Specificity of E2F1, E2F2, and E2F3 in Mediating Phenotypes Induced by Loss of Rb

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Abstract
The Rb/E2F pathway plays a critical role in the control of cellular proliferation. Here, we report that E2F1, E2F2, and E2F3 make major individual contributions toward the in vivo phenotypic consequences of Rb deficiency. In the developing lens of Rb1/−/− embryos, loss of E2F1, E2F2, or E2F3 reduces the unscheduled proliferation of fiber cells, with the loss of E2F3 having the most pronounced effect. In Rb-deficient retinas, all three E2Fs contribute equally to the ectopic proliferation of postmitotic neuronal cells. In contrast, E2F1 is unique in mediating apoptosis in both Rb1/−/− lenses and retinas. In the central nervous system, loss of E2F1 or E2F3 can almost completely eliminate the ectopic DNA replication and apoptosis observed in Rb1/−/− embryos, and loss of E2F2 partially reduces the unscheduled DNA replication and has no effect on apoptosis. These results provide clear evidence for functional specificity among E2Fs in the control of Rb-dependent proliferation and apoptosis in a tissue-specific manner.

Introduction
A large body of work suggests that the Rb tumor suppressor controls G1/S transitions of the cell cycle, at least in part, by regulating the activities of the E2F family of transcription factors. On mitogenic induction, cells enter G1, and Rb is inactivated by CDK3-mediated phosphorylation. This leads to the release and accumulation of E2F activities and the concomitant activation of expression of numerous gene products whose functions are intimately linked with S phase entry and cell cycle progression (reviewed in Refs. 1 and 2). Disruption of various components of the pathway controlling E2F accumulation, including Rb, can lead to loss of cell growth control and the development of various human cancers. It is striking that this pathway has been disrupted in essentially all human tumors examined to date.

Of the seven known E2F family members, E2F1, E2F2, and E2F3a protein levels oscillate during the cell cycle. The expression of this group of E2Fs peaks late in G1, and coincides with the activation of G1-S-specific genes (3). Consistent with an important role for these proteins in G1-S progression, their ectopic expression in quiescent cells leads to the activation of E2F target genes and drives cells to enter S phase (4, 5). On the basis of these properties, E2F1, E2F2, and E2F3a are thought to function as transcriptional activators. In contrast, the E2F3b, E2F4, and E2F5 genes are expressed throughout the cell cycle, and their protein products can be found in association with Rb and Rb-related proteins in growth-arrested G0 cells (6–8). These observations, together with the fact that MEFs deficient for E2F4 and E2F5 are insensitive to a p16INK4A-mediated cell cycle arrest (9), suggest that this group of E2F factors likely functions as transcriptional repressors important for driving cells into, or keeping them in, a G0-quiescent state.

Roles played by the various E2F family members in development have been studied by mouse knockout models. These models have unraveled important functions for the different E2Fs in the differentiation of multiple tissues and in full viability of the organism but have failed to demonstrate an in vivo role for E2Fs in the control of cell proliferation. E2F1−/− mice are viable but prone to various tumors. These mice show a defect in T-cell differentiation that is apparently because of a reduced capacity of their thymocytes to be appropriately activated to undergo programmed cell death (apoptosis; 10–12). E2F2−/− mice remain largely uncharacterized but are fully viable and do not show obvious developmental phenotypes (this study). E2F3−/− mice have reduced viability presumably because of a defect in proliferation in certain cell types, such as fibroblasts (13). E2F4−/− mice are viable but show defects in differentiation of erythrocytes and the gut epithelium (14, 15). The development of E2F5−/− embryos appears normal, but newborn mice de-
velop hydrocephalus because of a choroid plexus defect that results in excessive production of cerebrospinal fluid (16).

The functional relationship between Rb and E2Fs has also been studied in vivo. A major consequence of disrupting Rb in mice is unscheduled DNA replication and extensive apoptosis in the lens and CNS (17, 18). These mice die in mid-gestation because of defects in erythropoietic differentiation and the resulting anemia (17–19). Binding and inactivation of Rb by the DNA tumor virus oncogenes large T-antigen and E7 also lead to unscheduled proliferation and apoptosis of lens fiber cells (20, 21). The fact that Rb/H11002/E2F1 mice exhibit significantly less apoptosis than Rb/H11002 mice (22, 23) demonstrates that E2F1, at least in part, mediates the apoptosis resulting from loss of Rb. Consistent with these findings, overexpression of E2F1 in rat fibroblasts leads to the induction of apoptosis (24, 25).

Here, we report the targeted disruption of E2F3 in mice, which leads to abortion of both the E2F3a and E2F3b proteins. Analysis of E2F1/H11002/E2F2/H11002/E2F3 mice suggests that E2F1, E2F2, and E2F3 have largely redundant functions during early embryonic development but make major individual contributions toward the in vivo phenotypic consequences of Rb deficiency.

Results
Loss of E2F3 Results in Gestational Mortality. The E2F3 gene locus uses two separate promoters to regulate the expression of two distinct proteins, E2F3a and E2F3b, which differ only at the NH2 terminus (Ref. 7). To study the role for E2F3a and E2F3b in the control of cellular proliferation in vivo, we used Cre-loxP gene-targeting techniques to generate E2F3-deficient mice (Fig. 1B; “Materials and Methods”). Deletion of exon 3, which encodes the DNA binding domain common to both E2F3a and E2F3b, from targeted ES cells was verified at each step by Southern blotting and PCR analysis using the three primers labeled as A–C. LoxP sites are depicted as A–C. Southern blot analysis of DNA from embryos with the indicated genotypes. Genomic DNA was digested with EcoRV and hybridized with the Southern probe shown in A. D, PCR genotyping of offspring derived from heterozygous crosses. The 123-bp DNA marker is depicted as M with the fastest migrating band being 123 bp and the slowest migrating band being 369 bp. E, Western blot analysis of protein lysates derived from MEFs, using an E2F3–specific antibody from Santa Cruz Biotechnology (SC-879). F, E13.5 embryos with the indicated genotypes. G, H&E-stained sagittal section from E13.5 embryos with the indicated genotypes.
incidence of gestational mortality (Table 1). The few surviving $E2F3^{-/-}$ mice were small, and most died within 3 weeks of birth, consistent with a recent report describing the independent generation of $E2F3$ knockout mice (13). Analysis of $E13.5$, $E15.5$, or $E17.5$ embryos revealed that most $E2F3^{-/-}$ animals died between $E15.5$ and $E17.5$ (Table 1). Visual or histological examination of $E13.5$ $E2F1^{-/-}$, $E2F2^{-/-}$, and $E2F3^{-/-}$ embryos revealed no gross differences with their wt counterparts (Fig. 1, F and G and data not shown), although all three genes are known to be expressed at this embryonic stage of development (27).

**Loss of $E2F1$, $E2F2$, or $E2F3$ Does Not Affect Proliferation in the Developing Lens.** To directly determine the in vivo role of $E2F3$ and the possible contributions made by the other two members of the $E2F$-activating class, $E2F1$ and $E2F2$, toward the control of cell proliferation in vivo, we analyzed the developing lens of $E2F$ mutant embryos. We focused on the developing lens for several reasons: (a) $E2F1$, $E2F2$, and $E2F3$ are expressed in the lens and have been speculated to play a role in normal proliferation of lens epithelial cells; although $E2F1$, $E2F2$, $E2F3$, $E2F4$, and $E2F5$ are all expressed in epithelial lens cells, only $E2F1$, $E2F3$, and $E2F5$ are expressed in lens fiber cells (28); (b) each of these $E2Fs$ can specifically interact with Rb, and inactivation of Rb in mice leads to deregulated $E2F$ function and lens cell proliferation (20, 21, 29); and (c) the architectural organization of the lens, where spatially regulated proliferation and differentiation events are dependent on Rb, provides a unique in vivo system to study the cell cycle. The lens consists of a proliferative layer of epithelial cells that divide laterally until they reach the lens equator (Fig. 2A). At this point, epithelial cells exit the cell cycle and migrate to the internal side of the lens, where they become postmitotic and elongate vertically to form lens fiber cells that eventually fill the cavity of the lens vesicle (reviewed in Ref. 30).

Surprisingly, loss of any one of the three $E2Fs$ had no significant effect on the proliferation of lens epithelial cells ($P ≥ 0.1$ for all pairwise comparisons; Fig. 2, B and C). We also analyzed discrete regions of the lens epithelium to determine whether $E2Fs$ may be particularly important within confined regions of the lens epithelium. Thus, we performed the analysis described by McAvoy et al. (31, 32) in which proliferating cells contained within 10 degree increments, beginning at the equatorial region of the lens (0 degrees) and continuing toward the area facing the cornea (90 degrees), were counted separately. This analysis revealed no differences in proliferation between lens epithelial cells of $E2F3^{-/-}$ embryos and their wt siblings (Fig. 2D). In addition, $E2F$-deficient fiber cells exited the cell cycle and differentiated normally since no unscheduled DNA replication could be detected in these cells ($P ≥ 0.1$ for all pairwise comparisons; Fig. 2, B and C), and the differentiation-specific markers, $\beta$- and $\gamma$-crystallins, were expressed appropriately (data not shown). One interpretation of these data are that under normal circumstances, loss of a single $E2F$ member can be functionally compensated by the other related $E2F$ activities.

**$E2F3$ Makes a Major Contribution toward Proliferation in $Rb^{-/-}$ Embryos.** One major consequence of disrupting the Rb pathway in mice is unscheduled DNA replication in the lens and CNS (20–23, 29, 33). During normal differentiation of the lens, the Rb pathway is necessary to maintain the $G_0$ state of the differentiated lens fiber cells. Targeted disruption of components of the Rb regulatory pathway either by the overexpression of cyclins (34), inactivation of p57, p27 or Rb itself (29, 33), or inactivation of Rb by the viral onco-genes E7 and T-antigen (20–22) results in failure of lens epithelial and fiber cells to exit the cell cycle. Rb is thought to be required in maintaining a $G_0$ state by controlling the activities of $E2Fs$. On Rb loss, many putative $E2F$-responsive genes, such as cyclins A, B1, and E and the CDKs cdc2, cdk2, and cdk4, are abnormally up-regulated in lens fiber cells, resulting in increased proliferation (35). Considering that $E2F1$, $E2F2$, and $E2F3$ are the three growth-regulated activities capable of interacting specifically with Rb, and not with the other pocket-binding proteins p107 and p130, we sought to determine the relative contributions made by these $E2Fs$ toward the phenotypic consequences of Rb loss. Consistent with previous results, we found that a significant proportion of lens fiber cells in $E13.5$ $Rb^{-/-}$ embryos was unable to exit the cell cycle, as measured by BrdUrd incorporation (Fig. 3A). Analysis of embryos deficient for Rb and each of the three activating $E2Fs$ revealed a partial suppression of the unscheduled proliferation ($P < 0.001$ for each pairwise comparison). The fact that loss of $E2F3$ had the

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<th>Table 1</th>
<th>Loss of $E2F3$ leads to gestational lethality*</th>
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<tr>
<td>A.</td>
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<tr>
<td>Number of $E15.5$ embryos</td>
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<td>1</td>
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<tr>
<td>Number of $E17.5$ embryos</td>
<td>13</td>
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<tr>
<td>Observed ratio</td>
<td>1</td>
</tr>
<tr>
<td>Number of pups at weaning</td>
<td>68</td>
</tr>
<tr>
<td>Observed ratio</td>
<td>1</td>
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<tr>
<td>B.</td>
<td>$E2F2^{+/-}$</td>
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<tr>
<td>Number of pups at weaning</td>
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<td>Observed ratio</td>
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* The expected ratio for each data set is 1 (wt):2 (heterozygote):1 (homozygous mutant).
most pronounced impact suggests that E2F3 makes the major contribution toward the unscheduled proliferation of Rb<sup>-/-</sup> lenses (Fig. 3C). Moreover, observation of DAPI-stained lenses from Rb<sup>-/-</sup>E2F3<sup>-/-</sup> embryos by microscopy revealed a cellular arrangement that more closely resembles the typical "bow arrangement" found in properly differentiated lenses of wt embryos (Fig. 3).

Analysis of the proliferation zone of the epithelial cells revealed no differences in the number of proliferating cells between wt, Rb<sup>-/-</sup>, Rb<sup>-/-</sup>E2F1<sup>-/-</sup>, Rb<sup>-/-</sup>E2F2<sup>-/-</sup>, and Rb<sup>-/-</sup>E2F3<sup>-/-</sup> embryos (Fig. 3B and data not shown). However, analysis of the epithelial cells of the lens by the method of McAvoy et al. (31, 32) revealed a small increase in the number of proliferating cells in the transition zone between the lens epithelial and fiber cells of Rb<sup>-/-</sup> lenses relative to wt litter mates. In addition, the number of proliferating cells in the transition zone of Rb<sup>-/-</sup>E2F3<sup>-/-</sup> lenses was slightly lower than the ones in Rb<sup>-/-</sup> embryos, albeit not statistically significant (Fig. 3B). The number of replicating cells in the transition zone of Rb<sup>-/-</sup>E2F1<sup>-/-</sup> and Rb<sup>-/-</sup>E2F2<sup>-/-</sup> lenses was identical to the ones in Rb<sup>-/-</sup> embryos (data not shown). This is consistent with the results described in McCaffrey et al. (22), where the expression of an E7 transgene in the lens of wt or E2F1<sup>-/-</sup> mice resulted in an increase in the proliferation of epithelial cells in the transition zone.

Although the analysis of wt, Rb<sup>-/-</sup>, and Rb<sup>-/-</sup>E2F3<sup>-/-</sup> embryos revealed no obvious changes in the total percent-age of replicating cells of the retina (Fig. 3D), we did notice that Rb<sup>-/-</sup> embryos had increased numbers of BrdUrd-incorporating cells in an area of the retina that normally contains postmitotic neurons (Fig. 3E). Loss of E2F1, E2F2, and E2F3 from Rb<sup>-/-</sup> retinas resulted in a reduction in the number of neuronal retina cells undergoing unscheduled DNA replication.

In the CNS, as in the lens and retina, maintenance of a G<sub>0</sub> state in differentiated neurons is also dependent on Rb. Loss of Rb function in the CNS has been shown to result in elevated levels of free E2Fs and E2F target genes and unscheduled DNA replication (36). The patterns of expression of E2Fs in the CNS is complex and has been thoroughly described previously (27). Although E2F1, E2F2, and E2F3 expression is distributed widely over the brain and spinal cord during early embryonic development, by E12.5, their expression is down-regulated in postmitotic neurons of the intermediate and marginal zone but not in the ventricular zone of the brain. E2F3 is present at low levels in the ventricular zone and at higher levels in the differentiated neurons of the intermediate and marginal zones. To determine whether the activating E2Fs can mediate the unscheduled proliferation observed in Rb<sup>-/-</sup> brain tissues, we analyzed postmitotic neurons in the intermediate zone adjacent to the fourth ventricle of the brain of doubly null Rb/E2F embryos for their ability to replicate DNA. As reported previously (36), loss of Rb resulted in increased
proliferation of the normally postmitotic neurons (Fig. 4A). As in the lens fiber cells, there was a significant reduction in the number of proliferating neurons in \( \text{Rb}^{-/-} \), \( \text{E2F1}^{-/-} \), \( \text{Rb}^{-/-}\text{E2F2}^{-/-} \), and \( \text{Rb}^{-/-}\text{E2F3}^{-/-} \) embryos (Fig. 4B). Although all three activating E2Fs contributed to the unscheduled proliferation induced by loss of Rb, E2F1 and E2F3 made the most pronounced contribution to this phenotype; the percentage of BrdUrd-positive lens fiber cells from E13.5 embryos with the indicated genotypes. D, percentage of BrdUrd-positive cells in the epithelial retina of the indicated E13.5 embryos. E, percentage of BrdUrd-positive cells in the neuronal retinal cells of E13.5 embryos. For all of the bar graphs in this figure, the presented results are the average obtained from multiple sections derived from at least two embryos. Bars, SD.

**E2F1 and E2F3 Mediate Apoptosis in Rb\(^{-/-}\) Embryos in a Tissue-specific Manner.** Loss of Rb also leads to extensive apoptosis in cells of the CNS, the developing lens, and in photoreceptor cells of the retina (17, 18, 21, 23, 29, 37, 38). This is mediated, in part, by E2F1 as suggested by the significant suppression of apoptosis in \( \text{Rb}^{-/-}\text{E2F1}^{-/-} \) mice (23). Given that E2F1, E2F2, and E2F3 are expressed in the CNS, the developing lens, and the retina, we sought to determine whether apoptosis arising in \( \text{Rb}^{-/-} \) embryos is uniquely mediated via E2F1 or whether it could also be mediated by E2F2 and E2F3 (27, 28). Therefore, we compared the extent of apoptosis in the lens, retina, and CNS of \( \text{Rb}^{-/-} \), \( \text{Rb}^{-/-}\text{E2F1}^{-/-} \), \( \text{Rb}^{-/-}\text{E2F2}^{-/-} \), and \( \text{Rb}^{-/-}\text{E2F3}^{-/-} \) embryos. As expected, loss of Rb led to an increase in apoptosis observed in the CNS, the lens, and the retina (\( P = 0.0006; \) Figs. 5 and 6). Loss of E2F1 suppressed the apo-
E2Fs Mediate Rb Function in Vivo

Discussion

The Rb pathway is disrupted frequently during the progression of cancer (reviewed in Ref. 39). The tumor suppressor function of Rb is thought to be mediated, at least in part, through the regulation of the E2F transcription activators. Mice deficient for each of the three transcriptional activators (E2F1, E2F2, and E2F3) have been generated (reported here and in Refs. 10, 11, 13, and 26). We report that E2F1, E2F2, and E2F3 make major contributions toward the phenotypic consequences of Rb loss. Importantly, our findings begin to document in vivo evidence for functional specificity among these E2Fs.

Of the seven known E2F family members, the expression of E2F1, E2F2, and E2F3 is cell cycle regulated, with their levels peaking at the G1-S transition. The oscillating nature of their DNA binding activities during the cell cycle exactly coincides with the expression of known E2F target genes whose protein products are thought to be essential for entry into S phase. Moreover, the ectopic expression of these E2Fs can efficiently induce S phase entry in otherwise quiescent fibroblasts. A series of recent experiments have suggested that there is specificity in promoter occupancy by different E2Fs (40) and in the target genes that are transcriptionally regulated by E2F1, E2F2, and E2F3 (41, 42). In view of these findings suggesting a critical role for E2Fs in cell growth control, it is surprising that E2F1−/− and E2F2−/− MEFs have no measurable growth defect and that E2F3−/− MEFs have only a mild defect (13, 26). Additionally, the disruption of E2F1, E2F2, or E2F3 in mice does not significantly affect cellular proliferation in the developing lens, retina, or CNS, tissues known to undergo unregulated proliferation on disruption of Rb. Neither do their loss significantly alter the localization or levels of the late stage lens differentiation markers, γ- or β-crystallins (data not shown). This is consistent with the lack of an overall change in the appearance and cellularity of E13.5 E2F mutant embryos (Figs. 1 and 2 and data not shown). One interpretation of these data are that under normal circumstances, loss of a single E2F member can be functionally compensated by other related E2F activities. This notion of functional redundancy among E2Fs is substantiated by our recent findings, indicating that E2F1−/−E2F3−/− and E2F2−/−E2F3−/− embryos are early embryonic lethal and that MEFs lacking all three E2Fs (conditionally deleted via Cre-mediated recombination) have a severe growth defect (43). It will be important to determine

Apoptosis resulting from Rb deficiency (P ≤ 0.0001). In contrast to Rb−/−E2F1−/− embryos, lens fiber and postmitotic retina cells from Rb−/−E2F2−/− and Rb−/−E2F3−/− embryos contained similar or higher numbers of TUNEL-positive cells than in the Rb−/− cellular counterparts (P = 0.11 and 0.01, respectively), suggesting that apoptosis in the lens and the retina is mediated specifically through E2F1 (Fig. 5). The apparent increase in apoptosis in Rb/E2F3 double knockout lenses is interesting and might stem from an emphasis of the E2F1 apoptotic function in the absence of a competing E2F3 activity, albeit a greater number of samples will need to be analyzed to strengthen the statistical significance of this result. Strikingly, loss of either E2F1 or E2F3, but not E2F2, almost completely abolished the apoptosis observed in the CNS of E13.5 Rb−/− embryos (P < 0.001; Fig. 6). These results demonstrate that although E2F1 specifically mediates apoptosis in the lens and the retina, both E2F1 and E2F3 are required for the apoptosis arising in the CNS of Rb−/− embryos.

**Fig. 4.** E2F1, E2F2, and E2F3 mediate unscheduled proliferation in the CNS of Rb−/− embryos. In A, transverse sections of brains from E13.5 embryos with the indicated genotypes were stained by the BrdUrd incorporation assay. We analyzed sections from the posterior area of the brain, adjacent to the fourth ventricle, specifically the neurons from the inter- 

cellular zone. Bars, the ventricular area (v), where proliferation occurs normally, and the intermediate zone, composed of normally postmitotic cells. B, percentage of BrdUrd-positive cells in the CNS of embryos with the indicated genotypes. The presented results are the average obtained from multiple sections derived from at least three embryos. Bars, SD of multiple embryos.
whether ablation of multiple E2Fs from selected tissues, such as the lens, affects proliferation and differentiation in vivo.

In contrast to the lack of a strict requirement for individual E2F activities in the control of proliferation during normal embryonic development, we show that E2F1, E2F2, and E2F3 can make major contributions toward the phenotypic consequences of Rb deficiency (Figs. 3–6). Each of these E2F family members contributes toward the inappropriate proliferation of Rb−/− lens fiber cells and neurons of the CNS and the retina, with E2F3 making the most profound contribution to this phenotype in the lens (Figs. 3, A and C and 4, A and B). Whether E2F3 can mediate the unscheduled proliferation and/or apoptosis observed in Rb−/− embryos through E2F3a, E2F3b, or both remains to be determined. Perhaps most dramatic is the almost complete abolishment in the CNS, lens, and retina of Rb−/−E2F1−/− and in the CNS of Rb−/−E2F3−/− embryos (Figs. 5 and 6), suggesting important roles for E2F1 and E2F3 in the control of apoptosis. It appears that loss of the regulation imposed by Rb causes E2F function to become unchecked, leading to greatly exaggerated biological consequences, not unlike the unscheduled proliferation and/or apoptosis observed when these E2Fs are overexpressed in fibroblasts and in lens fiber cells (44). Our present results provide striking in vivo evidence that E2F1, E2F2, and E2F3 are important effectors of Rb function that must be kept under constant control for the maintenance of a postmitotic state.

Fig. 5. E2F1 mediates apoptosis in the lens of Rb−/− embryos. In A, transverse sections of lenses and retinas from E13.5 embryos with the indicated genotypes were stained by the TUNEL peroxidase assay and counterstained with DAPI. Arrows, selected apoptotic (TUNEL positive) cells. B, percentage of TUNEL-positive fiber cells. C, percentage of TUNEL-positive neuronal retinal cells. The presented results are the average obtained from multiple sections derived from at least two embryos. Bars, SD of multiple embryos.
E2Fs Mediate Rb Function in Vivo

Specificity of function for E2Fs can also be inferred from the present studies. Previous work demonstrated that E2F1 can in part mediate the apoptosis and proliferation induced by loss of Rb (22, 23). The work described here illustrates that there is specificity of function among E2Fs in mediating proliferation and apoptosis in vivo. Although the apoptosis arising in Rb<sup>−/−</sup> lenses and retinas is uniquely mediated by E2F1, all three E2Fs contribute to the control of proliferation in cells of these tissues (Fig. 3). These findings are consistent with the previous observation that overexpression of E2F1, but not E2F2–E2F5, can induce apoptosis in serum-starved rat fibroblasts and with recent findings linking E2F1, but not E2F2 or E2F3, in Myc-induced apoptosis of primary MEFs (4, 26). These results strongly suggest that, at least in the developing lens, apoptosis is not simply a consequence of the unscheduled proliferation resulting from the inactivation of Rb but likely reflects a specific signal mediated by E2F1. Our observations that E2F1 and E2F3, but not E2F2, are required to elicit an apoptotic signal in the CNS of Rb<sup>−/−</sup> embryos substantiates the concept of specificity among E2Fs. Clearly, E2F2, which has an identical spatial and temporal expression profile as E2F1 in the CNS and the retina (45), is not without an effect in these tissues, as its loss significantly reduces the amount of proliferation occurring in postmitotic Rb<sup>−/−</sup> CNS and retinal neurons.

The unique ability of E2F1 to elicit an apoptotic response in the developing lens and retina of Rb<sup>−/−</sup> embryos likely reflects its ability to regulate a specific set of target genes. Some evidence for specific gene target activation by E2Fs exists. The overexpression of the various E2Fs in rat embryo fibroblasts can lead to the activation distinct sets of genes (4). In these cells, E2F1, but not E2F2, can lead to the stabilization of p53 protein and apoptosis (46). In mouse embryo fibroblasts, the specific induction of the proapoptotic Apaf1 gene by E2F1 is required for the efficient execution of an apoptotic response (47), providing a further molecular basis for the unique function of E2F1 in apoptosis.

The accumulation of E2F1 is part of the normal cascade of events that occur during the stimulation of cellular proliferation. Several observations may help explain why cells induced to proliferate normally escape from E2F1-mediated cell death. In G<sub>0</sub> cells stimulated to reenter the cell cycle, E2F1 DNA binding activity accumulates sharply at the G<sub>1</sub>-S boundary, quickly decreases as cells progress through S phase, and remains low thereafter during cycling conditions (3). Thus, E2F1 activity in normal cells is tightly regulated and apparent only at specific points during the cell cycle. On the other hand, in the absence of Rb, E2F1 DNA binding activity accumulates to higher levels than normally found during G<sub>1</sub>-S and persists throughout the cell cycle. Such unresolved E2F activity might lead to the inappropriate persistent activation of target genes or to the activation of targets normally not activated during S phase and the ensuing catastrophic consequence of apoptosis. Furthermore, the accumulation of E2F1 during a mitogenic response normally occurs in conjunction with the parallel activation of survival signals, such as those mediated by the phosphatidylinositol 3'-kinase/Akt pathway, allowing cells to proliferate and escape an apoptotic fate. Indeed, the apoptosis resulting from the overexpression of E2F1 in quiescent fibroblasts can be largely abrogated by the addition of serum. Thus, E2F1-mediated apoptotic signals could be viewed to represent a checkpoint for proper cell cycle exit/entry that must be negated by either direct binding to Rb or countered by the activation of appropriate survival signals.

Why does the loss of E2F3 rescue the apoptosis occurring in the CNS but not in the lens of Rb<sup>−/−</sup> embryos? Previous work using chimeric mice reconstituted from Rb<sup>+/−</sup> and Rb<sup>−/−</sup> embryonic stem cells suggested that although Rb’s function in the developing lens appears to be cell autonomous, its function in the CNS may be cell nonautonomous (37, 48). More recent studies of the developing CNS have defined a role for Rb in the suppression of apoptosis that is cell nonautonomous and a role for suppression of ectopic S phase that is cell autonomous (48). These findings raise the distinct possibility that suppression of apoptosis in the CNS of Rb/E2F3 double mutant embryos is a cell nonautonomous consequence. Hence, the developing lens may be a uniquely suited tissue in which to investigate the roles for E2Fs in the regulation of apoptosis and cell proliferation without confounding cell extrinsic effects. The replacement of specific E2Fs by other family members using knock-in strategies in
vivo might provide more definitive answers to this important issue of functional specificity among E2F family members.

Alternatively, our observation that loss of E2F3 eliminates most of the apoptosis occurring in the CNS, but not in the lens and retina of Rb−/− embryos, might imply that the induction of E2F-mediated apoptosis can be influenced by tissue-specific factors. Considering the complexity of the organization of gene promoters, it is not difficult to envision how additional tissue-specific transcription factors or coactivators might impact, by interacting directly or indirectly with specific E2Fs, on the regulation of E2F target genes responsible for mediating apoptosis, e.g., a putative CNS-specific factor might, by specifically interacting with E2F3, shift its gene target spectrum to include apoptotic-type target genes.

Our finding that E2F1 is the only activating E2F that can mediate apoptosis in the lens and retina is in conflict with a recent report showing a dramatic reduction in apoptosis in the lenses of Rb−/−/E2F3−/− mice (49). Although the genetic background of our studies is similar (C57BL/6 × 129/Sv), these differences might be because of the degree of inbreeding between our strains. In this regard, genetic modifiers have been evoked to explain differences in the severity of growth characteristics of cells lacking E2F3 (13) and might explain the differences observed in the lens.

Finally, we believe that the results presented here directly impact on our understanding and possible development of therapeutic treatments of cancer. Although in normal cells, loss of a single E2F member will likely have no effect on the control of cellular proliferation, under oncogenic circumstances, where the Rb pathway is disrupted, loss of an individual E2F activity may have profound consequences on the fate of the malignant cell. We speculate that targeting the disruption of a single E2F member in human cancers may have either a positive or negative outcome, depending on the tissue origin of the tumor and the ability of the E2F to affect proliferation and/or apoptosis in that tissue.

Materials and Methods

Generation of E2F3−/− Mice. To ablate E2F3 function, we targeted exon 3, which encodes the DNA binding domain that is common and essential for both E2F3a and E2F3b function. A triple LoxP targeting vector system was used to generate ES cells containing the modified E2F3 allele shown in Fig. 1B. Details for the generation of the targeting vector, the targeted inactivation of E2F3 from ES cells by homologous recombination, and the generation of E2F3 chimeras are described elsewhere (26) and can be obtained on request. All embryos were genotyped by PCR. PCR for the E2F3 gene was carried out using three primers as described in Fig. 1B; primers B (TGAATCATGGGAGAGGGACAG) and C (GATTGATTTCGAGGTGGTTCAGG) are specific for the wt allele and give rise to a 190-bp product, and primers A (GTGGCTGGTGGTTGTCAGG) and C are specific for the knockout allele, giving rise to a 290-bp PCR fragment. The E2F1−/− mice have been described (10, 11). The E2F2−/− mice have been generated recently, and a detailed phenotypic analysis is in progress (26). Double knockout embryos were generated by either intercrossing Rb−/−/E2F3−/− or, in some cases, by intercrossing Rb−/−/E2F3−/− with Rb+/−/E2F3−/− parents.

BrdUrd Incorporation and TUNEL Assays. BrdUrd assays were performed as described previously (21). Pregnant mice (13.5–15.5 days postcoitum) were injected i.p. with BrdUrd (100 μg/grams body weight) 2 h before sacrificing, and individual embryos were fixed in formalin. We analyzed at least three embryos of each genotype that were derived from at least two different litters (i.e., two different mothers). Sections (5 μm) were deparaffinized, rehydrated, digested with pepsin, and denatured in HCl. BrdUrd incorporation was detected using an anti-BrdUrd antibody (DAKO Co.) and a secondary FITC-antimouse monoclonal antibody (Vector Laboratories). Nuclei were counterstained with DAPI.

The TUNEL/peroxidase assay was done according to the manufacturer’s instructions (Invitrogen), except that cells were treated with 0.1% Triton X-100 for 10 min before proteinase K digestion, and the terminal deoxynucleotidyltransferase reaction was performed for 1.5 h. Nuclei were counterstained with DAPI. The percentage of apoptotic or proliferating cells was calculated by counting the total number of BrdUrd- or TUNEL-positive cells relative to the number of DAPI-stained cells. Approximately 150 epithelial cells, 300 fiber cells, and 300 neuronal cells from the retina were counted from each lens section. Approximately 400 cells from the immediate zone adjacent to the fourth ventricle were analyzed per brain section. In each case, at least four sections per embryo were analyzed. The lens epithelial cells were also analyzed as described (31, 32). Briefly, the lens was divided into equal quarters, and two of the quarters were divided using a protractor into 10-degree sections that ranged from −20° to 90°. Cells were counted from the −20° area, which corresponds to 20° beyond the lens equator, facing the retina, to the 90° position, which corresponds to the middle of the epithelial cell layer facing the cornea (Fig. 2A). Statistical analysis was performed by the two-tailed, unequal variance paired t test using the Microsoft Excel program.

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References


5 S. Field, personal communication.


