# Supplemental Data

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

**Xiaoliang L. Xu, Yuqiang Fang, Thomas C. Lee, Douglas Forrest, Cheryl Gregory-Evans, Dena Almeida, Aihong Liu, Suresh C. Jhanwar, David H. Abramson, and David Cobrinik**

# SUPPLEMENTAL FIGURES

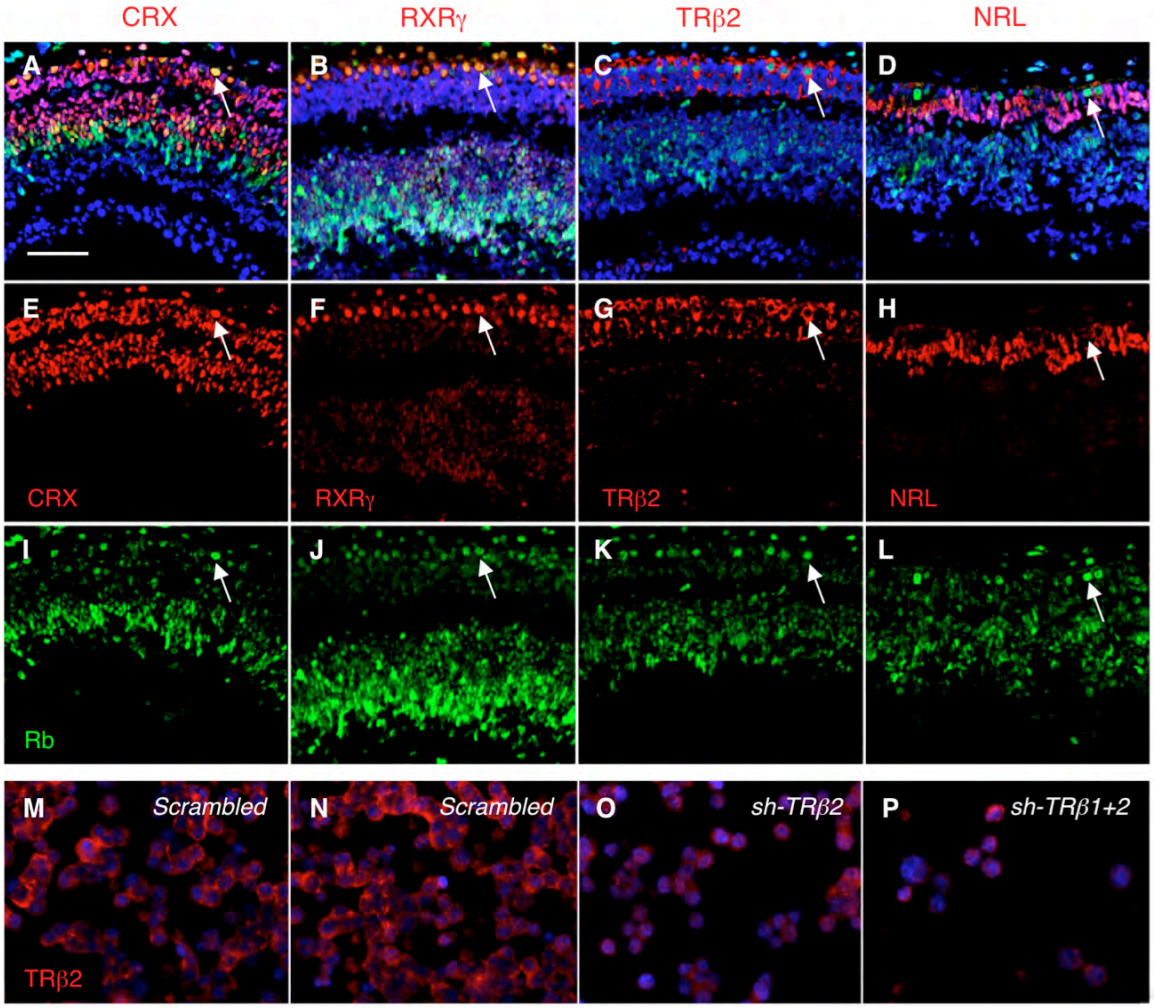


**Figure S1**

**(A-L) Co-expression of Rb and cone precursor markers in the developing human retina.** Photoreceptor precursor markers CRX, RXRγ, TRβ2, and NRL (red), Rb (green), and DAPI-stained DNA (blue) in gestational week 16 retina, displaying merged image (A-D), photoreceptor precursor markers alone (E-H), and Rb alone (I-L). Note that cells with prominent Rb (arrows) co-express cone markers CRX, RXRγ, and TRβ2, but not the rod marker NRL. Scale bar, 50 μm.

**(M-P) Cytoplasmic TRβ2 staining is diminished by TRβ2-specific shRNAs.** RB177 cells two weeks after transduction with a scrambled control shRNA (M, N), an shRNA against TRβ2 (O), or an shRNA against TRβ1 and TRβ2 (P); followed by staining for TRβ2 (red) and DAPI (blue), and imaging under the same conditions.

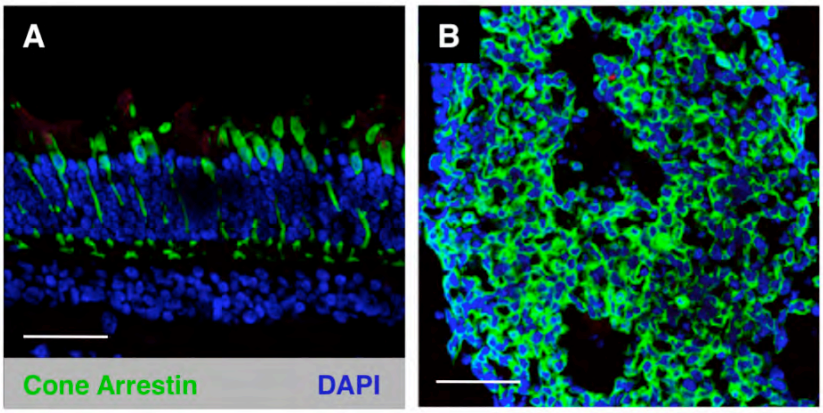


**Figure S2. Cone arrestin (green) expression in cone photoreceptors of a tumor-associated retina (A) and a representative retinoblastoma tumor (B).** DAPI staining of nuclei is shown in blue. Scale bars, 50 μm.

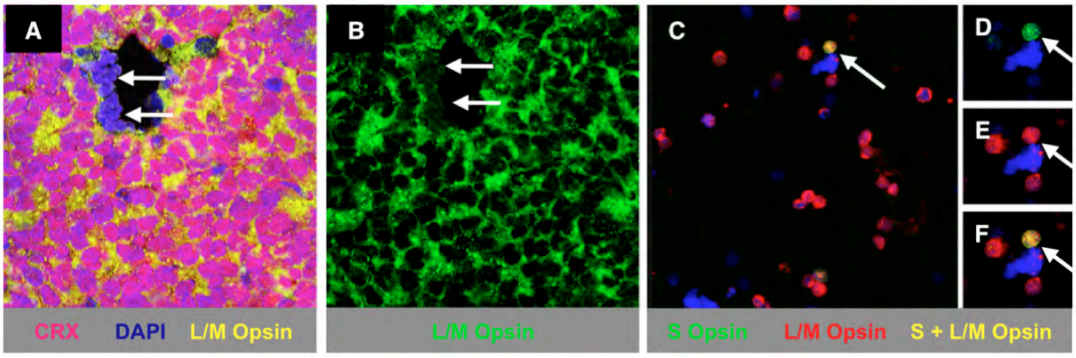


**Figure S3. Co-expression of L/M opsin with CRX and S opsin in retinoblastoma tumors.** **(A-B)** Retinoblastoma section co-stained for CRX and L/M opsin. (**A**) Merged image showing L/M opsin (yellow) surrounding $CRX^+$ nuclei (magenta), but not surrounding $CRX^-$ nuclei adjacent to a blood vessel (arrows). The section was stained sequentially with rabbit anti-L/M opsin and FITC-conjugated secondary antibody (green), followed by rabbit anti-CRX and Cy5-conjugated secondary antibody (red). Thus, CRX was marked by only Cy5 and displays as magenta when superimposed over DAPI-stained nuclei, and L/M opsin was marked by both FITC and Cy5 and displays as yellow. (**B**) The same section as in panel A, showing cytoplasmic L/M opsin (green) in the FITC channel. The results are representative of the three tumors examined.

(**C-F**) Co-expression of S and L/M opsin in dissociated retinoblastoma cells. A tumor was dissociated and cells stained for S opsin (green), L/M opsin (red), and DAPI (blue). (**C**) One S+L/M opsin-expressing cell (arrow), among a field of cells expressing only L/M opsin (red) or no opsin (blue). (**D-F**) Cell marked by arrow in (**C**) examined separately for S opsin (**D**), L/M opsin (**E**), or both (**F**). Among more than 4,000 cells examined, none had S but no L/M opsin expression.

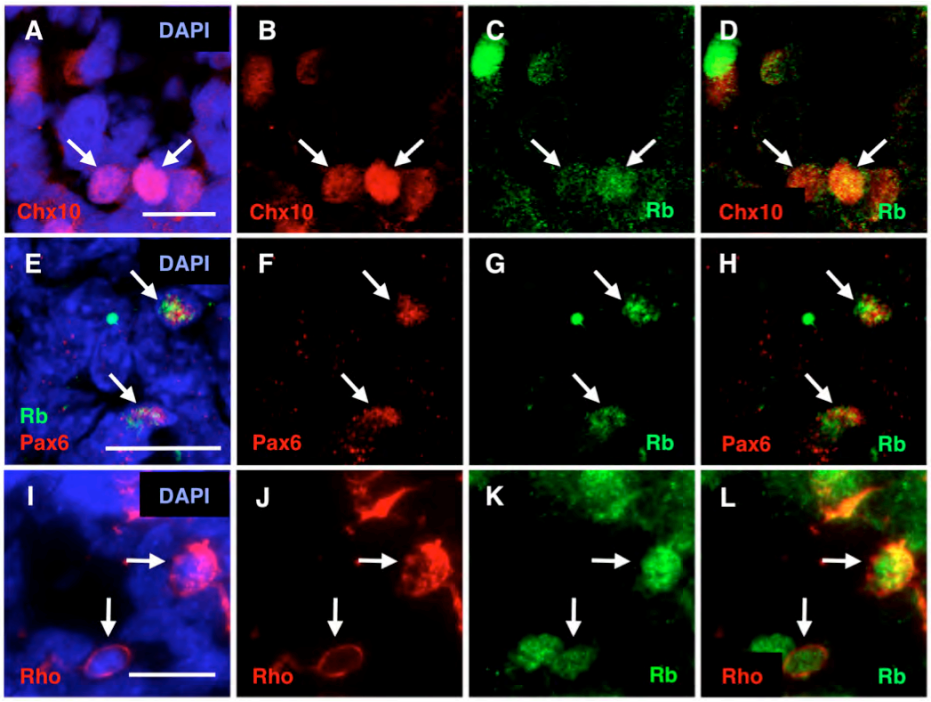


**Figure S4. Chx10$^{+}$, Pax6$^{+}$, and rhodopsin$^{+}$ cells in retinoblastomas co-express Rb.** Tumors were co-stained for Rb (green) together with Chx10 (A-D), Pax6 (E-H), or rhodopsin (I-L) (red) and DAPI (blue). Cells that stained for Chx10, Pax6, and rhodopsin also stained for Rb (arrows), whereas Rb was not detected in most of the Chx10$^{-}$, Pax6$^{-}$, or rhodopsin$^{-}$ cells. Scale bars, 20 μm.

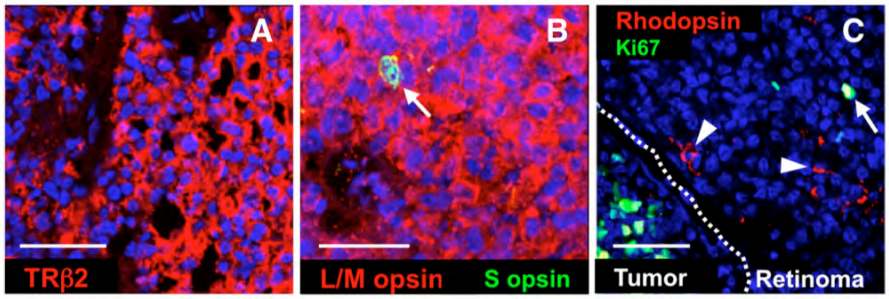


**Figure S5. Photoreceptor markers in a retinoma-like region.** One tumor had a region with retinoma-like histology and widespread expression of TRβ2 (A) and L/M opsin (B), and rare cells that co-expressed L/M and S opsin (B, arrow). Rhodopsin was detected in less than 1% of cells in the retinoma-like region (C, arrowheads), and not in the adjoining retinoblastoma tumor. The retinoma-like region also had rare Ki67$^{+}$ cells (C, arrow), but these did not co-stain for rhodopsin. Scale bars: 50 μm.

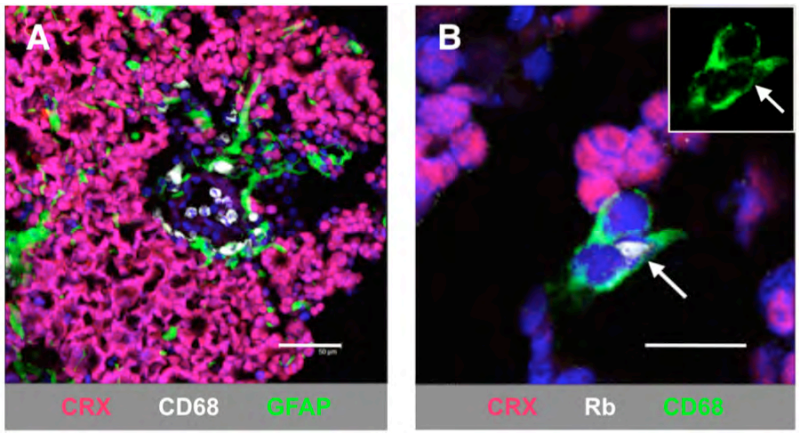


**Figure S6. Rb$^+$ and Rb$^-$ microglia in retinoblastoma.** (A) Retinoblastoma showing the majority of cells expressing CRX (red), and rare cells expressing the glial marker GFAP (green) and the microglia marker CD68 (white). As CD68 was stained prior to GFAP, the white signal specifically marks the position of the mouse anti-CD68 antibody. (B) A representative confocal image showing Rb (white) in one of three nuclei (arrow) surrounded by CD68$^+$ cytoplasm (green), indicative of Rb expression in a subset of CD68$^+$ microglia. As Rb staining was performed prior to CD68, the white signal specifically marks the position of mouse anti-Rb antibody. The inset shows cytoplasmic CD68 localization that is distinct from the nuclear Rb signal. DAPI stained nuclei are shown in blue. Scale bars: A, 50 μm; B, 20 μm.

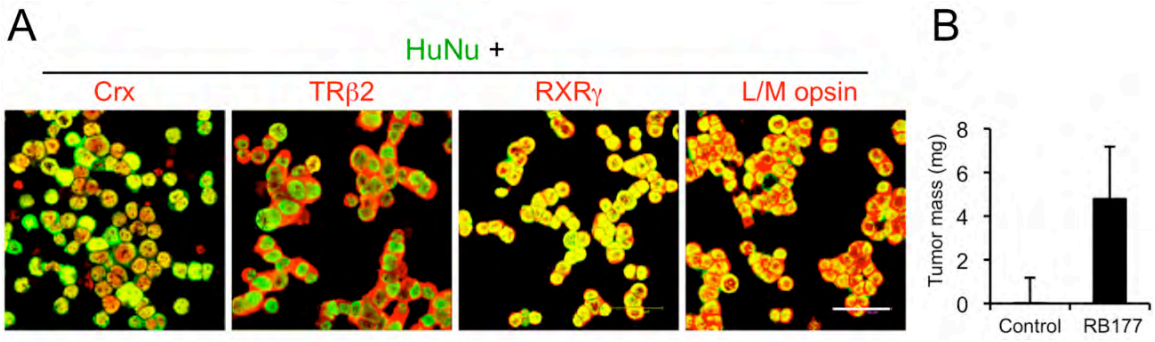


**Figure S7.** (A) RB177 cultures co-stained for human nuclear antigen (HuNu, green) and CRX, TRβ2, RXRγ, or L/M opsin (red) prior to subretinal xenograft. Among two coverslips each having > 4,000 cells, all HuNu$^+$ cells co-stained for each of the cone markers to produce a merged yellow signal, as well as a cytoplasmic red signal for L/M opsin and TRβ2. Rare HuNu$^-$ nuclei were TUNEL$^+$ and pyknotic, and concluded to be undergoing apoptosis. Scale bar, 50 μm. (B) Mean tumor mass 50 days after sub-retinal xenograft of 4 eyes with 1,000 cells, or mock engraftment of the control contra-lateral eyes.

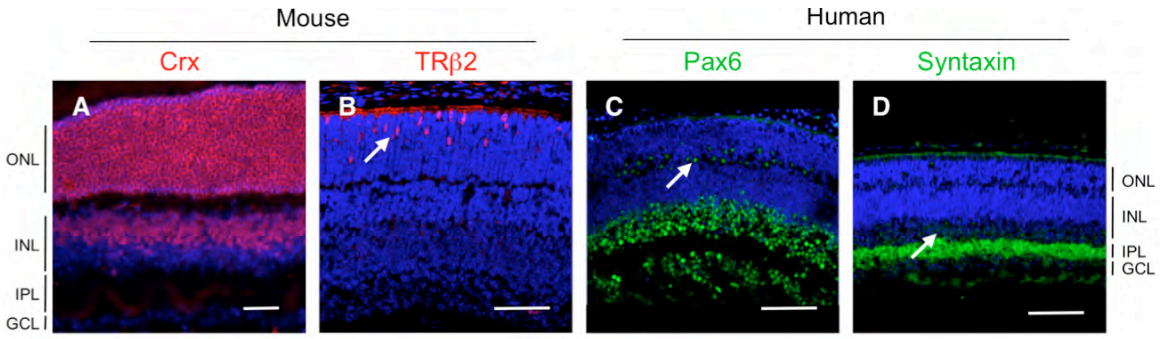


**Figure S8. A. Cross-species reactivity of Crx, TRβ2, Pax6, and syntaxin antibodies.** The same antibodies that were used in Figure 4 recognized the appropriate cell types in the normal mouse and human retinas. (A) Adult mouse retina showing Crx (red) in photoreceptors in the outer nuclear layer (ONL) and bipolar cells in the inner nuclear layer (INL). (B) Mouse P5 retina showing TRβ2 (red) in cone precursors (arrow). (C) Human wk 18 retina showing Pax6 (green) in horizontal cells (arrow), amacrine cells in the inner portion of the INL, and ganglion cells in the ganglion cell layer (GCL). (D) Human wk 18 retina showing syntaxin (green) in INL amacrine cells (arrow), in GCL ganglion cells, and in the neuronal processes in the inner plexiform layer (IPL). All retinas were co-stained with DAPI (blue). Scale bars: 50 μm.

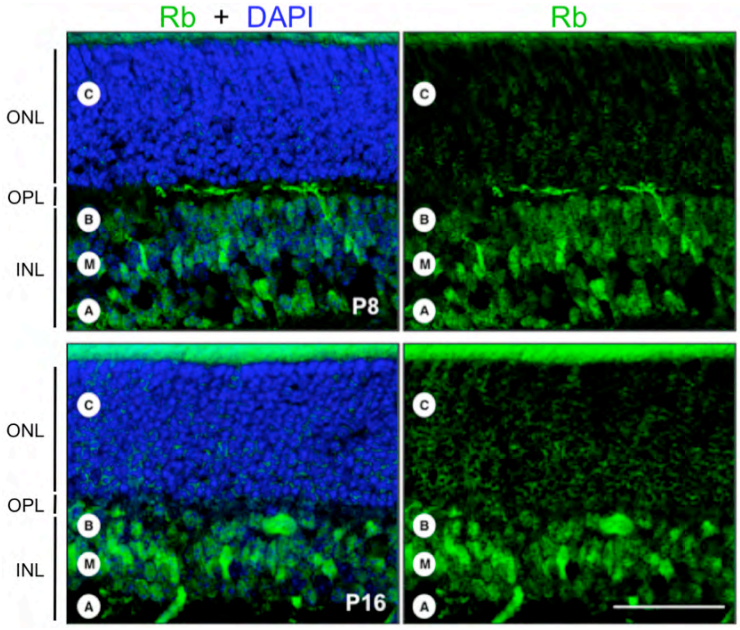


**Figure S9. Weak staining of Rb in mouse cone precursors at P8 (top) and P16 (bottom).** These ages bracket the onset of mouse L/M opsin expression at P11 (Szel et al., 1993), and display minimal Rb signal (green) in the outer nuclear layer (ONL) relative to the inner nuclear layer (INL) bipolar and Müller cells. Similarly, little or no Rb was detected in the outer nuclear layer at P0 or P5, or in the outer neuroblastic layer at E14 or E18 (data not shown), as previously reported (Spencer et al., 2005). Labels indicate approximate positions of cone (C), bipolar (B), Müller (M), and amacrine (A) cells. Scale bar, 50 μm.

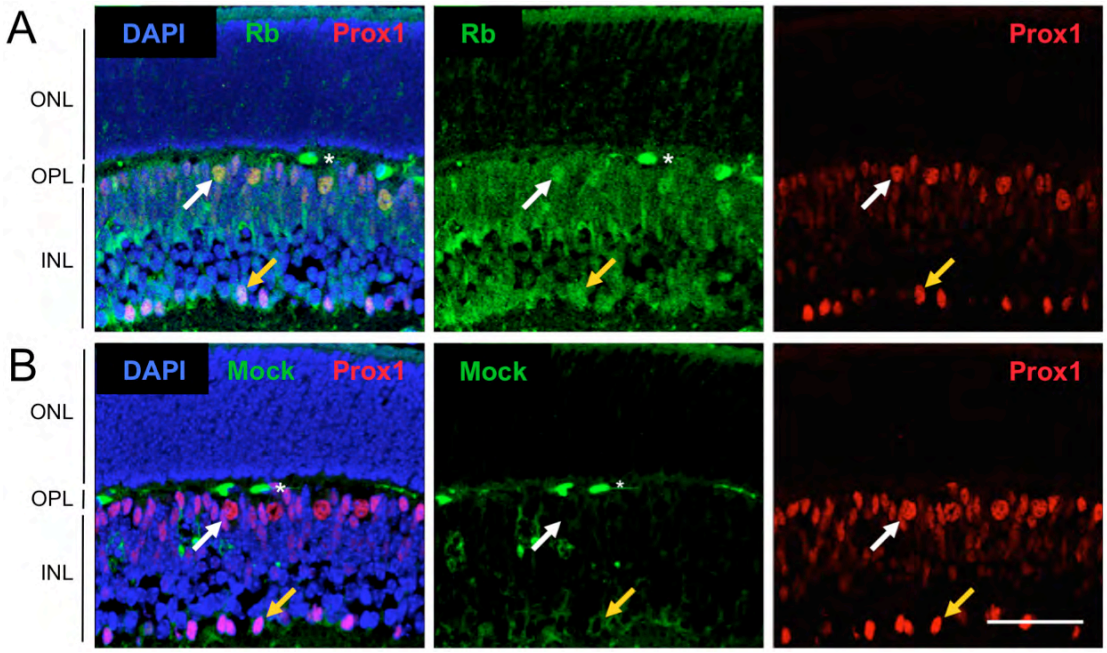


**Figure S10. Rb expression in mouse horizontal and amacrine cells.** P12 mouse retinal sections stained for Prox1 (red), and either stained (A) or mock stained (B) for Rb (green). White and yellow arrows indicate Prox1$^{+}$ horizontal and amacrine cells, respectively, and asterisks indicate a recurring antibody-independent signal (due to streptavidin-FITC binding) in the outer plexiform layer (OPL). Note Rb signal in Prox1$^{+}$ horizontal and amacrine cell nuclei (A), and antibody-independent signal in amacrine cell cytoplasm (yellow arrow in B). Scale bar, 50 mm.

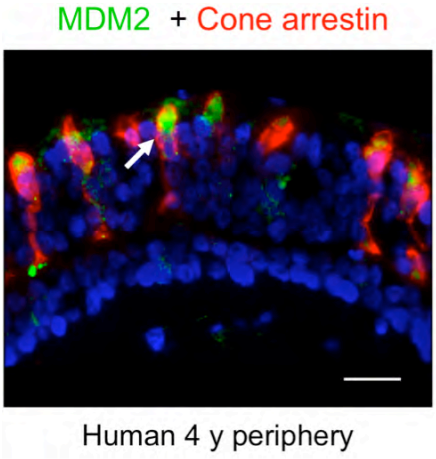


**Figure S11.** Uninvolved peripheral retina from a 4-year old retinoblastoma patient stained for MDM2 (green) and cone arrestin (red), showing prominent MDM2 expression in mature cones (arrow) but not other retinal cell types. Scale bars, 20 μm.

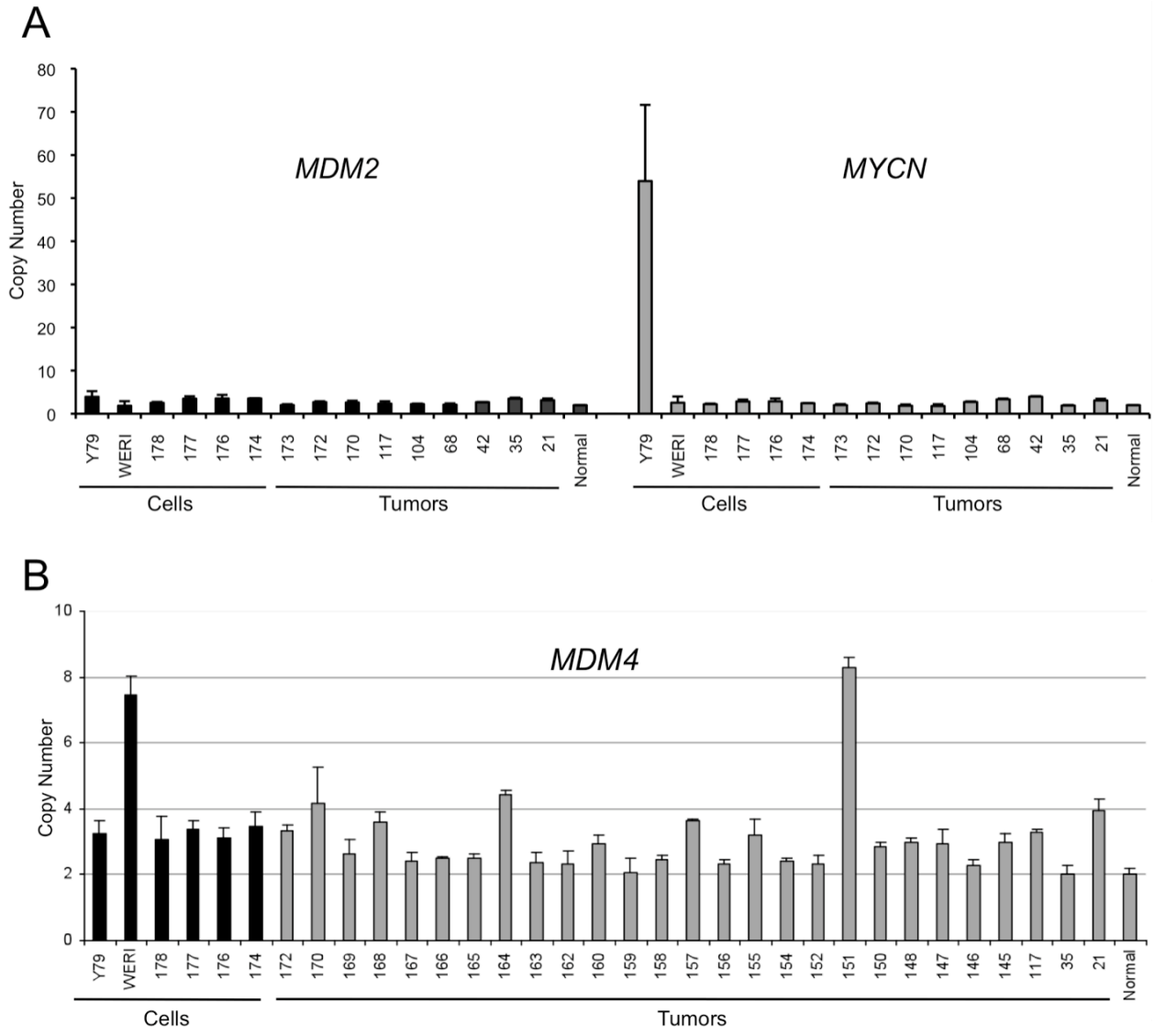


**Figure S12. *MDM2*, *MYCN*, and *MDM4* copy number in retinoblastoma cells and tumors.** Genomic DNA was isolated from established or early passage retinoblastoma cells, from retinoblastoma tumors, or from normal peripheral blood DNA, and copy number determined by qRT-PCR and normalization to the average of the *BRCA1* and *HNF4α* genes in each sample.

**(A) Near diploid *MDM2* and *MYCN* copy number in most retinoblastomas.** *MDM2* had less than a 2-fold increase in copy number in all samples, consistent with Laurie et al (2006). *MYCN* was amplified in Y79, but not in most tumors, consistent with an analysis of 87 primary retinoblastomas (Bowles et al., 2007).

**(B) *MDM4* copy number in retinoblastoma.** WERI cells had an *MDM4* copy number of ~7, corroborating the reported *MDM4* amplification (defined as copy number $\geq$ 5) in these cells (Laurie et al., 2006). However, the M*DM4* copy number ranged from 2 to 4 in most samples.

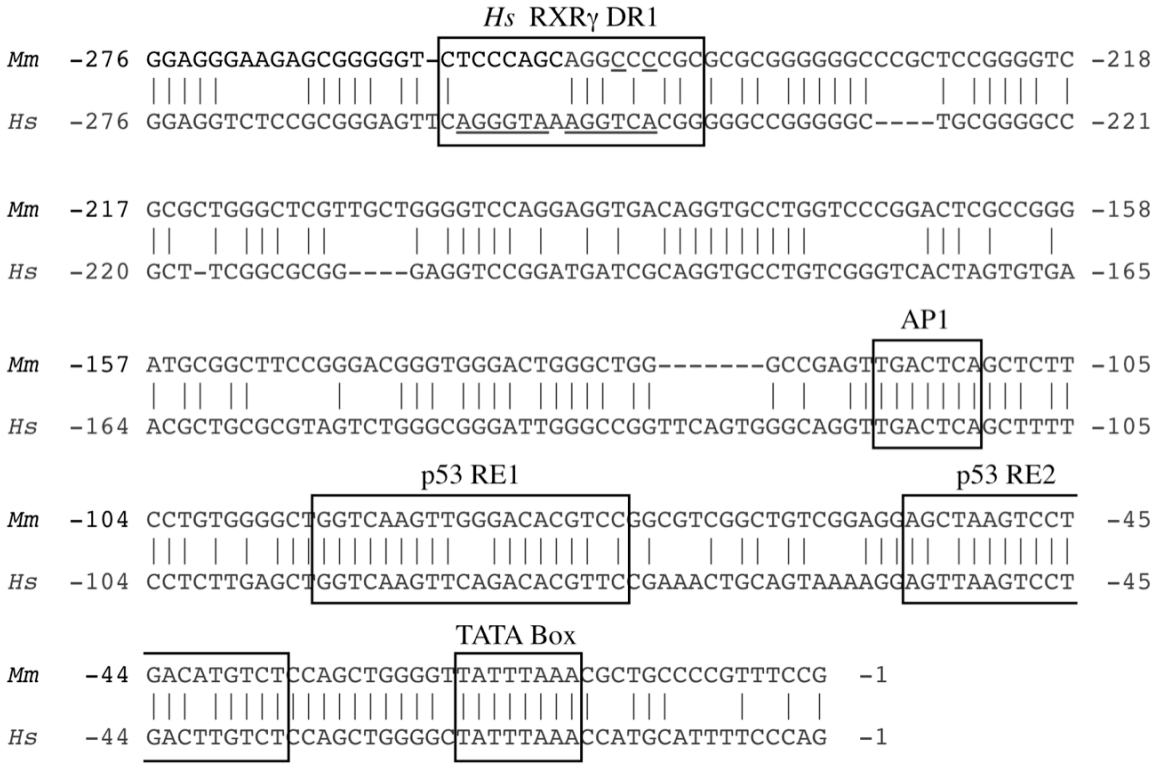


**Figure S13. Identification of an RXRγ DR1 element in the human but not mouse *MDM2* P2 promoter**. Human (*Hs*) and mouse (*Mm*) P2 promoter sequences were aligned using NCBI Blast2. The box labeled *Hs* RXRγ DR1 indicates an RXRγ consensus binding site (from Table 1 of Dowhan et al., 1994) in the human but not mouse promoter. A degenerate direct repeat separated by one base (the DR1 element) is underlined in the human sequence, and the two human-to-mouse substitutions in *P2-luc-ΔRXR* are underlined in the mouse sequence. Previously characterized AP1, p53 response, and TATA Box elements are also indicated. Numbers indicate nucleotides from the P2 transcription start site. The alignment is similar to that of Phelps *et al* (2003).

# SUPPLEMENTAL TABLES

## Table S1. Properties of Retinoblastoma Tumors in this Study

| Tumor | Age (mo) [a] | Laterality[b] | Differentiation[c] | Treatment[d] | PR Marker Quantitation[e] |
|---|---|---|---|---|---|
| 7 | *ND*[d] | *ND*[d] | + | *ND*[d] | |
| 8 | *ND*[d] | *ND*[d] | + | *ND*[d] | C, R, N |
| 18 | 8 | U | - | Carboplatin | R, N |
| 20 | 60 | U | + | *ND*[d] | R, N |
| 21 | 45 | U | + | None | |
| 26 | 18/18 | B | - | None | |
| 28 | 34 | U | +/- | None | A, N |
| 30 | 24 | U | - | *ND*[d] | C, R, N |
| 31 | 40 | U | + | None | C, R, N |
| 35 | 7 | U | + | None | A, S, N |
| 42 | 25 | U | + | None | C, T, L/M, N |
| 43 | 16 | B | - | *ND*[d] | |
| 44 | 24 | U | + | None | C, R, N |
| 49 | 24 | U | + | None | C, R, L/M, N |
| 51 | 36/20 | B | + | Ext Beam Rad<br>Cryotherapy | R, N |
| 55 | 20 | U | +/- | None | T, L/M, N |
| 56 | 125 | B | +/- | Carboplatin<br>Cryotherapy<br>Plaque Rad<br>Diathermy | R, L/M, S, N |
| 63 | 20 | B | - | Carboplatin | C, N |
| 64 | 23 | U | +/- | Carboplatin | L/M, S, N |
| 65 | 32/35 | B | + | None | L/M, A, S, N |
| 68 | 2 | B | +++ | None | C, T, L/M, S, N |
| 70 | 48 | B | +/- | *ND*[d] | A, N |
| 75 | 11 | B | - | Chemotherapy<br>PC<br>Plaque Rad<br>Cryotherapy<br>Laser | C, T, L/M, A, N |
| 87 | 8 | B | + | Carboplatin<br>Ext Beam Rad<br>Cryotherapy | T, N |
| 88 | 8 | B | +/- | Carboplatin<br>Ext Beam Rad<br>Laser | S, N |
| 92 | 24 | B | ++ | Chemo<br>Plaque Rad<br>Local chemo | C, L/M, S, N |
| 94 | 30 | U | + | Carboplatin | |
| 99 | 60 | U | - | *ND*[d] | C, T, N |
| 103 | 7 | U | + | Carboplatin<br>Laser | |
| 104 | 36 | U | +/- | *ND*[d] | T, L/M, S, N |
| 106 | 8 | U | + | None | T, N |

| | | | | | |
|---|---|---|---|---|---|
| 109 | 19 | B | +/- | Laser Cryotherapy | T, S, N |
| 110 | 10 | U | + | None | A, N |
| 114 | 25 | U | +/- | None | C, N |
| 115 | 12 | U | + | None | C, L/M, A, S, N |
| 117 | 37 | B | +++ | Local chemo Cryotherapy Laser | L/M, S, N |
| 119 | 4 | B | - | Carboplatin Laser | R, N |
| 122 | 12 | U | | None | |
| 135 | 29 | U | + | None | |
| 139 | 10 | U | - | None | T, R, L/M, A, S, N |
| 155 | 31 | U | +/- | None | L/M, N |
| 170 | 24 | U | + | None | C, A, N |
| 172 | 30 | U | - | None | C, A, S, N |
| 173 | 35 | U | + | Carboplatin Laser | S, N |
| 174 | 17 | U | + | None | |

Notes:

a. Age at enucleation in months. Two numbers indicate double enucleation

b. B, Bilateral; U, unilateral;

c. Indicates the percentage of cells forming rosettes in the most differentiated regions of each tumor, -, 0%; +/-, 0-1%; +, 1-10% ++, 10-50%; +++, >50%.

d. *ND*, data not available.

e. Tumors that were evaluated for the percentage of cells expressing each of the photoreceptor (PR) markers that are detailed in Table S2 and Figure 1P. C, Crx; T, TR$\beta$2; R, RXR$\gamma$; A, cone arrestin; L/M, L/M opsin; S, S opsin; N, NRL.

**Table S2. Retinal cell markers detected in retinoblastoma tumors.**

| Marker | Cell Types [a] | Tumors Analyzed | Tumors with (+) Cells (%) | % of (+) Cells in (+) Tumors (St. Dev.) [b] | $Rb^{+}$ [c] |
|---|---|---|---|---|---|
| CRX | C, R, B | 40 | 40 (100) | 98.1 (1.0) | N |
| RXRγ | C, G | 40 | 40 (100) | 97.6 (1.6) | N |
| TRβ2 | C | 40 | 40 (100) | 97.8 (1.2) | N |
| NRL | R | 40 | 1 (2.5) | < 0.01 | Y |
| Cone Arrestin | C | 22 | 22 (100) | 97.0 (1.5) | n.d. |
| L/M opsin | C | 22 | 22 (100) | 97.8 (1.4) | N |
| S opsin | C | 22 | 16 (73) | 0.28 (0.38) | N |
| Rhodopsin | R | 20 | 1 (5) | < 0.01 % | Y |
| Chx10 | P, B, M | 15 | 2 (13) | < 0.01 % | Y |
| Pax6 | P, G, A, H, Rpe | 17 | 5 (29) | < 0.01 % | Y |
| Syntaxin | A, H | 12 | 0 (0) | -- | |
| Brn3b | G | 8 | 0 (0) | -- | |
| Prox1 | P, H | 8 | 0 (0) | -- | |
| Nestin | P, M, As | 20 | 20 (100) | ~ 2 | Y (~90%) |
| GFAP | M, As | 20 | 20 (100) | ~ 2 | Y (~90%) |
| CD68 | Mi | 10 | 10 (100) | ~ 0.3 -1% | Y (~50%) |

[a] A, amacrine; As, astrocyte; B, bipolar; C, cone; G, ganglion cell; H, horizontal cell; M, Müller glia; Mi, microglia; P, retinal progenitor cell; R, rod; Rpe, retinal pigment epithelium.

[b] Mean percentages determined by evaluating >1,000 cells for each marker, in each of at least 10 representative tumors. The percentage of marker positive cells for each tumor was determined by counting all marker positive and negative cells in 1 – 3 micrographs, with each micrograph having ~700 - 1,100 cells. Values for GFAP and Nestin were estimated due to difficulty attributing positive-staining cellular processes to specific nuclei.

[c] Indicates whether cells expressing each marker also express Rb. N, No; Y, Yes; n.d., not determined.

## Supplemental Experimental Procedures

**Samples.** Human retinoblastoma tissue and fetal eyes were obtained with informed consent and under protocols approved by the Weill Cornell Medical College and Memorial Sloan-Kettering Cancer Center Institutional Review Boards. 40 tumors were randomly chosen from a bank of more than 100 samples (Table S1). Murine eyes were obtained and xenografts performed under protocols approved by the Weill-Cornell Institutional Animal Care and Use Committee. Retinal tumors from chimeric Rb/p130-deficient mice were as described (Dannenberg et al., 2004).

**Sample Preparation**

Human fetal and mouse retinas were prepared as described (Lee et al., 2006). Briefly, the cornea and lens were removed and eyes fixed in 4% paraformaldehyde (PFA) in PBS (PFA/PBS) overnight at 4°C, incubated in 30% sucrose/PBS overnight at 4°C, embedded in one part 30% sucrose/PBS and two parts optimal cutting temperature compound (OCT; Miles Laboratories, Elkhart, IN), frozen, sectioned at 5 to 10 $\mu$m, and cryosections affixed to poly-L-lysine coated slides. Mouse birth dates were designated P0.

Retinoblastoma tumors were removed from the globe and then processed as for retinal samples. Dissociated retinoblastoma cells were prepared after culturing freshly explanted tumors for one day in RB Culture Medium (see Retinoblastoma Cell Culture, below). Cells were dissociated by gentle triteration, spread on poly-L-lysine coated slides, and incubated in a humidified incubator at 5% $CO_2$ and 37° C for 3 h. The medium was removed and the cells fixed in 4% PFA/PBS, gently rinsed with TBS, dried, and stored at –20 °C. Mouse xenografts were from eyes removed immediately after carbon dioxide asphyxiation, punctured through the cornea, and embedded and sectioned as described for human retinas. Mouse and human tumors were compared using paraffin-embedded samples sectioned at 5 $\mu$m.

**Immunofluorescence analysis of frozen sections and dissociated tumor cells**

Immunofluorescence staining was performed as described (Lee et al., 2006), with the following modifications.

Preparation of frozen sections

*For all staining of dissociated tumor cells, staining of human and mouse (P8-P42) retinas (except rabbit anti-N-Myc), and cone arrestin and HuNu staining of retinoblastomas*: Sections were post fixed in 4% PFA/PBS for 5 min, washed in 0.15 M NaCl/20 mM Tris (pH 8.0; TBS), treated with

1 mM EDTA/TBS for 5 min at room temperature, washed with TBS, treated with 1% sodium borohydrite in deionized water for 5 min, and washed with TBS.

*For all other staining of retinoblastoma frozen sections, mouse retina (E14-P5), and rabbit N-Myc staining of human and mouse retina*: Sections were post fixed in 4% PFA/PBS for 5 min, washed with TBS, dried for 10 min at 37 °C, heated in 250 ml 1mM EDTA for 20 min at 95-100 °C (by microwave at high power for 2.5 min followed by heating at low power for 17.5 min), treated with 1% sodium borohydrite in deionized water for 5 min, and washed with TBS.

Immunostaining of frozen sections

*For staining with mouse antibodies to Rb, HuNu, CD68, Pax6, syntaxin, Ki67, cone arrestin, MDM2, and N-MYC*: Sections were treated with ABC kit reagent A (Vector Laboratories, Burlingame, CA) in TBS for 15 min, washed in TBS, treated with ABC kit reagent B (Vector Laboratories) in TBS for 15 min, washed in TBS, blocked and permeabilized in 2.5% horse serum, 2.5% donkey serum, 2.5% human serum, 1% nonfat milk, 1% BSA, 0.1% Triton-X-100, and 0.05% Tween-20 in TBS (block 1) for 20 min, incubated in the above mouse primary antibodies in block 1 overnight (3-4 days for Rb and Mdm2 staining of mouse retina) at 4°C, and washed in TBS. They were then incubated in biotinylated horse anti-mouse antibody (BA-2000, Vector Laboratories; 1:135) in block 1 for 30 min, washed in TBS, washed in 0.1 M sodium bicarbonate and 0.15 M NaCl pH 8.2, with 10 mM HEPES (balanced saline), incubated with FITC-conjugated streptavidin (Vector Laboratories; 1:175) in balanced saline, and washed with TBS.

*For co-staining analyses with all other antibodies, or when Pax6, CD68, or Ki67 were co-stained with Rb:* On completing the first staining reaction as described above, sections were incubated in block 1 for 20 min, incubated overnight with primary antibody in block 1, washed in TBS, incubated for 30 min in block 1 with Cy3 or Cy5 conjugated secondary antibody, and washed in TBS. Sections were then stained with 4', 6'-diamino-2-phenylindole (DAPI) in TBS, and analyzed by inverted immunofluorescent microscopy (Axiovert 35, ZEISS, Germany) or confocal microscopy (DMIRE2, Leica, Wetzlar, Germany).

The specificity of all antibodies used in co-staining analyses was confirmed by staining in parallel with control mouse or rabbit IgG. The specificity of other antibodies was confirmed by staining in parallel with a same species control or with no primary antibody.

**Immunofluorescence analysis of paraffin-embedded sections**

Human retinoblastoma and mouse retinal tumor sections were deparaffinized by incubating in citrus clearing solvent three times for 3 min each, hydrated in a decreasing ethanol gradient, washed with TBS, heated in 1mM EDTA at high power for 3 min and at low power for one hour to maintain a temperature of >95°C, treated with sodium borohydrite, washed with TBS, and stained as for frozen samples.

**Antibodies**

Mouse antibodies to Rb for mouse and human tissue (G3-245, 554136, BD-PharMingen, San Diego, CA; 1:100) or for human tissue (Rb-Ab5, D36802, Calbiochem, San Diego, CA; 1:50), Pax6 (P3U1, Developmental Studies Hybridoma Bank, Iowa City, IO; 1:30), Syntaxin (HPC-1, S0664, Sigma, St. Louis, MO; 1:100), Ki67 (clone B56, 550609, Becton Dickenson, Franklin Lakes, NJ; 1:50), HuNu (MAB1281, Chemicon, Temecula, CA; 1:100), GFAP (Clone G-A-5, G3893, Sigma; 1:50), rhodopsin (Adamus et al., 1988) (B6-30 ascites, gift of P. Hargrave; 1:6,000), cone arrestin (Wikler et al., 1997) (7G6, gift of Peter R. MacLeish; 1:500), and CD68 (PGM1, M0876, Dako, Glostrup Denmark; 1:100), MDM2 (SMP-14, Santa Cruz Biotechnology; 1:50), and N-Myc (EMD Biosciences NCM II 100; 1:200).

Rabbit antibodies to CRX (Hodges et al., 2002, 1:50 after purification on Econo-Pac serum IgG columns (Bio-Rad, Hercules, CA)), Prox1 (AB11941-100, Abcam, Cambridge, MA; 1:300), Nrl (Swain et al., 2001) (gift of Anand Swaroop; 1:1,000), TRβ2 (Ng et al., 2009) (1:2,000), RXRγ (E4331, Spring Bioscience, Fremont, CA; 1:1.5), cleaved caspase 3 (#96643, Cell Signaling Technology, Danvers, MA; 1:100), L/M opsin (Wang et al., 1992) (JH492, 1:6,000) and Blue (S) opsin (Wang et al., 1992) (JH455, 1:6,000, gifts of Jeremy Nathans), N-Myc (Cell Signaling Technology #9405), Nestin (AB5922, Chemicon; 1:200), and Pax6 (CRP, Denver PA; 1:100, for co-staining of Rb and Pax6). Biotin labeled antibody to phosphorylated histone 3 (16-189, Upstate Biotechnology, Millipore, Billerica, MA; 1:200).

Goat antibodies to Brn-3b (C-19, SC-6026, Santa Cruz, CA; 1:200) and Blue (S) opsin (SC14363, Santa Cruz; 1:50, for co-staining of L/M and S opsin).

Sheep antibody to Chx10 (N-terminal antibody, X1180P, Exalpha Biologicals, Maynard, MA; 1:400).

Secondary antibodies For mouse antibodies to Rb, cone arrestin, HuNu, syntaxin, MDM2, N-Myc, and some Pax6, CD68, and Ki67 staining, we used biotin-conjugated horse anti-mouse (BA-2000, 1:135), followed by FITC-conjugated streptavidin (SA-5001, Vector Laboratories;

1:175). Occasionally, biotin-conjugated goat anti-rabbit (BA-1000, 1:135), followed by FITC-conjugated streptavidin (SA-5001, Vector Laboratories; 1:175) was used for TRβ2, RXRγ, and N-Myc staining. FITC-conjugated streptavidin (SA-5001, Vector Laboratories; 1:175) was used for co-staining of biotin-conjugated anti-phosphohistone H3. Cy3-conjugated donkey anti-sheep (713-165-147, Jackson; 1:150) was used for Chx10. Cy5-conjugated Donkey anti-goat (705-175-147, Jackson, 1:150) and Alexa 488-conjugated donkey anti-goat (A-11055, Molecular Probes, Invitrogen; 1:200) were used for S opsin and Brn-3b. Cy3 (715-165-150 and 711-165-152) or Cy5 (715-175-151 and 711-175-152)-conjugated donkey anti-mouse or anti-rabbit (Jackson Laboratories, Bar Harbor, Maine; 1:150) were used for all other staining.

**Rb + CRX or Rb + GFAP immunofluorescence staining followed by *RB1* fluorescent in situ hybridization (FISH)**

We identified and examined two retinoblastomas (RB109 and RB64) with one deleted *RB1* allele and one retained *RB1* allele among 10 samples. Five-micron thick cryosections were stained with Rb-Ab5 (1:50) followed by biotin-conjugated horse anti-mouse and FITC-conjugated streptavidin as described above. Sections were then stained with CRX antibody (1:50) or GFAP antibody (1:50), followed by Cy5-conjugated donkey anti-rabbit or donkey anti-mouse secondary antibodies. Fifteen confocal images of each section (4-6 sections of each tumor) were obtained to identify $Rb^{-}$,$CRX^{-}$ or $Rb^{-}GFAP^{+}$ cells, as well as cells with other immunophenotypes.

The immunostained and imaged sections were probed by FISH for *RB1* using Vysis LSI 13q14.3 D13S319 (*RB1*) SpectrumOrange, LSI 13q34 SpectrumAqua, and CEP 12 (12p11.1-q11) SpectrumGreen Multi-color Probes (32-191025, Vysis Inc. Des Plaines, IL). LSI 13q34 SpectrumAqua probe was used as an internal hybridization control (Elnenaei et al., 2003). Briefly, the immunostained and imaged sections were washed twice for 10 min in PBS at room temperature, followed by a pepsin (1%, in HCl, pH 1.5) digestion at 37°C for 5~8 min, and dehydrated in an ethanol gradient (70%, 90%, 100%, each 2 min), and air dried for 10 min. The probes were combined and added to the air-dried slides, and the slides cover-slipped and sealed with rubber cement. Sections with the probes were co-denatured for 5 min at 94 °C and hybridized overnight at 37°C in a humidified chamber. The slides were washed in 0.4×SSC/0.3%NP-40 for 2 min at 77 °C and 2×SSC/0.1%NP-40 for 1 min at room temperature respectively under a dark light. After post-hybridization washes, the slides were counterstained with DAPI and cover-slipped. Locations originally imaged for Rb and CRX staining, or for Rb and GFAP staining, were re-imaged under the Zeiss immunofluorescence microscope equipped with Meta-systems Isis software, and FISH signals for cells of interest evaluated. On Rb and

CRX co-stained sections, $Rb^{-}$,$CRX^{-}$, $Rb^{+}$,$CRX^{-}$, and $Rb^{-}$,$CRX^{+}$ cells with two 13q34 FISH signals were selected and the number of *RB1* FISH signals recorded. The aqua 13q34 probe signal was redesignated as green for display purposes. FISH analyses were performed by XLX, and confirmed by an investigator (DC), who was blinded to sample identities.

**Retinoblastoma xenografts**

Xenografts were performed on 3-week-old athymic (nude) mice (Taconic, Hudson, NY). RB170 retinoblastoma was cultured as described in *Retinoblastoma cell culture*, below. Cells were collected after 11 days, dissociated by pipetting, and resuspended in the above medium at 1 x $10^5$ cells/µl and held on ice. For all xenografts, mice were anaesthetised by intra-peritoneal injection of a ketamine (final concentration; 10mg/ml) and xylazine (final concentration; 1mg/ml) mixture (0.01ml/g mouse weight), and with Alcaine (proparacaine HCL) ocular surface anesthesia. Under a surgical microscope, a 30 gauge sharp needle was used to make two holes through the sclera, one into the intravitreal space to reduce intraocular pressure; and one tangentially through the sclera into the sub-retinal space for injection (MacLaren et al., 2006). 2 x $10^5$ cells in 2 $\mu$l medium were injected through the second hole into sub-retinal space, with some cells potentially entering the vitreous, using a 1.5 cm, 33 gauge blunt end microinjection needle (7803-05, Hamilton, Reno, NV). After injection, eyes were covered with ophthalmic bacitracin ointment.

For serial xenografts, cells were initially engrafted to one eye of each of 11 mice. After 60 days, mice were sacrificed by $CO_2$ asphyxiation and the eyes enucleated. Extraocular tissue was removed and eye mass determined. Tumor mass was calculated by the mass of the engrafted eye minus the average mass of control uninjected eyes. Several eyes were divided into two parts. One part was cultured for 1 day and used for a second xenograft into 4 eyes. The other part was fixed in 4% PFA in TBS overnight, incubated in 30% sucrose/PBS overnight at 4°C, embedded in one part 30% sucrose/PBS and two parts OCT, frozen, and sectioned at 5 to 10 $\mu$m. After 60 days, eyes from the second xenograft were treated as for the first xenograft, with a portion cultured for one day and used for tertiary xenografts into six eyes, and the tertiary grafts were examined after 48 days. Xenograft sections were co-stained with antibodies to HuNu (imaged as green) and either CRX, RXRγ, TRβ2 or L/M opsin (imaged as red), and the proportion of $HuNu^{+}$ cells that lacked detectable CRX, RXRγ, TRβ2 or L/M opsin was determined by inspection of at least four confocal images.

**Copy number analysis**

DNA was isolated with QiaAMP DNA Mini kit (Qiagen), and relative mRNA levels determined in triplicate by qPCR using QuantiTect SYBR Green PCR Kit (Qiagen) on an Applied Biosystems ABI 7900HT Sequence Detection System. The DNA content of *MDM2*, *MYCN*, and *MDM4* was normalized to the average of the *BRCA1* and *HNF4a* reference genes in each sample and compared to a peripheral blood DNA. qPCR primers were:

| Gene | Sense Primer | Anti-sense Primer |
|---|---|---|
| MDM2 | GACTATTCTCAGCCATCAACTTCTAG | TCTTCCCTTTCAAACTCTTTCACATC |
| MYCN | TGATGAAGATGATGAAGAGGAAGATG | CAGTGATGGTGAATGTGGTGAC |
| MDM4 | AGGAAGGATTGGTATTCAGATTGTTC | TAACGACAGGAGCCGAAATGG |
| BRCA1 | TGAACCTATAAGCAGCAGTATAAGC | AAGACTTCCTCCTCAGCCTATTC |
| HNF4A | GGAGCCGTCGCCACAATC | CTAGATAACTTCCTGCTTGGTGATG |

**Retinoblastoma cell culture**

Primary retinoblastomas were explanted to RB culture medium consisting of IMDM (Invitrogen, Carlsbad, CA) with 15% FBS (ATCC, Manassas, VA), 2mM glutamine (Mediatech Inc., Herndon, VA), 0.1% Fungen (Invivogen, San Diego, CA), 5μM Plasmocin (Invivogen), 55μM beta-mercaptoethanol (Invitrogen), and 10μg/ml insulin (I5500, Sigma, St. Louis MO) as described (DiCiommo et al., 2004), sometimes growing on a feeder cells derived from tumor-associated glia, at 37° C in a humidified incubator with 5% $CO_2$.

**shRNA constructs**

Effective pLKO lentiviral shRNA vectors from the TRC library (Open Biosystems) were for *MDM2*: TRCN0000003376, TRCN0000003377, and TRCN0000003380; for *TRβ*-common: TRCN0000020344; for RXRγ: TRCN0000021639 and TRCN0000021640; and for *MYCN*: TRCN0000020695 and TRCN0000020696, and in the text they are designated by pLKO- followed by the name of the target gene and the last 3 digits of the above identifiers. The pLKO scrambled control was Addgene plasmid 1864 (Sarbassov et al., 2005). The TRβ2 shRNA vectors pLKO-TRβ2-99-119 and pLKO-TRβ2-251-271; and the *CDKN2A*$^{ARF}$ shRNA vectors pLKO-CDKN2A-144-164 and pLKO-CDKN2A-331-351 were designed using siDirect (http://genomics.jp/sidirect/) and the TRC-recommended cloning strategy and sub-cloning conditions (www.addgene.org/pgvec1?f=v&cmd=showfile&file=protocols) with deoxyoligonucleotides for DNA-directed RNAi (Integrated DNA Technologies), and are designated in the text according to the first shRNA target nucleotide RefSeq positions. shRNA sequences extending from the pLKO AgeI to EcoRI site, with numbers designating RefSeq positions of targeted sequences (underlined) were:

TR$\beta$2-99-119:
5' ACCGGGGGTTACAGCGGTTGTGATGCCTCGAGGCATCACAACCGCTGTAACCCTTTTTGAATTC

TR$\beta$2-251-271:
5' ACCGGGCCAACCTGAATATTTCACTGACTCGAGTCAGTGAAATATTCAGGTTGGCTTTTTGAATTC

pLKO-CDKN2A-144-164:
5' ACCGGCTGGAGGCGGCGAGAACATGGAGTACTCCATGTTCTCGCCGCCTCCAGTTTTTGAATTC

pLKO-CDKN2A-331-351:
5' ACCGGGCAGCCGCTTCCTAGAAGACCAGTACTGGTCTTCTAGGAAGCGGCTGCTTTTTGAATTC.

**Lentivirus production and retinoblastoma cell infections**

Concentrated pLKO lentiviral shRNA vectors were produced in 293T cells using pVSVg and helper constructs, and Lipofectamine 2000 (InVitrogen). Virus harvested 48 and 72 h after transfection was combined, concentrated ~100-fold by centrifugation and resuspension in growth medium, and 100-300 μl of concentrated virus used to infect 5 x $10^5$ retinoblastoma cells suspended in 500 μl of growth medium, in the presence of 4 μg/ml polybrene. Infected cells were diluted five-fold in growth media after 24 h, and cells were selected with 1.4 – 3 μg/ml puromycin for 48-72 h, starting 48 h after infection, and subsequently fed every 2 days by replacing two-thirds of the medium.

**Knockdown analyses**

Reverse transcription and quantitative real time PCR (qRT-PCR) Total RNA was isolated from puromycin-selected cells using RNeasy Mini Kit (Qiagen) or GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). RNA reverse transcription was performed with ImProm-II™ Reverse Transcription System (Promega). Primers were designed by Beacon Designer software (Premier Biosoft International). Relative mRNA levels were determined by qRT-PCR using QuantiTect SYBR Green PCR Kit (Qiagen) on an Applied Biosystems ABI 7900HT Sequence Detection System. Two samples for each group were collected and evaluated in triplicate and normalized to β-actin mRNA quantitated in parallel. qRT-PCR primers were:

| Gene | Sense Primer | Anti-sense Primer |
|---|---|---|
| TRβ2 | AAACTAGAAACTGAACCAGGGAAAC | GCATCACAACCGCTGTAACC |
| MDM2 | GACTATTCTCAGCCATCAACTTCTAG | TCTTCCCTTTCAAACTCTTTCACATC |
| β-actin | GCAAGCAGGAGTATGACGAGTC | CAAGAAAGGGTGTAACGCAACTAAG |
| CDKN2A$^{ARF}$ | CCTGGAGGCGGCGAGAAC | CGGGATGTGAACCACGAAAACC |
| MYCN | TGATGAAGATGATGAAGAGGAAGATG | CAGTGATGGTGAATGTGGTGAC |
| RXRγ | AGCGATGACCACTCTTGTTAG | TCGTCAGTTCATGTTCCTCTC |
| CDKN1A (p21) | CCCCTTTCCTGGACACTCAG | CACCCTGCCCAACCTTAGAG |
| 14-3-3 | GAGCCATGGAGAGAGCCAGT | AGAGCAGGTTTCGCTCTTCG |

Immunoblotting Infected and puromycin selected cells were gently pelleted, suspended in PBS, re-pelleted, stored at -80º C, and lysed in ELB (150 mM NaCl, 50 mM HEPES pH 7.4, 0.1% NF40, 5 mM EDTA, 2mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 10 mM NaF, 1 μg/ml aprotinin). Proteins were separated on 4–15% Ready Gel Tris-HCl (BioRad), transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences), and membranes incubated with antibodies to MDM2 (SMP-14, Santa Cruz Biotechnology; 1:200), TRβ2 (1:2,000), p14ARF (4C6/4, Cell Signaling Technology; 1:1,000), α-tubulin (DM1A, K4805, Sigma; 1:1,000), or γ-tubulin (GTU-88, T6557, Sigma; 1:500), and detected with HRP-conjugated sheep anti-mouse (NXA931, 1:25,000) or anti-rabbit (NA934, 1:150,000, Amersham Biosciences), using ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and HyBlot CL X-Ray film (Denville Scientific Inc).

TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) was performed 8 days after the initial lentivirus infection. Cells were attached to poly-L-lysine coated cover slips by culturing for 3 h in complete medium without insulin, fixed with 4% paraformaldhyde in TBS for 5 min, rinsed with TBS, dried at 37 ˚C for 30 min, and stored at -20 ˚C. TUNEL was performed using the Trevigen TumorTACS™ *In Situ* Apoptosis Detection Kit (4815-30-K), modified as follows. Cover slips were rinsed in 1X TBS for 5 min, treated for 30 min with Cytonin, washed two times in deionized water for 2 min each, immersed in methanol for 5 min, washed in 1X TBS for 1 min, immersed in 1X TdT Labeling Buffer for 5 min, incubated for 60 min at 37 ˚C in a humidity chamber with 50 $\mu$l of Labeling Reaction Mix (made with 5$\mu$l 10X buffer 4 (New England Biolabs), 5 $\mu$l 2.5mM CoCl2 solution, 0.4 $\mu$l 1 mM Bio-16-dUTP (Roche), 0.5 $\mu$l 20 units/ml terminal deoxynucleotidyl transferase (TdT, New England Biolabs), and 39 $\mu$l $dH_2O$), immersed in 1X TdT Stop Buffer for 5 min, and washed twice for 2 min each in 1X TBS. Samples were then covered with 100 $\mu$l of FITC-conjugated streptavidin (SA-5001, Vector Laboratories, 1:175), and incubated for 20 min at room temperature in a humidified chamber, washed twice for 2 min each in 1X TBS, stained with DAPI for 5 min, washed in $dH_2O$, and mounted onto glass slides using Vectashield mounting medium (Vector Laboratories).

Xenografts of TRβ2 knockdown cells. 2 x $10^5$ Y79 cells were transduced with pLKO-TRβ2-99, pLKO-TRβ2-344, or the pLKO scrambled control, selected in puromycin as above, engrafted, and analyzed after 50 days, as described in *Retinoblastoma xenografts*.

**shRNA-resistant *MDM2* cDNA constructs and cells**

*UGINZ* lentiviral expression vector. *UGINZ* was derived from and resembles *CMV-GIN-Zeo* (Open Biosystems), but has an *Ubiquitin C* promoter in place of the *CMV* promoter and has the *mir* region removed. It was constructed as follows: First, the AfeI-XbaI *CMV* promoter segment of an Open Biosystems *CMV-GIN-Zeo* shRNA construct was replaced with an NheI-AfeI *EF1α* promoter fragment, and the *mir* region HpaI-MluI fragment excised to produce *EGINZΔ*, and the GFP cDNA was replaced with polylinker sequences to produce *EINZΔ*. Second, the PacI-BsrGI *Ubiquitin C* promoter + *eGFP segment* of *pFUGW* (Lois et al., 2002) was used to replace the XbaI-BsrGI *CMV* promoter + *Turbo GFP* segment of *pGIPZ-NSC* (Open Biosystems) to produce *UGIPZ*. Finally, the SfiI-XbaI *EF1α* promoter segment of *EGINZΔ* was excised and replaced by the equivalent SfiI-XbaI *Ubiquitin C* promoter fragment from *UGIPZ*, to produce *UGINZ*.

An shRNA-resistant *MDM2* cDNA was constructed using pcDNA3-*MDM2* (Buschmann et al., 2000) as a template for mutagenesis, using Quick-Change II-XL (Stratagene), and the mutagenic primer CTATTCTCAGCCATCAACTAGCTCCAGCATTATTTATAGCAGCC (mutated positions underlined) and its reverse complement, to change MDM2 codons Ser406 (TCT→AGC) and Ser407 (AGT→TCC) within the TRCN0000003380 target sequence, CTCAGCCATCAACTTCTAGTA, to make *pcDNA3-MDM2-sh380R*.

*UINZ-MDM2-sh380R* was produced by excising the sh380-resistant *MDM2* cDNA from *pcDNA3-MDM2-sh380R* using a BamHI site at -8 relative to the exon 3 translation start site and a BglII site in the 3'UTR, and inserted into the BamHI site of *EINZΔ* to produce *EINZΔ–MDM2-sh380R***.** A SpeI-SphI fragment containing the *MDM2-sh380R* cDNA was then transferred from *EINZΔ–MDM2-sh380R* to the equivalent position of XbaI-SphI digested *UGINZ*, thereby replacing the *GFP* with *MDM2-sh380* cDNA, to produce *UINZ-MDM2-sh380R*.

RB177 cells expressing *UGINZ*, and *UINZ-MDM2-sh380R* were made by producing high titer lentivirus as described for pLKO vectors above, and selecting infected RB177 cells with 500 μg/ml G418 for 7 days.

**Luciferase Assays**

RB177 cells were plated at $2.5X10^5$ cells per well of a 24-well dish, in IMDM plus 10% FBS, and transfected with 2μl of lipofectamine 2000 (InVitrogen) 0.04μg of pRL-TK (Promega), and 0.8 μg of either pGL3 (Promega), *P2-Luc* (pGL3-*HDM2-luc*-02 of (Phelps et al., 2003)), or *P2-Luc-ΔRXR*, which was made using the mutagenic oligonucleotide

GGAGGTCTCCGCGGGAGTTCAGGGTAAAGGCCCCGGGGGCCGGGGGCT (mutated bases underlined) and its reverse complement, using the QuikChange II XL site directed mutagenesis kit (Stratagene). Transfected cells were re-fed at 24 hours, harvested at 72 hours, collected by centrifugation, washed with 1ml PBS, resuspended and lysed in 100 µl PLB (Promega) and the *Ranila* and *firefly* luciferase activities measured using the Stop-and-Glow system and ProMega luminometer.

**Chromatin Immunoprecipitation (ChIP) assays**

ChIP assays were performed as described (Soutoglou and Talianidis, 2002), using rabbit antibodies H-105 (Santa Cruz sc-25737) and Y-20 (Santa Cruz sc-555) at 5 µg/ml, and primer pairs MDM2-P2-RXR1 and MDM2-P2-RXR2, giving PCR products from -317 to -239 and from -264 to -180, respectively, relative to the P2 transcription start site, and *MDM2* exon 12 and *HNF4-a* primers, as follows:

| Gene | Sense Primer | Anti-sense Primer |
|---|---|---|
| MDM2-P2-RXR-1 | GATTTCGGACGGCTCTCG | CCCGTGACCTTTACCCTGAA |
| MDM2-P2-RXR-2 | CGGGAGTTCAGGGTAAAGGTC | GACAGGCACCTGCGATCAT |
| MDM2 ex 12 | GACTATTCTCAGCCATCAACTTCTAG | TCTTCCCTTTCAAACTCTTTCACATC |
| HNF4-α | GGAGCCGTCGCCACAATC | CTAGATAACTTCCTGCTTGGTGATG |

## SUPPLEMENT REFERENCES


Adamus, G., Arendt, A., Zam, Z.S., McDowell, J.H., and Hargrave, P.A. (1988). Use of peptides to select for anti-rhodopsin antibodies with desired amino acid sequence specificities. Pept Res *1*, 42-47.

Bowles, E., Corson, T.W., Bayani, J., Squire, J.A., Wong, N., Lai, P.B., and Gallie, B.L. (2007). Profiling genomic copy number changes in retinoblastoma beyond loss of RB1. Genes Chromosomes Cancer *46*, 118-129.

Buschmann, T., Fuchs, S.Y., Lee, C.G., Pan, Z.Q., and Ronai, Z. (2000). SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. Cell *101*, 753-762.

Dannenberg, J.H., Schuijff, L., Dekker, M., van der Valk, M., and te Riele, H. (2004). Tissue-specific tumor suppressor activity of retinoblastoma gene homologs p107 and p130. Genes Dev *18*, 2952-2962.

DiCiommo, D.P., Duckett, A., Burcescu, I., Bremner, R., and Gallie, B.L. (2004). Retinoblastoma protein purification and transduction of retina and retinoblastoma cells using improved alphavirus vectors. Invest Ophthalmol Vis Sci *45*, 3320-3329.

Dowhan, D.H., Downes, M., Sturm, R.A., and Muscat, G.E. (1994). Identification of deoxyribonucleic acid sequences that bind retinoid-X receptor-gamma with high affinity. Endocrinology *135*, 2595-2607.

Elnenaei, M.O., Hamoudi, R.A., Swansbury, J., Gruszka-Westwood, A.M., Brito-Babapulle, V., Matutes, E., and Catovsky, D. (2003). Delineation of the minimal region of loss at 13q14 in multiple myeloma. Genes Chromosomes Cancer *36*, 99-106.

Hodges, M.D., Vieira, H., Gregory-Evans, K., and Gregory-Evans, C.Y. (2002). Characterization of the genomic and transcriptional structure of the CRX gene: substantial differences between human and mouse. Genomics *80*, 531-542.

Laurie, N.A., Donovan, S.L., Shih, C.S., Zhang, J., Mills, N., Fuller, C., Teunisse, A., Lam, S., Ramos, Y., Mohan, A., *et al*. (2006). Inactivation of the p53 pathway in retinoblastoma. Nature *444*, 61-66.

Lee, T.C., Almeida, D., Claros, N., Abramson, D.H., and Cobrinik, D. (2006). Cell cycle-specific and cell type-specific expression of Rb in the developing human retina. Invest Ophthalmol Vis Sci *47*, 5590-5598.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

MacLaren, R.E., Pearson, R.A., MacNeil, A., Douglas, R.H., Salt, T.E., Akimoto, M., Swaroop, A., Sowden, J.C., and Ali, R.R. (2006). Retinal repair by transplantation of photoreceptor precursors. Nature *444*, 203-207.

Ng, L., Ma, M., Curran, T., and Forrest, D. (2009). Developmental expression of thyroid hormone receptor beta2 protein in cone photoreceptors in the mouse. Neuroreport, in press.

Phelps, M., Darley, M., Primrose, J.N., and Blaydes, J.P. (2003). p53-independent activation of the hdm2-P2 promoter through multiple transcription factor response elements results in elevated hdm2 expression in estrogen receptor alpha-positive breast cancer cells. Cancer Res *63*, 2616-2623.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Soutoglou, E., and Talianidis, I. (2002). Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. Science *295*, 1901-1904.

Spencer, C., Pajovic, S., Devlin, H., Dinh, Q.D., Corson, T.W., and Gallie, B.L. (2005). Distinct patterns of expression of the RB gene family in mouse and human retina. Gene Expr Patterns *5*, 687-694.

Swain, P.K., Hicks, D., Mears, A.J., Apel, I.J., Smith, J.E., John, S.K., Hendrickson, A., Milam, A.H., and Swaroop, A. (2001). Multiple phosphorylated isoforms of NRL are expressed in rod photoreceptors. J Biol Chem *276*, 36824-36830.

Szel, A., Rohlich, P., Mieziewska, K., Aguirre, G., and van Veen, T. (1993). Spatial and temporal differences between the expression of short- and middle-wave sensitive cone pigments in the mouse retina: a developmental study. J Comp Neurol *331*, 564-577.

Wang, Y., Macke, J.P., Merbs, S.L., Zack, D.J., Klaunberg, B., Bennett, J., Gearhart, J., and Nathans, J. (1992). A locus control region adjacent to the human red and green visual pigment genes. Neuron *9*, 429-440.

Wikler, K.C., Rakic, P., Bhattacharyya, N., and Macleish, P.R. (1997). Early emergence of photoreceptor mosaicism in the primate retina revealed by a novel cone-specific monoclonal antibody. J Comp Neurol *377*, 500-508.