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***RB1*, development, and cancer**

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Abstract

The *RB1* gene is the first tumor suppressor gene identified whose mutational inactivation is the cause of a human cancer, the pediatric cancer retinoblastoma. The twenty five years of research since its discovery has not only illuminated a general role for *RB1* in human cancer, but also its critical importance in normal development. Understanding the molecular function of the *RB1* encoded protein, pRb, is a long-standing goal that promises to inform our understanding of cancer, its relationship to normal development, and possible therapeutic strategies to combat this disease. Achieving this goal has been difficult, complicated by the complexity of pRb and related proteins. The goal of this review is to explore the hypothesis that, at its core, the molecular function of pRb is to dynamically regulate the location specific assembly or disassembly of protein complexes on the DNA in response to the output of various signaling pathways. These protein complexes participate in a variety of molecular processes relevant to DNA including gene transcription, DNA replication, DNA repair, and mitosis. Through regulation of these processes, *RB1* plays a uniquely prominent role in normal development and cancer.

Keywords

retinoblastoma; stem cells; chromatin; differentiation; epigenetics

Introduction

If there is one lesson to be learned from the preceding three decades of cancer research, it is that there are a large variety of genetic, epigenetic, and chromosomal changes that accumulate in cancer cells. If only a small number of these changes actually drive malignant behavior, the possible combinations of contributing molecular alterations are dizzying. This molecular heterogeneity is perhaps why even the most successful therapeutic regimens fail in a significant fraction of patients, bedeviling efforts to understand and effectively treat cancer. How can this molecular complexity be distilled into important general principles that would advance understanding and predict successful therapeutic strategies?

Confronted by complexity, scientists have traditionally sought refuge in simple model systems. Simple systems are easier to study, and the general principles gleaned are often relevant to the more complicated cases. In the world of human cancer research, perhaps the simplest “model” system is the pediatric eye cancer retinoblastoma. A subset of retinoblastoma cases are clearly hereditary, with susceptibility transmitted as a simple autosomal dominant trait. The remaining cases arise sporadically. Hereditary cases are diagnosed earlier and exhibit tumors in both eyes while sporadic cases are diagnosed later and typically only occur in one eye. Mathematical modeling of the time to diagnosis suggests hereditary retinoblastoma as a one hit phenomenon while sporadic retinoblastoma

is a two hit phenomenon (Knudson, 1971). This now famous “two-hit hypothesis” suggests that genetic mutation of both alleles of a single gene is the cause (Comings, 1973). The molecular cloning of the *Rb1* gene has verified the essential features of this hypothesis (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Mutational inactivation of both *Rb1* alleles is both necessary and rate limiting, but likely not sufficient, for the genesis of retinoblastoma. The molecular etiology of retinoblastoma, therefore, is perhaps the simplest among all human cancers. In addition to its causative role in retinoblastoma, deregulation of the molecular pathway in which *Rb1* functions occurs with substantial frequency in virtually every other type of cancer where it has been examined (Hanahan and Weinberg, 2000; Sherr and McCormick, 2002). Thus the possibility of learning important general principles relevant to cancer from a detailed molecular understanding of *Rb1* has long been its siren song.

The relative genetic simplicity of retinoblastoma, however, belies the significant functional complexity of the *Rb1* encoded protein (pRb). The first cellular function described for pRb, and the most thoroughly studied, is as a negative regulator of the cell cycle (Goodrich et al., 1991). Loss of pRb-mediated cell cycle control is frequently observed in cancer (Malumbres and Barbacid, 2001; Weinberg, 1995). As cancer is a disease of abnormal cell proliferation, it makes intuitive sense that the key function underlying pRb mediated tumor suppression is cell cycle regulation. However, pRb loss also has profound effects on many other cellular processes relevant to cancer including differentiation (Korenjak et al., 2005; McClellan et al., 2007b; Nguyen and McCance, 2005), survival (Chau et al., 2003; Delston et al., 2006; Hallstrom et al., 2009), senescence (Ben-Porath et al., 2005; Liu et al., 2004), and genome stability (Knudsen et al., 2006) to name a few. This functional complexity is mirrored by the variety of molecular interactions involving pRb. *Rb1* protein interacts with a large and steadily growing list of cellular proteins, and an even greater number of genes. Deriving satisfying general cancer principles from the study of pRb thus remains elusive.

The goal of this review is to explore the emerging hypothesis that the core molecular function of pRb is to dynamically regulate the location specific assembly or disassembly of protein complexes on the DNA. In essence pRb serves as an adaptor, physically linking sequence specific DNA binding proteins with other proteins that influence chromatin and DNA in various ways. The pRb protein interactions central to this function are regulated by post translational modification, and these modifications represent the output of different signaling pathways. *Rb1* protein thus integrates the output of signaling pathways and translates them into genome location specific changes in protein complexes that influence a variety of molecular processes relevant to DNA including gene transcription, DNA replication, DNA repair, and mitosis. Through its regulation of these important processes, *Rb1* plays a uniquely prominent role in normal development and cancer.

The pocket proteins

Rb1 is the founding member of a small gene family that also includes the *Rb11* (p107) and *Rb12* (p130) genes. Of these three family members, *Rb1* is clearly the focus of attention because of its well documented function as a tumor suppressor gene. Despite the fact that they share partially overlapping functions, *Rb11* and *Rb12* are rarely mutated in human cancer. Most of the experiments we will review here are focused on *Rb1*. For more coverage of the similarities and differences among the different pocket proteins, the reader is referred to comprehensive reviews on the gene family (Classon and Dyson, 2001; Claudio et al., 2002).

The proteins encoded by these three genes are structurally related with their defining feature being the “pocket,” a structural domain required for their established cellular and molecular

functions. The pocket is composed of two highly conserved and well ordered subdomains separated by a less structured spacer and followed by a similarly unstructured tail (Figure 1). The structure of the sub domains has been solved by x-ray crystallography (Kim et al., 2001; Kim and Cho, 1997; Lee, 2002; Lee et al., 1998; Liu and Marmorstein, 2007; Xiao et al., 2003). Each sub domain bears similarity to the cyclin box fold (Kim and Cho, 1997). Thus the overall structure of the pocket includes tandem cyclin box folds separated by a spacer and followed by an unstructured tail. Cyclin folds are well characterized structures involved in mediating protein-protein interactions. The unstructured carboxy terminal tail of pRb also contributes to intermolecular protein interactions and can make intramolecular contacts with the tandem cyclin fold (Dick and Dyson, 2003; Rubin et al., 2005; Welch and Wang, 1993). These intramolecular contacts compete for access to a tandem cyclin fold protein interaction surface, thereby regulating intermolecular protein interactions. Interestingly, a similar tandem cyclin fold structure is present in the amino terminal half of pRb (Hassler et al., 2007). It too mediates intermolecular interactions with cellular proteins as well as intramolecular interactions with the pocket tandem cyclin fold. The amino terminal tandem cyclin fold thus adds additional protein interaction surfaces through which pRb can function and be regulated.

Threading analysis suggests that all members of the *Rb1* family have an analogous dual tandem cyclin fold structure (Hassler et al., 2007). However, it is clear that p107 and p130 (47% identity) are more closely related to each other than either one is to pRb (<21% identity). The greatest degree of similarity is within the pocket tandem cyclin fold (30–40% identity). The amino terminal tandem cyclin fold and the unstructured spacers and carboxy terminal tails are not as well conserved (10–20% identity). Differences in the dual tandem cyclin folds as well as the unstructured regions that make intramolecular contacts with the tandem cyclin folds conceivably could account for some of the functional differences between the pocket proteins. However, why pRb is often mutated in human cancer while p107 and p130 are not is an unresolved question.

It is clear from structure/function studies conducted over the last two decades that both the tandem cyclin folds and the unstructured tail that comprise the pocket are required for activity in cell culture based assays that have been used to define various aspects of pRb cellular function, including assays relevant to the cell cycle, differentiation, and apoptosis. The amino terminal tandem cyclin fold domain of pRb is much less studied, likely because it is generally not required for activity in such assays. However, there is evidence that the amino terminal tandem cyclin fold is required for normal function *in vivo*, although this conclusion is controversial (Goodrich, 2003). A small number of *Rb1* missense point mutants have been identified from retinoblastoma patients that specifically affect the amino terminal tandem cyclin fold while sparing the carboxy terminal pocket. There are also a number of cellular proteins that interact specifically with the amino terminal tandem cyclin fold, some of which also play a role in functions regulated by pRb. It seems likely that additional amino terminal tandem cyclin fold interacting proteins will be discovered as more study is devoted to this domain. Finally, the amino terminal tandem cyclin fold is conserved among pocket protein orthologues in different species (Hassler et al., 2007).

The multiple protein interaction surfaces available within the overall pRb structure suggest it can interact with more than one protein at a time, making it ideally suited to serve as an adaptor. Function as an adaptor molecule is also in keeping with one of the defining characteristics of the pocket proteins, their ability to interact with a large variety of cellular and viral proteins (Goodrich, 2006; Morris and Dyson, 2001). Over 150 different pRb protein interactions have been documented in the literature to date, although the biological relevance of most of them has not been validated. The proteins that p107 and p130 interact with have not been well characterized. Nonetheless it is clear they can interact with some,

but not all, of the proteins that interact with pRb. A significant majority of the proteins that interact with pRb are involved, in some way, in the regulation of RNA transcription and gene expression. Other protein interaction partners have roles in different processes involving DNA including DNA replication, chromosome condensation, and DNA repair. Thus it is likely that the pocket protein adaptor is a tool used in a number of biological contexts where location specific changes in protein complex assembly or activity are important.

The pRb/E2F cell cycle switch

The hypothesis that pRb functions as an adaptor is based on the paradigm of its well characterized interaction with the sequence specific DNA binding E2F transcription factors and the role of this complex in regulating cell proliferation. *E2F1* is the founding member of the E2F gene family (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992); the family is currently comprised of eight structurally related transcription factors, five of which (E2F1-5) interact with the pocket proteins (Chen et al., 2009a). E2Fs have traditionally been sub-classified as either transcriptional activators or repressors. Of the E2Fs that interact with pocket proteins, E2F1-3 are activators. They share a winged-helix DNA binding domain, a leucine zipper, a transcriptional activation domain, and marked box domains that specify binding to their obligate heterodimerization partners, the DP proteins (*TFDP1-3*). Within the transcriptional activation domain is a region required for pocket protein interaction. *E2F1-3* proteins are most commonly found in complex with pRb, although p107 and p130 can bind E2F1-3 under certain conditions (Lee et al., 2002). *E2F4-5* proteins are classified as transcriptional repressors. They have an overall structure similar to E2F1-3, but lack a nuclear localization signal and instead contain a nuclear export signal. Localization to the nucleus appears to depend on heterodimerization with DP proteins and interaction with pocket proteins. E2F4-5 are most frequently found in complex with p107 and p130, although pRb can also be found in complex with E2F4. The sub-division of E2F1-5 into activators and repressors is based largely on in vitro studies, reporter gene assays for example, and may not always reflect their actual role in vivo.

When bound to E2Fs, pocket proteins are able to negatively regulate transcription of E2F target genes by at least two different mechanisms. The transcriptional activation domain of E2F1 interacts with general transcription factors and histone acetylases that are necessary for transcriptional activation (Emili and Ingles, 1995; Hagemeier et al., 1993; Lang et al., 2001). Due to their overlapping interaction surfaces within the transcriptional activation domain, pRb competes with these factors for binding E2F thereby blocking transcriptional activation (Pearson and Greenblatt, 1997). Pocket proteins can also bind a host of factors that influence chromatin structure including histone deacetylases, histone methyltransferases, histone demethylases, DNA methyltransferases, and histone remodeling complexes (Table 1). In some cases such as the histone deacetylases, binding to pRb appears to be important for recruiting these factors to particular sites on the DNA (Brehm et al., 1998; Luo et al., 1998; Muzumdar et al., 2007). In other cases like the histone H4K20 methyltransferases SUV420H1 and SUV420H2, interaction with pRb appears to stabilize their localization to a specific location rather than to specify it (Gonzalo et al., 2005). pRb can also displace chromatin modifying factors from promoters (Benevolenskaya et al., 2005). For a comprehensive review of pRb interactions with such chromatin modifying factors, see (Frolov and Dyson, 2004). The typical net effect of pRb at these E2F bound promoters is alteration to a more closed chromatin structure that represses gene transcription. E2Fs regulate many genes important for the cell cycle (Ren et al. 2002; Ishida et al. 2001) and are required for a normal cell cycle in some cells (Humbert et al., 2000; Wu et al., 2001). Thus by both blocking E2F transcriptional activation and actively silencing expression of E2F target genes, pRb restrains cell proliferation.

Cyclin-dependent kinases (CDKs) can phosphorylate pRb in vitro. The phosphorylation status of pRb in cells correlates with the cyclic activity of CDKs during the cell cycle, and pRb is phosphorylated on a dozen or more sites that match the consensus target sites for proline directed kinases like CDKs. These observations suggest CDKs are physiological kinases for pRb. Phosphorylation sites are scattered throughout the length of pRb, but invariably cluster in the unstructured regions rather than the cyclin folds (Figure 1). The overall organization of the phosphorylation sites is similar among the different pocket proteins. While less is known about the phosphorylation of p107 and p130, regulation is likely to be analogous to that of pRb (Leng et al., 2002). Phosphorylation inhibits pRb/E2F complex formation (Dymlacht et al., 1994) and pRb-mediated cell cycle arrest activity (Connell-Crowley et al., 1997). How phosphorylation regulates other of the myriad pRb protein interactions is not well known, but most of the interactions studied to date are favored when pRb is relatively unphosphorylated.

High affinity pRb/E2F interaction involves two major contacts. One is between the transcriptional activation domain of E2F and the pocket tandem cyclin fold of pRb. This contact is modulated by two clusters of phosphorylation sites, S608/S612 and T356/T373. S608/S612 is located within the unstructured spacer between the pocket dual cyclin folds, and phosphorylation of these residues promotes an intramolecular interaction between the spacer and the dual cyclin fold that inhibits access to the E2F binding surface (Burke et al., 2010). T356/T373 is located within the linker between the amino terminal and pocket tandem cyclin folds. Phosphorylation of these residues induces an intramolecular interaction between the two tandem cyclin folds, also interfering with the interaction between pRb and the E2F transcriptional activation domain. The other major contact is between the flexible carboxy terminal tail of pRb and the marked box domains of the E2F/DP heterodimers. This contact is also modulated by two clusters of phosphorylation sites, S788/S795 and T821/T826. S788/S795 phosphorylation directly destabilizes one set of interactions while T821/T826 phosphorylation induces an intramolecular interaction between the tail and the dual cyclin fold that indirectly destabilizes the remaining interaction (Rubin et al., 2005).

All cell cycle CDKs that have been tested are able to phosphorylate pRb in vitro, and they do so with some site specificity (Connell-Crowley et al., 1997; Takaki et al., 2005; Zarkowska and Mittnacht, 1997). Other proline directed serine/threonine kinases can also phosphorylate pRb including the p38 stress activated kinase 1 (Hou et al., 2002; Nath et al., 2003), ERK1/2 (Guo et al., 2005), AMP-activated protein kinase (Dasgupta et al., 2009), protein kinase C beta (Suzuma et al., 2002), Aurora B (Nair et al., 2009), Chk1/2 (Inoue et al., 2007), and Raf-1 (Dasgupta et al., 2004). The particular subsets of sites phosphorylated by these kinases are overlapping, but typically different. While some phosphorylation sites appear to be critical for regulation (Connell-Crowley et al., 1997; Knudsen and Wang, 1996), how phosphorylation at individual sites or combinations of sites regulates pRb protein interactions and function has not been clearly defined (Barrientes et al., 2000; Brown et al., 1999; Knudsen and Wang, 1997). While dephosphorylation of pRb may be dynamic and occur throughout the cell cycle, pRb is converted to a largely unphosphorylated form as cells transit mitosis (Durfee et al., 1993; Ludlow et al., 1993).

These observations define the pRb mediated cell cycle switch that currently serves as the paradigm for *Rb1* tumor suppressor function (Figure 2). In its unphosphorylated active state, pRb binds to E2F at the promoters of E2F regulated cell proliferation genes. *Rb1* protein prevents assembly of protein complexes required for transcriptional transactivation and instead assembles alternative complexes that alter local chromatin structure to silence gene expression. Mitogenic signaling pathways impinge on pRb by altering its phosphorylation status. These phosphorylation events alter pRb protein interactions resulting in derepression and activation of E2F target cell cycle genes, including cyclins. CDK activity is enhanced

increased cyclin levels, and increased CDK activity augments pRb phosphorylation forming a positive feedback loop. This positive feedback amplifies the mitogenic signals, inactivates the pRb mediated block to cell proliferation, and commits cells to a round of cell division. The switch is also subject to negative feedback control as the *Rb1* gene itself is subject to transcriptional control by pRb/E2F (Burkhart et al., 2010). Increases in E2F transcriptional transactivation increase *Rb1* expression which, in turn, dampens E2F activity. Positive and negative feedback control makes the pRb/E2F switch bistable (Yao et al., 2008). Negative feedback keeps the initial threshold of mitogenic stimulation necessary to derepress and activate transcription of E2F target genes high. Positive feedback through activation of CDK activity lowers the threshold of mitogenic stimulation required to maintain the switch in an “on” position. Thus the switch tends to exist in either an off or on state, spending less time in intermediate states. As cells transit mitosis, pRb is dephosphorylated. This resets the switch, making mitogenic stimulation necessary again for continued cell proliferation.

It is important to note that relatively normal cell cycles occur in the complete absence of the pocket protein family (Sage et al., 2000). Rather than execution of the cell cycle, their role is in regulating entry into and transit through different phases of the cell cycle. Thus pocket proteins translate the output of signaling pathways into binary go/no go decisions on whether to enter and traverse the cell cycle. This switch is important in many biological contexts that require coordination with the cell cycle including DNA damage responses, cellular differentiation, cell death signaling, contact inhibition, and tissue morphogenesis among others. Loss of pRb alters the dynamics of this switch, making the conditions necessary for cell proliferation in these different contexts more permissive. Permissive cell proliferation is presumably a major reason why pRb loss has profound effects on tumorigenesis.

While the pRb/E2F cell cycle switch is an intuitively satisfying explanation for *Rb1* mediated tumor suppression, there is evidence that the switch is more versatile and can be used to regulate gene expression in biological contexts unrelated to cell cycle control. For example, compound loss of *E2F3* rescues a cell cycle independent defect in neuronal migration observed in *Rb1* null mice, presumably because the pRb/E2F switch regulates genes like neogenin with known roles in neuronal migration (McClellan et al., 2007a). Similarly, compound loss of *E2F3a* rescues a cell cycle independent defect in the differentiation of retinal starburst amacrine cells that occurs in *Rb1* deficient mice (Chen et al., 2007). *Rb1* loss also causes a defect in retinal rod cell differentiation that appears to be unrelated to the cell cycle (Zhang et al., 2004). Interestingly, the pRb/E2F switch is also used to regulate metabolic responses. pRb/E2F regulates insulin secretion by pancreatic β cells through modulation of *KIR6.2* expression, a gene that encodes a K^+ ATP channel that controls membrane polarization (Annicotte et al., 2009). CDK4 is activated in response to glucose in these cells, resulting in pRb phosphorylation, liberation of pE2F, and increased *KIR6.2* transcription. This feedback loop involving the pRb/E2F switch thus contributes to blood glucose homeostasis. Such examples likely under represent the significance of cell cycle independent functions of the pRb/E2F switch as it is often difficult to disentangle direct effects from the indirect effects caused by deregulated cell proliferation. Thus it appears the pRb/E2F switch has been co-opted to regulate gene expression for a number of different purposes. How the pRb/E2F complexes are specifically directed to regulate unique subsets of genes in these different biological contexts remains unknown.

Evolutionary conservation of the switch

E2F and pocket proteins are conserved through evolution, with orthologues present in vertebrates, invertebrates, plants, and unicellular algae. The invertebrate orthologues most thoroughly studied are from *Drosophila melanogaster* and *Caenorhabditis elegans* where the

reduced number of E2F and pocket protein family members has facilitated analysis. Studies of these model organisms have not only verified the evolutionary conservation of the pocket protein cell cycle switch, but have also yielded new potential roles for the switch in DNA replication and cell fate commitment.

Drosophila contains two pocket protein family members (*RBF1-2*), one activating E2F (*dE2F1*), one repressor E2F (*dE2F2*), and one DP partner for E2F (*dDP*) (van den Heuvel and Dyson, 2008). Activating *dE2F1* is essential for normal cell proliferation in flies (Duronio et al., 1995), foreshadowing an analogous cell proliferation requirement for activating *E2F1-3* observed in mouse fibroblasts (Wu et al., 2001). *RBF1* is required for embryogenesis in *Drosophila*, and *RBF1* deficient cells exhibit deregulated *dE2F1* target gene expression, loss of normal cell cycle control, and increased apoptosis (Du and Dyson, 1999). These phenotypes are broadly similar to those observed in mice lacking *Rb1* (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Phenotypes observed in *RBF1* deficient flies can be rescued by mutations in *dE2F1*, in particular mutations that specifically compromise its ability to transactivate transcription (Du, 2000). This observation suggests that the ability of pRb to block E2F transactivation, rather than its ability to actively silence gene expression, is paramount for control of cell proliferation in *Drosophila*. There is also evidence from studies in *Drosophila* that the pRb/E2F switch is responsive to signaling pathways. For example, Notch signaling promotes release of dE2F1 from RBF1, expression of dE2F1 cell cycle target genes, and cell proliferation (Baonza and Freeman, 2005).

Caenorhabditis elegans contains a single *Rb1* like orthologue (*lin-35*), a single DP gene (*DPL-1*), and three possible E2F-like genes (*EFL-1*, *EFL-2*, *F49E12.6*). In worms, the pRb/E2F switch appears to have a less prominent role in the regulation of cell proliferation. *Lin-35* mutant worms have subtle cell cycle defects (Grishok and Sharp, 2005; Ouellet and Roy, 2007), and the canonical pRb/E2F cell cycle switch is only revealed upon combining multiple mutations in switch components (Boxem and van den Heuvel, 2001; Boxem and van den Heuvel, 2002; Fay et al., 2002; Park and Krause, 1999). Rather, studies in *C. elegans* have emphasized a role for the pRb/E2F switch in cell fate commitment. Mutation of *lin-35*, *dpl-1*, or *efl-1* all have similar phenotypes (Ceol and Horvitz, 2001; Page et al., 2001) suggesting they function as a unit to repress gene transcription (Kirienko and Fay, 2007). Thus in worms, it appears the ability of the pRb/E2F switch to actively silence gene expression may be most important for the cell fate commitment phenotypes observed. Of particular interest is that pRb/E2F may be involved in specifying the distinction between germline and somatic gene expression patterns. Germline specific expression patterns in worms are maintained by a number of different mechanisms including epigenetic chromatin modifications and RNA interference (Bender et al., 2006; Fong et al., 2002; Ketting et al., 1999; Sijen and Plasterk, 2003). Mutation of *lin-35* causes a soma to germline transformation in expression pattern that is mediated, at least in part, by chromatin modifying factors like the MES-4 histone methyltransferase (Wang et al., 2005). In *C. elegans*, the pRb/E2F switch appears to facilitate a soma associated gene expression pattern by maintaining repressive chromatin at germline specific genes. Loss of this switch presumably leads to a relaxed chromatin environment permissive for transcription of these germline genes.

The essential features of the pRb/E2F cell cycle switch, including its role in regulating chromatin structure and gene expression, are also conserved in plants (Sanchez Mde et al., 2008; Shen, 2002). *Arabidopsis thaliana* contains a single *Rb1* related gene (*RBR*). As in animals, studies in *Arabidopsis* have reinforced the notion that pocket proteins have context dependent roles in regulating both cell fate commitment and cell proliferation. RNA interference induced knockdown of *RBR* expression causes a defect in the ability of stem cells to commit to a differentiating lineage (Borghi et al., 2010; Wildwater et al., 2005). This

initially results in expansion of the stem cell pool, but ultimately compromises stem cell maintenance causing an arrest of plant development. Increased expression of *RBR* has the opposite effect, decreasing the number of multipotent stem cells due to over commitment to differentiating lineages. While the phenotype of these plants is associated with disruption of the normal patterns of cell proliferation, the effects of *RBR* on stem cells appear to be primarily in cell fate commitment rather than deregulated cell proliferation. Loss of *RBR* also compromises gametogenesis (Chen et al., 2009b; Ebel et al., 2004; Ingouff et al., 2006). In this case, however, defects are attributed primarily to deregulated cell proliferation.

A comparative analysis of the pRb and pE2F function in evolutionarily divergent species reinforces the hypothesis that the pRb/E2F switch functions to control assembly and disassembly of protein complexes at specific regions of the genome. The switch has been adapted for various context dependent uses. A primary function is in the control of gene expression, and the genes it regulates can directly influence cell proliferation, differentiation, migration, and metabolic homeostasis to name a few.

Variations on the switch

The complexity of the pRb/E2F switch is considerable given that the above discussion has summarized a small subset of the literature published on the topic. This complexity brings to mind the principle that evolution often builds upon a good thing. Once a useful molecular tool is built to satisfy a particular need, it is often re-used to solve new problems in different biological contexts. By creating variations on the basic tool, its repertoire of possible functions can be enlarged. As an adaptor, pRb is ideally suited for functional diversity. Increasing the number of sequence specific DNA binding factors it interacts with will increase the variety of locations where it can be recruited. By increasing the variety of proteins it interacts with at those locations, the possible biological effects can be expanded and more finely tuned. The possible combinatorial permutations increase functional diversity further still.

Rb1 protein can interact with a number of sequence specific DNA binding transcription factors beyond E2F including MyoD (Gu et al. 1993), C/EBPs (Chen et al., 1996b), NF-IL6 (Chen et al., 1996a), ATF2 (Kim et al., 1992), Pax8 (Miccadei et al., 2005), CBFA1/RUNX2 (Thomas et al. 2001), Tbx2 (Vance et al., 2010), nuclear hormone receptors (Batsche et al., 2005; Lu and Danielsen, 1998; Singh et al., 1995), and GATA1 (Kadri et al., 2009). In a number of cases, loss of *Rb1* compromises transcriptional activation and differentiation that is dependent on such transcription factors. Thus in contrast to the effects of the canonical pRb/E2F switch in blocking gene expression, pRb can also function as a transcriptional coactivator for lineage specific transcription factors. For example, pRb augments differentiation into muscle cells by binding muscle specific transcription factors like MyoD and augmenting their ability to drive expression of genes required for muscle differentiation and function (Gu et al., 1993; Novitch et al., 1996; Novitch et al., 1999; Schneider et al., 1994; Zacksenhaus et al., 1996). Likewise, *Rb1* can function as a coactivator for the CBFA1/RUNX2 and the C/EBP transcription factors that participate in osteogenic and adipogenic differentiation, respectively (Chen et al., 1996b; Thomas et al., 2001). Importantly, the differentiation defects that result appear to be unrelated to cell proliferation. For example, adipogenesis defects are independent of cell proliferation since compound loss of *E2F4* rescues cell proliferation defects, but does not rescue differentiation defects (Landsberg et al., 2003).

It seems likely that additional interactions between pRb and tissue specific transcription factors necessary for differentiation may exist. *Rb1* deficiency in vivo affects the differentiation of a relatively large number of distinct tissues and cell types. *Rb1* null mice

die in midgestation (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992) with defects in the eye lens, brain, peripheral nervous system, muscle, liver, placenta, and the hematopoietic system (Iavarone et al., 2004; Novitsch et al., 1996; Spike et al., 2004; Tsai et al., 1998; Wu et al., 2003; Zacksenhaus et al., 1996). Conditional *Rb1* loss in adult mice affects the differentiation of additional cell types and tissues including the epidermis (Balsitis et al., 2003; Ruiz et al., 2004), melanocytes (Yu et al., 2003), hair cells (Sage et al., 2005), prostate (Maddison et al., 2004), lung (Wikenheiser-Brokamp, 2004), cerebellum (Marino et al., 2003), pituitary (Vooijs et al., 1998), small intestine (Guo et al., 2009), and retina (Chen et al., 2004; MacPherson et al., 2004; Zhang et al., 2004) among others. The mechanisms responsible for these defects have not been completely defined. While the effects of deregulated E2F and cell proliferation probably contribute, it is possible that disruption of pRb interactions with currently unidentified tissue specific transcription factors also play a part in these phenotypes.

Rb1 function is thus extended by interaction with additional sequence specific transcription factors that recruit pRb to additional genome locations in different biological contexts. The observation that pRb can function as a transcriptional coactivator indicates that the net effect of pRb recruitment is not always to repress transcription. It is currently not clear how pRb functions as a transcriptional coactivator. One possible mechanism is that pRb can bind and block the activity of differentiation inhibitors like EID-1 (MacLellan et al., 2000; Miyake et al., 2000) and Id2 (Iavarone et al., 1994; Iavarone et al., 2004). EID-1 binds and inhibits p300 histone acetylase activity that normally functions as a coactivator for transcription factors like MyoD. When bound by pRb, EID-1 is targeted for proteolytic degradation. In this case, pRb functions as an inhibitor of an inhibitor of a transcriptional coactivator. In a similar way, pRb also inhibits the Id2 differentiation inhibitor. Id2 functions as a dominant negative helix-loop-helix protein that heterodimerizes with basic helix-loop-helix transcription factors. Heterodimerization with Id2 prevents these transcription factors from binding their E box response elements on DNA, thus preventing transcriptional activation. Locally recruited pRb can compete for binding Id2, thus shifting the equilibrium away from the inhibitory Id2/bHLH interaction. Thus pRb recruitment may disassemble inhibitory interactions that prevent transcriptional activation.

It is also conceivable that pRb may coactivate transcription by altering chromatin structure. While many repressive chromatin modifying factors are recruited or potentiated by pRb, it is also possible that pRb may oppose their activity. RBP2 is a histone demethylase specific for di- and tri-methylated H3K4. Tri-methylated H3K4 typically marks chromatin that is permissive for transcription (Christensen et al., 2007; Iwase et al., 2007; Klose et al., 2007). Removal of this methyl mark by RBP2 is expected to tip the balance in favor of repressive chromatin. Consistent with this, knockdown of RBP2 expression facilitates the transcription of genes necessary for differentiation in some contexts (Benevolenskaya et al. 2005). Interaction with pRb displaces RBP2 from promoters of genes such as osteocalcin, thus facilitating transcriptional activation. While pRb interaction opposes transcriptional repression by RBP2 at some promoters, they actually work together to activate transcription at other promoters. For example, binding of pRb and RBP2 to the *BRD2* and *BRD8* promoters coincides with increased expression of both native and reporter genes (Benevolenskaya et al., 2005). The mechanisms underlying this observed transcriptional activation are not currently known.

Such observations make clear that it is currently difficult to predict what the net effect of pRb will be on gene expression. While pRb recruitment typically results in transcriptional repression, there clearly are exceptions where pRb is required for robust transcriptional activation. Given that the preponderance of studies to date examine the effects of pRb/E2F interaction at cell cycle related genes, examples where pRb functions as a transcriptional

activator may be under represented in the published literature. It is not well understood why pRb functions to repress transcription at some locations, but functions as a transcriptional activator at others. What is becoming clear is that the effects of pRb recruitment to a particular location are highly context dependent, probably reflecting the diversity of proteins present within the local environment. Current efforts to map the genome location of pocket proteins, transcription factors, chromatin modifying factors, and histone marks will likely inform these issues, especially when correlated with gene expression profiling.

***Rb1* function beyond transcription**

Studies in *Drosophila* have provided some of the earliest clues that the pRb/E2F switch, or variations of the switch, may be used for purposes beyond transcriptional regulation. While it is clear the switch can influence DNA replication indirectly through the regulation of gene expression, in a number of biological contexts the effects of pRb/E2F loss appear to be too rapid to be accounted for by changes in gene expression. A more direct role for pRb/E2F in DNA replication is suggested by the observation that *Drosophila* RBF and dE2f are found in complex with the origin recognition complex (DmORC) during chorion gene amplification (Bosco et al., 2001). Elimination of the RBF/dE2F complex by *RBF* or *dE2F* mutation increases DNA amplification and genomic replication without detectable effects on E2F target gene expression (Bosco et al., 2001; Royzman et al., 1999). Thus pRb/E2F appears to directly inhibit the activity DmORC.

While it is not well understood how pRb/E2F inhibits DmORC, some possibilities have been suggested. DNA replication initiation during *Drosophila* chorion gene amplification is associated with acetylation of histones at the origin (Hartl et al., 2007). Chromatin structure is known to regulate replication origin activity in *Drosophila* (Aggarwal and Calvi, 2004). Loss of RBF results in the persistence of acetylated histones and increased DNA replication, perhaps due to lack of histone deacetylase activity normally recruited by pRb. This suggests that the pRb/E2F adaptor is used in this case to alter chromatin structure for regulation of DNA replication rather than gene transcription. Other possible mechanisms have been suggested by mammalian systems. For example, pRb interacts with replication factors such as MCM7 and replication factor C, blocking activation of the pre-replication complex (Mukherjee et al., 2010; Pennaneach et al., 2001; Sterner et al., 1998). *Rb1* protein has also been suggested to impede the loading of the DNA replication processivity factor PCNA (Angus et al., 2004). Consistent with these findings, pRb can inhibit DNA replication in vitro, and this pRb mediated activity is regulated by CDK phosphorylation (Gladden and Diehl, 2003; Sterner et al., 1998). Thus targeting pRb to replication origins may impede assembly or activation of protein complexes necessary for initiation and elongation of DNA replication.

Convincing evidence has recently been accumulating that pRb may also play a direct role in mitosis. Loss of pRb has long been known to compromise genome stability, and one component of this instability is reflected in the appearance of aneuploidy. Chromosome losses occur with a frequency three orders of magnitude higher in embryonic stem cells lacking pRb compared to wild type cells (Zheng et al., 2002). Acute loss of pRb in human or mouse primary fibroblasts causes supernumerary centrosomes, aneuploidy, and micronuclei (Amato et al., 2009; Iovino et al., 2006). Both loss of normal spindle assembly checkpoint control and defects in centrosome homeostasis can contribute to such effects. The pRb/E2F switch does regulate a number of genes involved in these processes (Ishida et al., 2001; Ren et al., 2002), and deregulated expression of these genes does contribute to chromosome instability observed upon pRb loss. For example, the mitotic checkpoint gene *MAD2* arrests cells in metaphase by blocking activation of the anaphase promoting complex that is required for destruction of the chromosome cohesion regulator Securin. *MAD2* expression is

controlled by the pRb/E2F switch, and *MAD2* over expression resulting from pRb loss leads to aneuploidy and tumorigenesis (Hernando et al., 2004; Sotillo et al., 2007).

While transcriptional effects of the pRb/E2F switch are clearly important for chromosome stability, pRb also plays a direct, non-transcriptional role in chromosome structure and mitosis. Loss of *Drosophila* RBF causes a defect in normal chromosome condensation in mitotic cells; chromosomes in these animals contain regions of condensed chromatin interspersed with regions of uncondensed chromatin. Both genetic and biochemical data indicate this effect is mediated by direct interaction of pRb with the condensin II complex subunit CAP-D3 (Longworth et al., 2008). RBF appears to be required for optimal loading of CAP-D3 onto chromosomes. This function appears to be specific for CAP-D3 as other condensin subunits localize normally to chromosomes in the absence of RBF. As the two condensin complexes load to distinct regions of chromatin, the specificity of the pRb/CAP-D3 interaction may explain the region specific chromatin condensation defect.

This pRb/CAP-D3 interaction is also observed in human and rodent cells (Longworth et al., 2008; Manning et al., 2010; van Harn et al., 2010). RNAi mediated silencing of *Rb1* in immortalized rat pigment epithelial cells causes a number of subtle mitotic defects including delayed progression through mitosis, an increased intercentromeric distance, premature loss of sister chromatid cohesion, a defect in chromosome congression, and lagging chromosomes in anaphase. These defects are associated with a deficiency in the loading of cohesin and CAP-D3 to centromeric locations. The net effect of these defects is to cause chromosome missegregation at a frequency comparable to that observed in human tumor cell lines, suggesting that chromosome instability upon pRb loss may contribute to tumorigenesis.

Many chromatin regulators contain the so called “LXCXE” amino acid motif that binds pRb through an interaction surface in the pocket tandem cyclin fold. Alanine substitutions at three critical amino acids in this pRb interaction surface disrupts interactions with LXCXE containing proteins, and a knock-in *Rb1* allele containing these mutations has been created in the mouse (Isaac et al., 2006). Mice homozygous for the mutant allele are viable without pronounced cell cycle or differentiation defects. However cells from these mice have subtle defects in chromatin structure, particularly a reduction in trimethylated H4K20 at pericentric heterochromatin. The mutant pRb is unable to bind CAP-D3, and mutant cells show defects in chromosome loading of CAP-D3 and other condensin II components. Because of these defects, mutant cells take longer to complete mitosis and exhibit aneuploidy. Importantly, the mutant *Rb1* allele exacerbates tumorigenesis in *TP53* deficient mice (Coschi et al., 2010). The median time to death is reduced in these double mutant mice, compared to mice deficient only for *TP53*, and tumors from these mice exhibit increased numbers of chromosomal changes.

These observations reinforce the hypothesis that pRb has direct functions in organizing chromosome structure during mitosis, and these functions are independent of the canonical pRb/E2F switch. Importantly, loss of these functions in mitosis can contribute directly to tumorigenesis. While these studies demonstrate that pRb plays a role in recruiting condensin II complexes, other mechanisms of regulating chromosome condensation during mitosis exist (Takemoto et al., 2009). Further, it is clear that pocket proteins are not strictly required for mitosis, but rather contribute to the fidelity of the process. It is currently unknown how pRb is located to particular regions of chromosomes during mitosis to assist in recruitment of condensin complexes, or whether pRb has additional effects on the function of these complexes once recruited to the chromosome. A possibly relevant observation is that pRb is also known to directly interact with the targeting subunits of E3 ubiquitin ligases like SCF^{skp2} and APC^{cdh1} (Binne et al., 2007; Ji et al., 2004) and such interactions are important

for tumorigenesis (Wang et al., 2010). Regulated protein degradation is required for proper execution of mitosis. Thus pRb can recruit biochemical activities that may be relevant to mitosis.

Most of the examples reviewed here have emphasized the effects of pRb on local chromatin structure and gene expression. The effects of pRb deficiency on chromosome structure during mitosis suggest it may also contribute to the organization of chromatin domains over larger regions (Longworth and Dyson, 2009). The emerging roles of pRb in cellular senescence are consistent with this possibility. Cellular senescence is a physiological response to a variety of stresses that results in long term or permanent cessation of cell proliferation. It provides an important barrier to tumorigenesis (Prieur and Peeper, 2008). *Rb1* and *TP53* represent two key tumor suppressor gene pathways that are required for enforcing this senescence response (Courtois-Cox et al., 2008). It is not obvious whether pRb is essential for the initiation or maintenance of senescence, or both. Loss of all three pocket protein family members is sufficient to trigger cell cycle reentry in senescent mouse embryonic fibroblasts (Sage et al., 2003), suggesting they are at least required for the maintenance. Ectopic expression of pRb in some cell lines can induce a senescence response, suggesting pRb may also be involved in initiation (Hinds et al., 1992; Huang et al., 1988; Templeton et al., 1991). The molecular mechanisms underlying pRb's role in cellular senescence are not completely defined, but the pRb/E2F switch appears to be involved. The pRb/E2F switch regulates a subset E2F target genes, particularly those involved in DNA replication, specifically in the context of cellular senescence (Chicas et al., 2010). The regulation of these genes is unique to pRb among the pocket proteins.

Of note is that in order to enforce the stability of the non-proliferative state, cellular senescence is accompanied by global reorganization of chromatin domains. In particular, senescence is associated with the appearance of microscopically visible foci of heterochromatin called senescence associated heterochromatin foci (SAHF) (Narita et al., 2003). These foci are highly enriched for trimethylated H3K9, the methyl-lysine binding proteins HP1, the histone variant macroH2A, chromatin regulators HIRA and ASF1a, and high mobility group A proteins (Funayama et al., 2006; Narita et al., 2006; Zhang et al., 2005). These factors participate in the formation of the transcriptionally repressive facultative heterochromatin at SAHF. A number of the genes within SAHF are E2F target genes, and both pRb and marks of heterochromatin can be found at these genes. As discussed above, pRb can interact with a number of chromatin modifying factors involved in SAHF formation, including the H3K9 methylase SUV39H1, HP1, and histone deacetylases (Nielsen et al., 2001; Vaute et al., 2002).

These observations have led to the hypothesis that pRb can specify the regional organization of chromatin into SAHF during senescence. Recruitment of pRb to specific genomic location potentiates H3K9 methylation that attracts HP1. HP1 recruitment is augmented by local histone deacetylases. HP1 facilitates the formation of local heterochromatin as well as heterochromatin spreading (Verschure et al., 2005). This hypothesis is supported by evidence indicating that loss of pRb changes the dynamics of HP1 localization, the sensitivity of chromatin to Micrococcal nuclease digestion, and changes in histone H1 phosphorylation (Herrera et al., 1996; Siddiqui et al., 2007). These changes are consistent with a more open chromatin structure in the absence of pRb. Thus pRb may participate in the organization of chromatin structure in contexts as diverse as cellular senescence and mitosis.

Rb1, stem cells, and the cell of tumor origin

How does pRb loss initiate retinoblastoma? Given the diversity of pRb function reviewed, the answer is certainly not obvious. As much of this functional diversity is context dependent, it is difficult to learn how pRb loss initiates tumorigenesis without knowing the type of cell it is lost in. Thus it is useful to know the cell of tumor origin. Cancer is thought to be a clonal disease in an evolutionary sense. It arises from a single ancestor cell that undergoes rounds of genetic and epigenetic alteration followed by selection, eventually evolving into a malignant cancer. Thus identifying the ancestor cell of origin for a particular type of cancer is important as it allows identification of the initiating genetic mutations and how those mutations affect the cells most relevant to tumor initiation.

In the case of retinoblastoma, the initiating genetic mutation is known. Despite the simple molecular etiology of retinoblastoma, however, the cell of retinoblastoma origin has remained controversial for more than a hundred years since the initial attempts at assignment based on similarity to photoreceptors (Wintersteiner, 1897). The crux of the problem is that the cell of origin is inferred from the morphological, molecular, and functional properties of the presenting tumors, and retinoblastoma exhibits features of multiple cell types. Recent work has provided support for the hypothesis that a photoreceptor is the cell of origin (Xu et al., 2009), but other work has suggested that an interneuron (Johnson et al., 2007; Laurie et al., 2006) or a progenitor cell is the tumor originating cell (Kyritsis et al., 1984). This is not just due to the mixture of multiple cell types within a heterogeneous tumor, as a recent unpublished examination of human and mouse retinoblastoma suggests individual retinoblastoma cells simultaneously exhibit molecular, cellular, and neurochemical features of multiple cell types (M. Dyer, personal communication). The cells express markers that are specific for progenitor, interneuron, and photoreceptor cells. The expression of such markers is normally mutually exclusive. This suggests it may not be possible to infer the retinoblastoma cell of origin from examination of retinoblastoma cells as they take on the identity of multiple cell lineages. What can be inferred, however, is that retinoblastoma cells have lost the ability to establish or maintain differentiated gene expression patterns associated with these distinct cell lineages. Thus we are left to guess the cell of retinoblastoma origin, but this guess is informed by the knowledge that it will most likely be a cell where pRb loss has the greatest potential to disrupt cell fate commitment and elaboration of lineage specifying gene expression programs.

We have reviewed evidence that supports the hypothesis that the core function of pRb is to serve as a signaling pathway responsive adaptor that regulates the assembly or activity of protein complexes at specific locations in the genome. Most typically, this results in alteration of local and regional chromatin structure to a more closed conformation that is repressive for gene transcription. Based on this, we suggest that a relatively uncommitted stem or progenitor cell is the most likely candidate for the retinoblastoma cell of origin. Loss of pRb is expected to cause a loosening of local and regional chromatin making it more permissive for transcription. We surmise that stem or progenitor cells are more sensitive to such effects as they exhibit a neutral chromatin structure at important lineage specifying genes. This neutral chromatin exhibits histone modifications associated with both permissive and repressive chromatin suggesting it is poised for either transcriptional activation or repression in accordance with cell fate commitment (Hemberger et al., 2009; Mohn and Schubeler, 2009; Sang et al., 2009). Loss of pRb in such cells is expected to tip the balance toward transcriptional activation by preventing stable transcriptional repression. This would potentially allow simultaneous expression of genes that are normally restricted to distinct lineages. Loss of pRb in more committed, differentiated cells would be expected to have less effect on gene expression patterns as permissive or repressive chromatin states are reinforced by multiple redundant mechanisms, some of which may not be impacted by pRb

loss. Thus the relative plasticity of stem or progenitor cells, as reflected in the programming of their chromatin structure, may make them particularly sensitive to pRb loss.

This is not to say that loss of pRb cannot initiate retinoblastoma in lineage committed cells. Indeed, mouse models of retinoblastoma have been created that are comprised of interneurons with a striking degree of morphological, molecular, and functional differentiation (Ajioka et al., 2007). Rather, we suggest the probability of transformation decreases with increasing lineage commitment and differentiation. Of course the lower probability of transformation may be offset by larger numbers of lineage committed cells. One potentially salient observation is that retinoblastoma is never observed in adults, even if they have inherited an *Rb1* mutation (the penetrance of hereditary retinoblastoma is very high, but not 100%). This suggests the cell of retinoblastoma origin exists only during a narrow window of retinal development, past which the cells change or differentiate in such a way as to make them resistant to the effects of *Rb1* loss. This argues against the candidacy of cell types that persist into adulthood for the cell of retinoblastoma origin. While we favor this interpretation, there may be something unique about the pediatric retinal microenvironment that facilitates the genesis of retinoblastoma. In this case, the cell tumor origin persists into adulthood, but the microenvironment cannot support neoplastic transformation and tumorigenesis.

Beyond retinoblastoma, small cell lung cancer (SCLC) exhibits the highest rate of *Rb1* mutation in human cancers, up to 90% (Wistuba et al., 2001). Hereditary retinoblastoma patients do exhibit an increased risk of lung cancer (Marees et al., 2008). Thus *Rb1* mutation may be an initiating event in small cell lung carcinoma. Consistent with this notion, compound loss of pRb and p53 in the mouse lung generates tumors with strong resemblance to human SCLC (Meuwissen et al., 2003). SCLC express genes characteristic of the neuroendocrine lineage like *ASCL1*, synaptophysin, and *NCAM1*. They also express markers of stem/progenitor cells (Meuwissen et al., 2003; Moreira et al., 2010). Interestingly, *Rb1* loss is required for this unique SCLC phenotype as mouse lung tumors retaining pRb are phenotypically adenocarcinomas. Thus in more than one type of human tissue, stem or progenitor cells may be particularly sensitive to loss of pRb in the context of tumorigenesis.

There is a fair amount of current evidence that supports the hypothesis that stem or progenitor cells are uniquely susceptible to pRb loss. As reviewed above, studies in plants and invertebrates have demonstrated that pRb deficiency causes defects in stem cell pool size and cell fate commitment. There are hints that similar effects occur upon *Rb1* loss in vertebrate stem and progenitor cells. For example, *Rb1* null mouse embryonic stem cells show defects in differentiation in vitro (Papadimou et al., 2005). Loss of pRb in mouse trophoblast stem cells causes an abnormal expansion of this stem cell pool (Wenzel et al., 2007). Such defects are not observed when pRb is ablated in more differentiated trophoblast derivatives. Loss of pRb causes stem or progenitor cell related defects in a number of additional mouse tissues including the skin (Ruiz et al., 2004), bone (Gutierrez et al., 2008), small intestine (Guo et al., 2009), and blood (Macleod, 2008). However, the effects of pRb loss on stem/progenitor cells are likely context dependent. *Rb1* status does not apparently affect the rescue of differentiating prostate tissue by transplantation of stem cell enriched embryonic urogenital sinus (Day et al., 2002). Other pocket protein family members, like p107, may also serve the predominant role in regulating stem cells in some biological contexts (Vanderluit et al., 2004).

There is also evidence that stem or progenitor cells are particularly sensitive to pRb loss in the context of neoplastic transformation. In the mouse, the effects of conditional *Rb1* deletion have been examined in a number of different tissues. Typically this is not sufficient

for tumorigenesis, but tumors often do occur when *Rb1* deletion is combined with other mutations, commonly deletion of *TP53*. For example, conditional deletion of floxed *Rb1* and *TP53* alleles using a probasin promoter based Cre transgene causes metastatic prostate cancer (Zhou et al., 2006). Analogous to retinoblastoma, individual prostate tumor cells express markers of multiple lineages, in this case both luminal epithelial and neuroendocrine lineages. The probasin promoter is androgen responsive and maximally expressed in differentiated prostate epithelial cells in post-pubescent males. Despite frequent deletion of *Rb1* and *TP53* and neoplastic changes in these differentiated epithelial cells, lethal tumors first arise in an anatomical region enriched for prostatic stem cells (Zhou et al., 2007). Early neoplastic lesions in this region co-express stem cell, epithelial, and neuroendocrine markers, as do a small number of normal cells that exist there. These observations suggest that prostatic stem or progenitor cells are the cell of origin for the first lethal tumors, and thus that they are more sensitive to neoplastic transformation upon pRb loss than differentiated epithelial cells. In a similar situation, deletion of *Rb1* and *TP53* in the skin causes squamous cell carcinoma that first arises in the hair follicle, an anatomical location enriched with epidermal stem cells (Martinez-Cruz et al., 2008). Early neoplastic lesions in the hair follicle express stem cell markers. It has also been observed that deletion of *Rb1* and *TP53* in brain stem cells, but not mature astrocytes, causes brain tumors (Jacques et al., 2010). The resulting tumors have a relatively undifferentiated neuroectodermal phenotype. Mouse models of osteosarcoma (Berman et al., 2008) and gastrointestinal tract cancer (Kucherlapati et al., 2008) have provided additional evidence consistent with the involvement of stem, progenitor, or immature precursor cells. These observations reinforce the notion that there may be an inverse relationship between the degree of cellular differentiation and susceptibility to loss of pRb.

***Rb1* and tumor progression**

Rb1 is clearly important for the initiation of retinoblastoma and likely for other tumors like SCLC. Mouse models have demonstrated that *Rb1* deletion can contribute to the initiation of tumorigenesis in a wide variety of tissues. For many common human malignancies, however, it is clear that *Rb1* mutation is a rather late event in tumor progression (Burkhart and Sage, 2008). This indicates *Rb1* mutation does not typically contribute to tumor initiation in some cancers. Assuming late *Rb1* mutation is a driver of malignant behavior, why is it selected for late, rather than early, in tumor progression?

One possibility is that a cell type susceptible to tumor initiation upon pRb loss does not exist in a particular tissue, or may be too rare to support a significant probability of tumorigenesis. In such tissues, tumorigenesis is initiated in an *Rb1* resistant cell by other genetic alterations. Once the cell is transformed, however, it may become sensitive to the effects of pRb loss, thus providing selection for acquisition of *Rb1* mutations that further drive tumor progression. A common feature of tumorigenesis is progressive loss of differentiated features. If this is accompanied by increased plasticity in the programming of chromatin structure, then loss of pRb may be expected to have greater effect on gene expression patterns in such cells than in the original cells from which transformation is initiated. This possibility has not been tested as the cell types susceptible to transformation upon pRb loss have not been well characterized in most tissues, and because mouse models that test the effects of pRb loss in pre-existing neoplastic lesions currently do not exist. However, pRb has been implicated in a number of processes that are particularly relevant to the progression of pre-existing tumors. For example, autophagy is induced in hypoxic tumors. The pRb/E2F switch regulates a number of genes important for hypoxia induced autophagy (Polager et al., 2008; Tracy et al., 2007). The pRb/E2F switch also regulates genes involved in angiogenesis (Gabellini et al., 2006). Loss of pRb could thus alleviate hypoxic stress as tumors grow and also influence the responses to that stress. We have noted above that pRb functions to

enforce cellular senescence and the fidelity of mitosis. Increased chromosome instability and loss of senescence responses in the absence of pRb will also facilitate tumor progression.

Another possibility is that there may be selection against *Rb1* mutation early in tumorigenesis that is ultimately relaxed later in tumorigenesis. This would suggest a paradoxical oncogenic role for pRb in early stages of tumorigenesis in some tissues. Surprisingly, there is experimental precedent for such an oncogenic role for pRb. E2F1 activity is a well known mediator of apoptotic cell death in response to pRb loss (Tsai et al., 1998), and the regulation of E2F1 mediated apoptosis may be a normal physiological function of pRb during the DNA damage response (Inoue et al., 2007; Markham et al., 2006). E2F1 mediated apoptosis appears to be regulated by a unique interaction surface in the pRb carboxy terminal tail (Dick and Dyson, 2003). Thus in some contexts, pRb mediated suppression of apoptosis may facilitate the survival of neoplastic cells, particularly since they are prone to DNA damage (Halazonetis et al., 2008). Consistent with this, constitutive activation of pRb in the mouse mammary gland initiates tumorigenesis (Jiang and Zacksenhaus, 2002). Further, pRb appears to be required for transformation by some oncogenes, in some cases (Williams et al., 2006).

Summary

Here we have reviewed the hypothesis that the core function of pRb is as a signaling pathway responsive adaptor that facilitates the assembly or disassembly of location specific protein complexes on the DNA. *Rb1* protein interacts with a number of different sequence specific DNA binding factors that determine its location. The presence of pRb affects the composition or activity of protein complexes at those locations in a variety of ways. It can recruit new proteins to the location or stabilize their occupancy. The primary example of this is the recruitment of chromatin modifying factors like histone deacetylases. Recruitment of such factors results in the alteration of chromatin structure, typically to a closed state that is repressive for transcription. *Rb1* protein can also displace proteins from a location, or inhibit the activity of a protein at that location. In this way pRb can function as a transcriptional coactivator by blocking the effects of transcriptional inhibitors. In sum, pRb is a major regulator of chromatin structure that establishes gene expression patterns necessary for cell cycle control, cell fate commitment, cellular differentiation, and development. This function is presumed to be a key reason why *Rb1* mutation has such profound effects on normal development and cancer.

While the function of pRb as a programmer of chromatin structure is a major theme, another theme is the diversity of pRb function. The pRb adaptor is a tool that has been co-opted for use in many disparate biological contexts relevant to DNA metabolism. For example, the pRb adaptor is not only used to control local chromatin structure at individual genes, but it has also been adapted for use in the regional organization of chromatin structure that is important for mitosis and cellular senescence. The pRb adaptor has also been used to influence DNA replication and DNA repair, either indirectly through modulation of chromatin or directly by modulating the protein complexes that carry out these biochemical processes. As an adaptor, the range of its potential functions is quite large given the number of DNA binding factors it interacts with, the number of DNA modifying proteins it interacts with, the number of signaling pathways that impinge upon these interactions, and the potential combinatorial permutations.

An important implication that follows from this functional diversity is that the consequences of pRb loss will be highly context dependent. As an adaptor, pRb function will be largely defined by the subset of proteins available for interaction in a particular cell or sub-cellular location, and the signals impinging on that cell which influence pRb post-translational

modification. The state of chromatin in a cell at the time of pRb loss will also heavily influence the resulting effects. Chromatin structure is regulated by multiple and redundant mechanisms. As cells become committed to a particular differentiated cell type, their chromatin tends to become progressively fixed to stably maintain the necessary gene expression pattern. Thus the impact of pRb loss on gene expression is likely to be greater in a cell with a more plastic chromatin state. The diverse, context dependent functions of pRb suggest there may be no universal mechanism of pRb mediated tumor suppression. How pRb loss impacts tumorigenesis is likely to differ in different tissues.

An important motivation for studying *Rb1* is that it will result in effective new approaches for the diagnosis and treatment of a range of human cancer. This has not been realized as quickly as once hoped, possibly because of the large diversity of pRb function. However, with an increasing understanding of the core pRb functions, the context dependent variations on these functions, and the increasingly sophisticated tools available to study these functions, this goal remains in sight. Another key motivation for studying *Rb1* is the possibility of discerning important general cancer principles from the study of a case with simple molecular etiology. From one perspective, the dizzying functional diversity of pRb renders this a false hope. From another perspective, what we have learned about *Rb1* is echoed in important principles emerging in the field of cancer research. Both the developing fields of cancer stem cells and cancer epigenetics (Gupta et al., 2009; Maenhaut et al., 2010; Sharma et al., 2010) are directly relevant to *Rb1*. Perhaps, we are beginning to see the forest for the trees.

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Figure 1. Structural organization of the *Rb1* encoded protein

The schematic identifies major structural and functional elements including the “pocket,” the dual tandem cyclin folds (A1+B1, A2+B2), the unstructured tail (C), and threonine (T) and serine (S) residues whose phosphorylation regulates intermolecular and intramolecular protein interactions. The colored regions mark the positions of the five alpha helices that are characteristic of cyclin folds. Not shown are additional structural elements (i.e. alpha helices) that contribute to overall pRb structure. The schematic is approximately to scale.

Figure 2. The pRb/E2F cell proliferation switch

The pRb/E2F switch is bistable, existing primarily on one of two states, either permissive or non-permissive for cell proliferation. Bistability is established by both positive and negative feedback loops (see text). In its unphosphorylated state, pRb complexes with E2F transcription factors at gene promoters, blocks the ability of E2F to recruit transcriptional coactivators like histone acetylases (HAT), and recruits corepressors like histone deacetylases (HDAC). These coactivators and corepressors alter chromatin to a more open or condensed states, respectively. Mitogenic kinases phosphorylate pRb, altering its ability to interact with E2F and transcriptional corepressors. The resulting transcriptional derepression of genes like cyclins increases cyclin dependent kinase activity (CDK). Increased CDK activity, in turn, augments pRb phosphorylation thus creating a positive feedback loop that amplifies the mitogenic signal and triggers commitment to a round of cell division. Negative feedback is provided by the fact that the *Rb1* promoter is itself regulated by E2F. As cells transit mitosis, pRb is dephosphorylated, resetting the switch to a non-proliferative state.

Figure 3. Stem/progenitor cells and sensitivity to pRb loss

The figure summarizes the hypothesis that there exists an inverse correlation between differentiation and sensitivity to pRb loss. Stem/progenitor cells are relatively undifferentiated and have a neutral chromatin state at key lineage specifying regulatory genes. Chromatin at such genes is characterized by the simultaneous presence of protein complexes and chromatin marks characteristic of both open (shapes marked +) and closed chromatin (shapes marked -). pRb is typically associated with factors that create a closed chromatin state repressive for transcription (although there are notable exceptions). Loss of pRb in such cells is expected to have a large effect on gene expression as it will tip the balance in favor gene expression. In more differentiated cells, chromatin is more rigidly committed to an open or closed state by multiple, redundant mechanisms. Loss of pRb in such cells is expected to have less effect on the expression of stably repressed genes as there are redundant mechanisms in place to maintain the closed chromatin state.

Table 1pRb interacting genes products involved in chromatin regulation¹

Gene ²	Biochemical activity; role in chromatin biology	References
HDAC1, 2, 3	Histone deacetylase; transcriptional repression	(Brehm et al., 1998; Lai et al., 2001; Lai et al., 1999; Luo et al., 1998; Magnaghi-Jaulin et al., 1998)
SIRT1	NAD-dependent protein deacetylase; transcriptional repression	(Wong and Weber, 2007)
ARID4A (RBP1)	Component of Sin3-histone deacetylase complexes; imprinting, transcriptional repression	(Defeo-Jones et al., 1991; Fattaey et al., 1993)
PA2G4 (EBP1)	RNA binding protein, component of HDAC containing complexes; transcriptional repression	(Xia et al., 2001)
EP300	Histone acetyltransferase; transcriptional activation	(Chan et al., 2001)
KAT5 (TIP60)	Histone acetyltransferase; transcriptional activation	(Leduc et al., 2006)
SUV420H1, 2	Histone H4 methyltransferase; organization of heterochromatin	(Gonzalo et al., 2005; Isaac et al., 2006)
SUV39H1	Histone H3 methyltransferase; organization of heterochromatin, transcriptional repression	(Nielsen et al., 2001; Vandel et al., 2001)
RBBP5	Component of the MLL1/MLL Histone H3 methyltransferase complex; transcriptional activation	(Saijo et al., 1995)
KDM1A (LSD1)	Histone H3 demethylase; transcriptional repression or activation	(Chau et al., 2008)
KDM5A (RBP2)	Histone H3 demethylase; transcriptional repression or activation	(Benevolenskaya et al., 2005; Defeo-Jones et al., 1991; Fattaey et al., 1993)
DNMT1	DNA methyltransferase; transcriptional silencing	(Robertson et al., 2000)
CBX1, 3, 5 (HP1)	Methyl-Lysine binding protein; organization of heterochromatin, transcriptional repression	(Nielsen et al., 2001)
MORF4L1 (MRG15)	Methyl-Lysine binding protein; component of histone acetylase and deacetylase complexes, transcriptional regulation	(Leung et al., 2001)
L3MBTL	Methyl-Lysine binding protein; nucleosome compaction	(Trojer et al., 2007)
RBBP7 (RpAp46)	Histone binding protein; component of multiple chromatin assembly, remodeling, and transcriptional repressor complexes	(Qian and Lee, 1995)
RBBP4 (RpAp48)	Histone binding protein; component of multiple chromatin assembly, remodeling, and transcriptional repressor complexes	(Qian et al., 1993)
SMARCA4 (BRG1)	ATP-dependent helicase, component of the SWI/SNF chromatin remodeling complex	(Dunaief et al., 1994)
SMARCA2 (BRM)	ATP-dependent helicase, component of the SWI/SNF chromatin remodeling complex	(Strober et al., 1996; Trouche et al., 1997)
NCAPD3	Subunit of the condensin II complex, organization of chromosome condensation and cohesion during mitosis	(Longworth et al., 2008; Manning et al., 2010)
SMC4	Part of the ATPase subunit of condensin complexes, chromosome condensation during mitosis	(Longworth et al., 2008)
RBBP8 (CtIP)	Interacts with CTBP/Polycomb transcriptional repressor complexes	(Dahiya et al., 2001; Meloni et al., 1999)

¹ This list is restricted to proteins where evidence supports direct binding to pRb. Additional proteins with roles in chromatin biology interact with pRb indirectly as part of multi-protein complexes. Other chromatin modifying complexes appear to interact primarily with other members of the pocket protein family (i.e. DREAM and LINC complexes).

² Common alias in parenthesis.