

# E2F1 and p53 Are Dispensable, whereas p21<sup>Waf1/Cip1</sup> Cooperates with Rb to Restrict Endoreduplication and Apoptosis during Skeletal Myogenesis

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We describe temporal and genetic analyses of partially rescued Rb mutant fetuses, *mgRb:Rb*<sup>-/-</sup>, that survive to birth and reveal specific defects in skeletal muscle differentiation. We show that in the absence of Rb, these fetuses exhibit increased apoptosis, *bona fide* endoreduplication, and incomplete differentiation throughout terminal myogenesis. These defects were further augmented in composite mutant fetuses, *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup>, lacking both Rb and the cyclin-dependent kinase inhibitor p21<sup>Waf1/Cip1</sup>. Although E2F1 and p53 mediate ectopic DNA synthesis and cell death in several tissues in Rb mutant embryos, both endoreduplication and apoptosis persisted in *mgRb:Rb*<sup>-/-</sup>:*E2F1*<sup>-/-</sup> and *mgRb:Rb*<sup>-/-</sup>:*p53*<sup>-/-</sup> compound mutant muscles. Thus, combined inactivation of Rb and p21<sup>Waf1/Cip1</sup> augments endoreduplication and apoptosis, whereas E2F1 and p53 are dispensable during aberrant myogenesis in Rb-deficient fetuses. © 2000 Academic Press

**Key Words:** Rb; p21; E2F1; p53; cell cycle; apoptosis; endoreduplication; myogenesis.

## INTRODUCTION

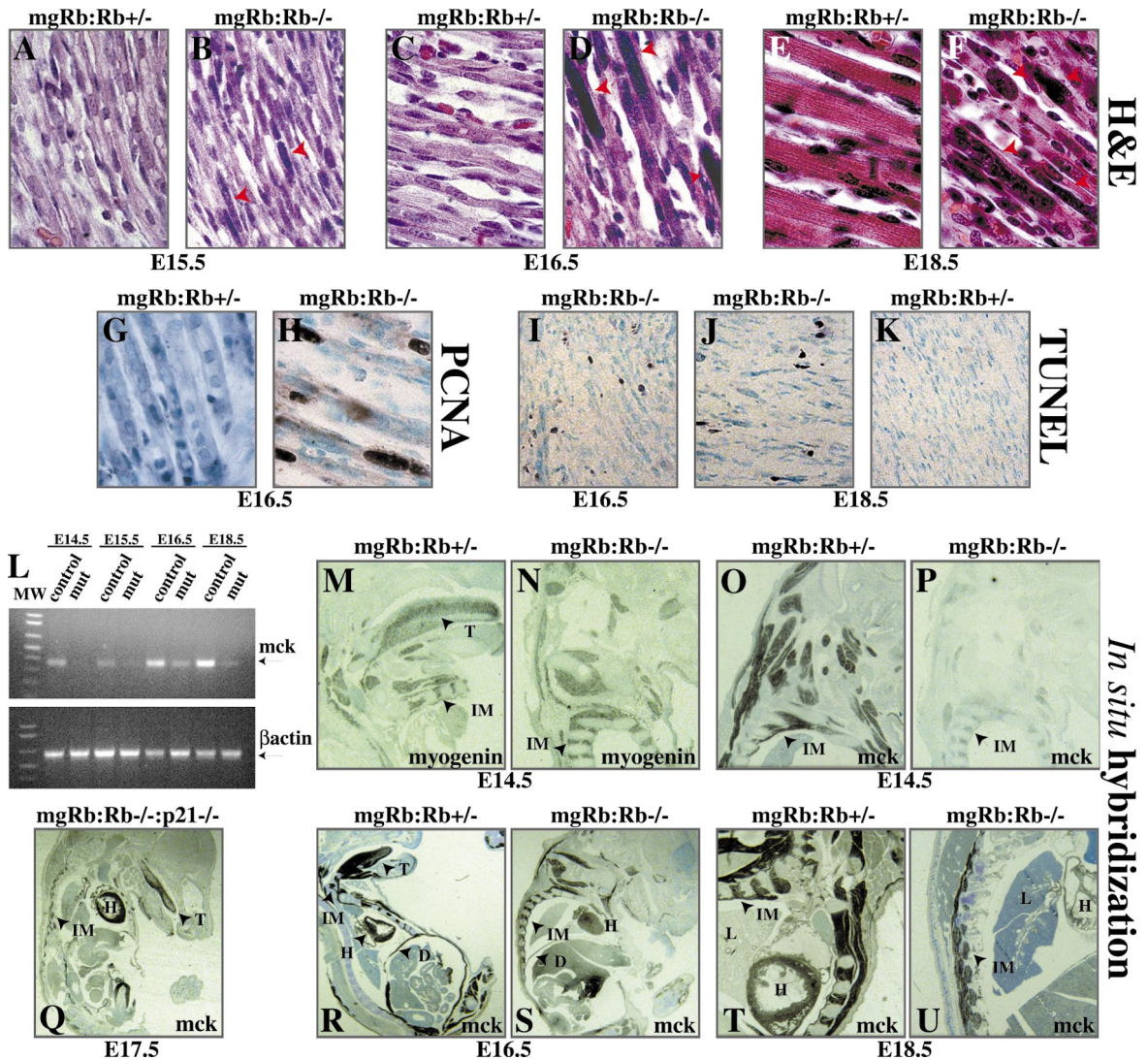
The retinoblastoma tumor suppressor, Rb, is a transcriptional cofactor whose phosphorylation and activity oscillate during the cell cycle under control of cyclin-dependent kinases (Cdks) (Sherr and Roberts, 1999). The Cdks and their associated G1 cyclins are subject to noncatalytic inhibition by specific cyclin-dependent kinase inhibitors (CKIs; e.g., p16) and universal CKIs (e.g., p21, p27, p57) (Sherr and Roberts, 1995). During most of the G1 phase of the cell cycle, Rb suppresses the expression of genes required for progression into, and execution of, the DNA replication phase by forming specific complexes with certain transcription factors, such as E2F1 (Nevins *et al.*, 1997;

Dyson, 1998). Following its phosphorylation prior to a restriction point late in G1, Rb dissociates from its partners and the progression into S phase is allowed. In addition to S phase, E2F1 also induces apoptosis when overexpressed *in vitro* (DeGregori *et al.*, 1997; Wu and Levine, 1994). Free E2F1 can transcriptionally activate p19<sup>ARF</sup>, which stabilizes p53 by debilitating the function of its regulator MDM2, thereby linking mutations in Rb to p53-dependent apoptosis, at least in some tissues (Bates *et al.*, 1998; Pomerantz *et al.*, 1998). Indeed, Rb and p53 are often inactivated in human cancer, leading to deregulation of the cell cycle and survival of the transformed state (Gottlieb and Oren, 1998).

Mice lacking functional Rb die at midgestation and exhibit ectopic DNA synthesis, apoptosis, and incomplete differentiation during neurogenesis, erythropoiesis, and lens development (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992, 1994; Macleod, 1999; Mulligan and Jacks, 1998). This spectrum of defects is consistent with the restricted pattern of expression of Rb during embryogenesis in the nervous system, lens, liver, and skeletal muscles (Jiang *et al.*, 1997). Studies on the role of Rb during terminal

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**FIG. 1.** Temporal analysis of aberrant myogenesis in Rb mutant embryos. (A–F) H&E staining of intercostal muscles from Rb mutant fetuses and control littermates (original magnification 100 $\times$ ). Large nuclei, indicated by arrowheads, are evident as early as E15.5 and accumulate during late fetal development. (G, H) PCNA staining of back muscles (100 $\times$ ). Mutant muscles contain PCNA-positive nuclei within myotubes. (I–K) TUNEL analysis of neck muscles reveals cell death throughout terminal myogenesis (40 $\times$ ). (L) RT-PCR analysis of MCK gene expression in E14.5–E18.5 mgRb:Rb $^{-/-}$  fetuses and wild-type littermates with  $\beta$ -actin as internal control. (M–P, R–U) *In situ* hybridization analysis of mgRb:Rb $^{-/-}$  mutant fetuses and control littermates, using  $^{35}$ S-labeled MCK or myogenin antisense riboprobes. Shown are bright-field images in which silver grains appear as black dots. (Q) Expression of MCK in E17.5 mgRb:Rb $^{-/-}$ :p21 $^{-/-}$  double-mutant fetus. Abbreviations: H, heart; D, diaphragm; L, lung; IM, intercostal muscles; T, tongue.

myogenesis, which occurs after Rb $^{-/-}$  embryos die, were made possible in fetuses partially rescued to birth by a Rb minigene (Zacksenhaus *et al.*, 1996). At embryonic day (E) 17.5, these mutant fetuses express the transgenic Rb protein in the brain but not in muscles or other tissues (Zacksenhaus *et al.*, 1996). The partially rescued mgRb:Rb $^{-/-}$  fetuses exhibit severe skeletal muscle defects, including increased cell death and shorter myotubes, ectopic DNA synthesis, the appearance of giant nuclei in residual

myotubes, and expression of early skeletal muscle structural genes (myosin heavy chain and cardiac actin) but not late muscle markers (MCK and MRF4).

Inactivation of both Rb and E2F1 results in extension of life span by several days and inhibition of apoptosis in lens and central nervous system (CNS) but to a lesser extent in the peripheral nervous system (PNS) (Tsai *et al.*, 1998). Apoptosis in the lens and CNS, but not the PNS, is also mediated by a p53-dependent pathway (Macleod *et al.*,



1996). Accordingly, mutations in p53 increase the spectrum of tumors in Rb+/-:p53-/- double mutants relative to Rb+/- heterozygote mice (Williams *et al.*, 1994a). In addition to apoptosis, p53 can also induce cell cycle arrest by direct transcriptional activation of the Cdk inhibitor p21<sup>Waf1/Cip1</sup>. p53-dependent activation of p21 following  $\gamma$ -irradiation leads to cell cycle arrest by inhibiting phosphorylation of Rb (Brugarolas *et al.*, 1999). Notably,  $\gamma$ -irradiation or overexpression of p21 *in vitro* induces cell cycle arrest in Rb-positive cells but endoreduplication in cells lacking Rb (Niculescu *et al.*, 1998). p21 (as well p16) can also suppress apoptosis during myoblast differentiation *in vitro* through Rb or other pathways (e.g., AKT; Wang and Walsh, 1996; Fujio *et al.*, 1999).

Since components of the Rb pathway exhibit tissue-specific effects, the analysis of the Rb pathway in particular tissues is important in understanding normal development as well as neoplastic transformation of specific cell lineages. To determine the role of E2F1, p53, and p21<sup>Waf1/Cip1</sup> in the aberrant myogenesis in mgRb:Rb-/- embryos, we performed a temporal and genetic analysis on compound mutant mice lacking Rb and E2F1, p53, or p21<sup>Waf1/Cip1</sup>. Our results show that ectopic DNA synthesis and apoptosis in Rb-deficient muscles are mediated by a pathway, yet to be defined, which is independent of E2F1 or p53. Inactivation of both Rb and p21<sup>Waf1/Cip1</sup> leads to increased endoreduplication and apoptosis, indicating that these two negative regulators cooperate to facilitate cell cycle exit during terminal myogenesis.

## MATERIALS AND METHODS

### Intercrosses and Genotyping

The mgRb:Rb+/- mice and p53-/-, E2F1-/-, and p21-/- knockout mice were described previously (Brugarolas *et al.*, 1995; Field *et al.*, 1996; Jacks *et al.*, 1994; Zacksenhaus *et al.*, 1996). For timed pregnancy, the morning of vaginal plug observation was considered E0.5. In the experiments described below, we analyzed three to six double-mutant embryos, mgRb:Rb-/- mutants, and control littermates in every assay. All mutant embryos were genotyped in duplicates using DNA extracted separately from limb and tail biopsies. Tissues were treated overnight with proteinase K (400  $\mu$ g/ml) at 55°C. The DNA was extracted once with phenol:chloroform (1:1) by shaking for 1 h in a mixer (Fisher; Model 5432) followed by centrifugation, isopropanol precipitation, one wash in 70% ethanol, and resuspension in 100  $\mu$ l TE buffer (10 mM Tris · Cl, pH 8.0, 1 mM EDTA). PCRs included DNA 2  $\mu$ l, MgCl<sub>2</sub> (25 mM) 3  $\mu$ l, PCR buffer (10 $\times$  100 mM Tris · HCl, pH 8.3, 500 mM KCl) 5  $\mu$ l, dNTP (10 mM) 1  $\mu$ l, gelatin (2 mg/ml) 2.5  $\mu$ l, primers (200 ng/ $\mu$ l) 1  $\mu$ l  $\times$  2, in 50- $\mu$ l reactions. PCR conditions included 30 cycles of 1 min each at 94°C (denaturation), 55–60°C (annealing), and 72°C (extension). Primers for mgRb, Rb, E2F1, and p21 were as described (Brugarolas *et al.*, 1995; Field *et al.*, 1996; Zacksenhaus *et al.*, 1996). The primers for p53 were p53-1 (5'-GTA TCT GGA AGA CAG GCA GAC) and p53x7 (5'-GAT GGT GGT ATA CTC AGA GCC), for wild-type allele, and neo-2 (5'-TCC TCG TGC TTT ACG GTA TCG) and p53x7 for mutant allele.

### Histology and Immunohistochemistry

Embryos were embedded in 4% paraformaldehyde in PBS, dehydrated, paraffin embedded, and cut at 4–5  $\mu$ m for hematoxylin and eosin (H&E) staining, fluorescence *in situ* hybridization (FISH), and image cytometry and 8  $\mu$ m for TUNEL, immunohistochemistry, and *in situ* hybridization. Immunohistochemical analysis of myosin heavy chain (Fast) and PCNA was performed with monoclonal antibodies (Sigma) at 1:300 dilution followed by biotin-labeled anti-mouse and ABC kit from DAKO.

### RNA *In Situ* Hybridization and Reverse Transcription-PCR

*In situ* hybridization analysis for Rb, MCK, myogenin, p21, and other markers was performed as described (Jiang *et al.*, 1997). All probes were used at  $2 \times 10^5$  cpm/ $\mu$ l with the exception of cardiac-actin and MCK probes, which were used at  $5 \times 10^4$  cpm/ $\mu$ l. The slides were coated with emulsion and exposed for 10–20 days. RT-PCR analysis (25 cycles) of MCK was performed with forward primer EZ211 (5'-CAG ACA AGC ATA AGA CCG) at nt 379 and reverse primer EZ212 (5'-TTG TCG TCG TGC CAG ATG) at nt 737, yielding a fragment of 359 bp.

### Apoptosis Analysis

Sections were deparaffinated and hydrated by two changes of xylenes, 5 min, followed by 100, 96, 90, and 80% ethanol and ddH<sub>2</sub>O for 3 min each. For nuclear condensation analysis, sections were treated with Hoechst 33258 (Sigma; 0.5 mg/ml in water) for 30 min, washed, and observed under fluorescence microscope. For TUNEL analysis, we used the following modified procedure (Gavrieli *et al.*, 1992). For nuclei stripping, slides were immersed in 1 $\times$  PBS for 5 min and then incubated for 2 min with 5  $\mu$ g/ml proteinase K in PBS. The sections were rinsed 1  $\times$  1 min and 2  $\times$  5 min in PBS and then 2  $\times$  5 min in ddH<sub>2</sub>O. For H<sub>2</sub>O<sub>2</sub> treatment, sections were covered with 3% H<sub>2</sub>O<sub>2</sub> for 15 min and then rinsed with ddH<sub>2</sub>O twice for 2 min and once for 5 min. For equilibration, the sections were covered with 1 $\times$  TdT buffer with Na-cacodylate (supplied with the TdT enzyme; Boehringer Mannheim) and BSA 0.25  $\mu$ g/ $\mu$ l using Parafilm as coverslips for 30 min. For the reaction, the equilibration buffer was removed and replaced by the same buffer containing 10  $\mu$ M Bio-16-dUTP (Boehringer Mannheim) and 0.25 U/ $\mu$ l TdT (Boehringer Mannheim) with the same coverslips in humid atmosphere for 60 min at 37°C. For termination of the reaction, the slides were washed twice for 15 min in prewarmed 4 $\times$  SSC buffer at 37°C. The slides were further washed 2  $\times$  5 min in PBS and 2  $\times$  5 min in PBS-T (PBS plus 0.1% Triton X-100) at room temperature. ABC reagent (DAKO) was prepared according to the manufacturer's instructions in PBS-T and added for 30 min. Slides were washed 2  $\times$  5 min in PBS-T and 2  $\times$  5 min in PBS. One milliliter of DAB (3',3'-diaminobenzadine) solution was made by mixing 50  $\mu$ l of DAB (2.5 mg/ml in 0.1 M NaPO<sub>4</sub>, pH 7.6), 23  $\mu$ l of 1% NiCl<sub>2</sub>, 1  $\mu$ l of H<sub>2</sub>O<sub>2</sub> in NaPO<sub>4</sub>, pH 7.6. Slides were treated with DAB for 10 min and washed extensively with tap water. Counterstaining with methyl green (0.5% w/v in 0.1 M Na-acetate, pH 4.0) was done for 30 s, followed by several washes with tap water, 2  $\times$  30 s in 100% butanol and 2  $\times$  3 min in xylenes. The slides were mounted with Permount (Fisher).

## Bone Staining

Embryos were fixed in 95% ethanol. Following genotype analysis by PCR, the skin and viscera were carefully removed and the skeleton was incubated in 1 vol of acetic acid, 4 vol of 95% ethanol, and 7.5 mg Alcian blue (Sigma) per 50 ml to stain ossification centers (Lufkin *et al.*, 1992). After 24 h, the solution was removed, and the skeleton was washed in 95% ethanol for 1 h and then with 2% (original protocol) or 1% (modified protocol) KOH for 24 h. Staining of cartilage in Alizarin red (Sigma) in 1% KOH was done overnight followed by clearing in 20% glycerol, 1% KOH for a week with daily changes. Skeletons were finally stored in 50% glycerol, 50% ethanol.

## Image Cytometry

Sections of 5  $\mu\text{m}$  were dewaxed, rehydrated, and then stained by a standard Feulgen technique using the Quantitative DNA Staining Kit (Becton-Dickinson Cellular Imaging System, San Jose, CA; Cat. No. 54100140). Image cytometry analysis was performed with the CAS 200, a video-based interactive image cytometer (Becton-Dickinson) (Bacus and Bacus, 1994; Berezowsky *et al.*, 1995). After instrument calibration using CAS control slides provided by the manufacturer, microscopic fields of muscle tissue were randomly selected. Within each field, all the isolated nuclei were picked manually. In order to get the largest number of nuclei from each field, manually drawn boundaries were necessary in many cases to assist the software in picking up single nuclei. Ten to twenty fields were selected and more than 300 nuclei for each section were analyzed in total. The images of selected nuclei were digitized and the optical densities and images recorded. The integrated optical density of each Feulgen-stained nucleus was considered proportional to the amount of DNA per nucleus. The optical density was converted into the weight of DNA, and a histogram of all processed nuclei, as well as a scatter plot, was generated by the software. For a better comparison, the processed nuclei for each sample were subclassified into five groups based on their DNA content: class 1 (less than or equal to 12 pg, diploid to tetraploid), class 2 (between 12 and 24 pg), class 3 (between 24 and 48 pg), class 4 (between 48 and 96 pg), and class 5 (more than 96 pg).

## Fluorescence in Situ Hybridization (FISH)

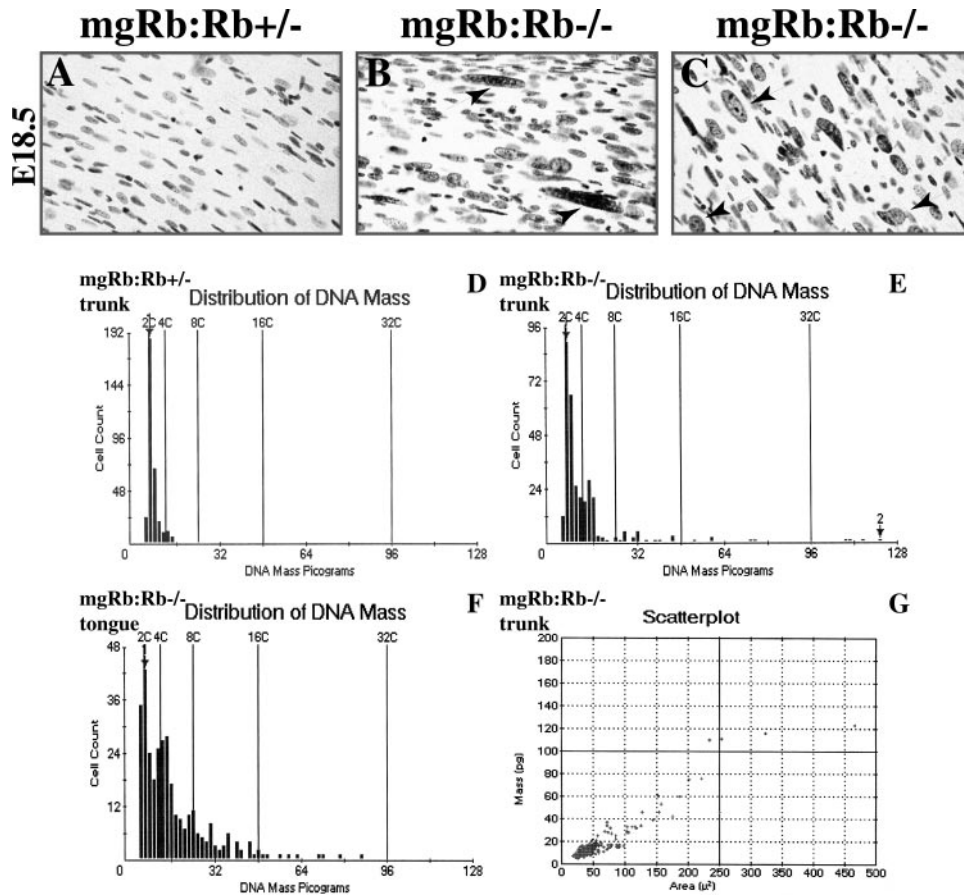
Genotype analysis of mouse Y chromosome by PCR with primers specific to the *Sry* gene was performed with forward primer TDY1, 5'-GAC TGG TGA CAA TTG TCT AG, and reverse primer TDY2, 5'-TAA AAT GCC ACT CCT CTG TG, with male samples generating a product of 292 bp (Gubbay *et al.*, 1990). The mouse Y chromosome paint probe (biotin-labeled) (Breneman *et al.*, 1993) was purchased from Cedarlane Laboratories (Cat. No. 1187-YMB-02). All other reagents were from Oncor (tissue kit, S1337-TC; detection kit biotin-FITC, S1333-BF; and hybridization kit, S1340-Kit). The procedures were performed as recommended by the manufacturers with minor modifications (available upon request). The slides were finally mounted with 9  $\mu\text{l}$  of DAPI (2.5 ng/ml) and 3  $\mu\text{l}$  of PI (0.1  $\mu\text{g}/\mu\text{l}$ ) in anti-fade and observed under fluorescence microscope (Axioscope II; Zeiss).

## RESULTS

### Temporal Analysis of Aberrant Myogenesis in Rb Mutant Fetuses

We previously described the generation and analysis of partially rescued Rb mutant fetuses, in which the neurogenic defect in Rb<sup>-/-</sup> embryos is specifically rescued by a Rb minigene, mgRb (Zacksenhaus *et al.*, 1996). Recent analysis of Rb promoter-lacZ transgenic mice (Jiang *et al.*, 2000) indicates that the same Rb promoter used to generate the mgRb minigene can direct transgene expression exclusively to the developing nervous system but not to other tissues, such as liver and skeletal muscles, where endogenous Rb is normally expressed (Jiang *et al.*, 1997). The mgRb:Rb<sup>-/-</sup> fetuses can therefore be viewed as null for Rb throughout myogenesis. Our previous analysis identified major skeletal muscle defects in E17.5–E18.5 mgRb:Rb<sup>-/-</sup> fetuses. To address the temporal consequences of Rb loss during terminal myogenesis, mgRb:Rb<sup>-/-</sup> embryos were analyzed from E13.5 to E18.5 (Fig. 1). H&E staining revealed that large nuclei accumulated within myotubes; there were very few large nuclei at E13.5–E14.5 but they were clearly detected by E15.5 (Figs. 1A and 1B); and by E18.5 more than 20% of the myotube nuclei appeared abnormally enlarged (Fig. 1F). The formation of large nuclei was accompanied by ectopic DNA synthesis within the myotube as detected by staining with antibodies specific to the proliferating cell nuclear antigen PCNA (Figs. 1G and 1H, also see below). Thus, in the absence of Rb, myotubes are unable to permanently withdraw from the cell cycle and instead reenter S phase and accumulate large nuclei. Concomitant with this abnormal cell cycle exit, there was a marked increase in cell death throughout terminal myogenesis (Figs. 1I–1K).

The cellular abnormalities described above were accompanied by specific reduction in expression of the late muscle marker, muscle creatine kinase (MCK) (Fig. 1L), but not early muscle markers such as myosin heavy chain (MHC), myogenin, and cardiac actin (see below). Some low levels of MCK transcripts were, however, detectable by RT-PCR analysis at E16.5–E18.5 even in mutant fetuses (Fig. 1L). To determine whether the reduction in MCK gene expression affected all or a subset of myotubes, we performed *in situ* hybridization analysis on sagittal sections of mutant and control littermates. Expression of MCK was uniformly reduced in mgRb:Rb<sup>-/-</sup> mutant embryos at E13.5–E15.5 (Figs. 1O and 1P and data not shown), but progressively elevated and readily detectable in E16.5–E18.5 fetuses (Figs. 1R–1U). In contrast, expression of cardiac actin (not shown) and myogenin (Figs. 1M and 1N) was unaffected as determined by *in situ* hybridization on adjacent sections. The results indicate that Rb is required for the proper transcriptional activation of MCK during myogenesis but despite the excessive apoptosis and ectopic DNA synthesis, a low level of kinase expression was gradually attained during late fetal development even in the absence of Rb.



**FIG. 2.** *mgRb:Rb-/-* myotubes undergo endoreduplication and become polyploid. (A–C) Images of E18.5 muscle sections of control (A) and *Rb* mutant fetuses (B, C) stained by the Feulgen technique. Note the disorganized appearance and abnormally large nuclei in mutant muscles (arrows). (D–F) Image cytometry analysis of E18.5 control (D) and *Rb* mutant muscles from trunk (E) and tongue (F). The *Rb* mutant muscles contain nuclei with DNA mass of  $2N$  to  $>32N$ . (G) Scatter-plot analysis of E18.5 *mgRb:Rb-/-* mutant fetuses revealing direct relationship between large nuclei and DNA mass. (H–M) Fluorescence *in situ* hybridization analysis with Y chromosome painting of myotube nuclei of E18.5 control and mutant fetuses. Fetuses were genotyped with primers for the mouse Y-chromosome-specific *Sry* gene and analyzed as described under Materials and Methods. (H) A control female fetus showing no positive signal. (I) A wild-type male fetus showing single focus of staining per nuclei along the myotubes. (J) DAPI staining of the FISH image shown in (K). (K–M) Three examples of *mgRb:Rb-/-* male mutant fetuses with large nuclei (demarcated) containing multiple Y chromosomes.

### ***Nuclei in mgRb:Rb-/- Myotubes Undergo Endoreduplication and Become Polyploid***

The giant nuclei in the *Rb*-deficient myotubes could reflect nonspecific DNA repair/synthesis or polyploidy due to genuine endoreduplication. To address the nature of these nuclei, we first stained histology sections with Feulgen reagent, which provides a quantitative, linear staining in proportion to the amount of DNA in the nuclei (see Materials and Methods). Microscopic observations readily detected large nuclei in muscle areas but not in other tissues in mutant fetuses (Figs. 2A–2C). Image cytometry analysis revealed that nuclei from normal E18.5 skeletal muscles displayed a DNA content of  $2N$ – $4N$  (Fig. 2D). This DNA distribution represented postmitotic nuclei in myo-

tubes and mitotic nuclei from myoblasts and other cell lineages that overlapped the muscle fibers. In contrast, a fraction of the nuclei from *mgRb:Rb-/-* muscles had a DNA content of more than  $4N$  (Figs. 2E and 2F). Remarkably, some nuclei contained more than  $32N$  chromosomes, presumably due to more than five successive DNA replications without intervening mitosis. A scatter-plot analysis comparing DNA mass and nuclear size revealed a linear relationship (Fig. 2G), thus establishing that the nuclei with excess DNA are the giant nuclei.

We next used FISH to determine whether the excess DNA in the large nuclei in *mgRb:Rb-/-* fetuses was organized into multiple, distinct chromosomes. Embryos were sexed by PCR with primers for the mouse



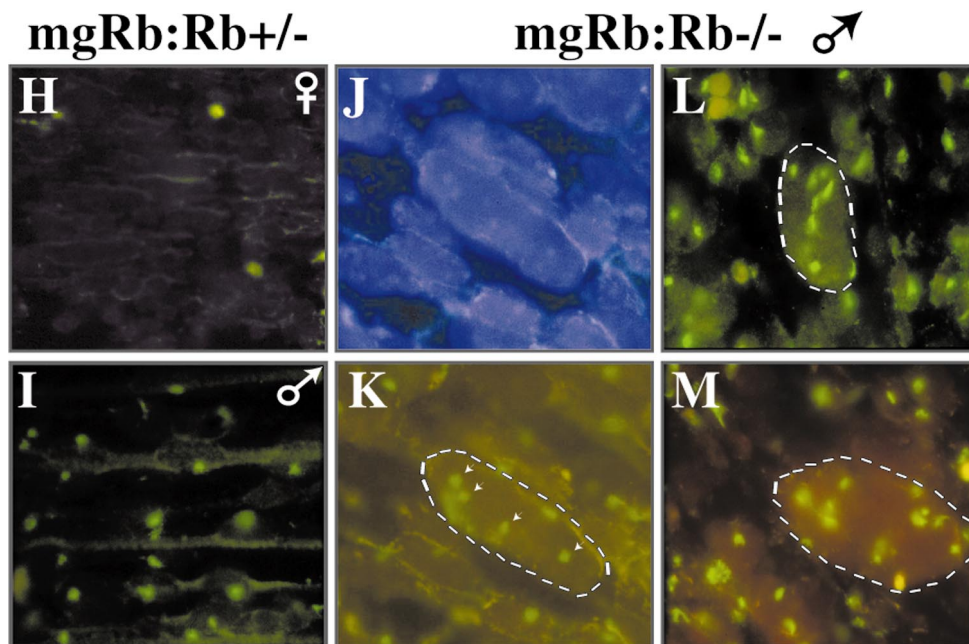


FIG. 2—Continued

Y-chromosome-specific *Sry* gene (Gubbay *et al.*, 1990, see Materials and Methods). Tissue sections were then hybridized with a biotin-labeled Y chromosome paint and treated with DAPI plus propidium iodide (PI) to stain the Y chromosomes and nuclei, respectively. Sections from female fetuses produced no signal (Fig. 2H), whereas normal male fetuses revealed a regular pattern of nuclei along the myotube with a single focus of staining per nucleus (Fig. 2I). In contrast, large nuclei from *mgRb:Rb*<sup>-/-</sup> male fetuses contained several foci of staining, indicative of multiple Y chromosomes (Figs. 2J–2M). Thus, in the absence of Rb, nuclei in residual myotubes are unable to permanently withdraw from the cell cycle and instead undergo endoreduplication and become polyploid.

### Increased Endoreduplication and Apoptosis in Composite Mutant Fetuses Lacking both Rb and p21<sup>Waf1/Cip1</sup>

The observed endoreduplication in *mgRb:Rb*<sup>-/-</sup> muscles could be mediated by a G2 block exerted by p21<sup>Waf1/Cip1</sup>, as ectopic expression of this CKI leads to endoreduplication in Rb-deficient cells but not Rb-positive cells *in vitro* (Niculescu *et al.*, 1998). Furthermore, p21<sup>Waf1/Cip1</sup> was readily detected by *in situ* hybridization in skeletal muscles of normal fetuses and might be even slightly elevated in *mgRb:Rb*<sup>-/-</sup> mutant littermates (Figs. 3A–3B). To determine whether endoreduplication in *mgRb:Rb*<sup>-/-</sup> fetuses was mediated by p21<sup>Waf1/Cip1</sup>, we generated and analyzed *mgRb:Rb*<sup>-/-</sup>:p21<sup>-/-</sup> compound mutant fe-

tuses. Some *mgRb:Rb*<sup>-/-</sup>:p21<sup>-/-</sup> fetuses harvested at E18.5 appeared unwell with patches of hemorrhage, indicating that loss of both genes had an adverse effect on development. We therefore recovered *mgRb:Rb*<sup>-/-</sup>:p21<sup>-/-</sup> mutant fetuses at E16.5–E17.5 (frequency 7/117 = 5.9%), at stages when the compound mutants appeared viable and grossly indistinguishable from *mgRb:Rb*<sup>-/-</sup> single-mutant littermates. Additional compound mutant fetuses were generated by mating mutant mice homozygous for p21<sup>Waf1/Cip1</sup> (i.e., *mgRb:Rb*<sup>+/-</sup>:p21<sup>-/-</sup>).

PCNA staining of the *mgRb:Rb*<sup>-/-</sup>:p21<sup>-/-</sup> double-mutant muscles readily detected large positive nuclei within myotubes, indicating that endoreduplication occurred even in the absence of p21<sup>Waf1/Cip1</sup> (Figs. 3E and 3F). To quantify the effect of p21 on endoreduplication, we performed image cytometry analysis on single and compound mutant muscles (Fig. 3G). On average, there were over twice as many nuclei with 32N chromosomes (class 4) and nine times more nuclei with >32N chromosomes (class 5) in *mgRb:Rb*<sup>-/-</sup>:p21<sup>-/-</sup> tongue muscles compared with *mgRb:Rb*<sup>-/-</sup> littermates (Fig. 3G).

Immunohistochemical analysis with antibody specific to MHC revealed that myotubes in E16.5 *mgRb:Rb*<sup>-/-</sup>:p21<sup>-/-</sup> fetuses were generally shorter and more disorganized than in single-mutant fetuses (Figs. 4A–4C). Expression of MCK was also reduced in the compound mutant fetuses (Fig. 1Q). Some large nuclei in the *mgRb:Rb*<sup>-/-</sup>:p21<sup>-/-</sup> embryos appeared to collapse (Figs. 4C, 4F, 4I, and 4L, red and yellow arrowheads; Fig. 3F, arrow) and may represent retraction of the myotubes, apoptosis, or mitotic

catastrophe. Nuclear staining with the fluorescent dye Hoechst 33258 revealed multiple nuclei with chromatin condensation in *mgRb:Rb*<sup>-/-</sup> and *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> muscles but very few in wild-type fetuses (Figs. 4G–4I). Both apoptosis and mitotic catastrophe involve nuclear and chromatin condensation but the former is distinguished by DNA degradation, the hallmark of programmed cell death (Chan *et al.*, 1999, and references therein). *In situ* analysis of DNA degradation (TUNEL) revealed that some, but not all, of the collapsed nuclei were TUNEL-positive (Fig. 4L), thus precluding unequivocal definition of these collapsed nuclei. There appeared to be more cell death in *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> muscles than in *mgRb:Rb*<sup>-/-</sup> mutants. To quantify cell death, apoptotic nuclei were scored in several areas from three single mutants and three compound mutants at E16.5 (Fig. 4J). A moderate but significant increase in the frequency of apoptotic nuclei was found in muscles of *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> double-mutant relative to *mgRb:Rb*<sup>-/-</sup> single-mutant fetuses (*t* test 0.005; *t* critical = 2.776). On average there were 60% more apoptotic nuclei in muscle areas of Rb-deficient fetuses when *p21* was absent. Thus, the combined loss of Rb and *p21*<sup>Waf1/Cip1</sup> during myogenesis leads to relatively more ectopic DNA synthesis, which is accompanied by increased cell death.

### Enhanced Skeletal Defects in *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> Compound Mutant Fetuses

The *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> double-mutant fetuses exhibited abnormal posture and hunchback that appeared more severe than in *mgRb:Rb*<sup>-/-</sup> fetuses. To test the skeletons of *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> fetuses, we performed Alizarin red and Alcian blue staining to reveal bones and cartilage, respectively. The skeletons of *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant embryos were fragile and readily dissociated under standard conditions, but were successfully obtained under lower concentrations of KOH (1% instead of 2% in the standard protocol—see Methods and Materials).

The limb bones in the *mgRb:Rb*<sup>-/-</sup> and *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant embryos were moderately shorter compared with those of control littermates (Figs. 5B vs 5A). This defect cannot be explained merely as a delay in development because the timing of ossification of the metacarpal and phalangeal bones of limbs (Figs. 5A and 5B), a faithful indicator of developmental stages, was comparable in the mutant and wild-type animals. The humerus process (deltoid tuberosity) was significantly less developed in mutant fetuses and in some mutants was completely missing (Figs. 5A–5D, arrows). At higher magnification, the bones (e.g., scapula, Figs. 5E and 5F) of the *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> double-mutant fetuses appeared more perforated and the boundaries between the ossification center and the cartilage were not as distinct as in wild-type littermates. In wild-type and *mgRb:Rb*<sup>-/-</sup> mutant fetuses the ribs were connected to the sternum at an angle, whereas in the double-mutant fetuses the ribs joined the sternum perpendicularly (Figs.

5G–5J). The upper region of the xiphoid process at the end of the sternum was abnormal in both *mgRb:Rb*<sup>-/-</sup> and *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant embryos (Figs. 5H and 5J, arrows). In the double-mutant fetuses there was also abnormal ossification of the sternum (Fig. 5J, upper arrow). The abnormalities in the deltoid tuberosity and the sternum likely represent indirect consequences of the muscle deficiency in Rb-deficient fetuses, as similar bone defects are also observed in myogenin-deficient mice (Hasty *et al.*, 1993). Thus, the increased skeleton abnormalities in *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> double mutants compared with *mgRb:Rb*<sup>-/-</sup> single mutants further support the conclusion that inactivation of *p21*<sup>Waf1/Cip1</sup> further augments the muscle defects in Rb-deficient fetuses.

### Endoreduplication and Apoptosis in Skeletal Muscles of Rb-Deficient Fetuses Are Independent of E2F1 and p53

In the lens and CNS but not PNS of Rb mutant embryos, both ectopic DNA synthesis and apoptosis are mediated by E2F1- and p53-dependent pathways (Macleod *et al.*, 1996; Tsai *et al.*, 1998). To test the roles of E2F1 and p53 in endoreduplication and apoptosis in Rb-deficient muscles, we generated composite *mgRb:Rb*<sup>-/-</sup>:*p53*<sup>-/-</sup> and *mgRb:Rb*<sup>-/-</sup>:*E2F1*<sup>-/-</sup> fetuses. The *mgRb:Rb*<sup>-/-</sup>:*E2F1*<sup>-/-</sup> mutant fetuses were readily obtained (6/74 = 8.1%), while *mgRb:Rb*<sup>-/-</sup>:*p53*<sup>-/-</sup> embryos were recovered at a lower frequency (6/137 = 4.4%), in agreement with the lethal effect of p53 loss on some female embryos (Sah *et al.*, 1995).

The double-mutant fetuses exhibited a hunchback appearance and only a subtle response to stimulation, similar to *mgRb:Rb*<sup>-/-</sup> single-mutant fetuses (Zacksenhaus *et al.*, 1996), suggesting that the absence of E2F1 or p53 did not further rescue the mutant embryos or the muscle defects. Accordingly, histology and PCNA-staining analyses revealed no obvious differences in the morphology of the skeletal muscles or the frequency of polyploid nuclei between the single- and the double-mutant fetuses (data not shown). Indeed, image cytometry analysis indicated that the loss of neither E2F1 nor p53 had any measurable effect on aberrant entry of Rb mutant muscles into an endoreduplicating cycle (Fig. 6A). TUNEL analysis during terminal myogenesis in compound *mgRb:Rb*<sup>-/-</sup>:*p53*<sup>-/-</sup> and *mgRb:Rb*<sup>-/-</sup>:*E2F1*<sup>-/-</sup> mutant fetuses revealed that apoptosis was also not affected by the absence of E2F1 or p53 (Figs. 6B–6G). In contrast, apoptosis in the lens of *mgRb:Rb*<sup>-/-</sup>:*p53*<sup>-/-</sup> fetuses was reduced over 10-fold (Figs. 6H–6I), in accord with previous reports (Morgenbesser *et al.*, 1994; Tsai *et al.*, 1998). We conclude that in contrast to the lens and CNS but similar to the PNS, Rb-deficient skeletal muscles undergo ectopic DNA synthesis and apoptosis through a pathway, yet to be defined, which is independent of E2F1 and p53.

## DISCUSSION

### Control of Myogenesis by Rb

In the absence of Rb, initial myoblast fusion and formation of multinucleated myotubes are near normal until E15.5–16.5. Thereupon, myotubes become relatively shorter presumably due to the accumulative effects of the excessive apoptosis and endoreduplication that occur during terminal myogenesis in the Rb-deficient fetuses. Expression of MCK but not several other muscle markers was significantly reduced in E13.5–E15.5 embryos but gradually elevated thereafter (Fig. 1). Thus, Rb is required for permanent withdrawal from the cell cycle, otherwise apoptosis and endoreduplication ensue, and for transcriptional activation of a subset of muscle genes (Fig. 7A). Although the expression of MCK never reaches wild-type levels, the gradual increase in MCK gene expression in Rb mutant fetuses from E13.5 to E18.5, despite the ectopic DNA synthesis, excessive apoptosis, and muscle degeneration, is somehow puzzling. Other factors, perhaps p107 and p130, may partially compensate for Rb loss. Alternatively, Rb may be required to coordinate several aspects of cell cycle exit and terminal differentiation but many Rb<sup>-/-</sup> cells may survive the initial crisis and continue to differentiate, albeit less effectively, even in the absence of Rb. The latter scenario is consistent with the observations that Rb<sup>-/-</sup> cells can contribute efficiently to most adult tissues in chimeric mice (Maandag *et al.*, 1994; Williams *et al.*, 1994b).

Rb was shown to interact directly with myogenic factors such as MyoD and myogenin (Gu *et al.*, 1993) and the adipogenic factor C/EBP (Chen *et al.*, 1996) and to positively participate in transcriptional activation. These interactions are, however, difficult to reproduce (Zhang *et al.*, 1999b) and the mechanisms by which Rb cooperates with these factors are ill defined. Whereas a complex containing Rb, E2F1, and an E2F-recognition site can readily be demonstrated experimentally, analogous complexes containing Rb, MyoD (or C/EBP), and the appropriate DNA binding sites have not been observed (Chen *et al.*, 1996; Gu *et al.*, 1993). Recently, a stable protein–DNA complex, containing Rb, the bHLH protein tal-1, and other factors, that controls erythroblast gene expression has been documented, directly implicating Rb in regulating not only cell cycle exit but also differentiation in a physiological context (Vitelli *et al.*, 2000). Rb was also shown to bind Pax3, an earlier muscle factor that controls MyoD expression in body muscles (Wiggin *et al.*, 1998). Analysis of Rb<sup>-/-</sup> fibroblasts engineered to express myogenic factors indicates that transcriptional activation by MEF2C is also affected (Novitch *et al.*, 1999). However, there are clearly some differences between fibroblasts expressing myogenic factors and myoblasts. For example, MyoD:Rb<sup>-/-</sup> fibroblasts do not fuse well and fail to express both MCK and MHC (Gu *et al.*, 1993; Novitch *et al.*, 1996), whereas Rb<sup>-/-</sup> myoblasts do form myotubes and exhibit near-normal expression of MHC *in vitro*

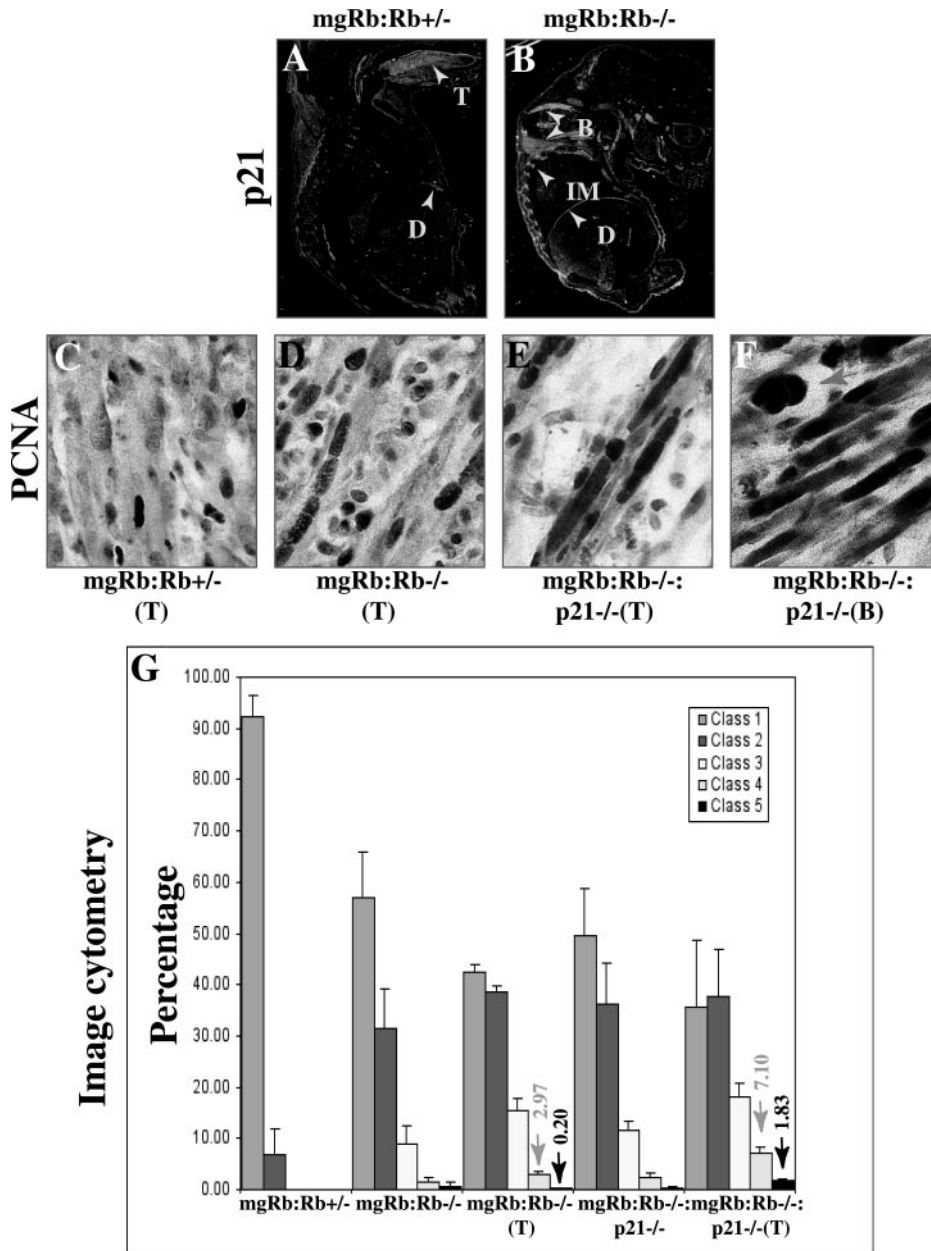
(Schneider *et al.*, 1994) and *in vivo* (Figs. 4A and 4B). Other pathways may impinge upon MCK gene expression in the absence of Rb. It has recently been shown that Rb-deficient fibroblasts have elevated levels of activated Ras and that suppression of Ras activity in these fibroblasts restores their ability to induce MyoD-responsive genes (Lee *et al.*, 1999). However, so far, we were unable to detect elevated GTP-loaded RAS in mgRb:Rb<sup>-/-</sup> muscles (Y. Liu and E. Zacksenhaus, unpublished data). Our observation that Rb controls the normal expression profile of MCK *in vivo* should encourage additional analysis to explore the mechanisms of this regulation in Rb mutant mice and myoblasts.

### Cooperative Effects of Rb and p21<sup>Waf1/Cip1</sup> during Myogenesis

We demonstrated herein that nuclei in Rb-deficient myotubes undergo *bona fide* endoreduplication leading to the accumulation of DNA up to 32N chromosomes and more (Fig. 2). Endoreduplication in human is rare and occurs only in certain specialized lineages such as megakaryocytes (Zimmet *et al.*, 1997). More often, endoreduplication is associated with genomic instability, loss of cell cycle control, and neoplastic transformation. Although the loss of Rb normally leads to ectopic DNA synthesis or apoptosis, there might be scenarios in which the transition through G2 is inhibited in Rb mutant cells, leading to endoreduplication and genomic instability. Such a scenario was demonstrated in Rb mutant cells overexpressing p21<sup>Waf1/Cip1</sup> or irradiated to induce p53 and endogenous p21<sup>Waf1/Cip1</sup> (Niculescu *et al.*, 1998). In both cases endoreduplication occurred only in cells lacking Rb. Rb was also implicated in limiting rereplication and aneuploidy in human cells treated with microtubule inhibitors (Khan and Wahl, 1998). Thus, the endoreduplication observed in Rb mutant muscles *in vivo* represents a pathological process that may occur during the progression of cancer.

Although p21<sup>Waf1/Cip1</sup> can induce endoreduplication in Rb mutant cells *in vitro*, our genetic analysis revealed that despite its high expression in mgRb:Rb<sup>-/-</sup> myotubes, inactivation of this CKI does not prevent endoreduplication in mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> muscles. In fact, mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> mutant embryos exhibited increased polyploidy and apoptosis, reduced MCK gene expression and muscle mass, and more severe bone defects (Figs. 3–5 and 7A). The elevated cell death in mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> muscles may be triggered by the increased cell proliferation that occurs when both Rb and p21 are inactivated (Fig. 3) and/or may reflect an anti-apoptotic effect of p21. The enhanced cell proliferation despite the increased cell death in mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> mutant muscles is consistent with the accelerated tumorigenicity observed in Rb<sup>+/-</sup>:p21<sup>-/-</sup> mutant mice (Brugarolas *et al.*, 1998). Embryos lacking both p21 and p57 demonstrate muscle defects very similar to those in Rb mutant fetuses, suggesting that Rb may be a major target of these CKIs (Zhang *et al.*, 1999). However,

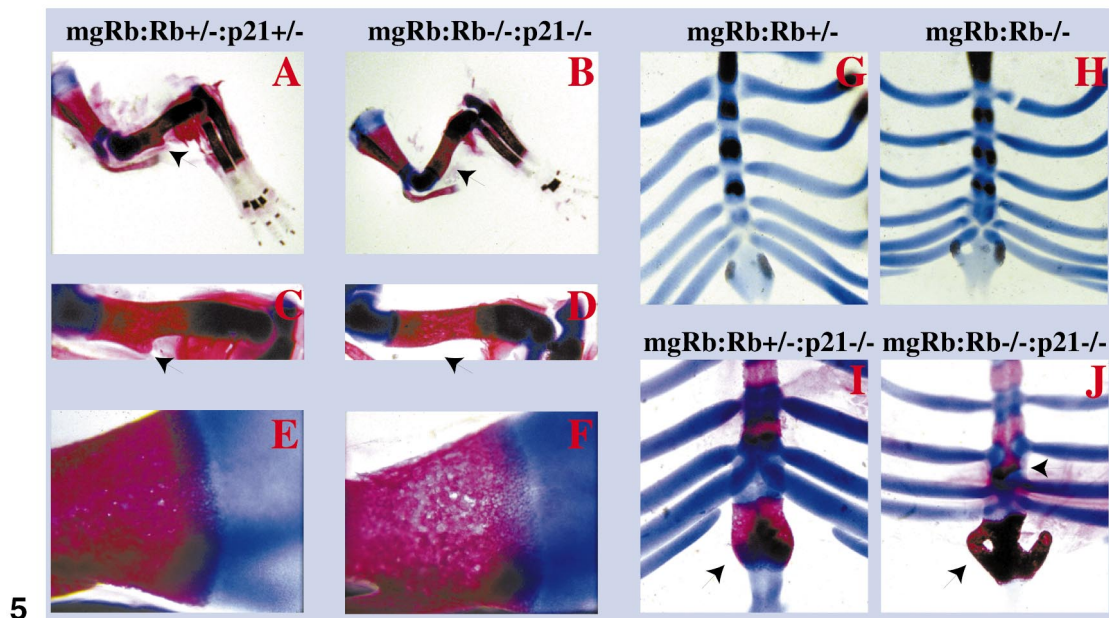
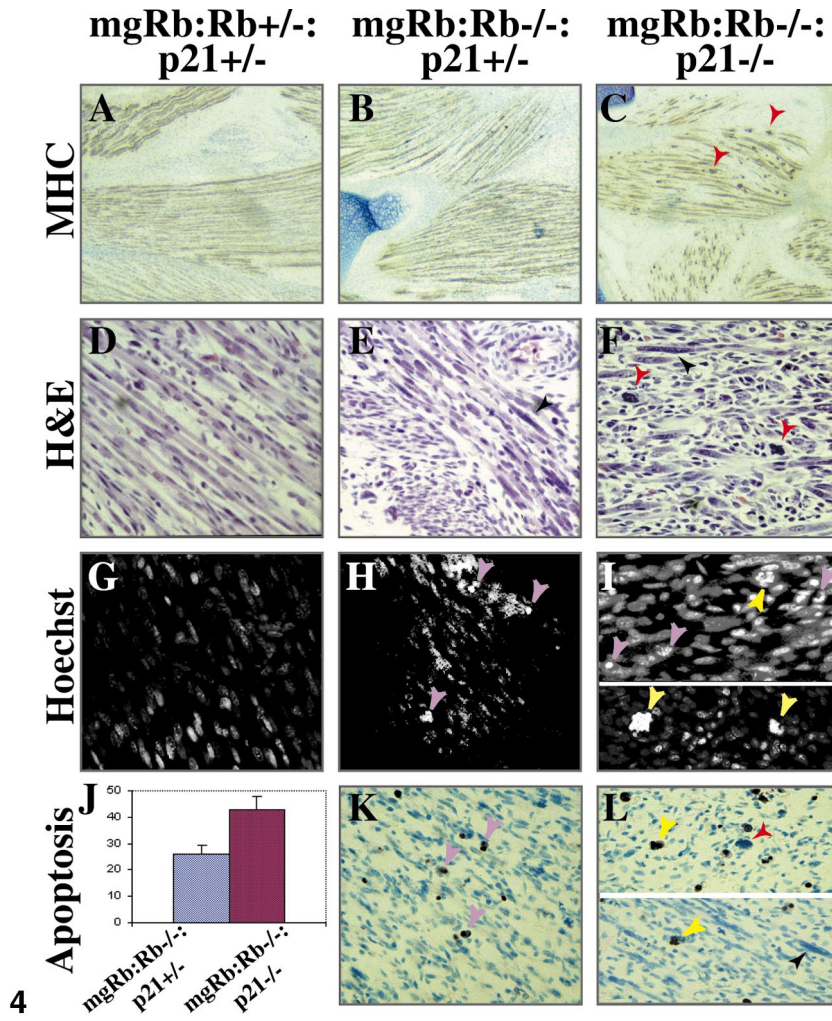


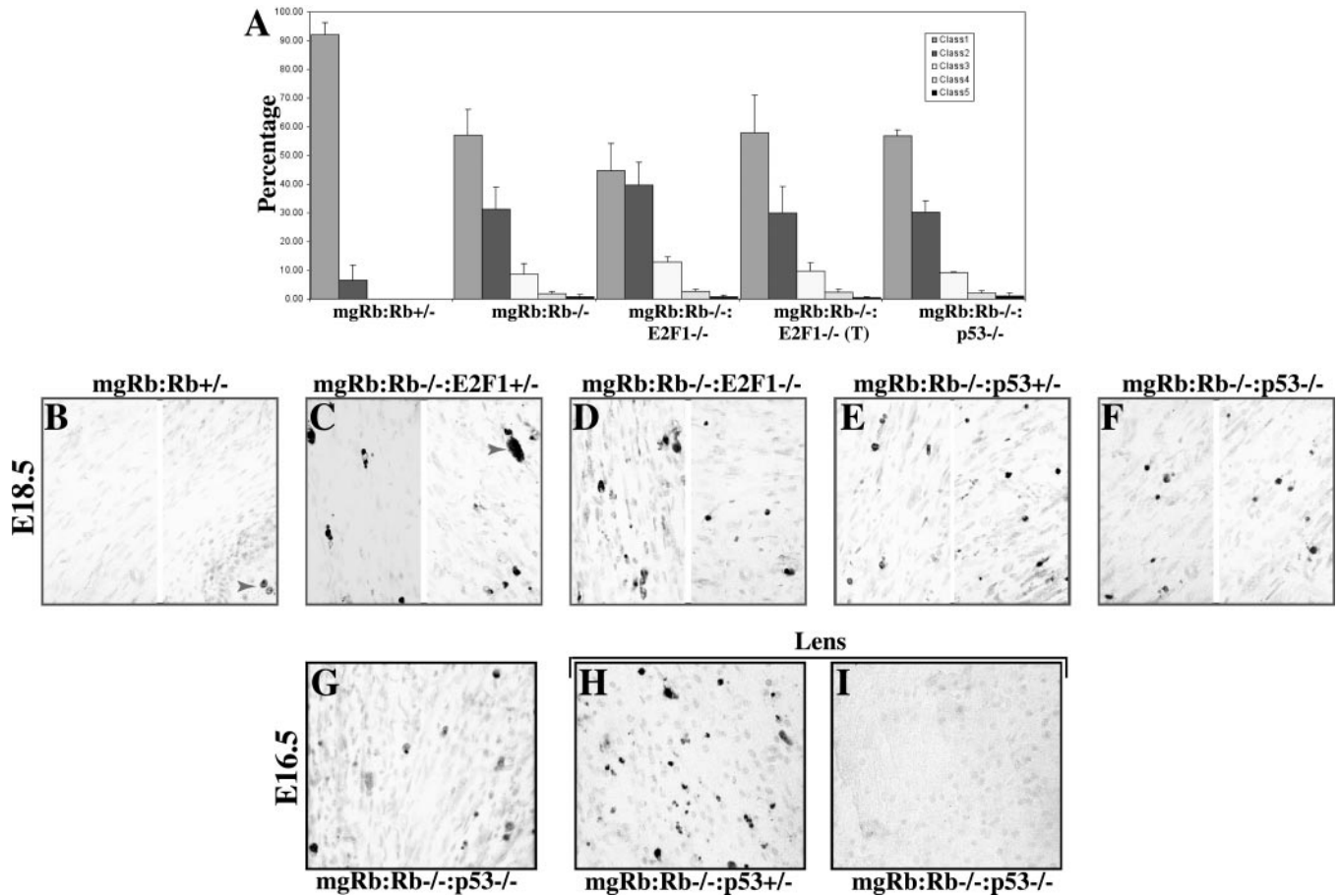


**FIG. 3.** Compound mutant fetuses lacking both Rb and p21<sup>Waf1/Cip1</sup> exhibit increased endoreduplication. (A, B) RNA *in situ* hybridization of p21 in a E17.5 mgRb:Rb<sup>+/-</sup> control embryo (A) and a mgRb:Rb<sup>-/-</sup> mutant littermate (B). Abbreviations: B, back muscles; IM, intercostal muscles; D, diaphragm; T, tongue. (C-F) Compound mutant fetuses, defective in both Rb and p21, exhibit increased PCNA-positive nuclei within myotubes. Arrow in F indicates a “collapsed” nucleus. (G) Quantitative image cytometry analysis. Class 1 corresponds to nuclei with 2N–4N chromosomes and classes 2 to 5 represent polyploid nuclei (see Materials and Methods). The mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> double-mutant fetuses exhibited increased numbers of 32N and >32N nuclei compared to mgRb:Rb<sup>-/-</sup> single mutants at E16.5. The increased endoreduplication is most evident in the tongue (T). Numbers indicate the percentage of nuclei in the respective class.

the enhanced muscle phenotype in mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> mutant embryos suggests that p21 likely regulates other factors in addition to Rb. Thus, p21<sup>Waf1/Cip1</sup> may modulate

the activity of cyclin E/Cdk2 (Brugarolas *et al.*, 1998; Mal *et al.*, 2000) or cyclin-independent Cdk2 activity (Gil-Gomez *et al.*, 1998), the Rb-related proteins p107 or p130, myogenic

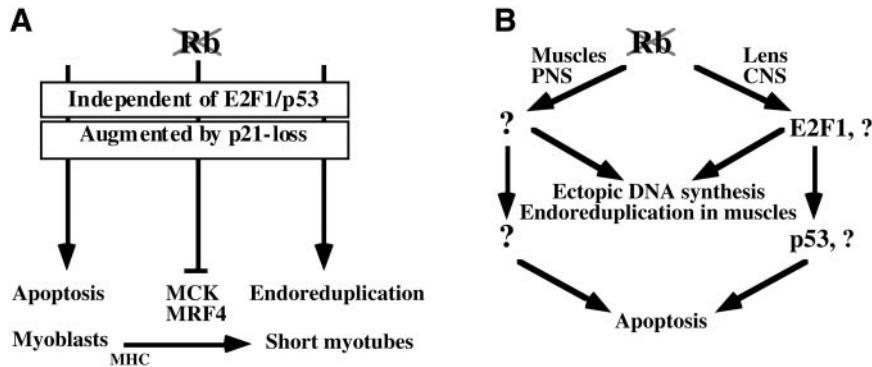




**FIG. 4.** Compound mutant fetuses lacking both Rb and *p21*<sup>Waf1/Cip1</sup> exhibit reduced muscle mass and increased apoptosis. (A-C) Immunohistochemical staining of E16.5 Rb mutant fetuses with antibodies reactive to myosin heavy chain (fast). The myotubes in *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant muscles are shorter, are less organized, and contain many collapsed nuclei (red arrowheads) (original magnification, 10×). (D-F) H&E staining demonstrating giant (black arrows) and collapsed (red arrowheads) nuclei in E16.5 *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant muscles (40×). (G-I) Fluorescence microscopy of muscle sections stained with Hoechst 33258. Purple arrows point to condensed nuclei; yellow arrows indicate large collapsed nuclei. (J-L) TUNEL analysis showing increased apoptosis in E16.5 *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant muscles compared with *mgRb:Rb*<sup>-/-</sup> littermates. Both TUNEL-positive (yellow arrowheads) and TUNEL-negative (red arrowhead) collapsed nuclei are observed in *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant (L, top and bottom). Purple arrows indicate apoptotic nuclei of regular size. (I) The number of TUNEL-positive nuclei represents the average from three different areas counted at 40× magnification from three independent *mgRb:Rb*<sup>-/-</sup> and *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> fetuses.

**FIG. 5.** Compound mutant fetuses lacking both Rb and *p21*<sup>Waf1/Cip1</sup> exhibit more severe skeletal defects. Skeletons were stained with Alizarin red (ossification centers) and Alcian blue (cartilage). (A, B) Limbs of *mgRb:Rb*<sup>+/+</sup>:*p21*<sup>+/+</sup> and *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> compound-mutant fetuses at E17.5. The mutant limb is smaller, but the ossification centers in the metacarpal and phalangeal bones indicate a similar developmental stage. (C, D) Higher magnification of the humerus demonstrating lack of deltoid tuberosity in mutant fetuses (arrows). (E, F) Higher magnification of the scapula of E16.5 *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant and control littermate fetuses. The mutant scapula appears more brittle/perforated and lacks defined boundaries between bone and cartilage. (G, H) Sternum of *mgRb:Rb*<sup>-/-</sup> mutant and control littermate at E16.5. (I, J) Sternum of *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> mutant and control littermate at E17.5. In both pairs, the xiphoid process at the end of the sternum is abnormally developed in the mutant fetuses (arrows). Note the abnormal ossification of the sternum in the double-mutant fetus (upper arrow in J) and that the ribs join the sternum at 90° in the double mutant but at a smaller angle in control or single-mutant fetuses. The reduced concentrations of KOH used to obtain intact skeletons of the more fragile *mgRb:Rb*<sup>-/-</sup>:*p21*<sup>-/-</sup> double-mutant fetuses yielded incomplete clearing of the skeleton preparations in A-D and I-J.





**FIG. 7.** Distinct pathways downstream of Rb in muscles and other tissues. The genetic analysis described herein establishes the roles of the Cdk inhibitors p21<sup>Waf1/Cip1</sup>, E2F1, and p53 in aberrant myogenesis in Rb mutant fetuses. (A) Inactivation of Rb results in apoptosis; endoreduplication; reduced expression of MCK and MRF4, but not MHC; and reduced muscle mass and shorter myotubes. These defects are augmented by the loss of p21<sup>Waf1/Cip1</sup> but not by loss of E2F1 or p53. (B) At least two pathways exist downstream of Rb. In lens and CNS, E2F1 and p53 mediate ectopic DNA synthesis and apoptosis. In skeletal muscles and PNS, inappropriate DNA synthesis and cell death occur through a pathway(s), yet to be defined, which is independent of E2F1 and p53.

factors (Zhang *et al.*, 1999a), or survival factors such as AKT (Fujio *et al.*, 1999).

### **E2F1- and p53-Dependent and Independent Apoptotic Pathways Downstream of Rb**

We demonstrated herein that both endoreduplication and apoptosis in Rb mutant muscles are independent of E2F1 and p53 (Fig. 6). In accord with our results, recent analysis has demonstrated that myoblasts and other cell types can undergo p53-independent apoptosis *in vitro* (Cerone *et al.*, 2000; Fimia *et al.*, 1998; Agah *et al.*, 1997). These and other studies (Macleod *et al.*, 1996; Tsai *et al.*, 1998) indicate that there are at least two pathways that control cell cycle progression and apoptosis in Rb-deficient mice (Fig. 7B). In the CNS and lens, ectopic DNA synthesis and programmed cell death are mediated through E2F1- and p53-dependent pathways. In the PNS, loss of E2F1 reduces to some degree inappropriate entry into S phase, but there is still substantial cell proliferation and apoptosis in the Rb-E2F1 double mutants (Tsai *et al.*, 1998). Thus, in the PNS and skeletal muscles, the E2F1/p53 pathways do not operate or are redundant. Interestingly, we have recently found that Apaf1 is required for apoptosis in the CNS and lens but less so in PNS and skeletal muscles (Z. Guo, T. Mak, and E. Zacksenhaus, unpublished data). The elucidation of this E2F1/p53/Apaf1-independent pathway(s) is important as it may be disrupted during the progression of tumors in which the p53 pathway is intact. Curiously, however, p53 is often inactivated in rhabdomyosarcoma, a pediatric cancer of skeletal muscles (Diller *et al.*, 1995), indicating that p53 may play an important role in muscle physiology at least in some contexts that are presumably different from the cell death observed during myogenesis in Rb-deficient mice.

### **Rb and Bone Development**

Most of the bone defects observed in the mgRb:Rb<sup>-/-</sup> fetuses, which are further augmented in mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> compound mutants, likely reflect an indirect consequence of muscle degeneration, as they are also observed in myogenin knockout mice. The relatively shortened limbs and brittle/pierced appearance of the bones in mgRb:Rb<sup>-/-</sup>:p21<sup>-/-</sup> fetuses (Fig. 5) may, however, represent an autonomous role for Rb in bone development. A requirement for Rb in bone development would be in accord with the increased risk for osteosarcoma in individuals with germ-line mutations in Rb (Weichselbaum *et al.*, 1988). Notably, ectopic expression of Rb in the Rb mutant osteosarcoma cell line Saos-2 induces differentiation (Sellers *et al.*, 1998), indicating that Rb can be a limiting factor in osteoblast differentiation. Mutant mice devoid of both p107 and p130 display increased chondrocyte density, delayed endochondral bone formation, and short limbs (Cobrinik *et al.*, 1996). In this regard, it will be of interest to test mgRb:Rb<sup>-/-</sup>:p107<sup>-/-</sup> and mgRb:Rb<sup>-/-</sup>:p130<sup>-/-</sup> mutant fetuses for synergistic effects of Rb and its relatives on development of the bone as well as skeletal muscles and other tissues.

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## REFERENCES

- Agah, R., Kirshenbaum, L. A., Abdellatif, M., Truong, L. D., Chakraborty, S., Michael, L. H., and Schneider, M. D. (1997). Adenoviral delivery of E2F-1 directs cell cycle reentry and p53-independent apoptosis in postmitotic adult myocardium in vivo. *J. Clin. Invest.* **100**, 2722–2728.
- Bacus, J. W., and Bacus, J. V. (1994). A method of correcting DNA ploidy measurements in tissue sections. *Mod. Pathol.* **7**, 652–664.
- Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L., and Vousden, K. H. (1998). p14ARF links the tumour suppressors RB and p53. *Nature* **395**, 124–125.
- Berezowsky, J., Zbieranowski, I., Demers, J., and Murray, D. (1995). DNA ploidy of hydatidiform moles and nonmolar conceptuses: A study using flow and tissue section image cytometry. *Mod. Pathol.* **8**, 775–781.
- Breneman, J. W., Ramsey, M. J., Lee, D. A., Eveleth, G. G., Minkler, J. L., and Tucker, J. D. (1993). The development of chromosome-specific composite DNA probes for the mouse and their application to chromosome painting. *Chromosoma* **102**, 591–598.
- Brugarolas, J., Bronson, R. T., and Jacks, T. (1998). p21 is a critical CDK2 regulator essential for proliferation control in Rb-deficient cells. *J. Cell Biol.* **141**, 503–514.
- Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T., and Hannon, G. J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**, 552–557.
- Brugarolas, J., Moberg, K., Boyd, S. D., Taya, Y., Jacks, T., and Lees, J. A. (1999). Inhibition of cyclin-dependent kinase 2 by p21 is necessary for retinoblastoma protein-mediated G1 arrest after gamma-irradiation. *Proc. Natl. Acad. Sci. USA* **96**, 1002–1007.
- Cerone, M. A., Marchetti, A., Bossi, G., Blandino, G., Sacchi, A., and Soddu, S. (2000). p53 is involved in the differentiation but not in the differentiation-associated apoptosis of myoblasts. *Cell Death Differ.* **7**, 506–508.
- Chan, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999). 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* **401**, 616–620.
- Chen, P. L., Riley, D. J., Chen, Y., and Lee, W.-H. (1996). Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev.* **10**, 2794–2804.
- Clarke, A. R., Maandag, E. R., Van Roon, M., Van der Lugt, N. M. T., Van der Valk, M., Hooper, M. L., Berns, A., and Te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development. *Nature* **359**, 328–330.
- Cobrinik, D., Lee, M. H., Hannon, G., Mulligan, G., Bronson, R. T., Dyson, N., Harlow, E., Beach, D., Weinberg, R. A., and Jacks, T. (1996). Shared role of the pRb-related p130 and p107 proteins in limb development. *Genes Dev.* **10**, 1633–1644.
- DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc. Natl. Acad. Sci. USA* **94**, 7245–7250.
- Diller, L., Sexsmith, E., Gottlieb, A., Li, F. P., and Malkin, D. (1995). Germline p53 mutations are frequently detected in young children with rhabdomyosarcoma. *J. Clin. Invest.* **95**, 1606–1611.
- Dyson, N. (1998). The regulation of E2F by pRB-family proteins. *Genes Dev.* **12**, 2245–2262.
- Field, S. J., Tsai, F. Y., Kuo, F., Zubiage, A. M., Kaelin, W. G., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85**, 549–561.
- Fimia, G. M., Gottifredi, V., Bellei, B., Ricciardi, M. R., Tafuri, A., Amati, P., and Maione, R. (1998). The activity of differentiation factors induces apoptosis in polyomavirus large T-expressing myoblasts. *Mol. Biol. Cell* **9**, 1449–1463.
- Fujio, Y., Guo, K., Mano, T., Mitsuuchi, Y., Testa, J. R., and Walsh, K. (1999). Cell cycle withdrawal promotes myogenic induction of Akt, a positive modulator of myocyte survival. *Mol. Cell. Biol.* **19**, 5073–5082.
- Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* **119**, 493–501.
- Gil-Gomez, G., Berns, A., and Brady, H. J. (1998). A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis. *EMBO J.* **17**, 7209–7218.
- Gottlieb, T. M., and Oren, M. (1998). p53 and apoptosis. *Semin. Cancer Biol.* **8**, 359–368.
- Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell* **72**, 309–324.
- Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., and Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature* **346**, 245–250.
- Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J. M., Olson, E. N., and Klein, W. H. (1993). Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501–506.
- Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992). Effects of an Rb mutation in the mouse. *Nature* **359**, 295–300.
- Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. (1994). Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.* **4**, 1–7.
- Jiang, Z., Gou, Z., Saad, F., Ellis, J., and Zacksenhaus, E. (2000). The Rb promoter directs transgene expression exclusively to the nervous system. Submitted for publication.
- Jiang, Z., Zacksenhaus, E., Gallie, B. L., and Phillips, R. A. (1997). The retinoblastoma gene family is differentially expressed during embryogenesis. *Oncogene* **14**, 1789–1797.
- Khan, S. H., and Wahl, G. M. (1998). p53 and pRb prevent rereplication in response to microtubule inhibitors by mediating a reversible G1 arrest. *Cancer Res.* **58**, 396–401.
- Lee, E. Y. H. P., Chang, C. Y., Hu, N., Wang, Y. C. J., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and hematopoiesis. *Nature* **359**, 288–294.
- Lee, E. Y.-H. P., Hu, N., Yuan, S.-S. F., Cox, L. A., Bradley, A., Lee, W.-H., and Herrup, K. (1994). Dual roles of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Genes Dev.* **8**, 2008–2021.
- Lee, K. Y., Ladha, M. H., McMahon, C., and Ewen, M. E. (1999). The retinoblastoma protein is linked to the activation of Ras. *Mol. Cell. Biol.* **19**, 7724–7732.

- Lufkin, T., Mark, M., Hart, C. P., Dolle, P., LeMeur, M., and Chambon, P. (1992). Homeotic transformation of the occipital bones of the skull by ectopic expression of a homeobox gene. *Nature* **359**, 835–841.
- Maandag, E. C., van der Valk, M., Vlaar, M., Feltkamp, C., O'Brien, J., van Roon, M., van der Lugt, N., Berns, A., and te Riele, H. (1994). Developmental rescue of an embryonic-lethal mutation in the retinoblastoma gene in chimeric mice. *EMBO J.* **13**, 4260–4268.
- Macleod, K. (1999). pRb and E2f-1 in mouse development and tumorigenesis. *Curr. Opin. Genet. Dev.* **9**, 31–39.
- Macleod, K. F., Hu, Y., and Jacks, T. (1996). Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. *EMBO J.* **15**, 6178–6188.
- Mal, A., Chattopadhyay, D., Ghosh, M. K., Poon, R. Y., Hunter, T., and Harter, M. L. (2000). p21 and retinoblastoma protein control the absence of DNA replication in terminally differentiated muscle cells. *J. Cell Biol.* **149**, 281–292.
- Morgenbesser, S. D., Williams, B. O., Jacks, T., and DePinho, R. A. (1994). p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature* **371**, 72–74.
- Mulligan, G., and Jacks, T. (1998). The retinoblastoma gene family: Cousins with overlapping interests. *Trends Genet.* **14**, 223–229.
- Nevins, J. R., Leone, G., DeGregori, J., and Jakoi, L. (1997). Role of the Rb/E2F pathway in cell growth control. *J. Cell. Physiol.* **173**, 233–236.
- Niculescu, A. B., Chen, X., Smeets, M., Hengst, L., Prives, C., and Reed, S. I. (1998). Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Mol. Cell Biol.* **18**, 629–643.
- Novitsch, B. G., Mulligan, G. J., Jacks, T., and Lassar, A. B. (1996). Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J. Cell Biol.* **135**, 441–456.
- Novitsch, B. G., Spicer, D. B., Kim, P. S., Cheung, W. L., and Lassar, A. B. (1999). pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. *Curr. Biol.* **9**, 449–459.
- Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Cordon-Cardo, C., and DePinho, R. A. (1998). The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **29**, 713–723.
- Sah, V. P., Attardi, L. D., Mulligan, G. J., Williams, B. O., Bronson, R. T., and Jacks, T. (1995). A subset of p53-deficient embryos exhibit exencephaly. *Nat. Genet.* **10**, 175–180.
- Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in RB<sup>-/-</sup> muscle cells. *Science* **264**, 1467–1471.
- Sellers, W. R., Novitsch, B. G., Miyake, S., Heith, A., Otterson, G. A., Kaye, F. J., Lassar, A. B., and Kaelin, W. G. J. (1998). Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. *Genes Dev.* **12**, 95–106.
- Sherr, C. J., and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9**, 1149–1163.
- Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: Positive and negative regulators of G1-phase progression. *Genes Dev.* **13**, 1501–1512.
- Tsai, K. Y., Hu, Y., Macleod, K. F., Crowley, D., Yamasaki, L., and Jacks, T. (1998). Mutation of E2f-1 suppresses apoptosis and inappropriate S phase entry and extends survival of Rb-deficient mouse embryos. *Mol. Cell* **2**, 293–304.
- Vitelli, L., Condorelli, G., Lulli, V., Hoang, T., Luchetti, L., Croce, C. M., and Peschle, C. (2000). A pentamer transcriptional complex including tal-1 and retinoblastoma protein downmodulates c-kit expression in normal erythroblasts. *Mol. Cell Biol.* **20**, 5330–5342.
- Wang, J., and Walsh, K. (1996). Resistance to apoptosis conferred by CDK inhibitors during myocyte differentiation. *Science* **273**, 359–361.
- Weichselbaum, R. R., Beckett, M., and Diamond, A. (1988). Some retinoblastomas, osteosarcomas, and soft tissue sarcomas may share a common etiology. *Proc. Natl. Acad. Sci. USA* **85**, 2106–2109.
- Wiggan, O., Taniguchi-Sidle, A., and Hamel, P. A. (1998). Interaction of the pRB-family proteins with factors containing paired-like homeodomains. *Oncogene* **16**, 227–236.
- Williams, B. O., Remington, L., Albert, D. M., Mukai, S., Bronson, R. T., and Jacks, T. (1994a). Cooperative tumorigenic effects of germline mutations in Rb and p53. *Nat. Genet.* **7**, 480–484.
- Williams, B. O., Schmitt, E. M., Remington, L., Bronson, R. T., Albert, D. M., Weinberg, R. A., and Jacks, T. (1994b). Extensive contribution of Rb-deficient cells to adult chimeric mice with limited histopathological consequences. *EMBO J.* **13**, 4251–4259.
- Wu, X., and Levine, A. J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl. Acad. Sci. USA* **91**, 3602–3606.
- Zackenhause, E., Jiang, Z., Chung, D., Marth, J., Phillips, R. A., and Gallie, B. L. (1996). pRb controls cell proliferation, differentiation and death of skeletal muscle cells and other lineages during embryogenesis. *Genes Dev.* **10**, 3051–3064.
- Zhang, J. M., Wei, Q., Zhao, X., and Paterson, B. M. (1999a). Coupling of the cell cycle and myogenesis through the cyclin D1-dependent interaction of MyoD with cdk4. *EMBO J.* **18**, 926–933.
- Zhang, J. M., Zhao, X., Wei, Q., and Paterson, B. M. (1999b). Direct inhibition of G(1) cdk kinase activity by MyoD promotes myoblast cell cycle withdrawal and terminal differentiation. *EMBO J.* **18**, 6983–6993.
- Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J. W., and Elledge, S. J. (1999). p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev.* **13**, 213–224.
- Zimmet, J. M., Ladd, D., Jackson, C. W., Stenberg, P. E., and Ravid, K. (1997). A role for cyclin D3 in the endomitotic cell cycle. *Mol. Cell Biol.* **17**, 7248–7259.

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