Gelsolin dysfunction causes photoreceptor loss in induced pluripotent cell and animal retinitis pigmentosa models

Citation for published version:
Megaw, R, Abu-Arafeh, H, Jungnickel, M, Mellough, C, Gumiak-Witke, C, Witke, W, Zhang, W, Khanna, H, Mill, P, Dhillon, B, Wright, AF, Lako, M & Ffrench-Constant, C 2017, 'Gelsolin dysfunction causes photoreceptor loss in induced pluripotent cell and animal retinitis pigmentosa models', Nature Communications, vol. 8, no. 271, 271. https://doi.org/10.1038/s41467-017-00111-8

Digital Object Identifier (DOI):
10.1038/s41467-017-00111-8

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Nature Communications

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Gelsolin dysfunction causes photoreceptor loss in induced pluripotent cell and animal retinitis pigmentosa models

Roly Megaw¹, Hashem Abu-Arafeh¹, Melissa Jungnickel², Carla Mellough³, Christine Gurniak⁴, Walter Witke⁴, Wei Zhang⁵, Hemant Khanna⁵, Pleasantine Mill², Baljean Dhillon⁶, Alan F. Wright², Majlinda Lako³ & Charles ffrench-Constant¹

Mutations in the Retinitis Pigmentosa GTPase Regulator (RPGR) cause X-linked RP (XLRP), an untreatable, inherited retinal dystrophy that leads to premature blindness. RPGR localises to the photoreceptor connecting cilium where its function remains unknown. Here we show, using murine and human induced pluripotent stem cell models, that RPGR interacts with and activates the actin-severing protein gelsolin, and that gelsolin regulates actin disassembly in the connecting cilium, thus facilitating rhodopsin transport to photoreceptor outer segments. Disease-causing RPGR mutations perturb this RPGR-gelsolin interaction, compromising gelsolin activation. Both RPGR and Gelsolin knockout mice show abnormalities of actin polymerisation and mislocalisation of rhodopsin in photoreceptors. These findings reveal a clinically-significant role for RPGR in the activation of gelsolin, without which abnormalities in actin polymerisation in the photoreceptor connecting cilia cause rhodopsin mislocalisation and eventual retinal degeneration in XLRP.
The rod photoreceptor enhances processing of visual stimuli by compartmentalising proteins critical for phototransduction within its outer segment (OS). The OS emerges from the distal end of the connecting cilium (CC), with membrane extensions folding to form thousands of disc-like processes that stack to form the body of the OS. The CC is therefore a highly specialised primary cilium whose protein composition is unique to the retina. Up to 10% of OS discs are renewed every day and, with all photoreceptor proteins being synthesised in the cell’s inner segment (IS), this rate of OS turnover requires high levels of protein trafficking from the IS to (and across) the CC to maintain homeostasis. Indeed, up to 1000 molecules of rhodopsin are trafficking through the 0.3 μm-wide CC in human photoreceptors per second. Breakdown of this cilia trafficking results in protein mislocalisation and, eventually, photoreceptor death. Such photoreceptor degeneration is the hallmark of retinitis pigmentosa (RP), a heterogenous group of inherited retinal dystrophies affecting 1 in 3000 people. RP causes severe visual loss and blindness. RFGR mutations account for 70–90% of XLRP and 10–15% of all RP. Whilst its exact function remains unknown, RPGR localises to the base of the CC where it was proposed to play a role in trafficking of rhodopsin to the OS based on knockout mouse models. In addition, depletion of RPGR in cell lines and mice increases actin polymerisation. To define RPGR’s role in photoreceptor maintenance and to investigate the molecular pathogenesis of XLRP, we generated induced pluripotent stem cells (iPSCs) from patients with RPGR type XLRP (RPGR/XLRP).

Here, we show RPGR-mutant, iPSC-derived photoreceptor cultures display increased actin polymerisation, a phenotype also observed in the RPGR knockout mice that show rhodopsin mislocalisation and photoreceptor degeneration. RPGR binds to and activates the actin-severing protein gelsolin, an interaction that is lost in RPGR-mutant (XLRP) cells, and Gelsolin knockout mice also show abnormalities of actin polymerisation and rhodopsin localisation. Activated Gelsolin rescues the ciliogenic defect observed in RPGR-depleted cells. We conclude, therefore, that RPGR mutations in XLRP lead to defective gelsolin activation and defects in actin regulation and rhodopsin trafficking within the photoreceptor CC.

**Results**

**iPSCs as a novel model for RPGR/XLRP.** Induced pluripotent stem cells (iPSCs) were generated from fibroblasts of two brothers carrying an RPGR mutation (g.ORF15+689–692del4) using previously described methods. Control iPSCs were generated from an unaffected son/nephew. All iPSC clones expressed markers of pluripotency, as demonstrated with qPCR (Supplementary Fig. 1c) and immunostaining (Supplementary Fig. 1d). iPSC lines could differentiate into all three major cell lineages: ectoderm, mesoderm and endoderm (Supplementary Fig. 1e). To generate photoreceptors from these iPSC clones, a three-dimensional retinal differentiation protocol was optimised. Successful patterning of free floating embryoid bodies resulted in spontaneous outpouching of optic vesicles (Fig. 1a, b), which invaginated into optic cup-like structures (Fig. 1c), as previously described. After 100 days, retinal pigment epithelium (RPE) emerged on the outer surface of the cups (Fig. 1d) while inside an organised layer of photoreceptors emerged, expressing several maturity-associated markers (Fig. 1e, f). These cells displayed cilia with the classic “9+0” microtubule doublet organisation characteristic of primary cilia (Fig. 1g, h) and also were associated with additional membranous, disc-like material produced and deposited between the melanosome-containing RPE cells (Fig. 1j). RPGR, as previously reported, localised to this emerging CC that joins the cell body to the rod OS (Fig. 1i). We concluded that human photoreceptors, derived from iPSCs, could therefore be used to study the molecular pathogenesis of RPGR/XLRP.

**RPGR regulates actin polymerisation in hiPS derived and rodent photoreceptors.** We first asked whether the abnormalities in the actin cytoskeleton reported in RPGR-depleted cell lines are present in iPSC-derived photoreceptors from RPGR/XLRP subjects. Increased actin polymerisation was observed in cultured iPSC-derived photoreceptors of both patients compared to the control (Fig. 2a–d, Supplementary Fig. 3a). The iPSC-derived RPGR/XLRP photoreceptor cultures (CB10 and HB02) showed a 2.7-fold (SEM 0.127; p = 0.0001, n = 3) and 2.49-fold (SEM 0.34; p = 0.0081, n = 3) increase in actin polymerisation, respectively.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** Three-dimensional patterning of induced pluripotent stem cells produces mature photoreceptors. Free-floating aggregates grown using a retinal differentiation protocol self-organise into budding spheroids with a single bud shown in a (arrow) and multiple buds from one spheroid in b. Buds invaginate (c) to form optic cups which mature over 100 days (d). Pigmented retinal pigment epithelium (RPE) emerges externally (arrow; d) while a radial arrangement of Recoverin (e) and Rhodopsin (f) expressing photoreceptors organise internally, forming an outer nuclear-like layer. Electron microscopy studies reveal a classic “9+0” microtubule doublet formation in these cilia (g, inset h shows cross-section). RPGR is present in photoreceptor cilia (arrow; i) whilst electron microscopy shows the production of membranous material, as required for outer segment formation (arrow, j). Scale bars: 400 μm (b); 200 μm (a); 50 μm (c, d); 10 μm (e, f); 100 nm (g); 200 nm (h); 5 μm (i); 1 μm (j)
photoreceptors were compared to control (panels a, b). See text for details of quantification of pixel intensities. Nuclei stained with Hoechst (blue).

The mean phosphorylation level of cytoskeletal regulatory proteins was higher in RPGR/XLRP photoreceptors compared to control photoreceptors (red dots; 0.092±0.0033, n=56 proteins). This was significantly more than the mean ratio of phosphorylation levels of non-cytoskeletal proteins when RPGR/XLRP photoreceptors were compared to control (blue dots; -0.008±0.01, n=583 proteins, unpaired, two-tailed t-test, p=0.004 (** p < 0.01)).

RPG mutations ameliorate the activation of the actin-severing protein Gelsolin. Next, we performed a cytoskeletal-focused phoshoarray in an attempt to determine the mechanism by which RPGR regulates actin turnover. We observed in RPGR/XLRP photoreceptor cultures that coflin, a protein whose role in regulating actin polymerisation appears important for ciliogenesis14 and which localises to the photoreceptors in mature wild-type retina (Supplementary Fig. 5), showed 3.16-fold higher levels of phosphorylation of Serine 3, a post-translational modification that inhibits binding to and treadmilling/depolymerisation of F-actin15 (Fig. 4a). This result was confirmed by western blot analysis of repeat cultures of both RPGR/XLRP lines compared to control (Fig. 4b; blot density measurement control MB02 22.544% vs patient CB10 40.364% vs patient HB02 37.092%). This post-translational modification of coflin is normally inhibited by the actin-severing protein gelsolin16, suggesting that loss of gelsolin activity might be responsible for the increased actin polymerisation and photoreceptor abnormalities seen in the RPGR-mutant photoreceptors. In support of this, it has been shown that fibroblasts from gelsolin knockout mice show similar increased actin fibre formation17 as seen in our cultured iPSC-derived RPGR/XLRP photoreceptors and RPGR-knockdown lines6. Moreover, gelsolin has been shown to regulate cilia formation, as evidenced by degeneration of outer ear stereocilia in gelsolin KO mice18 and the identification of gelsolin in an siRNA screen for genes required in ciliogenesis19. We therefore examined gelsolin activation in human RPGR/XLRP photoreceptors. Gelsolin occurs in two forms; a closed, inactive form and an active form which only the activated form will bind22, 23. When F-actin was incubated with knockdown cell lysates to compete for gelsolin binding (Supplementary Fig. 6), we observed a significant increase in phosphorylation of cytoskeletal regulatory proteins in the diseased photoreceptor cultures compared with non-cytoskeletal proteins (−0.008±0.01, n=583 proteins, p=0.004) (Fig. 2e, Supplementary Data1). These results revealed a significant increase in phosphorylation of cytoskeletal regulatory proteins in the diseased photoreceptor cultures as compared to control. The mean ratio of phosphorylation level in diseased vs control cells was 0.092±0.0033 (n=56 proteins) compared with non-cytoskeletal proteins (−0.008±0.01, n=583 proteins, p=0.004) (Fig. 2e, Supplementary Data 1). These results further support a disruption of cell signalling pathways regulating actin turnover in RPGR/XLRP mutant photoreceptors.

In parallel experiments to confirm a role for RPGR in actin turnover in the photoreceptor CC in vivo, we examined a recently reported Rpgr knockout (KO) mouse13. Significant photoreceptor degeneration was observed by 4 months of age (Fig. 3a–c). Prior to this, at 3 weeks of age, signs of photoreceptor stress were apparent as evidenced by increased GFAP immunolabelling throughout the radial length of Müller cells in the outer nuclear layer (ONL: Fig. 3d). In addition, the photoreceptors failed to correctly localise rhodopsin to the OS at this stage: instead it was trafficked to the outer plexiform layer and eventually remained in the peri-nuclear space (Fig. 3e, f). Cytoskeletal examination in murine retina during development (at postnatal day 2 and 10) revealed no difference between wild type and Rpgr KO photoreceptors (Supplementary Fig. 4), suggesting the initial photoreceptor development was RPGR-independent at stages where neonatal eyes are still closed and phototoxicity minimal. The degeneration in Rpgr-mutant photoreceptors seems to be linked to when eyes open at P14, as reactive gliosis is detected by P21 and by 2 months of age increased actin polymerisation was seen in the connecting cilia (Fig. 3g), all prior to photoreceptor loss. These results demonstrate that RPGR is required for actin regulation in the mature photoreceptor CC and for correct localisation of rhodopsin to its OS in vivo.

RPGR mutants are associated with reduced photoreceptor development, reactive gliosis and degeneration. In this study, we report that mutations in RPGR may have a role in actin polymerisation and photoreceptor development. The degeneration in RPGR mutants is not due to an absence of an alternative ciliogenesis pathway, as it occurs in the peri-nuclear space (Fig. 3e, f). Cytoskeletal examination in murine retina during development (at postnatal day 2 and 10) revealed no difference between wild type and Rpgr KO photoreceptors (Supplementary Fig. 4), suggesting the initial photoreceptor development was RPGR-independent at stages where neonatal eyes are still closed and phototoxicity minimal. The degeneration in Rpgr-mutant photoreceptors seems to be linked to when eyes open at P14, as reactive gliosis is detected by P21 and by 2 months of age increased actin polymerisation was seen in the connecting cilia (Fig. 3g), all prior to photoreceptor loss. These results demonstrate that RPGR is required for actin regulation in the mature photoreceptor CC and for correct localisation of rhodopsin to its OS in vivo.

**Fig. 2** RPGR-mutant, iPSC-derived, 3-dimensional photoreceptor cultures display perturbed actin regulation. a-d RPGR-mutant photoreceptors display increased actin polymerisation, as evidenced by increased phalloidin binding in the Recoverin-positive photoreceptors of patient-derived cultures (panels c, d) as compared to photoreceptors from the control patient (panels a, b). e The mean phosphorylation level of cytoskeletal regulatory proteins was higher in RPGR/XLRP photoreceptors compared to control photoreceptors (red dots; 0.092±0.0033, n=56 proteins). This was significantly more than the mean ratio of phosphorylation levels of non-cytoskeletal proteins when RPGR/XLRP photoreceptors were compared to control (blue dots; -0.008±0.01, n=583 proteins, unpaired, two-tailed t-test, p=0.004 (** p < 0.01)).
the Rpgr KO mouse photoreceptor compared to wild type (Images representative of 10 analysed).

Increased GFAP immunolabeling throughout the radial length of Müller cells in the ONL was observed (Fig. 5d). Further, degeneration of the photoreceptor-containing ONL was significant by 5 months of age (Fig. 5b, c). At earlier time points (2-month old), increased actin polymerisation was seen in the connecting cilia (Fig. 5e), akin to the Rpgr KO mouse. Together, these observations show that gelsolin regulates actin polymerisation in the photoreceptors and is required for both rhodopsin transport to the photoreceptor OS and photoreceptor maintenance.

Gelsolin KO mice phenocopy the retinal abnormalities seen in Rpgr KO mice. To confirm that the increased actin polymerisation and rhodopsin mislocalisation observed in Rpgr KO photoreceptors could be the consequence of loss of gelsolin activity, we examined the retina of the gelsolin KO mouse\(^{17}\). At 5 months of age, a similar phenotype was seen, with rhodopsin mislocalisation to the outer plexiform layer and peri-nuclear space (Fig. 5a). Increased GFAP immunolabeling throughout the radial length of Müller cells in the ONL was observed (Fig. 5d). Further, degeneration of the photoreceptor-containing ONL was significant by 5 months of age (Fig. 5b, c). At earlier time points (2-month old), increased actin polymerisation was seen in the connecting cilia (Fig. 5e), akin to the Rpgr KO mouse. Together, these observations show that gelsolin regulates actin polymerisation in the photoreceptors and is required for both rhodopsin transport to the photoreceptor OS and photoreceptor maintenance.

RPGR binds gelsolin and activated gelsolin can rescue RPGR-deficient cells. Having established that loss of gelsolin phenocopies key abnormalities of the Rpgr KO mouse, we next sought...
to determine whether there is a direct biochemical interaction between RPGR and gelsolin. Co-immunoprecipitation (Co-IP) of recombinant proteins revealed that gelsolin binds directly to the ubiquitous splice variant (RPGREx1-19) but not the retina-enriched RPGRORF15 isoform. The retinal-specific RPGRORF15 isoforms were pulled down with enriched RPGR ORF15 form of the protein (Supplementary Fig. 1). This report shows that RPGR functions in photoreceptors to regulate actin polymerisation and to activate the actin-cleaving protein gelsolin, since KO mice lacking RPGR lose cilia formation to wild-type levels (Fig. 6d). Concordant expression of the active, N-terminal half of gelsolin in this cell line restored cilia formation to wild-type levels (Fig. 6d), confirming that gelsolin activation can rescue RPGR loss.

**Discussion**

This report shows that RPGR functions in photoreceptors to activate the actin-cleaving protein gelsolin, since KO mice lacking gelsolin showed the same retinal phenotype as RPGR KO mice and human XLRP mutations in RPGR prevented the normal interaction between RPGR and gelsolin, reducing its activation and increasing actin polymerisation. Further support for the significance of this interaction comes from a report that families with a homozygous D187N (G654A) mutation in the human gelsolin (GSN) gene have RP, as well as manifestations of systemic amyloidosis resulting from either loss-of-function (e.g. RP) or deposition of amyloidogenic gelsolin fragments.

This activation of gelsolin is achieved by direct binding to RPGR, as evidenced by Co-IP studies showing the interaction of gelsolin with RPGR and by disruption of both this binding and subsequent activation of gelsolin in the presence of an XLRP mutation. Our results suggest the primary binding site appears to be in the ubiquitous splice variant (RPGREx1-19) rather than the retina-specific RPGRORF15 isoform. The finding that the pathogenic mutation that prevents this binding is in the RPGRORF15...
isofrom could be explained by the finding that the two RPGR isoforms form a complex in vivo (WZ and HK, unpublished observations), whereupon the mutant RPGR\textsuperscript{QRF15} prevents gelsolin binding to the RPGR\textsuperscript{E1} isoform.

There are two potential mechanisms by which the dysregulation of actin caused by abnormalities of gelsolin activation could lead to the mislocalisation of rhodopsin, photoreceptor stress and eventual degeneration characteristic of XLRP (Fig. 7). First, by disturbing cilia formation, which impacts on the maintenance of the modified cilium that comprises the photoreceptor. It is well recognised that actin exerts an influence on ciliogenesis and cilial maintenance\textsuperscript{19, 26–28}. Indeed, actin depolymerisation leads to lengthened, nascent, OS discs in photoreceptors\textsuperscript{29, 30}. Actin is localised to the distal portion of the CC in human photoreceptors where the ciliary membrane evaginates to form OS discs\textsuperscript{31–34}. These results suggest that RPGR facilitates OS disc budding or the completion of disc formation by locally regulating actin dynamics in a gelsolin-dependent manner. In keeping with this, the disorganised nature of discs reported in an Rprg-KO mouse\textsuperscript{59} occurs with a hyperstabilised actin cytoskeleton in both mutant postnatal mouse eyes and RPGR-mutant photoreceptor organoids characterised here. Additional support comes from a role of RPGR in facilitating the OS content of inositol polyphosphate INPP5E\textsuperscript{55}. INPP5E regulates the phosphoinositide content of the membrane, which in turn regulates the local lipid-actin cytoskeleton interface\textsuperscript{36}. Interestingly, recent work has demonstrated the highly localised role actin plays in excision of a membranous bud (ectosome) at the cilia tip\textsuperscript{37, 38}. The process, known as ectocytosis (or decapitation), serves as an alternative ciliary exit route for G-protein-coupled receptors. We speculate that photoreceptor disc formation may share a common ancestral mechanism with ectocytosis and that RPGR’s alternatively spliced ORF15 variant may have evolved to facilitate this.

Second, photoreceptor degeneration could occur if actin dysregulation functionally abrogates rhodopsin trafficking. In the photoreceptor, an actin bundle connects the periciliary membrane complex with the basal body at the base of the CC, along which the actin-based motor protein myosin VIIA appears to travel\textsuperscript{30, 39}. Myosin VIIA contributes to the active transport of visual pigments, including rhodopsin, into the CC\textsuperscript{39}. Support for a role of RPGR and gelsolin in cilary function and rhodopsin trafficking comes from work examining Usher Syndrome, a syndromic form of RP and deafness. This syndrome can be caused by whirlin (WHRN) mutations\textsuperscript{40}, coding for a protein that forms part of the usherin complex that regulates the actin filament network in the periciliary membrane complex\textsuperscript{41, 42}. Gelsolin is part of this WHRN complex in inner ear stereocilia\textsuperscript{18} where it is mislocalised away from stereocilia in the whirlin mutant mouse\textsuperscript{18}. WHRN has also been shown to interact with the RPGR ORF15 basic domain in photoreceptors\textsuperscript{43}, in keeping with a model whereby loss of WHRN perturbs the regulation of gelsolin-mediated actin turnover by RPGR, disrupting myosin VIIA-mediated rhodopsin transport at the periciliary membrane complex. RPGR also facilitates the cilary targeting of the small G protein, RAB8, which regulates vesicular trafficking during primary ciliogenesis and rhodopsin transport in the photoreceptor\textsuperscript{42}, supporting this model.

These models are not mutually exclusive, and further work will define their relative contributions to the pathologies of XLRP, as well as addressing the important translational implication of our study that pharmacomodulation or ectopic expression of activated gelsolin in the retina could slow or reverse the retinal degeneration observed in RPGR/XLRP disease.

**Methods**

**Antibodies and reagents.** Primary antibodies: mouse anti-OCT3/4 (R&D AF1759 10 µg/ml IF), goat anti-Nanog NL493-conjugated (R&D 967151; 1 in 10 IF), SOX2 (E Bioscience 14-9811-82; 1 in 100 IF), rabbit anti-SMA (Abcam AB5694; 1 in 100 IF), β-Tub (Abcam ab7751; 1 in 1000 IF), mouse anti-βF (Sigma A8452; 1 in 500 IF), rabbit anti-PAK (Covance PRR-278P; 1 in 200 IF), goat anti-CHX30 (Santa Cruz SC-21692; 1 in 500 IF), rabbit anti-RPGR (Atlas Antibodies HPA001593; 1 in 200 IF), mouse anti-RETP1 (Chemicon MAB5326; 1 in 1000 IF), phallolidin-Alexa487 (Life Technologies a2287; 1 in 200 IF), Hoechst, rabbit anti-GFAP (DAKO ZO334; 1 in 100 IF), mouse anti-α-tubulin (Sigma, T6793; 1 in 200 IF), rabbit anti-Gelsolin (Abcam 74420; 1 in 900 WB), rabbit anti-cofilin/p-Cofilin (Cell Signalling 3313 & 5175; 1 in 1000 WB), mouse anti-GAPDH (Chemicon MAB374; 1 in 1000 WB).
Fig. 6 Loss-of-function and gain-of-function experiments support a role for gelsolin in RPGR mediated cilia maintenance. a Immunoprecipitation (IP) of RPGR KD and gelsolin (lower panel) demonstrates an endogenous RPGR-gelsolin interaction in bovine retina, as evidenced by immunoblotting (IB) for the presence of gelsolin (86 kD) and the 127 kD retina-specific form of RPGR respectively in the immunoprecipitates. b Immunoprecipitation (IP) of RPGR KD followed by immunoblotting (IB) for RPGR reveals that the RPGR-gelsolin interaction is present in control iPSC-derived photoreceptors but is perturbed in both XLRP patient-derived RPGR-mutant cultures—note in the right hand set of three lanes that RPGR is present only in the IPs from the control patient. The lower panel shows a longer exposure of the immunoblot to show that both the constitutively-expressed (90 kD, encoded by exons 1-19, arrowhead) and retina-specific (127 kD—encoded by exons 1-14 and specific open reading frame ORF 15, arrow) forms of RPGR are present in the control and mutant cell lysates shown in the three left hand lanes. Far right panel shows equal amounts of gelsolin are pulled down from each sample by gelsolin IP. c siRNA-mediated RPGR knockdown. Note that expression of both the constitutively-expressed (90 kD—Ex 1-19) and retina-specific (127 kD—ORF 15) forms of RPGR are reduced by the siRNA while the non-specific band at 100 kD is unaffected in the hTERT-RPE cell line. d, e Immunolabeling of the hTERT-RPE cell line after siRNA-mediated RPGR knockdown reveals a loss of ciliogenesis (Mean percentage of ciliated cells 37.62%±3.113 vs 16.48%±3.762 (SEM)), unpaired two-tailed t-test, p = 0.0124; identified by acetylated α-tubulin immunoreactivity in green), as shown by a comparison of the control (left) and RPGR knockdown (centre) panels. The defect is rescued by overexpression of constitutively active gelsolin, as shown in the right panel (Mean percentage of ciliated cells 16.48%±3.762 vs 38.33%±5.349 (SEM), unpaired two-tailed t-test, p = 0.0288.) The tracks below and to the right of the micrographs in d show z-planes of the image to confirm the presence of elongated cilia. Scale bars: 10 μm (d)

Secondary antibodies (all Life Technologies; all 1 in 1000 dilution unless otherwise stated): donkey anti-mouse-Alexa488 (A21202), donkey anti-mouse-Alexa568 (A10037), donkey anti-goat-Alexa488 (A21432), donkey anti-goat-Alexa555 (A11055), donkey anti-sheep-Alexa488 (A11015), donkey anti-sheep-Alexa568 (A12099), donkey anti-rabbit-Alexa488 (A10042), donkey anti-rabbit-Alexa568 (A21206), Horseradish peroxidase rabbit (NA934V, GE Healthcare; 1 in 10,000 WB). Alexa568 (A21206), donkey anti-sheep-Alexa488 (A11015), donkey anti-mouse-Alexa488 (A21202), donkey anti-mouse-Alexa568 (A12099).

iPSC generation. Skin biopsies were performed under local anaesthetic (Xylocaine 2% with Adrenaline 100 micrograms/20 ml (1:200,000), AstraZeneca) using a sterile technique following ethical approval (REC Ref No 10/S1103/10, Lothian R&D Project No 2012/R/DER/01) and informed consent. Both patients and controls were aware of their use of their samples in research. Fibroblast cell lines developed in Dubeczko’s Modified Eagle Medium (Gibco) with 10% fetal calf serum (Gibco) and 1:500 Penicillin/Streptomycin (Gibco). From day 18 onwards, cells were maintained in DMEM: F-12 was supplemented with 10% FCS, 100 nm SAg and 1% Geltrek in DMEM/Ham’s F-12 (Gibco). Cells were counted after 25 days and hESC-looking colonies picked and expanded. Cells were maintained on Geltrek in E8 and passaged with EDTA (0.5 M, Invitrogen).

Photoreceptor differentiation. Three-dimensional optic cup structures protocols were adapted from previous protocols11, 12. Confluent iPSC colonies were dissociated using EDTA (0.5 M, Invitrogen), resuspended in Essential8 media (Gibco) and allowed to form embryoid bodies in non-adherent 10 cm petri dishes (Sterlin) at 37 °C on an orbital shaker (Stuart). Twenty-four hours later they began patterning for 12 days with reducing knockout serum replacement (KOSR; 20% for 5 days, 15% until day 9, 10% until day 37), 1:50 B27 (PAA), 5 ng/ml rhIGF-1 (Sigma), 3 μm IWR1e and 1% Geltrek in DMEM/Ham’s F-12 (Gibco). From day 12-18, cultures were incubated with reducing KOSR, 1:50 B27 (PAA), 5 ng/ml rhIGF-1 (Sigma), 10% FCS, 100 nm SAg and 1% Geltrek in DMEM/Ham’s F-12 (Gibco). From day 18-37, DMEM: F-12 was supplemented with 10% KOSR, 1:50 B27 (PAA), 5 ng/ml rhIGF-1 (Sigma) and 1% Geltrek. From day 37 onwards, DMEM: F-12 was supplemented with 1:50 B27 (PAA), 5 ng/ml rhIGF-1 (Sigma) and 1% Geltrek.

Animals. All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the United Kingdom Animals (Scientific Procedures) Act 1986 under project licence number P6CS15DC9 (Dr X Shu).

Histology and immunostaining. Mouse eyes13, 17 were fixed immediately after enucleation and corneal excision in 4% paraformaldehyde (Sigma-Aldrich) in PBS at room temperature (RT) for 1 h, infiltrated with sucrose for cryoprotection (15%, 30%) and embedded in OCT (CellPath). Complete sectioning of whole eyes was performed through the horizontal meridian. Sections (8–50 μm thickness) were collected. Three-dimensional iPSC-derived optic cup structures were fixed,
RPGR’s regulation of Gelsolin mediates Rhodopsin trafficking to the connecting cilium via actin-led Myosin VIIa transport

RPGR’s regulation of Gelsolin mediates outer segment disc budding / elongation via actin turnover

Healthy photoreceptor

Actin dysregulation in the connecting cilium ameliorates Rhodopsin trafficking, mislocalising it to the inner segment

Actin dysregulation at site of disc budding perturbs disc morphogenesis, mislocalising rhodopsin to the inner segment

RPGR-mutant photoreceptor

Fig. 7 Proposed model for RPGR mediated actin turnover in the photoreceptor connecting cilium. We propose two possible mechanisms of action for RPGR. First, RPGR’s regulation of actin turnover could mediate rhodopsin trafficking into the photoreceptor connecting cilium (left panels). An actin bundle connects the periciliary membrane complex to the basal body, along which Myosin VIIa actively transports visual pigments. Whirlin regulates this actin network at the connecting cilium base and interacts with both gelsolin and RPGR, suggesting a model whereby mutant RPGR disrupts the complex, perturbing actin turnover and compromising Myosin VIIa-mediated rhodopsin transport (left panels). The resulting rhodopsin mislocalisation to the inner segment results in cell stress and degeneration. Second, RPGR’s regulation of actin turnover could facilitate outer segment disc formation (right panels). Actin exerts an influence on both ciliogenesis and maintenance and is localised to the site of photoreceptor disc budding. The disorganised discs and hyperstabilised actin seen in the Rpgr knockout mouse supports a model whereby RPGR facilitates outer segment disc budding or the completion of disc formation by gelsolin-mediated actin turnover (right panels). The compromised disc morphogenesis seen with mutant RPGR would mislocalise rhodopsin to the inner segment, resulting in cell stress and degeneration.
cryoprotected, embedded and sectioned in a similar fashion. hTERT-RPE cells grown on slides were fixed in 4% paraformaldehyde for 30 min at RT.

Sections/cells were blocked/permeabilized with 2% NDS (Sigma), 2% BSA (Sigma) and 0.5% Triton X100 (Fisher) for 1 h at RT, washed with PBS, incubated with primary antibodies overnight at 4°C, washed in PBS, incubated with secondary antibodies for 60 min at RT, washed in PBS, incubated in Phalloidin for 30 min at RT, washed in PBS, incubated in Hoechst for 5 min at RT and mounted with coverslips using Fluoromount-G (Southern Biotech). Images were taken on a Leica SPE confocal microscope and analysed using the Imagent and ImagePro software. Images represent confocal projections, unless stated otherwise.

For actin levels, pixel intensity of phalloidin staining of standardised regions of interest (ROI) of confocal Z-stacks were measured by blinded assessors using ImageJ.

Assessors were blinded for all mouse retina measurements.

Electron microscopy. Three-dimensional optic cup structures were fixed in 2.5% glutaraldehyde (TAAB) in 0.1 M phosphate (PO) buffer at RT (2 h), washed (0.1 M PO buffer) and lipids stained with 1% osmium tetroxide (Electron Microscopy Science) (1 h). Samples were washed (PO buffer) prior to ethanol dehydration. Residual ethanol was removed with propylene oxide (TAAB) prior to overnight incubation in 5 g araldite CY212 (Agar Scientific) and 5 g dodecenyl succinic anhydride (DSSA, Agar Scientific). Sequential incubations in the above mix precluded to final embedding resin (11.5 g araldite CY212, 11 g DSSA, 0.55 ml benzyldimethylamine (Agar Scientific), 0.5 ml dibutylphthalate (Agar Scientific). Repeated exchange of embedding resin preceded 48 h in an oven (60°C).

RNA extraction and RT-PCR. mRNA was extracted from transfected cells using the RNeasy Minikit (Qiagen, Crawley, UK) before being reverse transcribed with the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer’s instructions. The resulting cDNA was used to perform RT-PCR with the LightCycler 480 (Roche) plus 10% FBS and penicillin/streptomycin. For testing Gelsolin-RPGR interaction, we transiently transfected the cells with plasmids encoding FLAG-tagged Gelsolin and GFP-tagged full length RPGR (const), RPGR (Δ8-19) and different RPGR domains (RPGR (Δ1-17), RPGR (Δ1-25), RPGR (Δ3-46), or RPGR (Δ66-102)). Details of these constructs have been described.

At the end of 48 h, cells were lysed in IP lysis buffer (25 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% Glycerol and Complete Protease Inhibitor (Roche)). The lysates were centrifuged to remove the debris and subjected to pulldown using GFP antibody crosslinked to AminoLink Plus Coupling Resin. The details of crosslinking have been described. The immunoprecipitate was washed three times with the IP lysis buffer and the samples were eluted in glycin lysis buffer (pH 2.8), neutralised using 1 M Tris pH 9.5 and analysed by SDS-PAGE and immunoblotting.

Gelsolin assay for determination of triton-insoluble F-actin. Cell cultures were lysed with buffer containing 120 mM PIPES, 50 mM HEPES, 1 mM MgCl2, 10 mM glucose, 20 mM EDTA, 0.1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and protease (Millipore) and phosphatase (Calbiochem) inhibitors. Initial lystate also contained 1.5% Triton-X100 to isolate Triton-soluble G-Actin (inactive/unbound Gelsolin). Lystate was vortexed (10 min) and spun (12,000 x g) for 10 min, all at 4°C. Triton-soluble supernatant (G-actin; inactive/unbound gelsolin) was removed and the pellet resuspended in lysis buffer containing 6% SDS prior to 10 min vortexing and centrifugation (12,000 x g) for 10 min, all at RT. Resulting supernatant contained F-actin (active/bound gelsolin). Lysates were then processed for western blotting.

Phospho protein arrays. Arrays were carried out as per manufacturer’s instructions (Fullmoon Bio). Briefly, 100 day old photoreceptor cultures (RPGR-mutant vs Control, n = 3) were lysed with extraction buffer containing Protease (Millipore) and Phosphatase (Calbiochem) inhibitors, purified and biotinylated. Array chips were blocked and biotinylated proteins allowed to couple to chips. Chips were washed, incubated with Cy3 streptavidin, washed and measured on Axon 4200 A slide scanner.

Transfection and cilia formation. Cells were transfected with Lipofectamine2000 (Invitrogen) using the reverse transfection protocol as per manufacturer’s instructions. 0.125 µl control/RPGR siRNA and 1 µl Lipofectamine2000 were diluted in 100 µl OptiMEM (Gibco) and incubated for 20 min in a 24-well plate at RT. Cells were plated on the siRNA/Lipofectamine mix at 3 x 10^5 cells/well. Twenty-four hours later the siRNA/Lipofectamine mix was removed and replaced by 1 ml of normal media, 2 µl Lipofectamine and 100 µl OptiMEM (preincubated for 20 h). Four hours later, the medium was replaced with serum-free medium for 24 h, after which cells were fixed, immunostained and assessed for cilia formation.

Data availability. All data generated or analysed during this study are included in this published article (and its Supplementary Information) or from the corresponding author upon reasonable request.

Received: 15 February 2016 Accepted: 2 June 2017

Published online: 16 August 2017

References
1. Young, R. W. The renewal of photoreceptor cell outer segments. J. Cell Biol. 33, 61–72 (1967).
2. Besharse J. in The Retina: A Model for Cell Biological Studies (eds Adler, R. & Farber, D.) 297–352 (Academic Press, 1986).
3. Megaw, R., Soares, D. & Wright, A. F. RPGR: its role in photoreceptor physiology, human disease, and future therapies. Exp. Eye Res. 138, 32–41 (2015).
4. Wright, A. F., Chakarova, C. F., Abd El-Aziz, M. M. & Bhattacharya, S. S. Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. Nat. Rev. Genet. 11, 273–284 (2010).
5. Hong, D. et al. A retinitis pigmentosa GTPase regulator (RPGR)-deficient mouse model for X-linked retinitis pigmentosa (RPS). Proc. Natl Acad. Sci. USA 97, 3649–3654 (2000).
6. Gakovic, M. et al. The role of RPGR in cilia formation and actin stability. Hum. Mol. Gen. 20, 4840–4850 (2011).
7. Rao, K. N. et al. Loss of human disease protein retinitis pigmentosa GTPase regulator (RPGR) differentially affects rod or cone-enriched retina. Hum Mol Genet. 25, 1345–1356 (2016).
8. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872 (2007).
9. Okita, K. et al. A more efficient method to generate integration-free human iPS cells. Nat. Methods 8, 409–412 (2011).
10. Nakano, T. et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10, 771–785 (2012).
11. Mellough, C. et al. IGF-1 signaling plays an important role in the formation of three-dimensional laminated neural retina and other ocular structures from human embryonic stem cells. *Stem Cells* 33, 2416–2430 (2015).

12. Huang, W. C. et al. RPRG-associated retinal degeneration in human X-linked RP and a murine model. *Invest. Ophthalmol. Vis. Sci.* 53, 5594–5608 (2012).

13. Zhang, C. et al. NuDC regulates actin dynamics and ciliogenesis by stabilizing coflin 1. *Cell Res.* 26, 239–253 (2016).

14. Ressad, F. et al. Kinetic analysis of the interaction of actin-depolymerizing factor (ADF)/cofilin with G- and F-actins. *J. Biol. Chem.* 273, 20894–20902 (1998).

15. Takahashi, K., Kanno, S. I. & Mizuno, K. Activation of cytosolic sphericin-1 photophase by gelsolin-generated soluble actin filaments. *Biochem. Biophys. Res. Commun.* 454, 471–477 (2014).

16. Kurokawa, Y. et al. Hermosatatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell 81*, 41–51 (1995).

17. Mbura, P. et al. Gelsolin plays a role in the actin polymerization complex of hair cell stereocilia. *PLoS ONE* 5, e11627 (2010).

18. Kim, J. et al. Functional genomic screen for modulators of ciliogenesis and cilium length. *Nature* 464, 1048–1051 (2010).

19. Cooper, J. et al. Microinjection of gelsolin into living cells. *J. Cell Biol.* 104, 491–501 (1987).

20. Burtnick, L., Urosev, D., Irobi, E., Narayan, K. & Robinson, R. Structure of the N-terminal tail of gelsolin bound to actin: roles in severing, apoptosis and FAF. *EMBO J.* 23, 2713–2722 (2004).

21. Cunningham, C. et al. Cell permeant polyphosphoinositide-binding peptides block cell motility and actin assembly. *J. Biol. Chem.* 276, 43390–43399 (2001).

22. Finkelstein, M., Etkovitz, N. & Breitbart, H. Role and regulation of sperm gelsolin prior to fertilization. *J. Biol. Chem.* 285, 39702–39709 (2010).

23. Nagaya, H. et al. Regulated motion of glycoproteins revealed by direct visualization of a single cargo in the endoplasmic reticulum. *J. Cell. Biol.* 180, 129–143 (2008).

24. Ardalan, M. R., Shoja, M. M. & Kiuru-Enari, S. Amyloidosis-related nephrotic syndrome due to a G654A gelsolin mutation: the first report from the middle east. *Nephrol. Dial. Transplant.* 22, 272–275 (2007).

25. Bershteyn., M., Atwood, S. X., Woo, W. M., Li, M. & Oro, A. E. MIM and TAZ activity and vesicle trafficking in the connecting cilium of mammalian photoreceptor cells. *Hum. Mol. Genet.* 17, 71–86 (2008).

26. Wright, R. N., Hong, D. & Perkins, B. Rprg/ORF15 connects to the Usher protein network through direct interactions with multiple whirin isoforms. *Invest. Ophthalmol. Vis. Sci.* 53, 1519–1529 (2012).

27. Miyatake, K., Kusakabe, M., Takahashi, C. & Nishida, E. ERK7 regulates whirin interactions with espin and modulates its actin-regulatory function: an insight into the mechanism of Usher syndrome type II. *Hum. Mol. Genet.* 21, 692–710 (2012).

28. Maurer, T. et al. A novel Usher protein network at the periciliary reloading point between molecular transport machineries in vertebrate photoreceptor cells. *Hum. Mol. Genet.* 17, 71–86 (2008).

29. Wolfrum, U. & Schmitt, A. Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells. *Cell Motil. Cytoskeleton.* 46, 95–107 (2000).

30. Arikawa, K. & Williams, D. S. Organization of actin filaments and immunococalocalization of alpha-actinin in the connecting cilium of rat photoreceptors. *J. Comp. Neurol.* 288, 640–646 (1989).

31. Chaitin, M. & Burnside, B. Actin filament polarity at the site of rod outer segment disk morphogenesis. *Invest. Ophthalmol. Vis. Sci.* 30, 139–342 (1999).

32. Williams, D. S., Linberg, K. A., Vaughan, D. K., Fariss, R. N. & Fisher, S. K. Disruption of microfilament organization and deregulation of disk membrane morphology by cytochalasin D in rod and cone photoreceptors. *J. Comp. Neurol.* 272, 161–176 (1988).

33. Phua, S. C. et al. Dynamic remodeling of membrane composition drives cell cycle through primary cilia excision. *Cell 168*, 264–279 (2017).

34. Wolfrum, U. & Schmitt, A. Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells. *Cell Motil. Cytoskeleton.* 46, 95–107 (2000).

35. Ebermann, I. et al. A novel gene for Usher syndrome type 2: mutations in the long isoform of whirin are associated with retinitis pigmentosa and sensorineural hearing loss. *Hum. Genet.* 121, 203–211 (2007).

36. Wang, L. et al. Whirin interacts with espin and modulates its actin-regulatory function: an insight into the mechanism of Usher syndrome type II. *Hum. Mol. Genet.* 21, 692–710 (2012).

Acknowledgements

We would like to acknowledge Dr Xinhua Shu (Glasgow Caledonian University) for kindly housing the Rpgr KO mice. We would also like to thank CB, HB, MB and KR for their generous tissue donation to generate the iPSCs. This work was supported by grants from the Wellcome Trust (R.M., H.A. by Grant Number 100470/Z/12/Z, C.F.-C. by an investigator award), Retinitis Pigmentosa Fighting Blindness (R.M., H.A., A.F.W., Grant Number G583), the Academy of Medical Sciences (R.M., M.I.; Grant Number SG014101), a Medical Research Council Programme Grant (A.F.W.), an ERC Fellowship (C.M., M.L.), a DFG Grant (C.G., W.W.; Grant Number SPI1464) and the NIH (W.Z., H.K.; Grant Number EY02372).

Author contributions

R.M., A.F.W. and C.F.-C. designed research; R.M., H.A.-A., M.J., C.G. and W.Z. performed research; R.M., H.A.-A., M.J., C.M., H.K., P.M., M.L., C.F.-C. analyzed data; R. M. and C.F.-C. wrote the manuscript; R.M., C.M., W.W., H.K., P.M., B.D., A.F.W., M.L. and C.F.-C. edited the manuscript.

Additional information

Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00111-8.

Competing interests: The authors declare no competing financial interests.

Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s) 2017