Subcellular localization of coagulation factor II receptor-like 1 in neurons governs angiogenesis

Jean-Sébastien Joyal1–4,10, Satra Nim4,10, Tang Zhu1–10, Nicholas Sitaras2,3, José Carlos Rivera1,2, Zhuo Shao4, Przemyslaw Sapieha2, David Hamel3, Melanie Sanchez4, Karine Zaniolo1, Manon St-Louis4, Johanne Ouellette4, Martin Montoya-Zavala3, Alexandra Zabeida1, Emilie Picard1, Pierre Hardy1, Vikrant Bhosle4, Daya R Varma4, Fernand Gobeil Jr6, Christian Beauséjour3, Christelle Boileau3, William Klein7, Morley Hollenberg8, Alfredo Ribeiro-da-Silva4, Gregor Andelfinger9 & Sylvain Chemtob1–4

Neurons have an important role in retinal vascular development. Here we show that the G protein–coupled receptor (GPCR) coagulation factor II receptor-like 1 (F2rl1, previously known as Par2) is abundant in retinal ganglion cells and is associated with new blood vessel formation during retinal development and in ischemic retinopathy. After stimulation, F2rl1 in retinal ganglion cells translocates from the plasma membrane to the cell nucleus using a microtubule-dependent shuttle that requires sorting nexin 11 (Snx11). At the nucleus, F2rl1 facilitates recruitment of the transcription factor Sp1 to trigger Vegfa expression and, in turn, neovascularization. In contrast, classical plasma membrane activation of F2rl1 leads to the expression of distinct genes, including Ang1, that are involved in vessel maturation. Mutant versions of F2rl1 that prevent nuclear relocalization but not plasma membrane activation interfere with Vegfa but not Ang1 expression. Complementary angiogenic factors are therefore regulated by the subcellular localization of a receptor (F2rl1) that governs angiogenesis. These findings may have implications for the selectivity of drug actions based on the subcellular distribution of their targets.

F2rl1 is a protease-activated receptor belonging to a family of GPCRs highly expressed throughout the central and the peripheral nervous system1. F2rl1, previously known as Par2, is particularly abundant in neurons, where it mediates pain, propagates inflammation and controls cell survival and the release of neurotransmitters1. F2rl1 is irreversibly activated by circulating and resident serine proteases that can cleave its N terminus and expose a tethered ligand or by synthetic peptides that mimic the tethered ligand2. In disease processes, F2rl1 integrates signals from proteases of the coagulation cascade and inflammatory cells to trigger repair by promoting survival and proliferation. In this context, F2rl1 also accelerates the revascularization of ischemic tissues3 and is proangiogenic in the retina4. In the developing retina, F2rl1 expression increases proportionally with vessel growth4.

The anatomic network of blood vessels and neurons evolves developmentally to match neuronal activity with tissue perfusion. This paradigm applies equally to the visual system, where the first appearance of visual-evoked potentials coincides with the development of the human outer vascular plexus (at 25–26 weeks of gestation)5. The increased metabolic demand and oxygen consumption of newly functioning neurons contribute to localized regions of hypoxia6, particularly at the vascular front of the developing retina, and hypoxia in turn triggers the release of angiogenic factors. Although retinal glial cells participate in the synthesis of hypoxia-driven vascular growth factors under physiologic7 and pathologic conditions8, a selective loss of Vegfa production in astrocytes9 or Müller cells10 is relatively dispensable during retinal vascular development. Conversely, loss of retinal ganglion cells (RGCs) in transgenic mice (Pou4f2lacZ-DTA+/SIX3-Cre) completely abrogates developmental retinal vascular growth while preserving a normal astrocytic network11. Hence, neurons have an important role in retinal vascular development by releasing angiogenic factors11. Analogously, peripheral sensory neurons govern arterial differentiation and vascular branching through vascular endothelial growth factor (Vegfa) secretion12.

GPCRs, such as F2rl1, classically signal at the plasma membrane or as internalized scaffolds that are subsequently recycled back to the cell surface or sorted for degradation. Many GPCRs have been identified at the cell nucleus, implying nuclear translocation of activated receptors13. The presence in the nucleoplasm of phospholipids14 and in the nucleus of membrane-associated signaling factors13, β-arrestin15, 1Department of Pediatrics, Centre Hospitalier Universitaire (CHU) Sainte-Justine Research Center, Université de Montréal, Montréal, Québec, Canada. 2Department of Ophthalmology, Hôpital Maisonneuve-Rosemont Research Center, Université de Montréal, Montréal, Québec, Canada. 3Department of Pharmacology, Université de Montréal, Montréal, Québec, Canada. 4Department of Pharmacology and Therapeutics, McGill University, Montréal, Québec, Canada. 5The Wyss Institute, Harvard University, Boston, Massachusetts, USA. 6Department of Pharmacology, Sherbrooke University, Sherbrooke, Quebec, Canada. 7Department of Systems Biology, University of Texas MD Anderson Cancer Center, Houston, Texas, USA. 8Department of Physiology and Pharmacology, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada. 9Department of Cardiology, CHU Sainte-Justine Research Center, Université de Montréal, Montréal, Québec, Canada. 10These authors contributed equally to this work. Correspondence should be addressed to S.C. (sylvain.chemtob@umontreal.ca), G.A. (gregor.andelfinger@recherche-ste-justine.qc.ca) or J.-S.J. (js.joyal@umontreal.ca).

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G protein–receptor kinases and regulators of G protein signaling, as well as the existence of nuclear membrane channels and nuclear bodies (with hydrophobic regions) and electron microscopy evidence of nuclear membrane invaginations, all point to an intranuclear network of membranes and hydrophobic regions that could harbor nuclear-localized GPCRs. Although translocation of GPCRs to the cell nucleus has been proposed, the mechanism of this translocation is unknown. Moreover, the in vivo physiologic consequences of nuclear-localized GPCR signaling have yet to be established.

In the process of elucidating the contribution of F2rl1 to retinal angiogenesis, we detected its presence at the cell nucleus, leading us to explore how the subcellular distribution of F2rl1 governs its effects on angiogenesis. We show that membrane-activated F2rl1 translocates from the plasma membrane to the cell nucleus using a microtubule-dependent shuttle requiring importin-β1 and Snx11. At the nucleus, activated F2rl1 recruits Sp1 to trigger Vegfa expression and, in turn, neovascularization.

RESULTS

F2rl1 is angiogenic in the developing retina

We first explored whether F2rl1 contributes to retinal vascular development. Compared to wild-type (WT) mice, F2rl1 knockout (F2rl1−/−) mice showed a modest delay in retinal vascular growth and reduced vascular density during early development (Supplementary Fig. 1a–c). Although these results are in part consistent with those reported previously, our findings diverge from those showing robust effects of F2rl1 stimulation on retinal angiogenesis (in vivo) and neurovascular cell migration (in vitro). To eliminate potential confounding compensatory mechanisms in F2rl1−/− mice, we knocked down F2rl1 using intravitreal injections of shRNA-encoded lentivirus (shF2rl1). Knockdown of F2rl1 in neurons of the ganglion cell layer significantly reduced developmental neovascularization, whereas treatment with the mouse F2rl1 agonist peptide (F2rl1-AP) SLIGRL triggered retinal neovascularization in WT but not F2rl1−/− animals (Fig. 1a and Supplementary Fig. 1a,d). In a model of ischemic retinopathy, exposure of mice to 75% O2 from postnatal day (P) 7–12 induced a marked vaso-obliteration, which was augmented by shF2rl1 and was diminished by SLIGRL. Physiologic revascularization of the central avascular retina was delayed by shF2rl1, resulting in an increased number of pathologic preretinal neovessels. Conversely, the F2rl1-AP (SLIGRL) improved normal retinal revascularization and in turn decreased the number of pathologic neovessels (Supplementary Fig. 2), confirming a pivotal role for F2rl1 in physiologic retinal angiogenesis.

F2rl1 was localized in RGCs and, to a much lesser extent, in endothelial cells, as reported previously, but was not present in the astroglia of the developing retina (Fig. 1b and Supplementary Fig. 3a–d). We confirmed the predominance of F2rl1 mRNA in RGCs by laser capture microdissection (LCM) of retinal layers (Fig. 1c); F2rl1 mRNA expression by LCM of retinal layers, n = 4 mice. INL, inner nuclear layer; NFL, nerve fiber layer. Scale bar, 20 µm. (d) Top, schematic of the experimental strategy. Pou4f2−/− mice containing a floxed-lacZ-stop–diphtheria A toxin cassette expressed selectively in RGCs were crossed with tamoxifen (Tm)-inducible Cre recombinase mice (CAGG-Cre-ERTM); the mice were injected with Tm at P1 to selectively ablate RGCs during vascular development, treated with SLIGRL at P4 and analyzed by flat mount at P6. Bottom, ß-galactosidase (ß-Gal) stains of retinal cross-sections. Scale bar, 20 µm. (e) Lectin-stained retinal flat mounts (P6) of Pou4f2−/− mice expressing Cre-ERTM or not and treated with vehicle (Veh) or SLIGRL (20 µM). The bar graph shows the changes in vascular area. Scale bar, 200 µm. n = 3–6. All error bars represent the s.e.m. **P < 0.01, ***P < 0.001 compared to WT or vehicle using one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test (c) or Student’s t test (a,e).
expression was substantially (3.3-fold) lower in superficial retinal vessels compared to RGCs and was negligible in the inner and outer nuclear layers (Fig. 1c). We then tested the angiogenic contribution of F2rl1 expressed in retinal ganglion cells. In transgenic mice in which RGCs are conditionally ablated during vascular development (Pou4f2CreERT2 mice; Fig. 1d), F2rl1-AP (SLIGRL) failed to promote angiogenesis (Fig. 1e), which is contrary to the results in transgenic mice with intact RGCs (CreERT2/− or Pou4f2CreERT2/−/+). There was also a trend towards decreased angiogenesis in RGC-deficient mice (Pou4f2lacZ-DTA/−/+CreERT2) compared to controls (Pou4f2lacZ-DTA/−/+cre−/−). Ex vivo, when RGCs (but not F2rl1-silenced RGCs) were activated by conditioned medium induced robust proangiogenic vascular sprouting in aortic explants from WT and F2rl1−/− mice (Supplementary Fig. 3e,f). In contrast, direct F2rl1 stimulation with SLIGRL of aortic explants was ineffective. Together these findings point to a key role for F2rl1 on RGCs in stimulating angiogenesis.

F2rl1 translocates to the nucleus

On closer observation using immunogold staining and electron microscopy of the inner retina, F2rl1 localized to the cell nucleus of RGCs (Fig. 2a); we detected no immunoreactivity in F2rl1−/− mice. We used numerous approaches to explore the cell-compartment origin of nuclear F2rl1. Live confocal video imaging of stimulated F2RL1-GFP–expressing HEK293 cell stained with anti-Flag FITC (green) and anti-LBR (red) confirming the presence of translocated Flag-F2RL1 at the nucleus. Incubation with immunogold-tagged antibody to Flag (immunogold) and stimulated with vehicle or SLIGKV (30 min). Shown are the whole cell (left) and high-magnification views of the plasma membrane and nucleus. Scale bars, 5 µm (left); 1 µm (center and right). (e) An isolated nucleus from a stimulated HEK293 cell stained with anti-Flag FITC (green) and anti-LBR (red) confirming the presence of translocated Flag-F2RL1 at the nucleus. Scale bar, 2 µm. Arrowheads indicate the presence of F2RL1 in the nucleus. (f) Purity of isolated nuclei confirmed by scanning electron microscopy at increasing magnifications. Scale bars, 30 µm (left); 2 µm (center); 0.5 µm (right). (g) FACS analysis (anti-Flag FITC) of the cell nuclei in e. (h) F2rl1 abundance in subcellular fractions of RGCs stimulated with vehicle or SLIGKV (30 min). The following markers were used to confirm the identity of each fraction: 1, clathrin, plasma membrane; 2, EEA1, early endosome (EE); 3, Rab9A, late endosome (LE); 4, lamin A/C, nucleus. The bar graph represents the relative expression of F2rl1 in the corresponding cell fraction. All error bars represent the s.e.m. n = 3–4 for all experiments. *P < 0.05, **P < 0.01 compared to vehicle using Student’s t test.
is associated with nuclear membranes. In addition, fractionation of RGC-5 into plasma membrane, nuclei and early and late endosome fractions confirmed a threefold to fourfold enrichment of endogenous F2Rl1 (52 kD) in the nuclear and late endosome fractions after stimulation (Fig. 2h and Supplementary Fig. 4a). Collectively, this evidence suggests that activated F2Rl1 translocates from the plasma membrane to the cell nucleus.

**Sorting partners shuttle F2rl1 on microtubules**

We next investigated the mechanism for nuclear F2rl1 translocation. The destination of endocytic cargo is influenced by receptor-targeting sequences, association with cargo proteins and cytoskeletal tracks and motors. The sorting of endosomes is location dependent, taking place in segregated compartments, as well as time dependent, involving precisely orchestrated cargo-protein interactions. In this respect, importins are pivotal nuclear transport proteins of the karyopherin family that recognize and bind nuclear localization targeting sequences.

They have an essential role in protein import across the nucleopor complex, including the nuclear translocation of integral membrane proteins. Classically, the adaptor protein importin-β binds to the cargo, whereas importin-β1 grants passage to the nucleus through the nucleopor complex; however, importin-β1 is necessary and could be sufficient for nuclear import. We found that importin-β1 colocalized and coimmunoprecipitated with stimulated F2rl1 in transfected HEK293 cells (Fig. 3a and Supplementary Figs. 4b and 6a,b). Silencing of importin-β1 (as well as silencing of importin-α3 or importin-α5) interfered with F2rl1 relocation to the nucleus (Fig. 3c and Supplementary Fig. 7).

In addition to importins, the role of sorting nexins (Snx) is becoming increasingly recognized in endosomal trafficking. Snx harbor a characteristic Phox domain and have been identified across phyla with conserved endocytic, endosomal-sorting and signaling functions. SNX1 and SNX2, along with other components of the mammalian retromer, contribute to retrieval of F2R (or PAR1).
from the endosome to the trans-Golgi network and prevent its lysosomal degradation. However, silencing of SNX1 or SNX2 did not affect the relocation of tagged F2RL1 to the nucleus (Fig. 3c and Supplementary Fig. 6d). Snx11 contains nuclear localization motifs; when expressed in HEK293 cells, Snx11-GFP was found at and around the nucleus, where it colocalized with the late endosomal marker M6P and importin-β1 (Supplementary Fig. 6c). After F2RL1 stimulation, SNX11 coimmunoprecipitated with F2RL1, along with importin-β1 (Fig. 3b and Supplementary Fig. 6a,b). In addition, knockdown of SNX11 abrogated the nuclear translocation of F2RL1-GFP, as detected by FACS analysis of isolated nuclei (Fig. 3c), and Flag-F2RL1 was correspondingly retained largely at or adjacent to the cell membrane, as assessed by confocal imaging (Supplementary Fig. 6d,e).

Sorting nexins and importins have been reported to shuttle on microtubules. Carrier-protein kinesins move cargo on microtubules towards the plasma membrane and contribute to endosomal sorting, whereas dyneins transit towards the juxtanuclear microtubule organizing center and nucleus. They also contribute to the nuclear import of regulatory proteins. We determined whether the microtubule–dynein complex contributes to F2RL1 nuclear trafficking. The intracellular distribution of F2RL1 and Snx11 overlapped the microtubule network, as revealed by line-scan analysis (α-tubulin; Fig. 3d,e). F2RL1 coimmunoprecipitated with dynein and α-tubulin after F2RL1-AP stimulation (Fig. 3b and Supplementary Figs. 4b and 6a,b). Concordantly, F2RL1 trafficking to the nucleus was abrogated...
by the microtubule disruptors colchicine and vinblastine but was independent of clathrin (dansyl cadaverine) and caveolin (dominant-negative dynein, DynIK44A) (Fig. 3f and Supplementary Fig. 6f). F2RL1 therefore travels to the cell nucleus through the microtubule network, and this trafficking involves importin-β1 (as well as importin-α3 and importin-α5) and Snx11.

We next examined which F2RL1 domains are required for nuclear translocation. We made mutations in four regions of the protein: the nuclear localization region (NLS) of either the first (1iL) or third intracellular loop (3iL), the β-arrestin interaction domain or the C terminus (Supplementary Fig. 8a). All F2RL1 constructs preserved functional stimulation-induced calcium transient and MEK-ERK or Akt phosphorylation activity, albeit with different dynamic profiles (Supplementary Fig. 8b,c). We studied mutant F2RL1-GFP–expressing cells stimulated with F2rl1–AP using FACS of isolated nuclei (Fig. 3g), subcellular fractionation (Supplementary Figs. 4a and 9a) and confocal microscopy (Supplementary Fig. 9b). Both the NLS and C-terminus truncation mutants of F2RL1 failed to translocate to the nucleus after stimulation with F2rl1–AP but instead remained at the plasma membrane (or endosomes) (Supplementary Fig. 9a,b). F2RL1-NLS mutants also failed to coimmunoprecipitate with importin-β1 after stimulation (Supplementary Fig. 9c). These findings suggest a prominent role for both the NLS and the C-terminal domains as scaffolds for the assembly of protein complexes that are required for F2RL1 nuclear translocation.

Nuclear F2rl1 recruits Sp1 to bind the Vegfa promoter

We next investigated the potential transcriptional activity of translocated F2rl1 at the cell nucleus. Plasma membrane receptors classically convey transcriptional effects through second messengers to remote nuclear transcription factors. Because we detected F2rl1 at the nucleus, we explored the possibility that it may form a transcriptional complex, as assessed by chromatin immunoprecipitation (ChIP) of stimulated F2RL1-GFP–expressing HEK293 cells with F2RL1-specific antibodies, followed by short-read sequencing (ChIP-Seq). To focus our analysis on the angiogenic role of F2RL1, we screened for F2RL1 binding to known angiogenic genes and discovered a hit covering a predicted Sp1 binding site of the Vegfa promoter (Supplementary Fig. 10a–c); we also identified binding of F2RL1 to other relevant angiogenic gene promoters (Supplementary Table 1). Using stringent filtering of Sp1 sites, we found that 227 genes were bound by F2RL1 (data not shown). We then subcloned the Sp1 consensus sequence recovered by the ChIP assay into three different reporter plasmids, which confirmed its transcriptional activity in response to SLIGKV stimulation;

Figure 6 Nuclear F2rl1 contributes to retinal neovascularization. (a,b) Vegfa expression after stimulation (P6) in retinal ganglion cell layer neurons (NeuN labeled) of WT mice injected intravitreally (P4) with vehicle or SLIGRL (scale bar, 20 μm; a), as confirmed by LCM and quantitative RT-PCR (b). n = 3–4 mice per group. (c) Localization of F2RL1, F2RL1-E3 and F2RL1-NLS mutants (all GFP tagged) in LV-infected retinas; in each case, lower and higher magnification images are shown in the top and bottom rows, respectively. NeuN (red), RGC-markers (green) and axonal marker. Scale bars, 20 μm. (d) F2RL1-E3 is concentrated at the nuclear membrane (or endosomes) (scale bar, 200 μm). (e) Confocal microscopy (dominant-negative dynein, DynIK44A) (scale bar, 20 μm). (f) Bar graphs showing changes in vascular area and vascular density at P6. n = 10–16 mice per group. (g) Vegfa and Ang1 mRNA expression in P6 retinas expressing F2RL1, F2RL1-E3 or F2RL1-NLS compared to GFP-transfected retinas (control). n = 3 mice per group. All error bars represent the s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control or LV.GFP using one-way ANOVA with Tukey’s multiple comparison test.
silencing of Sp1 abolished this effect (Supplementary Figs. 10d and 11a,b). We confirmed the relevance of our ChIP-Seq results to RGCs by showing that stimulated F2r1 bound to the Vegfa promoter region, as assessed by ChIP-PCR. Using primers encompassing our ChIP-Seq hit (Supplementary Fig. 10b), we tested whether F2r1 and Sp1 together form a transcriptional complex by performing a ChIP with F2r1-specific antibodies followed by re-ChIP with Sp1-specific antibodies. We observed a significant enrichment in Sp1-rich DNA fragments from the Vegfa promoter region of stimulated RGCs (Fig. 4a and Supplementary Fig. 10b); in control experiments, ChIP using protein A/G alone or using antibody to histone H3 failed to significantly retrieve the Vegfa promoter DNA fragments.

Consistent with a role in gene induction, native nuclear F2r1 of RGCs coimmunoprecipitated with phosphorylated Sp1 (active) in a time-dependent manner after F2r1-AP stimulation (Fig. 4b and Supplementary Fig. 10e). Similarly, F2R1-GFP, but not the F2R1-NLS–GFP mutant, was able to recruit phosphorylated Sp1 after stimulation (Supplementary Fig. 10f). Moreover, F2R1 interacted with Sp1-containing protein complexes that specifically bind Sp1 consensus DNA binding sites, as shown by electrophoresis mobility shift assay (EMSA; Fig. 4c and Supplementary Fig. 12). Silencing of Sp1 expression in RGCs abrogated F2r1-dependent Vegfa induction (Fig. 4d and Supplementary Fig. 10d) and cell proliferation (Supplementary Fig. 11c). Altogether, these data indicate that F2r1 forms a transcriptional complex with activated Sp1 at the nucleus that regulates Vegfa expression.

Expression of distinct genes controlled by F2r1 at the nucleus and plasma membrane

To examine F2r1 subcellular signaling specificity, we identified angiogenic genes regulated by F2r1 that did not show F2r1 binding in the nuclear ChIP-Seq assay (i.e., Ang1). In RGCs and stably transduced HEK293 cells, stimulation of F2r1 induced the expression of Ang1, in addition to that of Vegfa (Fig. 5a,b). To confirm that nuclear F2r1 induces Vegfa expression, we pretreated RGCs with SLIGKRV to induce F2r1 nuclear translocation and then isolated their nuclei at 20 and 45 min after stimulation. Treatment of the isolated nuclei with SLIGKRV resulted in time-dependent expression of Vegfa but not Ang1 (Fig. 5d). In contrast, stimulation of F2r1 in conditions that interfere with its nuclear translocation (cells transfected with the F2R1-NLS and C-truncation mutants, cells treated with the microtubule disruptor colchicine or cells in which Snx11 was silenced) prevented Vegfa expression but preserved that of Ang1 (Fig. 5a–c and Supplementary Fig. 10g). Hence, stimulated F2r1 that relocates to the cell nucleus induces Vegfa, whereas F2r1 at or close to the plasma membrane triggers Ang1 expression.

To further ascertain whether nuclear F2r1 exerts specific functions, we explored whether retention of F2r1 at the nucleus would reproduce these effects. Accordingly, we introduced a nuclear retention signal to the C terminus of F2R1. The E3 region of adenovirus type 5 targets transmembrane receptors to the nucleus40. F2R1-GFP fused to E3 (F2R1–E3) localized at the nucleus to the exclusion of the plasma membrane (Fig. 5e and Supplementary Fig. 13a). To stimulate nuclear F2R1–E3, we fused the internalization peptide sequence tat (GRKKRRQRRRPPQ) to SLIGKV (tat-SLIGKV). Tat-SLIGKV triggered calcium transients (Supplementary Fig. 13b) and induced Vegfa but not Ang1 expression in F2R1–E3–expressing cells (Fig. 5f). Conversely, in cells expressing the F2R1-NLS mutants that impede nuclear translocation, SLIGKV caused rapid calcium transients and increased Ang1 but not Vegfa expression (Fig. 5f and Supplementary Fig. 13b). In intact F2RL1–expressing cells, SLIGKV triggered calcium transients (Supplementary Fig. 13b), as well as expression of both Ang1 and Vegfa (Fig. 5a,b). These findings corroborate distinct functions for F2r1 at the plasma membrane and cell nucleus.

Nuclear F2r1 exhibits an angiogenic phenotype in vivo

We next investigated the differential effects of F2r1 localized to either the nucleus or plasma membrane of retinal ganglion neurons on retinal angiogenesis. During neovascularization, Vegfa causes differentiation, proliferation and migration of the vascular endothelium, whereas Ang1 fosters the maturation of newly formed vessels41. Intravitreally injected SLIGKRV resulted in localized production of Vegfa in the ganglion cell layer of WT (but not F2r1L−/−) mice, as assessed by immunofluorescence (Fig. 6a and Supplementary Fig. 14a) and quantitative RT-PCR of laser microdissected ganglion cell cell layers (Fig. 6b). In contrast, RGC-deficient mice (Pou4f2<sup>±/−</sup>DT1<sup>KO</sup>/Cre-ER<sup>tm</sup>) failed to produce Vegfa after F2r1 stimulation (Supplementary Fig. 14b). To distinguish plasma-membrane from nuclear F2R1 functions in vivo, we used F2r1L−/− mice infected with F2R1–encoded lentivirus injected intravitreally, as such a lentivirus has a propensity for targeting retinal ganglion cell layer neurons11,42. As anticipated, the F2R1-NLS mutant localized at the plasma membrane of ganglion cell layer neurons and was accordingly detected in their axons and dendrites populating the nerve fiber layer and inner plexiform layer, respectively. In contrast, F2R1–E3 was nearly exclusively localized to the nuclei of ganglion cell layer neurons (Fig. 6c and Supplementary Fig. 14,c,d). Intraretinally transfected native F2R1 increased the expression of both Vegfa and Ang1 and, in turn, promoted developmental angiogenesis (Fig. 6e–g). Retinas transfected with the F2R1–NLS mutant exhibited increased Ang1 but negligible Vegfa expression associated with curtailed retinal neovascularization (Fig. 6d–g); these observations are in line with the actions of Ang1 on vessel quiescence and microangiopathy41. Retinas transfected with F2R1–E3 showed decreased Ang1 but increased Vegfa expression, resulting in increased developmental angiogenesis (Fig. 6d–g).

DISCUSSION

In exploring the role of F2r1 in retinal angiogenesis, we found that unique functions of the receptor are segregated according to its subcellular distribution in neurons. We showed that F2r1 from the plasma membrane translocates to the nucleus on microtubules together with Snx11 and importins. At the nucleus, F2r1 forms a transcriptional complex with Sp1 that regulates Vegfa expression, whereas surface F2r1 triggers the expression of a complementary angiogenic gene, Ang1. Nuclear F2r1 in retinal ganglion neurons contributes to developmental angiogenesis, providing in vivo evidence for physiologic functions conferred by receptor subcellular localization (Supplementary Fig. 15).

The coagulation factors FVIIa and FXa are known agonists of F2r1. These serine proteases bind tissue factor to activate F2r1 and exert angiogenic effects33. The cytoplasmic domain of tissue factor modulates F2r1 signaling in vessels33. F2r1 is also widely distributed in neurons, which are devoid of tissue factor44. However, numerous tissue-specific serine proteases are able to activate F2r1; examples include members of the kallikrein-related peptidase family (such as KLK6)45, as well as a brain-derived trypsin-like serine protease (P22)46, β-trypasase47 and membrane-type serine proteases 1 and 3 (MT-SP1 and MT-SP3)48. Notably, MT-SP1 and F2r1 share a similar tissue distribution, have important roles in angiogenesis49 and are stimulated by hypoxia and inflammation50.
Early in the course of our studies, we detected F2r1L at the nuclei of retinal neurons. We used various approaches to show that the activated receptor reaches the nucleus by translocation from the plasma membrane: (i) live-confocal imaging of F2RL1-GFP nuclear trafficking; (ii) colocalization by confocal microscopy of FITC-tagged Flag-F2RL1 activated at the plasma membrane with the inner nuclear membrane (marked by LBR), as confirmed by electron microscopy; (iii) nuclear fluorescence of activated cell surface-tagged F2r1L, quantified by FACS and corroborated by cell fractionation; (iv) pharmacological blockade of F2r1L translocation to the nucleus with microtubule inhibitors; and (v) prevention of the nuclear translocation of F2RL1 by mutagenesis of its NLS or its C terminus. Therefore, unlike GPCRs that localize to the nucleus in an agonist-independent manner, F2r1L traffics to the nucleus after activation at the cell surface.

We found that translocation of F2r1L to the nucleus required microtubules. The microtubule motor dynein attaches to cargo proteins and powers long-range retrograde journeys to the nucleus; regulatory proteins and secondary messengers use microtubules to reach the cell nucleus. Numerous receptors, including transferrin and nerve growth factor receptor-1 (ref. 52), as well as the GPCRs cannabinoid-1 (ref. 53), somatostatin-2A (ref. 54) and DFrizzled-2 (ref. 21), associate with dynein and microtubules en route to the perinuclear region. Microtubules that are associated with the nuclear envelope allow the transition of proteins from early to late endosomes.

We also found that the two putative nuclear localization domains determine the intracellular destination of F2r1L. NLsS are recognized by importins to promote nuclear transport, which has been described for soluble as well as integral membrane proteins, including GPCRs. Proteins are ushered by importin-α and importin-β from the endoplasmic reticulum through the nuclear pore complex to reach the inner nuclear membrane. Native F2r1L, but not the F2r1L-NLS mutants, was able to recruit importin-β after activation. Truncation of the C terminus of F2r1L also abrogated its nuclear translocation. The C terminus harbors a binding site for β-arrestin-1, which can translocate to the nucleus and mediate gene transcription. However, mutation of the F2r1L docking site for β-arrestin-1 did not prevent nuclear translocation, suggesting that other proteins assemble on the F2r1L C terminus to promote its nuclear translocation.

The sorting nexin Snx1L was also needed for the nuclear translocation of F2r1L. Snx1L was abundantly expressed at the cell nucleus and, together with importin-β and dynein, forms a protein complex that is necessary for the nuclear translocation of F2r1L. The association of Snx4 with dynein to mediate the transport of F2r1L from early endosomes to perinuclear recycling endosomes is a recent example of the key role of Snx in long-range cargo transport. Snx1L and Snx3 are members of a Snx subfamily that lacks a BAR domain, distinguishing them from the prototypical mammalian retromer proteins Snx1 and Snx2. By analogy with Snx3 (ref. 55), we speculate that Snx1L may prevent lysosomal degradation of its endosomal cargo while traveling to the nucleus.

The transcriptional activity of GPCRs in isolated nuclei has been widely reported. Although traditional signaling machinery has been described at the nucleus, nuclear GPCR signaling remains ill defined. We found that F2r1L could form a nuclear transcriptional complex with the transcription factor Sp1, as shown by ChIP, communoprecipitation and DNA binding complex (EMSA) assays. This transcriptional complex in turn bound consensus sites for Sp1 and Sp3 (ref. 38) in the VEGFA promoter region, increasing its expression. All conditions tested that prevent F2r1L nuclear translocation also abolished VEGFA induction while preserving the transcription of plasma membrane F2r1L–regulated Ang1. Distinct angiogenic genes are therefore conditionally transcribed depending on the subcellular localization of F2r1L.

Developmental angiogenesis is finely regulated by cooperative angiogenic signals. Both VEGFA and Ang1 have pivotal functions in growth and maturation during vascular development: the former promotes differentiation, proliferation and migration of the vascular endothelium, whereas the latter recruits pericytes and stabilizes nascent vasculature. Deletion of either gene is embryonically lethal. Conditional suppression of Ang1 between embryonic day (E) 10.5 and E12.5 results in an increased overall number of vessels and disorganized vascular beds. We observed a similar phenotype in mouse retinas overexpressing nuclear F2r1L-E3, which produce relatively less Ang1 while maintaining high levels of VEGFA. Conversely, high levels of Ang1 in the presence of lower levels of VEGFA appeared to stunt retinal vascular growth in F2r1L-NLS mutant–expressing mice. Neuronal F2r1L therefore maintains the equilibrium between retinal vascular growth and maturation. Preventing nuclear translocation of neuronal F2r1L tilts the balance towards early vascular maturation and results in impaired vascular growth, whereas unopposed nuclear F2r1L activity triggers excessive VEGFA-mediated neovascularization. Hence, F2r1L separately governs two major angiogenic cues depending on its subcellular localization, segregating the regulation of complementary growth factors. We provide new evidence for the physiologic role of a nuclear GPCR in vivo that expands our understanding of GPCR signaling governed by subcellular localization. Our findings have implications for the design of more selective drugs based on the subcellular distribution of their targets.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS


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Cortactin activation by FVIIa/tissue factor and PAR2 promotes protease-activated receptor-2 stimulates angiogenesis and nuclear localization of prostaglandin E2 receptors. Proangiogenic effects of protease-activated receptor 2 are tumor Müller cell-derived VEGF is a significant contributor to retinal abnormal blood vessel development and lethality in embryos α.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
ONLINE METHODS

Animals. Mice were used in compliance with the Animal Care Committee of CHU Sainte-Justine and the Canadian Council on Animal Care guidelines. Wild-type (WT) C57BL/6 and F2rl1−/− (stock 004993) mice were obtained from Jackson Laboratory and used at P4, P6 and P10. Pou4f2lacZ-DTA/− (pou4f2) mice (provided by W.K.) contain a floxed-lacZ-stop–diphtheria A toxin cassette expressed selectively in RGCs (Pou4f2 or Brn3b promoter) and were crossed with tamoxifen (Tm)-inducible Cre recombinase mice (CAGG-Cre-ERTm, Jackson Laboratory, stock 004682) to generate temporal and geographical conditional RGC ablation. Pou4f2lacZ-DTA/− (Cre-ERTm) mice were infected with tamoxifen (50 µg intraperitonally) on P1 to selectively ablate RGCs during viral development (P3–P4). Intravitreal injections were performed with vehicle or SLIGRL (mouse F2rl1–AP; Elim Biopharm) at P4 or lentiviruses expressing GFP, GFP-fused F2RL1, F2RL1-ΔNLS, F2RL1-E3 or shRNA at P2. Retinas were collected at P6.

Cells. RGC-5 cells (mouse RGCs; kindly provided by N. Agarwal) were terminally differentiated in the presence of staurosporine (1 µM) for 12 h, which effectively confers a post-mitotic neuronal state. HEK293T and HEK293 cells (American Type Culture Collection) were cultured in DMEM (ATCC) supplemented with 10% FBS, as reported previously.

shRNAs and siRNAs. shRNA sequences against F2rl1, Snx1, Snx2 and Snx11 and control shRNAs were obtained from Open Biosystems, and siRNA sequences against importin-β1 and Sp1 and the scrambled siRNA were obtained from Ambion (Ambion Silencer TM siRNA Construction Kit).

F2RL1 mutant cloning strategies. The cytosolic tail of the adenosine type 5 receptor (A2A) was commercially cloned into a pCDNA3 plasmid (Top Gene Technologies) and subcloned into the BamHI and AgeI sites of the F2RL1–enhanced GFP (eGFP)–N1 vector (kindly provided by N. Bunnett) immediately after the cytosolic tail of F2RL1 and preceding eGFP. The Flag-F2RL1-HA11 vector was also kindly provided by N. Bunnett (Ambion Silencer TM siRNA Construction Kit).

LCM. Eyes were immediately embedded in optimal cutting temperature compound (OCT), frozen in liquid nitrogen and cross-sectioned (16 µm) onto a MembraneSlide 1.0 PEN NF (Zeiss). Sections were stained for isocitrate dehydrogenase (1:50 in 1 mM CaCl2) and dehydrated with 70%, 90% and 100% ethanol washes. Retinal vessels and layers were dissected using LCM with a Zeiss (Observer.Z1) Palm Microbeam LCM microscope system and collected directly into RNA-stabilizing buffer from the RNeasy Micro kit (Qiagen, Chatsworth, CA). RNA was extracted from microdissected tissues using the RNeasy kit (Qiagen), and real-time PCR was performed with the generated cDNA.

Microvascular sprouting from aortic explants. Microvascular sprouting experiments using aortic explants from WT and F2rl1−/− mice were performed as previously described. After infection with LV shScram or shF2rl1, RGCs were stimulated with vehicle or SLIGRL (24 h), and their cell medium (RGC-conditioned medium) was centrifuged and filtered (0.22 µm) to remove cell debris. Aortic rings obtained from WT C57BL/6 or F2rl1−/− aortas were sectioned into 1-mm rings and embedded in Matrigel for 48 h. RGC-conditioned medium or SLIGRL was incubated with the growth factor–reduced Matrigel-embedded aortic rings with nascent vascular sprouts and photographed daily. Vascular sprouting area was assessed using ImageJ.

Snx11-specific antibody production. Polyclonal antibodies to Snx11 were custom generated by Open Biosystems using the epitope CGWQAEQOSTHSVLKDGQ. After immunization, rabbit serum was purified by affinity chromatography.

Immunocytochemistry. Immunocytochemical experiments were performed as described previously with antibodies to F2rl1 (Invitrogen, 35-2300, 1:500) and its rodent cleavage-activation site sequence 30-GENSKGR-LSGLRDTDP-46 (B5 antibody, from M.H., 1:500) and GFP (Molecular Probes, A1122, 1:500), importin-β1 (Santa Cruz, C-19, 1:200), NeuN (Millipore, MAB377, 1:100), Thy1.1 (Millipore, MAB4106, 1:200), β3-intubulin (ECM Biosciences, TP1691, 1:500), and Vegfa (Santa Cruz Biotechnology, sc-152, 1:400) and with TRITC-labeled secondary antibodies (1:500). Negative controls were exposed to secondary antibodies. Sections were assessed by epifluorescence and confocal microscopy.

Immunofluorescence and confocal microscopy. F2RL1–GFP-expressing HEK293 cells were maintained at 37 °C, 0.5% CO2 for live imaging with a white laser Leica SXX5 multispectral-multiphoton confocal system. Nuclei of live cells were stained with Hoechst (2 µg ml−1) shortly before stimulation with SLIGKV
(20 μM). Other cells were fixed, permeabilized in the presence of taxol (5 μM) and blocked with the appropriate serum before incubation with antibodies; their nuclei were stained with DAPI. Confocal images of fixed cells were obtained using a Zeiss LSM 510 microscope.

Electron microscopy. Specimens for electron microscopy were prepared as described previously. A pre-embedding immunogold protocol was applied on the retinas of WT C57BL6 or F2rli−/− mice fixed by vascular perfusion with a mixture of 4% paraformaldehyde, 15% picric acid (v/v) and 0.1% glutaraldehyde in 0.1 M PB. For ultrastructural immunocytochemistry, tissues and cells were incubated with mouse monoclonal antibodies to F2rli (Invitrogen, 35-2300, 1:600) and subsequently with a goat anti-mouse IgG Fab fragment conjugated to 1.4-nm gold particles (1:200, Nanoprobes, 2002); tissue was then processed for silver enhancement using a Nanoprosys HQ Silver #2012 kit. Negative controls were performed on tissue sections from F2rli−/− retinas using conditions identical to those used for WT mice and by omitting the primary antibodies for experiments using cells. Specimens were examined with a transmission electron microscope (Philips CM120) equipped with a Gatan digital camera.

Scanning electron microscopy. The purity and integrity of HEK293 nuclear fractions were confirmed by high-resolution scanning electron microscopy, as reported previously.

RT-PCR. RT-PCR was performed on HEK293 cells, RGCs and dissected retinas. mRNA was extracted with TRIzol (Invitrogen), and synthesized cDNA was amplified using Taq DNA polymerase (Invitrogen, Molecular Probes). Primers were designed (Alpha DNA) for the sequences described in Supplementary Table 2. Results were normalized against cyclophilin A or 18S mRNA levels (QuantumRNA 18S universal Standard, Ambion).

Isolation of subcellular fractions and purified nuclei. Subcellular fractions and nuclei were isolated from RGCs and F2RL1-GFP– or F2Rli−/−-expressing HEK293 cells, stimulated with vehicle or agonist peptide (20 μM, 30 min) and treated with cycloheximide (50 mM) to arrest protein synthesis 2 h before stimulation. Confluent RGCs and stably transfected HEK293 cells (two 15-cm plates) were rinsed with PBS and harvested in 10 ml ice-cold homogenizing buffer (HB; 0.25 M sucrose, 25 mM KCl, 5 mM MgCl2 and 20 mM Tris-HCl, pH 7.4). Pelleted cells were resuspended in HB (0.8 ml, 4 °C) containing protease inhibitor cocktail (Complete, EDTA-free, Roche), homogenized with a 26.5-gauge needle (15 strokes) and centrifuged (700g, 10 min, 4 °C). The pellet contained the nuclear fraction, and the supernatant was used to isolate other subcellular fractions.

To isolate subcellular fractions, a discontinuous OptiPrep density gradient (Sigma-Aldrich, D1556) was prepared containing 2 ml of 2.5%, 3.5 ml of 10%, 3.5 ml of 20% and 3.5 ml of 30% OptiPrep solution. Non-nuclear lysate (0.9 ml) was mixed with 50% OptiPrep (2.1 ml), yielding a 35% OptiPrep solution loaded on the OptiPrep gradient. After ultracentrifugation (130,000g, 4 °C, 22 h), the OptiPrep gradient was separated into 13 fractions of decreasing density.

Similarly, the nuclear fraction (pellet) was resuspended in HB (1 ml, containing protease inhibitor cocktail) with OptiPrep (1 ml, 50%) to obtain a 25% OptiPrep solution that was loaded on a new Opti-grad tube (8 ml of 30% and 5.5 ml of 35% OptiPrep solution). After centrifugation (20,000g, 4 °C, 1 h), the OptiPrep gradient was separated into 13 fractions of decreasing density.

Corresponding fractions were then separated by SDS-PAGE and identified by immunoblot analysis of known plasma membrane (clathrin), early (EEA1) and late endosomes (Rab9A) or nuclear (lamin A/C) markers. Respective fractions were pooled to characterize the subcellular expression of F2rli after stimulation.

Flow cytometry. HEK293 cells stably transfected with F2RL1, Flag-F2RL1 and F2RL1 mutants (fused to GFP) were treated with SLIGKV (20 μM) for 30 min before nuclear isolation. HEK293 cells transfected with Flag-F2RL1 were first incubated with FITC-tagged antibody to Flag (1:200, Santa Cruz Biotechnology; sc-7787) for 15 min in DMEM (37 °C) before stimulation. The nuclear preparations were processed by flow cytometry using FACS Calibur, and the results were analyzed by CellQuest software (BD Biosciences).

Coimmunoprecipitation experiments and western blot analyses. HEK293 cells expressing GFP-tagged F2RL1, F2RL1-GFP mutants and eGFP-N1 (control; Clontech Laboratories) were lysed and immunoprecipitated (200 μg) using the magnetic GFP-Trap-M kit (Chromotek). Traditional coimmunoprecipitations were also performed using antibodies (2 μg) to F2rl1, Snx1, importin-β1, dynin (Abcam, ab6304), Sp1 (Cell Signaling, 5931) and IgG (R&D Systems, MAB0041, control) from soluble lysates of F2RL1-GFP– or SNX11-GFP–expressing HEK293 cells treated with SLIGKV (20 μM, 30 min). Immune complexes were immobilized by adding 50 μl of protein A (Sigma), washing three times with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ethylen glycol tetraacetic acid, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO3 and 1 μM ml−1 leupeptin) and incubating with 30 μl of Laemmli SDS-sample buffer (Boston BioProducts, BP-110R). Western blot analyses for F2rl1, Snxl1, Snx2, Snx11, importin-β, α-tubulin, dynin and Sp1 were performed as described. Total lysates represented 25% of the input (50 μg). Specific proteins were revealed with chemiluminescent reagent (PerkinElmer Life Sciences) and signal was exposed on X-ray films.

Measurement of Ca2+ signals. Cellular Ca2+ signals were measured using Fura-2 AM (Calbiochem) as described previously. Cells were washed with prewarmed HBSS and calcium buffer (2 ml), incubated with Fura-2 AM (4 μl, 1 h at 37 °C) and resuspended in HBSS and calcium buffer. Cell calcium signals were measured by spectrophotometry (LS50, PerkinElmer Life Sciences). The calcium concentrations were calculated according to the methods of Grynkiewicz et al.

ChiP and short-read sequencing. F2RL1-GFP–expressing HEK293 cells stimulated with SLIGKV (20 μM, 30 min) were fixed with formaldehyde (0.75%, 10 min) and quenched with glycine (125 mM) before nuclear isolation. Nuclei were sonicated using a S220 Covaris sonicator (3 min; Covaris), yielding fragment sizes of 300–350 bp. After centrifugation (14,000 r.p.m., 5 min), ChiP was performed on the supernatant that was then incubated overnight (4 °C) with F2rl1 antibodies (5 μg) together with protein A/G agarose beads (Sigma, P3296 and P9424) or beads alone; the remaining nuclear sample served as the input control. Isolated protein–DNA complexes were washed and digested with 6 ng/ml RNaseA (Fermentas, EN0531) and 0.01 μg/ml protease K (Fermentas, EO0491) (4 h, 65 °C). Precipitated ChiP DNA was isolated using a kit (GFX PCR DNA and Gel Band purification kit, Qiagen) and bioanalyzed with an Agilent DNA 1000 kit (Agilent Technologies). The ChiP DNA concentrations ranged from 20 to 40 ng/ml.

Short-read sequencing of ChiP DNA fragments (150 ng) was processed using the Solexa/Illumina Genome Analyzer II platform (McGill University and Genome Quebec Innovation Center). Short reads were mapped to a human reference genome (Feb. 2009, GRCh37/hg19) using the ELAND module of the Illumina Genome Analyzer Hx. Sequences that could not be mapped unambiguously or that mapped to simple or complex repeats were removed from the analysis. Results were converted to BED files (using the ChiP-Seq mini 2.0.1 suite) for processing and visualization in the UCSC Genome Browser (https://genome.ucsc.edu/). MACS 1.4.2 rc2 (ref. 77) settings were used for peak detection, and the output was filtered to remove peaks overlapping repeat and low-complexity regions by more than 50%, as well as peaks distant from potential transcriptional start sites (−10 kb to +500 bp). We used stringent filtering of overlapping Sp1 sites with a motif matrix corresponding to a CCCC[GT]CCCC sequence for Sp1. Key regulators were identified in the functional screen with motif matrices provided by PATCH 1.0, which uses TRANSFAC 6.01 to search for transcription factor patterns. The raw sequence data is publically available in GEO (accession code GSE58660).

To explore whether F2rli and Sp1 form a transcriptional complex in stimulated RGC nuclei (SLIGKV, 20 μM, 30 min), F2rl1 ChiP was followed by re-ChiP with Sp1-specific antibodies as described. Isolated protein–DNA complexes eluted from F2rli-ChiP were incubated with Sp1 antibodies (2 μg) and A/G agarose beads in re-ChiP buffer (25 μl, 37 °C, 30 min; 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1, and 10 mM dithiothreitol). After washing the agarose beads three times, re-ChiP DNA was eluted (50 μl; 1% SDS and 0.1 M NaHCO3) and quantified (Nanodrop 1000, Thermo Scientific).
Priming that encompass the peak discovered by ChIP-Seq were designed, and 1 ng of re-ChIP DNA was used to confirm enrichment of that peak by qRT-PCR.

**EMSA.** Crude nuclear protein preparations and EMSA were performed as described previously. Briefly, EMSA was carried out by incubating 3 × 10^6 counts per minute of 5′ end-labeled (32P) double-stranded oligonucleotides bearing high-affinity binding sites for the transcription factor Sp1 (5′-GATCATATCTGGGGGCGGAGACACAG-3′) with SLIGKV-stimulated (20 μM, 30 min) F2RL1-GFP–expressing HEK293 nuclear lysates. Supershift experiments were conducted by adding Sp1- and F2RL1-antibodies (400 ng) to the reaction mixtures.

**Luciferase assay.** HEK293 F2RL1-GFP–expressing cells were transfected with empty PGL3 vector (Promega), with a PGL3 vector containing a DNA fragment obtained by ChIP (Supplementary Fig. 11a), or with pCDNA3 LacZ (Invitrogen), with or without transfection with siRNA (0.6 μg) targeting Sp1 or a scrambled sequence. Each plate was transfected with Lipofectamine (Invitrogen) with 1 μg of PGL3 and pCDNA3 LacZ plasmids. A dual-luciferase reporter assay system (Promega) was used to monitor luciferase activity using a Sirius single-tube luminometer (Berthold). The pCDNA3 LacZ vector was co-transfected to monitor the transfection efficiency. All luciferase results are reported as relative light units (RLUs), representing the average Photinus pyralis firefly luminosity divided by the average β-galactosidase activity.

**Proliferation assay by [3H]thymidine incorporation.** F2RL1-GFP–expressing HEK293 cells (4 × 10^4 cells per well) in 24-well plates were transfected with scrambled or Sp1-targeting siRNA and stimulated with SLIGKV (20 μM) or vehicle for 12 h. [3H]Thymidine (1 μCi ml⁻¹) was then incubated with the cells (18 h). Lysed cells were transferred into a scintillation flask and analyzed by a liquid scintillation counter. The data represent the c.p.m. of [3H]thymidine incorporation normalized to the untreated control.

**Statistical analyses.** Data are presented as the means ± s.e.m. Two-tailed independent Student's t tests were used when comparing two groups, using Welch's correction where appropriate to account for significantly different variances. Comparisons between multiple groups were made using one-way analysis of variance followed by post-hoc Dunnett's or Tukey's multiple comparisons test among means. Gender did not affect our retinal phenotype and was therefore not considered. Values more than two standard deviations from the mean were considered outliers and were excluded. P < 0.05 was considered as statistically significant.