Zebrafish Rpgr is required for normal retinal development and plays a role in dynein-based retrograde transport processes

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Mutations in the human RPGR gene cause one of the most common and severe forms of inherited retinal dystrophy, but the function of its protein product remains unclear. We have identified two genes resembling human RPGR (ZFRPGR1, ZFRPGR2) in zebrafish (Danio rerio), both of which are expressed within the nascent and adult eye as well as more widely during development. ZFRPGR2 appears to be functionally orthologous to human RPGR, because it encodes similar protein isoforms (ZFRPGR2ORF15, ZFRPGR2ex1-17) and causes developmental defects similar to other ciliary proteins, affecting gastrulation, tail and head development after morpholino-induced knockdown (translation suppression). These defects are consistent with a ciliary function and were rescued by human RPGR but not by RPGR mutants causing retinal dystrophy. Unlike mammals, RPGR knockdown in zebrafish resulted in both abnormal development and increased cell death in the dysplastic retina. Developmental abnormalities in the eye included lamination defects, failure to develop photoreceptor outer segments and a small eye phenotype, associated with increased cell death throughout the retina. These defects could be rescued by expression of wild-type but not mutant forms of human RPGR. ZFRPGR2 knockdown also resulted in an intracellular transport defect affecting retrograde but not anterograde transport of organelles. ZFRPGR2 is therefore necessary both for the normal differentiation and lamination of the retina and to prevent apoptotic retinal cell death, which may relate to its proposed role in dynein-based retrograde transport processes.

INTRODUCTION

Retinitis pigmentosa (RP) is a progressive outer retinal dystrophy characterized by impaired dark adaptation, visual field defects and pigmentary retinopathy (1). RP affects 1/3500 individuals in most populations and can be inherited as an autosomal dominant, autosomal recessive, X-linked, mitochondrial or oligogenic trait (2). X-linked RP (XLRP) is one of the most severe forms of human retinal degeneration, as determined by age-of-onset and progression, which accounts for 6–20% of all RP cases (3–7). XLRP has been genetically mapped to at least five loci: RP2, RP3, RP23, RP24 and RP34 (http://www.sph.uth.tmc.edu/retnet/), but RP3 is the major subtype, accounting for 70–80% of affected families (8–10). The RP3 locus contains the Retinitis Pigmentosa GTPase Regulator (RPGR) gene, which is mutated in 10–20% of all RP patients, making it one of the commonest genetic causes of RP (5).

Human RPGR shows a complex pattern of alternative splicing (11–14). The gene was initially identified as containing 19 exons (RPGRex1-19), encoding a predicted 90 kDa protein (15,16). Mutations in RPGRex1-19 accounted for only 15–20% of XLRP patients, and subsequent studies revealed many more disease-causing mutations within one or more alternatively spliced transcripts containing an unusual carboxyl (C)-terminal exon called open reading frame 15 (ORF15; RPGRORF15) (10,12,17–19). Exon ORF15 encodes a repetitive glycine and glutamic acid-rich domain of unknown function and a basic C-terminal domain (ORF15C2) (17). This exon

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harbours a high frequency of microdeletions and premature termination codons (5,12,18,20,21). Over 290 RPGR<sup>ORF15</sup> mutations have been identified, the majority (95%) of which give rise to XLRP but some cause cone–rod dystrophy, cone dystrophy, atrophic macular degeneration (3%) or RP with primary ciliary dyskinesia (2%) (10,22).

The function of RPGR is unclear, although the N-terminal half of RPGR (exons 2–11) is structurally similar to the regulator of chromosome condensation (RCC1), a guanine nucleotide exchange factor for Ran, a small GTP-binding protein involved in nucleocytoplasmic transport and cell cycle control (15,23). RPGR<sup>ORF15</sup> is predominantly expressed in photoreceptor connecting cilia and centrosomes/basal bodies but expression has also been reported both in nuclei and in the outer segments of some species (24–29).

RPGRORF15 has been shown to co-immunoprecipitate in retinal extracts with a number of different axonemal, centrosomal/basal body and microtubular transport proteins, and its localization to basal bodies was shown to be dependent on the retrograde dynein–dynactin motor complex (26,27). However, it is not clear whether it is a direct participant in ciliary transport processes or simply a cargo.

Loss of RPGR function both in a mouse model and in human XLRP cause retinal degeneration with mislocalization of rod and cone opsins but normal photoreceptor morphogenesis (24,30). Only one RPGR mutant, the XLTRA2 dog, shows both abnormal photoreceptor development and progressive degeneration. Affected dogs show abnormal photoreceptor morphogenesis with misaligned outer segments at 1 month of age and reduced amplitude electroretinograms (ERGs) followed by a rapidly progressive photoreceptor degeneration (31–33).

The vertebrate neural retina shows strong evolutionary conservation (34). The anatomy, histology and function of the zebrafish retina closely resembles human retina, with the same major cell classes organized in the same laminar pattern. The zebrafish is therefore an increasingly attractive model for studying both retinal neurogenesis and degeneration (35). Several genetic defects of the zebrafish retina are similar to human disorders and some mutations affecting photoreceptor cells in zebrafish resemble RP and cone–rod dystrophies (36–37). The neurogenesis of the zebrafish retina starts very early: the optic primordium appears at about 11.5 h post-fertilization (hpf) and the optic eye cup is well formed by 24 hpf (38). Between 24 and 34 hpf, the retina undergoes rapid proliferation and consists of two main epithelial layers: the pigmented epithelium and the retinal neuroepithelium. Retinal ganglion cells (RGC) are the first to differentiate, becoming postmitotic starting at about 28 hpf (38,39) and RGC differentiation spreads rapidly into the temporal retina between 36 and 40 hpf (40). The inner nuclear layer (INL) starts to appear at 38 hpf and the first photoreceptors become postmitotic at about 43 hpf (39,40). Photoreceptors begin to differentiate at 50–54 hpf (39) and the first outer segments can be readily observed at 54–60 hpf (40). The lamination of ganglion cells occurs at 32–40 hpf and full lamination is established by 60 hpf. Zebrafish exhibit visual function by 72 hpf (40,41).

Here we use zebrafish as a model to investigate RPGR function. We first identified and then examined the function of zebrafish RPGR using antisense methodology (morphpolino oligonucleotides, MOs) to suppress zebrafish RPGR expression during early development. Zebrafish (ZF) RPGR deficient fish were found to exhibit a range of developmental abnormalities, similar to those seen with suppression of other ciliary proteins, followed by retinal degeneration associated with extensive apoptosis-like cell death. We also measured the movement of pigment granules (melanosomes) along microtubules in pigmentated zebrafish skin cells and found that RPGR is associated with retrograde (cytoplasmic dynein–dynactin complex) but not anterograde (kinesin-2) molecular motors and so appears to be a direct participant in ciliary transport processes.

RESULTS

Identification of zebrafish RPGR

A basic local alignment search tool (BLAST) search of the Ensembl zebrafish database was performed using human RPGR protein sequences (Acc. nos AAC50481.1 and DAA05713.1) to identify the zebrafish RPGR gene. This showed that the zebrafish RPGR gene is duplicated, with one copy on chromosome 9 and the other on chromosome 11.

The genomic sequence around each putative zebrafish RPGR was compared using human RPGR protein as a template in the GeneWise programme to identify the predicted zebrafish protein and gene sequences. On the basis of these predictions, specific primers were designed (Supplementary Material, Table S1) to clone zebrafish RPGR cDNA by reverse transcriptase–polymerase chain reaction (RT–PCR). The first putative zebrafish RPGR homologue, called ZFRPGR<sup>1ORF15</sup> (corresponding to rpgra in ZFIN, http://zfin.org/), encodes an open reading frame of 1697 amino acids and consists of at least 13 exons, spanning ~14 kb of genomic sequence on chromosome 9 (Fig. 1). The second putative RPGR homologue, ZFRPGR2 (corresponding to rpgrb in ZFIN, http://zfin.org/) on chromosome 11, has at least two isoforms (ZFRPGR2<sup>ORF15</sup> and ZFRPGR2<sup>ex1-17</sup>). The ZFRPGR2<sup>ORF15</sup> isoform is predicted to encode 1413 amino acids and to contain 14 exons, spanning ~16 kb. The ZFRPGR<sup>2ex1-17</sup> isoform encodes 708 amino acids and contains 17 exons, spanning ~19 kb (Fig. 1). Protein sequence comparisons showed a high degree of homology in the RCC1-like (RCCL) domains between zebrafish and human RPGR (Fig. 1), although there is relatively low identity at the level of full-length protein sequence (Supplementary Material, Fig. S1 and Table S2). Predicted ZFRPGR<sup>1ORF15</sup> and ZFRPGR2<sup>ORF15</sup> proteins both contain a glutamic acid- and glycine-rich domain similar to that in human RPGR<sup>ORF15</sup>. The ZFRPGR2<sup>ex1-17</sup> isoform is shorter than its mammalian homologue (which ranges from 815 to 1003 amino acids). The isoprenylation signal motif of human RPGR<sup>ex1-19</sup> was conserved in ZFRPGR2<sup>ex1-17</sup> (Supplementary Material, Fig. S1) (28).

Expression of zebrafish RPGR genes in early development and adult tissues

The temporal and spatial expression pattern of zebrafish RPGR genes during embryogenesis was first examined by RT–PCR,
using primers directed to the RCCL domains, which were therefore capable of detecting all major alternatively spliced isoforms. The ZFRPGR1*orf15 transcript was readily detected in 13-somite stage (15 hpf) embryos and throughout development up to 5 dpf. ZFRPGR2 (ZFRPGR2*orf15 and ZFRPGR2*ex1-17) was detected at the time of fertilization and persisted during gastrulation and through the tailbud and larval stages (Fig. 2A). ZFRPGR1 and ZFRPGR2 expression in adult tissues was examined in total RNA isolated from zebrafish kidney, muscle, ovary, liver, intestine, brain, eye, heart, skin and testis by RT–PCR. Both ZFRPGR1 and ZFRPGR2 were readily detected in the eye, but ZFRPGR1 was also detectable in muscle and weakly in ovary and testis, whereas ZFRPGR2 was detected in all tissues examined other than liver and kidney (muscle, ovary intestine, brain, heart, skin and testis) (Fig. 2B).

To determine the temporal and spatial expression pattern of ZFRPGR genes in the visual system during early development, whole-mount in situ hybridization was carried out on zebrafish embryos at stage 24 hpf. Both ZFRPGR1 and ZFRPGR2 showed similar expression patterns, with high dorsal transcript expression in the retina, and also in the brain and neural tube (Fig. 2C and D). To investigate the expression of ZFRPGR1 and ZFRPGR2 protein in adult zebrafish eyes, we performed

Figure 1. Identification of zebrafish RPGR genes. (A) Exon/intron structures of the ZFRPGR1*orf15, ZFRPGR2*orf15 and ZFRPGR2*ex1-17 transcripts. (B) Schematic structure of ZFRPGR1*orf15 (GU012647), ZFRPGR2*orf15 (GU012648) and human RPGR*orf15 proteins (DAA05713.1, drawn to scale). The RCC1-like domain (RCCL) and glutamic acid-rich domain (ORF15) are shown with the residue numbers. (C) Schematic structure of ZFRPGR2*ex1-17 (GU012649) and human RPGR*ex1-19 proteins (AAC50481.1).
Figure 2. Expression and localization of zebrafish RPGR. (A) Temporal expression of ZFRPGR1 and ZFRPGR2 genes detected by RT–PCR from total RNA extracted from oocytes and at different developmental stages of zebrafish. (B) ZFRPGR1 and ZFRPGR2 expression in different zebrafish tissues detected by RT–PCR. (C and D) Whole-mount RNA in situ hybridization analysis showing both ZFRPGR1 and ZFRPGR2 expression in the retina, brain and neural tube of embryos at 24 hpf. R, retina; L, lens. (E and F) Localization of ZFRPGR1 and ZFRPGR2 proteins in adult zebrafish photoreceptors, where the connecting cilia of rod cells (E) and cone cells (F) were labelled with anti-RPGR antibody (red). The outer segments of rod cells were labelled with anti-rhodopsin antibody (green in E), whereas cone cells were labelled with anti-Zrp-1 antibody (green in F). Nuclear layers were labelled with Toto-3 (blue in E and F). Photographs were at ×20 magnification. GL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.
immunostaining with a polyclonal antibody raised against a human RPGR peptide from a region that is conserved between human RPGR and ZFRPGR1 and 2 (Supplementary Material, Fig. S2). ZFRPGR (including ZRFPGR1 and ZFRPGR2) was expressed in the connecting cilium region of rod and cone photoreceptors but was only clearly evident in the longer outer segments of rods (Fig. 2E and F). In whole zebrafish adult eye lysates, we detected three RPGR-positive bands on western blots, corresponding to ZFRPGR1 ORF15, ZFRPGR2 ORF15 and ZFRPGR2 ex1-17 (Fig. 3F).

**Phenotype of morpholino-mediated ZFRPGR knockdown**

In order to evaluate whether the loss of RPGR function causes embryonic defects, we designed antisense morpholinos to suppress protein expression by disrupting translation due to targeting of the initiating methionine in either ZFRPGR1 or ZFRPGR2. Morpholinos that target ZFRPGR1 (MO1), ZFRPGR2 (MO2) or both together (MO1 + MO2) were injected into 1–2 cell stage embryos. As negative controls, either a standard negative control morpholino (CMO) or a mispair control morpholino (MO2 with five base modifications marked out of 25; mCMO2) was used at the same concentrations as either CMO-injected embryos. MO2-injected embryos displayed a small eye phenotype (mild phenotype, M) or very small eye with small head (severe phenotype, S). Most MO2 morphants also have shortened, curved tails. (E) The measurement of diameter (eye size) of the eyes from CMO-injected (control) morphants, MO2 injected morphants with a mild phenotype, and MO2 injected morphants with a severe phenotype. The mean eye size for control morphants is 205.7 μm, for MO2 morphants with a mild phenotype is 185.3 μm, for MO2 morphants with a severe phenotype is 149.7 μm. (F) Western blot analysis with anti-RPGR antibody in zebrafish adult eye lysates showed three bands, which are consistent with ZFRPGR1 ORF15, ZFRPGR2 ORF15 and ZFRPGR2 ex1-17. In the lysates prepared from MO2-injected embryos at 48 hpf, the control ZFRPGR2 ex1-17 (also present in adult eye) and presumed ZFRPGR2 ORF15 (arrowed) bands both disappear. The ZFRPGR2 ORF15 band seen in adult eye is not seen in whole embryos, probably because it is most strongly expressed in eye. The arrowed band seen in whole embryo control lysates may represent an alternatively spliced or post-translationally modified isoform.

Figure 3. Knockdown of ZFRPGR in zebrafish embryos by morpholinos. (A) At 13 hpf, ZFRPGR2 morpholino (MO2)-injected embryos displayed dark brown granules in the prominent horizontal crease of the optic primordium (arrowhead), whereas ZFRPGR1 morpholino (MO1)-injected and control morpholino (CMO)-injected embryos were normal. (B) At 24 hpf, the eye area of MO2 morphants exhibited a dark brown and unclear boundary between lens and neural retina compared with control and MO1 morphants (arrows show the eye area). (C and D) At 48 hpf, MO1-injected embryos were normal (N) as were CMO-injected embryos. MO2-injected embryos displayed a small eye phenotype (mild phenotype, M) or very small eye with small head (severe phenotype, S). Most MO2 morphants also have shortened, curved tails. (E) The measurement of diameter (eye size) of the eyes from CMO-injected (control) morphants, MO2 injected morphants with a mild phenotype, and MO2 injected morphants with a severe phenotype. The mean eye size for control morphants is 205.7 μm, for MO2 morphants with a mild phenotype is 185.3 μm, for MO2 morphants with a severe phenotype is 149.7 μm. (F) Western blot analysis with anti-RPGR antibody in zebrafish adult eye lysates showed three bands, which are consistent with ZFRPGR1 ORF15, ZFRPGR2 ORF15 and ZFRPGR2 ex1-17. In the lysates prepared from MO2-injected embryos at 48 hpf, the control ZFRPGR2 ex1-17 (also present in adult eye) and presumed ZFRPGR2 ORF15 (arrowed) bands both disappear. The ZFRPGR2 ORF15 band seen in adult eye is not seen in whole embryos, probably because it is most strongly expressed in eye. The arrowed band seen in whole embryo control lysates may represent an alternatively spliced or post-translationally modified isoform.
ZFRPGR2 deficiency affects retinal development

We carried out histological analysis of the retina from control and ZFRPGR2 deficient morphants at 72 hpf and found that normal retinal lamination could be readily observed in control morphants, with discrete ganglion cell layers (GL), INL and outer nuclear layers (ONL). By this stage, the outer segment of photoreceptors are normally formed but in ZFRPGR2-deficient morphants, lamination was defective, since the three cell layers (GL, INL and ONL) were absent (Fig. 4A and B) and rod photoreceptor outer segment formation (detected with anti-rhodopsin antibody) was absent (Supplementary Material, Fig. S5). Similarly, cone photoreceptor staining with Zpr-1 antibody was absent in mild (MO2M) and severe (MO2S) morphants (Fig. 4A). The development of the retinal pigmented epithelium (RPE) cells was unaffected in ZFRPGR2-deficient morphants, because the distribution and characteristic pigmentation of RPE was similar between control and ZFRPGR2-deficient larvae (Fig. 4B).

Knockdown of ZFRPGR2 leads to cell death

ZFRPGR2 morphants exhibited abnormally dark, non-transparent eyes as early as 24 hpf. Later, at 48 and 72 hpf, the eyes of MO2-injected embryos were either small with normal head size (‘mild’ phenotype) or very small with small heads (‘severe’ phenotype). Classification was quantitative, based only on eye size, with 175–200 μm (mild), 100–175 μm (severe) or >200 μm (normal) (Supplementary Material, Table S3). These phenotypes have previously been attributed to ongoing neurodegeneration as a result of apoptotic cell death (35,43), which was therefore investigated further.

Acridine orange is a vital dye reported to stain apoptotic cells but not necrotic cells in Drosophila (45) and is widely used to detect cell death in vivo in zebrafish (43,46–48). Control and ZFRPGR2-deficient embryos were incubated at 48 hpf in acridine orange solution which showed that the lack of ZFRPGR2 protein causes an increase in acridine orange accumulation in the retina (Fig. 5A). Cell death in the retina from control and ZFRPGR2-deficient fish was also examined in early larvae at 72 hpf by TUNEL staining, which showed that lack of ZFRPGR2 causes increased apoptotic cell death in the retina and in the brain of ZFRPGR2-deficient morphants showing the ‘severe’ small eye/head phenotype (Fig. 5B and C).

Knockdown of ZFRPGR2 delays retrograde intracellular transport

RPGR is associated with both retrograde (cytoplasmic dynein–dynactin complex) and anterograde (kinesin-2) molecular motors (27), but it is unclear whether it is a cargo or a direct participant in intracellular transport processes. One means of investigating this is to use zebrafish as a model system to monitor pigment granule (melanosome) movement along microtubules within non-ciliated pigmented skin cells (melanophores) in response to light or drugs that promote pigment granule aggregation or dispersion (49,50). If RPGR is solely a cargo in microtubule-based transport, then its suppression should not affect melanosomal or other organelar transport rates along microtubules. Melanosomes are lysosome-related organelles whose bi-directional translocation towards the cell centre (aggregation) or towards the cell periphery (dispersion) are carried out by different motor proteins.
The aggregation of pigment granules requires a minus-end-directed molecular motor (cytoplasmic dynein), whereas dispersion requires a plus-end-directed motor (kinesin-2) (50,51). Pigment aggregation (retrograde) or dispersion (anterograde) can be achieved within minutes after treatment with epinephrine and caffeine, respectively, due to their effects on cyclic AMP levels (50).

In our assay, embryos at 4 dpf displayed melanophore dispersion after overnight dark adaptation (Fig. 6A). When the embryos were treated with epinephrine, the melanosomes rapidly aggregated and the area of pigmentation was reduced (Fig. 6A). At the maximum aggregation endpoint, all pigment granules showed perinuclear accumulation of melanosomes (Fig. 6A). The time to reach this pigment aggregation endpoint was measured. CMO-injected embryos rapidly retracted their melanosomes (3.6 min), whereas ZFRPGR2 morphants with a ‘mild’ phenotype showed significantly delayed melanosome aggregation (9.83 min) and morphants with a ‘severe’ phenotype showed very slow melanosome retraction and only reached the endpoint after 30.65 min of exposure (Fig. 6B).

Anterograde melanosome transport was evaluated by the recovery time to reach full pigment dispersion after the early larvae were treated with caffeine. The recovery time for ZFRPGR2 morphants (mild or severe phenotype) was similar to that for the control larvae, suggesting that ZFRPGR2 does not influence anterograde transport (Fig. 6B).

**Rescue of ZFRPGR2 morphant phenotypes**

To confirm that the phenotypes described above were RPGR-specific and to test whether those phenotypes are relevant to human RPGR function, we co-injected human RPGR wild-type (RPGR<sup>ex1-19</sup>, RPGR<sup>ORF15</sup>) or mutant (RPGR<sup>ORF15c.2522delA</sup> or RPGR<sup>ORF15c.2650G>T</sup>) mRNA together with ZFRPGR2 morpholino (MO2) and scored the phenotypes at 72 hpf. Rescue was obtained with wild-type but not mutant RPGR mRNAs. The overall appearance of fully rescued embryos was similar to that of the control embryos, with eyes of normal size and normal body appearance (Fig. 7). Human RPGR<sup>ORF15</sup> produced the most efficient rescue (61%), whereas human RPGR<sup>ex1-19</sup> gave only a 20% rescue and two RPGR<sup>ORF15</sup> mutants, each of which causes XLRP in patients, showed a low level of rescue (14.6% for RPGR<sup>ORF15c.2522delA</sup>, 12% for RPGR<sup>ORF15c.2650G>T</sup>) (Fig. 7C).
whole-genome duplication occurred in teleosts, one of each gene pair often degenerates into a pseudogene or is lost from the genome due to its redundant function. However, if the duplicated genes acquire non-redundant functions, then both may be retained, but their spatiotemporal expression patterns or functions diverge over time (54). The latter outcome appears to be the case for the duplicated zebrafish RPGR genes, which show 26% (ZFRPGR1\textsubscript{ORF15}) to 34–43% (ZFRPGR2\textsubscript{ORF15}) amino acid identity overall and both of which are retained, although with different developmental and adult expression patterns.

Similar to mammalian and amphibian RPGR genes, both zebrafish RPGR-like genes are developmentally expressed, with ZFRPGR2 most similar to the pattern of RPGR expression in *Xenopus laevis* embryos, where expression was found in oocytes and early cleavage stage embryos as well as later stages of development. Whole-mount in situ hybridization analysis of ZFRPGR2 in 24 hpf embryos showed strongest expression in brain, eye and neural tube, reminiscent of amphibian embryos, where RPGR expression was first evident in the anterior neural plate, then in the neural tube and developing optic vesicles, brain, notochord and eye primordium, becoming increasingly localized to the eye and retina as development progressed (28). Within the adult eye, both ZFRPGR1 and ZFRPGR2 showed high dorsal transcript expression in the retina (Fig. 2C and D) and immunostaining showed expression of these proteins in the connecting cilia of rods and cones and the outer segments of rods (Fig. 2E and F).

The functions of the two zebrafish RPGR genes also appear to have diverged, because when specific MOs were used to effectively suppress both genes, only ZFRPGR2 suppression resulted in an abnormal developmental phenotype, similar to other ciliary proteins. A working hypothesis was therefore adopted in which ZFRPGR2 is the human RPGR orthologue, whereas ZFRPGR1 is a paralogue, with an unknown but divergent function. This conclusion is supported by the failure of native ZFRPGR1 to rescue ZFRPGR2-targeted (MO2) morphants (Fig. 3F).

The function of ZFRPGR2 was therefore studied in greater depth with particular attention to the ocular phenotype. The main finding in ZFRPGR2 morphants was that the eyes were smaller than in controls at 48 hpf (Fig. 3D–E) and at 72 hpf the retina showed both defective lamination and abnormal photoreceptors, lacking outer segments, which are normally present by 60 hpf (40) (Fig. 4A and B). Further insight into this observation was obtained by staining with acridine orange, which stains apoptotic but not necrotic cells (43,45,47,48), and showed that the number of apoptotic retinal neurons is significantly increased in ‘severe’ morphants (Fig. 5A). This was confirmed by quantification of apoptotic neurons following TUNEL staining, which showed progressively greater numbers of apoptotic neurons from ‘mild’ to ‘severe’ morphants (Fig. 5B and C). Cell death did not appear to affect other ocular structures, which suggested that it might be a consequence of abnormal retinal differentiation. Retinal lamination defects and/or increased developmental cell death have been observed in the context of genetic defects affecting transcription factors (e.g. math5, Prox1, NR2E3) (55–58), cell cycle regulators (e.g. Cdkn1b/c,
Cdk5) (59) and the intraflagellar transport protein IFT88 (60). A homozygous mutation in the mouse IFT88 gene causes abnormal photoreceptor differentiation, with reduced and disorganized outer segments but normal lamination, followed by progressive apoptotic degeneration (31,32). Similarly, the Rpgr\textsuperscript{XLPRA2} mutant dog has disorganized and shortened photoreceptor outer segments (although with normal retinal lamination) followed by a rapidly progressive degeneration (31,32). This contrasts with other ciliary proteins, which show progressive photoreceptor degeneration but normal retinal development (49). Cell death resulting from ZFRPGR2 knockdown could result from abnormal retinal development, as seen in Nr2e3 (55,57) or Cdkn1b/c mutants (59). Alternatively, abnormal retinal development (dysplasia) could itself result from a severe and early retinal degeneration, as appears likely with the RPGR\textsuperscript{XLPRA2} and

Figure 7. Rescue of the ZFRPGR2 knockdown phenotype using human RPGR mRNA. (A) Schematic representation of the domain structures of human RPGR\textsuperscript{ex1-19} and RPGR\textsuperscript{ORF15} and two RPGR\textsuperscript{ORF15} mutants (RPGR\textsuperscript{ORF15c-2522delA}, RPGR\textsuperscript{ORF15c-2650G>T}), showing their common RRC1-like domain (RCCL). (B) Rescue of the overall MO2 phenotype at 72 hpf by co-injected full-length human RPGR\textsuperscript{ORF15}. The rescued phenotypes included small eyes, small head and body length and curved tail. (C) Graphical representation of the percentage of ZFRPGR2 knockdown phenotypes rescued by co-injection with human RPGR\textsuperscript{ex1-19} (MO2+Wt ex1-19), RPGR\textsuperscript{ORF15} (MO2+Wt ORF15), RPGR\textsuperscript{ORF15} with c.2522delA mutation (MO2+c.2522delA) and RPGR\textsuperscript{ORF15} with c.2650G>T (MO2+c.2650G>T). The number of fish in each group varied from 30 to 45 (Supplementary Material, Table S4). Only normal human RPGR\textsuperscript{ORF15} produced significant rescue.
*IFT88* mutations. The small eye phenotype therefore appears to involve both abnormal retinal development (dysplasia) and extensive retinal cell death.

What mechanism could be responsible for the abnormal extraocular development of zebrafish embryos resulting from ZFRPGR*ORF15* suppression? Similar extraocular defects have been observed by transcript suppression of other ciliaopathy genes (42,61–64). Defective gastrulation or neurulation resulting from ciliopathies is thought to result from impaired planar cell polarity, in part due to abnormal Wnt signalling, which requires basal body/ciliary function (63,65). Hedgehog signalling has also been reported to require cilia (66), although this has been questioned in zebrafish (67). The type of developmental defect observed in ZFRPGR*ORF15* knockdowns, and in many other ciliary disorders, is associated with defective cell movement, rather than cell death or toxicity, and results in shortened body axis, broad notochord, thin extended somites and failure of tail extension. In some ciliopathies, small head and eyes are also observed (27), although the precise ocular phenotype has not been investigated. All of these changes were observed after knockdown of ZFRPGR*ORF15* consistent with a ciliary function for RPGR*ORF15*. These phenotypes were rescued by wild-type human RPGR*ORF15* but not by human RPGR*ORF15* mutants associated with retinal dystrophies, consistent with ZFRPGR2 being the orthologue of human RPGR*ORF15*. It remains unclear why similar non-ocular developmental abnormalities do not occur with mutations affecting mammalian forms of RPGR.

The function of ZFRPGR2 was further elucidated by the demonstration that its knockdown in zebrafish embryos resulted in disruption of the retrograde (minus-end-directed) movement of skin melanosomes along microtubules but did not disrupt anterograde movement. Pigmented cells can be used as a model for intracellular organelle transport (49,50). Fish melanophores contain hundreds of melanin-filled pigment granules, called melanosomes, which can aggregate at the centre of the cell or disperse throughout the cytoplasm, allowing the fish to change colour in response to environmental conditions. Both types of motor protein have been indirectly associated with RPGR*ORF15* previously by co-immunoprecipitation experiments (27). The melanosome transport results might suggest that RPGR*ORF15* has a direct role in microtubular transport rather than being an associated cargo. However, some caution is required, since there is wide variation in the function and supramolecular structure of microtubular transport motors in different cell types and contexts. Nevertheless, if RPGR is simply a cargo, one might expect that its abrogation using morpholinos would have no effect on transport per se, which is clearly not the case. There is also corroborating evidence that RPGR has a role in retrograde microtubular transport. RPGR is transported to the basal body in cultured cells by means of a retrograde dynein–dynactin motor complex. First, both the dynactin subunits p150*Ghosh* and p50-dynamin and the dynein intermediate chain were co-immunoprecipitated in retinal extracts together with RPGR (27). Secondly, inhibition of dynein function by overexpressing p50-dynamin abrogated the localization of RPGR-ORF15 to basal bodies (26,27). On balance, the results therefore argue that RPGR is not simply a transport cargo but is most directly involved in retrograde transport. This result is strikingly similar to the retrograde transport defects observed, when another group of ciliary proteins capable of causing retinal degeneration, BBS1–8, are abrogated in zebrafish (49,68). These proteins are not known to interact with RPGR, so the reason for this finding is unclear. One possibility is that different signalling molecules (e.g. in Wnt or Shh pathways) stimulate the loading and either retrograde or anterograde transport of specific complexes (69) and that RPGR and BBS proteins are concerned with different retrograde transport components.

The dynein light chain Tetex-1 was shown to recognize and bind both dynein intermediate chain and a rhodopsin C-terminus motif proposed to be involved in targeting this protein to apical membranes (70,71). RPGR*ORF15* mutants are known to be associated with ectopic localization of rod and cone opsins to the cell body and synapses, whereas rod photoreceptors have a reduced level of rhodopsin in outer segments (24,33,72). Although it is possible that RPGR is involved in the retrograde transport of opsins-containing vesicles to the basal body, delivery of rhodopsin is not influenced by microtubule depolymerizing agents (73) and the Arf4 ciliary targeting complex now appears to be more directly involved in rhodopsin C-terminal (VxPx) binding (74). Further work is therefore required to clarify the precise role of RPGR in retrograde transport.

**MATERIALS AND METHODS**

**Zebrafish strains and husbandry**

Zebrafish of the AB strain were maintained at 28.5°C on a 14 h light/10 h dark cycle. Fertilized eggs were obtained and grown in incubators and embryos were staged as described (34).

Reverse transcriptase–polymerase chain reaction

Total RNA was extracted from embryos at different stages with Trizol Reagent (Invitrogen). RNAs from different tissues of adult zebrafish were extracted using RNAeasy Mini kit (QIAGEN) according to the manufacturer’s instruction. Two micrograms of the resulting RNA sample was reverse transcribed with random primers using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The cDNA was then amplified according to standard protocols using Platinum Taq DNA polymerase (Invitrogen). PCR primers were manufactured by Sigma-Aldrich and are listed in Supplementary Material, Table S1.

Identification of zebrafish RPGR orthologues

The human RPGR amino acid sequences were compared with the translated protein sequences from zebrafish genomic sequence data using a BLAST. Gene predictions were made using Genewise (50) using the published human RPGR protein sequences as templates. These predictions were subsequently manually curated and refined using other sources of information available via the Ensembl website (http://www.ensembl.org/): including Ensembl’s own gene prediction models, de novo Genscan predictions, expressed sequence tag.
cDNA and protein homologies. On the basis of the resultant sequences, primers were designed to amplify the complete sequence of the zebrafish RPGR genes. PCR products were analysed by agarose gel electrophoresis and sequenced.

**In situ hybridization**

A 450 bp fragment of the zebrafish (ZF) RPGR1 gene and a 675 bp fragment of the ZFRPGR2 gene were amplified by PCR with the following primers: RPGR1, 5'-GATGCA GAAAAAGCCAAATCC-3' and 5'-CTATTACCTTTGTCT AGTGACG-3'; RPGR2, 5'-GCAAAAAGCTGGATCTG AAAAAAGAC-3' and 5'-CTTTATCCCTTGTTCCCCAG-3'. These fragments were cloned into pGEM-T easy vector (Promega) and used as templates for in vitro synthesis of antisense RNA probes. Whole-mount in situ hybridization was performed using a digoxigenin (DIG)-labelled antisense RNA probe was carried out as previously described (28). Briefly, embryos at the 24 hpf stage were fixed in 4% paraformaldehyde in PBS, hybridized with DIG-labelled riboprobe in a hybridization buffer (50% formamide, 5 x SSC, 50 mg/ml tRNA and 0.1% Tween-20) at 68 °C, followed by incubation with anti-DIG antibody conjugated with alkaline phosphatase and staining with the substrate (BCIP) to produce a purple insoluble precipitate. Embryos were stored in 70% glycerol in PBS for photography.

**Immunohistochemistry and histology**

Eyes from adult zebrafish were fixed in 4% paraformaldehyde in PBS overnight at 4 °C, washed with PBS twice (5 min each), the fixed eyes were then cryoprotected in 5% sucrose/PBS at 4 °C overnight. Eyes were embedded and rapidly frozen in dry ice; 10 μm thick sections were cut at −20 °C, mounted on superfrost slides, and air dried at room temperature for at least 2 h. Sections were blocked in 10% FCS, 2% BSA and 0.1% Triton X-100 in PBS for 1 h, then incubated for 1 h with primary antibodies diluted in blocking solution—rabbit anti-RPGR (Atlas Antibodies, Sweden) at 1.5 μg/ml, mouse anti-rhodopsin (ab5417, Abcam plc) at 2 μg/ml and Zpr-1 (Zebrafish International Resource Center, OR, USA) at 1:200 dilution. After washing, the sections were incubated with secondary AlexaFluor 594-conjugated anti-rabbit and AlexaFluor488-conjugated anti-mouse antibodies (Invitrogen). The nuclei were stained with Toto-3 (Invitrogen). The slides were mounted using the ProLong Gold Antifade Reagent (Invitrogen) and images were captured using a confocal microscope (LSM 510, Carl Zeiss Inc.).

For rhodopsin or Zpr-1 staining, control and ZFRPGR2 morpholino-injected morphants (72 hpf) were fixed in 4% paraformaldehyde in PBS, dehydrated, embedded in paraffin and sectioned at 5 μm thickness. The sections were dewaxed, blocked with 2% BSA–PBS, incubated with anti-rhodopsin antibody (1:200) or anti-Zpr-1 antibody (1:200), and subsequently incubated with Texas-Red conjugated secondary antibody. Sections were mounted in Vectashield (Vector Laboratories Ltd) containing 4-6-diamidino-2-phenylindole (DAPI). Images were captured using a Zeiss Axioplan II fluorescent microscope and analysed using IPLab software.

**Measurement of eye size and criteria for rescue**

Embryos were dechorionated and anaesthetized with Tracine. The phenotypes were recorded by photographing and photo prints used for eye measurement. Eye measurements were taken from the anterior to the posterior edge. The typical eye diameter of a 48 hpf embryo is about 275 μm. The eye diameter of 48 hpf morphants ranged from 100 to 200 μm. Morphants were divided into two classes—mild and severe—based on eye size, which was correlated with the severity of other abnormalities caused by morpholino injection. Morphants with eye size from 175 to 200 μm (small eye) always had slightly reduced heads and curved tails (‘mild’), whereas those with very small eyes (diameter 100–175 μm) (‘severe’) always showed severely reduced head size, a curved body and short tail, especially those morphants with eye size <150 μm, which always had no tail and an enlarged cardiac chamber.

For rescue experiments, morphants with eye sizes from 225 to 275 μm were classified as ‘rescued’. These embryos have nearly restored eyes and head size and straight bodies. Morphants with eye size <225 μm were regarded as ‘not rescued’, although the majority of them (eye size 200–225 μm) had straight but shorter bodies.

**Morpholino and mRNA injections**

Morpholino antisense oligonucleotides (MOs) were designed and synthesized by Gene-Tools. MOs targeted to translational start sites of both zebrafish RPGR genes included the following: MO1 for ZFRPGR1, 5'-CTGGTAGATTTTGTCTCTCA GTCAT-3'; MO2 for ZFRPGR2, 5'-TTTCTACCTTTGTTT CCTCACAGCAT-3'; standard control MO (CMO), 5'-CTTACCTACGTCAATAATTATA-3'; 5-base mismatch control for ZFRPGR2 (mCMO2), 5'-TTTTgATgTTgTGTTT CTTgACACCAT-3'. All MOs were solubilized in water and diluted to the required concentration for embryo microinjections. For the rescue, cDNAs including human RPGRex1-19, RPGRORF15, HRPGR ORF15 with c.2522delA and RPGR ORF15 with c.2650G > T (RPGRORF15c.2650G > T) (proteins coded by those cDNAs are shown in Fig. 7A) were cloned into pcPS2 vector. In vitro transcription of synthetic capped mRNA was performed using the SP6mMESSAGE mMACHINE kit (Ambion) according to the manufacturer’s instructions. Two nanolitres of MO or mixed MO/mRNA was injected into each 1–2 cell stage embryo. The final concentrations of MO and mRNA were 200 μM and 120 ng/μl, respectively.

**Detection of cell death**

Apoptotic cells in live 24- to 48-hpf-old embryos were detected by incubating the embryos in acridine orange solution (5 μg/ml) (43,47) for 30 min at room temperature, washed briefly five times in embryo medium. Cells loaded with dye were visualized immediately using a Zeiss Axioplan II
fluorescent microscope. Apoptotic cells in the retina/brain of 72-phf-old morphants were detected by the DeadEnd™ fluorometric tunnel system (Promega). Sections from 4% parafomaldehyde fixed and paraffin embedded morphants were washed with PBS, permeabilized with 20 μg/ml proteinase K, fixed again with 4% paraformaldehyde in PBS, then equilibrated with equilibration buffer. The sections were labelled with TdT reaction mix for 60 min at 37°C and the reactions were stopped with 2× SSC. After washing in PBS three times (5 min each), the specimens were mounted in Vectashield containing DAPI. Images were captured using Zeiss Axiosoplan II fluorescent microscope and analysed using IPLab software.

Western blot analysis
Zebrafish adult eyes, control and ZFRPGR2 morpholino-injected embryos (48 hpf) were lysed in a modified RIPA buffer (50 mM Tris–Cl, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.05% SDS and 2 mM EDTA) containing a protease inhibitor cocktail (Roche), 1 mM sodium-vanadate, 5 mM sodium-fluoride and 10 mM iodoacetamide. The lysates were centrifuged for 10 min at 16,200 g and the post-nuclear supernatants were collected. Equal amounts of protein were electrophoresed on 6% (for RPGR analysis) SDS–polyacrylamide gels and transferred to nitrocellulose membrane. Membranes were blocked in 5% non-fat dried milk in 0.1% Tween-20/PBS for 2 h at room temperature. Primary antibodies were used at 1:2000 (RPGR, HPA001593, Atlas Antibodies). HRP-conjugated secondary antibodies were used at 1:5000 dilution. Bound antibodies were visualized by ECL (Amersham Biosciences).

Melanosome transport assay
Control and ZFRPGR2 morpholino-injected larvae at 4 dpf were exposed to epinephrine (500 μg/ml, Sigma, E4375) in embryo medium. Melanosome retraction was continuously monitored under a microscope and the aggregation endpoint was scored when all melanosomes in the head and trunk were prenuclear. To evaluate melanosome dispersion, the larvae were washed with excess embryo medium immediately after epinephrine application, and then exposed to caffeine (1 mg/ml, Sigma, C0750). The dispersion was also continuously monitored and the endpoint scored.

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