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Human retinopathy-associated ciliary protein retinitis pigmentosa GTPase regulator mediates cilia-dependent vertebrate development

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Dysfunction of primary cilia is associated with tissue-specific or syndromic disorders. RPGR is a ciliary protein, mutations in which can lead to retinitis pigmentosa (RP), cone-rod degeneration, respiratory infections and hearing disorders. Though RPGR is implicated in ciliary transport, the pathogenicity of RPGR mutations and the mechanism of underlying phenotypic heterogeneity are still unclear. Here we have utilized genetic rescue studies in zebrafish to elucidate the effect of human disease-associated mutations on its function. We show that *rpgr* is expressed predominantly in the retina, brain and gut of zebrafish. In the retina, RPGR primarily localizes to the sensory cilium of photoreceptors. Antisense morpholino (MO)-mediated knockdown of *rpgr* function in zebrafish results in reduced length of Kupffer's vesicle (KV) cilia and is associated with ciliary anomalies including shortened body-axis, kinked tail, hydrocephaly and edema but does not affect retinal development. These phenotypes can be rescued by wild-type (WT) human RPGR. Several of the *RPGR* mutants can also reverse the MO-induced phenotype, suggesting their potential hypomorphic function. Notably, selected RPGR mutations observed in XLRP (T99N, E589X) or syndromic RP (T124fs, K190fs and L280fs) do not completely rescue the *rpgr*-MO phenotype, indicating a more deleterious effect of the mutation on the function of RPGR. We propose that RPGR is involved in cilia-dependent cascades during development in zebrafish. Our studies provide evidence for a heterogenic effect of the disease-causing mutations on the function of RPGR.

INTRODUCTION

Primary cilia are microtubule-based extensions of the plasma membrane generated by Intraflagellar transport (IFT) of precursor proteins in almost all post-mitotic cells (1). Once considered vestigial, the primary cilia are now recognized to perform diverse and essential cellular functions, including cell cycle, protein trafficking, embryonic axis patterning and photoreception (2–6). Defects in ciliary function are associated with syndromic disorders including Senior-Loken Syndrome, Bardet–Biedl Syndrome and Joubert Syndrome (7). Many of these disorders include retinal degeneration, RP and LCA (Leber congenital amaurosis) due to perturbed photoreceptor ciliary transport (7,8).

Mutations in retinitis pigmentosa GTPase regulator (RPGR) are a major cause of photoreceptor degenerative diseases, including X-linked and simplex forms of RP, cone-rod degeneration and atrophic macular degeneration (9–15). Some RPGR patients exhibit hearing dysfunction, respiratory infections and primary cilia dyskinesia (16–21). Multiple alternatively spliced isoforms of RPGR are widely expressed, at both RNA and protein levels (22–24). Two major isoforms of RPGR are detected in the retina: $RPGR^{1-19}$ (amino acids 815; exons 1–19) and $RPGR^{1\text{-}ORF15}$ (amino acid residues

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1152; exons 1–15 and part of intron 15) (10,13). Both isoforms share exons 1–15 (residues 1–635) and encompass a common N-terminal RCC1-like domain (RLD; residues 1–446) (10,13). Although RCC1 is a guanine nucleotide exchange factor for Ran GTPases (25), no such activity has yet been reported for RPGR. Mutations in exons 1–15 of RPGR account for almost 20% of XLRP with no disease-associated variations reported in exons 16–19 (12). The purine-rich RPGR exon ORF15 encodes a Glutamic Acid and Glycine (Glu–Gly) rich C-terminal domain; mutations in this exon account for additional 50–60% of XLRP (12,13).

The RPGR protein isoforms are prominently localized to the sensory cilia of photoreceptors (transition zone and basal bodies), human and monkey cochlea and to the nucleus, cilia and centrosomes of cultured cells (16,23,26–28). RPGR is shown to interact (directly or indirectly) with several ciliary proteins, including RPGR-interacting protein 1 (RPGRIP1), RPGRIP1-like (RPGRIP1L/NPHP8) (29) and CEP290/NPHP6 (2,3,23,27,30–32). Given that CEP290 and RPGRIP1L regulate ciliogenesis and cilia-dependent signaling (33,34), we hypothesize that RPGR is essential for primary cilia genesis or function. Studies using mouse and canine models of RPGR mutations demonstrate phenotypic heterogeneity and altered ciliary transport (27,35,36). Within this context, the mechanism(s) by which RPGR mutations cause diverse phenotypes in humans have not been defined at the biochemical or cellular level.

We and others have shown that knockdown of expression of ciliary proteins in zebrafish (*Danio rerio*) results in measurable structural and functional phenotypes, some of which are due to perturbed planar cell polarity (PCP) or vesicular trafficking (5,29,37–39). Ability of human mRNA to genetically rescue the ciliary phenotype serves as an excellent platform to assess the functional consequence of the human disease-causing mutations by comparing the efficacy of rescue to the wild-type counterpart. We have utilized the zebrafish system to examine the function of RPGR and pathogenicity of the retinopathy-associated mutations on its function. We show that RPGR is essential for cilia-dependent developmental pathways in zebrafish. Moreover, some human disease-causing RPGR mutations, which result in syndromic or isolated RP in patients, exhibit a loss-of-function effect while others seem to be hypomorphic. These studies provide novel insights into the function of RPGR in cilia-dependent pathways and reveal critical information about variable pathogenic consequences of the disease-causing mutations in RPGR on its function.

RESULTS

RPGR is expressed in zebrafish during development

Using BLAST analysis, we first identified an *rpgr* transcript (Accession number XM_680872.3), which bears 57% identity and 72% similarity to human $RPGR^{ORF15}$. This *rpgr* transcript consists of 14 exons with terminal exon encoding for purine-rich repeats (Fig. 1A). Reverse transcriptase-polymerase chain reaction (RT–PCR) analysis of RNA from zebrafish embryos revealed an expected *rpgr* cDNA product of 4.2 kb (Fig. 1A). Whole-mount immunofluorescence analysis using a previously reported rabbit polyclonal antibody (GR-P1) against human RPGR-RLD (27,40) revealed that RPGR is expressed predominantly in the eye, head and gut of 4 days post-fertilization (dpf) zebrafish embryos (data not shown). Further analysis of zebrafish embryos at 5 dpf revealed RPGR expression predominantly in the brain, optic chiasma and gastric epithelial layers (Fig. 1B and C). In the retina at 5 dpf or adult zebrafish, RPGR localizes predominantly in the inner segment/sensory cilium region of photoreceptors, where it co-localizes with acetylated α-tubulin (Fig. 1D; Supplementary Material, Fig. S1). The staining of RPGR in different tissues of zebrafish embryos can be blocked by pre-incubating with the specific antigen but not by a non-specific antigen (data not shown).

Knockdown of RPGR function causes ciliary defects in zebrafish

We then proceeded to evaluate the effect of knockdown of *rpgr* expression in zebrafish. Injection of translation-blocking morpholino (MO) against *rpgr* into wild-type (WT) embryos revealed a dose-dependent defect reminiscent of a ciliary phenotype (Fig. 2A). A 3 ng dose of the specific MO against *rpgr* resulted in ∼60% morphants after 4 dpf, as opposed to ∼10% with a mismatch morpholino (Mm) at the same dose (Fig. 2B). Embryonic lethality was observed at doses greater than 3 ng of *rpgr*-MO. Immunoblot and immunofluorescence analyses using the GR-P1 antibody confirmed knockdown of RPGR protein expression in 4–5 dpf morphants (Fig. 2C; Supplementary Material, Fig. S2). To directly correlate a ciliary defect due to knockdown of *rpgr* function in zebrafish, we analyzed the cilia of Kupffer's vesicle (KV), a ciliated organ involved in left-right axis patterning during zebrafish development (5,41–43). Staining with anti-acetylated α-tubulin revealed that although the number of cilia is not significantly altered, *rpgr*-MO embryos show significant decrease (∼50%) in the length of the KV cilia (Fig. 2D and E). These data indicate that RPGR is involved in the maintenance of the cilium and not ciliogenesis.

Ciliary dysfunction in zebrafish is frequently associated with convergent-extension (CE) defects, including body axis extension anomalies (5,29,38,44,45). We therefore analyzed *rpgr*-morphants for such anomalies. As shown in Figure 3A, *rpgr*-MO results in kinked tails in 60% of morphants. We also observed hydrocephaly in ∼30% and edema in ∼50% of the morphants, a phenotype also associated with ciliary dysfunction (46). We also assessed retinal morphology and trafficking of opsins in the 4–5 dpf abnormal embryos that exhibited other ciliary anomalies. Notably, the architecture of the retina and photoreceptors as well as transport of rhodopsin to outer segments seems unaltered in the defective embryos as compared to WT embryos (Supplementary Material, Fig. S2).

rpgr-MO phenotype can be rescued by human RPGR

We next investigated whether human WT RPGR can rescue *rpgr*-dependent cilia phenotype. We show that the effect of knockdown of *rpgr* can be rescued in a dose-dependent manner using mRNA encoding human $RPGR^{1-19}$ (NM_000328.2), $RPGR^{1\text{-}ORF15}$ (NM_001034853.1) and $RPGR^{1-15}$ (Fig. 3B). While 3 ng of *rpgr*-MO yielded 60%

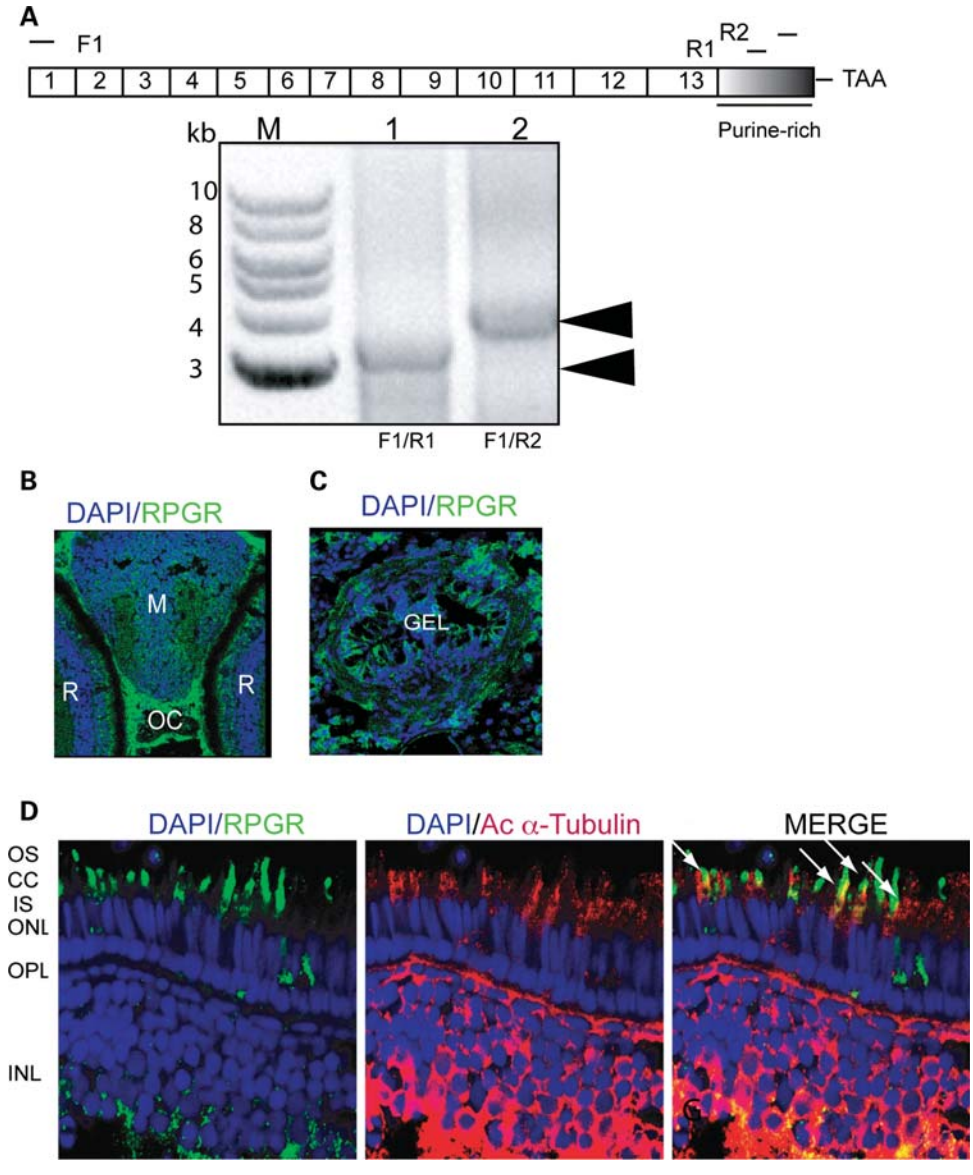

Figure 1. Expression of RPGR in zebrafish. (**A**) RT–PCR analysis of zebrafish total mRNA identifies the zebrafish *rpgr* cDNA (sequence verified). Primers (F: Forward, R: reverse) were designed based on the GenBank sequence XM_680872.3. Molecular weight marker (M) is shown in kilo bases (kb). (**B**–**D**) Immunohistochemistry of cryosections of embryonic zebrafish (5 dpf) head (B), gut (C) and retina (D) was performed using the GR-P1 antibody (green) against RPGR. RPGR expression can be detected in the midbrain (M), neurites of optic chiasma (OC) and in gastric epithelial layer (GEL). DAPI (blue) was used to stain the nuclei. R: Retina. (**D**) Retinal sections were co-stained with a ciliary marker acetylated α—tubulin (Ac α-Tubulin; red). Arrows (Merge; yellow) depict co-localization of RPGR at the photoreceptor cilium. IS, inner segment; OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer.

morphants, co-injection of 500 pg of human RPGR mRNA resulted in <10% morphants. These results suggest that the observed MO phenotype is specifically due to the knockdown of *rpgr*. Co-injection of human WT RPGR mRNA can rescue almost all of the phenotypes detected in *rpgr*-MO, including length of the KV cilia, as depicted in Figure 3B–D.

RP-associated mutations in RPGR exhibit hypomorphic and loss-of-function effect

We then utilized *rpgr*-MO as a platform to assess the pathogenic potential of selected RPGR mutations found in patients. We first examined selected missense mutations reported in the RLD of RPGR (Fig. 4A). Co-injection of human RPGR mRNA encoding mutants G60V (47), G165V or G173R (12) could rescue the *rpgr*-knockdown phenotype comparable to that by wt RPGR-encoding mRNA (~15% morphants), whereas RPGR-T99N (48) mutant did not rescue the phenotype (~47% defective embryos) (Fig. 4B and D). Specifically, the T99N mutant can partially rescue the kinked tail phenotype but not edema and hydrocephaly (Fig. 4D; Supplementary Material, Fig. S3),

Next, we assessed the effect of nonsense mutations in exons 1–15 or in exon ORF15 on the function of RPGR.

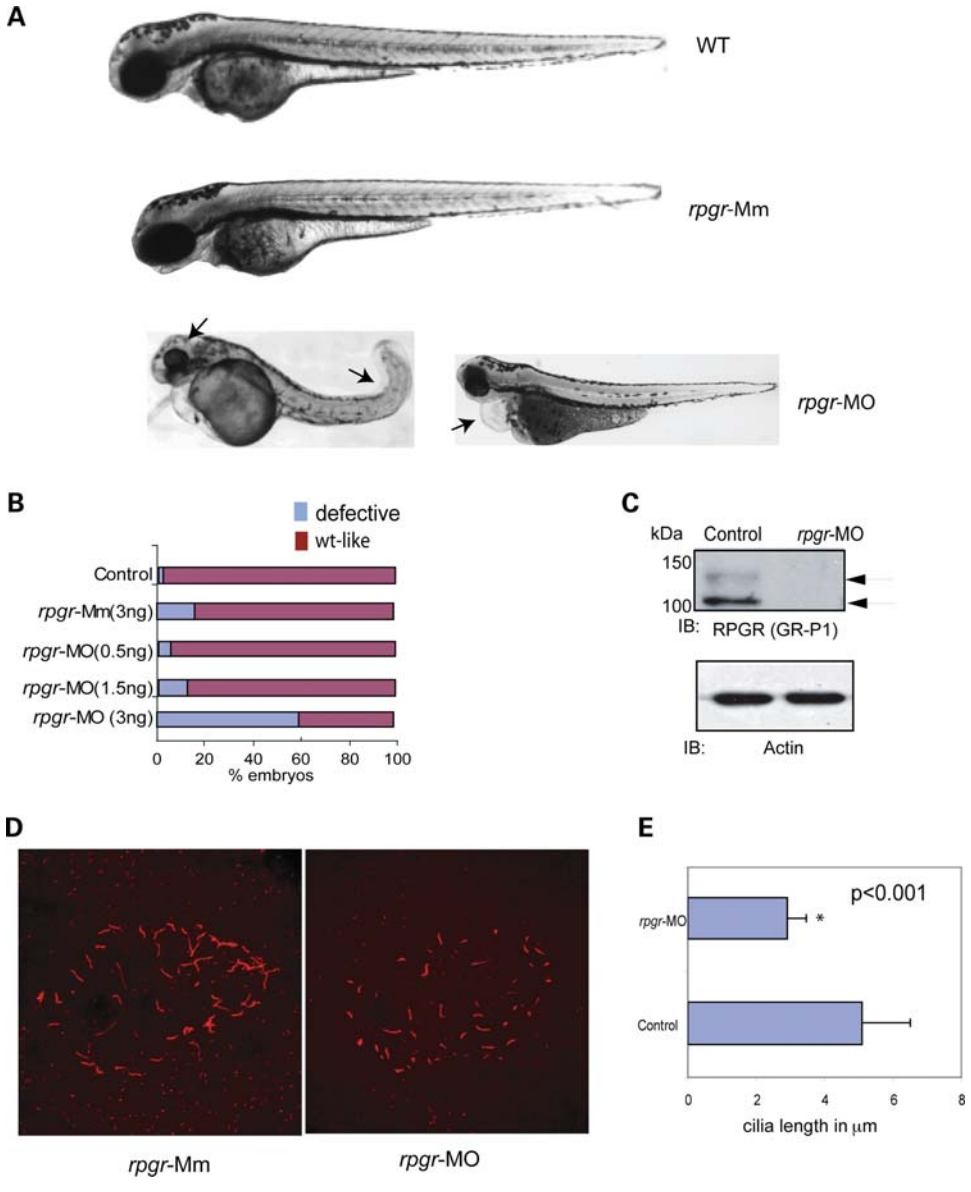

Figure 2. Knockdown of zebrafish RPGR causes developmental anomalies. (**A**) Injection of anti-sense morpholino (*rpgr*-MO) into zebrafish embryos results in developmental disorder, including shortened body axis and edema at 4 dpf. Uninjected wild-type (WT) and embryo injected with the 5 base mismatch (Mm) control (*rpgr*-Mm) are also shown. Arrows in the *rpgr*-MO panel depict the different morphological phenotypes observed in the defective embryos (left panel: hydrocephaly and kinked tail; right panel: edema). (**B**) The effect of silencing of *rpgr* is observed in a dose–dependent manner (at indicated concentrations) with increasing number percentage of defective embryos at increased concentrations of the morpholino. Data presented here are representative of at least three independent experiments with $n = 100$ for each group. (**C**) Immunoblot (IB) analysis of protein extracts of embryos injected with mismatch (control) or *rpgr*-MO shows considerable knock down of RPGR protein expression in the *rpgr*-MO group. Arrows indicate the specific immunoreactive bands observed with the rabbit polyclonal GR-P1 antibody. IB with actin was used as loading control. (**D**) Confocal microscopy image of KV cilia stained with anti-acetylated tubulin (red) in both control and *rpgr*-knockdown embryos. (**E**) Graphical representation of the length of the KV cilia in *rpgr*-MO relative to controls. A student's *t*-test was performed on the samples (total cilia in each group = 340) from six to eight embryos for each group.

A termination in exon 15 of RPGR (E589X; c.1765G > T) (12) did not seem to rescue any of the *rpgr*-MO phenotypes tested (∼65% defective embryos) (Fig. 4C and D; Supplementary Material, Fig. S3). Co-injection of mRNA encoding wt $RPGR^{1\text{-}ORF15}$, $RPGR^{1\text{-}ORF15}$ E853X [c.2557G > T] (49) or $RPGR^{1\text{-}ORF15}$ E1071X [c.3211G > T] (50) with *rpgr*-MO resulted in a comparable rescue (∼15% morphants) (Fig. 4C).

RPGR mutations associated with syndromic phenotype cannot rescue the *rpgr*-MO phenotype

We also assessed three truncation mutations in the RLD exhibiting syndromic phenotypes: T124 [G > T at splice site of intron 5] (14), K190 [631_IVS6 + 9del] (13) and L280 [845–846delTG] (15). These mutations are associated with sino-respiratory infections, pseudo-Usher syndrome and sperm defects in *RPGR* patients. None of these mutants

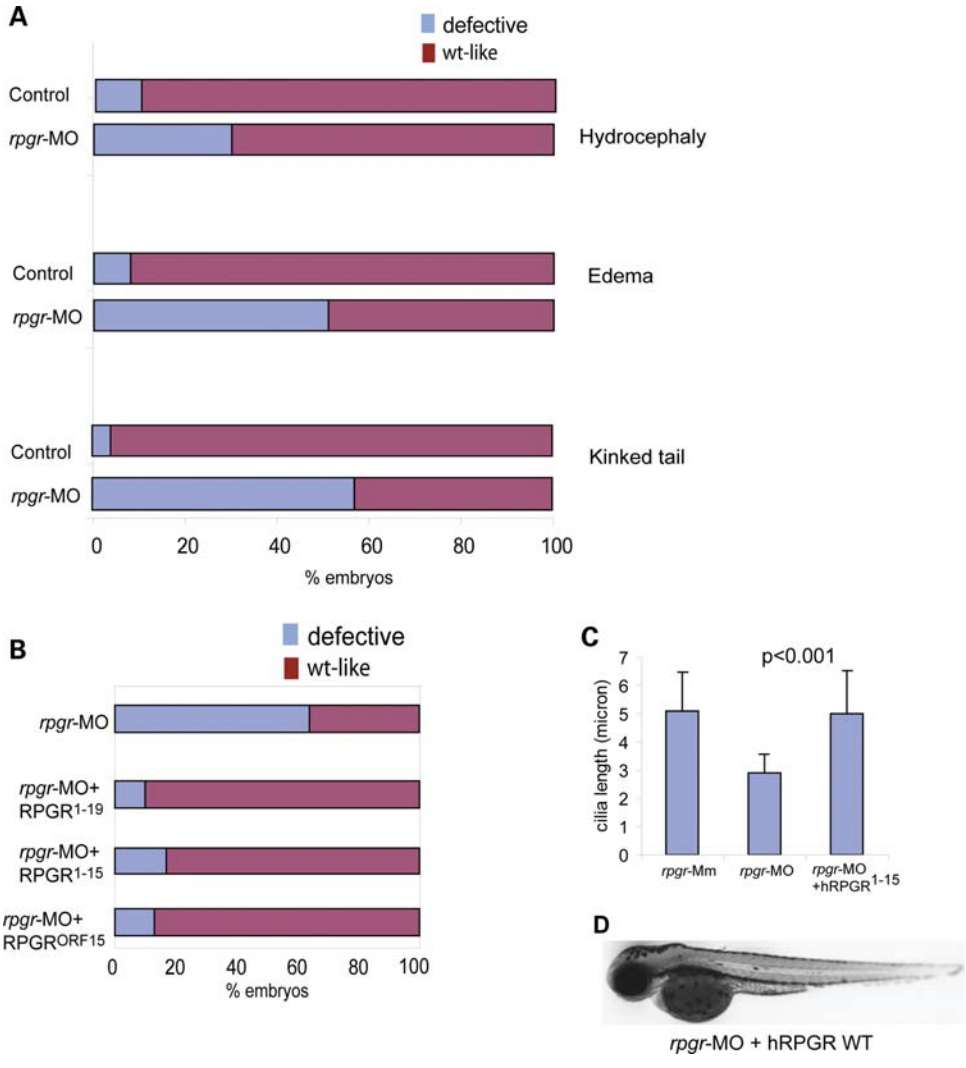

Figure 3. Phenotypic analysis of *rpgr*-morphants. (**A**) Embryos injected with *rpgr*-MO were scored for ciliary phenotypes including hydrocephaly, edema or tail extension (kinked tail) defects. (**B**) The *rpgr*-knockdown phenotype can be rescued by injecting human wt RPGR. Data are representative of three independent experiments and show the percentage of defective embryos observed when injected with either *rpgr*-MO alone or co-injected with human mRNA encoding $RPGR^{1-19}$, $RPGR^{1-15}$ or $RPGR^{1\text{-}ORF15}$ isoforms ($n = 100$). (**C**) Graphical representation of the length of the KV cilia in embryos injected with *rpgr*-Mm, *rpgr*-MO or *rpgr*-MO and mRNA encoding wt human RPGR. A student's *t*-test was performed on the samples (total cilia in each group = 340) from six to eight embryos for each group. (**D**) A representative image of an embryo co-injected with the specific MO against rpgr and hRPGR WT mRNA.

could rescue of the *rpgr*-MO phenotype (40–60% morphants); hence, these mutations appear to be detrimental to RPGR function (Fig. 4C and D). Notably, mutants K190X and L280X cannot rescue the kinked tail, edema and hydrocephaly due to knockdown of *rpgr* function, whereas T124X exhibited relatively less number of defective embryos. The expression levels of these mutant RPGR proteins *in vitro* were not altered, indicating that the mutations do not affect the stability of the RPGR protein (Supplementary Material, Fig. S3).

DISCUSSION

There is considerable clinical heterogeneity associated with retinal degenerative diseases due to mutations in ciliary proteins. Investigations on the impact of disease-causing mutations on the function of the causative protein have been difficult due to the lack of a platform to analyze protein function. In this study, we have utilized zebrafish development as a paradigm to elucidate the pathogenic potential of selected disease-associated mutations in RPGR. We show that RPGR mutations exhibit variable effects on its ability to rescue the *rpgr*-knockdown phenotype in zebrafish.

All patients carrying *RPGR* mutations exhibit photoreceptor degeneration; however, we did not detect any defects in photoreceptor development or opsin trafficking in *rpgr*-morphants by 4–5 dpf (Supplementary Material, Fig. S2). These results likely suggest that RPGR is not essential for retinal differentiation and development. Notably, RPGR patients and animal models exhibit normal photoreceptor development but undergo relatively late-onset photoreceptor degeneration and blindness (15,35,36). Hence, we cannot rule out an effect on photoreceptor survival in adult zebrafish, as also proposed with *bbs* genes (5). A lack of retinal phenotype in

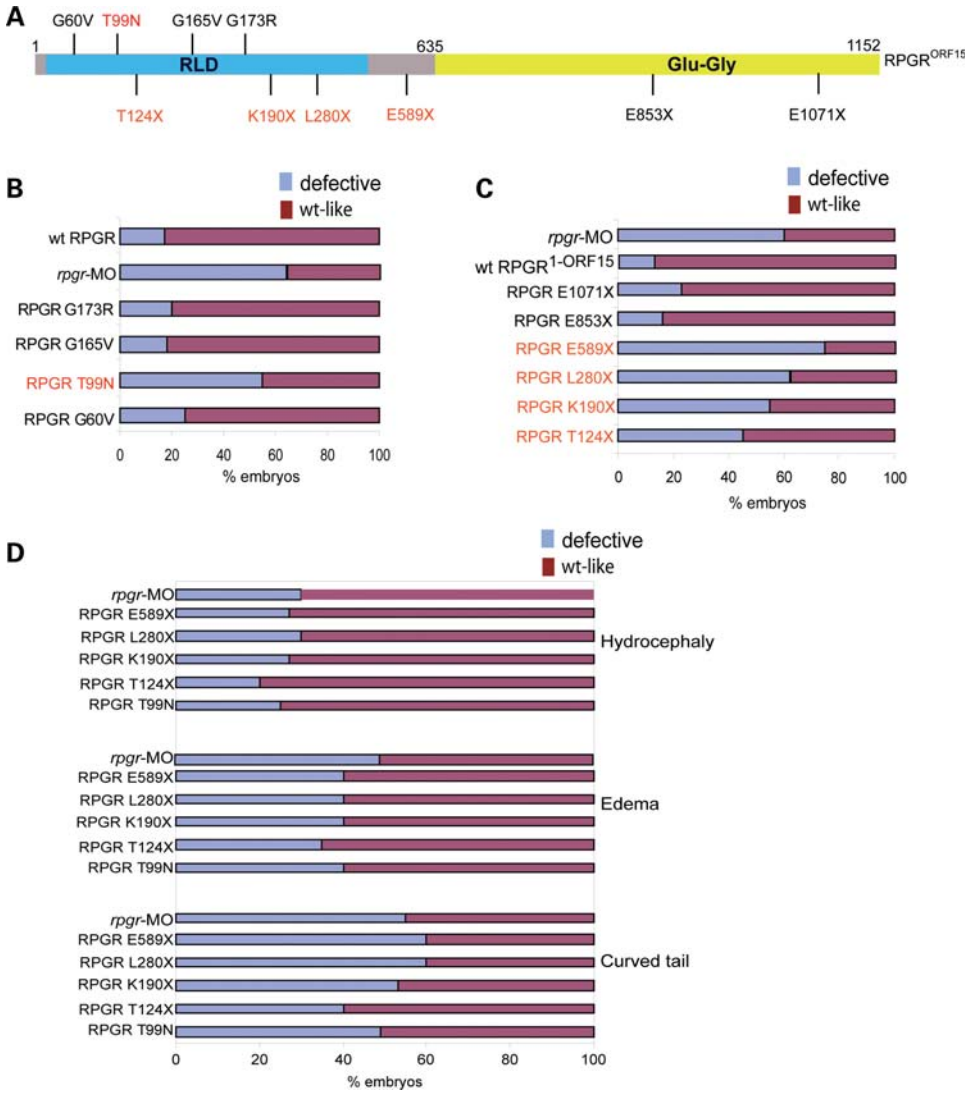

Figure 4. Rescue of *rpgr*-MO phenotype by mutant RPGR proteins. (**A**) Schematic of RPGR protein is represented showing the RLD and the C-terminal Glu–Gly rich domain (NM_001034853.1). Zebrafish embryos were injected with *rpgr*-MO or *rpgr*-MO plus the mRNA encoding indicated RPGR mutants (missense, **B** and truncations, **C**) followed by phenotypic analysis. (**D**) The deleterious mutants showed variable spectrum and incidence of ciliary defects in the morphants. Data are representative of three independent experiments.

rpgr-knockdown zebrafish embryos is not unexpected. Because of the duplication of the zebrafish genome (51), a functional redundancy of RPGR is highly likely. Tissue- and cell-type specific alternative isoforms of RPGR have also been reported in mice, bovine and human tissues. Hence, it is possible that the specific isoform of RPGR, investigated in the present study, may not play a role in retinal development. While this study was going on, we identified another potential RPGR isoform by *in silico* analysis of the updated genome of zebrafish. Further investigations are necessary to delineate the function of this isoform in retinal development in zebrafish.

Our studies provide *in vivo* evidence for the functional significance of the N-terminal domain of RPGR (encoded by exons 1–15) in ciliary functions (23). The T99 residue is highly conserved during evolution as well as in the RCC1 protein (10); hence, the T99N mutation may result in altered conformation, a detrimental effect on an as yet uncharacterized activity of RPGR, ciliary localization and/or interaction with other proteins. Truncation mutations in RPGR-RLD that are associated with a syndromic phenotype in patients exhibit an expected detrimental effect on protein function whereas the G173R mutation (associated with hearing loss, sinusitis and recurrent respiratory infections) (17) seems to retain partial function in zebrafish development.

Mutations in exon ORF15 exhibit a hypomorphic effect in our assays. These data are consistent with previous observations that ORF15-variants in patients are associated with a relatively mild phenotype (12,50,52). Moreover, the RPGR$^{1\text{-ORF15}}$ variants encode the RPGR-RLD as part of exons 1–15, which are sufficient to rescue the *rpgr*-MO phenotype. Intriguingly, animal models of exon ORF15 mutations

exhibit discordant phenotype (slow or rapid photoreceptor degeneration and a dominant gain of function effect) based upon the length of the Glu–Gly repeats encoded by exon ORF15 (36,53). We predict that relative dosage of the truncated ORF15-mutant proteins may alter the functional conformation of the RPGR protein, resulting in a mild versus severe phenotype in the cognate model system. Further studies are necessary to piece-together the complex association of mutations in exon ORF15 of RPGR with its function.

PCP proteins, which localize to cilia, control CE movements during axis elongation in vertebrates (44,54). Our data suggest that RPGR is essential for vertebrate embryonic development and complete loss of RPGR function during development likely results in lethality. Support of this hypothesis also comes from previous observations that RPGR is expressed in mouse embryonic stem cells (unpublished data), and that attempts to generate a complete loss of function mouse mutant of *Rpgr* have so far been unsuccessful. Moreover, the reported *Rpgr*-ko mouse (35) is not complete null because expression of alternative RPGR isoforms can be detected in the photoreceptors (23). However, the precise function of RPGR during vertebrate development remains to be established.

Why do RPGR mutations not exhibit lethality in humans? We suggest that a majority of RPGR mutations detected in RP patients are hypomorphs with reduced function. This functional retention seems to be sufficient to rescue the developmental phenotype. Only selected mutations in RPGR may severely compromise the protein function. Given high protein trafficking demands due to periodic outer segment disc shedding and renewal (55–57), even hypomorphic RPGR mutations have deleterious impact on the integrity of ciliary protein complexes and intracellular transport in photoreceptors. We predict that multiple isoforms of RPGR (generated by alternate splicing or with alternate promoter) may complement the detrimental effects of such mutations in extraretinal tissues in higher vertebrates during development.

Although previous studies have reported a predominantly retinal phenotype associated with RPGR-T99N and RPGR-E589X mutations, we propose that detailed clinical analysis of patients predicted to have deleterious mutations based on the zebrafish assays reported here should be performed to analyze potential heterogenic phenotype(s), such as olfactory dysfunction, which can assist in early diagnosis of the disease (58). We and others have shown that hypomorphic mutations in the ciliary protein CEP290 are associated with predominantly LCA and olfactory defects (58,59) whereas predicted null mutations cause Joubert Syndrome, Bardet–Biedl Syndrome and Meckel–Gruber Syndrome (38,60–63). Although olfactory dysfunction was not significantly observed in the RPGR patients tested (58), additional studies using patients with other mutations should be performed for such analyses.

Taken together, our work analyzes the effect of RPGR mutations on its function using a zebrafish model system. This system offers a platform to understand the behavior of mutant RPGR *in vivo* so that future therapeutic strategies can be appropriately designed to target a complete or partial loss of function of the mutant RPGR protein in patients with ciliary disorders.

MATERIALS AND METHODS

RT–PCR analysis, immunoblotting and immunohistochemistry

Total RNA was extracted from the 3 dpf embryos using Qiagen RNA extraction kit. RNA was reverse-transcribed to cDNA using oligo-dT. Two milliliters of cDNA was amplified for the full-length RPGR with the following primers:

F1: 5′-GCAGAGATGG CTGGAGAAAC-3′
R1: 5′-GCTTTTGTCCTCATCATCATTGCTC-3′
R2: 5′-AAAACATCATCCCAAAACTGC-3′.

Immunoblotting was performed using embryo lysates generated after treatment in radioimmunoprecipitation (RIPA) buffer. The cell lysates were resolved on SDS–PAGE and immunoblotting using appropriate antibodies. For retinal immunohistochemistry, eye sections from zebrafish embryos (4 or 5 dpf) were stained with appropriate antibodies. Fluorescent images were taken using confocal microscope (Leica).

Morpholino and mRNA injections

A translation blocking *rpgr*-MO (MO; 5′-CTTCTGTTTC TCCAGCCATCTCTGC-3′) and its 5 base mismatch morpholino (Mm; 5′-CTTgTGTTTgTCgAGCgATCTgTGC-3′) were purchased from Gene Tools Inc., diluted in Danieau's solution (5 mM HEPES pH 7.6, 58 mM NaCl. 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM calcium nitrate) and injected into WT zebrafish embryos at 1–8 blastomere stage at different concentrations. Different doses of morpholino were injected along with 0.1% Phenol Red into fertilized eggs from AB wild-type breeders at two-cell stage using Picospritzer II microinjector (General Valve Corporation). Embryos were analyzed at 2 dpf for hydrocephaly and 3 dpf for the curved tails and edema phenotypes. For rescue experiments, the RNA was transcribed from human $RPGR^{1-15}$, $RPGR^{1\text{-}ORF15}$ and $RPGR^{1-19}$ plasmids with appropriate primers using mMESSAGE mMACHINE kit (Ambion, Austin, TX) in combination with T7 RNA polymerase. RPGR constructs encoding mutant RPGR protein were used as template to generate appropriate mRNA encoding human mutations.

Staining of KV cilia in zebrafish embryos

Embryos at 10–12 somite stage were fixed in 4% paraformaldehyde and processed for staining as described (5). Embryos were incubated overnight with anti-acetylated α-tubulin (Sigma; 1:500 dilution) and GR-P1 (1:250 dilution) antibodies, washed with PBST for 2 h and incubated with anti-mouse Alexa Flour 594 and anti-rabbit Alexa Flour 488 (Molecular Probes). After further washing and removal of the yolk, the embryos were mounted with dorsal side facing upward with the mounting media. Z-stack images of KV cilia were taken using confocal microscope (Leica).

Phenotypic analysis of zebrafish embryos

Embryos were assessed for tail extension anomalies, hydrocephaly and edema essentially as described (29).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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