# STABILITY, ACTIVITY AND GENOME-WIDE ASSOCIATION OF THE *DROSOPHILA* RETINOBLASTOMA TUMOR SUPPRESSOR RBF1

By

Pankaj Acharya

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## ABSTRACT

STABILITY, ACTIVITY AND GENOME-WIDE ASSOCIATION OF THE *DROSOPHILA* RETINOBLASTOMA TUMOR SUPPRESSOR RBF1

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The Retinoblastoma protein (RB) is a well known tumor suppressor that controls cell cycle and developmentally regulated gene expression. Germ-line mutation of RB is closely linked with retinoblastoma in early childhood and osteosarcoma in adolescence, and is mutated in about half of all human cancers. During normal growth, interactions between E2Fs and RB family proteins, including the *Drosophila* RB homolog, Rbf1 are regulated by phosphorylation by cyclins and cyclin-dependent kinases (CDKs) and proteolytic destruction by the proteasome. 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 C-terminal instability element (IE) stabilized Rbf1, but sacrifice 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 the instability and activity relation is similar to that observed in the case of transcriptional activators such as VP16 and Myc.

Physical and functional targets of RB and its paralogs p107/p130 have been studied largely in cultured cells, but the full biological context of this family of proteins' activities will likely be revealed only in whole organismal studies. To identify direct targets of the major Drosophila RB counterpart in a developmental context, we carried out ChIP-Seq analysis of Rbf1 in the embryo. The association of the protein with promoters is developmentally controlled;

early promoter access is globally inhibited, while later in development Rbf1 was found to associate with promoter-proximal regions of approximately 2,000 genes. In addition to the conserved cell cycle-related genes, a wholly unexpected finding was that Rbf1 targets many components of the insulin, Hippo, JAK/STAT, Notch and other conserved signaling pathways. Rbf1 may thus directly affect output of these essential growth-control and differentiation pathways by regulation of receptor, kinase and downstream effector expression. Rbf1 was also found to target multiple levels of its own regulatory hierarchy. Bioinformatic analysis indicates that different classes of bound genes exhibit distinct promoter motifs, suggesting that the context of Rbf1 recruitment involves diverse transcription factors, which may allow for independent regulation of Rbf1 bound genes. Many of these targeted genes are bound by Rbf1 homologs in human cells, indicating that a conserved role of retinoblastoma proteins may be to adjust the set point of interlinked signaling networks essential for growth and development.

*To my Dad, Mr. Dhruba Prasad Acharya, my Mom, Mrs. Kopila Acharya,*

*my wife, Kunjana Acharya*

*my son, Anshuman Acharya*

***AND***

*To my mentor, Dr. David N. Arnosti*

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## KEY TO ABBREVIATION

| | |
|---|---|
| Akt | Protein Kinase B |
| AR | Androgen Receptor |
| ATP | Adenosine Triphosphate |
| BMI1 | B lymphoma Mo-MLV insertion region 1 homolog |
| BRG1 | BRM-related Gene product 1 |
| BRM | Brahma |
| CBP | CREB Binding Protein |
| CDK | Cyclin Dependent Kinase |
| CDKN2A | Cyclin Dependent Kinase Inhibitor 2A |
| CHD | Chromodomain Helicase DNA-binding |
| ChIP | Chromatin Immunoprecipitation |
| CHK2 | Checkpoint Homolog 2 |
| CMV | Cytomegalovirus |
| COP9 | Constitutive Photomorphogenic 9 |
| CSN | COP9 Signalosome |
| DCB | Dihydrocytochalasin B |
| dILP | Drosophila Insulin-like Peptides |
| 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 |
| E(bx) | Enhancer of bithorax |
| 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 |
| EBNA3C | Epstein-Barr virus nuclear antigen 3C |
| EZH2 | Enhancer of Zeste 2 |
| FOXO | Forkhead box-containing protein, O subfamily |
| GR | Glucocorticoid receptor |
| HCC | Hepatocellular carcinoma |
| HDAC | Histone Deacetylase |
| HPV E7 | Human Papilloma Virus Early 7 |
| IE | Instability Element |
| InR | Insulin Receptor |
| IRS | Insulin Receptor Substrate |
| ISWI | Imitation Switch |
| Ken | Ken and Barbie |
| L | Lobe |
| l(3)mbt | lethal (3) malignant brain tumor |
| L3MBTL1 | lethal (3) malignant brain tumor like 1 |
| LOH | Loss of Heterozygosity |

| | |
|---|---|
| MBD | Methyl-CpG-binding domain |
| 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 |
| NICD | Notch Intracellular Domain |
| NURF | Nucleosome Remodeling Factor |
| P/CAF | p300/CBP Associated Factor |
| PCNA | Proliferating Cell Nuclear Antigen |
| PDK1 | Phosphatidylinositol-Dependent Kinase 1 |
| PI3K | Phosphatidylinositol 3-Kinase |
| PIC | Pre-Initiation Complex |
| PIP2 | Phosphatidylinositol-4,5-biphosphate |
| PIP3 | Phosphatidylinositol-3,4,5-triphosphate |
| PP1 | Protein Phosphatase type 1 |
| PP2 | Protein Phosphatase type IIA |
| PPAR | Peroxisome Proliferator-Activated Receptor |
| PRC1L | Polycomb-repressive complex-1-like |
| PRC2 | Polycomb-repressive complex-2 |
| PTEN | Phosphatase and Tensin Homolog |
| Pzg | Putzig |
| RB, pRB | Retinoblastoma Protein |
| Rbf1 | Retinoblastoma Factor 1 |

| | |
|---|---|
| Rbf2 | Retinoblastoma Factor 2 |
| RBP1 | Retinoblastoma Binding Protein 1 |
| Rheb | Ras-Homolog Expressed in Brain |
| RNA | Ribonucleic Acid |
| S2 cells | Schneider 2 cells |
| S6K | p70 ribosomal S6 Kinase |
| SCF | Skp/Cullin/F-box complex |
| SIK2 | Salt Induced Kinase |
| SIRT1 | Sirtuin 1 |
| SMYD2 | SET and MYND domain containing 2 |
| Su(dx) | Suppressor of deltex |
| 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 |
| Tip60 | TAT interacting protein 60 |
| TKO | Triple Knock Out |
| TOR | Target of Rapamycin |
| TSC | Tuberous Sclerosis Complex |
| TSS | Transcriptional Start Site |
| UTX | Ubiquitously transcribed tetraticopeptide repeat, X chromosome |
| VP16 | Virion Protein 16 |

# CHAPTER I

# INTRODUCTION

## *I. Introduction*

Named after its role in pediatric retinoblastoma, the Retinoblastoma protein (pRB) is a tumor suppressor that controls transcription of cell cycle specific and developmental genes, and thereby regulates cell cycle, differentiation, apoptosis and growth [1-6]. *RB1,* the gene encoding this protein, was the first tumor suppressor gene to be cloned [7, 8]. Germline mutation of *RB1* is associated with retinoblastoma in childhood [9] and osteosarcoma in adolescence [10] and is one of the two most mutated genes in human cancers [11]. The E2F family of transcription factors recruit pRB to the target gene promoters, thereby repressing their transcription [12, 13], and controlling the cell cycle. pRB's cell cycle control is frequently disrupted in wide variety of cancers [11, 14-16]. Though less complex than its human counterpart, the *Drosophila* retinoblastoma network is conserved [17]. We utilized this less complex network to study the structure and function of Rbf1, the *Drosophila* ortholog of pRB, and its genome wide association with target gene promoters. In this study I describe research that has contributed to our understanding of pRB regulation in two important areas. First, we show that a degron, which we named IE (instability element), is present in the C-terminal region of Rbf1, and that the IE is also responsible for its activity on certain promoters. Second, I show that Rbf1 targets conserved signaling pathway genes that were previously not known to be regulated by this tumor suppressor protein, suggesting widespread involvement of cell-cycle links to central developmental signaling events.

### *II. The Retinoblastoma family of proteins or the 'pocket' proteins*

The Retinoblastoma protein (pRB) is a transcriptional corepressor that regulates a wide variety of cellular processes such as cell cycle, growth, differentiation, and apoptosis. The negative regulation of cell cycle is most well studied [1-4, 6, 18]. Apart from pRB, there are two other closely related proteins in mammals, p107 (or *RBL1*) and p130 (or *RBL2*). These three proteins are collectively also known as "pocket proteins" because of a conserved pocket region that consists of two conserved A and B subdomains, with a less conserved spacer between them [19]. The A and B domains are characterized by tandem cyclin folds [20-22], which are known to mediate protein-protein interactions. The pocket also contains a conserved "LxCxE binding motif" that interacts with LxCxE sequences present on several cellular proteins such as chromatin remodelers, replication factors, transcription factors, kinases and others. Several viruses produce proteins targeting this "LxCxE binding motif" of pRB to inactivate the tumor suppressor for their proliferation. These proteins include the Adenovirus E1A, SV40 TAg and HPV E7 gene products that function as viral oncoproteins to compete with cellular proteins for binding to pRB [21, 23].

Amino acid sequence alignment reveals that p107 and p130 are more similar to each other than either are to pRB [24]. The spacer sequence that separates the A and B boxes of the pocket region is longer in p107 and p130 than in pRB. Furthermore, the spacer contains a cyclin/cdk binding site in both p107 and p130 but not in pRB [25-27], which help these pocket proteins form stable complexes with cdks.

All three pocket proteins bind to heterodimers of E2F and DP transcription factors and control cell cycle progression. In mammals, there are nine E2Fs, E2F1-3a, E2F3b, E2F4-8 and three DPs, DP1, DP2 and DP4 [28]. Studies have shown that p107 or p130 deficient cells show

# Figure 1.1

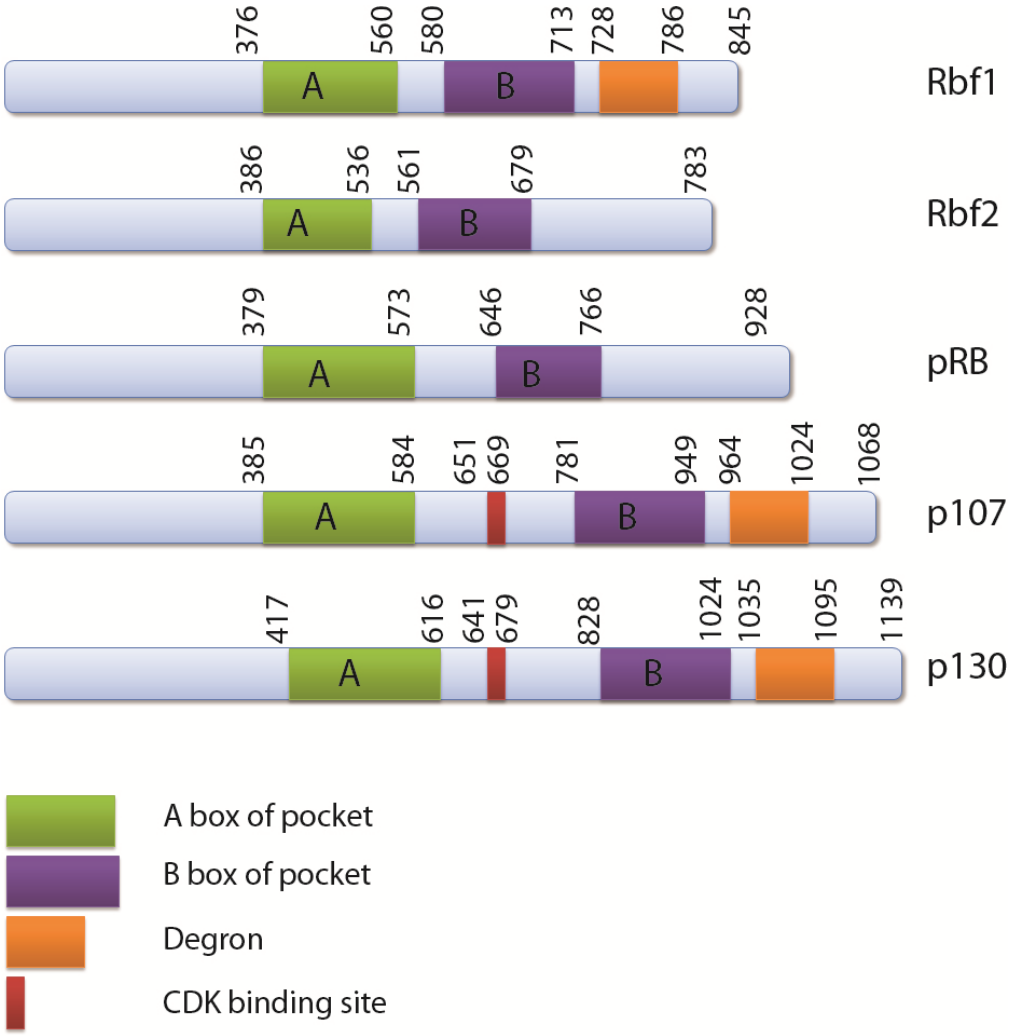


**Figure 1.1 Schematic drawings of *Drosophila* and human retinoblastoma family proteins.** Comparison of the structure of fly and human pRB family proteins is shown here. The A and B boxes of the pocket region are shown in green and purple respectively. A putative degron that is present in Rbf1, p107 and p130 is indicated in orange. A cyclin/CDK binding site found only in p107 and p130 is shown in red. The amino acid residue numbers are shown above the structure of each protein. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

similar cell cycle defects as pRB deficient cells, thus it seems that to a certain extent there is functional redundancy among these proteins [29]. Further *in vivo* evidence for functional redundancy is provided by mutant phenotypes of knockout mice; the developmental defects worsen when p107 [30] or p130 [31] deficiencies are combined with loss of pRB. Although functional redundancies exist among these three pocket proteins, pRB is by far the most important one with unique functions. Homozygous mutation in *RB1* gene is embryonic lethal [32], however, *p107* or *p130* mutant mice have normal development [33]. Although pRB, p107 and p130 bind to E2F transcription factors, they differ in preferences for E2Fs. pRB prefers to bind E2F1, E2F2, E2F3a, E2F3b and E2F4, while p107 and p130 prefer to bind E2F3b, E2F4 and E2F5 [28]. In a pRB and E2F4 null background, however, p107 and p130 regulate E2F1-3 [34].

Comparison of pRB in human, mouse, frog and chicken, shows high (66%) amino acid conservation in the A and B boxes of pocket region. However, the spacer region has only 33% identity. Interestingly, a 20 amino acid sequence (SKFQQKLAEMTSTRTRMQKQ) at the C-terminus is 100% conserved in pRB in all four organisms [35]. *Drosophila* has two retinoblastoma orthologs, Rbf1 and Rbf2 [36]. The overall sequence similarity puts Rbf1 closer to p107 and p130 than pRB, however Rbf1 is more similar to pRB because of the absence of a cyclin / cdk binding site in the spacer and sequence of the B box [37]. Rbf2 is more similar to Rbf1 than other mammalian pocket proteins, and shows closer similarity to mammalian p107 and p130 than pRB [36].

The RB-E2F network is much simpler in *Drosophila* than in mammals. There are only two E2Fs, E2F1 and E2F2 and only one DP. E2F1 is an activator and E2F2 is a repressor. Rbf1 binds to both E2F1 and 2 whereas Rbf2 binds to only E2F2 [36]. In *Drosophila,* a single copy of

Rbf1 is sufficient for normal growth and development. Null mutants in Rbf1 are larval sub-viable, never reaching the late pupal stages of development [38]. However, Rbf2 null mutant flies are viable and fertile. Mutation of Rbf2 in Rbf1 mutant background revealed that these two fly pocket proteins have some functional redundancy [39]. Some animals have even more reduced RB systems: *C. elegans* has only one pRB ortholog, lin-35. This gene is dispensable for viability, although the animals have a multivulval phenotype. By sequence homology, lin-35 is closer to Rbf1, p107 and p130 than pRB [40].

***III. Tissue expression patterns of the Retinoblastoma family of proteins***

Mammalian pRB, p107 and p130, despite their functional redundancies, are differentially expressed in tissues and during different stages of the cell cycle. The total pRB levels are abundant throughout the cell cycle; however the phosphorylation status changes. p130, on the other hand, is highly expressed in quiescent and differentiated cells, but at low levels in proliferating cells. p107 is expressed at low levels in non-proliferating cells, but the levels increase in the proliferating cells [41-47]. These three mammalian pocket proteins are expressed in dynamic patterns during mouse development [48, 49]. *pRB* transcripts are expressed during neurogenesis, hematopoiesis, myogenesis, lens development and in the ganglion cell layer of the embryonic retina before and during differentiation. In contrast, *p107*, but not *pRB* is expressed in the heart, lung, kidney and intestine. However, both *pRB* and *p107* are co-expressed in liver and the central nervous system [49]. In the central nervous system, *p107* is restricted to proliferating cells as mentioned in the previous studies [41, 43-46]; while, *pRB* is expressed in both proliferating and differentiating cells. Interestingly, *p130* transcripts are found at low levels throughout embryogenesis [49]. The retinoblastoma family proteins are also differentially

regulated in the mammary luminal epithelium of mice [48]. These studies underscore the distinct roles of pRB, p107 and p130 during development.

Similarly, the *Drosophila* counterparts, Rbf1 and Rbf2 are also differentially and co-expressed during development [36, 50]. Rbf1 protein levels are relatively unchanged throughout embryogenesis and also in larvae and adult flies, however, Rbf2 levels peak between four and ten hours of embryonic development before declining, and the protein is undetectable in adult flies [36, 50]. Proteins and transcripts of both Rbf1 and Rbf2 are ubiquitously expressed during early embryogenesis; however they are differentially expressed and confined to certain tissues during later stages. Both pocket proteins are confined to epidermis, gut and central nervous system during embryogenesis [50]. Interestingly, *rbf1* and *rbf2* are differentially expressed in central nervous system [50] consistent with the distinct expression patterns of *pRB* and *p107* in central nervous system [49]. These studies in mice and flies showing distinct expression patterns in developing embryo suggest that the pocket proteins play unique roles during development.

***IV. Regulation of the pocket proteins***

The retinoblastoma family proteins are regulated by various modifications, of which phosphorylation is the most important one [51-53]. Hypophosphorylated forms of pRB, p107 and p130 bind to E2F/DP and repress target genes during G0 and early G1 phase. Their phosphorylation states are progressively increased during the transition from G1 to S phase of cell cycle. This hyperphosphorylation causes dissociation of pocket proteins from E2F/DP, relieving transcriptional repression [51-53]. Phosphorylation of pRB, p107 and p130 is executed by Cyclin D/CDK4, Cyclin D/CDK6, Cyclin A/CDK2, Cyclin E/CDK2, CHK2 (checkpoint homolog 2) and RAF-1 (c-Raf) [51]. During late mitosis, pRB is dephosphorylated by phosphoprotein phosphatase type 1 (PP1), recycling it for regulation of subsequent cell cycles

[54, 55]. Another phosphatase, PP2A has been shown to dephosphorylate pRB, p107 and p130 [55-57]. Based on sequence analysis, there are 16 putative cyclin/CDK phosphorylation sites in human pRB [58, 59]. Upon mutation of 11 out of 16 cyclin/CDK phosphorylation sites, pRB becomes resistant to cyclin/CDK regulation, and is able to block cell proliferation in fibroblasts and tumor derived cell lines [58, 60, 61]. Out of those 11 sites, 7 are in the C terminal region [62]. It is hypothesized that a conformational change is created by the phosphorylation at the C terminus. This conformational change in turn displaces histone deacetylase (HDAC) bound to pRB and finally disrupts the E2F-pRB interaction [63]. Similarly, the *Drosophila* counterpart, Rbf1 has 5 putative cyclin/CDK phosphorylation sites, four of which are in the C terminal region. Serine/threonine to alanine point mutations of those sites rendered Rbf1 insensitive to cyclin/CDK regulation [64]. Taken together, phosphorylation is a very important mode of regulating the control of retinoblastoma family proteins during the cell cycle.

Another type of modification of human pRB is acetylation at lysines 873 and 874 [65-67]. DNA-damage-dependent acetylation at K873/874 interferes with pRB-E2F1 interaction, retains pRB in a hypophosphorylated state, keeps E2F bound to pRB pocket, however releases E2F1 bound to C-terminal region and allows E2F1 activity [65]. Acetylation of the C-terminus of pRB by p300/CBP, which is a histone acetyl transferase, blocks pRB phosphorylation by Cyclin E/CDK2 [66]. Not only p300, but also p300-Associated Factor (P/CAF) can acetylate pRB, resulting in pRB-mediated terminal cell cycle exit and induction of late myogenic gene expression [67]. A recent study showed that acetylation of pRB by P/CAF is required for its nuclear localization and keratinocyte differentiation [68]. SIRT1 (sirtuin 1) binds to pRB and mediates NAD dependent deacetylation. This deacetylase activity of SIRT1 ensures resumption of cell cycle when required by making pRB susceptible to cyclin/CDK mediated phosphorylation

[69]. Acetylation of pRB might be beneficial in differentiation and cellular stress, such as DNA damage, to make sure cell cycle is arrested. However, an alternative pathway for pRB acetylation is also possible: Tat interacting protein 60 (Tip60) targets pRB for proteasomal mediated degradation by acetylating its C-terminus. $p14^{ARF}$, an alternative reading frame product of the cyclin-dependent kinase inhibitor 2A (CDKN2A) locus, retards Tip60 induced acetylation of pRB [70]. $p14^{ARF}$ is a positive regulator or p53 tumor suppressor whereas $p16^{INK4a}$, another product of CDKN2A locus positively regulates pRB [71].

In addition to the above modifications, sumoylation is another type of modification in pRB. Sumoylation is a small ubiquitin-like modification of proteins and has been shown to play important roles in several biological processes and human diseases [72]. pRB can be sumoylated at lysine 720, close to the LxCxE binding motif [73]. The function of sumoylation in the context of pRB is not known, but in the case of p53 and RBP1 (retinoblastoma binding protein 1), this modification has been implicated in induction of senescence [74, 75].

Two recent studies showed that pRB can be methylated by Set7/9[76] and SMYD2 [77]. Set7/9, a histone mono-methyltranserase, is responsible for methylation of pRB at lysine 873, which in turn promotes transcriptional repression. K873 methylation also induces cellular senescence and differentiation [76]. However, SMYD2 methyltransferase methylates pRB at K860, which is in proximity with pRB acetylation sites (K873 and K874). This methylation at K860 is elevated in G0 phase, during cell cycle exit and during first stages of differentiation. Furthermore, SMYD2 mediated methylation promotes interaction between pRB and the corepressor L3MBTL1, a human homolog of *Drosophila* tumor suppressor l(3)mbt [77]. Taken together, methylation of pRB at different residues seems to enhance its tumor suppressor activity.

A final level of regulation involves protein turnover. Some studies have shown the importance of this event in the mammalian setting. Caspase cleaves pRB at consensus caspase-cleavage site $_{884}$DEADG$_{888}$ present towards the C-terminus. Absence of this site makes mice resistant to TNFα induced apoptosis and this in turn renders the mice prone to cancer [78]. The Ubiquitin-proteasome pathway is another major proteolytic pathway [79]. Ubiquitin is a conserved 76-amino acid peptide, which is covalently tagged to lysine residues. The polyubiquitylated proteins are destined for degradation [79]. pRB is ubiquitylated *in vitro* and *in vivo* by Mdm2, a ubiquitin ligase for p53, and targeted for degradation [80]. However, besides proteolysis, ubiquitin-tagged proteins are also involved in certain nonproteolytic cellular events, such as nuclear localization, endocytosis and cofactor recruitment [81].

In addition to the above mentioned modifications of retinoblastoma family of proteins, in chapter two, I describe our findings about a novel turnover-mediated regulation of *Drosophila* retinoblastoma tumor suppressor Rbf1. We found a degron-containing 59-amino acid region in the C-terminus of Rbf1 just C-terminal to the pocket's B box. Furthermore, this region is also critical for Rbf1's activity. Point mutations in certain amino acid residues made the protein lose its activity and accumulate to higher levels. We named the region as IE for instability element. We also found single point mutations in K774 that make the protein hyperactive, suggesting that this region has additional potential regulatory activities that do not affect protein levels. We extended our study to the human ortholog, p107 and found that its IE is responsible for its turnover. Our findings suggest that in addition to phosphorylation, acetylation, methylation, sumoylation, ubiquitylation and caspase cleavage, retinoblastoma proteins might be generally regulated by IE dependent turnover.

### *V. Role of retinoblastoma proteins in differentiation and development*

A variety of developmental phenotypes underscore the roles of pRB and related proteins in tissue differentiation processes. Ectopic expression of a genomic construct of pRB caused the transgenic mice to be dwarf by embryonic day 15 compared to their nontransgenic littermates [82]. Heterozygous $pRB^{+/-}$ mice develop normally and do not develop retinoblastoma. However, when pRB is completely knocked out from mice, they die between embryonic day 13 and 15 with several defects [4, 32, 83, 84]. $pRB^{-/-}$ embryos show defects in neural, hematopoietic and eye development that are caused by enhanced apoptosis [32, 83, 84]. In addition, $pRB^{-/-}$ embryos have abnormal placenta with excessive proliferation of trophoblast cells and severe disruption of the normal labyrinth architecture. Interestingly, wild-type placenta is sufficient to carry the *pRB* deficient embryos to term, however, they don't survive long after birth [85].

In contrast to the severe *RB* mutant effects, $p107^{-/-}$ or $p130^{-/-}$ mice show no apparent phenotypes [33, 86] suggesting that either these pocket proteins are not important for development or retinoblastoma family of proteins have overlapping functions. However, mice with the combination of $p107^{-/-}$ and $p130^{-/-}$ mutations are born with short limbs, short snout, reduced rib cage size and die soon after birth because of defective tracheal cartilage mediated respiratory failure [33]. This result confirms that the members of pocket family of proteins have overlapping functions. This notion is further supported by the fact that quiescent pRB-negative or p130-negative cells have elevated levels of p107 [29, 33]. Furthermore, triple knockout ($pRB^{-/-}$ ,$p107^{-/-}$,$p130^{-/-}$) cells are resistant to senescence-inducing signals such as DNA damage, contact inhibition, serum starvation, and they had shorter cell cycle compared to the wild type, single or double knock out cells [87, 88]. Interestingly $pRB^{-/-}$ MEFs that have elevated levels of p107 are

sensitive to senescence-inducing signals, however, acute loss of *pRB* causes increase in the number of cells entering S phase probably because there is a lag time between inactivation of *pRB* and induction of *p107* [89]. *pRB/p107* or *pRB/p130* null embryos have similar phenotypes as $pRB^{-/-}$ littermates, but die a couple of days earlier. This aggravating phenotype suggests that p107 and p130 can partially substitute for pRB during development [86, 90, 91]. Interestingly, some phenotypes exhibited by $pRB^{-/-}$ mice can be partially rescued in E2F1, E2F2 and E2F3 mutant backgrounds [92-94]. The current model is that E2F1, E2F2 and E2F3 have some redundancy and knocking out pRB causes these E2Fs to go unchecked, causing various biological consequences. Furthermore, these three E2Fs are significant regulators of pRB function and must be kept under control for normal development [92]. The differentiated roles of pocket proteins can also be inferred from gene expression changes. The majority of genes misexpressed in pRB-deficient cells include genes encoding DNA replication and cell cycle regulatory proteins, but the genes misexpressed in p107/p130-deficient cells are mostly devoid of DNA replication and cell cycle regulatory genes [95]. Taken together, the molecular and genetic evidence indicates that the three mammalian pocket proteins play critical and distinct roles during development.

Similar to the phenotype observed with the mammalian pRB, null mutants of *Drosophila rbf1* are larval lethal. The embryos do hatch into larvae, but die at early larvae stage [96]. Although Rbf1 is dispensable for early cell cycles, it plays an important role in controlling G1 to S phase transition during development. The lethality in *rbf1* null mutants is caused by the deregulation of the cell cycle and elevation of apoptosis in embryos [38, 96]. E2F1 and E2F2 are the transcription factors that recruit Rbf1 to the target gene promoters. E2F1 is an activator, so removing Rbf1 from the system is thought to activate target genes, many of which are involved

in the cell cycle. Conversely, overexpression of E2F1 should have the same effect. Nicholas Dyson's group showed that overexpression of E2F1 in the fly eye causes a rough eye phenotype, a result of disruption of eye development due to post-mitotic cells re-entering cell cycle [97]. This phenotype is further worsened by *rbf1* mutation [38] which supports the notion that Rbf1 suppresses E2F1 activity. Consistent with this notion, knocking down E2F1 in an *rbf1* background rescues the early larval lethality and late differentiation phenotypes [96].

Polyteny in *Drosophila* adds more complexity to the cell cycle and this process involves Rbf activity. Polyteny is caused by endoreduplication cycles in many larval and adult tissues [98]. Endoreduplication in a process involving more than one round of DNA replication without cytokinesis that leads to polyploidy. Giant chromosomes in *Drosophila* salivary glands are well known examples of result of endoreduplication [99]. *Drosophila* follicle cells undergo endoreduplication cycles to reach 16n ploidy. This is important for localized amplification of the chorion gene cluster, which is necessary for eggshell formation. Endoreduplication in follicle cells is regulated by Rbf1/E2F; in absence of Rbf1 or E2F or in presence of mutant E2F that cannot recruit Rbf1, the cells reach 32 ploidy [100].

Eye-specific overexpression of Rbf1 causes rough and small eye phenotypes. Rbf1 mutant proteins that are resistant to cyclin/CDK phosphorylation worsen the eye phenotypes [64]. In chapter II, I show that similar severe phenotypes are induced by a single point mutation towards the C-terminus makes Rbf1 hyperactive. Interestingly, *rbf2* mutant flies are viable and fertile and they only exhibit a phenotype in a background with reduced expression of *rbf1* [39]. This result suggests that Rbf1 and Rbf2 may have functionally redundant roles in development, similar to the mammalian counterparts. Molecular studies support this overlapping function; Rbf1 and Rbf2 co-occupy many of the same genes, and a knockdown study in *Drosophila* S2

cells, showed that some target genes show significant perturbations only when both proteins are knocked down [101].

### *VI. Role of retinoblastoma proteins in tumorigenesis*

Germline mutation of pRB is closely linked with retinoblastoma in early childhood [9] and osteosarcoma in adolescence [10] and is one of the two most mutated genes in a variety of sporadic and familiar human cancers [11]. Mutations in *p107* or *p130* were not observed in a recent genome wide study of somatic copy-number alteration in a wide variety of human cancers [102]. Although deletions of chromosomal regions containing p107 or p130 have been reported in cancers, these large deletions contain many other genes that may also play a role in tumorigenesis [102-105]. Further experimental evidence suggests that pRB is a potent tumor suppressor but p107 and p130 are not because mutations in *pRB* cause tumors to develop in chimeric mice but *p107*, *p130* or $p107^{-/-};p130^{-/-}$ mutant mice do not show any tumors [33, 86, 106]. However, *p107* or *p130* mutations in *pRB* null background worsen the tumor phenotype compared with *pRB* null mutation alone [86, 90, 91, 107-110], which indicates that they may act as tumor suppressor in certain contexts. In *Drosophila*, overexpression of the Notch ligand Delta in the eye causes enlarged eye phenotypes, which when coupled with activation of two Polycomb group proteins, Pipsqueak and Lola, induces metastatic tumors. This induction of metastatic tumors is associated with silencing of the *rbf1* gene by these Polycomb group epigenetic silencers [111]. This study corroborates Rbf1's role as a tumor suppressor because its silencing causes metastatic tumors in flies.

### *VII. Mechanisms of retinoblastoma protein-mediated transcriptional repression*

The retinoblastoma tumor suppressor protein is a transcriptional co-repressor that regulates cell cycle, differentiation, apoptosis and growth [1-6]. pRB and related pocket proteins

interact with DNA indirectly by binding sequence specific transcription factors, in particular the E2F family of transcription factors [28]. Bound to target genes, these corepressors use a variety of mechanisms to repress gene expression. One pathway involves direct antagonism of E2F protein activation. These corepressor proteins can physically bind to and block the C-terminal transactivation domain of E2Fs [112-115]. However, a R661W mutant pRB protein that is defective in binding E2F [2, 116] still retains some growth repressive activity in cell culture [117], which suggests that retinoblastoma tumor suppressor's interaction with E2F is not the only mechanism utilized to regulate transcription and cell growth. pRB does not act solely by blocking E2F activity; when it is tethered to a promoter, the corepressor can repress reporter gene activity independent of E2F [118].

This activity reflects the ability of retinoblastoma family proteins to mediate active repression of transcription by recruiting histone modifiers such as histone deacetylases and methylases [119-124]. Covalent modification of lysine residues on histones N-terminal tails with acetyl group is critical for gene activation [125]. This modification neutralizes the positive charge of histones causing the DNA to unwrap, hence opening the promoter for transcriptional initiation. This action is reversed by removal of the acetyl moiety by histone deacetylase (HDAC) complexes [126]. Because histone deacetylation causes the chromatin to compact, restricting access to transcription factors, it is considered to be a hallmark of repressed chromatin [127, 128]. Several groups have shown association of HDACs with mammalian retinoblastoma family proteins, and shown that these enzymes are recruited to promoters by pocket proteins to repress the target genes [119, 120, 127, 129-131]. In *Drosophila,* Rpd3, an HDAC, was shown to be associated with dREAM complex, a 700 kDa multi-subunit repressor complex containing nine proteins including Rbf, E2F2 and DP [132, 133]. Consistent with the interaction of

retinoblastoma proteins and HDACs, E2F target gene promoters are hypoacetylated during early G1 phase [134] when pRB family members are actively silencing these target genes.

Modification of certain lysine (and sometimes arginine) residues of histones with methyl group causes the genes to be either activated or repressed. H3K4, H3K36, H3K79, H3R2, H3R17, H3R26 and H4R3 methylation are the mark of activation and H3K9, H3K27, and H4K20 are the hallmarks of heterochromatin [125, 135]. SUV39H1 is a histone methylase that specifically methylates H3K9 [136], which helps recruit HP1 [137], a mark of silenced chromatin. pRB associates with SUV39H1 and HP1 on target gene promoters [122]. Loss of all three pRB, p107 and p130 showed increased acetylation of histone H3 and decreased trimethylation of H4K20 globally [138]. In the same study, the authors showed that the mammalian pocket proteins associate with the H4K20 tri-methylating enzymes Suv4-20h1 and Suv4-20h2, suggesting a role of pRB family proteins in H4K20 tri-methylation and promotion of heterochromatin formation [138]. Methylation reactions are also linked to the activity of the Polycomb complex proteins [139]. Polycomb proteins are critical for development and mutations in these proteins cause developmental defects leading to homeotic phenotypes [140, 141]. There are two Polycomb-repressive complexes (PRCs), PRC1 and PRC2. Studies show that the PRC2 recruits the PRC1 to promoters of target genes [142, 143]. pRB family proteins recruit Polycomb genes BMI1 (a subunit of PRC1L) and EZH2 (a catalytic subunit of PRC2) to the promoter of *p16* gene causing repression of *p16* gene expression through H3K27 tri-methylation by PRC2 and ubiquitination of H2A by PRC1L ubiquitin ligase complex [144]. In summary, these results suggest that the retinoblastoma tumor suppressor proteins use diverse chromatin modifying enzymes to effect gene silencing.

In mammals, pRB is involved not only in histone methylation but also in DNA methylation. DNA methylation occurs at CpG dinucleotides and is essential for normal development, and frequently perturbed in disease[145]. Methylation of cytosine is carried out by DNMT1, DNMT3a and DNMT3b [146-148], and this modification of the DNA provides a binding site for proteins possessing the methyl-CpG-binding domain (MBD). These proteins interact with HDACs, histone methyl transferases, and corepressors such as Sin3A to silence genes [149]. E2F target genes are known to be silenced via promoter hypermethylation in tumors and during terminal differentiation [145, 150-155]. Interestingly, DNMT1 co-purifies with pRB, E2F and HDAC1, and these proteins can synergistically repress transcription of a reporter gene [156].

RB corepressors are also found to interact with SWI/SNF remodeling complexes. These complexes play extensive roles in the regulation of gene expression. Unlike the above-mentioned chromatin modifying proteins, chromatin remodeling complexes hydrolyze ATP to move nucleosomes along DNA and exchange or remove nucleosomes [157]. Originally identified in yeast, SWI/SNF complexes are evolutionarily conserved and orthologs have been reported in flies, plants and mammals [157-159]. There are four chromatin remodeling complex families: SWI/SNF, INO80, ISWI and CHD [157], which are suggested to act by either a “twist-diffusion” model, in which the changes in DNA twist are propagated from linker DNA into the nucleosomal DNA, or “reptation bulge” mechanism whereby a portion of the nucleosomal DNA is displaced away from the histone core, to form a “bulge” which can then be propagated [160, 161]. In either case, the alterations in chromatin structure generated by chromatin remodeling complexes activate or repress gene transcription [162]. pRB, p107 and p130 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 [52, 163-166].

These interactions are conserved in *Drosophila*; and E2F1 genetically interacts with the components of BRM complex *brahma, moira* and *osa* [167]. Furthermore, BRM complex physically interacts with Rbf1 [168]. In addition, Rbf containing transcriptional repressor complex, dREAM, is shown to be associated with nucleosome remodelers such as ISWI and NURF301 [132] , another ATP dependent chromatin remodeler [169].

An additional level of RB activity is represented by interactions with general transcription factors. Activation of gene expression involves formation of a stable pre-initiation complex (PIC), in which co-activators recruit histone and nucleosome remodelers to promoter regions, facilitating the recruitment of RNA polymerase II and the general transcription factors to form the PIC [170]. The PICs are believed to be assembled in a step-by-step manner. First TFIID binds to TATA element followed by binding of TFIIA, TFIIB, TFIIF and RNA polymerase at transcription initiation site [171, 172]. pRB family proteins can directly disrupt the assembly of transcriptional initiation complexes by blocking the formation of TFIID-TFIIA complex [173]. pRB is also known to interfere with RNA polymerase III mediated transcription. TFIIIC complex binds to promoters of classes 1 and 2 genes, and then recruits TFIIIB. TFIIIB is an initiation factor that recruits RNA polymerase III. pRB disrupts the TFIIIC-TFIIIC interaction and prevents the formation of PIC [174-176]. However, pRB and RNA polymerase II co-occupy promoters of class 3 genes such as U6 snRNA, suggesting that it might not interfere with the formation of PIC [177].

In summary, pRB and Rbf1 regulate target genes by diverse means, including direct inhibition of E2Fs, recruitment of histone modifying proteins, recruitment of nucleosome

remodelers and preventing formation of PIC through interference with the basal transcription machinery.

***VIII. Link between transcriptional regulation and turnover of transcription factors***

Transcription factors that regulate cell growth, such as Myc, p53, Jun, Fos and E2F are unstable proteins [178-183] that are turned over through the ubiquitin proteasome pathway [184]. This pathway is one of two major intracellular proteolytic pathways in the cells [79]. Ubiquitin mediated protein turnover involves a three reaction cascade [185]. In the first reaction, the ubiquitin activating enzyme, E1, hydrolyses ATP and binds ubiquitin to form a complex of E1-ubiquitin. Then, E1 transfers the activated ubiquitin to one of several E2 conjugating enzymes. The E2-ubiquitin then forms a complex with an E3 ubiquitin ligase and a substrate protein, which is ubiquitylated at one or more lysine residues to generate a polyubiquitylated substrate. The polyubiquitylated substrate is targeted to the 26S proteasome, where it is deubiquitylated. The ubiquitins are recycled and the protein is degraded [185].

For transcriptional activity of the 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, proteolytic destruction is required for full transcriptional activity [184, 186, 187]. Interestingly, the transcriptional activation domains and degrons overlap in most of these unstable transcriptional activators [188]. Although the mechanism of how degradation and transcriptional activation are linked, a model proposed by Muratani and Tansey suggests that, when activators interact with general transcription machinery, they recruit ubiquitin ligases to the site of transcription. These ubiquitin ligases then ubiquitylate several factors such as the activators, RNA polymerase II and histones, which in

turn recruit the 26S proteasome. This results in turnover of activators and promotion of transcriptional elongation by RNA pol II [188].

Although established for activators, this instability-activity link has not been established for any repressors. In chapter II I discuss our findings that suggest there is an instability element (IE) in the C-terminus of Rbf1 that contains a degron and is critical for activity of the repressor. We also note that the instability mechanism is conserved in the p107 human homolog. This finding suggests that the turnover of Rbf1 is required for its 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.

RB instability is not only linked to activity, but is also regulated by diverse mechanisms. pRB is targeted to ubiquitin-dependent or ubiquitin-independent proteasome mediated turnover by several cellular and viral proteins [189]. MDM2 is a cellular oncoprotein and E3 ubiquitin ligase for p53 that is overexpressed in many human cancers [190], leading to p53 degradation in ubiquitin-proteasome pathway. Interestingly, David Livingston's group has shown that MDM2 also interacts with pRB [191]. Further study showed that MDM2, like viral oncoproteins, binds to hypophosphorylated pRB and the interaction blocks pRB-E2F binding, hence suppressing pRB's repression activity [192]. MDM2 interacts with C8 subunit of 20S proteasome and promotes pRB-C8 interaction [193]. Two different groups have demonstrated that MDM2 targets pRB to ubiquitin-dependent [80] and ubiquitin-independent [193] proteasome mediated turnover. Another cellular oncoprotein, gankyrin, is shown to interact with pRB and promote proteasome mediated turnover [194].

In addition to these cellular pathways, viral proteins have dramatic effects on RB stability. E7 viral oncoprotein of human papilloma virus (HPV) binds to the LxCxE binding

motif of pRB [195] targeting the protein to the ubiquitin-proteasome pathway [196, 197]. Another viral oncoprotein, human cytomegalovirus (CMV) pp71, is shown to promote degradation of pRB, p107 and p130 via ubiquitin-independent but proteasome-dependent pathway [198]. Epstein-Barr virus nuclear antigen 3C (EBNA3C) destabilizes pRB by forming a complex with pRB and SCF ubiquitin ligase [199]. Hepatitis C virus NS5B is also shown to interact with and down-regulate pRB [200]. Similar to pRB, p107 and p130 are also inactivated through proteasomal degradation by simian virus large T antigen (SV40 LTAg) [201]. All these above viral oncoproteins utilize LxCxE motif to bind to pRB family of proteins to target them for turnover. These studies demonstrate that regulation of pRB family protein levels is important for normal cellular growth, but their elevated turnover promotes tumorigenesis.

***IX. Promoter occupancy of pRB family proteins***

There are two forms of cell cycle, canonical and noncanonical. The noncanonical cell cycle involves DNA-synthesis (S-phase) followed by mitosis (M-phase) and in canonical cell cycle, S- and M-phases are separated by two gap phases, G1 and G2. The noncanonical forms of embryonic cell cycle are common during development of organisms requiring rapid embryogenesis, such as insects, amphibians and marine invertebrates [202]. In *Drosophila,* the first thirteen cell cycles are synchronous and consist of S-M cycles driven by maternal components that are independent of zygotic transcription. G2 phase appears in cell cycle fourteen and its length is regulated by developmental control. After completion of cell cycle sixteen, cells in different tissues undergo different forms of cell cycle, for example, epidermal cells exit cell cycle, whereas the cells in nervous system continue S-G2-M cycles. G1 phase is introduced in the seventeenth cell cycle [202]. Another variant of the cell cycle, endoreduplication (in which mitosis is not followed by cytokinesis), is common in many larval and adult tissues. Many

organisms contain some tissues with polyploid or polytene chromosomes, which is required for high metabolic activity and are a result of repeated endo cycles [98, 202]. Endo cycles and origin of replication in follicle cells are regulated by Rbf1/E2F in *Drosophila* suggesting a role of Rbf1 in regulating origin of replication activity, an S-phase event, and separate from its role as transcriptional corepressor [100].

pRB family of proteins are also required in tissue- and stage-specific manners, as seen in studies of different metazoan pRB family members. Although the protein is expressed in the early embryo, the *Drosophila* Rbf1 protein is not functionally required for early cell cycle regulation [36, 38, 50]. In the mouse, early embryonic requirements for pRB are restricted to the trophectoderm, although the protein is expressed in other tissues [85]. The *C. elegans lin-35* (pRB homolog) mutant shows a largely nonoverlapping set of genes that are misregulated in embryo, L1 and L4 larvae suggesting that the protein targets distinct genes in different developmental stages [203]. These and other studies emphasize that the multifarious functions of this protein family will require global studies in a developmental setting. A major objective along these lines is the identification of functional and physical target genes of pRB family corepressors.

pRB family of proteins are best known to regulate genes involved in cell cycle regulation, such as *PCNA* and *DNA polymerase α*. However, several studies have shown that the pocket proteins are also involved in regulation of other pathways, such as apoptosis, DNA repair, differentiation and development [91, 203, 204]. The diversity of pRB family-mediated regulation in a developmental context indicates that the regulation of pRB proteins may also be complex. We know, for instance, that Rbf1 is not involved in early cell cycle regulation in the embryo, despite the physical presence of the protein [36, 50]. One way to develop a better

understanding of how Rbf1 may be differentially regulated is to study target occupancy on a genome-wide level. It is well known that Rbf1 (or other pRB proteins) binds to target gene promoters via E2F to execute repression. Nicholas Dyson's group looked at the presence of Rbf1 at various Rbf1 target genes in S2 cell culture by transcriptomic analysis. Based on up or down regulation of E2F1 or DP or E2F2 knockdown background, the genes were classified into A through E genes [101]. Physical and functional targets of the MMB/dREAM complex, with which Rbf1 is also associated, were identified in *Drosophila* Kc cells by chromatin immunoprecipitation (ChIP) followed by tiling array analysis [205]. These studies suggest that Rbf1, and to a lesser extent Rbf2, interact with distinct classes of genes that show varying sensitivity to loss of Rbf and E2F proteins. Ferrari *et al* [206], studied genomewide temporal occupancy of human pRB, p107 and p130 on E2F target gene promoters in adenoviral E1A protein overexpressed human lung fibroblast cell line. The E1A protein does two important things; first it recruits p300, pCAF and H3K18ac to the promoters of cell cycle and growth genes causing transcriptional activation. Second, it recruits pRB family proteins to the promoters of antiviral genes causing transcriptional repression [206]. This observation of redeployment of pocket proteins in response to the expression of E1A protein suggests that these proteins switch targets depending upon different growth and developmental signals. A recent ChIP-seq study with human E2F4 protein identified about 16,000 E2F4 binding sites. Among those sites, about 20% were more than 20 kb away from any annotated transcriptional start sites (TSS) suggesting that E2F4 might function as a long range regulator. This study also suggests possible role of E2F4 in regulation of micro RNAs [207]. Because all three human pocket proteins are known to bind E2F4, the potential E2F4 functions suggested in this study might also hold for pRB, p107 and p130. Furthermore, another ChIP-on-chip and expression profiling study shows that p130

and E2F4 bind to a common set of widespread transcriptionally downregulated targets [208]. The genome-wide occupancy of pRB and p130 proteins was also recently reported in growing, quiescent and senescent human fibroblasts, indicating that these proteins bind to thousands of putative target genes [209]. However, until now no study has presented a picture of the genome-wide occupancy of pRB proteins in a whole organism. As discussed above, the study with E2F4 suggests it may be a long range regulator. Although pRB, p107 and p130 are known to bind E2F4, no study until now has suggested the pocket proteins behaving as long range repressors. A model has been proposed that E2Fs bind within a gene and activate the expression of non-coding antisense transcript, yielding gene silencing [210]. The question arises that is there a possibility of finding pRB family proteins binding to a non-promoter region and repressing gene silencing? Furthermore, do pocket proteins bind to promoters in the context of E2F sites only or other transcription factors also recruit them?

Using specific antibodies developed against the endogenous Rbf1 protein, I carried out ChIP with embryos and collaborated with Kevin White's lab for the utilization of massive parallel sequencing (ChIP-seq) to identify genome-wide targets of Rbf1 in developing *Drosophila* embryo. In chapter III, I describe my findings in which I show that there are many more non-cell cycle targets than genes involved in cell cycle. I also show that almost all of the Rbf1 binding occurs within 2 kb from the annotated TSS, suggesting that it is a short-range repressor. Two thirds of Rbf1 binding regions are devoid of E2F sites, suggesting that either the regions have poor E2F sites or there are other transcription factors recruiting Rbf1 to the promoters. Furthermore, I found that there is a distinct promoter composition in terms of transcription factor binding motifs.

***X. Involvement of RB family proteins in regulation of conserved signaling pathways***

Functional assays in model systems have provided evidence for complex regulatory relationships between RB proteins and conserved signaling pathways. One such pathway involves insulin signaling; metabolic signals propagated through this pathway control the activity of basic protein synthetic capacity of cells and hence tissue growth in response to nutritional status [211-213]. The protein components of this signaling pathway are shown in Figure 1.2. PDK4, a component of this pathway, is a major isoenzyme responsible for changes in pyruvate dehydrogenase complex activity. The PDK family (PDK1 through 4) is responsible for controlling insulin responsiveness and the efficacy of glucose utilization. PDKs are considered as nutrient sensors that transduce signals in response to nutrient status (shortage or abundance) [212, 214, 215]. A decrease in glucose levels induces muscle PDK4 expression to avoid being hypoglycemic [216-218]. Loss of *E2F1 in vivo* results in increased sensitivity to insulin stimulation and improvement in glucose oxidation and inactivation of *pRB* does the opposite [212]. This study suggests that E2F1-pRB complex is involved in maintaining glucose homeostasis in the body and insulin sensitivity thus linking pRB proteins in insulin signaling pathway. Another study also links pRB to insulin pathway, however, it suggests that pRB haploinsufficiency ameliorates insulin resistance [213]. This study in the mouse shows that partial deficiency in the pRB gene antagonizes development of obesity and associated metabolic disturbances by increasing fatty acid oxidation in these tissues through increase in the expression of peroxisome proliferator-activated receptor (PPAR)α in liver and PPARδ in skeletal muscle [213]. Deregulation of CDK/pRB pathway is shown to help mammary epithelial cells to cause desmoplasia via phosphorylation of c-Met tyrosine phosphorylation and stimulation of the phosphorylation of downstream signaling intermediates S6K and Akt [219]. In plants, the Arabidopsis S6K ortholog

# Figure 1.2

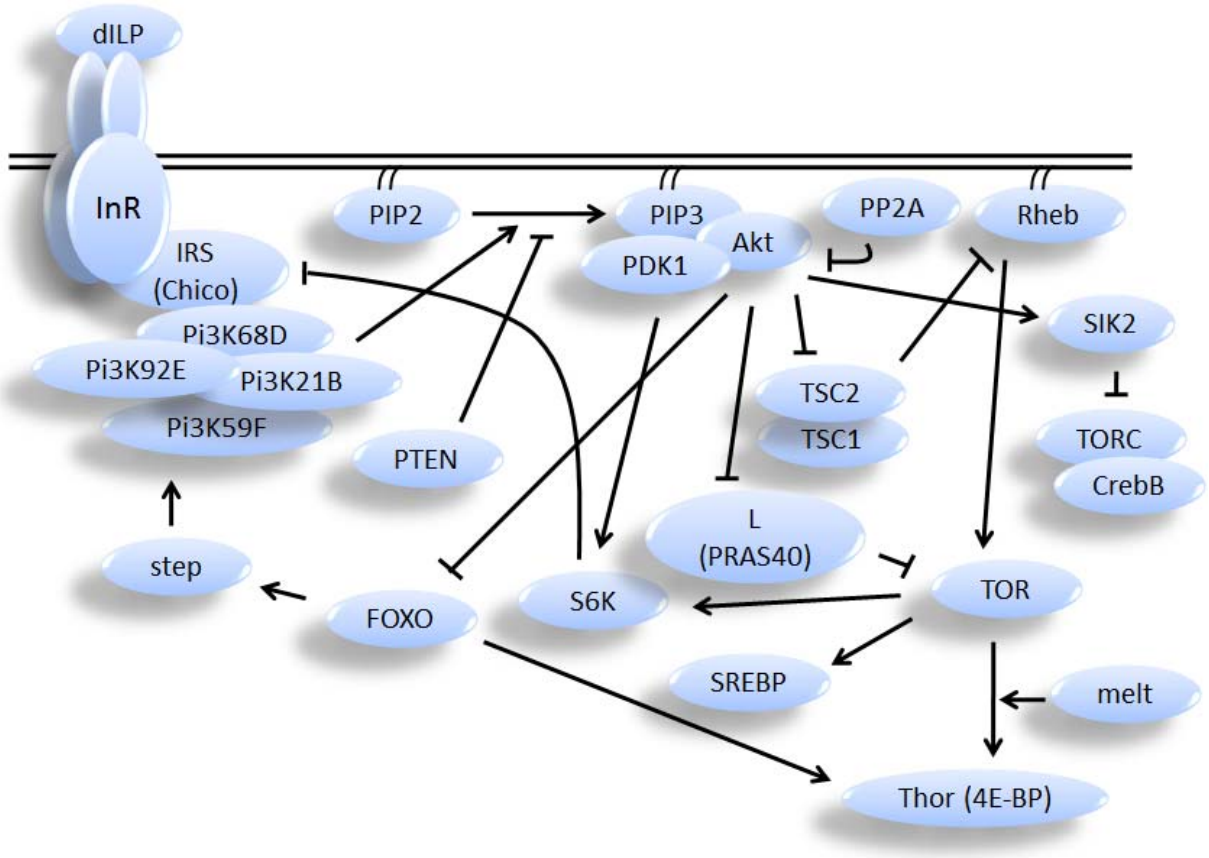


**Figure 1.2 Schematic drawing of insulin signaling pathway.** This is insulin signaling pathway in *Drosophila,* but the pathway is largely conserved. Insulin like peptide binds to membrane receptor and the signal is transduced via kinase cascade that involves phosphoinositol 3 kinase and downstream effectors such as FOXO, TSC and translational regulators including S6K and 4E-BP. Arrows indicate activation and bar-ended lines indicate inhibition.

S6K1 interacts with the Retinoblastoma-related 1(RBR1), Arabidopsis ortholog of pRB, through its LVxCxE motif. This S6K1-RBR1 complex helps nuclear transport of RBR1 and is involved in repression of cell cycle regulatory proteins such as E2FB, DPA, CDKB1;1 and CDKA [211]. These studies indicate a possibility of crosstalk between retinoblastoma and insulin pathways in animals and plants.

Links between insulin and TOR signaling and RB function have also been noted in *Drosophila*. A recent study from Wei Du's group showed that inactivation of *gigas*, a *Drosophila* ortholog of TSC2 (Tuberous sclerosis complex 2), in *rbf1* mutant background induces cell death. The authors claim that inactivation of *gigas* specifically kills *rbf1* mutant cancer cells under stress conditions without affecting normal cells [220]. These studies suggest close functional interrelation between the insulin signaling pathway and pRB proteins. However, no study has shown whether pocket proteins are involved in direct repression of the genes involved in this pathway. In Chapter III I show that Rbf1 binds at the promoter of several genes involved in this pathway and that Rbf1 directly represses insulin receptor, InR, in a cell culture study with InR-luciferase reporter.

Additional signaling pathways have been functionally related to RB proteins in knock out experiments. For instance, Wnt, MAPK, Ras and Notch pathways were found to be upregulated in pRB, p107 and p130 triple knock out hepatocellular carcinomas (TKO HCCs) [221]. A variety of genetic experiments link Notch and RB proteins. The protein components of this signaling pathway are shown in Figure 1.3. Expression of several components of the Notch signaling pathway was significantly increased in TKO HCCs. It was also shown that Notch signaling in HCC cells are regulated by E2F-directed transcription [221], suggesting that pRB proteins may be directly involved in regulation of this pathway. This activation of Notch signaling actually

seems to compensate for the loss of pRB proteins in promoting normal cell cycling, because blocking Notch signaling with DAPT (an inhibitor of γ–secretase that normally cleaves the internal domain of the Notch receptor during activation), accelerates the tumor progression [221]. Notch signaling is not confined to tumor suppression, because both tumor suppressive and oncogenic properties of Notch have been implicated in tumors [222]. A further link between Notch signaling and RB action comes from study of the fly eye. In *Drosophila*, overexpression of the Notch ligand Delta in the eye causes enlarged eye phenotypes. Further activation of two Polycomb group proteins, Pipsqueak and Lola, induces metastatic tumors, which is caused by epigenetic silencing of *rbf1* gene [111]. A further study also showed that Notch can induce proliferation of cells in *Drosophila* eye by inhibiting Rbf1 [223]. Notch-induced inactivation of RB protein provides a separate path to cell proliferation. A recent study showed that Notch activates the Hes-1 transcription factor, which represses expression of CKDN1B (p27) an inhibitor of the CDK2-Cyclin E complex. This kinase complex normally phosphorylates and inactivates pRB. Thus by inhibiting CKDN1B, Hes-1 promotes pRB hyperphosphorylation, leading to cell proliferation [224]. Another genetic connection between Notch and Rbf1 is noted in the context of UTX [225], an H3K27me3 demethylase that antagonizes Polycomb-mediated silencing. *utx* mutant tissue has a growth advantage over wild type tissue because of increased Notch activity, indicating that UTX is a Notch antagonist. Furthermore, the *utx* phenotype is overrepresented by the loss of *rbf1* [225]. Taken together, these studies suggest that there is negative cross-talk between pRB and Notch pathways. In Chapter III I show that Rbf1 is physically present on promoters of several components of Notch signaling pathway, providing a molecular explanation for negative feedback between pRB and Notch signaling pathways.

# Figure 1.3

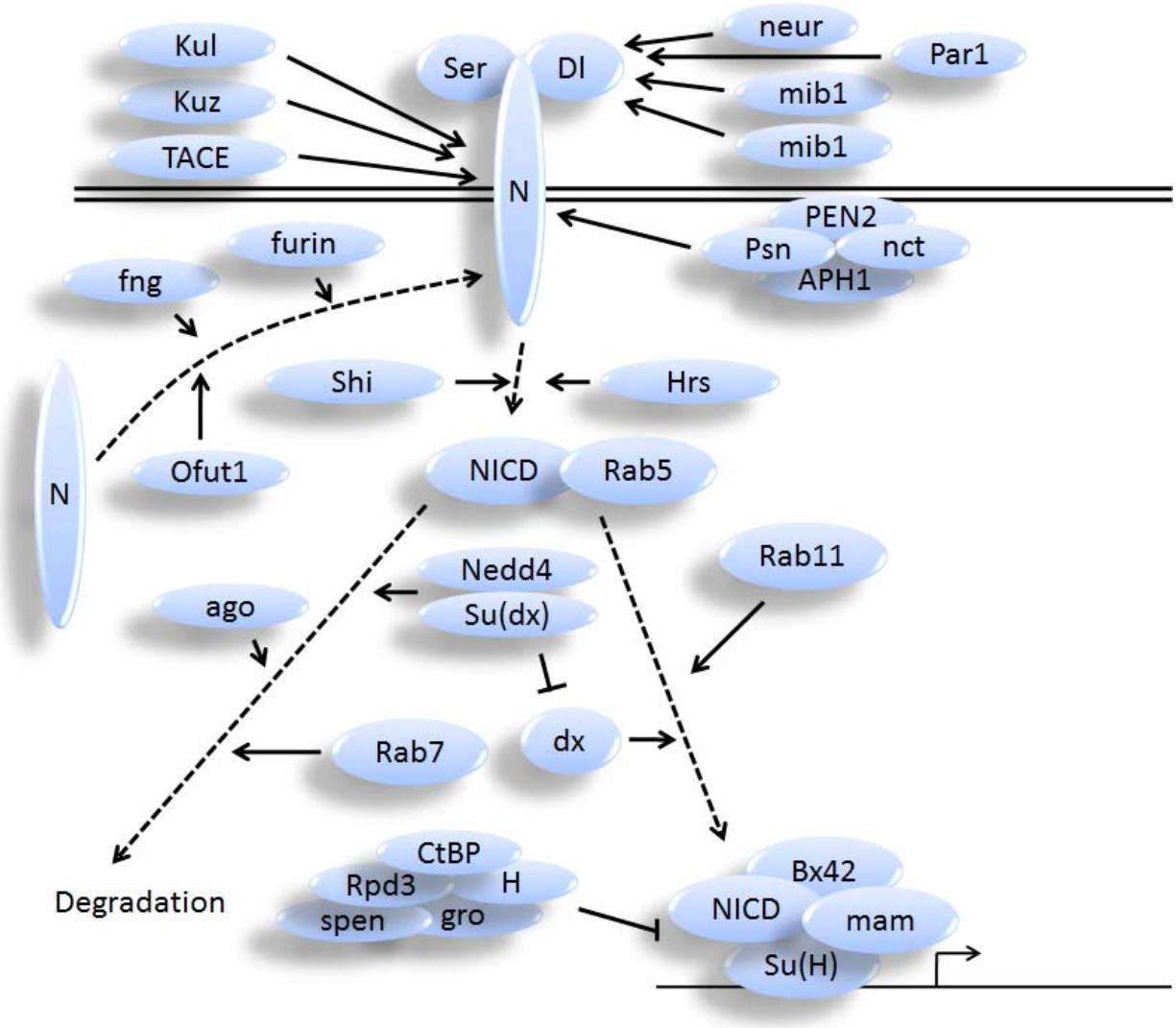


**Figure 1.3 Schematic drawing of Notch signaling pathway.** This is notch signaling pathway is in *Drosophila,* but the pathway is largely conserved. After the binding of Notch ligands (Serrate and Delta), the membrane-bound Notch receptor is cleaved extracellularly and intracellularly by several proteases. It is then translocated to the nucleus and forms an activator complex with proteins such as mastermind, and Su(H) and activates target genes. Arrows indicate activation and bar-ended lines indicate inhibition. Dashed arrows indicate translocation.

# Figure 1.4

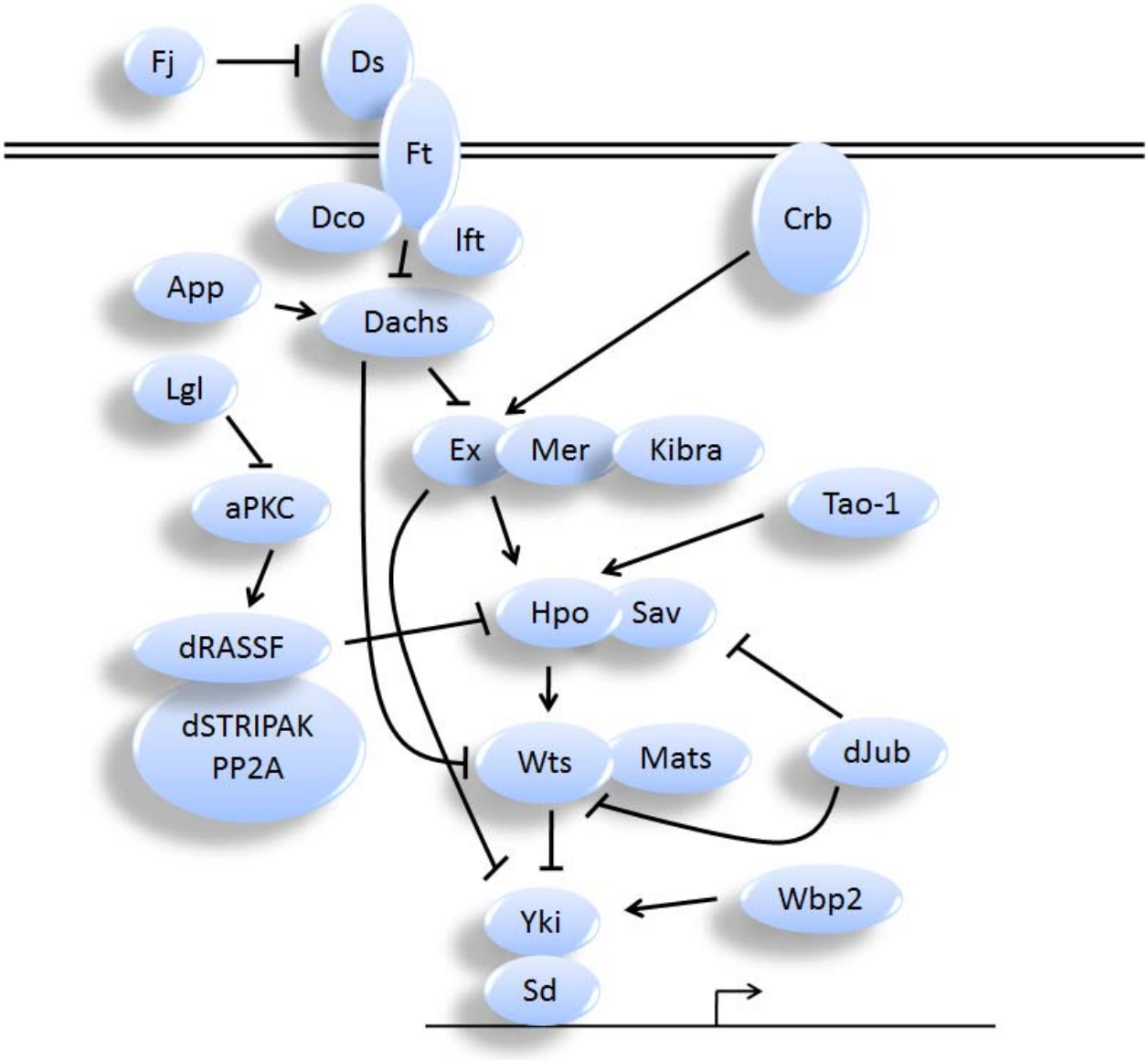


**Figure 1.4 Schematic drawing of Hippo signaling pathway.** This is hippo signaling pathway is in *Drosophila,* but the pathway is largely conserved. Hippo and Warts are the major kinases of this tumor suppressor pathway. These kinases regulate the target gene expression by controlling Yorkie/Scalloped transcription factors. Arrows indicate activation and bar-ended lines indicate inhibition.

Several studies have linked retinoblastoma tumor suppressor with the hippo tumor suppressor pathway, a recently discovered conserved signaling pathway that regulates organ growth [226-229]. The protein components of this signaling pathway are shown in Figure 1.4. Merlin, a component of hippo pathway, blocks pRB phosphorylation, and inhibiting the increase of cyclin D1 levels in NIH3T3 cells. Furthermore, Merlin represses an E2F activated luciferase reporter, possibly by the same pRB phosphorylation mechanism [230]. In another study cells were arrested at G1 in response to dihydrocytochalasin B (DCB) in a pRB proteins-dependent but p53-independent manner. Merlin levels were elevated in these treated cells, indicating a possibility of requirement of both Merlin and pocket proteins for DCB-induced arrest. Interestingly, pRB triple knock out (TKO), but not p53 null, MEFs die rapidly when exposed to DCB [231]. Merlin is positively regulated by pRB by repressing Rac1/Pak1, which suppress Merlin's activity. This way pRB regulates cell adhesion by helping Merlin promote cell adhesion [232]. This study suggests that loss of pRB weakens Merlin's activity which causes loss of cell-to-cell contacts eventually contributing to the later stages of metastasis [232]. Another study suggests that pRB and Hippo pathways may act synergistically in gene regulation [233]. A microarray analysis using RNA extracted from imaginal discs harboring *rbf1* and *wts* mutants suggests that Rbf and hippo pathways cooperate to regulate DNA replication and cell cycle genes. In the same study using reporter assay, authors show that overexpression of E2F1 and Yki, a component of hippo pathway, showed synergistic upregulation of some target genes compared to E2F1 or Yki alone. Interestingly this was squelched by cotransfection of Rbf1 overexpressing plasmid. The authors argue that in the absence of both Rbf1 and wts, hyperactivated E2F1 and Yki synergistically upregulate a novel set of genes and establish the distinct gene expression signature needed to overcome terminal cell cycle exit upon differentiation [234]. LATS2, a mammalian ortholog of

warts kinase and a component of mammalian hippo tumor suppressor pathway, is required for a senescent-like state and silencing of E2F target genes [235]. Interestingly, LATS2 is located at 13q11-q12 region of chromosome 13 which is close to 13q14.2 (where pRB is located) and loss of heterozygosity (LOH) of LATS2 is frequently observed in primary cancers along with pRB [235, 236]. LATS2 phosphorylates DYRK, a kinase that phosphorylates lin52 that is necessary for the assembly of DREAM complex, thus helping RB in its repressive activity [235].

All the above studies imply a connection between pRB pathway and other conserved signaling pathways. In Chapter III I show that *Drosophila* Rbf1 binds to promoters of many components of conserved signaling pathways providing a molecular mechanism by which this protein may direct alternative levels of signaling components, possibly setting different thresholds of signaling activity in a tissue specific manner.

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# CHAPTER II

## Paradoxical instability-activity relationship defines a novel regulatory pathway for Retinoblastoma proteins[1]


### 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.


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## 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 (Knudson, 1978; Classon and Harlow, 2002) attesting to the centrally important role for RB in proliferation control. Further analyses in mammals have revealed that other RB related proteins, p130 and p107, contribute to cell cycle governance, but the partitioning of cell cycle duties among family members is not well defined. Nonetheless, the RB family and their cognate regulatory networks are well conserved among metazoans, substantiating the physiological significance of RB family function (van den Heuvel and Dyson, 2008).

As potent regulators of cellular proliferation, the activities of RB family proteins are tightly regulated. The canonical pathway for RB family regulation is mediated by cyclin/Cdk complexes that phosphorylate pocket proteins at key points during the cell cycle. In response, phosphor-RB dissociates from E2F binding partners, and transcription of cell cycle related genes such as *PCNA* can initiate at the G1/S phase transition (Dyson, 1998). In addition to phosphorylation control, RB protein activities are also regulated by proteolysis. During *in vitro* differentiation of 3T3-L1 adipocytes, p130 levels are transiently decreased relative to p107 by a proteasome-mediated pathway, and this switch is associated with successful differentiation (Prince *et al.*, 2002). RB levels can be regulated by the Mdm2 ubiquitin ligase, better known for its control of levels of the p53 tumor suppressor, and in cancers overexpressing Mdm2, RB levels are diminished (Sdek *et al.*, 2004; Uchida *et al.*, 2005). The idea that altered RB protein levels contribute to disease etiology is further highlighted during infection by certain oncogenic

viruses that hijack the proteolytic process and induce RB family member turnover to relieve host control of cellular proliferation (Boyer *et al.*, 1996; Stubdal *et al.*, 1997). Together, these examples demonstrate that regulation of RB family protein levels are important for normal cellular growth, but that these processes are often deregulated in disease.

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

or whether this control is regulated during development. The CSN4 subunit of the COP9 signalosome co-occupies cell cycle regulated genes simultaneously with Rbf proteins, suggesting that processes affecting repressor stability are spatially and temporally linked to repressor function during gene regulation (Ullah *et al.*, 2007).

While proteasome-mediated destruction of cellular proteins is clearly linked to downregulation of factor activity, the converse relationship has also been described, notably, that the potency of transcriptional regulatory proteins is directly linked to processes that mediate their destruction. This somewhat paradoxical relationship has been described for a variety of eukaryotic transcriptional activator proteins, including c-Jun, c-Fos, Myc, E2F1, and Gal4, all of which harbor degradation signals in regions closely overlapping with their activation domains (Salghetti *et al.*, 1999; Salghetti *et al.*, 2000; Salghetti *et al.*, 2001). Synthetic constructs with multiple degradation domains exhibit higher levels of transcriptional activation, suggesting that the correspondence is not just coincidental (Salghetti *et al.*, 1999; Salghetti *et al.*, 2000). One proposed explanation for the tight correlation between protein lability and increased transcriptional potency posits that the proteasome, which is essential for turnover of ubiquitylated substrates, also mediates transcriptional activation functions directly (Gonzalez *et al.*, 2002; Ferdous *et al.*, 2007). 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 (Muratani and Tansey, 2003; Lee *et al.*, 2005; Collins and Tansey, 2006; Daulny *et al.*, 2008). 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 (Schejter and Shilo, 1989) between *Kpn*I and *Xho*I sites in three steps using PCR amplification of genomic DNA. Two Flag epitope tags were inserted immediately 5' of the *rbf1* stop codon into an *Xba*I site. The genomic construct of Rbf1 Δ728-786 was made by site-directed mutagenesis. For genes used in S2 cell culture transfection, *rbf1* cDNA was PCR amplified and various mutants produced by site-directed mutagenesis were cloned from pLD02906 (Keller *et al.*, 2005) into *Kpn*I and *Xba*I sites of pAX vector (Ryu and Arnosti, 2003). Two Flag epitope tags were inserted 5' of the stop codon. For misexpression in the fly, the constructs were cloned into *Kpn*I and *Xba*I sites of pUAST (Brand and Perrimon, 1993). For bacterial expression of GST fusion proteins, the pRSF Duet-1 vector (Novagen, 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 (Keller *et al.*, 2005).

***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 (Martinez and Arnosti, 2008), except that the chromatin (1 ml) was incubated with 5 µl (5 µg) of Flag antibody (Sigma; F7425) or 2 µl H3 antibody (Abcam, 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 (Gilbert *et al.*, 2006), and the offspring were screened for eye phenotypes. The $rbf^{14}$ mutant (stock number 7435) was obtained from the Bloomington Stock Center.

***Immunohistochemical Staining of Imaginal Discs***

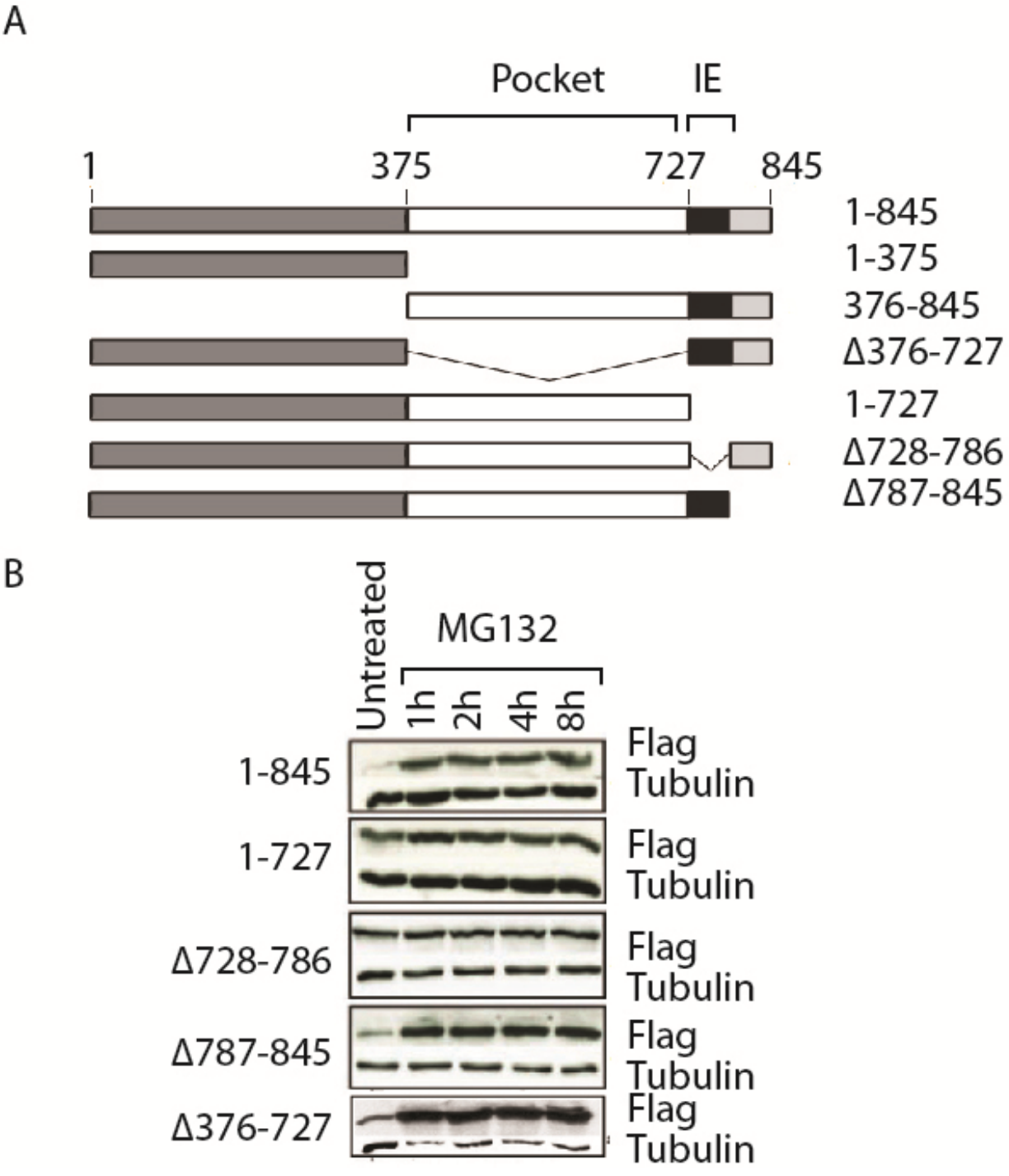
Imaginal discs were dissected in chilled PBS from third-instar larvae of *rbf1* and rbf1 Δ728-786 flies and fixed in 3.7% formaldehyde in 10 mM potassium phosphate, pH 6.8; 15 mM NaCl; 45 mM KCl; 2 mM $MgCl_2$ for 30 min at room temperature. Antibody detection was performed by diaminobenzadine staining 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 (Ullah *et al.*, 2007). 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 (Stevaux *et al.*, 2005). Furthermore, previous data suggested that endogenous Rbf1 levels fluctuate during embryogenesis (Keller *et al.*, 2005; Stevaux *et al.*, 2005). We initially examined the importance of the conserved central pocket domain, as well as the less-conserved N- and C-terminal regions (Figure 1A; Table 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 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 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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IE
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375
727
845
1-845
1-375
376-845
Δ376-727
1-727
Δ728-786
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B
Untreated
MG132
1h
2h
4h
8h
1-845
1-727
Δ728-786
Δ787-845
Δ376-727
Flag
Tubulin

**Figure 2.1 (cont'd.)**

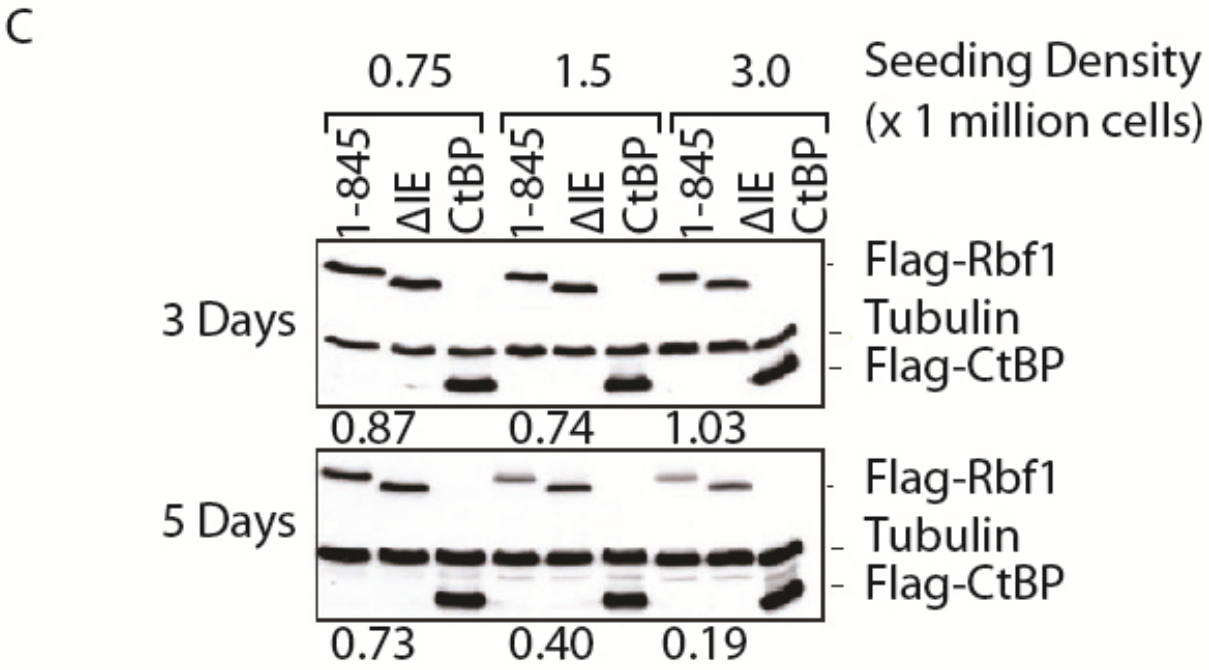
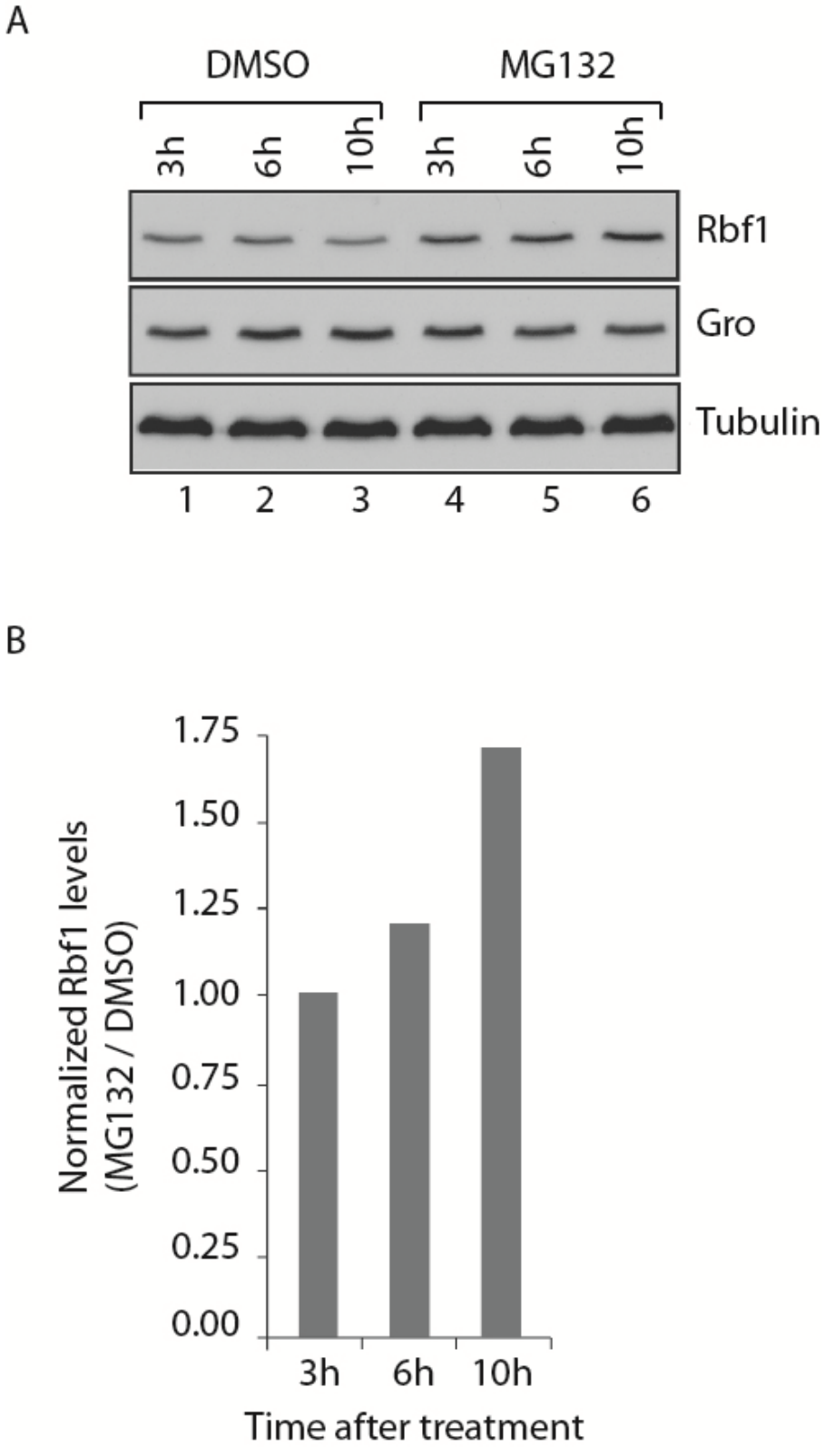


Figure 2.1 cont'd.

**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.

# Supplementary Figure 2.1



Supplementary Figure 2.1 cont'd.

**Supplementary Figure 2.1.** (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 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 (Supplementary Figure 1). 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; Table 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 2A). To determine whether the change in steady state levels is due to altered stability, we next tested whether the half-life of wild-type and mutant (4KA) Rbf1 proteins differed by treating S2 cells with the translational inhibitor cycloheximide. Three days after transfection at a point when our previous data indicated that Rbf1 (4KA) mutant protein was expressed at higher levels than wild-type Rbf1, S2 cells were treated with cycloheximide and Rbf1 protein levels subsequently measured at 0, 6, and 12 h (Figure 2B, 2C). 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 2D).

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 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 3A (right panel) and imaginal disc staining in Figure 3, 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 (Keller *et al.*, 2005), but the Rbf1 ΔIE mutant also shows staining in these posterior cells, suggesting an abnormal perdurance of the protein (Figure 3C, D). The marked difference between the steady-state levels of the two proteins in these contexts indicates that the wild-type Rbf1 protein is specifically destabilized in the proliferating and differentiating tissue of the imaginal discs. The tissue-specific stability of the Rbf1 wild-type and mutant proteins suggests that turnover of Rbf1 is a regulated event and is likely triggered by developmental signals. The cell density-dependent difference in protein accumulation for wild-type and IE-deleted Rbf1 proteins as described in Figure 1C also supports this hypothesis.

# Figure 2.2

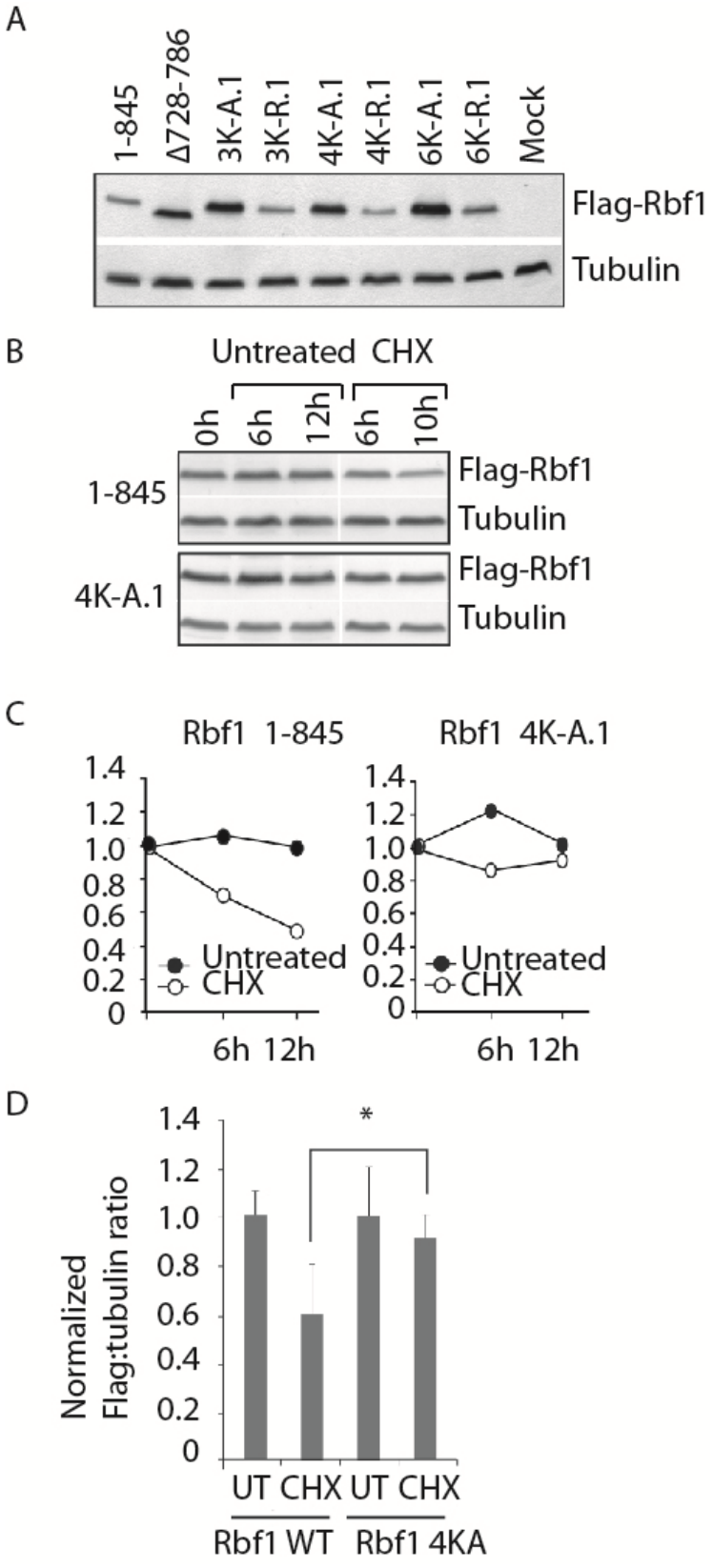

A
1-845
Δ728-786
3K-A.1
3K-R.1
4K-A.1
4K-R.1
6K-A.1
6K-R.1
Mock
Flag-Rbf1
Tubulin
B
Untreated
CHX
0h
6h
12h
6h
10h
1-845
Flag-Rbf1
Tubulin
4K-A.1
Flag-Rbf1
Tubulin
C
Rbf1 1-845
Rbf1 4K-A.1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Untreated
CHX
6h 12h
D
Normalized Flag:tubulin ratio
*
UT CHX UT CHX
Rbf1 WT
Rbf1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

Figure 2.2 cont'd.

**Figure 2.2.** 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 2.3

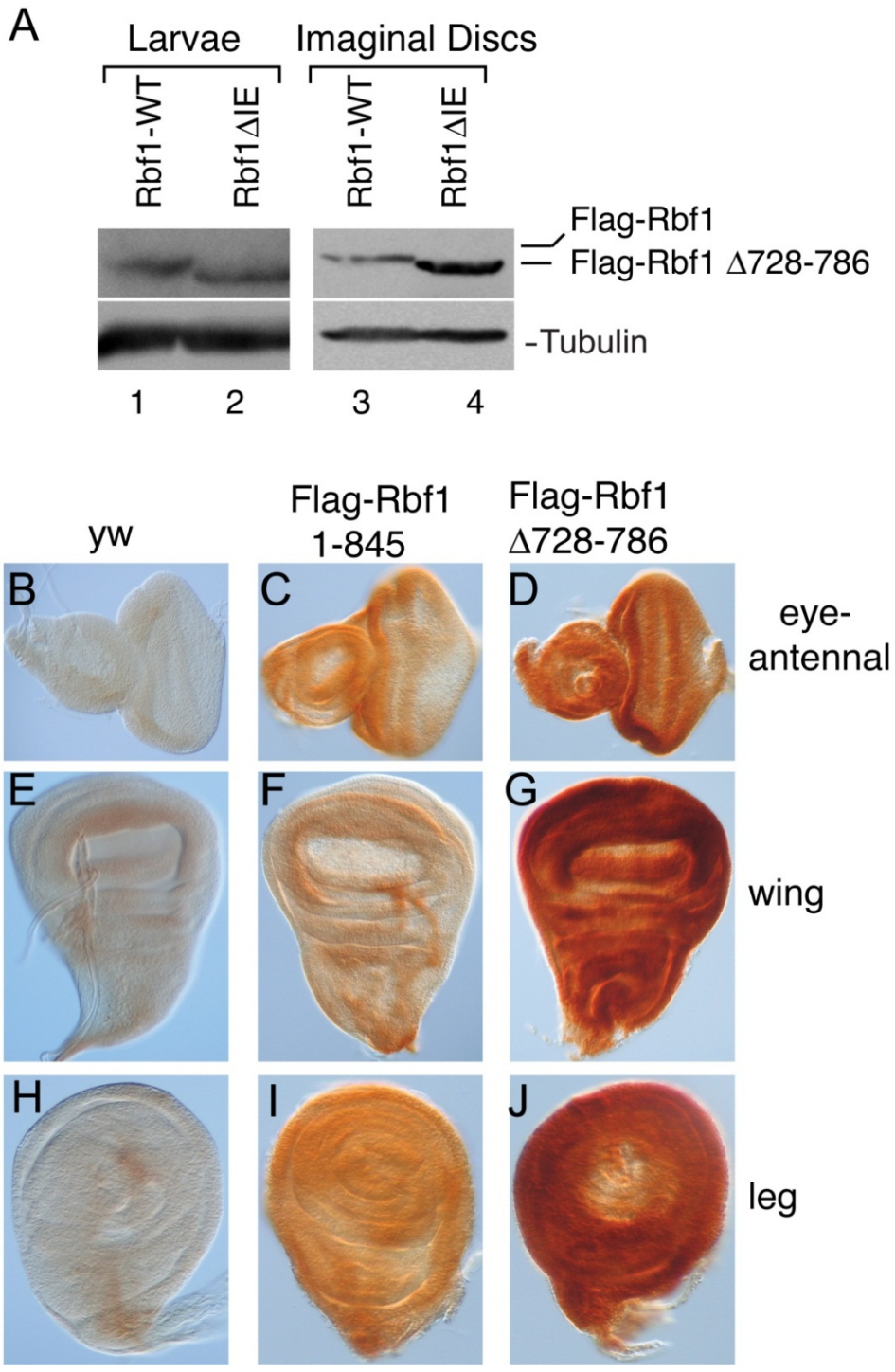

A
Larvae
Imaginal Discs
Rbf1-WT
Rbf1ΔIE
Rbf1-WT
Rbf1ΔIE
Flag-Rbf1
Flag-Rbf1 Δ728-786
-Tubulin
1
2
3
4
yw
Flag-Rbf1 1-845
Flag-Rbf1 Δ728-786
B
C
D
eye-antennal
E
F
G
wing
H
I
J
leg

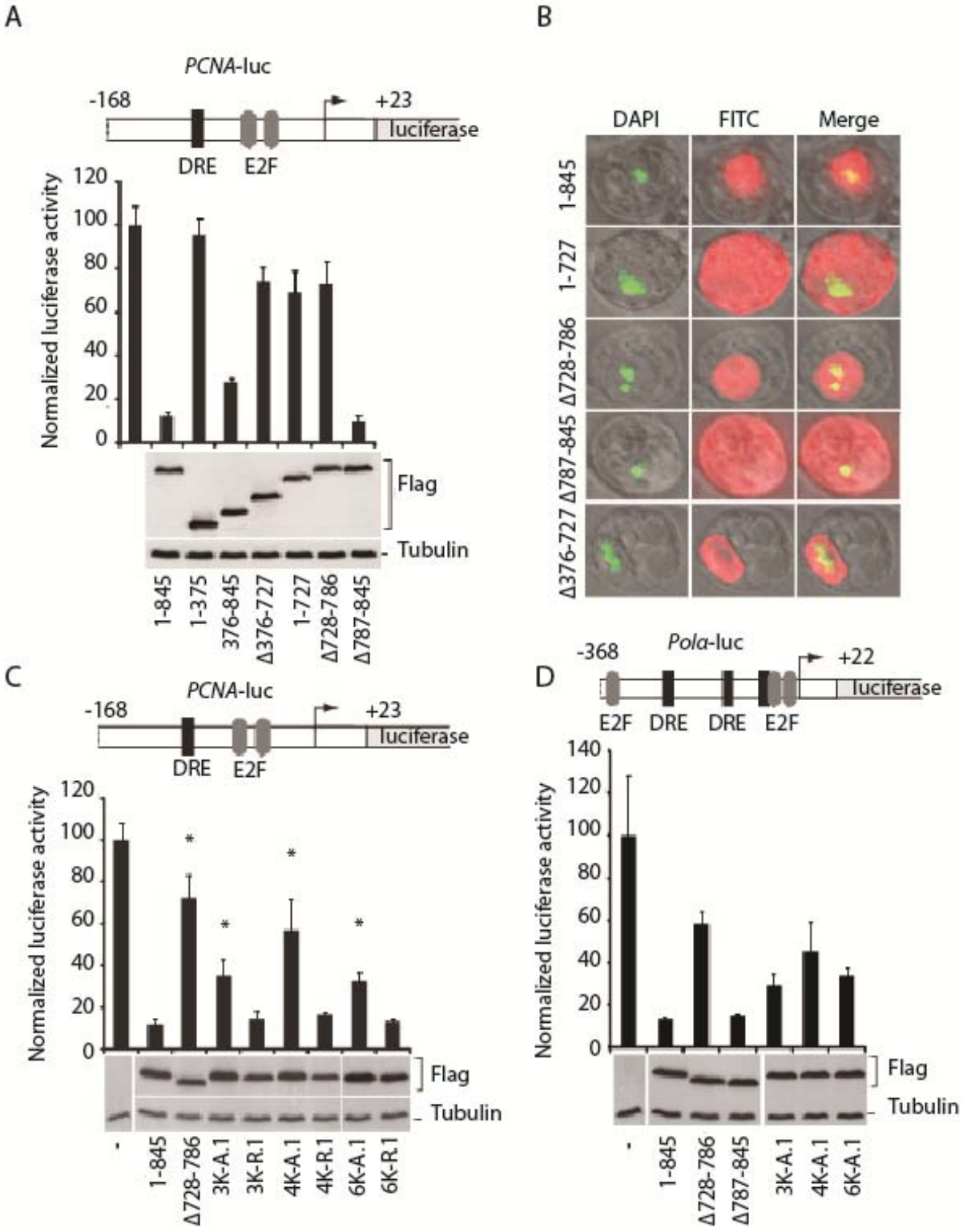
Figure 2.3 cont'd

**Figure 2.3.** 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. 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 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 (Stevaux *et al.*, 2002). As expected, proteins lacking the central pocket domain were inactive; this region of the protein is required for interaction with the E2F transcription factors that recruit Rbf1 to the promoter (Figure 4A). Removal of the N-terminal portion of the protein had only a mildly deleterious effect on repression, consistent with previous studies that suggested it is not required for transcriptional activity in vivo and in vitro (Hiebert *et al.*, 1992). In contrast, removal of portions of the entire C terminus revealed multiple effects. First, deletion of the IE region alone had a strong inhibitory effect on transcriptional repression, and this effect was just as severe as removal of the critical pocket domain. The Rbf1 ΔIE and pocket deletion mutant proteins did not exhibit aberrant localization, but remained in the nucleus (Figure 4B). 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. As observed for deletion of the entire IE (Δ728-786), removal of portions of this 59-aa region in blocks of 20 was

# Figure 2.4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+23
luciferase
DRE
E2F
Normalized luciferase activity
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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
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-368
+22
luciferase
E2F
DRE
DRE
E2F
Normalized luciferase activity
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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 2.4 cont'd

**Figure 2.4.** Rbf1 requires the IE for transcriptional repression. (A) Deletion of the IE (Δ728-786) or E2F binding pocket (Δ376-727) compromises transcriptional repressin 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 4A 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**. *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.

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 4C, Rbf1 proteins bearing multiple lysine to alanine substitutions were less effective repressors, even though these proteins 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 4D) (Dimova *et al.*, 2003). Deletion of the IE or point mutations within this region similarly reduced the repression activity on this promoter as well, indicating that the relationship between protein activity and instability is independent of promoter context. Taken together, these data strongly indicate that the ability of the Rbf1 protein to act as a transcriptional

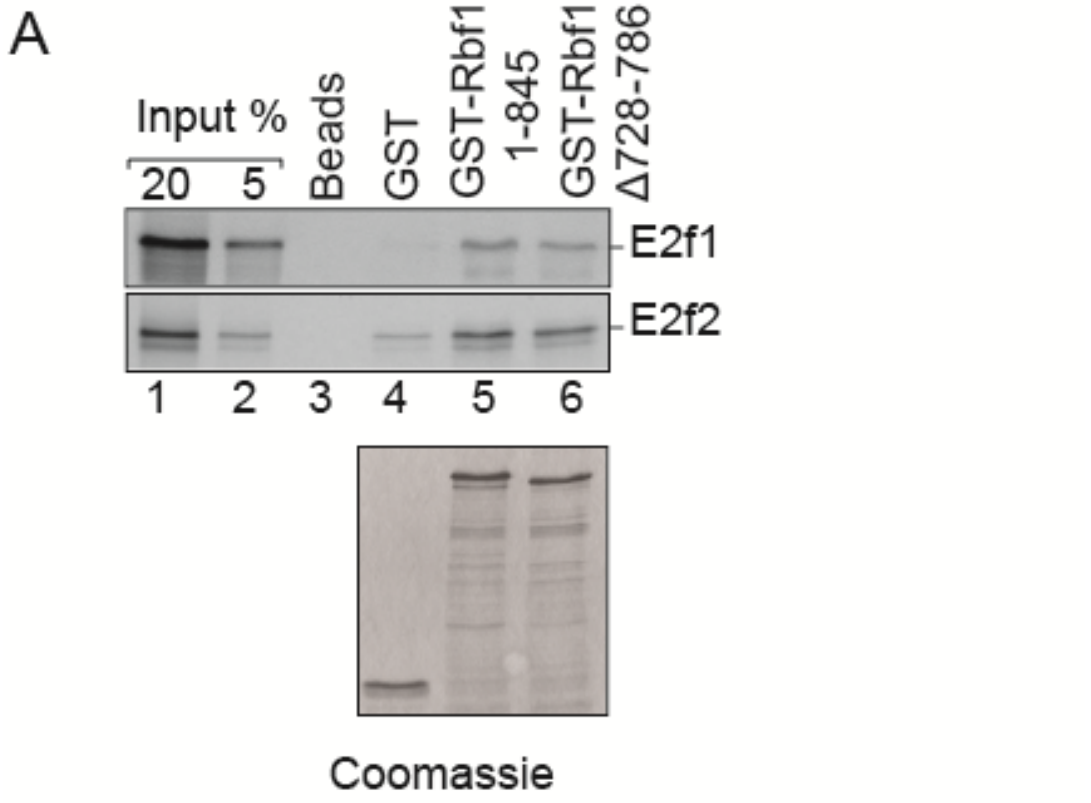
repressor is tightly associated with its instability, and that the IE in the Rbf1 C terminus is multifunctional, linking these two features.

***The Rbf1 IE Is Not Essential for E2F Interactions and Promoter Binding***

Previous studies have shown that both the pocket domains as well as the carboxy terminus of the human RB protein can make molecular contacts with E2F1 (Lee *et al.*, 2002; Xiao *et al.*, 2003; Rubin *et al.*, 2005). 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 5A, lanes 5 and 6). No interaction was observed with beads alone or GST protein (Figure 5A, 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 5B; top panel, lanes 3-6). These results show that the IE mutants retain a capacity to interact with both E2F1 and E2F2 proteins.

To assess whether the IE plays a role in Rbf1 promoter occupancy we performed chromatin immunoprecipitation (ChIP) assays using embryos expressing the Flag-tagged Rbf1 wild-type or ΔIE mutant to test for promoter binding of these proteins at the *DNA primase* promoter (Figure 5C). 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 2.5**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Input %
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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
+
-
+
+
+
+
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Heavy Chain
Flag
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4
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7
8

# Figure 2.5 cont'd.

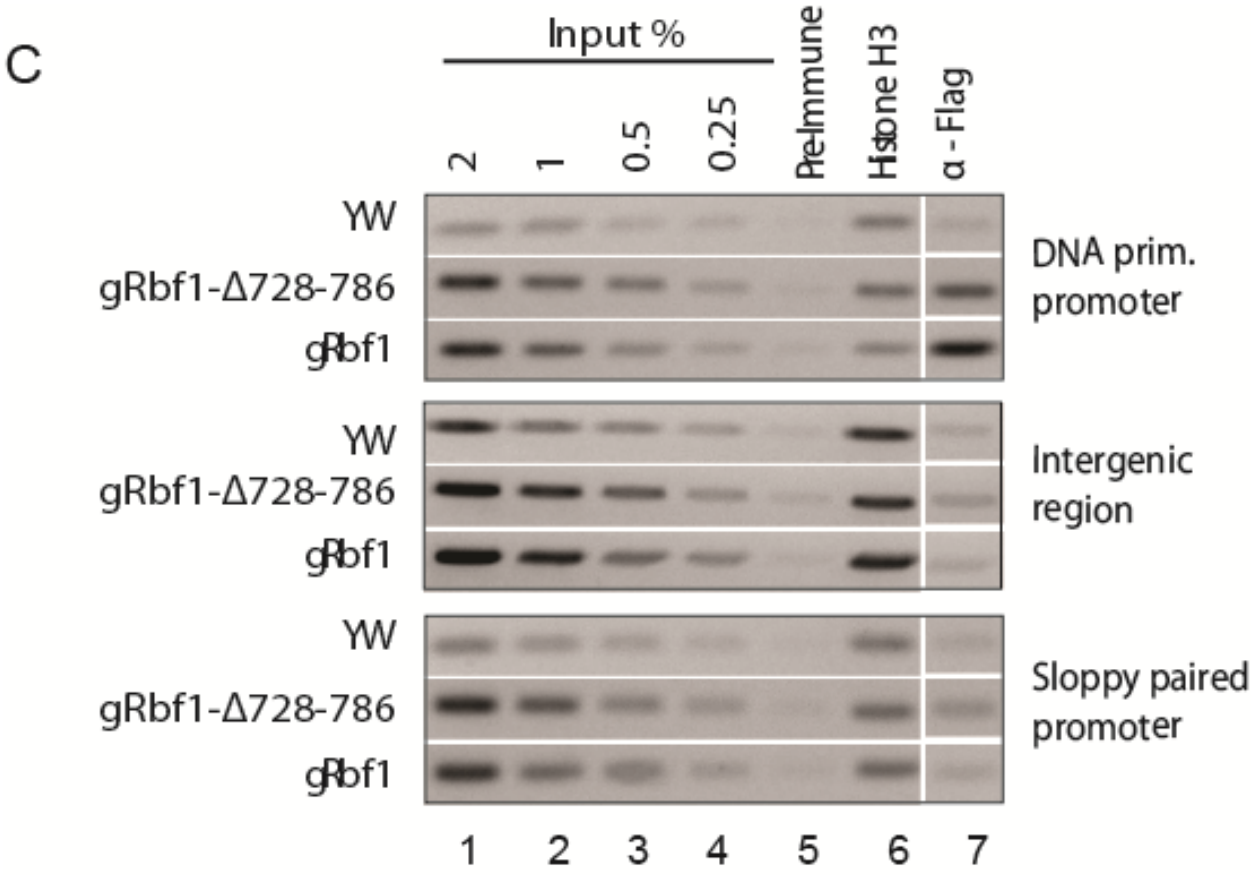



**Figure 2.5.** Rbf1 IE is not essential for E2F interactions and promoter binding. (A and B) Physical association between Rbf1 IE mutants and E2F proteins. (A) GST-Rbf1 and E2F interaction assay. Indicated GST fusion proteins were bound to radio-labeled E2F proteins and bound proteins were analyzed 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 2.5 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).

occupy promoters (Figure 5C; 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.

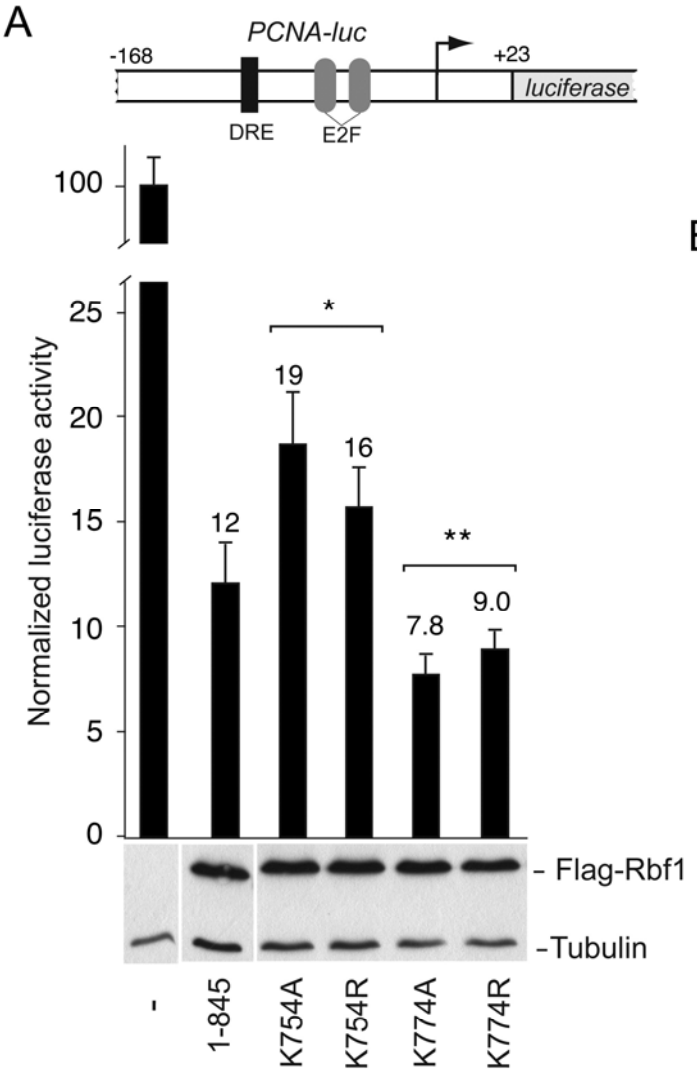
***The Rbf1 IE Is a Dual-Function Regulator of Repressor Potency***

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 6A, revealing three outcomes. In one case (K732), alanine substitution did not affect repressor potency and was indistinguishable from wild-type Rbf1. The second class of mutants were hypomorphic (K739, K740, K754), exhibiting modest but reproducible inhibitory effects on repression, consistent with these residues contributing a positive influence on repressor potency (Figure 6, 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 6, 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 7, 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 7, 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

# Figure 2.6




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 |

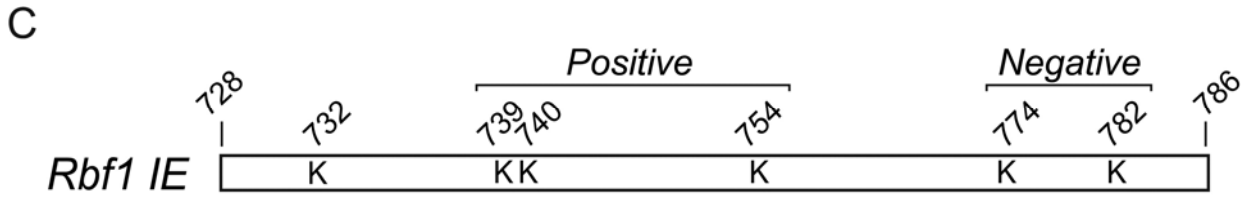


Figure 2.6 cont'd.

**Figure 2.6.** 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 2.7

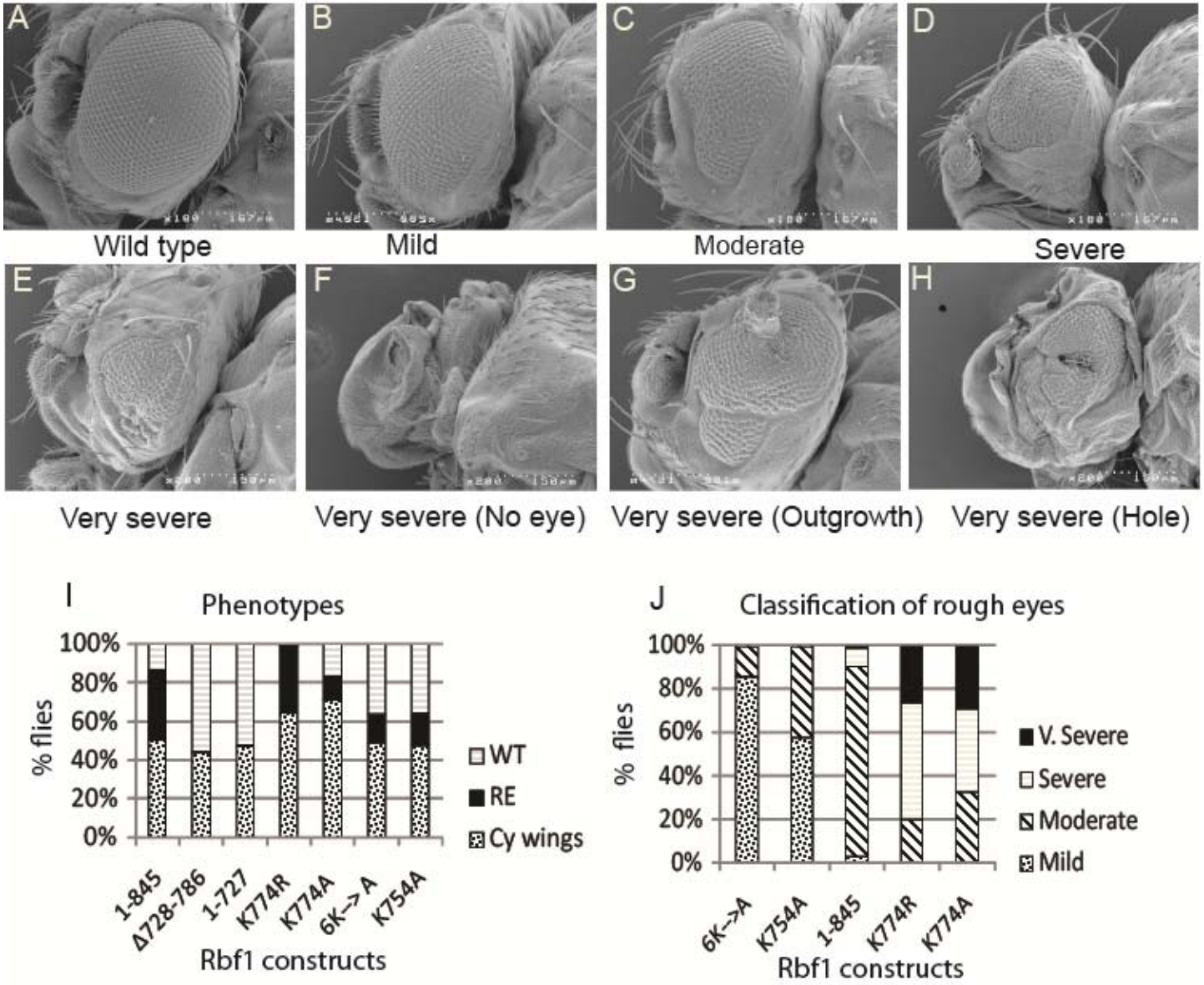

A
B
C
D
Wild type
Mild
Moderate
Severe
E
F
G
H
Very severe
Very severe (No eye)
Very severe (Outgrowth)
Very severe (Hole)
I
Phenotypes
100%
80%
60%
40%
20%
0%
% flies
WT
RE
Cy wings
1-845
Δ728-786
1-727
K774R
K774A
6K--> A
K754A
Rbf1 constructs
J
Classification of rough eyes
100%
80%
60%
40%
20%
0%
% flies
V. Severe
Severe
Moderate
Mild
6K-->A
K754A
1-845
K774R
K774A
Rbf1 constructs

Figure 2.7 cont'd.

**Figure 2.7.** 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.

outgrowths and fewer transgenic individuals were recovered relative to nonexpressing controls, suggesting lethality (Figure 7, 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 8A). To directly compare Rbf1 and p107, we transfected S2 cells with wild-type p107 and mutant forms in which conserved lysine and arginine residues were replaced with alanine, as well as a deletion of the region most similar to the Rbf1 IE (amino acids 964-1024). Similar to the stabilization effects noted with Rbf1, mutant p107 exhibited increased accumulation compared with the wild-type protein (Figure 8B), suggesting that the C-terminal region of p107 harbors an instability element that funnels p107 into similar turnover pathways even in this heterologous system.

# Figure 2.8

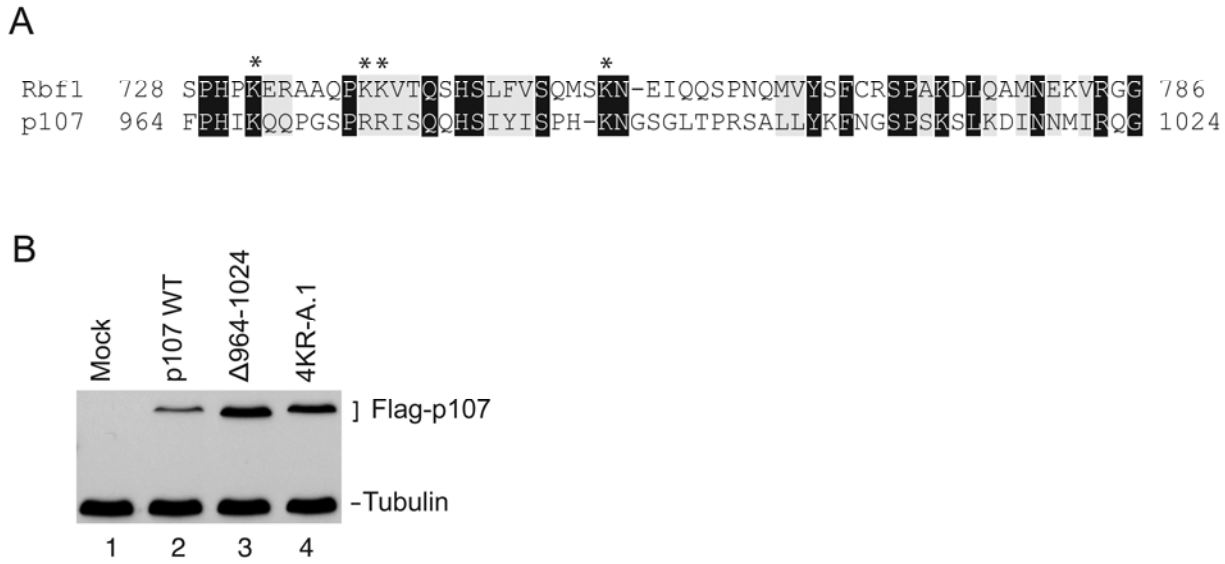



**Figure 2.8.** Mutations in the conserved IE of p107 enhance expression. (A) Similarities between Rbf1 IE and homologous region of p107, which is most similar to Rbf1. Asterisks mark basic residues mutated in each protein to stabilize expression. (B) Genes for Flag-tagged wild-type p107 or IE mutants 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 1, 3, and 4).

Not only is the turnover of Rbf1 required for effective gene regulation, but it appears that this turnover can be developmentally cued, presumably to be coordinated with the engagement of Rbf1 with regulation of the cell cycle (Figure 3). Highly proliferative imaginal disc tissue appears to provide one such context, where levels of wild-type, but not an instability element mutant, Rbf1 protein decrease sharply, presumably in response to the engagement of this protein during cell cycling. In the eye imaginal disc, the Rbf1 protein levels drop sharply in the posterior, where cells are becoming terminally differentiated. Presumably, Rbf1 is activated and consumed in the coordinated 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 (Salghetti *et al.*, 2001; Kim *et al.*, 2003). The activation domain of the VP16 protein was found to be subject to modification by ubiquitylation, enhancing the transcriptional potency of this factor as well as destabilizing it. This process is thought to affect other transcriptional activators as well (Salghetti *et al.*, 2000). The exact mechanism by which ubiquitylation enhances transcriptional activation is poorly understood. The ubiquitin tag may serve a dual purpose of facilitating interactions with the transcriptional machinery as well as attracting the 26S proteasome. Alternatively, the proteasome itself, or portions of this multi-protein complex, may directly enhance transcription; chromatin immunoprecipitation experiments have placed the "lid" of the proteasome on specific genomic locations (Gonzalez *et al.*, 2002; Ferdous *et al.*, 2007).

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

The C terminus of Rbf1 appears to represent a regulatory nexus for this protein; in addition to the instability/repression activity described here, key residues appear to provide a damper to modulate its overall activity (Figure 6), and phosphorylation within this region by cyclin kinases can inactivate the protein (Xin *et al.*, 2002). The deep conservation of residues

within the Rbf1 IE argues strongly for similar activities in mammalian pocket proteins; indeed, mutations of key residues in p107, the closest homolog of Rbf1, strongly stabilize the levels of this protein (Figure 8). In addition, the spectrum of mutations associated with the human retinoblastoma gene indicates that the C-terminal region correlating to the Rbf1 IE may similarly contain critical functions for the mammalian RB protein. One common class of genetic lesion associated with retinoblastomas are nonsense mutations that cause a truncation of the C terminus of the RB protein, and several cancer-associated missense mutations have similarly been mapped to the region corresponding to the Rbf1 IE (Lohmann, 1999).

Previous studies have shown that the RB C terminus interacts with the E3 ligase Skp2 and the anaphase promoting complex (APC/C) to regulate turnover of the p27 cyclin kinase inhibitor (Ji *et al.*, 2004; Binne *et al.*, 2007). This pathway has been suggested to represent a transcription-independent mechanism by which RB controls the cell cycle, and indeed RB was shown not to be subject to APC/C degradation (Binne *et al.*, 2007). Our results indicate that a clean separation of transcription and proteolytic control in the context of RB proteins may be oversimplified; here we see evidence for a separate route of proteolytic regulation that modulates transcriptional regulatory potential and protein stability of Rbf1, and possibly related mammalian pocket proteins. Interestingly, the regulation of this pathway may involve the evolutionarily conserved COP9 signalosome. Our previous biochemical studies indicated that the COP9 signalosome regulatory complex is physically associated with Rbf proteins and limits turnover of these repressors (Ullah *et al.*, 2007). From the results of the current study, we postulate that COP9 antagonizes the function of the Rbf1 IE, perhaps by blocking the access of ubiquitin-modifying E3 ligases that would otherwise potentiate Rbf1 activity and turnover. Alternatively, inhibition of E3 ligases may involve the enzymatic activity of COP9, whereby this complex

downregulates E3 ligases by deneddylation of their cullin subunits (Wei *et al.*, 2008). How the instability of pocket proteins potentiates their activities, and how these processes relate to developmental control of retinoblastoma family proteins and cancer, will be an area of active investigation.

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# CHAPTER III

## Evidence for autoregulation and cell signaling pathway regulation from genome-wide binding of the *Drosophila* retinoblastoma protein[2]


### Abstract

The retinoblastoma (RB) tumor suppressor protein is a transcriptional corepressor with essential roles in cell cycle and development. Physical and functional targets of RB and its paralogs p107/p130 have been studied largely in cultured cells, but the full biological context of this family of proteins' activities will likely be revealed only in whole organismal studies. To identify direct targets of the major Drosophila RB counterpart in a developmental context, we carried out ChIP-Seq analysis of Rbf1 in the embryo. The association of the protein with promoters is developmentally controlled; early promoter access is globally inhibited, while later in development Rbf1 is found to associate with promoter-proximal regions of approximately 2,000 genes. In addition to conserved cell cycle-related genes, a wholly unexpected finding was that Rbf1 targets many components of the insulin, Hippo, JAK/STAT, Notch and other conserved signaling pathways. Rbf1 may thus directly affect output of these essential growth-control and differentiation pathways by regulation of expression of receptors, kinases and downstream effectors. Rbf1 was also found to target multiple levels of its own regulatory hierarchy. Bioinformatic analysis indicates that different classes of bound genes exhibit distinct promoter motifs, suggesting that the context of Rbf1 recruitment involves diverse transcription factors, which may allow for independent regulation of Rbf1 bound genes. Many of these targeted genes are bound by Rbf1 homologs in human cells, indicating that a conserved role of retinoblastoma proteins may be to adjust the set point of interlinked signaling networks essential for growth and development.

[2]The work described in this chapter is resubmitted (after reviewer's comments) to Genome Research as the following manuscript: Pankaj Acharya, Nicolas Negre, John Johnston, Heming Wang, Yiliang Wei, Kevin P. White, R. William Henry, and David N. Arnosti

## Introduction

The retinoblastoma tumor suppressor protein (RB) is an evolutionarily conserved transcriptional corepressor that controls cell cycle, differentiation, development, autophagy and apoptosis (Lipinski and Jacks 1999; Bosco et al. 2001; Nevins 2001; Classon and Harlow 2002; Jiang et al. 2010). Germ-line mutations of RB are closely linked to retinoblastoma in early childhood and osteosarcoma in adolescence, and somatic mutations in the RB gene are extremely frequent in human cancers (Lohmann 1999; Sherr and McCormick 2002; Tang et al. 2008). The vertebrate RB protein and the related family members, p107 and p130, are recruited to promoters by interactions with E2F/DP heterodimers (van den Heuvel and Dyson 2008). Interactions between E2F transcription factors and RB family proteins are regulated by cyclin/CDK-directed phosphorylation during the cell cycle, and RB-E2F interactions can also be affected by viral proteins (Nevins 1992a; Nevins 1992b; Dick 2007; Flowers et al. 2010). Though less complex than its human counterpart, the *Drosophila* retinoblastoma network is functionally conserved and consists of two RB proteins, Rbf1 and Rbf2, two E2F proteins, E2F1 and E2F2, and one DP protein (Du and Pogoriler 2006). *Drosophila* Rbf proteins are regulated by phosphorylation, similar to the vertebrate RB proteins (Xin et al. 2002; Frolov et al. 2005). Rbf1 activity is also regulated during development by proteosome-dependent degradation, which is dependent on a C-terminal instability element that is simultaneously required for corepressor activity (Acharya et al. 2010). The instability mechanism is conserved in the p107 human homolog, indicating that this novel linkage between protein lability and repression function may be a general property of these proteins in multicellular organisms (Acharya et al. 2010).

RB proteins are involved in regulation of both canonical and noncanonical forms of the cell cycle. The canonical cycling involves the separation of DNA-synthesis (S-phase) and

mitosis (M-phase) by two gap phases, G1 and G2. However, during development, noncanonical mitotic programs are common. In *Drosophila,* the first thirteen embryonic cell cycles are synchronous, consisting of only S and M phases. G2 appears in cell cycle fourteen and G1 in cell cycle seventeen (Foe 1989; Edgar and O'Farrell 1990; Shibutani et al. 2007). Endoreplication (in which mitosis is not followed by cytokinesis), is another variant common in many larval and adult tissues (Spradling and Orr-Weaver 1987). Endoreduplication in follicle cells is regulated by Rbf1/E2F (Bosco et al. 2001). This diversity of cell cycle regulation suggests that Rbf1/2 and its partners may be differentially utilized or regulated in different settings. Consistent with this idea, Rbf1 stability is decreased in proliferating larval imaginal discs, and E2F1 is specifically turned over during early S-phase in embryos and larvae (Shibutani et al. 2007; Shibutani et al. 2008; Acharya et al. 2010).

RB proteins are also required in tissue- and stage-specific manners, as seen in studies of different metazoan RB family members. Although expressed, the Drosophila Rbf1 protein is not functionally required for early cell cycle regulation in the embryo (Du and Dyson 1999; Stevaux et al. 2002; Keller et al. 2005). In the mouse, early embryonic requirements for RB are restricted to the trophectoderm, although the protein is expressed in other tissues (Wu et al. 2003). The *C. elegans lin-35* (RB homolog) mutant shows a largely nonoverlapping set of genes that are misregulated in embryo, L1 and L4 larvae (Kirienko and Fay 2007). These and other studies emphasize that the multifarious functions of this protein family will require global studies in a developmental setting. A major objective along these lines is the identification of functional and physical target genes of RB family corepressors.

Among the best-characterized targets of RB family proteins are genes such as *PCNA* and *DNA pol alpha*, which are involved in cell cycle regulation, however, RB proteins also regulate a

variety of genes involved in other pathways, such as apoptosis, DNA repair, and differentiation (Lipinski and Jacks 1999; Classon and Harlow 2002; Kirienko and Fay 2007). In Drosophila, functional targets of Rbf and E2F proteins were identified by transcriptomic analysis of Drosophila S2 cells (Dimova et al. 2003). Physical and functional targets of the MMB/dREAM complex, with which Rbf1 is also associated, were identified in Drosophila Kc cells (Georlette et al. 2007). These studies suggest that Rbf1, and to a lesser extent Rbf2, interact with distinct classes of genes that show varying sensitivity to loss of Rbf and E2F proteins. Targets of mammalian RB, p107 and p130 proteins were identified by chromatin immunoprecipitation (ChIP) in human lung fibroblasts; the proteins are observed to be redeployed in response to expression of the RB-binding adenoviral E1A protein (Ferrari et al. 2008). The genome-wide occupancy of RB and p130 was also recently reported in growing, quiescent and senescent human fibroblasts, indicating that these proteins bind to thousands of putative target genes (Chicas et al. 2010). However, until now no study has presented a picture of the genome-wide occupancy of RB proteins in a whole organism during development. Using highly specific antibodies developed against the endogenous Rbf1 protein, we carried out ChIP to study Rbf1 protein occupancy through developmental time, and employed parallel sequencing (ChIP-seq) to identify genome-wide targets of Rbf1 in the Drosophila embryo. These results identify a diversity of potential Rbf1 targets and promoter composition, and suggest that in addition to known links to cell cycle, this protein may play a direct role in the control of numerous conserved signaling pathways that are linked to metabolic regulation and growth.

## Results

### *Rbf1 exhibits developmentally-regulated promoter occupancy*

In previous studies, we and others showed that Rbf1 protein is expressed throughout embryogenesis, although the corepressor is not required for early cell cycles (Du and Dyson 1999; Stevaux et al. 2002; Keller et al. 2005). To investigate possible temporal control of Rbf1 binding to target gene promoters, we performed ChIP using 0-6, 6-12, 12-18 and 18-24 hr old embryos. Enrichment of Rbf1 protein was studied at selected promoters from different classes of Rbf1-responsive genes, described previously (Dimova et al. 2003). In all cases, Rbf1 occupancy was low in early embryos with a peak in 12-18 hr embryos (Figure 1 and Supplementary Figure 1). These results indicate that Rbf1 promoter association is developmentally regulated; the lack of early Rbf1 association coincides with the rapid early cell cycles that lack G1 and G2 phases.

### *Characterization of genome-wide Rbf1 association*

Although many well-characterized targets of RB family proteins are genes involved in cell cycle regulation and DNA replication, genes involved in other processes are also functionally regulated by these corepressors (Classon and Harlow 2002; Dimova et al. 2003; Kirienko and Fay 2007). To develop a global understanding of the genomic targets Rbf1, we utilized ChIP-seq technology. We prepared chromatin from 12-18 hr embryos where robust signals had been detected by conventional ChIP (Figure 1 and Supplementary Figure 1), and generated separate DNA libraries from two Rbf1 immunoprecipitation experiments and one experiment using preimmune antibodies. The anti-Rbf1 libraries yielded ~14 and 18 million reads, while the preimmune serum library generated considerably fewer reads (1.2 million), as expected for non-specific interactions of the preimmune serum with the Drosophila chromatin.

# Figure 3.1

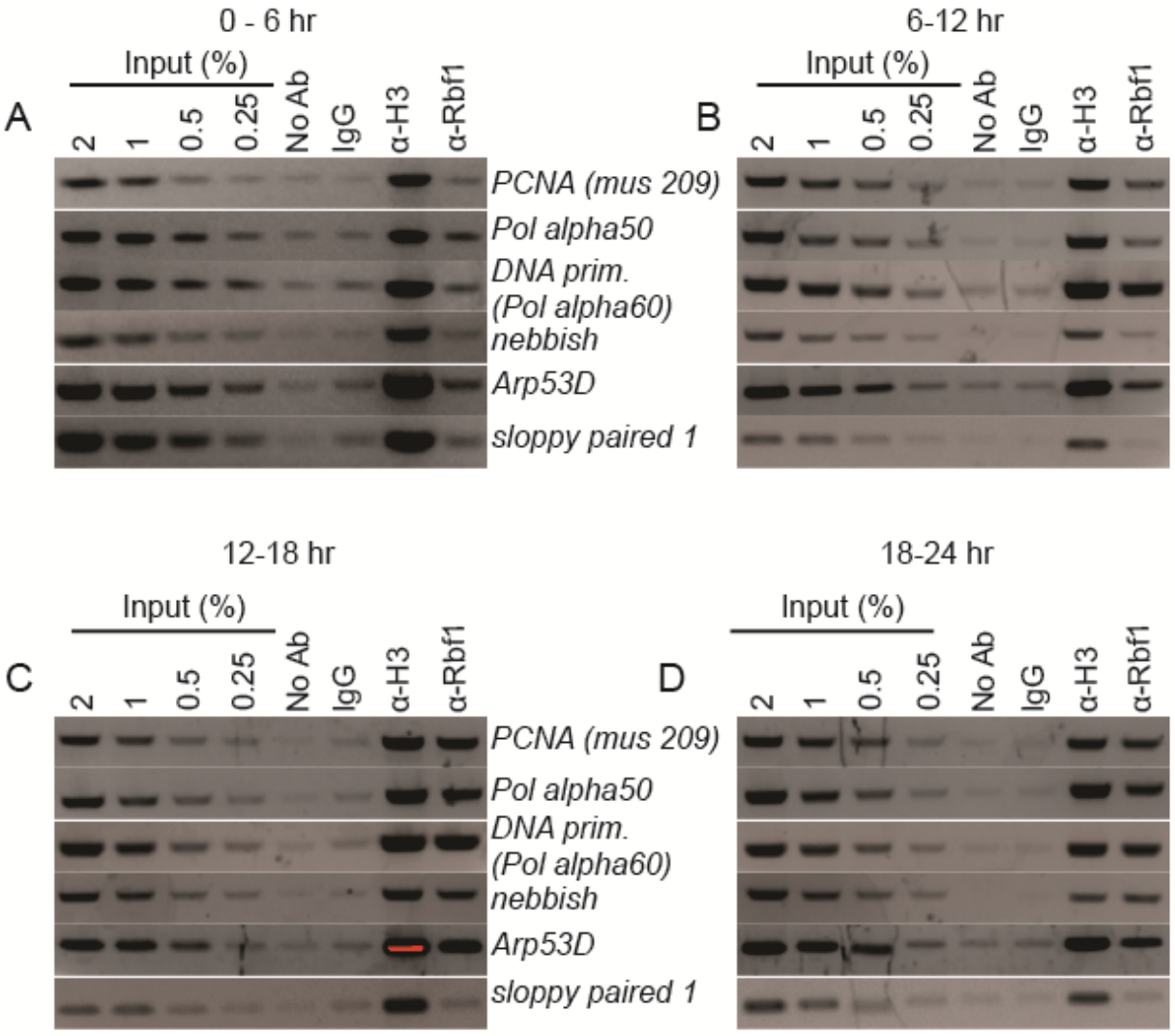
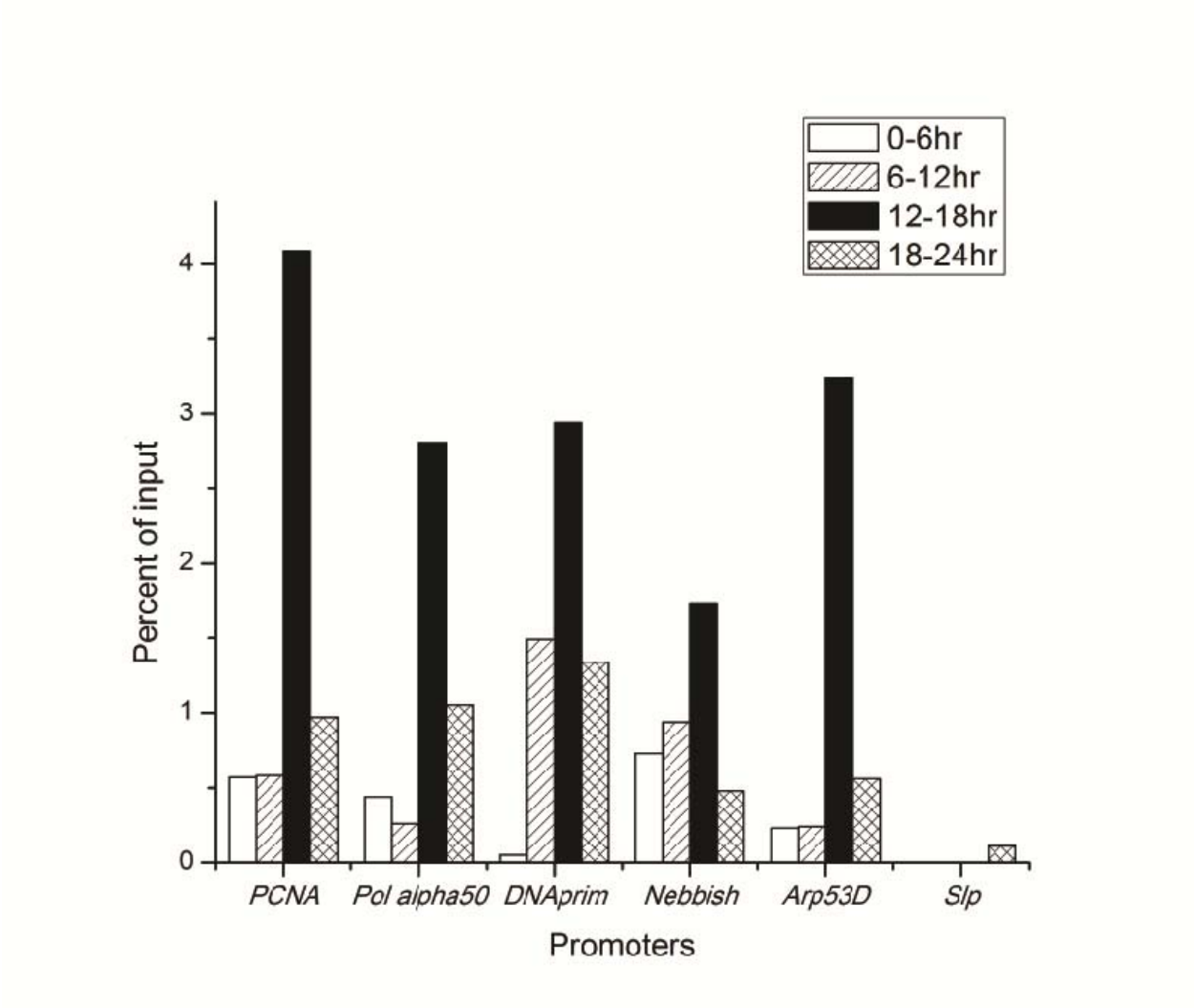

A
0 - 6 hr
Input (%)
2
1
0.5
0.25
No Ab
IgG
α-H3
α-Rbf1
PCNA (mus 209)
Pol alpha50
DNA prim.
(Pol alpha60)
nebbish
Arp53D
sloppy paired 1
B
6-12 hr
C
12-18 hr
D
18-24 hr

Figure 3.1 cont'd.

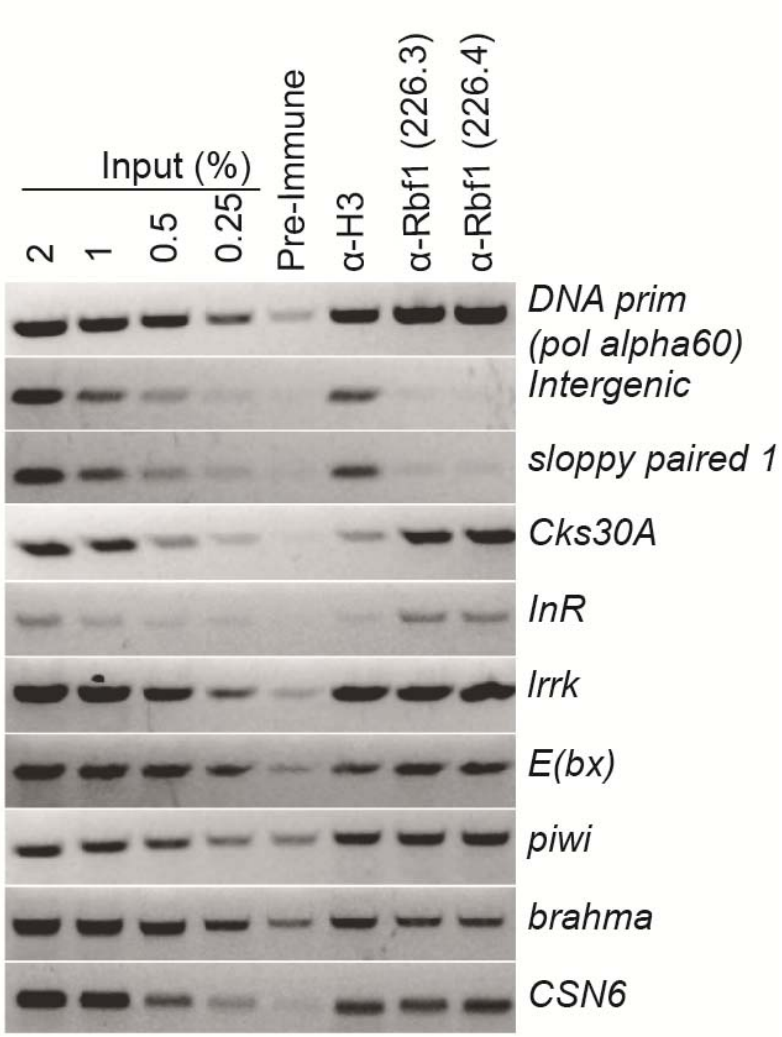
**Figure 3.1. Rbf1 exhibits dynamic promoter occupancy.** Rbf1 occupancy of regulated promoters measured by chromatin immunoprecipitation was low in 0-6 hr embryos and peaked at 12-18 hr. Formaldehyde cross-linked chromatin was prepared from embryos of different ages and immunoprecipitated using the indicated antibodies. No specific enrichment was found at a non-target gene promoter (*sloppy paired 1*). "No Ab", immunoprecipitation carried out without antibody; "IgG", nonspecific mouse polyclonal antibodies; "α-H3", anti-histone H3 antibody; "α-Rbf1", rabbit anti Rbf1 antibody.

# Supplementary Figure 3.1




**Supplementary Figure 3.1. Enrichment of Rbf1-bound promoters peaks at 12-18 hr.** Quantitation of enrichment of Rbf1-bound promoters shows that the dynamic promoter occupancy of Rbf1 is maximum at 12-18 hr. The PCR products shown in Figure 1 were measured on a Fuji LAS3000 imager and quantitated using Multi Gauge software (Fuji).

# Supplementary Figure 3.2



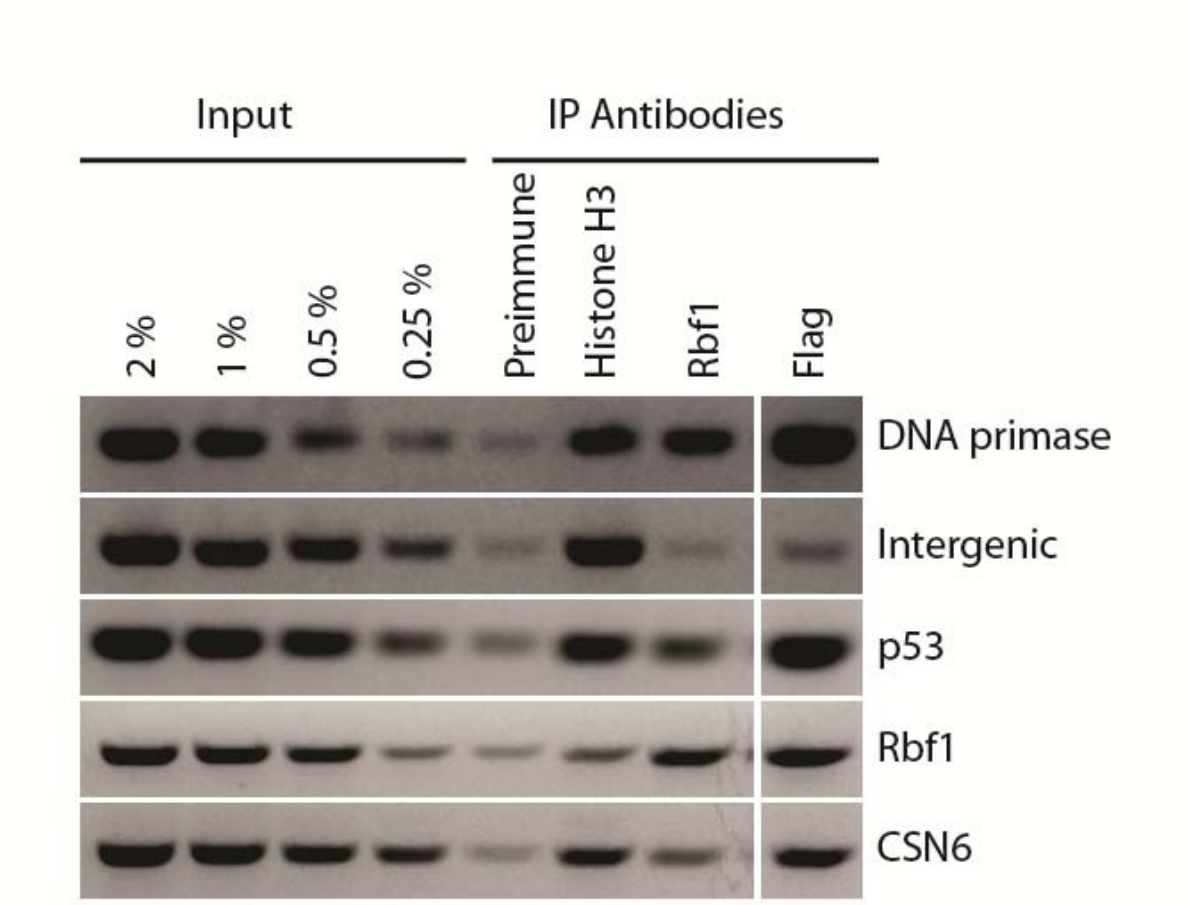

**Supplementary Figure 3.2. Validation of selected promoters for Rbf1 occupancy.** To independently assess enrichment of Rbf1 on novel target genes, several genes were selected and their enrichment in ChIPed chromatin was tested by PCR. *DNA prim* is a positive control; the intergenic region on chromosome 3 and *sloppy paired 1* are negative controls. The enrichment of the Rbf1 target gene promoters tested is significantly above the background. "Preimmune", serum from the rabbit used for later generation of α-Rbf1 antibody; "α-H3", anti-histone H3 antibody; "α-Rbf1 226.3" and "α-Rbf1 226.4", different bleeds of rabbit anti-Rbf1 antibodies.

About 60% of the reads were uniquely alignable to the *Drosophila melanogaster* genome; other reads were found to map to more than one site, or did not align at all using the strict criteria employed. 1236 peaks were found in both anti-Rbf1 immunoprecipitations, while an additional ~1000 peaks were also found, mainly in one of the two ChIP experiments that exhibited more robust peak intensities. The preimmune control ChIP results showed an even distribution of very low peaks as would be expected from nonspecifically precipitated material (data not shown). The overall low background found with the preimmune immunoprecipitation and reproducibility between the two biological replicates provides a high level of confidence for many of the peaks. Of the 1236 high-confidence peaks recovered in both of the biological replicate experiments, about 95% could be mapped to within 2 kb of predicted transcriptional start sites of known genes. These peaks were proximal to transcriptional start sites of 1890 genes. Additional strong candidate genes were found in the library that yielded 18 million reads, generating a total of 3188 genes.

***Rbf1 target genes indicate regulation of RB pathway at multiple levels***

Clear signals were observed on a number of promoters that we expected to find in this data set, including *DNA pol alpha*, *DNA primase, and PCNA,* known physical targets of Rbf1 (Stevaux et al. 2002; Dimova et al. 2003). To validate the process of Illumina sequencing and peak calling used to generate this data, we selected a set of promoters to independently analyze by direct PCR; positive and negative signals were confirmed in all cases (Supplementary Figure 2 and data not shown). We also carried out experiments with a different antibody to test the reproducibility of these ChIP results. Chromatin was prepared from flies harboring transgenic Flag-epitope tagged Rbf1, (Acharya et al. 2010) and immunoprecipitations were carried out using either anti-Rbf1 or Flag antibodies (Supplementary Figure 3). In each case, ChIP signals

# Supplementary Figure 3.3




**Supplementary Figure 3.3. Validation of specificity of Rbf1 antibodies.** To assess the specificity of Rbf1 antibodies, ChIP experiment was performed with embryos from transgenic flies harboring Flag epitope tagged Rbf1. Some genes identified in ChIP-seq analysis along with previously known target (*DNA primase)* and intergenic region were selected for PCR. A similar significant enrichment of the Rbf1 target gene promoters was noted for each antibody. "Preimmune", serum from the rabbit used for later generation of α-Rbf1 antibody; "α-H3", anti-histone H3 antibody; "α-Rbf1", rabbit anti-Rbf1 antibody; "Flag", anti-Flag antibody.

coincided exactly. We found that diverse classes of genes were targeted by Rbf1, including a set of genes that indicates that Rbf1 regulates its own functional output at multiple levels (Figure 2 and Supplementary Table 1 as in Acharya *et al*, 2012). The autoregulatory properties of Rbf1 were suggested by particular genes, such as *cyclin A*, *B3*, *E*, and *cdk4/6*, which encode the kinase complexes that downregulate Rbf activity, the *rbf1* gene itself, and *cyclin-dependent kinase subunit 30A,* a component of the Cdk1-cyclin B kinase complex that phosphorylates numerous proteins involved in DNA replication, translation, and chromatin structure (Holt et al. 2009). Consistent with this notion, negative feedback loops of regulation of *RB* and *p107* have been reported in mammalian cells (Burkhart et al. 2010a; Burkhart et al. 2010b). A peak was also associated with the 5' promoter of the *l(3)mbt* gene, whose protein product is a member of a conserved MMB/dREAM transcriptional regulatory complex that also involves the Rbf1 protein (Lewis et al. 2004). Other MMB/dREAM components were also targeted by Rbf1, consistent with autoregulation of the entire complex by Rbf1 and MMB/dREAM (Lewis et al. 2004; Tabuchi et al. 2011). Rbf1 peaks were also associated with additional chromatin-regulatory components, including the *E(bx)* gene, which encodes the NURF301 component of a SWI/SNF nucleosome remodeling complex that has been shown to antagonize the action of RB in *C. elegans* development (Andersen et al. 2006). These data indicate that Rbf1's direct transcriptional regulation is likely to control RB pathway output at five levels; Rbf activity via cyclins and kinases that directly phosphorylate the corepressor, production of *rbf1* transcripts, of cofactors that work together with Rbf proteins, of factors that antagonize Rbf activity, and kinases that are implicated in cell-cycle regulation of downstream genes. The potential effect of this regulatory structure is that changes in Rbf protein abundance or activity will reset levels of other components that would magnify or dampen the control of the entire Rbf regulon.

# Figure 3.2

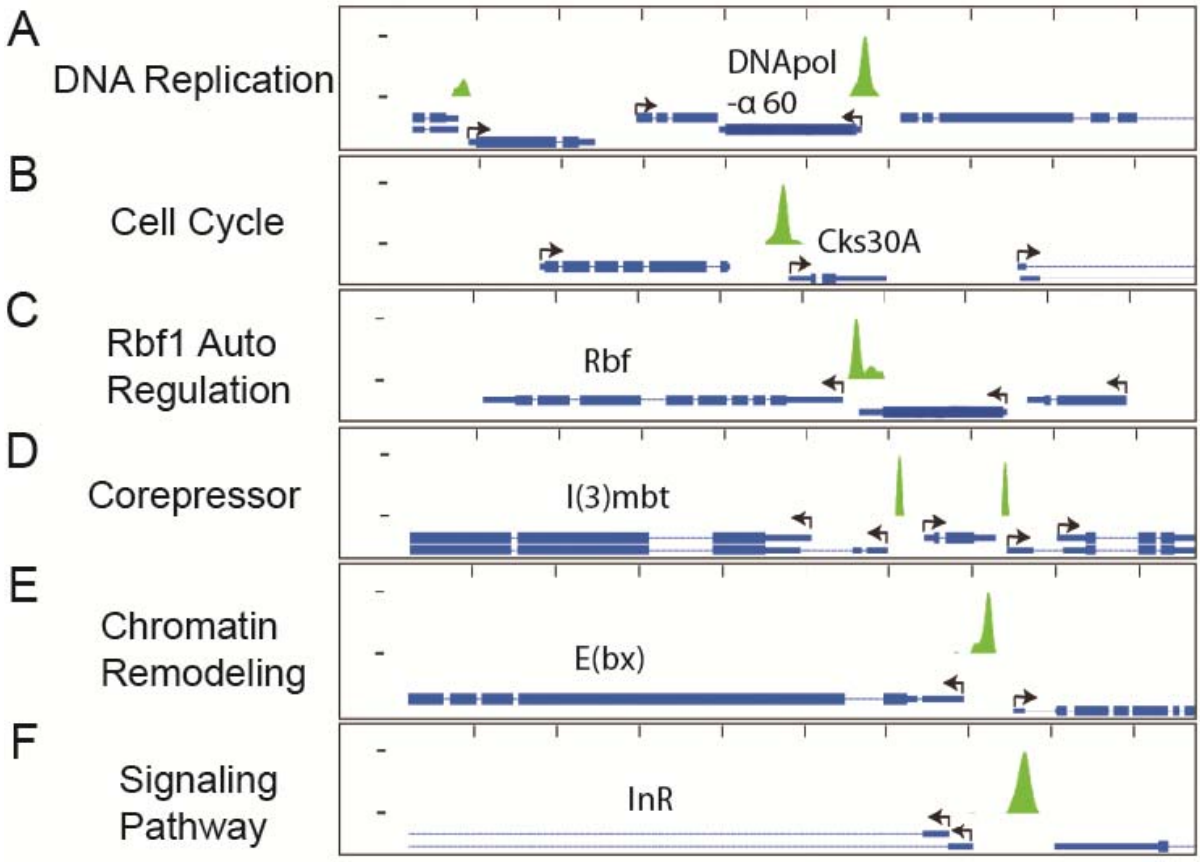

A
DNA Replication
DNApol
-α 60
B
Cell Cycle
Cks30A
C
Rbf1 Auto
Regulation
Rbf
D
Corepressor
l(3)mbt
E
Chromatin
Remodeling
E(bx)
F
Signaling
Pathway
InR

Figure 3.2 cont'd.

**Figure 3.2. Rbf1 promoter-proximal occupancy of diverse classes of genes suggests autoregulatory effects.** Strong peaks were noted on cell-cycle related genes, such as *DNApol-α60* and *cyclin-dependent kinase 30A* (A, B). Autoregulation is suggested by occupancy of the *Rbf* gene (C). The 5' region of the Rbf1-related corepressor *l(3)mbt* is also associated with Rbf1 (D). The promoter of the *dNURF 301/E(bx)* gene, a chromatin remodeling component for RB function in development, is also bound (E). Numerous components of cell signaling pathways, including the insulin receptor, *InR*, are also targeted by Rbf1 (F). Relative peak intensities are shown on the Y-axis. Representative individual peaks are visualized on the UCSC genome browser. Bent arrows indicate the direction of transcription of the genes and absence of arrows indicates 3' region of the gene. 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 target genes include multiple components of conserved signaling pathways***

One of the most striking observations about the roster of genes occupied by Rbf1 was its extensive and hitherto unappreciated occupancy of genes involved in essential, conserved signaling pathways. One of the strongest Rbf1 peaks is found proximal to the insulin receptor promoter (Fig. 2F). Further investigation of the 3188 gene data set revealed that Rbf1 peaks were extensively associated with other components of insulin signaling, including three of the four Drosophila PI3 kinase genes, S6 kinase, and Thor/4E-BP (Fig. 3 and Supplementary Table II as in Acharya *et al,* 2012). JAK/STAT signaling components identified as Rbf1 targets include the signal mediator JAK kinase, the STAT92E transcriptional effector, as well as regulators of this pathway, Ken, E(bx), Pzg and STAM. A large number of the genes in the Hippo growth-control pathway, including those for the central Hippo and Warts kinases, are bound by Rbf1. Components of the Notch, in particular regulatory proteases that process the Notch protein, were also found among Rbf1 target genes. Wingless, Hedgehog, NF-κB, TGF-β, TOR, EGFR/Ras, and JNK pathway components were also bound by Rbf1. In total, we identified 137 signaling pathway genes that were bound by Rbf1; 75 of these were clearly identified in both ChIP-seq samples, with a further thirteen present as peaks in both datasets, but just below the cutoff in one of the ChIPs. Association of Rbf1 with signal pathway genes was confirmed in additional biological ChIP experiments (Supplementary Figures 2 and 3, and data not shown). The promoter-proximal positions of most Rbf1 peaks, as well as the comparatively small number of regions bound by Rbf1 overall, suggest that these binding events are not simply the effect of large numbers of promiscuously bound Rbf1 proteins. Genes for almost half of insulin signaling and more than half of Hippo signaling components were occupied by the Rbf1 protein, compared with the genome-wide average of 22%, indicating a strong enrichment. The extensive physical

# Figure 3.3

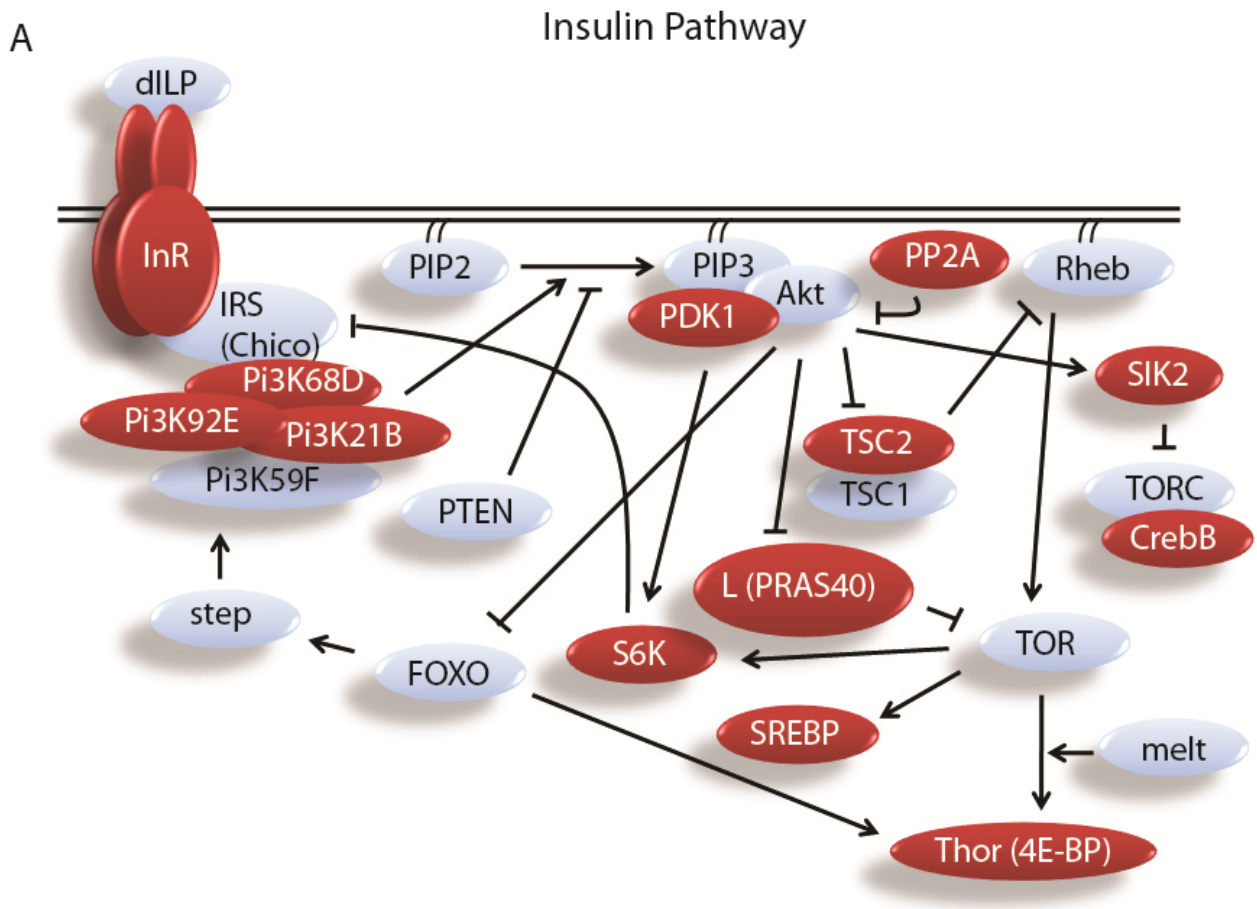

A
Insulin Pathway
dILP
InR
IRS
(Chico)
Pi3K68D
Pi3K92E
Pi3K21B
Pi3K59F
PIP2
PIP3
PDK1
Akt
PP2A
Rheb
SIK2
TSC2
TSC1
TORC
CrebB
PTEN
step
L (PRAS40)
S6K
TOR
FOXO
SREBP
melt
Thor (4E-BP)

**Figure 3.3 cont'd.**

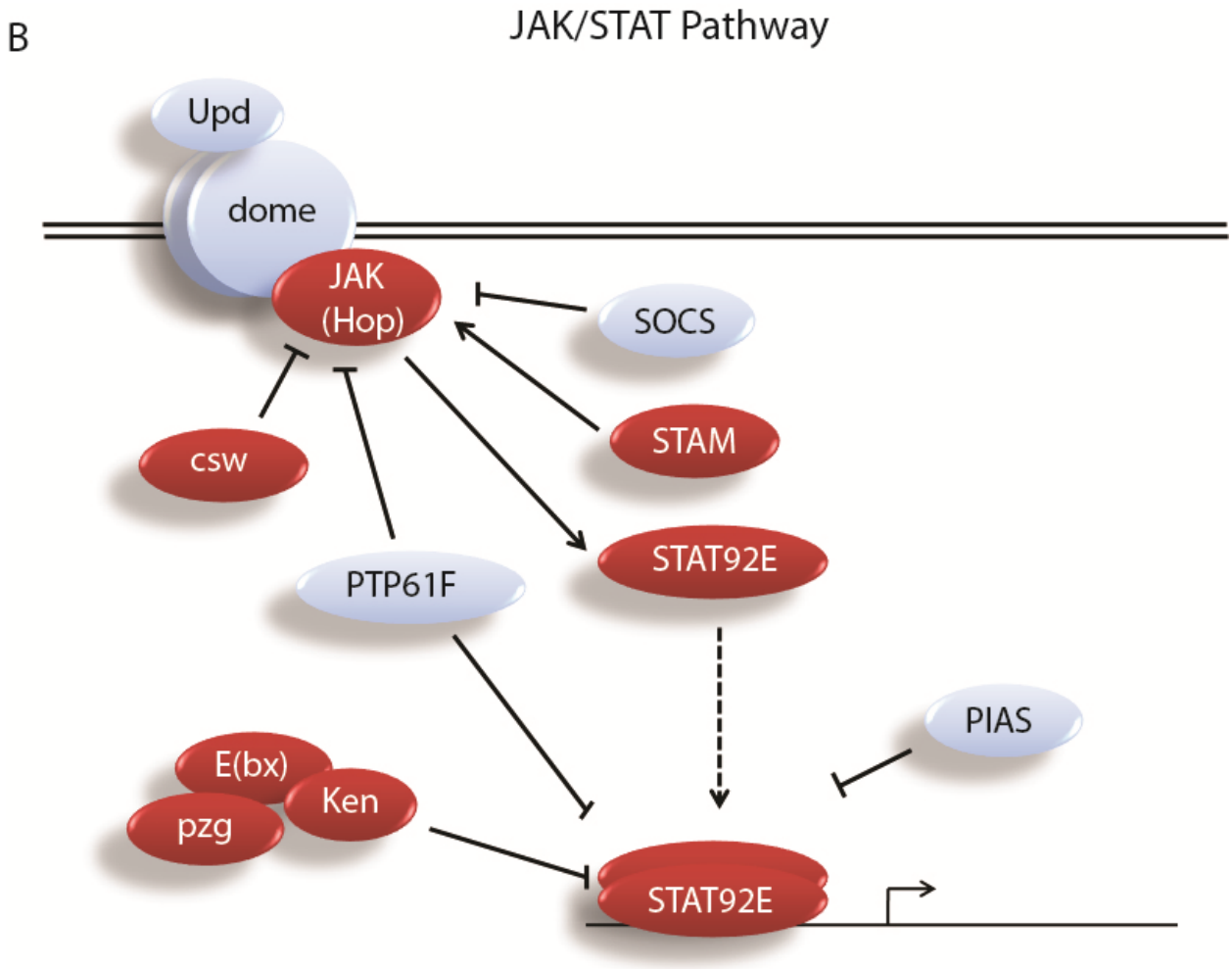


**Figure 3.3 cont'd.**

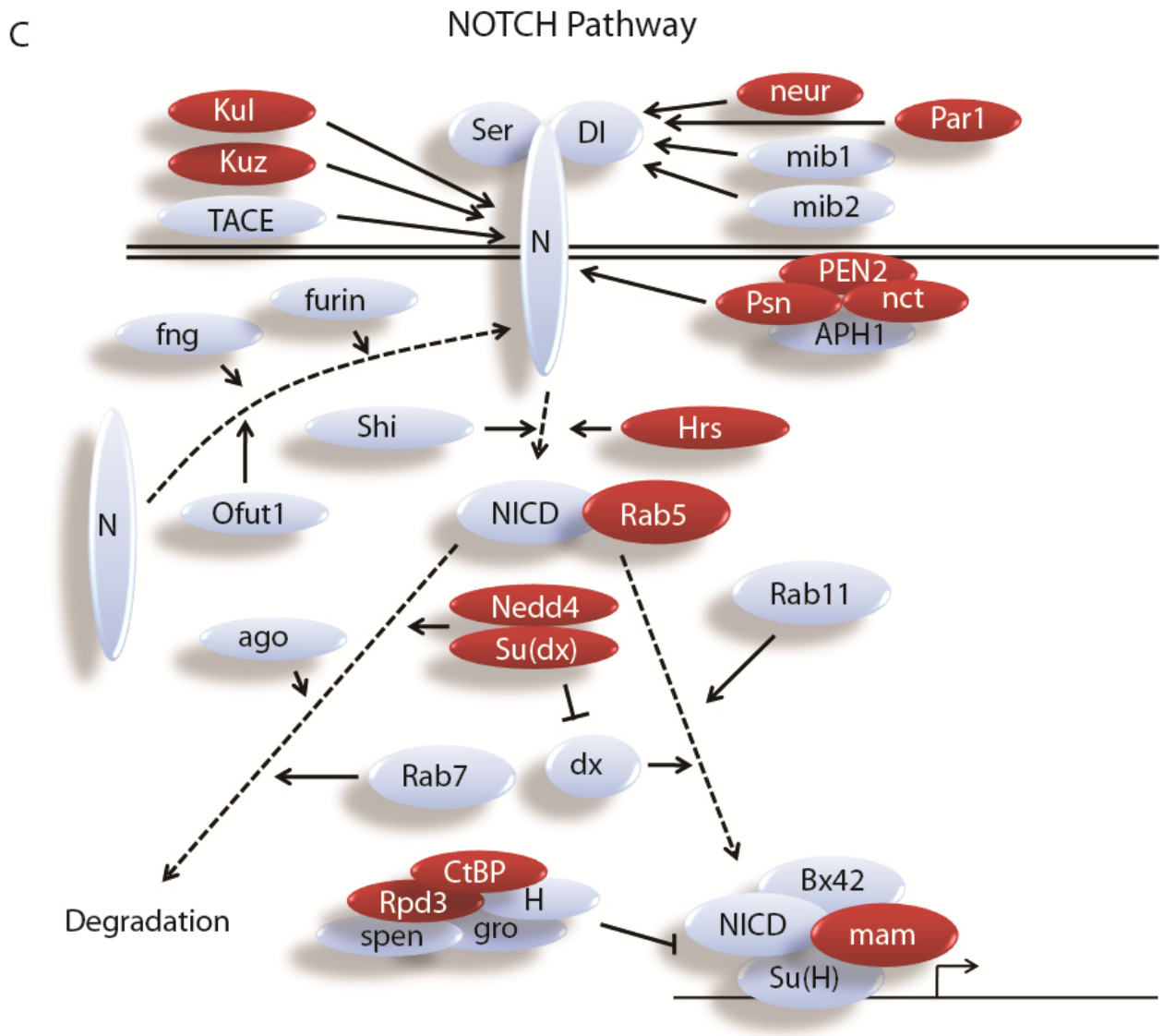


**Figure 3.3 cont'd.**

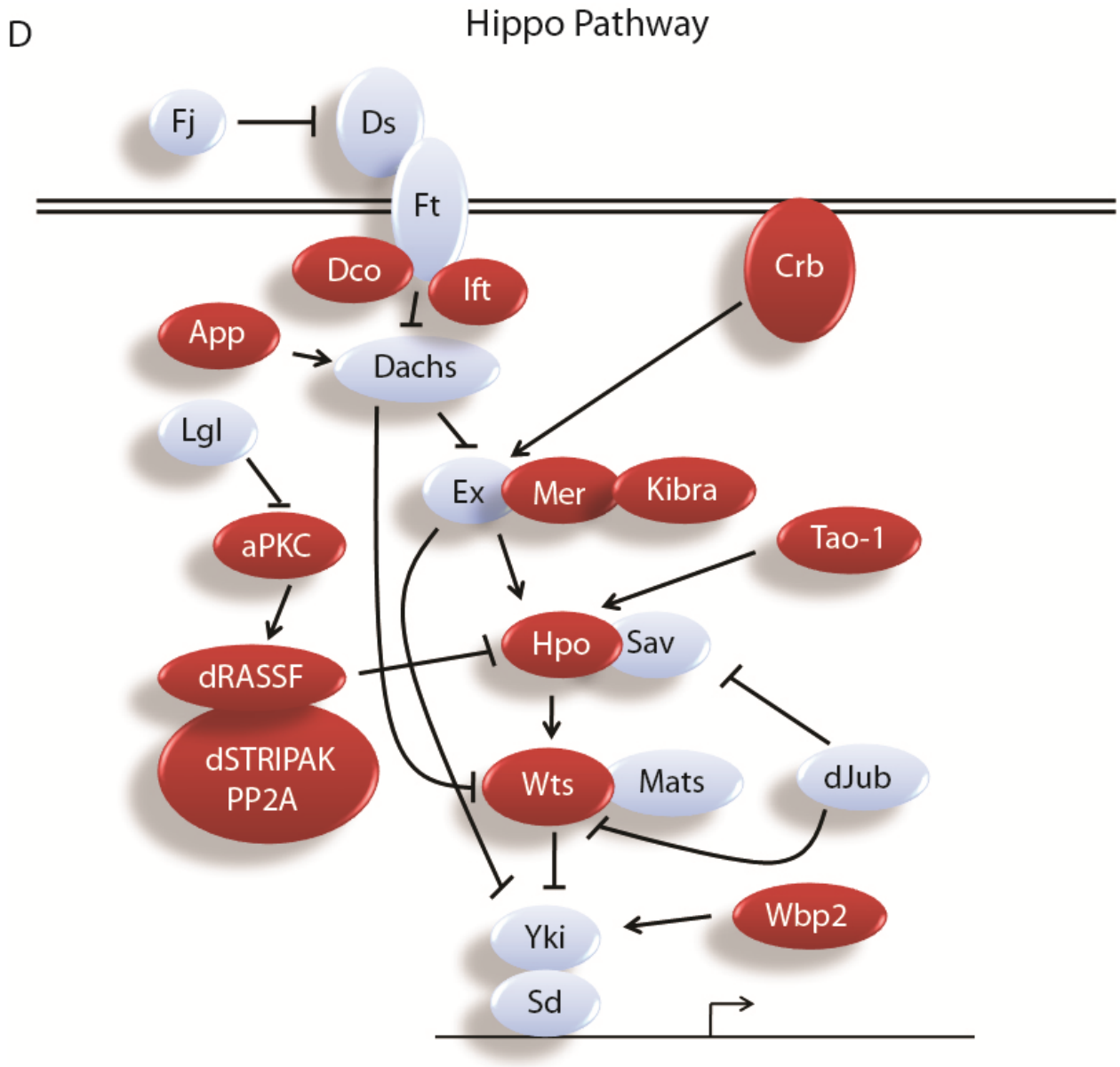
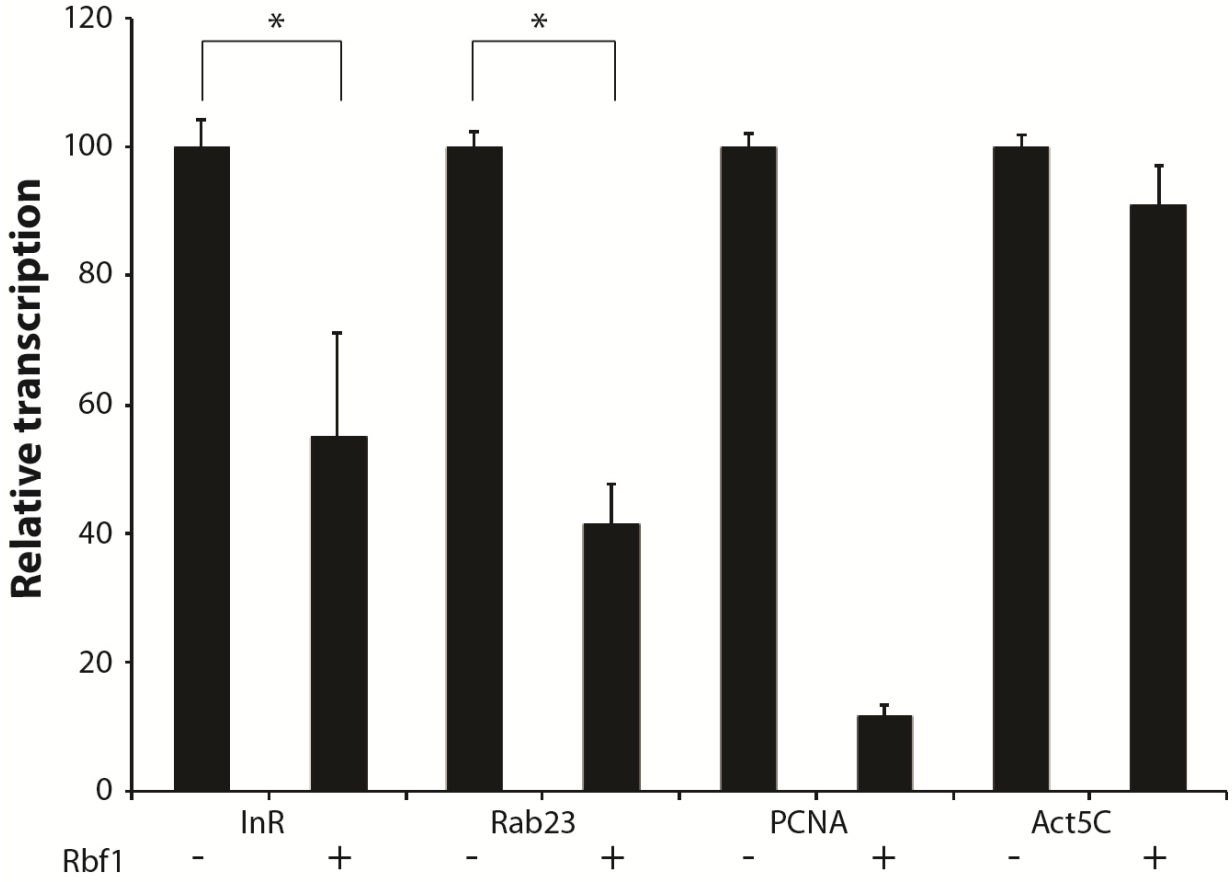


Figure 3.3 cont'd.

**Figure 3.3. Rbf1 occupies multiple nodes in conserved signaling pathways.** Genes in the insulin (A), JAK/STAT (B), Notch (C) and Hippo/Warts/Yorkie (D) signaling pathways were targeted at multiple levels by Rbf1. Proteins of genes targeted by Rbf1 are indicated in red. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

# Supplementary Figure 3.4




**Supplementary Figure 3.4. Repression of *InR* and *Rab23* promoters by Rbf1.** *Drosophila* S2 cells were cotransfected with *InR, Rab23*, *PCNA,* or *Act5C* luciferase reporters, with (+) or without (-) an Rbf1-expressing plasmid. Cells were harvested 72 h after transfection, and luciferase assay was performed. Results of four (*Act5c* luciferase), and six (*InR, Rab23,* and *PCNA* luciferase) biological replicates with three technical replicates each were pooled. Asterisks indicate $p < 0.0001$.

interaction between Rbf1 and components of diverse signaling pathways suggests a novel means by which cell cycle information may be integrated with the information related to metabolic status, organ and tissue size, and differentiation states. The occupancy of genes located at multiple levels of signaling, such as the insulin receptor and the S6 kinase genes indicate that Rbf1 may be in a position to regulate these pathways in a complex, multifactorial manner. To test whether Rbf1 indeed represses the some of these novel targets, we created *InR* (insulin signaling), and *Rab23* (hedgehog signaling) reporter constructs and measured their sensitivity to Rbf1 overexpression compared with *Act5C,* and *PCNA* controls. As expected, Rbf1 effectively repressed the *PCNA* reporter and not the *Act5C* reporter. Significantly, strong inhibition of *InR* and *Rab23* promoters was observed, indicating that Rbf1 can functionally regulate in S2 cells at least some of the novel targets identified (Supplementary Figure 4).

***Rbf1 exhibits a strong promoter-proximal targeting bias***

As noted above, most Rbf1 peaks were located within 2 kb of transcriptional initiation sites; mapped on a finer scale, we found that there was a strong preference for binding centered at -205 bp (Figure 4). Unlike other corepressors such as CtBP and Groucho, the strong preference of Rbf1 for the 5' ends of genes suggests that Rbf1 can only exhibit activity near the initiation site, or that transcription factors that it interacts with, such as E2F proteins, can only activate effectively from promoter-proximal locations. Interestingly, Rbf1-associated MMB/dREAM complex proteins are also found to bind in promoter-proximal locations; neither this complex nor Rbf1 alone appear to frequently interact with distal cis-regulatory sequences (Georlette et al. 2007). Rbf1 peak intensities (representing the number of sequences recovered for particular genomic positions) spanned approximately an order of magnitude, with relatively few outliers showing more than ten fold higher than the median value. Overall peak heights were

**Figure 3.4**

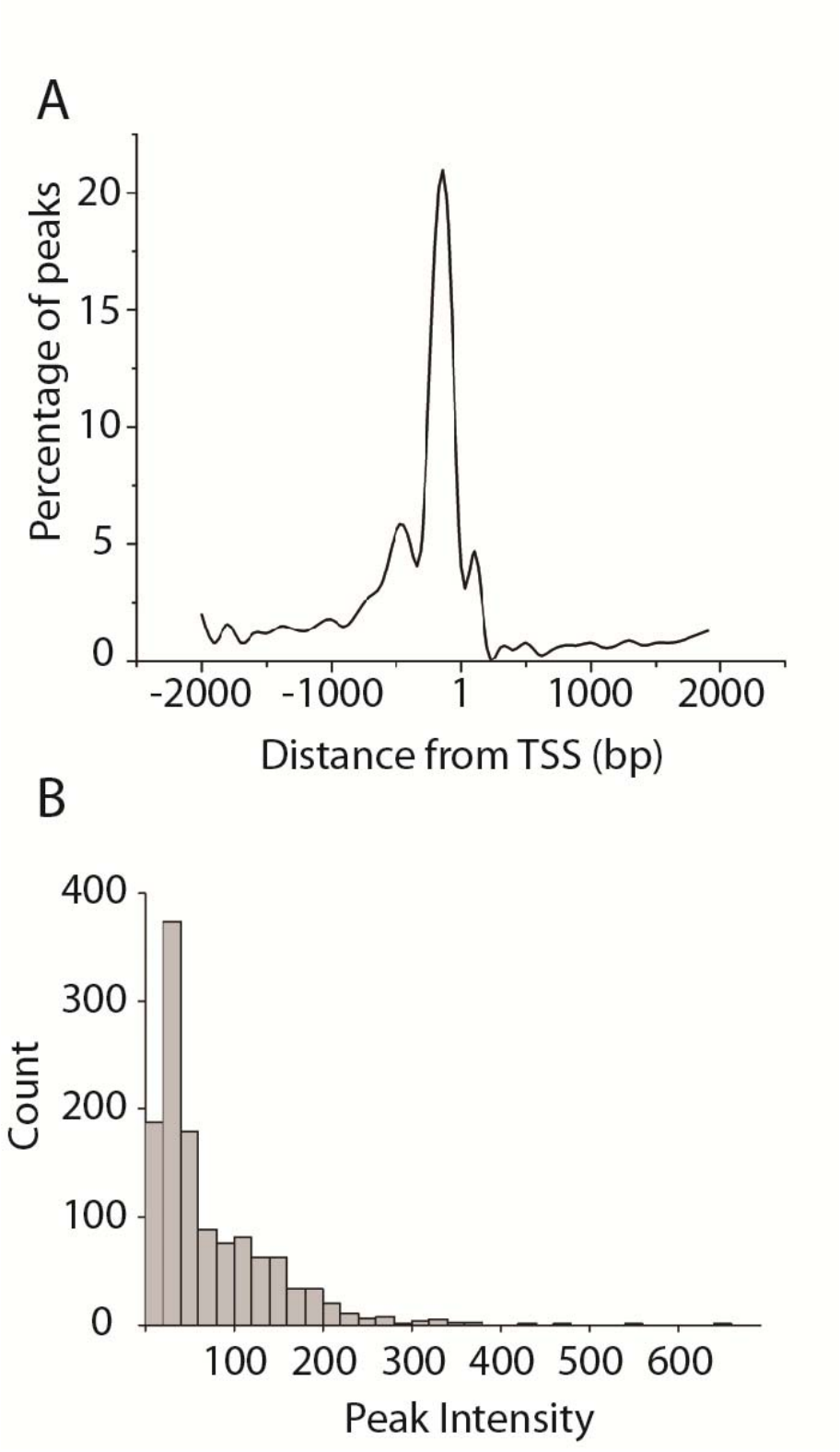

A
Percentage of peaks
20
15
10
5
0
-2000
-1000
1
1000
2000
Distance from TSS (bp)
B
Count
400
300
200
100
0
100
200
300
400
500
600
Peak Intensity

Figure 3.4 cont'd.

**Figure 3.4. Rbf1 exhibits a strong promoter-proximal targeting bias.** (A) The distribution of peaks relative to the nearest transcription start site (TSS). The majority of peaks are centered 205 bp 5' of the TSS. Distances were grouped into 100 bp bins and points fitted with a smooth curve. (B) Distribution of peak intensities. Most peaks had an intensity within a few fold of the average, although some peaks were >10 fold higher.

not correlated to the types of genes targeted, as genes in functionally related classes exhibited peaks of a range of intensities. As discussed below, the sequences underneath the peaks presented a very heterogeneous picture, indicating that different transcription factors may provide alternative pathways for recruiting Rbf1.

***Cell cycle and DNA replication related genes represent only a minority of bound sites***

The previous functional assessment of genes regulated by Rbf in cell culture had highlighted genes related to cell cycle and DNA replication, although a much broader spectrum of gene functionalities was indicated by analysis of genes regulated and bound by the Rbf-related MMB/dREAM complex (Dimova et al. 2003; Georlette et al. 2007). To characterize the nature of Rbf1 direct targets, we performed Gene Ontology (GO) analysis on genes associated with the highest confidence peaks using the DAVID annotation analysis system (Huang da et al. 2009). Out of 1890 Rbf1 target genes, 42% were enriched for GO terms. Rbf1 peaks were associated primarily with protein-coding genes, but 12 annotated noncoding RNA genes were also associated with the cofactor. Approximately one quarter of the genes were enriched for cell cycle and DNA replication categories, the areas that showed the most significant enrichment of all groups of genes (Figure 5 and Supplementary Table III as in Acharya *et al*, 2012). Other categories that were enriched included processes, such as chromatin modification and transcription, cellular systems, such as cytoskeleton, and developmental programs, including oogenesis and neurogenesis. A large number of smaller categories comprised 36% of the target genes. Previous studies have shown that RB family members have particular roles in distinct developmental settings, such as the role for RB in mouse trophectoderm development, vulval development in *C. elegans*, and osteoblast differentiation (Wu et al. 2003; Bender et al. 2007; Berman et al. 2008). The smaller number of non-cell cycle related genes previously found to be

# Figure 3.5

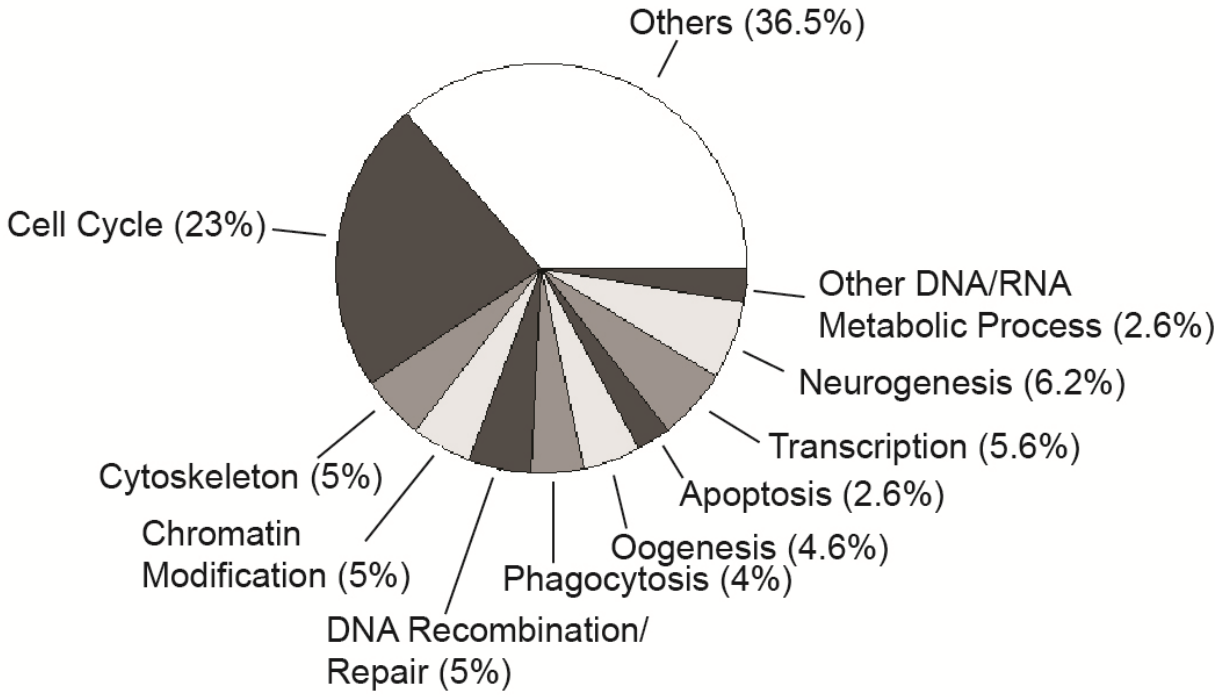



**Figure 3.5. Rbf1 target genes represent diverse Gene Ontology (GO) categories**. 42% of 1890 Rbf1 target genes were enriched for GO terms. Of these, only about a quarter were associated with Cell Cycle and DNA replication, while the majority of targets grouped into other gene regulatory and developmental processes. The GO terms are arranged in the pie chart in decreasing order of significance of enrichment from Cell Cycle and DNA replication to Other DNA/RNA Metabolic Process. "Others" indicates numerous smaller groups of enriched genes. The p-values for the categories are as follows: Cell Cycle, 7E-16, Cytoskeleton, 5E-13; Chromatin Modification, 2E-12, Recombination/Repair, 7E-09; Phagocytosis, 1E-03; Apoptosis, 5E-03; Transcription, 2E-02; Neurogenesis, 2E-02; and other DNA/RNA Metabolic Process, 4E-02.

functionally regulated by Rbf1, Rbf2 and E2F proteins indicate that perhaps in cell culture, many physiological targets of these proteins are relatively quiescent (Dimova et al. 2003). Our data suggest that taken in a developmental context, Rbf1 function may be distributed over a very wide set of diverse cellular processes.

***Enrichment of transcription factor motifs in Rbf1-bound peaks***

Rbf1 does not bind to DNA directly, but is instead recruited by transcription factors, generally of the E2F family. RB has been reported to interact with other types of transcription factors, including MyoD, NeuroD1, GATA1, and components of the RNA polymerase III basal transcriptional machinery (Gu et al. 1993; Felton-Edkins and White 2002; Batsche et al. 2005; Kadri et al. 2009). In consideration of the wide diversity of genes targeted by Rbf1, we sought to understand whether particular subsets of these genes would be characterized by distinct constellations of protein binding sites in the promoter proximal regions. We extracted sequences representing the 200 bp sequences from the center of each peak and performed *de novo* motif discovery analysis using MEME (Bailey and Elkan 1994). We sought the top five overrepresented motifs for 5 to 15-mers and noted that variations of four motifs occurred most frequently (Figure 6A). We searched the motifs in the JASPAR and TRANSFAC databases, and one of the motifs was similar to the E2F site, as expected. Another bears clear similarity to the DREF site, which is bound by the DNA Replication-related Element-binding Factor involved in expression of a wide variety of proliferation-related genes, and whose function in the context of cell cycle regulation has been investigated on the *PCNA* and *DNApol alpha* promoters (Yamaguchi et al. 1995; Takahashi et al. 1996; Seto et al. 2006). We also found a motif similar to the binding sequence of the mammalian Forkhead transcription factor FOXJ2, and one novel motif that did not closely match any other sequence in the database, which we designated RAM

**Figure 3.6**

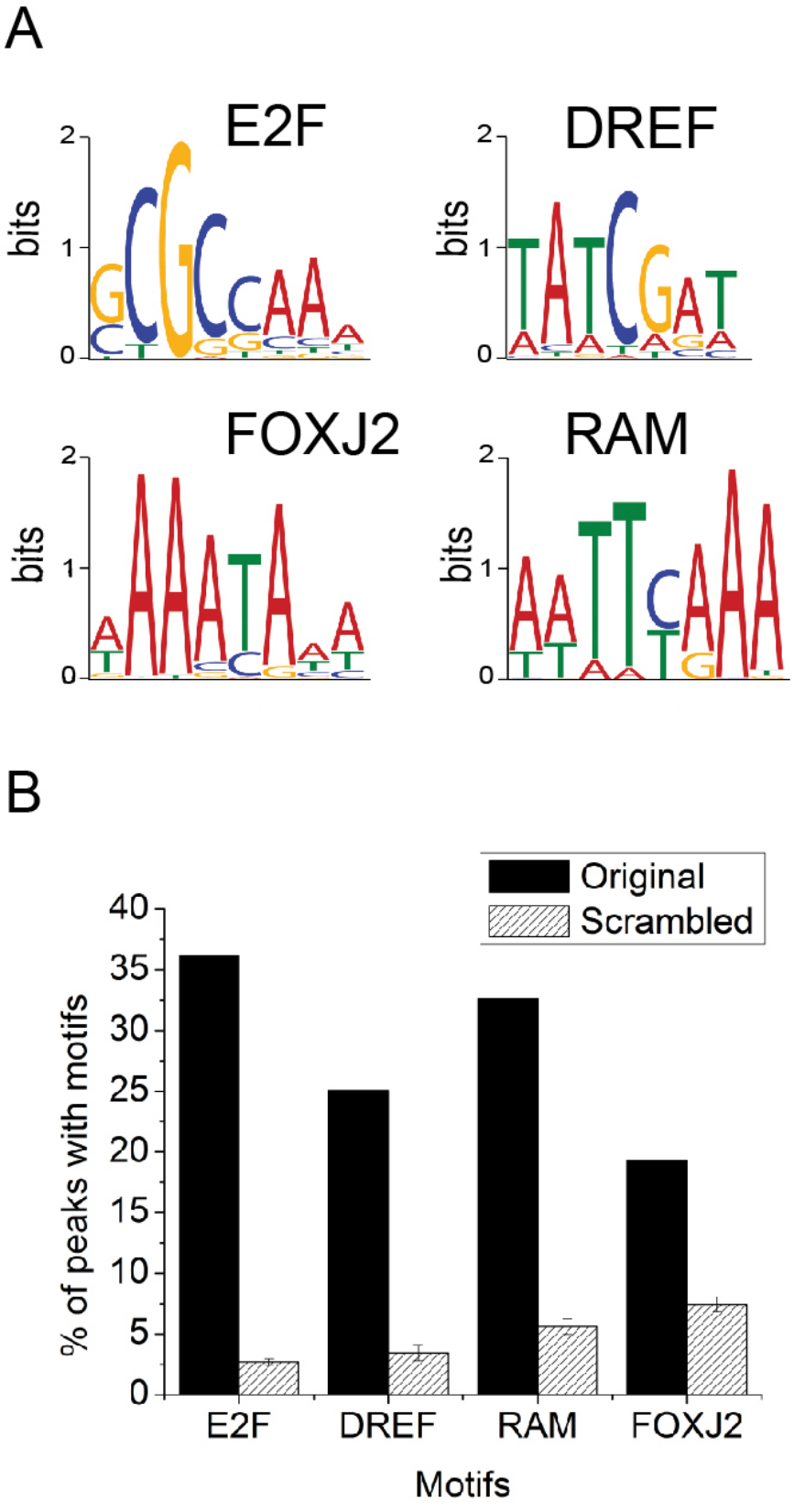
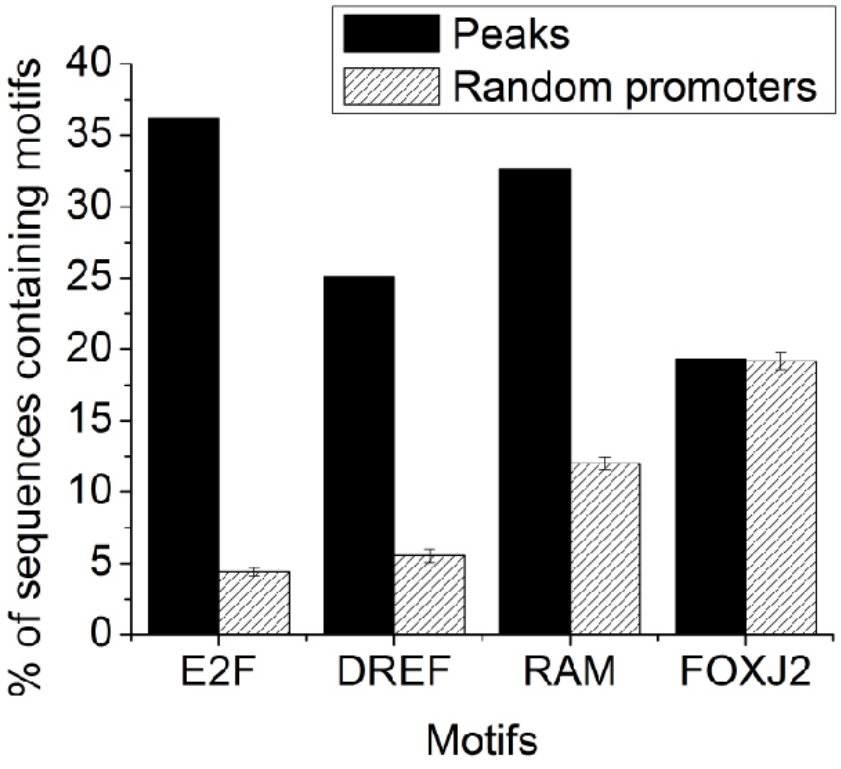

A
E2F
DREF
FOXJ2
RAM
bits
2
1
0
B
Original
Scrambled
% of peaks with motifs
40
35
30
25
20
15
10
5
0
E2F
DREF
RAM
FOXJ2
Motifs

**Figure 3.6 cont'd.**

C




D

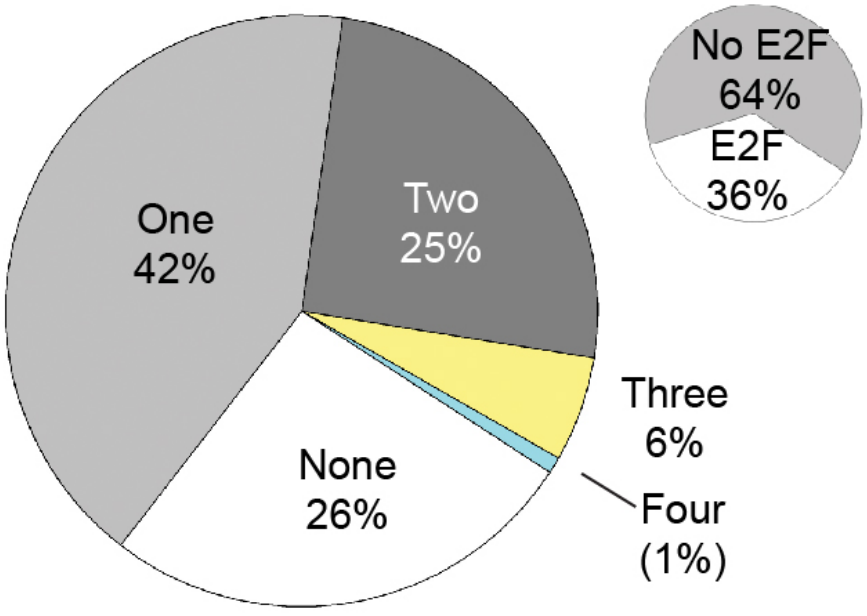
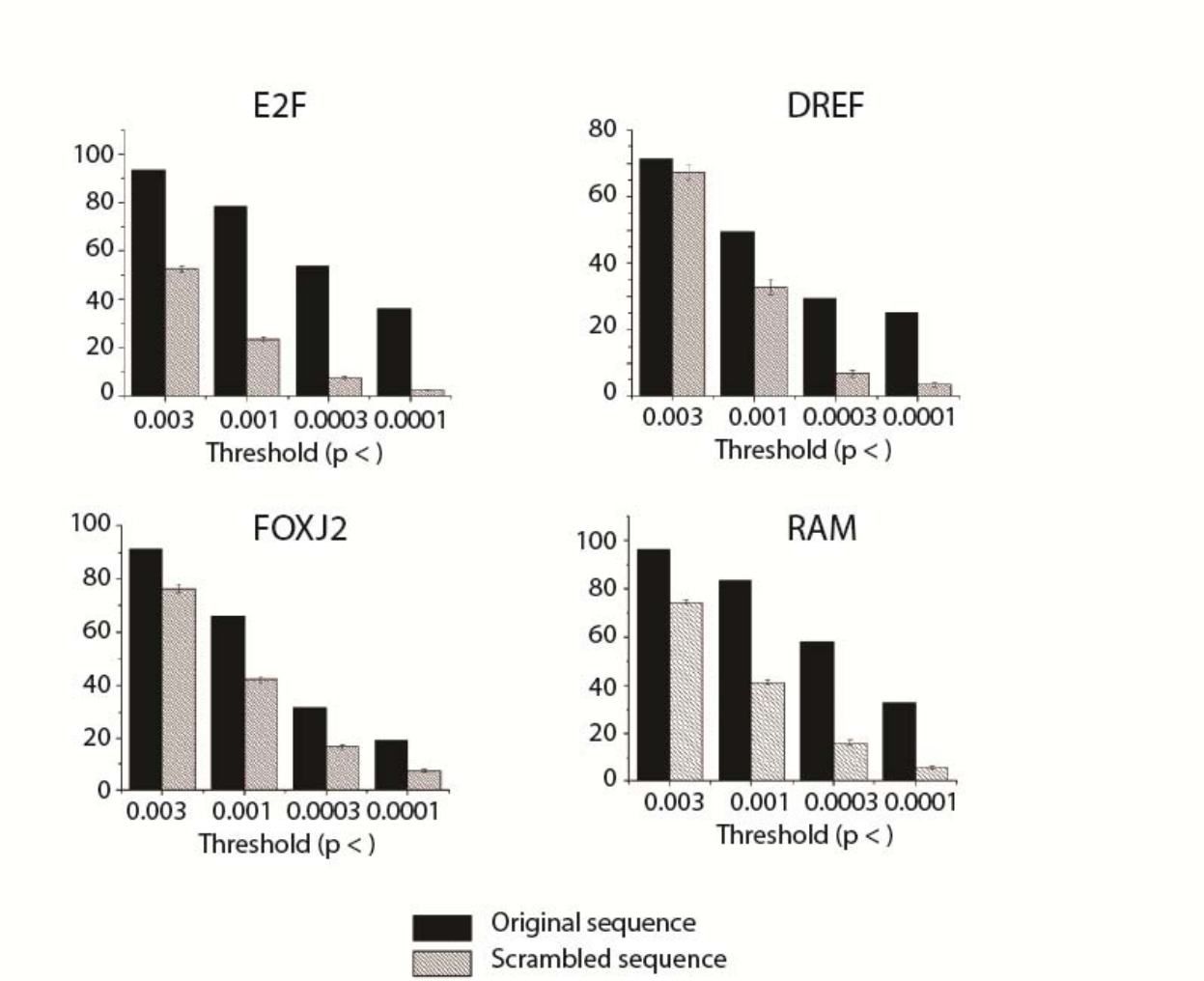


Figure 3.6 cont'd.

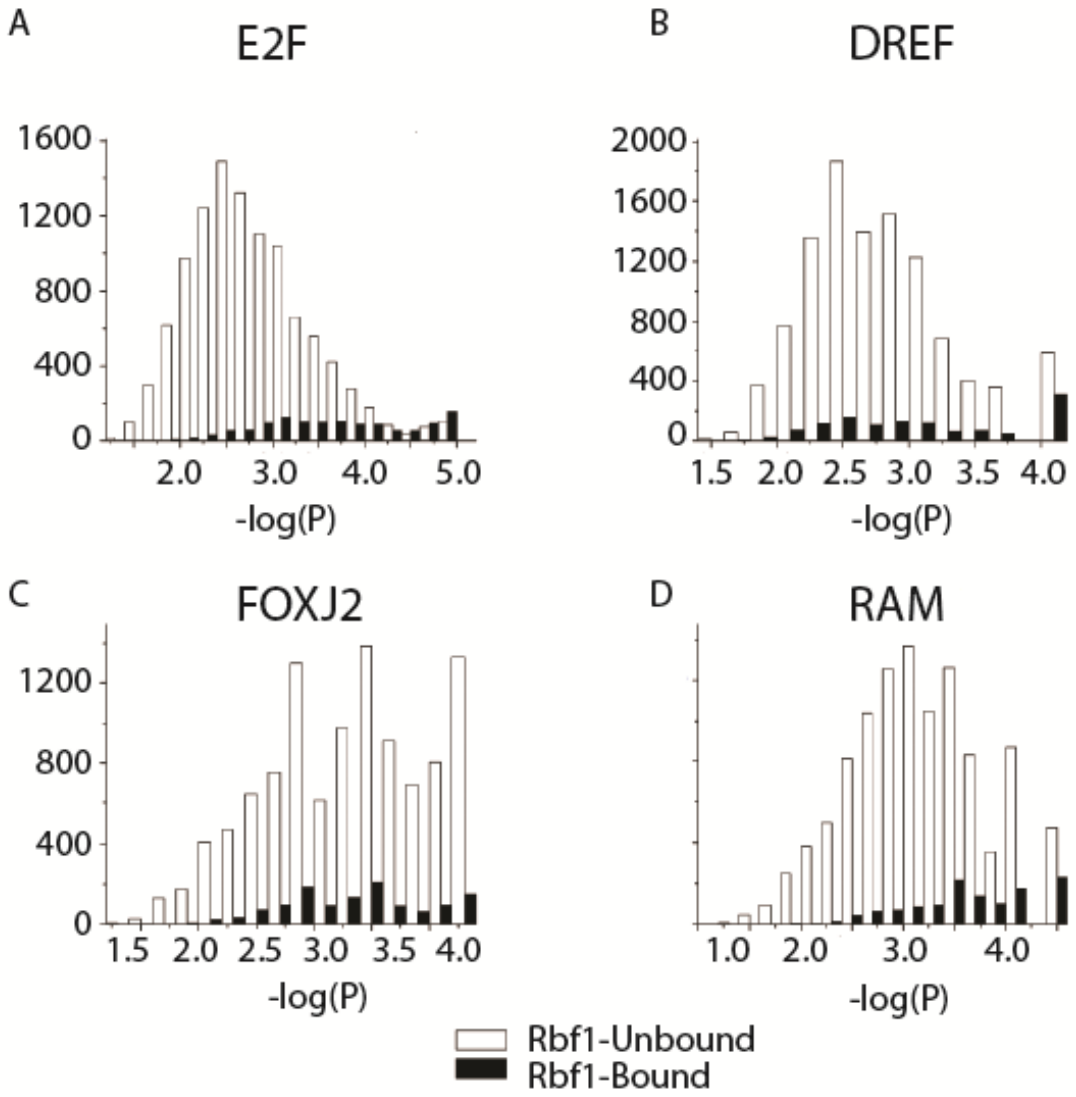
**Figure 3.6**. **Transcription factor motifs enriched in Rbf1-bound peaks.** (A) The four most overrepresented motifs identified by the MEME motif discovery tool, including one previously unknown motif (RAM). (B) Rbf1-associated motifs are highly enriched compared to average occurrence in DNA of the same A/T composition. The sequences under Rbf1 peaks were scrambled five times and specific motifs with $p < 0.0001$ were identified. E2F sites showed the highest level of enrichment in specifically bound regions compared to randomized DNA sequences. (C) E2F, DREF and RAM motifs preferentially associate with Rbf1-bound promoters. Presence of motifs in Rbf1-bound sequences was compared to presence in Rbf1-unbound promoters. FOXJ2 sites are not restricted to Rbf1-associated promoters, and may represent a motif for a broadly acting factor. Note that the canonical DREF sites are 8-mers (Yamaguchi et al. 1995). In our data the eighth nucleotide was not conserved. (D) Diversity of motif composition of peaks. 42% of total peaks contained only one of the four different motifs (E2F, DREF, FOXJ2 or RAM). A quarter of the peaks had a combination of two different motifs; 6%, a combination of three and 1% contained all four motifs. Only 36% or peaks had an identifiable E2F motif (small insert). Strikingly, a quarter of the peaks did not have any of the four motifs. The heterogeneity of sequences in Rbf1-bound peaks suggests that E2F may not be the only transcription factor that recruits Rbf1 to target gene promoters. Peaks used in this analysis were drawn from the 1236 bound regions found in both Rbf1 ChIP biological experiments. A peak with multiple E2F motifs, but no other motif types, was counted as one type of motif; a similar treatment applies for the other three motifs. "None" means the peaks did not contain any motifs for E2F, DREF, FOXJ2 or RAM. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

# Supplementary Figure 3.5




**Supplementary Figure 3.5**. **Determination of threshold for motif analysis**. To determine optimal p values to minimize false positive calls and provide reasonable sensitivity, MAST analysis was performed on sequences under peaks with different thresholds for E2F, DREF, FOXJ2 and RAM motifs (A-D). The analysis was repeated five times on sequences of identical A/T composition that had been scrambled. The threshold $p < 0.0001$ showed the highest fold enrichment, thus further analyses (in Figure 6 B, C and D) were carried out with this value.

# Supplementary Figure 3.6



Supplementary Figure 3.6 cont'd.

**Supplementary Figure 3.6**. **Prevalence of E2F-, DREF- and RAM-like motifs on Rbf-1 bound and not bound promoter regions**. Scores for each Rbf1-bound peak (black bars) and Rbf1-unbound 200 bp promoter region centered at -200 bp (white bars) obtained from MAST analysis show that Rbf1-bound promoters have better E2F, DREF and RAM motifs (in each case, Wilcoxon rank sum test $p < 2.2e-16$). However, there was no significant difference in scores for FOXJ2 motifs in Rbf1-bound or unbound promoters (Wilcoxon rank sum test $p = 0.16$). The significant enrichment of weak E2F, DREF, and RAM sites among bound genes suggests that there may be a higher fraction of Rbf1-bound promoters that utilize these proteins than indicated by the use of our stringent cutoff criteria. An alternative statistical analysis of the peaks indicated that the prevalence of these motifs in Rbf1-bound and -unbound promoters is significant (Supplementary Table IV as in Acharya *et al,* 2012).

(Rbf associated motif). The motifs occur much more frequently than they would in scrambled sequences of similar composition, indicating that these motifs have a high information value (Figure 6B). The threshold for calling each of the motifs ($p = 0.0001$) was selected to provide a high discrimination between the bound sequences and scrambled DNA of similar composition (Supplementary Figure 5). Promoters selected from those in the genome that did not exhibit Rbf1 binding were tested for frequency of these motifs; E2F, DREF, and RAM motifs were considerably less enriched on these promoters than on those bound by Rbf1, indicating that these sequences may play a role in recruiting of Rbf1, or coregulation of the associated promoters (Fig. 6C). FOXJ2 sequences were not preferentially enriched on Rbf1 bound promoters, although these sequences occur at a higher frequency than would be expected by chance, thus it is likely that these motifs are relevant to promoter function in general.

Regarding overall promoter composition, just under one half of the peaks contained at least one copy of one of the four motifs identified. A quarter of the peaks had a mixture of two of the motifs, and a small percentage had three or all four of the motifs (Fig. 6D). One-fourth lacked any of these motifs; these promoters may contain novel motifs that are not found in many genes, or they may contain low-affinity canonical sites that fell below the threshold used here (see Materials and Methods). Interestingly, of the peaks that contained recognizable motifs, only about a third contained the E2F motif, although this has been presumed to be the chief route by which Rbf proteins are recruited to promoters (Figure 6D insert). There may be other factors involved in recruiting Rbf1, or low-affinity E2F sites may be important on some genes. Indeed, Rbf1-bound regions as a group tend to be enriched in E2F-like sites (Supplementary Figure 6).

***Enrichment of motifs in different promoter subclasses***

In light of the diverse cellular processes represented among the targets of Rbf1, we studied whether different classes of genes exhibited distinct promoter composition (Fig. 7). Sorting genes by GO category, we noted that genes involved in Phagocytosis, Chromatin Modification and Cell Cycle were among those most highly enriched in E2F motifs, while Neurogenesis and Oogenesis GO categories are depleted of these motifs. The novel RAM motif co-occurred frequently with E2F sites, except for certain GO categories such as Chromatin Modification while DREF sites, which had been previously shown to help regulate cell cycle-related genes such as *PCNA* and *DNApol alpha*, were not overall enriched on this class of gene, instead showing strong association with genes involved in Apoptosis. FOXJ2 was also differentially distributed, showing some correlation with RAM sites, but it was not highly enriched in any subcategory (Fig. 7A). The overall impression obtained from this analysis is that Rbf1-bound regions vary strongly in their average composition; it is likely that functional classes of genes are coordinately regulated by unique combinations of factors that interact with these motifs. Factors responsible for Rbf1 recruiting may vary as well, possibly placing some Rbf1-bound promoters out of reach of the canonical cell-cycle regulatory pathways.

We also analyzed the association of motifs with groups of genes drawn from our set of Rbf1-associated promoters that are defined by other properties, rather than the GO categories identified by DAVID. The separate categories analyzed included a set of genes affected by RNAi knockdown of Rbf1, Rbf2, E2F1, and E2F2 in S2 cells (Dimova et al. 2003), genes misexpressed by knockdown of the l(3)mbt malignant brain tumor protein that interacts with Rbf-containing complexes (Janic et al. 2010), functional targets of the MMB/dREAM complex identified by knockdown in Kc cells, physical targets of the Rbf1-associated MMB/dREAM complex

# Figure 3.7

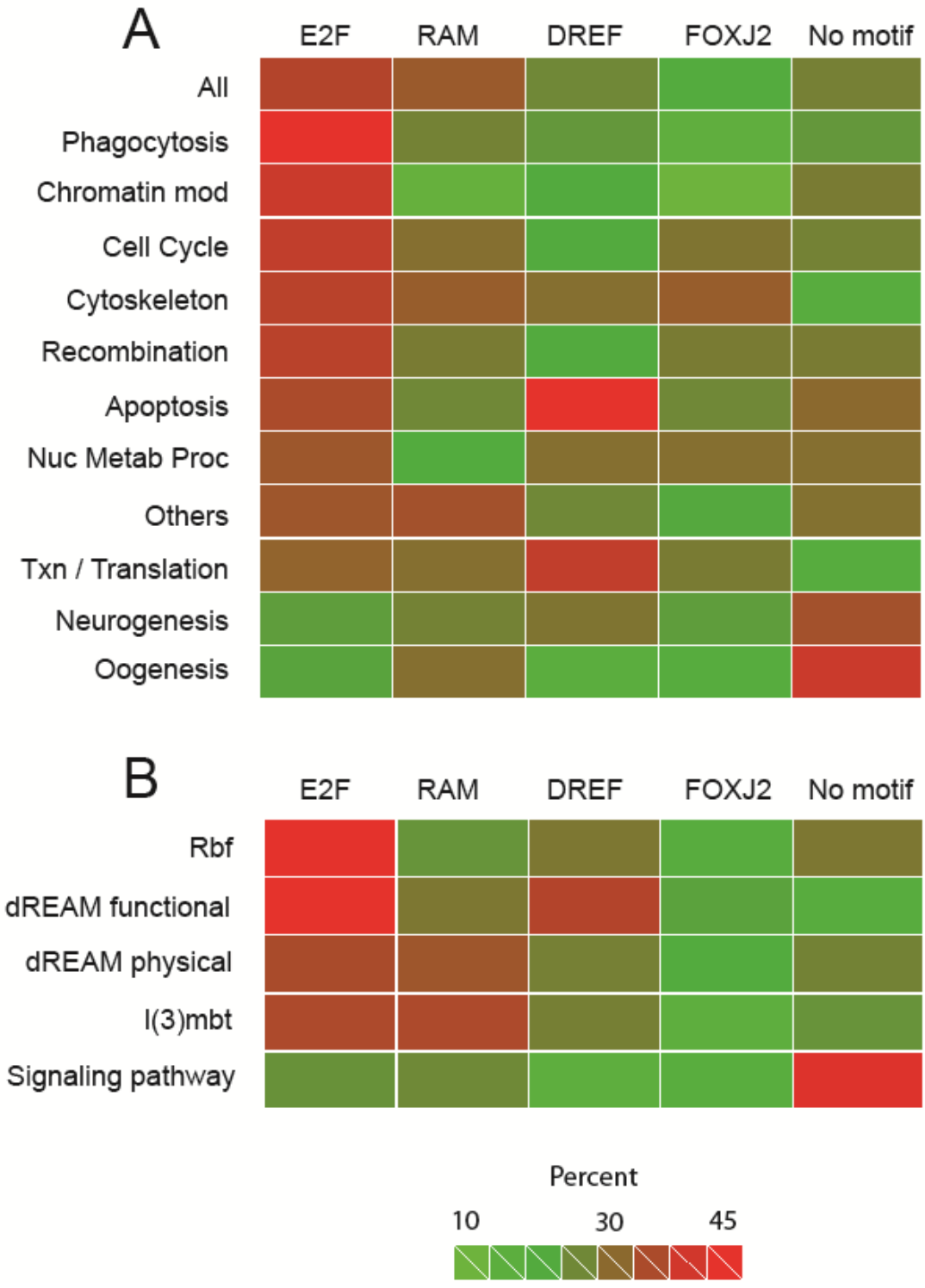

A
E2F
RAM
DREF
FOXJ2
No motif
All
Phagocytosis
Chromatin mod
Cell Cycle
Cytoskeleton
Recombination
Apoptosis
Nuc Metab Proc
Others
Txn / Translation
Neurogenesis
Oogenesis
B
E2F
RAM
DREF
FOXJ2
No motif
Rbf
dREAM functional
dREAM physical
l(3)mbt
Signaling pathway
Percent
10
30
45

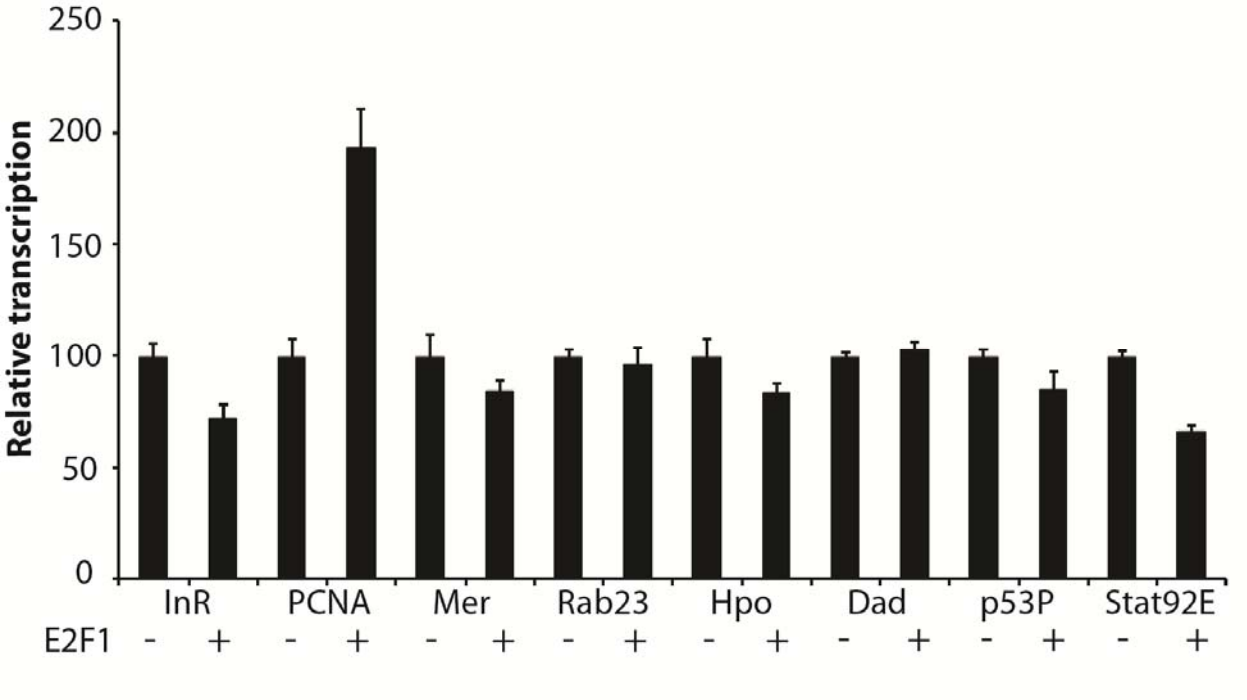
Figure 3.7 cont'd.

**Figure 3.7**. **Enrichment of Rbf1-associated motifs indicates distinct promoter subclasses in the Rbf1 regulon**. (A) Heat map for association of motifs with different GO categories. E2F sites were present in a significant fraction of bound regions as a whole, especially in GO categories Phagocytosis, Chromatin Modification, and Cell Cycle. Genes involved in Neurogenesis and Oogenesis tend to be depleted of E2F motifs. RAM sites tend to occur on a subset of E2F-containing sequences, but Chromatin Modification and Nucleotide Metabolic Process genes are depleted of RAM motifs. Apoptosis and Transcription/Translation genes are associated with DREF motifs. Chromatin Modification, Phagocytosis and Oogenesis genes are depleted of FOXJ2 motifs, while a larger fraction of Neurogenesis and Oogenesis genes lack all of the four motifs. (B) Heat map for association of motifs with selected functional and physical targets of Rbf1 including functional targets of Rbf in S2 cells (Rbf); functional and physical targets of the Rbf- and Myb- containing dREAM complex; functional targets of l(3)mbt, a corepressor and a binding partner of Rbf; and physical targets of Rbf1 identified in this study that are involved in signaling pathways. The E2F motif alone is preferentially associated with Rbf1 functional targets in S2 cells (Rbf) and dREAM functional targets, however, a combination of E2F and RAM sites is found preferentially on l(3)mbt functional and dREAM physical targets. Signaling pathway Rbf1 target genes are depleted of all four motifs, suggesting a distinct promoter signature. "Percent" indicates fraction of genes in a selected GO category containing at least one occurrence of the indicated motif within the Rbf1 peak. The category "all" represents all 1236 peaks present in both ChIP replicates. The category "other" represents small clusters of genes found to be overrepresented in numerous GO categories (see Fig. 5). "Signaling pathway" represents 136 genes found in diverse conserved Drosophila signaling networks.

Figure 3.7 cont'd.

For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

# Supplementary Figure 3.7




**Supplementary Figure 3.7**. **E2F responsiveness of promoters of selected genes in signaling pathways**. *Drosophila* S2 cells were cotransfected with *InR, PCNA, Merlin, Rab23, Hippo, Dad, p53-proximal,* or *Stat92E* luciferase reporters, with (+) or without (-) a plasmid overexpressing E2F1. Only *PCNA* luciferase expression was elevated by E2F1.

components identified by ChIP (Georlette et al. 2007), and the set of genes that we identified in this study that are components of conserved signaling pathways (Fig. 7B). Grouping genes in these five (non-exclusive) categories, we found that the E2F motif is especially enriched for functional targets of Rbf1 and MMB/dREAM identified in S2 cells. As noted above, the motif is associated with cell cycle-related genes, which would be expected to be expressed in these mitotically active cells. The E2F motif is also associated with phagocytosis-related genes, which may also be preferentially active in the S2 cell line, which has properties of hemocytes (insect macrophages). E2F sites were less enriched, and RAM sites were more enriched, on physical targets of MMB/dREAM and genes misexpressed in l(3)mbt brain tumor samples; these genes may represent developmentally regulated targets of Rbf1, as opposed to genes that are tightly integrated into regular mitotic control pathways (Lee et al. 2010). In the fifth group, genes involved in conserved signaling pathways, we noted that Rbf1-bound regions were depleted of all four motifs, suggesting a distinct promoter signature for these genes. Further bioinformatic analysis of the group as a whole did not find enrichment of new motifs, although weak E2F sites were found (data not shown).

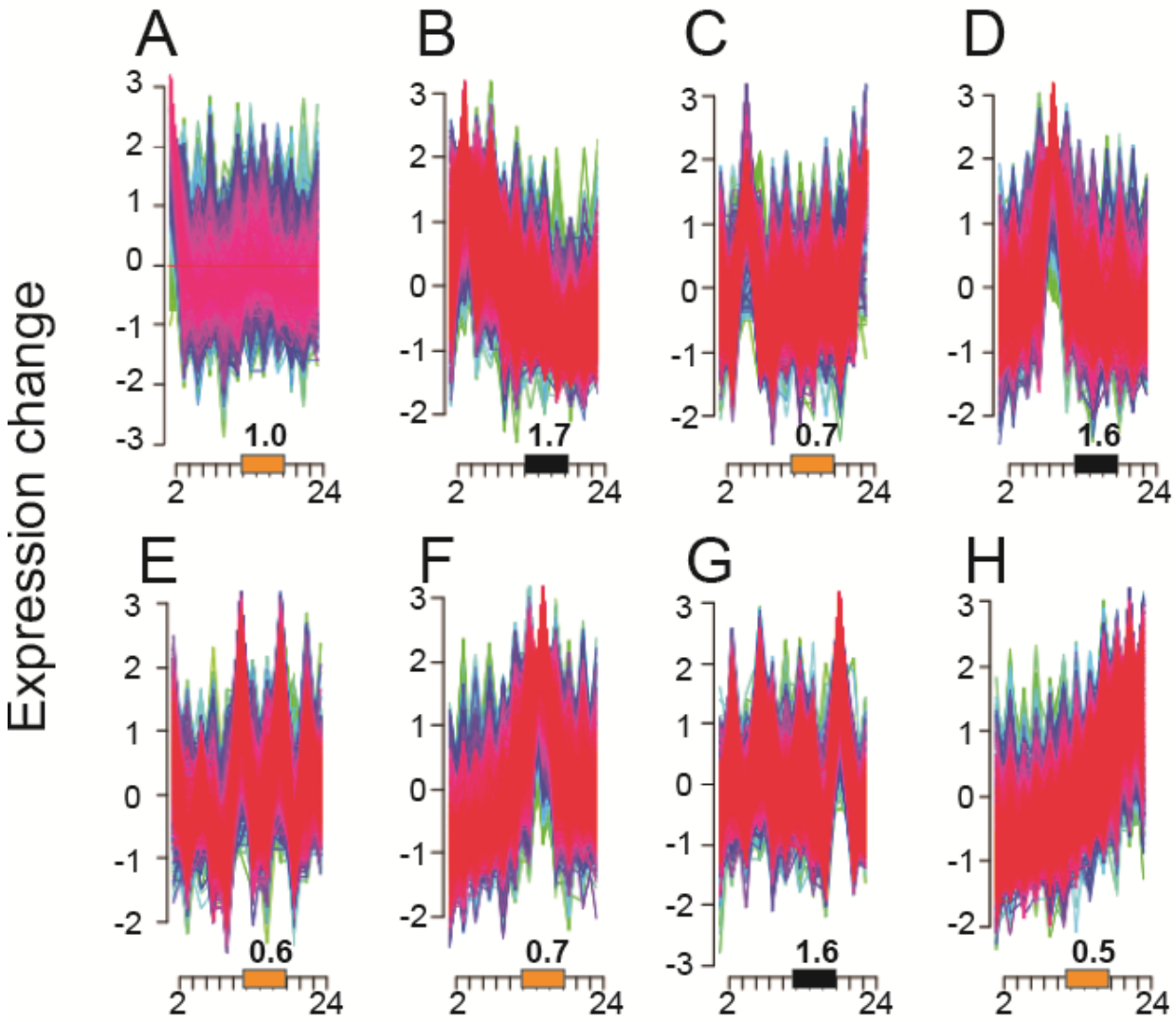
E2F proteins have been observed to bind diverse sequences *in vivo* (Bieda et al. 2006). To directly test the possibility that some of the promoters with weak or nonexistent E2F sites may nonetheless interact with this transcription factor, we overexpressed E2F1 and measured its ability to activate a diverse panel of signaling pathway gene promoters. E2F1 strongly activated the *PCNA* promoter as expected, however, none of the signaling pathway gene promoters tested were activated by E2F1, indicating that these promoters are E2F-independent (Supplementary Figure 7). Some promoters such as *InR* were slightly repressed, possibly because E2F1 may drive expression of *rbf1* gene itself. Taken together, we see evidence that Rbf1 binding takes

place in the context of a rich diversity of motifs among the different categories of genes targeted, suggesting that regulation of promoters by this corepressor may involve separate regulatory programs, consistent with recent studies (Dimova et al. 2003; Kirienko and Fay 2007; Lee et al. 2010).

***Correlation of Rbf1-target gene expression and Rbf1-binding***

The RB protein is generally thought to mediate transcriptional repression, but mutant studies have also identified genes that are downregulated upon loss of the protein (Dimova et al. 2003; Georlette et al. 2007; Kirienko and Fay 2007; Flowers et al. 2010). We therefore examined whether targeted promoters tend to show a loss of activity at the timepoint when we see peak Rbf1 binding, consistent with corepressor activity. Using recently described transcriptome data, from ModENCODE, we grouped embryonic expression of genes into eight distinct profiles using fuzzy c-means clustering, a method that identifies genes with the most similar patterns of change (Figure 8A-H and Supplementary Table VI as in Acharya *et al*, 2012). 54% of the genes bound by Rbf1 were enriched in three of these clusters, which all show downregulation of expression during the 12-18 hour time period for which the Rbf1 data was collected. To test whether this enrichment in certain clusters is significant, we randomly selected 2000 genes not targeted by Rbf1 and determined whether there was significant enrichment in any of the eight clusters. No significant enrichment was found for any of the five repetitions of this procedure (data not shown). The composition of genes in these three clusters showed a strong bias toward cell cycle-related genes (Fig. 8I), indicating that on these targets Rbf1 is likely to play a common repressive role. Thus, at least for a large fraction of targeted genes, Rbf1 is implicated in transcriptional repression.

# Figure 3.8


A
B
C
D
E
F
G
H
Expression change
3
2
1
0
-1
-2
-3
1.0
1.7
0.7
1.6
0.6
0.7
1.6
0.5
2
24

# Figure 3.8 cont'd.

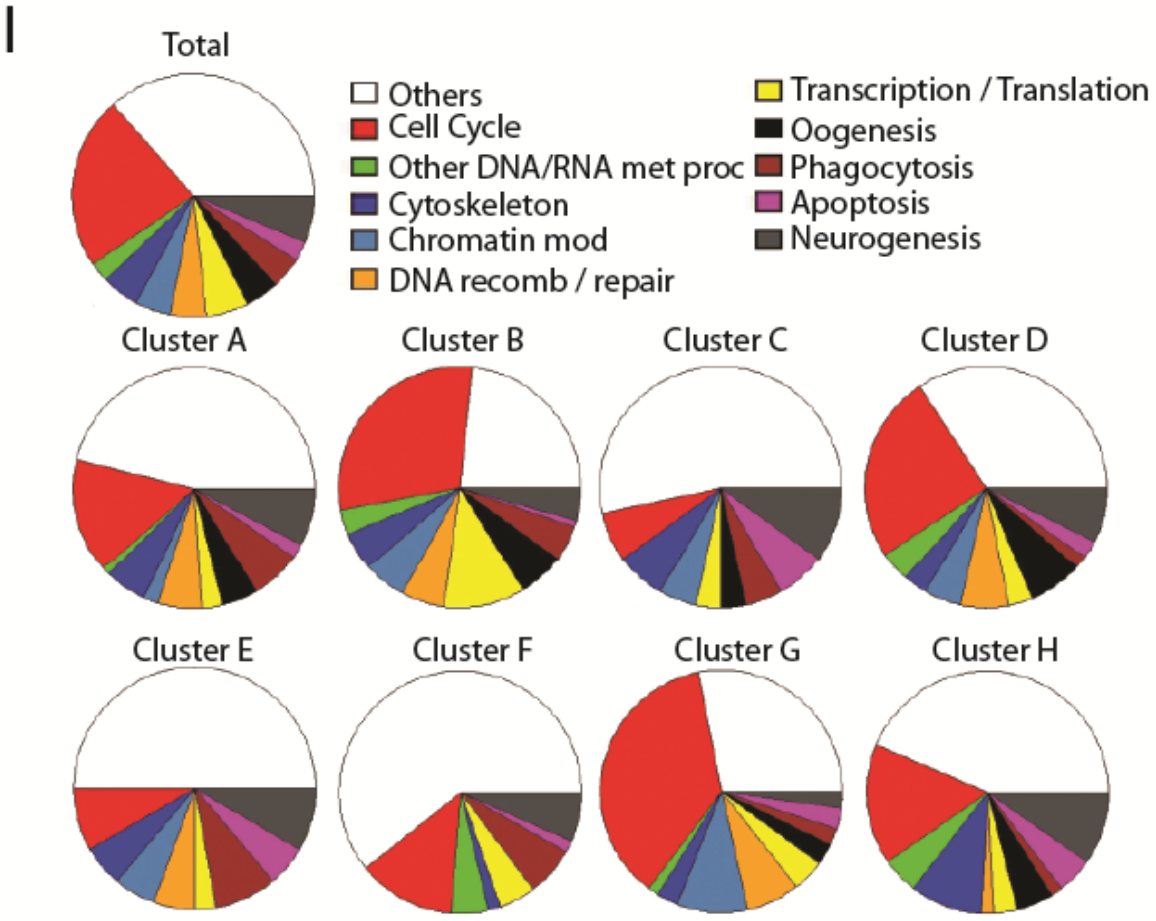


Figure 3.8 cont'd.

**Figure 3.8**. **Promoter occupancy by Rbf1 correlates with downregulation of target genes**. A-H show the expression patterns of all embryonic genes clustered into eight groups by similarity of expression. The Y-axis represents standard Z score (see Materials and methods) for the expression change. The X-axis represents age of the embryos in hours. The boxes indicate the 12 to 18 hr time window that was used for ChIP-seq. The enrichment of Rbf1 targets is indicated by numbers above the boxes. The Rbf1 target genes are enriched in clusters B, D and G, which comprise of 54% of the targets, and 33% of total genes. Genes in clusters B, D and G tend to be downregulated between 12 to 18 hours, consistent with a repressive role for Rbf1. The RNA-seq data for 0 to 24 hour embryos (in two hour windows) was obtained from modENCODE, converted to relative changes in expression, and grouped using fuzzy c-means clustering. Clusters are arranged according to timing of increased expression from early (A) through late (H). Distribution of GO categories of Rbf1 targets in clusters A-H are shown in I. Statistics for each cluster is shown in Supplementary Table VI (as in Acharya *et al* 2012). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Interestingly, some genes bound by Rbf1 (in particular cluster F) show upregulation during the 12 to 18 hr time window. The composition of these genes showed a strong bias away from cell cycle-related genes and are enriched in the highly heterogenous "other" GO category (Fig. 8I). This increase in expression may occur despite Rbf1 promoter occupancy because of the heterogeneity of cells in the embryos, so that only a fraction of nuclei have Rbf1 occupancy. Alternatively, genes that are highly active may be also be intermittently occupied by Rbf1, or Rbf1 may have true activator function in certain settings (Flowers et al. 2010).

***Divergence and Conservation of Rbf1 regulon***

To determine whether the genomic targets of the Drosophila Rbf1 protein represent deeply conserved regulatory interactions, we compared human orthologs of the Rbf1-occupied genes with those bound by human RB and p130 proteins in fibroblasts (Chicas et al. 2010). The 1890 Drosophila genes identified in our study correspond to 2310 human orthologs. We compared these genes to those bound by RB or p130 in growing, quiescent, or senescent fibroblasts (Figure 9). Close to half of the orthologs were identified as RB targets under at least one condition, while just over 60% of the orthologous genes were bound by p130 (Fig. 9A-E). Among the genes bound by RB or p130, the GO categories DNA Replication, Cell Cycle, DNA Damage/Repair and Chromatin Modification were under all conditions enriched. The GO category Cytoskeleton, which was found to be enriched in the Rbf1 targets in the Drosophila embryo, was actually depleted from genes bound by RB in growing and quiescent cells, however, it was enriched in genes bound by RB in senescent cells, as well as p130. This result indicates that certain categories of genes can be selectively occupied depending on the state of the cells, underscoring the differential regulation of subsets of RB/p130 targets. Finally, GO categories such as Oogenesis, Phagocytosis, and Neurogenesis that were overrepresented among

Drosophila targets were slightly or not at all enriched in the set of human genes. The common binding of genes involved in chromatin modification, cell cycle, DNA repair and replication by RB family members suggests that they represent deeply conserved functions of this family of proteins. Other categories of genes may represent lineage-specific innovations or tissue-specific binding interactions that are not present in the cell culture system.

The intriguing targeting of many conserved signaling genes by Rbf1 in the Drosophila embryo let us examine whether RB/p130 show similar binding preferences. The vast majority (106/137) of signaling components bound in the fly were also found to have RB and/or p130 at the promoter in human fibroblasts. These corepressors were found at 111 additional signaling pathway genes that lacked Rbf1 occupancy in the embryo (Supplementary Table IX as in Acharya *et al*, 2012). The high proportion of conserved signaling pathway genes targeted by RB family proteins suggests that this proposed regulatory connection may represent an essential link between multiple cellular components of growth control and differentiation.

# Figure 3.9

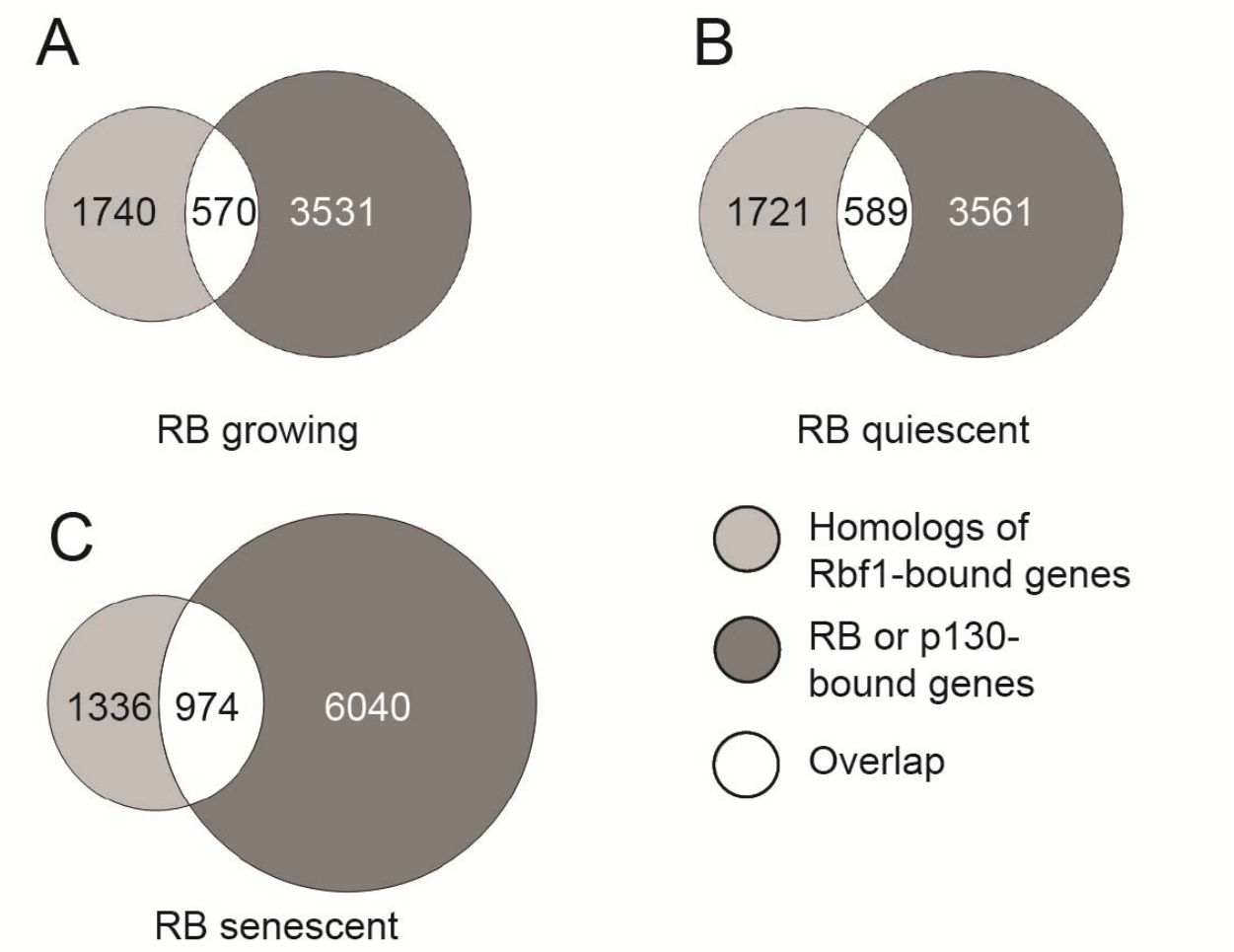
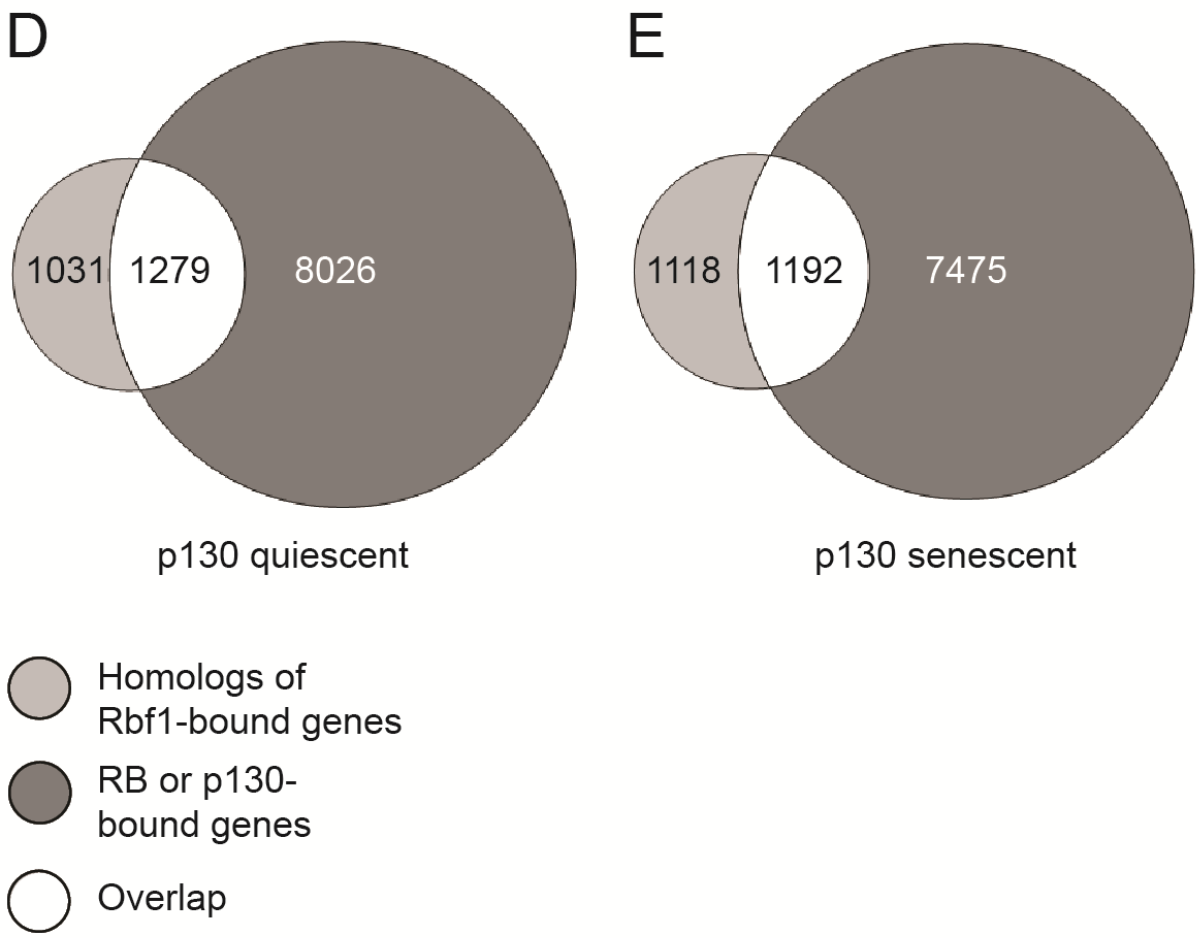

A
1740
570
3531
RB growing
B
1721
589
3561
RB quiescent
C
1336
974
6040
RB senescent
Homologs of Rbf1-bound genes
RB or p130-bound genes
Overlap

**Figure 3.9 cont'd.**



# Figure 3.9 cont'd.

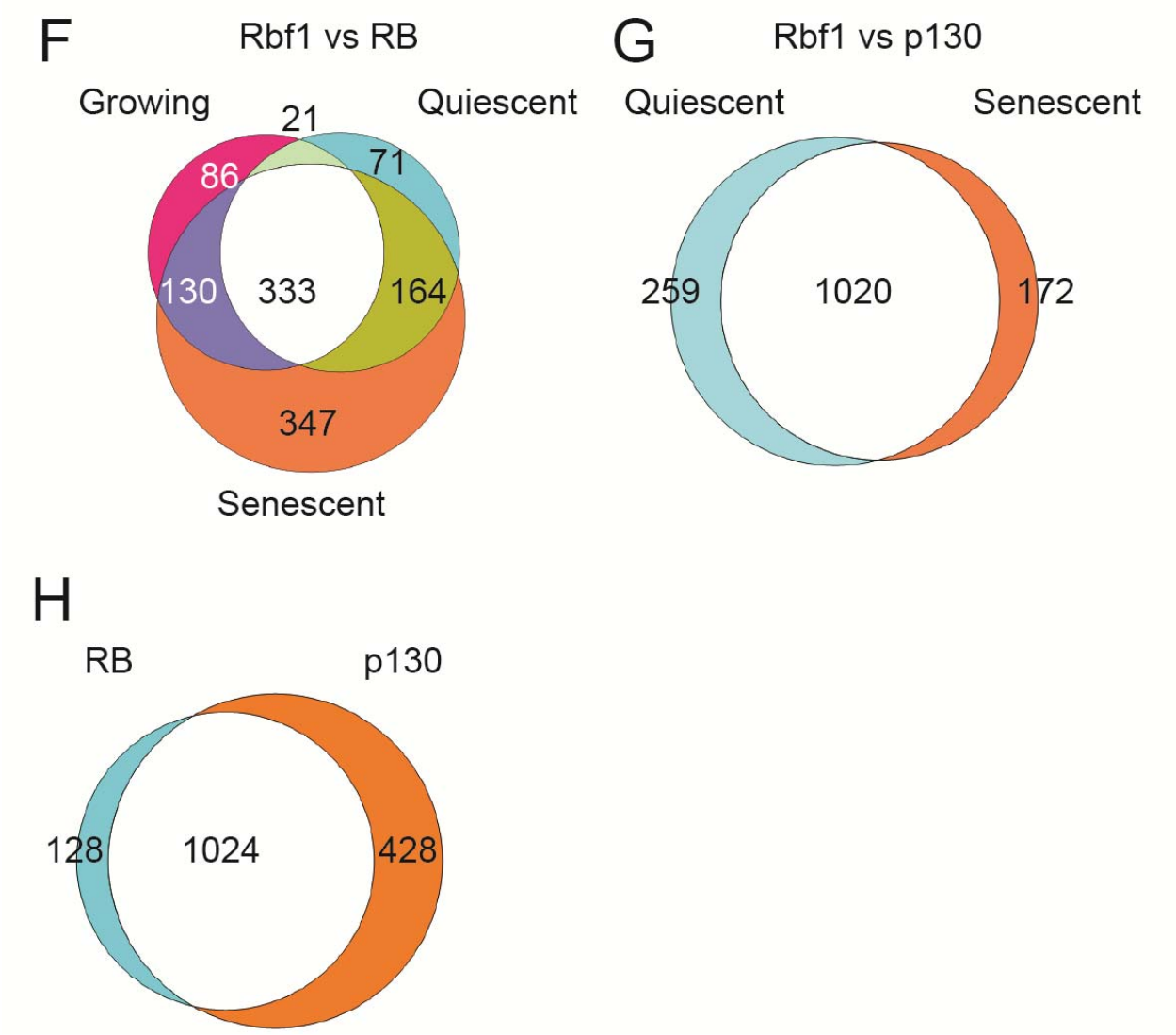


Figure 3.9 cont'd.

**Figure 3.9**. **Divergence and conservation of RB regulon**. 2310 identifiable human orthologs of Rbf1 targets were compared with RB targets in growing (A), quiescent (B) and senescent (C) cells and p130 targets in quiescent (D) and senescent (E) cells. The overlaps in (A), (B) and (C) were further compared with each other (F) and the overlaps in (D) and (E) were compared with each other (G). Comparison of the total genes in (F) and (G) with each other (H), shows that most of the targets of Rbf1 and RB, as well as Rbf1 and p130, are the same. In all overlaps in A-E, GO terms DNA Replication, Cell Cycle, DNA Repair, and Chromatin Modification were enriched, indicating that these genes may form a conserved ancient regulon of RB proteins. The overlap of human homologs of Rbf1 targets in *Drosophila* embryos and RB and p130 targets in human cell culture suggests that many genes have retained regulation by RB proteins since divergence of these organisms. Other categories of genes may represent divergence of RB family function, or context-dependent differences in binding. Human homologs for Rbf1 targets were compared with published RB and p130 targets in growing, quiescent and senescent human lung fibroblasts (Chicas et al. 2010). For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

**Discussion**

Our analysis of genomic occupancy of Rbf1, the major retinoblastoma protein in the Drosophila embryo, provides intriguing new pictures of activities of this conserved corepressor. Previous genetic studies of this factor showed that the protein is not required in early embryogenesis, despite the presence of this protein (Du and Dyson 1999; Stevaux et al. 2002; Keller et al. 2005). Consistent with this picture, our temporal analysis indicates that there is a widespread, perhaps universal regulation of Rbf1 binding during this period of development, limiting access to promoter regions. Although phosphorylation of RB proteins is a well-studied pathway that regulates contact of the corepressor with E2F proteins, there is no evidence that RB proteins from early embryos show a preferentially hyperphosphorylated, slower-migrating form, thus additional forms of regulation may be important for this developmentally controlled binding (Stevaux et al. 2002; Keller et al. 2005).

Our study identified 1890 promoters that are bound by Rbf1; this is an order of magnitude higher than the number of genes identified as functional targets of Rbf/E2F factors in RNAi experiments conducted on cultured S2 cells (Dimova et al. 2003). A large majority of the genes misregulated in S2 cells after RNAi knockdown of Rbf1 were bound by the corepressor in the embryo, indicating that many of these genes are indeed direct Rbf1 targets, but the question remains how to interpret the other identified binding events. Some may represent fortuitous associations that do not materially contribute to gene regulation, as has been suggested for some ion change data for each gene. We chose a fuzzy c-(Georlette et al. 2007; Li et al. 2008), the tight promoter localization puts the corepressor in a position that is very likely to influence basal promoter activity and indeed we show evidence that at least some of these promoters can be repressed by Rbf1. We favor the idea that only a fraction of the genes that we identified are

affected in cell culture because S2 cells do not represent the complex mixture of differentiated tissues that we sampled in the embryo. Activation of some of these genes may require co-stimulatory signals that are lacking in the cell culture system. Alternatively, the RNAi knockdown may have not been extensive enough to uncover the true scope of Rbf1 regulation.

One of the most surprising findings of our study is the extensive occupancy of multiple nodes of conserved signaling pathways by Rbf1 (Fig. 3 and Supplementary Table II). This aspect highlights one feature of RB biology that this ChIP-seq analysis in developing embryos has brought to the fore. Previous studies that examined the regulons or direct physical targets of RB proteins have not emphasized this striking aspect of the system (Dimova et al. 2003; Georlette et al. 2007; Kirienko and Fay 2007; Ferrari et al. 2008; Chicas et al. 2010), which may be partially due to the heavy reliance on cell culture systems for this information; certain promoters may only be bound in a developmental context. However, this aspect of RB biology may also have been overlooked in part because GO categories do not specifically identify individual signaling pathway genes. We reanalyzed the genes identified as RB and p130 targets in human fibroblasts and found that over 200 signaling pathway genes are bound in these cells (Chicas et al. 2010 and data not shown). An additional feature of the human cell data set is that RB and p130 in cultured cells appear to occupy a higher percentage of total promoters than does Rbf1 in the *Drosophila* embryo, which tends to obscure the enrichment of any particular set of genes.

There is abundant evidence linking RB and conserved signaling pathways. The Hippo growth control pathway has been recently found to control Rbf1 activity itself, suggesting that there are homeostatic feedback loops regulating Rbf and Hippo levels (Nicolay et al. 2011; Tschop et al. 2011). Previous functional studies linked RB regulation to individual components of the insulin signaling and S6 kinase pathways in mammals and plants (Hsieh et al. 2008;

Annicotte et al. 2009; Mercader et al. 2009; Henriques et al. 2010). Recent studies have also highlighted the functional interaction of RB regulatory pathways with insulin signaling; (Hsieh et al. 2008; Mercader et al. 2009; Henriques et al. 2010). The direct targeting of signaling component gene promoters by RB family members may provide one means for a molecular linkage of these conserved pathways.

For example, the integration of RB and insulin signaling would provide a means by which the sensitivity of this pathway would be controlled through differential expression of the insulin receptor, downstream kinases, and targets such as 4E-BP, a regulator of translation. Occupancy of target genes in the Wnt, Hh, EGFR, JNK, TGF-β, PI3K/Akt, insulin, AMPK, Notch, Hippo, JAK/STAT, NF-κB, and TOR pathways indicates that Rbf1 has the potential to exert broad and concerted regulation of multiple signaling systems. Of the ~300 genes that we identified as core constituents of these pathways in Drosophila, about 46% exhibit significant promoter-proximal signals for Rbf1 occupancy, more than twice the frequency for genes at large. It is possible that Rbf1 controls all promoters involved in signaling pathways in a unified manner, or that some promoters are especially sensitive to the levels/activity of Rbf1 protein; determining how promoters of individual components of these pathways respond to this corepressor will be a first step to quantitatively modeling the interaction of these systems. One feature of the signal transduction genes bound by Rbf1 is the relative paucity of genes encoding extracellular signaling proteins; despite rich representation of receptors and intercellular components, very few ligands involved in the signaling pathways were among the observed targets of Rbf1. Perhaps Rbf1 is involved more in setting the cellular response curves of these systems than the levels of signals impinging on a cell.

Analysis of the physical targets of Rbf1 point to a richer suite of regulatory mechanisms for this protein's output than has been previously indicated. Much attention has been focused on the role of reversible phosphorylation in regulation of RB activity, and other posttranslational forms of RB protein family regulation are well known, including proteolysis, methylation, and acetylation that control abundance, binding of regulatory factors, and nuclear localization. Our genomic analysis indicates that regulation of the RB pathway may extend to five levels of a functional hierarchy: through transcriptional control of cyclin and Hippo kinases that modify the protein and its function, through direct regulation of its own promoter, through regulation of proteins that work together with Rbf1 in some contexts to effect repression (MMB/dREAM and l(3)mbt), through regulation of downstream kinases that control meiotic and mitotic cell cycle, and lastly through regulation of the levels of proteins that can functionally antagonize Rbf1 repression, namely the E(bx)/NURF301 chromatin remodeling factor. These types of regulatory linkages are unlikely to be restricted to Drosophila, thus the picture that emerges of RB pathways is one of tightly interwoven connections, where transcriptional links mediated through this family of proteins are likely to play important roles in adjusting the set points of numerous signaling pathways.

The almost exclusive genomic binding of Rbf1 very close to transcriptional start sites indicates that Rbf1 associates with genes in a very different sort of way compared to the binding of other transcriptional cofactors. Groucho and CtBP corepressor proteins are very broadly distributed, with no predisposition to localize to the transcriptional start sites (modENCODE). In contrast, most of the E2F2-containing MMB/dREAM components are tightly linked with basal promoters (Georlette et al. 2007). We hypothesize that E2F proteins may be short-range activators that only function when bound close to the basal promoter, similar to Sp1 activator

proteins. To antagonize them, Rbf1 would be colocalized to these regions. However, many of the Rbf1-associated promoter regions that we identified lack high-affinity E2F sites, thus other short-range activators may also be involved. Alternatively, it is possible that the entire Rbf1 regulon consists of genes with compact promoter structures that do not use distantly-acting cis regulatory elements. Interestingly, in reanalyzing the data developed in Chicas *et al.* we note that about 70% of RB and p130 binding interactions are found within 1kb of the transcriptional start sites of genes, suggesting that promoter proximity is a conserved feature of the RB family of corepressors (Chicas et al. 2010).

Our bioinformatic analysis of the Rbf1-bound regions clearly indicates that the regions occupied by Rbf1 are heterogeneous, and that certain combinations of motifs are closely associated with functionally related genes (Fig. 7). In some cases, these motifs may recruit proteins that bind adjacent to Rbf1 to provide specialized responses, similar to the way that modulatory proteins in mammals bind near E2F sites to functionally differentiate subclasses of these promoters (Jin et al. 2006; Freedman et al. 2009). The Rbf-associated MMB/dREAM complex, which contains several DNA-binding proteins, provides one example of this context: the complex binds to about 70% of the genes targeted by Rbf1 in the embryo (Supplementary Table VIII as in Acharya *et al* 2012) (Georlette et al. 2007). Interestingly, few of these genes are involved in signaling pathways, suggesting that alternative Rbf1-containing complexes form on these promoters. An additional feature of the signaling pathway genes is their lack of high-affinity E2F motifs, or sequences resembling the other three overrepresented motifs found on the rest of the Rbf1 targets, suggesting that these genes may recruit Rbf1 by interactions with novel transcription factors. Alternatively, E2F may interact with these promoters via non-canonical sites (Bieda et al. 2006; Xu et al. 2007). Our functional testing for E2F1 responsiveness

(Supplementary Figure 7) strongly suggests that at least some of these alternative Rbf1 target genes are regulated by a distinct promoter grammar. A preliminary bioinformatic analysis of these signaling pathway promoter regions did not identify motifs common to the whole set, therefore it is possible that there are subclasses of motifs that are involved in setting a transcriptional "grammar" for individual pathways.

In summary, our genomic identification of Rbf1 targets in the Drosophila embryo provides the first view of this important class of corepressor in a whole animal; we find that in addition to a core of conserved genes related to RB protein function in cell cycle and DNA replication, the Rbf1 occupied genes are distributed among a diversity of functions. The complexity of binding regions occupied by Rbf1 among different classes of genes strongly indicates that this corepressor is involved in gene regulation in very different contexts, interacting with promoters that are occupied by distinct types of transcription factors. Such complexity would allow the development of independently-controlled groups of Rbf1 target genes. Much work remains in deciphering the "promoter grammar" of these regulatory regions. Most intriguingly, a high degree of enrichment of genes for conserved signaling pathways suggests that Rbf1 is directly involved in setting levels of components of these systems at multiple points; such regulation would change the sensitivity of signaling, which may vary from tissue to tissue. Identifying the functional significance of RB interactions with genes from these pathways will clarify new pathways of regulation of importance in development and disease.

## Materials and Methods

### *Fly stocks*

Embryos of a *Drosophila melanogaster* $yw^{67}$ strain were used for all chromatin immunoprecipitation assays.

### *Reporter constructs and luciferase assay*

To further analyze target genes bound by Rbf1, upstream promoter regions of *InR* from -1000 to -1, *Mer* from -600 to +400, *Rab23* from -900 to +100, *Hpo* from -600 to +61, *Dad* from -500 to +100, *p53-proximal* from -204 to +50, *Stat92E* from -500 to +152, and *Act5C* from -900 to +100 with respect to the transcriptional initiation sites were PCR amplified and cloned into *Xho*I and *Asc*I sites in pAC2T-luciferase vector (Ryu and Arnosti 2003). Each clone contained the portion of DNA bound by Rbf1 in the embryo. In addition, *PCNA*-luciferase reporter (Acharya et al. 2010) was also used as a positive control. *Drosophila* S2 cells were transfected using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. 1.5 million cells were transfected with 600 ng of one of the luciferase reporters, 250 ng of pRL-CMV Renilla luciferase reporter (Promega, Madison, WI), and 250 ng of pAX-*rbf1* (Acharya et al. 2010) or 20 ng of pIE4-*myc-E2F1* (Frolov et al. 2001). Cells were harvested 72h after transfection and luciferase activity was measured using Dual-Glo Luciferase assay system (Promega) and quantified using the Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA).

### *Chromatin Immunoprecipitation*

Chromatin Immunoprecipitations were conducted using *yw Drosophila melanogaster* embryos collected at room temperature and aged as indicated in Figure 1. For Supplementary

figure 3, embryos were collected from *Drosophila melanogaster* harboring Flag epitope tagged *rbf1*. For preparation of chromatin, embryos were collected, dechorionated with bleach and placed in a 50-ml tube with 9.4 ml of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.76 mM $KH_2PO_4$, pH 7.4). 0.6 ml of freshly prepared 25mM DSP [dithiobis(succinimidyl propionate)] was added to the embryos in this buffer, and the tube was shaken vigorously for 30 min at room temperature, centrifuged at 1500 rpm in a Beckman Allegra 6R clinical centrifuge for 5 min and supernatant was removed. Embryos were fixed for 15 min with vigorous shaking in a 50 ml tube in 9.2 ml crosslinking buffer (50mM HEPES [pH 7.6], 1mM EDTA, 0.5mM EGTA, 100mM NaCl), 0.81 ml of 37% formaldehyde and 30 ml heptane. The cross-linking reaction was stopped with 25 ml stop buffer (0.125M glycine, 0.01% Triton X-100 in phosphate-buffered saline [PBS]) while the tube was shaken vigorously for 15 min at room temperature. The supernatant was removed and the embryos were washed in 10 ml embryo wash buffer (10 mM HEPES[pH 7.6], 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.01% Triton X-100) for 10 min with vigorous agitation at room temperature. The supernatant was removed and embryos were resuspended in 5 ml of sonication buffer (10 mM HEPES[pH 7.6], 1mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate), transferred to a 15-ml tube and a proteinase inhibitor tablet (Roche complete mini, 11-836-153-001) was added. The embryos were sonicated for 20 s (60% duty cycle) and cooled on ice for 30 s a total of 12 times followed by three 30 s sonication and 30 s cooling cycles, using a Branson sonicator. Crude chromatin was centrifuged at 14000 rpm in a microcentrifuge at 4°C and supernatant was transferred to a 15-ml tube. An equal volume of room temperature 2X radioimmunoprecipitation assay (RIPA) buffer (2% Triton X-100, 280 mM NaCl, 20 mM Tris-HCl [pH 8.0], 2 mM EDTA) was added. The chromatin was precleared by adding 10 µl/ml of a 50% slurry containing an equal mixture of

agarose beads coupled to protein A and protein G (Millipore; equal volume of protein A and G beads were mixed) previously washed three times with 1 X RIPA buffer and blocked 2 hr at room temperature with 0.1 mg/ml bovine serum albumin and 0.2 mg/ml salmon sperm DNA. For immunoprecipitations, 1 ml of precleared chromatin was incubated with 5 µl preimmune, 5 µl Rbf1 antibody (Keller et al. 2005), 5 µl of Flag antibody (Sigma; F7425) as used in (Acharya et al. 2010) or 2 µl H3 antibody (Abcam, Cambridge, MA; 0.4 µg/ µl) overnight at 4°C. After overnight incubation, the samples were centrifuged at 14000 rpm in a microcentrifuge for 10 minutes at 4°C. Supernatant was taken in microcentrifuge tubes and 40 µl blocked beads (as described above) were added to each sample and incubated for 2 h (with rotating) at 4°C. The beads were centrifuged in a microcentrifuge at 1000 rpm for 1 min, washed twice with 1ml ice cold low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 150 mM NaCl), twice with ice cold high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl [pH 8.0], 500 mM NaCl) and twice with Tris-EDTA (10 mM Tris [pH 8.0], 1 mM EDTA). Chromatin was eluted at room temperature with 250 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min without shaking. Beads were centrifuged, the supernatant was transferred to a microcentrifuge tube, a second elution was performed, and supernatants were combined. 25 µl of 4M NaCl was added, and cross-links were reversed overnight at 65°C. The eluates were then incubated with 1 µl RNase A (10 mg/ml) at 37°C for 30 min. 10 µl 0.5 M EDTA, 20 µl 1 M Tris-Cl (pH 6.5), and 1 µl proteinase K (20 mg/ml) were then added and tubes incubated at 42°C for 1 h. DNA was extracted with phenol-chloroform and precipitated with equal volume of isopropanol, 3 M sodium acetate (final concentration 0.3M) and GlycoBlue pellet paint by centrifuging at 14000 rpm at 4°C. The pellets were carefully washed once with 70% ethanol, air dried and resuspended in 40 µl water. 2 µl of each ChIP sample was used for 31 cycles of PCR

for 0-6 hr embryos and 28 cycles of PCR for the DNA from other time points. The oligonucleotides used for PCR are listed in Supplementary table VIII (as in Acharya *et al,* 2012).

***Sequencing of immunoprecipitated DNA fragments***

The double-stranded DNA ends were repaired with T4 DNA polymerase, Klenow fragment and T4 PNK enzymes. After a second purification step, an adenine-residue was added with Klenow [3'>5' exo$^-$] enzyme and again purified on Quiaquick columns. Adapters from Illumina for LM-PCR were then ligated to the end of the DNA molecules. The product of the reaction was then run on a 2% NuSieve agarose gel and a band corresponding to 200 bp was extracted and purified. 20 cycles of PCR were performed using Phusion polymerase (Finnzyme F-530S) and the Illumina oligos, and the products were purified by gel electrophoresis. High throughput sequencing was performed on an Illumina Genome Analyzer with standard Illumina 36 cycles reaction kit. The DNA libraries generated (two Rbf1 and one preimmune) were sequenced in one lane each.

***Mapping the reads, peak finding and visualization***

We obtained 13,909,250, 18,070,094 and 1,247,796 reads for two anti-Rbf1 immunoprecipitation libraries and one control library respectively. The quality-filtered 36-bp short sequence reads were aligned to *Drosophila melanogaster* genome (Flybase build r5.28) using Bowtie 0.12.3 (Langmead et al. 2009) with default parameters except that sequences were required to map uniquely to the genome (setting –m 1). To identify Rbf1 bound regions, QuEST software (Valouev et al. 2008) version 2.4 was used with relaxed stringency. A peak was called if the ChIP to background enrichment was 2.5 fold. The peaks were visualized using the online version of UCSC genome browser. We identified 2187 and 1337 peaks in two biological anti-Rbf1 immunoprecipitation replicates; there were 1236 peaks that were present in both replicates,

which we termed Class A peaks. Additional 951 and 101 non-overlapping peaks are termed Class B and Class C peaks respectively (Supplementary Table I as in Acharya *et al*, 2012). Class A peaks were used for the all analyses except for the signaling pathway components (Figure 3) where Classes A, B and C peaks were used. Intensities of peaks in first replicate were higher (from 10 to 656) than in the second replicate (from 10 to 109). The intensities were on average higher for Class A peaks than for Class B, and higher for Class B than C. We list the higher intensities obtained from the first replicate experiment in Supplementary Table I (as in Acharya *et al*, 2012) for Class A peaks.

***Validation of ChIP-seq peaks***

To independently assess enrichment of Rbf1 on novel target genes, several genes were selected and their enrichment in ChIPed chromatin was tested by PCR (Supplementary Figure 2). The immunoprecipitated material that was also used for Illumina sequencing was used to validate the ChIP-seq peaks. The oligonucleotides used for PCR are listed in Supplementary Table VII as in Acharya *et al*, 2012.

***Determination of peak overlap in replicates, denovo motif discovery and motif analysis***

Peaks observed in the two Rbf1-immunoprecipitate experiments for which the maximum points were located within 200 bp of each other were considered to be overlapping peaks. For each peak region, 100 bp sequences on each side of the peak maximum height location were extracted using a Perl script (output_genomic_regions_from_calls.pl) that was obtained from QuEST website (http://www.stanford.edu/~valouev/QuEST). Motif discovery was performed using MEME suite version 4.3.0 (Bailey and Elkan 1994). The program was set to search for overrepresented 5 through 15 mer motifs separately. The top four overrepresented motifs identified for each k-mer were selected and compared against TRANSFAC and JASPAR

databases using the online version of TOMTOM (http://meme.sdsc.edu). In most cases, similar motifs were found for different lengths (5 to 15-mers); the motifs shown in Figure 6A represent the shortest version of the motifs found to be overrepresented. We determined the quality of individual motifs compared to the defined consensus sequences using MAST (MEME suite version 4.3.0) on the sequences extracted from peak regions. To determine the significance threshold that would provide the best discrimination between enriched motifs and chance sequences shown in Figure 6B, the sequences under the peaks were randomized five times and MAST was run for each scramble independently. A $p < 0.0001$ was found to provide the biggest difference between the percentage of randomized promoters containing the overrepresented motif and the percentage of Rbf1-bound regions. To determine whether the overrepresented motifs identified were enriched specifically on promoter regions associated with Rbf1 binding shown in Figure 6C, DNA sequences extending from -100 to -300 from one thousand randomly selected Drosophila promoters not bound by Rbf1 were used for background analysis using MAST, and this process was repeated for total of five different sets of one thousand non Rbf1-binding promoters.

***Gene ontology analysis***

Genes with transcriptional start sites (TSS) within 2kb from peak maximum were considered associated with the peak. 1169 out of 1236 Class A peaks were mapped to TSS of 1890 genes. The enrichment of gene ontology terms was performed using online tool DAVID (Huang da et al. 2009) and 321 original GO categories were identified. Using visual inspection, related GO terms such as cell cycle, mitotic cell cycle, meiotic cell cycle etc were pooled into one broader category Cell Cycle and DNA replication. In this way the GO terms with significant enrichment were pooled into a total of 10 different categories. Other terms with fewer genes and

less significant enrichment were grouped under the "others" category. We report in figure 5 the p-values for the most populous subcategory of each super category shown on the pie chart.

***Cluster analysis***

We used *Drosophila* RNAseq data from modENCODE (http://bit.ly/iSOyEy) from 0 to 24 hours (in two hour windows) and replaced the missing value (NA) with 0. By normalizing each gene with

$$\hat{x}_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{\frac{1}{n}\sum_{j=1}^{n}(x_{ij} - \bar{x}_i)^2}}, j = 1,...,n$$

we obtain the expression change data for each gene. We chose a fuzzy c-means clustering method (Dembele and Kastner 2003), which creates a fuzzy boundary for each cluster. By minimizing the objective function

$$J_m = \sum_{i=1}^{N}\sum_{j=1}^{C} u_{ij}{}^{m}\left\|x_i - c_j\right\|^2, 1 \le m \le \infty$$

where

$$u_{ij} = \frac{1}{\sum_{k=1}^{C}\left(\frac{\left\|x_i - c_j\right\|}{\left\|x_i - c_k\right\|}\right)^{\frac{2}{m-1}}}, \qquad c_j = \frac{\sum_{i=1}^{N} u_{ij}{}^{m} x_i}{\sum_{i=}^{N} u_{ij}{}^{m}}$$

we obtained the cluster index for each gene. In this study we chose *m=1.25, C=8.*

***Identification of human orthologs to genes bound in Drosophila by Rbf1***

The human orthologs of the 1890 genes associated with Class A peaks of Rbf1 in Drosophila were obtained from FLIGHT (http://flight.icr.ac.uk/). To determine conservation of RB family binding to conserved signaling pathway genes, the 295 genes listed on Supplementary Table IX (as in Acharya *et al*, 2012) were input into FLIGHT. An ortholog was considered bound in both fly and human if at least one member of an orthologous family was occupied in each species.

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# CHAPTER IV

## Conclusions and Future Perspectives

The studies that I describe in this dissertation elucidate novel mechanisms through which the *Drosophila* Rbf1 is regulated, and uncovers unprecedented sets of Rbf1 target. Here I discuss three main discoveries that we have made and their significance in the field of retinoblastoma biology. Firstly, the repression function is closely linked to the turnover of the pocket proteins. Secondly, Rbf1 appears to target many components of conserved signaling pathways, suggesting that retinoblastoma family proteins might have broader role in development than just controlling cell cycle genes. Thirdly, I will also discuss the possibility of E2F independent regulation of target genes by Rbf1 and the impact on "promoter grammar".

***Evidence of linkage between turnover and repression activity of Rbf1***

Several studies have shown that for the transcriptional activity of the activators such as p53, β-Catenin, Rpn4, Glucocorticoid receptor (GR), c-Jun, Hif1α, VP16, Myc, Gcn4, Ste12, Androgen receptor (AR), Estrogen receptor, Gal4 and Notch intracellular domain, proteolytic destruction is required [1-3]. These studies further pointed out that the transcriptional activation domains and degrons overlap in most of these unstable transcriptional activators [4]. It has been proposed that when activators interact with general transcriptional machinery, they recruit ubiquitin ligases to the site of transcription. These ligases then ubiquitylate several factors such as, the activators, RNA polymerase II and histones, which in turn recruit the 26S proteasome. This results in turnover of activators and promotion of transcriptional elongation by RNA pol II [4]. For the first time we reported that this instability-activity link is true for repressors too, as in the case of Rbf1 discussed in Chapter II [5]. We showed that the instability element (IE) present in the C-terminal region of Rbf1 is responsible for the activity and stability of the protein. We

also 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.

Our study initiates several interesting questions. Is IE an autonomous degron? Future studies with the IE or IE-inactivating lysine mutations, fused to other protein such as Gal4 would address this question. An assay with proteasome inhibition would test whether the IE is an autonomous degron. Additionally, tethering IE or its mutant version to a promoter would test whether the IE is sufficient for repression. In the same study described in Chapter II, we also showed that the orthologous region in p107 is also responsible for its accumulation in S2 cells. Similar studies with the orthologous regions of mammalian pocket proteins would further test how general is the mechanism of the IE among pRB proteins. This question is especially important because mutations in C-terminal region of pRB are associated with a variety of osteosarcomas [6, 7] and mutations in C-terminal region of p130 orthologous to IE are associated with lung tumors [8]. Furthermore, we have shown that Rbf1 is developmentally regulated and IE plays a role in it, so are the IEs of mammalian pocket proteins responsible for developmental regulation? Thus, the studies with IE to answer above mentioned questions would provide further insights in tumor biology.

We know that Rbf1 is degraded by proteasome mediated turnover and the IE mutants are resistant to it. However, we showed that certain lysine residues in the IE region are important for Rbf1's activity and turnover. We hypothesized that lysines may be ubiquitylation targets of E3 ubiquitin ligases and this process targets Rbf1 to proteasomal degradation. Interestingly, when we mutated those lysines to arginines conserving the charge, Rbf1 still was active and actively turned over. So the question is if Rbf1 is turnover by proteasome mediated turnover and IE is

also responsible for its turnover, how is IE mediating this regulation even when lysines are replaced with arginines (which are not ubiquitylated)? Ubiquitylation studies with full length and IE mutant Rbf1 (and mammalian pocket proteins) and GFP fused to IE or IE mutants can further elucidate whether the turnover is ubiquitin dependent or independent and whether IE is a target for ubiquitylation or serves as a docking site for ubiquitin ligases.

Previous studies from our group have shown that the COP9 signalosome (CSN) physically associates with Rbf2 and knockdown of individual CSN subunits destabilizes Rbf1 and Rbf2, suggesting that COP9 protects *Drosophila* pocket proteins from degradation [9]. Now we have two processes to stabilize Rbf1; first is COP9 mediated stabilization and second is IE mediated destabilization. What is the link between COP9 signalosome's role in stabilizing Rbf1 and IE? Is COP9 signalosome masking IE from E3 ligases and deletion of IE is sufficient to provide protection provided by COP9 signalsome? Or is the IE mutant Rbf1, which is stable, destabilized by CSN knockdowns? These questions can be answered by first finding out the Rbf1-COP9 interaction domain in Rbf1 followed by generation of CSN-binding deficient and IE combo mutant Rbf1. This would broaden our understanding of CSN-mediated regulation of Rbf protein stability.

***Targeting of various conserved signaling pathways by Rbf1***

One of the most astonishing findings of our study, as described in Chapter III, is previously unappreciated extensive occupancy of multiple nodes of conserved signaling pathways. Previous studies that examined the regulons or direct physical targets or functional targets of pRB proteins have not emphasized this striking aspect of the system [10-14], which may be partially due to the heavy reliance on cell culture systems of this information. Several studies have shown the link between retinoblastoma proteins and several conserved signaling

pathways [15-24]. As described in Chapter III, we showed possibility of direct regulation of several components of conserved signaling pathways, which further supports the involvement of pocket proteins in those pathways as found in studies from different groups. In addition, I showed that Rbf1 represses *InR*- (insulin signaling pathway) and *Rab23*- (hedgehog signaling pathway) luciferase reporters. This finding strongly suggests that Rbf1 targets these signaling pathway components and represses them.

Our finding raises an interesting question whether Rbf1 regulates other components of the conserved signaling pathways. Similar reporter assay in cell culture with promoters of those genes would answer this question. It might be possible that some of the pathways might require stimulation in order to show response, because cell culture system might lack those stimulatory signals. Some of the genes might also be developmentally regulated, and might not show response in cell culture. These might be reasons this feature of retinoblastoma tumor suppressor was not underscored in previous studies [10, 11, 13, 14]. In that case a reporter assay in fly tissues in an *rbf1* wild type or mutant background might be helpful to elucidate the function of the repressor on those genes. In addition, incorporation of Rbf2 in the assays may throw some light on this feature, as Rbf1 and Rbf2 share some functional redundancy.

Another intriguing finding that may have significant implication is my finding that Rbf1 binds the p53 promoter. Crosstalk between the pRB and p53 pathways has been well studied; inactivation of retinoblastoma proteins leads to upregulation of p53 [25-30]. My observation of physical presence of Rbf1 on the p53 promoter raises an important question, namely, does Rbf1 transcriptionally regulate p53? I and Jonas Pedersen (a Master's degree student in our lab) have conducted preliminary S2 cell experiments with *p53*-luciferase reporter, but thus far we have not seen significant repression of the gene by Rbf1. We may need to first stimulate transcription of

p53 using agents such as doxorubicin. Measuring endogenous p53 mRNA levels after Rbf1 and Rbf2 knockdown would also be helpful in understanding the above question. Furthermore, this study can be extended in *Drosophila* by generating *rbf1/rbf2* conditional knockout transgenic flies, knocking out the genes in a tissue specific manner and looking at the endogenous p53 levels. Alternatively, levels of *p53*-GFP can be visualized in *rbf1/rbf2* background tissues. Finally, extension of these studies in human cell lines would give a bigger picture on how pocket proteins regulate p53. These studies would help broaden our understanding on crosstalk between RB and p53 pathways.

***E2F-independent regulation of target gene promoters by Rbf1***

It is well known that RB family proteins do not bind to DNA directly, but are recruited to the target gene promoters by the E2F family of transcription factors [31, 32]. In my bioinformatics analysis described in Chapter III, I found that only a third of Rbf1 binding peaks had high affinity E2F binding sites. Does Rbf1 bind to the remaining promoters independently of E2F transcription factors? There are two possibilities, either those regions have poor E2F sites or maybe Rbf1 is recruited by factors other than E2Fs. Consistent with the latter possibility, we show that ectopic expression of E2F1 increases expression of a *PCNA*-luciferase reporter, which is known to be bound by E2F, but not of an *InR*-luciferase. This hypothesis would be bolstered by chromatin immunoprecipitation (ChIP) with Rbf1 and Rbf2 antibodies after E2F1, E2F2 or DP knockdown. If we can still observe Rbf signal on the promoters lacking strong E2F sites after E2F or DP knockdown, it would suggest that Rbf1 and/or Rbf2 are recruited to those promoters in an E2F-independent manner. This approach can identify target genes that are regulated by Rbf1 in E2F-independent manner, if any. A genomics approach is necessary to identify such targets globally. The Botchan lab has shown the global targets of dE2F2 [13], however, to my

knowledge nobody has reported genome-wide physical targets of dE2F1. Comparison of Rbf1 peaks that we identified with physical targets of dE2F1/2 may provide stronger evidence about how Rbf1 is recruited to the promoters. These studies may change the current notion that E2F- plays the main role in binding of retinoblastoma proteins.

My genome-wide study of Rbf1 revealed some interesting facts; however, it was done on 12 to 18 h embryos. As we know that a multitude of genes are dynamically expressed throughout the development of an animal, including Rbf1 and Rbf2. So how do the pocket proteins regulate the genes at different development stages and different tissues? A developmental ChIP-seq study with Rbf1 and Rbf2 would provide on the genes that are differentially regulated by these retinoblastoma proteins.

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# APPENDIX A

## Nuclear localization of Rbf1 mutants

In Chapter II, we showed that Rbf1 ΔIE mutant loses its activity. We also ruled out the possibility that the inactivity is not due its inability to localize to nucleus by immunocytochemistry (Figure 4B, Chapter II). To see if activity of other mutants is affected by exhibition of aberrant nuclear localization, I performed the same experiment (Chapter II, Materials and Methods) with other mutants. Except the Rbf1 1-727, a C-terminal mutant, all the other mutants were exclusively localized to the nucleus (Figure A.1).

# Figure A.1

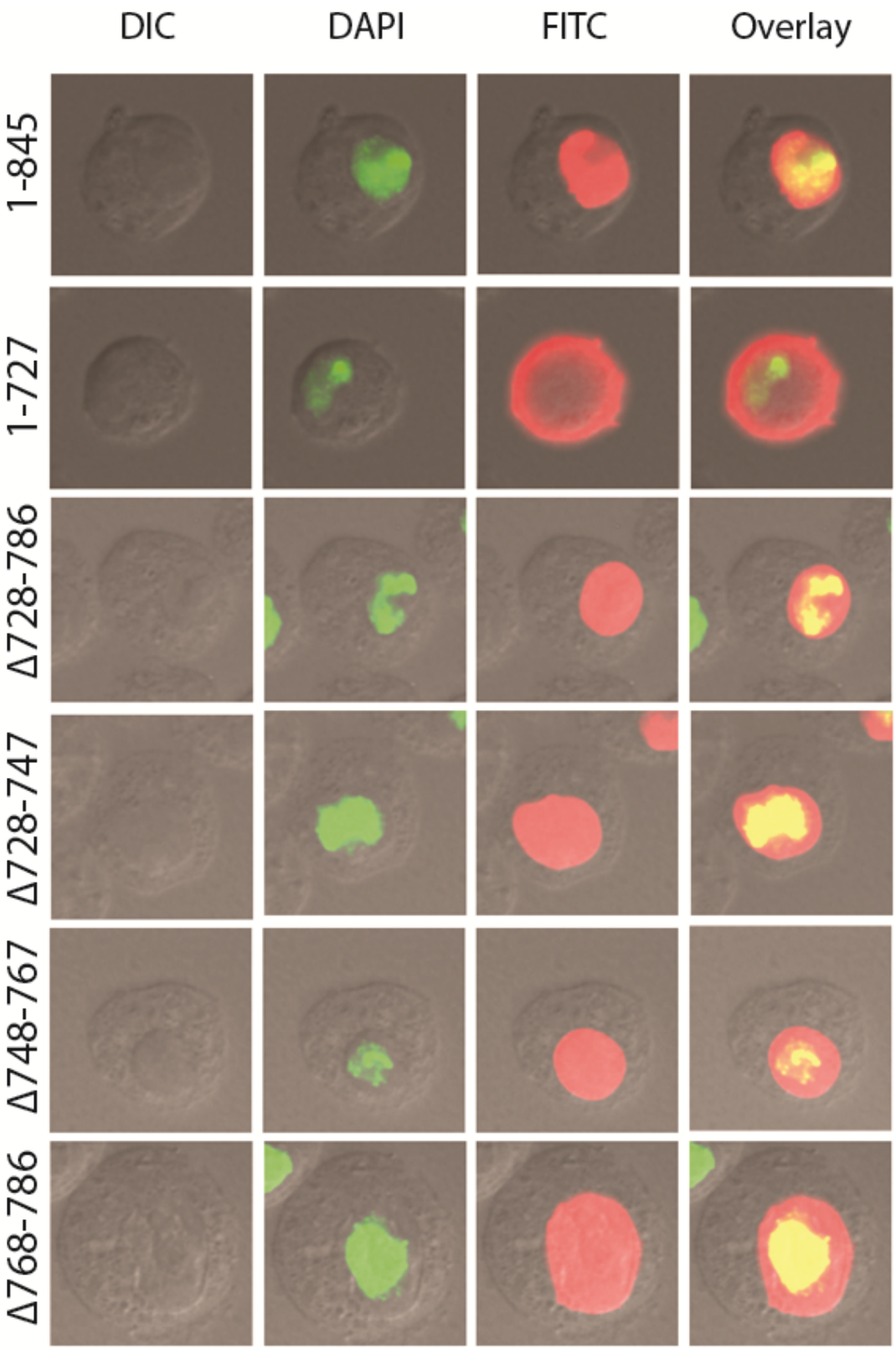
DIC
DAPI
FITC
Overlay
1-845
1-727
Δ728-786
Δ728-747
Δ748-767
Δ768-786

**Figure A.1 cont'd.**

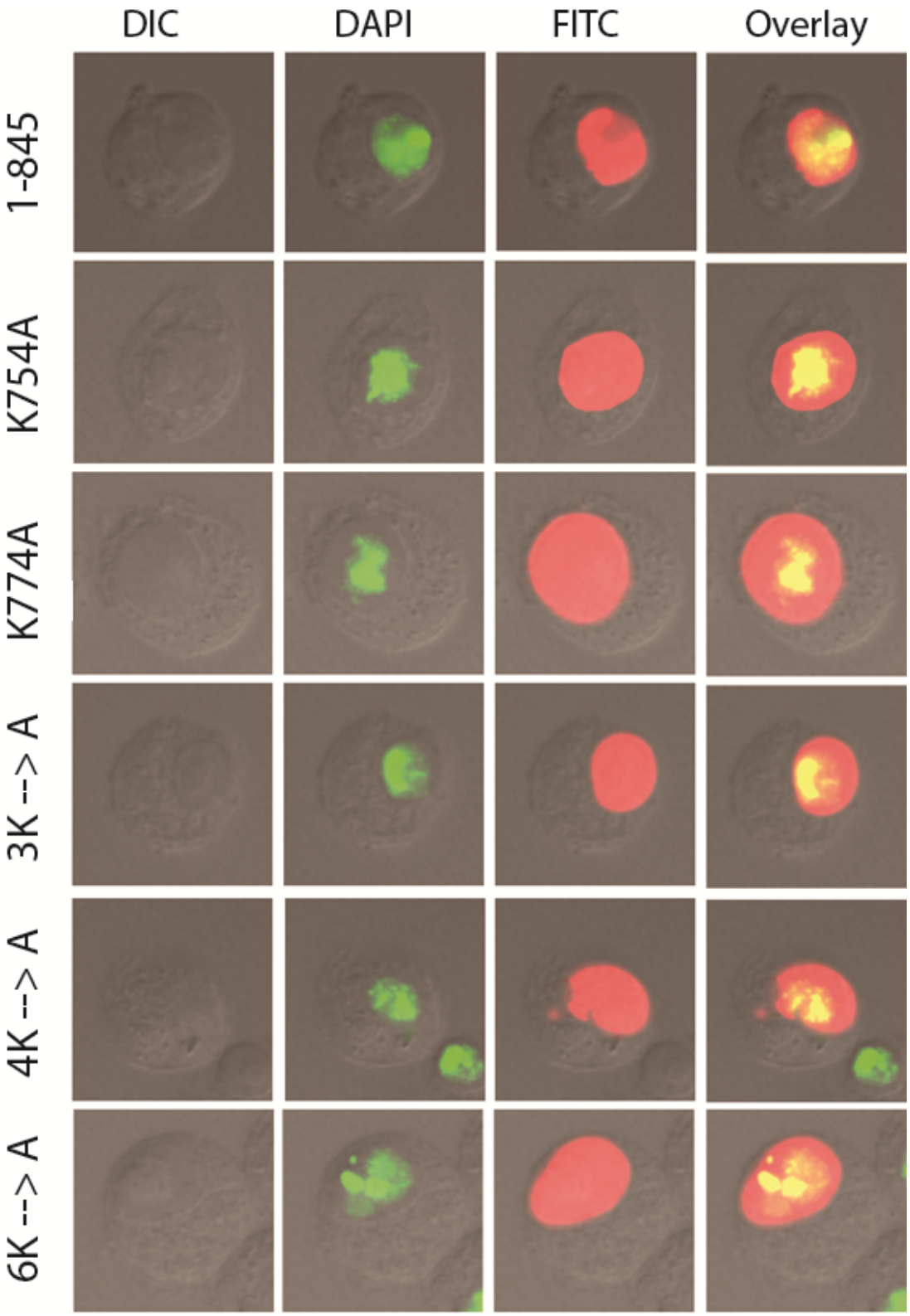

**Figure A.1 cont'd.**

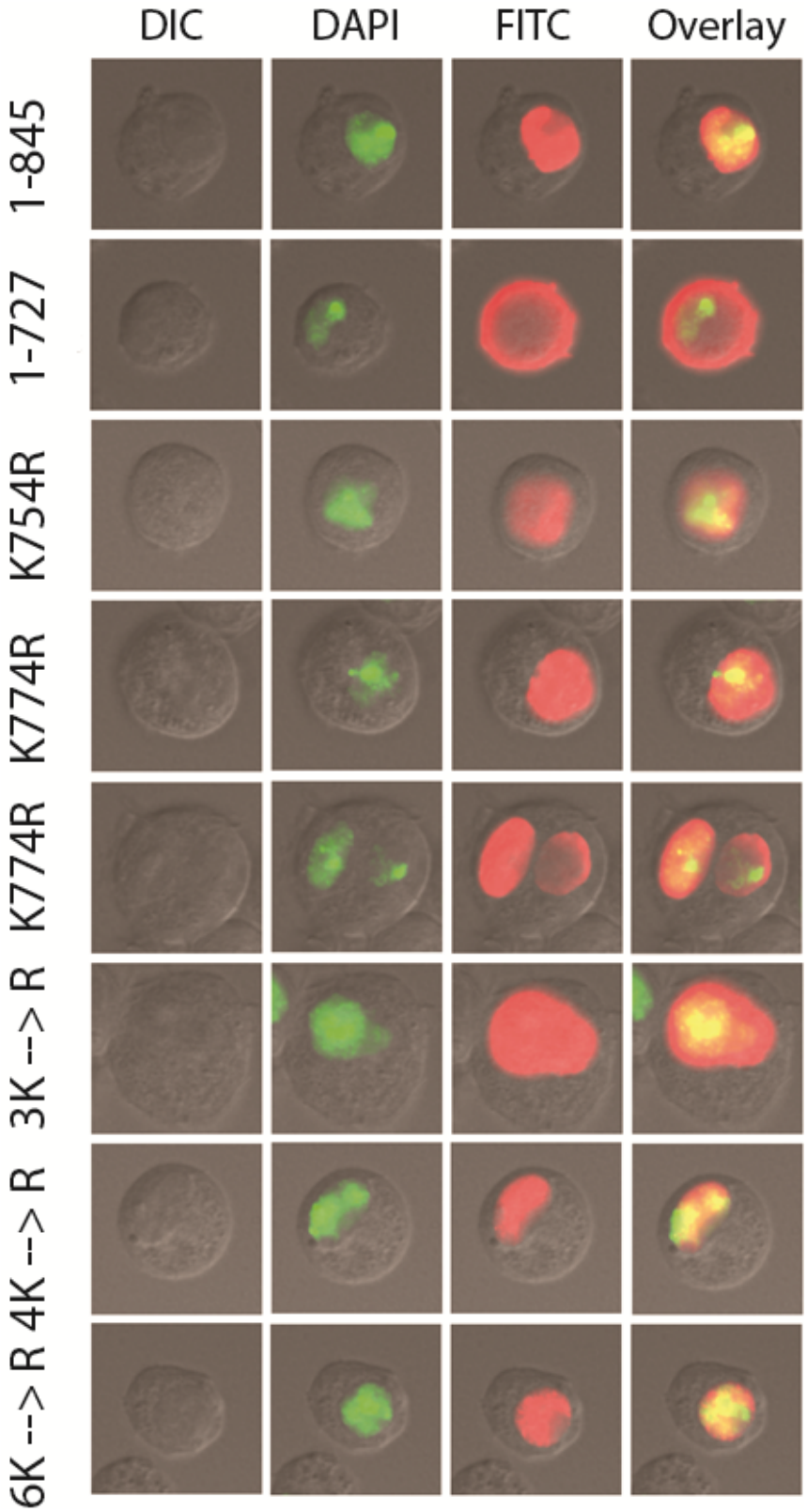

**Figure A.1 cont'd.**

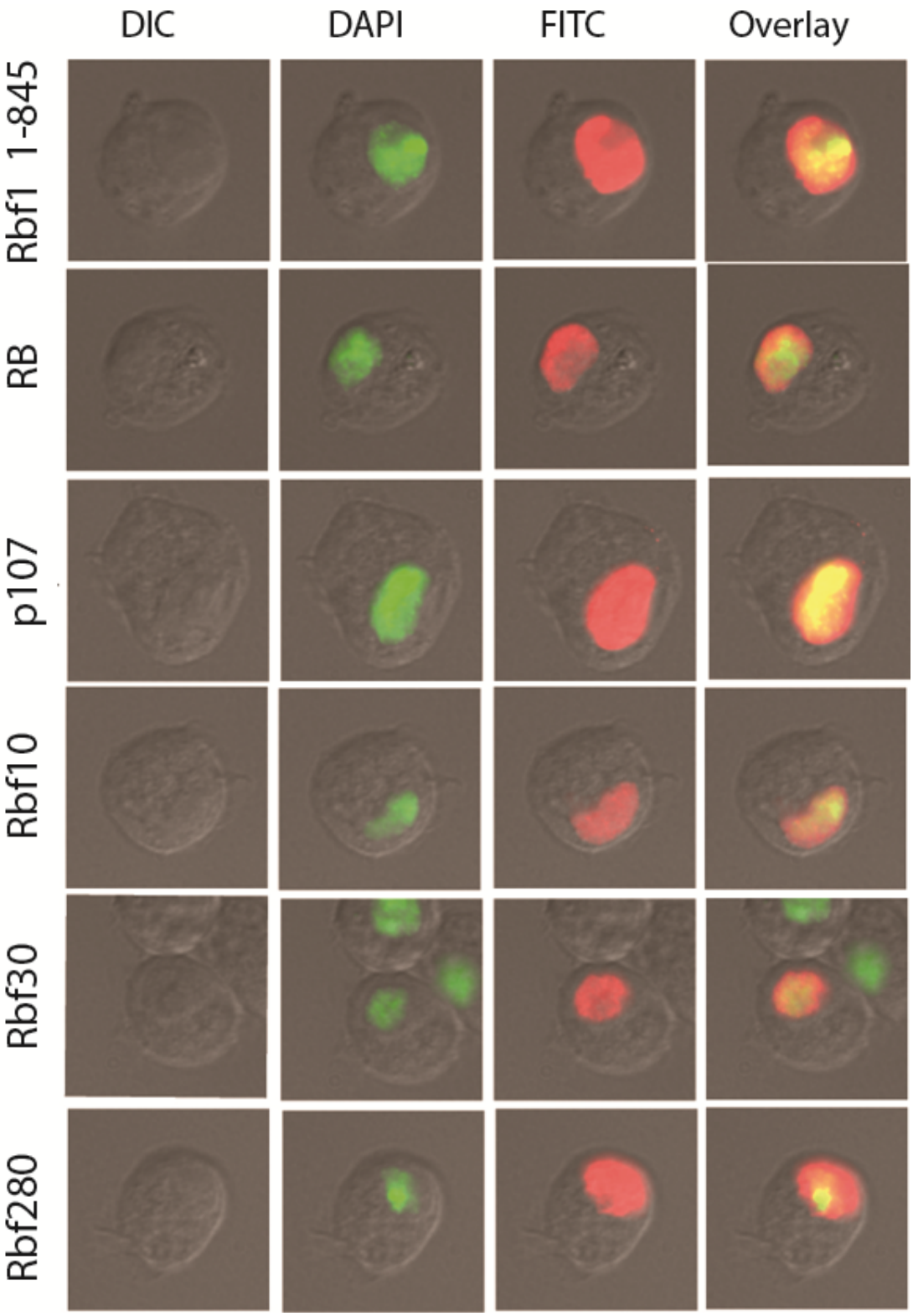

Figure A.1 cont'd.

**Figure A.1. Nuclear localization of Rbf1 mutants**. *Drosophila* S2 cells were transfected with flag-tagged Rbf1 (wild type or mutants) or RB or p107 and cultured for three days. Cells were then fixed and the intracellular localization of Rbf1 proteins was assayed by indirect immunofluorescence using the mouse monoclonal M2 anti-flag antibody followed by the anti-mouse IgG-FITC (red). Cells were counterstained with DAPI in order to visualize DNA (green). Merged images are shown on the right column. 1-845 is Rbf1 wild type; 1-727 is Rbf1 ΔC-terminus; Δ728-786 is Rbf1 ΔIE; Δ728-747, Δ748-767 and Δ768-786 are three small deletions within IE; 3K → A is K732A, K739A and K740A; 4K → A is K732A, K739A, K740A and K754A; 6K→ A is K732A, K739A, K740A, K754A, K774A and K782A; 3K → R is K732R, K739R and K740R; 4K → R is K732R, K739R, K740R and K754R; 6K→ R is K732R, K739R, K740R, K754R, K774R and K782R; RB, human pRB; p107, human p107; Rbf10 is S728A, S760A and S771A; Rbf30 is T715A, S728A, S760A and S771A; and Rbf280 is T356A, S728A, S760A and S771A. Rbf10, Rbf30 and Rbf280 are mutants reported in Xin *et al*[1]. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

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# APPENDIX B

## Generation of epitope tagged CNS genomic rescue constructs

We showed that COP9 signalosome associates with Rbf1 and Rbf2 and protects them from proteasomal mediated degradation [1] and that CSN4, a subunit of COP9 signalosome, co-occupies Rbf target genes together with Rbf1 and Rbf2. CSN4, CSN5 and CSN7 are found as monomers [2, 3], it might be possible that just CSN4 be present at Rbf target promoters or the whole complex is present. We hypothesized that "Entire COP9 complex co-occupies Rbf target gene promoters with Rbf." Since chromatin immunoprecipitation (ChIP) is considered a gold standard assay for investing promoter occupancy by any protein, I sought to perform ChIPs with COP9 subunits and Rbf1. In addition we also sought to map the interacting COP9 subunit with Rbf1 or Rbf2. To pursue above experiments, we a handle on the proteins would be handy, thus I constructed flag epitope tagged CSN2, CSN3, CSN4, CSN5, CSN6 and CSN8 genomic rescue contructs. Further, I generated transgenic flies for these subunits using P-element mediated germline transformation of *yw* flies.

### Materials and Methods

#### *Expression Constructs and Transgenic Lines*

To express CSN proteins under control of the endogenous regulatory sequences, a 6 kb genomic locus of CSN2 was cloned, extending from 2.1 kb upstream of first exon to 2.1 kb downstream stop (2.1 kb downstream end of the last exon) into pCaSpeR[4] between *Kpn*I and *Xho*I sites in two steps using PCR amplification of genomic DNA. A 5.5 kb locus of CSN3 (1.8 kb upstream of first exon to 1.4 kb downstream of the last exon), 4.5 kb locus of CSN6 (2 kb upstream of first exon to 1.3 kb downstream of the last exon), and 4.0 kb locus of CSN8 (1.8 kb upstream of first exon to 1.4 kb downstream of the last exon) were cloned into pCaSpeR between

*Kpn*I and *Xho*I sites in two steps using PCR amplification of genomic DNA. Similarly a 5.0 kb locus of CSN4 (1.7 kb upstream of first exon to 1.6 kb downstream of the last exon) was cloned into pCaSpeR between *Kpn*I and *Hind*III sites and a 4.5 kb locus of CSN5 (1.0 kb upstream of first exon to 2.2 kb downstream of the last exon), were cloned into pCaSpeR between *Eco*RI and *BamH*I sites in two steps using PCR amplification of genomic DNA. Two Flag epitope tags were inserted 5' of the stop codon. The 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.

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