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ARF Is Not Required for Apoptosis in *Rb* Mutant Mouse Embryos

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Summary

The retinoblastoma (*RB*) tumor suppressor gene occupies central roles in cell cycle control and tumor suppression [1]. Homozygous mutant (*Rb*$^{-/-}$) embryos die at E13.5–E15.5 [2–4], exhibiting extensive apoptosis and inappropriate S phase entry in the central and peripheral nervous systems, liver, and ocular lens [2–6]. Mice simultaneously mutant for *Rb* and other genes can be generated to assess the requirement for these genes in cell cycle control and apoptosis. Using such analysis, *E2f-1*, *E2f-3*, *p53*, and *Id2* have been identified as important regulators of cell cycle control and apoptosis in *Rb*$^{-/-}$ embryos [7–10]. Because unrestrained E2F activity in the absence of *Rb* function contributes to *p53*-dependent apoptosis in many systems [7, 9, 11–14], we wished to identify genes linking deregulated E2F activity to p53 activation and subsequent apoptosis. As a transcriptional target of E2F-1 [15–18], a regulator of p53 [19–21], and an important mediator of apoptosis [20–26], *ARF* was a strong candidate for such a role, especially since it can be upregulated in the absence of *Rb* [21]. From the analysis of *Rb/ARF* compound mutants we demonstrate that *ARF* is not an obligatory link between *Rb* inactivation and *p53*-dependent apoptosis.

Results and Discussion

ARF Is Not Required for Apoptosis or Inappropriate S Phase Entry in *Rb*-Deficient Lens or CNS

In normal wild-type E13.5 embryos, epithelial fiber cells of the ocular lens are postmitotic and exhibit negligible levels of apoptosis (Figures 1A, 1D, and 1G) [6]. Accordingly, levels of TUNEL and BrdU staining in wild-type and *ARF* mutant samples were comparable (data not shown). *Rb* mutant embryos exhibited intense incorporation of BrdU indicating inappropriate S phase entry (Figure 1H), and TUNEL staining revealed the presence of apoptotic cells (Figures 1B and 1E). While *E2f-1* and *p53* are required for apoptosis in *Rb* mutant lens [6, 7], *ARF* loss suppressed apoptosis by only 18% and had no effect on inappropriate S phase entry (Figures 1C, 1F, 1I, and 3). These results are broadly consistent with the report of Pomerantz et al. (1998) [30] using *Rb/Ink4a*^{ex2,3} double mutants, which concluded that *ARF* may partially contribute to *p53*-dependent apoptosis in the lens. Importantly, this *Ink4a* mutation inactivates both *ARF* and *p16Ink4a*, making it difficult to separate their individual contributions, in this earlier analysis. The mice used in our study contain an *ARF*-specific mutation that leaves *p16Ink4a* intact [27].

We also examined the requirement for *ARF* in apoptosis in the *Rb*-deficient CNS, a process also strongly *p53*- and *E2f-1*-dependent [7, 9]. Low levels of *ARF* can be detected by RT-PCR analysis in the wild-type CNS tissue at E13.5, but expression levels were not significantly elevated in *Rb*$^{-/-}$ samples (data not shown). Moreover, there was no functional requirement for *ARF* in apoptosis in the *Rb*$^{-/-}$ CNS as assessed by TUNEL-staining mid-sagittal sections through the hindbrain of E13.5 *Rb/ARF* double mutant embryos. Levels of apoptosis were indistinguishable from those observed in *Rb* mutant embryos (Figures 2B and 2C), and *ARF* mutant samples exhibited only background levels comparable to those in wild-type controls (Figures 2A and 3A and data not shown). As in the ocular lens, *ARF* loss had no effect on inappropriate S phase entry observed in *Rb*-deficient CNS (Figures 2E, 2F, and 3).

The *p53* Pathway Is Activated in *Rb/ARF* Double Mutant CNS

Because it was expected that *ARF* would be important for *p53*-dependent apoptosis in the CNS and lens, it was necessary to establish that the *p53* pathway was still activated and functional in the *Rb/ARF* double mutants. Western blot analysis confirmed that p21 expression is significantly upregulated in *Rb* mutant brain extracts relative to wild-type controls [9] (Figure 4A, lanes 1–3) in a *p53*-dependent manner (Figure 4A, lane 6). Importantly, this upregulation persisted in *Rb/ARF* double mutants (Figure 4A, lanes 4 and 5), while p21 levels in *ARF* mutants were comparable to those in wild-type controls. (Figure 4A, lanes 1 and 7). Furthermore, p53 DNA binding activity continues to be elevated in the *Rb/ARF* double mutants and is comparable to levels observed in *Rb* mutants (Figure 4B, compare lanes 7 and 8 to 11 and 12). This evidence demonstrates that the *p53* pathway is activated in *Rb/ARF* double mutant animals, indicating that *ARF* is dispensable for p53 activation in the context of *Rb* deficiency in vivo.

Apoptosis and Excessive S Phase Entry Are Exacerbated in *Rb*-Deficient PNS Ganglia by Inactivation of *ARF*

While apoptosis in *Rb*-deficient lens and CNS is *p53* dependent, *Rb* mutants exhibit phenotypes that are *p53* independent as well, such as the embryonic lethality of *Rb/p53* double mutant embryos and the extensive apoptosis and excessive S phase entry observed in trigeminal and dorsal root PNS ganglia [9]. Indeed, in this

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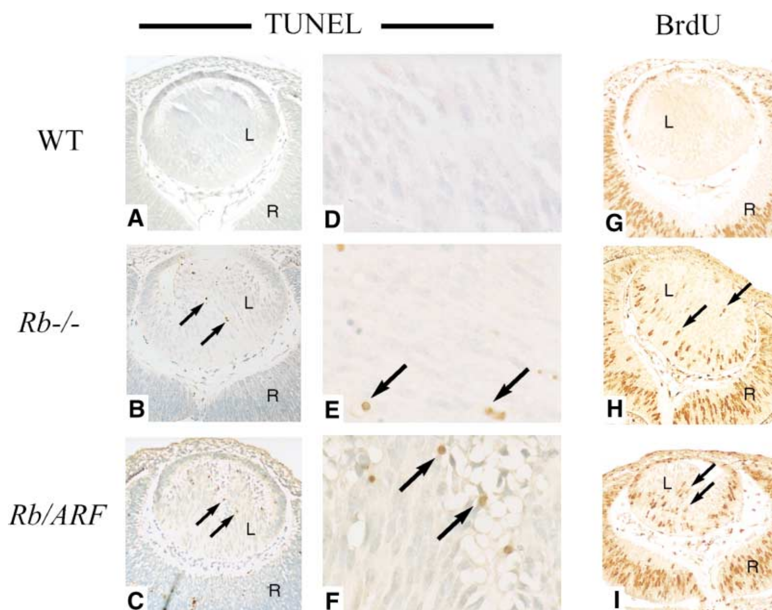

Figure 1. Effects of *ARF* Mutation on Apoptosis and S Phase Entry in the *Rb*-Deficient Ocular Lens

Transverse sections through the ocular lens (L) and retinas (R) of E13.5 embryos were stained for apoptosis (TUNEL protocol) in (A)–(F) or for S phase (BrdU incorporation) in (G)–(I). (A–C) TUNEL-stained sections through ocular lens (L) and retina (R) at 40×. (D–F) TUNEL-stained sections through ocular lens and retina at 100× magnification. Note the presence of darkly stained apoptotic cells (arrows) in *Rb* mutant lens (B and E) which persist in *Rb/ARF* double mutant samples (C and F). Wild-type controls exhibit negligible background staining (A and D). (G–I) BrdU immunohistochemistry on similar sections at 40×. Both *Rb* mutant (H) and *Rb/ARF* double mutant samples (I) show similarly extensive aberrant S phase entry in the lens fiber cell compartment (arrows), but only background staining is observed in wild-type controls (G).

tissue, increased apoptosis and inappropriate S phase entry were observed in *Rb/p53* embryos compared to *Rb* mutant embryos [9]. Similarly, we observed that both apoptosis and inappropriate S phase entry were exacerbated in the PNS ganglia of *Rb/ARF* double mutants relative to *Rb* mutant tissues (Figure 3, see also the Supplementary Material available with this article online). Apoptosis was increased ~1.5-fold, whereas S phase entry was increased ~1.4-fold (Figure 3). It may be that ARF functions primarily to restrict cell cycle progression in *Rb*-deficient PNS ganglia, and the resultant elevation of S phase entry in *Rb/ARF* double mutants causes higher levels of apoptosis. Furthermore, the fact that these phenotypes were exacerbated in both *Rb/p53* and *Rb/ARF* embryos may indicate that ARF functions upstream of p53 in this context.

Conclusions

The complexity of the *Rb* pathway makes it surprising that a handful of proximal genes, such as *p53*, *E2f-1*, and *E2f-3*, have been identified as critical mediators of apoptosis in the absence of *Rb* [7–9]. Here we evaluated the possibility that *ARF* would directly link deregulated E2F activity to *p53*-dependent apoptosis. Because *ARF* can be transcriptionally regulated by E2F-1 [15–18, 28] and is important for the stabilization of p53 in response to oncogenic signals [16, 17, 20, 21, 29], it seemed plausible that deregulated E2F activity resulting from *Rb* inactivation would upregulate *ARF* expression, leading to p53 stabilization and apoptosis. Furthermore, multiple tumor models [22–26] and cell culture systems [20, 21] support the notion that *ARF* plays a pivotal role in *p53*-dependent apoptosis.

Our data instead demonstrate that *ARF* is not required for the *p53*-dependent apoptosis in *Rb*-deficient mouse embryos (Figures 1–3). We conclude that *ARF* does not form an obligatory link between *Rb* inactivation and *p53*-dependent apoptosis in vivo. Perhaps due to the very low levels of *ARF* expression in the developing embryo, we were also not able to demonstrate transcriptional induction of ARF in $Rb^{-/-}$ CNS. However, in a choroid plexus brain tumor model in which apoptosis occurs in an *E2f-1*- and *p53*-dependent manner [12, 13], Tolbert et al. [33] have shown that *ARF* is transcriptionally induced but dispensable for apoptosis. Importantly, in both our studies and those of Tolbert et al., p53 is activated and is transcriptionally competent as assessed by target gene induction and DNA binding activity in the absence of *ARF* function (Figure 4 and [33]).

In our studies, *ARF* loss modestly suppressed apoptosis in the lens (Figure 3A), which is consistent with the results of Pomerantz et al. (1998) [30], who used *Rb/Ink4a*^{ex2,3} double mutant embryos lacking both *p16Ink4a* and *ARF*. While the suppression of apoptosis observed by Pomerantz et al. was quantitatively larger (50%–60% versus 18%), the difference may be explained by the observation that *Rb/Ink4a*^{ex2,3} double mutant lens have 25% more nuclei, whereas our samples of *Rb/ARF* double mutant lens averaged 25% fewer nuclei than *Rb* mutant controls (data not shown). This implies that *p16Ink4a* loss might have conferred a growth advantage to these cells even in the absence of *Rb*. Our results, using animals with an *ARF*-specific mutation that retains wild-type *p16Ink4a* [27], indicate that *ARF* plays a minor role in regulating apoptosis, an effect that may have been overestimated in the *Rb/Ink4a*^{ex2,3} embryos [30]. All of these results indicate that *ARF*-independent pathways downstream of aberrant E2F activity are responsible for p53 activation and subsequent apoptosis in the developing *Rb* mutant embryo.

While the nonequivalence of *ARF* inactivation and *p53* inactivation has been demonstrated in mice heterozygous for *Patched* (*Ptch*) [31], our results are the first demonstration in vivo that *ARF* is not a required activator of *p53*-dependent apoptosis in *Rb*-deficient animals.

We have also demonstrated that loss of *ARF* exacerbates the apoptosis and excessive S phase entry in *Rb*-deficient PNS ganglia, indicating that ARF might nega-

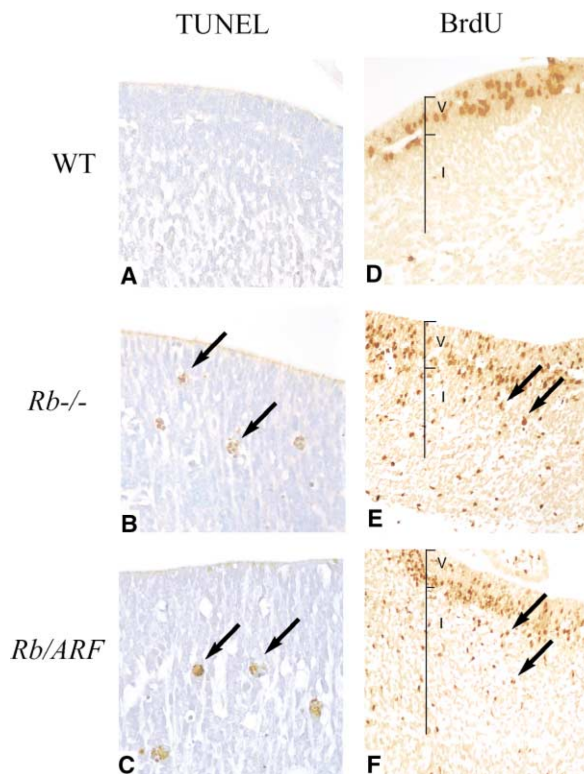

Figure 2. Effects of *ARF* Mutation on Apoptosis and S Phase Entry in the *Rb*-Deficient CNS

Mid-sagittal cross-sections through the fourth ventricle and hindbrain of E13.5 embryos stained for apoptosis (TUNEL protocol) in (A)–(C) or for S phase entry (BrdU incorporation) in (D)–(F). (A–C) TUNEL-stained sections though hindbrain at 40× magnification. Note the presence of darkly stained apoptotic cells (arrows) in *Rb* mutant samples (B) which are minimally changed in *Rb/ARF* double mutant samples (C) and absent in wild-type controls (A). (D–F) BrdU immunohistochemistry on CNS sections at 40×. Wild-type tissue demonstrates S phase entry only in cells in the ventricular zone (V). In contrast, *Rb* mutant tissues (E) and *Rb/ARF* tissues (F) both show excessive and ectopic S phase entry into the intermediate zone (I) (arrows), indicating that loss of *ARF* does not affect this phenotype.

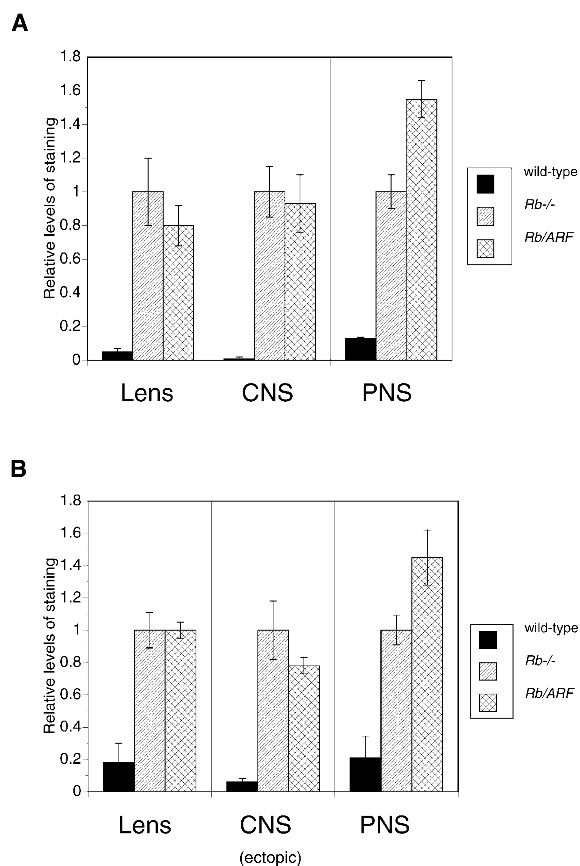

Figure 3. Quantitative Analysis of Apoptosis and S Phase Entry in the Ocular Lens, CNS, and PNS Ganglia of Wild-Type, *Rb* Mutant, and *Rb/ARF* Double Mutant E13.5 Embryos

(A) Loss of *ARF* does not significantly affect apoptosis in the *Rb*-deficient ocular lens or CNS but exacerbates it in PNS ganglia. *Rb* mutant lens exhibit high levels of apoptosis (normalized to 1) that are minimally affected (18%) by further loss of *ARF*. Apoptosis in the *Rb* mutant CNS is unaffected by further inactivation of *ARF*. Even though *Rb* mutant samples exhibit significantly increased levels of apoptosis above wild-type background in PNS, *Rb/ARF* samples show a further increase, by ~1.5-fold, in the level of apoptosis.

(B) Mutation of *ARF* minimally affects inappropriate S phase entry in *Rb*-deficient ocular lens and CNS but significantly exacerbates it in PNS ganglia. *Rb* mutant lens and CNS tissue show excessive and ectopic S phase entry far above background wild-type levels. This is significantly increased in *Rb/ARF* double mutant PNS ganglia by ~1.4-fold. Note that counts in the CNS and PNS were normalized to the area of the tissues within each section; however, counts in ocular lens were normalized to the total number of nuclei within the tissue of each section to facilitate comparison with Pomerantz et al. (1998) [30].

tively regulate proliferation in this context. The increased apoptosis might be a consequence of this increased proliferation. Furthermore, because this exacerbation was also observed in *Rb/p53* embryos [9], it may be that ARF is an upstream regulator of p53 in this tissue.

Establishing the pathways that link defects in proliferation and differentiation that accompany disruption of the *Rb* pathway to the activation of *p53*-dependent apoptosis are critical for our understanding of normal development, tumorigenesis, and the response of tumor cells to many anticancer agents. Thus, it will be important to elucidate the pathway leading to p53 induction and cell death in $Rb^{-/-}$ embryos.

Experimental Procedures

Mice

Rb/ARF compound mutant animals were generated by breeding $Rb^{+/-}$ mice to $ARF^{-/-}$ mice to generate compound mutants. $Rb^{+/-}$; $ARF^{-/-}$ and $Rb^{+/-}$; $ARF^{+/-}$ mice were intercrossed to generate the *Rb/ARF* double mutants ($Rb^{-/-}$; $ARF^{-/-}$). The morning of plug detection was considered E0.5. Pregnant females were labeled for 1 hr with 30 μg/kg body weight BrdU and 3 μg/kg body weight FdU. Embryos were harvested and fixed in 10% formalin (3.7% formaldehyde solution in PBS) for 48 hr. Tissues were processed and imbedded in paraffin blocks from which 4–6 μm sections were cut.

TUNEL and Immunohistochemistry

Apoptosis was assessed using the TUNEL assay [32]. Sections were rehydrated, blocked in 3% H_2O_2, processed in proteinase K, and incubated with rTdT (GIBCO) and biotin-16-dUTP (Boehringer-Mannheim). A mixture of BrdU (5-bromo-2′-deoxyuridine; Sigma) and FdU (5-fluoro-2′-deoxyuridine; Sigma) was injected intraperito-

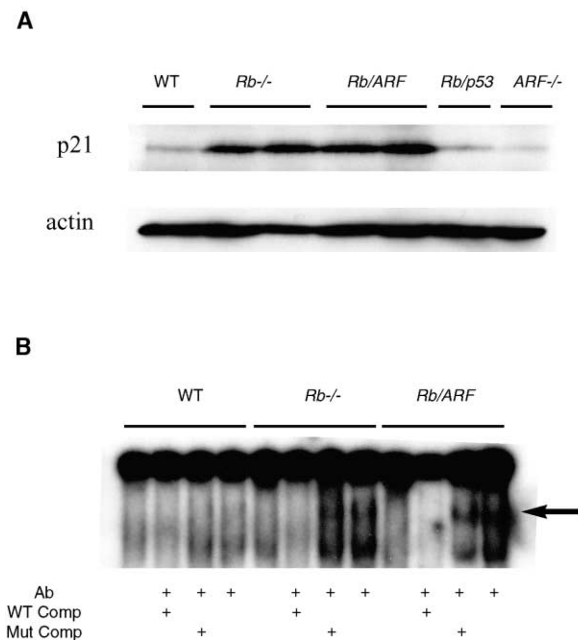

Figure 4. The *p53* Pathway Is Activated in *Rb/ARF* Double Mutant CNS

(A) Western blot analysis for p21CIP1 demonstrates that expression is significantly upregulated in *Rb* mutant samples (lanes 2 and 3) relative to wild-type and *ARF* mutant controls (lanes 1 and 7, respectively). This upregulation is indicative of activation of the *p53* pathway, as it is absent in *Rb/p53* double mutant samples (lane 6), and it persists in *Rb/ARF* double mutants at levels comparable to those in *Rb* mutants (lanes 4 and 5). The blot was stripped and reprobed for actin as a loading control.

(B) EMSA demonstrates that *p53* DNA binding activity in whole cell extracts from *Rb/ARF* E13.5 brains using a radiolabeled *p53* consensus binding site oligonucleotide is upregulated (lanes 11 and 12) compared to wild-type controls (lanes 3 and 4) and comparable to levels observed in *Rb* mutant samples (lanes 7 and 8). Two levels of specificity for *p53* are included: binding activity is dependent upon presence of a *p53* C-terminal-specific polyclonal antibody (Geneka Biotechnology) and remains in the presence of 100× excess of unlabeled mutant (Mut comp) oligonucleotide competitor but not in a 100× excess of unlabeled wild-type oligonucleotide (WT comp).

neally (30 μg and 3 μg/gm body weight, respectively) 1 hr prior to sacrifice. Sections were rehydrated, blocked in 3% H_2O_2, processed in pepsin, HCl, and incubated with a mouse monoclonal anti-BrdU antibody (Becton Dickinson). All immunohistochemistry employed the ABC peroxidase detection system (Vector Laboratories). TUNEL- or BrdU-positive cells were counted in the entire tissue and normalized for tissue size. Darkly staining cells were counted, and then tissue sizes were estimated using a hemacytometer from which numbers describing stained cells per unit tissue area were determined. For CNS tissue, only ectopic BrdU positivity in the intermediate zone was scored. For ocular lens, TUNEL and BrdU positivity were corrected by the number of nuclei in the entire lens section.

Electrophoretic Mobility Shift Assay

Microdissected brains were lysed in buffer (1% NP-40, 100 mM NaCl, 100 mM Tris-HCl [pH 8.0], Complete Protease Inhibitor Cocktail [Roche]) for 30 min at 4°C and the supernatant collected following centrifugation. A double-stranded oligonucleotide sequence (AGC TGGACATGCCCGGGCATGTCC) was end labeled with 32P-γ-ATP by T4 polynucleotide kinase (NEB) and gel purified. Extracts were incubated with or without polyclonal anti-*p53* antibody (Nushift *p53* murine, Geneka Biotechnology) first for 30 min at 4°C and then for another 30 min in the presence of radiolabeled probe at 4°C. For some samples, a 100× excess of unlabeled wild-type or mutant (AGCTGGA**TCGC**CCCGGGCATGTCC) competitor oligonucleotide was introduced at this second incubation. Protein-DNA complexes were resolved on 4.5% polyacrylamide gels (0.38 M glycine, 2 mM EDTA, 50 mM Tris-HCl,[pH 8.5]) for 4–6 hr at 160V, dried, and exposed to film.

Western Blot Analysis

Protein extracts were isolated by either lysing tissues in buffer (1% NP-40, 100 mM NaCl, 100 mM Tris-HCl [pH 8.0], Complete Protease Inhibitor Cocktail [Roche]) for 30 min at 4°C and collecting the supernatant following centrifugation or by extraction from TRIZOL Reagent (GIBCO BRL) as per the manufacturer's protocols into 1% SDS. Total protein was electrophoretically separated by SDS-PAGE (10%–12.5%), transferred to PVDF membrane (Immobilon P, Millipore), and probed with the following antibodies to the following proteins at the indicated dilutions: p21 (clone F-5) (1:2000, Santa Cruz) and actin (1:4000, Santa Cruz). HRP-conjugated anti-mouse secondary antibody was used at 1:5000 (Jackson Immunochemicals), anti-goat secondary was used at 1:10,000 (Santa Cruz), and blots were subjected to enhanced chemiluminescence (ECL+, Amersham) and exposed to film (Kodak X-OMAT).

Supplementary Material

Supplementary Material including a figure that shows that loss of *ARF* exacerbates apoptosis and excessive S phase entry in the *Rb*-deficient PNS ganglia is available at http://images.cellpress.com/supmat/supmatin.htm.

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