**Objective**—Increasing evidence suggests that bone morphogenetic protein (BMP) signaling regulates angiogenesis. Here, we aimed to define the function of BMP receptors in regulating early postnatal angiogenesis by analysis of inducible, endothelial-specific deletion of the BMP receptor components Bmpr2 (BMP type 2 receptor), Alk1 (activin receptor-like kinase 1), Alk2, and Alk3 in mouse retinal vessels.

**Approach and Results**—Expression analysis of several BMP ligands showed that proangiogenic BMP ligands are highly expressed in postnatal retinas. Consistently, BMP receptors are also strongly expressed in retina with a distinct pattern. To assess the function of BMP signaling in retinal angiogenesis, we first generated mice carrying an endothelial-specific inducible deletion of Bmpr2. Postnatal deletion of Bmpr2 in endothelial cells substantially decreased the number of angiogenic sprouts at the vascular front and branch points behind the front, leading to attenuated radial expansion. To identify critical BMPR1s (BMP type 1 receptors) associated with BMPR2 in retinal angiogenesis, we generated endothelial-specific inducible deletion of 3 BMPR1s abundantly expressed in endothelial cells and analyzed the respective phenotypes. Among these, endothelial-specific deletion of either Alk2/acvr1 or Alk3/bmpr1a caused a delay in radial expansion, reminiscent of vascular defects associated with postnatal endothelial-specific deletion of BMPR2, suggesting that ALK2/ACVR1 and ALK3/BMPR1A are likely to be the critical BMPR1s necessary for proangiogenic BMP signaling in retinal vessels.

**Conclusions**—Our data identify BMP signaling mediated by coordination of ALK2/ACVR1, ALK3/BMPR1A, and BMPR2 as an essential proangiogenic cue for retinal vessels.

**Visual Overview**—An online visual overview is available for this article. (Arterioscler Thromb Vasc Biol. 2017;37:657-663. DOI: 10.1161/ATVBAHA.116.308422.)

**Key Words:** angiogenesis • BMP signaling • retina • vertebrate development

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Bone morphogenetic protein (BMP) signaling has been implicated as a key regulator for angiogenesis. Depending on the nature of the ligands, BMP signaling can either promote or inhibit angiogenesis: proangiogenic BMP2/4 augments vessel sprouting in a matrigel plug assay, and stimulation with BMP2 promotes angiogenic responses such as filopodia extension and migration in human umbilical vein endothelial cells by inducing the expression of target genes, such as MYOX. In addition, its zebrafish orthologue, Bmp2b, functions as the predominant angiogenic cue for veins. Similar proangiogenic effects have been demonstrated for BMP6. In contrast, BMP9 and BMP10 induce quiescence of endothelial cells and, therefore, function as antiangiogenic cues. Consistent with the idea that BMP9 and BMP10 modulate the homeostasis of mature vessels, it has been shown that BMP9 can promote differentiation of endothelial progenitors during ischemic neovascularization. © 2017 The Authors. Arteriosclerosis, Thrombosis, and Vascular Biology is published on behalf of the American Heart Association, Inc., by Wolters Kluwer Health, Inc. This is an open access article under the terms of the Creative Commons Attribution Non-Commercial-NoDerivs License, which permits use, distribution, and reproduction in any medium, provided that the original work is properly cited, the use is noncommercial, and no modifications or adaptations are made.
On the cell membrane of the signal receiving cells, BMP ligands interact with tetra-heteromeric receptor complexes composed of 2 BMP type 1 receptors (BMPR1s) and 2 BMP type 2 receptors (BMPR2s). The signaling specificity of each BMP ligand is determined by the interaction between BMP ligand and its cognate BMPR1 since BMPR2 can only serve as a low-affinity receptor. Therefore, BMPR1s are essential for the outcomes of BMP signaling. In the mammalian genome, 4 BMPR1s, Alk1/Acvrl1, Alk2/Acvr1, Alk3/Bmpr1a, and Alk6/Bmpr1b, have been annotated. Previous work showed that endothelial-specific deletion of Alk1 generates exuberant angiogenesis, indicating that Alk1 (activin receptor-like kinase) is likely to mediate antiangiogenic BMP9/10 signaling in endothelial cells. Considering that Alk2, Alk3, and Alk6 bind to BMP2, BMP4, and BMP6 in other circumstances, these receptors are likely to mediate proangiogenic BMP signaling. However, since global deletion of these receptors leads to early embryonic lethality, surprisingly little is known about the individual function of these BMPR1s in endothelial cells. In addition, previous attempts to elucidate the role of each BMPR1 in endothelial cells failed to provide comprehensive analyses because of the lack of suitable endothelial-specific Cre driver lines. Most importantly, manipulations of BMPR1 in endothelial cells were not performed in the same way in the same vascular bed in previous studies, making it difficult to determine the role of each BMPR1s in endothelial cells. In this study, we investigated the function of each BMPR1 in angiogenesis using postnatal mouse retinal vessels to better understand the molecular and cellular underpinning of proangiogenic BMP signaling. To assess the contribution of BMPs in retinal angiogenesis, we showed BMP signaling reporter activity in the early postnatal retina and found that BMP6 and BMP7 are the most abundant BMP ligands in that organ. We found that several BMPR1s were expressed in distinct regions of the retinal vasculature. Conditional deletion of Bmpr2 and 3 highly expressed BMPR1s, Alk1/Acvrl1, Alk2/Acvr1, and Alk3/Bmpr1a, in endothelial cells of postnatal mice showed that mice deficient for Alk2/Acvr1 or Alk3/Bmpr1a partially phenocopied the vascular defects of mice with an endothelial-specific deletion of Bmpr2. Taken together, our data indicate that the proangiogenic BMP signaling mediated by ALK2 and ALK3 receptors, likely in conjunction with BMPR2, is essential for proper retinal angiogenesis.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Diverse BMP Ligands and Receptors Are Expressed During Retinal Angiogenesis

To determine the role of proangiogenic BMP signaling, we first examined the expression of GFP (green fluorescent protein) in retinal vessels of BMP responsive elements (BRE)-GFP mice, where the expression of GFP is regulated by BRE isolated from the Id1 promoter. As previously reported, BRE-GFP expression was widely detected in P5 retinal vessels (Figure 1A). Within the vascular front, BRE-GFP was highly expressed in both tip and stalk cells (Figure 1B). While the expression of BRE-GFP in stalk cells can be attributed to the ALK1-mediated antiangiogenic BMP signaling activity, it is not clear how BRE-GFP expression in tip cells is induced.

To identify which BMP ligands and receptors induce BRE-GFP expression in the retinal vessels, we performed in situ hybridization analyses on mouse retinas and quantitative reverse transcriptase polymerase chain reaction using cells isolated from postnatal retinas at different developmental stages (Figure 1C; Figure I in the online-only Data Supplement). A number of proangiogenic BMP ligands were highly expressed during the stages, coincident with extensive retinal angiogenesis (Figure 1C; Figure II in the online-only Data Supplement). In particular, the expression of BMP6 and BMP7 was increased over 2-folds. In contrast, expression of BMP9 and BMP10, which are ligands for ALK1 and are produced by liver and heart, respectively, were not detected in the retina (Figure I in the online-only Data Supplement). However, given that they are delivered by circulation, it is likely that the mature BMP9 and BMP10 protein are present in the retina. In endothelial cells, only 3 BMPR1s, ALK1, ALK2, and ALK3, but not ALK6, were highly expressed. Therefore, ALK6 was excluded from further analyses (Figure III in the online-only Data Supplement).

Next, we analyzed the expression of BMPR2 and the 3 BMPR1s that are abundant in endothelial cells, ALK1/Acvrl, ALK2/Acvr1A (activin type 1 receptor A), and ALK3/Bmpr1A by immunostaining in retinas (Figure 1D through 1G and data not shown). BMPR2 staining is vascular and appears in all vessels, with heightened expression at the vascular front (Figure 1D and 1D’). Interestingly, each BMPR1 has distinct expression pattern; ALK1 and ALK3 proteins are found in both plexus and vascular front, with ALK1 being enriched in the arteries of the plexus region. ALK2 staining is highly enriched in veins in the plexus region and not associated with the vascular front or arteries (Figure 1E through 1G and 1E’ through 1G’). These findings show that proangiogenic BMP ligands and receptors are highly expressed in blood vessels during retinal development, and the receptors have distinct expression patterns. Therefore, it is likely that BRE-GFP reporter activity in tip cells at the vascular front results from the activity of proangiogenic BMP ligands.

BMPR2 Activity Promotes Retinal Angiogenesis

To examine the requirement for proangiogenic BMP signaling during retinal angiogenesis, we generated mice carrying an inducible, endothelial-specific Bmpr2 deletion by crossing Bmpr2<sup>Cre<sup>ER</sup></sup> and Cdh5<sup>(Pac)Cre<sup>ERT2</sup></sup> mice. Given that BMPR2 is the predominant binding partner for BMPR1s mediating...
proangiogenic BMP activity, we postulated that deletion of Bmpr2 is likely to attenuate signaling outcomes downstream of most proangiogenic BMP ligand engagement during retinal angiogenesis. Consistent with our hypothesis, endothelial cell deletion of Bmpr2 significantly decreased the radial expansion of the retinal vessels (Figure 2A and 2C), indicating that BMP signaling is essential for retinal angiogenesis. Bmpr2fl/fl; Cdh5(Pac)CreERT2 mice also displayed a significant reduction in vascular density (percent vascularized area) compared with that of wild-type littermates (Figure 2B and 2C). These data show that proangiogenic BMP signaling mediated by BMPR2 is a critical positive regulator of retinal angiogenesis.

**Figure 2.** BMPR2 is essential for retinal angiogenesis. A, Representative overview of P5 retinal vessels taken from inducible endothelial-specific knock out of Bmpr2fl/fl; Cdh5(Pac)CreERT2 (right) and their phenotypic wild-type (WT) littermates (left); scale bar, 500 μm. Mean radial expansion of the retinal vessels in P5 Bmpr2fl/fl retinas was decreased to 78.9±9.8% of WT littermates. B, Representative overview of plexus region of P5 retina in Bmpr2fl/fl; Cdh5(Pac)CreERT2 (right) and their phenotypic wild-type littermates (left). Mean vascular density in the plexus region of P5 retina in Bmpr2fl/fl; Cdh5(Pac)CreERT2 (right) and their phenotypic wild-type littermates (left). Mean vascular density in the plexus region of P5 retina in Bmpr2fl/fl; Cdh5(Pac)CreERT2 (right) and their phenotypic wild-type littermates (left). C, Quantification of radial expansion and vascular density (percent vascularized area) in Bmpr2fl/fl; Cdh5(Pac)CreERT2 (green) and Bmpr2fl/fl; Cdh5(Pac)CreERT2 mice injected with 100 μg tamoxifen at P1; retinas were assayed P5 (n=5). *P<0.05. Statistical significance was assessed using a Student unpaired t test.
Endothelial-Specific Deletion of Alk2/ACVRL1A or Alk3/BMPR1A Recapitulates Vascular Defects of Endothelial-Specific Deletion of Bmpr2

Next, we investigated critical BMPR1 interactions mediating proangiogenic BMP signaling during retinal angiogenesis. Endothelial deletion of Alk1, Alk2, or Alk3 during postnatal retinal angiogenesis was induced by crossing homozygous mice carrying a floxed allele of each BMPR1 and Cdh5(Pac)CreERT2 mice (Figure IV in the online-only Data Supplement). As previously reported,9,27 tamoxifen-mediated deletion of Alk1 in endothelial cells at P1 did not exhibit severe defects in radial extension, but caused exuberant angiogenesis in developing retinas when compared with phenotypic wild-type littermates and, therefore, substantially increased vascular density (percent vascularized area; Figure 3A and 3D). In Alk1<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> mice, the vascular phenotype was more pronounced in the vascular front than in the plexus region (Figure 3A). Considering the distinct phenotypes between Bmpr2<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> and Alk1<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> mice, it is unlikely that ALK1 is involved in proangiogenic BMP signaling mediated by BMPR2.

In contrast, tamoxifen-mediated deletion of Alk2 or Alk3 in endothelial cells at P1 led to a substantial reduction in the radial expansion of retinal vessels (Figure 3B and 3C; Figure IV in the online-only Data Supplement), reminiscent of the defects in Bmpr2<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> mice (Figure 2A). In addition, vascular density was substantially reduced in tamoxifen-injected Alk2<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> or Alk3<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> retinas compared with that in wild-type littermates (Figure 3D). These findings show that both ALK2 and ALK3 promote retinal angiogenesis.

Figure 3. ALK2 and ALK3 promotes retinal angiogenesis. A–C, Representative overview of retinal vessels taken from inducible endothelial-specific knock out of Alk1<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> (A), Alk2<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> (B), or Alk3<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> (C) and their phenotypic wild-type (WT) littermates. Mice were injected with 50 μg tamoxifen at P1, retinas were assayed P5; scale bar, 500 μm. Both radial expansion and vascular density in the plexus region were significantly increased in P5 Alk1<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> retinas (101±6.7% for radial expansion and 155±6.2% for vascular density compared with WT littermates). By contrast, deletion of either Alk2 or Alk3 significantly decreased both radial expansion (63.2±17.1% for Alk2<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> retinas and 56.4±14.2% for Alk3<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> retinas compared with WT littermates) and vascular density in the plexus region (42.3±15.9% for Alk2<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> retinas and 61.9±12.8% for Alk3<sup>fl/fl</sup>;Cdh5(Pac)Cre<sup>ERT2</sup> retinas compared with WT littermates). Areas within the white rectangle in middle column are shown in higher magnification (right). While radial expansion was similarly affected by deletion of either Alk2 or Alk3, vascular density in the plexus region is more severely affected by the deletion of Alk2 than Alk3. Endothelial cells are visualized by anti-isolectin B4 (IB4) staining. D, Quantification of radial expansion and vascular density (percent vascularized area) in inducible endothelial-specific knockout of each BMPR1 (pink) and their littermates (green; n=5). P<0.05. Statistical significance was assessed using a Student unpaired t test. ALK indicates activin receptor-like kinase 1.
are important for proper retinal vessel morphogenesis, likely in conjunction with BMPR2.

Endothelial-Specific Deletion of Alk3/BMPR1A and Bmpr2 but Not Alk2/ACVR1A Affect Angiogenic Sprouts at the Vascular Front

Considering that both Alk2 and Alk3 deletion in endothelial cells significantly delayed radial expansion but only Alk3 is expressed at the vascular front, we hypothesized that ALK2 and ALK3 receptors would differentially affect angiogenic sprouts. To examine this notion, we first examined the phenotype of endothelial deletion of Bmpr2 and found that lack of Bmpr2 significantly decreased the number of angiogenic sprouts (Figure 4A). Endothelial deletion of Alk3/BMPR1A similarly decreased the number of angiogenic sprouts present at the vascular front when compared with phenotypic wild-type littermates (Figure 4B and 4C), whereas deletion of Alk2 did not significantly alter the number of angiogenic sprouts at the vascular front. Considering that the vascular coverage (percent vascularized area) was significantly decreased in retinas of mice with deletion of either receptor (Figure 3B through 3D), it is likely that different BMPR1 receptors mediate proangiogenic BMP signaling at the vascular front and behind the front.

Discussion

Our results identify BMPR2/ALK2 and BMPR2/ALK3 as key receptors that mediate proangiogenic BMP signaling in the early postnatal retina and reveal regional differences among BMPR1s by analysis of parallel genetic experiments in a defined vascular bed. Deletion of the common BMPR2 receptor reduced vascular sprouting and density. Deletion of ALK3, which is ubiquitously expressed in retinal endothelial cells, also dramatically reduced vascular sprouting and density, while loss of ALK2, which is enriched behind the vascular front, did not significantly affect sprouting but reduced overall vessel density. Therefore, we propose that spatially regulated BMPR1 expression fine-tunes endothelial cell responses to proangiogenic BMP ligands in development. Since expression of BMP ligands selective for ALK2 and ALK3 is elevated during retinal angiogenesis, it is tempting to speculate that BMP6/7–ALK2/3–BMPR2 signaling axis may provide essential input for the developing retina.

Since the phenotype of endothelial-specific deletion of Bmpr2 is distinct from the vascular phenotype caused by endothelial-specific deletion of Alk1, it is likely that BMPR2-mediated proangiogenic signaling is dominant over interactions with ALK1 in the early postnatal retina. In contrast, ALK2 and ALK3 seem to be essential for BMPR2-mediated proangiogenic signaling. These receptors function similarly but nonredundantly behind the vascular front in mediating branching as deletion of either type 1 receptor partially phenocopies the phenotype of Bmpr2 deletion and results in reduced branching. This finding suggests that both ALK2/BMPR2 and ALK3/BMPR2 complexes contribute to branching morphogenesis, which is consistent with our previous finding that proangiogenic BMP signaling leads to increased branching in

Figure 4. ALK3/BMPR2 signaling is required for the formation of angiogenic sprouts in the vascular front. A, Representative image of vascular front in postnatal day P5 Bmpr2fl/fl;Cdh5(Pac)CreERT2(A), Alk2fl/fl;Cdh5(Pac)CreERT2(B), or Alk3fl/fl;Cdh5(Pac)CreERT2(C) endothelial cells are visualized by anti-isolectin B4 (IB4) staining and their phenotypic wild-type (WT) littermates. Green arrows point to the angiogenic sprouts in the vascular front (n=5); scale bar, 200 μm. D, Quantification of angiogenic sprouts. The number of angiogenic sprouts in the vascular front was measured by dividing the number of angiogenic sprouts by the total length of vascular front (see Methods in the online-only Data Supplement for detail). While the number of angiogenic sprouts in the vascular front was mildly decreased by 20% in P5 Alk2fl/fl retinas (8.8±0.2 compared with 10.8±1.9 in WT littermates) and by 83% in Alk3fl/fl retinas (5.3±0.4 in Alk3fl/fl retinas compared with 11.2±0.9 in WT littermates), P<0.05. Statistical significance was assessed using a Student unpaired t test. ALK indicates activin receptor-like kinase 1.
vitro and in vivo. Angiogenic sprouting at the vascular front is likely to selectively use ALK3/BMPR2 complexes since genetic deletion of Alk2 did not significantly affect angiogenic sprouting. This is consistent with the expression patterns of the receptors in the early postnatal retina as ALK3 but not ALK2 reactivity was found at the vascular front. ALK2 reactivity is enriched in veins, while ALK3 expression is not enriched in larger vessels, suggesting potential differences in ALK2- and ALK3-mediated proangiogenic BMP signaling. ALK1, which does not seem to be essential for BMPR2-mediated proangiogenic BMP signaling, may provide an additional regulation for vessel remodeling and homeostasis at later stages.

Previously, it has been shown that BMPR1s can promiscuously form heterodimeric complex. For instance, it has been shown that ALK1 and ALK2 can form heterodimers under certain circumstances. Similarly, ALK2 and ALK3 are proposed to form heterodimers to mediate BMP2 signaling. Therefore, it is possible that they may influence the signaling property of each other. Although we cannot rule out this possibility because of the lack of appropriate reagents, given the distinct expression pattern of BMPR1s, we think that the vascular phenotype caused by endothelial-specific deletion of single BMPR1 is likely to represent the unique signaling property of each receptor.

Our analyses indicate that ALK3/BMPR2-mediated BMP signaling regulates angiogenic sprouting at the vascular front, whereas both ALK3/BMPR2 and ALK2/BMPR2 regulate branching behind the vascular front. This is consistent with our finding that BMP signaling, as read out by BRE-EGFP (enhanced green fluorescent protein), is active throughout the retinal vasculature and suggests that proangiogenic BMP signaling regulates both sprouting and branching. These results are consistent with a previous report describing ubiquitous expression of BRE-EGFP in retinal vessels.

Our work presents compelling evidence that proangiogenic BMP signaling is important for angiogenesis. Here we show that expression of type I receptors, specifically ALK2 and ALK3, may explain the different effects of BMP signaling on developing vessels, and they each likely complex with BMPR2 to mediate proangiogenic effects in blood vessels in a nonredundant way. While the molecular underpinning of the phenotypic differences between Alk2;Cdh5(Pac)CreERT2 and Alk3;Cdh5(Pac)CreERT2 mice needs further analyses, careful phenotypic comparisons between these mice will help elucidate how angiogenesis is regulated in development and disease and may provide useful information in developing therapeutic strategies.

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Disclosures

None.

References


**Highlights**

- Proangiogenic bone morphogenetic protein signaling is essential for retinal angiogenesis.
- Alk2/Acvr1 and Alk3/Bmpr1a relays distinct aspects of proangiogenic effects of bone morphogenetic protein.
Alk2/ACVR1 and Alk3/BMPR1A Provide Essential Function for Bone Morphogenetic Protein–Induced Retinal Angiogenesis

Heon-Woo Lee, Diana C. Chong, Roxana Ola, William P. Dunworth, Stryder Meadows, Jun Ka, Vesa M. Kaartinen, Yibing Qyang, Ondine Cleaver, Victoria L. Bautch, Anne Eichmann and Suk-Won Jin

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ONLINE MATERIALS AND METHODS

Genetic experiments and pharmacological inhibition
All animal procedures were performed in accordance with Yale University and University of North Carolina Institutional Animal Care and Use Committee guidelines. The following mouse strains were used: \textit{Alk1}^{fl/fl}, \textit{Alk2}^{fl/fl}, \textit{Alk3}^{fl/fl}, \textit{Bmpr2}^{fl/fl} and \textit{Cdh5(Pac)Cre}^{ERT2} and BRE-gfp mice. For endothelial-cell-specific loss-of-function of BMPRs, mice carrying homozygous floxed alleles of BMPR were interbred with \textit{Cdh5(Pac)Cre}^{ERT2} mice. All of the mouse strains were back-crossed into the C57Bl/6J background. Genetic modifications were induced by intraperitoneal injections with 30 or 100\(\mu\)g tamoxifen (Sigma) at P1 and P2.

Isolation of mouse endothelial cell and manipulation of mouse retina
Mouse lung endothelial cells (MLECs) for qRT-PCR analyses were isolated as described previously (Magee et al. 1994; Sakurai et al. 2002). In total, six P6 pups were euthanized by decapitation. The lungs were removed and placed in a 1.5mL eppendorf tube. Lungs were finely minced with sharp scissors, then the tissue was digested with 2mg/mL collagenase type I in PBS at 37˚C for 45 min. The digest was passed through a 75\(\mu\)m cell strainer to remove undigested tissue fragments. Cells were pelleted at 400 g for 5 minutes, resuspended in 2% FBS containing PBS with magnetic beads-coated rat anti-mouse CD31 (PECAM-1) antibody (Ab) (BD PharMingen). After incubation on room temperature for 12 min, the cells were placed on the magnet for 5 min and unbound cells were removed. The bound cells were resuspended in medium and plated onto a 0.1% gelatin-coated dish. Mouse retina dissection and manipulation was performed as previously reported.

Immunofluorescence (IF)
The following antibodies were used: anti-ALK1 (1:100, R&D Systems), anti-ALK2 (1:100, Proteintech), anti-ALK3 (1:100, Thermo), anti-BMPR2 (1:100, BD), anti-PECAM-1 (1:200, BD) and isolectin-B4 (1:100, BD). Alexa Fluor 488, 555 and 647 donkey secondary antibodies were from Invitrogen. Retina immunostaining was carried out with littermates processed simultaneously under the same conditions. To analyze and quantify the retina vascular phenotype, the eyes were fixed in 4% PFA for 2 hours at 4˚C and dissected as previously reported (REF). After washing the retinas were permeabilized using PBS-T (0.1% Triton X-100 containing PBS) and incubated overnight at 4˚C in PBS-T containing biotinylated isolectin B4 (IB4) and the primary antibodies. Then the retinas were incubated with fluorophore-conjugated antibodies and with Alexa Fluor 488/555/647 (Invitrogen), and mounted with Fluorescence Mounting Medium (Dako). Confocal microscopy was performed with Leica SP5 confocal microscopes. ImageJ (NIH) was used for the data analysis. The figures were assembled using Adobe Photoshop. The only adjustments used in the preparation of the figures were for brightness and contrast.

RT-PCR
For gene expression analysis, the total RNA was isolated with RNeasy Mini kits (QIAGEN) and 1 \(\mu\)g total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermofisher scientific) following the manufacturer instructions. The cDNAs were amplified using the FG Power SYBR Green PCR Master Mix (Applied Biosystems) in an Eppendorf Mastercycler gradient. The relative expression differences represent the means of the results obtained from at least three-independent experiments.

Statistical analysis
Data are expressed as mean \(\pm\) SEM. Comparisons between groups were made using a two-tailed Student t test. Prior to the student t test, we have used the Levene's test to examine whether the assumptions and conditions for t test were violated, and therefore, could not be used to determine the differences between groups. We found that knockout mice and phenotypic wild-type siblings are statistically indistinguishable groups for a number of unrelated parameters. In all parameters we used, the F values were significantly smaller than the Table F value at \(\alpha=0.001.\) Our statistical analyses demonstrated that knockout mice and their phenotypic wild-type siblings have similar population
variances, and therefore, the assumptions and conditions for t test was not violated. This result supports our approach to employ t test to perform statistical analyses.
ONLINE MATERIALS AND METHODS REFERENCES


