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Molecular dissection of cone photoreceptor-enriched genes encoding transmembrane and secretory proteins

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Abstract

Cone photoreceptors mediate color perception and daylight vision through intricate synaptic circuitry. In most mammalian retina, cones are greatly outnumbered by rods and exhibit interdependence for functional maintenance and survival. Currently, we have limited understanding of cone-specific molecular components that mediate response to extrinsic signaling factors or are involved in communication with rods and other retinal cells. To fulfill this gap, we compared the recently-published transcriptomes of developing S-cone-like photoreceptors from the *Nrl*^{-/-} mouse retina with those of rods and identified candidate genes responsible for cone cell functions and communication. We generated an *in silico* expression profile of 823 genes that encode candidate transmembrane and secretory proteins and are up-regulated in *Nrl*^{-/-} cone photoreceptors compared to wild type cones. *In situ* hybridization analysis validated high expression of seven of the selected candidate genes in the *Nrl*^{-/-} retina. To examine their relevance to cone function, we performed *in vivo* knockdown of *Epha10* in the *Nrl*^{-/-} retina and demonstrated aberrant morphology and mislocalization of the photoreceptor cell bodies. Thus, the receptor tyrosine kinase Ephrin type-A receptor 10 appears to influence cone morphogenesis. Our studies reveal novel cone-enriched genes involved in interaction of cones with other retinal cell types and provide a framework for examining molecular pathways associated with intercellular communication.

Graphical abstract

Inter-cellular interactions are critical for morphogenesis and homeostasis. Papal et al. mined transcriptome data to identify cone-enriched genes that encode transmembrane and secretory proteins. Knockdown of *Epha10* resulted in aberrant cone phenotype and validated our approach. These studies provide the framework for dissecting molecular components underlying cone function and survival.

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Author Contributions

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Conflicts of Interest Statement

The authors have declared no conflict of interest.

Keywords

Cell communication; Signaling pathway; Retina; Transcriptome; Gene expression

Introduction

Communications among cells and with the extracellular milieu are essential for development and homeostasis. Mechanisms implicated in cellular communications involve direct interactions through cell-cell contact and/or via the release of soluble factors (Gerdes & Pepperkok, 2013). Adhesion proteins are the molecular components associated with direct cell contacts. One such group of proteins are connexins that form gap junctions connecting cellular cytoplasm and allowing direct transmission of ions and small molecules (Dbouk, Mroue, El-Sabban, & Talhouk, 2009; Evans & Martin, 2002; Herve & Derangeon, 2013). Other cell adhesion proteins include cadherins that can form tight junctions, integrins which bind extracellular matrix components, and proteins of the immunoglobulin superfamily implicated in fine tuning of cell adhesion (Byron, Morgan, & Humphries, 2010; Gumbiner, 1996; Maitre & Heisenberg, 2013). Indirect communication relies on the secretion of extracellular vesicles (e.g., exosomes) or soluble factors that can act over short or long distances and bind to cell surface receptors (Bang & Thum, 2012; Hwang, 2013), which include ligand-gated ion channels, receptor tyrosine kinases, and G-protein coupled receptors. Interactions between adhesion molecules located on adjacent cells and binding of ligands to their respective receptors can trigger intracellular signals that influence multiple physiological processes and the loss of cell-cell communications can lead to a wide range of diseases in humans (Gnanapavan & Giovannoni, 2013; Uyemura, Takeda, Asou, & Hayasaka, 1994; Wechsler-Reya & Barres, 1997; Wei & Huang, 2013).

The retina is the photosensitive structure at the back of the eye that converts photons into neuronal signals which are then perceived as images in the brain. Five different classes of neurons and one type of glia are organized in the retina into three nuclear and two plexiform layers, which contain the cell bodies and the synapses of retinal cells, respectively. Photoreceptors are located in the outer nuclear layer (ONL) and mediate the first step in the visual transduction process by capturing light. Rod photoreceptors are highly sensitive and implicated in night vision, whereas cone photoreceptors are responsible for daylight vision

and are essential for high acuity and color perception (Sung & Chuang, 2010). Although rods dominate the retina of most mammals, including mice and humans, constituting over 95% of all photoreceptors; cones are more abundant in the central region (macula) of primates and are critical for human vision.

During retinal development, the differentiation of retinal progenitors into the appropriate cell types and the establishment of the visual circuitry are a multistep process that requires intrinsic and extrinsic signals (Livesey & Cepko, 2001; Reese, 2011). Moreover, cells are interconnected and in constant communication within the adult retina. Cone photoreceptors establish connections with different subtypes of cone bipolar neurons and horizontal cells for transmission and integration of visual signals (Hoon, Okawa, Della Santina, & Wong, 2014; Missaire & Hindges, 2015; Seung & Sumbul, 2014). Cones are tightly packed within the ONL and electrically coupled to rods via gap junctions, allowing the transmission of scotopic signals through the photopic pathway in low-light conditions (Asteriti, Gargini, & Cangiano, 2017; O'Brien, Chen, Macleish, O'Brien, & Massey, 2012; Tsukamoto, Morigiwa, Ueda, & Sterling, 2001). Importantly, secondary death of cone photoreceptors in retinitis pigmentosa is, in part, correlated to the loss of Rod Derived Cone Viability factor (Rdcvf), which is secreted by rod photoreceptors and protects cone cells (Ait-Ali et al., 2015; Leveillard et al., 2004).

Here, we have taken advantage of the cone-only retina of mice deficient in the neural retina leucine zipper NRL (*Nrl*^{-/-} mice) (Mears et al., 2001) to identify genes for transmembrane and secretory proteins. Instead of rods, photoreceptors in the *Nrl*^{-/-} retina acquire molecular and functional characteristics of S-cones (Akimoto et al., 2006; Daniele et al., 2005; Nikonov et al., 2005; Yoshida et al., 2004). By comparing the transcriptomes of *Nrl*^{-/-} S-cone-like photoreceptors (SCLs) and wildtype (WT) rods (Kim et al., 2016), we identified cone-enriched genes encoding plasma membrane and secretory proteins and performed *in situ* hybridization studies for seven of them. *In vivo* knockdown of one of the genes, Ephrin type-A receptor 10 (*Epha10*), by electroporation of shRNA in newborn mouse retina, provided strong evidence in support of its physiological relevance and validated our strategy for investigating molecular components involved in intercellular interactions.

Methods and Materials

Animals

All animal procedures were approved by the Animal Care and Use Committee of the National Eye Institutes (Animal study protocol 650). Mice were housed in an atmosphere-controlled environment (temperature: 22°C +/- 2°C, humidity: 30-70%), under a 12 hours dark/12 hours light cycle and supplied with food and water *ad libitum*. Food, water and nesting material were changed weekly. The absence of the *rd8* mutation in the colony was assessed by PCR. *Nrl*^{-/-} mice have been described previously (Mears et al., 2001; Akimoto et al., 2006) and were backcrossed onto C57BL/6J more than 10 times. All mice were maintained in the National Institutes of Health (NIH) animal care facilities.

***In situ* hybridization**

C57BL/6J wild type and *Nrl*^{-/-} retina of either sex were collected at postnatal day (P) 28 after euthanasia using CO₂ atmosphere. A hole was made in the center of the cornea using a 26-gauge needle, and eyes were placed in 4% paraformaldehyde (PFA) for 15 min. The cornea was then dissected and the eye cups incubated in 4% PFA for 1 hour at room temperature before cryoprotection in 20% and 30% sucrose-phosphate-buffered saline (PBS) at 4°C for 1 hour and overnight, respectively. Eye cups were then quickly frozen in Shandon™ M-1 Embedding Matrix (Thermo Fisher Scientific) and sectioned at 12µm.

In situ hybridization of sections was performed using RNAscope Multiplex Fluorescent Assay (Advanced Cell Diagnostics), according to the manufacturer's instructions with minor modification. Briefly, slides were post-fixed for 30 min in 4% PFA at room temperature, washed in PBS, incubated in Target Retrieval solution at 100°C for 5 minutes and then incubated in Protease III at 40°C for 30 minutes. RNAscope probes were hybridized for 2 hours at 40°C, followed by amplification steps. Staining was visualized using a LSM 880 confocal scanning microscope (Zeiss). C57BL/6J (n=2; retina from 2 different mice), *Nrl*^{-/-} (n=2; retina from 2 different mice). Detailed information of the probes used in this study is provided in table 1.

Immunostaining

For immunostaining, *Nrl*^{-/-} and CD1 wildtype mice of both sexes were recovered at P28 after euthanasia using CO₂ atmosphere. Eyecups were processed as described previously. 40µm-thick retinal cryosections were blocked in PBS containing 5% Donkey serum and 0.3% Triton X-100 (PBST) for 1 hour at room temperature and then incubated overnight at 4°C with primary antibody diluted in PBST. Sections were then washed 3 times with PBS and incubated with secondary antibody and 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 1 hour at room temperature. After 3 washes in PBS, the sections were mounted in Fluoromount-G (SouthernBiotech). Images were captured using a LSM 880 confocal scanning microscope (Zeiss). For electroporated *Nrl*^{-/-} mice, the groups were as follows: control (9 retina, 5 independent experiments), shRNA Epha10_1 (7 retina, 3 independent experiments), shRNA Epha10_2 (5 retina, 3 independent experiments). For CD1 retina, the groups were as follows: control (4 retina, 2 independent experiments), shRNA Epha10_1 (4 retina, 2 independent experiments), shRNA Epha10_2 (4 retina, 2 independent experiments).

Antibodies

Detailed information of primary antibodies used in this study is provided in Table 2. Green fluorescent protein (GFP) staining was enhanced using polyclonal rabbit anti-GFP antibody from Torrey Pines Biolabs (1:1000) or chicken anti-GFP antibody from Abcam (1:1000). A previously characterized (Sun et al., 2010) polyclonal chicken anti-blue cone opsin antibody (a gift from Dr. Tiansen Li, N-NRL, National Eye Institute, Bethesda, MD 20892) was used (1:1000) to label S-cone photoreceptors. M- and S-cone photoreceptors were labeled using rabbit polyclonal antibody anti-cone arrestin (1:100) from Millipore.

Sub-retinal injection

Sub-retinal injections followed by electroporation were performed as described (Matsuda et al., 2004). Briefly, postnatal day (P)1 *Nrl*^{-/-} or CD1 mouse pups of either sex were anesthetized on ice and their eyelids were opened using a 30-gauge needle. An incision was made into the sclera at the nasal part of the retina, and 0.4µl of shRNA construct or control vector was injected using a Hamilton syringe. We injected a final concentration of 4µg/µl plasmid in PBS containing 0.5µg/µl of pUB-GFP vector as a fluorescent reporter and 0.1% fast green to monitor the injection process. Retinas were recovered at P21 for immunostaining. Sex of the animals was not assessed upon recovery.

Small hairpin RNA (shRNA) constructs

Detailed information for shRNAs is provided in Table 3. Construct Epha10_1 was obtained from Sigma-Aldrich. Construct Epha10_2 target sequence was designed using siRNA Wizard v3.1 (InvivoGen, <http://www.invivogen.com/sirnazard/siRNA.php>). The target sequence was subcloned into a pLKO1-TRC vector (Addgene, <http://www.addgene.org>, RRID:SCR_002037) between EcoRI and NcoI restriction sites.

Gene selection

Transcriptomes of *Nrl:GFP;Nrl*^{+/+} and *Nrl:GFP;Nrl*^{-/-} flow-sorted photoreceptors (Kim et al., 2016) (GEO accession # GSE74660). Selection of transmembrane and secreted genes was performed by Ensembl BioMart (Kinsella et al., 2011) (<http://www.ensembl.org>, RRID:SCR_002344), using Ensembl genes 91 database and the GRCm38.p5 genome.

Quantification of electroporated cells

GFP positive (GFP+) cells within the outer nuclear layer (ONL) were quantified unblinded using maximum intensity projections of z-stack images by ImageJ v1.46 (<https://imagej.nih.gov/ij>, RRID:SCR_003070). The regions too close to the injection site were not used for quantification. We used the single point tool and ROI Manager to identify the location of GFP+ cell bodies. First, the outer and inner boundaries of the ONL were assigned based on DAPI staining. The position of a GFP+ cell body was then determined by measuring the distances between the approximate center of the cell body and the outer (d1) and inner (d2) edges. To account for its curvature, the retina was divided into various relatively linear portions and the position of the GFP+ cells was determined for edges of each portion. A cell was assigned to the outer portion of the ONL (OONL) when d1 was less than d2; otherwise, it was assigned to the inner portion of the outer nuclear layer (IONL). Finally, the number of GFP+ cells in the OONL and the IONL were counted, respectively. Total GFP+ cells were also determined in all layers for each image. For *Nrl*^{-/-} injected mice, the groups were as follows: control (n=8 retina, 5 independent experiments, 708 GFP+ cells); Epha10_1 (n= 7 retina, 3 independent experiments, 467 GFP+ cells), Epha10_2 (n=5 retina, 3 independent experiments, 426 GFP+ cells). For CD1 injected retina, the groups were as follows: control (n=4 retina, 2 independent experiments, 481 GFP+ cells), shRNA Epha10_1 (4 retina, 2 independent experiments, 304 GFP+ cells), shRNA Epha10_2 (n=4 retina, 2 independent experiments, 285 GFP+ cells).

Distribution of cone photoreceptors in CD1 electroporated retina

Cone photoreceptors distribution (as indicated by cone arrestin positive cells) within the OONL and IONL was determined unblinded, as described previously for GFP+ cells. The regions too close to the injection site were not used for quantification. Control (4 retina, 2 independent experiments, 169 cone arrestin + cells), shRNA Epha10_1 (4 retina, 2 independent experiments, 183 cone arrestin + cells), shRNA Epha10_2 (n=4 retina, 2 independent experiments, 196 cone arrestin + cells).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, USA, RRID:SCR_002798). Data distribution was assessed using Shapiro-Wilk test and confirmed by computing a QQ-plot. Normally-distributed data were analyzed using one-way ANOVA. To compare the proportion of GFP+ cone cells in the OONL and IONL between control and shRNA electroporated retina, we used a one-way AVOVA followed by Bonferroni post-hoc test. To compare the proportion of electroporated cone photoreceptors among all GFP+ cells between control and shRNA injected retina, the data were analyzed using a one-way AVOVA followed by Bonferroni post-hoc test. Finally, to compare the proportion of cone arrestin+ photoreceptors present in the OONL and IONL between control and shRNA CD1 electroporated retina, we also used a one-way AVOVA followed by Bonferroni post-hoc test. A p-value <0.05 was considered significant (depicted by * in the figures).

Results

Identification of genes overexpressed in S-cone-like photoreceptors of *Nrl*^{-/-} retina

Taking advantage of the published transcriptomes of *Nrl-GFP:Nrl*^{+/+} rods and *Nrl-GFP:Nrl*^{-/-} SCLs (Kim et al., 2016) at distinct stages of photoreceptor maturation (Postnatal day (P)6, P10, P14, P28), we identified 2662 differentially-expressed genes with higher expression (fold change ≥ 2, false discovery rate ≤ 0.01) in at least one time point in SCLs (Figure 1a). We further filtered for genes encoding plasma membrane or secreted proteins applying the Gene Ontology (GO) term annotations plasma membrane (GO: 0005886) and extracellular region (GO:0005576) and selected genes with human homologs (Figures 1a and b). This analysis yielded 823 genes showing higher expression in SCLs compared to rods (Figure 1c). We finally selected seven genes for further investigation based on 2 additional criteria: (i) little or no study in the retina, and (ii) suggested or demonstrated functions in other (non-retina) tissues (Figure 2).

Validation of expression of selected genes encoding transmembrane proteins

Four of the selected genes – Protogenin (*Prtg*), Immunoglobulin superfamily member 11 (*Igsf11*), Leucine rich repeat and fibronectin type III domain containing 2 (*Lrtn2*), and Ephrin type-A receptor 10 (*Epha10*) – are predicted to encode transmembrane proteins (Figure 2a). We analyzed their expression during WT retinal development, from embryonic day (E)11 to P28. *Prtg* expression decreased dramatically between E11 and P28 whereas *Igsf11*, *Lrtn2* and *Epha10* demonstrated higher expression as development proceeded

(Figure 3a). *Igsf11* and *Epha10* transcripts were expressed early in retinal development starting at E14, in contrast to *Lrfr2*, whose expression level remained minimal until P4. Our photoreceptor transcriptome analysis indicated that all four genes were upregulated in SCLs while their expression either decreased (*Prtg* and *Igsf11*) or remained relatively low (*Lrfr2* and *Epha10*) in rod cells (Figure 3a). In order to determine the expression patterns of these genes, we performed *in situ* hybridization analysis. All tested genes showed enrichment of transcripts in the ONL of adult (P28) *Nrl*^{-/-} retina compared to the WT retina (Figure 3b). Interestingly, *Prtg*, *Lrfr2* and *Epha10* were enriched in cells located in the outermost part of the ONL, where cone nuclei are generally located. *Igsf11*, *Lrfr2* and *Epha10* expression was also detected in other retinal cells (Figure 3b). Our results validate distinct expression of *Prtg*, *Lrfr2* and *Epha10* in cone versus rod photoreceptors and an enrichment of *Igsf11* transcripts in *Nrl*^{-/-} retina compared to the WT retina.

Developmental expression of *Vasn*

We analyzed the expression of Vasorin (*Vasn*) that encodes for a transmembrane protein whose extracellular domain can be released after cleavage, producing an active soluble protein (Figure 2b). RNA-seq data showed that *Vasn* is stably expressed during early retinal development and upregulated at P4 (Figure 4a). Notably, *Vasn* is expressed in both rods and SCLs until P6, but from P6 to P28, its expression is decreased in rods and increased in cone cells (Figure 4a). *In situ* hybridization of retinal sections confirmed the enrichment of *Vasn* in the ONL of *Nrl*^{-/-} retina, although transcripts were also detected in the ONL of WT retina and in other cell layers, indicating that this gene does not exhibit photoreceptor-specific expression (Figure 4b).

Developmental expression of genes encoding secretory proteins

We then examined the expression of Tuftelin1 (*Tuft1*) and *1190002N15Rik*, two genes encoding secretory proteins (Figure 2c). The *1190002N15Rik* transcripts decreased in the retina (from E12 to P6) and the purified rod photoreceptors (from P2 to P10), but progressively increased in SCLs between P6 and P28 (Figure 5a). We observed a dramatic increase in *Tuft1* expression from P14 to P28 in WT retina. *Tuft1* is downregulated during maturation of rods but exhibits higher expression in SCLs between P4 and P28 (Figure 5a). *In situ* hybridization studies validate the enrichment of both genes in the ONL of *Nrl*^{-/-} retina (Figure 5b).

In vivo knockdown of *Epha10* in the *Nrl*^{-/-} retina

To gain insights into the physiological function of Ephrin type-A receptor 10 in the retina, we knocked down the expression of *Epha10* by electroporation of two distinct shRNAs (*Epha10_1* and *Epha10_2*) at P1 in *Nrl*^{-/-} retina (Figure 6). Retina was examined 20 days after injection (at P21). A GFP expression plasmid (pUB-GFP) was co-injected to identify electroporated cells. We examined the localization of cell bodies of photoreceptors electroporated with a control vector (n= 8 retinas, 4 independent experiments) and with two different shRNA constructs (n= 7 retinas, 3 independent experiments for the first shRNA; n= 5 retinas, 3 independent experiments for the second). We consistently detected mislocalization of the electroporated photoreceptor cell bodies in *Epha10*-knockdown retina compared to those injected with an empty control vector (Figures 6a and b). Cell bodies of

the GFP positive cells (GFP+) in Epha10_1 shRNA retina were mainly positioned in the innermost part of the ONL (IONL), whereas the GFP+ cell bodies in the control injected retina were spanning the whole outer nuclear layer. These observations were confirmed using the second shRNA, Epha10_2. The results of statistical analysis performed using one-way ANOVA are: IONL groups (Control: M=0.464, SD=0.1168; Epha10_1: M=0.6509, SD=0.0751; Epha10_2: M=0.7691, SD=0.06; $F_{2,17}=18.25$, $p<0.0001$), followed by a Bonferroni post hoc test (Epha10_1: $p=0.0022$, 95% CI [-0.304, -0.070]; Epha10_2: $p<0.0001$, 95% CI [-0.434, -0.176]). Sex of the animals was not distinguished. In conclusion, knockdown of *Epha10* in *Nrt*^{-/-} retina affects the positioning of electroporated photoreceptor cell bodies (Figure 6c). In addition to cell body mislocalization, a few of the Epha10_1 transfected cells also reveal an aberrant inner segment morphology (Figure 6b, arrowheads).

In situ hybridization results demonstrate that Epha10 is also expressed in the inner nuclear layer (see Figure 3b). In order to determine whether the knockdown of *Epha10* affected retinal development, we quantified the percentage of electroporated cone cells among all GFP+ cells. The results of statistical analysis using one-way ANOVA are: groups (Control: M=0.7468, SD=0.1377; Epha10_1: M=0.787, SD=0.1055; Epha10_2: M=0.6922, SD=0.1344; $F_{2,17}=0.8206$, $p=0.4569$), followed by Bonferroni post hoc test (Epha10_1: $p>0.9999$, 95% CI [-0.201, 0.121]; Epha10_2: $p=0.9173$, 95% CI [-0.123, 0.232]). We didn't observe any difference between the percentage of GFP+ photoreceptors of *Nrt*^{-/-} control- and shRNA-injected retina (Figure 6d).

***In vivo* knockdown of *Epha10* in the CD1 wildtype retina**

Finally, we predicted that the cone mislocalization in the *Nrt*^{-/-} retina was solely due to the knockdown of *Epha10*. To test this hypothesis, we knocked down the expression of *Epha10* in P1 wildtype CD1 retina where rods, being the dominant photoreceptor type, will be primarily electroporated. We then examined the localization of GFP+ rod electroporated and cone photoreceptors cell bodies in these retina 20 days post-injection (Figure 7a and 7b). Statistical analysis results using one-way ANOVA are: IONL groups (Control: M=0.4291, SD=0.0775; Epha10_1: M=0.4303, SD=0.0699; Epha10_2: M=0.4958, SD=0.0982; $F_{2,9}=0.8521$, $p=0.4582$), followed by a Bonferroni post hoc test (Epha10_1: $p>0.999$, 95% CI [-0.158, 0.156], Epha10_2: $p=0.5670$, 95% CI [-0.224, 0.090]). We didn't observe any significant difference in the localization of rod photoreceptor cell bodies electroporated with the empty control vector and the two shRNA constructs (Figure 7c). Moreover, the localization of cone photoreceptor cell bodies as indicated by cone arrestin labelling was similar between control and shRNA electroporated retina with the vast majority of cone photoreceptor cell bodies located in the OONL (Figure 7d). Statistical analysis was performed using one-way ANOVA: [OONL groups (Control: M=0.8966, SD=0.1257; Epha10_1: M=0.8459, SD=0.1394; Epha10_2: M=0.8105, SD=0.0521; $F_{2,9}=0.5922$, $p=0.5733$), followed by a Bonferroni post hoc test (Epha10_1: $p>0.999$, 95% CI [-0.163, 0.264], Epha10_2: $p=0.6142$, 95% CI [-0.127, 0.300]).

Discussion

Despite their low abundance, cone photoreceptors make a disproportionately critical contribution to human vision, and cone degeneration is a major cause of untreatable blindness. However, organization of cones within the remarkable laminated architecture of the retina and their functional interactions with other cell types have not been adequately explored at the molecular level. We are beginning to delineate the molecular complexities associated with the coupling of cones with rods through gap junctions and cone synaptic organization (Sarria et al., 2018; Ueno et al., 2018), however, we have limited understanding of the mechanisms associated with cone interactions. As a prelude to identify components associated with cone interactions and communication, we took advantage of the *Nrt^{-/-}* cone-only mouse model to screen for genes enriched in SCLs. Although, the retinal architecture in this mouse model is disrupted by the presence of rosettes; SCLs are almost identical to WT S-cones (Daniele et al., 2005; Nikonov et al., 2005), their molecular and functional similarity has permitted us to elucidate cone development and function. Here, we have identified several genes that are predicted to encode transmembrane or secreted proteins and may potentially participate in cone morphogenesis, communications and functions. We present detailed investigations of expression for seven of these genes – *Prig*, *Igsf11*, *Lrfrn2*, *Epha10*, *Vasn*, *119002N15Rik* and *Tuft1*.

Retinal photoreceptors utilize oxygen very efficiently (Kooragayala et al., 2015) and are exquisitely sensitive to oxygen levels. Interestingly, while *Nrt^{-/-}* retina exhibits signs of oxidative stress (Daniele et al., 2005; Roger et al., 2012), three of the genes we analyzed can be induced by hypoxia: *Vasn* (Choksi et al., 2011), *119002N15Rik* (Huang et al., 2014) and *Tuft1* (Saarikoski, Rivera, & Hankinson, 2002). However, the expression of the hypoxia-induced transcription factor (*Hif1a*) is reduced in *Nrt^{-/-}* retina, suggesting that the up-regulation of these genes in SCLs is not likely due to hypoxia. Vasorin (encoded by *Vasn*) is present both in the plasma membrane and mitochondria and modulates TGF β signaling and cell survival (Choksi et al., 2011; Ikeda et al., 2004). The human homolog of *119002N15Rik*, called Hypoxia and Akt-induced stem cell factor (*Hasf*), is reported to have a cytoprotective function (Huang et al., 2014). Tuftelin 1 (*Tuft1*) is induced by Nerve Growth Factor (NGF) and is associated with neurite outgrowth (Leiser et al., 2011). Therefore, we propose that these three genes may be involved in cone photoreceptor homeostasis and survival. Additional studies will be necessary to test this hypothesis.

Cone photoreceptors form complex synaptic contacts with multiple cone bipolar and horizontal cells. Immunoglobulin superfamily member 11 (*Igsf11*), leucine rich repeat and fibronectin type III domain containing 2 (*Lrfrn2*) and Ephrin type-A receptor 10 (*Epha10*) encode for transmembrane proteins involved in synapse formation. IgSF11 is an adhesion molecule that regulates synaptic transmission and plasticity. It interacts with the postsynaptic proteins PSD95 and the AMPA receptors subunits GluR1 and GluR2 to recruit them to sites of homophilic IgSF11 adhesion (Jang et al., 2016; Suzu et al., 2002). Interestingly, we also detected *Igsf11* mRNA in the inner retina, suggesting that the protein may be present pre- and post-synaptically and enable correct positioning of AMPA receptors in cone bipolar cells. LRFN2 is a member of the Leucine Rich repeat superfamily and plays a role in synapse formation and neurites outgrowth in neuronal cultures (Wang, Seabold, &

Wentholt, 2008). Alike *Igsf11*, some studies indicate that *Lrtn2* can interact with PSD95, AMPA and NMDA receptors (Ko et al., 2006) *Lrtn2* is also expressed in the inner retina and can form homomeric complexes (Seabold et al., 2008), suggesting a similar role as *Igsf11*.

Protogenin (PRTG), like *Igsf11*, is a protein of the immunoglobulin superfamily but implicated in the maintenance of early retinal progenitor competence state (La Torre, Georgi, & Reh, 2013). High expression of *Prtg* in the SCLs of the *Nrl*^{-/-} retina suggests yet underdetermined function of this gene in mature cone photoreceptors.

EPHA10 is part of the Eph receptor family, which is involved in embryonic development and neurogenesis as well as in adult neurogenesis (Kania & Klein, 2016). *Epha10* is reportedly upregulated in *rd7* mice (Corbo & Cepko, 2005) that carry a mutation in *Nr2e3*, leading to expression of S-cone genes in rods and rosette-like structures in the ONL (Akhmedov et al., 2000; Chen, Rattner, & Nathans, 2006). Enrichment of *Epha10* in the SCL-rich retina of *Nrl*^{-/-} mice and mislocalization of cone cell bodies by *Epha10* knockdown is indicative of its role in correct lamination and cone positioning within the ONL. The absence of phenotype when the gene is knockdown in wildtype CD1 mice confirms the cell specific and cell autonomous role of this gene in cone cells. Given the established role of Ephrins in morphogenesis including cell positioning (Kania & Klein, 2016), *Epha10* is likely associated with synapse formation by guiding the localization of the cone terminal. *Epha10* is largely uncharacterized in the retina and the brain though its over-expression is detected in breast cancer tissues where it is suggested to be involved in cell proliferation. The lack of availability of a commercial antibody for *Epha10* did not allow us to examine its localization in the retina. Furthermore, we cannot exclude the possibility whether sex affects the severity of the phenotype observed since the sex information was not obtained for the samples used for quantification. Further studies are in progress to elucidate the function of *Epha10* during retinal development, and especially during cone morphogenesis.

We hypothesize that the transmembrane proteins and secretory factors identified here would likely mediate the influence of microenvironment by activating or inhibiting specific signaling pathways by contributing to the formation of junctions with adjacent cells, interaction with rods and/or selection of specific bipolar cells for synapse formation. For example, such proteins can serve as receptors for signaling molecules secreted by Muller glia or microglia particularly under stress and disease conditions. Although not investigated in this study, rod photoreceptors also express specific sets of transmembrane and secretory proteins, some generated through alternative promoter or including alternative exons (Kim et al., 2016; Popova, Salzberg, Yang, Zhang, & Barnstable, 2017).

In summary, we have identified several cone-enriched genes that potentially contribute to interaction of cones with other cell types. Our studies thus provide a framework for investigating cone communication and tissue homeostasis. Some of the genes identified here could serve as candidates for mutation screening of retinal diseases.

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Significance Statement

Intercellular interactions are critical for development and homeostasis. In the mammalian retina, cone photoreceptors are embedded among rods and form the first visual synapse. We identified genes encoding transmembrane and secretory proteins by comparing transcriptomes of rods and S-cones-like cells of *Nrt^{-/-}* retina and validated the cone-enriched expression of seven genes by *in situ* hybridization. *In vivo* knockdown of one of the genes, *Epha10*, altered cone morphology and cell body localization within the retina. Our studies should assist in elucidating how cone photoreceptors interact and communicate with other retinal cell types and provide candidate targets for retinal disease and therapy.

Figure 1: Study design and transcriptome dynamics of genes in rod and S-cone like photoreceptors

(a) Gene selection flow chart. FSPR: Flow sorted photoreceptors. (b) Schematic representation of a membrane showing the gene ontology (GO) cellular component terms used for selecting candidate genes. (c) Hierarchical clustering, heatmap of transcripts with fold change ≥ 2 and false discovery rate ≤ 0.01 from rod and S-cone like photoreceptors at postnatal day (P)6, P10, P14 and P28. Clusters containing the selected genes are shown on the right. When multiple transcripts are produced from a single gene, the highest expressed transcript is used to represent the gene expression.

Figure 2: Protein structure of the selected genes

SMART predicted structures of proteins encoded by the seven selected genes: (a) transmembrane protein (b) transmembrane protein with active soluble ectodomain, which can be cleaved to produce a biologically active soluble peptide (c) secreted proteins. The protein encoded by the highest expressed transcript in *Nrt^{-/-}* photoreceptors is shown here.

Figure 3: Validation of RNA-seq data for selected genes encoding transmembrane protein in retina

(a) Gene expression profiles of *Prtg*, *Igslf11*, *Lrln2* and *EphA10* in WT retina (top) and in flow-sorted rod and S-cone-like photoreceptors (bottom) at different developmental time points. FSPR: flow-sorted photoreceptors; CPM: counts per million. The y-axis represents average CPM $\pm$ the standard deviation (SD). (b) Validation of mRNA expression by *in situ* hybridization at postnatal day 28 in WT and *Nr1*^{-/-} retina. White punctate dots represent the signal of the ISH probe. Nuclei staining is indicated by DAPI. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Bar = 50 μ m.

Figure 4: Developmental expression of *Vasn* encoding a transmembrane protein with a soluble ectodomain

(a) *Vasn* expression in WT retina (top) and in flow-sorted rod and S-cone-like photoreceptors (bottom) at different developmental time points. FSPR: flow-sorted photoreceptors; CPM: counts per million. Y-axis represents average CPM $\pm$ the standard deviation (SD). (b) Validation of mRNA expression by *in situ* hybridization at postnatal day 28 in WT and *Nrt*^{-/-} retina. White punctate dots represent the signal of the ISH probe. Nuclei staining is indicated by DAPI. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Bar = 50 μ m.

Figure 5: Developmental expression of selected genes encoding secretory proteins

Gene expression profile of *1190002N15Rik* and *Tuft1* genes which encode for secreted proteins (a) in WT retina (top) and in flow-sorted rod and S-cone-like photoreceptors (bottom) at different developmental time points. FSPR: flow-sorted photoreceptors; CPM: counts per million. The y-axis represents average CPM $\pm$ the standard deviation (SD). (b) Validation of mRNA expression by *in situ* hybridization at postnatal day 28 in WT and *Nrf*^{-/-} retina. White punctate dots represent the signal of the ISH probe. Nuclei staining is indicated by DAPI. ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Bar = 50 μ m.

Figure 6: *In vivo* knockdown of EphA10 in the *Nr1*^{-/-} retina

The *Nr1*^{-/-} mouse retina at P1 was electroporated with pUB-GFP and shRNA empty vector or shRNA against EphA10. (a-b) The retina was harvested at P21 and examined for GFP fluorescence (green), S-opsin immunoreactivity (red) and DAPI staining (blue). (a) ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Bar = 20 μ m (b) higher magnification images of (a). Arrowheads indicate cone morphological differences between control and EphA10_1 injected retina. OONL: outer portion of the outer nuclear layer. IONL: inner portion of the outer nuclear layer. Bar = 10 μ m (c) The fraction of electroporated (GFP+) cell bodies in the outer nuclear layer is calculated in retina recovered at P21. OONL: outer portion of the outer nuclear layer. IONL: inner portion of the outer nuclear layer. Data are represented as mean with standard deviation. Control, n= 8 retina; shRNA EphA10_1, n=7 retina, p=0.0022; shRNA EphA10_2, n=5 retina, p<0.0001.

p<0.01, **p<0.0001 by one-way ANOVA followed by Bonferroni correction post-hoc test. (d) Fraction of S-cone like electroporated cells in the retina recovered at P21. Control,

n= 8 retina; shRNA Epha10_1, n=7 retina, $p>0.9999$; shRNA Epha10_2, n=5 retina, $p=0.9173$ by one-way ANOVA followed by Bonferroni correction post-hoc test. NS: Not significant.

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Figure 7: *In vivo* knockdown of EphA10 in the wildtype CD1 mice

Wildtype CD1 mouse retina at P1 was electroporated with pUB-GFP and shRNA empty vector or shRNAs against EphA10. (a-b) The retina was harvested at P21 and examined for GFP fluorescence (green), cone arrestin immunoreactivity (red) and DAPI staining (blue). (a) ONL: outer nuclear layer; INL: inner nuclear layer; GCL: ganglion cell layer. Bar = 20 μ m (b) higher magnification images of (a). CARR: Cone arrestin. OONL: outer portion of the outer nuclear layer. IONL: inner portion of the outer nuclear layer. Arrows indicate GFP + rod electroporated nuclei, whereas asterisks point to cone photoreceptors nuclei. Bar = 10 μ m. (c) The fraction of electroporated (GFP+) cell bodies in the outer nuclear layer in the retina recovered at P21. OONL: outer portion of the outer nuclear layer. IONL: inner portion of the outer nuclear layer. The data are represented as mean with standard deviation (SD). Control, n= 4 retina; shRNA EphA10_1, n=4 retina, $p>0.9999$; shRNA EphA10_2, n=4 retina, $p=0.5670$ by one-way ANOVA followed by Bonferroni correction post-hoc test. (d)

The fraction of cone photoreceptors cell bodies in the outer nuclear layer in the retina recovered at P21. CARR+: Cone arrestin positive. OONL: outer portion of the outer nuclear layer. IONL (inner portion of the outer nuclear layer). Data are represented as mean with standard deviation (SD). Control, n= 4 retina; shRNA Epha10_1, n=4 retina, $p>0.9999$; shRNA Epha10_2, n=4 retina, $p=0.6142$ by one-way ANOVA followed by Bonferroni correction post-hoc test. NS: Not significant.

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Table 1:Probes used for *In situ* hybridization

Name	GeneID (NCBI)	Probe name	Catalog number	Probe targeted region	Number of probe pairs (ZZpairs)
Igsf11	NM_170599.2	Mm-Igsf11-3UTR	451131	1639 - 3100	20
Epha10	NM_001256432.1	Mm-Epha10-tv1	451111	1740 - 3389	20
Tuft1	NM_011656.3	Mm-Tuft1	518471	146 - 1160	20
1190002N15Rik	NM_001033145.2	Mm-1190002N15Rik	451101	812 - 1851	20
Prtg	NM_175485.4	Mm-Prtg	451171	630 - 1638	20
Lrfn2	NM_027452.3	Mm-Lrfn2	451141	542 - 2041	20
Vasn	NM_139307.3	Mm-Vasn	450791	610 - 1954	20

Table 2:

Primary antibodies information

Name	Description of immunogen	Source, host species, catalog No., RRID	Concentration used
GFP (Green Fluorescent Protein)	Recombinant full length GFP	Torrey Pines Biolabs, rabbit polyclonal, TP401, AB_10013661	1:1000 (IHC)
GFP (Green Fluorescent Protein)	Recombinant full length GFP	Abcam, chicken polyclonal, ab13970, AB_300798	1:1000 (IHC)
Blue cone opsin	Mouse peptide: CRKPMADESDVSGSQ KT	Custom made, chicken polyclonal affinity purified	1:1000 (IHC)
Cone-Arrestin	Epitope within 12 amino acids from the C-terminal end	Millipore, rabbit polyclonal, AB15282, AB_1163387	1:100 (IHC)

Abbreviations: IHC, immunohistochemistry

Table 3:

shRNA sequences

shRNA name	Clone ID	Target sequence
Epha10_1	TRCN0000362111	TCGGTGCGTGTCTACTACAAG
Epha10_2	-	GTGCCCTTGGCTAGGTCTATCT