Reduced mTORC1-signaling in retinal ganglion cells leads to vascular retinopathy

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Abstract

Background: The coordinated wiring of neurons, glia and endothelial cells into neurovascular units is critical for central nervous system development. This is best exemplified in the mammalian retina where interneurons, astrocytes and retinal ganglion cells sculpt their vascular environment to meet the metabolic demands of visual function. Identifying the molecular networks that underlie neurovascular unit formation is an important step towards a deeper understanding of nervous system development and function.

Results: Here, we report that cell-to-cell mTORC1-signaling is essential for neurovascular unit formation during mouse retinal development. Using a conditional knockout approach we demonstrate that reduced mTORC1 activity in asymmetrically positioned retinal ganglion cells induces a delay in postnatal vascular network formation in addition to the production of rudimentary and tortuous vessel networks in adult animals. The severity of this vascular phenotype is directly correlated to the degree of mTORC1 down regulation within the neighboring retinal ganglion cell population.

Conclusions: This study establishes a cell nonautonomous role for mTORC1-signaling during retinal development. These findings contribute to our current understanding of neurovascular unit formation and demonstrate how ganglion cells actively sculpt their local environment to ensure that the retina is perfused with an appropriate supply of oxygen and nutrients.

KEYWORDS

endothelial cells, mTORC1, Raptor, retinal ganglion cells, vascular retinopathy

INTRODUCTION

The coordinated development of neurovascular units is critical for the function of the central nervous system (CNS). This is best exemplified in the mouse retina where retinal progenitor cells (RPCs) undergo an orchestrated differentiation program to produce six neuronal and one glial cell-type that become positioned within three stratified layers: (1) rod and cone photoreceptors reside in the outer nuclear layer, (2) interneurons (bipolar, horizontal, and amacrine cells) and Müller glia are localized within the inner nuclear layer while (3) retinal ganglion cells (RGCs) and displaced amacrine cells are situated in the ganglion cell layer (GCL). In parallel, the retinal vasculature is produced by
sprouting angiogenesis in sequential order. Optic stalk-derived astrocytic precursor cells (APCs) first enter from the optic nerve head and migrate peripherally across the inner surface of the retina to form a mature glial scaffold. Endothelial cells then enter the retina in the same manner and radially navigate along this astrocytic template to form three interconnected vascular networks: (1) the superficial plexus that neighbors the GCL and the (2) intermediate and (3) deep plexus that straddle the inner nuclear layer. This coordinated neurovascular interaction involving neurons, astrocytes and endothelial cells ensures that the retina is perfused with an appropriate supply of oxygen and nutrients to meet the metabolic demands of phototransduction.

Growing evidence derived from mouse models demonstrates that retinal neurons are directly involved in sculpting their vascular environment. For example, a reduction in RGC number and activity leads to the irregular development of the neighboring superficial plexus. In a similar manner, genetic ablation of either horizontal or amacrine cells reveals that these neurons are required for deep and intermediate plexus formation while spontaneous activity of these interneurons is required to maintain the function of the adjacent intraretinal vascular network. Moreover, long-term neurovascular uncoupling ultimately affects retinal homeostasis and drives the progressive development of vascular pathologies. This is best exemplified by diseases such as diabetic retinopathy and retinopathy of prematurity which are amongst the leading causes of visual impairment and blindness. The generation of novel mouse models that identify the signaling complexes involved in the establishment and maintenance of neurovascular units are therefore crucial to broaden our understanding of retinal development and disease.

The mammalian target of rapamycin complex 1 (mTORC1) is a central signaling hub that is assembled from regulatory (mTOR, Raptor and mLST8) and inhibitory components (PRAS40 and Deptor). This complex modulates fundamental cellular processes with Raptor functioning as an adaptor protein that recruits canonical mTORC1 substrates. One such downstream effector is S6K1 whose phosphorylation of the S6 ribosomal protein (pS6) represents an experimental marker of active mTORC1 signaling. Recent studies in mouse models demonstrate that the precise spatiotemporal regulation of retinal development is driven in part by intracellular and extracellular cues that converge on the mTORC1 signaling axis. For example, precocious activation of the mTORC1 pathway leads to accelerated neurogenesis and retinal hypertrophy while genetic ablation of this signaling axis leads to sustained apoptosis that commences at postnatal day (P) 6 and the consequent development of asymmetric RGC mosaics. While this aberrant ganglion cell positioning leads to visual pathway dysfunction, it is currently unknown whether the observed reduction of mTORC1-signaling within the asymmetric RGCs population also influences neurovascular unit formation in a cell nonautonomous manner.

The work presented in this study therefore addresses the roles of RGC-derived mTORC1-signaling during retinal vascular development. Using a novel mouse model, we demonstrate that reduced pathway activity in asymmetrically positioned RGCs triggers the delayed development of the neighboring superficial plexus in postnatal mice. Moreover, this reduction in mTORC1 activity also leads to the production of tortuous vascular networks in adult mice with vessel crossing and vessel leakage being readily apparent. Our findings therefore contribute to the current understanding of neuronal and endothelial cell cross-talk and demonstrate how retinal neurons signal through mTORC1 to sculpt the neighboring vascular environment.

2 | RESULTS

2.1 | Conditional deletion of Rptor in retinal progenitor cells leads to a reduction in mTORC1-signaling and underlies the development of asymmetric retinal ganglion cell mosaics

We recently reported the creation of a retinal-specific Rptor mouse model (Lhx2-Cre:Rptorf/f) that exhibited a distinct spatial pattern of target allele recombination within RPCs (Figure 1). In this previous study, we demonstrated that progenitors generating the dorso temporal retina exhibited an 80% level of Rptor-ablation while RPCs that established the remaining retina (written as pan-ventronasal) displayed an allele deletion rate of 50% (Figure 1A,B). This novel spatial pattern of Rptor recombination consequently led to contrasting reductions in mTORC1-signaling within Brn3+ RGCs that in turn influenced their mosaic positioning (Figure 1C,1J). Interestingly, the severity of this asymmetric phenotype was directly correlated to the degree of Rptor ablation within a given retinal region. This was best exemplified by the most severe Brn3+ RGC mosaic disarray being consistently observed in the dorso temporal retina due to a high level of Rptor deletion and absence of mTORC1-signaling within this domain. Moreover, this novel phenotype was fully penetrant in Lhx2-Cre:Rptorf/f mice and was independent of both sex and genetic background.

2.2 | Lhx2-Cre:Rptorf/+ mice present with vascular retinopathy

We previously demonstrated that precocious activation of the mTORC1 pathway during retinogenesis impacted the
development of adjacent ocular tissues in a cell non-autonomous manner. The distinct spatial pattern of Rptor-ablation observed within the retina of Lhx2-Cre:Rptorf/f mice therefore provided an ideal opportunity to investigate how contrasting reductions in mTORC1-signaling within irregularly positioned RGCs (dorsotemporal vs pan-ventronasal) influenced the development of the neighboring retinal vasculature.

We first examined the posterior eye segment of control and Lhx2-Cre:Rptorf/f mice at 10 weeks of age by a combination of fundoscopy and fluorescein angiography (Figure 2). The retina of control mice displayed a characteristic vascular network comprised of even calibre vessels that uniformly radiated out from the optic nerve head (Figure 2A) to elaborate dense capillary beds throughout the retina (Figure 2B). Moreover, longitudinal angiography demonstrated an intact brain-retinal barrier due to an absence of fluorescein leakage (Figure 2C-E). In contrast, Lhx2-Cre:Rptorf/f mice exhibited an asymmetric mixed calibre vascular network (Figure 2F,G) with vessel crossing (Figure 2F,G, arrows) and tortuosity (Figure 2F, G, arrowheads) being readily evident in the pan-ventronasal retina. Moreover, the ischemic dorsotemporal domain was completely devoid of any vascular network (Figure 2F,G, asterisks). Vessel leakage and the appearance of several distinct fluorescein foci was also observed in Lhx2-Cre:Rptorf/f mice following longitudinal angiography which was correlated with these ischemic areas and was indicative of a compromised brain-retinal barrier (Figure 2H-J, arrows).

Given the profound vascular deficits observed in the posterior eye segment of adult Lhx2-Cre:Rptorf/f mice, we next performed a detailed assessment of the superficial, deep and intermediate plexus by isolectin-B4 staining (Figure 3). These analyses were conducted on both the dorsotemporal and pan-ventronasal retina in order to assess whether the contrasting reductions in mTORC1-signaling and correlative RGC mosaic disarray observed in these domains influenced the appearance of the neighboring vascular networks. Flat mounted retina taken from control mice at 7 weeks of age demonstrated that the superficial plexus extended all the way to the retinal periphery (Figure 3A) with the optic nerve head exhibiting equal numbers of radially arranged arteries (5.9 ± 0.38; mean ± SEM) (Figure 3B, green arrowheads and Figure 3Q) and veins (5.4 ± 0.42; mean ± SEM) (Figure 3B, red...
Moreover, all plexus layers elaborated dense capillary beds throughout the whole of the dorsotemporal (Figure 3C-E) and pan-ventronasal (Figure 3F,H) regions. Contrastingly, the retina of Lhx2-Cre:Rptorf/f mice contained a compromised superficial network (Figure 3I) with the optic nerve head exhibiting a reduced number of irregularly positioned arteries (1.9 ± 0.35; mean ± SEM) (Figure 3J, green arrowheads and Figure 3Q) and veins (2.1 ± 0.30; mean ± SEM) (Figure 3J, red arrowheads and Figure 3R) with a greater number of major vessel crossing being readily observable (Figure 3S). Moreover, the ischemic dorsotemporal region was devoid of any vascularization (Figure 3I, arrowheads and Figure 3K,M) while the plexus layers in the pan-ventronasal retina appeared sparser than control littermates (Figure 3N,P). These apparent vascular differences were therefore quantified using AngioTool with all analyses being limited to the superficial plexus to eliminate experimental bias. Both retinal (Figure 3T) and vessel (Figure 3U) areas were reduced in Lhx2-Cre:Rptorf/f (cKO) mice and in combination resulted in an overall decrease in the percent vascular coverage (Figure 3V). Moreover, decreases in vessel junction (Figure 3W) and end point (Figure 3X) numbers also contributed to the sparse appearance of the superficial plexus in mutant animals.

### 2.3 Delayed endothelial cell network formation underlies the observed vascular retinopathy

Formation of the retinal vasculature commences during the first postnatal week of mouse development and encompasses the gradual regression of the hyaloid vessels followed by the sequential migration of astrocytes and endothelial cells into the retina from the optic nerve. Given that the Lhx2-Cre transgene is expressed within the optic stalk (precursor to the optic nerve) we first determined whether the Rptor allele underwent recombination within the APCs of mutant animals (Figure 4). Retina were therefore harvested from control and Lhx2-Cre:Rptorf/f mice and immunohistochemical analyses demonstrated that a comparable number of PDGFRα-expressing APCs were observed in the optic nerve head and basal retina of both groups during embryogenesis (Figure 4A,D, arrowheads). Moreover, these PDGFRα+ cells exhibited widespread pS6S240/244 reactivity indicative of canonical mTORC1-signaling (Figure 4B,C,E,F, arrowheads). In a similar manner, all migrating Pax2+ astrocytes displayed comparable patterns of pS6S240/244 immunoreactivity during postnatal development (Figure 4G,L, arrowheads). Interestingly,
we observed an increase in the number of migrating Pax2+ astrocytes in mutant animals (Figure 4M) that formed well-ordered reticular networks across the basal surface of the dorso-temporal and pan-ventro-nasal retina (Figure 4N,Q). However, similar levels of GFAP expression were observed in both control and Lhx2-Cre::Rptor<sup>fl/fl</sup> animals (Figure 4R). Taken together, we concluded that the Rptor allele did not undergo recombination within the APCs of mutant animals and that these retinal astrocytes exhibited canonical mTORC1-signaling in a similar manner to control mice. In addition, despite an increase in number these glial cells exhibited migratory behavior that was similar to control mice during postnatal development.

Given the above astrocytic analyses, we reasoned that the vascular retinopathy observed in mutant animals

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**FIGURE 3** Legend on next page.
therefore arose due to a delay in endothelial cell radial migration. Moreover, we postulated that this phenotype was primarily driven by the reduced mTORC1-signaling observed within the neighboring asymmetric RGC population. To address this hypothesis, retina were harvested at several postnatal ages that coincided with aberrant RGC mosaic formation in Lhx2-Cre:Rptorf/mice and isolectin-B4 flat mount analyses were performed to visualize the developing vascular networks (Figure 5). Control animals exhibited an absence of the hyaloid vasculature and a rudimentary endothelial cell network that surrounded the optic nerve head at P1 (Figure 5A). The superficial plexus then formed over the first week (P3, P6, and P9) as these endothelial cells radially migrated towards the retinal periphery (Figure 5B,D). Vessels from the newly formed superficial plexus then migrated into the retina during the second and third postnatal weeks to form the deep (P15) (Figure 5E, arrowhead in inset) and intermediate plexus (P21) (Figure 5F, arrowheads in inset), respectively. Contrastingly, despite a comparable absence of hyaloid vessels we observed that vascular growth was markedly delayed in Lhx2-Cre:Rptorf/mice shortly after birth (P1 and P3) (Figure 5G,H) and a rudimentary endothelial cell network only appeared in the central retina at P6 (Figure 5I). Interestingly, this initiation of endothelial cell radial migration in Lhx2-Cre:Rptorf/mice preceded the aberrant programmed cell death previously reported for this model.3,30 Moreover, no superficial plexus ever formed within the dorso-temporal region (Figure 5J-L, arrowheads) while the remaining pan-ventronasal retina developed a tortuous vascular network that gradually reached the retinal periphery over the course of the first postnatal week (P9) (Figure 5J). The deep (Figure 5K, arrowhead in inset) and intermediate vascular layers (Figure 5L, arrowheads in inset) then formed in this region by the end of the third postnatal week. This delayed migratory phenotype was subsequently quantified with all analyses being limited to the superficial plexus to avoid experimental bias. Both retinal (Figure 5M) and vessel (Figure 5N) areas were reduced in Lhx2-Cre:Rptorf/mice which in combination resulted in an overall reduction in the percentage area occupied by the vascular network at all ages analyzed (Figure 5O).

2.4 | The delay in superficial plexus development correlates with abnormal tip cell behavior

Irregular endothelial tip cell behavior is a common mechanism underlying the production of disorganized vascular networks.37 Given that we observed a delay in superficial plexus formation in postnatal Lhx2-Cre:Rptorf/mice we performed an assessment of endothelial tip cell morphology at the growing vascular front during postnatal development (Figure 6). These analyses were conducted on both the dorso-temporal and pan-ventronasal retina in order to assess whether the contrasting reductions in mTORC1-signaling and correlative RGC mosaic disarray observed in these regions influenced localized tip cell behavior. High resolution imaging of control retinas revealed that the tip cells in both quadrants extended numerous long filopodia which was indicative of an active migratory phenotype (Figure 6A,B, arrowheads in insets). In contrast, the dorso-temporal retina of Lhx2-Cre:Rptorf/mice exhibited a sparse endothelial cell network (Figure 6C, inset) while the pan-ventronasal domain contained a coalesced vascular front (Figure 6D, arrowhead in inset). Moreover, both regions had a reduced number of angiogenic tip cells. The delay in superficial plexus development correlated with abnormal tip cell behavior, as the vessels from the newly formed superficial plexus were subsequently found to be disorganized (Figure 6E). This observation was further supported by the fact that the vessels from the newly formed superficial plexus were subsequently found to be disorganized (Figure 6F). These findings suggest that the delay in superficial plexus development is due to a delay in tip cell migration and angiogenesis.
of migratory tip cells at their vascular fronts which was indicative of a stalled or arrested migratory phenotype (Figure 6E).

Tissue-derived VEGF is the master regulator of endothelial tip cell migration during vascular development.38 We therefore reasoned that the stalled or arrested migratory phenotype observed at the vascular front of Lhx2-Cre: Raptor<sup>fl/fl</sup> retina might be due in part to aberrant VEGF levels arising as a result of aberrant mTORC1-signaling originating from the underlying RGCs. The expression of VEGF isoforms (VEGFA, VEGFB, and VEGFC) and their associated receptors (VEGFR1, VEGFR2, and VEGFR3) were
therefore examined in retina harvested from both control and mutant animals. These analyses were performed on total RNA harvested from (1) P6 retina since VEGF-driven endothelial tip cell sprouting and vascular remodeling occur dynamically at this age and (2) retina harvested from 7 week old mice since the original vascular retinopathy phenotype was characterized at this age (Figure 3). We observed that all three VEGF isoforms were differentially expressed in mutant animals at P6 with VEGFA being downregulated while both VEGFB and VEGFC transcripts were increased (Figure 6F). Contrastingly, changes to receptor levels (VEGFR2 and VEGFR3) were observed at 7 weeks of age while VEGF isoform and VEGFR1 expression remained similar (Figure 6G).

3 | DISCUSSION

This study describes a cell nonautonomous role for mTORC1 signaling during retinal neurovascular development. Lhx2-Cre mediated Rptor-ablation leads to a reduction in mTORC1 pathway activity within asymmetrically positioned RGCs and triggers (1) a delay in vascular development, (2) the production of tortuous arteries and veins, and (3) an increase in vessel crossing and leakage. These phenotypes were fully penetrant and consistently bilateral with their severity being directly correlated with the degree of mTORC1 down regulation within the neighboring RGC population. This was best exemplified in the dorsotemporal retina which was devoid of any vascular network due to the absence of mTORC1 pathway activity in the irregularly positioned RGCs residing in this region. Our work therefore agrees with previous studies and demonstrates that RGCs strongly influence retinal vasculature development via cell nonautonomous mechanisms and implicates the involvement the mTORC1 pathway in this process.

The superficial plexus in the pan-ventronasal retina of adult Lhx2-Cre:Rptor(−/−) mice displayed a rudimentary structure that was best characterized by a reduced number of tortuous arteries and veins. Such gross abnormalities presumably arose shortly after birth since the critical window for the initial patterning of the retinal vasculature commences during this early postnatal window.41,42 Our observations are also consistent with various comparable mouse retinopathy models where decreased vascular complexity and tortuous vessels were consistently observed.43-46 Moreover, major vessel tortuosity often leads to long-term vision loss due to blood flow instability and hemodynamic disturbances.47-49 We therefore believe that the gross aberrant vasculature observed in Lhx2-Cre: Rptor(−/−) mice contribute to the visually mediated behavioral deficits previously reported for this mouse model.30

Continuous apoptosis is the driving mechanism that underlies asymmetric RGC mosaic development in Lhx2-Cre:Rptor(−/−) mice.30 Given that similar neuronal loss precedes the appearance of vascular pathology in a variety of similar mouse models18,19,21 we postulate that the aberrant RGC apoptosis occurring in mutant animals also contributes to the vascular retinopathy observed in our study. However, it should be noted that changes in programmed cell death cannot fully explain the vascular phenotype observed since initial endothelial cell migration occurred just prior to the onset of apoptosis in the retina of Lhx2-Cre:Rptor(−/−) animals.30 Moreover, RGCs within severely ischemic retinal regions secrete repulsive guidance cues in an attempt to deviate the growing vascular front away from the lesioned area.50 It is therefore conceivable that the failure of vascular development within the ischemic dorsotemporal retina of Lhx2-Cre:
mice may result from the asymmetrically positioned RGCs establishing a repulsive front in an attempt to divert metabolic resources away from their surroundings.1

All three VEGF isoforms (VEGFA, VEGFB, and VEGFC) and their receptors (VEGFR2 and VEGFR3) were differentially expressed in postnatal Lhx2-Cre:Rptorβ/β mice. Given that neuronal-derived VEGF contributes to the development of the neighboring vascular network,51,52 we therefore believe that this aberrant postnatal VEGF signaling contributes in part to the delayed superficial plexus formation observed in mutant animals. In addition, our observations are also consistent with a previous study where alterations in VEGF isoform ratios directly influenced vessel morphogenesis in the retina.53 The reduced number of disorganized arteries and veins in Lhx2-Cre:Rptorβ/β animals is also reminiscent to that observed in VEGF mutant mice54 and adds further support to our hypothesis that the observed changes in VEGF expression contribute to the vascular retinopathy phenotype of Lhx2-Cre:Rptorβ/β mice could be as a direct response to injury given the known neuroprotective roles of VEGF.55,56

Although the astrocytic template in Lhx2-Cre:Rptorβ/β mice exhibited intact mTORC1 signaling, the stalled

Figures 5 A-L. Time-course isolectin-B4 staining of flat mounted retina taken from control and Lhx2-Cre:Rptorβ/β postnatal mice to visualize the developing vascular plexus. Control animals exhibited a rudimentary endothelial cell network that surrounded the optic nerve head shortly after birth (A). The superficial vascular plexus then formed during the first postnatal week as these proliferative endothelial cells migrated radially towards the retinal periphery (B-D). Vessels from the newly formed superficial vascular plexus then migrated into the retina to form the deep and intermediate plexus during the second (E, arrowhead in inset) and third (F, arrowheads in inset) postnatal weeks, respectively. Contrastingly, vascular growth was markedly delayed shortly after birth in Lhx2-Cre:Rptorβ/β mice (G-H) and a rudimentary endothelial cell network was only observed in the central retina of mutant animals towards the end of the first postnatal week (I). No superficial vascular plexus ever formed within the dorso-temporal region (J-L, arrowheads) while the remaining retina developed a tortuous endothelial cell network that reached the retinal periphery and formed the deep and intermediate vascular layers (K-L, arrowheads in insets) by the end of the third postnatal week. M-O, Quantification of superficial vascular plexus development in control and Lhx2-Cre:Rptorβ/β mice during postnatal development. Total retinal (M) and vessel areas (N) were significantly reduced that consequently lead to a reduced vascular network area (O) in mutant mice compared to control littermates. The number of retina analyzed at each age was as follows: P1 (control, n = 10; Lhx2-Cre:Rptorβ/β, n = 10); P3 (control, n = 10; Lhx2-Cre:Rptorβ/β, n = 10); P6 (control, n = 10; Lhx2-Cre:Rptorβ/β, n = 10); P9 (control, n = 11; Lhx2-Cre:Rptorβ/β, n = 11); P15 (control, n = 10; Lhx2-Cre:Rptorβ/β, n = 7) and P21 (control, n = 12; Lhx2-Cre:Rptorβ/β, n = 7). All data represents the mean ± SEM. All statistical differences were calculated using an unpaired two-tailed Student’s t test. P values are denoted as follows: ***P ≤ .001 and ****P ≤ .0001. Scale bar: (A-L, main images) 500 μm; (E-F and K-L, inset images) 25 μm. D, dorsal; GCL, ganglion cell layer; INL, inner nuclear layer; N, nasal; ONL, outer nuclear layer; P, postnatal; T, temporal; V, ventral.
appearance of the endothelial tip cells suggested that there were subtle molecular differences in the structure of this glial framework. This was reminiscent of a recent study where mTORC1 inactivation in cortical neurons induced astrocytic hypertrophy due to anomalous cell-to-cell communication. We therefore propose that the astroglial template in Lhx2-Cre:Rptorf/+ mice could be similarly influenced in part by paracrine molecules originating from the neighboring mutant RGCs during postnatal development. Such signaling factors could include contact-mediated or secreted guidance cues which are critical for the detailed spatial patterning of astrocytic networks within the developing retina. Alternatively, the axons of the asymmetric mutant RGCs could also contribute to the proposed differences in the fine tuning of the overlying astrocytic template given that these projections directly influence the direction and patterning of the glial framework during retinal development.

In conclusion, this study establishes a cell non-autonomous role for the mTORC1 pathway during neurovascular development. While a reduction in RGC number is the main driving mechanism underlying the vascular phenotype of Lhx2-Cre:Rptorf/+ retina we also cannot eliminate the contribution of both Rptor-deficient interneurons and Müller glia in aberrant plexus formation given their integral roles during plexus formation. Notwithstanding, our observations have broad applicability for neurovascular coupling throughout the nervous system in addition to the generation of future therapies that target debilitating neurodegenerative and neurological conditions.
4 | EXPERIMENTAL PROCEDURES

4.1 | Animals

Animal experiments were approved by the Animal Review Board at the Court of Appeal of Northern Norrland in Umeå (#A36-2018). The derivation and genotyping of Tg(Lhx2-Cre)1Lcar transgenic mice (written as Lhx2-Cre) and Rptortm1.1Dmsa floxed mice (written as Rptor+/- or Rptorf/f) have been previously described.35,70 The genotype of all animals was determined by Polymerase chain reaction (PCR) analysis of genomic DNA extracted from ear punch biopsies.71 Breeding Lhx2-Cre:Rptor+/- and Rptorf/f mice generated all experimental animals. The morning of the vaginal plug was considered as embryonic day (E) 0.5. Both males and females were used for experimental analyses and littermates lacking the Lhx2-Cre transgene were used as controls. All experiments were conducted on both 129/Sv:CBA:C57BL/6 and 129/Sv:CBA:C57BL/6:NMRI mixed genetic backgrounds. No phenotypic penetrance variability was observed within or between these genetic background strains.

4.2 | Polymerase chain reaction

Genomic qPCR: An orientation mark was burnt at the dorsal pole of the eye using a heated 27-gauge needle prior to enucleation. The cornea and lens were removed and a radial incision was made from the orientation mark along the dorsal axis towards the optic disc. The pigment epithelium, iris and ciliary body were then peeled away and the retina was further dissected by radial incisions along the temporal, ventral, and nasal axes to yield four equal tissue quadrants. Genomic DNA was extracted from each retinal quadrant71 and used as the template for qPCR using the FastStart Universal SYBR Green Master (Roche) and CFX Connect Real-Time System (BioRad). All reactions were performed in duplicate using oligonucleotides designed against exons 4 and 6 of the mouse Rptor gene (NCBI 74370). The level of product generated by the target primer pair (exon 4) was normalized against that amplified by the reference primer pair (exon 4). qRT-PCR: Retina were dissected out from enucleated eyes and then snap frozen in liquid nitrogen. Total RNA was isolated from the retina using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). The resulting templates were then analyzed using the SsoAdvanced Universal SYBR-Green Supermix (Bio-Rad) in a CFX Connect Real-Time System (BioRad). All reactions were performed in duplicate using either validated primer pairs57 or PrimePCR Custom Array Plates (Bio-Rad). The level of product generated by the target primer pairs (GFAP, VEGFA, VEGFB, VEGFC, VEGFR1, VEGFR2, and VEGFR3) were normalized against that amplified by the reference primer pair (GAPDH or TBP). All validated oligonucleotide sequences are given in Table 1. The RefSeq accession numbers and unique assay ID for each PrimePCR assay are listed in Table 2.

4.3 | Immunohistochemistry

Cryosection analyses: embryonic heads were fixed in 4% (w/v) PFA in PBS for 2 h on ice. The harvested tissues were equilibrated overnight at 4°C in 30% (w/v) sucrose in PBS and embedded in OCT compound (Sakura Finetek). Flat mount analyses: an orientation mark was burnt at the dorsal pole of the eye using a heated 27-gauge needle prior to enucleation. Isolated eyes were then placed in separate wells of a 48-well plate and fixed in 4% (w/v) PFA in PBS for 10 min on ice. The cornea...
and lens were subsequently removed before the retina was dissected out. Four incomplete radial incisions were then made along the dorsal, temporal, ventral, and nasal axes to yield four petals attached to one another at the central optic disc region. A separate incision was also placed in the dorsonasal domain for orientation purposes. Retina were then fixed for a further 45 min in 4% (w/v) PFA in PBS on ice. Immunohistochemistry was performed on cryosections (10 μm) or flat mount retina as previously described. All antibodies and lectins used in this study are listed in Table 3.

4.4 | Fundoscopy and fluorescein angiography

Fundus imaging and fluorescein angiography were conducted using a Micron IV Retinal Imaging System (Phoenix Research Laboratories). Pupils were first dilated by the topical addition of 10% (w/v) phenylephrine and 5% (v/v) tropicamide. The mice were then anesthetized by an intraperitoneal injection of Hypnorm (0.079 mg/ml fentanyl citrate and 2.5 mg/ml fluanisone) and Midazolam (1.25 mg/ml) at 10 μl/g and placed onto an adjustable heated stage. Hypromellose gel (3.2 mg/ml) was placed onto the cornea and the heated stage was stereoscopically adjusted until the cornea came into contact with the Micron IV objective. Fundus images were then captured at various focal depths and illumination settings using Discover Software (Phoenix Research Laboratories).

4.5 | Image analyses

All immunohistochemical images were captured using an LSM 710 confocal microscope (Zeiss). Voronoi domain analyses were performed using customized macros written in Fiji as previously described. Vascular networks were quantitatively analyzed using AngioTool. All figures were compiled using Adobe Photoshop.

4.6 | Statistical analyses

All statistical analyses were performed blind to genotype using Prism9 (GraphPad Software). All raw data was first analyzed for Gaussian distribution using a Shapiro-Wilk normality test. An unpaired two-tailed Student’s t test or a Mann-Whitney test was used to determine all statistical significances. Error bars in all figures represent the SE of the mean (SEM). The number of animals analyzed (n) for each statistical test are given in the respective figure legend. P values are indicated as follows: *P ≤ .05, **P ≤ .01, ***P ≤ .001, ****P ≤ .0001.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.
AUTHOR CONTRIBUTIONS
Iwan Jones: Conceptualization; data curation; formal analysis; investigation; methodology; resources; software; validation; visualization; writing-original draft; writing-review & editing. Anna-Carin Häglund: Conceptualization; data curation; formal analysis; methodology; resources; software; validation; visualization; writing-original draft; writing-review & editing. Leif Carlsson: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing-original draft; writing-review & editing.

DATA AVAILABILITY STATEMENT
All raw data and imaging scripts are available from the corresponding author upon request.

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