Knockout of RP2 decreases GRK1 and rod transducin subunits and leads to photoreceptor degeneration in zebrafish

Fei Liu1,†, Jiaxiang Chen1,†, Shanshan Yu1,†, Rakesh Kotapati Raghupathy2, Xiliang Liu1, Yayun Qin1, Chang Li1, Mi Huang1, Shengjie Liao1, Jiuxiang Wang1, Jian Zou3, Xinhua Shu2, Zhaohui Tang1,* and Mugen Liu1,*

1Key Laboratory of Molecular Biophysics of Ministry of Education, Department of Genetics and Developmental Biology, College of Life Science and Technology, Huazhong University of Science and Technology, 1037 Luoyu Road, Wuhan, Hubei 430074, PR China, 2Department of Life Sciences, Glasgow Caledonian University, Glasgow G4 0BA, UK and and 3Institute of Translational Medicine, Zhejiang University, 268 Kaixuan Road, Zhongxin Beilou, Hangzhou, 310029 Zhejiang, PR China

*To whom correspondence should be addressed at: Department of Genetics and Developmental Biology, College of Life Science and Technology, 1037 Luoyu Road, Wuhan, P.R. China. Tel: +86 2787794549; Fax: +86 2787794549; Email: lium@hust.edu.cn (M.L.); zh_tang@hust.edu.cn (Z.T.)

Abstract
Retinitis pigmentosa (RP) affects about 1.8 million individuals worldwide. X-linked retinitis pigmentosa (XLRP) is one of the most severe forms of RP. Nearly 85% of XLRP cases are caused by mutations in the X-linked retinitis pigmentosa 2 (RP2) and RPGR. RP2 has been considered to be a GTPase activator protein for ARL3 and to play a role in the traffic of ciliary proteins. The mechanism of how RP2 mutations cause RP is still unclear. In this study, we generated an RP2 knockout zebrafish line using transcription activator-like effector nuclease technology. Progressive retinal degeneration could be observed in the mutant zebrafish. The degeneration of rods’ outer segments (OSs) is predominant, followed by the degeneration of cones’ OS. These phenotypes are similar to the characteristics of RP2 patients, and also partly consistent with the phenotypes of RP2 knockout mice and morpholino-mediated RP2 knockdown zebrafish. For the first time, we found RP2 deletion leads to decreased protein levels and abnormal retinal localizations of GRK1 and rod transducin subunits (GNAT1 and GNB1) in zebrafish. Furthermore, the distribution of the total farnesylated proteins in zebrafish retina is also affected by RP2 ablation. These molecular alterations observed in the RP2 knockout zebrafish might probably be responsible for the gradual loss of the photoreceptors’ OSs. Our work identified the progression of retinal degeneration in RP2 knockout zebrafish, provided a foundation for revealing the pathogenesis of RP caused by RP2 mutations, and would help to develop potential therapeutics against RP in further studies.
retinitis pigmentosa (XLRP) is one of the most severe forms of RP, characterized by early-onset and rapid progression of visual impairment. Mutations in the X-linked retinitis pigmentosa 2 (RP2, OMIM *300757) gene account for 7–18% of XLRP cases (5,6).

RP2 was identified in 1998 (7). It consists of five exons encoding a ubiquitously expressed 350-amino acid polypeptide, which contains an N-terminal tubulin folding cofactor C-like (TBCC) domain and a C-terminal nucleoside diphosphate kinase-like (NDPK) domain. So far, more than 70 RP2 mutations have been reported and over half of them are located in exon 2 (8). Currently, the mechanism of how RP2 mutations cause retinal degeneration is still not fully understood.

In human retinas, RP2 associates with the plasma membrane of photoreceptors, including the outer segments (OSs), inner segments, cell bodies and synapses (9). The membrane localization of RP2 relies on the myristoylation of Gly2 and palmitoylation of Cys3 at the N-terminal. RP2 has also been reported to locate to the cilium and the Golgi apparatus in cultured cell lines (10–12). Several proteins have been identified to interact with RP2, including tubulin (13), ADP-ribosylation factor-like 3 (ARL3) (14), polycystin 2 (11), importin j2 (10), GÎ1 [â subunit of rod transducin] (15) and N-ethylmaleimide sensitive factor (NSF) (16). Among them, the small GTPase ARL3 is the best-studied RP2 relevant protein. Structural analysis and biochemical research revealed that RP2 accelerates the GTPase activity of ARL3, which dissociates the small GTPase ARL3 from the prenylated cargos from UNC119 (14,17–21). The Cys3 at the N-terminal. RP2 has also been reported to interact with RP2, including ADP-ribosylation factor-like 3 (ARL3) (14), polycystin 2 (11), importin j2 (10), GÎ1 [â subunit of rod transducin] (15) and N-ethylmaleimide sensitive factor (NSF) (16). Among them, the small GTPase ARL3 is the best-studied RP2 relevant protein. Structural analysis and biochemical research revealed that RP2 accelerates the GTPase activity of ARL3, which dissociates the ARL3-GTP-UNC119 complex after ARL3-GTP releases myristoylated cargos from UNC119 (14,17–19). The UNC119 homologous protein, PDE6D, which is another ARL3 effector, can bind prenylated proteins and plays a role in maintaining the GRK1 and PDE6 catalytic subunits in mouse retinas (20–22). The particular correlation between RP2 and PDE6D has not been confirmed experimentally until now. Deletion of ARL3 (23), UNC119 (24) and PDE6D (22) all leads to retinopathy in mice. Furthermore, RP2 has been reported to facilitate the membrane association of GNB1 in ARPE19 cells (15). Up to now, two mouse models of RP2 have been described in 2013 (25) and recently in 2014 (26). Progressive retinal degeneration with a cone predominantly affected pattern was identified in both RP2 defective models without any obvious physical defect.

In recent years, zebrafish is becoming a useful animal model due to the convenience of genetic manipulation and the high rate of spawning. Additionally, the similar structure of retina and the cone-dominant vision of zebrafish (similar to humans) makes it an important model for human retinal disorders (27,28). In zebrafish, there is only one orthologue of RP2, containing 6 exons coding 376 amino acids. Morpholino-mediated knockdown of RP2 in zebrafish leads to severe early-onset retinal dysplasia (29,30) and pronephric cysts (11), with body curvature, left–right symmetry defects and heart looping defects, implying the dysfunction of ciliium. The high amino acid identity (67%; Supplementary Material, Fig. S1) and the rescue data in zebrafish sh with 80% identity (Fig. 1A and Supplementary Material, Fig. S2B), indicating RP2 is expressed in both rod and cone photoreceptor cells with mainly plasma membrane localization from the synapses to the OSs. In other types of retinal cells, the signal of RP2 was much weaker. Furthermore, we performed immunostaining in adult WT and del5 mutant zebrafish using the anti-zebra RP2 antibody. In del5 mutant group or RP2 peptide blocking group, no specific signal could be detected, whereas in WT group, we observed similar retinal localization of RP2 as mentioned earlier (Fig. 1E). These results demonstrate that the knockout of RP2 in this study is effective. In the rest of the research, we considered the homozygous del5 zebrafish as an RP2 null animal model.

Truncation mutations in RP2 have been reported to cause protein degradation or aggregation in insoluble fractions with normal mRNA levels in lymphoblastoid cell lines derived from RP2 patients (32). Recently, Schwarz et al. reported that in patient (R120X) iPSC-derived RPE cells, mutant RP2 protein could not be detected and the mRNA level of RP2 R120X transcript was also decreased (33), which is consistent with the situation of our RP2null zebrafish. In addition, we did not observe apparent developmental defects such as microphthalmia, body curvature, hydrocephalus and pericardial effusion in RP2null zebrafish, as have been reported in RP2 knockout zebrafish previously (11,29,30). These severe
developmental defects of RP2 morphants might be caused by the side effects of morpholino injection. The retina laminations of RP2null zebrafish were also unaffected at the age of 1 month (Supplementary Material, Fig. S3), suggesting that RP2 is seemingly not essential for the early development of zebrafish retina.

RP2null zebrafish show progressive retinal degeneration with a rod predominantly affected pattern

As reported previously, RP2 mutations cause XLRP in humans (34,35) and rod-cone dystrophy in mice (25,26). We first tested the visual impairment in our RP2null zebrafish. Electoretinography
(ERG) was carried out in 7 dpf (days post-fertilization) zebrafish. The scotopic b-wave amplitudes of RP2null zebrafish decreased by about 30% when compared with WT controls (Fig. 2), indicating the existence of a mild vision loss at an early age.

Next, we performed histological analysis to evaluate the morphology of the retinas. At the age of 2 months old, no obvious difference were observed between WT and RP2null zebrafish, except for the increased pigment granules in the OS layer of the RP2null zebrafish (Supplementary Material, Fig. S4A). In 7-months-old RP2null retinas, the abnormal pigmentation was also evident, and the OSs were shortened remarkably when compared with WT control (Supplementary Material, Fig. S4B), suggesting the presence of retinal degeneration.

After that, we wondered whether both the rods and cones were affected and when the abnormities of photoreceptors became obvious. Anti-rhodopsin (4D2) antibody and the peanut lectin (PNA) were used to label the OSs of rods and cones, respectively, in immunofluorescence analysis. At the age of 1 month, the OSs of rods (Fig. 3A, top panel) and cones (Fig. 3B, top panel) exhibited little or no difference between WT and RP2null zebrafish. In 2-months-old RP2null retinas, the OSs of rods were mildly shorter than that of WT controls with increased pigment deposition (Fig. 3A, mid-top panel). At 4 months of age, we found remarkable reductions in the length of rods’ OS in RP2null zebrafish with a ∼80% level of WT controls (Fig. 3A, mid-bottom panel and Fig. 3C). Meanwhile, the cones’ OS also showed aberrant morphology without decrease in number (Fig. 3B, mid-bottom panel). Furthermore, in 7-months-old RP2null retinas, the OS degeneration of rods increased (∼70% level of WT controls in OS’s length; Fig. 3A, bottom panel and Fig. 3C), and the loss of cones’ OS became evident (Fig. 3B, bottom panel, Fig. 3D and Supplementary Material, Fig. S5).

To look at the ultrastructure of the OSs of RP2null zebrafish, we performed transmission electron microscopy assay on the 10-month-old WT and RP2null zebrafish. The rods’ OS almost disappeared in the mutant retina (Fig. 4B and B′) when compared with WT control (Fig. 4A and A′). Among the remaining OSs of the RP2null retina, some of them showed normal morphology and disc stacking (Fig. 4C and C′), while some of them were shortened and disorganized (Fig. 4D and D′). Taken together, our observations demonstrated that deletion of RP2 leads to progressive retinal degeneration (noticed as early as 2 months old), affects both the rods and cones, but mainly the rods are influenced in early stages in zebrafish.

Deletion of RP2 does not affect the retinal localizations of the cone’s opsins

Mislocalization of the cone opsins has been reported to be a possible reason of the RP2-associated retinal degeneration (25). In this study, we investigated the retinal localizations of the four cone opsins by immunofluorescence assay. The cone opsins localized to the OSs normally in the 1 and 5.5 months old RP2null retinas (Supplementary Material, Fig. S6), which is the same as the normal transport of rhodopsin as shown in Figure 3A. Similar result has been found in another RP2 knockout mouse model (26). Thus, the mistrafcking of cone opsins is unlikely to be responsible for the retinal degeneration in our RP2 knockout zebrafish.

RP2 knockout results in decreased protein levels of GRK1, GNAT1 and GNB1 in zebrafish

Because the major interacting partner of RP2 is ARL3, a small GTPase playing an important role in dissociating the myristoyl-binding protein UNC119 or the prenyl-binding protein PDE6D from their cargos, we considered that RP2 might participate in the intracellular trafficking of myristoylated or prenylated retinal proteins.

Figure 2. Mild visual impairment in the RP2null larval detected by ERG analysis. (A) Representative traces of ERG of WT and RP2null zebrafish at 7 dpf. The arrows indicate the start and the end of light stimulation, respectively. (B) Comparison of b-wave amplitudes between WT (n = 10) and RP2null (n = 11) zebrafish using two-tailed Student’s t-test. The result was shown as mean ± SD. **P < 0.01.
such as the subunits of transducin and phosphodiesterase 6 (PDE6) complex in the phototransduction cascade. GRK1 (rhodopsin kinase) is a well-documented prenylated protein (36) involved in the phosphorylation and inactivation of rhodopsin in the light signal transduction process, and is also a disease-causing gene of autosomal recessive stationary night blindness (37). GNAT1, the alpha subunit of rod transducin, is a myristoylated protein that binds to UNC119. In UNC119-deleted mice, the transport of...
GNAT1 between the inner segment and the OS was delayed (38). Mutations in GNAT1 have been reported to cause autosomal dominant and recessive stationary night blindness (39,40). The beta subunit of rod transducin, GNB1, has been proven to be an RP2 interacting protein, and its membrane association could be reduced by RP2 RNAi in ARPE19 cells (15,33). For these reasons, we were curious about whether the transport in GRK1, GNAT1 and GNB1 were affected by RP2 ablation in zebrafish.

First of all, we detected the protein levels of GRK1, GNAT1 and GNB1 in our RP2null zebrafish at the age of 10 days, 2 and 4 months. For GRK1, the protein levels of RP2null groups decreased to ∼50% of WT controls from 10 dpf to 4 mpf (months post-fertilization; Fig. 5A and B). For GNAT1, in RP2null zebrafish, the protein levels were reduced from ∼40% (10 dpf) to 30% (4 mpf; Fig. 5A and C), showing an age-related decline pattern. For GNB1, although the protein level of 10 dpf stage was unchanged, significant reductions (∼50%) could be detected at 2 and 4 months of age in RP2null eyes (Fig. 5A and D). The gamma subunit of rod transducin, GNGT1, is also a prenylated protein (41) like GRK1. But we have not obtained suitable antibody against zebrafish GNGT1 for western blot yet and could not test it.

In addition, IFT20 (a component of the intraflagellar transport complex) has been reported to be involved in the trafficking of rod and cone opsins from the Golgi body to the base of the cilium (42), and its cellular localization is disrupted in the RNAi and RP2 mutant retinal pigment epithelium cells (12,33). In this study, we detected the protein levels of IFT20 in 4-month-old WT and RP2null eyes by western blot, and found that unlike GRK1, GNAT1 and GNB1, IFT20 was unaffected by RP2 knockout (Supplementary Material, Fig. S7), which is consistent with the normal localizations of rhodopsin and cone opsins in the RP2null zebrafish.

To further find out whether the down-regulation of these proteins happened on transcriptional level or post-translational level, we performed quantitative RT-PCR analysis to detect the mRNA levels of these genes in 2-months-old WT and RP2null eyes. Except for RP2, the mRNA levels of GRK1, GNAT1 and GNB1 were unchanged or even up-regulated (Fig. 5E). These results indicated that post-translational mechanism might be responsible for the reductions of GRK1, GNAT1 and GNB1 caused by RP2 elimination.

Abnormal retinal localizations of GRK1, GNAT1 and GNB1 in the RP2null zebrafish

After confirming the reductions of protein levels of GRK1 and rod transducin subunits, we examined their retinal localizations in 1- and 5.5-months-old WT and RP2null retinas by immunofluorescence assay. At the age of 1 month, the fluorescence signal of GNAT1 was markedly reduced in RP2null retinas (almost background fluorescence left) when compared with WT retinas (Fig. 6A, middle panel). Meanwhile, in RP2null retinas, the signals of GRK1 and GNB1 were mildly decreased with misplaced GRK1 still could be seen (arrows in Fig. 6B, upper panel), and the declines of the GNAT1 and GNB1 were obvious in the RP2 knockout zebrafish (Fig. 6B, middle and bottom panels). Additionally, the retinal localizations of the cone opsins kinase GRK7 were unaffected by RP2 knockout at the age of 2 and 4.5 months (Supplementary Material, Fig. S8), suggesting that the rod degeneration is predominant in our RP2null zebrafish. Based on the above-mentioned results, we speculated that RP2 might play a role in maintaining the protein level of GRK1 and rod transducin complex, which is important for the function and survival of the rod cells. The abnormal protein levels or retinal localizations of these proteins might be one of the pathogeneses of the rod predominant retinal degeneration caused by RP2 mutations probably.

Ablation of RP2 causes aberrant distribution of the farnesylated proteins in the retina

Recently, mistraficking of prenylated proteins has been reported to be the cause of RP2 (26). Using an antibody recognizing the farnesyl and geranylgeranyl groups (prenyl groups), we performed...
immunofluorescence assay on the 2- and 6-months-old retinal sections from WT and RP2null zebrafish to investigate the distribution of the total farnesylated proteins. We found that in WT retinas, the signals mainly localized to the photoreceptors from the outer plexiform layer to the base of inner segment and were faint in the OS. The fluorescence of the peripheral retina was stronger than that of the middle retina and central retina one by one (Fig. 7A and B, left panels). In 2-month-old RP2null retina (early stage of retinal degeneration), although no obvious difference could be seen in the peripheral and middle regions, the reduction of the signals near inner segment in the central region was evident (Fig. 7A). Furthermore, in 6-month-old RP2null retina (middle stage of retinal degeneration), the signals near inner segment were mostly lost in all the three regions (Fig. 7B). These results implied that deletion of RP2 might affect the trafficking of farnesylated proteins in the retina, which might be involved in the progress of the retinal degeneration in our RP2 knockout zebrafish.

**Discussion**

Retinitis pigmentosa refers to a group of progressive retinal dysfunction and degeneration diseases with irreversible loss of vision. In the hope of increasing the understanding of RP2-related RP and establishing an animal model for rapid screening of therapies, we generated an RP2 knockout zebrafish line, identified the progression of retinal degeneration in the RP2 deficient model and investigated the function of zebrafish RP2 protein in vivo in this study.

Mutations of human RP2 are thought to cause early-onset night blindness and subsequent peripheral and central visual loss, usually accompanied by high myopia with a semi-dominant
In 4-month-old RP2null retinas, the OS degeneration of rods bewhisphenotype identiFes with the presentations of rod-cone dystrophy. Our RP2null zebraFish agree with the observations from the gene-trapped well as the RP2 interaction protein GNB1. These results partly

and the prenylated protein GRK1 are both down-regulated, as

out zebraFsh, we found that the myristoylated protein GNAT1

occurs at the age of 2 months (25). However, in the gene-trapped eFects in ERG recording early in their lives, suggesting a diag-

nosis of rod-cone dystrophy (8). In the exon2-deleted RP2 mice, significant mislocalization of cone opsins rather than rod opsins occurs at the age of 2 months (25). However, in the gene-trapped RP2 mice, both the cone opsins and rhodopsin target normally to OSs. Instead, mistrafcking of cone PDE6 and GRK1 is more evident than rod PDE6 (26). These results, combined with the phenotype identiFed in the two RP2 knockout mice, accord with the presentations of rod-cone dystrophy. Our RP2null zebraFsh mentioned above show normal retina development and correct localization of rhodopsin and cone opsins at the age of 1 month. Mild deterioration of the rods’ OSs can be noted from as early as 2 months of age, whereas the cones’ OSs are not affected.

In 4-months-old RP2null retinas, the OS degeneration of rods be-

comes apparent, yet the cones are moderately affected. Further-

more, in 7-month-old RP2null retinas, both rods and cones show aggravated OS degeneration. Our observations prefer the dia-

nosis of typical RP to rod-cone dystrophy, which is much less severe than the phenotype of RP2 morphants described previously (29,30). The presence of ciliopathies such as pronephric cysts and heart looping defects in RP2 knockdown zebraFsh (11) has not been checked yet in our RP2null zebraFsh.

As a GTPase activator protein for ARL3, RP2 is speculated to play an important role in the correct localization of myristoy-

lated or prenylated retinal proteins through the ARL3-UNC119 or ARL3-PDE6D complex for several years (46–49). In the RP2 knock-

out zebraFsh, we found that the myristoylated protein GNAT1

and the prenylated protein GRK1 are both down-regulated, as well as the RP2 interaction protein GNB1. These results partly agree with the observations from the gene-trapped RP2 mouse model, which shows declined level of GRK1 in immunofluores-

cence assay but with normal protein levels of GRK1 and GNAT1 in western blot assay (26). The three models both show normal transport of rhodopsin, although the OSs of rods are dramatically shortened in late stages. The gene-trapped RP2 mice exhibit a slow progression of retinal degeneration with a ~15% reduction in the retina thickness at 2 years of age. These may explain the normal levels of GRK1 and GNAT1 in 1-month-old RP2-trapped mouse retinas. Furthermore, there is only one opsin kinase (GRK1) in mouse while human and zebraFsh have two opsin kinases, GRK1 and GRK7 (speciFed for the phosphorylation of cone opsins) (50).

The retinal localization of GRK7 is not changed in 2- and 4.5-

months-old RP2null zebraFsh eyes (Supplementary Material, Fig. S8). This difference may partly explain the rod-dominant phenotype of RP2 patients and RP2 knockout zebraFsh and the cone-dominant phenotype of RP2 knockout mice. Besides, we also found abnormal distribution of the prenylated proteins in RP2 mutant retinas, suggesting that RP2 may play a role in the traf-

Fcking of prenylated retinal proteins. The exact mechanism of how RP2 regulates GRK1, GNAT1 and GNB1, whether RP2 interacts with them and if ARL3 is involved in requires further studies in animal and cell line models. According to the best of our knowledge, we guessed that the post-translational modiFedation (such as prenyla-

tion and myristoylation) and intracellular trafFcking of these pro-

teins may be the key to answer these questions.

Our work provides important cues for revealing the function of RP2 in vivo and the pathogenesis of RP caused by RP2 muta-

tions. In addition, zebraFsh is a low-cost laboratory animal model with high rate of spawning and fast development speed. Coupled with the facts that the zebraFsh genome has been se-

quenced completely and many of the genes and pathways are conserved between human and zebraFsh, we believe that our RP2null zebraFsh, the first RP zebraFsh model constructed by TALEN technology, will be a useful platform for large-scale screening of therapies against RP in future.
Materials and Methods

Zebrafish maintenance and breeding

The study was approved by the Ethics Committee of Huazhong University of Science and Technology. Laboratory inbred wild-type AB line of zebrafish was employed. The zebrafish were placed in recirculating water system (pH 6.6–7.4, 26–28.5°C) with a daily cycle of 14 h of light and 10 h of dark. Adult zebrafish were fed three times per day with fresh brine shrimps, whereas baby fish were fed three times daily with live paramecia after 5 dpf, paramecia mixed with brine shrimps after 10 days and only the brine shrimps after 30 dpf. For mating, males and females (1:1 or 1:2) were moved to crossing cages in the early evening and left undisturbed until the following morning. The males and females in the crossing cage were separated with a plastic divider that was removed in the morning before spawning. The eggs were collected and transferred to a Petri dish in embryo medium, and then kept in an incubator (∼28.5°C) for 72 h until the larvae were hatched.

TALEN construction and microinjection

The online tool TAL Effector-Nucleotide Targeter (TALE-NT) (51) was used to design pairs of TAL effectors (TALE) for the fused endonucleases to target the zebrafish RP2. The targeted sequences are 5′ GTGTAAGCTGGCATT3′ (TALE-L) and 5′ CTGAAAGAACA-CACTGCCT3′ (TALE-R). The assembly of customized TALEs was accomplished using the FastTALE™ TALEN kit (Sidansai Biotechnology, China) according to the operating manual. TALEN mRNAs were in vitro transcribed and purified using mMESSAGE mMACHINE® kit (Ambion). A pair of TALEN mRNAs was mixed and microinjected into one-cell stage eggs of zebrafish.

Mutant zebrafish screening

Ten embryos were collected from each dish of injected eggs at 48 hpf in order to extract a mixed genomic DNA. The effect of the TALEN-mediated mutagenesis was tested by restriction enzyme (AvaII, see Fig. 1A) digestion analysis. The rest embryos of the effective groups were raised to sexual maturity and cross-fertilized with WT fish to balance the genetic background and generate F1 zebrafish. F1 zebrafish were brought up to 2 months and examined using the PCR-restriction fragment length polymorphism (PCR-RFLP) method. The heterozygotes were further subjected to DNA sequencing to identify the exact mutations. Males and females carrying the same mutation mated with each other to make homozygotes. Homozygotes were identified using the PCR-RFLP method and further confirmed by DNA sequencing. Their offsprings were employed in the further research.

Quantitative PCR

Total RNAs were extracted from 2 mpf WT and RP2null zebrafish eyes using RNAiso Plus reagent (Takara), and reverse-transcribed...
into cDNA using MMLV reverse transcriptase (Invitrogen) and oligo(dT) primer (Takara). The cDNA was served as templates to detect the mRNA levels of the specified genes by quantitative PCR, which were performed on the StepOnePlus™ real-time PCR System (Life Technologies) using AceQ™ qPCR SYBR® Green Master Mix (Vazyme). The data were analysed using the 2-ΔΔ Ct method in the StepOne software (version 2.3).

Significance was determined by two-tailed Student’s t-test. Primer sequences for RP2 (NM_213446), actb1 (NM_131031), GRK1a (NM_001034181), GRK1b (NM_001017711), GNAT1 (NM_131868), GNB1a (NM_212609) and GNB1b (NM_213481) were presented in Supplementary material, Table S2.

Table 1. List of primary antibodies used in this study

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ZIRC, Zebrafish International Resource Center; WB, western blot; IF, immunofluorescence.
at the concentration of 5 µg/ml. The sections were then rinsed three times with PBS, and mounted with a glycerol-based liquid mountant under coverslips. Fluorescence images were acquired using a confocal laser-scanning microscope (FluoView™ FX1000 confocal microscope, Olympus Imaging). The quantitation of the images was performed using the ImageJ software.

Transmission electron microscopy

Adult zebrafish eyes were enucleated and fixed in 2.5% glutaraldehyde in 0.1 M PBS buffer (pH 7.0) overnight at 4°C. After three washes (15 min each) with 0.1 M PBS buffer, the eyes were further fixed in 1% osmium tetroxide in 0.1 M PBS buffer for 2 h at RT. After three washes (15 min each) with 0.1 M PBS buffer, the eyes were dehydrated in 50, 70, 80, 95 and 100% ethanol successively (20 min each), and incubated in acetone for 20 min at RT. The eyes were treated with 50% (1 h), 75% (3 h) and 100% (overnight) epoxy resin (mixed with acetone, w/v), and then heated at 70°C overnight. Embedded eyes were sliced to ultrathin sections (70 nm) using a Reichert-Jung ultramicrotome (Leica). Sections were stained with 3% uranyl acetate and 3% lead citrate for 15 min and visualized with a transmission electron microscope system (HT7700, Hitachi).

Supplementary Material

Supplementary Material is available at HMG online.

Acknowledgements

We are grateful to Dr Yafeng Liu for his great help in the zebrafish ERG measurement, to Professor David R. Hyde (University of Notre Dame) and Professor Fulton Wong (Duke University, School of Medicine) for the kind gift of the zebrafish UV and blue opsin antibodies and to Professor Stephan C.F. Neuhauss (University of Zurich) for the kind gift of the zebrafish GRIK7a antibody.

Conflict of Interest statement. None declared.

Funding

This work was supported by the National Natural Science Foundation of China (nos. 31071106, 81270983, 81371064, 31471199 and J1103514), the Research Fund for the Doctoral Program of Higher Education of China (20120142110007), 'Program of Introducing Talents of Discipline to Universities' by Ministry of Education of PR China (B08029), and National Key Technology R&D Program in the 12th Five Year Plan of China (2012BAI09900). The work was also supported by the Rosetrees Trust, UK Fight for Sight and National Research Center (X.S.).

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