Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation

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To investigate the requirement for pRb in myogenic differentiation, a floxed Rb allele was deleted either in proliferating myoblasts or after differentiation. Myf5-Cre mice, lacking pRb in myoblasts, died immediately at birth and exhibited high numbers of apoptotic nuclei and an almost complete absence of myofibers. In contrast, MCK-Cre mice, lacking pRb in differentiated fibers, were viable and exhibited a normal muscle phenotype and ability to regenerate. Induction of differentiation of Rb-deficient primary myoblasts resulted in high rates of apoptosis and a total inability to form multinucleated myotubes. Upon induction of differentiation, Rb-deficient myoblasts up-regulated myogenin, an immediate early marker of differentiation, but failed to down-regulate Pax7 and exhibited growth in low serum conditions. Primary myoblasts in which Rb was deleted after expression of differentiated MCK-Cre formed normal multinucleated myotubes that did not enter S-phase in response to serum stimulation. Therefore, Rb plays a crucial role in the switch from proliferation to differentiation rather than maintenance of the terminally differentiated state.

Introduction

The development of skeletal muscle in mammals provides a powerful system with which to study the molecular regulation of genesis, growth, and differentiation of stem cells during embryonic and regenerative myogenesis (Parker et al., 2003). Importantly, the activity of the bHLH transcription factor family of myogenic regulatory factors (MRFs) is tightly coupled to cell cycle control, and the study of this regulation has provided important insights into the cellular mechanisms that regulate cell growth versus differentiation or apoptosis (Walsh and Perlman, 1997; Yee et al., 1998; Puri and Sartorelli, 2000).

MRFs are subject to regulation that acts to couple MRF activity to the cell cycle. Hypophosphorylated pRb was suggested to bind MyoD and this association to be required for MyoD-mediated activation of E-box–containing muscle-specific promoters (Gu et al., 1993). However, direct binding between pRb and MyoD has been ruled out by in vivo and in vitro assays (Zhang et al., 1999a,b). Therefore, pRb likely potentiates MyoD activity via an indirect mechanism not involving binding Rb to MyoD.

Proliferating myoblasts express Id, activated Cdk4, low levels of hyperphosphorylated Rb, and free E2Fs as well as E2Fs complexed largely with p107. Activated Cdks and Id both stimulate cell cycle progression. In contrast, myotubes express high levels of p21 and hypophosphorylated pRb. Despite the abundance of hypophosphorylated pRb, p130–E2F4 complexes are the predominant E2F complexes in the myotube (Corbeil et al., 1995). Terminal differentiation and protection against apoptosis is maintained by Cdk inhibitors (p21, p27, etc.) and high expression of hypophosphorylated pRb (Jiang et al., 2000; Peschiaroli et al., 2002; Ho et al., 2004). Together, these data suggest that during myogenic differentiation pRb plays a role distinct from the conventional repression of E2F transcriptional activity.

Newborn mice lacking pRb exhibit multiple deficits including severe deficiencies in the formation of skeletal muscle (Zacksenhaus et al., 1996; de Bruin et al., 2003; Wu et al., 2003). Studies using MyoD-converted pRb-deficient embryonic fibroblasts have suggested that Rb is essential for...
both MyoD and MEF2 transcriptional activity, as well as maintaining the terminally differentiated state (Schneider et al., 1994; Novitch et al., 1996, 1999). Although pRb-deficient fibroblasts transfected with MyoD become myogenic and express early muscle markers such as myogenin, expression of late markers such as myosin heavy chain (MHC) is reduced. In addition, serum restimulation of these differentiated pRb-deficient myoblasts results in BrdU incorporation and, thus, S-phase entry and DNA synthesis. However, these cells are unable to enter mitosis. Moreover, forced expression of MyoD in a variety of \( Rb^{+/−} \) fibroblastic cells results in apoptosis that appears to be p21 dependent (Peschiaroli et al., 2002). In the absence of N-\( ras \), pRb-deficient embryos exhibit normal muscle differentiation without apoptosis, suggesting a role for signaling downstream of N-\( ras \) in provoking cell death in \( Rb^{+/−} \) muscle (Takahashi et al., 2003).

Rb plays a key role in controlling cell cycle progression through the G1 restriction point for entry into S-phase (Stevaux and Dyson, 2002). During myogenic differentiation, proliferating myoblasts must also exit the cell cycle from the G1 phase, before the restriction point (Perry and Rudnick, 2000). Therefore, it can be hypothesized that pRb plays an analogous role in myoblasts by regulating the switch from proliferation to differentiation.

To investigate the requirement for pRb in myogenic differentiation, we examined the proliferation and differentiation potential of primary myoblasts in which a floxed \( Rb \) allele was deleted either before or after differentiation. Our experiments unequivocally establish that pRb is required for progression of the differentiation program and not for maintenance of the differentiated state.

**Results**

\( Rb^{+/−}:Myf5-Cre \) mice die at birth with severe muscle deficits

To investigate the requirement for pRb in myogenesis, mice carrying a floxed \( Rb \) allele (Marino et al., 2000) were interbred with \( Myf5-Cre \) knockin mice (Tallquist et al., 2000) or \( MCK-Cre \) transgenic mice (Wang et al., 1999). The \( Myf5-Cre \) allele faithfully recapitulates the expression pattern of the endogenous \( Myf5 \) gene and is uniformly expressed in all proliferating myoblasts (Tallquist et al., 2000). In contrast, the \( MCK-Cre \) transgene is not expressed in myoblasts but is up-regulated in differentiated multinucleated skeletal myotubes (Wang et al., 1999; Andrechek et al., 2002).

\( Rb^{+/−} \) males were crossed with \( Rb^{wt}:Myf5-Cre \) females to generate \( Rb^{+/−}:Myf5-Cre \) progeny. Notably, no viable \( Rb^{+/−}:Myf5-Cre \) mice were identified after genotyping over 95 offspring. Examination of newborn litters revealed the expected Mendelian proportion of \( Rb^{+/−}:Myf5-Cre \) pups. However, the newborn pups lacking pRb in myoblasts were motionless, became cyanotic, and failed to survive. Therefore, we concluded that \( Rb^{+/−}:Myf5-Cre \) mice exhibited a phenotype simi-
lar to that of other Rb knockout mouse models (Lasorella et al., 2000; de Bruin et al., 2003).

Histological examination of skeletal muscle revealed the presence of severe differentiation deficits (Fig. 1, A–F). Hind limb muscles exhibited a dramatic reduction in mass with a complete absence of mature fibers compared with littermate controls (n = 3 independent animals; Fig. 1, compare A with B). In addition, the morphology of the residual muscle fibers in the Rb\(^{f/f}\):Myf5-Cre mice was short and irregular in shape (Fig. 1 D). Moreover, the long and orderly parallel arrangement of the fibers typically seen in the wild-type controls was absent in the Rb\(^{f/f}\):Myf5-Cre muscle (Fig. 1, compare C with D). These results confirm the well-established requirement for pRb in myogenesis.

The severe deficit in muscle tissue development led us to question whether the residual muscle fibers were undergoing appropriate differentiation. Therefore, immunofluorescent staining of the MHC terminal differentiation marker, and of desmin—a marker for myoblasts and newly formed fibers—was performed (Fig. 2, A–H). Desmin expression was significantly diminished in both hind limb and intercostal muscles in the Rb\(^{f/f}\):Myf5-Cre mice in comparison with the level of staining in the control sections (n = 3 independent animals; Fig. 2, compare A and C with E and G). Interestingly, MHC expression was considerably diminished in limb muscle culture, but was less affected in intercostal muscles (Fig. 2, F and H). Together, the histological and immunofluorescent analyses support the notion that Rb\(^{f/f}\):Myf5-Cre pups die at birth due to severe deficiencies in skeletal muscle that impede ventilation, leading to rapid cyanosis and death.

Rb\(^{f/f}\):MCK-Cre mice are viable and apparently normal

Genetic crosses between Rb\(^{f/f}\) and Rb\(^{f/f}\):MCK-Cre mice resulted in completely viable and healthy Rb\(^{f/f}\):MCK-Cre mice in the expected Mendelian ratio. Rb\(^{f/f}\):MCK-Cre mice were similar in size to wild-type littersmates, from birth to late adulthood, and appeared normal in all respects. Skeletal muscle-specific expression of the MCK-Cre transgene was tested by crossing MCK-Cre mice with the R26R3 Cre-inducible LacZ reporter line of mice. Single fibers isolated from R26R3:MCK-Cre mice displayed robust X-Gal staining, confirming the proper expression of the MCK-Cre transgene (Fig. 3 H). To quantitatively assess the efficiency of Rb gene deletion in Rb\(^{f/f}\):MCK-Cre mice, we calculated the Rb\(^{f/f}\):Rb\(^{flox}\) allele ratios using densitometry. These allele ratios were used to determine the percentage of the unexcised Rb\(^{flox}\) allele in pooled single muscle fiber preparations (n = 3 independent animals). As expected, we detected low levels of the unexcised Rb\(^{flox}\) allele; this result is likely due to the presence of satellite cells on the muscle fibers (~1–2% Rb\(^{flox}\) remaining in Rb\(^{f/f}\):MCK-Cre fibers; Fig. 3 I). Additionally, protein levels of pRb were examined by Western blot analysis of pooled single fibers from Rb\(^{f/f}\):MCK-Cre and Rb\(^{f/f}\) littermate controls (200 fibers per animal). Levels of pRb were readily detectable in the control fibers but were below the limit of detection in the Rb\(^{f/f}\):MCK-Cre fibers (Fig. 3 J). The extremely low levels of pRb in the Rb\(^{f/f}\):MCK-Cre fibers precluded any possibility of comparing fold differences by this method. Our histological analysis of the hind limb skeletal muscle revealed no gross abnormalities in the skeletal muscle fibers of Rb\(^{f/f}\):MCK-Cre mice in comparison with littermate controls (n = 3 independent animals; Fig. 3, compare A–C with D–F). The fiber calibers of Rb\(^{f/f}\):MCK-Cre and littermate controls (n = 3) were on average similar (unpublished data). The normal appearance of the skeletal muscle phenotype prompted us to examine the regenerative capacity of the Rb\(^{f/f}\):MCK-Cre skeletal muscle using cobra venom-derived cardotoxin. After cardotoxin-induced injury to the tibialis anterior muscle, Rb\(^{f/f}\):MCK-Cre mice demonstrated no deficit in the regeneration of the damaged muscle tissue (n = 3 independent animals; unpublished data). Together, these data suggest that pRb is not required for the maintenance or regeneration of differentiated skeletal muscle.
Rb-deficient primary myoblasts exhibit altered cell cycle kinetics

To investigate the cellular basis for the muscle deficits in newborn Rbf/f:Myf5-Cre mice, we isolated primary myoblasts from Rbf/f adult muscle. To generate null mutations in Rb, we infected primary myoblasts isolated from Rbf/f mice with Cre-expressing adenovirus (Ad-Cre; Anton and Graham, 1995). Myoblasts were infected with Lac-Z–expressing adenovirus (Ad-Lac-Z) as a control.

Adenoviral infection and expression of Cre resulted in the complete excision of the floxed region of Rb as assessed by 32P end-labeled PCR genotyping and Western blot analysis (Fig. 4, A and B). Our method of 32P end-labeled PCR genotyping was capable of detecting an Rbflox:Rbexcised allele ratio as low as 10^-4 when amplified from 20 ng of total DNA (unpublished data). Moreover, pRb protein was below the level of detection by Western blot analysis in Ad-Cre–infected Rb/f myoblasts (n = 3 independent isolates and more than three infections per isolate). Additionally, pRb protein was undetectable by immunofluorescence in the nuclei of the Ad-Cre–infected myoblasts after 2 d in differentiation media (DM), when pRb is normally expressed at high levels (n = 3; Fig. 4 C). Ad-Cre–infected myoblasts appeared to be smaller and more compact than the control-infected cells. Our visual assessment was validated by flow cytometry analysis that confirmed the actual decrease in average size of pRb-deficient myoblasts relative to controls (unpublished data).

Cells with null mutations in Rb display altered cell cycle kinetics consistent with the role for pRb as an important G1/S checkpoint regulator (Herrera et al., 1996; Classon et al., 2000). Therefore, we examined the growth characteristics of pRb-deficient primary myoblasts. We observed increased growth kinetics with a 2.5-fold decrease in the average doubling time (n = 6). The myoblasts exhibited a 17% reduction in the G0/G1 population (50% compared with 60% for control and Rbf/f:Ad-Cre, respectively) and a 30% increase in S-phase populations (20% compared with 30% for control and Rbf/f:Ad-Cre, respectively) of an asynchronously dividing pool of cells. Despite the compact morphology and altered cell cycle characteristics, pRb null primary myoblasts under subconfluent growth conditions showed no obvious evidence of senescence or cell death after a high number of passages (n = 3, >15 passages). Together, these data indicate that pRb-deficient myoblasts exhibit a relatively subtle phenotype associated with an enhanced proliferative potential. Therefore, these data suggest that the perinatal death of pups was not due to proliferation deficiencies of the myogenic progenitors.

Rb-deficient primary myoblasts are incapable of differentiation

Primary myoblasts can be quickly and efficiently induced to exit the cell cycle and initiate the differentiation program by exposure to low serum conditions. To investigate the differentiation potential of primary myoblasts lacking pRb, we ex-
Figure 4. Adenoviral delivery of Cre recombinase into Rb floxed primary myoblasts completely eliminates pRb expression. (A) 32P end-labeled PCR genotype analysis of the floxed Rb locus revealed complete excision. Primers flanking the LoxP sites in the Rb locus were used to amplify DNA samples from Ad-Lac-Z control- and Ad-Cre–infected Rb flox homozygous (Rb+/−) primary myoblasts from three independent isolates (Rb+/− A, Rb+/− B, and Rb+/− C). (B) Immunoblot detection of pRb from control- and Ad-Cre–infected primary myoblast protein extracts indicated a complete absence of pRb. Whole cell protein extract was harvested from primary myoblasts in growth media (GM) and after 1 d in DM (D1). (C) Immunofluorescent staining of control- and Ad-Cre–infected Rb+/− primary myoblasts. Infected primary myoblasts were PFA fixed after 2 d in DM and stained with antibody to pRb.

pRb is not required for maintenance of differentiation

posed Ad-Cre–infected Rb+/− myoblasts to low serum conditions using standard procedures (Sabourin et al., 1999). We observed a rapid loss of cell viability (Fig. 5) together with an inability of the remaining cells to form multinucleated myotubes (see Fig. 6, compare C with F).

Detection of nuclear fragmentation by TUNEL revealed apoptotic nuclei beginning to accumulate 6 h after serum reduction (Fig. 5, A–F). TUNEL staining was detected in a markedly higher number of pRb-deficient myoblasts by 6 h in low serum media (6.7 ± 2.4% compared with 22.3 ± 1.52% for control and Rb+/−:Ad-Cre, respectively). The elevated levels of apoptosis gradually diminished the total cell numbers in direct relation to the length of time spent under low serum conditions (n = 3 independent isolations and infections; ~25% compared with ~85% cell death in controls and Rb+/−:Ad-Cre, respectively, after 5 d in low serum).

Interestingly, the small number of cells that did survive the low serum conditions appeared incapable of differentiation. The surviving pRb-deficient primary cells were adherent and formed elongated mononuclear myocytes that failed to fuse with closely neighboring myocytes to form multinucleated myotubes (Fig. 4 C, bottom left).

We conducted immunofluorescent staining experiments to examine the cellular expression and localization patterns of MyoD, myogenin, and MHC (n = 3 independent isolations and infections; Fig. 6, A–L). MyoD is expressed in proliferating myoblasts and is down-regulated during differentiation. Myogenin is not expressed in proliferating myoblasts and is up-regulated in mononuclear cells as an early response gene during induction of differentiation. Finally, MHC is up-regulated relatively late in the differentiation program and is typically detected in multinucleated myotubes (Sabourin et al., 1999).

Under growth conditions, MyoD was localized to the nuclear compartment and similarly expressed in almost all of the pRb-deficient and wild-type primary myoblasts (Fig. 6, A and D). In addition, high levels of MyoD were detected in the nuclei of the pRb-deficient and wild-type myoblasts by day 1 of differentiation (Fig. 6, B and E). After differentiation, MyoD protein was down-regulated to low levels in virtually all the nuclei of wild-type multinucleated myotubes (Fig. 6 C). In contrast, ~36% of the pRb-deficient Rb+/−::Ad-Cre myocytes continued to express high levels of MyoD (Fig. 6 F). Western blot analysis showed that both wild-type and mutant myoblasts expressed similar levels of MyoD in subconfluent growth conditions (Fig. 7 C). However, by days 3 and 5 of differentiation, mutant cells had failed to down-regulate MyoD (n = 3; Fig. 7 C).

Myogenin was expressed at typically low levels in subconfluent cultures of wild-type and Rb mutant primary myoblasts. Up-regulation of myogenin expression occurred after 1 d of differentiation in both wild-type and pRb-deficient cells to about equal levels (Fig. 6, G, H, J, and K). However, by day 5 of differentiation, myogenin protein was detectable in only 17% of wild-type nuclei, whereas ~50% of Rb-deficient cells exhibited robust nuclear expression (Fig. 6, I and L). Western blot analysis revealed that myogenin was not detectable in either wild-type or mutant myoblasts in growth conditions (Fig. 7 C). However, by days 3 and 5 of differentiation, mutant cells had failed to down-regulate myogenin (n = 3; Fig. 7 C). Therefore, pRb-deficient myoblasts entered the differentiation program, as evidenced by the up-regulation of myogenin, which is an immediate early marker of commitment for differentiation (Bergstrom et al., 2002).

After 1 d of differentiation, cytoplasmic MHC expression was detected by immunofluorescence in newly formed wild-type myotubes, whereas little such expression was detected in mutant cells (Fig. 6, compare B with E). By day 5 of differentiation, robust cytoplasmic MHC staining was evident.
in wild-type multinucleated myotubes (Fig. 6 C). In contrast, the pRb-deficient cells were incapable of completing the differentiation program, as evidenced by the absence of multinucleated myotubes expressing MHC (Fig. 6 F). Western blot analysis confirmed the very low level expression of MHC in Rbf/f:Ad-Cre-derived cells (Fig. 7 C). Together, these data suggest that the perinatal death of Rbf/f:Myf5-Cre mice is due to an intrinsic defect in differentiation of the myogenic progenitors.

Pax7 is typically expressed at high levels in proliferating myoblasts and is rapidly down-regulated upon induction of differentiation (Seale et al., 2000). Western blot analysis re-
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revealed that pRb-deficient primary myoblasts exposed to differentiation medium fail to down-regulate Pax7 (Fig. 7 C). Together, these data suggest that the low numbers of surviving pRb null myoblasts are able to initiate differentiation, but subsequently fail to properly regulate the progression of the myogenic program required for the completion of myogenic differentiation.

Primary myoblasts lacking pRb fail to arrest upon induction of differentiation

To elucidate the cell cycle and differentiation phenotype of the Ad-Cre–infected Rb<sup>−/−</sup> myoblasts, we performed a series of RNA and protein expression analyses for cell cycle and differentiation markers. RNA expression levels of cyclins and cdk's were analyzed under growth conditions and serum withdrawal differentiation conditions (n = 3). Because of RNase protection, pRb-deficient primary myoblasts fail to down-regulate cyclinA2, cyclinB1, cdk1, cdk2, and cdk4 mRNAs in response to serum withdrawal (Fig. 7 A and B).

Mutant pRb-deficient cells displayed increased levels of p107 protein under growth conditions in comparison with the wild-type cells (Fig. 7 C). In addition, postdifferentiation mutant cells continued to express high levels of p107 protein relative to wild-type myotubes (n = 3; Fig. 7 C). However, the levels and pattern of p130 expression appeared normal (n = 3; Fig. 7 C). Therefore, pRb-deficient primary myoblasts display a similar cell cycle phenotype to that of MyoD-transfected pRb-deficient fibroblasts (Schneider et al., 1994; Novitch et al., 1996, 1999).

To investigate whether pRb-deficient myoblasts were appropriately withdrawing from the cell cycle upon induction of differentiation, we performed BrdU incorporation experiments on newly differentiated cultures. Wild-type cells displayed no incorporation of BrdU in cultures exposed for 1 h to BrdU, after 4 d of differentiation (Fig. 8 A). In contrast, ~25% of Rb<sup>−/−</sup>:Ad-Cre myoblasts present after a 4-d exposure to differentiation medium incorporated BrdU (Fig. 8 C). In addition, Rb<sup>−/−</sup>:Ad-Cre myocytes also resumed cell division and exhibited an ~50% increase in total cell numbers after reexposure to growth medium for 24 h (Fig. 8 D). These data are consistent with the hypothesis that Rb-deficient myoblasts display an intrinsic
failure of the G1 restriction point necessary for the switch to terminal myogenic differentiation.

**Primary myoblasts from Rbf\(^{fl}\):MCK-Cre mice exhibit normal differentiation**

To investigate whether pRb is required to maintain the terminally differentiated state, we derived primary myoblasts from Rbf\(^{fl}\):MCK-Cre mice and examined their growth and differentiation characteristics. The MCK-Cre transgene has been used extensively to efficiently and completely excise floxed alleles in terminally differentiated cardiac and skeletal muscle (Wang et al., 1999; Zisman et al., 2000; Kim et al., 2001; Andrechek et al., 2002; Norris et al., 2003). We verified the efficiency of Rb gene deletion in the Rbf\(^{fl}\):MCK-Cre mice by quantitating the allele ratios by 32P end-labeled PCR (100% Rbf\(^{lox}\) allele present during growth vs. <1% Rbf\(^{lox}\) allele remaining by day 5 of differentiation; Fig. 9 A). In addition, Rb protein levels were undetectable by Western blot analysis in all three isolates of Rbf\(^{fl}\):MCK-Cre myoblasts by day 5 of differentiation (Fig. 9 B). Finally, we did not detect pRb-positive nuclei in multinucleated MHC-expressing Rbf\(^{fl}\):MCK-Cre myotubes (Fig. 9 C).

**Figure 9.** MCK-Cre transgene efficiently eliminates pRb expression in Rbf\(^{fl}\):MCK-Cre differentiated primary myotubes. (A) 32P end-labeled PCR genotype analysis of the floxed Rb locus. Primers flanking the LoxP sites in the Rb locus were used to amplify DNA samples from Rbf\(^{fl}\):MCK-Cre primary myoblasts during growth (GM) and after 5 d in differentiation conditions (D5) in three independent isolates (Rbf\(^{fl}\):MCK-Cre 1–3). (B) Immunoblot detection of pRb from Rbf\(^{fl}\):MCK-Cre primary myoblast protein extracts. α-Tubulin protein levels were used as loading controls. Whole cell protein extract was harvested from primary myoblasts in growth media (GM) and after 5 d in DM (D5). (C) Immunofluorescent staining of Rbf\(^{fl}\):MCK-Cre control and Rbf\(^{fl}\):MCK-Cre primary myotubes. Myotubes were PFA fixed after 5 d in DM, stained with antibodies to pRb (FITC) and MHC (rhodamine), and counterstained with DAPI.
Interestingly, this late-stage elimination of pRb did not result in the up-regulation of p107 as observed in Rb\textsuperscript{fl/fl}:Ad-Cre cultures (compare Fig. 10 B with Fig. 7 C). Consistent with the normal differentiation of Rb\textsuperscript{fl/fl}:MCK-Cre primary myoblasts, MyoD and myogenin levels were subject to normal modulation (Fig. 10 B). Thus, functional pRb is critically involved in the cascade of regulatory events required for progression through myogenic differentiation, but pRb is not required for the maintenance of the terminally differentiated state.

**Discussion**

We have used mouse genetics to demonstrate that the completion of skeletal myogenic differentiation is dependent on pRb function. The loss of pRb in myogenic precursor cells precludes their ability to properly couple cell cycle exit with the progression of differentiation after the induction of myogenin (Figs. 5–7). Importantly, pRb function was not required for maintaining the terminally differentiated state of myotubes in vitro (Figs. 8 and 10) or for the formation of completely differentiated skeletal muscle tissue in vivo (Fig. 3).

Recent studies have demonstrated that many of the developmental defects observed in the initial Rb knockout studies were primarily caused by a pRb-dependent extraembryonic defect in placental development (Wu et al., 2003). Functional rescue of placental defects by the generation of aggregation chimeras with wild-type tetraploid donor embryos results in Rb\textsuperscript{-/-} pups that survive to birth without neuronal or erythroid phenotypes. Notably, the newborn Rb\textsuperscript{-/-} pups exhibited ectopic S-phases and apoptosis in the lens, together with extensive deficiencies in skeletal muscle differentiation (de Bruin et al., 2003). The muscle phenotype in these animals is strikingly reminiscent of the Rb\textsuperscript{fl/fl}:Myf5-Cre muscle phenotype. Importantly, our experiments provide the formal genetic proof that pRb is required in a cell-autonomous manner for skeletal muscle development.

Ad-Cre–injected Rb\textsuperscript{fl/fl} primary myoblasts could not properly form multinucleated MHC-expressing myotubes (Fig. 6). Additionally, Rb\textsuperscript{fl/fl}:Ad-Cre myoblasts were unable to down-regulate cyclin, cdk's, p107, Pax7, MyoD, and myogenin when withdrawn from serum (Fig. 7). However, upon induction of differentiation, Rb\textsuperscript{fl/fl}:Ad-Cre myoblasts up-regulated myogenin expression, which is an immediate early marker of commitment for differentiation (Bergstrom et al., 2002). Therefore, we conclude that Rb\textsuperscript{fl/fl}:Ad-Cre myoblasts enter the differentiation program but fail to progress, as evidenced by continued Pax7 expression, cell division, and failure to form multinucleated myotubes.

Activation of myogenin transcription is directly mediated by MyoD binding to E-boxes located in the proximal myogenin promoter in response to differentiation-inducing signals (Gerber et al., 1997; Bergstrom et al., 2002). Therefore, these data are consistent with the suggestion that myogenin induction occurs independently of Rb transcriptional induction during myogenic differentiation.

During muscle differentiation, pRb becomes hypophosphorylated and mRNA and protein levels increase ∼10-fold (Martelli et al., 1994; Corbeil et al., 1995). MyoD activation during differentiation is responsible for the transcriptional activation of the Rb (Martelli et al., 1994). More recent studies demonstrate that MyoD activation of Rb transcription requires a cyclic AMP–responsive element in the Rb promoter, which binds CREB protein (Magenta et al., 2003). Moreover, CREB is phosphorylated during myogenic differentiation and recruits MyoD, p300, and pCAF to the Rb promoter (Magenta et al., 2003). Therefore, both Rb and myogenin are immediate early targets of MyoD–mediated transcriptional activation during the switch to myogenic differentiation. Our experiments demonstrate that pRb activity is absolutely required for MyoD-mediated cell cycle exit and completion of the differentiation program, but not for activation of myogenin.

The severe myogenic differentiation defect seen in the Rb\textsuperscript{fl/fl}:Myf5-Cre mice (Figs. 1 and 2) and the Rb\textsuperscript{fl/fl}:Ad-Cre primary myoblasts (Figs. 5–7) was in stark contrast with the normal phenotype observed in the Rb\textsuperscript{fl/fl}:MCK-Cre skeletal muscle from which pRb was eliminated after the completion of differentiation (Figs. 3 and 9). Furthermore, primary myoblasts isolated from Rb\textsuperscript{fl/fl}:MCK-Cre mice were also able to properly differentiate into multinucleated MHC-positive myotubes. The Rb\textsuperscript{fl/fl}:MCK-Cre myotubes lacking pRb did not incorporate BrdU when restimulated with growth media for 24 h (Fig. 8 F). However, Rb\textsuperscript{fl/fl}:Ad-Cre myocytes were fully capable of incorporating BrdU and resuming cell division upon growth media restimulation (Fig. 8 D). The persistence of activated cdk2 (Fig. 10 C) and an abundance of its regulatory subunit cyclin E (Fig. 10 C) observed in the restimulated Rb\textsuperscript{fl/fl}:Ad-Cre myocytes, but not in the Rb\textsuperscript{fl/fl}:Ad-
Lac-Z and Rb\textsuperscript{flx}:MCK-Cre myotubes, are indicative of the actively proliferating state of the restimulated Rb\textsuperscript{flx}:Ad-Cre myocytes (Cenciarelli et al., 1999). Interestingly, serum restimulation of Rb\textsuperscript{flx}:MCK-Cre myotubes results in the induction of p107 protein (Fig. 10 C). However, despite the induction of p107 in differentiated myotubes, the cell cycle continues to be checked. Up-regulation of cell cycle genes has previously been reported in serum-treated terminally differentiated C2C12 myotubes. Serum restimulation of C2C12 myotubes induces immediate early genes such as c-fos, c-jun, c-myec, and Id-1 (Tiainen et al., 1996a). Moreover, cell cycle genes such as cyclin D1 and cdc2 were similarly serum-induced to levels comparable to that of proliferation (Jahn et al., 1994), yet no evidence of cell cycle reentry was ever observed. Therefore, Rb\textsuperscript{flx}:MCK-Cre myotubes are capable of inhibiting DNA synthesis and maintaining the terminally differentiated state in the absence of pRb.

The maintenance of the terminally differentiated state is likely the effect of multiple mechanisms that may include p130–E2F4 complexes (Corbeil et al., 1995; Takahashi et al., 2000), cyclin D3 complexes (Cenciarelli et al., 1999), p21 activity (Jiang et al., 2000; Mal et al., 2000), and inhibition of cyclin D1 expression (Skapek et al., 1995; Latella et al., 2001). Notably, forced expression of E2F1 in differentiated C2C12 myotubes is insufficient to induce ectopic DNA synthesis (Tiainen et al., 1996a). However, the expression of viral oncoproteins such as E1A (Tiainen et al., 1996b) and SV40 large T antigen (Gu et al., 1993) in C2C12 myotubes can induce ectopic DNA synthesis, but these oncoproteins are both known to inactivate multiple checkpoint mechanisms (Helt and Galloway, 2003). Therefore, terminal differentiation is a very stable state that appears tolerant of inactivation of multiple pathways. A high proportion of pRb-deficient myoblasts undergo apoptosis in response to induction of differentiation. Interestingly, attenuation of N-cadherin appears tolerant of inactivation of single pathways.

A high proportion of pRb-deficient myoblasts undergo apoptosis in response to induction of differentiation. Interestingly, attenuation of N-cadherin appears to be checked. Up-regulation of cell cycle genes has previously been reported in serum-treated terminally differentiated C2C12 myotubes. Serum restimulation of C2C12 myotubes induces immediate early genes such as c-fos, c-jun, c-myec, and Id-1 (Tiainen et al., 1996a). Moreover, cell cycle genes such as cyclin D1 and cdc2 were similarly serum-induced to levels comparable to that of proliferation (Jahn et al., 1994), yet no evidence of cell cycle reentry was ever observed. Therefore, Rb\textsuperscript{flx}:MCK-Cre myotubes are capable of inhibiting DNA synthesis and maintaining the terminally differentiated state in the absence of pRb.

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A high proportion of pRb-deficient myoblasts undergo apoptosis in response to induction of differentiation. Interestingly, attenuation of N-cadherin appears tolerant of inactivation of single pathways.

In this paper, we have demonstrated that pRb is essential for progression to terminal myogenic differentiation. Several pRb-dependent mechanisms have been suggested to play important roles in the regulation of myogenesis. Dominant negative N-ras enhances MyoD-mediated expression from an MCK reporter construct in Rb\textsuperscript{−/−} fibroblasts (Lee et al., 1999). Additionally, N-ras\textsuperscript{−/−}:Rb\textsuperscript{−/−} compound mutant muscle appears to be relatively normal, suggesting that N-ras pays a role in mediating the defect in differentiation of pRb-deficient myoblasts (Takahashi et al., 2003). Other studies have suggested that dynamic interactions between pRb, HDAC1, and MyoD play an important role (Puri et al., 2001). Alternatively, pRb inhibition of Id2 has been suggested to potentiate MyoD activity (Lasorella et al., 2000). Clearly, the importance of these interactions must be reassessed under a unified context in light of our findings.

We have demonstrated that pRb is required during the early stages of differentiation in order to properly control cell cycle exit and regulate the progression of the differentiation program. However, maintenance of myogenic terminal differentiation does not require pRb. Identifying the early pRb-dependent downstream regulatory network could potentially be valuable in developing highly efficient stem cell therapeutics for a broad range of myopathies. Future studies will address the mechanisms and the downstream targets of pRb regulation during the early stages of skeletal muscle differentiation.

Materials and methods

Mice and cell culture

Mice carrying the Loxp-targeted Rb allele (Rb\textsuperscript{flx}; provided by M. Vooijs and A. Berns, Netherlands Cancer Institute, Amsterdam, Netherlands; Marino et al., 2000) were backcrossed into the BALB/c and FVB strains and bred with either Myf5-Cre mice (provided by P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA; Tallquist et al., 2000) or transgenic MCK-Cre mice (provided by R. Kahn, Joslin Diabetes Center, Boston, MA; Wang et al., 1999). Primary myoblasts were derived from lower hind limb skeletal muscle of 4–8-wk-old mice as described previously (Sabourin et al., 1999). All primary myoblast isolations were expanded and enriched to 99% purity (assessed by desmin and MyoD staining) before experimental use. Primary myoblasts were differentiated in 5% horse serum in DME for the duration of time indicated in the figure legends. Rb\textsuperscript{flx}:MCK-Cre myoblasts were differentiated for 3 d in 5% horse serum, followed by 2 d in 5% horse serum in DME supplemented with 5 mg/ml cytosine arabinoside. Cytosine arabinoside was used to eliminate dividing mononuclear cells in culture. For adenoviral infections of primary myoblasts, 300,000 cells per 60-mm dish were seeded and infected with Ad-Cre or Ad-Lac-Z at 5 multiplicity of infection for 1 h using established techniques (Parks et al., 1999). To assess the efficiency of Cre-mediated excision, genomic DNA was isolated from Ad-Cre–infected myoblasts and was PCR genotyped using primers that flank the LoxP-targeted Rb locus (Marino et al., 2000).

Single fiber isolation

A single-fiber isolation procedure was performed on the extensor digitorum longus muscle of 8-wk-old Rb\textsuperscript{flx}:MCK-Cre and Rb\textsuperscript{flx/+} control littermates as described previously (Rosenblatt et al., 1995). 20 fibers were lysed in DNA lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 µg/ml proteinase K), and the DNA was precipitated by isopropanol and washed once in 70% ethanol. 200 fibers were lysed in protein extraction buffer (50 mM Tris-HCl, pH 7.4, 0.1% Triton X-100, 5 mM EDTA, 250 mM NaCl, and 50 mM NaF supplemented with protease inhibitors (0.1 mM Na3VO4, 1 mM PMSF, and 10 µg/ml leupeptin).

\textsuperscript{32}P End-labeled PCR and densitometry analysis

Rb\textsuperscript{flx} forward primer was end labeled with γ-[\textsuperscript{32}P]ATP (Amersham Biosciences) by T4 kinase (Invitrogen) in a final reaction volume of 20 µl. Rb\textsuperscript{flx} PCR was performed under standard conditions using 0.4 µl of end-labeled primer in each 10-µl PCR reaction. 20 ng DNA was used as a starting template in each PCR reaction. Radiolabeled PCR products were resolved on a 7% denaturing acrylamide gel and exposed on film. Band densities were quantified using ImageJ analysis software. Rb\textsuperscript{flx}/Rb\textsuperscript{flx} allele ratios were determined by densitometry analysis, and an allele ratio standard curve was constructed. Sensitivity of detection was determined by decreasing the total ratio of Rb\textsuperscript{flx} DNA to Rb\textsuperscript{flx} DNA in 10-fold steps.

BrdU-pulsed cell cycle analysis

Exponentially growing asynchronous Ad-Cre– and Ad-Lac-Z–infected Rb\textsuperscript{flx} primary myoblasts were pulsed with BrdU for 20 min at a concentration of
30 μM. Myoblasts were trypsinized, pelleted, and washed twice in ice-cold PBS. Myoblasts were stained for BrdU incorporation and total DNA content using the BrdU Flow Kit (BD Biosciences) according to the manufacturer’s instructions. Flow cytometry was performed on a FACStar (Beckman Coulter).

**TUNEL assay**
Ad-Cre- and Ad-Lac-Z-infected Rb<sup>fl/fl</sup> primary myoblasts were grown on collagen-coated 4-well polystyrene chamber slides (Lab-Tek). Cells were fixed with 4% formaldehyde in PBS at the 6-h differentiation time point. TUNEL assays were performed using the ApoAlert DNA Fragmentation Kit (CLON-TECH Laboratories, Inc.) according to the manufacturer’s instructions. TUNEL staining was analyzed and photographed on an Axioscope microscope (Carl Zeiss Microimaging, Inc.) equipped with a UV source and FITC, rhodamine, and HOECHST detection filters.

**RNase protection and Western analysis**
Total RNA was extracted from Ad-Cre and Ad-Lac-Z Rb<sup>fl/fl</sup> primary myoblasts using the RNeasy Mini-Kit (QIAGEN). RNase protection assay was performed using the RiboQuant kit along with the multi-probe template sets m-CYC-1 and m-CC-1 (BD Biosciences). 2 μg of total RNA from each time point was used for the ribo-probe hybridization incubation. The RNase protection assay was completed according to the manufacturer’s instructions.

Primary myoblasts were harvested and lysed in RIPA extraction buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 150 mM NaCl, and 50 mM NaF supplemented with protease inhibitors (complete; Roche) on ice. Western blots were probed with the following antibodies: to pRb, 1:1,000 (41302; BD Biosciences); to p107, 1:500 (150; Santa Cruz Biotechnology, Inc.); to p130, 1:2,000 (ab6545-100; Ab-Cam); to Pax7, 1:10 (Developmental Studies Hybridoma Bank [DSHB]); to myogenin, 1:10 (F5D; DSHB); to MyoD, 1:1,000 (c-20; Santa Cruz Biotechnology, Inc.); to Myc, 1:10 (MF-20; DSHB); to cyclin E, 1:000 (06-459; Upstate Biotechnology); to cdk2, 1:1,000 (M2; Santa Cruz Biotechnology, Inc.); and to α-tubulin, 1:3,500 (T 9026; Sigma-Aldrich). Secondary detection was performed with HRP-conjugated antibodies (Bio-Rad Laboratories). Membrane-bound immune complexes were visualized by ECL Plus kit (Amersham Biosciences).

**Immunostaining**
Tissue samples were fixed in 4% PFA at 4°C overnight. Samples were washed in PBS for 30 min and processed for paraffin embedding. Sections were dewaxed in two washes of Citri-Solv for 5 min each and rehydrated down to room temperature and transferred into PBS. Sections were stained with antibodies to desmin (1:200, clone D33; DakoCytomation) and to M-cyclin (1:10, M2; Santa Cruz Biotechnology, Inc.) and to cyclin E, 1:1,000 (06-459; Upstate Biotechnology); to cdk2, 1:1,000 (M2; Santa Cruz Biotechnology, Inc.); and to α-tubulin, 1:3,500 (T 9026; Sigma-Aldrich). Secondary detection was performed with HRP-conjugated antibodies (Bio-Rad Laboratories). Membrane-bound immune complexes were visualized by ECL Plus kit (Amersham Biosciences).

**DNA synthesis assay**
Primary myoblasts were grown on collagen-coated 4-well polystyrene chamber slides. Cells were induced to differentiate in DM for 4 d. Before fixation, cells were pulsed with 30 μM BrdU for 1 h or restimulated in growth media supplemented with 30 μM BrdU for 24 h. Fixed cells were processed for BrdU staining using the BrdU kit in situ staining kit (BD Biosciences) according to the manufacturer’s instructions. The final HRP-streptavidin incubation was omitted and replaced with fluorescein-conjugated streptavidin (1:200; Vector Laboratories). Cells were washed twice in PBS and double stained using rabbit antymyosin skeletal muscle (1:500, M7523; Sigma-Aldrich). Anti–rabbit rhodamine-conjugated secondary antibody was used to detect the myosin antibody.

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