Rb is required for retinal angiogenesis and lamination

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Abstract
Retinoblastoma tumor suppressor (Rb) promotes cell cycle exit, survival, differentiation, and tumor suppression in the retina. Here, we show it is also essential for vascularization and lamination. Despite minimal effects on Hif1α target expression, intraretinal vascular plexi did not form in the 

\[ \text{Rb}^{-/-} \]

murine retina. Deleting adenovirus E2 promoter binding factor 3 (E2f3), which rescues starburst amacrine cell differentiation, or E2f2, had no effect, but deleting E2f1, which promotes neuronal cell cycle exit and survival, restored retinal vasculature. We specifically linked cell loss to the defect because removing Bax rescued rod and bipolar neurons and the vasculature, but not cell cycle exit. Despite rescuing 

\[ \text{Rb}^{-/-} \]

neurons, Bax deletion exacerbated a delay in outer retina lamination, and exposed a requirement for Rb in inner retina lamination. The latter resembled Sem5 or FAT atypical cadherin 3 (Fat3) mutants, but expression of Sem5/Fat3 pathway components, or that of Neogenin, which perturbs migration in the 

\[ \text{Rb}^{-/-} \]

cortex, was unchanged. Instead, lamination defects correlated with ectopic division, and were E2f1-dependent, implicating the cell cycle machinery. These in vivo studies expose new developmental roles for Rb, pinpoint aberrant E2f1 and Bax activity in neuronal death and vascular loss, and further implicate E2f1 in defective lamination. Links between Rb, angiogenesis and lamination have implications for the treatment of neovascularization, neurodegeneration and cancer.

Introduction
Angiogenesis is a critical step in development and disease and is regulated by pro-angiogenic and anti-angiogenic factors1. In mice, the retinal vasculature consists of three interconnected parallel vascular plexi. A superficial vascular plexus (SVP) in the nerve fiber layer (NFL) develops from the optic nerve head and progresses radially to the peripheral retina between postnatal day 0 (P0) and P8. Subsequently, vessels sprout vertically into the retina. Around P7, sprouting vessels descend and advance into the outer plexiform layer (OPL) to establish the deep vascular plexus (DVP). Around P11, the DVP vessels ascend into the inner plexiform layer (IPL) and form the intermediate vascular plexus (IVP)2-4. Previous studies revealed that Frizzled-4 (Fzd4), Lrp5, Norrin, and Tetraspanin 12 (Tspan12) are required for intraretinal vascular development3,5,6, and retinal Hif1α is required for IVP development7. Retinal neurons form the neurovascular unit to interact with endothelial cells8,9. Retinal ganglion cells (RGCs) are essential for the SVP development5,10, photoreceptors are important for the development of the intraretinal vascular plexus11,12, and amacrine and horizontal cells are critical for developing and maintaining the intraretinal vasculature4.

The retinoblastoma tumor suppressor (Rb) plays a major role in regulating cell cycle and other cellular processes by interacting with adenovirus E2 promoter binding factors (E2fs)13. The Rb/E2f pathway plays critical...
roles in angiogenesis. For example, Rb binds Hif-1α and enhances its transcriptional activity. Atypical E2fs (E2F7/8) bind Hif-1α to stimulate Vegfa induction. As such, deletion of Rb1, or E2f7/8, or Hif1 in mice all result in vascular defects in the placenta and early embryonic lethality.

The retina comprises three distinct nuclear layers (GCL, ganglion cell layer; INL, inner nuclear layer; and ONL, outer nuclear layer), separated by two synaptic layers (OPL and IPL). Retinal lamination is guided by many different cues, such as Dscam, semaphorins (Sema), and plexins. Proper development of retinal lamination is important for physiological retinal responses and function. Recent studies have revealed that Rb can regulate neuronal migration and cortical lamination.

Previously we reported that Pax6 alpha enhancer Cre (α-Cre)-mediated Rb gene knockout (KO) in mouse retina cause ectopic cell division, cell death, and differentiation defects. α-Cre is active from embryonic day 10 (E10) in the peripheral progenitors of the temporal and nasal retina. Employing this model we now implicate Rb in formation of the two intraretinal vascular plexi, as well as lamination of the outer and inner retina. We show that the angiogenesis defect is related to E2f1 and Bax-induced retinal cell death, whereas the lamination defects are cell death-independent and instead correlate with ectopic cell division.

**Results**

**Rb is required for the development of intraretinal capillaries and retinal lamination**

We examined Rbα/α-Cre and Rbfl/− retinas between P7 and P60. To mark the RbKO area, we utilized Cre reporter Z/Red mice which express the red fluorescent protein upon Cre-mediated recombination. Retinal whole-mount staining revealed that at P18 and later time points, the density of retinal blood vessels was much lower in the peripheral RbKO area of Rbα/α-Cre retinas than that in the same areas of the Rbfl/− retinas (wild-type [WT] control), and the WT areas of Rbfl/−Cre retinas (Fig. 1a). Killing RGCs in the embryonic retina blocks the SVP formation in the postnatal tissue, as does Math5 deletion, which prevents the genesis of 95% RGCs. Surprisingly, in the Rbfl/α-Cre retina, where 80% RGCs are deleted in the P0 peripheral retina, the SVP formed normally (Fig. 1b–d). This finding suggests that the reduced RGC density in the periphery and/or that the RGC signals in the central retina are sufficient to promote peripheral vascular outgrowth. The SVP remained intact at later stages (Fig. 1b–d), thus peripheral RGCs are also not required to maintain these vessels. At P18 the RbKO area had a normal SVP, but almost entirely lacked the IVP and DVP (Fig. 1c), as confirmed by histological staining of retinal sections (Fig. 1d). In WT P18 retinal sections, isolectin B4-positive (IB4+) cells were found in the NFL, IPL, and OPL, corresponding to the SVP, IVP, and DVP, respectively. However in RbKO retinal sections, IB4+ cells were only found in the NFL, but not in the IPL and OPL (Fig. 1d). In the P60 RbKO retina, the IVP and DVP were still absent, indicating that the defect was not transient due to delayed development (Fig. 1b).

We also examined the lamination of Rb-deficient retinas. At P0, the IPL had already formed between neuroblast layer (NBL) and GCL in WT and Rbfl/− retinas (Fig. 1e); thus, although Rb is required for maturation of a subset of amacrine cells, most of the IPL forms in its absence. At P8, the OPL had formed between the ONL and INL, in the WT but not Rbfl/− retina (Fig. 1e). At P18, the OPL had also formed in the Rbfl/− retinas (Fig. 1d), suggesting that Rb deficiency delayed OPL formation, likely due to fewer rod and bipolar cells. This phenotype is similar to the migration and lamination defects in the developing Rbfl/− cortex. We concluded that Rb is required for the development of intraretinal capillary plexi and formation of the OPL.

**A subset of angiogenesis regulators is down-regulated following Rb loss**

The α-Cre transgene is restricted to retinal cells, indicating a non-cell autonomous effect of Rb loss on angiogenesis. As the DVP and IVP originate from the SVP at about P7 and P11, respectively, the defects should begin at around P7. Notably, P7 is the peak time for ectopic cell division and cell death of the Rbfl/− retina. As Rb can bind Hif-1α and enhance its transcriptional activity, and thus Hif-1α activity. On the other hand, non-Hif-1α targets such as Norrin, Fzd4, and Tie2, which are...
Fig. 1 Rb is required for the development of intraretinal vascular plexi and the OPL. 

a) Isolectin B4 (IB4) staining of P18 whole-mount retinas of Rb<sup>fl/fl</sup> (WT control) and Rb<sup>fl/fl</sup>;Z/Red<sub>α-Cre</sub> (RbKO in red areas) mouse. Selected areas are blown up to show the vascular density. 
b) Whole-mount retinas of the indicated ages and genotypes were stained for IB4. 
c) Confocal images of IB4-stained SVP, IVP, and DVP of P18 whole-mount retinas of the indicated genotypes. 
d) IB4 (green) and DAPI (blue) staining of P18 retinal sections of WT or Rb<sup>ko</sup> retina. 
e) Horizontal sections of indicated ages and genotypes were stained for nuclear (DAPI, blue), mitosis (PH3, green at P0, red at P8). Arrows in d and e indicate the position of outer plexiform layer (OPL). The dotted lines in a–c indicate the boundary between WT (in the center) and RbKO areas (in the periphery). NBL neuroblast layer, ONL outer nuclear layer, INL inner nuclear layer, IPL inner plexiform layer, GCL ganglion cell layer. Scale bar is 50 µm.
important for intraretinal vascular capillaries, were reduced in \(Rb^{-/-}\) retina at P7 (Fig. 2b, c).

Inactivating E2f1, but not E2f2 or E2f3, rescues the vascular and lamination defects of RbKO retina

E2f1 can suppress or promote angiogenesis in different conditions; however, we have not found any retinal vascular or lamination defects in E2f1KO, or E2f2KO, or E2f3KO;\(;\alpha\)-Cre mice. We reported that in the RbKO retina, E2f1 mediates ectopic division and cell death, E2f3 disrupts starburst amacrine cell (SAC) differentiation, and while E2f2 does not cause defects in the RbKO retina, it mediates the ectopic division and death of cones of Rb/\(p107\) double KO (DKO) retina. To define whether any E2f contributes to the vascular defects, we crossed \(Rb^{f/f};\alpha\)-Cre mice with E2f1 KO, or E2f2 KO, or E2f3f/f mice (Fig. 3a, b).

We used the AngioTool software to analyze the vessel coverage, average vessel length, and lacunarity of vascular plexi. This analysis confirmed that the RbKO retina had a normal SVP, but the vascular density and average vessel length were much reduced in IVP and DVP (Fig. 3c–f).

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**Fig. 2** Gene expression changes in the Rb-null retina and the effect of E2f1 or Bax loss. **a** Gene list enrichment analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) 2016 datasets in Enrichr of RbKO-regulated retinal DEGs at P8 (\(-\log 10(P)\)). Dotted line indicates adjusted \(p < 0.05\). **b** Heatmap of relative expression level of selected genes of P8 RbKO and WT retina, based on the microarray analysis. **c**–**f** Real-time RT-PCR analysis of angiogenesis genes (**c, d**) and the E2f family (**e, f**) at P7, P18 retinas of the indicated genotypes, respectively. Error bars represent SD of measurements from three animals, and asterisks indicate a significant difference between the WT and the indicated genotypes (*\(p < 0.05\); **\(p < 0.01\), one-way ANOVA followed by Bonferroni correction).
Fig. 3 E2f1 mediates the RbKO-induced retinal angiogenesis and lamination defects. a P18 retinal sections of the indicated genotypes were stained for nuclear (DAPI, blue), vascular endothelium cells (IB4, green), and amacrine cells (Calretinin, red). White arrows indicate Calretinin+ tracks in the IPL; loss of the outer two tracks reveals the starburst amacrine cell defect. b P18 whole-mount retinas of indicated genotypes were stained for IB4 to label vasculature. Dotted lines indicate the boundary between WT (center) and RbKO (peripheral) areas. c Confocal images of IB4-stained SVP, IVP, and DVP of P18 whole-mount retinas of indicated genotypes. Pseudo-colors were used to differentiate these three plexi. d–f Quantification of vessel coverage (d), average vessel length (e), and lacunarity (f) by the AngioTool software. Error bars represent SD of measurements from at least three animals and asterisks indicate significant differences between retinas of RbKO and the indicated genotypes (*p < 0.05, **p < 0.01, one-way ANOVA followed by Bonferroni correction). Scale bar is 50 μm. ONL outer nuclear layer, INL inner nuclear layer, GCL ganglion cell layer, ON optic head.
Deleting E2f3 rescued Calretinin\(^{+}\) SAC processes in the Rb\(^{-/-}\) IPL (Fig. 3a), as before\(^{27}\), but neither that nor E2f2 loss affected angiogenesis (Fig. 3b–f). In stark contrast, removing E2f1, which does not rescue the SAC defect (Fig. 3a), completely reversed the loss of both IVP and DVP, and returned vessel coverage and length as well as lacunarity to WT levels (Fig. 3b–f). Even removing one E2f1 allele had a marked effect (Fig. 3b–f). E2f1KO also restored Fzd4 and Tie2 mRNA levels in the Rb\(^{-/-}\) retina at P7 (Fig. 3b), which may account for the rescue of retinal vascular defects. These data are reminiscent of the dose-dependent effects of E2f1 on abnormal cell division and death in the RbKO retina\(^{36}\).

The lamination defects were rescued by either E2f1\(^{+/−}\) or E2f1\(^{−/−}\), but neither E2f2 nor E2f3 loss. These results indicate that whereas E2f2 or E2f3 activity, or disrupted SAC differentiation are not involved, E2f1 drives the vascular and lamination defects in the RbKO retina. This is slightly different from the developing Rb\(^{-/-}\) cortex, in which either E2f1\(^{-/-}\) or E2f2\(^{-/-}\) rescues the lamination defects\(^{25,40}\). E2f1 drives both ectopic division and cell death in the RbKO retina\(^{37}\), thus we next sought to define which of these cellular defects contributes to vascular disruption and retinal lamination defects.

**Bax contributes to cell death and retinal vasculature defects**

The Bcl-2 family member Bax mediates neuronal apoptosis\(^{41}\), including physiological retinal apoptosis\(^{12,43}\) and neuronal death in the Rb/p107 DKO brain\(^{24}\). Thus, we tested whether Bax drives cell death in the RbKO retina. Bax\(^{-/-}\) did not affect ectopic division in the RbKO retina at P2 or P8, and actually elevated proliferation at P18 (Fig. 4a, b). All ectopic division had ceased by P30 indicating Rb-independent means of cell cycle exit. The increase in dividing cells at P18 might result from improved survival of ectopically proliferating Rb\(^{-/-}\) cells at earlier times, and indeed apoptosis was markedly reduced in the Rb/Bax DKO retina at P2, and P8 when cell death peaks (Fig. 4c–d). Thus, Bax drives cell death in the Rb\(^{-/-}\) retina.

Next, we defined which death-prone Rb\(^{-/-}\) cell types are rescued by BaxKO. Rb loss causes death of most rod bipolar and RGCs, and many rods\(^{36}\). Bax loss restored RGCs to WT numbers, and also suppressed rod bipolar cell death considerably (Fig. 4e, f). Rhodopsin staining and measurement of ONL thickness indicated partial rescue of rod photoreceptors (Fig. 4e, g). Thus, Bax is a major, but not the sole contributor to apoptosis in Rb\(^{-/-}\) neurons. Its role is greater in the inner (ganglion, bipolar cells) than outer retina (photoreceptors).

Next, we asked whether BaxKO affects the disrupted vasculature of the Rb\(^{-/-}\) retina. Strikingly, we observed both IVP and DVP in the P18 Rb/Bax DKO retina (Fig. 5a–c). Quantification in the IVP revealed that BaxKO increased vessel coverage and length, and reduced lacunarity essentially to WT levels (Fig. 5d–f). The extent of the defects was greater in the RbbKO DVP; Bax loss did not restore vascularity to WT levels, but did elevate vessel coverage and length, and dramatically reduced lacunarity (Fig. 5d–f). In line with these cellular effects BaxKO rescued the reduction of Fzd4, Norrin, and Tie2 expression in P7 Rb\(^{-/-}\) retina, and actually induced Vegfa and Cxcr4 expression considerably by P18 (Fig. 2c, d). We also observed some increase in vasculature in the Bax\(^{-/-}\) relative to WT retina (Fig. 5c–e). These data suggest that the modest effects of Bax loss on natural neuronal pruning (Fig. 4b)\(^{43}\) promote vessel formation in the developing retina.

In summary, without reducing ectopic division, deleting Bax promotes survival of Rb\(^{-/-}\) cells, most prominently in the inner retina, which correlates with increased expression of angiogenic factors and dramatic rescue of the IVP and DVP.

**Inactivating Bax exposes new roles for Rb in retinal lamination**

The striking rescue of cell death in the Rb/Bax DKO retina also exposed two roles for Rb in retinal lamination. The first affected OPL formation. OPL forms in the P8 WT retina, but not in the RbKO tissue until P18 (Figs. 1e and 4a, c, e). We assumed that this delay reflects the loss of rods and bipolar cells, and thus expected it to be rescued in the DKO retina (Fig. 4e–g). Unexpectedly, no OPL was evident in the DKO retina at P8 and P18 (Fig. 4a, c, e), which was confirmed by staining for synaptic vesicle proteins synaptotagmin 1 (Syt1)\(^{44}\) and synaptic vesicle glycoprotein 2a (SV2a)\(^{45}\) (Fig. 6a).

In contrast to the uniform OPL staining in P8 WT or BaxKO retina, SV2A\(^{+}\) synapses were disorganized in the RbbKO or DKO retinas (Fig. 6a). Syt1 staining was better ordered in the P18 RbKO retina, even though the M opsins\(^{+}\) cones and Onecut2-positive (OC2\(^{+}\)) horizontal cells were almost superimposed, but it remained disorganized in the DKO tissue, even though positioning of the cone and horizontal cells was restored (Fig. 6a). By P30, a Syt1\(^{+}\) OPL was evident in the DKO retina, although still less uniform than in other genotypes (Fig. 6a). In the Rb\(^{-/-}\) cortex, Neogenin 1 (Neog1) induction causes migration and lamination defects\(^{25,40}\). However Neog1 was not induced in the Rb\(^{-/-}\) retina (Figs. 2b and 6b). Other migration regulators that were induced in the Rb\(^{-/-}\) cortex, including Sema3d, ApoE, CCK, Twist1, and Twist2\(^{26}\), were also not altered in the Rb\(^{-/-}\) retina (Fig. 2b).

Potentially, ectopic division directly perturbs OPL formation, because its appearance correlated with a large reduction in ectopically dividing Ki67\(^{+}\) cells in the DKO.
retina between P18 and P30, and in the \( \text{Rb}^{-/-} \) retina between P8 and P18 (Fig. 4a). Furthermore, in the boundary between central \( \alpha\text{-Cre}^- \) and peripheral \( \alpha\text{-Cre}^+ \) retina, OPL disruptions coincided precisely with Ki67+ cells (Fig. 6c, arrows), disordered SV2a vesicles coincide with dense Ki67 staining (Fig. 6c).

Cyclin E can bind and sequester cyclin-dependent kinase 5 (Cdk5), which plays important roles in neuronal migration and lamination by phosphorylating doublecortin (Dcx)46–48. As an E2f target, cyclin E increased in the \( \text{Rb}^{-/-} \) retina, while Cdk5 and Dcx levels had barely changed (Figs. 2b and 6b). In P8 BaxKO retina, Dcx and Cdk5 were most prominently expressed in the cell bodies and dendrites of horizontal and amacrine cells, while cyclin E was most evident in the OPL and less so the IPL and cytoplasm of some cells (Fig. 6d). In P8 \( \text{Rb}/\text{Bax} \) DKO retina the expression pattern of Dcx and Cdk5 was similar, but cyclin E was induced in many retinal cells.
These data are consistent with the notion that cyclin E in ectopically dividing cells may interfere with Cdk5 function and contribute to the lamination defects in the Rb KO and Rb/Bax DKO retina.

In addition to delayed OPL formation, there was an unanticipated “outer misplaced plexiform layer” (OMPL) in P8 DKO retinas, which split the INL (Figs. 4a, c, e and 6a, e). It was not observed in either the Rb KO or Bax KO retinas (Fig. 6a). Excess cell production cannot explain this phenomenon since, at best, Bax loss restored the missing retinal cell types (Fig. 4f, g), retinal thickness was similar at all time points between DKO and control retinas, and Bax deletion had no effect on the numbers of Rb−/− death-resistant cone, horizontal, amacrine, and Müller cells (Fig. 6e–g). Many amacrine cells flanked the OMPL, as indicated by Calretinin and Ap2α staining (Fig. 6e, h). A similar OMPL was observed in Fat3−/−, Sema5A−/−, and Sema5B−/− retinas, in which amacrine cells are bipolar, leading to new synaptic contacts with bipolar cells22,23. Indeed, some amacrine cells in the DKO INL were bipolar and had ectopic dendrites that stratified in the OMPL (Fig. 6h, arrows).

In view of the phenotypic similarities between Rb/Bax DKO and Fat3 or Sem5 KO retinas, we assessed the mRNA levels of Fat3 and Fat5A & B, as well as those of Dachsous (Dchs), a Fat3 ligand, and Four jointed box 1 (Fjx1), which modifies Fat activity. The levels of Dchs, Fat3, and Sema5A were not different between WT, RbKO,
Fig. 6 (See legend on next page.)
BaxKO, and DKO retina at three different time points, and while Fjx1 and Sema5B were slightly increased in DKO retinas, this was also the case in the BaxKO control which has no OMPL (Fig. 6h). The occurrence of the OMPL in the DKO, where ectopic division is enhanced, and its rescue in the Rb/E2f1 DKO retina, where ectopic division is blocked, suggest that it may also be linked to excess activity of cell cycle machinery. Irrespective, these data expose a new role for Rb in coordinating retinal lamination with phenotypic similarities to Fat3-deficient and Sema5-deficient retinas.

**Discussion**

Rb is critical in the retina as it promotes cell cycle exit, neuronal survival, SAC differentiation, and tumor suppression. Here we exposed new roles in retinal angiogenesis and lamination. The vasculature was rescued by deleting E2f1 or Bax, pinpointing neuronal loss rather than ectopic division in the phenotype. Rescuing death-prone neurons with Bax deletion exposed new roles for Rb in OPL and INL formation. These data expand our insight into the multi-faceted functions of Rb in retinal development.

To separate defects linked to division or death, we focussed on Bax because it mediates the death of Rb/p107 DKO neurons in the brain. Indeed, Bax loss rescued most rod bipolar and RGCs, and many rods, but had no major effect on ectopic cell division. Rb loss delays the formation of OPL from P8 to P18; we assumed this defect was due to rod and bipolar cell loss. However, while deleting Bax rescued many Rb−−/− rods and bipolar cells, it did not rescue the OPL defect. Despite the disorder, synaptic proteins were present, and Neogenin, which is induced and disrupts lamination in the Rb/p107-null cortex, was not elevated in the Rb-null retina.

The OPL defect may be related to ectopic cell division because E2f1KO promoted cell cycle exit and rescued OPL defects, but deleting Bax, which increased ectopic division due to extended cell survival, further delayed OPL genesis. Moreover, at the boundary of Cre expression, sporadic OPL disruption correlated perfectly with interposed ectopically dividing cells. The eventual appearance of a narrow OPL correlated with delayed cell cycle exit at P18 or P30 in the Rb-null or Rb/Bax-null retina, respectively. Potentially, over-active cell cycle machinery disrupts formation of the synaptic layers. One candidate, cyclin E, can sequester Cdk5 which is important for lamination, and we confirmed high levels of this E2f1 target in ectopically dividing Rb-null and Rb/Bax-null cells. Future work will address whether reducing cyclin E or elevating Cdk5 ameliorates the OPL defect.

The unnatural OMPL that split the Rb/Bax-null INL at P8 resembles the phenotype of Fat3−−/−, and Sema5A−−/−; Sema5B+/−/− retinas, suggesting that Rb may affect these adhesive and repulsive cues. We did not observe major changes in their expression. Cell cycle enzymes might also contribute to this phenotype as the OMPL separated postmitotic amacrine cells in the inner INL from ectopically dividing amacrine cells in the outer INL (Fig. 4a).

Our work also exposes a critical role for Rb in the formation of intraretinal vasculature. In theory, Rb could influence angiogenesis through its positive effects on Hif1, and/or by promoting survival of cells essential for vessel formation. Our results support the latter because rescuing death-prone Rb−−/− neurons with Bax deletion restored the IVP and DVP. Hif1 targets were relatively unaffected by Rb loss, perhaps due to induction of E2f7/8, known to augment Hif1 activity, and instead other angiogenic regulators, such as Norrin, Fzd4, and Tie2, were down-regulated.

Excitatory retinal neurons, including RGCs and photoreceptors, can drive and regulate retinal angiogenesis. Specifically, RGCs are required for the SVP development, and photoreceptors are required for the DVP development. Surprisingly, Rb deficiency did not affect the SVP development, even though Rb−−/− mouse retinas lose most RGCs. In prior studies RGCs were depleted from the entire retina, but in our case cell death is confined to the periphery, so conceivably the signals in the intact central retina are sufficient to drive the SVP development in the periphery.
Clearly, this was not the case for the IVP and DVP, which showed dramatic loss in the Rb-null retina. There is a very close relationship between the number of photoreceptors and vessel profiles in the DVP. As there are still many rods in the Rb−/− retina, their reduction may not explain all the vascular defects. Rod bipolar cells may affect the IVP development because these cells are essentially absent in Rb−/− P18 retina. E2f1KO rescued all the cell death of rods and bipolar cells, and completely rescued the vascular defects. Bax loss rescued about 80% of rod bipolar cells and 50% of rods, but rescued about 80% of IVP coverage and 50% of DVP coverage which, while not definitive, suggests a potential role for the bipolar cells.

Amacrine and horizontal cells are required for generating and maintaining the intraretinal vasculature. However, in our study, amacrine and horizontal cells survive Rb deficiency, but intraretinal vascular plexi still could not develop, indicating that these inhibitory neuron-regulated pro-angiogenic factors are not sufficient for the retinal vascular development.

Our work exposes new roles for Rb in the developing retina, pinpointing aberrant E2f1 and Bax activity as key drivers of defects in neuronal survival and angiogenesis, and further implicating E2f1 in aberrant lamination. The Rb/E2f pathway is commonly involved in many diseases, such as most human cancers, retinal degeneration, diabetic retinopathy, and neuronal degeneration. Abnormal cell survival, proliferation, angiogenesis, and lamination are common features of these diseases. Thus, our findings will enhance our understanding of the pathogenesis and optimize the treatment strategies for these diseases in the future.

### Materials and methods

#### Mouse strains and genotyping

Mice were treated according to institutional and national guidelines. All procedures were performed in compliance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and visual research. a-Cre mice (P. Gruss), Rbfl/fl mice (A. Berns), E2f1−/− mice (M. Greenberg), E2f2−/− mice (G. Leone), E2f3−/− mice (G. Leone), Z/Red (Jackson Laboratory, stock#005438), and Bax−/− (Jackson Laboratory, stock#002994) were maintained on a mixed (NMRI × C57/BL6 × FVB/N × 129sv) background.

Bax-null males are infertile, to generate Rbfl/fl Bax−/− a-Cre males we first generated Rbfl/fl Bax−/− a-Cre males and Rbfl/fl Bax−/− females, and inter-bred them. Of 96 pups we obtained 50 with a-Cre and 48 with Bax−/− alleles as predicted, but only 5 pups (5.2%) with Bax−/− a-Cre, far less than the expected 24 pups (25%, Table 1), suggesting that a-Cre and Bax alleles may be on the same chromosome. We screened pups for a crossover event to generate a-Cre and Bax alleles on the same chromosome. We obtained Rbfl/fl, Bax−/− a-Cre females, and bred them with Rbfl/fl Bax−/− males to generate experimental littersmates.

Mice of different genotypes were compared within the same litter and across a minimum of three litters. We have not noted any phenotypic differences in separate litters. Genotyping was performed as before and the primers for genotyping Z/Red mice are primers olMR3847 and olMR4110 for transgene (208 bp), and olMR7338 and olMR7339 for internal positive control (324 bp). The primers for genotyping Bax−/− mice are primers olMR0661 and olMR0662 for mutant allele (507 bp), and olMR0661 and olMR0663 for WT allele (304 bp). The sequences of the above primers are listed in Jackson Laboratory genotyping protocols (www2.jax.org).

#### Histology, immunofluorescence, and measurements

Eyeballs were fixed in 4% paraformaldehyde for 1 h at 4 °C, embedded in OCT (TissueTek 4583), frozen on dry ice, and cut into 12–14 μm sections on Superfrost slides. For immunohistochemistry, the retinal sections were dried at room temperature and incubated in blocking solution (0.5% normal donkey serum, 0.03% Triton X-100 in 1× phosphate-buffered saline (PBS)) for 1 h, then were incubated with primary antibodies such as active caspase-3 (Cell Signaling Technology, 9661), Ap2 (Abcam, ab18723), glutamine synthetase (Millipore, MAB302), Ki67 (BD Science Pharmingen), cyclin E (Upstate, 07-687), Dcx (Abcam, ab18723), glutamine synthetase (Millipore, MAB302), Ki67 (BD Science Pharmingen), 550609), M ops (C. M. Craft and X. Zhu, University South California), Oncut2 (R&D System, AF6294), phoshpo-histone H3 (Santa Cruz, SC-8566), protein kinase Ca (Sigma, P5704), rhodopsin (Santa Cruz, SC-57433), SV2a (R. Janz, The University of Texas-Houston Medical School), and Syt1 (Abcam, ab13259). Vascular endothelial cells were labeled by fluorescein isothiocyanate (FITC)-conjugated isoelectric B4 (Sigma, L2895). Antigen retrieval was performed as described by boiling sections in citric acid (H-3300, Vector Lab). Primary antibodies or labeled cells were visualized using donkey anti-mouse, donkey anti-rabbit, and donkey anti-goat antibodies conjugated with...
Alexa-488, Alexa-568, or Alexa-647 (1:1000; Molecular Probes). Nuclei were counter-stained with 4', 6-diamidino-2-phenyindole (DAPI; Sigma) and mounted with Mowiol mounting medium.

For whole-mount staining, eyeballs were enucleated and incubated for 30 min in 4% paraformaldehyde in PBS. With a dissection microscope, a circumferential incision was made around the limbus, followed by removal of the anterior segment, lens, and vitreous body. The retinas were incubated at 4°C with FITC-conjugated isoelectin B4 (Sigma, L2895) and DAPI in PBS for 1–2 days. After briefly washing with PBS, radial cuts were made to divide the retina into four quadrants to flatten the retina, and flat retinas were mounted with Mowiol.

Stained sections and slides were analyzed using a Zeiss Axio Imager Z2 fluorescence microscope and Nikon C1si confocal microscope. Image J 1.50b with cell counter plugin (https://imagej.nih.gov/ij/) was used for cell counting following the online guide. The positive cells of Brn3, PKCa, active caspase-3, Ki67, and cell-type markers (including Rb, PKCa) were counted manually. The thickness of ONLs was measured by the microscope program. For vascular blood vessel analysis, representative images were analyzed using the AngioTool software (https://ccrod.cancer.gov/confluence/display/ROB2/Home) to quantify the vessel coverage (percentage of area covered by IB4+ endothelial cells), average vessel length, and lacunarity (distribution of the gap sizes surrounding the object).

**Microarray dataset selection and analysis**

The dataset GSE86372 at NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/) was used to compare WT vs. RbKO mouse P8 retinas, which include three WT and three RbKO mouse P8 retinas. The data were analyzed by GEO2R from the GEO website. The genes, of which expression fold changes are >2 or <0.5, and adjusted p < 0.05 were selected as the RbKO-related DEGs. Totally 677 DEGs were identified. The heatmap was generated using Heatmapper\(^{55}\). The function enrichment of DEG was performed using Enrichr\(^{56,54}\), the pathways with adjusted p < 0.05 were chosen to report.

**RNA extraction, RT, and quantitative real-time PCR**

Total RNA was isolated from dissected peripheral retina using the TriPure isolation reagent (Roche, USA) or RNaseasy mini kit (Qiagen) followed by digestion with RNase-Free DNase (DNA-free\(^{TM}\), Thermo Fisher Scientific) to remove DNA contamination. First-strand cDNA was synthesized from 0.2–0.5 µg of total RNA using the RT reagent kit with gDNA Eraser (TaKaRa, China) or SuperScript II first-strand synthesis system (Invitrogen). PCR primers are listed in Supplementary Table 1. Real-time quantitative PCR was performed using the qTOWER 2.2 PCR machine (Analytik Jena, Germany) or C1000 touch Thermal Cycler (Bio-Rad, USA). Tests were run in duplicate on three separate biological samples with EvaGreen PCR Supermix (SsoFastTM, Bio-Rad Laboratories, Singapore) or SYBRGreen PCR Master Mix (Applied Biosystems). PCR consisted of 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 55°C for 30 s. An additional cycle (95°C, 15 s) generated a dissociation curve to confirm a single product. Values obtained for test RNAs were normalized to β-actin mRNA levels.

**Statistical analysis**

All data were presented as mean ± SD. Statistical analysis was undertaken using the GraphPad Prism software (GraphPad Prism Software, Inc., San Diego, CA, USA). The results were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons. The threshold for significance was set at p < 0.05.

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D.C. and R.B. designed the study and interpreted data. Y.Z., R.W., L.Z., Y.C., S.L., C. L., Y.W., L.X., J.Z., and D.C. performed the experiments. D.C. and R.B. wrote the paper and all authors contributed to editing.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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**Supplementary Information**

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