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REVIEW

pRb: master of differentiation. Coupling irreversible cell cycle withdrawal with induction of muscle-specific transcription

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The protein product of the retinoblastoma (RB) gene is necessary for the completion of the muscle differentiation program and for myogenic basic helix–loop–helix-dependent transcription. In fact, in addition to induction and maintenance of permanent cell cycle withdrawal through negative regulation of E2F-responsive genes involved in proliferation, pRb also plays a positive role in the activation of muscle-specific genes. In pRb−/− myocytes, the expression of late myogenic markers is defective and myoblast fusion into myotubes occurs without irreversible cell cycle exit. This evidence demonstrates only a partial functional redundancy between pRb and its relatives p107 and pRb2/p130, as these pRb−/− multinucleated cells, which display p107 levels higher than normal myotubes, respond to mitogens with cell cycle re-entry and DNA synthesis. At the molecular level, pRb myogenic functions are mediated by cooperation with MyoD, Myocyte enhancer factor 2 (MEF2), High mobility group box protein-1 (HBP1) and histone deacetylase1, affecting chromatin configuration and tissue-specific transcription, and by post-translational modification in response to intracellular signaling cascades.

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Introduction

More than 50 years ago, it was observed that a children's tumor occurred sporadically in some patients, but it was inherited in others (Falls and Neel, 1951). This tumor was the retinoblastoma (RB), and it was hypothesized by Knudson (1971), in his 'two-hit hypothesis', that RBs arise as a result of two mutations, which inactivate both the alleles of an RB susceptibility gene. The intensive study to highlight the molecular mechanisms underlying this tumor led to the identification and to the cloning of the *RB1* gene (Friend *et al.*, 1986), which represents a key molecule in cell cycle regulation and the prototype of tumor suppressor genes.

pRb gives its name to the RB family of proteins (pRBs), also referred to as 'pocket proteins', which negatively regulate progression from G0 through to G1 and into S phase. Three members belong to this family, pRb, p130 and p107, which share high homology in the conserved pocket domain through which they bind to cellular factors and viral oncoproteins. The expression patterns of pRBs differ during the G0/G1/S phase transition, with p130 expressed during G0, p107 in S phase and pRb at a fairly steady level throughout the cell cycle (Classon and Dyson, 2001).

The pocket proteins are regulated, in part, via phosphorylation by cyclin/cyclin-dependent kinase (cdk) complexes, including cyclin D/cdk4 (or CDK6), cyclin E/cdk2 and cyclin A/cdk2 (Dyson, 1998) (see Figure 1). The regulation of pRb by phosphorylation is the best understood.

pRb is a nuclear protein, which is hypophosphorylated in resting or G0-arrested cells, when it actively represses proliferation. When cells need to proceed through the cell cycle, mitogenic signals will lead to the subsequent activation of the cyclin D/cdk4 and 6, cyclin E/cdk2 and cyclin A/cdk2 complexes, which will increasingly phosphorylate pRb during progression through G1. pRb will be kept in a hyperphosphorylated state until late in mitosis, leading to its inactivation and degradation. pRb phosphorylation by cdks 4 and 6 is regulated by the cdk inhibitor p16, which ensures that no phosphorylation of pRb will be obtained, until the cell needs to proceed through the cell cycle. Cells possess a complex pathway regulating the G1/S transition, accomplished by several proteins, and the alteration of each of those proteins may be relevant in malignant transformation.

The phosphorylation state of the RB family members is important for their biological activity, as hypophosphorylated pRBs inhibit proliferation through their association with other proteins.

A principal target of pRBs is represented by the E2F family of transcription factors (E2Fs), which regulate proliferation-associated genes (Nevins, 1992). Early

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studies suggested that hypophosphorylated pRBs bind to and inhibit the E2F transactivation domain, and that pRBs phosphorylation releases E2Fs to allow the expression of genes that mediate S-phase entry (Flemington *et al*., 1993; Helin and Ed, 1993). E2F-binding sites are found in the promoters of many genes whose function is important for cell proliferation or whose products drive cell cycle progression (see Figure 1). E2Fs form heterodimeric complexes containing one subunit encoded by the E2F family and a subunit encoded by the DP family. There are seven E2F family members (E2F-1–E2F-7) and two DP family members (DP1 and 2). E2F-1 to E2F-5 associate with RB family members, whereas E2F-6 and E2F-7 act independently of pRBs.

Depending on their role in the cell cycle, the E2F family is often subdivided into activators (E2F-1, E2F-2 and E2F-3a) and repressors (E2F-4, E2F-5 and E2F-6). Although it might seem simplistic, a separation between activators and repressors could be useful, and this distinction is reinforced by the pattern of interactions between E2Fs and pRB family members. E2F-1, E2F-2 and E2F-3a are strong transcriptional activators that are preferentially bound and inhibited by pRb. E2F-1 through -3a are generally absent or expressed at low levels in quiescent cells, and are induced to high levels and stimulate gene expression in late G1 (Dyson, 1998). A distinct E2F-3b isoform is expressed throughout the cell cycle, and is implicated in forming transcriptional repressor complexes with pRb in G0 (Leone *et al*., 2000), and in forming pocket protein-independent repressor complexes in both resting and normally proliferating cells (Aslanian *et al*., 2004; Ginsberg, 2004). In contrast, E2F-4 and the generally less abundant E2F-5 are poor transcriptional activators, in part, owing to their lack of a nuclear localization signal. E2F-4 and -5 are expressed throughout the cell cycle, but in G0 and early G1 they are bound and recruited to the nucleus by p107 and p130, and form transcriptional repressor complexes. The activation of G1 cyclin/cdk complexes leads to phosphorylation of p130 and p107 and to the release of E2F-4 and 5 from E2F-regulated promoters, where they repress transcription. Once E2F-4 and -5 have been released from E2F-responsive promoters, activator E2Fs will allow for the expression of E2F target genes.

The control of gene expression mediated by pRBs can be exerted by their binding directly to the activation domain of the activator E2Fs, consequently blocking the activity of this domain (Flemington *et al*., 1993; Helin and Ed, 1993). Transcription regulation may also be achieved through chromatin modification mediated by pRBs (see Figure 1). Accessibility to a certain promoter by RNA polymerase depends on the chromatin structure, tightly regulated by chemical modifications of histones, which can be targeted by a variety of post-translational modifications, including acetylation, ubiquitylation, phosphorylation, methylation and others (Lachner *et al*., 2003). Such changes are thought to regulate gene expression, and the status of any given nucleosome reflects the concerted action of localized-modifying and de-modifying enzymes. Lysine acetylation and deacetylation are among the best-studied histone modifications. Histones are generally hyperacetylated at the promoters of actively transcribed genes, because of the repulsive forces arising between histones and DNA, following hyperacetylation, which allow a looser chromatin structure. *Vice versa*, hypoacetylated histones are responsible for gene silencing owing to the increased attraction to DNA, resulting in a tighter chromatin conformation, which is then not accessible to the transcription machinery. Many transcription regulators recruit histone acetyltransferase (HAT) and histone deacetylase (HDAC) activities. DNA methylation may also play a role in controlling transcription, although it does not cause a change in the overall charge of the nucleosome. Methylation may represent a signal for the recruitment of repressive enzymes, which maintain promoters in a silenced state. Pocket proteins may contribute to transcriptional repression of E2F-responsive promoters through recruitment of a variety of chromatin-modifying activities to E2F-responsive promoters (Frolov and Dyson, 2004), such as type I HDACs, which contribute to a more compact chromatin structure.

pRb expression is upregulated in many tissue types undergoing terminal differentiation and it is interesting to speculate about the possible role of high level expression of active pRb in regulating tissue-specific transcription factors. There is growing interest in this area following the identification, on the basis of microarray data, of new classes of E2F target genes that are clearly associated with differentiation (Muller *et al*., 2001; Dimova *et al*., 2003). The extent to which pRb regulates tissue-specific differentiation through E2Fs is not entirely known because both pRb and E2Fs have been reported to interact independently with tissue-specific transcription factors. It is not clear how the interaction of pRb with E2Fs affects the interaction of E2Fs with tissue-specific transcription factors or *vice versa*. Briefly, we can summarize the involvement of pRb in several systems of differentiation, including eye lens, brain, peripheral nervous system, muscle, liver, placenta, hematopoietic system, epidermis, melanocytes, hair cells, liver, prostate, lung, cerebellum, pituitary and retina (reviewed by Goodrich, in this issue). In this review, we will focus on the involvement of pRb in muscle differentiation.

pRb gene expression in muscle cells

The combinatorial activity of the muscle-specific basic helix–loop–helix (bHLH) and the MEF2 transcription factors promotes the expression of myogenic genes during the transition from undifferentiated myoblasts to differentiated myotubes (for a review see Puri and Sartorelli, 2000). Transcription is achieved by the MyoD binding to its cognate sequences (E-box, CANNTG), and consequent recruitment of HATs, SWI/SNF chromatin-remodeling complexes and polymerase II-activating kinases (Simone *et al*., 2004b; Giacinti *et al*., 2006) (Figure 1). Furthermore, MyoD activates pRb

and p21 gene expression (Martelli et al., 1994; Halevy et al., 1995) to shoot down the cell cycle machinery. However, cells from embryos in which the *MyoD* gene has been inactivated undergoing terminal differentiation without abnormalities in the program of permanent cell cycle arrest (Parker et al., 1995), indicating that functional redundancy must exist among bHLH factors. As Myf5 has the same range of activity of MyoD in both embryos and cultured cell lines (Weintraub, 1993), it is likely that Myf5 can fulfill the antiproliferative activities of MyoD in MyoD−/− mice. This evidence demonstrates that the interplay between myogenic bHLH, cdk inhibitors and pRb contributes to induce permanent cell cycle exit in differentiating myocytes by counteracting the proliferative potential of cyclin/cdks and pRb targets, E2F family members (see Figure 1).

In MyoD-proficient cells, MyoD stimulates transcription from the *RB* promoter by an E-box-independent mechanism (Martelli et al., 1994). Consistently with this finding, full activation occurs in the absence of the basic DNA-binding domain, whereas the HLH region is required. When differentiation is induced, cAMP-responsive element binding protein (CREB) transcription factor is upregulated and phosphorylated on serine 133, a critical residue for CREB-CBP/p300 association and transcription activation. After that step, CREB is able to recruit a multiprotein complex containing MyoD and the HATs p300 and PCAF on *RB* promoter to induce gene expression (Magenta et al., 2003).

The engagement of HATs draws attention to the balance between acetylation and deacetylation of histone and non-histone proteins that controls gene expression in a variety of cellular processes, with transcription being activated by HATs and silenced by HDACs. An overall histone hyperacetylation following exposure to deacetylase inhibitors (DI) has been observed at certain regulatory loci, supporting the presence of both HATs and HDACs in close proximity within these regions. Indeed, both p300 and HDAC1 were detected by chromatin immunoprecipitation (ChIP) on the promoter region of *CDKNIA* in murine fibroblasts and myoblasts (Simone et al., 2004a). Histone deacetylase1 can suppress MyoD-dependent transcription (Puri et al., 2001), but increasing amounts of relative p300 levels were able to relieve transcriptional repression of MyoD caused by HDAC1 overexpression, with an efficiency comparable to DI (Simone et al., 2004a). The crucial role of the acetylation/deacetylation balance in the transcription of *RB* during myogenesis is underscored by the fact that pretreatment of myoblasts with DI, before induction of differentiation, results in a significant upregulation of *RB* expression (Iezzi et al., 2004).

Post-translational modification modulates pRb function

The MyoD-mediated induction of myogenesis is accompanied by the downregulation of cyclin D1, E and A and the upregulation of cdk inhibitors to impose the permanent cell cycle exit. This step is critical because overexpression of cyclin/cdks has been reported to inhibit the activity of MyoD by different modalities (Figure 1). For instance, overproduction of cyclin E and A, together with their associated kinase, cdk2, inhibit MyoD function through the hyperphosphorylation and subsequent inactivation of pRb (Skapek et al., 1996; Guo and Walsh, 1997). In addition, cdk2 (and cdk1) phosphorylates MyoD on serine 200, thereby

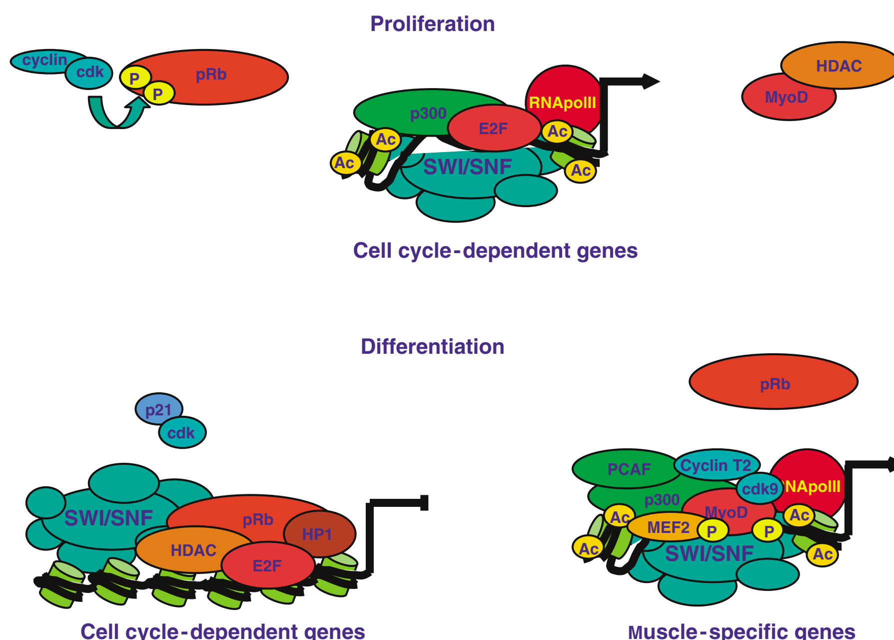

Figure 1 Schematic representation of the transcriptional complexes regulating gene expression in proliferating and differentiating muscle cells.

triggering its degradation (Song *et al*., 1998; Kitzmann *et al*., 1999). Finally, cyclin D1 overexpression promotes the nuclear accumulation of cdk4, which in turn binds the C-terminus of MyoD and prevents its binding to the DNA (Zhang *et al*., 1999a, b). Thus, in differentiating myocytes, MyoD-mediated expression of the cdk inhibitor p21 inhibits the residual cyclin/cdk activity (Walsh and Perlman, 1997), and prevents the formation of E2F complexes containing the kinase cyclin E/cdk2 and cyclin A/cdk2 (Puri *et al*., 1997), which are possibly involved in the initiation of DNA synthesis. The positive feedback loop is then accomplished by the hypophosphorylated form of pRb that co-activates MyoD resulting in further upregulation of expression of p21 and of genes necessary for continued differentiation.

Unlike other cyclin/cdk complexes, cyclin T2a/cdk9 levels and activity are not downregulated during muscle differentiation (Simone and Giordano, 2001). Furthermore, MyoD-mediated recruitment of cyclin T2/cdk9 on muscle-specific regulatory regions activates transcription, whereas inhibition of cdk9 kinase activity prevents the activation of the myogenic program (Simone *et al*., 2002b; Giacinti *et al*., 2005). Intriguingly, cdk9 is able to phosphorylate pRb *in vitro*, and this kinase activity peaks at 96 h after the induction of the differentiation program in C2C12 cells (Grana *et al*., 1994; De Luca *et al*., 1997; Bagella *et al*., 1998). Phosphopeptide analysis of p56/pRb after phosphorylation by cdk9, compared to that mediated by cdk2 and cdk1, indicates that, at least *in vitro*, the three cdks share several target phosphosites, but cdk9 affects only serine residues (De Luca *et al*., 1997). By studying the composition of cyclin T/cdk9 complexes, we identified pRb as a cdk9-interacting protein in murine C2C12 myoblasts, in murine NIH3T3 fibroblasts and in two human tumor cell lines, HeLa and Jurkat. Cyclin T2/cdk9 binds to pRb, involving residues 129–195 of cdk9, the first 642 amino acids of cycT2 and the C-terminal region of the RB protein (835–928), and phosphorylates the pRb region spanning amino acids 793–834. This region contains at least three proline-directed serines (sp), S795, S807 and S811, which have been reported to be phosphorylated *in vivo* and which could be targeted by the cdk9 complex (Simone *et al*., 2002a). At present, we still do not know if this phosphorylation could take place *in vivo*, as for the cdk9-mediated MyoD phosphorylation at serine 37 (Simone and Giordano, manuscript in preparation), but an indirect suggestion of specificity is given by the fact that in the same assays cdk9 fails to phosphorylate histone H1, a common target for cdc2-related kinase family members.

In addition to phosphorylation, pRb undergoes other post-translational modifications, such as acetylation, that affect its biological functions. Both p300 and PCAF can acetylate pRb on the C-terminus (Chan *et al*., 2001; Nguyen *et al*., 2004), and during myogenesis this modification is required for pRb-dependent terminal cell cycle exit and the induction of muscle-specific transcription.

Molecular mechanisms of pRb function

pRb can influence the myogenic program through several mechanisms in addition to the control of E2F activity (see Figure 1). A transcriptional synergism between MyoD and pRb over the course of myogenesis has been reported by several studies (Gu *et al*., 1993; Novitch *et al*., 1996; Sellers *et al*., 1998). It has been demonstrated that expression of pRb is able to significantly upregulate MyoD transcriptional activity and induce expression of late muscle differentiation markers, *MHC* and *MCK* (Gu *et al*., 1993). Expression of either p107 or p130 in pRb-deficient muscle precursors can to some extent augment MyoD transcriptional activity and upregulate expression of *MHC* and *MCK*, but functional pRb is necessary for their full induction. For example, pRb−/− MEFs induced to differentiate into muscle by expression of MyoD are able to express early muscle markers (such as myogenin and MEF2) but have reduced levels of late genes (Novitch *et al*., 1996). The importance of the interactions of pRb with non-E2F factors has been best demonstrated through analysis of pRb mutants incapable of binding E2F family members or induction of G1/S arrest, but still able to augment MyoD transcriptional activity and induce tissue-specific gene expression (Sellers *et al*., 1998). This evidence suggests that at least some of the differentiation-specific targets interact with pRb through mechanisms other than binding to the A/B pocket domain. Whether this effect is the result of a direct interaction with the muscle-specific bHLH or is mediated through the modulation of the cell cycle and apoptosis remains controversial. It is unclear if direct MyoD/pRb interaction takes place in muscle cells (Gu *et al*., 1993). Recently, nuclear magnetic resonance and mass spectrometry studies clearly demonstrated that this interaction does not occur even in an *in vitro* system, where the employment of pure proteins unambiguously demonstrated no direct protein–protein interaction between the pocket domain of pRb and the bHLH region of MyoD (Smialowski *et al*., 2005).

However, pRb is essential in promoting functional cooperation between MyoD and MEF2, which, along with the bHLH family, is required for muscle-specific gene expression (Novitch *et al*., 1999). Without pRb, MyoD induces accumulation of MEF2, which remains transcriptionally inactive, but the requirement for pRb can be partially bypassed by co-expression of MyoD and constitutively active MEF2 (Novitch *et al*., 1999). Moreover, muscle differentiation mediated by MyoD and activated MEF2 is further augmented by co-expression of chimeric E2F1-pRb protein, which blocks cell cycle progression, suggesting that both pRb-mediated induction of muscle gene expression and pRb-dependent cell cycle exit contribute to full myogenesis.

The fact that hypophosphorylated pRb accumulates upon muscle differentiation, exceeding the levels of E2F, suggests that pRb might have other targets during the myogenic program, similarly to some Rb mutants defective in E2F binding that are still able to induce tissue-specific gene expression and promote differentia-

tion when expressed in the Rb−/− cells (Sellers *et al*., 1998). One of these targets, HBP1, appears to also play a role in terminal cell cycle exit and its coordination with the onset of tissue-specific gene expression (Sellers and Kaelin, 1996). HBP1 is a transcriptional repressor belonging to the HMG family of transcription factors that specifically interacts with p130 and pRb, but not with p107 (Tevosian *et al*., 1997), and it is induced during muscle differentiation with approximately the same kinetics as pRb. Forced expression of HBP1 in the presence of pRb leads to cell cycle arrest and, unexpectedly, blockage of the myogenic program (Shih *et al*., 1998). This event can be overcome by the co-expression of MyoD, myogenin or pRb. It has been proposed that at low pRb/HBP1 ratios, cells exit the cell cycle but pRb-mediated muscle-specific transcription is blocked. As pRb/HBP1 ratios augment, bHLH activity is increased and full differentiation proceeds. The transient block of myogenesis allows cells to increase levels of hypophosphorylated pRb leading to the inactivation of E2F-dependent genes. As HBP1 levels also increase, HBP1/pRb complexes can bind to and further inactivate promoters of these genes. The sequential action of E2F/pRb and HBP1/pRb complexes could potentially lock the transcriptional repression of mitotic genes definitively and confer irreversible cell cycle withdrawal.

Another family of proteins cooperating with pRb to control progression through the cell cycle is the HDACs, which in concert with E2F, pRb and the chromatin remodeling complex SWI/SNF are able to repress the expression of cyclin E at the G_1/S phase boundary of the cell cycle (Zhang *et al*., 2000; Simone, 2005). The physical interaction between the hypophosphorylated form of pRb and HDAC1 in growth-arrested cells have suggested an additional mechanism of cooperation between pRb and MyoD in the absence of a direct interaction (see Figure 1). In fact, once hypophosphorylated in response to cdk inactivation by serum withdrawal, pRb disassembles HDAC1 from MyoD, thereby removing its inhibitory effect on myogenic transcription (Puri *et al*., 2001). As a consequence, the HDAC1–pRb complex in myotubes can both allow accumulation of hyperacetylated MyoD and block the E2F-dependent transcription of genes, which antagonizes myogenesis. Thus, the hypophosphorylated form of pRb can switch HDAC1 from being a direct repressor to an indirect activator of muscle-specific transcription.

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