Rb and N-ras Function Together To Control Differentiation in the Mouse

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The product of the retinoblastoma tumor suppressor gene (Rb) can control cell proliferation and promote differentiation. Murine embryos nullizygous for Rb die midgestation with defects in cell cycle regulation, control of apoptosis, and terminal differentiation of several tissues, including skeletal muscle, nervous system, and lens. Previous cell culture-based experiments have suggested that the retinoblastoma protein (pRb) and Ras operate in a common pathway to control cellular differentiation. Here we have tested the hypothesis that the proto-oncogene N-ras participates in Rb-dependent regulation of differentiation by generating and characterizing murine embryos deficient in both N-ras and Rb. We show that deletion of N-ras rescues a unique subset of the developmental defects associated with nullizygosity of Rb, resulting in a significant extension of life span. Rb−/−; N-ras−/− skeletal muscle has normal fiber density, myotube length and thickness, in contrast to Rb−/− deficient embryos. Additionally, Rb−/−; N-ras−/− muscle shows a restoration in the expression of the late muscle-specific gene MCK, and this correlates with a significant potentiation of MyoD transcriptional activity in Rb−/−; N-ras−/−, compared to Rb−/− myoblasts in culture. The improved differentiation of skeletal muscle in Rb−/−; N-ras−/− embryos occurs despite evidence of deregulated proliferation and apoptosis, as seen in Rb−/− deficient animals. Our findings suggest that the control of differentiation and proliferation by Rb are genetically separable.

The loss of tumor suppressor gene function is a common event in the development of human cancer. The retinoblastoma gene (Rb) has served as the paradigm for the study of this class of genes (67, 68). Mutations resulting in the inactivation of Rb are found in a large fraction of human cancers of both mesenchymal and epithelial origin (57, 62). An understanding of how the retinoblastoma protein (pRb) exerts its tumor-suppressive action can be gained from knowledge of the biological and molecular consequences of its inactivation.

pRb participates in the control of cell cycle progression, apoptosis, and differentiation. How it exerts effects on proliferation is well understood. This appears to be achieved by its regulated interaction with the E2F family of transcription factors (14, 56, 63). E2F can bind to and promote the expression of a number of genes involved in cell cycle progression (e.g., DHFR). pRb, by binding to E2F, can inhibit the transactivation function of E2F. In addition, pRb is targeted to promoters through its interaction with E2F, and its association with other factors involved in altering chromatin structure (e.g., histone deacetylase and BRG1) leads to active repression of genes involved in cell proliferation (e.g., cyclin E). The ability of pRb to influence cell death also appears to involve its regulation of E2F (13, 56, 63).

How pRb regulates differentiation is poorly understood. Most differentiation programs involve withdrawal from the cell cycle, but the participation of pRb appears to extend beyond an ability to merely facilitate the process by inhibiting E2F and cell cycle progression. Naturally occurring mutants of pRb can be identified that retain tumor suppressor activity and the ability to promote differentiation when assayed in vitro despite having lost the capability to bind to E2F (28, 58). Consistent with this, pRb influences the activity of a number of transcription factors known to participate in different differentiation processes, such as MyoD, the glucocorticoid receptor, C/EBF1, and C/EBPB (3, 4, 11, 43, 44, 59, 61). Also, cell culture studies have demonstrated a key role for pRb in myogenesis, osteogenic differentiation, and adipogenesis (3, 6, 11, 43, 54, 58, 61).

Mouse genetics have been employed to better understand the physiological functions of pRb (35, 66). Mice heterozygous for Rb succumb to pituitary tumors (15, 17, 19), while inactivation of both Rb alleles results in embryos that die in midgestation (5, 19, 31, 71). These embryos are characterized by...
defects in erythroid, neuronal, and skeletal muscle differentiation, and ectopic S-phase entry and apoptosis are observed in the central nervous system (CNS), peripheral nervous system (PNS), lens, and skeletal muscle (5, 19, 31, 32, 71). The contribution of deregulated E2F activity to these phenotypes has been assessed with compound embryos lacking Rb and E2F-1 or E2F-3 (21, 64, 72). These embryos live longer than their Rb−/− counterparts, and this has been attributed to a partial restoration of fetal liver erythropoiesis. Additionally, loss of either E2F-1 or E2F-3 can suppress the deregulated proliferation and apoptosis to significant but varying degrees in the CNS, PNS, and lens. Importantly, the lens of Rb-deficient embryos also shows signs of aberrant differentiation that are not rescued by loss of E2F-1, suggesting again that Rb’s influence on cell cycle progression and apoptosis is genetically separable from its regulation of differentiation (36). A notable aspect of these studies is the observation that the extended life span of Rb−/−; E2F-1−/− and Rb−/−; E2F-3−/− embryos reveals additional phenotypes, including developmental defects in the lung and heart, and more pronounced defects in skeletal muscle. Abnormalities in skeletal muscle differentiation are also observed in Rb−/− mice with a partial reconstitution of Rb (71) and in Rb−/−; Id2−/− mice (30). Indeed, respiratory failure resulting from the complete lack of muscle fibers in the diaphragm is thought to be responsible for their neonatal death. Thus, Rb appears to play important roles in differentiation of several tissues throughout embryonic development. Genetic dissection of Rb-dependent pathways holds promise for a greater understanding of its role in development and may shed light on how its inactivation contributes to the genesis of tumors of diverse histological origin.

The ras genes are the most frequently mutated proto-oncogenes in human cancer (1, 24). Like pRb, Ras is thought to play important roles in differentiation and proliferation, and there has been considerable progress in elucidating the downstream effectors of Ras. Many of the transcription factors influenced by pRb, e.g., MyoD, glucocorticoid receptor, and C/EBPβ, are also influenced by Ras (16, 26, 27, 45, 48–50, 55). Recent efforts have been devoted to understanding the individual roles of the three ras genes (H-ras, K-ras, and N-ras) in the mouse. Mice nullizygous for H-ras or N-ras, or both, are developmentally normal, but homozygous deletion of K-ras results in lethality during gestation (22, 25). A role for N-ras during embryogenesis was revealed by the observation that K-ras−/−; N-ras−/− embryos die during gestation, suggesting that K- and N-ras have partially overlapping functions (22). Testing for genetic interactions suggests a means of revealing other functional roles for N- and H-ras.

We demonstrated previously that cultured fibroblasts derived from Rb-deficient embryos possess abnormally high levels of Ras activity and showed that the ability of pRb to negatively regulate Ras was linked to its effects on differentiation (33). To test the physiological relevance of these observations, we have generated embryos lacking both Rb and N-ras. We find that loss of N-ras reverses many of the differentiation defects observed in both mid- and late-gestational Rb-deficient embryos, despite the absence of obvious effects on proliferation and cell death. Our analyses suggest that N-ras operates with Rb in the control of cellular differentiation.

**Materials and Methods**

Mouse strains. Parental Rb−/− and N-ras−/− mice were maintained on a mixed genetic background (C57BL/6 × 129/Sv and C57BL/6 × 129/Ola, respectively) and intercrossed to generate subsequent founders. Rb−/−; N-ras−/− females were crossed with Rb−/−; N-ras−/−; Rb−/−; N-ras−/−, or Rb−/−; N-ras−/− males. Timed pregnancies were established by the detection of a plug, taken as embryonic day 0.5 (E0.5). Mice and embryos were genotyped by PCR with genomic DNA extracted from tails and yolk sacs, respectively, as previously described (19, 65). All animal experimentation was performed at the Dana-Farber Cancer Institute Animal Resource Facility in accordance with the guidelines of the National Institutes of Health.

**Histology and immunohistochemistry.** Embryos were fixed in Bouin’s solution, rinsed with 70% ethanol, and embedded in paraffin for sectioning. Sections (6 μm) were stained with hematoxylin and eosin (H&E). Alternatively, sections were incubated with a monoclonal antibody (MY-32, Sigma) to myosin heavy chain (MHC) following deparaffinization and rehydration. To identify proliferating cells, bromodeoxyuridine (BrdU) was injected intraperitoneally (33 μg per mouse) 1 h prior to sacrifice. Fixed embryos were rinsed with 70% ethanol and embedded in paraffin, from which sections were cut. After rehydration, the endogenous peroxidase activity was quenched with 3% H2O2–10% methanol in phosphate-buffered saline (PBS) (pH 7.4). Sections were then treated successively with 0.05 mM trypsin, 2 N HCl, and PBS (pH 6.0). After blocking with 6% goat serum, sections were incubated with an anti-BrdU mouse monoclonal antibody (B44; Becton Dickinson) in the presence of 0.5% Tween 20 in PBS (pH 7.4). Bound primary antibody (MHC and BrdU) was detected with the ABC mouse peroxidase detection system (Santa Cruz Biotechnology). For the analysis of cell death, embryos were fixed in formalin (3.7% formaldehyde in PBS), from which tissue sections were generated. Apoptosis was measured by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay with the Apotag mouse peroxidase plus system (Intergen). Counterstaining was with 0.5% methyl green in 0.1 M sodium acetate (pH 4.0) or methanol.

**Analysis of gene expression.** RNA was extracted from homogenized carcasses of live E14.5 embryos by using the RNeasy minikit (Qiagen). Northern blot analyses for MyoD and myogenin were performed as described previously (60). Radiolabeled antisense riboprobes were prepared by using the Lign’Sirne and MAXScript in vitro transcription kits (Ambion). Multiplex RNase protection assays were performed with RPAIII (Ambion) in accordance with the manufacturer’s instructions. Probes correspond to the following sequences: muscle creatine kinase (MCK), nucleotides 808 to 1283 of the sequence under GenBank accession number X03233; and α-actin, nucleotides 739 to 979 of the sequence under GenBank accession number X03672.

**MEFs and transcriptional transactivation assays.** Mouse embryo fibroblasts (MEFs) were prepared from live E12.5 embryos as described previously (9) and plated in six-well dishes at 105 cells per well in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum. MEFs were transfected as described in the legend to Fig. 3, using Fugene 6 (Roche). At 24 h after transfection, the cell medium was changed to DMEM containing 2% horse serum (Sigma) and 10 μg of insulin per ml. Luciferase and β-galactosidase activities were assayed 72 h later (33). The plasmids pCSA-MyoD (43), MCK-Luc (44), MEF2-luc (44), pCXN2-H-rasV12 (52), pBPT2R-3b (38), and pCMV-H-gal (29) had been described previously. pBabe-N-ras was constructed by PCR amplification with primers 5′-CCCGGATCCGCCGACCATGACTGATACAAACTGTTGG-3′ and 5′-CCCCGATATTCGGTATACACACATGGCCATATCC-3′ and pZIPNeoSV(X)-EE-N-ras (23) as a template, followed by digestion with BamHI and EcoRI. The sequence was confirmed after subcloning into pBabe-puro.

**Infections and immunostaining.** MEFs were infected with medium containing MDM virus from pBabe-MyoD (43)-transfected EcoPack-293 cells (Clontech/BD Biosciences). The viral titer was estimated from the efficiency of colony formation following selection with 4 μg of puromycin per ml. Two thousand CFU/g of puromycin per ml. Two thousand CFU/ml of bacteria were plated in six-well dishes at 105 cells per well in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum. At 24 h after infection, the medium was changed to DMEM containing 2% horse serum (Sigma) and 10 μg of insulin per ml. Luciferase and β-galactosidase activities were assayed 72 h later (33). The plasmids pCSA-MyoD (43), MCK-Luc (44), MEF2-luc (44), pCXN2-H-rasV12 (52), pBPT2R-3b (38), and pCMV-H-gal (29) had been described previously. pBabe-N-ras was constructed by PCR amplification with primers 5′-CCCGGATCCGCCGACCATGACTGATACAAACTGTTGG-3′ and 5′-CCCCGATATTCGGTATACACACATGGCCATATCC-3′ and pZIPNeoSV(X)-EE-N-ras (23) as a template, followed by digestion with BamHI and EcoRI. The sequence was confirmed after subcloning into pBabe-puro.

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RESULTS

Survival of embryos. We first determined if deletion of N-ras prolonged the survival of Rb-deficient embryos. Rb and N-ras heterozygotes were bred and the resulting Rb+/−; N-ras+/− mice were intercrossed. No viable Rb−/−; N-ras−/− pups were recovered. Subsequently, Rb+/−; N-ras+/− females were crossed with Rb+/−; N-ras+/+, Rb+/−; N-ras+/−, or Rb+/−; N-ras−/− males, and embryonic viability during a time course of gestation was assessed by the detection of a beating heart (Table 1). Death of embryos lacking Rb alone was first noted at E12.5 (30%). Consistent with previous results, the majority of Rb-deficient embryos (65%) were dead by E14.5, with no viable embryos being recovered at E15.5 or beyond. Remarkably, most Rb+/−; N-ras−− embryos (75%) were alive at E16.5, with some living as long as E17.5 (33%). Rb−/−; N-ras−/− embryos showed an intermediate level of survival. Rb+/−; N-ras−/− embryos were macroscopically indistinguishable from their wild-type littermates at E13.5 (Fig. 1A and B). Rb+/−; N-ras−/− embryos continued to display a remarkably normal appearance at a later stage in development (E15.5), although they were noticeably smaller (Fig. 1D and E). N-ras−/− embryos were indistinguishable in appearance from their wild-type littermates (data not shown). Together, these data indicate that loss of N-ras can significantly prolong the life span of Rb-deficient embryos, suggesting the possibility that a genetic interaction between these genes may reverse key developmental defects associated with Rb loss.

Skeletal muscle development. Our previous cell culture-based work linked pRb and Ras to the control of myogenic differentiation, and thus we initially focused on the analysis of skeletal muscle. Rb-deficient embryos display a number of defects in skeletal muscle differentiation, many of which are observed as early as E13.5, and these are exacerbated in late gestational Rb compound mutants (see the introduction). We began by assessing whether N-ras loss might influence the development of Rb-deficient muscle by directly comparing the axial muscle from live E14.5 wild-type, N-ras−/−, Rb−/−, and Rb−/−; N-ras−/− littermates. Thoracic skeletal muscle from Rb−/− embryos showed a dramatic reduction in fiber density compared to that from wild-type and N-ras−/− embryos upon H&E staining of tissue sections (Fig. 2A to C). This defect was even more apparent in sections immunostained with an antibody to MHC (Fig. 2E to G and M). Myotubes were also found to be shorter and thinner in Rb-deficient skeletal muscle (Fig. 2G and N). Strikingly, Rb−/−; N-ras−/− axial muscle clearly displayed normal fiber density, myotube length, and thickness (Fig. 2D, H, M, and N). The presence of abnormal large nuclei associated with ectopic DNA synthesis was detected at low frequency (~1 to 2%) by H&E and BrdU staining in Rb−/−; N-ras−/− and Rb−/− muscle, consistent with previous findings (Fig. 2D and O and data not shown) (21, 71). Additionally, apoptotic cells (i.e., TUNEL positive) were seen in the skeletal muscle of Rb-deficient and Rb−/−; N-ras−/− embryos, unlike their wild-type and N-ras−/− counterparts (Fig. 2I to L and P). Together, these results suggest that loss of N-ras prevents the reduction in muscle fiber density and abnormal myotube formation seen in E14.5 Rb-deficient skeletal muscle, while having no impact on ectopic proliferation or cell death.

Given the relatively normal appearance of Rb+/−; N-ras−/− skeletal muscle at E14.5, we extended our gross histological examination to the expression of muscle-specific genes. For this analysis, total RNA derived from the carcasses of live E14.5 embryos was used. MyoD and myogenin, both of which are expressed during early stages of muscle development (53), were expressed at comparable levels in Rb+/−; N-ras+/−, Rb+/−; N-ras−/−, Rb−/−; N-ras+/−, and Rb−/−; N-ras−/− embryos as determined by Northern blot analysis (Fig. 2Q). MCK, a late marker of muscle differentiation, is normally expressed from E13 onward (38). RNAse protection assay for MCK revealed almost no expression in Rb-deficient (Rb−/−; N-ras−/−) embryos (Fig. 2R), consistent with a previous report on Rb-deficient embryos with partial reconstitution of Rb (71). By contrast, normal levels of MCK transcripts were detected in Rb−/−; N-ras−/− embryos. MCK is a transcriptional target of MyoD, and the activity of this transcription factor requires pRb for its full activation (44). In this context, pRb acts in part by cooperating with MyoD to promote the transcriptional activity of MEF2C, which also participates in the transcriptional induction of MCK (44). We therefore assessed the influence of N-ras loss on the activity of MyoD and MEF2C in cells nullizygous for Rb. MEFs were generated from wild-type, Rb+/−, N-ras−/−, Rb−/−,...
and \( Rb^{-/-} \); \( N\text{-}ras^{-/-} \) embryos and transfected with a MyoD-encoding plasmid, and the activity of an MCK promoter-reporter construct was measured under culture conditions known to induce myogenic differentiation. The activity of MyoD was significantly reduced in \( Rb^{-/-} \)-deficient myoblasts. By contrast, \( Rb^{-/-}; N\text{-}ras^{-/-} \) myoblasts possessed levels of activity similar to those seen in wild-type, \( Rb^{+/+} \), and \( N\text{-}ras^{-/-} \) cells (Fig. 3A). Similar observations were found with a MEF2 promoter-reporter construct (Fig. 3B). To rule out the possibility that \( N\text{-}ras \)-independent mechanisms might be responsible for the restoration of MyoD and MEF2C activity in \( Rb^{-/-}; N\text{-}ras^{-/-} \) MEFs, we reconstituted \( N\text{-}ras^{-/-} \) expression. Expression of wild-type \( N\text{-}ras \) in \( Rb^{-/-}; N\text{-}ras^{-/-} \), but not \( Rb^{+/+} \), MEFs significantly reduced the activity of both the MCK (Fig. 3C) and MEF2 (Fig. 3D) reporters, suggesting that loss of \( N\text{-}ras \) and not other genetic events potentiates the activity of MyoD and MEF2C function in vitro.

The analyses described above provided a direct comparison between wild-type, \( Rb^{-/-} \), and \( Rb^{-/-}; N\text{-}ras^{-/-} \) skeletal muscle from matched litters. The next question was whether the improved skeletal muscle development observed in E14.5 \( Rb^{-/-} \); \( N\text{-}ras^{-/-} \) embryos persisted during later stages of embryonic development. We found that the lengths and thicknesses of myotubes in \( Rb^{-/-} \); \( N\text{-}ras^{-/-} \) and wild-type thoracic muscle from sagittal sections of E17.5 embryos were largely indistinguishable as judged by MHC immunostaining (Fig. 4A and B). A similar analysis of transverse sections though the cervical skeletal muscle revealed that the densities of the fibers in \( Rb^{-/-} \); \( N\text{-}ras^{-/-} \) and wild-type muscle were also similar (Fig. 4C and D). However, the fibers appeared to be somewhat disorganized in \( Rb^{-/-}; \ N\text{-}ras^{-/-} \) muscle. Nevertheless, from the area occupied by the fibers in transverse sections and the length of the myotubes seen in sagittal sections, these data suggest that the axial muscle masses (proportional to body size) were similar in \( Rb^{-/-} \); \( N\text{-}ras^{-/-} \) and wild-type embryos. Normal fiber density and MHC staining were also seen in intercostal muscle from sagittal sections of \( Rb^{-/-}; \ N\text{-}ras^{-/-} \) embryos (Fig. 4E and F). A similar analysis of the diaphragm revealed normal levels of MHC, although a slightly reduced...
Fiber density was apparent (Fig. 4G to J). The presence of cells with abnormally large nuclei still persisted in Rb\(^{+/−}\)/H11002; N-ras\(^{+/−}\)/H11002; N-ras\(^{+/−}\)/H11002 embryos; skeletal muscle (Fig. 4K and L). These results suggest that loss of N-ras allows for near-normal development of Rb-deficient skeletal muscle during embryogenesis.

**Ectopic S-phase entry and death in the CNS.** E13.5 Rb\(^{+/−}\) embryos are characterized by extensive proliferation and cell death in normally postmitotic regions of the CNS, PNS, and lens (5, 19, 31). We therefore sought to determine if loss of N-ras affected these phenotypes. Pregnant females at 13.5 days of term were injected intraperitoneally with BrdU, and DNA synthesis was assessed by immunological detection of incorporated BrdU in tissue sections. BrdU-positive cells were abundant in the intermediate zone of the hindbrain (CNS) in both Rb\(^{+/−}\) and Rb\(^{+/−}\); N-ras\(^{+/−}\) embryos (Fig. 5A to D and I). Similar results were obtained from our analysis of the fiber cell compartment of the developing lens and dorsal root ganglion of the PNS (Fig. 5I and data not shown). Apoptosis was assessed by TUNEL staining. As described previously, Rb\(^{+/−}\) embryos showed an elevated level of cell death in the lens, the PNS, and the cortical region around the fourth ventricle of the brain (CNS) compared to wild-type and N-ras\(^{+/−}\) embryos (Fig. 5E, F, and G and data not shown). Rb\(^{+/−}\); N-ras\(^{+/−}\) mutants showed a degree of apoptosis similar to that found in Rb-deficient embryos (Fig. 5G, H, and I and data not shown). Together, these results indicate...
that the ectopic proliferation and apoptosis characteristic of Rb-deficient embryos are not altered by the concomitant loss of N-ras.

**Lung and heart development.** Given the improved survival and development of Rb−/−; N-ras−/− embryos, we conducted a full histological analysis of these animals. We focused initially on the lung, since two previous reports had revealed an essential role for Rb in the development of this organ during late gestation (64, 72). Specifically, well-defined terminal bronchioles and primitive alveoli were not observed in embryos lacking Rb and either E2f-1 or E2f-3. Our analysis of E17.5 Rb−/−; N-ras−/− embryos revealed a normal appearance for the lung (Fig. 6A and B). Consistent with these findings, the space occupied by the lung in the intrathoracic cavity of Rb−/−; N-ras−/− embryos was comparable to that observed in wild-type littermates (data not shown). In addition, the hearts of Rb−/−; E2f-3−/− embryos have been reported to possess reduced cardiac muscle fiber density, resulting in a marked thinning of the heart wall.

FIG. 2—Continued.
This defect was not apparent in Rb/H11002/H11002/N-ras/H11002/H11002 embryos (Fig. 6C and D). Analysis of other organs did not reveal any defects beyond those described for Rb-deficient embryos.

DISCUSSION

To understand the functions of Rb, much effort has been devoted to characterizing the biological consequences of its inactivation in the mouse. Rb+/− embryos die in midgestation with defects in a restricted set of tissues. Results from various attempts to rescue this embryonic lethality have revealed that Rb is also required at later stages in development in several tissues. The diversity of defects exhibited in Rb-deficient embryos suggests that pRb engages several downstream effector pathways to affect a multitude of biological outcomes. Our results demonstrate that loss of N-ras significantly extends the life span of Rb-deficient embryos and that the effect of N-ras loss on the phenotypes associated with Rb nullizygosity extends to several differentiation programs, including skeletal muscle differentiation and possibly development of the lung and heart.
Below we discuss the effects of N-ras loss on the phenotypes associated with deficiency in Rb.

**Skeletal muscle differentiation.** The first extensive analysis of Rb-deficient skeletal muscle was made by using Rb<sup>−/−</sup> embryos in which Rb expression had been partially restored (21, 71). Importantly, the transgene used, although effective in extending life span, does not direct expression of Rb to the muscle (20), affording those workers the ability to study skeletal muscle differentiation in the absence of Rb at later stages of gestation. Defects in skeletal muscle were readily apparent at the time that Rb-deficient embryos normally die, and they became more pronounced at later stages of gestation, during which time the skeletal muscle continues to develop. Rb-deficient skeletal muscle was characterized by reduced fiber density, abnormal myotube formation, and length and lack of expression of late markers of differentiation. Additionally, apoptotic cells and ectopic S-phase entry with evidence of endoreduplication were noted. So severe was the muscle defect that respiratory failure is thought to be the cause of death. Similar skeletal muscle phenotypes were noted following loss of Rb and E2f-1, E2f-3, or Id2 (30, 64, 72). A complete lack of muscle fibers and MHC staining was noted in the diaphragms of Rb<sup>−/−</sup>; Id2<sup>−/−</sup> neonates, suggesting that these mice also died of respiratory failure (30). The lack of an effect of Id2 loss on any of the muscle phenotypes is not surprising given that it is not expressed in this tissue. This, however, is not the case for E2f-1 and E2f-3. The inability of E2f-1 or E2f-3 deficiency to ameliorate any of the muscle phenotypes, while clearly rescuing defects in other tissues, has led to the notion that the pathway(s) by which pRb influences skeletal muscle development may involve a positive differentiation function of pRb (64). Consistent with this, in vitro studies indicate that the transcriptional induction of certain muscle-specific genes by MyoD requires Rb (11, 43). However, with the myriad of defects observed in Rb<sup>−/−</sup> skeletal muscle in vivo, it is unclear whether there is a single underlying mechanism.

Our results begin to address these issues. Loss of N-ras significantly improved myotube formation and muscle fiber density in both mid- and late-gestational Rb-deficient muscle. In addition, the expression of a late marker of differentiation, MCK, was restored to wild-type levels. By contrast, cell death and ectopic S-phase entry still persisted in Rb<sup>−/−</sup>; N-ras<sup>−/−</sup> skeletal muscle. These results indicate that the various phenotypes that characterize Rb-deficient muscle are genetically sep-
FIG. 4. Effect of N-ras loss on skeletal muscle development in E17.5 Rb-deficient embryos. (A to D) Thoracic (A and B) and cervical (C and D) skeletal muscle, immunostained with an antibody to MHC and counterstained with methyl green, derived from sagittal sections of E17.5 embryos of the indicated genotype from the same litter. Panels A and B show longitudinal sections of the muscle, while panels C and D show transverse sections through the muscle fibers. Magnification, ×20. (E to J) Immunostained and counterstained sections of intercostal muscle between the fourth and fifth ribs (E and F) and the diaphragm (G to J) derived from sagittal sections of embryos of the indicated genotype. Represented are the transverse sections of each muscle group. Magnifications, ×20 (E and F), ×10 (G and H), and ×40 (I and J). (K and L) Longitudinal sections through the fibers of thoracic muscle from sagittal sections of E17.5 embryos derived from the same litter with the indicated genotype were stained with H&E. Note the presence of abnormal large nuclei in Rb<sup>−/−</sup>; N-ras<sup>−/−</sup> muscle (arrow). Magnification, ×40.
Further, they suggest that deregulated proliferation and apoptosis are likely not responsible for the inability of certain aspects of the muscle differentiation program to proceed during embryogenesis. Our data suggest that multiple Rb-dependent pathways influence skeletal muscle differentiation and that the genetic interaction between Rb and N-ras controls a subset of these.

Several studies have documented that constitutively active Ras can block the transcriptional functions of MyoD (26, 45), and this appears to occur through multiple effector pathways (48–50). These findings are consistent with our previous demonstration that aberrant activation of Ras following loss of Rb is linked to the defect in MyoD transcriptional function in Rb-deficient cells (33). Our genetic analysis in cell culture suggests that loss of N-ras (effectively reducing the levels of active Ras) in Rb-deficient fibroblasts restores the activity of MyoD, which participates in the induction of MCK during myogenesis. Further, our results reveal that activity of MEF2C, which cooperates with MyoD in the transcription of the MCK gene (44), is also restored in Rb−/−; N-ras−/− myoblasts. Together with our
observation that Rb<sup>−/−</sup>; N-ras<sup>−/−</sup> embryos show normal levels of MCK expression, these data suggest that the ability of N-ras loss to rescue several of the skeletal muscle defects manifest in Rb-deficient embryos is mediated at least in part through a cell-autonomous rescue of MyoD and MEF2C function.

Recently, it has been shown that Rb-deficient placentas are abnormal and that this contributes to certain developmental defects that characterize Rb<sup>−/−</sup> embryos (69). Specifically, Rb-deficient embryos supplied with wild-type extraembryonic cells can be carried to term and die soon after birth. Analyses of these embryos revealed that the erythroid, CNS, and PNS defects were largely rescued (69). By contrast, defects in certain tissues, such as skeletal muscle (69; G. Leone, personal communication) and the lens (69) were not rescued. Further, respiratory failure due to defects in the muscle fibers in the diaphragm is responsible for the neonatal death of these animals (G. Leone, personal communication). This suggests that Rb-dependent placental abnormalities do not contribute to the skeletal muscle defects and thus the genetic interaction between Rb and N-ras described here is confined to the embryo proper. Consistent with this interpretation, Rb<sup>−/−</sup> embryos in which Rb expression has been partially restored by creation of transgenic animals (71) or where there is a restoration of Rb function in extraembryonic tissues (69) share many features, suggesting that in the former placental defects were not present. However, in both of these genetically modified mice skeletal muscle defects are still manifest, indicating that abnormalities in extraembryonic cell lineages do not contribute to Rb-dependent skeletal muscle phenotypes.

Separation of differentiation and proliferation control. Studies in mammalian cell culture have placed pRb both upstream and downstream of Ras. Inhibition of Ras activity brings about G<sub>1</sub> arrest, and this is dependent on the presence of functional pRb as well as E2F-4 and E2F-5 (10, 34, 41, 47). In addition, ectopic expression of cyclin D1 and its catalytic partner Cdk4 can reverse the arrest induced by inhibition of Ras (47). Given that cyclin D1 is a transcriptional target of the Ras/mitogen-activated protein kinase (MAPK) pathway, it has been suggested that pRb is a downstream target of Ras signaling.

Inactivation of pRb by simian virus 40 large T antigen has been shown to result in elevated Ras activity (33, 51). In addition, Rb-deficient mouse embryonic fibroblasts possess elevated Ras activity compared to their wild-type counterparts (33). Previously, we showed that the ability of pRb to regulate Ras activity was linked to pRb’s positive control of certain differentiation processes (33). For example, the activity of the glucocorticoid receptor, which is known to require pRb for activation (59), was induced in Rb-deficient cells by the inhibition of Ras activity. In this regard, it is interesting that Rb-deficient lungs lacking E2f-1 or E2f-3 show impaired development, resulting in an atelectic appearance similar to that observed in glucocorticoid receptor-deficient mice (7, 64, 72). This phenotype is not observed in Rb<sup>−/−</sup>; N-ras<sup>−/−</sup> embryos, suggesting the possibility that loss of N-ras ameliorates the Rb-deficient lung defect through an influence on glucocorticoid receptor transcriptional functions. Likewise, we reported that inhibition of aberrant Ras activity in Rb-deficient myo-
ally active in the expression of a late marker of differentiation (33). Importantly, these in vitro findings are in agreement with our in vivo analysis demonstrating that the expression of MCK, a transcriptional target of MyoD, is restored in Rb⁻/⁻; N-ras⁻/⁻ muscle and our observation that MyoD is more transcriptionally active in Rb⁻/⁻; N-ras⁻/⁻ MEFs than in their Rb⁻/⁻ counterparts. More recently, others have linked the elevated Ras and MAPK activity in Rb-deficient MEFs to their inability to undergo adipose conversion (12). Together, these data suggest that pRB can act antagonistically upstream of Ras to influence certain differentiation processes in vivo without an apparent effect on the cell cycle.

Studies with the nematode Caenorhabditis elegans have revealed some striking similarities to the signaling between pRB and Ras in mammalian cells. C. elegans Ras (LET-60) function is not essential for proliferation during embryogenesis and the four larval stages but is required later in development for the establishment of distinct cell fates (70). The C. elegans genes lin-35, eff-1, and dpl-1 encode proteins similar to mammalian pRB, E2F, and DP, respectively. Genetic epistasis tests indicate that each of these proteins acts upstream of or in parallel to the Ras/MAPK pathway during postembryonic vulval induction (2, 37). No discernible effects on cell proliferation were observed. In a related study, mutations in eff-1 and dpl-1 were shown to cause defects in embryonic polarity in a cell cycle-independent manner (46). In addition, it was demonstrated that EFL-1 and DPL-1 exert their effects by negatively regulating MAPK activity, suggesting that EFL-1–DPL-1 complexes operate upstream of the Ras/MAPK pathway (46). These data parallel those for mammalian systems in that they identify an antagonism between pRB/E2F- and Ras-mediated signaling. They also suggest that pRB functions upstream of Ras to influence differentiation.

A question that arises when comparing the nematode and mammalian studies is whether E2F and Ras operate in common or different pathways. Our results pertaining to the CNS, lens, and skeletal muscle shed some light on this. Loss of E2F-1 or E2F-3 significantly reverses the ectopic S-phase entry and apoptosis observed in the CNS and lens of Rb-deficient embryos (64, 72), while loss of N-ras has no impact on these phenotypes. On the other hand, loss of N-ras can ameliorate many but not all of the abnormalities in Rb-deficient skeletal muscle, while deficiency in E2F-1 or E2F-3 has no apparent effect (64, 72). Thus, in certain tissues a genetic interaction between Rb and E2F-1 or E2F-3 is not apparent while that between Rb and N-ras is and vice versa. These observations suggest that N-ras and E2F do not operate in a common signaling pathway but rather that the genetic interactions between Rb and E2F-1 or -3 and between Rb and N-ras indicate a bifurcation in Rb function. Consistent with this interpretation, cell culture-based experiments have led to the suggestion that Rb engages at least two tumor suppressor functions that can be genetically dissociated, one linked to E2F-dependent cell cycle progression and the other linked to control of Ras activation and differentiation (33, 58).

Our results indicate the existence of a novel, physiologically important genetic interaction between Rb and N-ras during mammalian embryogenesis. N-ras participates in several of the early and late developmental defects brought about by loss of Rb. We suggest that the ability of Rb to function upstream of ras represents an evolutionarily conserved signaling pathway that impinges on several differentiation programs.

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