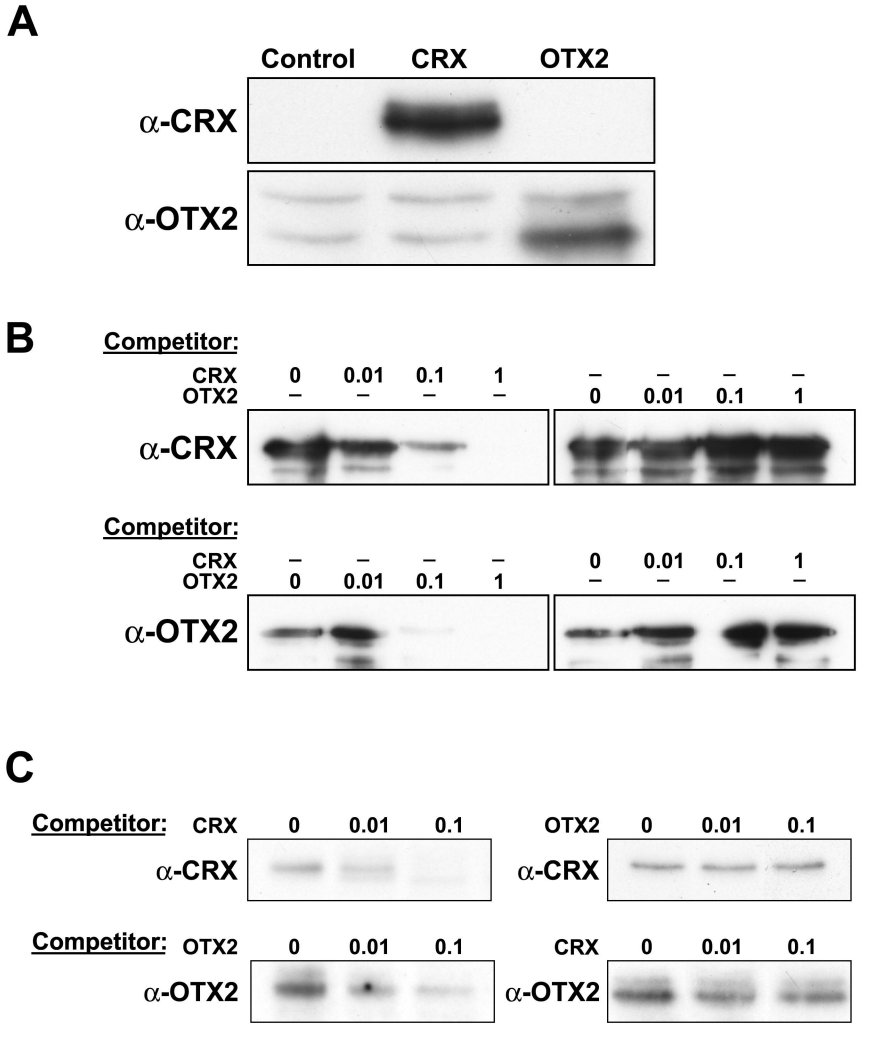
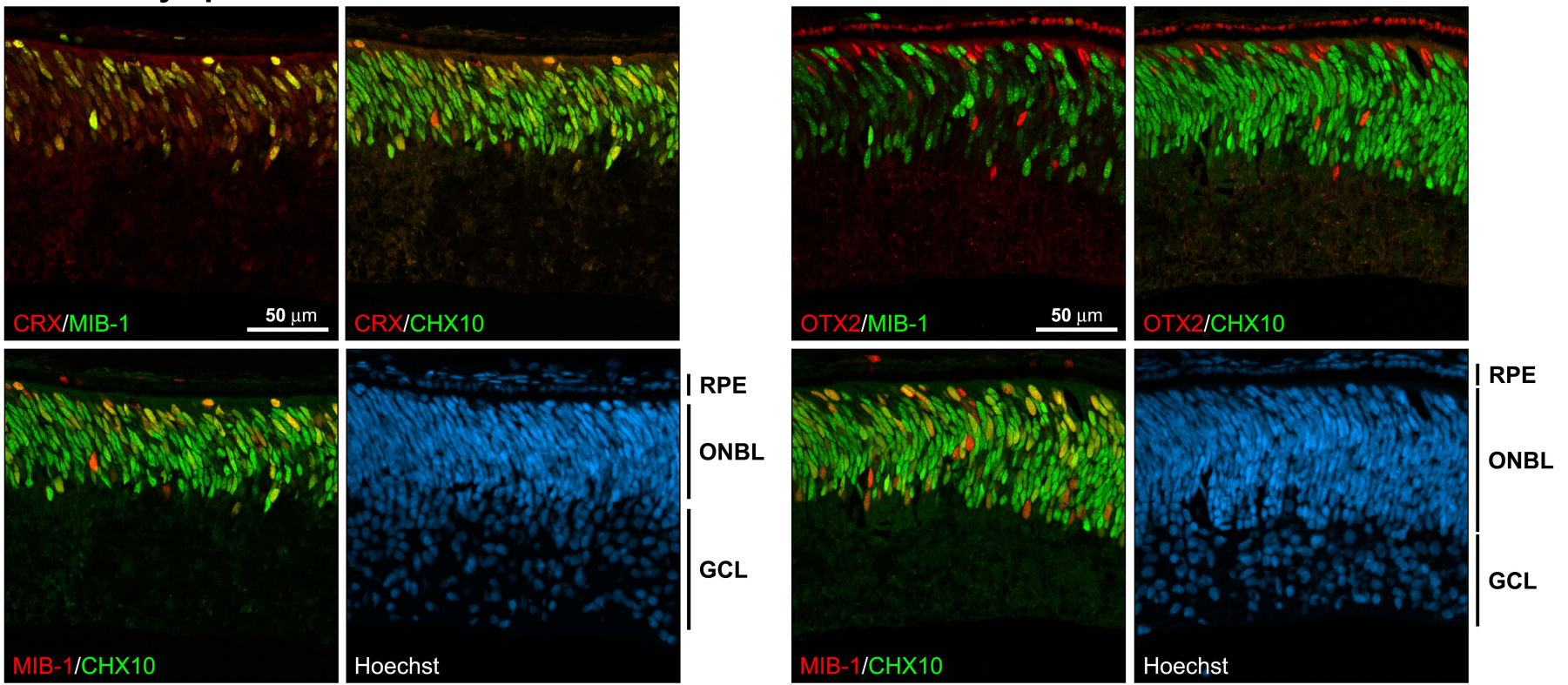
Supplementery Figure 1



Supplementary Figure 1. Experiments addressing the specificity of the anti-CRX and anti-OTX2 antibodies. (A) Western blot analysis of HeLa cells transfected with CRX and OTX2 expression constructs. HeLa cells were transfected with empty pcDNA3 (empty), CRX and OTX2 expression constructs by calcium phosphate-mediated DNA transfection. Forty-eight hours after removal of the DNA, cells were lysed and protein extracts electrophoresed in duplicate 12% SDS-PAGE gels. Proteins were transferred to a PVDF membrane and immunostained with anti-CRX antibody (top) or anti-OTX2 antibody (bottom). (B) Competition experiments using recombinant GST-CRX and GST-OTX2 peptides. Primers were used to amplify the C-terminal halves of the CRX and OTX2 proteins. CRX and OTX2 cDNA products were inserted into the pGEX-4T3 vector and recombinant GST-CRX and GST-OTX2 proteins purified from bacteria using glutathione Sepharose 4B. The concentration of GST-CRX and GST-OTX2 was 0.63 μg/ml and 0.27 μg/ml, respectively. 0.21 μg of GST-CRX or 0.27 μg of GST-OTX2 were run on SDS-acrylamide gels in quadruplicate and transferred to a PVDF membrane. Individual strips were incubated with anti-CRX or anti-OTX2 antibody in the absence of competitor, or in the presence of 0.01 μl (6.3 ng), 0.1 μl (63 ng) or 1 μl (0.63 μg) GST-CRX, or 0.01 μl (2.7 ng), 0.1 μl (27 ng) or 1 μl (0.27 μg) GST-OTX2. GST-CRX, but not GST-OTX2, competed effectively with anti-CRX antibody, whereas GST-OTX2, but not GST-CRX, competed effectively with anti-OTX2 antibody. (C) Competition experiments using WERI-Rb1 whole cell lysates to assess the specificity of the anti-CRX and anti-OTX2 antibodies. Whole cell lysates (50 μg/lane) prepared from WERI-Rb1 were loaded in 12 lanes, electrophoresed through a 12% SDS-PAGE gel and transferred to PVDF membranes. Individual lane strips were

incubated with anti-CRX or anti-OTX2 antibody in the absence of competitor, or in the presence of 0.01 μl (6.3 ng), 0.1 μl (63 ng) or 1 μl (0.63 μg) GST-CRX, or 0.01 μl (2.7 ng), 0.1 μl (27 ng) or 1 μl (0.27 μg) GST-OTX2. GST-CRX, but not GST-OTX2, competed effectively with anti-CRX antibody, whereas GST-OTX2, but not GST-CRX, competed with anti-OTX2 antibody


13 week human fetal retina
Near ciliary epithelium
CRX/MIB-1
50 µm
CRX/CHX10
OTX2/MIB-1
50 µm
OTX2/CHX10
MIB-1/CHX10
Hoechst
RPE
ONBL
GCL
MIB-1/CHX10
Hoechst
RPE
ONBL
GCL

Supplementary Figure 2. CRX and OTX2 expression in the peripheral region of normal human fetal retina at 13 weeks gestation. The peripheral region near the ciliary epithelium represents the least differentiated part of the retina. Tissue sections from paraformaldehyde-fixed human fetal retina at 13 weeks gestation were triple-stained with either rabbit anti-CRX antibody or rabbit anti-OTX2 antibody, MIB-1 antibody and sheep anti-CHX10 antibody. The signal was detected using donkey anti-rabbit Alexa 555 secondary antibody (CRX, OTX2), donkey anti-mouse Alexa 647 secondary antibody (MIB-1) or donkey anti-sheep Alexa 488 secondary antibody (CHX10). Abbreviations: RPE, retinal pigmented epithelium; ONBL, outer neuroblastic layer; GCL, ganglion cell layer.

Supplementary Figure 3

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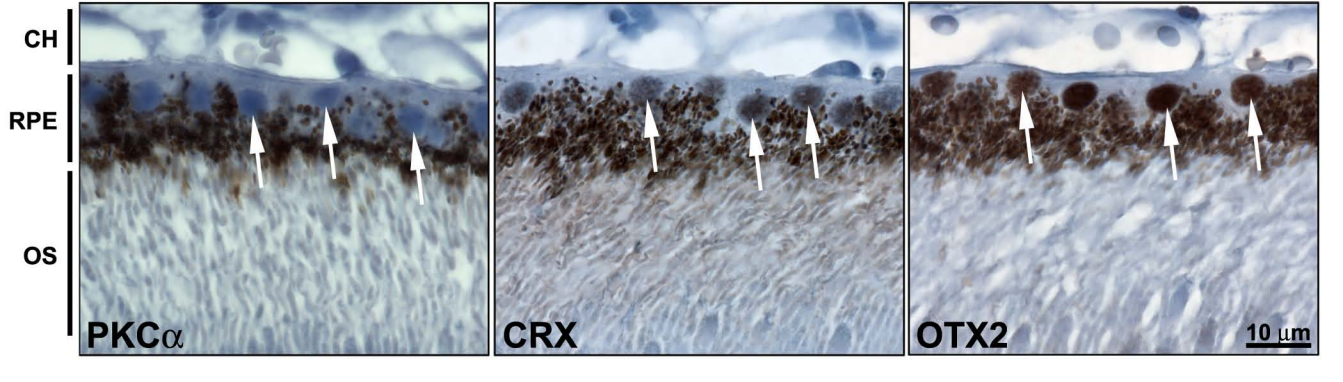

Supplementary Figure 3. Expression of CRX, OTX2 and PKCα in retinal pigmented epithelial cells. The sections shown in Fig. 4A and 4B have been magnified to demonstrate strong immunostaining of OTX2 and weak immunostaining of CRX in the RPE. Immunostaining was also carried out using antibody to the rod bipolar marker PKCα. The arrows point to the nuclei of RPE cells. Abbreviations: OS, outer segments; RPE, retinal pigment epithelium; CH, choroid.

Supplementary Figure 4

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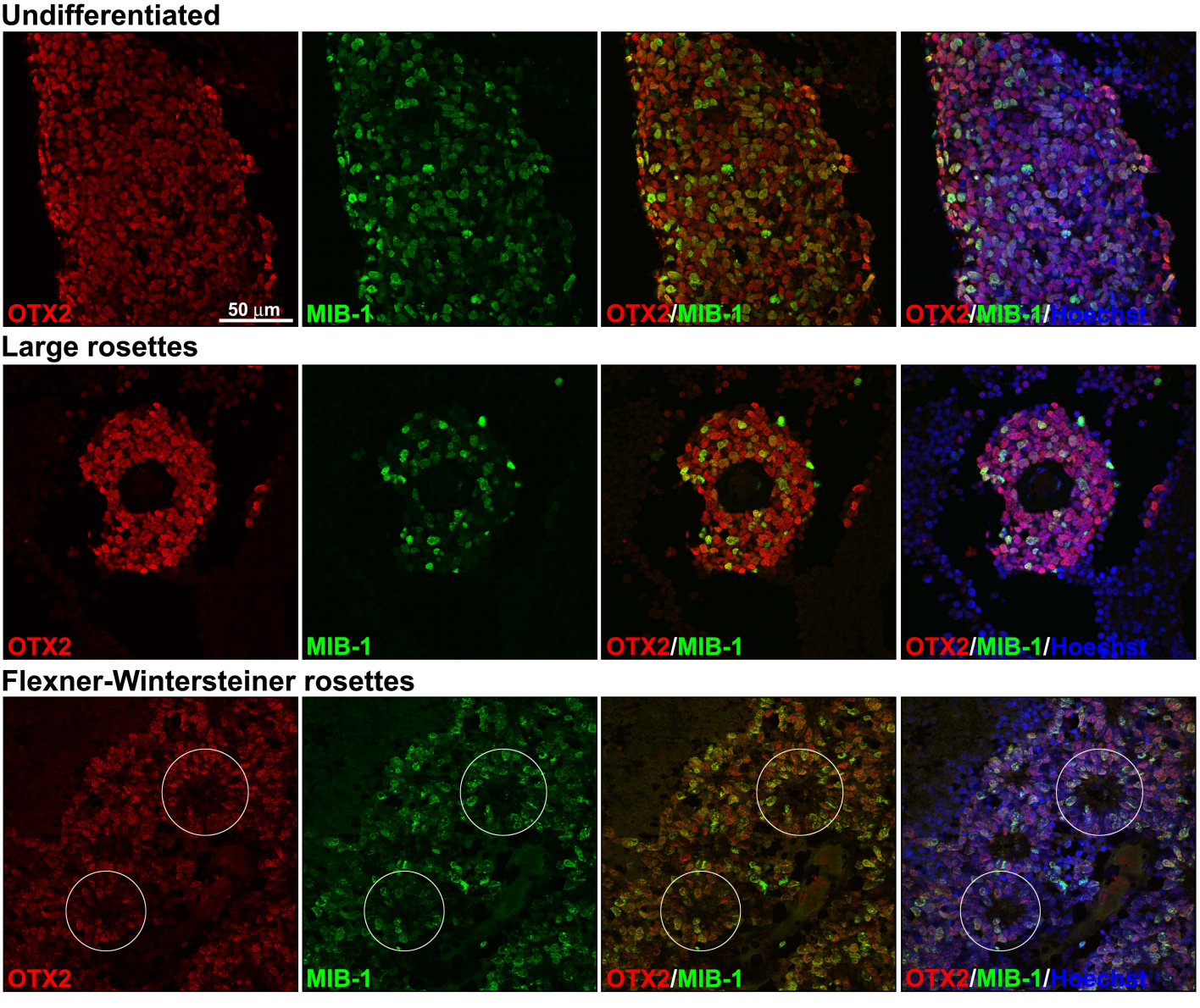

Supplementary Figure 4. Co-staining of OTX2 and MIB-1 in retinoblastoma tumors. Tissue sections from retinoblastoma tumors (undifferentiated tumor from patient 48292 – top panels; large rosettes from patient 48292 – middle panels; Flexner-Wintersteiner rosettes from patient 45376 - bottom panels, were co-stained with anti-CRX and MIB-1 antibodies. Positive signals were detected using Alexa 488-conjugated donkey anti-rabbit (OTX2) and Alexa 555-conjugated donkey anti-mouse (MIB-1) secondary antibodies. Sections were counterstained with Hoescht 33342 to visualize nuclei and mounted with FluorSave reagent. The yellow color in OTX2/MIB-1 merged panels demonstrates co-immunostaining. Although OTX2-positive cells are more abundant than MIB-1-positive cells, the great majority of MIB-1-positive cells express OTX2.