

## *E2f3a* and *E2f3b* Contribute to the Control of Cell Proliferation and Mouse Development<sup>∇†</sup>

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The *E2f3* locus encodes two Rb-binding gene products, E2F3a and E2F3b, which are differentially regulated during the cell cycle and are thought to be critical for cell cycle progression. We targeted the individual inactivation of *E2f3a* or *E2f3b* in mice and examined their contributions to cell proliferation and development. Chromatin immunoprecipitation and gene expression experiments using mouse embryo fibroblasts deficient in each isoform showed that E2F3a and E2F3b contribute to G<sub>1</sub>/S-specific gene expression and cell proliferation. Expression of other *E2f3a* or *E2f3b* was sufficient to support E2F target gene expression and cell proliferation in the absence of other E2F activators, *E2f1* and *E2f2*, suggesting that these isoforms have redundant functions. Consistent with this notion, *E2f3a*<sup>-/-</sup> and *E2f3b*<sup>-/-</sup> embryos developed normally, whereas embryos lacking both isoforms (*E2f3*<sup>-/-</sup>) died in utero. We also find that *E2f3a* and *E2f3b* have redundant and nonredundant roles in the context of *Rb* mutation. Analysis of double-knockout embryos suggests that the ectopic proliferation and apoptosis in *Rb*<sup>-/-</sup> embryos is mainly mediated by *E2f3a* in the placenta and nervous system and by both *E2f3a* and *E2f3b* in lens fiber cells. Together, we conclude that the contributions of E2F3a and E2F3b in cell proliferation and development are context dependent.

The *retinoblastoma* gene (*RB*) was the first tumor suppressor identified. Inheritance of a mutant *RB* allele leads to early childhood retinoblastoma and predisposes patients to a variety of other malignancies (29). In addition, somatic mutation in *RB* is a frequent event observed in small-cell lung carcinoma and breast cancer (9, 30). Mice with mutation in a single *Rb* allele, however, do not develop retinoblastoma but instead develop pituitary and thyroid tumors (3, 14, 16). The underlying basis for this difference in tumor spectrum between humans and mice remains unclear. Consistent with having tumor suppressor function, loss of both copies of *Rb* in mice leads to defects in proliferation, apoptosis, and differentiation (3, 14, 16). *Rb*<sup>-/-</sup> embryos have widespread ectopic proliferation and apoptosis in the central nervous system (CNS), peripheral nervous system (PNS), placenta, and a number of other tissues. In addition, cells of neuronal, erythroid, and skeletal muscle lineage fail to terminally differentiate. Consequently embryos suffer from anemia, systemic edema, and placental dysfunction and die at embryonic day 13.5 (E13.5) or soon after.

The Rb tumor suppressor pathway is believed to play a critical role in the control of cellular proliferation by regulating

E2F transcriptional activities. To date, eight different E2F family members have been identified (4, 7, 18, 21). Among these, E2F3 has emerged as a critical Rb-binding factor important for mediating many of Rb's functions during embryonic development and tumorigenesis (24, 35, 36). For instance, loss of *E2f3* profoundly suppresses ectopic proliferation and inappropriate apoptosis in the CNS, PNS, and lens fiber cells of *Rb*<sup>-/-</sup> embryos. Moreover, development of pituitary tumors in *Rb*<sup>+/-</sup> mice lacking one or two copies of *E2f3* is decreased, but surprisingly the incidence and invasiveness of medullary thyroid carcinomas are increased (35).

The molecular basis for why E2F3 is uniquely important for mediating Rb function may be related to the complexity of the *E2f3* genomic organization (17). The *E2f3* locus drives the expression of two related gene products, E2F3a and E2F3b, through the use of two distinct promoters (1). As a result, *E2f3a* and *E2f3b* have unique first exons encoding 122 and 6 N-terminal amino acids, respectively. Other than at their N termini, these two related proteins share the same coding sequence, including regions important for specific DNA binding, transactivation, and association with the *Retinoblastoma*-encoded tumor suppressor protein and related pocket proteins. The control of their expression is complex and involves both transcriptional and posttranscriptional mechanisms (1). E2F3b protein is expressed in quiescent cells and remains constant throughout the cell cycle, whereas E2F3a protein accumulates maximally at the G<sub>1</sub>/S transition (17). The interesting dichotomy of the *E2f3* locus in relation to the control of cell proliferation remains to be clearly understood. To rigorously evaluate the individual contributions of the two isoforms in the control of cell proliferation and development, we specifically targeted the inactivation of *E2f3a* or *E2f3b* in mice. Here, we provide evidence suggesting that both E2F3a and

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E2F3b contribute to the activation of E2F target gene expression and cell proliferation but that E2F3a is the main isoform that contributes to the unscheduled proliferation, apoptosis, and early lethality observed in *Rb*<sup>-/-</sup> embryos.

#### MATERIALS AND METHODS

**MEF proliferation and ChIP analysis.** For analysis of cell proliferation, passage 2 mouse embryo fibroblasts (MEFs) with the indicated genotypes were plated and counted every day for 6 days. To bring cells to quiescence, cultures at 40 to 50% confluence were incubated in Dulbecco modified Eagle medium containing 0.2% serum for 60 h. Synchronized cells were then stimulated to proliferate by the addition of Dulbecco modified Eagle medium supplemented with 15% fetal bovine serum (FBS) and 10  $\mu$ M bromodeoxyuridine (BrdU). At the desired time points, cells were then fixed with methanol and acetone (1:1), processed for immunofluorescence using anti-BrdU antibody (Ab-3; Oncogene), and counterstained with 4,6-diamidino-2-phenylindole (DAPI). A minimum of 500 DAPI-positive nuclei were scored for each time point. For chromatin immunoprecipitation (ChIP) experiments, approximately  $10 \times 10^6$  cells were harvested for each antibody reaction. Formaldehyde was added directly to the culture medium at a final concentration of 1%. Cross-linking was allowed to proceed for 10 min at room temperature and was then stopped by the addition of glycine to a final concentration of 0.125 M. Cross-linked cells were washed twice with phosphate-buffered saline and scraped off the plate, and lysates were sonicated in order to shear the genomic DNA into fragments of 200 to 1,000 bp. Four micrograms of anti-E2F3 antibody (sc-876x) was added to each sample and incubated overnight at 4°C. Antibody-protein-DNA complexes were recovered by adding 60  $\mu$ l of salmon sperm DNA-protein G agarose slurry and incubated for 1 h at 4°C. Following extensive washing, complexes were eluted and cross-linking was reversed by heating the samples at 65°C for 4 h. The eluted material was purified and resuspended in 30  $\mu$ l of water. Quantitative real-time PCR (RT-PCR) was performed with 1  $\mu$ l of DNA and 100 nM primers diluted to a final volume of 25  $\mu$ l in Sybr green reaction mix (Bio-Rad). Accumulation of fluorescent products was monitored by RT-PCR using the Bio-Rad iCycler PCR machine. Reactions were performed in triplicate, and values were normalized using the threshold cycle number from the total input sample.

**RT-PCR.** Total RNA from E13.5 *Rb*<sup>-/-</sup> or *Rb*<sup>+/+</sup> placentas and MEFs was isolated by using Trizol (Invitrogen) according to the manufacturer's instruction. cDNA was synthesized from 2  $\mu$ g of total RNA using Superscript III reverse transcriptase (Invitrogen) at 50°C for 60 min. After the completion of the reverse transcription, the final volume was brought to 100  $\mu$ l by adding 80  $\mu$ l of water. RT-PCR was performed in the Bio-Rad iCycler PCR machine. Each PCR mixture contained 0.5  $\mu$ l of cDNA template and 100 nM of gene-specific primers in a final volume of 25  $\mu$ l of Sybr green reaction mix (Bio-Rad). Each quantitative PCR yielded only the expected amplicon as determined by the melting-temperature profiles of the final products and by gel electrophoresis. Standard curves were generated using cDNA to determine the linear range of amplification for each primer pair. Reactions were performed in triplicate, and relative amounts of cDNAs were normalized to the *Gapdh* gene. Primers used for RT-PCR have been described previously (21).

**BrdU staining and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis.** Pregnant females (13.5 days post-coitum) were injected intraperitoneally with BrdU (100  $\mu$ g/gram of body weight) 2 h prior to harvesting of embryos. Desired tissues from placentas and embryos were fixed in 10% neutral buffered formalin upon harvesting, and paraffin-embedded sections (5  $\mu$ m) were used for immunohistochemistry analysis after deparaffinization. Anti-BrdU antibody (Dako Co.; MO-0744) and Vectastain Elite ABC reagent (Vector Labs) were used to detect BrdU incorporation according to the manufacturer's instructions, followed by hematoxylin counterstaining. For lens fiber cells, we used a secondary antibody conjugated with Alexa 594 and DAPI for nuclear staining.

Apoptotic cells were determined using TUNEL assays according to the manufacturer's protocol (Chemicon S7101), except that the terminal deoxynucleotidyltransferase enzyme was incubated at room temperature for 1.5 h and the antidigoxigenin conjugate was incubated at room temperature for 1 h. All slides were counterstained with hematoxylin. Quantification of proliferation and apoptosis was achieved by calculating the percentage of cells with positive signals in the desired tissue sections.

**Giemsa staining.** Peripheral blood samples (4  $\mu$ l) were spread on slides. Giemsa staining (Sigma; diluted 1:20) was performed according to the manufacturer's protocol to determine whether red blood cells were nucleated. The same embryos were used both for hematoxylin and eosin (H&E) staining and for immunohistochemical analysis.

#### RESULTS

**E2F3 isoforms contribute to cell proliferation in MEFs.** The *E2f3* locus plays a critical role in the control of cell proliferation. Ablation of both *E2f3a* and *E2f3b* (*E2f3*<sup>-/-</sup>) in MEFs profoundly decreases E2F target gene expression and cell proliferation (11). To evaluate the individual contributions of each isoform in the control of cell proliferation, we investigated the molecular consequences of inactivating *E2f3a* and *E2f3b* in MEFs. The targeted inactivation of *E2f3a* and *E2f3b* in mice was described previously (Fig. 1A) (26). MEFs were derived from *E2f3a*<sup>+/-</sup> or *E2f3b*<sup>+/-</sup> intercrosses, and the specific ablation of E2F3a or E2F3b proteins was confirmed by Western blot assays using total E2F3-specific antibodies (Fig. 1B). Standard proliferation assays revealed a measurable defect in the proliferation rate of *E2f3a*<sup>-/-</sup> and *E2f3b*<sup>-/-</sup> MEFs relative to *E2f3*<sup>+/+</sup> littermate control cells (Fig. 1C and D). We also analyzed the ability of quiescent *E2f3a*<sup>-/-</sup> and *E2f3b*<sup>-/-</sup> MEFs to enter the cell cycle in response to mitogenic signals. S-phase entry was monitored by the incorporation of BrdU into genomic DNA. These assays also revealed a slight delay in the entry of *E2f3a*<sup>-/-</sup> and *E2f3b*<sup>-/-</sup> MEFs into the cell cycle, relative to *E2f3*<sup>+/+</sup> MEFs (Fig. 1C and D).

Given the established roles for E2F family members in the control of cell cycle-dependent gene expression, we measured the levels of known E2F target genes in isoform-specific mutant MEFs that were stimulated to enter the cell cycle. Consistent with previous reports, expression of E2F-responsive genes such as *cdc6*, *cyclin E*, *mcm3*, *pol $\alpha$* , *dhfr*, and *pcna* in wild-type MEFs peaked at the G<sub>1</sub>/S transition (Fig. 1E and F, *E2f3a*<sup>+/+</sup> and *E2f3b*<sup>+/+</sup>; see also Fig. S1 in the supplemental material). In contrast, this serum-dependent induction of E2F target gene expression was significantly delayed in *E2f3a*<sup>-/-</sup> and *E2f3b*<sup>-/-</sup> MEFs and peak levels never reached those observed in littermate control cells. Together, these observations and recent work by Danielian and colleagues (5) suggest that E2F3a and E2F3b proteins individually contribute to the growth factor-dependent activation of E2F target genes during the G<sub>1</sub>/S transition and to the proliferative capacity of MEFs.

**Proliferation of MEFs with a single E2F isoform.** Considerable functional redundancy has been observed among members of the E2F activator subclass (26). Perhaps the most striking example of redundancy was demonstrated by previous studies comparing the proliferative capacities of MEFs deficient in *E2f1*, *E2f2*, and *E2f3*, singly or in combination. These studies showed that loss of *E2f3* (*E2f3a* and *E2f3b*) significantly compromised the proliferation of MEFs but the combined loss of *E2f1*, *E2f2*, and *E2f3* completely abrogated proliferation (33). To examine the potential redundancy among these E2Fs, we generated fibroblasts from E13.5 *E2f1*<sup>-/-</sup> *E2f2*<sup>-/-</sup> *E2f3a*<sup>-/-</sup> and *E2f1*<sup>-/-</sup> *E2f2*<sup>-/-</sup> *E2f3b*<sup>-/-</sup> embryos and once again examined E2F target gene expression and cell proliferation under the same conditions as described above. While entry into S phase was delayed and proliferation was compromised in *E2f1*<sup>-/-</sup> *E2f2*<sup>-/-</sup> *E2f3a*<sup>-/-</sup> and *E2f1*<sup>-/-</sup> *E2f2*<sup>-/-</sup> *E2f3b*<sup>-/-</sup> (TKO) MEFs relative to littermate-derived control cells (Fig. 2A and B), these TKO cells did not proliferate appreciably slower than cells lacking only in *E2f3a* or *E2f3b*. These results suggest that one of the two *E2f3* isoforms is sufficient to support cell proliferation (Fig. 2A and B). While

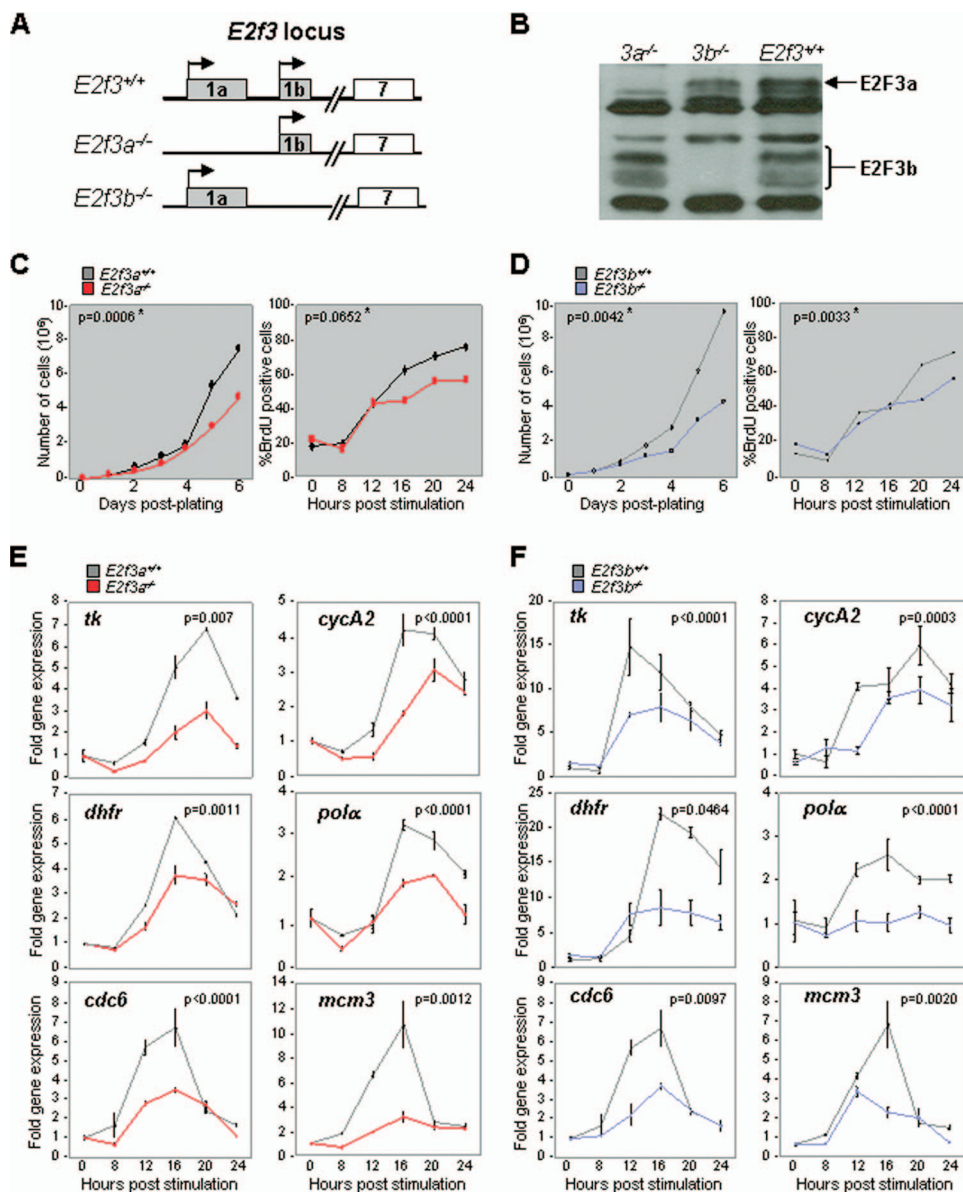


FIG. 1. E2F3a and E2F3b contribute to transcriptional activation. (A) Illustration of the *E2f3* locus and the *E2f3a* and *E2f3b* targeted alleles. (B) Western analysis of E2F3a and E2F3b in cell extracts derived from *E2f3*<sup>+/+</sup>, *E2f3a*<sup>-/-</sup>, and *E2f3b*<sup>-/-</sup> MEFs. (C and D) (Left panels) Exponential growth curves of control (*E2f3a*<sup>+/+</sup> or *E2f3b*<sup>+/+</sup>; gray), *E2f3a*<sup>-/-</sup> (red), and *E2f3b*<sup>-/-</sup> (blue) MEFs. (Right panels) BrdU incorporation of control (*E2f3a*<sup>+/+</sup> or *E2f3b*<sup>+/+</sup>; gray), *E2f3a*<sup>-/-</sup> (red), and *E2f3b*<sup>-/-</sup> (blue) MEFs. Quiescent MEFs with the indicated genotypes were restimulated with medium containing 15% FBS and assessed for BrdU incorporation at the indicated time points. Three independent experiments for proliferation assays, BrdU incorporation, and gene expression were performed in triplicate. The *P* value (\*) comes from all three independent experiments. (E and F) RT-PCR analysis of E2F target gene expression. Total RNA from MEFs harvested as described for panels C and D was used to measure the levels of expression of the indicated E2F target genes. The y axis represents the average induction in gene expression, where the expression level at the 0-h time point is equal to 1. Statistical analyses for proliferation assays, BrdU incorporation, and gene expression were performed by using generalized linear models. The interaction “group by time” was included in both models. The *P* values shown in the plots correspond to the total “group” effect, which indicates the interactions of “group by time.”

some variation was observed among different MEF preparations of the same genotype, we could reproducibly measure a delay and attenuation in the expression of many E2F target genes as TKO cells were stimulated to enter the cell cycle (Fig. 2C and D; see also Fig. S2 in the supplemental material). From these experiments we conclude that *E2f3a* and *E2f3b* have redundant functions and that expression of either one is suf-

ficient to support E2F target gene expression and cell proliferation.

**E2F3a and E2F3b act as transcriptional activators.** The *E2f3a* and *E2f3b* genes are differentially expressed during the cell cycle. *E2f3a* mRNA levels are low in quiescent cells, increase in late G<sub>1</sub>, and peak at G<sub>1</sub>/S, whereas *E2f3b* mRNA levels are constitutively expressed throughout the cell cycle.

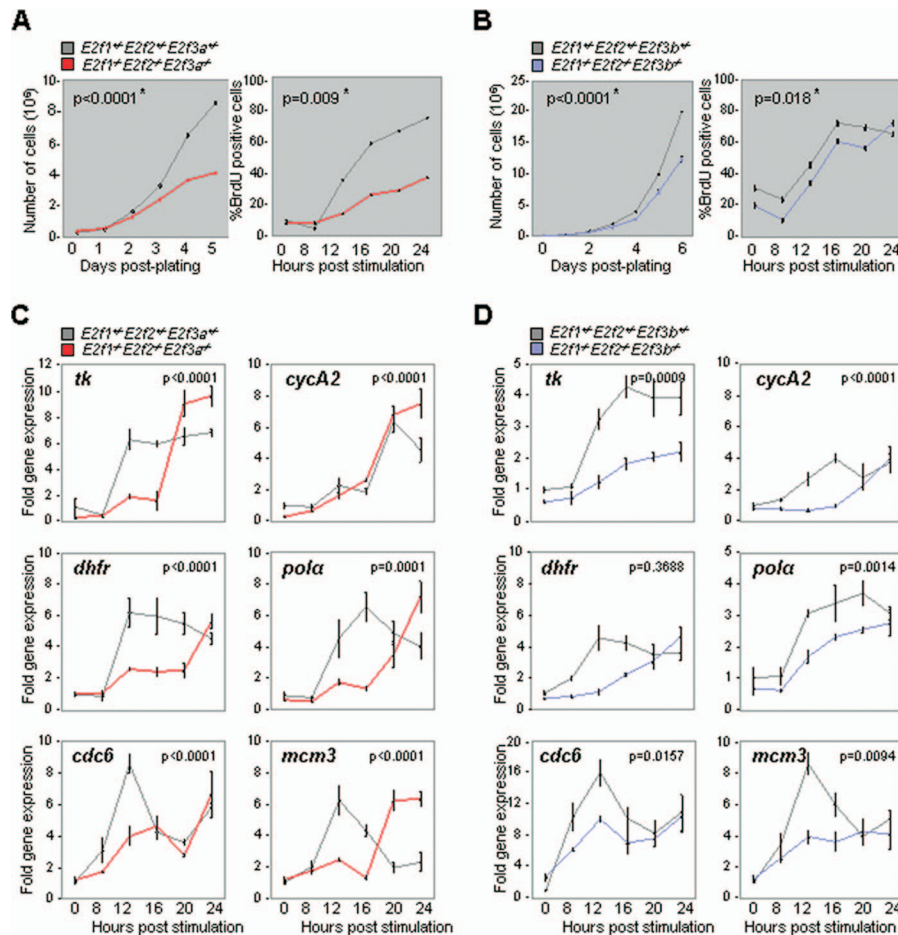


FIG. 2. *E2f3a* and *E2f3b* are sufficient to support cellular proliferation. (A and B) (Left panels) Growth curves of control (gray), *E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3a<sup>-/-</sup>* (red), and *E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3b<sup>-/-</sup>* (blue) MEFs. Passage 2 MEFs with the indicated genotypes were plated in duplicate and counted every day for 6 days. (Right panels) BrdU incorporation in control (gray), *E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3a<sup>-/-</sup>* (red), and *E2f1<sup>-/-</sup>E2f2<sup>-/-</sup>E2f3b<sup>-/-</sup>* (blue) MEFs. (C and D) RT-PCR analysis of E2F target gene expression from MEFs harvested as described for panels A and B. The statistical analyses for proliferation assays, BrdU incorporation, and gene expression were performed as described for Fig. 1. Three independent experiments were performed for proliferation assays, BrdU incorporation, and gene expression. The *P* value comes from all three independent experiments.

Consistent with their expression patterns, previous biochemical studies described E2F3a as a transcription activator that functions at G<sub>1</sub>/S and E2F3b as a repressor that functions during G<sub>0</sub> (17). To determine the extent to which E2F3a and E2F3b proteins might contribute to the control of E2F target gene expression during the cell cycle, we performed ChIP assays on wild-type, *E2f3a<sup>-/-</sup>*, and *E2f3b<sup>-/-</sup>* MEFs with antibodies that recognize both isoforms. The specificity of these antibodies was confirmed by comparing the recruitment of E2F3 proteins to two target promoters, *cdc6* and *E2f1*, in *E2f3<sup>+/+</sup>* and *E2f3<sup>-/-</sup>* cells (Fig. 3A). Using these antibodies, we could detect E2F3-specific proteins on target promoters during G<sub>0</sub> in *E2f3<sup>+/+</sup>* cells (Fig. 3B, 0-h samples). Consistent with the selective expression of *E2f3b* in quiescent cells, the G<sub>0</sub>-specific recruitment of total E2F3 was markedly reduced in *E2f3b<sup>-/-</sup>* cells but was unaltered in *E2f3a<sup>-/-</sup>* MEFs. The recruitment of E2F3 proteins to target promoters increased two- to threefold when *E2f3<sup>+/+</sup>* cells were stimulated to enter G<sub>1</sub>/S (Fig. 3B, 18-h samples). This increase was abolished in *E2f3a<sup>-/-</sup>* cells, indicating that E2F3a was the major isoform

recruited at G<sub>1</sub>/S. But clearly, E2F3b continued to occupy E2F binding sites on target promoters at G<sub>1</sub>/S since an equal amount of E2F3 protein could be detected in *E2f3a<sup>-/-</sup>* cells at G<sub>0</sub> and G<sub>1</sub>/S. From these assays we conclude that E2F3b is the principal isoform bound to target promoters in quiescent cells and that both E2F3a and E2F3b contribute to the loading of target promoters during G<sub>1</sub>/S. Exogenous expression of E2F3a or E2F3b activated an E2F-responsive luciferase reporter to an equal extent (Fig. 3C). Together, these findings are consistent with their role in transcription activation at G<sub>1</sub>/S and provide a molecular basis for their redundant roles in cell proliferation.

Previous work suggested that E2F3b functions as a transcriptional repressor (17). The fact that loss of *E2f3b* did not result in widespread derepression of classic E2F target genes, especially during G<sub>0</sub>, is inconsistent with a general model for E2F3b in transcriptional repression. One possibility for the normal levels of E2F target gene expression in quiescent *E2f3b<sup>-/-</sup>* MEFs is the potential for E2F4, which is known to function as a repressor during G<sub>0</sub>, to compensate for the absence of E2F3b. ChIP assays using E2F4-specific antibodies in

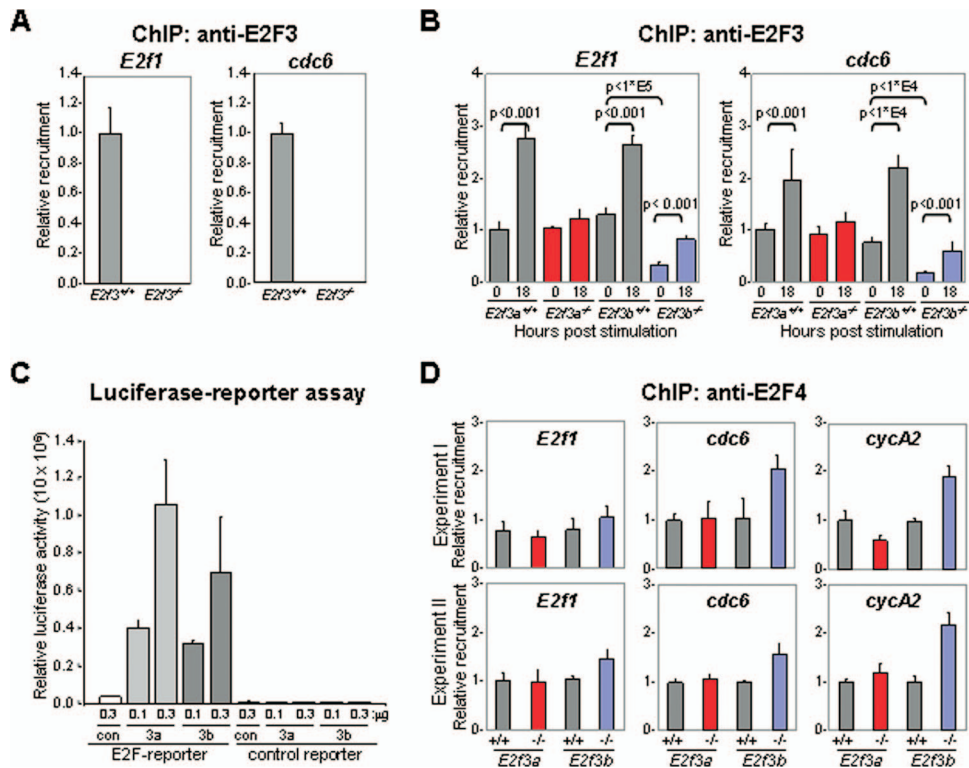


FIG. 3. E2F3a and E2F3b proteins can bind and activate E2F-responsive promoters. (A) Specificity of anti-E2F3 antibodies used in ChIP assays. Control ChIP assays using anti-total E2F3 antibodies in proliferating *E2f3*<sup>+/+</sup> (gray) or *E2f3*<sup>-/-</sup> (white) MEFs. (B) ChIP assays of cell lysates from synchronized *E2f3a*<sup>+/+</sup> or *E2f3b*<sup>+/+</sup> (gray), *E2f3a*<sup>-/-</sup> (red), and *E2f3b*<sup>-/-</sup> (blue) MEFs using antibodies against total E2F3. Immunoprecipitated DNA was measured by RT-PCR using primers flanking the E2F binding sites on the *E2f1* and *cdc6* promoters. RT-PCR was performed in triplicate, and cycle numbers were normalized to 1% of the input DNA. Results are shown as induction relative to the quiescent (0-h) *E2f3a*<sup>+/+</sup> sample; 18 h, quiescent MEFs stimulated with 15% FBS for 18 h. Statistical analyses for ChIP analysis were performed for two independent experiments using the Student *t* test method, and *P* values are indicated within the graph. (C) E2F3a and E2F3b proteins can activate E2F-responsive promoters. Cells were cotransfected with the thymidine kinase *Renilla* luciferase reporter plasmids (as an internal control), with either an E2F-responsive reporter (E2F-reporter) or a control reporter construct (control reporter), and with 0.1 μg or 0.3 μg of a vector expressing E2F3a (3a), E2F3b (3b), or an empty control (con). Relative luciferase reporter activity was internally normalized to the *Renilla* luciferase activity. (D) E2F4 recruitment to E2F target promoters during quiescence. ChIP assays of cell lysates from synchronized wild-type (*E2f3a*<sup>+/+</sup> and *E2f3b*<sup>+/+</sup>; gray), *E2f3a*<sup>-/-</sup> (red), and *E2f3b*<sup>-/-</sup> (blue) MEFs using antibodies against E2F4 (sc-1082).

*E2f3b*<sup>-/-</sup> MEFs revealed a 1.5- to 2-fold increase in the loading of E2F4 to at least some E2F target promoters (Fig. 3D). Given the relatively high abundance of E2F4 in MEFs, as determined by gel shift assays (13, 17), this increase in E2F4 loading could represent a compensatory mechanism in quiescent cells to repress E2F target genes in the absence of E2F3b.

**Loss of *E2f3a* extends the life span of *Rb*<sup>-/-</sup> embryos.** One physiological circumstance where E2Fs have been shown to contribute to the control of proliferation *in vivo* is when there is a limiting amount of the *Rb* tumor suppressor protein. Loss of *E2f1* or *E2f3* (*E2f3a* and *E2f3b*) can suppress the unscheduled proliferation and apoptosis observed in *Rb*<sup>-/-</sup> embryos and extend their viability from approximately E13.5 to E17.5 (24, 25, 36). To determine which E2F3 isoform contributes to the phenotypes caused by the loss of *Rb*, we set up intercrosses between *Rb*<sup>+/-</sup>; *E2f3a*<sup>+/-</sup> mice and either *Rb*<sup>+/-</sup>; *E2f3a*<sup>+/-</sup> or *Rb*<sup>+/-</sup>; *E2f3a*<sup>-/-</sup> mice (Table 1), as well as intercrosses between *Rb*<sup>+/-</sup>; *E2f3b*<sup>-/-</sup> and *Rb*<sup>+/-</sup>; *E2f3b*<sup>-/-</sup> mice (Table 2). Genotypic analysis of the resulting embryos at various stages of gestation is summarized in Tables 1 and 2. In contrast to *Rb*<sup>-/-</sup> and *Rb*<sup>-/-</sup>; *E2f3b*<sup>-/-</sup> embryos, which died by E13.5, *Rb*<sup>-/-</sup>;

*E2f3a*<sup>-/-</sup> embryos were found alive as late as E17.5, suggesting that loss of *E2f3a* suppresses the lethality of *Rb* mutant embryos to the same extent as does loss of *E2f3* (36).

**Loss of *E2f3a* suppresses placental abnormalities in *Rb*<sup>-/-</sup> embryos.** Previous work demonstrated that the extraembryonic function of *Rb* is critical for embryonic viability (6, 34). Indeed, the specific deletion of *Rb* in extraembryonic trophoblast cells was sufficient to cause embryonic lethality (31). Conversely, *Rb*<sup>-/-</sup> fetuses supplied with a wild-type placenta could be carried to term. Therefore, we reasoned that the significant extension of *Rb*<sup>-/-</sup> embryonic viability by loss of *E2f3a* could be due to the suppression of *Rb* mutant placental phenotypes. To investigate this possibility, we first analyzed the expression of *E2f3a*, *E2f3b*, and a number of known E2F target genes in *Rb*<sup>-/-</sup> placentas. Loss of *Rb* resulted in a specific increase of *E2f3a* expression, without affecting *E2f3b* expression, and a concomitant increase in E2F target genes (Fig. 4A and B). These observations raised the possibility that *E2f3* isoforms may differentially contribute to *Rb* mutant placental phenotypes.

We thus compared placental development in *Rb*<sup>-/-</sup>, *Rb*<sup>-/-</sup>;

TABLE 1. Genotypic analysis of embryos derived from intercrosses between *Rb*<sup>+/-</sup>; *E2f3a*<sup>+/-</sup> mice and either *Rb*<sup>+/-</sup>; *E2f3a*<sup>+/-</sup> or *Rb*<sup>+/-</sup>; *E2f3a*<sup>-/-</sup> mice<sup>a</sup>

Genotype	No. of embryos at day:								
	E13.5		E15.5		E16.5		E17.5		
	Found	Expected	Found	Expected	Found	Expected	Found	Expected	
<i>Rb</i> <sup>+/+</sup>									
<i>3a</i> <sup>+/+</sup>	6	9	12	9	—	—	2	1	
<i>3a</i> <sup>+/-</sup>	20	15	15	13	6	3	3	3	
<i>3a</i> <sup>-/-</sup>	7	9	9 <sup>A</sup>	9	7	5	4	6	
<i>Rb</i> <sup>+/-</sup>									
<i>3a</i> <sup>+/+</sup>	11	13	16 (1)	19	—	—	5	3	
<i>3a</i> <sup>+/-</sup>	33 (1)	31	31 (1)	26	5	7	4	6	
<i>3a</i> <sup>-/-</sup>	16 (1)	22	11	18	8	8	12	13	
<i>Rb</i> <sup>-/-</sup>									
<i>3a</i> <sup>+/+</sup>	4 (2)	7	0 (9) <sup>B</sup>	9	—	—	0 (1)	1	
<i>3a</i> <sup>+/-</sup>	14 (5)	15	2 (9)	13	0	3	0 (2)	3	
<i>3a</i> <sup>-/-</sup>	6 (2)	9	5 (3) <sup>C</sup>	9	2 (4)	5	1 (7)	6	
Total	128		124		32		41		

<sup>a</sup> All embryos, including dead embryos, were genotyped. The numbers of genotyped dead fetuses are indicated in parentheses. —, no embryos with this genotype were found. Chi-square analysis was used for association (superscript capital letters): A,  $P > 0.1$  compared to *Rb*<sup>+/+</sup> mice; B,  $P < 0.005$  compared to *Rb*<sup>+/+</sup> mice; C,  $P < 0.005$  compared to *Rb*<sup>-/-</sup> mice.

*E2f3a*<sup>-/-</sup>, and *Rb*<sup>-/-</sup>; *E2f3b*<sup>-/-</sup> embryos. Consistent with previous observations, histological examination of *Rb*<sup>-/-</sup> placentas by H&E staining revealed a severe disruption of labyrinth architecture, with extensive accumulation of densely packed trophoblast cells at the expense of maternal and fetal blood spaces (Fig. 5A). This increase in cellularity was, at least in part, due to an increase in proliferation of trophoblast cells (Fig. 5A to C; see also Fig. S3 in the supplemental material). Remarkably, *Rb*<sup>-/-</sup>; *E2f3a*<sup>-/-</sup> placentas appeared relatively normal, with few clustered trophoblast and blood cells populating the ample and well-distributed supply of blood spaces present within the labyrinth cell layer. In contrast, the inactivation of *E2f3b* did not ameliorate the placental abnormalities of *Rb* mutant embryos (Fig. 5A; see also Fig. S3 in the supplemental material). Quantification of BrdU-positive trophoblast cells showed that loss of *E2f3a*, but not *E2f3b*, suppressed much of the ectopic proliferation evident in *Rb*<sup>-/-</sup> placentas (Fig. 5B and C). Although we cannot completely rule out other potential functions, such as differentiation (2), these data suggest that elevated and unharnessed E2F3a activity contributes to the ectopic trophoblast proliferation and severe disruption of placental architecture in *Rb* mutant embryos.

**E2f3a is the major isoform contributing to *Rb* mutant phenotypes in the CNS and PNS.** During embryonic development, Rb controls proliferation, apoptosis, and cell differentiation through cell-autonomous and nonautonomous mechanisms (6, 19, 22, 32, 34). To determine the extent to which E2F3 isoforms contribute to these processes in developing fetuses, we analyzed *Rb*<sup>-/-</sup>; *E2f3a*<sup>-/-</sup> and *Rb*<sup>-/-</sup>; *E2f3b*<sup>-/-</sup> fetal tissues of the nervous system that are typically affected by the simple loss of *Rb*, including neurons of the CNS and PNS.

In the hindbrain of the CNS, proliferation of neuronal progenitors is restricted to a region lining the ventricles that is known as the “ventricular zone.” As progenitors are induced to differentiate, they migrate from the ventricular zone into the intermediate and marginal zones, exit the cell cycle, and exhibit a terminal differentiation program of gene expression. The functional integrity of postmitotic neurons requires the timely exit of the cell cycle and maintenance of a G<sub>0</sub> state. The Rb tumor suppressor protein is critical for establishing and maintaining this G<sub>0</sub> state. Indeed, *Rb* inactivation results in increased proliferation and apoptosis of neurons that should normally be postmitotic (3, 14, 16). Along with this unscheduled proliferation and apoptosis, loss of Rb function results in

TABLE 2. Genotypic analysis of embryos derived from *Rb*<sup>+/-</sup>; *E2f3b*<sup>-/-</sup> intercrosses<sup>a</sup>

Genotype	No. of embryos at day:							
	E13.5		E15.5		E16.5		E17.5	
	Found	Expected	Found	Expected	Found	Expected	Found	Expected
<i>Rb</i> <sup>+/+</sup> <i>3b</i> <sup>-/-</sup>	13	14	—	—	17	14	—	—
<i>Rb</i> <sup>+/-</sup> <i>3b</i> <sup>-/-</sup>	30 (2)	28	—	—	24	28	—	—
<i>Rb</i> <sup>-/-</sup> <i>3b</i> <sup>-/-</sup>	9 (5) <sup>A</sup>	14	—	—	0 <sup>B</sup>	14	—	—
Total	59				41			

<sup>a</sup> All embryos, including dead embryos, were genotyped. The numbers of genotyped dead fetuses are indicated in parentheses. —, no embryos with this genotype were found. Chi-square analysis was used for association (superscript capital letters): A,  $P > 0.1$  compared to *Rb*<sup>+/+</sup> mice; B,  $P < 0.001$  compared to *Rb*<sup>+/+</sup> mice.

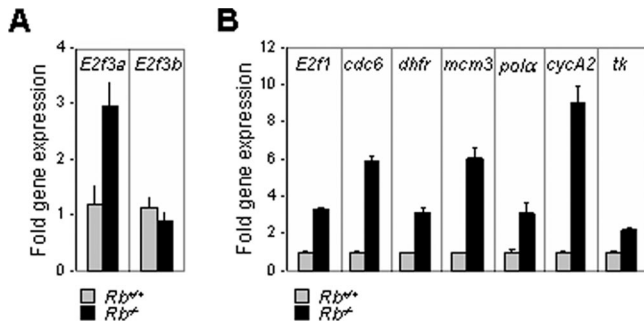


FIG. 4. (A) Analysis of *E2f3a* and *E2f3b* expression in *Rb*<sup>+/+</sup> and *Rb*<sup>-/-</sup> placentas by RT-PCR. (B) Gene expression analysis of known E2F target genes in *Rb*<sup>+/+</sup> and *Rb*<sup>-/-</sup> placentas by RT-PCR.

elevated levels of free E2Fs and increased expression of E2F target genes known to be involved in the control of cell proliferation (20). To determine whether E2F3a and/or E2F3b contributes to the unscheduled proliferation observed in *Rb*<sup>-/-</sup> neuronal tissues, we analyzed postmitotic neurons in the intermediate zone adjacent to the fourth ventricle of the hind-brain of doubly null *Rb/E2F* embryos for their ability to replicate DNA. As reported previously (20), loss of *Rb* resulted in increased neuronal proliferation, which was accompanied by a wave of apoptosis (Fig. 6A to F). The additional inactivation of *E2f3a*, but not *E2f3b*, significantly suppressed the unscheduled proliferation of neurons in the fourth ventricle of the CNS (Fig. 6A and C). Similarly, deletion of *E2f3a* significantly suppressed the massive apoptosis arising in the CNS of *Rb*-deficient embryos (Fig. 6A and E). These results suggest that *E2f3a* is a particularly important mediator of *Rb* mutant phenotypes manifested in the CNS, but these findings do not show a direct role of either *Rb* or *E2f3a* in these tissues.

We next analyzed proliferation and apoptosis in the PNS. While proliferation of neurons in the dorsal root ganglia was significantly reduced in *Rb*<sup>-/-</sup>; *E2f3a*<sup>-/-</sup> embryos relative to *Rb*<sup>-/-</sup> embryos, apoptosis was not affected (Fig. 6B, D, and F). In contrast, loss of *E2f3b* did not suppress either the ectopic proliferation or apoptosis in these *Rb*<sup>-/-</sup> neurons of dorsal root ganglia. Together, these results demonstrate that *E2f3a* is the main isoform responsible for mediating the tissue-specific ectopic proliferation and apoptosis observed in *Rb*-deficient embryos.

**Limited role of individual *E2f3* isoforms in *Rb*<sup>-/-</sup> lens fiber cells and erythrocytes.** We also analyzed lens development and erythropoiesis, two additional processes severely affected by the loss of *Rb*. By E10.5 the developing eye has already formed into a semihollow sphere of cells that have anterior and posterior orientation, with posterior cells lining the interior of the eye. Proliferative epithelial cells at the anterior side continue to divide laterally until they reach the lens equator, at which point they exit the cell cycle and begin to migrate toward the inside of the lens. E13.5 postmitotic lens cells then express copious amounts of crystallin proteins and elongate vertically to form lens fiber cells that eventually fill the cavity of the lens vesicle (15). *Rb* is an important negative regulator of proliferation that is necessary for cell cycle exit at the lens equator (10, 23). The inactivation of *Rb* not only leads to ectopic proliferation of lens fiber cells within the cavity of the lens vesicle but

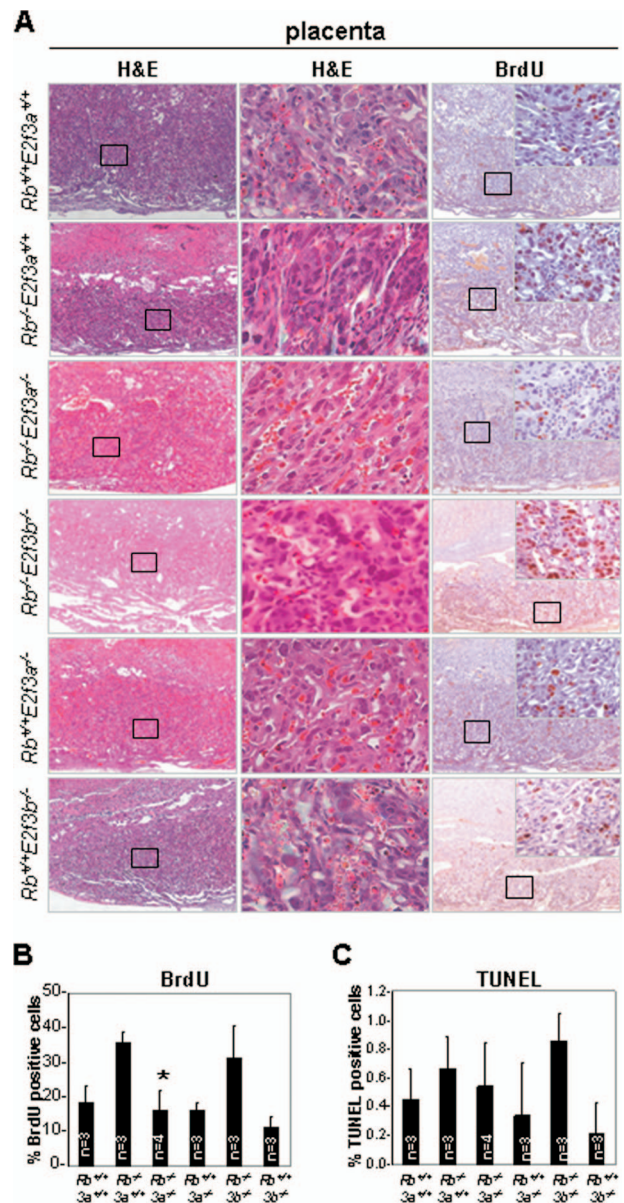


FIG. 5. Loss of *E2f3a*, but not *E2f3b*, rescues placental defects in *Rb*<sup>-/-</sup> embryos. (A) Placenta sections (5  $\mu$ m) from E13.5 *Rb*<sup>+/+</sup>; *E2f3a*<sup>+/+</sup>, *Rb*<sup>-/-</sup>; *E2f3a*<sup>+/+</sup>, *Rb*<sup>-/-</sup>; *E2f3a*<sup>-/-</sup>, *Rb*<sup>-/-</sup>; *E2f3b*<sup>-/-</sup>, *Rb*<sup>+/+</sup>; *E2f3a*<sup>-/-</sup>, and *Rb*<sup>+/+</sup>; *E2f3b*<sup>-/-</sup> embryos were stained with H&E, processed for immunohistochemical analysis using anti-BrdU antibodies, or processed for TUNEL analysis (not shown). (B) Quantification of BrdU-positive trophoblast cells from embryos with the indicated genotypes. 3a, *E2f3a*; 3b, *E2f3b*. \*, *t* test, *P* = 0.0076. (C) Quantification of TUNEL-positive trophoblast cells in embryos with the indicated genotypes. At least two different sections were evaluated per placenta.

also results in apoptosis and failure of nuclei to properly migrate to the center of the vesicle, leading to a disorganized lens cavity filled with nuclei (Fig. 7A to C). In contrast to the loss of both E2F3 isoforms, which was previously shown to restore normal proliferation and lens architecture in *Rb*<sup>-/-</sup> embryos, the individual inactivation of *E2f3a* or *E2f3b* did not significantly suppress ectopic proliferation and apoptosis, nor did it restore lens architecture (Fig. 7A to C). From these results we

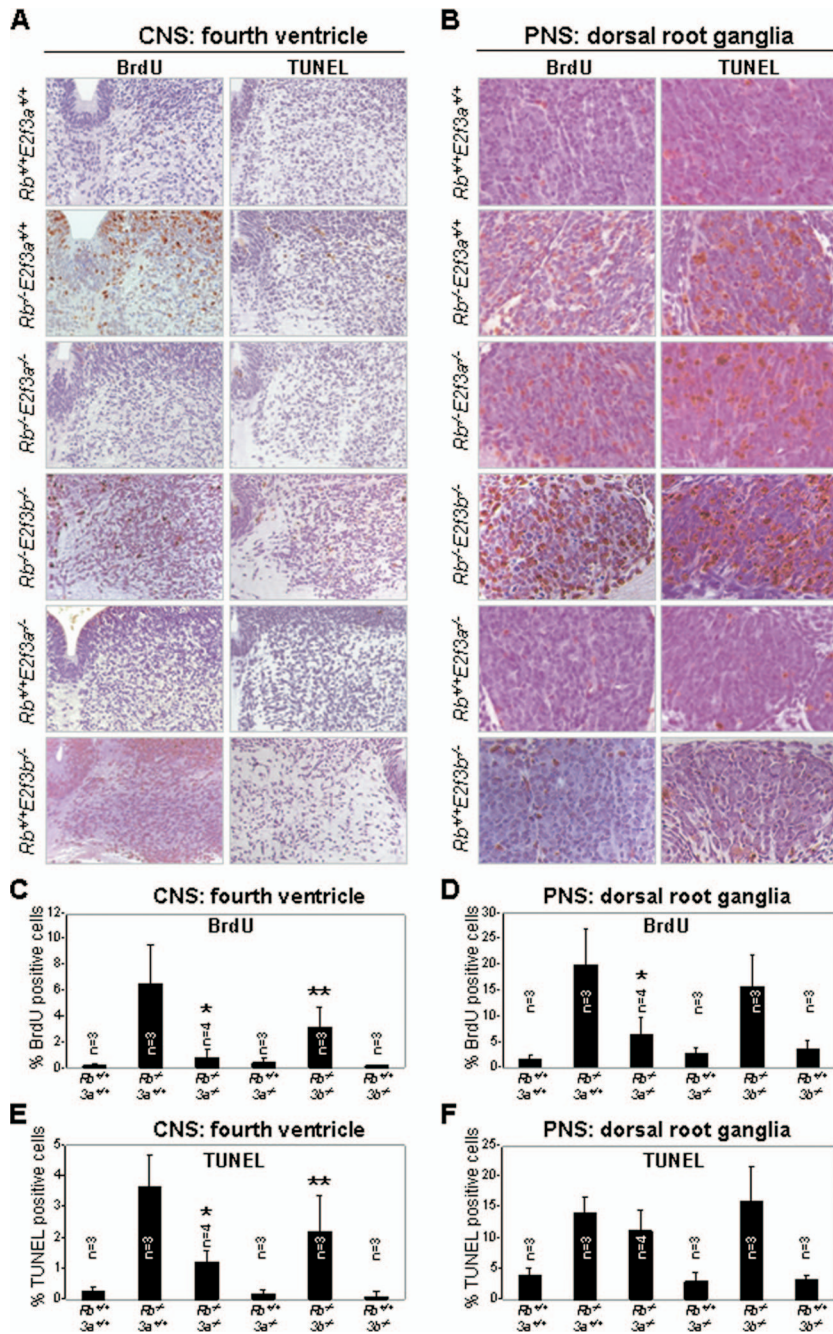


FIG. 6. Analysis of proliferation and apoptosis in the CNS and PNS of *Rb<sup>+/+</sup>; E2f3a<sup>+/+</sup>*, *Rb<sup>-/-</sup>; E2f3a<sup>+/+</sup>*, *Rb<sup>-/-</sup>; E2f3a<sup>-/-</sup>*, *Rb<sup>-/-</sup>; E2f3b<sup>-/-</sup>*, *Rb<sup>+/+</sup>; E2f3a<sup>-/-</sup>*, and *Rb<sup>+/+</sup>; E2f3b<sup>-/-</sup>* embryos. (A) BrdU and TUNEL staining in the fourth ventricle of the hindbrain of the CNS derived from E13.5 embryos with the indicated genotypes. (B) BrdU staining and TUNEL assays in dorsal root ganglia of the PNS in embryos with the indicated genotypes. (C) Loss of *E2f3a* suppresses proliferation in the CNS of *Rb<sup>-/-</sup>* embryos. Percentage of BrdU-positive cells in hindbrain sections of embryos with the indicated genotypes. *3a*, *E2f3a*; *3b*, *E2f3b*. \*, *t* test, *P* = 0.03; \*\*, *t* test, *P* = 0.16. (D) Loss of *E2f3a* suppresses proliferation in the PNS of *Rb<sup>-/-</sup>* embryos. Percentage of BrdU-positive cells in dorsal root ganglion sections of embryos with the indicated genotypes; \*, *t* test, *P* = 0.03. (E) Loss of *E2f3a* suppresses apoptosis in the CNS of *Rb<sup>-/-</sup>* embryos. Quantification of TUNEL-positive cells in the fourth ventricle of the hindbrains of embryos with the indicated genotypes; \*, *t* test, *P* = 0.0017; \*\*, *t* test, *P* = 0.18. (F) Loss of *E2f3a* does not suppress apoptosis in the PNS of *Rb<sup>-/-</sup>* embryos. Quantification of TUNEL-positive cells in dorsal root ganglia of embryos with the indicated genotypes. At least two different sections were evaluated per embryo.

conclude that *E2f3a* and *E2f3b* both contribute to the disruption of lens development in *Rb<sup>-/-</sup>* embryos.

Impaired red blood cell differentiation leading to anemia is another hallmark phenotype of *Rb*-knockout mice. This role of

*Rb* is mediated through both cell-autonomous and nonautonomous functions in liver-specific macrophages (12, 27, 28). Consistent with previous observations, there were few enucleated red blood cells in E13.5 *Rb<sup>-/-</sup>* embryos (Fig. 8A and B),



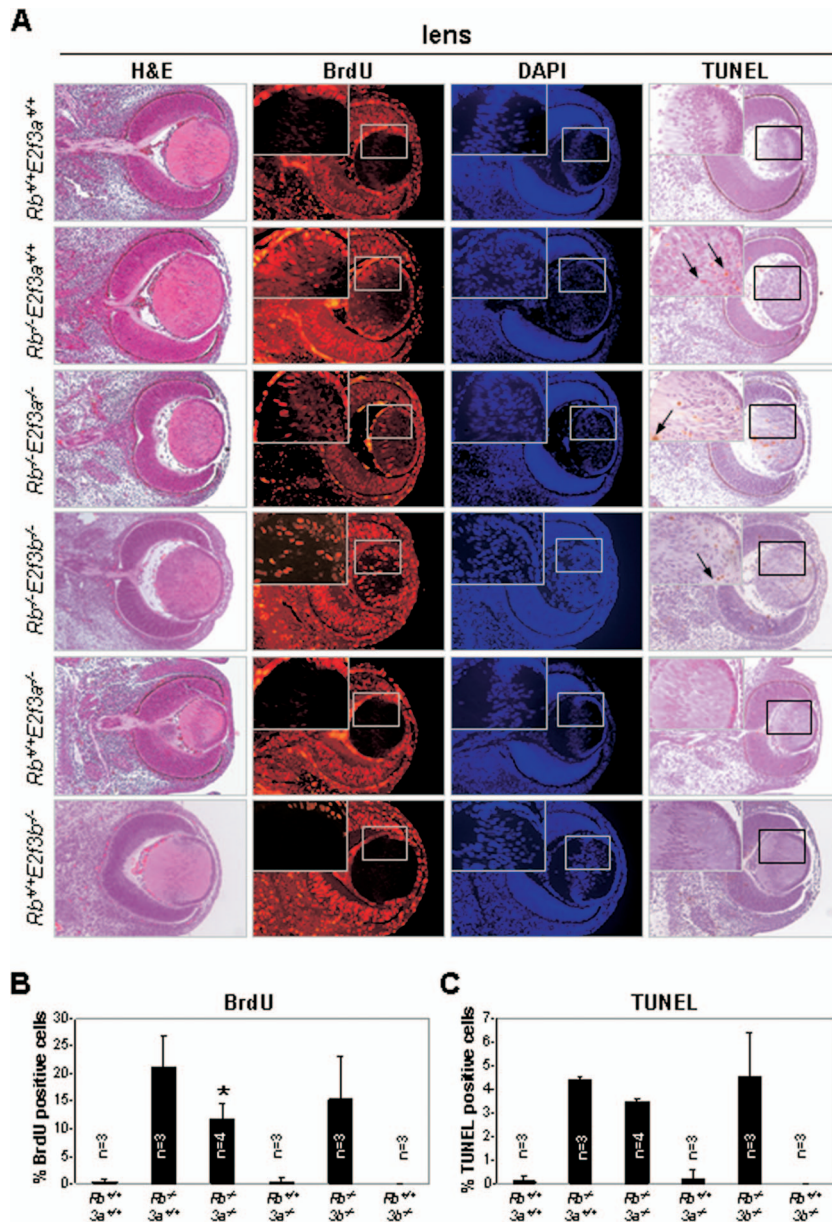


FIG. 7. Analysis of proliferation and apoptosis in lenses from *Rb<sup>+/+</sup>; E2f3a<sup>+/+</sup>*, *Rb<sup>-/-</sup>; E2f3a<sup>+/+</sup>*, *Rb<sup>-/-</sup>; E2f3a<sup>-/-</sup>*, *Rb<sup>-/-</sup>; E2f3b<sup>-/-</sup>*, *Rb<sup>+/+</sup>; E2f3a<sup>-/-</sup>*, and *Rb<sup>+/+</sup>; E2f3b<sup>-/-</sup>* embryos. (A) Sections of paraffin-embedded lenses from embryos with the indicated genotypes were used for H&E, anti-BrdU immunofluorescence, and DAPI staining as well as for TUNEL analysis. Arrows indicate apoptosis-positive cells. At least two different sections were evaluated per lens. (B) Quantification of BrdU-positive lens fiber cells from embryos with the indicated genotypes. *3a*, *E2f3a*; *3b*, *E2f3b*. \*, *t* test,  $P = 0.065$ . (C) Quantification of TUNEL-positive lens fiber cells in embryos with the indicated genotypes.

and this defect was not corrected by the loss of either *E2f3a* or *E2f3b* (Fig. 8A and B). Our observation that loss of *E2f3a* extended the life span of *Rb* mutant embryos without alleviating the anemia is consistent with the notion that E2F2 is the key downstream target of *Rb* in erythroblasts and that lethality of *Rb<sup>-/-</sup>* embryos is not linked to anemia (8, 34).

## DISCUSSION

The *E2f3* locus encodes two Rb-binding proteins, E2F3a and E2F3b, thought to be important for mouse development and cell proliferation. *E2f3a* expression is regulated during the cell

cycle with maximum expression in G<sub>1</sub>/S, while *E2f3b* expression is constitutive throughout the cell cycle. Previous studies analyzed the inactivation of both *E2f3* isoforms (11, 24, 33, 36), but the extent to which their individual functions contribute to mouse development and cell proliferation remained unclear. To investigate their functions, we targeted the specific inactivation of *E2f3a* and *E2f3b* in mice. In contrast to the ablation of both *E2f3a* and *E2f3b*, which resulted in embryonic lethality, inactivation of either isoform alone had no measurable effect on mouse embryonic development. On the other hand, loss of *E2f3a* or *E2f3b* significantly rescued selected phenotypes ob-

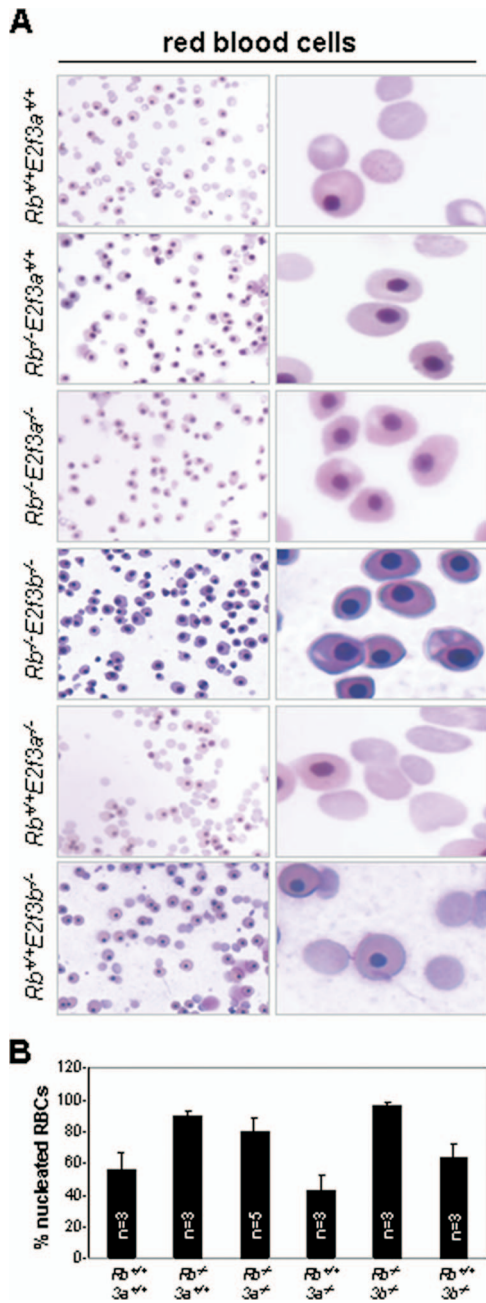


FIG. 8. Analysis of defective enucleation of peripheral red blood cells from *Rb<sup>+/+</sup>; E2f3a<sup>+/+</sup>, Rb<sup>-/-</sup>; E2f3a<sup>+/+</sup>, Rb<sup>-/-</sup>; E2f3a<sup>-/-</sup>, Rb<sup>-/-</sup>; E2f3b<sup>-/-</sup>, Rb<sup>+/+</sup>; E2f3a<sup>-/-</sup>, and Rb<sup>+/+</sup>; E2f3b<sup>-/-</sup>* embryos. (A) Peripheral blood smears from E13.5 embryos with the indicated genotypes were analyzed by Giemsa staining. (B) Percentage of enucleated red blood cells (RBCs) in embryos with the indicated genotypes. 3a, *E2f3a*; 3b, *E2f3b*.

served in *Rb* mutant embryos. The data would suggest that E2F3a and E2F3b proteins contribute to the control of proliferation in *Rb* mutant embryos in a tissue-specific manner, with E2F3a playing a major role in the placenta and nervous system and with E2F3a and E2F3b having critical roles in the lens. The suppression of placental phenotypes in *Rb-E2f3a* compound-knockout embryos likely accounts, at least in part, for

the rescue of fetal phenotypes. This notion is consistent with previous findings showing that *Rb* mutant fetuses supplied with a wild-type placenta could be carried to term (34).

Currently, it is thought that E2F3a acts as a transcription activator. Given that E2F3b complexes specifically with Rb in quiescent cells, it has been assumed that E2F3b functions as a repressor (17). Consistent with functional redundancy among repressors, ChIP experiments revealed that in quiescent cells, an increase in E2F4 recruitment to target promoters may compensate for loss of E2F3b. However, it is difficult to envision how E2F3b would contribute, in collaboration with E2F3a, to the proliferation of *Rb<sup>-/-</sup>* lens fiber cells by repressing gene expression. Given that loss of both *E2f3a* and *E2f3b*, but not either alone, can suppress proliferation in these cells, it is more likely that E2F3b, like E2F3a, contributes by activating gene expression in this cellular context. In this view, either E2F3a or E2F3b activity would be sufficient to promote unscheduled proliferation in *Rb* mutant lenses. Several additional lines of in vivo evidence now suggest that E2F3a and E2F3b proteins can function in the same capacity in other cell types. Analysis of MEFs deficient in various E2F family members showed that either E2F3a or E2F3b can rescue the proliferation defect of cells lacking all known activators (*E2f1*, *E2f2*, and *E2f3a/b*) (33). Moreover, recent analysis of knock-in mice showed that expression of *E2f3b* from the *E2f3a* locus (*E2f3a<sup>3bki</sup>*) suppressed all the postnatal phenotypes associated with the inactivation of *E2f1* and *E2f3a* (26). Finally, results from this study show that expression of either E2F3 isoform (*E2f1<sup>-/-</sup> E2f2<sup>-/-</sup> E2f3a<sup>-/-</sup>* and *E2f1<sup>-/-</sup> E2f2<sup>-/-</sup> E2f3b<sup>-/-</sup>*) is sufficient to support cell proliferation. We therefore suggest that E2F3b is important for normal cell cycle progression and, like E2F3a, functions as a transcription activator of E2F-responsive genes.

Why then is E2F3b protein loaded onto target promoters in quiescent cells? The association of E2F3b with target promoters during G<sub>0</sub> could be viewed as a mechanism for cells to quickly respond to growth-stimulatory signals by having E2F3b loaded and poised to activate gene expression upon mitogenic induction. In G<sub>0</sub> cells, E2F3b exists as a complex with Rb and hence is blocked from activating gene expression. Phosphorylation of Rb as cells are stimulated to enter the cell cycle would release Rb from E2F3b and allow it to activate transcription. The accumulation of E2F3a protein later in G<sub>1</sub> would then ensure that the amount of total E2F3 activity, composed of E2F3a and E2F3b proteins, is available to maximally activate gene expression at the G<sub>1</sub>/S transition.

We also find that *E2f3a* and *E2f3b* have redundant and nonredundant roles in the context of *Rb* mutation, with *E2f3a* playing a more dominant role in most *Rb<sup>-/-</sup>* tissues affected. It is notable that loss of *E2f3a*, *E2f3b*, or both did not decrease cell proliferation in *Rb* mutant tissues below levels normally found in wild-type tissues. In other words, basal levels of proliferation were not affected in *Rb-E2f3a* or *Rb-E2f3b* compound-knockout embryos. Because the inactivation of *E2f3a* or *E2f3b* in an otherwise normal embryo failed to affect basal levels of proliferation and apoptosis, we conclude that the in vivo roles for these two isoforms are manifested mainly in the context of inactivation of *Rb*. The contribution of E2F3a and E2F3b to *Rb* mutant phenotypes in developing embryos may thus represent a special circumstance that could be relevant to the cancer phenotype. We suggest that E2F3a, and to a lesser

extent E2F3b, is a critical component of the Rb tumor suppressor axis.

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