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Retinoblastoma Has Properties of a Cone Precursor Tumor and Depends Upon Cone-Specific MDM2 Signaling

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SUMMARY

Retinoblastomas result from the inactivation of the *RB1* gene and the loss of Rb protein, yet the cell type in which Rb suppresses retinoblastoma and the circuitry that underlies the need for Rb are undefined. Here, we show that retinoblastoma cells express markers of postmitotic cone precursors but not markers of other retinal cell types. We also demonstrate that human cone precursors prominently express MDM2 and N-Myc, that retinoblastoma cells require both of these proteins for proliferation and survival, and that MDM2 is needed to suppress ARF-induced apoptosis in cultured retinoblastoma cells. Interestingly, retinoblastoma cell *MDM2* expression was regulated by the cone-specific RXRγ transcription factor and a human-specific RXRγ consensus binding site, and proliferation required RXRγ, as well as the cone-specific thyroid hormone receptor-β2. These findings provide support for a cone precursor origin of retinoblastoma and suggest that human cone-specific signaling circuitry sensitizes to the oncogenic effects of *RB1* mutations.

INTRODUCTION

Retinoblastoma is a childhood retinal tumor that has provided numerous insights into human cancer biology. For instance, retinoblastoma was one of the first malignancies to be recognized as having hereditary features, and it engendered the characterization of one of the first tumor suppressors genes to be cloned, *RB1* (Weller, 1937; Friend et al., 1986). In turn, the retinoblastoma protein, Rb, was found to have crucial roles in cell-cycle control and differentiation, and to govern a signaling pathway that is inactivated in most if not all human cancers (Weinberg, 1995; Cobrinik, 2005; Skapek et al., 2006). However, despite the general significance of Rb in cancer biology, the basis for its crucial role in retinoblastoma pathogenesis has not been defined.

Retinoblastomas are thought to result from the inactivation of *RB1*, either with somatic inactivation of both *RB1* alleles, or with a germline *RB1* mutation and somatic inactivation of the second allele in hereditary cases. Biallelic *RB1* mutations may initially result in the production of benign retinomas, with subsequent genetic changes mediating malignant transformation (Dimaras et al., 2008). Germline *RB1* mutations predispose to an average of five retinoblastomas, usually bilaterally, and with most forming in the first year (Abramson and Gombos, 1996), but predispose to only an ~0.5% chance of developing other cancers per year (Kleinerman et al., 2005). The exceptionally high rate of retinoblastoma arising from the minuscule retinal cell population implies that the tumors derive from a cell type that is unusually sensitive to the loss of Rb function.

One way that a cell may be sensitized to the loss of Rb is by having signaling circuitry that fails to respond to Rb loss with appropriate countermeasures. Indeed, at least two cellular responses to Rb loss impede tumorigenesis in other settings, but evidently fail to do so in the cells from which retinoblastomas arise. First, in diverse settings including explanted mouse retinas, Rb loss is compensated by increased expression of the Rb-related p107 (Donovan et al., 2006). Second, loss of Rb can deregulate E2F transcription factors and elicit E2F-dependent apoptosis (Chen et al., 2007). Among other effects, deregulated E2Fs induce expression of a *CDKN2A* isoform that encodes ARF, which inhibits MDM2 and promotes p53-mediated apoptosis (Iaquinta and Lees, 2007). As Rb-deficient retinas

and premalignant retinomas have greatly increased *CDKN2A*^{ARF} expression (Laurie et al., 2006; Chen et al., 2007; Dimaras et al., 2008), it appears that the ARF-induced apoptotic response may be impaired in early stages of retinoblastoma tumorigenesis.

To gain insight into the circuitry that sensitizes to Rb loss, we sought to relate features of retinoblastoma to human retinal development. As retinoblastomas form as early as 21 weeks of gestation (Maat-Kievit et al., 1993), we initially sought clues to this circuitry by evaluating Rb's developmental expression pattern. We found that Rb is expressed in a cell cycle-dependent manner in retinal progenitor cells, but is not evident in the early postmitotic precursors of the different retinal neurons. However, Rb was detected in older, maturing retinal precursors, and at exceptionally high levels in maturing cone precursor cells (Lee et al., 2006).

Rb's prominent expression in cone precursors was intriguing, as cultured retinoblastoma cells generally express cone markers (Bogenmann et al., 1988), but not glial markers as was once believed (Virtanen et al., 1988), and the tumors have cone but not rod phototransduction activities (Hurwitz et al., 1990). In addition, retinoblastomas are topographically distributed across the retina in a pattern that mimics that of L/M cones (Munier et al., 1994). Nevertheless, the significance of the cone circuitry has been unclear, as cone features were not consistently detected in retinoblastomas in situ, and cells with properties of other retinal cell types are also present in the tumors (Gonzalez-Fernandez et al., 1992; Nork et al., 1995). Moreover, mice with targeted loss of Rb and Rb-related proteins produced tumors with amacrine or horizontal cell, but not cone cell, features (Robanus-Maandag et al., 1998; Chen et al., 2004; Dannenberg et al., 2004; MacPherson et al., 2004; Ajioka et al., 2007). Thus, in the current study, we examined the relevance of cone-specific signaling circuitry to retinoblastoma tumorigenesis.

RESULTS

Widespread Expression of L/M Cone Photoreceptor Markers in Retinoblastoma Tumors

To assess the cellular phenotypes of retinoblastoma cells, we stained a panel of tumors with retinal cell type-specific markers. The panel included tumors displaying a range of differentiation states, with and without prior chemotherapy, and from bilaterally and unilaterally affected patients (Table S1 available online).

As mature photoreceptor features were previously detected in only a subset of retinoblastomas, we initially examined whether the tumors more generally express proteins that are characteristic of immature photoreceptor precursors. These included CRX, which is specific to cones, rods, and bipolar cells (Bibb et al., 2001); RXRγ, which is specific to cones and ganglion cells (Mori et al., 2001); TRβ2, which is specific to cones (Ng et al., 2001); and NRL, which is specific to rods (Swain et al., 2001) (Figures 1A–1D and S1).

CRX, RXRγ, and TRβ2 were detected in the vast majority of cells in each of 40 tumors, including >95% of cells in ten quantitatively evaluated samples, whereas NRL generally was not detected (Figures 1E–1H and 1P, and Table S2). CRX, RXRγ, and TRβ2 were expressed in all tumor regions, and in morphologically differentiated cells as well as in proliferating cells that express Ki67 or phosphorylated histone H3 (Figures 1I–1L and data not shown). In keeping with their neoplastic status, CRX$^+$, RXRγ^+, and TRβ2$^+$ tumor cells lacked detectable Rb (Figures 2A and 2B), whereas CRX$^+$, RXRγ^+, and TRβ2$^+$ cone precursors in the central retina had prominent Rb expression (Figures 1A–1D and S1). In addition, the vast majority of cells in each of 22 retinoblastomas expressed cone-specific arrestin (Figures 1P and S2).

As coexpression of CRX, RXRγ, TRβ2, and cone arrestin was indicative of a cone phenotype, we examined whether retinoblastoma cells resemble short wavelength-sensitive S cones, or the developmentally distinct long and medium wavelength-sensitive L/M cones, by staining 22 tumors for S and L/M opsins. L/M opsin was detected in >95% of cells in each of the tumors, including proliferating Ki67$^+$ cells and each of ~4000 CRX$^+$ cells evaluated in a costaining analysis (Figures 1M, 1P, and S3). In contrast, S opsin was detected in only 0.1%–2% of cells in 16 (73%) of the samples, and always in cells that coexpressed L/M opsin (Figures 1N and 1P). As S and L/M opsins are transiently coexpressed in developing L/M but not S cones (Cornish et al., 2004) (Figure 1O), these findings indicate that retinoblastomas largely consist of cells that have an L/M cone precursor phenotype.

Cells within Retinoblastomas that Lack Cone Markers Express Rb and/or Retain Wild-Type *RB1* Alleles

We next examined whether retinoblastoma cells also express markers of retinal neurons other than cones. In these analyses, we costained samples for cell type-specific markers and for Rb, in order to identify nonneoplastic Rb$^+$ cells. We detected no tumor cells expressing the amacrine and horizontal cell-specific syntaxin, the ganglion cell-specific Brn-3b, or the horizontal, amacrine, bipolar, and progenitor cell-specific Prox1 (Table S2). Occasional tumors had clusters of cells that expressed the retinal progenitor cell- and bipolar cell-specific Chx10 or the progenitor, ganglion, horizontal, amacrine, or pigment epithelium cell-specific Pax6. However, the Chx10$^+$ and Pax6$^+$ cells coexpressed Rb (Figure S4) and were concluded to derive from the normal retina. Similarly, a cluster of cells expressing the rod-specific rhodopsin was detected in one tumor, yet these cells also coexpressed Rb (Figure S4). One sample had a highly differentiated retinoma-like region in which the majority of cells expressed cone markers and <1% expressed rhodopsin (Figure S5). However, these cells also seemed to be nonneoplastic, as no rhodopsin$^+$ cells costained for Ki67. Thus, in this series, neoplastic retinoblastoma cells expressed markers of cones but not other retinal neurons.

We also examined retinoblastomas for expression of glial and progenitor cell markers. Each of 20 tumors had cells that coexpressed glial fibrillary acidic protein (GFAP) and nestin (Table S2). The vast majority of these cells coexpressed Rb (Figures 2A–2F), consistent with their being nonneoplastic astrocytes or Müller glia. However, Rb was not detected in rare GFAP$^+$ cells (Figure 2K), or in rare cells that lacked both GFAP and the cone marker CRX (Figures 2A–2C, arrowhead).

To assess whether the rare CRX$^-$,Rb$^-$ or GFAP$^+$,Rb$^-$ cells derive from *RB1*-mutated tumor cells, we examined whether they retained wild-type *RB1* alleles. We first identified tumors in which one of the *RB1* alleles was deleted, then immunostained and recorded the positions of CRX$^-$,Rb$^-$ and GFAP$^+$,Rb$^-$ cells, and then defined their *RB1* status using two-color fluorescence