A Genetic modification that reduces ON-bipolar cells in hESC-derived retinas enhances functional integration after transplantation

**Highlights**
- Deletion of ISL1 in hESC-retinas resulted in a reduced number of ON-bipolar cells.
- Photoreceptors in ISL1−/− hESC-retinas achieved functional maturation in vivo.
- ISL1−/− hESC-retinas showed better host-graft contact with putative synapses.
- ISL1−/− hESC-retinas better restored RGC light responsiveness in degenerated retina.

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A Genetic modification that reduces ON-bipolar cells in hESC-derived retinas enhances functional integration after transplantation

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SUMMARY

Pluripotent stem cell (PSC)-derived retinal sheet transplanted in vivo can form structured photoreceptor layers, contact with host bipolar cells, and transmit light signals to host retinas. However, a major concern is the presence of graft bipolar cells that may impede host-graft interaction. In this study, we used human ESC-retinas with the deletion of Islet-1 (ISL1) gene to achieve the reduced graft ON-bipolar cells after xenotransplantation into end-stage retinal degeneration model rats. Compared with wild-type graft, ISL1/−/− hESC-retinas showed better host-graft contact, with indication of host-graft synapse formation and significant restoration of light responsiveness in host ganglion cells. We further analyzed to find out that improved functional integration of ISL1/−/− hESC-retinas seemed attributed by a better host-graft contact and a better preservation of host inner retina. ISL1/−/− hESC-retinas are promising for the efficient reconstruction of a degenerated retinal network in future clinical application.

INTRODUCTION

Photoreceptor cell replacement therapy for retinal degenerative diseases including retinitis pigmentosa (RP) and age-related macular degeneration (AMD) is considered an attractive approach to restore visual function. The advance of innovative technology to generate self-organizing retinal organoids from mouse and human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) enabled retinal tissue transplantation for end-stage retinal degeneration, with a stable supply of retinal cells that are qualitatively comparable with fetal retinal tissue or cells (Eiraku et al., 2011; Kuwahara et al., 2015, 2019; Meyer et al., 2009; Nakano et al., 2012; Reichman et al., 2014; Zhong et al., 2014). Transplantation of hESC/iPSC-retinal tissue-sheet (retinal sheet, hereafter) or cells has been conducted in animal models of end-stage retinal degeneration to show functional potential to elicit light responses in host retinal ganglion cells (RGC) and light-guided behaviors (Iraha et al., 2018; Mandai et al., 2017; McLelland et al., 2018; Ribeiro et al., 2021; Tu et al., 2019; Zerti et al., 2021). Based on these proof-of-concept studies, we recently initiated the first-in-human clinical research to confirm the safety of hiPSC-retinal sheet transplantation in patients with RP.

Photoreceptor transplantation into the subretinal space can be conducted either in the form of a cell suspension or as retinal sheet. In the end-stage host retina, transplantation of purified photoreceptors is advantageous in forming direct contact with host bipolar cells, whereas retinal sheet transplantation is reported to be associated with longer graft survival, low immunogenicity, and photoreceptor maturation with a layered structure with formation of outer segments (OS)-like structures as also confirmed by electron microscopy (Assawachananont et al., 2014; Iraha et al., 2018; Shirai et al., 2016; Tu et al., 2019; Yamasaki et al., 2021). However, the ESC/iPSC-retinal sheet also provides retinal inner cells, which may simultaneously benefit and hinder the host-graft integration. Müller glia are important for the development of OS structures, glutamate uptake, and recycling of visual pigments, mostly for cone photoreceptors (Bringmann et al., 2009; Wang and Kefalov, 2011). Horizontal cells participate in ribbon synapses and contribute to the integration and regulation of the signals from photoreceptors, and the deletion of horizontal cells leads to photoreceptor degeneration (Janssen-Bienhold et al., 2012). On the other hand, bipolar cells in the retinal sheet seemed to impede host bipolar cells contacting and forming synapses with graft photoreceptors (Assawachananont et al., 2014).

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Based on these observations, in order to enhance host-graft contact by reducing graft bipolar cells while retaining the cellular and structural benefits of the retinal sheet, we recently reported the knockout (KO) effect of Bhlhb4 and Isl1 genes that are related to the fate-determination and maturation of bipolar cells, in mouse ESC/iPSC-retinal sheet (Matsuyama et al., 2021). Previous studies showed that Bhlhb4 gene deletion specifically reduced rod bipolar cells (Bramblett et al., 2004), whereas Isl1 conditional KO mice presented a considerable reduction of ON-bipolar cells, cholinergic amacrine cells, and RGC (Elshtoray et al., 2007). Consistently, using mouse ESC/iPSC-retinas, we observed a drastic reduction of rod bipolar cells in both KO graft lines and of subsets of cone bipolar cells in Isl1 KO (Isl1−/−) retina grafts, both with enhanced host-graft synaptic formation, signal-to-noise ratio in RGC response, and improved light responsive behavior (Matsuyama et al., 2021). In the current study, in view of clinical application, we deleted the ISL1−/− gene to confirm the reproducibility of these features using hESC-retinas. We found a marked reduction in ON bipolar cells while sparing photoreceptors, Müller glia, and horizontal cells in vitro and/or in vivo after transplantation and a significant improvement in host RGC light responses in the transplanted retina with ISL1−/− hESC-retinas than with wild-type hESC-retinas using multiple electrode array system. We also identified expression of number of synaptic marker proteins at possible host-graft contact site. Furthermore, we analyzed the factors that may affect functional integration of the graft and found that a good host-graft contact as well as a good preservation of host inner retina may contribute to improved function of ISL1−/− hESC-retinas after transplantation.

RESULTS

Generation of an ISL1−/− retinal organoid from human ESCs

We obtained ISL1 KO (ISL1−/−) clones as summarized in Figures S1A–S1C using Crx::Venus reporter hESC line (KhES-1), in which photoreceptor precursor cells and mature photoreceptors express Venus fluorescence (Nakano et al., 2012). ISL1−/− hESC clones were routinely maintained with the expression of PSC markers (Figure S1D). Retinal organoids were differentiated from ISL1−/− hESCs in a similar manner to wild-type (WT) hESCs, which were positive for a retinal progenitor cell (RPC) marker Chx10 at differentiation day (DD) 15 (Figures 1A, 1B, and S1E). The differentiated organoids of WT and ISL1−/− hESC showed the characteristic appearance of continuous neuroepithelium that expressed early born RGC marker Brn3 on the ISL1 responsive behavior (Matsuyama et al., 2021). In the current study, in view of clinical application, we deleted ISL1 (Figure 1G).

The proportions of the subtypes of retinal cells in the early stage of WT and ISL1−− hESC-retinas (~DD58) was similar, including Chx10+/Crx− RPC (30%–40%), Pax6++ RGCs and amacrine cells (AC) (20%–30%), and...
As to bipolar cells, Gox / ISL1 and found positive for ISL1 in the WT hESC-retina (Figure S3C). We also checked the presence of ISL2 in WT of retinal cells including the photoreceptor precursor cells in the early stage. The RBPMS (pan-RGC) and PKC cells (Figures S4C–S4E).

Secretagogin+ cone bipolar cells were observed in both retinas at DD240 (Figures 1M–1P and S4A–S4B). These ISL1+/ON/rod-bipolar cells were present in the Ku80+ WT but not in ISL1+/hESC-retina. (K and L) Note that host bipolar dendrites were observed surrounding the graft photoreceptors (yellow arrows). A few Chx10+/PKC+ cells were present in HuNu positive graft cells in ISL1+/hESC-retina (white arrows). DD, differentiation day; TP, transplantation; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer.

Crx+:Venus+ photoreceptors (10%–20%) that were mostly Rxry+ cone photoreceptor precursor cells at this stage (Figures 1H and S3B); this indicates that deletion of the ISL1 gene does not affect the differentiation of retinal cells including the photoreceptor precursor cells in the early stage. The RBPMS (pan-RGC) and Calretinin (subsets of RGC and AC) were also positive in the emerging inner layer where some cells were found positive for ISL1 in the WT hESC-retina (Figure S3C). We also checked the presence of ISL2 in WT and ISL1+/hESC-retinas to find that ISL2+ cells were decreased among RGC in ISL1+/hESC-retina up to DD60 but were similarly observed among Rxry+ cone photoreceptor precursor cells in both WT and ISL1+/hESC-retinas at DD74 and after (Figure S3).

Loss of ISL1 leads to drastic reduction of ON-bipolar cells in long-term in vitro culture

In order to observe the effect of ISL1 deletion on late-born retinal cells such as photoreceptors and bipolar cells in human retinas, we observed WT and ISL1+/hESC-retinas at around DD240. The appearances of WT and ISL1+/hESC-retinas were similar, presenting fluffy cilium-like structures covering the apical surface (Figures 1I and S3D). Cnx+:Venus+/Recoverin+ photoreceptors were located in most apical and middle layers in both WT and ISL1+/hESC-retinas (Figure 1J). The apically located photoreceptors developed inner-/outer-segment (IS/OS)-like structures expressing PRPH2 (Figures S4A–S4B). On FCM analysis, Cnx+:Venus+ populations formed two groups based on Venus intensity (Cnx+:Venus+ and +), of which Cnx+:Venus++ cells mostly expressed Recoverin and PRPH2 similarly in WT and ISL1+/hESC-retinas (Figures 1K and S4A–S4B). These Cnx+:Venus++ photoreceptors included cells positive for cone and rod photoreceptor markers S- and L/M-opsin, cone arrestin, and rhodopsin, and the majority of Cnx+:Venus++ photoreceptors also expressed a synaptic marker PSD95 (Figure S4B). The characteristic localization of the cells positive for these markers was also confirmed by immunohistochemistry (IHC) (Figure S4A). The fraction of Cnx+:Venus++ photoreceptors was about 40%, with a Nr1+ rod to Rxry+ cone photoreceptor ratio of about 1:1 (n = 4; >10 sheets for each) (Figures S4C–S4F). At around DD240, RPCs (Chx10+/Cnx+:Venus+, Chx10+/Sox2+, Pax6+/Sox2+) were identified in about 5% of the hESC-retinas but there were few Ki67+ proliferating cells (Figures S4C–S4E).

As to bipolar cells, Gox+ ON-bipolar cells, PKCα+ or L7+ rod bipolar cells were present in Cnx+:Venus+ population by FCM and in the inner layer of the organoids in WT but absent or very infrequent in ISL1+/hESC-retinas at DD240 (Figures 1M–1P and S4A–S4B). Secretagogin+ cone bipolar cells were observed in both lines but as a very small population (Figures S4A–S4B). In spatial and temporal analyses, Gox+ and PKCα+ cells were observed from DD167 in WT hESC-retinas, which were also Chx10+, suggesting that these are mature ON/rod-bipolar cells. We observed very few Gox+ and PKCα+ cells but a substantial number of Chx10+ cells in ISL1+/hESC-retinas (Figure S5A), these may include premature retinal cells or some Müller glia. Altogether, deletion of ISL1 gene in hESC-retina reduced the number of ON/rod-bipolar cells without affecting the development of photoreceptors.

ISL1+/hESC-derived retina survived and matured after transplantation into a rat model of retinal degeneration

Next, we transplanted retinal sheet into the subretinal space of a nude rat model of the end-stage retinal degeneration with few remaining photoreceptors, SD-Foxn1 Tg (S334ter) 3LavRrrc, at 16–25 postnatal weeks (Seiler et al., 2014; Tu et al., 2019). We dissected the Cnx+:Venus+ retinal epithelium around DD60 to prepare a strip sheet of approximately 0.5 mm width for transplantation (Figure 2A and S5B). After transplantation, the graft formed multiple Cnx+:Venus+ photoreceptor rosettes as observed by in vivo color fundus imaging (Figures 2B and S5C). At day 180 post-transplantation (i.e., ~DD240), IHC image presented graft outer nuclear layer (ONL)-like structures expressing rhodopsin or S- and L/M-opsin in hESC-retinas of both WT and 2 ISL1+/ clones of 330A16 and 330A19 (Figures 2C–2D and S5G).
receptors in transplanted used here were checked using adult monkey retina (Figure S6A). These observations suggest that phototransduction proteins were mostly PKCα rod bipolar cells (Figure SSD). The photoreceptor rosettes in the WT graft were densely surrounded by graft Goα ON-bipolar or PKCα+ rod bipolar cells, whereas those in the ISL1+/hESC graft had no surrounding graft ON/rod-bipolar cells, which visualized host ON/rod-bipolar cells extending their dendrites into graft photoreceptors (Figures 2G–2J and S5–S5I). Although HuNu+ /PKCα+ cells were few, a very small number of HuNu+/Chx10+/PKCα+ nonbipolar cells were still observed in ISL1+/− grafts (Figures 2K and 2L). Host bipolar cells often extended their long PKCα+ dendrites to contact graft photoreceptors (Figures 2J and 2L).

Glutamate synthase (GS)+ Müller glia and Calbindin+ horizontal cells were similarly present in both WT and ISL1+/− grafts (Figures S5E–S5F). Interestingly, GS+ Müller glia were observed stretching throughout the entire graft, whereas human GFAP was only positive in the graft near the host RPE, indicating the limited activation of graft Müller glia distal to the integration site. This was consistent with our previous observation (Tu et al., 2019; Yamasaki et al., 2021).

Collectively, ISL1+/− hESC-retina transplant presented reduced number of bipolar cells yet retaining the essential components such as photoreceptors, Müller glia, and horizontal cells in the organized structure with matured rod and cone photoreceptors.

Photoreceptors of the ISL1+/− hESC-retina mature functionally after transplantation

We then estimated photo-responsive potential of mature photoreceptors in ISL1+/− hESC-retina after transplantation by confirming the presence of several key proteins that are involved in phototransduction signaling by IHC in PRPH2+/− mature graft photoreceptors (Figures 3A and 3B). In rat retinas six months after transplantation, graft photoreceptor rosettes formed the IS/OS-like structure in the internal space, which were positive for the rod phototransduction-pathway-related markers GRK1, S-arrestin, GNAT1, PDE6a/b, and CNGB1 (Figures 3Ba–3Bq); the cone phototransduction-pathway-related markers cone arrestin GNAT2, PDE6H, and CNGB3 (Figures 3Bh–3Bm), and the rod and cone phototransduction pathway-related markers including GUCY2D, GUCY2F, GUCA1A, and GUCA1B (Figure 3Bn–3Bq).

In addition, we assessed the extracellular microenvironment in ISL1+/− hESC-retina after transplantation by immunostaining the interphotoreceptor matrix (IPM) components inside the rosette cavity. Interestingly, the internal space of graft photoreceptor rosettes, which was separate from the RPE, expressed IPM including IMPG1 (SPACR), IMPG2 (SPARCAN), IRBP, CD44, versican, and brevican, suggesting that these IPM were secreted by graft photoreceptors and/or Müller glia (Figure 3Bs–3Bx). IPM proteins were expressed in most of the photoreceptor rosettes; however, the phototransduction protein GNAT1 was typically observed in mature OS-like structures in relatively large rosettes (Figures 3C and 3D). All antibodies used here were checked using adult monkey retina (Figure S6A). These observations suggest that photoreceptors in transplanted ISL1+/− hESC-retinas not only express functional proteins but also may create a supportive environment for them to function.

Improved contact efficiency between host bipolar and graft photoreceptors in ISL1+/− hESC-retina

As the deletion of bipolar cells seemed to enhance host-graft contact, we evaluated host bipolar-graft photoreceptor contact by immunostaining Recoverin, PKCα, and Ku80 to classify the contact patterns into 3 types: Poor, graft bipolar cells are present between host bipolar cells and graft photoreceptor rosettes, with no apparent host-graft contact. Fair, some host bipolar cells reach toward graft photoreceptors,
although graft bipolar cells are present between graft photoreceptors and host bipolar cells. Good, most graft photoreceptors are bordering host bipolar cells (Figure 4A). The quantitative data, as the rate of observed contact type (poor, fair, good) by sample, demonstrated about half the rosettes in WT hESC-retinas had poor contact, whereas good contact was observed in about half of the rosettes in ISL1<sup>−/−</sup> hESC-retinas (Figure 4B). We modeled these ordinal data (poor < fair < good) assuming an underlying metric variable with a normal distribution with cutoff thresholds for poor = 0.5 and good = 2.5 and estimated its mean and standard deviation considering graft group (WT or ISL1<sup>−/−</sup>), host sex, and sample bias using hierarchical Bayesian modeling (Figure 4B). The distributions of predicted mean for WT and ISL1<sup>−/−</sup> and the distribution of predicted values, i.e. the expected distribution when taking into account the standard deviation (sd), were calculated (Figure 4C). The rosette contact data had an overall mean of approximately 2 with sd 0.6. There was a clear and substantial effect of the group (difference of WT and ISL1<sup>−/−</sup> was not immediately apparent in samples due to both the large sd of the distribution and large sample size. We also evaluated IS/OS growth by the staining patterns of the phototransduction protein GNAT1 in graft photoreceptors. The growth of OS expressing GNAT1 was graded into three patterns (Figure 4I): Poor, unclear polarization of GNAT1 and no apparent IS/OS structures; Fair, sparse presence of polarized GNAT1 positive IS or OS; Good, GNAT1 positive OS within the central space of the rosette. A summary of collected data alongside model predictions, as the proportion of observed IPL pattern (poor, fair, good) by sample, are shown with modeling analysis (Figures 4F–4H). IPL preservation data have an overall mean of approximately 2.9 ± 1.3 with a small effect of group (Figure 4H group; difference of WT and ISL1<sup>−/−</sup> hESC-retina is −0.9 [95% interval: −1.2 to −0.6]), strongly indicating that ISL1<sup>−/−</sup> hESC-retinas had better contact compared with WT hESC-retinas (Figure 4D). Host sex did not influence rosette contact, and sample bias was relatively small (sample sd is about 0.2).

The ISL1<sup>−/−</sup> hESC-retina better preserved the host IPL structure but may have fewer rod outer segments than WT

Although we have not observed any undesirable proliferation of graft retinas after transplantation, grafted retinal cells sometimes migrated into host retinas, causing varying degrees of disorganization of the structure of the host retina (Figure 2I, red arrow). Thus, we evaluated the degree of host IPL disorganization in areas adjacent to photoreceptor rosettes. We stained choline acetyltransferase (ChAT) and categorized the IPL state into poor, fair, or good based on the pattern of the two IPL lines that were usually present (Figure 4E): Poor, both IPL lines were disrupted. Fair, one of the IPL lines was disrupted. Good, both IPL lines were preserved. The summary of collected data alongside model predictions, as the proportion of observed IPL pattern (poor, fair, good) by sample, are shown with modeling analysis (Figures 4F–4H). IPL preservation data have an overall mean of approximately 2.9 ± 1.3 with a small effect of group (Figure 4H group; difference of WT and ISL1<sup>−/−</sup> hESC-retina is −0.9 [95% interval: −1.9 to 0.2]). Overall, host retinas transplanted with ISL1<sup>−/−</sup> hESC-retinas seemed to have less disrupted IPL structure but the difference was not immediately apparent in samples due to both the large sd of the distribution and large sample biases.

We also evaluated IS/OS growth by the staining patterns of the phototransduction protein GNAT1 in graft photoreceptors. The growth of OS expressing GNAT1 was graded into three patterns (Figure 4I): Poor, unclear polarization of GNAT1 and no apparent IS/OS structures; Fair, sparse presence of polarized GNAT1 positive IS or OS; Good, GNAT1 positive OS within the central space of the rosette. A summary of collected data alongside model predictions and modeling analysis is presented in Figures 4J–4L; OS growth data have an overall mean of approximately 2.9 with sd 1. There is a small but credible effect of group (Figure 4L group; difference between WT and ISL1<sup>−/−</sup> hESC-retina is 0.4 [95% interval: 0.0 to 0.8]); this indicates that, although OS growth was mostly good in both groups, retinas transplanted with ISL1<sup>−/−</sup> hESC-retinas seemed to have shorter/fewer IS/OS structures although the effect was subtle.

Presence of multiple synaptic components suggested a formation of host-graft synapses after transplantation of ISL1<sup>−/−</sup> hESC-retina

With the ISL1<sup>−/−</sup> hESC-retina graft, in the absence of graft rod bipolar cells, we could clearly identify PKCα<sup>+</sup> host rod bipolar dendrites reaching toward the photoreceptors, which allowed us to confirm multiple synaptic components at host-graft synapses. Crx::Venus<sup>+</sup>-grafted photoreceptors expressed ribbon synapse components at host-graft synapses.
Figure 5. Host-graft synaptic formation of transplanted photoreceptors derived from ISL1−/− hESC-retina

(A and B) Presynaptic marker CtBP2 was localized on the margin of the Cx::Venus+/Recoverin+ photoreceptors and the dendritic tips of Ku80/PKCα+ host bipolar cells (yellow arrows).

(C) Outer Plexiform layer-like structures formed with synaptophysin (Syn) around the rosette.

(D–F) Presynaptic proteins pikachurin, CtBP2, PSD95, and LRIT3 were present at the dendritic tips of PKCα+ host rod bipolar cells. (G) Postsynaptic marker CACNA1S were coupled with pikachurin.
marker CtBP2 at the tips of host bipolar dendrites (Figure 5A) (Matthews and Fuchs, 2010; Tom Dieck et al., 2005). Host rod bipolar cells often extended dendrites even through the occasionally present graft inner cells (Ku80+/Recoverin−) to reach graft photoreceptors (Figure 5B). Transplanted photoreceptors also exhibited synaptophysin, the synaptic vesicle protein, around ONL-like rosette structures, suggesting the synaptic maturation of graft photoreceptors (Figure 5C). Another synaptic protein pikachurin, which contributes to precise synaptic interaction as a dystroglycan ligand, was found to localize in the cleft of CtBP2-labeled, horseshoe-shaped synaptic ribbons around the graft rosette at the tips of PKCα+ dendrites (Figure 5D) (Sato et al., 2008). Presynaptic protein PSD95 also colocalized with pikachurin at the photoreceptor terminals (Figure 5E). LRT3, a synapse protein involved in coordination of the transsynaptic communication between the rod and rod bipolar cells (Hasan et al., 2019), was observed with CtBP2 at the dendrite tips of a host rod bipolar cell (Figure 5F). We further observed postsynaptic proteins CACNA1S and mGluR6 on the host bipolar dendrite tips (PKCα or cone bipolar Secretagogin) coupled with pikachurin, CtBP2, and PNA (cone pedicle marker) to form putative rod and cone synaptic complexes at the margin of graft photoreceptor rosettes (Figures 5G–5J). High-magnification images show that mGluR6 puncta were located adjacent to CtBP2 and peanut agglutinin (PNA), suggesting the presence of host-graft synaptic connectivity between the rod and rod bipolar cells (Hasan et al., 2019), was observed with CtBP2 at the dendrite tips of a host rod bipolar cell (Figure 5F). We further observed postsynaptic proteins CACNA1S and mGluR6 on the host bipolar dendrite tips (PKCα or cone bipolar Secretagogin) coupled with pikachurin, CtBP2, and PNA (cone pedicle marker) to form putative rod and cone synaptic complexes at the margin of graft photoreceptor rosettes (Figures 5G–5J). High-magnification images show that mGluR6 puncta were located adjacent to CtBP2 and peanut agglutinin (PNA), suggesting the presence of host-graft synaptic complexes (Figure 5I” and 5J). Finally, we determined whether horizontal cells, a component of the typical triad photoreceptor synapse, are involved in the formation of host-graft photoreceptor synapses. Calbindin+/Ku80+ graft horizontal cells were present in the ISL1−/− hESC-retina, and Calbindin−/− axons or dendrites either from host (Ku80+) or graft (Ku80−) horizontal cells surround the graft rosette (Figure 5K). We observed a possible synaptically assembled of PKCα+ host bipolar cell, Calbindin+ horizontal cell dendrites, and CtBP2+ graft photoreceptor terminals (Figure 5L). All the synaptic antibodies used here were checked in adult nude rat retina (Figure 5F). These data suggest that photoreceptors in ISL1−/− hESC-retinas are highly capable of forming functional synapses with host bipolar cells.

**ISL1−/− hESC-retinas elicited better light responses in host RGCs than WT hESC-retinas**

Multi-electrode array (MEA) recordings of the transplanted rat retinas were conducted at 6 weeks old or later (8–10 months after transplantation), where almost no RGC light responses were observed with the scotopic-mesopic light stimuli (10.56 log photons/cm²/s, referred to as weak stimuli hereafter) (Tu et al., 2019). To assess the light responses derived from the transplanted hESC-retinas, the freshly isolated retina was mounted with the grafted area centered at the MEA electrodes and the optic nerve disc within sight, as in the example shown in Figure 6A. The Crx::Venus+ rosettes of WT or ISL1−/− hESC graft were confirmed to have bright green fluorescence after recordings (Figure 6B). The peri-stimulus time histograms (Figure 6C) and raster plots (Figure 6D) of this sample suggest the host RGC light responses were spatially correlated with the graft coverage, consistent with other transplanted retinas of both WT and ISL1−/− hESC graft. For most transplants, retinas from the opposite, nontransplanted eyes were used as the age-matched control. Presence of light responses sensitive to the mGluR6 blocker L-AP4 in transplanted retinas implied functional synaptic transmissions between photoreceptors and bipolar cells (Figure 6E). In contrast, relatively few light responses were detected in control retinas, mostly with strong (12.84 log photons/cm²/s) and super-strong (15.48 log photons/cm²/s) stimuli, which possibly originated from residual cone photoreceptors, if any, or melanopsin-expressing RGCs that were not sensitive to L-AP4 blockade. Note that the super-strong stimuli were only applied at the end of experiments for all samples to confirm their viability.

The population averages of RGC spiking frequency revealed a higher baseline firing rate in control retinas (Figure 6E), especially in the presence of L-AP4. Figure 6G shows the distribution of averaged spontaneous firing rate (spontaneous spiking before light stimulation). The spontaneous activity has a lognormal distribution, as we have reported previously (Matsuyama et al., 2021). We estimated the effect of different parameters (group, sex, L-AP4 treatment, stimulus strength, and sample bias) on the spontaneous activity using hierarchical Bayesian inference. The overall mean (log) of the firing frequency distribution was 1.68 (95% interval: 1.66 to 1.70), which is equivalent to 5.4 Hz, with sd 1.02 (95% interval: 1.01 to 1.02). The mean is indicated in Figure 6G by the vertical line. The group has a clear effect on spontaneous spiking (Figure 6H group), with transplanted retinas showing less spontaneous firing (difference of control and WT is 0.7 [95% interval: 0.4 to 0.9] and control and ISL1−/− is 0.5 [95% interval: 0.3 to 0.8]). There was no effect of...
Figure 6. Light responses of transplanted retina by MEA recordings

(A–D) Representative recordings of transplanted (ISL1+/−) hESC-retina using MEA system. Transplanted rat retina (A) was mounted on the MEA probe with the Crx::Venus+ hESC-retina centered on electrode area (B) indicated by red boxes. (C) Peri-stimulus time histogram of host RGC spikes with responses to strong light stimuli after washout of L-AP4. Detailed raster plots from three channels (highlighted in black, red, and green) are shown in (D).

(E) Comparison of light responses across different conditions and genotypes. Average firing rate is shown for each stimulus intensity level. (F) Heatmap showing the fraction of RGC types for different functional cell types across various conditions.

(G–J) Distribution of firing rates across different stimulus intensities and genotypes. Density plots illustrate the distribution of firing rates for weak, medium, and strong stimuli.
Figure 6. Continued
(E) Population averages of transplanted retinas with WT and ISL1−/− HESC-retinas and nontransplanted control retinas. Thin lines represent the sample average and thicker lines represent the group averages for the respective L-AP4 treatment condition (before, L-AP4, after) and light stimulation (weak, medium, strong, super-strong).
(F) Breakdown of the functional RGC types detected in both transplanted and control retinas.
(G) Distribution of RGC spontaneous firing (logHz). Vertical lines indicate the estimated overall mean (1.68). Bars summarize recorded data, whereas lines and ribbon plots show the mode and 95% compatibility interval of model posterior predictions.
(H) Posterior distribution of model parameters for RGC spontaneous activity.
(I) Summary of RGC response probability with light stimulation. Dots and bars show per sample summary of collected data with lines showing the Clopper and Pearson binomial 95% confidence interval. Violin plots show the model posterior predictions.
(J) Posterior distribution of model parameters for RGC responsiveness. Note that values represent log odds. These data in this figure was collected from a total of 47 retinas (16 control, 13 WT, and 18 ISL1−/− graft transplanted retinas).

host sex (Figure 6H sex). Similar to our previous finding in mice (Matsuyama et al., 2021), there was a small but distinct effect of stimulus strength, with spontaneous activities increased after brighter stimulation (Figure 6H stimulus). We also found that spontaneous activity clearly increased upon L-AP4 blockade (Figure 6H L-AP4 treatment). Finally, there was a relatively large effect of sample (Figure 6H sample, sample sd estimated to be 0.4 [95% interval: 0.3 to 0.5]).

To better characterize and compare the light responsiveness in transplanted retinas with robust RGC spontaneous firing, we classified the detected host RGCs, in consideration of their sensitivity to L-AP4 blockade and recovery after L-AP4 washout, into unresponsive, onXoff, on, and adapted on types (Figure 6F; see STAR Methods for further details) (Matsuyama et al., 2021). Most of the responding cells in transplanted retinas were on or adapted on types with similar on to adapted on ratios in both types of transplanted retinas. Figure 6I summarizes cell response rate (i.e., the probability that each detected cell would not be unresponsive) per sample upon different levels of light stimulation. We modeled the response probability using logistic regression that took into consideration of the group, sex, stimulus, and sample bias as predictors. The overall mean (log odds) was −3.2 (95% interval: −3.4 to −3.0). Figure 6J shows the posterior estimates of parameters. There was a clear and substantial effect of group (Figure 6J group, control < WT < KO (ISL1−/−)), difference of control and WT is −1.4 (95% interval −2.6 to 0), control and KO (ISL1−/−) is −3.2 (95% interval −4.4 to −1.9), and WT and KO (ISL1−/−) is −1.7 (95% Interval −3.1 to −0.6). There was no appreciable effect of host sex (Figure 6J sex). Light responses were clearly increased in medium (12.16 log photons/cm²/s) and strong light stimulation compared with weak stimulation (Figure 6J stimulus, difference of weak and medium was −1.2 [95% interval −1.3 to −1.1], weak– and strong was −1.2 [95% interval −1.4 to −1.1]). Sample bias was substantial, with sample sd estimated to be 1.7 (95% interval 1.3–2.2) (Figure 6J sample). In addition to the above predictors, we used the average spontaneous firing rate during L-AP4 treatment as a covariate and found that it was inversely correlated with light responsiveness (Figure 6J spontaneous freq).

Light responsiveness after transplantation positively correlated with host-graft contact rate and well-preserved host IPL

So far, we have observed some differences in our immunohistological characterization (rosette contact, IPL preservation, and IS/OS growth) and in light responsiveness by electrophysiology, and it was therefore intriguing to elucidate which are the important factors that affect the functional output after transplantation. For this purpose, we further estimated correlation coefficients (ρ) among the different features, using the sample estimates from the respective analyses: light response probability for light responsiveness, mean (log) of the spontaneous firing for the analysis of spontaneous activity, and the mean of the underlying metric variable for rosette contact, OS growth, and IPL preservation analyses. We used the Student’s t-distribution for the bivariate distribution, as the number of available samples was relatively small (21 samples). The number of estimated degrees of freedom for the Student’s t-distribution was 18.8 (95% interval: 5.8–46.5). Pair plots in Figure 7 shows a summary of the relationships between features (lower) and estimated correlation coefficients (upper). Spontaneous activity did not seem to be correlated with any feature. On the other hand, rosette contact (ρ =−0.4 [95% interval 0.0 to 0.7]), OS growth (ρ =−0.5 [95% interval −0.7 to −0.1]), and IPL preservation (ρ =0.5 [95% interval 0.1 to 0.7]) were weakly to moderately correlated to light responsiveness. Note that contact and IPL were positively correlated, whereas OS growth was negatively correlated. Rosette contact was positively correlated to IPL (ρ =−0.4 [95% interval 0.0 to 0.7]) and negatively correlated to OS elongation (ρ =−0.4 [95% interval −0.7 to −0.1]). Finally, there was a potential weak negative correlation between IPL preservation and OS growth (ρ =−0.2 [95% interval −0.5 to 0.2]). Overall, the
cis-retinal for visual pigment recovery, was strongly expressed inside the rosette structures (Palczewski et al., 1999). It is unclear if the amount of 11-retinal when kept distant from the RPE. The retinoid transporter IRBP, known to transport all-retinoids to the photoreceptor space in normal retina, by expressing several IPM proteins within the rosette cavity even under a xenotransplantation condition. It is noteworthy that graft photoreceptors in rosettes may create a microenvironment similar to that of in vivo host retina, indicating that nonintegrated photoreceptors were relatively prone to degenerate after transplantation.

DISCUSSION

Our work demonstrated proof-of-concept regarding the therapeutic approach using combination of gene engineering and regenerative medicine for retinal degeneration, i.e., the deletion of a key cell fate regulator gene to achieve an ideal type of retinal graft consisting mostly of essential cell types in a structured form upon maturation after transplantation. The deletion of ISL1<sup>−/−</sup> gene in hESC retinas resulted in a reduced number of ON-bipolar cells, the second neurons that receive signals from photoreceptors, sparing the subsequent photoreceptor cells, Müller glia, horizontal cells, and facilitated functional host-graft integration after transplantation; this was consistent with our previous observation using genetically engineered mouse ESC/iPSC retina grafts with the deletion of Isl1 or Bhlhb4 gene (Matsuyama et al., 2021).

In this study, we further accessed detailed phenotypes of ISL1 gene deletion in hESC-retina to pursue possible benefits in view of future clinical application. Mature graft photoreceptors in an organized layer expressed phototransduction cascade proteins after transplantation, assuring the function of these grafts as photoresponsive transplants. These ISL1<sup>−/−</sup> hESC-retinas resulted in increased host-graft contact with improved light responsiveness in the host RGCs compared with the WT hESC-retinas. Moreover, the host-graft synapse formation was confirmed by the presence of multiple synaptic markers using IHC even under a xenotransplantation condition.

It is noteworthy that graft photoreceptors in rosettes may create a microenvironment similar to that of interphotoreceptor space in normal retina, by expressing several IPM proteins within the rosette cavity even when kept distant from the RPE. The retinoid transporter IRBP, known to transport all-trans-retinal and 11-cis retinal for visual pigment recovery, was strongly expressed inside the rosette structures (Palczewski et al., 1999). It is unclear if the amount of 11-cis retinal delivered inside the rosettes is enough for retinoid cycles, but our MEA data at least showed that transplanted retinas were able to respond to light repetitively after isolation from RPE, without rod/cone opsin recovery by the addition of 9-cis retinal, suggesting some compensation may exist in the graft rosette environment. Interestingly, some rosettes exhibited ONL-like structures with a thinner photoreceptor layer on the RPE side, mimicking the correct orientation of ONL (Figure 2L). We previously reported that most of the rosettes that remained 2 years after xenotransplantation in the monkey model had a hemispherical shape with the opening facing the host RPE, potentially allowing retinoic acid cycling to happen (Tu et al., 2019). We also observed here and previously that the expression of GFAP or HLA class I was only enhanced on the RPE side of graft rosettes that failed to integrate with the host retina, indicating that nonintegrated photoreceptors were relatively prone to degenerate after transplantation (Yamasaki et al., 2021).

Lastly and most importantly, our MEA analysis together with the immunohistochemical analysis suggest the presence of essential factors for graft-driven retinal reconstruction. As we initially expected, the restoration of light responsiveness correlated positively with the host-graft contact rate that potentially resulted from the reduction of graft inner cells in ISL1<sup>−/−</sup> hESC-retinas, which, surprisingly and beneficially, correlated to better-preserved host IPL organization. In contrast, the thick layers of graft retinal inner cells that were sometimes observed on the RPE side in WT hESC-retinas may hinder visual pigment recycling via RPE in graft photoreceptors. These findings possibly explain the better anatomical and functional reconstruction of rat retinas transplanted with ISL1<sup>−/−</sup> hESC-retina compared with those seen with WT hESC-retina. However, WT hESC-retina was better than ISL1<sup>−/−</sup> hESC-retina in terms of their GNAT1<sup>+</sup> IS/OS morphology. We previously reported that thinning of the graft photoreceptor layer (ONL) by transplantation of the graft at later days of differentiation possibly resulted from a subsequent reduction in graft inner cells, implying a supportive role of inner cells for photoreceptor
survival and maturation. We therefore suggested that there was a necessary trade-off between ONL thickness and host-graft direct contact (Shirai et al., 2016). Here again, the negative correlation between the IS/OS status and RGC responsiveness may imply this trade-off. However, better performance by ISL1−/− hESC-retinas may imply that these transplanted retinas already have an excess functional photoreceptors compared with host-graft synapse formation and that currently host-graft contact and host IPL preservation are the most critical requirements for functional graft integration.

In the current study, the retinal cell profiles were not much different between the WT and ISL1−/− hESC-retinas at the time of transplantation but resulted in different phenotypes after transplantation. A beneficial tip of gene engineering may lie in the case of progenitor cell transplantation as seen in our case, where the graft progenitor cells at the time of transplantation further differentiate and mature, when the engineered gene plays a role to realize the pre-set phenotype sometime after transplantation. By this approach, we were also able to retain the organized structure of the retina transplants, which would be difficult to achieve by purification of graft cells before transplantation. Hence, our approach here would provide a new conceptual approach for utilizing gene engineering in regenerative medicine.

Limitations of the study
Since the report on material transfer from graft photoreceptors to remaining host photoreceptors, whether the reconstructed RGC responses are genuinely derived from the graft photoreceptors or from the rescued host photoreceptors has been a matter of concern (Pearson et al., 2016; Santos-Ferreira et al., 2016; Singh et al., 2016). We believe that immunostaining of multiple synapse markers at host-graft cell contact site are highly suggestive of the presence of some host-graft synaptic connections, but the evidence to directly show how much of these synapses contribute to what part of signaling output by host RGC is yet technically challenging. Other approaches including 3-dimensional electron microscopy or possibly some visual tracing of neuronal-cell activity transmission, for example, may provide further evidence. At present, however, we believe that evident improvement of physiological contact between host bipolar and graft photoreceptor cells associated with a substantial improvement of RGC light responses provides a promising possibility for retinal network reconstruction by cell therapies.

STAR★METHODS
Detailed methods are provided in the online version of this paper and include the following:

● KEY RESOURCES TABLE
● RESOURCE AVAILABILITY
  ○ Lead contact
  ○ Materials availability
  ○ Data and code availability
● EXPERIMENTAL MODEL AND SUBJECT DETAILS
  ○ Human ES cell line
  ○ Rat model
● METHOD DETAILS
  ○ Establishment of ISL1−/− hESC line
  ○ Retinal differentiation and long-term culture
  ○ Flow cytometry
  ○ Transplantation into retinal degeneration model nude rats
  ○ Multi-electrode array (MEA) recording
  ○ Immunohistochemistry (IHC)
  ○ Quantification analysis
  ○ Statistical analysis

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103657.

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AUTHOR CONTRIBUTIONS
M. M. and M.T. designed and supervised the study. S. Y. and T. H. generated the iSL1−/− hESCs. S. Y. and M. H. conducted FCM and IHC analysis. M. M. and J. S. conducted transplantation surgeries. S. Y. and T. M. conducted quantitative analysis. A. Ku., S.Y., A. Ki., and T. K. developed the protocol for 3D retinal differentiation culture from feeder-free hPSCs. H-Y. T. and T. M. conducted MEA recording and data analysis. S. Y., H-Y. T., T. M., and M. M. wrote the manuscript. All authors reviewed and approved the final manuscript.

DECLARATION OF INTERESTS
S. Y., M. H., A. Ku., A. Ki., and T.K. are employed by Sumitomo Dainippon Pharma Co., Ltd. S. Y., M. M., and M. T. are co-inventors on patent applications.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Goat Polyclonal anti-Arrestin 3 (Cone arrestin) | Novus Biologicals | Cat#NBP1-37003  
RRID:AB_2060085 |
| Mouse monoclonal anti-Brevican | BioLegend | Cat#820101  
RRID:AB_2564837 |
| Goat Polyclonal anti-Bm3 | Santa Cruz Biotechnology | Cat#sc-6026  
RRID:AB_673441 |
| Mouse monoclonal anti-CACNA1S | Millipore | Cat#MAB427  
RRID:AB_2069582 |
| Rabbit polyclonal anti-Calbindin | Abcam | Cat#ab108404  
RRID:AB_10861236 |
| Rabbit polyclonal anti-Calretinin | Millipore | Cat#ab5054  
RRID:AB_2068506 |
| Rat monoclonal anti-CD44 | Abcam | Cat#ab119348  
RRID:AB_10902529 |
| Goat polyclonal anti-Choline Acetyltransferase | Millipore | Cat#ab144P  
RRID:AB_2079751 |
| Mouse monoclonal anti-Chx10 | Santa Cruz Biotechnology | Cat#sc-365519  
RRID:AB_10842442 |
| Sheep polyclonal anti-Chx10 | Exalpha Biologicals | Cat#K1180P  
RRID:AB_2314191 |
| Mouse monoclonal anti-CNGB1 | Millipore | Cat#abMN2429 |
| Goat polyclonal anti-CNGB3 | Novus Biologicals | Cat#abBP2-75087 |
| Rabbit polyclonal anti-Crx | Takara Bio Inc. | Cat#M231 |
| Mouse monoclonal anti-CtBP2 | BD Bioscience | Cat#612094  
RRID:AB_2994931 |
| Rabbit polyclonal anti-GNAT1 (Gα1) | Santa Cruz Biotechnology | Cat#sc-389  
RRID:AB_2294749 |
| Rabbit polyclonal anti-GNAT2 (Gα2) | Santa Cruz Biotechnology | Cat#sc-390  
RRID:AB_2279097 |
| Mouse monoclonal anti-G Protein Goαz | Millipore | Cat#abMAB3073  
RRID:AB_94671 |
| Rabbit polyclonal anti-GRK1 | Novus Biologicals | Cat#abBP2-55226 |
| Rabbit polyclonal anti-GUCA1A (GCAP1) | Novus Biologicals | Cat#abBP2-55158 |
| Rabbit polyclonal anti-GUCA1B (GCAP2) | Novus Biologicals | Cat#abBP2-68721 |
| Rabbit polyclonal anti-GUCY2D | Proteintech | Cat#S5127-1-AP  
RRID:AB_10804281 |
| Rabbit polyclonal anti-GUCY2F | Proteintech | Cat#25252-1-AP  
RRID:AB_2879989 |
| Mouse monoclonal anti-Glutamine Synthetase (GS) | Millipore | Cat#abMAB302  
RRID:AB_2110656 |
| Rabbit polyclonal anti-IMPG1 | Novus Biologicals | Cat#abBP2-57461 |
| Rabbit polyclonal anti-IMPG2 | Novus Biologicals | Cat#abBP2-54954 |
| Rabbit polyclonal anti-IRBP (RBP3) | Proteintech | Cat#ab14352-1-AP  
RRID:AB_2096956 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse monoclonal anti-Islet-1 | Developmental Studies Hybridoma Bank (DSHB) | Cat#40.2D6
RRID:AB_528315 |
| Goat polyclonal anti-Islet-1 | R&D Systems | Cat#AF1837
RRID:AB_2126324 |
| Sheep polyclonal anti-Islet-2 | R&D Systems | Cat#AF4244
RRID:AB_2296113 |
| Rabbit monoclonal anti-Ku80 (human specific) | Cell Signaling Technology | Cat#2180
RRID:AB_2218736 |
| Goat polyclonal anti-Ku80 (human specific) | R&D Systems | Cat#AF5619
RRID:AB_2218619 |
| Rabbit polyclonal anti-U/M Opsin (Opsin, Red/Green) | Millipore | Cat#AB5405
RRID:AB_177456 |
| Rabbit polyclonal anti-LRIT3 | Novus Biologicals | Cat#NBP1-83895
RRID:AB_11039569 |
| Rabbit polyclonal anti-L7/Pcp2 | Takara Bio Inc. | Cat#M202 |
| Rabbit polyclonal anti-mGluR6 | Novus Biologicals | Cat#NLS4655
RRID:AB_343723 |
| Mouse monoclonal anti-Nanog | Millipore | Cat#MABD24
RRID:AB_11203826 |
| Rabbit polyclonal anti-Oct3/4 | Santa Cruz Biotechnology | Cat#sc-9081
RRID:AB_2167703 |
| Mouse monoclonal anti-Pax6 | BD Pharmingen | Cat#61462
RRID:AB_10715442 |
| Rabbit polyclonal anti-PDE6a | Novus Biologicals | Cat#NBP1-87312
RRID:AB_11009970 |
| Rabbit polyclonal anti-PDE6b | Novus Biologicals | Cat#NB120-5663
RRID:AB_792693 |
| Rabbit polyclonal anti-PDE6H | Novus Biologicals | Cat#NBP2-68659 |
| Rabbit polyclonal anti-Pikachurin | Abcam | Cat#ab91314
RRID:AB_10861100 |
| Mouse monoclonal anti-PKCα | Novus Biologicals | Cat#NB600-201
RRID:AB_10003372 |
| Goat polyclonal anti-PKCα | R&D Systems | Cat#AF3340
RRID:AB_2168552 |
| Lectin peanut agglutinin (PNA), Alexa Fluor 647 conjugate | Thermo Fisher Scientific | Cat#L32460 |
| Mouse monoclonal anti-Peripherin-2 (PRPH2) | Millipore | Cat#MABN293 |
| Rabbit polyclonal anti-Peripherin-2 (PRPH2) | Proteintech | Cat#18109-1-AP
RRID:AB_10665364 |
| Mouse monoclonal anti-PSD95 | BioLegend | Cat#10401
RRID:AB_2564750 |
| Mouse monoclonal anti-RBPMS | Novus Biologicals | Cat#NBP2-45551 |
| Rabbit polyclonal anti-Recoverin | Proteintech | Cat#10073-1-AP
RRID:AB_2178005 |
| Mouse monoclonal anti-Rhodopsin | Millipore | Cat#MABN15
RRID:AB_10807045 |
| Mouse monoclonal anti-Rxrγ | Santa Cruz Biotechnology | Cat#sc-365252
RRID:AB_10850062 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Mouse monoclonal anti-S arrestin | Novus Biologicals | Cat#NBP2-25161 |
| Sheep polyclonal anti-Secretagogin | BioVendor | Cat#RD184120100 RRID:AB_2034062 |
| Goat polyclonal anti-S-Opsin (Opsi, Blue) | Santa Cruz Biotechnology | Cat#sc-14363 RRID:AB_2158332 |
| Mouse monoclonal anti-Stem123 (human specific GFAP) | Takara Bio Inc. | Cat#Y40420 RRID:AB_2833249 |
| Goat polyclonal anti-Synaptophysin | R&D Systems | Cat#AF5555 RRID:AB_2198864 |
| Mouse monoclonal anti-Versican | Millipore | Cat#MABT161 |
| Alexa Fluor 488 Donkey Anti-Mouse IgG (H+L) | Thermo Fisher Scientific | Cat#A21202 RRID:AB_141607 |
| Alexa Fluor 546 Donkey Anti-Mouse IgG (H+L) | Thermo Fisher Scientific | Cat#A10036 RRID:AB_2534012 |
| Alexa Fluor 647 Donkey Anti-Mouse IgG (H+L) | Thermo Fisher Scientific | Cat#A31571 RRID:AB_162542 |
| Alexa Fluor 488 Donkey Anti-Rabbit IgG (H+L) | Thermo Fisher Scientific | Cat#A21206 RRID:AB_2535792 |
| Alexa Fluor 546 Donkey Anti-Rabbit IgG (H+L) | Thermo Fisher Scientific | Cat#A10040 RRID:AB_2534016 |
| Alexa Fluor 647 Donkey Anti-Rabbit IgG (H+L) | Thermo Fisher Scientific | Cat#A31573 RRID:AB_2536183 |
| Alexa Fluor 488 Goat Anti-Mouse IgM (H+L) | Thermo Fisher Scientific | Cat#A10680 RRID:AB_2534062 |
| Alexa Fluor 546 Donkey Anti-Sheep IgG (H+L) | Thermo Fisher Scientific | Cat#A21098 RRID:AB_2535752 |
| Alexa Fluor 647 Donkey Anti-Sheep IgG (H+L) | Thermo Fisher Scientific | Cat#A21447 RRID:AB_2535864 |
| Mouse monoclonal anti-Chx10, Alexa Fluor 647 conjugate | Santa Cruz Biotechnology | Cat#sc-365519 AF647 RRID:AB_10842442 |
| Mouse monoclonal anti-Islet-1, PE conjugate | BD Biosciences | Cat#562547 RRID:AB_11154592 |
| Mouse monoclonal anti-Ki67, Alexa Fluor 647 conjugate | BD Biosciences | Cat#558615 RRID:AB_647130 |
| Mouse monoclonal anti-Nrl, Alexa Fluor 647 conjugate | Santa Cruz Biotechnology | Cat#sc-374277 AF647 RRID:AB_10991100 |
| Mouse monoclonal anti-Pax6, Alexa Fluor 647 conjugate | BD Biosciences | Cat#562249 RRID:AB_11152956 |
| Mouse monoclonal anti-RXRγ, Alexa Fluor 647 conjugate | Santa Cruz Biotechnology | Cat#sc-365252 AF647 RRID:AB_10850062 |
| Mouse monoclonal anti-Sox2, BV421 conjugate | BioLegend | Cat#656114 RRID:AB_2566262 |
| Mouse IgG1k isotype control, APC conjugate | BioLegend | Cat#400120 RRID:AB_2888687 |
| Mouse IgG2ax isotype control, APC conjugate | BioLegend | Cat#400220 RRID:AB_326468 |

Chemicals, peptides, and recombinant proteins

Knockout Serum Replacement | Thermo Fisher Scientific | Cat#10828-028 |
FBS | Thermo Fisher Scientific | Cat#10270-106 |

(Continued on next page)
## REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| N2-Supplement       | Thermo Fisher Scientific | Cat#17502-048 |
| LM511-E8 matrix     | Matrixome | Cat#892011 |
| Y-27632             | Wako Pure Chemical Industries, Ltd. | Cat#039-24591 |
| SB431542            | Sigma-Aldrich | Cat#S4317 |
| SAG                 | Enzo Biochem Inc. | Cat#ALX-270-M001 |
| BMP4                | R&D Systems | Cat#314-8P-050 |
| GSK-3 Inhibitor     | Wako Pure Chemical Industries, Ltd. | Cat#038-24681 |
| SU5402              | Wako Pure Chemical Industries, Ltd | Cat#197-16731 |
| Neuron Dissociation Solution S | Wako Pure Chemical Industries, Ltd | Cat#297-78101 |
| Stem Fit AK03N      | Ajinomoto | Cat#AK03N |
| DMEM/F-12 Glutamax  | Thermo Fisher Scientific | Cat#10565-018 |
| IMDM GlutaMAX       | Thermo Fisher Scientific | Cat#31980-097 |
| Ham’s F-12 Nutrient Mixture | Thermo Fisher Scientific | Cat#11765-062 |
| CD lipid concentrate | Thermo Fisher Scientific | Cat#11905-031 |
| 1-thioglycerol      | Sigma-Aldrich | Cat#M6145 |
| DMSO                | Wako Pure Chemical Industries, Ltd | Cat#037-24053 |
| citrate buffer      | Thermo Fisher Scientific | Cat#AP-9003-125 |
| 4% paraformaldehyde (PFA) | Wako Pure Chemical Industries, Ltd | Cat#30525-89-4 |
| 4',6-Diamidino-2-phenylindole, dihydrochloride (DAPI) | Thermo Fisher Scientific | Cat#D1306 |
| bovine serum albumin | Sigma-Aldrich | Cat#A4161 |
| L-AP4               | Wako Pure Chemical Industries, Ltd | Cat#016-22083 |
| Opsinamide          | Sigma-Aldrich | Cat#AA92593 |

### Critical commercial assays

- **Human Stem Cell Nucleofector™ Kit**
  - Lonza
  - Cat#VPH-5012

### Experimental models: Cell lines

- **KhES1 Crx::Venus reporter line**
  - Nakano et al., 2012
  - HES0653
- **Ilset-1/-KhES1 Crx::Venus reporter line**
  - This paper
  - N/A

### Experimental models: Organisms/strains

- **SD-Foxn1 Tg (S334ter) 3LavrRrc nude rats**
  - Rat Research Resource Center
  - RRRRC# 539

### Software and algorithms

- **CED spike 2 (version 7.2)**
  - CED
  - NA
- **MC_Rack**
  - Multi Channel System
  - https://www.multichannelsystems.com/software/mc-rack
- **FlowJo v10**
  - BD
  - http://www.flowjo.com/
- **Fiji/ImageJ**
  - National Institutes of Health (NIH)
  - http://fiji.sc
- **IMARIS 8.4**
  - Oxford Instruments
  - https://imaris.oxinst.com
- **R**
  - R Core Team, 2020
  - https://cran.r-project.org
- **ZEN imaging software (blue edition)**
  - Carl Zeiss
  - https://www.zeiss.de/mikroskopie/produkte/mikroskopsoftware/zen-lite.html
- **Code (stan) for statistical analyses**
  - Github
  - https://github.com/matsutakehoyo/KO-graft

### Other

- **USB-MEA60-up-system**
  - Multi channel Systems
  - NA
- **Nucleofector™ 2b**
  - Lonza
  - Cat#AAAB-100
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michiko Mandai (michiko.mandai@riken.jp).

Materials availability
Materials generated in this study are available from the lead contact’s laboratory upon request.

Data and code availability
- The code used for statistical analyses is available in a github repository (https://github.com/matsutakehoyo/KO-graft).
- The datasets supporting the current study have not been deposited in a public repository because of the large size of the data but are available from the corresponding author on request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human ES cell line
hESC (KHeS-1) line (RIKEN BioResource Center, Cell Number: HES0653) was used in accordance with hESC research guidelines of the Japanese government. hESCs express Venus under the control of Crx promoter (Nakano et al., 2012).

Rat model
SD-Foxn1 Tg (S334ter) 3LavRrrc nude rats were obtained from the Rat Resource and Research Center for transplantation study (Seiler et al., 2014).

Animal transplantation experiments were performed in accordance with local guidelines and the ARVO statement on the use of animals in ophthalmic and vision research. All experimental protocols were approved by the RIKEN Biosystems Dynamics Research Ethics Committee and were conducted according to guidelines for animal experiments of the RIKEN. Rats of 16-25 postnatal weeks were used for transplantation. MEA data was analyzed using a total of 31 rats (14 males and 17 females) after transplantation. Among these, 22–23 transplanted retinas were also used for quantitative analysis by immunohistology.

METHOD DETAILS

Establishment of ISL1−/− hESC line
hESCs were maintained using iMatrix511 (Matrixome) and Stem fit (AK03N, Ajinomoto, Japan) feeder free culture (Nakagawa et al., 2014). The Cas9 and puromycin-resistance gene bearing plasmid pSpCas9(BB)-2A-Puro (Addgene), with a pair of CRISPR/Cas9 guide RNAs designed so as to delete the first and second exons of the ISL1 gene (Sequence ID: CCAACTCCGCCGGCTTAAAT, GGGAGGTTAATACTTCGGAG), was transfected to the hESCs by electroporation (Nucleofector IIb, program B-016, Lonza). Transfected hESCs were cultured in a six-well plate (1.0 × 10^3 cells per well; AGC Techno Glass, Japan) coated with iMatrix511 in the presence of 10 μM Y-27632 (ROCK inhibitor, Wako Pure Chemical Industries, Japan) in Stem Fit. One day after inoculation, the medium was replaced without Y-27632. Thereafter, the hESCs were cultured for six days in the presence of 0.5 ng/mL puromycin for puromycin selection. The successfully transfected colonies were picked up and the genomic DNA was analyzed using PCR primers designed around the target site. We then established two clones with ISL1 gene deletion (No. 330A16 line and No. 330A19 line). ISL1 gene-deleted hESC clones were analyzed by Sanger sequencing to confirm disruption of the ISL1 gene.

Retinal differentiation and long-term culture
Retinal differentiation was conducted following the modified SFEBq method we recently reported (Kawahara et al., 2019; Yamasaki et al., 2021) (Nukaya et al.; WO19/017,492, 054,514, 054,515). In brief, subconfluent hESCs were treated with 5 μM SB431542 (TGFβ receptor inhibitor, Sigma-Aldrich) and 300 nM SAG (Smoothened agonist, Enzo Biochem) from 24 h prior to differentiation. Cells were then dissociated into...
single cells using TrypLE Select Enzyme (Thermo Fisher Scientific), suspended in 100 µL serum-free culture medium with 10 µM Y-27632, and cultured at 1.2 × 10⁶ cells per well in low cell adhesion 96-well V-bottomed plates (Sumitomo Bakelite). On differentiation day (DD) 3 after initiation of suspension culture, aggregates were treated with 1.5 nM recombinant human BMP4 protein (R&D Systems). Aggregates on DD14 were transitioned to RPE induction medium with 3 µM CHIR99021 (GSK3β inhibitor, Wako Pure Chemical Industries) and 5 nM SU5402 (FGF signaling pathway inhibitor, Wako Pure Chemical Industries) for 3–4 days in a 90-mm low adhesion culture dish (Sumitomo Bakelite). Subsequently, aggregates were cultured in the maturation culture (Yamasaki et al., 2021) (Nukaya et al. in preparation; WO2019017492A1, WO2019054514A1). The medium was exchanged every 3–4 days.

Flow cytometry

The differentiated retinal cell population was analyzed by staining the intracellular markers for each retinal neuron type. hESC-retina was dissociated using Neuron Dissociation Solution (Wako Pure Chemical Industries) (20-30 min at 37°C) followed by a repeated wash with PBS, and dissociated cells were fixed using Fixation/Permeabilization Solution Kit (BD Biosciences) or transcription factor buffer Kit (BD Biosciences) at 4°C for 20 min. Cells were permeabilized with Perm/Wash buffer, centrifuged, and resuspended in Perm/Wash buffer for a reaction with BV421- or Alexa Fluor 647-conjugated antibodies for 60 min at 4°C. For some antibodies without conjugated fluorochrome, the second antibody was applied at 4°C for 60 min. Then cells were washed twice with Perm/Wash buffer and resuspended in 2% FBS/PBS buffer for FCM analysis using FACS Canto II (BD Biosciences). The antibodies used in this analysis are listed in key resources table. FCM data were analyzed using FlowJo software (BD Biosciences).

Transplantation into retinal degeneration model nude rats

For graft preparation, hESC-retinas were cut into small pieces of approximately 0.5 mm width, indicated by Crx::Venus fluorescence, using micro scissors. Before transplantation surgery, nude rats with retinal degeneration at 16–25 postnatal weeks were anesthetized with ketamine hydrochloride (40–80 mg/kg) and Xylazine (5–10 mg/kg), or by inhalation of 3–5% isoflurane. Pupils were dilated using MydrinP (Santen Pharmaceutical, Japan). The glass capillary (1-000-0500, Drummon, Alabama, USA) for transplantation was pulled with the P-97/IVF puller (SUTTER INSTRUMENT, California, USA), followed by cutting and sharpening using a microgrinder (EG-400, Narishige, Tokyo, Japan). The glass capillary was attached to the microelectrode holder (MPH310, World Precision Instruments., FL, USA) on a 6.3 mm electrode handle (2505, World Precision Instruments.), connected to a 10 µL micro-syringe (1701LT, Hamilton, MA, USA) with an extension tube. hESC-retinas were loaded into the capillary tip and gently transplanted into the subretinal space of rats under a surgical microscope.

Multi-electrode array (MEA) recording

Nude rats transplanted with hESC-retina were used for multi-electrode array (MEA) recording at 14–15 months old (i.e., 8–10 months after transplantation). The procedure of MEA recording with a USB-MEA60-Up-System (Multi Channel Systems) and offline spike sorting are described in detail below (Matuyama et al., 2021; Tu et al., 2019). Rats were dark-adapted for 1–3 days before dissection under a dim red LED that peaked at a wavelength of 700 nm. Isoflurane or sevoflurane inhalation was used for both initial anesthetization and overdose euthanasia. The freshly harvested eyecups were kept in oxygenated Ames’ medium (Sigma-Aldrich) constantly supplied with 95% O₂ and 5% CO₂ in the dark until use. The retina was carefully isolated from the sclera and the graft was identified at the subretinal side by its voluminous, spotted appearance. After removing the residual vitreous, the retina was minimally trimmed to keep its integrity and immediately mounted with the RGC side down and the grafted area centered on the electrode area. From then, the retina was constantly supplied with oxygenated Ames’ medium perfused at 3–3.5 mL/min. The retina was allowed to recover for at least 20 min. Full-field light stimulation at different intensities (weak: 10.56, medium:12.16, strong: 12.84 log photons/cm²/s) was generated with a white LED (N500, Nichia Corp., Tokushima, Japan) without background illumination. Each 20 s recording with 1 s stimulation in the middle was repeated three times as a set, and each set of stimulation was repeated before, during and after the 10 µM L-AP4 (agonistic blocker mGluR6, Wako Pure Chemical Industries) treatment. Much brighter stimulation at 15.48 log photons/cm²/s (super-strong) was applied only at the very end of each experiment for each sample for retinal viability confirmation. To suppress the intrinsic photosensitivity of melanopsin expressing cells, 10 µM opsinamide (Opt4 Antagonist; Sigma-Aldrich) was added to the perfusion medium from the beginning of preparation. After recording, the retina was harvested and fixed with 4% PFA at room temperature for immunohistochemistry.
MEA data were collected at a 20 kHz sampling rate without applying a filter. Recorded spikes were sorted offline, using the automatic template formation and spike matching algorithm of Spike 2 (version 7.2, CED) with a few minor modifications, including a $-20 \mu$V threshold, a 5% tolerance for maximal amplitude change, and a Butterworth band-pass filter (200-2800Hz) together with DC offset removal. Recordings with the same stimulus intensity taken before, during and after L-AP4 treatment were merged to follow the spike trains from the same set of cells during the 3–4 h recording of each sample. The detected cells (i.e., spike sources) were then categorized into unresponsive, onXoff, on and adapted on types (see also Figure 6F) according to their light response pattern during the whole recording procedure.

**Immunohistochemistry (IHC)**

WT and ISL1$^{-/-}$ hESC-retinas and transplanted retinas were fixed in 4% paraformaldehyde at 4°C for 15–60 min and cryoprotected in 30% sucrose/PBS overnight. After MEA recording, transplanted retinas were fixed in 4% paraformaldehyde for 15 min at room temperature for cryo-section immunostaining. hESC-retinas were embedded in OCT compound (Sakura Finetek Japan) and sectioned at 12 μm thickness. Sections were washed with 0.3% Triton X-100/PBS prior to heat-induced epitope retrieval using citrate buffer (Thermo Fisher Scientific) and blocked in 3% BSA/0.3% Triton X-100/PBS for 1 h at room temperature. Primary antibodies diluted in 3% BSA/0.3% Triton X-100/PBS were incubated at 4°C overnight, followed by secondary antibodies for 1 h at room temperature together with DAPI. Tertiary antibodies such as Alexa Fluor 488-conjugated Ku80 were then applied after washing with 0.05% Tween/PBS. All images were obtained using BZX810 (Keyence) fluorescence microscopy, TCS SP-8 (Leica Camera) or LSM880 (Carl Zeiss) laser scanning confocal microscopes, and offline processed using IMARIS and Zen Blue (Carl Zeiss) imaging software.

**Quantification analysis**

For immunohistological features, host-graft contact and IS/OS patterns were quantified based on the categorization indicated in the figures and text, by observing 6–67 rosettes in 1–7 images for each sample. ChAT analysis to determine IPL preservation was assessed in the area over the graft rosettes by observing 5–50 rosettes in 1–6 images for each sample.

**Statistical analysis**

We used full Bayesian statistical inference with MCMC sampling for statistical modeling using Rstan (Stan Development Team. 2017. RStan: the R interface to Stan. R package version 2.16.2. http://mc-stan.org). Our data are multi-dimensional with a natural hierarchical structure, with observations having multiple possible predictors, such as group (control, WT, and KO (ISL1$^{-/-}$)) effects, sex (female or male) effects, inter-sample variation, and other predictors. We estimated the distribution of these effects using hierarchical Bayesian linear regression models. Note that posterior estimates do not represent a simple pooling of data for a particular set of predictor combinations (for example comparing population means); rather they represent the effect of predictors while considering the data as a whole.

We show the posterior distributions of parameters, which indicate the probability for the value of the parameter given the data, with 95% compatibility intervals (confidence intervals) and mean indicated above. Whenever a possible effect or difference between parameters is indicated we estimate the magnitude and credibility of the effect from the distribution of the difference between parameter values. Whenever a difference is indicated we therefore indicate the fraction of the area over zero, which indicates the confidence that we have that there is a difference. Stan scripts for the models are provided in a GitHub repository (https://github.com/matsutakehoyo/KO-graft).

**Analysis of rosette contact, IPL preservation, and OS elongation.** Rosette contact, host IPL preservation, and photoreceptor OS elongation were quantified using an ordinal scale with three categories (for example bad, poor, fair). We estimated the effects of different predictors assuming an underlying metric variable with a normal distribution following (Kruschke, J. Doing Bayesian data analysis: A tutorial with R, JAGS, and Stan. Physical Review E 70, (2014)). Since there are only three categories, we fixed the cutoff intervals to 1.5 and 2.5 and estimated the mean and standard deviation of the underlying metric variable.
Thus, the category for each observation $y_i$ is described by

$$y_i \sim \text{Categorical}(\gamma_1, \gamma_2, \gamma_3)$$

$$\gamma_{1,i} = p(\gamma = 1, \mu, \sigma) = \Phi\left(\frac{1.5 - \mu}{\sigma}\right)$$

$$\gamma_{2,i} = p(\gamma = 2, \mu, \sigma) = \Phi\left(\frac{2.5 - \mu}{\sigma}\right) - \Phi\left(\frac{1.5 - \mu}{\sigma}\right)$$

$$\gamma_{3,i} = p(\gamma = 3, \mu, \sigma, \theta_2) = 1 - \Phi\left(\frac{2.5 - \mu}{\sigma}\right)$$

$$\mu_i = \beta_0 + \beta_{grp} + \beta_{sex} + \beta_{amp}$$

Where $\gamma_k \in \{1, 2, 3\}$ represent the probability of observing each of the categories ($\sum \gamma = 1$). $\gamma_1$ correspond to the area under the normal to the left of 1.5, $\gamma_2$ is the area of the curve between 1.5 and 2.5, and $\gamma_3$ is the area of the normal to the right of 2.5. $\mu$ and $\sigma$ represent the mean and the standard deviation of the underlaying metric variable. We assume equal standard variation across predictors for simplicity, however, we note that similar results were obtained with models where the effects of different predictors on the standard deviation were taken into account. $\beta_0$ represents the overall mean, and the effects of different predictors were calculated as a deviation from this mean with the sum-to-zero constraint on each of the predictors ($\sum \beta_{\text{predictor}} = 0$). For the effect of the sample we estimated hyperparameters from the data; otherwise we used generic weakly informative priors (Normal(0,1)) for predictors. So for $\beta$

$$\beta_{grp} \sim \text{Normal}(0, 1)$$
$$\beta_{sex} \sim \text{Normal}(0, 1)$$
$$\beta_{amp} \sim \text{Normal}(0, \sigma_{amp})$$
$$\sigma_{amp} \sim \text{Normal}(0, 1)$$
$$\sigma \sim \text{Normal}(0, 1)$$

**RGC spontaneous firing (9 s recording before the 1 s stimuli).** We analyzed the distribution of spontaneous firing, by calculating the spontaneous firing rate before light stimulation (9 s). The distribution of spontaneous firing rate closely follows a lognormal distribution, as shown in Figure 6. We therefore modeled the influence of parameters on the mean log spontaneous firing. For the spontaneous firing analysis, we used 60396 observations from 23828 cells collected across 47 retinas. The number of observations in this analysis is higher than the number in the light response analysis, as we consider the spontaneous activity before, during, and after L-AP4 treatment, in addition to the different stimuli.

The spontaneous firing rate is then described by

$$y_i \sim \text{lognormal}(\mu_i, \sigma)$$

$$\mu_i = \mu_0 + \mu_{grp} + \mu_{sex} + \mu_{cnd} + \mu_{amp}$$

$\text{grp} \in \{\text{control, WT, KO}\}$

$\text{sex} \in \{\text{female, male}\}$

$\text{cnd} \in \{\text{before, L - AP4, after}\}$

$\text{stm} \in \{\text{weak, medium, strong}\}$

where $y_i$ is the log spontaneous firing rate for a particular observation $i$. $\mu_0$ and $\sigma$ are the overall logmean and logsd of the lognormal distribution. We assume a common $\sigma$ to simplify the model. The effects of different predictors were calculated as a deviation from this mean with the sum-to-zero constraint on each of the predictors ($\sum \mu_{\text{predictor}} = 0$).

We used a flat prior for $\sigma$ and a vague prior derived from the data for the overall mean ($\mu_0$).

$$\mu_0 \sim \text{Normal (log}(\bar{y}), 1)$$
$$\sigma \sim \text{Uniform}(0, \text{Inf})$$

For the effect of sample, we estimated the hyperparameter ($\sigma_{amp}$) from the data; otherwise we used generic weakly informative priors (Normal(0,1)).

$$\mu_{grp} \sim \text{Normal}(0, 1)$$
$$\mu_{sex} \sim \text{Normal}(0, 1)$$
$$\mu_{cnd} \sim \text{Normal}(0, 1)$$
The response types to a 1 s stimulus for each cell in each condition were first described as unresponsive, transient ON, sustained ON, delayed ON, ON suppression, OFF, ON-OFF and hypersensitive separately (see also Matsuyama et al., 2021), by comparing the onset and termination timing of their spiking frequency changes in response to the stimulation. Cells were then grouped as “flight” (unresponsive), “on”, “adapted on” or “onXoff” types based on their response patterns across all three conditions (before, during and after L-AP4). In principle, “on” cells showed L-AP4 sensitive ON (including sustained ON) responses throughout the whole recording, while “adapted on” cells had their ON responses (mostly sustained ON) responses after recovery from L-AP4. Although cells with OFF responses were also included, the rarely found “onXoff” type mostly consists of cells showing ON and OFF responses that were both sensitive to L-AP4 blockade, suggesting an ON-dependent OFF pathway involvement in the synaptic inputs to these cells. Note that delayed ON and hypersensitive responses were seldom observed and proved independent of the synaptic inputs; they were therefore assigned to the “flight” unresponsive type. Responses that were not consistent across the three replicate recordings were disregarded.

We collected 19,662 observations from 19,662 cells collected across 47 retinas. Note that responses from the same set of cells to different stimulus strengths are treated as different cells. We then modeled the probability of observing a light response (either on, onXoff or adapted on) using robust logistic regression. The probability of observing a light response \( y_i \) is

\[
y_i \sim Bernoulli(\theta_i)
\]

\[
\theta_i = \frac{1}{Z} guess + (1 - guess) \logistic(\beta_0 + \beta_{grp} + \beta_{stm} + \beta_{sex} + \beta_{imp} + \beta_{spt} \times t)
\]

\[
grp \in \{\text{control, WT, KO}\}
\]

\[
sex \in \{\text{female, male}\}
\]

\[
stm \in \{\text{weak, medium, strong}\}
\]

where \( y_i \) is either 0 (no light response) or 1 (light responsive) and \( \theta_i \) is the probability that the cell will respond to the light stimulus. \( \beta_0 \) represents the overall mean and the effects of different predictors were calculated as a deviation from this mean with the sum-to-zero constraint on each of the predictors \( \sum \beta_{\text{predictor}} = 0 \). \( \beta_{spt} \) representing the effect of spontaneous firing, and \( t \) the spontaneous firing rate of each cell. The spontaneous firing rate during L-AP4 treatment was taken as the most representative value for the cell spontaneous frequency, as this condition represents the cells’ firing without input from the outer retina.

The prior for the overall mean (\( \beta_0 \)) was estimated from the data (in log odds, probability of response: \( p_{\text{wt}} \)) with sd of 1.

\[
\beta_0 \sim Normal(\logit(p_{\text{wt}}), 1)
\]

For the effect of the sample, we estimated the hyperparameter (\( \sigma_{imp} \)) from the data, otherwise we used generic weakly informative priors (Normal(0,1)).

\[
\beta_{grp} \sim Normal(0, 1)
\]

\[
\beta_{sex} \sim Normal(0, 1)
\]

\[
\beta_{stm} \sim Normal(0, 1)
\]

\[
\mu_{imp} \sim Normal(0, \sigma_{imp})
\]

\[
\sigma_{imp} \sim Normal(0, 1)
\]

For the guess parameter, which represents outliers, we followed the recommendation from Kruschke (Kruschke, 2014) for robust logistic regression (Chapter 21.3). Using Beta(1, 9) which emphasizes small values, reflecting our expectation that the proportion of outliers is small. In particular, Beta(1, 9) gives values greater than 0.5 a very small but non-zero probability.

\[
guess \sim Beta(1, 9)
\]
Feature correlation analysis. Correlation among RGC spontaneous activity, RGC light responsiveness, rosette contact, photoreceptor OS elongation, and IPL preservation was analyzed by extending Pearson’s correlation test to a Bayesian framework. We used sample estimates from the respective analyses and estimated their correlation assuming a bivariate distribution. A total of 21 samples, where information was available for all the analyzed features, was used for this analysis. For the bivariate distribution we used the Student’s t distribution instead of the normal distribution and estimated the degrees of freedom \( \nu \) from the data to implement robustness.

\[
x \sim \text{MultiStudent}_t(\nu, \mu, \Sigma)
\]

The correlation and standard deviation between two features is given by the Variance-Covariance matrix \( \Sigma \), which was estimated by Cholesky decomposition

\[
\Sigma = LRL
\]

where \( L \) is the diagonal matrix with standard deviations (\( \sigma \)) and \( R \) is the correlation matrix. \( R \) is further decomposed to two triangular matrices

\[
R = L_{\text{corr}} \cdot L_{\text{corr}}^T
\]

For the mean and standard deviations we used weakly informative priors.

\[
\mu \sim \text{Normal}(0, 1) \quad \sigma \sim \text{Normal}(0, 1)
\]

For the correlation matrix we used the LKJ correlation distribution with \( \eta = 2 \) which is moderately skeptical of extreme correlations near \(-1 \) or \( 1 \).

\[
L_{\text{corr}} \sim \text{LKJcorr}(2)
\]

We followed Stan’s recommendations for the degrees of freedom of the Student’s t distribution.

\[
\nu \sim \text{Gamma}(2, 0.1)
\]