cell culture system suggesting that the function of the degron, at least in protein turnover, is conserved in the mammalian RB family proteins (*7*).

The most important question that this study raised was what might be the advantage of maintaining such a paradoxical relationship between repressor turnover and potency? In case of activators, it has been speculated that such a regulation could be important for rapid reprogramming of transcriptional patterns. Similarly, by having the destruction of repressors hard-wired into their repression activity, it may be possible to rapidly and consistently “clear the promoter decks” to allow a fresh transcriptional program to take over. Thus, this property may render Rbf1 transcriptional programs dynamic and sensitive to rapid changes in external environmental conditions. This may also be a mechanism to ensure that repeated rounds of repression, from a particular promoter, requires a constant supply of fresh repressors. Thus,

through this pathway, the cells may control the amount of repression that each molecule of the repressor can achieve. This property may also serve to prevent the accumulation of excess repressor that could potentially lead to squelching of the corepressors that may be needed at other genomic sites.

This study raised an intriguing question as to how might the repression activity of Rbf1 be linked to its destruction pathways. The exact mechanism by which the Rbf1 IE invokes a proteasome mediated degradation pathway is still not clear. One possibility was that the IE harbors lysine residues which are targeted for ubiquitination; a process that may stimulate Rbf1 degradation. The ubiquitination of Rbf1, similar to the case of activators, may then potentiate its repression activity while also serving to recruit the proteasome system for Rbf1 destruction. Interestingly, the Rbf1 IE harbors three evolutionarily conserved lysine residues (K732, K754 and K774) which could potentially be targeted for ubiquitination. We tested this possibility by replacing these lysines with arginine, thereby conserving the charge but blocking their potential ubiquitination. Interestingly, the lysine-to-arginine substitution mutations did not impact either Rbf1 repression or its steady-state levels indicating that these lysines are not direct ubiquitination targets and that the Rbf1 IE invokes a proteasome response through some other mechanism. Another possibility is that the positive charges of these lysine residues enable them to recruit an Rbf1 specific E3 ligase which targets the protein for ubiquitination outside of the IE. To test this possibility, it becomes imperative to identify the Rbf1 specific E3 ligase which is not known yet. Once the Rbf1 E3 ligase is identified, it will be interesting to test whether the inhibition of its ligase activity also counter intuitively inhibits Rbf1 repression potency.

Another cellular factor that may serve to connect Rbf1 degradation to its repression activity could be the COP9 signalosome complex (CSN). Previous studies from our group

showed that COP9 protects Rbf1 from its proteasome mediated degradation. Interestingly, this study also indicated that COP9 is co-enriched with Rbf1 at promoters of cell cycle genes but the physiological consequences of this feature is not known (*8*). Several studies have indicated that apart from its ability to regulate protein stability, COP9 has a role in transcriptional regulation. CSN2 was originally discovered as a corepressor of the thyroid hormone receptor (*9*). CSN5, also known as JAB1 (Jun Activating Binding Protein), stabilizes cJun transcription factor complexes, thereby increasing the specificity of target gene activation (*10*). CSN5 functions as a cofactor in MYC oncogene mediated transcriptional activation of several genes that promote cell proliferation (*11*). CSN5 has also been shown to have an antagonistic effect on the transcriptional activity of p53 by destabilizing p53 (*12*). By ChIP analysis, mouse CSN8 has been shown to be directly involved in the transcriptional regulation of cell cycle-related genes such as *Ccnd2* and *Cdk4* (*13*). Moreover, the global analysis of transcription profiles for *Drosophila csn4* and *csn5* mutants indicated that the CSN acts as a transcriptional repressor during fly development (*14*). Together, these reports indicate that CSN might play a direct role in gene expression regulation. Based on the above evidence, it was proposed that COP9 may serve to extend Rbf1 protein lifespan and thus enable stable repression of Rbf1 target promoters. However, in light of our recent findings, it may also be possible that in some contexts, COP9 may be capable of negatively interfering with Rbf1 repression by protecting it from its degradation by the proteasome. COP9 has been shown to physically associate with the proteasome and the SCF E3 ligases (*15*). Thus, it is possible that COP9 may antagonize the function of Rbf1 IE, perhaps by blocking interaction with an E3 ligase, resulting in a stabilized yet weak Rbf1 repressor. Future studies should focus on determining the Rbf1 domain that mediates its interactions with COP9. It will be interesting to test whether COP9 mediates its

stabilization effects on Rbf1 through its IE domain. It is possible that COP9 competes with an E3 ligase to bind to Rbf1 IE region and thereby prevents Rbf1 degradation. The answers to these possibilities would broaden our understanding of CSN-mediated regulation of Rbf protein stability.

In this study, we analyzed Rbf1 repression activity on only two of its target genes, *PCNA* and *Polα*, which are both involved in DNA replication and cell-cycle progression. In future, it will be interesting to determine whether the instability-activity relationship in Rbf1 is a generalized property on its numerous target genes which operate in diverse cellular processes. In Chapter III, we show evidence that this may not be the case as the Rbf1 IE mutation did not affect its repression activity on some of its signaling pathway target genes.

Our study raised several more interesting questions. First, does the Rbf1 IE function as an autonomous degron? Second, is the IE capable of functioning as an independent repression domain? Third, are the functions of the IE conserved in mammalian RB family proteins? Last but not the least, does the IE contributes to tumor suppression by the mammalian RB family proteins? The answers to these and several more such questions came in our subsequent analysis that is presented in Chapters III and IV.

Rbf1 ubiquitination stimulates its gene-specific repression function

In Chapter III, we provide further mechanistic insight into the paradoxical instability-activity relationship in Rbf1 that was described in Chapter II. In this study, we showed that the Rbf1 IE functions as an autonomous degron that stimulates both Rbf1 ubiquitination and repression activity. We further showed that the Rbf1 IE mediated ubiquitination could be one of

the mechanisms through which Rbf1 discriminates between its diverse gene sets that are involved in seemingly divergent cellular processes such as cell-cycle progression and apoptosis.

Our studies showed that the deletion of Rbf1 degron leads to a substantial reduction, but not complete loss, of Rbf1 ubiquitination. This data suggests that there are perhaps other domains in Rbf1 that are involved in its ubiquitination. It may also be possible that the deletion of the IE results in weaker or transient interaction with the E3 ligase which results in reduced ubiquitination and inefficient degradation of Rbf1. It was interesting to note that even in the context of GFP-IE chimeras, the IE lysine residues were still relevant for regulating the steady state levels of these fusion proteins. This suggested that the Rbf1 IE lysines modulate IE function through intermolecular interactions rather than intramolecular events. In the future, it will be interesting to see whether these lysines contribute to the ubiquitination of GFP-IE proteins or alternatively may be involved in proteasome-targeting of the ubiquitinated substrates. Such analysis will help elucidate the mechanism by which the lysines in the Rbf1 degron modulate protein turnover.

It will also be interesting to determine the nature of ubiquitin modification that is involved in regulation of Rbf1 turnover and activity. Previous studies have shown that the status of protein ubiquitination determines protein fate. The canonical poly-ubiquitin lysine 48 chain targets proteins for 26S proteasome-mediated degradation, whereas poly-ubiquitin chains formed via lysine 63 target proteins for diverse cellular activities (*16-18*). Future studies should focus on determining whether the mono- or poly-ubiquitination of Rbf1 contributes to its degradation and repression activities. This can be achieved by identifying the ubiquitination sites in Rbf1 through proteomic approaches and then analyzing the effects of mutations at these sites on Rbf1 turnover

and repression. The availability of this information will allow the development of specific inhibitors of RB family ubiquitination in order to modulate its function.

We also showed evidence that Rbf1 IE mediated ubiquitination plays a role in the repression of cell-cycle target genes but not in repression of the cell signaling genes. The analysis presented in this chapter is limited to the study of Rbf1 repression on only two of its target genes, *PCNA* and *InR*, which operate in different cellular processes. Thus, these findings need to be extended to several more Rbf1 target promoters to show that there is indeed a pattern to the selective usage of Rbf1 IE in potentiating its repression activity in a gene-specific manner. Recent genome–wide association studies have uncovered a plethora of Rbf1 target genes that are involved in very diverse cellular processes. One of the most interesting findings of this study was the previously unappreciated large-scale association of Rbf1 with multiple targets of conserved signaling pathways. Studies elucidating the importance of Rbf1 IE to the repression of these genes will be critical to our understanding of their regulation by Rbf1.

Rbf1 degron dysfunction enhances ectopic S-phase entry

In Chapter IV, we describe our interesting observation that Rbf1 mutants lacking the IE domain enhance G1-to-S phase progression when overexpressed in *Drosophila* S2 cells, suggesting that this class of mutations provides a gain-of-function for DNA synthesis. These findings bring to light an unprecedented class of RB family mutations that may have the potential to convert the tumor suppressor into an oncoprotein.

Our data indirectly indicates that mutations within the Rbf1 degron may provide a distinct growth advantage to cells, perhaps by stabilizing and exacerbating dE2F1 activation potential. Whether this S-phase stimulatory effect of the Rbf1 Δdegron mutation results in

enhanced cellular proliferation remains to be seen. This can be tested by a simple cell counting experiment where a competition is set up between two cell populations over expressing either Rbf1 WT or the Δdegron mutant proteins. In future, it will also be very interesting to determine whether this gain-of-function property is conserved in the mammalian RB family proteins and whether it has the potential to stimulate mammalian cells into a hyper proliferative state. These findings are especially relevant in the context of cancer cells where such RB family mutations may act as driver genes in oncogenesis.

Based on our observation that Rbf1 Δdegron mutant can enhance cell-cycle progression, we hypothesized that mutations within RB family degron are selected for in human cancers. Consistent with this idea, several studies have identified mutations in the evolutionarily conserved C-terminal domains of RB and p130 in human cancers suggesting that perturbations in this domain play an important role in disease. Mutations in C-terminal region of RB are associated with a variety of osteosarcomas (*19, 20*) and mutations in C-terminal region of p130 orthologous to Rbf1 IE are associated with non-small cell lung cancer (*21*). It is interesting to note that several of the p130 degron mutations that were associated with lung cancer were found in the conserved basic residues in this region that we found to be important for the regulation of Rbf1 turnover and activity. In future studies, it will be interesting to determine the contributions of these mutations to p130 biology and their relevance in human cancer.

Acknowledgements

I thank my advisor, Dr. Henry and my committee member, Dr. David Arnosti for their helpful discussions.

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