Cooperation between Rb and Arf in suppressing mouse retinoblastoma

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Retinoblastoma is a pediatric cancer that has served as a paradigm for tumor suppressor gene function. Retinoblastoma is initiated by RB gene mutations, but the subsequent cooperating mutational events leading to tumorigenesis are poorly characterized. We investigated what these additional genomic alterations might be using human retinoblastoma samples and mouse models. Array-based comparative genomic hybridization studies revealed deletions in the CDKN2A locus that include ARF and P16INK4A, both of which encode tumor suppressor proteins, in both human and mouse retinoblastoma. Through mouse genetic analyses, we found that Arf was the critical tumor suppressor gene in the deleted region. In mice, inactivation of one allele of Arf cooperated with Rb and p107 loss to rapidly accelerate retinoblastoma, with frequent loss of heterozygosity (LOH) at the Arf locus. Arf has been reported to exhibit p53-independent tumor suppressor roles in other systems; however, our results showed no additive effect of p53 and Arf coinactivation in promoting retinoblastoma. Moreover, p53 inactivation completely eliminated any selection for Arf LOH. Thus, our data reveal important insights into the p53 pathway in retinoblastoma and show that Arf is a key collaborator with Rb in retinoblastoma suppression.

Introduction

Human retinoblastoma is initiated by mutation of both alleles of RB, but other changes are required for malignancy (1, 2). RB is a central regulator of proliferation that acts to inhibit E2F transcription factors through direct inhibition and through recruitment of chromatin-modifying enzymes (3). Recent evidence suggests that human retinoblastoma may be derived from a benign lesion, retinoma, caused by RB inactivation (4). Indeed, nonproliferative retinoma, but not retinoblastoma, was correlated with high levels of the RB family member p130, suggesting that, in the absence of RB, other family members can enforce cell cycle exit and inhibit tumorigenesis (4). Studies of retinoma using human samples are limited in that retinomas can only be examined in cases in which late-stage retinoblastoma leads to removal of the eye. Early-stage human retinomas cannot be readily accessed for histological or molecular analyses, making it difficult to know whether retinoma truly represents a precursor lesion to retinoblastoma.

Mouse models based on Rb deletion can provide insight into tumor origins and progression. Loss of Rb and p107 leads to retinoblastoma that arises with long latency and incomplete penetrance (5, 6). Examination of secondary alterations may provide insight into the nature of the switch to malignant retinoblastoma. Human retinoblastomas exhibit consistent regions of chromosomal alteration, including 6p and 1q gains, suggesting that RB loss may not be sufficient for human retinoblastoma (1, 2). Several candidate genes have been described. For example, NMYC is amplified in human and murine retinoblastoma (1, 2, 6). The p53 pathway may also play a role in human retinoblastoma. One study reported that the p53 pathway is evaded through amplifications in MDM4 (7). Another found MDM2 to be highly expressed (8). The latter study suggested that intrinsically high MDM2 levels synergize with RB loss in promoting retinoblastoma. It has also been reported that Arf is highly expressed in human retinoblastoma (7). This is consistent with Arf being an E2F target gene that would be expected to be deregulated upon RB inactivation (9, 10). Whether Arf has any functional role in retinoblastoma suppression is unknown. Efforts are being undertaken to develop activators of the p53 pathway as potential treatment for retinoblastoma (7, 11). However, the importance of this pathway for retinoblastoma is unclear. In this study, we use genomic analyses and mouse genetics to explore roles for Arf and for other p53 pathway components in retinoblastoma.

Results

Human retinoblastoma has been proposed to arise from benign retinoma (4). We studied a mouse model of retinoblastoma initiated by retina-specific Rb deletion on a background of a null mutation in the Rb-related gene p107. In this model, Cre is expressed in the mid-periphery to far-periphery of the retina through the use of the Pax6 α enhancer Cre transgenic allele (12), and retinoblastoma arises with partial penetrance and long latency (6). Rb and p107 loss during development of the retina leads to extensive cell death prior to detection of retinoblastoma, although certain cell types survive Rb/p107 deletion (5, 6, 13). We examined Rb<sup>lox/lox</sup>:p107<sup>−/−</sup>;Pax6 α Cre (Rb/p107DKO) retinas from mice that did not develop retinoblastoma to determine whether premalignant lesions could be detected. As Sox2 and syntaxin mark the vast majority of murine retinoblastoma cells (14), we stained for these markers in Rb/p107DKO retinas at 1 year of age. In addition to observing retinal degeneration, we also detected the presence of nonproliferative lesions that resembled retinoblastoma based on Sox2/syntaxin expression (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI61403DS1). These observations suggest that a cell cycle block may form a barrier to murine reti-

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noblastoma, as suggested in human retinoblastoma studies (4). A block to tumorigenesis overcome through secondary mutations may involve suppression of cell death or bypass of a cell cycle arrest. The p53 tumor suppressor has been implicated in both apoptosis and cell cycle arrest but is not directly mutated in human retinoblastoma. To better understand the blocks to tumorigenesis that must be overcome through secondary alterations, we performed genomic analyses.

Array-based comparative genomic hybridization analyses of p53 pathway alterations in murine and human retinoblastoma. Using array-based comparative genomic hybridization (array-CGH) data from 21 Rb/p107DKO retinoblastomas, including 14 primary and 7 metastatic tumors, we focused on alterations that might disrupt known p53 pathway components. MDM4 has been implicated as genetically altered in human retinoblastoma (7). Murine Mdm4 is located at 1qE4. Of the 21 murine retinoblastomas, no high-level amplifications or focal alterations including Mdm4 were observed, although gains of the entire chromosome 1 were found in 7 out of 21 murine retinoblastomas. We did observe 1 Rb/p107DKO retinoblastoma with a focal Mdm2 amplification that correlated with high Mdm2 expression (Figure 1A and Supplemental Figure 2). A previous study indicated

![Figure 1](http://www.jci.org)
that 65% of human retinoblastomas exhibit increased \textit{MDM4} copy number (7). However, that study did not define the size of the region of amplification. We examined \textit{MDM4} copy number alterations in human retinoblastoma samples using array-CGH. Out of 37 human retinoblastomas, 2 exhibited focal amplifications (1.6 Mb, 9.2 Mb) (Figure 1B). In addition, 17 out of 37 retinoblastomas exhibited gain of the long arm of 1q, which included \textit{MDM4} at 1q32 (Supplemental Figure 3). Thus, both human and murine retinoblastomas exhibited large regions of chromosomal gain that included \textit{MDM4}, and a small subset (2 out of 37) of human retinoblastomas exhibited high-level focal \textit{MDM4} amplification.

**CDKN2A deletions in a subset of murine and human retinoblastomas.** Thus far unexplored in genetic analyses of retinoblastoma, the \textit{MDM2} inhibitor \textit{Arf} is an activator of the p53 pathway. Our genomic analyses of murine retinoblastoma revealed the occurrence of recurrent 4qC4-5 deletions in 3 out of 21 \textit{Rb/p107DKO} mice (Figure 1A). The deletion included the \textit{Cdkn2a} locus, encoding both \textit{p16INK4a} and \textit{Arf} (15). Both bona fide tumor suppressors, \textit{p16INK4a} and \textit{Arf} have different promoters
and first exons but share exons 2 and 3, translated using differing reading frames (15). The minimal deleted region across 3 tumors was 7.5 Mb. Real-time PCR analyses showed that 2 out of the 3 deletions were homozygous, while the third was consistent with a hemizygous mutation or of a mixed oligoclonal tumor (Supplemental Figure 4A). We also used real-time PCR to confirm that both p16 and Arf are expressed in tumors of both p16DKO and Rb/p107DKO mice. (B) Pie charts showing the frequency of p53-related focal genetic alterations in murine Rb/p107DKO and human retinoblastoma. Charts do not include whole chromosome or chromosome arm gains or losses.

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**Figure 3**

Inactivation of p53 abrogates selection for Arf LOH in murine retinoblastoma. (A) Observational curves showing time to first appearance of retinoblastomas in the anterior chamber of the eyes of Rb/p107/p53TKO and Rb/p107/p53TKO;Arf–/–. (B) Southern blot analysis showing that 0 out of 13 Rb/p107/p53TKO;Arf–/– retinoblastomas exhibit LOH for Arf. (C) Sequencing traces showing direct p53 mutation in a subset of murine retinoblastomas from Rb/p107DKO animals. (D) Pie charts showing the frequency of p53-related focal genetic alterations in murine Rb/p107DKO and human retinoblastoma. Charts do not include whole chromosome or chromosome arm gains or losses.

Arf, p16INK4a functions upstream of the entire RB family, RB, p107, and p130 as a CDK inhibitor. If p16INK4a were the critical gene in the deleted region, this would suggest that even in the absence of pRB and p107, selection for regulators of p130 activity may be important. If Arf were the critical gene, this would implicate another component of the p53 pathway beyond MDM2/MDM4 as being important for retinoblastoma or might indicate p53-independent effects. We sequenced exons 1α and 1β as well as exon 2 of the Cdkn2a locus in 19 mouse retinoblastomas and 37 human retinoblastomas to determine whether either p16 or Arf are inactivated through subtle mutations. In analyses of both human and murine primary retinoblastomas, no p16/Arf point mutations or indels were detected. Thus, as in many tumor types (16), p16/Arf inactivation in retinoblastoma occurred through large deletions without subtle mutations.

**Mouse genetic analysis of Cdkn2a locus in retinoblastoma.** We proceeded with mouse genetic analyses to assess whether either p16INK4a or Arf were important retinoblastoma suppressors. We performed compound mutant analyses using the Rb/p107/DKO model. We generated Rb/p107DKO;Arf–/+; Rb/p107DKO;Arf–/-, and Rb/p107DKO;p16–/- cohorts and followed mice for tumor development. Mice were monitored for first appearance of externally visible retinoblastoma in the anterior chamber of the eye. When retinoblastoma was initiated by loss of both Rb and p107, inactivation of either one or both alleles of p16 led to a rather modest acceleration of late-stage tumorigenesis (Figure 2A). Over 300 days, 2 out of 22 (9%) Rb/p107DKO control mice, compared with 12 out of 32 (38%) Rb/p107DKO;Arf–/- animals and 14 out of 36 (39%) Rb/p107DKO;Arf–/- animals developed retinoblastoma with anterior chamber invasion. Of the mice that developed retinoblastoma, the average time to visible tumor was 255 days in Rb/p107DKO controls, 185 days in Rb/p107DKO;Arf–/-, and 179 days in Rb/p107DKO;Arf–/- cohorts. The effect of p16 loss in promoting retinoblastoma is much weaker than that of strong Rb/p107 cooperating mutations, such as p53 deletion or the microRNA miR-17-92 overexpression, previously reported (7, 14). Nonetheless, the mild retinoblastoma-promoting effect of p16INK4a deletion supports the notion that CDK inhibitors can exhibit tumor suppressor function in retinoblastoma (14, 17).

**Arf is a potent retinoblastoma suppressor in mouse models.** We also examined the role of Arf in retinoblastoma suppression using the Rb/p107DKO model. We note that Arf-null mice exhibit failure in regression of the hyaloid vessels that are normally transiently present during eye development (18). The Arf–/- defect
leads to secondary degeneration of the retina and complicates analyses of Arf homozygous animals. Since Arf heterozygote mice exhibit normal hyaloid regression (18), we compared Rb/p107DKO;Arf+/+ cohorts with Rb/p107DKO;Arf+/– cohorts and followed them for retinoblastoma (Figure 2B). In contrast to the modest effects of p16 inactivation in the Rb/p107DKO model, germline inactivation of a single allele of Arf led to rapid, usually bilateral, retinoblastoma (Figure 2, B and C). In the control Rb/p107DKO cohort, 6 out of 27 (22%) mice developed retinoblastoma (average time until visible tumor, 178 days). In contrast, 27 out of 33 (82%) of the Rb/p107DKO;Arf+/– mice developed retinoblastoma (average time until visible tumor, 151 days). Analyses of late-stage progression revealed a subset of late tumors that progressed to invade the brain (Figure 2C). Southern blot analyses revealed loss of heterozygosity (LOH) of the Arf allele in 13 out of 23 primary tumors and 2 out of 3 metastases (Figure 2D and data not shown). Most samples with Arf LOH showed complete loss of the Arf allele (Figure 2D). Rare cases of Arf LOH, in which some residual wild-type allele was present, reflect either the presence of wild-type contaminating cells in the tumor or Arf deletion in most but not all tumor cells. These studies reveal selection for Arf inactivation and show that Arf is a clear tumor suppressor in retinoblastoma.

Rb/p107/p53/Arf tumorigenesis analyses. Arf may suppress retinoblastoma through the canonical p53 pathway via inhibition of MDM2, or Arf may exert p53-independent effects. p53-independent functions of Arf have been revealed in other systems through observations of synergistic effects of combined Arf and p53 mutation on tumorigenesis in mice (19). We generated Rb/p107/p53 triple knockout (Rb/p107/p53TKO) animals, with or without heterozygous inactivation of Arf. Rb/p107/p53TKO mice developed rapid retinoblastoma (Figure 3A), similar to results previously reported (20). When we compared the time to late-stage retinoblastoma development in Rb/p107/p53TKO mice with that in Rb/p107/p53TKO;Arf+/– mice, we found no difference (Figure 3A). Our observation of frequent LOH of Arf in Rb/p107DKO;Arf+/– retinoblastomas provided a powerful means to test whether selection for Arf inactivation still occurs when the p53 pathway is disrupted. We isolated retinoblastomas from Rb/p107/p53TKO;Arf+/– animals and performed LOH analyses for Arf via Southern blot. We found that, in the absence of p53, 0 out of 13 retinoblastomas exhibited Arf LOH (Figure 3B). Thus, dele-

Figure 4

Inactivation of p53 increases proliferation in the Rb/p107DKO retina without suppressing cell death. (A) Histology, Ki67 and phospho-histone H3 (PH3) immunostaining and TUNEL analyses showing retinal phenotypes of the following genotypes: Cre-negative control, Rb/p107DKO, and Rb/p107DKO;p53lox/lox. Arrows indicate TUNEL positive nuclei. Scale bar: 270 μm (first row); 50 μm (second, third, fourth, and bottom rows); 20 μm (TUNEL insets). (B) Quantification of Ki67, phospho-histone H3, and TUNEL data. n = 4–5 animals per data point. CON, control; DKO, Rb/p107DKO; TKO, Rb/p107/p53TKO. Error bars refer to standard deviation. P values shown are from Student’s t test.
tion of p53 completely abrogates the selective pressure to inactivate Arf. Moreover, we found no evidence that Arf functions in a p53-independent fashion to suppress retinoblastoma.

Direct p53 mutation in murine retinoblastomas. Despite the reported absence of p53 mutation in human retinoblastomas (21, 22), the strong effect of p53 loss in accelerating retinoblastoma in mice raised the possibility that p53 might be directly mutated in murine retinoblastoma. Thus, we sequenced the region of p53 subject to frequent mutation, exons 5 to 9 in Rb/p107DKO retinoblastomas. Three out of thirty-five retinoblastomas harbored direct p53 mutations. Two were heterozygous R270H mutations, while the third was a heterozygous 4-bp frameshift mutation (Figure 3C). R270H corresponds to one of the most frequently mutated p53 sites in human tumors (R273H) and has been shown to exhibit dominant-negative and potential gain-of-function properties in mice (23, 24). This report is the first evidence to our knowledge of direct p53 mutations in mouse retinoblastoma. Direct p53 mutation was specific to murine retinoblastoma, as sequencing of exons 5 to 9 of a panel of 37 human retinoblastomas did not reveal p53 mutations. Altogether, we observed Arf deletion, MDM2 amplification, or direct p53 mutation in approximately 28% of Rb/p107DKO murine retinoblastomas (Figure 3D). This provides genetic evidence that the p53 pathway is important for suppressing retinoblastoma in mice. We did not find evidence of direct p53 mutation in human retinoblastoma, in which focal alterations in known p53 pathway components were less frequent (~11%) than in the murine model (Figure 3D).

p53 inactivation increases proliferation in Rb/p107DKO retina. Previous studies have demonstrated that inactivation of p53 accelerates retinoblastoma in mice (7, 20), but there have been no in vivo analyses of early lesions to assess the nature of p53 function that is important. To determine how p53 loss promotes retinoblastoma, we compared the phenotype of Rb/p107DKO retinas with that of Rb/p107/p53TKO retinas. Examination of retinas at postnatal day (PND22) revealed that homozygous p53 inactivation significantly increased proliferation in the Rb/p107DKO model, as assayed by both Ki67 and phospho-histone H3 staining (Figure 4, A and B). We also performed TUNEL staining to quantify apoptosis. We found that inactivation of p53 did not suppress apoptosis in the retina (Figure 4, A and B). Thus, our data support the notion that, even in the absence of Rb, a cell cycle block forms a barrier to retinoblastoma that is overcome through secondary genetic alterations.

With evidence that the p53 pathway engagement leads to cell cycle inhibition to prevent retinoblastoma, we investigated whether nonproliferative Rb/p107DKO retinas in older animals (~1 year) exhibited evidence of senescence. We performed senescence-associated β-galactosidase staining to examine senescence in retinas from Rb/p107DKO animals that lacked retinoblastoma. We found no senescence-associated β-galactosidase staining in control or Rb/p107DKO nonproliferative retinas at 1 year of age (Supplemental Figure S). Senescent cells typically exhibit a senescence-associated secretory phenotype (SASP), with increased expression of proinflammatory factors (25). We also examined SASP transcripts in control adult retinas, Rb/p107DKO nonproliferative retinas, and Rb/p107/p53TKO retinoblastomas (Supplemental Figure 6). Of the 8 SASP factors examined, only 1 (lgfbp7) was upregulated in the Rb/p107DKO retinas compared with control retinas. Despite upregulation of lgfbp7 in Rb/p107DKO retinas, similar high levels remained in retinoblastomas that exhibited deletion of p53. Thus, the block to tumorigenesis that is overcome through p53 mutation is distinct from a classical senescence response.

Discussion

It has been proposed that evasion of the p53 pathway through MDM4 amplification is important for retinoblastoma in humans (7). It is clear that strong overexpression of MDM4 can promote retinoblastoma in mice (26), but our study reveals that most cases of increased MDM4 copy number in human retinoblastoma arise through low-level gain of the whole long arm of 1q (Supplemental Figure 2). A novel mouse model that exhibits low levels of MDM4 overexpression (27) will be an important tool to test the notion that low levels of increases in MDM4 expression (i.e., via 1q gain) may be oncogenic in retinoblastoma. With a low frequency of focal, high-level MDM4 amplifications in human retinoblastoma (2 out of 37 samples), we explored whether other components of the p53 pathway might be genetically altered in human retinoblastoma and mouse models. We report, for the first time to our knowledge, p16INK4a/Arf deletions in both human and murine retinoblastoma. Our mouse genetic analyses showed particularly strong tumor suppressive ability for Arf in a murine retinoblastoma model.

The strong effect of Arf loss in promoting murine retinoblastoma (Figure 2B) suggests that Arf is the major tumor suppressor in the large deleted region. Arf only appeared to be inactivated through deletions, as we found no Arf-specific mutations in retinoblastoma. Moreover, the Arf promoter is not silenced via methylation in human retinoblastoma (28). It is important to note that, in human and murine retinoblastomas with Arf deletion, the region of loss invariably included p16INK4a and p15Ink4b as well. p16INK4a loss alone led to a modest acceleration of Rb/p107DKO retinoblastoma (Figure 2A), and we have not investigated the possibility that co-mutation of Arf together with p16INK4a and/or p15Ink4b might exhibit cooperative effects in retinoblastoma suppression. Previous work indicated that upregulation of a number of CDK inhibitors (including p16Ink4a and p15Ink4b) occurs in response to Rb/p107 deletion in the retina (14). Also, deletion of another CDKI, Cdkn1a, led to increased proliferation in the Rb/p107DKO retina in vivo (14). This result supports the notion that CDK inhibitors may act as tumor suppressors in the context of Rb deletion. It will be interesting for future work to assess the roles of CDK inhibitors as retinoblastoma suppressors in the context of Arf mutation.

Arf has been shown to exhibit p53-independent activities in sperm development in mice (29). It has been hypothesized that p53-independent activities of Arf could contribute to the tumor suppressor function of Arf (reviewed in ref. 30). Indeed, mice lacking both Arf and p53 develop a wider spectrum of tumors than that found in Arf-deficient or p53-deficient mice (19). We asked whether p53 and Arf loss would synergize to promote retinoblastoma. We found no additive effect of Arf and p53 codeletion on the incidence of murine retinoblastoma. Moreover, the absence of p53 completely eliminated the selective pressure to inactivate the wild-type copy of Arf. Our data support the idea that Arf functions through the canonical p53 pathway in retinoblastoma. We also report direct p53 mutation and focal MDM2 amplification in Rb/p107DKO murine retinoblastoma. Importantly, Arf deletion, MDM2 amplification, and p53 mutation were mutually exclusive in the murine tumors. Also, MDM4 amplification and CDKN2A
deletion were not found in the same human retinoblastomas. Alterations in the same pathway at multiple levels across different tumors underscore the importance of this pathway in murine retinoblastoma models.

Human retinoblastoma has been proposed to arise from a benign lesion, termed retinoma (4). It was shown that retinoma, adjacent to normal retina and contiguous with malignant retinoblastoma, exhibited homozygous RB deletion but was nonproliferative (4). A limitation of human retinoma studies is that the lesions are studied in the presence of late-stage retinoblastoma that resulted in removal of the eye. The possibility that retinoma could represent a regressed region of retinoblastoma cannot be ruled out. Using mouse models, we could identify lesions that resembled murine retinoblastoma based on marker analyses but were nonproliferative. We hypothesize that murine retinoma-like lesions would have progressed to retinoblastoma had secondary mutations impaired the ability of the Rb/p107DKO cells to arrest/exit the cell cycle. Previous work has shown that many Rb/p107DKO cells can exit the cell cycle after an extended period of inappropriate proliferation during retinal development (5, 13) (also see Figure 4). In this study, we found that p53 deletion strongly promotes retinoblastoma, but without suppression of apoptosis (Figure 4). Instead, we found that p53 loss increased proliferation in the Rb/p107DKO retina. Thus, the proliferation machinery may need to be altered through multiple mutations in a single tumor, beyond Rb or Rb/p107 mutation alone, for full disruption of the pathway. Recent studies investigating the miR-17-92 microRNA cluster in retinoblastoma revealed that miR-17-92 overexpression also promoted retinoblastoma via effects on proliferation control (14). Interestingly, suppression of p21Cip1 was implicated as conferring some of the effects of miR-17-92. As p21Cip1 is a known p53 target, it will be interesting for future work to assess whether p21Cip1 also plays a role in mediating the retinoblastoma-suppressing effects of p53. In human retinoblastoma, focal high-level amplification of MDM4 or deletion of CDKN2A was relatively infrequent. We hypothesize that the ultimate effect of these alterations is a bypass of a proliferative block to tumorigenesis. Higher resolution analyses of the retinoblastoma genome may reveal additional mutated genes that act in the same pathway.

We have shown that the CDKN2A locus is deleted in a subset of murine and human retinoblastomas. We demonstrated that Arf exhibits potent tumor suppressor activity in mouse models and that the tumor-suppressing effects of Arf require p53 to be intact. We found that p53 loss increases proliferation in the Rb/p107DKO retina without suppressing cell death. We believe that our study provides new insights into the ultimate effect of these alterations is a bypass of a proliferative block to tumorigenesis. Higher resolution analyses of the retinoblastoma genome may reveal additional mutated genes that act in the same pathway.

Methods

Animals. Rb/p107DKO mice were bred with p16+/–, Arf+/–, or p53+/–/– strains to generate the compound mutant mice used in these studies. Rbmice/b and p107−/− mice were from Tyler Jacks (Massachusetts Institute of Technology, Cambridge, Massachusetts, USA), p16+/–, Arf+/–, and p53+/–/– mice were obtained from the NCI MMHCC repository. These strains were originally generated by Ron Depinho, MD Anderson Cancer Center, Houston, Texas, USA (p16–/– Cdcn2a exon 1α deletion); Charles Sherr, St. Jude Children’s Research Hospital, Memphis, Tennessee, USA (Arf–/– Cdhna2a exon 1β deletion); and Anton Berns, Netherlands Cancer Institute, Amsterdam, the Netherlands (p53lox). Independent Rb/p107DKO cohorts were generated for the different crosses using breeding strategies that produced littermate Rb/p107DKO controls and p16 or Arf mutant cohorts. Animals were maintained on a genetic background. For tumor studies, animals were monitored visually for the presence of retinoblastoma that exhibited invasion into the anterior chamber of the eye.

Array-CGH. DNA was isolated from human and murine primary retinoblastomas using phenol-chloroform extractions. Murine array-CGH was performed using the ROMA platform, as described previously, using 84 K arrays (6, 31). Human retinoblastoma array-CGH was performed on 32 human retinoblastomas using custom Nimblegen 135 K arrays. The regions surrounding CDN2A and MDM4 were tiled a medium distance of approximately 600 bp per probe, with a backbone spacing of 27 kb per probe through most of the genome. We also performed human ROMA analyses on an additional 5 human retinoblastomas using 84 K ROMA arrays.

Southern blot analyses. Genomic DNA was isolated from retinoblastoma or tail samples and digested with BglII. Standard Southern protocols were used, with an Arf exon 1β probe for hybridization, as described previously (32).

Immunoblot/immunohistochemistry/TUNEL analyses. Western blots were performed as described previously (14). For BrdU analyses, eyes were fixed in Bouin’s solution overnight and processed through paraffin, and BrdU immunostaining was performed, as described previously (14). Immunohistochemistry and TUNEL were performed on eyes fixed in 4% paraformaldehyde as described previously (14). Antibodies used were as follows: Sox-2 (Santa Cruz Biotechnology Inc., Y-17), Syntaxin (Sigma-Aldrich), Arf (Abcam), Ki67 (Abcam), Active Caspase-3 (Cell Signaling Technology), Phospo-Histone H3 (Millipore), and BrdU (BD Biosciences). TUNEL assays were performed using the In Situ Death Detection Kit (Roche).

Real-time PCR. For expression analyses, RNA was isolated with TRIzol reagent (Invitrogen), and cDNAs were generated using SuperScript II (Invitrogen). SYBR green real-time PCR was performed using a DNA Engine Opticon (MJ Research). The ΔACT method was used. Primer sequences are shown in Supplemental Table 1.

p53 and CDKN2A sequencing. Exons 5 through 9 of murine and human p53 and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified. PCR products were sequenced using Sanger sequencing with the following primers for CDKN2A and all exons of CDKN2A were PCR amplified.
Senescence assays. Senescence-associated β-galactosidase staining was performed on fresh frozen retinas and control kidney tissue, as described previously (34). Briefly, 10-μm slides were fixed for 10 minutes in 4% PFA in PBS, washed with PBS, and stained for 14 hours in stain solution (pH 6.0). Slides were counterstained with eosin.

Statistics. Two-tailed Student’s t test was used for comparisons of different groups. A P value of less than 0.05 was considered significant. Error bars correspond to standard deviation.

Study approval. All protocols that used human retinoblastoma material were approved by both the Johns Hopkins Homewood Institutional Review Board (Baltimore, Maryland, USA) and the Massachusetts Eye and Ear Infirmary Human Studies Committee (Boston, Massachusetts, USA). All protocols involving mice were approved by the Carnegie Institution Animal Care and Use Committee (Baltimore, Maryland, USA).

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