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Role of the Retinoblastoma Tumor Suppressor Protein in Cellular Differentiation

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Abstract The retinoblastoma protein (pRb105) is a true tumor suppressor as deregulation of the Rb pathway by either mutation of pRb105 itself or other proteins in the pathway, such as p16INK4a, occur in most cancers. This prototypical family member, along with the related p107 and p130, are involved in the control of cell cycle regulation, but pRb105 has also been shown to be involved in tissue development and differentiation. This prospective will discuss the increasing evidence for a role of pRb105 in cellular differentiation and the fact that various cancers, which contain mutant pRb105, or mutations in proteins in the pRb105 pathway, are perhaps a result of deregulation of differentiation. J. Cell. Biochem. 94: 870–879, 2005. © 2005 Wiley-Liss, Inc.

Key words: Rb; tumor suppressor; cellular differentiation

The *Rb* gene family consists of three members, *pRb105*, *p107*, and *p130*, all known to encode proteins that share extensive homology in a conserved domain interface termed the "pocket" region [Harbour and Dean, 2000a]. Collectively referred to as pocket proteins, pRb105, p107, and p130 are believed to function primarily as regulators of the mammalian cell cycle. Initial insights into the role of pocket proteins were provided by studies demonstrating that viral oncogenes such as the adenoviral E1A, SV40 large T antigen, and the high risk human papillomavirus E7 proteins can target pRb family members. Significantly, the oncogenic potential of these viral proteins correlates with their ability to bind to and disrupt the pocket regions of pRb105, p107, and p130 [Dyson, 1998]. Subsequent experiments revealed that over-expression of all three pocket proteins in cells can induce growth arrest in the G_1 phase of the cell cycle [Dyson, 1998]. These early studies helped define the role of the *Rb* gene family as suppressors of cellular growth and proliferation. However, it is becoming clear that one member of the family, pRb105, is also involved in development and differentiation of various tissues, making this member of the family somewhat unique. Initially, a brief discussion of pRb105 and control of the cell cycle will be followed by a prospective on pRb105 in cancer and differentiation.

pRb105 AND CELL CYCLE CONTROL

The Rb family have overlapping functions in the control of cell cycle progression and perhaps the most well described molecular characteristic of pocket proteins is their common ability to interact with the E2F family of transcription factors [Dyson, 1998]. The E2F family comprise a group of at least 7 DNA binding proteins that coordinate the expression of various genes important for cell cycle progression [Dyson, 1998]. E2F sites are found in the promoter region of many if not all genes required for cellular proliferation. In addition, ectopic expression of E2Fs can initiate DNA synthesis [Dyson, 1998]. The association of Rb members with E2F factors typically results in the inhibition of E2F activity, and repression of cell cycle regulated

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gene expression [Dyson, 1998]. Thus the anti-mitogenic characteristic of pocket proteins is at least partially linked to E2F binding.

Because pRb105 was found to interact with the E2F transactivation region, it was initially assumed that inhibition of E2F activity occurred via biophysical interference of the E2F transcriptional domain [Ross et al., 1999; Lee et al., 2002]. However, with recent advances in chromatin biology, it has become increasingly clear that transcriptional events are also regulated by co-factors that remodel chromatin structure. Accordingly, pRb and its relatives can bind to co-repressors with known chromatin remodeling activity. These include histone deacetylases (HDACs), histone methyltransferases, DNA methylases, and DNA remodeling ATPase [Zhang and Dean, 2001]. Pocket protein recruitment of such enzymes to E2F factors can lead to the formation of an active repressor complex that facilitates the condensation of surrounding nucleosomes [Stevaux and Dyson, 2002]. Consequently, the promoter chromatin is rendered less accessible to other transcriptional activators and gene expression is repressed.

The anti-proliferative function of pocket proteins and their ability to repress E2F activity are highly regulated by post-translational modification. pRb105, for instance, contains up to 16 putative phosphorylation sites with 5 of these detected in vivo [Lees et al., 1991], while the phosphorylation patterns of p107 and p130 remain to be fully mapped. Although the function of individual site phosphorylation remains unclear, such protein modifications are believed to modulate the interactions occurring between pocket proteins and various biochemical targets. Importantly, E2F binding is inhibited when pocket proteins are phosphorylated, a status that oscillates throughout the cell cycle [Dyson, 1998]. This is best exemplified by the regulation of pRb105, which remains in an active hypophosphorylated state that promotes stable E2F binding and growth inhibition in the G_0 or G_1 phases of the cell cycle. As cells progress into S phase, pRb105 gradually becomes hyperphosphorylated, thus releasing E2F activity and inducing DNA synthesis.

The phosphorylation of pRb105 as well as p107 and p130 is mediated primarily by cyclin-dependent kinases (CDKs) [Lin et al., 1991; Hinds et al., 1992]. These include the cyclin E-CDK2 and cyclin D-CDK4/6 complexes that sequentially catalyze pocket protein phosphorylation at multiple sites [Harbour and Dean, 2000b]. These kinase complexes are in turn inhibited by the Cip1/Kip1 and INK4A class of cyclin-dependent kinase inhibitors [Vooijs and Berns, 1999]. Dephosphorylation of pRb can be performed by the protein phosphatase 1 (PP1) under various anti-mitogenic conditions [Durfee et al., 1993; Ludlow et al., 1993]. As such, pocket proteins are linked to a complex network that controls cell cycle progression, further delineating a growth-suppressing pathway that converges on *Rb* family members. This model is supported by studies in which the cytostatic effects of upstream components of the *Rb* pathway such as the CDK inhibitor $p16^{INK4A}$, also requires intact pocket protein function [Lukas et al., 1995; Medema et al., 1995].

One important correlate of the "linear *Rb* pathway" hypothesis is that *pRb105*, *p107*, and *p130* may possess genetically redundant or overlapping functions, particularly with regards to cell cycle control. The in vitro phenotype of murine cells in which *Rb*, *p107*, and *p130* have been individually or collectively knocked-out, has provided partial evidence for this model. Germline deletion of *pRb105* results in a shorter G_1 phase of the cell cycle but does not completely ablate growth restriction, as $pRb105^{-/-}$ fibroblasts are still responsive to certain arrest signals such as contact inhibition [Jacks et al., 1992; Lee et al., 1992]. While $p107^{-/-}$ and $p130^{-/-}$ cells do not display any cell cycle defects [Cobrinik et al., 1996], $p107^{-/-}/p130^{-/-}$ double knockout cells possess a hyperproliferative phenotype similar to that of *pRb105* null fibroblasts [Classon and Dyson, 2001]. Ultimately, targeted inactivation of all three *Rb*-related genes is necessary to cause complete abrogation of G_1 arrest and promote cellular immortalization [Dannenberg et al., 2000; Sage et al., 2000]. Taken together, these results indicate that p107 and p130 are redundant for cell cycle regulation, and that their growth inhibitory capabilities overlap with pRb105 in vivo.

Because of their overlapping biological properties, it might be surprising to note that pocket proteins do possess some distinct biochemical characteristics. Indeed, pRb family members have different binding capacities for various E2F partners: pRb105 displays a higher affinity for E2F-1, 2, and 3 during the G_1 phase of the cell cycle, p130 almost exclusively interacts with E2F-4 and E2F-5 during quiescence or G_0,

and p107 binds more readily to E2F-4 but upon S phase [Dyson, 1998]. Additionally, the formation of particular pocket protein/E2F complexes correlates with different expression patterns of pRb105, p107, and p130 throughout the various phases of the cell cycle [Classon and Dyson, 2001]. It has therefore been suggested that pocket proteins can repress different subsets of genes involved in proliferation [Dyson, 1998]. Recent molecular experiments do support this notion, as distinct pRb-containing repressor complexes can assemble at the promoters of different E2F regulated genes in vivo [Takahashi et al., 2000; Wells et al., 2000].

Potentially reconciling these biochemical distinctions with a redundant genetic role in growth arrest, is the remarkable finding that a given pRb-related gene product can functionally compensate for the loss of another. In the absence of *pRb105* for instance, p107 levels are increased and the latter can now partially substitute for loss of *pRb105* with regard to cell cycle arrest. The mechanism behind this compensation is due to transcriptional activation of the *p107* promoter, since it contains E2F sites and thus is normally restricted by pRb105 [Dyson, 1998; Sage et al., 2000]. Another example of functional compensation can be seen in $p107^{-/-}/p130^{-/-}$ cells, where pRb can now preferentially bind E2F-4 due to altered stoichiometric conditions [Mulligan and Jacks, 1998]. Consequently, pocket proteins not only possess intrinsic functions that overlap, but they also acquire compensatory activities under abnormal physiological situations. The combined activity of pRb105, p107, and p130 has been postulated to form the basis of a potent tumor suppressive pathway that safeguards against the onset of carcinogenesis.

pRb105 AND CANCER

Because all three pocket proteins can restrict cell growth in vitro, it has generally been presumed that they can act individually or in concert as suppressors of tumor progression in vivo. Deregulation of the *Rb* signaling pathway is in fact a hallmark of most sporadic human tumors, since mutations in the *pRb105* gene itself or components of the *Rb* pathway are detected in nearly all cancerous specimens [Hanahan and Weinberg, 2000]. Deletions, point mutation and promoter methylation in the $p16^{INK4A}$ gene are found in numerous cancers; amplification of *cyclin D1* characterizes breasts, thyroid, and neck tumors, while mutations in *CDK4* correlates with melanoma [Vooijs and Berns, 1999]. Interestingly, in the majority of glioblastomas, cancers with $p16^{INK4A}$ mutations lack concomitant lesions in *pRb105* and vice versa [Shapiro et al., 1995]. As a result, it has been proposed that the *pRb105* pathway is linear, and not all components of this pathway need be abrogated during tumorigenesis. Significantly, the oncogenic potential of certain DNA tumor viruses correlates with their ability to target pRb105, p107, and p130 [Dyson, 1998], further lending credence to the idea that disruption of all three members is associated with tumor formation.

In spite of the apparent similarities between pocket proteins, however, multiple studies performed in animal models now reveal physiological differences that distinguish pRb105 from p107 and p130 [Classon and Dyson, 2001]. Most noteworthy is the fact that pRb105 is the only pocket protein known to exhibit features of a bona-fide tumor suppressor in vivo. Accordingly, *pRb105* heterozygous mice ($pRb105^{+/-}$) are pre-disposed to the onset of pituitary and thyroid cancer [Clarke et al., 1992; Jacks et al., 1992] and tumorigenesis in these animals is tightly linked to loss of the remaining *pRb105* allele. Conversely, $p107^{+/-}$, $p130^{+/-}$, or even heterozygous; homozygous combinations ($p107^{-/-}/p130^{+/-}$ and $p107^{-/+}/p130^{-/-}$) are virtually devoid of any tumor phenotype [Lipinski and Jacks, 1999]. The absence of tumor pathology is intriguing, given the observation that cells isolated from $p107^{-/-}/p130^{-/-}$ embryos display proliferative defects in culture [Classon and Dyson, 2001]. The lack of any carcinogenic phenotype following loss of *p107* or *p130* may, however, be influenced by genetic background [LeCouter et al., 1998b] and/or the occurrence of functional compensation. This is illustrated once again by the interplay between pRb105 and p107 in a well-characterized mouse tumor model of retinoblastoma. While mutations in *pRb105* are inevitably required for retinoblastoma in children, loss of *pRb105* alone does not cause retinal dysplasia in chimeric mice [Jacks et al., 1992]. $pRb105^{+/-}/p107^{-/-}$ animals on the other hand, develop retinoblastoma at high incidence, exhibiting classical *pRb105* loss of heterozygosity (LOH), and a pathology similar to that seen in the equivalent human disease [Robanus-Maandag et al., 1998].

Although these findings might reflect species-dependent requirements, they also indicate that p107 may yet function as a tumor suppressor, but only in the absence of a functional *pRb105* allele.

With such observations in mind, it now seems clear the Rb family members are not biologically equivalent. Further confounding the picture is the fact that pocket proteins not only regulate the cell cycle, but are also implicated in the control of apoptosis [Chau and Wang, 2003] and cellular differentiation [Lipinski and Jacks, 1999; Vooijs and Berns, 1999]. Which of these biological functions then, might be required to prevent tumorigenesis? Significant insight into this question has been provided from the first heritable models of murine retinoblastoma [Chen et al., 2004; MacPherson et al., 2004; Zhang et al., 2004]. As loss of *pRb105* in the retinal lens causes massive apoptosis, it was initially thought that retinoblastoma arose from cells that harbored additional mutations facilitating cell survival. Recent findings, however, clearly demonstrate that the carcinogenic phenotype observed in pRb105$^{-/-}$/p107$^{-/-}$ mice is attributed to the aberrant expansion of a distinct subset of retinal precursor cells that were naturally resistant to apoptosis and escaped terminal differentiation-associated growth arrest [Chen et al., 2004]. Consequently, although cell cycle control, apoptosis and differentiation are normally coupled, the deregulation of cell-type specific differentiation may be the initiating carcinogenic event, subsequently giving rise to a very distinct hyper-proliferative population of cells. If the differentiation state of certain cell types is more causally linked to cancer, this would then highlight the distinct tumor suppressive activity of pRb105, which, as discussed below, plays a dominant role in the development of many tissues.

pRb105 IN DEVELOPMENT AND DIFFERENTIATION

In addition to revealing a unique role for *pRb105* in tumor suppression, genetic manipulation in animal models has demonstrated a particular requirement for *pRb105* during various stages of metazoan development and cellular differentiation. Germline deletion of *pRb105* in mice causes embryonic lethality between 13 and 15 days of mid-gestation [Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992] whereas *p107*$^{-/-}$ or *p130*$^{-/-}$ mice are seemingly viable [Cobrinik et al., 1996]. There was a high incidence of apoptosis in tissues, particularly in the extra-embryonic tissues of the placenta, the brain and erythropoietic system. Simultaneous disruption of *p107* and *p130* is compatible with embryonic development up to birth, but neonatal death eventually occurs due to the shortened limbs and rib bones of *p107*$^{-/-}$/*p130*$^{-/-}$ mice [Cobrinik et al., 1996]. More recent reports, however, would suggest that the penetrance of *p107*/*p130* null phenotypes is dependent on genetic background [LeCouter et al., 1998a,b]. Be that as it may, the earlier lethal outcome of germline *pRb105* mutations, indicate that pRb105 possesses indispensable functions that do not overlap with p107/p130. Significantly, the distinct developmental anomalies in *pRb105*$^{-/-}$ mice are associated with a lack of specific tissue differentiation. These include defects in erythropoiesis, lens, and skeleton muscle differentiation [Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992]. Recent work where there is tissue-specific knockout of pRb105 has shown that it is also required for the normal differentiation of the skin and in its absence the differentiation is delayed and parakeratosis (persistence of cell nuclei at the top of the epithelium) is present [Balsitis et al., 2003; Ruiz et al., 2004]. Although some of these *pRb105*$^{-/-}$ embryonic tissues can initiate the expression of earlier lineage specific markers, they generally fail to reach a completely differentiated state. In addition, loss of *pRb105* induces ectopic mitosis and pronounced cell death, particularly in cells of the central and peripheral nervous system [Lipinski and Jacks, 1999; Vooijs and Berns, 1999].

Interestingly, elegant experimentation in the mouse demonstrates that some of the abnormalities observed in *pRb105*$^{-/-}$ animals can be attributed to non-cell autonomous functions. In one study, pRb105 defects were attributed to the failure of the extra-embryonic tissues and using different genetic approaches to produce mice with a normal placenta, demonstrated that mice can go to full term [Wu et al., 2003]. The extended survival was attributed to an absence of apoptosis in the brain and erythropoietic survival and is consistent with other work [Lipinski et al., 2001; MacPherson et al., 2003], which concluded that the cell death in *pRb105*$^{-/-}$ mice is partially caused by apoptosis of erythroid cells, leading to hypoxia in the

brain. Nevertheless, in most of these genetic systems, mice did have defects of lens and skeleton muscle differentiation supporting a cell-autonomous requirement for pRb105 in the differentiation of these tissues. Therefore, pRb105 is involved in cell and non-cell autonomous effects on cell survival and differentiation.

Because pRb105 represses E2F-1 transcription, and given that uncontrolled E2F activity contributes to hyper-proliferation as well as programmed cell death, attempts to rescue the *pRb105* null phenotype were performed by introducing a compound mutation of *E2F-1*. The resulting $pR105b^{-/-}/E2F\text{-}1^{-/-}$ mice no longer exhibit many of the apoptotic effects and have an extended lifespan [Tsai et al., 1998]. Moreover, the frequency of tumor formation is reduced in $pRb105^{+/-}/E2F\text{-}1^{-/-}$ mice confirming that E2F is an important downstream effector of pRb105 [Yamasaki et al., 1998]. Nevertheless, a simultaneous *E2F* mutation in the context of $Rb^{+/-}$ does not completely eliminate tumorigenesis and $Rb^{+/-}/E2F\text{-}1^{-/-}$ animals eventually die, owing to additional defects revealed because of their extended, yet still limited, lifespan. These E2F-1 independent abnormalities are most notable during pulmonary and muscular development [Yamasaki et al., 1998]. The disrupted myogenic phenotype is consistent with previous observations made in mice carrying a hypomorphic allele of *pRb105* [Zacksenhaus et al., 1996], and is characterized by the impaired differentiation of muscle cells. Consequently, important function(s) of the pRb105 tumor suppressor are not restricted to the regulation of E2F activity during cell cycle progression.

In vitro tissue culture models also support the notion that pRb105 possesses a unique cell-autonomous role in mediating differentiation. This is best demonstrated by the requirement for *pRb105* during skeletal myogenesis [Gu et al., 1993; Schneider et al., 1994]. Three coordinated yet distinct biological events are known to occur during muscle differentiation in culture. The first involves an initial growth arrest characterized by the upregulation of the CDK inhibitor $p21^{cip1}$ [Halevy et al., 1995; Parker et al., 1995], tightly linked to repression of proliferative genes such as those transactivated by E2F [Sabourin et al., 1999]. This early phase of the myogenic program does not seem to require pRb105, as other pocket proteins can substitute for loss of *pRb105* in inducing growth arrest and $pRb105^{-/-}$ myoblasts are capable of expressing early markers of differentiation [Schneider et al., 1994; Novitch et al., 1996]. Following this acute growth arrest, a more permanent cell cycle withdrawal is considered necessary for the later stages of differentiation. This then renders myotubes conducive to the expression of late markers including the myosin heavy chain (MyHC). In the absence of *pRb105*, cells exhibit delayed expression of late differentiation markers [Schneider et al., 1994; Novitch et al., 1996]. Furthermore, because $Rb105^{-/-}$ myoblasts cannot maintain a post-mitotic state following differentiation cues, they are susceptible to mitogenic re-stimulation, which under these conditions results in apoptosis [Novitch et al., 1996; Zacksenhaus et al., 1996]. Significantly, the aberrant myogenic phenotype associated with *pRb105* ablation does not occur in cells lacking *p107*, *p130*, or even both together [Novitch et al., 1996]. Thus, pRb105 seems to fill a more specific role throughout the later phases of terminal differentiation.

Although tumor suppressor genes have likely evolved to regulate normal tissue homeostasis rather than to exclusively prevent cancer, many believe that it is the specific disruption of their functions during differentiation that accounts for tumorigenesis [Harris, 2004]. Remarkably, several mutant alleles of *pRb105* isolated from tumors corroborate this model. These mutants are defective in inducing differentiation in vitro, yet retain their ability to repress E2F transcription and cause cell cycle arrest [Sellers et al., 1998]. As such, the ability of pRb105 to regulate differentiation does not require stable binding to E2F and is independent of acute G_1/S block. In light of these findings, the identification of E2F independent effectors of pRb is expected to provide novel insight on the link between differentiation and carcinogenesis. Consequently, a slew of studies have provided evidence that pRb105 potentially associates with over 100 different proteins [Classon and Harlow, 2002]. Although the physiological significance of most of these complexes remains to be determined, the existence of such a large number of pRb-containing complexes implies that distinct mechanisms must exist to coordinate pRb105 functions with a wide variety of targets, in response to specific physiological signals.

One potential regulatory process is via post-translational modulation. Multiple phosphorylation events for instance, are known to inhibit

the activity of pRb105. This process has been reviewed extensively in the past, especially in the context of E2F repression [Dyson, 1998]. Another modification of pRb105 has also been observed, where pRb105 is acetylated, although initially the biological outcome was unclear [Chan et al., 2001]. Recently though, work suggests that acetylation may be important for the functional role pRb105 has in differentiation of mouse muscle and human epithelial cells [Nguyen et al., 2004]. This modification is mediated by the co-activators p300 [Chan et al., 2001; Nguyen et al., 2004] and the p300-associated factor (P/CAF) [Nguyen et al., 2004], which target ε-amino lysine residues in C-terminal domain of human pRb105. Importantly, the C-terminus of pRb shares little homology with p107 and p130, and lysine acetylation is not observed in these proteins, providing a basis for the control of pRb-specific functions during differentiation. In addition, the acetylation of pRb105 was required for the permanent arrest of cells and for subsequent activation of transcription which directed late differentiation pathways. Furthermore, inhibition of acetylation does not affect the ability of pRb105 to repress E2F-1 activity or cellular proliferation, and this would be consistent with the fact that repression of transcription could be separated from the differentiation function of pRb105 [Sellers et al., 1998].

Preliminary efforts to determine the precise downstream effectors of pRb105 mediated differentiation have led to an idea that pRb105 can potentiate the activity of tissue specific transcription factors [Lipinski and Jacks, 1999]. This was first postulated when pRb105 was demonstrated to activate the myogenic factor MyoD [Gu et al., 1993]. MyoD is part of a family of basic-helix-loop transcription factors necessary for muscle specific gene expression [Lassar et al., 1989; Davis et al., 1990]. Subsequently, pRb105 was found to co-activate other lineage specialized factors, including the CCAAT/enhancer-binding protein (C/EBP) in models of adipogenesis [Chen et al., 1996] and the osteogenic protein CBFA-1 during bone differentiation [Thomas et al., 2001]. In all of these examples, pRb105-mediated co-activation may be the outcome of its de-phosphorylation, activation, and finally interaction with a given tissue specific transcription factor [Gu et al., 1993; Chen et al., 1996; Thomas et al., 2001]. However, the physiological relevance of such E2F independent complexes remains highly controversial, particularly in models of muscle differentiation [Li et al., 2000].Alternative hypotheses stipulate that inhibitors of muscle specific transcription are sequestered by pRb, leading to "de-repression" of MyoD activity. MyoD inhibitors found in complex with pRb include the class I histone deacetylases (HDACs 1–3). HDACs associate with MyoD under proliferative conditions and co-repress differentiation specific gene expression [McKinsey et al., 2001; Puri et al., 2001]. At the onset of differentiation, HDACs can relocate to pRb105 containing complexes, thus relieving MyoD activity and promoting pRb105 mediated repression [Puri et al., 2001]. Another inhibitor of differentiation is the recently identified E1A-like inhibitor of differentiation 1 (EID-1), a small protein (187 amino acids), which has two acidic domains and a LXCXE Rb binding motif at the C-terminal end [MacLellan et al., 2000; Miyake et al., 2000]. The functions of EID-1 are unclear and the only binding partner apart from pRb105, is the p300 co-activator and short heterodimer partner (SHP), a orphan member of mammalian nuclear receptor family, which can inhibit various nuclear receptors through binding EID-1 and inhibiting co-activation by p300/CBP [Bavner et al., 2002]. One observation is that for normal differentiation of muscle cells, EID-1 is required to be degraded. This degradation appears to be mediated by the ubiquitin ligase, MDM2, which binds to the C-terminus of pRb105, while EID-1 binds to the B-pocket and a complex of pRb105, EID-1, and MDM2 is found in mouse muscle cells induced to differentiate [MacLellan et al., 2000; Miyake et al., 2000]. In addition, pRb acetylation promotes MDM2 binding [Chan et al., 2001; Nguyen et al., 2004] and the subsequent degradation of EID-1 [Nguyen et al., 2004], thus providing a mechanistic explanation for the differentiation-specific regulation of pRb activity by acetylation. Such models favoring an indirect co-activating function of pRb may be of greater physiological relevance, because HDACs and EID-1 are ubiquitously expressed and could restrict differentiation in other tissues. A summary of the components required for muscle differentiation and their functions are presented in the Table I. Many of these components may also be required for the differentiation of other cell types, including keratinocytes.

TABLE I. Determinants of Muscle Cell Differentiation

Protein	Role in differentiation	Mechanism of action
MyoD	Cell arrest and commitment; early/late marker expression	Master muscle-specific transcriptional switch
pRb105	Acetylation dependent cell arrest and commitment; late markers expression	E2F independent transcriptional activation (directly/indirectly) of MyoD transcription
CBP/p300	Cell arrest and commitment; early/late marker expression	Acetylation of MyoD, pRb105 other targets; chromatin remodeling
P/CAF		
Histone deacetylase (HDAC I, II, and III)	Retain cells in cycling state; inhibit expression of differentiation markers	Deacetylate MyoD. Chromatin remodeling to repress transcription; EID-1 has unknown function in inhibiting cell differentiation
E1A-like inhibitor of differentiation (EID-1)		

PERSPECTIVE AND UNANSWERED QUESTIONS

As a prototypical tumor suppressor, pRb105 can fulfill three separate functions: cell cycle regulation, inhibition of apoptosis, and differentiation. Despite the complexity of pRb105 regulation, there is now good biochemical as well as genetic evidence lending credence to the idea that its multiple functions can be independently regulated. This seems particularly true for the role of pRb105 in differentiation. While a complete elucidation of all the functions of pRb105 during differentiation is lacking, it is required for tissue specific development and the functions are separate from its ability to bind and repress E2F transcriptional activity [Sellers et al., 1998; Nguyen et al., 2004]. The other family members, p107 and p130, cannot initiate the permanent arrest of cells and are unable to active differentiation-specific transcription factors. p107 and p130 therefore, seem unable to induce a differentiation phenotype, at least in certain systems such as myogenesis, osteogenesis, and adipogenesis [Sellers et al., 1998; Novitch et al., 1999; Thomas et al., 2001]. Consequently, pRb105 represents a potentially crucial link between the process of tissue specific homeostasis and cancer.

Just how pRb105 stimulates differentiation-specific transcription is uncertain, but there appears to be a requirement for the post-translational modifications of hypophoshorylation and, more recently, acetylation. In addition, given that pRb acetylation occurs at least in part at ε-amino group of lysine residues, it is likely to be highly regulated. What then, is the signal for acetylation during the early phase of differentiation? It may be that pRb105 is in a balanced state between acetylation and deacetylation and that the balance is shifted to acetylation under stimuli that induce the differentiation state, further intimating that pRb could be a substrate for particular deacetylase complexes under non-differentiating conditions. This is supported by work showing that the deacetylase inhibitor trichostatin A (TSA) promotes differentiation of muscle cells [Iezzi et al., 2004], and keratinocytes [Saunders et al., 1999]. An important question that arises from these results is whether the inhibition of pRB105 acetylation is important for the development of cancer. While acetylases like P/CAF are not typically mutated in cancers, the loss or even reduced levels of CBP/p300 has consequences for development and cancer [Iyer et al., 2004]. Since p300 binds P/CAF and has been shown to increase the level of acetylation of pRb105, loss of p300 may have consequences through the pRb105 pathway.

Traditionally, pRb105 has been viewed as a regulator of transcription, and while this might be the eventual output of its function, recent work would suggest that pRb105 also controls protein stability. A growing number of molecular circuits that regulate protein turnover involve Mdm2 [Ganguli and Wasylyk, 2003], and, more importantly, are often deregulated in tumor cells [Chin et al., 1998; Sherr, 2004]. By connecting an Mdm2/EID-1 protein degradation network to the pRb105 tumor suppressive pathway, the data provides additional insight into the relationship between protein stability, differentiation, and cancer. The role of EID-1 in cell cycle and proliferation is unknown, although at the molecular level EID-1 has been shown to bind p300 and inhibit the transactivating function of p300, a known co-activator of MyoD, the major transcription regulator of muscle differentiation. EID-1 binds to the CH1 and CH3 regions of p300, which are domains that bind many transcription factors, and

inhibits the acetylase activity of the co-activator. The inhibition of the acetylation function of CBP/p300 is one obvious mechanism by which EID-1 inhibits differentiation. However, CBP/p300 acetylate other proteins under different conditions and so the wholesale inhibition might be expected to have catastrophic affects for cells, especially as the loss of one allele of CBP/p300 can lead to cancer and developmental abnormalities [Iyer et al., 2004]. EID-1 then represents a potentially significant target of pRb105 function and might be a bonafide oncogene. Over-expression of EID-1 alone is in fact capable of either ablating or delaying cellular differentiation [MacLellan et al., 2000; Miyake et al., 2000]. As such, one might anticipate discovering *EID-1* gene amplifications and/or decreased EID-1 protein turnover in cancerous tissues. In the end, the physiological relevance of molecules that are targeted for degradation by pRb105 remains to be validated in vivo.

Clearly, tumor suppressor genes such as *pRb105* have evolved in order to act as regulators of normal tissue homeostasis rather than to prevent cancer. As such, the pathological outcome of mutations in a given tumor suppressor gene is context dependent. One of the factors that influence carcinogenesis is the developmental or differentiation state of a particular tissue. After all, studies with *pRb105* have taught us that germline mutations do not necessarily recapitulate loss of gene function in somatic cells, especially with regard to cancer phenotypes. Accordingly, *pRb105* and many recently identified tumor suppressor genes are not only involved in cell growth and proliferation, but possess independent functions that are essential for tissue differentiation. It ensues that genes previously characterized in models of metazoan development will likely turn out to play significant roles in the onset of cancer. Finally, multiple biochemical factors are likely to contribute to the overall functions of tumor suppressors such as pRb105, and how their activity is coordinated will likely include tissue specific as well as more conserved mechanisms.

The *retinoblastoma* gene was the first tumor suppressor to be cloned and, as a result, has been the subject of intense experimental scrutiny over the past decade. And yet despite all that that has been said and done, the study of pRb105 function will undoubtedly continue to enlighten our understanding on the fundamental link between tumor suppressor genes, tissue differentiation, and cancer biology.

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