Integrated Stability and Activity Control of the *Drosophila* Rbf1 Retinoblastoma Protein\(^*\)

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**Background:** Rbf1 C-terminus regulates protein turnover and potentiates its transcriptional activity.

**Results:** Rbf1 protein levels and activity are differentially regulated by phosphorylation sites.

**Conclusion:** Rbf1 turnover and activity processes are functionally separable, both influencing tissue development.

**Significance:** This study highlights the importance of coupled control of Rbf1 turnover and activity, with implications for similar regulation of mammalian RB proteins in development and cancer.

**ABSTRACT**

Retinoblastoma (RB) family transcriptional corepressors regulate diverse cellular events including cell cycle, senescence, and differentiation. The activity and stability of these proteins are mediated by post-translational modifications, however we lack a general understanding of how distinct modifications coordinately impact both of these properties. Previously, we showed that protein turnover and activity are tightly linked through an evolutionarily conserved C-terminal instability element (IE) in the *Drosophila* RB-related protein Rbf1; surprisingly, mutant proteins with enhanced stability were less, not more active. To better understand how activity and turnover are controlled in this model RB protein, we assessed the impact of Cyclin-Cdk kinase regulation on Rbf1. An evolutionarily conserved N-terminal threonine residue is required for Cyclin-Cdk response, and showed a dominant impact on turnover and activity, however specific residues in the C terminal IE differentially impacted Rbf1 activity and turnover, indicating an additional level of regulation. Strikingly, specific IE mutations that impaired turnover but not activity induced dramatic developmental phenotypes in the *Drosophila* eye. Mutation of the highly
Retinoblastoma stability and activity

conserved K774 residue induced hypermorphic phenotypes that mimicked the loss of phosphorylation control; mutation of the corresponding codon of the human RBL2 gene has been reported in lung tumors. Our data supports a model in which closely intermingled residues within the conserved IE govern protein turnover, presumably through interactions with E3 ligases, and protein activity, via contacts with E2F transcription partners. Such functional relationships are likely to similarly impact mammalian RB family proteins, with important implications for development and disease.

INTRODUCTION

The RB tumor suppressor protein governs a plethora of cellular pathways, including cell cycle progression, cellular differentiation, regulation of apoptosis, and maintenance of genomic stability (1). Consistent with this central regulatory role, RB inactivation is widely observed in a broad range of human cancers, contributing to both cancer initiation and progression. As a potent tumor suppressor, RB is subject to tight control through multiple regulatory pathways that are also often disrupted in disease (2). In particular, phosphorylation of RB, and the related family members p107 and p130, is recognized as a common mechanism that differentially influences RB family function (3,4). The canonical pathway for RB phosphorylation in cell cycle progression involves cyclical activity of cyclin-dependent kinases (Cdk). RB inactivation during G1-S transition requires sequential phosphorylation by different Cyclin-Cdk complexes; CycD-Cdk4 is active in mid to late G1, CycE-Cdk2 in late G1, and CycA-Cdk2 in S phase (3,5). In response to DNA damage, RB can also be phosphorylated by other kinases including p38 MAP kinase, which is activated in response to stress stimuli (6) and the checkpoint kinases, Chk1 and Chk2, which influence RB function on apoptotic genes (7). Thus, RB function is modulated in response to signaling through multiple pathways to generate distinct molecular outputs.

Levels of RB family proteins fluctuate during the cell cycle, raising an intriguing question about the connection between RB activity and stability. Upon entry into S phase in HeLa cells and human fibroblasts, as RB is functionally inactivated by phosphorylation, its expression level is dramatically increased, indicating a correlation between phosphorylation status and stability (8,9). Protein stability is often controlled by regulated association of E3 ligases, but precisely how phosphorylation may affect RB and RB family member stability is not understood. Our studies of the Drosophila Rbf1 protein suggest that lability of this protein is closely correlated with repression activity, and that the protein is destabilized at particular developmental times. Understanding how RB family protein turnover is integrated into the physiological context of gene regulation is thus a major objective of current studies.

The best understood function for phosphorylation of RB family proteins involves regulated interactions between RB and its E2F/DP partners. Cyclin-Cdk modification of specific sites within RB induces intramolecular conformational changes that directly modulate accessibility of the E2F1/DP1 interaction interface (10). Additionally, various phosphorylation sites exert distinct effects on cofactor binding (11,12). For example, RB phosphorylation by CycD-Cdk4 at T821/T826 induces dissociation of LXCXE-containing co-regulatory proteins, whereas S807 and S811 phosphorylation disrupts c-Abl tyrosine kinase binding (13). Interestingly, in response to DNA damage, RB is phosphorylated by the checkpoint kinases at S612, which rather than reducing E2F1 interactions actually enhances binding.
This particular modification also strengthens rather than alleviates RB anti-apoptotic activity (7). RB phosphorylation is thus not uniformly inactivating, but rather can have diverse and even opposing effects on RB function and cell fates.

It is less understood how protein stability of RB family proteins is influenced by specific phosphorylation events. RB is regulated by Mdm2, an E3 ubiquitin ligase best known for its regulation of the p53 tumor suppressor (14,15). Mdm2 preferentially targets hypophosphorylated RB through a C-terminal region (residues 785-803) in vitro, suggesting that phosphorylation antagonizes the Mdm2-RB interaction (16). However, p38 MAPK-directed phosphorylation of RB at S567 can trigger an interaction between RB and Hdm2 (human homolog of Mdm2) to induce RB degradation and cellular apoptosis, indicating the phosphorylation can have opposing effects on RB stability (6). Interestingly, the Mdm2 binding region in RB contains two critical phosphorylation sites S788 and S795, phosphorylation of which also interferes with binding of the E2F1 transactivation domain to the C-terminus of RB (17). Therefore, an intriguing possibility is that phosphorylation of the RB C-terminus may simultaneously affect protein stability and activity through interference with E3 ligase and E2F binding.

In Drosophila, the RB-E2F network and the corresponding regulatory pathways are highly conserved, attesting to the physiological importance of phosphorylation and proteolytic controls (18). The transcriptional activity of the Drosophila RB family protein Rbf1 is regulated by CycD-Cdk4 and CycE-Cdk2 kinase complexes (19). Additionally, levels of the Rbf1 protein are controlled by a developmentally regulated turnover process that involves the function of the COP9 signalosome, a conserved complex that controls E3 ligase activity and proteasome-dependent protein degradation (20). Our previous studies indicate that a C-terminal instability element (IE) of Rbf1 influences ubiquitination, turnover rate, and Rbf1 repression activity, suggesting a tight correlation between Rbf1 stability and activity (21,22). The wild-type, labile form of Rbf1 is fully active in repressing E2F1-dependent promoters, while stabilized mutant forms are not, even though they retain E2F binding activity.

Because there is a lack of understanding of how pathways for turnover and activity control are integrated in RB proteins and how phosphorylation influences these two processes, we have focused on the regulation of the Rbf1 model protein to assess functional capacities in cellular and developmental settings. Here we show that Cyclin-Cdk kinases both stabilize as well as inactivate wild-type Rbf1, and demonstrate that specific sites within the IE and N-terminal regions play interconnected roles in these processes. By analysis of directed mutant forms, we uncover evidence of dual roles for specific serine residues in turnover and activity, likely reflecting interactions with E2F complexes and E3 ligases. Our results decisively separate the requirement for lability for Rbf1 function, although ubiquitination may still play a role in modulating repression activity levels. Our study underscores the functions of the Rbf1 IE as a nexus connecting phosphorylation control of stability and activity pathways, a mechanism that is likely conserved in RB family proteins.

EXPERIMENTAL PROCEDURES

Expression Constructs

Generation of Rbf1 WT and mutant expression constructs was described previously (21). The mutations of phosphorylation sites in Rbf1 were made by site-directed mutagenesis using a Quick-Change™ strategy (Stratagene). To
generate Cyclin and Cdk expression constructs, *Cyclin D, Cyclin E, Cdk2 and Cdk4* cDNA were PCR-amplified from respective pOT constructs (DGRC) and cloned into the *KpnI* and *NotI* or *NotI* and *XbaI* sites of the pAX vector (23). Two Flag epitope tags were inserted 5’ of the stop codon. To generate Rbf1 expression constructs used in the fly eye assays, Rbf1 WT and mutants were cloned into pUASTattB (24). The plasmids were then injected by Rainbow Transgenics into the 51D site on the second chromosome of *yw* flies to generate transgenic lines.

**Western Blot Analysis**

*Drosophila* S2 cells cultured in Schneider’s Drosophila Medium with 10% fetal bovine serum were transfected using Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol. 1.0 million cells were transfected with 100 ng of pAX-Rbf1. For Cyc-Cdk overexpression assays, 100 ng of pAX-Rbf1 was cotransfected with 200 ng of pAX-CyclinE or pAX-CyclinD and 400 ng of pAX-Cdk2 or pAX-Cdk4. Cells were harvested five days after transfection by transferring to a 1.7 ml microfuge tube, centrifugation at 10,000 rpm in an Eppendorf centrifuge 5415C for one min at room temperature, and lysed by freezing at -80°C for five min and thawing at 37°C for one min a total of three times, after addition of 50 µl of lysis buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 1% Triton X-100). To every 10ml of lysis buffer was added a Protease Inhibitor Cocktail Tablet (Roche). The lysate was then centrifuged at 10,000rpm for one min at room temperature and the supernatant was used in Bradford assays and Western blot assays. For the Cyc-Cdk overexpression assays, RIPA buffer (Cell Signaling Technology) was used to lyse cells. Total protein levels were measured by Bradford assays. To measure Rbf1 protein levels, 50 µg S2 cell lysates were run on 12.5% SDS-PAGE gels, transferred to PVDF membranes and probed with M2 anti-Flag antibody (mouse monoclonal, 1:10,000, Sigma, F3165) and anti-tubulin (mouse monoclonal, 1:10,000, Iowa Hybridoma Bank). Antibody incubation was performed for overnight at 4°C for the primary antibody and one hr at room temperature for the secondary antibody in TBST (20 mM Tris-Cl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) with 5% non-fat dry milk, washed three times, five min each, after primary and secondary antibody incubation. Blots were developed using HRP-conjugated secondary antibodies (Pierce) and SuperSignal West Pico chemiluminescent substrate (Pierce). For determination of Rbf1 protein half-life under the CycE-Cdk2 overexpression condition, 1.0 million S2 cells were transfected with 100 ng of pAX-Rbf1, 200 ng of pAX-CycE and 400 ng pAX-Cdk2 or 600 ng of pAX empty vector. After five days of culturing, cells were treated with 100 µM cycloheximide for the indicated times.

**Phos-tag analysis of proteins**

For Phos-tag SDS-PAGE assay, cells were lysed in lysis buffer containing 1 mM Na$_3$VO$_4$ and 10 mM NaF. To detect Rbf1 protein phosphorylation, 5% of total S2 cell lysates were run on 7.5% SDS-PAGE gels containing 37 µM Phos-tag ligand (Phos-tag™ AAL-107, Wako Chemicals) (25). The electrophoresis was performed under constant current at 10 mA at 4°C for 21 hours. The gel was soaked and transferred to PVDF membrane as described (25).

**Luciferase Reporter Assays**

For *PCNA*-luciferase assays, 1.5 million Schneider S2 cells were transfected with 600 ng of *PCNA*-luciferase reporter (26), 200 ng of pAX-Rbf1 WT, 200 ng of pAX-CyclinE or pAX-CyclinD and 400 ng of pAX-Cdk2 or pAX-Cdk4. Cells were harvested three days after
transfection and luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega) and quantified using the Veritas microplate luminometer (Turner Biosystems).

**Fly Assays**

Heterozygous lines harboring the wild-type or mutant *rbf1* forms in the pUAST vector were crossed with flies containing an *eyeless-Gal4* / SM2 CyO driver (27) and the offspring containing both driver and UAS-*rbf1* transgenes (70-400 flies per construct) were screened for eye phenotypes. Expression of *rbf1* forms in wing was accomplished by crossing to a *beadex-Gal4* driver. Representative wings were mounted and imaged by electron microscopy.

**RESULTS**

The instability element (IE) harbors phosphorylation sites critical for controlling Rbf1 stability and activity. *Drosophila* Rbf1 protein is subject to phosphorylation control by CycD-Cdk4 and CycE-Cdk2 during cell cycle progression, leading to cell cycle-dependent modulation of its transcriptional repression activity (19). In the mammalian system, RB family proteins are inactivated by phosphorylation in a cell-cycle-dependent manner, associated with fluctuation of protein levels, suggesting a tight correlation between their phosphorylation status, activity and stability (9). To understand how phosphorylation may affect stability of Rbf1, we examined the effect of expression of exogenous Cyclin-Cdk complexes on wild-type Rbf1 in *Drosophila* S2 cells. We cotransfected CycD-Cdk4 or CycE-Cdk2 with Flag-epitope tagged Rbf1. The activity of Rbf1 under these conditions was assayed on a PCNA-luciferase reporter (Fig. 1A). As expected, Rbf1 was inactivated substantially by both Cyc-Cdk complexes, consistent with the known effect of Cyc-Cdk-mediated phosphorylation on Rbf1 (19). Intriguingly, the steady-state levels of Rbf1 were increased under the condition of Cyc-Cdk overexpression (Fig. 1B). Overall levels of Rbf1 protein increased, and an additional slower-migrating band appeared in the presence of overexpressed Cyc-Cdk. Increases in protein levels were directly attributable to a change in stability, as revealed by half-life measurements of the protein in cycloheximide treated cells (Fig. 1C).

The previously observed correlated effects of mutations on protein stability and activity suggested that the C-terminal IE, which regulates both features, may be a direct target of Cyc-Cdk-mediated phosphorylation (22). Indeed, previous phosphoproteomic studies have indicated that S760 and S771 of Rbf1 are modified in vivo, as are related sites in RB family members (19,28-34). In addition, S728 and S760 appear to be conserved as phosphorylation sites in all three vertebrate RB proteins, and these two sites in RB are specifically targeted by CycD-Cdk4 (12,35). Therefore, S728 and S760 in Rbf1 are likely to be regulated by CycD-Cdk4. Although Rbf1 S771 is conserved only in p107 and p130, it is a Cdk concensus site SPXK (Fig. 2A). We therefore examined whether these three serine residues are critical for Rbf1 stability by changing them to either aspartate or alanine. Mutant Rbf1 proteins harboring the phosphomimetic Ser-to-Asp mutations accumulated to about twice the level of the wild-type Rbf1 protein, similar to the effect of mutating four lysines in the IE to alanine, which we previously characterized as a stabilizing change (Fig. 2B). In contrast, the mutant form with unphosphorylatable Ser-to-Ala changes was expressed at about half the level of the wild-type protein (Fig. 2B). As previously demonstrated for the increased stability of Rbf1 proteins bearing specific mutations in lysine residues of
the IE (21, 22), the modification of these serine residues appears to influence Rbf1 stability, providing a regulatable switch to turn up or down Rbf1 protein levels. To test whether the IE is sufficient to respond to overexpression of Cyc-Cdk kinases, we also analyzed GFP-IE chimeras, which we have shown are destabilized by the presence of the IE (22). Expression of Cyc-Cdk kinases did not affect levels of these chimeras, suggesting that they are unable to recognize this part of Rbf1 alone, although the isolated IE is fully functional as a degron, suggesting it can interact with E3 ligases (data not shown).

In light of the previously identified close connection between Rbf1 lability and activity, we tested whether the stabilizing phosphomimetic Ser-to-Asp mutations in the Rbf1 IE would also inactivate Rbf1. We assayed the stabilized Rbf1 3SD and destabilized Rbf1 3SA mutants in transfection assays, using the PCNA-luciferase reporter gene (Fig. 2C). Surprisingly, both mutant forms showed strong repression activity, similar to that of wild-type Rbf1. As expected, the stabilized Rbf1 4KA, included as a negative control, had impaired repression activity. Thus, the C-terminal phosphorylation sites that influence Rbf1 stability are not essential for repression activity, and these mutations appear not to disrupt interaction with the partner E2F factors. We next tested whether these three serines are important for functional inactivation by Cyc-Cdk kinases. The activities of Rbf1 3SD and 3SA were only slightly reduced by this treatment, in contrast to the strong effect on wild type Rbf1, suggesting that the serine residues within the IE are indeed important for full responsiveness to Cyc-Cdk-mediated phosphorylation (Fig. 2D). The partial responsiveness of the Rbf1 mutants does suggest that modification of other phosphorylation sites may additionally impact activity. These results indicate that these conserved serines of the IE are important for regulation of the protein by Cyc-Cdk kinases, but are not intrinsically required for repression activity.

**The N-terminal T356 plays a dominant role in Cyc-Cdk-mediated stabilization of Rbf1.** Phosphorylation of T373 in the N-terminus of RB induces a conformational change that allows the N-terminus to bind to the pocket region, blocking E2F association and possibly relieving RB repression (36). However, it is largely unknown whether T373 is also critical for the regulation of RB stability and whether its potential roles in stability and activity are functionally linked. This residue is one of the few phosphorylation sites that are absolutely conserved in all mammalian and *Drosophila* RB family proteins (Fig. 2A), and T373 in RB is targeted by both CycD-Cdk4 and CycE-Cdk2 (12). In Rbf1, T356 is homologous to RB T373, therefore we tested whether Rbf1 T356 would be critical for the phosphorylation control of both activity and stability by mutating this residue to either aspartate or alanine (Fig. 3A, B). Unlike the distinct effects of Ser-Ala and Ser-Asp mutations in the IE, both Thr-to-Asp and Thr-to-Ala destabilized Rbf1, suggesting that T356 also affects Rbf1 stability, and that either mutation affects the steady-state levels of the protein by preventing phosphorylation. In this case, the aspartate substitution is unlikely to be phosphomimetic. To test whether phosphorylation affects stability of an Rbf1 T356 mutant, we assayed Rbf1 response to overexpressed Cyc-Cdk with T356D alone or in combination with the three Ser-to-Asp mutations in the IE (Fig. 3C). Strikingly, these two mutant proteins were resistant to Cyc-Cdk-mediated stabilization, consistent with a model in which phosphorylation of T356 facilitates phosphorylation of other sites in Rbf1. The
already stabilized Rbf1 3SD mutant became even more abundant upon Cyc-Cdk treatment, suggesting that phosphorylation of additional sites may further contribute to Rbf1 stabilization. Similar results were observed for Rbf1 T356A and 3SA mutant proteins (data not shown). We conclude that Cyc-Cdk stabilizes Rbf1 through two distinct regions; phosphorylation of T356 is required for a full response, and may facilitate subsequent phosphorylation events. Consistent with this model, Cyc-Cdk overexpression induced a shift in the mobility of the wild-type Rbf1 protein, while mobility of a mutant form in which T356 and the three serines within the IE were changed to aspartate did not shift in response to Cyc-Cdk overexpression (Fig. 3D).

**A role for T356 in Cyc-Cdk-mediated inactivation of Rbf1.** The separation of Rbf1 turnover and transcriptional activity with respect to the IE led us to ask whether the regulation mediated by T356 has common effects on Rbf1 repression activity. We measured the transcriptional activity of Rbf1 T356D/A and Rbf1 4D/A in response to Cyc-Cdk overexpression (Fig. 4A, B). These mutants showed robust repression activity, similar to the WT Rbf1. The supposed phosphomimetic T356D change is evidently not sufficient to interfere with E2F interactions and subsequent repression. However, full derepression upon Cyc-Cdk expression was only observed for the T356D mutant, but not for the T356A mutant. Combination of T356 mutations with those removing S728, S760, and S771 generated mutant proteins that were fully functional but not at all responsive to Cyc-Cdk inhibition (Fig. 4A, B). These findings indicate that the T356D substitution probably supplies sufficient negative charge to permit conformations required for further inhibitory phosphorylation. Phosphorylation of T356 appears to play distinct roles in regulation of stability and activity: it is essential for the former, but contributes only tangentially to phosphorylation-mediated inactivation.

**Phosphorylation mediates Rbf1 stabilization independently of the lysine residues in the IE.** Mutations of Ser-to-Asp and Lys-to-Ala within the IE both stabilize Rbf1, therefore we asked whether the roles of these residues might be interdependent, or if they would contribute in an additive fashion to regulate Rbf1 degradation. We compared steady-state levels of proteins with 4KA (stabilizing) mutations to those with 4KA plus 3SA (destabilizing) mutations. In the absence of ectopically expressed Cyc-Cdk complexes, the levels of both were similar, and higher than the wild-type Rbf1 protein (Fig. 5A, B). However, overexpression of Cyc-Cdk kinases led to higher levels of the 4KA mutant, but not the 4KA3SA mutant, indicating that modification of these serines is still possible, and can contribute to protein stabilization independently (Fig. 5C). Together, these data suggest that lysines and serines are involved in distinct regulation mechanisms both of which influence Rbf1 stability in a nonredundant but cumulative manner. We propose that these residues may both be involved in modulating interactions of the Rbf1 IE with an E3 ligase (Fig. 8E).

**Rbf1 responsiveness to Cyc-Cdk-mediated inactivation correlates with Rbf1 potency in Drosophila eye development.** To assess the physiological importance of the phosphorylation sites under developmental settings, we expressed these mutant forms of Rbf1 in eye imaginal discs using an *eyeless*-Gal4 driver system (Fig. 6A). As shown in our previous studies, the wild-type Rbf1 induced mild and moderate eye phenotypes, and the mutant form of Rbf1 lacking the IE had no obvious effect on eye development (Fig. 6B). The T356D mutant, which like the wild-type protein was inactivated by Cyc-Cdk in luciferase...
assays, induced slightly more severe phenotypes than wild-type Rbf1. In contrast, Rbf1 3SD and 4D mutants exhibited very severe phenotypes, including complete loss of the eye. These proteins were noted to be resistant to Cyc-Cdk inactivation in cell culture assays. Similarly, the T356A mutant caused severe to very severe phenotypes. Further mutation of serines in the IE did not appreciably alter this strong phenotype. Overall, the spectrum of eye phenotypes correlates well with the responsiveness of each mutant protein to Cyc-Cdk-mediated inactivation of transcriptional repression, supporting the idea that Cyc-Cdk-mediated phosphorylation is a key mechanism to restrain Rbf1 activity under physiological conditions. We did, however, see evidence that protein stabilization may also play an important role. The stabilized Rbf1 3SD mutant protein had a similar spectrum of eye phenotypes as the destabilized 3SA mutant, however 3SD exhibited a higher percentage of lethality than 3SA. Similarly, 4D, which is expressed at a wild-type level, also caused a much higher percentage of lethality than destabilized 4A (Table 1). These data indicate that in the context of developing tissues, Rbf1 protein levels may also be a critical factor, in addition to the protein responsiveness to the Cyc-Cdk control. Although certain aspects of the phenotypes were similar for different classes of mutant proteins at the level of morphological disruptions, we think that it is likely that individual impacts on gene expression may differ. Molecular assessment of the impact of each of these changes in Rbf1 are necessary to determine whether certain promoter complexes containing Rbf1 are differentially impacted by lesions affecting protein stability.

To assess the activity of these proteins in a distinct setting, we overexpressed Rbf1 in wing imaginal discs using the beadex driver. Consistent with the eye misexpression assay, the wild-type Rbf1 and T356D caused a similar degree of wing deformation. However, the Cyc-Cdk-resistant 3SD mutant exhibited a much stronger wing phenotype with a drastically reduced size (Fig. 6C). In both tissues, introduction of point mutations into serines of the IE resulted in hypermorphic phenotypes, which may reflect reduced cell proliferation and misregulation of apoptotic control genes (R. Mouawad, unpublished observations). Consistent with this view, previous study of effects caused by overexpressed Rbf1 4A mutant in eye and wing imaginal discs suggested that this mutant inhibits proliferation (19). Our data support the idea that Rbf1 responsiveness to the Cyc-Cdk control is critical for normal Rbf1 functioning in tissue development.

**K774 in the IE is critical for response to Cyc-Cdk-mediated phosphorylation.** The hypermorphic 3SD and 4D mutants phenocopy a previously identified mutant K774A, suggesting a potential link between the K774A mutation and effects of phosphorylation (Fig. 6B and C). Therefore, we tested whether K774 is important for the response of Rbf1 to Cyc-Cdk overexpression. We examined the K774A mutant protein level and activity in response to Cyc-Cdk overexpression (Fig. 7A, B). Whereas the wild-type Rbf1 was stabilized and inactivated by Cyc-Cdk overexpression, the K774A mutant showed a muted response, suggesting that the K774A mutation reduces susceptibility of Rbf1 to Cyc-Cdk control of Rbf1 stability and activity. Mutants expressing proteins with combined 3SD or 3SA and K774A substitutions exhibited even stronger phenotypes than 3SD or 3SA alone, suggesting that K774A impacts more than just modifications of the three serines in the IE (Fig. 6B). The K774A phenotype may reflect effects mediated by modification of both N-terminal and IE residues.
DISCUSSION

RB family proteins play significant roles in diverse cellular events, including cell cycle progression, cellular differentiation and apoptosis (1). Similar to the network of post-translational modifications of histone proteins that control chromatin dynamics, as well as those affecting function and stability of transcription factors such as p53, diverse modifications of the RB corepressor are thought to exert multiple levels of control (3). In particular, phosphorylation serves as a key mechanism of transmitting upstream regulatory signals into functional outputs, channeling RB activity into diverse pathways. However, the roles of specific phosphorylation patterns are not yet fully understood, in particular how signals may simultaneously impact protein function and stability. In this study, we focus on the role of control of protein stability of the Drosophila Rbf1 protein, showing how the C-terminal IE allows responses to Cyc-Cdk modifications that affect both stability and activity, aspects known to impact RB family proteins in human disease.

Our study reveals how integration of signaling can influence Rbf1 on multiple levels and through distinct but functionally linked regions of the protein. The N-terminal T356 phosphorylation site functions as a critical switch for Rbf1 protein stability and repression activity, which upon phosphorylation may serve as a priming event for other phosphorylation events, including in the IE (Fig. 8). The modification of T356 may induce a conformational change, similar to that reported for mammalian RB in vitro. In that protein, phosphorylation of the homologous residue (T373) induces a global conformational change, facilitating the N-terminus binding to the pocket domain, thereby blocking pocket domain interactions with the E2F transactivation domain (TD) (36). The presence of this phosphorylation site is sufficient to allow inactivation of RB in human and rodent cells (37). However, the physiological relevance of T373 in the regulation of RB is not clear, as modifications of other residues may also be sufficient to impart functional regulation. Previous studies have not specifically examined whether the functional control of RB through this residue also impacted protein accumulation, although expression levels are affected in some cases by mutation of Cdk sites (37). We found that the T356A mutation severely impaired Rbf1 responsiveness to the Cyc-Cdk control and caused dramatic eye phenotypes, and propose that there are at least two consequences to Cyc-Cdk targeting of additional phosphorylation sites in the Rbf1 pocket domain and the IE: disruption of E2F interactions, and downregulation of E3 ligase binding, which would impact Rbf1 function and stability (Fig. 8A, B). Recent studies indicate that CycD-Cdk4/6 phosphorylation of mammalian RB in early G1 leads to distinct monophosphorylation at multiple sites mediated by CycE-Cdk2 later in the cell cycle, with different functional consequences (38). With respect to protein stability and activity, both Cyc-Cdk complexes have a similar impact on Rbf1 in this study; whether this is a difference seen in the Drosophila system remains to be seen. The overexpression of kinase complexes may enable promiscuous modifications, thus the nature of the kinase complexes that control the functions tested here should be interpreted with caution.

Our previous studies demonstrate that the C-terminal IE in Rbf1 is crucial for both protein turnover and repression activity (21,22). We propose that this multifunctional region serves as a surface for protein-protein interactions that regulate contacts with E2F factors for activity and E3 ligases for turnover. Mutations of the
lysines would both weaken E3 ligase binding to block the degradation pathway and impair E2F binding, attenuating transcriptional repression (Fig. 8). Here, we uncovered a second set of residues in the IE involved in the linkage of stability and activity. Based on our analysis of mutant forms of Rbf1, we propose that phosphorylation of the serine residues in the IE block E3 and E2F associations to stabilize and inactivate the protein respectively (Fig. 8C). The Rbf1 IE, including the three serine residues studied here, is highly conserved in p107 and p130, implying a similar IE-mediated control of stability and activity. Indeed, we have shown that a p107 mutant lacking the IE accumulates in cells to a much higher level than the wild-type p107 (21). The C-terminal regions of RB proteins appear to play essential regulatory roles, for instance, a regulatable interaction between RB and the Marked Box (MB) of E2F1 (17). How general such interactions may be, and whether they occur only in the context of certain E2F partners, is unknown. We propose that Rbf1 may similarly use phosphorylation of residues within the C terminus to modulate E2F contacts, at the same time as such modifications alter the interaction potential with E3 ligases (Fig. 8C). Whether this coupling of turnover and activity is a general property of RB family proteins will be an important avenue for future exploration.

Our previous model of the paradoxical instability/activity relationship observed for Rbf1 suggests that these two processes are tightly linked, and that ubiquitination driven by the IE contributes to both protein degradation and repression activity (22). Here we show that these two pathways can be separated. Ser-to-Asp mutations only affect the Rbf1 degradation pathway, but do not disrupt repression activity. We propose that these mutations specifically block E3 ligase binding to Rbf1 to reduce the level of ubiquitination, but do not disrupt E2F interactions as actual phosphorylation would. The separable effects of mutations in Rbf1 on protein turnover and repression activity suggest that ubiquitination of Rbf1 is not always essential for Rbf1 activity, even though this modification does enhance the protein’s function in some contexts (22). The ubiquitin modification may help recruit transcription cofactors to achieve maximal repression activity on some genes. Alternatively, the proteasome during its association with ubiquitinated Rbf1 may also directly contribute to transcriptional repression. There may be situations in which ubiquitination may be important for tuning Rbf1 activity as well as protein levels. In rapidly dividing cells, where Rbf1 will be required repeatedly over a short period of time, activity, but not turnover, may be controlled by phosphorylation and dephosphorylation of specific residues (such as those in the pocket) so that the protein can be recycled efficiently. In contrast, in differentiating cells Rbf1 may be ubiquitinated and degraded to ensure a rapid depletion when it is no longer needed.

Within the Rbf1 IE, lysine and serine residues appear to have distinct roles in the regulation of the protein; four of these lysines (732, 739, 740, 754) may directly facilitate interactions with both E2F and E3 ligases (Fig. 8C). Whether this coupling of turnover and activity is a general property of RB family proteins will be an important avenue for future exploration.
is in a KDL sequence, it is insufficient to enable phosphorylation-mediated downregulation of the IE’s degron activity when this domain by itself is linked to GFP. More importantly, this lysine, unlike the four mentioned above, cannot be substituted with a similarly charged arginine residue (21), suggesting that it may be modified, similar to the targeting of RB K810 for methylation in response to DNA damage, which blocks subsequent RB phosphorylation (40). Intriguingly, acetylation of mouse p130 K1079, which is equivalent to Rbf1 K774, enhances Cdk4-mediated phosphorylation in vitro (41). This residue of p130 (K1083 in human) has been reported to be mutated in lung cancers (42). Although mutations on this residue were not detected in other studies (43), some mutations on the neighboring residues which may share the same functional roles as K1079 in the IE of p130 have been observed (44). RB family member proteins are traditionally described as tumor suppressors, whose loss of activity contributes to cellular transformation. The dominant effects of mutations within conserved regions of the Rbf1 IE suggest that similar lesions associated with human cancer may represent dominant gain-of-function changes that contribute to disease.

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Retinoblastoma stability and activity

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FOOTNOTES

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The abbreviations used are: RB, Retinoblastoma; IE, instability element; PCNA, proliferating cell nuclear antigen; Cdk, Cyclin-dependent kinase

FIGURE LEGENDS

FIGURE 1. Drosophila Rbf1 is subject to Cyc-Cdk-mediated stabilization and inactivation. (A) Wild-type Rbf1 was coexpressed with CycD-Cdk4 or CycE-Cdk2 in S2 cells and its repression activity was assayed on the PCNA-luciferase reporter gene. Rbf1-mediated repression is reduced by Cyc-Cdk. Bar graph shows average of four biological replicates. (B) The wild-type Rbf1 protein level with or without coexpression of Cyc-Cdk was assayed by Western blotting using anti-Flag antibody in this and subsequent experiments. Rbf1 levels are significantly increased in the presence of overexpressed Cyc-Cdk. Bar graph represents average of eight biological replicates. (C) Rbf1 protein
Retinoblastoma stability and activity

stability is increased with concomitant expression of CyclinE and Cdk2. In this representative experiment, Rbf1 protein exhibited a half-life of 6 hours, vs. 12 hours in the presence of Cyc-Cdk. Similar two-fold or greater differences were noted in five independent experiments. Protein levels were quantitated by photon-capture analysis with a Fuji LAS-3000 Imager and normalized to tubulin levels. In subsequent figures, data represent at least three biological replicates in all luciferase activity and Western blot assays. Error bars indicate standard deviation.

FIGURE 2. Conserved serine and threonine residues critical for Rbf1 protein stability and repression activity. (A) Schematic diagram of the wild-type Rbf1 and sequence alignment of N-terminal region and the IE in Rbf1 and human RB family proteins. The threonine and three serine residues subject to mutagenesis are indicated by numbers (356, 728, 760 and 771). Identical residues are colored in blue and similar residues are in aquamarine. Known phosphorylation sites are indicated in orange. The four lysines in the IE critical for protein stability are shown in boldface. (B) Rbf1 protein expression assayed by Western blot, comparing wild-type (WT), a stabilized 4KA mutant (21), and Ser-to-Asp or Ser-to-Ala mutants affecting serine residues 728, 760, and 771. Phosphomimetic S-D mutations stabilize Rbf1, whereas unphosphorylatable S-A mutations are associated with lower expression levels. (C) Rbf1 transcriptional activities, assayed using the PCNA-luciferase reporter gene. Both 3SA and 3SD mutants retain wild-type repression activity. The 4KA mutant exhibits low activity, as previously shown (21). (D) Responsiveness of 3SD and 3SA to Cyc-Cdk overexpression was assayed on the PCNA-luciferase reporter gene. The activity of Rbf1 WT is significantly decreased by Cyc-Cdk overexpression (P<0.01), while 3SD and 3SA mutants show a more modest but still significant decrease in repression activity (P<0.05). Asterisks indicate p < 0.01.

FIGURE 3. N-terminal T356 is a key residue for the regulation of Rbf1 protein levels. (A) Both Rbf1 mutants with Thr-to-Asp or Thr-to-Ala are destabilized in S2 cells, shown in Western blot assays. (B) Schematic diagram of Rbf1 mutants with the N-terminal Thr-to-Asp mutation and the C-terminal Ser-to-Asp mutations. (C) Rbf1 WT and 3SD levels are increased by expression of Cyc-Cdk, whereas Rbf1 mutants with a Thr-to-Asp mutation (T356D and 4D) show reduced responses. The difference between the fold increase of WT and T356D or WT and 4D under the condition of CycE-Cdk2 overexpression is significant (p = 0.01). Samples were run on different gels and representative data are shown. (D) Stabilization of Rbf1 under conditions of Cyc-Cdk overexpression is associated with direct modification of the protein, and is dependent on conserved threonine/serine residues. Wild-type Rbf1 protein exhibits a mobility shift when run on the PhosTagTM gel system, indicative of phospho-protein, associated with its increased abundance. The Rbf1 4D mutant exhibits no shift, and no significant increase in protein level.

FIGURE 4. T356 is critical for Cyc-Cdk-mediated inactivation of transcriptional repression activity. (A) PCNA-luciferase assays were used to measure Rbf1 repression activity. Rbf1 WT and Rbf1 T356D are inactivated by Cyc-Cdk, whereas Rbf1 4D shows no response to Cyc-Cdk overexpression. (B) Rbf1 T356A is partially resistant to Cyc-Cdk inactivation, whereas Rbf1 4A is completely resistant. Asterisks indicate p < 0.01.
Retinoblastoma stability and activity

FIGURE 5. Conserved serine residues within the IE influence Rbf1 stability independently of the lysine residues in the IE. (A) Schematic diagram of Rbf1 mutants with Lys-to-Ala and Ser-to-Ala mutations in the IE. (B) Western blot of Rbf1 proteins expressed in S2 cells. S-A mutations do not further significantly change level of stabilized Rbf1 4KA mutant. (C) Protein levels of stabilized Rbf1 4KA are further increased by Cyc-Cdk overexpression. However, the mutant with combined K-A and S-A mutations is unresponsive to Cyc-Cdk overexpression. The difference between the fold increase of WT and 4KA3SA under the condition of CycE-Cdk2 overexpression is significant (p<0.05).

FIGURE 6. Dramatic eye developmental defects induced by IE-mutant Rbf1 proteins. (A) Representative *Drosophila* eyes exhibiting wild-type, mild, moderate, severe and very severe phenotypes induced by expression of *rbf1* genes in the eye imaginal disc using an *eyeless* Gal4 driver. (B) Pie charts show percentage of flies exhibiting different eye phenotypes caused by Rbf1 overexpression. A mutant lacking the IE (∆IE) had no observable effect on eye development, while the wild-type Rbf1 (WT) caused a high percentage of mild and moderate phenotypes. The T356 mutant exhibited a slightly stronger phenotype than Rbf1 WT, and other mutants had much more pronounced effects, with two-thirds to three-quarters exhibiting severe to very severe phenotypes. 70-400 flies were scored for each line. (C) Wing developmental phenotypes upon expression of Rbf1 proteins using a beadex driver. Overexpression of wild-type and IE mutant forms of Rbf1 resulted in wing phenotypes whose relative severities matched those observed for the eye phenotypes.

FIGURE 7. K774 influences Cyc-Cdk control of both Rbf1 protein levels and transcriptional activity. (A) Western blot analysis of Rbf1 WT and K774A mutant. The latter is partially resistant to Cyc-Cdk-mediated stabilization. The Rbf1 WT and K774A have similar levels of expression without Cyc-Cdk overexpression. The difference between the fold increase of WT and K774A under the condition of CycE-Cdk2 overexpression is significant (p<0.05). (B) PCNA-luciferase assay of Rbf1 WT and K774A mutant transcriptional repression activity. Activity of the K774A mutant was less affected by Cyc-Cdk overexpression. Asterisks indicate p < 0.01.

FIGURE 8. Model for the phosphorylation-mediated functional inactivation and stabilization of Rbf1. (A) Unphosphorylated Rbf1 binds to E2F1 through Pocket-Transactivation domain (TD) and C-terminal domain-Marked Box (MB) interactions. E3 ligase interactions through the IE depend on specific lysine residues. (B) Phosphorylation of T356 by Cdk induces a partial conformational change that may serve as a priming event for other phosphorylation events in the pocket and the IE. (C) When the C-terminus is phosphorylated, Rbf1 interactions with E2F1 and the E3 ligase are inhibited, leading to inactivation and to stabilization. (D) Lysine K774 in the IE influences Cdk targeting of phosphorylation sites to affect Rbf1 sensitivity toward Cyc-Cdk for stability and activity. (E) Model for distinct roles of conserved lysine and serine residues in IE for Rbf1 stabilization; the positive charge of lysines plays a direct role in interactions with E3 ligase. Upon phosphorylation of conserved serines, this interaction is weakened. Loss of lysines reduces the interaction, while loss of serines would remove the possibility of downregulating the E3-Rbf1 interaction.
Table 1. Lethality caused by overexpression of different forms of Rbf1.

| Strains | Genotype | Ratio |
|---------|----------|-------|
|         | UAS-Rbf1/ey-Gal4 | UAS-Rbf1/CyO |       |
| WT      | 376      | 365   | 1.03  |
| ΔIE     | 208      | 232   | 0.90  |
| T356D   | 150      | 262   | 0.57  |
| 3SD     | 106      | 263   | 0.40  |
| 4D      | 66       | 210   | 0.31  |
| T356A   | 133      | 145   | 0.92  |
| 3SA     | 149      | 262   | 0.57  |
| 4A      | 94       | 112   | 0.84  |
| K774A   | 90       | 256   | 0.35  |
| 3D+K774A | 45      | 133   | 0.34  |
| 3A+K774A | 159     | 292   | 0.54  |

Lethality of flies with the UAS-Rbf1/ey-Gal4 genotype is assessed by calculating the ratio of UAS-Rbf1/ey-Gal4 to UAS-Rbf1/CyO. Crosses with a ratio considerably less than one indicate a deleterious effect on survival.
Figure 2

A

Retinoblastoma stability and activity

B

C

D

Normalized Protein Levels

Normalized Luciferase Activity

 WT 4KA 3SD 3SA

Rbf1 Tubulin

CycD+Cdk4 - - + - - + -
CycE+Cdk2 - - - + - - +
Figure 3

A

B

C

D

Retinoblastoma stability and activity
Figure 5

A

WT

Pocket

Flag

S

SS

KKKK

4KA

Pocket

Flag

S

SS

AAAA

4KA3SA

Pocket

Flag

A

AAAA

B

Normalized Protein Levels

WT

4KA

4KA3SA

Rbf1

Tubulin

C

Normallized Protein Levels

WT

4KA

4KA3SA

CycD+Cdk4

- + -

CycE+Cdk2

- - +

Rbf1

Tubulin
Figure 6

A

Normal  Mild  Moderate
Severe  Very Severe (tiny eye)  Very Severe (no eye)

B

ΔIE  WT  T356D  T356A
3SD  3SA  4D  4A
K774A  3D+K774A  3A+K774A

C

Normal  ΔIE  WT
T356D  3SD  K774A
Figure 7

A

Normalized Protein Levels

| Protein        | WT | + | - | K774A |
|----------------|----|---|---|-------|
| CycD+Cdk4      |    | + | - |       |
| CycE+Cdk2      |    |   | + |       |
| Rbf1           |    |   |   |       |
| Tubulin        |    |   |   |       |

B

Normalized Luciferase Activity

| Luciferase Activity | WT | + | - | K774A |
|--------------------|----|---|---|-------|
| CycD+Cdk4          |    | + | - |       |
| CycE+Cdk2          |    |   | + |       |

* p < 0.05
Figure 8

A

E3

Cdk

Rbf1

TD

E2F1

MB

B

E3

Cdk

Rbf1

TD

E2F1

MB

C

E3

X

Rbf1

TD

E2F1

MB

D

Cdk

K774

Rbf1

TD

E2F1

MB

E

E3

Rbf1IE

Cdk

E3

Rbf1IE
