Eys<sup>+/−</sup>; Lrp5<sup>+/−</sup> Zebrafish Reveals Lrp5 Can Be the Receptor of Retinol in the Visual Cycle

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**HIGHLIGHTS**

- Eys<sup>+/−</sup>; Lrp5<sup>+/−</sup> zebrafish showed mild photoreceptor degeneration
- Microarray analysis identified dramatical decrease of rbp1a expression
- Rbp1a protein was colocalized with Lrp5 protein at the microvilli of the RPE
- Rbp1a directly bound to the C-terminal intracellular region of Lrp5 in vitro
**SUMMARY**

Vision is essential for vertebrates including humans. Sustained vision is accomplished by retinoid metabolism, the “visual cycle,” where all-trans retinol (atROL) is incorporated into the retinal pigment epithelium (RPE) from photoreceptors presumably through decade-long missing receptor(s). Here, we show that the LDL-related receptor-5 (Lrp5) protein is linked to the retinol binding protein 1a (Rbp1a), the transporter of atROL in the visual cycle, by generating and analyzing the digenic eyes shut homolog+/--; lrp5+/-/C0 zebrafish, the same form of gene defect detected in a human case of inherited retinal degeneration. Global gene expression analysis followed by genetic study clarified that rbp1a played a role downstream of lrp5. Rbp1a protein was colocalized with Lrp5 protein at microvilli of RPE cells. Furthermore, Rbp1a directly bound to the C-terminal intracellular region of Lrp5 in vitro. Collectively, these results strongly suggest that Lrp5 is a potent candidate of the receptor of atROL in the visual cycle.

**INTRODUCTION**

In the vertebrate retina, two types of photoreceptors mainly mediate vision: rods and cones (Kawamura and Tachibanaki, 2008). Upon light absorption by the visual opsins, their chromophore, 11-cis retinal, is converted to all-trans retinol (atROL) and is released from rods and cones. Sustained vision, essential for vertebrates including humans, is accomplished by retinoid metabolism, the “visual cycle” (Lamb, 2013; Sparrow et al., 2010), where atROL is incorporated into the adjacent retinal pigment epithelium (RPE), isomerized to the 11-cis isomer, and transported back to photoreceptors. The uptake of atROL by RPE cells is thought to be mediated by a membrane receptor (Sparrow et al., 2010); however, the entity has been unknown for a decade.

Human eyes shut homolog (EYS) gene (Online Mendelian Inheritance in Man no. 612424), a homolog of the Drosophila eyes shut/spacemaker (eys) (Abd El-Aziz et al., 2008; Collin et al., 2008), is a major causative gene of nonsyndromic autosomal recessive retinitis pigmentosa (RP) (Abd El-Aziz et al., 2008; Hosono et al., 2012; Iwanami et al., 2012), and later analyses using next-generation sequencing (NGS) extended that mutations in EYS also cause cone-rod dystrophy (CRD) (Katagiri et al., 2014; Pierrache et al., 2019). RP, the most common genetic disorder among the inherited retinal dystrophies, is characterized by progressive visual loss caused by degenerative abnormalities of rods, and CRD is initiated by degenerative abnormalities of cones. Therefore, human genomics suggests that EYS protein function(s) crucially contribute to both rods and cones.

Previous studies using zebrafish ortholog si:ch211-69e5.1 or eys from our and other laboratories indicate that the full-length Eys protein may be important for Eys protein function(s) in terms of its pathogenesis (Lu et al., 2017; Takita et al., 2019) and that dysfunction of ciliary pocket of cones observed in eye-/- zebrafish may be the cause of photoreceptor degeneration (Yu et al., 2016); however, exact molecular function of Eys in photoreceptors is still not well understood.

Recently, a possibility emerged from a human case report in a Chinese family by a targeted NGS approach that digenic heterozygous form of mutations in the low-density lipoprotein (LDL)-related receptor-5 (LRPS) and EYS genes (EYS+/-; LRP5+/-) might cause RP (Gao et al., 2017). LRPS protein, a member of the LDL receptor superfamily, is best known as a co-receptor for Wnt ligands involved in the wingless (Wnt) signaling pathway on cell membranes, which is essential for the development of vascular endothelial cells, Müller cells, and retinal interneurons (Tamai et al., 2000). LRPS is also reported to be involved in cholesterol...
and glucose uptake (Chin et al., 2015; Fujino et al., 2003), and its possible involvement in vitamin A (i.e., atROL) system is also suggested (Figuerola et al., 2000).

LRP5 is known to cause familial exudative vitreoretinopathy (FEVR) (Gong et al., 2001; Li et al., 2018; Yang et al., 2012); however, the form of the mutation in LRP5 found in the pedigrees of FEVR is typically autosomal recessive (Li et al., 2018; Yang et al., 2012) and no mutations have been linked to RP so far (https://sph.uth.edu/retnet/). Mutations causing RP in EYS are also believed to be typically recessive (Iwannami et al., 2012). Therefore, it was necessary to examine the possible pathogenesis of the digenic EYS+/−; LRP5+/− mutations, apparently a rare form of mutation, using animal models carrying the same form of mutations. More importantly, because the digenic mutations in peripherin (PRPH2) and ROM1 cause RP and they physically interact with each other (Goldberg and Molday, 1996; Kajiwara et al., 1994), we presumed that it would not be unreasonable to also assume a similar scenario, that EYS and LRP5 proteins may interact with each other and the reduced expression levels of normal EYS and LRP5 proteins may not be sufficient enough to sustain the maintenance of photoreceptor function in patients, which may cause RP. If this hypothesis is correct, it could help elucidate the molecular function(s) of EYS and/or LRP5 proteins.

Here, we show that the digenic eys+/−; lrp5+/− heterozygous mutations cause mild photoreceptor degeneration. Global gene expression analysis of the eys+/−; lrp5+/− eye by the microarray reveals the remarkable decrease in the expression level of the retinol binding protein 1a (rbp1a) gene, which encodes the transporter of atROL in the RPE in the visual cycle (Lamb, 2013; Sparrow et al., 2010), and therefore it appears to be the hallmark of the digenic mutations. We further show that Lrp5 protein is a strong candidate for the receptor of atROL being involved in the uptake of atROL with Rbp1a in the RPE in the visual cycle.

RESULTS
Digenic eys+/−; lrp5+/− Zebrafish Shows Signs of Mild Photoreceptor Degeneration

In the previous human case report (Gao et al., 2017), the possibility that EYS+/−; LRP5+/− might cause RP is reported based on single male patient from single family. To examine whether the digenic heterozygous mutations would lead photoreceptor degeneration under experimental conditions, we first generated zebrafish mutants carrying digenic eys+/−; lrp5+/− mutations by crossing the eys+/−;1907 (eys+/−) and lrp5+/−;11097 (lrp5+/−) mutant lines (Figure 1A, arrows; Figures S1A and S1B; obtained from the Zebrafish International Resource Center [ZIRC]) as a complementary genetic study using tractable animal models. At 14 months post fertilization (mpf), we sectioned the eyes and measured the thickness of the photoreceptor cell layer (PRCL) and number of nuclei in the outer nuclear layers (ONL). The thickness of the PRCL and the number of nuclei in the ONL of the eys+/−; lrp5+/− mutants were 10% and 16% reduced compared with that of wild-type PRCL or ONL, respectively (Figure 1B, middle and right panels, respectively). The result showed mild photoreceptor degeneration in the digenic eys+/−; lrp5+/− mutant retina. In addition, thickness of the RPE layer was 14% thinner in the eys+/−; lrp5+/− eye than in the wild-type eye (Figure 52A). No reduction in the thickness of PRCL or number of nuclei in the ONL was observed in the single eys+/− or lrp5+/− mutant eye (Figures S2B and S2C). Therefore, retinal changes were not due to either of the single mutations, but rather due to the digenic mutations.

We also performed TUNEL staining using 8 mpf eye sections to examine apoptosis in the PRCLs (Figure S3). In wild-type adult eyes, we detected almost no apoptotic cells in all of the cell layers in the retina or retinal pigment epithelium (RPE) (Figure S3A). In contrast, in the digenic eys+/−; lrp5+/− mutant eyes (Figure S3C), apoptotic cells were sparse but were detected in the central region of PRCL, RPE cell layer, retinal ganglion cell layer, and inner nuclear layer. In the peripheral regions, apoptosis was intensely observed in all of the cell layers. These results also support the hypothesis that EYS+/−; LRP5+/− cause RP in humans, which was proposed in the previous study (Gao et al., 2017).

Identification of rbp1a as a Remarkably Downregulated Gene in the eys+/−; lrp5+/− Mutant Eyes

In previous studies in the eys−/− zebrafish, apoptosis is observed at 6, 8, and 14 mpf and the ratio of apoptosis increases with age (Lu et al., 2017; Yu et al., 2016). Also, the expression levels of marker proteins appear normal at least until 2 mpf. Therefore, we performed microarray analysis at 3.5 mpf so as to capture such candidate genes that would show remarkable decrease in the expression levels, caused more directly by the dysfunction of the genes in the initial phase rather than other genes in which marked decrease in the
expression levels can be attributed to the secondary effect caused by the increased apoptosis in PRCL and other layers in the retina, which could mask crucial genes essential for photoreceptors.

In the eys+/−; lrp5+/− eyes, 0.89% (529 genes) of total genes (59,384) were upregulated and 0.80% (473 genes) were downregulated (Figures 2A–2C). Among the genes, mhc2a1 was the one that was most downregulated (Figure 2D, fold change, 0.0159). mhc2a1 is one of the major histocompatibility complex (MHC) class II genes that are known to be critical for the cellular immune response (Bander et al., 1997) and are related to apoptotic pathways; the main function is to present processed antigens (Holling et al., 2004). Therefore, it appeared that the decrease in the expression level of mhc2a1 was more related to cell survival in response to the abnormality in the digenic eys+/−; lrp5+/− heterozygous mutations than was related to specific Eys and/or Lrp5 function(s).
Figure 2. Global Gene Expression Analysis of the Digenic eys+/−; lrp5+/− Zebrafish Eyes Identifies the retinol binding protein 1a (rbp1a) Gene.

(A and B) About 1.69% (1,002 genes) were 2-fold changed among the 59,384 transcript clusters (genes) in the eys+/−; lrp5+/− zebrafish eyes compared with the eyes of wild-type siblings (A). About 0.80% (473 genes) of the total were 2-fold downregulated and 0.89% (529 genes) were 2-fold upregulated (B, green and red spots, respectively).

(C) About 3.31% (1,966 genes) were 2-fold changed, 1.69% (1,002) genes were 2-fold downregulated, and 1.76% (1,044 genes) were 2-fold upregulated in the eys−/−, lrp5−/−, or eys+/−, lrp5+/− eyes compared with the eyes of wild-type siblings (left, middle, and right panels, respectively). Among 0.80% (473 genes) 2-fold downregulated in the eys+/−; lrp5+/− eyes (middle panel), 21.1% (100 genes) were overlapped with the eyes of eys−/− siblings and 14.6% (108 genes) were overlapped with the eyes of lrp5−/− siblings.

(D) Genes whose expression levels were dramatically changed were extracted. “Fold change” in the fourth column indicates the ratio (the bare value of the signal for eys−/−; lrp5−/− divided by that for the wild-type). The ratio for si:ch211-119o8.7 (the rbp1a gene, third and fourth rows) was identified as the gene remarkably downregulated in the eys−/−; lrp5−/− eyes (fold changes, 0.0524 (ID: 13304984) and 0.0620 (13083422)). “Signal for eys−/−; lrp5−/−” (second column) and “Signal for wild type” (third column) are shown in (log2).

| ID          | Signal for eys−/−; lrp5−/− (log2) | Signal for wild type (log2) | Fold change | Gene symbol; Accession |
|-------------|----------------------------------|----------------------------|-------------|------------------------|
| 132826334   | 1.67                             | 7.65                       | 0.159       | rme22c1-NM_131895      |
| 132936308   | 4.11                             | 9.14                       | 0.305       | ?                      |
| 133049694   | 5.08                             | 9.33                       | 0.524       | si-ch211-119o8.7 (rbp1a) |
| 133048322   | 3.37                             | 7.38                       | 0.660       | si-ch211-119o8.7 (rbp1a) |
| 131249368   | 2.67                             | 6.66                       | 0.627       | GENDSCAN0000000398112 |
| 133033896   | 5.03                             | 9.68                       | 0.748       | ?                      |
| 133048368   | 2.14                             | 8.07                       | 0.794       | LOC103011364            |
| 132991181   | 4.72                             | 4.31                       | 0.643       | xab1161a7               |
| 131259372   | 2.09                             | 6.18                       | 0.633       | LOC1000000764           |
| 132015603   | 3.71                             | 9.91                       | 0.683       | ?                      |
| 132037116   | 1.07                             | 4.37                       | 0.1110      | ?                      |
| 132084966   | 3.56                             | 6.61                       | 0.1150      | rbp17                 |
| 13205714    | 9.68                             | 9.68                       | 0.1286      | rbp17                 |
| 132048856   | 6.68                             | 12.08                      | 0.1388      | age                    |
| 132049762   | 5.22                             | 8.12                       | 0.1332      | age                    |
Figure 3. The *rbp1a* Gene Plays a Role Downstream of the *lrp5* Gene

(A) qPCR analysis showed that the expression level of the *rbp1a* gene was remarkably decreased in the *lrp5*+/− (−11%) and digenic *eys*+/−; *lrp5*+/− (−7%) eyes while moderately decreased in the *eys*−/− eyes (−51%) compared with wild-type eyes at 3.5 mpf.
These lines of evidence convinced us to pursue the levels of rbp1a. We then confirmed the downregulation of the related genes in the initial phase. (Bonilha et al., 2004; Huang et al., 2009), eyes were next co-stained with the antibody to MOESIN (MSN) in the outer retina (Figures 4A, S5A, and S5B). As Rbp1a is known to be localized to the microvilli in the RPE localization in the adult eye by immunohistochemistry. Immunopositive signal for Rbp1a was observed in the Eys and/or Lrp5 proteins. First, we raised an antibody against zebrafish Rbp1a protein and examined its genetic interaction between the Eys and/or Lrp5 proteins. The gene that was second-most downregulated in the digenic eyes compared with wild-type siblings was s:ch211-1190a8.7 (rbp1a, rbp1.2) (Figure 2D, fold changes, 0.0524 and 0.0620). RBP1 protein (i.e., CRBP) is well known to be involved in the transport of atROL in the RPE in the visual cycle (Lamb, 2013; Sparrow et al., 2010). In addition, Rbp1a was shown to cause photoreceptor outer segment degeneration under vitamin A-deficient diet conditions (Ghyselinck et al., 1999). Furthermore, in zebrafish, there is a paralog, rbp1b (rbp1.1). The expression level of rbp1b determined by cap analysis of gene expression (CAGE) (Takita et al., 2019) is 12.8% of that of rbp1a, indicating that rbp1a is the predominant gene expressed in the eye (Figure S2D). Also, the expression level of rbp1b was also 20% reduced in the digenic eye (Figure S2E). These lines of evidence convinced us to pursue the rbp1a gene as the most relevant among the candidates for the related genes in the initial phase.

We also stained the eye with anti-fibrillary acidic protein (GFAP) and anti-glutamine synthetase (GS) to visualize the processes of Müller cells. The upper-most intensities of the signal were observed in the lower part of ONL where nuclei of rods and UV cones are located (Figures S5E and S5F). Co-stained with the antibody to Rbp1a also clarified that the upper signals were located within the microvilli of RPE cells, whereas the lower signals were located within the processes of Müller cells (Figure S5G, arrows and arrowheads, respectively).

We confirmed the downregulation of rbp1a by quantitative real-time PCR (qPCR). The expression levels of rbp1a in the eyes+/−, lrp5+/−, ey+/−, and lrp5−/− mutant eyes were 6.8%, 51.4%, 10.9% compared with wild-type eyes, respectively (Figure 3A). Besides, no change was observed in the expression level of prom1a or prom1b (Figures 3B and 3C, respectively). The qPCR results were in good agreement with the microarray results.

We further sought to determine how the remarkable downregulation of the gene might be related to the Eys and/or Lrp5 proteins. First, we raised an antibody against zebrafish Rbp1a protein and examined its localization in the adult eye by immunohistochemistry. Immunopositive signal for Rbp1a was observed in the outer retina (Figures 4A, S5A, and S5B). As Rbp1a is known to be localized to the microvilli in the RPE (Bonilha et al., 2004; Huang et al., 2009), eyes were next co-stained with the antibody to MOESIN (MSN) in the outer retina (Figures 4B, C, and D). We also stained the eye with anti-fibrillary acidic protein (GFAP) and anti-glutamine synthetase (GS) to visualize the processes of Müller cells. The upper-most intensities of the signal were observed in the lower part of ONL where nuclei of rods and UV cones are located (Figures S5E and S5F). Co-stained with the antibody to Rbp1a also clarified that the upper signals were located within the microvilli of RPE cells, whereas the lower signals were located within the processes of Müller cells (Figure S5G, arrows and arrowheads, respectively).

Colocalization of Lrp5 and Rbp1a Proteins in the Outer Retina

Because the Rbp1 protein is known to play an important role in the visual cycle (Lamb, 2013; Sparrow et al., 2010), we next sought to determine how the remarkable downregulation of the gene might be related to the Eys and/or Lrp5 proteins. First, we raised an antibody against zebrafish Rbp1a protein and examined its localization in the adult eye by immunohistochemistry. Immunopositive signal for Rbp1a was observed in the outer retina (Figures 4A, S5A, and S5B). As Rbp1a is known to be localized to the microvilli in the RPE (Bonilha et al., 2004; Huang et al., 2009), eyes were next co-stained with the antibody to MOESIN (MSN) in the outer retina (Figures 4B, C, and D). We also stained the eye with anti-fibrillary acidic protein (GFAP) and anti-glutamine synthetase (GS) to visualize the processes of Müller cells. The upper-most intensities of the signal were observed in the lower part of ONL where nuclei of rods and UV cones are located (Figures S5E and S5F). Co-stained with the antibody to Rbp1a also clarified that the upper signals were located within the microvilli of RPE cells, whereas the lower signals were located within the processes of Müller cells (Figure S5G, arrows and arrowheads, respectively).

Direct Binding of Rbp1a Protein and C-Terminal Intracellular Region of Lrp5 Protein In Vitro

The colocalization of Rbp1a and Rbp1b proteins suggests that they may form a complex at the microvilli of the RPE, indicative of possible involvement of Lrp5 in the uptake of atROL. In order to test the possibility
Figure 4. Lrp5 and Rbp1a Proteins Are Colocalized in the Outer Retinas and Bind Directly In Vitro.
(A) Staining of Rbp1a protein was observed in the outer retina (arrowheads). Nuclei were stained with propidium iodide (PI) (red).
(B and C) Rbp1a signal overlapped well with MOESIN (MSN), a marker of the microvilli of the RPE, and strong signal was observed at the tip (arrowheads), which suggested that Rbp1a was concentrated in the tip of the microvilli in the RPE.
The rbp1a Gene Plays a Role Downstream of the lrp5 Gene in the Eye

The expression level of the rbp1a gene was remarkably decreased (~10%) compared with the wild-type siblings in the lrp5/C0 adult eyes (Figure 3A). To examine the possibility that the rbp1a and lrp5 genes could interact with each other in the eye, rbp1a-/- zebrafish was generated by the CRISPR-Cas9 system (Figure S1C) and the expression level of the lrp5 gene in the rbp1a-/- eyes was measured by qPCR at 3.5 mpf. The qPCR analysis showed that the expression level of lrp5 was not remarkably changed in the rbp1a-/- adult eyes compared with the wild-type siblings (~142%) (Figure 3D). The genetic study clarified that rbp1a-/- played a role downstream of lrp5 rather than genetically interacting with each other.

DISCUSSION

In the present study, we showed photoreceptor degeneration and remarkably decreased expression level of the rbp1a gene in the digenic eys/C0; lrp5/C0 zebrafish. Localization studies revealed that the Rbp1a protein is colocalized with the Lrp5 protein at the microvilli of the RPE. We showed the direct binding of Rbp1a to the C-terminal intracellular region of Lrp5 in vitro.

Photoreceptor Degeneration and Implication for Possible Treatment for Digenic eys/C0; lrp5/C0 Mutations

In this study, we showed in zebrafish that the thickness of the PRCL in the eys/C0; lrp5/C0 mutants is mildly reduced compared with that of the wild-type PRCL. The results using the zebrafish model strongly support the previous human genomic analysis that EYS/C0; LRPS/C0 might cause RP, which is based on one patient from a single family. In addition, because the RPE layer was thinner and apoptosis was also observed in the RPE layer, the RPE was degenerating as well as the photoreceptors in the eys/C0; lrp5/C0 mutants.

We next showed by global gene expression analysis that the expression level of rbp1a is remarkably decreased in the eys/C0; lrp5/C0 mutant eye. Rbp1 protein is involved in the transport of atROL in the RPE in the visual cycle. Furthermore, in mice, knockout of the ortholog, Rbp1, causes photoreceptor outer segment abnormalities under vitamin A-deficient diet conditions (Ghyselinck et al., 1999). Therefore, vitamin A ingestion may help prevent or slow the progression of photoreceptor degeneration in the digenic eys/C0; lrp5/C0 heterozygous mutations in humans.

Lrp5 Is a Strong Candidate for the Receptor of atROL

It has not been clear whether the uptake of atROL on the apical surface of the RPE in the visual cycle is mediated by a protein docking or receptor-mediated protein interaction(s) (Cook et al., 2017). On the basal side of the RPE, uptake of atROL is mediated by “stimulated by retinoic acid gene 6” (STRA6), the receptor for retinol binding protein (Kawaguchi et al., 2007). Accordingly, STRA6 is reported not to be expressed on the apical surface of the RPE (Kawaguchi et al., 2007); however, some researchers have postulated a similar mechanism on the apical side of the RPE (Sparrow et al., 2010).
Interestingly, Lrp5 protein is colocalized with Rbp1a protein at the microvilli of the RPE in the outer retina and Rbp1a is directly binds to the C-terminal intracellular region of Lrp5 in vitro (Figures 4D and 4F). We also showed that rbp1a plays a role downstream of lrp5 in a genetic study (Figures 3A and 3D). Previous studies show that LRPS is involved in uptake of small molecules such as cholesterol (Fujino et al., 2003) and glucose (Chin et al., 2015); in the case of glucose, the mechanism is thought to be independent of the canonical LRPS function where LRP5 is the frizzled co-receptor of WNT signaling. Furthermore, immunohistochemical approach using various human, mouse, and Rhesus monkey tissues suggests that LRPS is expressed in such cell types that transport, store, and metabolize retinoids (Figueroa et al., 2000). In the visual cycle, RBP1 functions in transporting atROL in the cytoplasm at the microvilli of the RPE. Taken together, these results lead us to propose that LRPS may be involved in the uptake of atROL at the microvilli of the RPE, and RBP1 is the downstream effector transporting atROL in the visual cycle (Figure 5, orange and light green in the right bottom, respectively). It would be of interest in future studies to examine the possible binding of atROL, its possible interaction with inter-photorceptor retinol binding protein (IRBP), the possible presence of coreceptor(s) (e.g., peropsin [RRH] [Cook et al., 2017]), and other possible transport pathway(s) of atROL from photoreceptors (Figure 5, shown with “?”). In addition, the signals that were Rbp1a (and Lrp5) positive but MSN negative in the outer retina (Figures 4A, 4C, 4D, and 5S) could be attributed to the processes of Müller cells (Figures 5S–SSG) (Bok et al., 1984; Bunt-Milam and Saari, 1983), which may suggest a possible involvement of RBP1 and LRPS in the cone-specific visual cycle as well as the canonical visual cycle (Wang and Kefalov, 2011) and, therefore, would also be an interesting subject for future studies.

Insights into Understanding Functional Role(s) of Vertebrate Eys

In previous reports using zebrafish, immunohistochemical studies show that Eys protein is localized near connecting cilium/transition zone in photoreceptors (Liu et al., 2020; Messchaert et al., 2018; Yu et al., 2016) and the disruption of ciliary pocket in cones is observed in eyes+/− (Yu et al., 2016). In Drosophila, a negative type of genetic interaction (Baryshnikova et al., 2010; Shen et al., 2017) is shown between Eys and Prominin-1 (Prom1) genes in photoreceptors (Gurudev et al., 2014; Nie et al., 2012).

Based on the reports, we initially examined the possibility of a negative genetic interaction of eys and prom1a and/or prom1b, the zebrafish orthologs of Prom1, in the adult eye by qPCR using eys+/− and prom1a+/−; prom1b+/− zebrafish eyes (Figures S1A, S1D, and S1E). Unfortunately, we were not able to observe any negative genetic interactions at least in the eye of zebrafish under the experimental conditions in this study (Figures 3B, 3C, and 3E). The results may reflect differences between vertebrate and invertebrate photoreceptors (Lamb, 2013). Future studies would be necessary to investigate whether their interaction at the genetic level could be observed in the zebrafish eyes having mutations at different positions on the eys, prom1a, and prom1b genes and/or by using photoreceptors. Confirmation of their binding at the protein level would be more rigorous, straightforward, and therefore promising.

On the other hand, we showed that the expression levels of the rbp1a gene and the Rbp1a protein are decreased in the eys+/− zebrafish eye for the first time, although the degrees were milder than those in the eys−/−; lrp5−/− zebrafish eye. Genetic studies suggest that the remarkable decrease in the expression level of rbp1a is not induced by lrp5 alone but that eys also contributes to the striking reduction (Figures 3A and S2F). Our present data may suggest a possible function of Eys in the visual cycle. Because it is believed that the ciliary pocket is the site of endocytosis and exocytosis (Ghossoub et al., 2011), it may be possible that Eys-deficiency may somehow impair release of atROL from cones. Whether this is related to dysfunction of the ciliary pocket or this is another possible contribution of Eys function is an interesting subject in future studies.

The Fate of Retinoid Metabolites in the Mutant Eyes and Possible Functional Implications of the Proteins

Because flaw of retinoids would be supposed to be impaired due to shortage of Rbp1a protein within the RPE, we postulate that atROL would probably be accumulated in the eye in the eys+/−; lrp5+/− mutant compared with wild-type eye. In the lrp5−/− mutant eye, atROL may be accumulated in the eye also presumably as a result of failure of incorporation of atROL by the RPE. atROL may be accumulated between photoreceptors and RPE (i.e., inter-photoreceptor matrix, IPM) in the lrp5−/− mutant. In the eys−/− mutant eye, it may be possible that atROL may be accumulated in the eye also if Lrp5 and Eys would physically...
interact with each other because atROL could not be incorporated into the RPE from IPM. Alternatively, it may be possible that atROL may be accumulated in photoreceptors if Eys would be involved in the retinoid metabolism within photoreceptors because atROL could not correctly be released from photoreceptors.

In the present study, we shed light on the decade-long missing link in vision by analyzing the digenic eys+/−; lrp5+/− mutations. With recent great advances and lower costs of NGS technology, which have made it far easier to detect digenic (or even more, i.e., polygenic) mutations, a combination of detection and comprehensive analysis of polygenic inheritance could be a powerful tool to dissect molecular networks such as

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Figure 5. A Proposed Model of LRPS Function in the Visual Cycle
LRP5 protein (shown in orange in right bottom) is a strong candidate for the receptor of atROL by forming a complex with RBP1 protein (the transporter of atROL, shown in light green in right bottom) and may be involved in the uptake of atROL in the RPE in the visual cycle. The flow of retinoids is shown by red arrows and the unknown possible interactions are indicated with "?". atROL, all-trans retinol; ABCA4, ATP binding cassette subfamily A member 4; IRBP, interphotoreceptor retinol binding protein (also known as RBP3, retinol binding protein 3); LRAT, lecithin retinol acyltransferase; LRPS, LDL receptor related protein 5; RBP1, retinol binding protein 1 (also known as CRBP, cellular retinol binding protein); RDH, retinol dehydrogenase; RLBP1, retinaldehyde binding protein 1 (also known as CRALBP, cellular retinal binding protein); RPE65, retinoid isomerohydrolase RPE65; STRA6, stimulated by retinoic acid gene 6.
protein-protein interactions and protein complexes not only in vision but also in other fields of biology and medicine.

Limitations of the Study
We did not examine electroretinogram (ERG) in the eys+/−; lp5+/− zebrafish because detailed examination of electrophysiology of the mutant would have been secondary support for the main conclusions of this study. We did not examine retinoid contents in the eys+/−; lp5+/− zebrafish eye, partly due to travel limitations under the current circumstances, and therefore did not include such data. In addition, the short variants, EYS-1-3 and EYS-1-4 (Takita et al., 2019), are not affected in the eyssa31957 mutants, and therefore, other possible function(s) for EYS owing to the variants are not unnecessarily omitted.

Resource Availability
Lead Contact
Further information and requests should be directed to and will be fulfilled by the corresponding author, Yuko Seko (seko-yuko@rehab.go.jp).

Data Availability
Microarray data are deposited in the GEO with the accession number GSE160663. Nucleotide sequence of zebrafish rbp1a is deposited in the DDBJ with the accession number LC579941.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101762.

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AUTHOR CONTRIBUTIONS
S.T. conceived and designed the study, performed the experiments, analyzed the data, and wrote the draft. Y.S. supervised the study. S.T. and Y.S. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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REFERENCES
Abd El-Aziz, M.M., Barragan, I., O’Driscoll, C.A., Goodstadt, L., Pirmorad, S., Mena, M., Pernas, J.I., El-Ashry, M.F., Safieh, L.A., et al. (2008). EYS, encoding an ortholog of Drosophila spacemaker, is mutated in autosomal recessive retinitis pigmentosa. Nat. Genet. 40, 1285–1287.

Banyshnikova, A., Costanzo, M., Kim, Y., Ding, H., Koh, J., Toufighi, K., Youn, J.Y., Ou, J., San Luis, B.J., Bandyopadhyay, S., et al. (2010). Quantitative analysis of fitness and genetic interactions in yeast on a genome scale. Nat. Methods 7, 1017–1024.

Berryman, M., Franck, Z., and Bretscher, A. (1993). Ezrin is concentrated in the apical microvilli of a wide variety of epithelial cells whereas moesin is found primarily in endothelial cells. J. Cell Sci 105 (Pt 4), 1025–1043.
Bok, D., Ong, D.E., and Chylil, F. (1984). Immunocytochemical localization of cellular retinal binding protein in the rat retina. Invest. Ophthalmol. Vis. Sci. 25, 877–883.

Bonilha, V.L., Bhattacharya, S.K., West, K.A., Sun, J., Crabb, J.W., Rayborn, M.E., and Hoflyfield, J.G. (2004). Proteomic characterization of isolated retinal pigment epithelium microvilli. Mol. Cell Proteomics 3, 1119–1127.

Bunt-Milam, A.H., and Saari, J.C. (1983). Immunocytochemical localization of two retinoid-binding proteins in vertebrate retina. J. Cell Biol. 97, 705–712.

Chin, E.N., Martin, J.A., Kim, S., Falakhraddeen, S.A., and Alexander, C.M. (2015). Lrp5 has a Wnt-independent role in glucose uptake and growth for mammary epithelial cells. Mol. Cell Biol. 36, 871–885.

Collin, R.W., Littin, K.W., Kleereng, B.J., van den Born, L., Koenekoop, R.K., Zonneveld, M.N., Bliskland, E.A., Strom, T.M., Hoyng, C.G., den Holland, A.I., and Cremers, F.P. (2008). Identification of a 2 Mb human ortholog of Drosophila eyes shut/spacemaker that is mutated in patients with retinitis pigmentosa. Am. J. Hum. Genet. 83, 594–603.

Cook, J.D., Ng, S.Y., Lloyd, M., Eddington, S., Sun, H., Nathan, J., Bok, D., Radu, R.A., and Travis, G.H. (2017). Peripom transduces transit of vitamin A from retina to retinal pigment epithelium. J. Biol. Chem. 292, 21407–21416.

Figueroa, D.J., Hess, J.F., Ky, B., Brown, S.D., Sandig, V., Hermanowski-Vosatka, A., Twells, R.C., Todd, J.A., and Austin, C.P. (2000). Expression of the type I diabetes-associated gene LRP5 in macrophages, vitamin A system cells, and the Islets of Langerhans suggests multiple potential roles in diabetes. J. Histochem. Cytochem. 48, 1357–1368.

Fujino, T., Asaba, H., Kang, M.J., Ikeda, Y., Sone, H., Takada, S., Kim, D.H., Ioka, R.X., Ono, M., Tomoyoi, Y., et al. (2003). Low-density lipoprotein receptor-related protein 5 (LRP5) is essential for normal cholesterol metabolism and glucose-induced insulin secretion. Proc. Natl. Acad. Sci. U S A 100, 229–234.

Gao, F.J., Zhang, S.H., Chen, J.Y., Xu, G.Z., and Wu, J.H. (2017). Digenic heterozygous mutations in EYS/LRP5 in a Chinese family with retinitis pigmentosa. Doc Ophthalmol. 132, 2049–2063.

Gong, Y., Sleen, R.B., Fukai, N., Rawadi, G., Roman-Roman, S., Repta, A., Wang, H., Cundy, T., Girolami, F.H., Levin, D., et al. (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. Cell 107, 513–523.

Gurudev, N., Yuan, M., and Knust, E. (2014). chopin, prominin, eyes and shunts form a genetic network controlling the apical compartment of Drosophila photoreceptor cells. Biol. Open 3, 332–341.

Holling, T.M., Schooten, E., and van Den Elsen, P.J. (2004). Function and regulation of MHC class II molecules in T-lymphocytes: of mice and men. Hum. Immunol. 65, 282–290.

Hosono, K., Ishigami, C., Takahasah, M., Park, D.H., Hiro, Y., Nakashini, H., Ueno, S., Yokoi, T., Hikoya, A., Fujita, T., et al. (2012). Two novel mutations in the EYS gene are possible major causes of retinitis pigmentosa in the Japanese population. PLoS One 7, e31036.

Huang, J., Possin, D.E., and Saari, J.C. (2009). Localizations of visual cycle components in retinal pigment epithelium. Mol. Vis. 15, 223–234.

Iwanami, M., Oshikawa, M., Nishida, T., Nakadomari, S., and Kato, S. (2012). High prevalence of mutations in the EYS gene in Japanese patients with autosomal recessive retinitis pigmentosa. Invest. Ophthalmol. Vis. Sci. 53, 1033–1040.

Kajiwara, K., Berson, E.L., and Dryja, T.P. (1994). Digenic retinitis pigmentosa due to mutations at the unlinked peripherin/RDS and ROM1 loci. Science 264, 1604–1608.

Katagiri, S., Akahori, M., Hayashi, T., Yoshitake, K., Gekka, T., Ikeo, K., Tsuneoka, H., and Iwats, T. (2012). Autosomal recessive cone-rod dystrophy associated with compound heterozygous mutations in the EYS gene. Doc Ophthalmol. 128, 211–217.

Kawaguchi, R., Yu, J., Honda, J., Hu, J., Whitelegge, J., Ying, P., Winta, P., Bok, D., and Sun, H. (2007). A membrane receptor for retinol binding protein mediates cellular uptake of vitamin A. Science 315, 820–825.

Kawamura, S., and Tachibanaaki, S. (2008). Rod and cone photoreceptors: molecular basis of the difference in their physiology. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 150, 369–377.

Kim, N., Stiegler, A.L., Cameron, T.O., Hallock, P.T., Gomez, A.M., Huang, J.H., Hubbard, S.R., Dustin, M.L., and Burden, S.J. (2012). Lrp4 is a receptor for Agrin and forms a retinitis pigmentosa. Cell 153, 334–342.

Lamb, T.D. (2013). Evolution of phototransduction, vertebrate photoreceptors and retina. Prog. Retin. Eye Res. 36, 52–119.

Li, J.K., Li, Y., Zhang, X., Chen, C.L., Yao, Q.F., Pei, Z., Zhang, Q., Zhao, P., and Liu, J. (2018). Spectrum of variants in 389 Chinese probands with familial exudative vitreoretinopathy. Invest. Ophthalmol. Vis. Sci. 59, 5368–5381.

Liu, Y., Yu, M., Shang, X., Nguyen, M.H.H., Balakrishnan, S., Sager, R., and Hu, H. (2020). Eyes shut homolog (EYS) interacts with matriglycan of O-mannosyl glycans whose deficiency results in EYS mislocalization and degeneration of photoreceptors. Sci. Rep. 10, 7795.

Lu, Z., Hu, X., Liu, F., Soares, D.C., Liu, Y., Yu, S., Gao, M., Han, S., Qin, Y., Li, C., et al. (2017). Ablation of EYS in zebrafish causes mislocalisation of outer segment proteins, F-actin disruption and cone-rod dystrophy. Sci. Rep. 7, 46098.

Messchaert, M., Dona, M., Broekman, S., Peters, T.A., Corral-Serrano, J.C., Slijkerman, R.W.N., van Wijk, E., and Collin, R.W.J. (2018). Eyes shut homolog is important for the maintenance of photoreceptor morphology and visual function in zebrafish. PluOSs 70, e1002789.

Nie, J., Mahato, S., Mustill, W., Tipping, C., Bhattacharya, S.S., and Zelhof, A.C. (2012). Cross species analysis of Prominin reveals a conserved cellular role in invertebrate and vertebrate photoreceptor cells. Dev. Biol. 371, 312–320.

Pieprasche, L.H.M., Messchaert, M., Thiaden, A., Haer-Wagman, L., de Jong-Hesse, Y., van Zelst-Stams, W.A.G., Collin, R.W.J., Klaver, C.C.W., and van den Born, L.I. (2019). Extending the spectrum of EYS-associated retinal disease to macular dystrophy. Invest. Ophthalmol. Vis. Sci. 60, 2049–2063.

Shen, J.P., Zhao, D., Sasik, R., Luebeck, J., Birmingham, A., Bojorquez-Gomez, A., Licon, K., Kleeper, K., Petin, D., Beckett, A.N., et al. (2017). Combinatorial CRISPR-Cas9 screens for de novo mapping of genetic interactions. Nat. Methods 14, 573–576.

Sparrow, J.R., Hicks, D., and Hamel, C.P. (2010). The retinal pigment epithilium in health and disease. Curr. Mol. Med. 10, 802–823.

Takita, S., Miyamoto-Matsui, K., and Seko, Y. (2019). Intra- and interspecies comparison of EYS transcripts highlights its characteristics in the eye. FASEB J. 33, 9422–9433.

Tamaa, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J.P., and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. Nature 407, 530–535.

Wang, J.S., and Kefalov, V.J. (2011). The cone-specific visual cycle. Prog. Retin. Eye Res. 30, 115–128.

Yang, H., Li, S., Xiao, X., Wang, P., Guo, X., and Zhang, Q. (2012). Identification of FZD4 and LRP5 mutations in 11 of 49 families with familial exudative vitreoretinopathy. Mol. Vis. 18, 2438–2446.

Yu, M., Liu, Y., Li, L., Natale, B.N., Cao, S., Wang, D., Amack, J.D., and Hu, H. (2016). Eyes shut homolog is required for maintaining the ciliary pocket and survival of photoreceptors. J. Biol. Chem. 288, 40624–40630.
Supplemental Information

*eyes*+/−; *lrp5*+/− Zebrafish Reveals Lrp5 Can Be the Receptor of Retinol in the Visual Cycle

Shimpei Takita and Yuko Seko
Figure S1. Zebrafish mutant lines used. Related to Figures 1-4 and S2-S4.
(A) eys$^{sa31957}$ carries a nonsense mutation (premature stop codon) C>A (arrows, Y2440X in EYS-1-1 (Takita et al., 2019)).
(B) lrp5$^{sa11097}$ carries a nonsense mutation C>A (arrows, Y284X in Lrp5 variant 1 (Lrp5-1; XP_009296098.1)).
(C) rbp1$^{-/-}$ carries a deletion of 6 nucleotides (CCTAGA, nucleotide position 169-174 of NM_001114899.1) and an insertion of a nucleotide (G) in the deleted region resulting in a frameshift after the first 38 amino acids of NP_001108371.1.
(D) prom1a$^{-/-}$ carries a deletion of 13 nucleotides (nucleotide position 358-370 of XM_021480968.1 or transcript variant 1 of prom1a (prom1a-1)) resulting in a frameshift immediately after the start codon (first 13 amino acids of XP_021336643.1).
(E) prom1b$^{sa13905}$ carries a nonsense mutation G>A (arrows, position 354 of XM_021481233.1 or transcript variant 1 of prom1b (prom1b-1)) resulting in a truncation after the first 14 amino acids in the signal peptide sequence region (W15X in Prom1b variant 1 (Prom1b-1; XP_021336908.1)).
(F) lrp5 (Ct)$^{-/-}$ carries a deletion of 25 nucleotides (CCACCACCCCTGCAGCACAGACGT, nucleotide position 4761-4785 of XM_009297823.3 or transcript variant 1 of lrp5 (lprp5-1)) and an insertion of 17 nucleotides (GTGAATGGCACGGTACG) resulting in frameshift after position 1538 of Lrp5-1.
Figure S2. Effects of single and digenic mutations on the retina and change in the expression levels of rbp1a (rbp1.2) and rbp1b (rbp1.1). Related to Figures 1, 2, and 3.

(A) The RPE layer was 14% thinner in the eys<sup>+</sup>/; lrp5<sup>+</sup> eye (wildtype, 84.9 ± 0.7 µm; eys<sup>+</sup>/; lrp5<sup>+</sup> mutants 73.3 ± 5.2, n = 3). Data are represented as mean ± SD. *p = 0.0186.

(B and C) Significant changes in the thickness of PRCL or number of nuclei in the ONL were not observed among wildtype, eys<sup>+</sup>/, and lrp5<sup>+</sup> zebrafish. (47.59 ± 3.3 µm for wildtype, 45.5 µm for eys<sup>+</sup>/, and 47.83 ± 1.3 µm for lrp5<sup>+</sup> PRCLs; 60 ± 1.4 for wildtype, 58 for eys<sup>+</sup>/, and 59 ± 1.4 for lrp5<sup>+</sup> ONLs; n = 2, 1, and 2 for wildtype, eys<sup>+</sup>/, and lrp5<sup>+</sup> zebrafish, respectively). Scale bar, 10 µm. Data are represented as mean ± SD. NS, not significant.

(D) The expression levels of the rbp1a (rbp1.2) and rbp1b (rbp1.1) genes by cap analysis of gene expression (CAGE) (counts per million) was 46.16 and 5.92, respectively.

(E) The expression levels of rbp1b (rbp1.1) was about 20% reduced in the eys<sup>-/</sup>, lrp5<sup>-/</sup>, and eys<sup>+</sup>/; lrp5<sup>-/</sup> eyes (eys<sup>-/</sup>, 74.0 ± 2.5%; lrp5<sup>-/</sup>, 79.1 ± 4.8%; eys<sup>+</sup>/; lrp5<sup>-/</sup>, 81.4 ± 12.9%, n=3) by qPCR. Data are represented as mean ± SD.

(F) The expression levels of rbp1a (rbp1.2) were 28.4 ± 3.6% in the lrp5<sup>-/</sup> eye and 76.0 ± 1.9% in the eys<sup>-/</sup> eye compared to the wildtype (n = 3). Data are represented as mean ± SD. **p < 0.005, ***p < 0.0001.
Figure S3. Photoreceptor degeneration in the digenic eys<sup>+</sup>-; lrp5<sup>-/-</sup> zebrafish at 8 mpf detected by TUNEL staining. Related to Figure 1.

(A-D) Apoptotic cells (green) were observed in the photoreceptor cell layer (PRCL) in the digenic eys<sup>+</sup>-; lrp5<sup>-/-</sup> zebrafish (C) while no apoptotic cells were observed in PRCL in wildtype zebrafish (A). Panels on the left half show central-ventral regions and panels on the right half show central-dorsal regions. Peripheral regions are intensely labeled. Apoptotic cells were also observed in the PRCLs of the eys<sup>-/-</sup> and lrp5<sup>-/-</sup> eyes (B and D respectively). Nuclei were stained with propidium iodide (PI) (red). GCL, ganglion cell layer; INL, inner nuclear layer; PRCL, photoreceptor cell layer; RPE, retinal pigment epithelium. Scale bars, 100 µm.
Figure S4. Immunohistochemical analysis of Rbp1a expression in the digenic eys<sup>+/−</sup>; lrp5<sup>+/−</sup> zebrafish adult eyes. Related to Figure 2.

(A-D) Upper left panels and their plot profiles (left and right ends are indicated by red arrowheads) show the signal for Rbp1a. Both the signal intensity and the number of spots were decreased in the digenic eys<sup>+/−</sup>; lrp5<sup>+/−</sup> eyes (C) compared to wildtype eyes (A). The expression levels of Rbp1a protein were also decreased in the eys<sup>−/−</sup> and lrp5<sup>−/−</sup> eyes (B and D, respectively). In the eys<sup>−/−</sup> eyes, the number of Rbp1a-positive microvilli appeared to be more affected than the signal intensity. The fine structures of the concentrated regions indicated by Rbp1a (green) may suggest that the structures underwent slightly differently alteration between the mutant retinas (right panels). Nuclei were stained with propidium iodide (PI) (red). Scale bars, 20 µm in left panels and 2 µm in right panels.
A. Wildtype

B. rbpa1−/

C. Lrp5

D. lrp5 (Ct)−/

E. GS

F. GFAP

G. GFAP, Rbp1a, Merge, Merge + DIC

Legend:

- RPE
- PRCL
- GCL
- INL
- Arrowheads indicate specific areas of interest.
Figure S5. Validation of specificity of the anti-Rbp1a and anti-Lrp5 antibodies and clarification of Müller glial processes in the zebrafish adult eyes. Related to Figure 4.

(A and B) Immunopositive signals (green) were detected in the outer retina in wildtype eyes (A, arrowheads). Immunopositive signals were not detected in the same region (B, arrowheads) nor in other region in the outer retina in the *rbp1a*^{-/-} eyes. Nuclei were stained with propidium iodide (PI) (red).

(C and D) Immunopositive signals (green) were detected in the outer retina in wildtype eyes (C, arrowheads). Immunopositive signals were not detected in the same region (D, arrowheads) nor in other region in the outer retina in the *lrp5 (Ct)*^{-/-} eyes.

(E - G) The processes of Müller cells were stained with anti-GFAP and anti-GS antibodies. The processes were also co-stained with the antibody to Rbp1a to compare the location within the outer retina. Arrows and arrowheads indicate the location of the microvilli of RPE cells and the processes of Müller cells, respectively. Nuclei were stained with PI (red). Ct, C-terminal region; DIC, differential interference contrast; GFAP, fibrillary acidic protein; GS, glutamine synthetase. Scale bars, 20 µm in A, B, C, D, E, and F, and 5 µm in G.
Table S1. Primer sequences for screening and genotyping of zebrafish mutants. Related to Figures 1, 2, and 3.

| Gene | 5' Primer Forward | 5' Primer Reverse |
|------|-------------------|-------------------|
|  
| dreys | Dreys_Fw7530      | GTTTCAATGCTCTAGGAGGGCCA |
|      | Dreys_g_Rev1      | ACGGTAATATGCAAAAGTGCGT |
| lrp5 | Drlrp5_g_Fw1      | AGGATCCACAAATGCTACAGATGTT |
|      | Drlrp5_g_Rev1     | ACCTGACCTGAGTGCAAAACAG |
| rbp1a| Drrbp1_Fw_g1      | ACGTGGATGCATTTTGTAGAGT |
|      | Drrbp1_Rev243     | ATGTCGTAATCTCCTAAATGTGGTT |
| prom1a| prom1a_Fw190   | TGATCATCATGACTCGCTCGCC |
|      | prom1a_Rev538     | GTTGGGCTGAAACCAAAAGAGG |
| prom1b| Drprom1b_Fw30    | AGGTCACACAACACTCAGCTC |
|      | Drprom1b_Rev504   | CCCCATGGGACCTGGTCATAA |
| Gene   | Forward Primer          | Reverse Primer          |
|--------|-------------------------|-------------------------|
| actb2  | Dractb2_Fw282           | GAGGTATCCTGACCCTGAAGTA  |
|        | Dractb2_Rev361          | GTGATGCCAGATCTTTCTCCATA |
| rbp1a  | Drrbp1_Fw116            | AACTGGGAAGAGGATAAGTTGG  |
|        | Drrbp1_Rev424           | ACAGACTCTCTATTTCCACATGT |
| rbp1b  | Drrbp1b_Fw115           | GGAAAATGATCTCCAAACGAC   |
|        | Drrbp1b_Rev296          | CTCCTGACCAACTAAAGTGCC   |
| lrp5   | Drlrp5_Fw757            | ACTCACCATTGACCTTGACGAG  |
|        | Drlrp5_Rev1210          | AGTGCAGCAAGTAAGTGAGCA   |
| prom1a | Drprom1a_Fw1674         | TTACCCTGTGCTGTATGATTGT  |
|        | Drprom1a_Rev1869        | AAAGCTGTATGACTCCATCTGA  |
| prom1b | Drprom1b_Fw1489         | AGGGAACAGACTAAAGGGATTG  |
|        | Drprom1b_Rev1570        | AACCAATAATCTCTGACCTGA   |
| eys (5') region | Dreys_Fw1586   | CTTGTCTCAACGCTGGCATCTG |
|        | DrEYS_Rev1816           | AGGCTAAGCATGCGCTTCCAA   |
| eys (3' region) | Dreys_Fw7856   | CTCCCATCAACATAAGGCCACAG |
|        | Dreys_Rev8096           | ACATCTAACTCTTTGAGCTAG   |
| eys (splice variant 3) | Dreys_splicing_Fw5646 | GTGTGTGGCCTGAAGTCACCCTA |
|        | Dreys_splicing_Rev1     | GGAAAACAGAAACATTCAGGCTTA |
### Table S3. Primer sequences for cloning of zebrafish $rbp1a$ and $lrp5$ genes. Related to Figure 4.

|      | Primer 1 (forward) | Primer 2 (reverse) |
|------|--------------------|--------------------|
| $rbp1a$ | Drrbp1_Fw7         | ATGTTGAAACATCAGCGCA |
|       | Drrbp1_Rev888      | AAGAATTGTGCAGGTTTTTATTG |
| $lrp5$  | Drlrp5_EcoRI_F4370-2 | TCGGTACCCGCTTTCCGAATTCCAGAGAGTCGTGTG |
|       | Drlrp5_Ct5_Rev     | CAATGCCTGAGGAGTCTGTGCAGGGCG |
Transparent Methods

Zebrafish care

All experiments with zebrafish (*Danio rerio*) in this study were performed in accordance with the institutional guidelines and all experimental protocols were approved by the Institutional Animal Care and Use Committee of National Rehabilitation Center for Persons with Disabilities Research Institute. The fish were kept under 14 hr light/10 hr dark cycle at 28.5°C. When necessary, the fish were anesthetized by putting them in ice-chilled water and decapitated during the light period.

Generation of *eys*+/−, *lrp5*+/−, *eys*-/−, and *lrp5*-/− zebrafish

The *eys*sa31957 (*eys*+/−) and *lrp5*sa11097 (*lrp5*+/−) mutant lines (isolated by Zebrafish Mutation Project (https://www.sanger.ac.uk/resources/zebrafish/zmp/) (Kettleborough et al., 2013)) were obtained from the Zebrafish International Resource Center (ZIRC). *eys*sa31957 carries a nonsense mutation (premature stop codon) C>A (Figure S1A; Y2440X in EYS-1-1 (Takita et al., 2019)) resulting in the truncation of the last 467 amino acids (Figure 1A, arrow in zebrafish EYS). *lrp5*sa11097 carries a nonsense mutation C>A (Figure S1B; Y284X in Lrp5 variant 1, XP_009296098.1, or Lrp5-1; Figure 1A, arrow in zebrafish LRP5). These heterozygous mutants were crossed to generate *eys*+/−; *lrp5*+/−, *eys*-/−, or *lrp5*-/− mutants. The primer pairs used for genotyping are listed in Table S1.
**Generation of rbp1a⁻/⁻ zebrafish**

The sequence in exon 2 within the lipocalin domain (position 157-179, CAGCTACCTCATCCTAGATAAGG, of NM_001114899.1) was chosen for the target of CRISPR/Cas9-mediated rbp1a gene knockout.

Six μM gRNA was formed by mixing the equimolar of custom-synthesized crRNA and tracrRNA (Integrated DNA technologies (IDT)) with the following parameters: 95°C for 5 min, 85°C for 1 sec, 85-25°C at -2°C/sec, 25°C for 30 sec, 25-16°C at 0.1°C /sec and hold at 16°C. Cas9 protein (Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT) (Vakulskas et al., 2018)) was diluted to 1 μg/μL (~ 6.11 μM) with Injection Buffer and stored at -20°C until use. Same volume of the gRNA and Cas9 protein were then incubated at 37°C for 10 min to form ctRNP prior to injection. ~ 2-3 nL of the ctRNP in 0.15 M KCl solution containing 0.05% phenol red was injected into zebrafish eggs at one-cell stage. The eggs were raised in Raising Medium at 28°C.

At 24 hpf, small pool of the injected embryos was subjected to gene editing. Each embryo was lysed by heating at 95 °C for 30 min in 20 μL of alkaline lysis buffer (25 mM NaOH, 0.25 mM EDTA) (Meeker et al., 2007). Then the solution was neutralized by 1/10th volume of neutralization buffer (1 M Tris-HCl, pH 7.4). The samples were next
subjected to genomic PCR with KOD FX Neo (Toyobo, Osaka, Japan) as follows after being held at 94°C for 2 min: 98°C for 10 sec, then at 55°C for 25 sec and finally at 72°C for 25 sec. The reactions were repeated for 35 cycles. Amplification was checked with 1% agarose gel electrophoresis (Nacalai Tesque, Kyoto, Japan). The primer pair used is listed in Table S1. The PCR product was then subjected to heteroduplex mobility assay (HMA). Heteroduplex DNA was formed using the same parameter that was used in forming the gRNA. Samples were separated on a non-denaturing 10% polyacrylamide gel [10% acrylamide (37.5:1), 1x TBE, 0.1% ammonium persulfate, 0.1% TEMED] and run in 1x TBE buffer. Remaining zebrafish in the pools of embryos were further raised to adulthood and crossed with wildtype to generate F1. Genomic DNA was extracted from the F1 zebrafish and genomic PCR was performed with the same primer pair in Table S1. The second round of PCR with the same primer pair was performed by ExTaq (TaKaRa, Kusatsu, Japan) using the PCR product as a template and the subsequent PCR product was purified with NucleoSpin gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). The purified PCR product was cloned into pTAK-2 vector (Biodynamics Laboratory Inc., Tokyo, Japan) and the sequences of mutations in the *rbp1a* gene was analyzed by DNA sequencing with T7 or SP6 primer. An individual that had a deletion of 6 nucleotides (CCTAGA, nucleotide position 169-174 of NM_001114899.1) and an insertion of a
nucleotide (G) in the deleted region (Figure S1C), which leads frameshift after the first 38 amino acids of NP_001108371.1 resulting in the premature stop codon, was chosen for the establishment of the rbp1a knockout line.

**Generation of prom1a<sup>−/−</sup>; prom1b<sup>−/−</sup> zebrafish**

The target of the CRISPR/Cas9-mediated prom1a gene knockout was An immediately downstream from the translation start site in the signal peptide sequence region (position 349-371, GTGCTGGGGCTTGACGTCCGGGG, of XM_021480968.1 or transcript variant 1 of prom1a (prom1a-1) that all known 13 transcript variants possess in their N-termini of coding sequences. An individual that had a deletion of 13 nucleotides (Figure S1D; nucleotide position 358-370 of prom1a-1), which leads frameshift immediately after the start codon (first 13 amino acids of XP_021336643.1), was chosen for the establishment of the prom1a knockout line. The primer pair used for the genomic PCR to confirm the mutation and for subsequent genotyping is listed in Table S1.

The prom1b<sup>sa13905</sup> (prom1b<sup>−/−</sup>) zebrafish were obtained from the ZIRC. prom1b<sup>sa13905</sup> carries a nonsense mutation G>A (Figure S1E; W15X in Prom1b variant 1 (Prom1b-1; XP_021336908.1)) resulting in the truncation after the first 14 amino acids in the signal peptide sequence region (position 354 of XM_021481233.1 or transcript variant 1 of
prom1b (prom1b-1)). All known 8 transcript variants possess the nonsense mutation in their N-termini of coding sequences. The primer pair used for genotyping is listed in Table S1. The prom1a+/−; prom1b+/− zebrafish were generated by crossing prom1a+/−; prom1b+/− mutants.

**Genotyping**

Caudal fins were lysed by heating at 95 °C for 30 min in 20 μL of alkaline lysis buffer (25 mM NaOH, 0.25 mM EDTA) (Meeker et al., 2007). The solution was neutralized by 1/10th volume of neutralization buffer (1 M Tris-HCl, pH 7.4). The samples were subjected to genomic PCR as follows after held at 94°C for 2 min: 98°C for 10 sec, then at 55°C for 30 sec and finally at 72°C for 30 sec. The reactions were repeated for 35 cycles. Amplification was checked with 1% agarose gel (Nacalai Tesque) and the PCR products were purified with NucleoSpin gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). Direct sequencing was performed with the purified PCR products using BigDye Terminator v3.1 Cycle Sequencing Kit and 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

**Observation of the PRCL in the digenic eys+/−; lrp5+/− and wildtype zebrafish eyes**
Light-adapted 6 or 14 mpf adult eyes were fixed and embedded in a mixture of OCT compound (Tissue tech) as described (Takita et al., 2016). The adult eyes were cryosectioned at 12 μm thickness. After dried for 1 hr at room temperature, the sections were washed three times with phosphate-buffered saline (PBS; 10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, 2.7 mM KCl, pH 7.3) containing 0.1% Triton X-100 (PBST) for 5 min. Then the sections were stained with propidium iodide (PI; 1 μg/mL in PBS) for 1h at room temperature in darkness. After the staining, the sections were washed three times with PBS for 5 min and mounted with VECTASHIELD mounting medium (Vector Laboratories).

The zebrafish eyes were imaged with inverted confocal microscope (LSM510 PASCAL, Zeiss) with Fluar 5×/0.25 or Plan-Apochromat 20×/0.8 NA objective lens.

**Detection of apoptotic cells**

Apoptosis in the mutant adult eyes was examined by TUNEL staining using *In Situ* Cell Death Detection Kit, Fluorescein (Roche) essentially according to the manufacturer’s instruction. Briefly, adult eyes at 8 mpf fixed with 4% paraformaldehyde (PFA) in PBS for 8 h were cryosectioned at 12 μm thickness and dried for 1 hr. The sections were washed twice with PBS for 5 min. Then the sections were fixed with 4% PFA in PBS for
20 min. After washed with PBS for 30 min, the samples were permeabilized with 0.1% (w/v) Triton X-100/0.1% sodium citrate for 2 min on ice and washed twice with PBS for 5 min. The sections were incubated with 50 μL of TUNEL reaction mixture in a humidified chamber for 1 hr at 37°C in darkness. The reaction mixture was washed away three times with PBS for 5 min and the sections were embedded with VECTASHIELD (Vector Laboratories) and observed with inverted confocal microscope (LSM510 PASCAL, Zeiss) with EC Plan-Neofluar 10×/0.3 NA objective lens.

**Immunohistochemistry**

Adult retinas were immunostained basically according to the method described previously (Takita et al., 2016). Rabbit anti-zebrafish Rbp1a and Lrp5 antibodies were raised by GL Biochem (Shanghai, China). Antigen for anti-zebrafish Rbp1a was a synthetic peptide, 42KEIVQNGDLYIKTTLTFRNYD63, of zebrafish Rbp1a protein (NP_001108371.1). Antigen for anti-zebrafish Lrp5 was a synthetic peptide, 1551DYTTSRWKSNKYYMDLNSDSP1572, of zebrafish Lrp5 protein (XP_009296098.1). To validate the specificity of the anti-Lrp5 antibody, lrp5 (Ct)-/ was generated using CRISPR/Cas9 system and used for the immunohistochemistry. The sequence (Figure S1F; position 4757-4779, CCCCCACCACCCCCCTGCAGCAC, of
XM_009297823.3 or transcript variant 1 of *lrp5* (*lrp5-1*) immediately upstream of the sequence encoding the antigen was chosen as the target to minimize any potential reactions that might hinder the proper validation: other potential minor wildtype *lrp5* transcript variant(s) and/or cryptic splicing that could escape the intended knockout of the gene as a result of the knockout.

The antibodies were purified with Protein A HP SpinTrap (Cytiva, Madison, Wisconsin, USA) and used for immunohistochemistry (anti-Rbp1a antibody, 1:75000 dilution; anti-Lrp5 antibody, 1:500000 dilution). For double staining of antibodies raised in the same species, the antibodies were labeled with Ab-10 Rapid HiLyte Fluor™ 555 Labeling Kit (Dojindo Molecular Technologies, Inc.) prior to use. Microvilli of RPE cells were visualized with rabbit anti-MOESIN (MSN) antibody (Proteintech, 16495-1-AP; 1:1000 dilution). The processes of Müller cells were visualized with rabbit anti-GFAP (Agilent (Dako), Z0334; 1:4000 dilution) and mouse anti-GS (Merck, MAB302 (clone GS-6); 1:1000 dilution) antibodies. Alexa Fluor 488 goat anti-rabbit antibody (1:500 or 1:1000 dilution) was purchased from Thermo Fisher Scientific.

The sections were embedded with VECTASHIELD (Vector Laboratories) and observed with inverted confocal microscope (LSM510 PASCAL, Zeiss) with EC Plan-Neofluar 40×/1.3 NA objective lens. Z-stack images (captured with a 0.45 μm step size)
were acquired, maximum intensity projection (MIP) was generated from the Z-stack images with ZEN 2011 Black Edition (Zeiss), and generated images were checked manually. Representative MIP images were selected for image presentation.

**Microarray analysis**

Total RNA was isolated from light-adapted 3.5 mpf adult three zebrafish as described (Takita et al., 2016). Briefly, zebrafish were sacrificed in the ice-cold fresh-water three hours after light onset. Eyes were then detached and the water and blood that remained were quickly wiped away as much as possible. The eyes were homogenized with 200 μL of TRI Reagent (Merck) and total RNA was isolated according to the manufacturer’s instructions. The concentration was quantified spectrophotometrically and microarray was performed with 250 ng of the total RNA on Genechip Zebrafish Gene 1.0 ST Array (Thermo Fisher Scientific). Acquired data were analyzed by Transcriptome Analysis Console (TAC) 4.0.1 (Thermo Fisher Scientific).

**qPCR**

First-strand cDNA was synthesized from 1 μg of the saved total RNA used for microarray by reverse transcription at 50°C for 2 hr using SuperScript IV reverse
transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) primed with the supplied random primer and cDNAs were stored at -25°C until use. Isolation of total RNA and cDNA synthesis for other mutants were performed similarly. To measure the expression levels of rbp1a, rbp1b, lrp5, prom1a, prom1b, or eys mRNA, the average expression level of each mRNA was obtained from three samples (n = 3) with Platinum™ SYBR™ Green qPCR SuperMix-UDG (Thermo Fisher Scientific). The primer pairs used are listed in Table S2.

**Binding assay**

Full-length rbp1a and C-terminal intracellular region of lrp5 were amplified from zebrafish eye cDNA by RT-PCR using the primer pairs listed in Table S3, cloned into pTAK-2 vector (BioDynamics Laboratory Inc.), and sequenced. The rbp1a fragment was fused with EGFP-HAT and subcloned into pTAK-2 vector. After the sequence was verified, the insert was excised with EcoRI and SalI and ligated to the corresponding site of pGEX-5X-1 (Cytiva) to obtain an N-terminally GST-fused and C-terminally EGFP-HAT-fused product (pGEX-5x-1-Drerbp1a-EGFP encoding GST-Rbp1a-EGFP).

The lrp5 fragment was fused with mCherry and subcloned into pTAK-2 vector. After the sequence was verified, the insert was excised with EcoRI and SalI and ligated to the
corresponding site of pMAL-c2E (New England Biolabs) to obtain an N-terminally MBP-fused and C-terminally mCherry-fused product (pMAL-c2E-Drelrp5(C-terminal)-mCherry encoding MBP-Lrp5(intracellular)-mCherry). pMAL-c2E for MBP expression was previously described (Takita et al., 2016).

Each plasmid was transformed into *E. coli* BL21 (DE3) for expression. A suspension of *E. coli* expressing the GST-Rbp1a-EGFP, MBP, or MBP-Lrp5(intracellular)-mCherry protein was sonicated and the supernatant was collected. The supernatant was loaded to Glutathione Sepharose-4B (Cytiva) or amylose beads (New England Biolabs) and GST-Rbp1a-EGFP was further purified according to the manufacturer’s instruction. Purified GST-Rbp1a-EGFP and MBP- or MBP-Lrp5(intracellular)-mCherry-bound amylose beads were kept at 4°C until use.

The purified GST-Rbp1a-EGFP (20 μL) was incubated in 200 μL of PBS containing 5 μL of the amylose beads pre-bound with either MBP or MBP-Lrp5(intracellular)-mCherry for 24 h at 4°C. Then the beads were centrifuged at 2000 g for 2 min at 4°C. The precipitated beads were washed three times with 200 μL of PBS and mounted on a slide with VECTASHIELD (Vector Laboratories) for observation. Fluorescence on the beads was observed under an inverted confocal microscope (LSM 510 PASCAL, Zeiss) with Plan-Apochromat 20×/0.8 NA objective lens.
Image analysis

The thickness of PRCL in wildtype or the mutant eyes was measured based on the previous study (Lu et al., 2017). Briefly, the region 100 μm away from the optic nerve head was chosen and the thickness of PRCL was measured with ZEN 2011 Black Edition (Zeiss). The thickness of eys<sup>+/−</sup>; lrp5<sup>+/−</sup> mutant PRCL was compared with age-matched wildtype. Three zebrafish from each group were used. The number of nuclei in the ONL in wildtype or the mutant eyes was counted manually using the same image acquired for the measurement of the thickness of PRCL.

Fluorescence on the surface of the beads in Figure 4F was analyzed with ImageJ (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD). The images were converted to 8-bit representations. The beads then were enclosed manually with oval selection and the mean for the area was measured. Background signal determined from the area where there were no bead was subtracted from the mean. The resulting value was assigned as the fluorescence for a single bead.

Acquired images shown in Figure S4 were analyzed with ImageJ. After signal intensity of each pixel in the image was converted to an 8-bit representation, plot profiles were generated with the line plot from the left red arrowhead to the right red arrowhead.
Statistical analysis

For the measurements of the thickness of PRCL and number of nuclei in the ONL in wildtype or mutant zebrafish and the expression levels of *rhp1a*, *rhp1b*, *lrp5*, *prom1a*, *prom1b*, or *eys* mRNA, data are presented as mean ± standard deviation (SD), and levels of significance (*P* value, Student's t-test) are indicated in the figure legends.
**Supplemental References**

Kettleborough, R.N., Busch-Nentwich, E.M., Harvey, S.A., Dooley, C.M., de Bruijn, E., van Eeden, F., Sealy, I., White, R.J., Herd, C., Nijman, I.J., *et al.* (2013). A systematic genome-wide analysis of zebrafish protein-coding gene function. Nature 496, 494-497.

Lu, Z., Hu, X., Liu, F., Soares, D.C., Liu, X., Yu, S., Gao, M., Han, S., Qin, Y., Li, C., *et al.* (2017). Ablation of EYS in zebrafish causes mislocalisation of outer segment proteins, F-actin disruption and cone-rod dystrophy. Sci Rep 7, 46098.

Meeker, N.D., Hutchinson, S.A., Ho, L., and Trede, N.S. (2007). Method for isolation of PCR-ready genomic DNA from zebrafish tissues. Biotechniques 43, 610, 612, 614.

Takita, S., Miyamoto-Matsui, K., and Seko, Y. (2019). Intra- and interspecies comparison of EYS transcripts highlights its characteristics in the eye. FASEB J 33, 9422-9433.

Takita, S., Wada, Y., and Kawamura, S. (2016). Effects of NDRG1 family proteins on photoreceptor outer segment morphology in zebrafish. Sci Rep 6, 36590.

Vakulskas, C.A., Dever, D.P., Rettig, G.R., Turk, R., Jacobi, A.M., Collingwood, M.A., Bode, N.M., McNeill, M.S., Yan, S., Camarena, J., *et al.* (2018). A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. Nat Med 24, 1216-1224.