A containing 0.5 M NaCl and once with buffer A to remove any MAP kinase and MEK activity associated with the P100 fraction (*15*). The washed membranes were rinsed with buffer A, resuspended in 50 μl buffer A containing NP40 (1%), mixed, incubated on ice for 10 min, and centrifuged. The supernatant was removed and the sedimented NP40-insoluble material was resuspended in 50 μl of buffer A. Portions (10 μl) of the resuspended material and supernatant were placed into a reaction with 5 μl (1 μg) of MEK, 5 μl (1 μg) of MAP kinase [as a glutathione-S-transferase (GST) fusion protein] and 5 μl of Mg^{2+}-ATP (40 mM and 0.5 mM, respectively). Portions (10 μl) of each sample were also incubated in identical reactions omitting either MEK or MAP kinase and MEK. After 30 min at 30°C, 10 μl of each reaction was diluted into 40 μl of ice-cold buffer C and 10 μl of the diluted reaction was incubated with 5 μl (16 μg) of MBP, 25 μl of buffer C, 10 μl of Mg^{2+}-ATP (50 mM and 0.5 mM, respectively), with γ-[^{32}P]ATP (specific activity, 1,200 cpm/pmol). After 10 min at 30°C, portions (40 μl) were removed and spotted on 2 cm × 2 cm squares of phosphocellulose paper and immersed in 75 mM of orthophosphoric acid. After washing 4 times in phosphoric acid and once in acetone, the papers were dried and counted in scintillation fluid (1 ml). The control incubations omitting either MEK or MAP kinase and MEK showed that only small amounts of MAP kinase and MEK remained associated with the P100 fraction after washing. These counts (<4000 cpm) were subtracted from those incorporated into MBP in the incubation with MEK and ERK to arrive at a measure of Raf activity present in the samples. Because the NP40-soluble and insoluble fractions were taken through identical assays, the Raf activities are directly comparable.

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Reversal of Terminal Differentiation Mediated by p107 in $Rb^{-/-}$ Muscle Cells

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The terminal differentiation of mammalian muscle cells requires the tumor suppressor retinoblastoma protein (Rb). Unlike their wild-type counterparts, multinucleated myotubes from mouse cells deficient in Rb ($Rb^{-/-}$) were induced by serum to reenter the cell cycle. Development of the myogenic phenotype in $Rb^{-/-}$ cells correlated with increased expression of p107, which interacted with myogenic transcription factors. Serum-induced cell cycle reentry, on the other hand, correlated with decreased p107 expression. Thus, although p107 could substitute for Rb as a cofactor for differentiation, it could not maintain the terminally differentiated state in $Rb^{-/-}$ myotubes.

Terminal differentiation in mammalian cells comprises two interdependent and normally irreversible biological phenomena: withdrawal from the cell cycle and phenotypic differentiation. The tumor suppressor Rb, a member of the viral oncoprotein-binding pocket protein family, is a regulator of cell cycle progression (*1*). Rb accumulates during embryonic development and cell differentiation and thus may participate in the terminal differentiation of various cell lineages (*2*). The cell type–specific function of Rb may derive from interaction with cell-specific regulatory molecules (*3*). Skeletal myogenesis involves the direct interaction of Rb with muscle-specific basic helix-loop-helix (bHLH) factors of the MyoD family (*4*). Although the exact mechanism is unclear, interaction of Rb and myogenic bHLH factors mediates the permanent withdrawal of muscle cells from the cell cycle and the activation of myogenic differentiation.

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Mice that are genetically deficient in Rb ($Rb^{-/-}$) die in fetal life from defective development in the hematopoietic and central nervous systems but have histologically normal skeletal musculature (*5*). Cultured skeletal muscle cell lines derived from $Rb^{-/-}$ mouse cells developed mature multinucleated myotubes, but unlike their wild-type counterparts, these myotubes from $Rb^{-/-}$ cells could synthesize DNA after restimulation with serum growth factor–rich medium. Serum induced most (>75%) nuclei within $Rb^{-/-}$ myotubes to asynchronously reenter the cell cycle, which we documented by immunostaining for the nucleoside analog 5-bromodeoxyuridine (BrdU) incorporated into nascent genomic DNA (Fig. 1A). Within 24 hours, many of these nuclei had visibly condensed chromatin, suggestive of entry into mitotic prometaphase (Fig. 1B). Serum growth factors also induced the expression of the proliferating cell nuclear antigen (PCNA) (*6*) in the nuclei of $Rb^{-/-}$ (Fig. 1D) but not wild-type myotubes (Fig. 1C). Wild-type myotubes did not respond to growth factor stimulation with the induction of D-type cyclin synthesis (*7*) (Fig. 1E), but the $Rb^{-/-}$ myotubes did (Fig. 1F). Activity of the cyclin-dependent kinase $p34^{cdc2}$, which distinguishes cycling from quiescent cells, also reappeared in cellular extracts of serum-stimulated $Rb^{-/-}$ but not wild-type myotubes (*8*). Thus, distinct from wild-type myotubes, the terminally differentiated state in $Rb^{-/-}$ myotubes was reversible by restimulation with serum growth factors.

The molecular defect in the terminal differentiation of these cells could be rescued by expression of Rb from a single gene copy in myotubes derived from hemizygous ($Rb^{+/-}$) teratoma cells (Fig. 1G) or by reconstitution of $Rb^{-/-}$ muscle cells with wild-type human Rb (Table 1). The possibility that Rb might be required in other cell lineages that are destined to terminal differentiation was examined in heterogeneous populations of differentiated $Rb^{-/-}$ teratoma cells. Like the myotubes, phenotypically mature $Rb^{-/-}$ neuronal, cardiac muscle, and other cell types synthesized DNA when stimulated with growth factor–rich serum (Fig. 1H). This suggested that homozygous deletion of Rb in mouse cells might cause a global failure of authentic terminal differentiation.

The behavior of $Rb^{-/-}$ skeletal muscle cells suggested that a different molecule

Table 1. Reconstitution of Rb or constitutive expression of p107 rescues the defect in terminal differentiation in CC42 ($Rb^{-/-}$) muscle cells, and transfection of p107 induces growth arrest in RD rhabdomyosarcoma cells. CC42 or RD cells were transiently transfected with pCMV-p107 (*9*), pCMV-p107DE-HAtag (*9*), or pCMV-Rb (*16*), as in (*16*), then stimulated for 24 hours with 20% FCS in the presence of BrdU and double immunostained with BrdU and either p107, HA-tag (p107DE), or Rb mAbs (*21*). For each plasmid and a corresponding control, the number of transfected marker-positive cells that had incorporated BrdU was counted and the percent inhibition of DNA synthesis calculated as in (*22*). Each result is representative of transfections performed in triplicate.

Transfected protein	Cells counted (number)	Inhibition of DNA synthesis (%)
	CC42 ($Rb^{-/-}$) cells	
p107	276	88
p107DE	302	2
Rb	254	85
	RD cells	
p107	125	74
p107DE	141	0

expressed in these cells might compensate for the myogenic but not the permanent cell cycle arrest function of Rb. Therefore, p107, which is both structurally and functionally similar to Rb (*9*, *10*), was examined in these cells. Protein blots of wild-type C2C12 and $Rb^{-/-}$ CC42 mouse skeletal muscle cells probed with p107- or Rb-specific monoclonal antibodies (mAbs) showed that p107 was present in growing C2C12 myoblasts but was undetectable during myogenic differentiation (Fig. 2A). In contrast, p107 protein remained present (or increased) during myogenic differentiation in $Rb^{-/-}$ CC42 cells (Fig. 2A). The Rb protein was undetectable in C2C12 myoblasts but appeared in the inactive hyperphosphorylated form (pRb^{phos}) within 24 hours of induction of differentiation, and it was detected exclusively in the active hypophosphorylated form (pRb) after 96 hours (Fig. 2A) (*2*, *4*). In addition to these protein blots, immunostaining confirmed that nuclear p107 was induced during myogenic development in $Rb^{-/-}$ CC42 cells (*8*), much like Rb in developing wild-type muscle cells (*2*).

A reciprocal pattern of p107 and Rb expression in skeletal muscle cells was also documented by RNA blot (Fig. 2B). Two distinct p107 mRNA species were detected in both wild-type and mutant myoblasts, with the larger species predominating in CC42 cells. With myogenic development, the amount of p107 mRNA diminished in C2C12 cells but remained high (or increased) in differentiated CC42 cells. Within 24 hours of inducing differentiation, C2C12 cells had increased Rb mRNA to a stable high level. After 72 hours of induced differentiation, the expression of mRNA encoding the myogenic transcription factors MyoD and myogenin and the sarcomeric protein myosin heavy chain (MHC) were comparable in wild-type and mutant myotubes (*11*). That the expression of regulatory molecules other than p107 was not significantly affected by genetic deficiency in Rb was shown by the steady expression of mRNA encoding p53 in developing wild-type and $Rb^{-/-}$ muscle cells (Fig. 2B).

The compensatory pattern of p107 expression in mutant cells suggested that, like Rb in wild-type myotubes, this pocket protein might associate with the myogenic bHLH factors present in $Rb^{-/-}$ myotubes. Therefore, we examined the ability of glutathione-S-transferase (GST) fusion proteins containing adenovirus E1A oncoprotein or MyoD, Id, or E2-2 HLH protein sequences to specifically bind in vitro–translated p107 (Fig. 3A). The labeled p107 protein bound E1A and MyoD GST fusion proteins with high efficiency, but it did not significantly bind the GST leader polypeptide, Id, or E-protein GST fusion proteins. Thus, p107 exclusively bound the myogenic subclass of bHLH proteins in vitro. To confirm and extend this interaction, we performed a reciprocal binding experiment using in vitro–translated myogenin and immobilized p107, Rb, and E-protein GST fusion proteins (Fig. 3B). Although myogenin was not retained on the GST leader polypeptide, it bound to the p107, Rb, and E-protein GST fusion proteins with similar efficiency.

In skeletal myotubes, myogenin protein is predominantly found in soluble high molecular weight complexes that do not bind to the E-box DNA sequence (*12*). To provide evidence that p107 can be a biological partner of myogenin in such complexes, we incubated equal amounts of CC42 ($Rb^{-/-}$) myotube extract with an

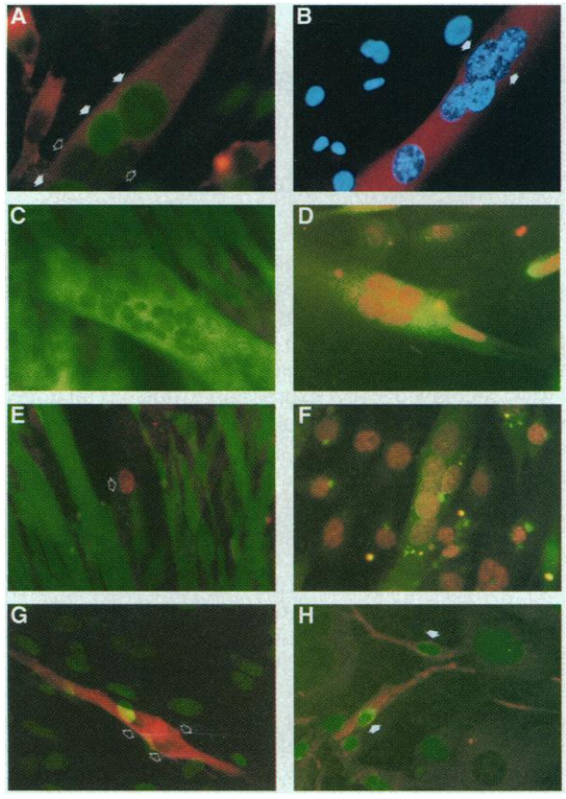

Fig. 1. Terminal differentiation is reversible in myotubes derived from $Rb^{-/-}$ cells (*21*). (**A**) CC42 ($Rb^{-/-}$) myotubes were induced with 20% FCS for 24 hours in the presence of 10 μM BrdU as in (*22*) then double immunostained with MHC (rhodamine, red) and BrdU (fluorescein, green) mAbs; closed and open arrows indicate myotube nuclei that were active or inactive in DNA synthesis, respectively. (**B**) CC42 ($Rb^{-/-}$) myotubes induced as in (A), immunostained with MHC mAb (rhodamine), and counterstained with the dye Hoechst 33258 (blue) to visualize condensed chromosomes (arrows). (**C**) C2C12 (Rb wild-type) and (**D**) CC42 ($Rb^{-/-}$) myotubes induced for 6 hours in 20% FCS and double immunostained with PCNA (rhodamine) and MHC (fluorescein) mAbs. (**E**) C2C12 (Rb wild-type) and (**F**) CC42 ($Rb^{-/-}$) myotubes induced for 6 hours in 20% FCS and double immunostained with cyclin D antiserum (rhodamine) and MHC mAb (fluorescein); open arrow indicates a residual proliferative myoblast in the differentiated C2C12 cell culture. (**G**) Myotube in hemizygous ($Rb^{+/-}$) teratoma cell culture treated as in (A) then double immunostained with MHC (rhodamine) and BrdU (fluorescein) mAbs; open arrows indicate nuclei of myotube that were not active in DNA synthesis. (**H**) Neuronal cell nuclei (arrows) that were active in DNA synthesis in $Rb^{-/-}$ teratoma cultures treated as in (A), then double immunostained with neural cell–specific intermediate filament (rhodamine) and BrdU (fluorescein) mAbs.

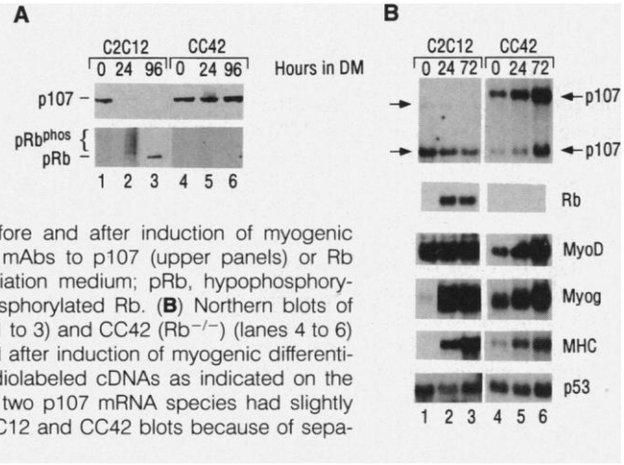

Fig. 2. Differential regulation of p107 and Rb expression during myogenic differentiation in wild-type and $Rb^{-/-}$ cultured muscle cells (*23*). (**A**) Protein immunoblots of C2C12 (Rb wild-type) (lanes 1 to 3) and CC42 ($Rb^{-/-}$) (lanes 4 to 6) whole cell extracts before and after induction of myogenic differentiation, detected with mAbs to p107 (upper panels) or Rb (lower panels). DM, differentiation medium; pRb, hypophosphorylated Rb; pRb^{phos}, hyperphosphorylated Rb. (**B**) Northern blots of C2C12 (Rb wild-type) (lanes 1 to 3) and CC42 ($Rb^{-/-}$) (lanes 4 to 6) cell poly(A)$^+$ RNA before and after induction of myogenic differentiation, hybridized with the radiolabeled cDNAs as indicated on the right (Myog, myogenin). The two p107 mRNA species had slightly different mobilities on the C2C12 and CC42 blots because of separation on different gels.

unrelated control or myogenin mAb and analyzed the precipitates by protein blot with a p107 mAb (Fig. 3C). The myogenin mAb specifically coimmunoprecipitated p107 from this extract. The reciprocal experiment was uninformative in CC42 cells because the mAb to human p107 failed to cross-react with murine p107 under nondenaturing conditions. Therefore, human RD rhabdomyosarcoma cells (*13*) were used instead. Equal amounts of RD cell extract were incubated with an unrelated control, p107, or Rb mAb, and the immunoprecipitates were analyzed by protein blot with a myogenin mAb. Both Rb and p107 mAbs specifically coimmunoprecipitated myogenin from the RD extract (Fig. 3D). Thus, in RD rhabdomyosarcoma cells, which comprise a population of proliferating tumor cells intermixed with occasional differentiated skeletal muscle cells, myogenin was detected in complexes with either Rb or p107. Together, these binding data suggest that p107 might function in place of Rb as a partner of myogenic bHLH factors in differentiated $Rb^{-/-}$ myotubes.

Although p107 might substitute for Rb in the myogenic differentiation of $Rb^{-/-}$ cells, myogenesis in cultured cells supported by p107 was incomplete, as the terminally differentiated state achieved by these mutant cells was reversible (Fig. 1). The inability of p107 to mediate permanent cell cycle arrest in $Rb^{-/-}$ cells might have resulted from intrinsic functional differences between the p107 and Rb molecules (*9*, *10*, *14*) or from differences in the mode of regulation of these two genes and their gene products.

We tested the first possibility by examining the collaboration between p107 and myogenic factors in Saos-2–myogenin cells, a permanently transfected subline of human Saos-2 Rb-deficient osteosarcoma cells (*15*). These cells constitutively express high amounts of mouse myogenin controlled by the cytomegalovirus promoter

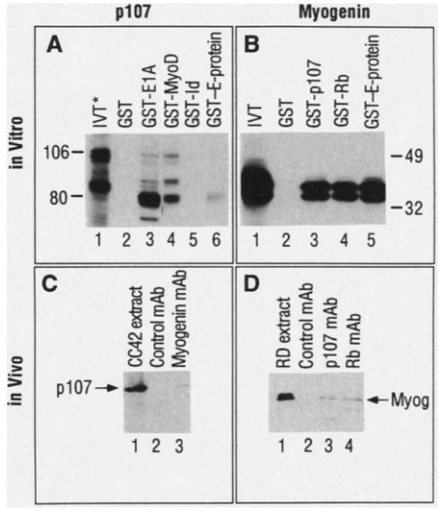

Fig. 3. Specific binding of p107 and myogenic bHLH factors in vitro and in CC42 ($Rb^{-/-}$) myotubes (*26*). (**A**) In vitro–translated ^{35}S-labeled p107 was mixed with agarose bead–immobilized GST or GST fusion proteins as shown, and bound p107 polypeptides were eluted and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. IVT*, in vitro–translated p107 (lane 1) that was run on a separate gel and therefore has slightly different mobility compared with that in lanes 3 and 4. (**B**) Same experiment as in (A) with in vitro–translated ^{35}S-labeled myogenin, which characteristically runs as a doublet, and GST fusion proteins as shown. Molecular sizes in (A) and (B) are indicated in kilodaltons. (**C**) CC42 ($Rb^{-/-}$) myotube extract was immunoprecipitated with control or myogenin mAbs in nondenaturing conditions, extensively washed, and the precipitates analyzed by protein blot with a p107 mAb; direct protein blot of CC42 extract with p107 mAb is shown in lane 1. (**D**) RD cell extract was immunoprecipitated with control, p107, or Rb mAbs and the precipitates analyzed by protein blot with a myogenin mAb; direct protein blot of RD extract with myogenin mAb demonstrates the myogenin doublet (lane 1).

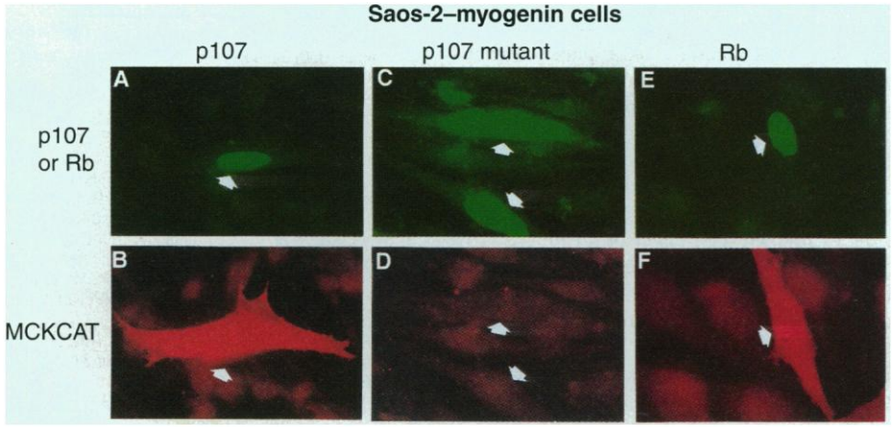

Fig. 4. Transient transfection of p107 or Rb permits activation of a myogenic factor–dependent reporter gene in Saos-2–myogenin cells. Saos-2–myogenin cells, which were derived from human Rb-deficient Saos-2 cells by permanent transfection of the mouse myogenin expression plasmid pCMV-myogenin (*8*), were transiently transfected with 10 μg of p3300MCKCAT (*17*) and 10 μg of either pCMV-p107 (**A** and **B**) (*9*), pCMV-p107DE-(HAtag) (mutant) (*9*) (**C** and **D**), or pCMV-Rb (**E** and **F**) (*16*). Cells were transfected exactly as in (*16*), induced for 72 hours in DM, then double immunostained with rabbit antiserum to CAT (rhodamine, red, lower panels) and with either p107, HAtag (p107DE), or Rb (fluorescein, green, upper panels) mAbs (*21*). The upper arrow in (C) and (D) indicates a cluster p107DE-positive cells. Identical results were obtained with transfections performed in triplicate.

Table 2. Permanent transfection of human p107 activates terminal differentiation in C2C12 myoblasts despite serum-rich culture conditions. C2C12 myoblasts in 20% FCS were permanently transfected with pCMV-p107 (HA-tag) (*9*) and selected for 2 weeks in G418 (500 μg/ml). Twenty-four random colonies were subcloned and their progeny scored for the expression of transfected p107 by immunostaining (− = no expression; +++++ = high expression), for the suppression of macroscopic colony formation (− = colonies with >2000 cells; + = colonies with <20 cells), and for the acquisition of morphological features of differentiated skeletal muscle cells such as elongation or multinucleated myotube formation by light microscopy (− = no differentiation; + = differentiated features present). Under identical conditions, colonies of C2C12 myoblasts transfected with pCMV vector alone (control) exhibited neither growth suppression nor myogenic differentiation. Identical results were obtained in two separate experiments. $G418^R$, G418-resistant.

$G418^R$ clone	Transfected p107 expression	Growth suppression	Myogenic differentiation
1	−	−	−
2	+/−	+/−	−
3	++	+	+
4	+/−	+/−	−
5	−	−	−
6	−	−	−
7	++	+	+
8	−	−	−
9	++	+	−
10	+/−	−	+/−
11	++++	+	+
12	+++	+	−
13	++	+/−	+/−
14	−	−	−
15	+++	+	+
16	−	−	−
17	++++	+	+
18	+++++	+	+
19	+++++	+	+
20	++++	+	+
21	−	−	+/−
22	−	−	−
23	−	−	−
24	+	+/−	−
Control (pCMV)	−	−	−

(pCMV) but express only low amounts of endogenous p107 that does not substitute for Rb in functional assays (*9*, *16*). When transfected alone in Saos-2–myogenin cells, MCKCAT (muscle creatine kinase–chloramphenicol acetyltransferase), a myogenic factor-dependent reporter gene construct (*17*), was silent (8). Cotransfected p107 could, however, restore the transactivation function of myogenin, shown by the induction of MCKCAT reporter activity in Saos-2–myogenin cells that expressed transfected p107 (Fig. 4). Though each Saos-2–myogenin cell that activated MCKCAT expressed transfected p107, only low efficiency was achieved, consistent with the established resistance of tumor cells to myogenic transdifferentiation (*18*). In control experiments, Saos-2–myogenin cells cotransfected with a pocket domain deletion mutant of p107 (p107DE) (9) did not activate MCKCAT (Fig. 4), whereas Rb yielded results identical to p107 (Fig. 4). Therefore, activation of the muscle-specific MCK reporter gene required either cotransfected p107 or Rb, and both were equally effective (*19*). These data show that myogenin and p107 can together mediate the activation of muscle genes in Rb-deficient cells and might, therefore, explain the myogenic differentiation of $Rb^{-/-}$ mouse embryos.

To test whether overexpression of p107 might enhance the terminal differentiation of rhabdomyosarcoma cells, we transiently transfected RD cells (*13*) with the p107 expression plasmid. DNA synthesis was blocked in 74% of the p107-transfected RD cells, but not in cells transfected with the deletion mutant p107DE (Table 1). In addition to the growth arrest, transfection of p107 significantly enhanced the ability of RD sarcoma cells to form multinucleated myotubes (8), which occurs at exceedingly low frequency in native populations of RD cells (*13*). Thus, forcible expression of p107 induced cell cycle arrest and potentiated muscle differentiation in rhabdomyosarcoma cells.

Similarly, transfection of p107 in undifferentiated C2C12 myoblasts activated terminal muscle differentiation, in a manner that was dominant over inhibitory signals from growth factors in the environment (Table 2). C2C12 myoblasts were stably transfected with the p107 expression plasmid and examined for transfected marker expression, growth ability, and for myogenic differentiation. As in the RD cells, forcible expression of p107 in wild-type C2C12 myoblasts accelerated the process of terminal differentiation. The divergent biological behavior of $Rb^{-/-}$ and wild-type myotubes was unlikely, therefore, to have resulted from the failure of p107 to functionally replace Rb in the terminal differentiation of CC42 myotubes.

An alternative hypothesis for the failure of p107 to mediate terminal differentiation in CC42 muscle cells was that although p107 could be a surrogate for Rb in myogenic differentiation, it could not induce bona fide terminal differentiation because its expression is deinduced by challenge with serum. When terminally differentiated C2C12 myotubes were restimulated with serum, the expression of Rb remained constant (Fig. 5, A and B) and this Rb was maintained in its active form (*4*), thereby locking these muscle cells in the terminally differentiated state. In contrast to Rb in wild-type muscle cells, the expression of p107 in $Rb^{-/-}$ myotubes was rapidly deinduced in response to serum growth factors, unlocking these cells from growth arrest and permitting reentry into the cell cycle (Fig. 5, C and D).

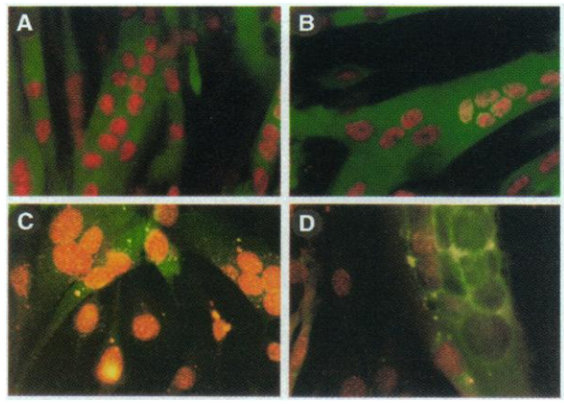

Fig. 5. Reentry of $Rb^{-/-}$ but not wild-type myotubes into the cell cycle correlates with the differential serum inducibility of Rb or p107 expression in wild-type or mutant cells, respectively. C2C12 (Rb wild-type) myotubes before (**A**) or after (**B**) a 6-hour induction with 20% FCS were double immunostained with Rb (rhodamine, red) and MHC (fluorescein, green) mAbs. CC42 ($Rb^{-/-}$) myotubes before (**C**) or after (**D**) serum induction were double immunostained with p107 (rhodamine) and MHC (fluorescein) mAbs. As in serum-stimulated CC42 myotubes (D), p107 was barely detectable by immunostaining in CC42 myoblasts growing in serum-rich medium (*8*).

Direct evidence that the serum-responsive behavior of p107 gene expression is responsible for the reversal of terminal differentiation in $Rb^{-/-}$ myotubes was obtained by transfection of a plasmid that expressed human p107 controlled by a serum nonresponsive viral promoter in CC42 cells. When constitutively expressed, p107 efficiently induced permanent growth arrest and thus rescued the mutant terminal differentiation phenotype in $Rb^{-/-}$ muscle cells (Table 1). The effect of serum growth factors on p107 abundance in $Rb^{-/-}$ myotubes is biologically equivalent to the expression of the pocket protein–inactivating SV40 or polyoma virus T antigens in C2C12 myotubes (*4*, *20*). In both cases, the de novo acquisition of a tumor suppressor protein–deficient state offers an explanation for the return of end-stage differentiated cells into the cell cycle.

Taken together, our results indicate that Rb and p107, despite their similarities, have distinct physiological roles in developing skeletal muscle cells. The p107 protein, whose biosynthesis is highly sensitive to growth factors in the environment, appears to be involved in cell cycle regulation of actively proliferating cells and normally may not participate in the process of terminal differentiation. In contrast, Rb, whose expression and activation by dephosphorylation are induced during cellular differentiation but are insensitive to growth factor restimulation, is required for acquisition and maintenance of the terminally differentiated state. The uncoupling of multinucleated myotube formation from permanent cell cycle arrest described here in $Rb^{-/-}$ mouse muscle cells appears to be relevant to other terminally differentiating cell lineages as well (Fig. 1). When applied to other systems, this observation might permit a wide variety of highly differentiated yet self-renewable lines to be established from mouse cells that are functionally deficient in terminal differentiation because of genetic deficiency in Rb.

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21. $Rb^{-/-}$ and $Rb^{+/-}$ embryonic stem cells (*5*) were grown as subcutaneous teratomas in severe combined immunodeficiency disease mice. Teratomas were explanted after 2 weeks, enzymatically disrupted, and recultured in monolayers in 20% fetal calf serum (FCS) with 1% rat serum in Dulbecco's minimum essential medium (DMEM). Myotube formation was induced by depletion of growth factors in 5% horse serum in DMEM, and myotubes were subcloned and recultured in 20% FCS for isolation of residual myoblasts. Identical results were obtained in at least three independently cloned $Rb^{-/-}$ teratoma-derived myoblast lines. Immunostaining and BrdU incorporation were done as in (*4, 22*) with the following mAbs and antisera: Rb mAb (PMG3-245; Pharmingen), p107 mAb (SD9), MHC mAb (MF20), myogenin mAb (F5D), 12CA5 hemagglutinin (HA) tag mAb (*9*), bacterial chloramphenicol acetyltransferase (CAT) antiserum (5-Prime 3-Prime), fluorescein-conjugated BrdU mAb (Boehringer), pan-neuronal filament mAb (SMI-311; Sternberger Monoclonals), PCNA mAb Z049 (Zymed), and cyclin D antiserum (UBI); secondary antisera were goat rhodamine-conjugated antibodies to rabbit Ig (Boehringer), goat fluorescein-conjugated antibodies to mouse Ig (Boehringer), rabbit rhodamine-conjugated antibodies to mouse Ig (Dako), or various goat antibodies to specific isotypes of mouse Ig conjugated to fluorochromes (Boehringer). Nuclei were counterstained with Hoechst 33258 (Sigma).
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23. Protein blots were done as in (*4*) with whole cell extracts in EBC buffer with Rb (PMG3-245) and p107 (SD9) mAbs and the ECL detection system (Amersham). RNA blots were done with polyadenylated RNA prepared as in (*24*) with Nytran (Schleicher & Schuell); equivalent loading was verified by methylene blue staining of the filters as in (*24*). The following complementary DNA (cDNA) fragments were used as probes: full-length human Rb from pCMV-Rb (*16*), 1.2-kb (Bam HI–Dra III) 5′-subfragment of human p107 from pCMV-p107 (*9*), and full-length murine MyoD and myogenin, rat skeletal MHC, and human p53, as described in (*25*).
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26. The GST binding experiments and coimmunoprecipitation protein blots were done as in (*4*). GST-Id contained full-length mouse Id and GST–E-protein contained the bHLH domain of human E2-2.
27. We thank H. te Riele and A. Berns for providing the $Rb^{-/-}$ and $Rb^{+/-}$ embryonic stem cells, M. Ewen for GST-p107, W. Kaelin for pCMV-Rb, J. Buskin for pMCKCAT, B. Vogelstein for pCMV-Neo-Bam, N. Dyson for SD9 mAb, W. Wright for F5D mAb, D. Bader for MF20 mAb, and E. Ferris for technical assistance. Supported by grants from NIH, the Muscular Dystrophy Association of America, the Howard Hughes Medical Institute, and the MERCK/American Federation for Clinical Research (J.W.S.).

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Inhibition of Hepatic Chylomicron Remnant Uptake by Gene Transfer of a Receptor Antagonist

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The low density lipoprotein receptor-related protein (LRP) has been proposed to mediate in concert with the LDL receptor (LDLR) the uptake of dietary lipoproteins into the hepatocytes. This hypothesis was tested by transient inactivation of LRP in vivo. Receptor-associated protein (RAP), a dominant negative regulator of LRP function, was transferred by an adenoviral vector to the livers of mice lacking LDLR ($LDLR^{-/-}$). The inactivation of LRP by RAP was associated with a marked accumulation of chylomicron remnants in $LDLR^{-/-}$ mice and to a lesser degree in normal mice, suggesting that both LDLR and LRP are involved in remnant clearance.

Apolipoprotein E (apoE) and apoB48 (a truncated form of apoB100) are constituents of chylomicron remnants, the carriers of dietary cholesterol. Whereas apoB48 is primarily a structural component of these lipoproteins, apoE is required for their receptor-mediated endocytosis in the liver (*1, 2*). Two hepatic receptors are known to bind apoE-containing lipoproteins: LDLR and LRP. Experiments performed in vitro and in intact animals demonstrate that LDLR binds to and takes up chylomicron remnants (*3, 4*). However, humans, rabbits, and mice lacking functional LDLR do not accumulate chylomicron remnants in their circulation (*5, 6*). It was therefore suggested that LRP also is involved in chylomicron remnant clearance and that it substitutes for LDLR when the latter is deficient (*7, 8*). This proposed function of LRP has been impossible to prove in a physiological experiment because embryos lacking LRP fail to develop in utero (*9*). Embryonic lethality probably occurs because LRP is a multifunctional receptor that not only participates in lipid metabolism but also is involved in the uptake of extracellular proteases, the protease inhibitor α_2-macroglobulin, and other macromolecules (*10, 11*). This precludes the analysis of remnant metabolism in animals that are genetically deficient in LRP.

One way to circumvent this problem is to administer a specific inhibitor of LRP. A candidate inhibitor is a receptor-associated protein (RAP) of approximately 39 kD that copurifies with LRP (*10*). When added to cultured cells or to purified receptor, RAP binds to LRP at multiple sites and blocks ligand-receptor interaction (*12–15*). In the current study we used an adenovirus vector to carry a RAP complementary DNA (cDNA) into the liver of adult mice in order to test the hypothesis that RAP overproduction would inactivate LRP and cause chylomicron remnants to accumulate in the circulation. Recombinant adenoviruses have proven to be efficient gene transfer vectors that preferentially target hepatocytes when injected intravenously into animals (*6, 16, 17*). As recipient animals we have used wild-type (+/+) mice and mice that lack LDL receptors ($LDLR^{-/-}$) as a result of targeted gene disruption. The latter mice have elevated levels of LDL but no gross accumulation of chylomicron remnants when fed a normal Chow diet (*6*).

Recombinant adenoviruses containing either the RAP cDNA (AdCMV-RAP) or the *Escherichia coli* β-galactosidase gene (AdCMV–β-Gal) (*18*) were intravenously injected into wild-type or $LDLR^{-/-}$ mice (*19*). Five days after the injection of the virus, we assessed LRP activity by monitoring the ability of the mice to clear an LRP-specific ligand, ^{125}I-labeled α_2-macroglobulin (^{125}I-α_2M), from their circulation. Other animals were injected with ^{125}I-labeled asialofetuin (^{125}I-ASF), which enters the cell through the asialoglycoprotein re-

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