

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.

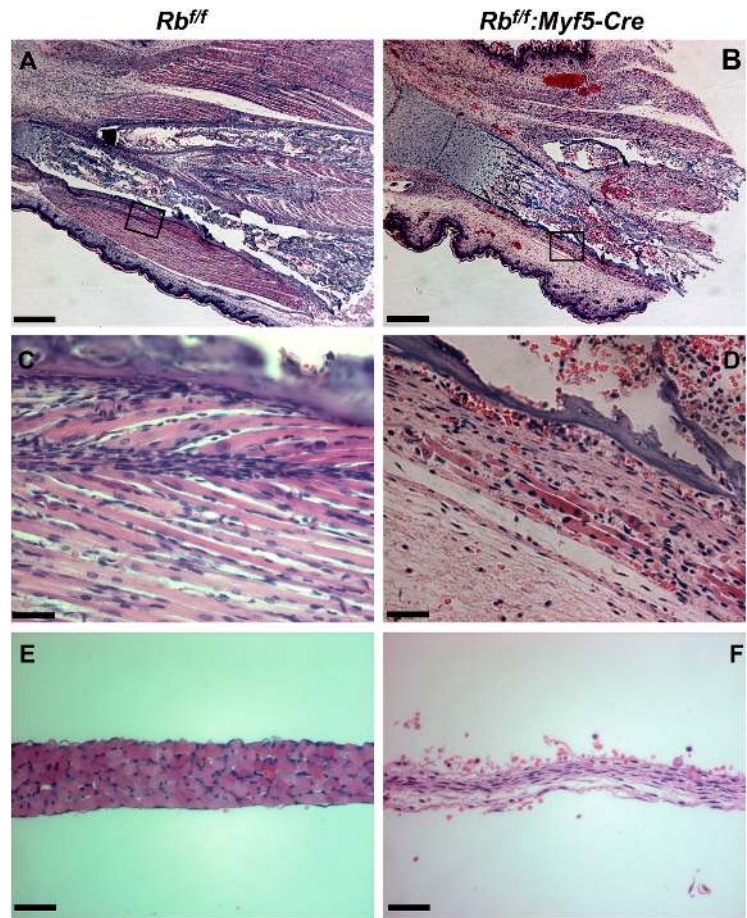
Newborn mice lacking pRb exhibit multiple deficits including severe deficiencies in the formation of skeletal muscle (Zacksenhaus et al., 1996; de Bruin et al., 2003; Wu et al., 2003). Studies using MyoD-converted pRb-deficient embryonic fibroblasts have suggested that *Rb* is essential for

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Abbreviations used in this paper: Ad-Cre, Cre-expressing adenovirus; Ad-Lac-Z, Lac-Z-expressing adenovirus; DM, differentiation media; MHC, myosin heavy chain; MRF, myogenic regulatory factor.

Figure 1. **Severely impaired myogenesis in P0 $Rb^{fl}:Myf5-Cre$ mice.** Hematoxylin and eosin staining of paraffin-embedded longitudinal sections through the hind limb of control (A and C) and $Rb^{fl}:Myf5-Cre$ (B and D) mice. Hematoxylin and eosin staining of cross sections through diaphragm of control (E) and $Rb^{fl}:Myf5-Cre$ (F) mice. Bars: (A and B) 400 μm ; (C–F) 50 μm .



both MyoD and MEF2 transcriptional activity, as well as maintaining the terminally differentiated state (Schneider et al., 1994; Novitsch 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* al-

lele 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

$Rb^{fl}:Myf5-Cre$ 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^{fl} males were crossed with $Rb^{fl}:Myf5-Cre$ females to generate $Rb^{fl}:Myf5-Cre$ progeny. Notably, no viable $Rb^{fl}:Myf5-Cre$ mice were identified after genotyping over 95 offspring. Examination of newborn litters revealed the expected Mendelian proportion of $Rb^{fl}:Myf5-Cre$ pups. However, the newborn pups lacking pRb in myoblasts were motionless, became cyanotic, and failed to survive. Therefore, we concluded that $Rb^{fl}:Myf5-Cre$ mice exhibited a phenotype simi-

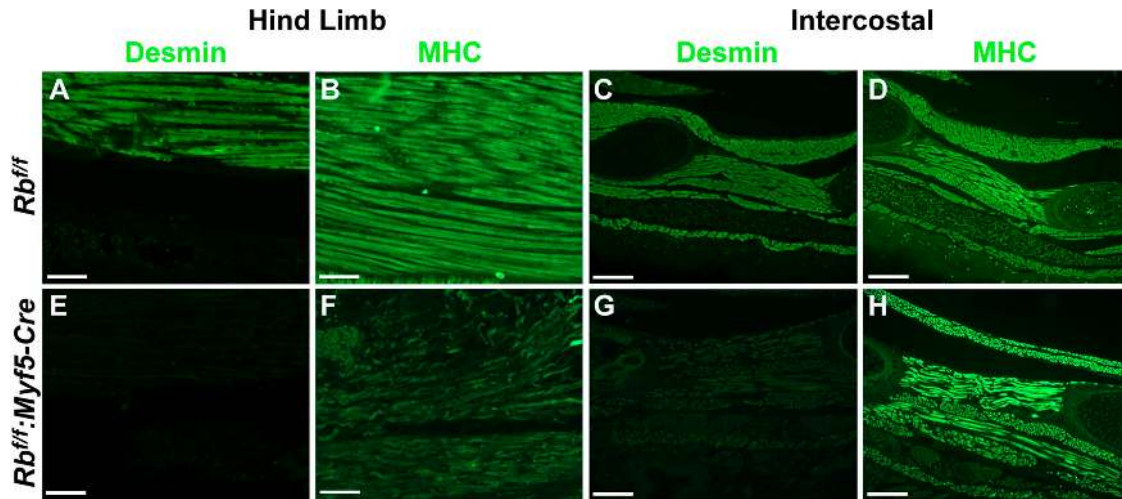


Figure 2. **Abnormal skeletal muscle differentiation in P0 $Rb^{fl/fl}:Myf5-Cre$ mice.** Immunofluorescent staining of longitudinal sections through the hind limb of control (A and B) and $Rb^{fl/fl}:Myf5-Cre$ (E and F) mice with antibodies to desmin and myosin heavy chain (MHC). Immunofluorescent staining of intercostal muscles of control (C and D) and $Rb^{fl/fl}:Myf5-Cre$ (G and H) mice with antibodies to desmin and MHC. Bars: (A, B, E, and F) 50 μm ; (C, D, G, and H) 100 μm .

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 $Rb^{fl/fl}:Myf5-Cre$ 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 $Rb^{fl/fl}:Myf5-Cre$ muscle (Fig. 1, compare C with D). These results confirm the well-established requirement for pRb in myogenesis.

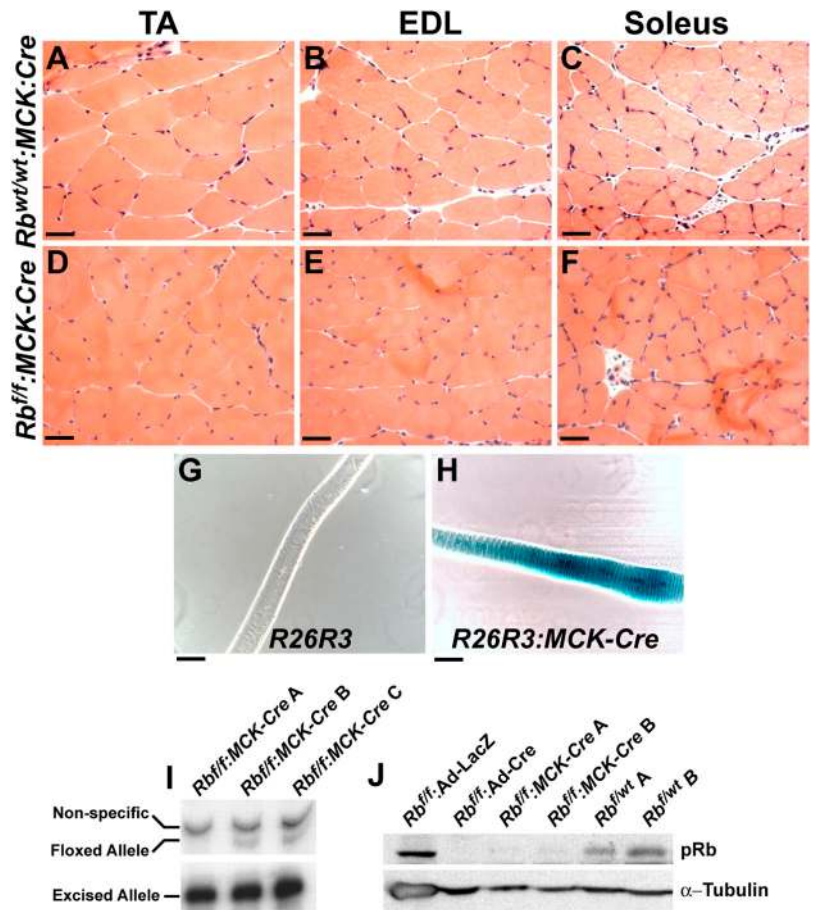
The severe deficit in muscle tissue development led us to question whether the residual muscle fibers were undergoing appropriate differentiation. Therefore, immunofluorescent staining of the MHC terminal differentiation marker, and of desmin—a marker for myoblasts and newly formed fibers—was performed (Fig. 2, A–H). Desmin expression was significantly diminished in both hind limb and intercostal muscles in the $Rb^{fl/fl}:Myf5-Cre$ 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 musculature, but was less affected in intercostal muscles (Fig. 2, F and H). Together, the histological and immunofluorescent analyses support the notion that $Rb^{fl/fl}:Myf5-Cre$ pups die at birth due to severe deficiencies in skeletal muscle that impede ventilation, leading to rapid cyanosis and death.

$Rb^{fl/fl}:MCK-Cre$ mice are viable and apparently normal

Genetic crosses between $Rb^{fl/wt}$ and $Rb^{fl/wt}:MCK-Cre$ mice resulted in completely viable and healthy $Rb^{fl/fl}:MCK-Cre$ mice in the expected Mendelian ratio. $Rb^{fl/fl}:MCK-Cre$ mice were similar in size to wild-type littermates, 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 $Rb^{fl/fl}:MCK-Cre$ mice, we calculated the $Rb^{fl/wt}:Rb^{excised}$ allele ratios using densitometry. These allele ratios were used to determine the percentage of the unexcised $Rb^{fl/wt}$ allele in pooled single muscle fiber preparations ($n = 3$ independent animals). As expected, we detected low levels of the unexcised $Rb^{fl/wt}$ allele; this result is likely due to the presence of satellite cells on the muscle fibers ($\sim 1\text{--}2\%$ $Rb^{fl/wt}$ remaining in $Rb^{fl/fl}:MCK-Cre$ fibers; Fig. 3 I). Additionally, protein levels of pRb were examined by Western blot analysis of pooled single fibers from $Rb^{fl/fl}:MCK-Cre$ and $Rb^{fl/wt}$ 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 $Rb^{fl/fl}:MCK-Cre$ fibers (Fig. 3 J). The extremely low levels of pRb in the $Rb^{fl/fl}:MCK-Cre$ 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 $Rb^{fl/fl}:MCK-Cre$ mice in comparison with littermate controls ($n = 3$ independent animals; Fig. 3, compare A–C with D–F). The fiber calibers of $Rb^{fl/fl}:MCK-Cre$ 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 $Rb^{fl/fl}:MCK-Cre$ skeletal muscle using cobra venom-derived cardiotoxin. After cardiotoxin-induced injury to the tibialis anterior muscle, $Rb^{fl/fl}:MCK-Cre$ 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.

Figure 3. *Rb^{fl/fl}:MCK-Cre* mice have normal skeletal muscle. Hematoxylin and eosin staining of frozen cross sections of control muscles (A–C) and *Rb^{fl/fl}:MCK-Cre* muscles (D–F). TA, tibialis anterior; EDL, extensor digitorum longus. Phase-contrast micrograph of X-Gal–stained single muscle fibers of *R26R3* control (G) and *R26R3:MCK-Cre* (H) mice. (I) ³²P end-labeled PCR genotype analysis of the floxed *Rb* locus revealed 98–99% excision. ³²P end-labeled PCR was performed on DNA samples extracted from a pool of 20 single fibers from three independent mice (*Rb^{fl/fl}:MCK-Cre* A, *Rb^{fl/fl}:MCK-Cre* B, and *Rb^{fl/fl}:MCK-Cre* C). (J) pRb immunoblot analysis of single-fiber protein extracts reveals virtually no detectable pRb. α -Tubulin protein levels were used as loading controls. Protein was extracted from a pool of 200 single fibers from *Rb^{fl/fl}:MCK-Cre* A and *Rb^{fl/fl}:MCK-Cre* B mice, and from *Rb^{fl/wt}* A and *Rb^{fl/wt}* B littermate controls. Protein extracts from Ad-Lac-Z– and Ad-Cre–infected *Rb^{fl/fl}* primary myoblasts were used as positive and negative controls, respectively. Bars, 50 μ m.



Rb-deficient primary myoblasts exhibit altered cell cycle kinetics

To investigate the cellular basis for the muscle deficits in newborn *Rb^{fl/fl}:Myf5-Cre* mice, we isolated primary myoblasts from *Rb^{fl/fl}* adult muscle. To generate null mutations in *Rb*, we infected primary myoblasts isolated from *Rb^{fl/fl}* 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 *Rb^{fl/fl}:Rb^{excised}* 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^{fl/fl}* 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 ($\sim 60\%$ compared with $\sim 50\%$ for control and *Rb^{fl/fl}:Ad-Cre*, respectively) and a 30% increase in S-phase populations ($\sim 20\%$ compared with $\sim 30\%$ for control and *Rb^{fl/fl}: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-

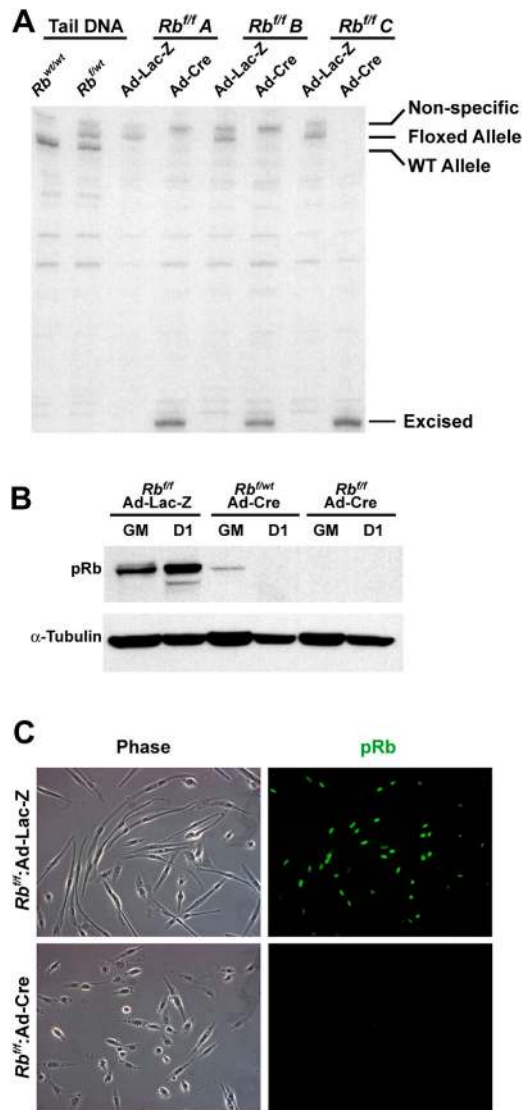


Figure 4. Adenoviral delivery of Cre recombinase into *Rb* floxed primary myoblasts completely eliminates pRb expression. (A) ^{32}P end-labeled PCR genotype analysis of the floxed *Rb* locus revealed complete excision. Primers flanking the *LoxP* sites in the *Rb* locus were used to amplify DNA samples from Ad-Lac-Z control- and Ad-Cre-infected *Rb* floxed homozygous (*f/f*) primary myoblasts from three independent isolates (*Rb*^{f/f} A, *Rb*^{f/f} B, and *Rb*^{f/f} 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*^{f/f} primary myoblasts. Infected primary myoblasts were PFA fixed after 2 d in DM and stained with antibody to pRb.

posed Ad-Cre-infected *Rb*^{f/f} 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 ($6.7 \pm 2.4\%$ compared with $22.3 \pm 1.52\%$ for control and *Rb*^{f/f}: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; $\sim 25\%$ compared with $\sim 85\%$ cell death in controls and *Rb*^{f/f}: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).

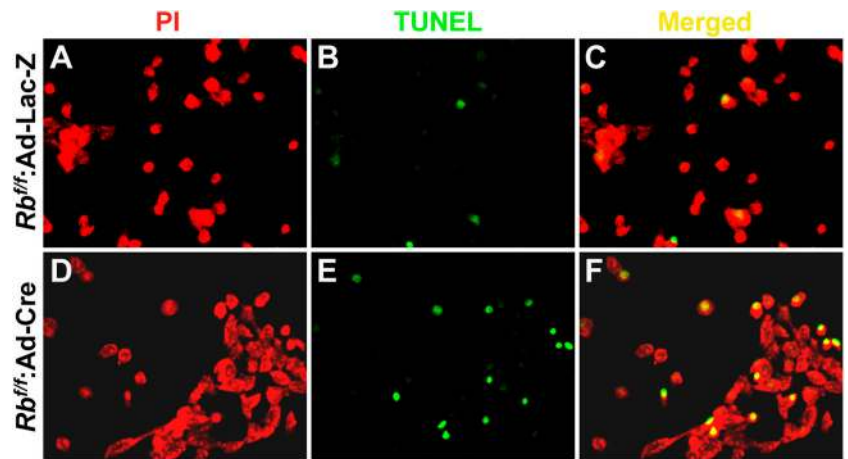
We conducted immunofluorescent staining experiments to examine the cellular expression and localization patterns of MyoD, myogenin, and MHC ($n = 3$ independent isolations and infections; Fig. 6, A–L). MyoD is expressed in proliferating myoblasts and is down-regulated during differentiation. Myogenin 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, $\sim 36\%$ of the pRb-deficient *Rb*^{f/f}: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 $\sim 50\%$ 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

Figure 5. **pRb null primary myoblasts exhibit high rates of apoptosis in response to serum withdrawal.** Apoptotic cell death was assessed by fluorescein-conjugated TUNEL analysis after 6 h in DM on control- (A–C) and Ad-Cre-infected (D–F) *Rb^{fl/fl}* primary myoblasts. Primary myoblasts were counterstained with propidium iodide (PI).



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 *Rb^{fl/fl}:Ad-Cre*-derived cells (Fig. 7 C). Together,

these data suggest that the perinatal death of *Rb^{fl/fl}: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-

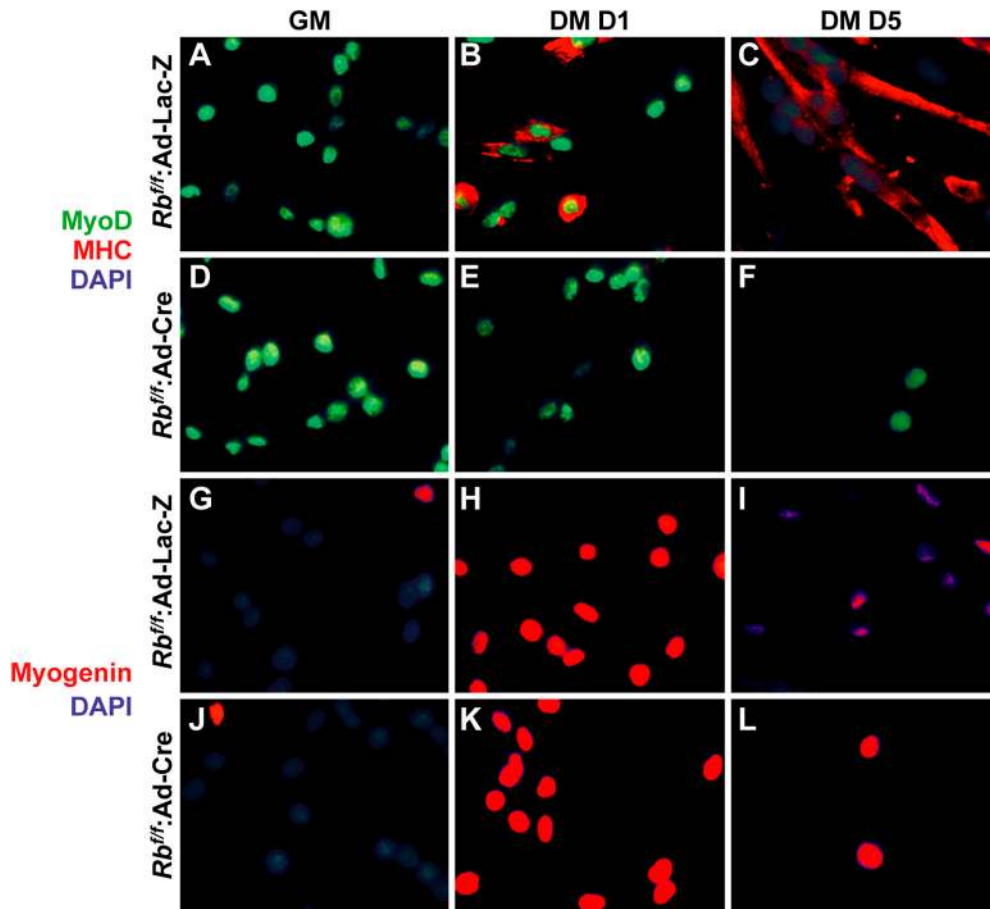


Figure 6. **pRb null primary myoblasts are incapable of forming terminally differentiated multinucleated myotubes.** Double immunofluorescent staining of control- (A–C) and Ad-Cre-infected (D–F) *Rb^{fl/fl}* primary myoblasts with antibodies to MyoD (FITC) and MHC (rhodamine). Immunofluorescent staining of control- (G–I) and Ad-Cre-infected (J–L) *Rb^{fl/fl}* myoblasts with antibody to myogenin (rhodamine). Nuclei were counterstained with DAPI. Infected primary myoblasts were PFA fixed and stained at the indicated time points. GM, growth; DM D1, differentiation day 1; DM D5, differentiation day 5.

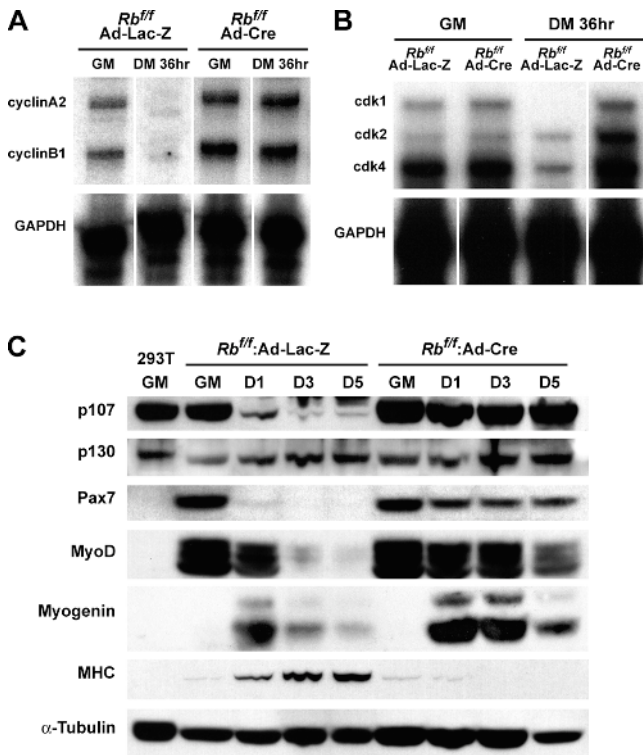


Figure 7. Absence of terminal differentiation in pRb null primary myoblasts correlates with an inability to down-regulate markers of proliferation and early markers of myogenic differentiation. (A) RNase protection assay for *cyclinA2* and *cyclinB1* mRNA in control- and Ad-Cre-infected *Rb^{fl/fl}* primary myoblasts. (B) RNase protection assay for *cdk1*, *cdk2*, and *cdk4* mRNA in control- and Ad-Cre-infected *Rb^{fl/fl}* myoblasts. GAPDH mRNA levels were used as loading controls. Total RNA was harvested from infected myoblasts at the indicated time points (A and B). GM, growth; DM 36hr, differentiation 36 h. (A and B) White lines indicate that intervening lanes have been spliced out. (C) Immunoblot differentiation time course analysis of p107, p130, Pax7, MyoD, myogenin, and MHC in control- and Ad-Cre-infected *Rb^{fl/fl}* myoblasts. α -Tubulin protein levels were used as loading controls. Whole cell protein extract was harvested at the indicated time points. GM, growth; D1, differentiation day 1; D3, differentiation day 3; D5, differentiation day 5. Protein extract from 293T cells was used as a nonmyogenic control.

vealed 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^{fl/fl}* myoblasts, we performed a series of RNA and protein expression analyses for cell cycle and differentiation markers. RNA expression levels of *cyclins* and *cdks* were analyzed under growth conditions and serum withdrawal differentiation conditions ($n = 3$). Because of RNase protection, pRb-deficient primary myoblasts fail to

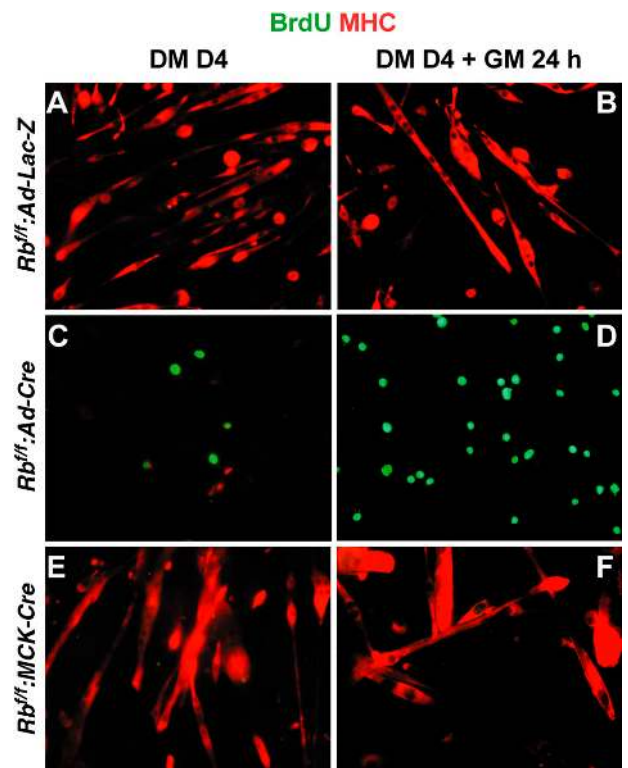


Figure 8. Serum restimulation is unable to induce DNA synthesis in terminally differentiated *Rb^{fl/fl}:MCK-Cre* myotubes. Double immunofluorescent staining for BrdU (FITC) and MHC (rhodamine) in *Rb^{fl/fl}:Ad-Lac-Z*-infected (A and B), *Rb^{fl/fl}:Ad-Cre*-infected (C and D), and *Rb^{fl/fl}:MCK-Cre* (E and F) primary myoblasts. Primary myoblasts were cultured in DM for 4 d and were either BrdU pulsed for 1 h before fixation (DM D4) or restimulated with BrdU-supplemented growth media for 24 h before fixation (DM D4 + GM 24 h).

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; Novitsch 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, $\sim 25\%$ of *Rb^{fl/fl}:Ad-Cre* myoblasts present after a 4-d exposure to differentiation medium incorporated BrdU (Fig. 8 C). In addition, *Rb^{fl/fl}:Ad-Cre* myocytes also resumed cell division and exhibited an $\sim 50\%$ 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

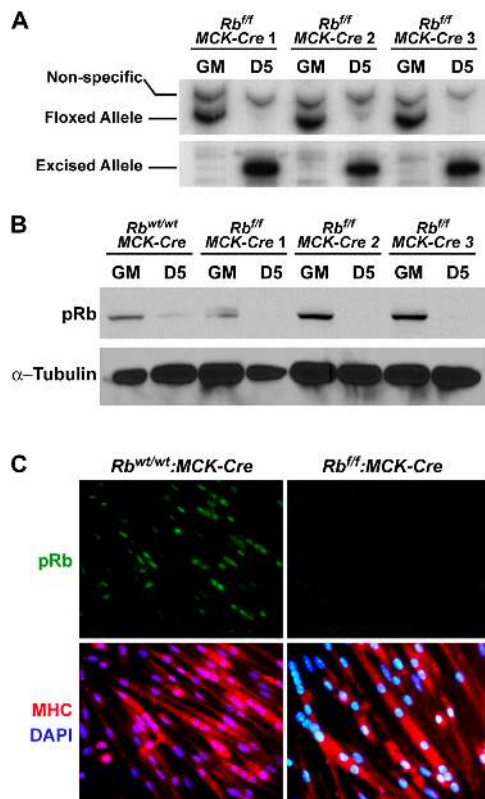


Figure 9. MCK-Cre transgene efficiently eliminates pRb expression in $Rb^{fl/fl}$:MCK-Cre differentiated primary myotubes. (A) ^{32}P end-labeled PCR genotyping analysis of the floxed *Rb* locus. Primers flanking the *LoxP* sites in the *Rb* locus were used to amplify DNA samples from $Rb^{fl/fl}$:MCK-Cre primary myoblasts during growth (GM) and after 5 d in differentiation conditions (D5) in three independent isolates ($Rb^{fl/fl}$:MCK-Cre 1–3). (B) Immunoblot detection of pRb from $Rb^{wt/wt}$:MCK-Cre control and $Rb^{fl/fl}$: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 $Rb^{wt/wt}$:MCK-Cre control and $Rb^{fl/fl}$: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.

failure of the G1 restriction point necessary for the switch to terminal myogenic differentiation.

Primary myoblasts from $Rb^{fl/fl}$:MCK-Cre mice exhibit normal differentiation

To investigate whether pRb is required to maintain the terminally differentiated state, we derived primary myoblasts from $Rb^{fl/fl}$: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 $Rb^{fl/fl}$:MCK-Cre mice by quantitating the allele ratios by ^{32}P end-labeled PCR (100% $Rb^{fl/fl}$ allele present during growth vs. <1% $Rb^{fl/fl}$ allele remaining by day 5 of differentiation; Fig. 9 A). In addition, *Rb* protein levels were undetectable by Western blot

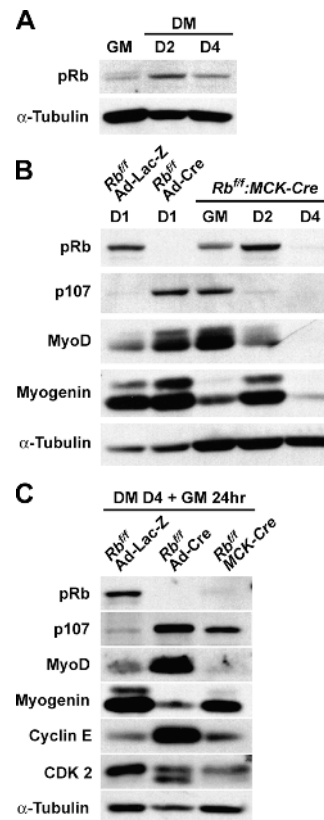


Figure 10. Late-stage removal of pRb in differentiated $Rb^{fl/fl}$:MCK-Cre myotubes does not result in the up-regulation of p107, MyoD, or myogenin protein levels in low serum conditions. (A) Immunoblot for pRb in wild-type primary myoblasts. (B) Immunoblot differentiation time course analysis of pRb, p107, MyoD, and myogenin in $Rb^{fl/fl}$:MCK-Cre primary myoblasts. Whole cell protein extract was harvested from $Rb^{fl/fl}$:MCK-Cre and wild-type myoblasts at the indicated time points. (C) Immunoblot analysis of pRb, p107, MyoD, myogenin, cyclin E, and cdk2. Whole cell protein extracts were harvested from $Rb^{fl/fl}$:Ad-Lac-Z, $Rb^{fl/fl}$:Ad-Cre, and $Rb^{fl/fl}$:MCK-Cre after 4 d in differentiation media, followed by 24 h in growth media. α -Tubulin protein levels were used as loading controls (A–C).

analysis in all three isolates of $Rb^{fl/fl}$:MCK-Cre myoblasts by day 5 of differentiation (Fig. 9 B). Finally, we did not detect pRb-positive nuclei in multinucleated MHC-expressing $Rb^{fl/fl}$:MCK-Cre myotubes by day 5 of differentiation (Fig. 9 C).

$Rb^{fl/fl}$:MCK-Cre primary myoblasts were indistinguishable from wild-type primary myoblasts in appearance, expression of myoblast-specific (*Pax7* and *Myf5*) and cell cycle (*cyclinA2*, *cyclinB1*, *cdk1*, *cdk2*, and *cdk4*) regulators, and growth rate (unpublished data). Importantly, $Rb^{fl/fl}$:MCK-Cre primary myoblasts were fully capable of differentiating into robust multinucleated MHC-expressing myotubes that were apparently normal ($n = 3$ independent isolates; Fig. 9 C).

The levels of pRb over 4 d of differentiation were examined by Western blot analysis to allow us to determine the temporal kinetics of pRb elimination by the *MCK-Cre* transgene ($n = 3$; Fig. 10 B). Levels of pRb in wild-type and mutant cells were similar in growth media and after 2 d in DM (Fig. 10, compare A with B). However, *MCK-Cre*-mediated deletion of the $Rb^{fl/fl}$ allele resulted in the complete ablation of pRb after 4 d of differentiation (Fig. 10, compare A with B).

Interestingly, this late-stage elimination of pRb did not result in the up-regulation of p107 as observed in *Rb^{fl/fl}:Ad-Cre* cultures (compare Fig. 10 B with Fig. 7 C). Consistent with the normal differentiation of *Rb^{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.

Serum stimulation does not induce DNA synthesis in *Rb^{fl/fl}:MCK-Cre* myotubes

The presence of pRb has been suggested to be required for inhibiting de novo DNA synthesis in response to mitogen restimulation in differentiated myocytes derived from *Rb^{-/-}* teratoma, derived myocytes, and MyoD-transfected fibroblasts (Schneider et al., 1994; Novitch et al., 1996). These experiments suggested that myotubes require pRb for the suppression of mitogen-activated ectopic S-phases. Therefore, we investigated the effect of serum stimulation on myotubes lacking pRb that were derived from *Rb^{fl/fl}:MCK-Cre* primary myoblasts.

Primary myoblasts were cultured in DM for 4 d and either BrdU pulsed for 1 h before fixation or stimulated with growth media containing 20% FCS and supplemented with BrdU for 24 h before fixation (Fig. 8). Our experiments revealed that stimulation of *Rb^{fl/fl}:MCK-Cre* myotubes for 24 h with growth media did not result in any myotube nuclei entering S-phase, as evidenced by an absence of BrdU incorporation (Fig. 8 F). Moreover, serum stimulation did not result in up-regulation of MyoD and cyclin E proteins in *Rb^{fl/fl}:MCK-Cre* myotubes (Fig. 10 C). However, levels of p107 were reinduced in the *Rb^{fl/fl}:MCK-Cre* myotubes to approximately half the levels present in the *Rb^{fl/fl}:Ad-Cre* myocytes (Fig. 10 C). Additionally, higher levels of cyclin E protein and an activated form of cdk2 were also present in the restimulated *Rb^{fl/fl}:Ad-Cre* myocytes but not in the *Rb^{fl/fl}:Ad-Lac-Z* and *Rb^{fl/fl}:MCK-Cre* myotubes (Fig. 10 C). Therefore, these experiments indicate that pRb is not required to maintain the terminally differentiated state in myotubes that are derived from primary myoblasts.

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^{-/-}* pups that survive to birth without neuronal or

erythroid phenotypes. Notably, the newborn *Rb^{-/-}* 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^{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-infected *Rb^{fl/fl}* primary myoblasts could not properly form multinucleated MHC-expressing myotubes (Fig. 6). Additionally, *Rb^{fl/fl}:Ad-Cre* myoblasts were unable to down-regulate *cyclins*, *cdk*'s, p107, Pax7, MyoD, and myogenin when withdrawn from serum (Fig. 7). However, upon induction of differentiation, *Rb^{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^{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^{fl/fl}:Myf5-Cre* mice (Figs. 1 and 2) and the *Rb^{fl/fl}:Ad-Cre* primary myoblasts (Figs. 5–7) was in stark contrast with the normal phenotype observed in the *Rb^{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^{fl/fl}:MCK-Cre* mice were also able to properly differentiate into multinucleated MHC-positive myotubes. The *Rb^{fl/fl}:MCK-Cre* myotubes lacking pRb did not incorporate BrdU when restimulated with growth media for 24 h (Fig. 8 F). However, *Rb^{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^{fl/fl}:Ad-Cre* myocytes, but not in the *Rb^{fl/fl}:Ad-*

Lac-Z and *Rb^{flf}:MCK-Cre* myotubes, are indicative of the actively proliferating state of the restimulated *Rb^{flf}:Ad-Cre* myocytes (Cenciarelli et al., 1999). Interestingly, serum restimulation of *Rb^{flf}: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-myc*, 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^{flf}: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 single pathways.

A high proportion of pRb-deficient myoblasts undergo apoptosis in response to induction of differentiation. Interestingly, attenuation of N-ras signaling has been suggested to rescue this apoptotic response of *Rb* mutant myoblasts (Takahashi et al., 2003). The set of genes activated by mitogens via the Ras–MAPK pathway are very similar to the genes activated after E2F induction (Muller et al., 2001). Moreover, forced expression of E2Fs results in high rates of apoptosis (Stevaux and Dyson, 2002), and elevated rates of apoptosis in the cortex of *Rb^{-/-}* embryos are attenuated by crossing into *E2F1* and *E2F3* mutant backgrounds (Ziebold et al., 2001). Several proapoptotic genes including *caspase 3* have been suggested to represent E2F target genes (Muller et al., 2001). Notably, *caspase 3* is up-regulated upon myogenic differentiation and is required for normal progression of the differentiation program (Fernando et al., 2002). Therefore, it is interesting to speculate that pRb may play a protective role in preventing an apoptotic response to activated *caspase 3* during myogenic differentiation.

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^{-/-}* fibroblasts (Lee et al., 1999). Additionally, *N-ras^{-/-}:Rb^{-/-}* compound mutant muscle appears to be relatively normal, suggesting that N-ras

plays 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 floxed*; provided by M. Vooijs and A. Berns, Netherlands Cancer Institute, Amsterdam, Netherlands; Marino et al., 2000) were backcrossed into the BALB/cj 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^{flf}: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^{flf}:MCK-Cre* and *Rb^{flwt}* 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).

³²P End-labeled PCR and densitometry analysis

Rb flox forward primer was end labeled with γ-³²PATP (Amersham Biosciences) by T4 kinase (Invitrogen) in a final reaction volume of 20 µl. *Rb* flox 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^{flox}:Rb^{excised}* 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^{flox}* DNA to *Rb^{excised}* DNA in 10-fold steps.

BrdU-pulsed cell cycle analysis

Exponentially growing asynchronous Ad-Cre- and Ad-Lac-Z-infected *Rb^{flf}* primary myoblasts were pulsed with BrdU for 20 min at a concentration of

30 μ M. 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^{fl/fl}* myoblasts were grown on collagen-coated 4-well poly-L-lysine 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 (CLONTECH Laboratories, Inc.) according to the manufacturer's instructions. TUNEL staining was analyzed and photographed on an Axioskop 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^{fl/fl}* 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 μ g 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 (c-18; 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 MHC, 1:10 (MF-20; DSHB); 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).

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 in graded alcohol washes. Antigen retrieval was performed by immersing sections in boiling citrate buffer (0.21% wt/vol citric acid monohydrate in dH₂O) for 10 min with continuous agitation. Slides were allowed to cool down to room temperature and transferred into PBS. Sections were stained with antibodies to desmin (1:200, clone D33; DakoCytomation) and to MHC (1:10, MF-20) using the fluorescent MOM kit (Vector Laboratories).

Primary myoblasts were grown on collagen-coated 4-well poly-L-lysine chamber slides (Lab-Tek). Cells were fixed with 2% PFA and permeabilized with 0.2% Triton X-100. Cells were stained with the following antibodies: to MyoD, 1:200 (M-318; Santa Cruz Biotechnology, Inc.); to MHC, 1:10 (MF-20); to myogenin, 1:10 (F5D); and to pRb, 1:250 (41302). Secondary detection was performed with fluorescein- or rhodamine-conjugated antibodies (CHEMICON International, Inc.). Cells were counterstained with DAPI and mounted in aqueous fluorescent mounting media (DakoCytomation). Bright-field, phase, and fluorescent images were viewed using an Axioplan2 fluorescent microscope (Carl Zeiss MicroImaging, Inc.) and digitally acquired (AxioCam or Axiovision 3.1; Carl Zeiss MicroImaging, Inc.) at room temperature. Image-processing software (Photoshop 7; Adobe) was used to overlay images and enhance color and clarity. Images were viewed through air-objective lenses (40 \times /0.75; 20 \times /0.5; 5 \times /0.12 [Carl Zeiss MicroImaging, Inc.]).

DNA synthesis assay

Primary myoblasts were grown on collagen-coated 4-well poly-L-lysine chamber slides. Cells were induced to differentiate in DM for 4 d. Before fixation, cells were pulsed with 30 μ M BrdU for 1 h or restimulated in growth media supplemented with 30 μ M BrdU for 24 h. Fixed cells were processed for BrdU staining using the BrdU 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 antimyosin skeletal muscle (1:500,

M7523; Sigma-Aldrich). Anti-rabbit rhodamine-conjugated secondary antibody was used to detect the myosin antibody.

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