Von Hippel–Lindau (Vhl) protein inhibits hypoxia-inducible factor (Hif), yet its deletion in murine retina does not cause the extensive angiogenesis expected with Hif induction. The mechanism is unclear. Here we show that retinoblastoma tumor suppressor (Rb1) constrains expression of Hif target genes in the \(Vhl^{-/-}\) retina. Deleting Rb1 induced extensive retinal neovascularization and autophagic ablation of photoreceptors in the \(Vhl^{-/-}\) retina. RNA sequencing, ChIP and reporter assays showed Rb1 recruitment to and repression of certain Hif target genes. Activating Rb1 by deleting \(cyclin D1\) induced a partial defect in the retinal superficial vascular plexus (SVP). Unexpectedly, removing Vhl suppressed retinoblastoma formation in murine \(Rb1/Rbl1\)-deficient retina, but generated subretinal vascular growths resembling retinal angiomatous proliferation (RAP), and retinal capillary hemangioblastoma (RCH). Most stromal cells in the RAP/RCH-like lesions were Sox9+, suggesting a Müller glia origin, and expressed Lgals3, a marker of human brain hemangioblastoma. Thus, the Rb family limit Hif target gene expression in the \(Vhl^{-/-}\) retina, and removing this inhibitory signal generates new models for RAP and RCH.
Rb1/Rbl1/Vhl loss induces mouse subretinal angiomaticous proliferation and hemangioblastoma

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Abstract (169 words)

Von Hippel–Lindau (Vhl) protein inhibits hypoxia-inducible factor (Hif), yet its deletion in murine retina does not cause the extensive angiogenesis expected with Hif induction. The mechanism is unclear. Here we show that retinoblastoma tumor suppressor (Rb1) constrains expression of Hif target genes in the Vhl−/− retina. Deleting Rb1 induced extensive retinal neovascularization and autophagic ablation of photoreceptors in the Vhl−/− retina. RNA sequencing, ChIP and reporter assays showed Rb1 recruitment to and repression of certain Hif target genes. Activating Rb1 by deleting cyclin D1 induced a partial defect in the retinal superficial vascular plexus (SVP). Unexpectedly, removing Vhl suppressed retinoblastoma formation in murine Rb1/Rbl1-deficient retina, but generated subretinal vascular growths resembling retinal angiomatous proliferation (RAP), and retinal capillary hemangioblastoma (RCH). Most stromal cells in the RAP/RCH-like lesions were Sox9+, suggesting a Müller glia origin, and expressed Lgals3, a marker of human brain hemangioblastoma. Thus, the Rb family limit Hif target gene expression in the Vhl−/− retina, and removing this inhibitory signal generates new models for RAP and RCH.
Introduction

Retinal neovascularization is associated with multiple disorders such as retinopathy of prematurity, diabetic retinopathy and age-related macular degeneration (AMD) (1, 2). Retinal angiomatous proliferation (RAP) is one form of neovascular AMD, which is a leading cause of blindness in the world (3). Neovascular AMD also includes two other forms, typical choroidal neovascularization (CNV) and polypoidal choroidal vasculopathy (PCV). RAP arises from deep retinal capillaries and develops from intra-retinal (stage I), to subretinal (stage II), and finally to choroidal neovascularization and retinal–choroidal anastomosis (stage III) (4). Pathologically RAP is an intra-retinal angiomatic complex within the outer retina (5, 6). While RAP follows a different natural course and response to treatment from typical CNV, few animal models are established to allow us to fully understand the pathogenesis or to test new treatments for late stage disease, even though there are several mouse models of early stage RAP (7-11).

Retinal capillary hemangioblastoma (RCH) is the hallmark lesion of ocular von Hippel–Lindau (Vhl) disease (12). This multisystem tumor syndrome is caused by germ line Vhl gene mutations, and also associated with many other tumors, such as brain hemangioblastoma and clear cell renal cell carcinomas (CCRCC) (13). Vhl protein is a component of the E3 ubiquitin ligase complex that targets hypoxia-inducible factor α (Hifα) for proteasomal degradation. Hif is the master regulator of the response to hypoxia, as Hifα accumulates and induces the transcription of genes involved in adaptation to hypoxia, including Bnip3, Epo and Vegfa (14). Angiogenesis is critical for adaptation to hypoxia and retinal development, and also influences or drives many retinal diseases. In murine newborn eyes, the hyaloid vessels in the vitreous regress and the retinal vessels develop to supply blood to the retina (15). The retinal vasculature consists of three parallel vascular plexi, including superficial vascular plexus (SVP) in the nerve fiber layer (NFL), deep vascular plexus (DVP) in the outer plexiform layer (OPL), and intermediate vascular plexus (IVP) in the inner plexiform layer (IPL) (16).

The origin of the true tumorigenic cell type in RCH and RAP is controversial. This reflects the dearth of human samples available for histopathological analyses, limiting the vast majority of studies to observations in intact human eyes. In RCH, early work referred to an astrocytic component (17). More recently, but with only three cases, two groups reported that GFAP+ glial
cells contribute to human RCH (18) (19). Thus, cell of origin of human RCH needs further investigation.

Deleting Vhl in murine retina delays regression of hyaloid vessels and inhibits retinal angiogenesis, which depend on the associated high levels of Hif1α (20, 21). Paradoxically, therefore, high Hif does not induce RCH, but instead inhibits retinal angiogenesis in the murine Vhl⁻⁻ retina. Deleting Vhl in all retinal cells and vascular endothelial cells using UBC-Cre<sup>ER</sup> also suppresses retinal angiogenesis (22). Deleting Vhl in specific retinal cell types, such as amacrine or horizontal interneurons (23), rods (24), cones (25), or RPE (retinal pigment epithelium) cells (26), also does not induce RCH. While removing Vhl in murine hemangioblasts using Scl-Cre<sup>ER</sup> triggered some features of early stage RCH, including dilated tortuous vessels, vascular leakage and foamy stromal cell clusters; histologically it did not cause any neovascularization or hemangioblastoma structures in the retina (27). Thus, it remains unclear why the Vhl⁻⁻ retina lacks vascular overgrowth and RCH. One possibility is that inhibitors interfere with Hif activity. Indeed a SNP microarray study implicated several cell cycle genes in the pathogenesis of hemangioblastoma, including the retinoblastoma repressor (Rb1) (28). Inactivating Vhl plus additional tumor suppressors (Rb1 and Tp53) can induce CCRCC (29). However, inhibitors that blocks angiogenesis in the Vhl⁻⁻ murine retina are unknown.

In addition to angiogenesis, autophagy is also important for hypoxia adaptation, as it captures and degrades intracellular components to sustain metabolism and homeostasis (30). Hypoxia-induced autophagy requires the constitutive expression of Becn1 and Atg5, and the Hif1-dependent expression of Bnip3/Bnip3L (30, 31). Autophagy has been observed in human RCH (32), but its role in the Vhl⁻⁻ murine retina is unclear.

Retinoblastoma is the most common pediatric intraocular tumor. Rb1 regulates cell cycle, cell death and many other cellular processes in part by inhibiting the E2f transcription factors (33). Loss of Rb1 and its relative Rbl1 (also called p107) in the retina causes mouse retinoblastoma (34-36). In the Rbl1/Rbl1 null retina, all differentiating cells divide ectopically, excitatory neurons (ganglion cells, bipolar cells, rods and cones) undergo apoptosis, while inhibitory neurons (horizontal and amacrine cells) and Müller glia survive, and retinoblastoma arises from the amacrine cell lineage (34). Rb1 can bind to the promoter and repress the expression of Bnip3,
a Hif1 target gene (37); and there are E2f binding sites in the promoter of several pro-angiogenic
Hif targets (38), raising the possibility that Rb1 might be the unknown inhibitor that counters Hif
activity in the Vhl⁻/⁻ retina. However, there is also in vitro work suggesting that Rb1 binds and
potentiates Hif1 (39), although this has not been reproduced by others. We showed that Rb1 is
required for the formation of intra-retinal vascular plexi (40). Nevertheless, this defect is indirect,
as it is rescued by deleting Bax and blocking neuronal cell death (40). Thus, whether Rb1 has a
separate direct role in constraining Hif activity and angiogenesis is unclear.

The Ccnd1 gene encodes cyclin D1, a regulatory subunit of the holoenzyme that phosphorylates
and inactivates Rb1. The retina has high levels of cyclin D1 that keep Rb1 inactive in retinal
progenitors (41). Ccnd1⁻/⁻ mice exhibit a hypocellular retina due to premature cell cycle exit of
retinal progenitor cells (41). Whether cyclin D1 influences retinal angiogenesis is unknown.

We utilized genetics, RNA sequencing, ChIP, and reporter assays to address these questions.
Strikingly, Rb1 deletion in the Vhl⁻/⁻ retina triggered both extensive retinal neovascularization and
photoreceptor autophagy. These Rb1 functions were linked to direct binding and repression of
Hif target genes in the retina. Moreover, activating Rb1 in Ccnd1⁻/⁻ retina induced a SVP defect.
Unexpectedly, Vhl loss suppressed murine retinoblastoma by inhibiting ectopic division in the
cell-of-origin, allowing us to assess the effect of removing both Rb1/Rbl1 and Vhl on
angiogenesis without the confounding effects of tumorigenesis. Notably, the Rb1/Rbl1/Vhl triple
null retina exhibited subretinal lesions that resemble human late stage RAP (5, 6) and RCH (12).
Most stromal cells in the RAP/RCH-like lesions were Sox9⁺ and galectin Lgals3⁺, suggesting a
Müller glia origin. Our study reveals new roles for Rb1 in constraining Vhl null-induced retinal
angiogenesis and autophagy, establishes a mouse model of RCH and late stage RAP, and links
the Rb pathway to vessel growth associated with neovascular AMD and Vhl disease.
Results

Rb1 suppresses angiogenesis in the Vhl null retina

To delete floxed (istencia) Rb1 or Vhl alleles (20, 21, 34) we used the α-Cre transgene, which is active from embryonic day 10 (E10) in peripheral retinal progenitors (42). We bred α-Cre;Rb1<sup>f/f</sup> (Rb1KO) mice with Vhl<sup>f/f</sup> mice, and quantified vessel growth using the endothelial marker isolectin B4 (IB4). As reported before (20, 21), α-Cre;Vhl<sup>f/f</sup> (VhlKO) mice demonstrated increased level of Hif1α protein (Supplementary Figure 1), delayed regression of hyaloid vessels and retinal angiogenesis, but unlike VHL patients, lacked any evidence of vascular overgrowth except a few vessels invade into the outer nuclear layer (ONL) (Figure 1A, B). Indeed, the protein levels of Vegfa and Kdr were normal (Supplementary Figure 1). In the Rb1/Vhl double knockout (DKO) retina, hyaloid vessel regression was still delayed, but strikingly, there were many new blood vessels in the peripheral retina (Figure 1A, B). These vessels penetrated throughout all retinal layers to form a dense capillary bed, and there was no normal SVP, IVP or DVP (Figure 1B). To compare the vascular density to other genotypes, we used vascular images taken from similar depths below the GCL (ganglion cell layer) to represent the IVP and DVP for the DKO retina. The vascular density was 3-4 times higher than in WT retina (Figure 1B, C).

These new blood vessels began to form at around P8, likely originated from both the existing retinal vasculature and the persistent hyaloid vessels (Supplementary video 1), as they were linked to hyaloid vessels (Figure 1D, arrow heads) and the DVP in the central retina (Figure 1D, arrows). In the middle of the new vessel bed, there were many IB4<sup>+</sup> “grape-like” clusters of endothelial cells (Figure 1E, 1F). As the delayed hyaloid vessels blocked the fluorescein signal (Figure 1G), the retinal blood vessels of live animal in FFA (fundus fluorescein angiography) were not clear, but neovascularization and vascular leakage could be documented at late stages (Figure 1H, arrows). Retro-orbital injection of FITC-dextran (43) demonstrated that the new vessels were leaking, and clearly shunted to the persistent hyaloid vessels (Figure 1I). These new vessels persisted for at least 2 years (Figure 1J).

Rb1 loss alone causes apoptosis of certain retinal neurons, which indirectly impedes angiogenesis (40). Cell loss is unlikely to explain the excessive angiogenesis in the Rb1/Vhl null retina. However, in addition, Rb1 loss causes a transient period of ectopic division of all retinal
cell types, and disrupts lamination (34, 40). To examine whether any of these $Rb1$ null phenotypes might explain the excessive vasculature in the $Rb1/Vhl$ null retina we took two genetic approaches. The $Rb1^{+/−}$ retina is wild type (34), thus we assessed the effect of removing one $Rb1$ allele on angiogenesis. Notably, this subtle genetic manipulation still induced retinal neovascularization in the $Vhl^{−/−}$ retina (Figure 1A-C). Moreover, vessel density was lower than in the $Rb1/Vhl$ DKO retina, revealing a dose-dependent role for $Rb1$ in limiting angiogenesis. Second, we assessed the effect of $E2f1$ on angiogenesis. $Rb1$ binds and inhibits $E2f$, and deleting $E2f1$ rescues all the ectopic cell division, cell death and lamination defects seen in the $Rb1$ null retina (40, 44). Importantly, $E2f1$ deletion did not rescue neovascularization in the $Rb1/Vhl$ DKO retina (Figure 1A-C). Together, these $Rb1^{+/-}$ and $E2f1^{−/−}$ data suggest that $Rb1$ inhibits vessel growth in the $Vhl^{−/−}$ retina independent of its roles in controlling cell division, apoptosis or lamination.

**Rb1 suppresses loss of Vhl null photoreceptors**

The above data show that deleting $Rb1$ unleashes the proangiogenic effects associated with $Vhl$ loss. Potentially, therefore, $Rb1$ also suppresses other phenotypes in this context. For example, while the number of all retinal cell types were normal in P18 $VhlKO$ retina (Figure 2A-D), there is modest loss of photoreceptors in the $Vhl^{−/−}$ retina around P30 (20). But whether $Rb1$ limits this phenotype is unknown. To assess this issue, we stained the P18 $Rb1/Vhl$ DKO retina with cell type-specific markers. As in the $Rb1^{−/−}$ tissue, the DKO retina was missing ganglion (Pou4f2$^+$) and rod bipolar (Pka$^+$) cells (Figure 2A-B, Supplementary Figure 2). Whereas the $Rb1^{−/−}$ retina retained many rod (Rho$^+$) and all cones (Arr3$^+$) as reported before (34), all photoreceptors were absent in the DKO tissue (Figure 2A-B, Supplementary Figure 2). To exclude the possibility that these neurons never formed in the DKO retina, we stained P0 DKO retina with Pou4f2, Crx (precursors of rod, cone, bipolar cells) and Thrb2 (precursor of cones) (supplementary Figure 3). Obviously all these neurons formed in the DKO retina. Thus, all $Rb1/Vhl$ null excitatory neurons are generated but then die, indicating that $Rb1$ and $Vhl$ loss are synthetic lethal to photoreceptors. Similar to the $Rb1^{−/−}$ tissue, the P18 $Rb1/Vhl$ DKO retina retained inhibitory amacrine (Ap2a$^+$) and horizontal (Onecut2$^+$) neurons, as well as Müller glia (Sox9$^+$), and while the DKO tissue had
similar numbers of horizontal cells, there were modest increases in the proportion of amacrine cells (~2-fold) and Müller glia (~1.5-fold) (Figure 2C-D, Supplementary Figure 2). These increases paralleled increased Mki67 and phospho-histone H3 (PH3) indexes in the P18 DKO retinas (Supplementary Figure 4A-B). While Müller cells kept dividing at P18, amacrine cells were most post-mitotic at this point, suggesting that their slightly elevated numbers may reflect ectopic division at an early time point (Supplementary Figure 4C-D). In summary, therefore, while deleting Rb1 and Vhl modestly increases the number of two INL cell types, it has a profound effect on both angiogenesis and photoreceptor survival.

**Rb1 binds and represses Hif target genes in the retina**

A unifying explanation for the effects on angiogenesis and photoreceptor survival may be that Rb1 directly represses Hif targets in the retina. Hif can induce autophagy genes (31), thus if Rb1 indeed represses these and other Hif targets, then photoreceptor cell death in the Rb1/Vhl null retina may be autophagic. In the Rb1−/− retina, neurons die by apoptosis (34, 44), but notably Rb1/Vhl DKO did not increase, and instead reduced cleaved caspase 3+ cells at P8 or P18 by ~2-fold (Figure 2E-F). Western blots of P14 retinal lysates confirmed this reduction (Figure 3A-B). Analysis of several autophagy regulators revealed that while Becl1 was not affected in the DKO retina, Atg5, Atg7, Bnip3 and Bnip3L were induced and the MAP1LC3B II/I ratio increased (Figure 3A-B). Thus, the dramatic loss of photoreceptors in the DKO tissue is due to both autophagy and apoptosis, supporting the idea that Rb1 may repress retinal Hif targets. VhlKO alone also induced autophagy without apoptosis in the retina (Supplementary Figure 1A-B), and likely autophagy causes the photoreceptor loss in the Vhl−/− retina at late stage (20, 21). One allele of Rb1 was sufficient to suppress the expression of Atg5, Atg7 and Bnip3L in the Vhl−/− retina (Supplementary Figure 1A-B), confirming Rb1 can directly inhibit Hif-induced autophagy. Indeed, Rb1+/−/Vhl−/− retina had less rod photoreceptors than Vhl−/− retina (Figure 2A-B).

To further understand why Rb1/Vhl loss induces extensive retinal angiogenesis and photoreceptor death, we performed RNA sequencing from P14 WT, Vhl−/−, Rb1+/−/Vhl−/− and Rb1−/− Vhl−/− retinas. We analyzed differentially expression genes (DEGs), and identified 1393 Vhl−/−-related DEGs compare to WT. There were 820 down- and 573 up-regulated genes (Supplementary Table 1). Gene list enrichment indicated that, the most enriched down-regulated DEGs were in the pathways of ECM-receptor interaction; some photoreceptor genes were also
slightly down-regulated, including Grk1, Slc24a1, Gnat1 and Cngb1. The most enriched up-regulated DEGs were in the pathways of ribosome biogenesis and oxidative phosphorylation, consistent with previous report that Vhl inhibits ribosome biogenesis (45) and the vascular defects in the Vhl<sup>−/−</sup> retina (Figure 1A-C). Only a few Hif pathway genes were slightly up-regulated in P14 Vhl<sup>−/−</sup> retina including Nos3 and Hmox1 (Supplementary Table 1), consistent with the presence of an inhibitor of Hif targets.

We further identified 890 Rb1<sup>+/+</sup>Vhl<sup>−/−</sup>-related DEGs and 4686 DKO-related DEGs, compare to Vhl<sup>−/−</sup> retinas (Supplementary Table 1). For DKO retinas, the most enriched down-regulated DEGs were in the pathways of oxidative phosphorylation and phototransduction, the most enriched up-regulated DEGs were in the pathways of cell cycle, ECM-receptor interaction, PI3K-Akt and Hif1 (Figure 3C-D). For Rb<sup>+/+</sup>/Vhl<sup>−/−</sup> retinas, the most enriched down-regulated DEGs were in ribosome biogenesis, and the most enriched up-regulated DEGs were in the pathways of ECM-receptor interaction, Hif1 and PI3K-Akt. Notably, cell cycle genes were not enriched in the Rb<sup>+/+</sup>/Vhl<sup>−/−</sup> retinas (Figure 3C-D). These RNAseq data support the notion that Rb family members constrain induction of Hif1 targets in the absence of Vhl.

RT-PCR results confirmed altered expression of several Hif targets, such as Bnip3, Epo, Tek, Vegfa and Kdr. Deleting Rb1 in Vhl<sup>−/−</sup> retina increased expression of these genes 1.7 to 2.7 fold (Figure 3E). Deleting one Rb1 allele had an intermediate effect, indicating a dose dependent regulation of Hif target genes (Figure 3E). The changes in protein levels of Vegfa and Kdr also supported this notion (Figure 3A-B, Supplementary Figure 1).

To test whether Rb1 directly regulates these genes in the retina we ran ChIP assays. It was difficult to obtain enough pure knockout material from the peripheral Cre-positive region of the α-Cre;Vhl<sup>+/−</sup> retina, thus we assessed Rb1 binding in WT retinal explants left untreated or exposed to CoCl2, which mimics hypoxia and induces Hif1α accumulation (Supplementary Figure 5) (46). Consistent with a previous analysis in fetal liver (37), Rb1 was bound to the promoters of several Hif targets such as Bnip3, Epo, Kdr, Tek and Vegfa (Figure 3F). CoCl2 treatment did not increase Rb1 binding (Figure 3F), suggesting that Hif1α might not directly recruits Rb1 to these targets, instead and most likely, Rb1 binds E2f which was recruited to the promoter of these targets, and recruits some cofactors that can bind and inhibit Hif protein or HRE (Hif responsive element) (37).
Dual luciferase reporter assay using 293T cells indicated that Rb1 can partially repress the transcription activity of the 198bp-promoter of Vegfa gene (~15% reduced compared to empty vector), the 938bp-promoter of Kdr gene (~55% reduced compared to the empty vector) and the 337bp-promoter of Tek gene (~35% reduced compared to the empty vector). Rb did not affect the shorter 213bp-promoter of Kdr (Figure 3G), suggesting a requirement for upstream regions. The result with the Vegfa 198bp-promoter-luc reporter is consistent with results showing that E2f1 can regulate a similar reporter (47).

Together, the above genetic and molecular analyses reveal Rb1 inhibits a subset of Hif1 target genes, providing a logical mechanism to explain why the Vhl−/− retina does not exhibit vessel overgrowth phenotypes, such as RAP or RCH.

**Rb1 suppresses the superficial vascular plexus in the Ccnd1−/− retina**

To further examine the effect of Rb1 on Hif targets, we asked whether activating Rb1 represses these genes. Hif expresses in WT retinal progenitors (21) and is required for the retinal vascular development (48). There are very high levels of cyclin D1 in retinal progenitors, which activate Cdk4 and Cdk6 kinase that phosphorylate and inhibit Rb1 (41). Deleting Ccnd1 activates Rb1 and we hypothesized that it may inhibit Hif action and impede angiogenesis in the developing retina. Indeed, Ccnd1−/− mice had retinal angiogenesis defects: There was almost no DVP or IVP, and the SVP had not reached to the peripheral retina by P18 (Figure 4A-D).

Ccnd1 loss reduces retinal progenitor proliferation, so these vascular effects might be an indirect consequence of hypo-cellularity rather than Rb1-mediated repression of Hif targets, similar to the rd1 retinal degeneration retina (49). To examine that possibility, we took three approaches. First, horizontal sections of P18 Ccnd1−/− retina were stained for all seven retinal cell types (Supplementary Figure 6). While the retinal thickness reduced as reported (41), clearly all retinal cell types formed and properly laminated, excluding the possibility that neuronal developmental delay or defects caused the vascular defects.

Second, we studied the peripheral α-Cre; E2f1−/−;E2f2−/−;E2f3ff retina, as removing E2f1-3 also reduces retinal progenitors and to an even greater extent than Ccnd1−/− (50). Notably, both SVP and DVP developed normally, although the IVP developed late (Figure 4A-D). Thus, severe
effects on the SVP and DVP are uniquely associated with $Ccn1^{-/-}$ and not with hypo-cellularity 
per se.

Third, we tested directly whether suppression of angiogenesis in the $Ccn1^{-/-}$ retina requires Rb1. There was no difference of body weight between $Ccn1^{-/-}$ and $\alpha$-Cre;$Ccn1^{-/-};Rb1^{+/+}$ mice (Supplementary Figure 7), indicating the overall development is similar between these genotypes. The $Ccn1/Rb1$ null retina still lacked the DVP and IVP but strikingly, it had a completely normal SVP (Figure 4A-D). Differentiating $Rb1^{-/-}$ retinal cells undergo ectopic division and some cell types die, but it is unlikely that these events promotes SVP development, because Bax-driven cell death in the $Rb1^{-/-}$ retina actually impedes IVP and DVP development (40). To validate that interpretation we further assessed the effect of $Rb1$ heterozygosity, which does not perturb any aspect of retinal development (34). As with $Rb1$ loss, there was a complete rescue of the SVP in the $Ccdn1^{-/-}Rb1^{+/+}$ retina (Figure 4A-D). Thus, the SVP defects in $Ccdn1^{-/-}$ retina were caused by activated Rb1. Notably, the expression of several Hif targets was significantly repressed in $Ccn1^{-/-}$ retina, which was rescued by removing one or two copies of $Rb1$, whereas only $Bnip3$ was slightly reduced in the $E2f1^{-/-}$ null retina that had a normal SVP (Figure 4E). The mechanism of the DVP/IVP defects in the $Ccdn1^{-/-}$ retina remain unclear, but may reflect inhibition of Hif1 targets by Rb1 relatives or Rb1-independent functions of cyclin D1, such as transcriptional regulation of Notch pathway (51).

In summary, multiple genetic and molecular analysis suggest that inhibition of Rb1 by cyclin D1 in retinal progenitors is critical to permit Hif to promote normal SVP development.

**Vhl is required for retinoblastoma in the $Rb1/Rbl1$ null retina**

Since Rb1 inhibited Hif target in the $Vhl^{-/-}$ retina, removing another Rb family member, such as Rbl1, might further enhance vascular overgrowth. Before addressing that hypothesis, we first asked whether Vhl affects any phenotypes caused by $Rb1/Rbl1$ loss. The most striking difference between $Rb1$ and $Rb1/Rbl1$ loss is that only the latter causes murine retinoblastoma (34, 35). Vhl is a potent tumor suppressor in other tissues, suggesting that $Vhl$ deletion might enhance retinoblastoma penetrance (13). However, whereas both Hif1$\alpha$ and Hif2$\alpha$ promote angiogenesis, the latter promotes while the former inhibits cancer progression (52). Notably, long-term
analysis of a large cohort of mutant mice revealed that the \textit{Rb/Rbl1/Vhl} triple knockout (TKO) retina is resistant to retinoblastoma (Figure 5A).

All seven retinal cell types in \textit{Rbl1/Rbl1DKO} retina fail to exit the cell cycle and divide ectopically (34, 44). Even though there were many Mki67$^+$ dividing cells at P8 and P18 (Supplementary Figure 4), they gradually exited cell cycle when they fully developed, thus they were not tumor cells. Indeed, there were almost no Mki67$^+$ cells at P138 in \textit{TKO} retina (Supplementary Figure 8). These data imply predominance of Hif1$\alpha$ in the cell-of-origin of retinoblastoma. Prior analysis of nascent tumors revealed that \textit{Rbl1/Rbl1} null murine retinoblastoma arises from ectopically dividing amacrine cells, which have an intrinsically high resistance to apoptosis (34). Therefore, we were interested in any effects of \textit{Vhl} loss on this lineage.

First, we assessed whether \textit{Vhl} loss compromised the survival of the tumor cell-of-origin. The \textit{Rbl1/Rbl1} null retina loses all ganglion, rod, cone, and rod bipolar cells, but retains ectopically dividing amacrine, horizontal and Müller cells (34). The \textit{TKO} retina lost the same cell types as the \textit{Rbl1/Rbl1} null tissue (Figure 2A-B). In addition, while \textit{Vhl} loss reduced the extent of cleaved caspase 3$^+$ cells, akin to the \textit{Rbl1/Vhl DKO} retina (Figure 2E-F), there was increased autophagy, as Atg5, Atg7, Bnip3/Bnip3L and the MAPLC3B II/I ratio increased (Figure 3A-B). Notably, however, the \textit{TKO} retina retained amacrine, horizontal and Müller cells (Figure 2C-D), indicating that \textit{Vhl} is not required for the survival of the retinoblastoma cell-of-origin.

Indeed, there were actually more amacrine cells, and more Müller glia when \textit{Vhl} was missing (Figure 2C-D). Cell specific analysis of Mki67$^+$ cells revealed that whereas both amacrine and Müller cells were dividing in the \textit{Rbl1/Rbl1} null retina, by P18 Müller glia were the major dividing cell type in the \textit{TKO} retina, whereas amacrine cells and horizontal cells were largely quiescent (Supplementary Figure 4C-D). These features resemble the effect of \textit{Vhl} loss in the \textit{Rbl1$^-$} retina (Supplementary Figure 4C-D).

The requirement for \textit{Vhl} to maintain ectopic division in \textit{Rbl1/Rbl1} null amacrine cells explains the absence of tumorigenesis in the \textit{TKO} tissue. In line with these cellular effects, RNA sequencing analysis identified 4077 \textit{TKO}-related DEGs, compare to \textit{Rbl1/Rbl1 DKO} retinas (Supplementary Table 1). The most enriched down-regulated DEGs were in the pathways of
DNA replication and cell cycle, the most enriched up-regulated DEGs were in the pathways of ribosome biogenesis, PI3K-Akt, ECM-receptor interaction and Hif1(Figure 5B, 3D). Indeed, TKO retina had lower expression of cell cycle genes, such as Cdk2, Ccne1, E2f1 and Tyms, although the expression of Myc family and some cell death genes (Bax, Becn1 and Bbc3) were similar between the two genotypes (Figure 5C).

Vhl loss can cause a senescent-like phenotype in mouse embryonic fibroblasts (53). However, we did not identify any signs of senescence in VhlKO, Rb1/Vhl DKO and Rb1/Rbl1/Vhl TKO retinas (Figure 5D). It will be interesting to deduce how Vhl loss influences cell cycle gene expression, but our major goal here was to address the role of the Rb family in constraining Vhl−/−-induced angiogenesis. The complete absence of cancer in the TKO retina removed a confounding factor in the pursuit of this goal.

**Rbl1/Rbl1/Vhl TKO retina forms RCH/RAP-like lesions**

Next, therefore, we asked whether deleting Rbl1 affects vessel growth in the Rbl1/Vhl null retina. Indeed, IB4 staining revealed that all 51 eyeballs of TKO mice we analyzed form RCH-like lesions after P10 (Figure 6A-B). Human RCH generally locates at the inner retinal surface, but the RCH-like lesions described here were always in the subretinal space, resembling stage II-III RAPs (5, 6). FFA revealed subretinal vascular leakage at late stages, even though the images were not clear as the fluorescein was blocked by the persistent hyaloid vessels (Figure 6C, arrows). Histologically, the RAP lesion was a vascular mass between the neuronal retina and the RPE layer (Figure 6D). RPE cells beside RAPs often degenerated, as there were many RPE vacuoles (Figure 6D, asterisks). Thus in some areas, RAPs could penetrate the RPE layer, and grow into the choroid, likely to form retinal–choroidal anastomosis (Figure 6D, arrow heads). Indeed, there were almost no RPE65+ RPE cells around RAP lesions (Figure 6E). Inside the RAP lesion, similar to RCH, there were many vacuolated “foamy” stromal cells (Figure 6D, arrow), likely due to Vhl deficiency induced abnormal lipid metabolism, also seen in the CCRCC and liver hemangioblastoma (54). The foamy stromal cells are considered as the true tumor cells in hemangioblastoma (12).

Next, we sought to define the cellular origin of the foamy stromal cells in the RAP/RCH-like lesions. They were mainly composed of Sox9+ cells, without any Ap2a+ amacrine cells,
suggesting that they originated from Müller glia (Figure 7A). Consistent with this notion, we found that these lesions expressed high levels of Lgals3 (Figure 7A), which is mainly expressed in Sox9+ Müller glial cells, but not Ap2a+ amacrine or Aif1+ (also called Iba1) microglial cells in WT retina (Figure 7B). Western blot and RT-PCR also confirmed this finding (Figure 3A-B, 5C). Lgals3 is expressed in all stromal cells of human cerebellar hemangioblastoma (55). High magnification images showed that Lgals3+ cells were the stromal cells, and they never co-stained with IB4+ endothelial cells (Figure 7A, blow-up). Lgals3 also marks active microglial cells (56), but co-staining with Aif1 indicated that while some microglia were present, most cells in the RAP lesions were Aif1-negative, further supporting a Müller glia origin (Figure 7A).

RPE cells also express Sox9 (57), thus it was possible that RPE cells are the cellular origin of the RAP lesions. However, four findings countered this idea. First, there were almost no RPE65+ cells in RAP lesions (Figure 6E). Second, RPE cells did not express Lgals3 (Figure 7B, arrow). Third, laser capture of micro-dissected RAP lesions followed by PCR analysis of genomic DNA confirmed Cre-mediated recombination of Rbl in the RAP lesions, confirming the retinal origin of the lesions (260 bp band in Figure 7C). Some un-recombined bands (670bp) may derive from blood vascular cells. Finally, we used Z/Red Cre-indicator mice to lineage-trace knockout cells (58), and found many DsRed+ cells in the RAP lesions, indicating that the cell-of-origin derives from the Cre-expressing retinal cells (Figure 7D). Thus, deleting Vhl in the Rbl/Rbl1 null retina prevented amacrine cell transformation, but induced RCH/RAP-like lesions that originate from Müller glial cells.
Discussion

The Vhl<sup>−/−</sup> retina does not exhibit vascular overgrowth such as RAP or RCH (20, 21), but it is unclear how Hif target expression is suppressed. Our data now pinpoint Rb1 as the critical angiogenic inhibitor. Thus, the Rb1/Vhl DKO retina exhibits striking levels of angiogenesis. Multiple pieces of evidence argue that this is not an indirect consequence of other cellular effects of Rb1 loss. First, Bax-driven apoptosis in the Rb<sup>−/−</sup> retina actually impedes vessel growth (40). Second, Rb1-heterozygosity does not perturb retinal development (34), but stimulates vessel growth in the Vhl<sup>−/−</sup> retina. Third, deleting E2f1 completely rescues ectopic division, apoptosis and abnormal lamination in the Rb1<sup>−/−</sup> retina (40), yet it does not reduce the excessive vessel growth in the DKO retina. Fourth, Hif can also stimulate autophagy, and removing Rb1 and Vhl triggers autophagic death of photoreceptors. Fifth, deleting Rb1 induces several Hif targets that drive both angiogenesis and autophagy. Finally, ChIP assays show that Rb1 protein binds to the promoters of several Hif targets in vivo, and reporter assays indicated that Rb1 could partially inhibit their transcription. Together, these data present a strong case that Rb1 directly inhibits Hif target expression in the retina, and explains why vascular growth is constrained in the Vhl<sup>−/−</sup> murine tissue. In addition, activating Rb1 in Ccnd1<sup>−/−</sup> retina suppresses Hif target expression and impedes SVP development, suggesting that Rb1 is necessary and sufficient to suppress Hif targets.

Our data indicate that in the Vhl<sup>−/−</sup> retina, Rb1 is haploinsufficient with respect to blocking either angiogenesis or autophagy. This finding is intriguing given that the Rb1<sup>−/+</sup> retina is normal (34). Rb1 is one of the classical tumor suppressor genes which are thought to require mutation or loss of both alleles to facilitate tumour progression (59). However, it has become clear that haploinsufficiency may contribute to tumorigenesis without loss of heterozygosity, such as is the case for Brca1, Brca2 and Tp53 (59). Rb1 haploinsufficiency has been associated with increased genomic instability in premalignant osteoblasts (60) and fibroblasts (61). Potentially, therefore, some of the phenotypes in VHL patients might be related to partial reduction in Rb1 levels.

We find that Vhl loss induces retinal autophagy, which is the likely reason for delayed, partial loss of photoreceptors in the Vhl<sup>−/−</sup> retina (20, 21). Rb1 or Rh/Rbll loss triggers apoptosis of certain retinal neurons (34, 35), but we find that Vhl loss partially suppresses apoptosis in the Rb1<sup>−/−</sup> and Rh/Rbll null retina. This suppression may be linked to enhanced autophagy (62). Our
data suggest that the combination of residual apoptosis plus enhanced autophagy contributes to synthetic lethality in Rbl/Vhl DKO rods and cones. It is also intriguing that inhibitory neurons and Müller glia can escape apoptosis or autophagy, but excitatory neurons die in both conditions. This finding has important implications in understanding the pathogenesis of retinal diseases, which have common risk factors such as smoking, which can inactivate Rbl (63), and oxidative stress, which can activate autophagy (64). In addition, while Vhl loss can induce cell cycle arrest of Rbl null or Rbl/Rbl null amacrine cells, it seems to enhance division in the Müller glia. This could contribute to reactive gliosis in some vascular retinal diseases.

Although Vhl is a well-known tumour suppressor, its deletion completely suppresses retinoblastoma in the Rbl/Rbl null retina. These tumours arise from ectopically dividing amacrine cells (34), so the quiescence of Rbl/Rbl/Vhl null amacrine cells as early as P18 provides a logical explanation for tumour suppression. While both Hif1α and Hif2α are stabilized in Vhl null retina, Hif1α is the major mediator of retinal phenotypes (20, 21). In many instances Hif1α acts as a tumour suppressor while Hif2α promotes tumour progression (52). For example, Hif2α, rather than Hif1α, is the key driver of renal cancer progression (65). Thus, it is possible Hif1α inhibits retinoblastoma initiation in the Rbl/Rbl null retina.

The absence of cancer in the Rbl/Rbl/Vhl TKO retina was an advantage, as it meant that any new vascular phenotypes relative to the TKO tissue could not be an indirect consequence of tumorigenesis. Deleting all three genes generated a mouse model with subretinal RCH or RAP-like lesions. Scl-CreER induced Vhl deletion in hemangioblasts can generate some features of early stage RCH, however histologically there are no hemangioblastoma structures and neovascularization in the retina, except some foamy stromal cell clusters (27). Vhl loss alone does not induce kidney Vhl disease, but Rbl/p53/Vhl triple knockout induces a CCRCC mouse model (29). Similarly, our results show that the RCH-like lesions can originate from retinal cells, but Vhl loss alone may not be sufficient. The foamy cells in the RCH-like lesions we detected originate from Müller glia, which express high levels of Sox9. Consistent with a report that Lgals3 is a diagnostic marker for brain hemangioblastoma (55), we also found RCH-like lesions express Lgals3, indicating that it is also a possible biomarker for diagnosing human RCH. As most eyes of RCH patients do not need to be enucleated, it has remained unclear whether human RCH has a glial component. An early study referred to an astrocytic component of RCH (17).
More recently, using histologic, immunohistochemical and ultrastructural methods to study three cases where samples were available, two groups reported that GFAP+ glial cells are a major component of human RCH (18) (19). The cell of origin of human RCH needs further investigation, but our work supports the notion that foamy stromal cells may originate from Müller glia.

The RCH/RAP-like lesions located in the subretinal space, and can induce degeneration of the RPE and invade into choroid. These features are similar to advanced stage III human RAP. Early stage RAP is observed in several mouse mutants, including mice with ONL-specific Vegf overexpression (7), inactivation of Vldlr (9) or Srf (10), and also in Bst (8) and NRV2 mutant mice (11). The retinal neovascularization in these mice is similar to stage I-II RAPs, and may be related to local Vegf up-regulation. However, there are no obvious stromal cells, and no angiomatic complexes in the later stages, which are quite different from the pathological changes in late stage human RAP (5, 6). For the same reasons as RCH, few reports have described the cellular features of human RAP. Recent studies found that RAP is a circumscribed intra-retinal angiomatic complex within the outer part of the neurosensory retina, composed of endothelial cells and stromal cells. Endothelial cells within the RAP lesion stained positively for VWF and VEGF (5, 6). However, retinal cell markers (neuronal or glial) were not stained in the RAP lesion, thus it remains unclear whether there is a glial component. Our work suggests that glia is a major component of RAP-like structures in mice. Based on these data, it will be interesting for the field to revisit the question of the origin of stromal cells in human RAP.

In summary, our work exposes previously unrecognized roles for the Rb family in constraining retinal angiogenesis and autophagy, and establishes a new mouse model of RCH/RAP, which will be a useful tool in dissecting the pathogenesis of these disorders, optimizing therapeutic strategies, and stimulating further work to address the origins of stromal cells in the human disease.
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 (ARVO) statement for the use of animals in ophthalmic and visual research. α-Cre mice (P. Gruss), Ccnd1−/− mice (Jackson Laboratory, stock# 002935), E2f1−/− mice (M. Greenberg), E2f2−/− mice (G. Leone), E2f3−/− mice (G. Leone), Rbl1f/f mice (A. Berns), Rbl1−/− (p107−/−) mice (M. Rudnicki), Vhl−/− mice (Jackson Laboratory, stock#004081) and Z/Red mice (Jackson Laboratory, stock#005438) were maintained on a mixed background. Different genotypes were compared within the same litter and across at least three litters. We have not noted any phenotypic differences in separate litters. Genotyping was performed as before and the Jackson laboratory guideline.

Histology, immunofluorescence and measurements: Eyeballs were fixed for 1 hour at 4°C in 4% paraformaldehyde, embedded in OCT (TissueTek 4583), frozen on dry ice and cut into 12-14 µm sections on Superfrost slides. The following antibodies were used: Aif1 (Wako 019-19741), Ap2a (Santa Cruz SC-8975), Calb1 (Sigma C9848), Cleaved caspase 3 (Cell Signaling Technology 9661), Arr3 (Millipore AB15282), Crx (CY Gregory-Evans, Imperial College School of Medicine, London, UK)(66), DsRed (Clontech 632496), Glul (Millipore, MAB302), Lgals3 (Santa Cruz SC-19283), Mki67 (BD science Pharmingen 550609), Oncocut2 (R&D system AF6294), Pax6 (DSHB, AB-528427), Phospho-histone H3 (Santa Cruz SC-8656), Pou4f2 (Santa Cruz SC-6062), PKCa (Sigma P5704), Rhodopsin (Santa Cruz SC-57433), RPE65 (Abcam ab13826), Sox9 (Millipore AB5535), Thrb2 (D Forrest, NIH)(67). Vascular endothelial cells were labelled by FITC conjugated-IB4 (Sigma, L2895). Antigen retrieval was performed as described (44). 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 DAPI (Sigma, D9542) and mounted with Mowiol. Validations of the primary antibodies are provided on the manufacturers’ websites or in the referenced citations.

For whole-mount staining, eyeballs were enucleated and incubated for 30 minutes in 4% paraformaldehyde. The retinas were incubated at 4°C with FITC conjugated-IB4 (Sigma, L2895)
and DAPI in PBS for 1-2 days. After briefly washes 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 was used for cell counting. The positive cells of active caspase 3, Mki67, PH3 and cell type markers were counted manually. For vascular blood vessel analysis, representative images were analyzed using the AngioTool (NCI) to assess the density of the vascular plexus. In brief, at least three ×200 magnification images (320×320 μm fields of view [FOV] per retina) per eye and three eyes from the same genotypes of different litters were counted. To compare the vascular density of the Rb1/Vhl DKO retina to other genotypes, we used vascular images taken from similar depth below the GCL to represent the IVP and DVP for the Rb1/Vhl DKO retina.

To generate 3D image for retinal vasculature, 40 z-stack images were taken using the Nikon C1si confocal microscope, and Image J was used to convert these z-stack images into a 3D video.

**Senescence-associated β-galactosidase staining:** Human lung cancer A549 cells (ATCC® CCL-185™) on cover-slips (with or without pre-treatment of etoposide 1uM for 4 days) and frozen horizontal retinal sections were stained for beta-galactosidase at pH 6.0 using the senescence-associated β-galactosidase staining kit (Cell signaling, #9860). Color images were taken using an Olympus BX61 microscope. For each genotype, at least 3 retinas were analyzed.

**H&E staining for frozen sections:** Slides were rinsed once with 1×PBS, and then stained with Meyers Hematoxylin for 5 minutes. They were then rinsed with running tap water for 4 minutes, dehydrated to 95% ethanol by dipping for 10-20 seconds in alcoholic Eosin, and then dehydrated through at least 2 more changes of 95% ethanol, and 100% ethanol. They were then cleared through three changes of Xylene, and mounted on coverslips with Permount (Fisher Scientific, SP15-100). Color images were taken using an Olympus BX61 microscope.

**RNA extraction, Reverse Transcription and Quantitative Real-Time PCR:** Total RNA was isolated from peripheral retina (α-Cre expression area) using the Trizol Reagent (Invitrogen) or RNeasy mini kit (Qiagen) followed by digestion with RNase-Free DNase (DNA-free™, Thermo Fisher Scientific) to remove DNA contamination. After quantification by a Nanodrop (NanoDrop Technologies, USA), first-strand cDNA was synthesized from 0.2-1μg 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 2. 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 seconds, annealing and extension at 55°C for 30 seconds. An additional cycle (95°C, 15 seconds) generated a dissociation curve to confirm a single product. Values obtained for test RNAs were normalized to β-actin mRNA levels.

**RNA sequencing:** Total RNAs were extracted from dissected alpha-Cre expression peripheral retinas using TRIzol (Invitrogen) and treated with RNase-free DNase I (New England Biolabs, Beverly, MA) to remove genomic DNA. The yield of total RNA was assessed using Nanodrop (Thermo). The cDNA libraries were prepared using the Illumina TruSeq RNA sample preparation kit and the qualities were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For sequencing, the cDNA libraries were loaded on an Illumina HiSeq 2500 at Biomaker (Beijing, China). The raw sequence reads in fastq format were processed and analyzed as previously reported (68, 69). Briefly, the sequencing quality was first assessed using FastQC and poor quality 5’ end reads were trimmed using a Perl script and then mapped onto mouse genome (mm9) using Tophat2, allowing for up to two mismatches as default settings. Reads were mapped onto multiple genomic locations were discarded and a custom R script was used to calculate the RPKM of each gene and obtain expression profile of each sample. The expression fold change of each coding gene of mutated retinas (including Vhl−/−, Rb1+/−/Vhl−/−, Rb1/Vhl DKO, Rb1/Rbl1 DKO, Rb1/Rbl1/Vhl TKO) retina compared to WT retina was calculated using the following formula: Fold change=(RPKM+1)mutated/(RPKM+1)wt. The genes, of which expression fold changes are greater than 1.54 or less than 0.65, were selected as the mutated-related differentially expression genes (DEG). The heatmap was generated using Heatmapper (70). The function enrichment of DEG was performed using Enrichr (71, 72), the pathways with adjusted p<0.05 were chosen to report.

**Ex vivo retinal explant cultures and Chromatin immunoprecipitation (ChIP):** Retinal explants obtained from postnatal day 8 wild type C57/BL mice were cultured using a modified
protocol from previously described methods. Briefly, both eyes were enucleated and retinas were
carefully peeled away from the retinal pigment epithelium, and radial cuts were made to flatten
the retina. The flattened retina was transferred to the membrane of a Millicell insert (Millipore,
PICM03050) with the photoreceptors facing down. The insert was placed into the wells of a 6-
well plate (Costar 3516, Corning), each contained 1300μl of retinal explant media, which was
replaced every 2 days, and was maintained in a 37°C incubator with 5% CO2 for 3 days. The
retinal explant basal medium was serum-free and made from Neurobasal A, DMEM/F12
medium, and N2/ B27 supplements (Life Technologies, USA). Cultured explants were exposed
to 300 μM CoCl2 (Sigma, France) treatment, or kept as PBS-treated controls. The ChIP assay
was performed using Magna ChIPTM A/G Chromatin Immunoprecipitation Kit (Millipore
Temecula, USA) according to the manufacturer's instructions. Briefly, retinal explants treated
with or without CoCl2 were cut into small pieces and then cross-linked for 20 min by addition of
formaldehyde to a final concentration of 1%. The cross-linking was stopped by adding 1/20
volume of 2.5 M glycine and lysed by SDS lysis buffer. Then cell lysate was sonicated and
immunoprecipitated with antibodies specific to Rb1 (1:100, Cell Signaling Technology, 9309) or
negative control IgG. After protein/DNA complexes were eluted, reverse cross-linked to free
DNA and purified, the specific DNA fragments were quantified using real-time PCR and
normalized to input from the same sample. The primer sequences for the promoters analyzed are
provided in Supplementary Table 3.

**Plasmid construction and reporter assays:** pREP4-Luc vector was from K Zhao (NIH) (73).

A 445bp mouse DNA fragment from -198 to +247 around Vegfa TSS (transcriptional start site)
was amplified using the primers Vegfa-p500-f1 and Vegfa-p500-r1; A 402bp mouse DNA
fragment from -213 to +189 around Kdr TSS was amplified using the primers Vegfr2-p400-f1
and Vegfr2-p400-r1 ; A 1127bp mouse DNA fragment from -938 to +189 around Kdr TSS was
amplified using the primers Kdrf 1127 and Vegfr2-p400-r1; a 597bp DNA fragment from -337 to
+260 around Tek TSS was amplified using the primers Tie2-p500-f1and Tie2-p500-r1. The
sequences of the above mentioned primers are as follow:

- Vegfa-p500-f1 (5’-CCCGGTACCGtttgaaggggtcacagat-3’, Kpn I site underlined)
- Vegfa-p500-r1 (5’-GGCAAGCTTAAACCCTGGGCAGATTTAAG-3’, Hind III site underlined)
- Vegfr2-p400-f1 (5’-CCCGGTACCTgctctcagatgcgacttg-3’)
Vegfr2-p400-r1 (5’-GGCAAGCTTAAGTCACAGAGCGGTATGC-3’)
Kdrf 1127 (5’-CCCGGTACCacccgtttctcttgctcc-3’)
Tie2-p500-f1 (5’-CCCGGTACCGctgagagctgacctcaacc-3’)
Tie2-p500-r1 (5’-GGCAAGCTTATGGTCCACTCGCTTTGTT-3’)

The amplified DNA fragments were digested with Kpn I and Hind III, ligated to Kpn I/Hind III-digested pREP4-Luc to generate Vegfa-198bp-Luc, Kdr 213bp-luc, Kdr 938bp-luc and Tek-337bp-luc. The inserts were confirmed by DNA sequencing. pSG5L-HA-RB and pSG5L vectors were from William Sellers (Broad Institute, Cambridge, USA) (74). 293T cells (ATCC® CRL-11268™) were transfected with the pREP4-Luc vectors and the pSG5L-HA-RB or pSG5L vectors using Lipofectamine 2000 (Invitrogen). In transient assays, 0.01 μg of renilla Luc plasmid was included to normalize transfection efficiency. Dual Luc assays were performed using Dual-Luciferase® Reporter Assay system (Promega, E1901) as described (75).

**Laser capture and recombined PCR of floxed Rb1 gene:** RAP lesions were dissected from 10μm H&E sections using the Zeiss PALM MicroBeam system and captured into the buffer (1 mM EDTA; 20 mM Tris [pH 8]) containing 2 mg/ml proteinase K. Cells were incubated at 55°C overnight in 10μl of capture buffer followed by heat inactivation of proteinase K at 99°C for 10 min. 2 μl of the buffer was used for each PCR. The PCR primers are Rb18 (5’-GGCGTGTGCATCAATG-3’) and Rb212 (5’-GAAAGGAAAGTCAGGGACATTGGG-3’).

**Western blotting:** Peripheral retinas were homogenized with a 30gauge needle 5-10 times in 1x cell lysis buffer (Cell Signaling #9803) with 0.1mM PMSF, 1μg/ml aprotinin, 1μg/ml leupeptin. Proteins were separated by SDS-PAGE, transferred to nitrocellulose or PVDF membranes and analyzed using ODYSSEY Infrared Imaging System (LI-COR) with antibodies against Active caspase 3 (Cell Signaling Technology 9661), Atg5 (Santa Cruz SC-33210), Atg7 (Santa Cruz SC-33211), Becn1 (Cell Signaling 349S), Bnip3 (Cell Signaling 3769), Bnip3L (Santa Cruz SC-28240), Hif1α (Millipore, 04-1006), Kdr (Millipore, 07-716-1), Lgals3 (Santa Cruz SC-19283), MAP1LC3B (Cell Signaling 2775), Vegfa (Santa Cruz SC-507) and β-actin (A5441, Sigma).
**Retro-orbital injection of FITC-dextran:** FITC-dextran (Sigma-Aldrich, FD2000S) was dissolved in ddH₂O at a concentration of 50 mg/ml, centrifuged at 10,000×g for 5 min, and collected as the supernatant. P18 mice were anesthetized with an intraperitoneal (IP) injection of 4.6% chloral hydrate (2.5 ml/kg). 0.15 ml of FITC-dextran was then injected into the lateral canthus of the left orbit using a 1 ml syringe and 30-gauge needle. Eyeballs were enucleated 1 min after the injection. Retinal whole-mounts were prepared and flattened with a coverslip, and were photographed with Zeiss Axio Imager Z2 fluorescence microscope.

**Fundus fluorescein angiography (FFA):** Fluorescein angiography was performed using a Micron III retinal imaging system or Heidelberg retina angiograph (HRA) system. P14-P18 mice were anesthetized with an intraperitoneal (IP) injection of 4.6% chloral hydrate (2.5 ml/kg), and the pupils were dilated by 0.1% tropicamide solution. Fluorescein images were captured at different time points after IP injection of 0.1 ml of 2% fluorescein sodium (Novartis).

**Statistical analyses:** All data were presented as mean ± SD or mean ± SEM. Kaplan–Meier survival curves and statistical analysis was performed 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, and student’s t test for comparisons between two groups. The p value of KM curves was calculated by log-rank (Mantel-Cox) test.

**Study approval:** All animal procedures were reviewed and approved by the Ethical Review Committee of Animal Research of West China Hospital, Sichuan University, Chengdu, Sichuan province, China (AUP# 2015001B), and performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and visual research.
Author contributions

DC designed the study, directed the project, and interpreted the data. RW, XR, HK, ZL, YC, YW, LX, TY, CL and DC performed the experiments. SH and AN helped on FFA analysis. RB provided partial funds, reagents and guidance to this project. DC and RB wrote the paper and all authors contributed to editing.

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References


Figure legends

Figure 1. Retinal neovascularization in the Rb1/Vhl DKO and Rb1+/Vhl− retinas. A. IB4 staining of P18 wholemount retinas of indicated genotypes. Selected areas are blown up to show the vascular density. B. IB4 (green) and DAPI (blue) staining of P18 retinal sections of indicated genotypes. C. Quantification of vessel branching points per fields of view [FOV] by AngioTool software. D. IB4 staining of the P8 Rb1/Vhl DKO wholemount retina. The hyaloid vessels and DVP layers were shown. Arrow heads indicate that new blood vessels link to hyaloid vessels. Arrows indicate new blood vessels link to retinal DVP. E. IB4 staining of the P14 Rb1/Vhl DKO wholemount retina. Blow-up shows the grape-like clusters of endothelial cells. F. IB4 (green) and DAPI (blue) staining of the P14 Rb1/Vhl DKO retinal sections shows the grape-like clusters of endothelial cells. G-H, FFA images of the P14 Rb1/Vhl DKO eye to show delayed regression of hyaloid vessels (G) and retinal vascular leakages (H, arrows). I. Retinal wholemount of P18 Rb1/Vhl DKO mice after retro-orbital injection of FITC-dextran, to show retinal vascular leakages. J. IB4 staining of P730 (2 years old) Rb1/Vhl DKO wholemount retina. Error bars represent SD of measurements from three retinas (n=3) and asterisks indicate significant differences between WT and indicated genotypes (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction). The dotted lines in A, D, E, I and J indicate the boundary between no Cre expression (in the center) and alpha-Cre expression areas (in the periphery). ONL: outer nuclear layer; INL: inner nuclear layer; IVP: intermediate vascular plexus; GCL: ganglion cell layer. Scale bar is 50µm in F; 100µm in B; 200 µm in A, D, E, I, J.

Figure 2. Rb1KO and VhlKO are synthetic lethal to retinal photoreceptors. A, C, E: P8 or P18 horizontal retinal sections of indicated genotypes were stained for nuclear (DAPI, blue), vascular endothelial cells (IB4, green) and rods (Rho, red, A), cones (Arr3, red, A), amacrine cells (Ap2a, red, C), Müller cells (Sox9, red, C), apoptosis (active caspase 3, red, E). B, D, F: Quantification of rods, cones, bipolar cells and ganglion cells (B), amacrine cells, horizontal cells and Müller cells (D), apoptosis (F). Error bars represent SD of measurements from three retinas (n=3) and asterisks indicate significant differences between WT and indicated genotypes (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction). ONL: outer nuclear layer; INL: inner nuclear layer; RAP: Lesions of retinal angiomatous proliferation. Scale bar is 50µm.

Figure 3. Rb1 directly inhibits Vhl null-induced angiogenesis and autophagy. A. Representative Western blots of indicated proteins in retinas of indicated genotypes. B. The quantification of total protein level relative to β-actin in A. C. Gene list enrichment analysis using KEGG 2016 datasets in Enrichr of Rb1+/Vhl− and Rb1/Vhl DKO-regulated DEGs (−log10(P)). Dotted line, P< 0.05. D. Heatmap of top
seven pathways of Vhl, Rb1, Rbl1-regulated DEGs by RNA sequencing. E. The relative mRNA levels of indicated genes and indicated genotypes were analyzed by RT-PCR. F. ChIP using Rb1 antibody at the promoter of indicated genes and treatments in WT retinal explant cultured from P8 to P11. The enrichment of Rb1 was quantified using qPCR and normalized to input. G. Luciferase reporter assay. 293T cells were transfected with indicated luciferase-reporter plasmid, pSG5L-HA-RB or pSG5L plasmid by Lipofectamine. Renilla Luciferase plasmid was included to normalize for transfection efficiency. Error bars represent SD (in B, G) or SEM (in E, F) of measurements from three retinas (B, E, F; n=3) or three assays (G; n=3) and asterisks indicate significant differences between WT and indicated genotypes in B, E; or between IgG No CoCl2 treatment and other indicated treatments in F, between pSG5L-HA-RB and pSG5L vector in G (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction).

**Figure 4. Hyperactive Rb1 inhibits the superficial vascular plexus in Ccnd1−/− retina.** A, IB4 staining of P18 wholemount retinas of indicated genotypes. Selected areas are blown up to show the vascular development. Bidirectional arrows indicate the spreading distance of vascular vessels from optic disk (OD). Dashed arrows indicate area without blood vessels. B. Quantification of the spreading distance of vascular vessels from optic disk (OD) in A of indicated genotypes. C. IB4 staining of the SVP, IVP and DVP of P18 wholemount retinas of indicated genotypes. D. Quantification of vessel branching points per fields of view [FOV] in (C) by AngioTool software. E. The relative mRNA levels of indicated genes and indicated genotypes were analyzed by RT-PCR. Error bars represent SD (in B, D) or SEM (in E) of measurements from three retinas (n=3) and asterisks indicate significant differences between WT and indicated genotypes (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction). The dotted lines in A indicate the boundary between no Cre expression (in the center) and alpha-Cre expression areas (in the periphery). SVP: superficial vascular plexus; IVP: intermediate vascular plexus; DVP: deep vascular plexus. Scale bar is 200 µm.

**Figure 5. VhlKO suppresses retinoblastoma formation in the Rb1/Rbl1DKO retinas.** A. Kaplan–Meier (KM) curves to assess the effect of Vhl loss on tumorigenesis in the Rb1/Rbl1 DKO retina. p values compare the indicated genotypes (log-rank Mantel-Cox test). B. Gene list enrichment analysis using KEGG 2016 datasets in Enrichr of Rb1/Rbl1/Vhl TKO-regulated DEGs (−log10(P)). Dotted line, P< 0.05. C. The relative mRNA levels of indicated genes and indicated genotypes were analyzed by RT-PCR. D. P18 horizontal retinal sections of the indicated genotypes and human lung cancer A549 cells treated with (positive control) or without (negative control) etoposide were stained for SA-β-gal (senescence associated (SA)-beta-galactosidase). Error bars represent SEM of measurements from three retinas (n=3) and asterisks indicate significant differences between WT and indicated genotypes (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction). Scale bar is 200µm in B.
**Figure 6.** *Rb1/Rbl1/Vhl* TKO induces retinal capillary hemangioblastoma (RCH)-like and retinal angiomatous proliferation (RAP)-like lesions. A. IB4 staining of *Rb1/Rbl1/Vhl* TKO wholemount retinas of indicated ages. B. Horizontal sections of *Rb1/Rbl1/Vhl* TKO retinas of indicated ages were stained for nuclear (DAPI, blue) and vascular endothelial cells (IB4, green). C. FFA images of P18 WT or *Rb1/Rbl1/Vhl* TKO retinas. Arrow indicates the vascular leakages from the subretinal RAP-like lesion. D. H&E staining of the RAP lesions. The two big arrows indicate that RAP invades into the sub-RPE space. E. P14 horizontal sections of WT and *Rb1/Rbl1/Vhl* TKO retinas were stained for nuclear (DAPI, blue), vascular endothelial cells (IB4, green) and retinal pigment epithelium cells (RPE65, red). The dotted lines in A indicate the boundary between no Cre expression (in the center) and alpha-Cre expression areas (in the periphery). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. RAP: Lesions of retinal angiomatous proliferation. RPE: retinal pigment epitheliun. Scale bar is 50μm.

**Figure 7.** The cellular composition of the RCH-like and RAP-like lesions in the *Rb1/Rbl1/Vhl* TKO retina. A. P14 horizontal TKO retinal sections were stained for nuclear (DAPI, blue), vascular endothelial cells (IB4, green), amacrine cells (Ap2a, red), Müller cells (Sox9, red), Müller/microglial cells (Lgals3, red) and microglial cells (Aif1, red). Blow-up shows that Lgals3⁺ cells are stromal cells, but not IB4⁺ vascular endothelial cells. B. P14 horizontal WT retinal sections were stained for nuclear (DAPI, blue), Müller/microglial cells (Lgals3, green), amacrine cells (Ap2a, red), Müller cells (Sox9, red), and microglial cells (Aif1, red). Arrow indicates RPE cells also express low levels of Sox9, but not Lgals3. C. Genomic DNA extracted from the laser capture micro-dissected (LCM) RAP lesions on slide, were amplified by PCR for the floxed alleles of exon19 of *Rb1* gene. Primers are Rb212 and Rb18. The 670bp band represents the un-recombined allele, 260bp band represents recombined allele. D. P14 horizontal [*a-cre; Rb1<sup>f/f</sup>; Rbl1<sup>/-</sup>; vhl<sup>f/f</sup>; Z/red] retinal sections were stained for nuclear (DAPI, blue), vascular endothelial cells (IB4, green) and DsRed (Red). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. RAP: Lesions of retinal angiomatous proliferation; RPE: Retinal pigment epithelium. Scale bar is 50μm.
Figure 1

**A**  
WT  |  a-Cre; Rb1\textsuperscript{+/+} |  a-Cre; Rb1\textsuperscript{+/−} |  a-Cre; Vhl\textsuperscript{−/−} |  a-Cre; Rb1\textsuperscript{−/−}; Vhl\textsuperscript{−/−} |  a-Cre; Rb1\textsuperscript{−/−}; E2f1\textsuperscript{−/−}; Rb1\textsuperscript{−/−}; Vhl\textsuperscript{−/−}  
--- | --- | --- | --- | --- | ---  
P18, IB4 Wholemont  
Blow-up (IVP)  

**B**  
ONL  |  DAPI/IB4  
--- | ---  
ONL  |  INL  |  GCL  |  ONL  |  INL  |  GCL  |  ONL  |  INL  |  GCL  |  ONL  |  INL  |  GCL  

**C**  
Branching points/FOV  
|  SVP |  IVP |  DVP |  
--- | --- | --- |  **p<0.05** |  **p<0.01** |  ***p<0.001***  

**D**  
α-Cre; Rb\textsuperscript{1/2}; Vhl\textsuperscript{−/−}  
P8, DAPI/IB4  
Hyaloid vessels  
DVP  

**E**  
P14, IB4  
Blow-up  

**F**  
P14, IB4  
P14, FFA  
P14, FFA  
P18, FITC-Dextran  
P730, DAPI/IB4  

**Figure 1. Retinal neovascularization in the Rb1/Vhl\textsuperscript{DKO} and Rb1\textsuperscript{+/−}/Vhl\textsuperscript{+/−} retinas.** A, IB4 staining of P18 wholemount retinas of indicated genotypes. Selected areas are blown up to show the vascular density. B, IB4 (green) and DAPI (blue) staining of P18 retinal sections of indicated genotypes. C. Quantification of vessel branching points per fields of view [FOV] by AngioTool software. D, IB4 staining of the P8 Rb1/Vhl DKO wholemount retina. The hyaloid vessels and DVP layers were shown. Arrow heads indicate that new blood vessels link to hyaloid vessels. Arrows indicate new blood vessels link to retinal DVP. E, IB4 staining of the P14 Rb1/Vhl DKO wholemount retina. Blowup shows the grape-like clusters of endothelial cells. F, IB4 (green) and DAPI (blue) staining of the P14 Rb1/Vhl DKO retinal sections shows the grape-like clusters of endothelial cells. G-H, FFA images of the P14 Rb1/Vhl DKO eye to show delayed regression of hyaloid vessels (G) and retinal vascular leakages (H, arrows). I, Retinal wholemont of P18 Rb1/Vhl/DKO mice after retro-orbital injection of FITC-dextran, to show retinal vascular leakages. J, IB4 staining of P730 (2 years old) Rb1/Vhl DKO wholemount retina. Error bars represent SD of measurements from three retinas (n=3) and asterisks indicate significant differences between WT and indicated genotypes (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction). The dotted lines in A, D, E, I and J indicate the boundary between no Cre expression (in the center) and alpha-Cre expression areas (in the periphery). ONL: outer nuclear layer; INL: inner nuclear layer; IVP: intermediate vascular plexus; GCL: ganglion cell layer. Scale bar is 50µm in F; 100µm in B; 200 µm in A, D, E, I, J.
Figure 2. **Rb1KO and VhlKO are synthetic lethal to retinal photoreceptors.** A, C, E: P8 or P18 horizontal retinal sections of indicated genotypes were stained for nuclear (DAPI, blue), vascular endothelial cells (IB4, green) and rods (Rho, red, A), cones (Arr3, red, A), amacrine cells (Ap2a, red, C), Müller cells (Sox9, red, C), apoptosis (active caspase 3, red, E). B, D, F: Quantification of rods, cones, bipolar cells and ganglion cells (B), amacrine cells, horizontal cells and Müller cells (D), apoptosis (F). Error bars represent SD of measurements from three retinas (n=3) and asterisks indicate significant differences between WT and indicated genotypes (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction). ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. RAP: Lesions of retinal angiomatous proliferation. Scale bar is 50µm.
Figure 3. Rb1 directly inhibits Vhl null-induced angiogenesis and autophagy. A. Representative Western blots of indicated proteins in retinas of indicated genotypes. B. The quantification of total protein level relative to β-actin in A. C. Gene list enrichment analysis using KEGG 2016 datasets in Enrichr of Rb1+/Vhl−/− and Rb1+/Vhl−/−DKO-regulated DEGs (−log10(p)). Dotted line, P < 0.05. D. Heatmap of top seven pathways of Vhl, Rb1, Rb1-regulated DEGs by RNA sequencing. E. The relative mRNA levels of indicated genes and indicated genotypes were analyzed by RT-PCR. F. ChIP using RB antibody at the promoter of indicated genes and treatments in WT retinal explant cultured from P8 to P11. The enrichment of Rb1 was quantified using qPCR and normalized to input. G. Luciferase reporter assay. 293T cells were transfected with indicated luc-reporter plasmid, pSG5L-HA-RB or pSG5L plasmid by Lipofectamine. Renilla Luc plasmid was included to normalize for transfection efficiency. Error bars represent SD (in B, G) or SEM (in E, F) of measurements from three retinas (B, E, F; n=3) or three assays (G; n=3) and asterisks indicate significant differences between WT and indicated genotypes in B, E; or between IgG No CoCl2 treatment and other indicated treatments in F, between pSG5L-HA-RB and pSG5L vector in G (*p<0.05, **p<0.01, one-way ANOVA followed by Bonferroni correction).

A

C

KEGG2016 Up-regulated enriched pathways [-log10(p)]

Cell cycle

ECM

Focal adhesion

Pathways in cancer

Protein digestion

PI3K-Akt signaling

AGE-RAGE signaling

Complement

HIF-1 signaling

D

WT

VhlKO

Rb1+/−;VhlKO

Rb1+/−;VhlKO DKO

Rb1+/−;VhlKO TKO

Cell cycle

Phototransduction

Hif pathway

Oxidative phosphorylation

PI3K-Akt pathway

ECM pathway

Ribosome biogenesis

Z-Score

E

WT

Vhl−/

Rb1+/−;Vhl−/

Rb1+/−;Vhl−/−

% Input

F

IgG-No CoCl2

IgG-CoCl2

Rb Ab-No CoCl2

Rb Ab-CoCl2

% Input

G

pSG5L

pSG5L-HA-RB

Relative luciferase activity

VEGFa-298bp-Luc

Kdr-214bp-Luc

Kdr-388bp-Luc

Tek-337bp-Luc

Relative luciferase activity
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