Structural and functional plasticity of subcellular tethering, targeting and processing of RPGRIP1 by RPGR isoforms

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Summary

Mutations affecting the retinitis pigmentosa GTPase regulator-interacting protein 1 (RPGRIP1) interactome cause syndromic retinal dystrophies. RPGRIP1 interacts with the retinitis pigmentosa GTPase regulator (RPGR) through a domain homologous to RCC1 (RHD), a nucleotide exchange factor of Ran GTPase. However, functional relationships between RPGR and RPGRIP1 and their subcellular roles are lacking. We show by molecular modeling and analyses of RPGR disease-mutations that the RPGR-interacting domain (RID) of RPGRIP1 embraces multivalently the shared RID of RPGR1–19 and RPGRORF15 isoforms and the mutations are non-overlapping with the interface found between RCC1 and Ran GTPase. RPGR disease-mutations grouped into six classes based on their structural locations and differential impairment with RPGRIP1 interaction. RPGRIP1α1 expression alone causes its profuse self-aggregation, an effect suppressed by co-expression of either RPGR isoform before and after RPGRIP1α1 self-aggregation ensue. RPGR1–19 localizes to the endoplasmic reticulum, whereas RPGRORF15 presents cytosolic distribution and they determine uniquely the subcellular co-localization of RPGRIP1α1. Disease mutations in RPGR1–19, RPGRORF15 or RID of RPGRIP1α1, singly or in combination, exert distinct effects on the subcellular targeting, co-localization or tethering of RPGRIP1α1 with RPGR1–19 or RPGRORF15 in kidney, photoreceptor and hepatocyte cell lines. Additionally, RPGRORF15, but not RPGR1–19, protects the RID of RPGRIP1α1 from limited proteolysis. These studies define RPGR- and cell-type-dependent targeting pathways with structural and functional plasticity modulating the expression of mutations in RPGR and RPGRIP1. Further, RPGR isoforms distinctively determine the subcellular targeting of RPGRIP1α1, with deficits in RPGRORF15-dependent intracellular localization of RPGRIP1α1 contributing to pathomechanisms shared by etiologically distinct syndromic retinal dystrophies.

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Key words: protein targeting, RPGRIP1, RPGR, protein aggregation, degeneration, photoreceptor, kidney cells

Introduction

X-linked retinitis pigmentosa 3 (XIRP3) is one of the most severe and predominant forms of retinitis pigmentosa (RP) leading to the progressive degeneration of photoreceptors (Breuer et al., 2002; Hartong et al., 2006; Sandberg et al., 2007). The XIRP3 locus encodes at least two major isoforms of the retinitis pigmentosa GTPase regulator (RPGR), RPGR1–19 and RPGRORF15 (Ferreira, 2005; Meindl et al., 1996; Vervoort et al., 2000). RPGR1–19 is encoded by 19 exons of XIRP3 (Meindl et al., 1996), whereas RPGRORF15 is produced from the alternate retention of the purine-rich intron 15 leading to an RPGR isoform with a terminal and extended exon 15 (Vervoort et al., 2000). Hence, RPGR1–19 and RPGRORF15 share an N-terminal domain but have distinct C-terminal domains. The N-terminal domain contains several well-defined internal repeats (Ferreira, 2005; Meindl et al., 1996), which are highly homologous to the β-propeller repeats of the regulator of chromosome condensation 1 protein (RCC1), a nuclear nucleotide exchange factor for Ran GTPase (Renault et al., 2001; Renault et al., 1998). On the other hand, the unique C-terminal domain of RPGR1–19 is 230 residues long and contains an isoprenylation motif (Ferreira, 2005; Sharon et al., 2003; Vervoort et al., 2000), which was reported to target RPGR1–19 to the Golgi apparatus (Yan et al., 1998). The C-terminal domain of RPGRORF15 instead comprises a stretch of 516 residues and is highly acidic (Ferreira, 2005; Meindl et al., 1996), which was reported to target RPGR1–19 to the Golgi apparatus (Yan et al., 1998). The C-terminal domain of RPGRORF15 is localized to the outer segment and connecting cilium of photoreceptors (Brunner et al., 2010; Mavlutyutov et al., 2002). RPGRORF15 is an isoform of critical biological and clinical relevance, because the majority of the mutations causing XIRP3 are found in the C-terminal domain of RPGRORF15 and mutations were never found in the sequence encoding the unique C-terminal domain of RPGR1–19 (Breuer et al., 2002; Ferreira, 2005; Sharon et al., 2003; Vervoort et al., 2000). Missense mutations in the shared RCC1-homologous
domain (RHD) of RPGR ORF15 lead to strong disease expression and some even cause syndromic visual phenotypes, while pathogenic mutations in ORF15 domain of RPGR ORF15 reflect always frame-shift mutations caused by small insertions or deletions and these are thought to constitute hypomorphic alleles leading to milder disease expression (Breuer et al., 2002; Iannaccone et al., 2003; Iannaccone et al., 2004; Sandberg et al., 2007; Sharon et al., 2003; Zito et al., 2003; Zito et al., 1999). However, the biological bases for such effects remain elusive.

To gain insights into the biological functions and molecular mechanisms underlying the pathogenesis of XIRP3, two interacting substrates of RPGR were identified. These are the δ-subunit of PDE (also named PrBP/δ) (Linari et al., 1999) and the retinitis pigmentosa GTPase regulator interacting protein 1 (RPGRIP1) (Boylan and Wright, 2000; Hong et al., 2001; Roepman et al., 2000a). Although no human mutations are known to affect PrBP/δ, its genetic ablation in the mouse causes slowly progressing rod/cone dystrophy (Zhang et al., 2007). By contrast, human mutations in RPGRIP1 cause Leber congenital amaurosis (LCA), a visual disorder typically characterized by the rampant degeneration of photoreceptors (den Hollandier et al., 2008; Dryja et al., 2001; Gerber et al., 2001). Moreover, ablation of Rpgrip1 expression in the mouse recapitulates well the human disease by strongly suppressing the formation of the outer segments of photoreceptors and causing the rapid degeneration of these neurons and ultimately, blindness (Won et al., 2009). Rpgr1 encodes various protein isoforms with differential expression across tissues (Ferreira, 2005; Roepman et al., 2000a) and the expression of some RPGRIP1 isoforms are pharmacologically modulated in mouse models of Fabry’s disease (Moore et al., 2007). Among the RPGRIP1 isoforms identified, a large ~175 kDa isoform, RPGRIP1α1, is specifically expressed in the retina and it is present in the connecting cillum and outer segments of photoreceptors, where it partially co-localizes with RPGR (Brüner et al., 2010; Castagnet et al., 2003; Ferreira, 2005; Mavlyutov et al., 2002; Roepman et al., 2000a).

A conserved domain of RPGRIP1α1, the RPGR-interacting domain (RID), interacts with the RHD shared by RPGR ORF15 and RPGR ORF15, and some RP- and LCA-causing mutations in either RHD or RID were shown to impair the interaction between the two (Lu et al., 2005; Roepman et al., 2000a). Among other domains, RPGRIP1α1 has two protein kinase C conserved region 2 (C2)-like motifs (C2-N and C2-C) adjacent to its C-terminal RID (Ferreira, 2005; Roepman et al., 2005). The C2-C region of RPGRIP1 interacts with nephrocystin-4 (NPHP4) and mutations in such region cause LCA likely by disrupting the interaction between RPGRIP1 and NPHP4 (Roepman et al., 2005). By contrast, mutations in NPHP4 cause either familial juvenile nephronophthisis or the ocular-renal disease, Senior-Løken syndrome (SLSN) (Mollet et al., 2002; Otto et al., 2002). Further, heterozygous and LCA-distinct mutations outside the RID of RPGRIP1 were found recently to increase the susceptibility to or cause various forms of glaucoma (Fernandez-Martinez et al., 2011). Evidence also indicates that the components of the RPGRIP1 complex, PrBP/δ and RPGR, apparently associate or form high-order complexes with Rab13, Arl2, H-Ras, Rheb, Rhox6, Rab8a, nephrocystin-5 (NPHP5) or CEP290/nephrocystin-6 (NPHP6) (Chang et al., 2006; Hanzal-Bayer et al., 2002; Murga-Zamalloa et al., 2010; Otto et al., 2005). In addition, genetic modifiers modulate the expression of XIRP3, because similar mutations in Rpgr can cause discordant phenotypes in the human (Banin et al., 2007; Walia et al., 2008), the penetrance of the disease varies greatly among species (Brüner et al., 2010; Hong et al., 2000; Jacobson et al., 1997; Zeiss et al., 1999; Zhang et al., 2002), allelic heterogeneity in XIRP3 differentially affect rod and cone photoreceptor neurons (Demirci et al., 2002; Sharon et al., 2003; Yang et al., 2002), and RPGRIP1, RPGRIP1L and NPHP5 (IQCB1) can act as genetic modifiers of the clinical expression of XIRP3 in the human (Fahim et al., 2011).

The lack of outer segment compartments in rod and cone photoreceptor neurons in the absence of the expression of RPGRIP1 hints that RPGRIP1 and some of its partners are determinant to support the subcellular targeting of critical components necessary for the elaboration of outer segments of photoreceptors, the subcellular structures which are essential to photoreception and phototransduction (Won et al., 2009). In contrast to some of RPGRIP1 partners, the RPGRIP1-dependent subcellular processes appear to be functionally redundant for kidney function (Mollet et al., 2002; Otto et al., 2002; Wiik et al., 2008; Won et al., 2009; Won et al., 2011). However, nothing is known about the nature and identity of the molecular and subcellular processes which the RPGRIP1 interactome plays a role in, even though abundant genetic evidence supports vital functions of components of the RPGRIP1 interactome in photoreceptor neurons, kidney tubular cells or both and of genetic interactions between RPGRIP1 and its components in cell/tissue and allelic-dependent fashions (Fahim et al., 2011). Likewise, little is known about the structural and temporal hierarchical organizations and functional relationships among any of the components of the RPGRIP1 assembly complex. The acquisition of such knowledge is critical to define the biological roles of RPGRIP1 and its partners, to understand essential and cell-type-dependent molecular and subcellular processes affected by disease mutations in components of the RPGRIP1 interactome and ultimately, to the understanding of the pathobiology of etiologically distinct syndromic retinal diseases.

Since the core components and processes underlying the subcellular sorting and targeting of newly synthesized proteins are typically conserved among cultured mammalian cells (Pelham, 1989; Pfeffer and Rothman, 1987; Warren and Mellman, 1999), the expression (or knock-down) of wild-type and mutant proteins, even with selective tissue expression, in cultured cells often provide unique or complementary mechanistic insights into their roles and mutations therein in regulating specific subcellular sorting, targeting and/or tethering processes critical to the function of selective tissues or cells. The impairment of these processes are typically difficult to dissect in animal disease models due to confounding secondary phenotypes or downstream cumulative effects caused by mutations leading to the functional impairment of such proteins. Here, we investigate the predicted and experimental structural and functional effects of an array of disease-related mutations on the interplay between RPGRIP1α1 and two isoforms of RPGR in three distinct mammalian cell-types in culture. The data support RPGR
isomers are critical to determine distinctively the subcellular targeting of RPGRIP1\(x_1\) and suppression of intracellular deposits of RPGRIP1\(x_1\). A model emerges whereby the combinatorial action of cell-type selective accessory factors together with the RPGRIP1 interactome contribute to the modulation of the tethering, targeting and processing of the RPGRIP1 interactome and selective expression of disease-mutations affecting its components.

**Results**

**Molecular modeling of the RCC1-homologous domain (RHD) of RPGR to RCC1**

The alignment of RPGR to RCC1 reveals that five complete blades (B2-B6) and two half blades (B1-C/D and B7-A/B) are fully conserved in the RHD of RPGR (supplementary material Fig. S1). Structural and biochemical evidence indicates that the mechanism of nucleotide release from Ran GTPase by RCC1 is distinct from other nucleotide exchange factors (Renault et al., 2001). In RCC1, a protruding and rigid \(\beta\)-wedge loop in blade 3 (residues 146–156) wedges between switch II and the P-loop of Ran GTPase and the structural conservation of the loop is the major determinant in destabilizing the nucleotide binding to Ran GTPase (Renault et al., 2001). Two key residues, F146 and R147, are important to the stabilization of the \(\beta\)-wedge loop in RCC1, and they are absent in the RHD of RPGR (supplementary material Figs S1, S2A,B).

To gain further insight into the nature of the interaction of the conserved RHD of the RPGR isoforms (Fig. 1A) with the RHD of RPGRIP1\(x_1\) (Fig. 2A) and other potential partners, we generated a homology model structure of the RHD based on the known structure of RCC1 (Fig. 1B). The RCC1 template structure has a pseudo-sevenfold symmetry with a propeller-like shaft made up of seven blades (B1–B7) consisting of four-stranded anti-parallel \(\beta\)-sheet. Due to the lack of homology in the C-terminal end of RHD of RPGR (residue 370 onwards) we are unable to predict using homology modeling the propeller structure of B1-A/B and B7-C/D or how the circular closure of the propeller in RPGR is achieved. There is strong homology in the propeller shaft \(\beta\)-sheet region, in particular the invariant glycine residues seen in each propeller of RCC1 are conserved in RPGR, which provides reassurance about the integrity of our model in this region. The probable errors or variations in the model are in the loop regions connecting the propellers due to insertions and deletions between the template and model. Another concern which needs to be noted is that RCC1 has a non-variant \(cis\)-proline at the beginning of each \(\beta\)-sheet-D in each of the seven propellers, this proline is not conserved in B1 or B2 in RPGR and so it is possible this short \(\beta\)-sheet is not identical in RPGR in these two regions. The protruding \(\beta\)-wedge in RCC1, which is a structural determinant for the nucleotide release from the P-loop of Ran GTPase (Renault et al., 2001), is distorted in RPGR, because of non-homologous residues and more importantly the deletion of two residues, F146 and R147, whose interactions with the D182 and hydrophobic core of Ran GTPase, respectively, are critical to the stabilization of the loop (supplementary material Figs S1, S2A,B).

Then, we mapped structurally to the RHD of RPGR all RP-associated missense mutations (Breuer et al., 2002; Demirci et al., 2002; Iannaccone et al., 2003; Sharon et al., 2003; Vervoort et al., 2000; Yang et al., 2002; Zito et al., 2003; Zito et al., 1999) (Fig. 1C) and the residues of RCC1 that make contact with Ran GTPase (Fig. 1C, supplementary material Fig. S2A–D). The predicted effect(s) of missense and disease mutations in RHD of RPGR were examined based on their nature and location in RHD or RCC1 structures (Fig. 1C, supplementary material Table S1) and the degree of conservation of the affected RHD residues in RPGR with RCC1 and several other RCC1-homologous proteins (Fig. 1D, supplementary material Fig. S3). This led to the grouping of the mutations in three main classes based on their predicted effects: i) class I comprises substitutions leading to the misfolding of a particular region/domain (e.g. G60V, G215V, G320R), ii) class II consists of mutations causing the destabilization of the tertiary structure due to the disruption of intramolecular interactions between blade structures/domains (e.g. H98Q, T99N, C250Y, D258S, C302Y) and iii) class III comprises mutations leading to the disruption of potential protein-protein interactions, because of their location in exposed loops (e.g. R127G, F130C, G173R, P235S, G275S, E285G, I289V). These mutations are localized throughout all six blades (B1–B6) (Fig. 1C, supplementary material Table S1). Notably, the majority of RP-associated missense mutations, in particular those of class III, form an exposed surface that is distinct from that made by the thirty residues interfacing with Ran GTPase in RCC1 and of which only two residues are conserved between RCC1 and RPGR (Fig. 1C,D, supplementary material Fig. S2C,D). These two residues are central at the top surface of the shaft of RPGR/RCC1, whereas the remaining outline a surface at the upper and outer boundaries of the shaft. Further, even when considering that parts of B1 and B7 are missing from the model, it is clear that there are significant differences in the electrostatic potential of the surfaces of RPGR and RCC1 (supplementary material Fig. S4). Hence, these analyses support that the residues mediating the interactions between RHD of RPGR and RPGRIP1\(x_1\), and a putative GTPase, are mutually exclusive.

**Disease-mutations differentially impair the direct interaction between RPGR and RPGRIP1**

The RPGRIP1\(x_1\) interacts with NPHP4 and the RHD of RPGR isoforms via two distinct domains, C2 and RID (Fig. 2A) (Roepman et al., 2000a; Roepman et al., 2005; Roepman et al., 2000b). To examine the functional effects of disease-causing mutations in \(\alpha\)IRP3 (Breuer et al., 2002; Demirci et al., 2002; Iannaccone et al., 2003; Sharon et al., 2003; Vervoort et al., 2000; Yang et al., 2002; Zito et al., 1999), we generated five classes of mutations in two constructs, N-RPGR and RPGR\(\alpha\)RF15. N-RPGR comprises the RHD and \(\delta\)-PDE-interacting domain (PID) shared by all RPGR isoforms, whereas RPGR\(\alpha\)RF15 is an isoform comprising N-RPGR and the unique ORF15 domain (Fig. 2B). Mutations of classes I, II and III, are predicted by molecular modeling to exert different roles in the RHD of RPGR (supplementary material Table S1). Class IV mutations are located in poorly characterized regions upstream of the RHD (e.g. F17S, G43R, G43E), whereas class V comprises a missense, native or artificial nonsense mutations in the PID region of N-RPGR. Finally, mutations of class VI comprise disease frame-shift mutations in the unique acidic ORF15 domain of the RPGR\(\alpha\)RF15 that cause its premature termination.

We employed quantitative yeast two-hybrid assays to analyze first the effects of the various classes of mutations in the interaction between RID of RPGRIP1\(x_1\) and wild-type or mutant constructs of N-RPGR (Fig. 2C,D) or the RPGR\(\alpha\)RF15 isoform (Fig. 2E). As shown in Fig. 2C, all mutations of classes I-IV
impaired significantly and differently the interaction between N-RPGR and RID of RPGRIP1 and the spectrum of the severity effects of mutations was relatively wide. For example, in class III mutations G173R had a strong effect while G275S had only a mild effect. G173R had the strongest effect among all classes of mutations analyzed. In fact, the G173R stands out clinically among all mutations examined because it is the only missense mutation known to be associated with syndromic RP, such as

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**Fig. 1. Molecular modeling of the RCC1-homologous domain (RHD) of RPGR and disease-associated mutations to RCC1.**

(A) Schematic diagram of RPGR isoforms, RPGR_{1–19} and RPGR_{ORF15}, and domains thereof. RPGR_{1–19} and RPGR_{ORF15} share seven N-terminal repeats, where most human missense disease mutations are localized. This region is homologous to RCC1 (RHD). The N-terminal repeats 1 and 7 of RHD are partially conserved with RCC1. RPGR_{1–19} and RPGR_{ORF15} have distinct C-terminal domains. Acidic (AD) and basic (BD) domains, nucleotide-binding (NB) and isoprenylation (CAAX) motifs, are shown. (B) Comparison of the ribbon diagram of the RHD model structure (left) to the seven propeller blades of RCC1 structure (middle) and superimposition of RHD and RCC1 structures (right). Blades are numbered B1–B7. Superimposed ribbons of RHD of RPGR and RCC1 are depicted in red and yellow, respectively. RHD of RPGR presents incomplete B1 and B7 blades and well-defined B2–B6 blades. The structures are axial views along the central shaft of the propeller structure. (C) Functional and colored ball representations of X-linked retinitis pigmentosa type 3 (XlRP3) mutations in RHD of RPGR. Left and right images are perpendicular and axial views of the central shaft of the propeller structure, respectively. Mutations are colored and mapped based on their predicted and functional effects on RHD (right table). Positions of green and white residues are those known to interface with Ran GTPase in RCC1 and among these, E116 and L268 are conserved between RHD and RCC1. Note that none of the XlRP3 mutations overlaps with the Ran GTPase-interacting interface and many, in particular those predicted to mediate protein–protein interactions, are in exposed loops. (D) Mapping of XlRP3-associated mutations to RHD by colored ball representation and based on the degree of conservation of the mutated residues with RCC1-homologous domains of other proteins. The position of poorly and semi-conserved residues tend to correlate with those predicted to mediate protein–protein interactions.
partial hearing loss and increased susceptibility to infections of the upper respiratory tract (Iannaccone et al., 2003; Iannaccone et al., 2004; Zito et al., 2003). G173 is also conserved among all RCC1 homologues (supplementary material Fig. S3). Class IV mutations located within the first 50 residues of N-RPGR (upstream the RHD) had also a significant effect. The V36F at the N-terminal nucleotide-binding region of RPGR severely impaired its interaction with RID of RPGRIP1α, and there was a significant difference between G43R and G43E, with the former causing a much weaker interaction of N-RPGR with RID. Finally, it is important to note that the truncation, Q236X, in N-RPGR, still retained some capacity to associate with RID of RPGRIP1α, supporting further that the interaction between the RHD of RPGR and RID of RPGRIP1 is multivalent.

Then we examined class V mutations comprising natural nonsense, missense and frame-shift mutations (e.g. R412X, G436D, 468RNQICX) in PID of N-RPGR, a region poorly conserved between N-RPGR and the 7th homologous repeat of RCC1 (Fig. 2D) and critical to the interaction of RPGR1_19 with δPDE (PrBP/δ) (supplementary material Fig. S5). No differences were found between the wild-type and mutant N-RPGR and also the splice variant, RPGRORF15A (Kirschner et al., 1999), on their ability to interact with RID of RPGRIP1α. Finally, we examined the effect of pathogenic frame-shift mutations of class VI in the ORF15 domain of RPGRORF15 (Breuer et al., 2002; Sharon et al., 2003) (Fig. 2E). The frame-shift mutations lead to truncated products of different lengths of RPGR ORF15. We found that the shorter the sequence of the ORF15 in RPGR ORF15, the stronger was the impairment of the interaction between RPGR ORF15 and RID of RPGRIP1α. These mutations define a new class of mutations, which are unique to RPGR ORF15 and exert an indirect effect on the interaction of RHD of RPGR ORF15 with RPGRIP1α.

Interestingly, these observations correlate positively with the spectrum of the severity of RP phenotypes reported in patients with hypomorphic mutations throughout ORF15 sequence (Sharon et al., 2003).
RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} determine distinct localizations of RPGRIP\textsubscript{1–19}

We had previously observed that expression of fluorescent tagged RPGRIP\textsubscript{1–19} alone produced RPGRIP\textsubscript{1–19} aggregates (unpublished results), a phenomenon that could be overcome by lowering the expression of RPGRIP\textsubscript{1–19} from constructs lacking polyadenylation signals to promote mRNA decay (Lu et al., 2005). Hence, we examined the roles of RPGR isoforms tagged at the N-terminal end with monomeric red fluorescent protein (mRFP) in determining the subcellular localization and aggregation of RPGRIP\textsubscript{1–19} tagged with yellow fluorescent protein (YFP) at its N-terminus and expressed from standard mammalian expression vectors. First, we assessed the subcellular localization of RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} when singly expressed. RPGR\textsubscript{1–19} was reported previously to localize to the Golgi apparatus with mannosidase II (Yan et al., 1998). However, we found that multiple markers of the Golgi, such as mannose-6-phosphate receptor, giantin and mannosidase II, did not show co-localization or co-distribution with RPGR\textsubscript{1–19} (supplementary material Fig. S6A). Then, we examined whether RPGR\textsubscript{1–19} localized to the endoplasmic reticulum (ER), because the CAAX motif of a number of proteins and shared by RPGR\textsubscript{1–19} is also known to target such proteins to endomembranes of the ER network (Apolloni et al., 2000; Choy et al., 1999; Michaelson et al., 2002). As shown in Fig. 3A (upper panel), RPGR\textsubscript{1–19} co-localized and was extensively co-distributed in live cells with the ER-tracker dye, ER-Tracker Green (glibenclamide Bodipy FL). Likewise, we found in fixed cells that RPGR\textsubscript{1–19} subcellular distribution paralleled extensively that observed for ER proteins with the KDEL-retention signal (Pelham, 1990), but less with calreticulin with which RPGR\textsubscript{1–19} interfaces at restricted domains of the ER (supplementary material Fig. S6B). Hence, RPGR\textsubscript{1–19} distribution overlaps extensively with the endomembrane system of the ER network, but not of the Golgi apparatus. In contrast to RPGR\textsubscript{1–19}, RPGR\textsubscript{ORF15} subcellular localization was instead dispersed throughout the cytoplasm in live and fixed cells and neither co-distributed nor co-localized with any ER and Golgi markers (Fig. 3A, lower panel; supplementary material Fig. S7A–B). In addition, we found no evidence of proteolytic processing of RPGR\textsubscript{ORF15}, because a RPGR\textsubscript{ORF15} construct containing mRFP and HSV tags at its N- and C-terminal ends, respectively, presented always co-localization of the tags (data not shown). To validate further the partition of RPGR\textsubscript{1–19} and RPGR\textsubscript{ORF15} between distinct subcellular compartments, we performed biochemical and subcellular fractionation of cytosolic, membrane, nuclear and cytoskeleton fractions of \( \sim 6 \) million COS7 cells transfected with RFP-RPGR\textsubscript{1–19}, RFP-RPGR\textsubscript{ORF15} or RFP alone. As shown in Fig. 3B, RFP-RPGR\textsubscript{1–19} was mostly present in the membrane fraction, whereas RFP-RPGR\textsubscript{ORF15} and RFP alone were present exclusively in the cytosolic fraction together with an unknown cytosolic protein marker cross-reacting with the RFP antibody. Hence, the distinct C-terminal domains of RPGR isoforms determine their unique subcellular partitioning and localization to the ER endomembrane network or cytosolic compartments.

Then, we determined the effects of co-expression of RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} with RPGRIP\textsubscript{1–19} on the subcellular targeting and localization of these proteins. As shown in Fig. 3C, expression of RPGRIP\textsubscript{1–19} alone leads to the genesis of profuse intracellular deposits of RPGRIP\textsubscript{1–19} throughout the cytoplasm. However, RPGRIP\textsubscript{1–19} co-expression with RPGR\textsubscript{1–19} did not alter the localization of RPGR\textsubscript{1–19} in the ER network, but it caused the targeting and retention of RPGRIP\textsubscript{1–19} to this network, where it always co-localized perfectly with RPGR\textsubscript{1–19} in all co-transfected cells (Fig. 3D, upper panel). In addition, co-expression of RPGR\textsubscript{1–19} with RPGRIP\textsubscript{1–19} prominently suppressed the formation of intracellular aggregates of RPGRIP\textsubscript{1–19} (Fig. 3D, upper panel). On the other hand, co-expression of RPGR\textsubscript{ORF15} with RPGRIP\textsubscript{1–19} did not affect the pan-intracellular distribution of RPGR\textsubscript{ORF15}, but it promoted a similar distribution of RPGRIP\textsubscript{1–19}, its broad and perfect co-localization with RPGR\textsubscript{ORF15} and complete suppression of formation of intracellular deposits of RPGRIP\textsubscript{1–19} (Fig. 3D, lower panel). Further, these RPGR-dependent effects were specific for the RPGR isoforms, because the expression of RFP alone from the same but empty RFP expression vector had no competing or non-specific expression effects on RPGRIP\textsubscript{1–19} localization and self-aggregation in kidney- and photoreceptor-derived cell lines, such as COS7 (supplementary material Fig. S8A) and 661W cells (supplementary material Fig. S8B). Hence, RPGRIP\textsubscript{1–19} has no effect on the localization of RPGR\textsubscript{1–19} and RPGR\textsubscript{ORF15}, whereas RPGR\textsubscript{1–19} and RPGR\textsubscript{ORF15} determine the targeting of RPGRIP\textsubscript{1–19} to distinct subcellular compartments.

Co-expression of mutant RPGR isoforms with wild-type RPGRIP\textsubscript{1–19} or vice-versa does not affect their subcellular co-localization in COS7 cells

The prior findings support that disease mutations impair to various degrees the direct interaction between RPGRIP\textsubscript{1–19} and RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} (Fig. 2C–E) and that each RPGR isoform critically determines distinct subcellular locations of RPGRIP\textsubscript{1–19} (Fig. 3D). Further, prior genetic and physiological evidence support the disease mutations affecting components of the RPGRIP1 interactome are expressed in cell-type and species-specific context-dependent fashions (e.g. photoreceptors and tubular kidney cells) (Ferreira, 2005; Mollet et al., 2002; Otto et al., 2002; Wiik et al., 2008; Won et al., 2011). Hence, we examined the effect(s) of disease mutations in the localization of RPGR isoforms upon single expression of these or the effects of co-expression of wild-type or mutant RPGR isoforms and RPGRIP\textsubscript{1–19} on the subcellular localization of the latter among mammalian cell lines of distinct tissue origins (Figs 4–7, supplementary Fig. S10). The usage of distinct cells lines allows the analysis of the effects of potential and inherent cell-type selective factors or differential expression levels of ectopic proteins in the subcellular co-localization and targeting of the RPGR-RPGRIP\textsubscript{1–19} complex and expression of mutations therein. We began first with the kidney cell line, COS7 cells, because this cell line lacks expression of RPGRIP1 and it presents good transfection efficiency and ectopic expression levels of proteins (supplementary material Fig. S9). These features allow subcellular and biochemical studies to be carried out in parallel. We first examined the role of RHD-PID, which contains only the N-terminal regions shared by RPGR\textsubscript{1–19} and RPGR\textsubscript{ORF15} (Fig. 4). Expression of RHD-PID (E463X) alone led to its pan-intracellular dispersion, while its co-expression with RPGRIP\textsubscript{1–19} led to a similar co-localization and subcellular pattern of RHD-PID with RPGRIP\textsubscript{1–19}. Then, we examined the effect(s) of mutations, G275S and G173R, in RPGR\textsubscript{1–19}, that were shown to impair the interaction of RHD with RID of RPGRIP\textsubscript{1–19}, with the latter having the strongest effect among all mutations examined (Fig. 2B), a feature that correlates directly
Fig. 3. Expression and subcellular localization of RFP-RPGR1–19, RFP-RPGR ORF15 and YFP-RPGRIP1α in COS7 cells. (A) RFP-RPGR1–19 is restricted to the ER network in live cells (upper panel). RFP-RPGR1–19 co-localizes with the ER-tracker dye and its subcellular co-distribution extensively overlaps with that of the ER dye (upper panel). RFP-RPGR ORF15 is dispersed throughout the cytoplasm and neither co-localizes nor co-distributes with the ER network of live cells stained with ER-tracker dye (lower panel). Inset pictures are an enlarged view of boxed areas. Scale bar, 13.3 μm. (B) Immunoblot of cytosolic (C), membrane (M), nuclear (N) and cytoskeleton (Ck) fractions of COS7 cells transfected with RFP-RPGR1–19, RFP-RPGR ORF15 or RFP alone. RFP-RPGR1–19 is mostly present in the membrane fraction with the ER marker, calreticulin, whereas RFP-RPGR ORF15 and RFP alone are exclusively present in the cytosolic fraction with the cytosolic markers, GADPH and a cytosolic protein (Ns Prot) which cross-reacts non-specifically with the anti-RFP antibody. RanBP2 (Nup358), calreticulin, acetylated α-tubulin and GADPH, are markers for the nuclear, ER, cytoskeleton and cytosolic fractions, respectively. IB, immunoblot. (C) Expression of YFP-RPGRIP1α alone leads to the formation of profuse intracellular deposits (inset picture represent other cells with aggregates of YFP-RPGRIP1α). Scale bar, 20 μm. (D) Co-expression of RFP-RPGR1–19 (upper panel) or RFP-RPGR ORF15 (lower panel) with YFP-RPGRIP1α in COS7 cells determines the RFP-RPGR isoform-dependent co-localization of YFP-RPGRIP1α to the ER network (upper panel) or throughout the cytoplasm (lower panel) and the perfect co-localization of either RFP-RPGR isoform with YFP-RPGRIP1α. Co-expression of either RFP-RPGR isoform with YFP-RPGRIP1α also suppresses completely the formation of intracellular deposits of YFP-RPGRIP1α. Inset pictures are enlarged views of boxed areas depicting the line scan used to plot two-channel fluorescent intensities graphs (right column, upper panel, 26.1 units=1 μm; right column, lower panel, 11.5 units=1 μm). The two-channel fluorescent intensity profile of either RFP-RPGR isoform and YFP-RPGRIP1α overlap perfectly. Scale bar: 26 μm, inset pictures, 6 μm. Images in (A), (C) and (D) are representative of cells examined.
with the broadest and strongest clinical expressivity of this mutation (Iannaccone et al., 2003; Iannaccone et al., 2004; Zito et al., 2003). As shown in Fig. 4, neither G275S nor G173R mutation in RPGR1-19 alone affected its localization to the ER, whereas their co-expression with RPGRIP1α led to the targeting of RPGRIP1α to such subcellular region, where it co-localized with either mutant RPGR1-19 protein. Finally, we probed the role of the mutations, G173R in RHD and E230fs in ORF15, of RPGRORF15 (Fig. 4). Again, expression of either of the mutant RPGRORF15 construct alone did not change the pan-intracellular distribution of mutant RPGRORF15, although the E230fs mutation promoted the formation of unique and large vesicular bodies containing RPGRORF15 throughout the cytoplasm of many but not all cells. Co-expression of E230fs mutant RPGRORF15 constructs with RPGRIP1α abolished the formation of the large RPGRORF15-vesicular bodies. In addition, both mutant RPGRORF15 constructs promoted the dispersion of RPGRIP1α throughout the cytoplasm and its co-localization with either mutant RPGRORF15 protein. Another prominent property shared by the mutant RPGR constructs examined was that they still retained the ability to suppress the formation of self-aggregates of RPGRIP1α.
We also examined the effects of two mutations, D1114G and ΔE1279, in the RID of RPGRIP1α1. The D1114G in RPGRIP1 causes LCA, while the role of ΔE1279 in disease is still ambiguous (Dryja et al., 2001; Gerber et al., 2001). Regardless, we have shown previously that D1114G completely suppresses the interaction of RID of RPGRIP1α1 with the RHD shared by the RPGR isoforms, whereas the ΔE1279 enhances such interaction (Lu et al., 2005). Moreover, these mutations affect the subcellular partitioning of RPGRIP1α1 when singly expressed at reduced levels in cells (Lu et al., 2005). Hence, we examined the effect of these mutations in the localization of RPGRIP1α1 upon co-expression with either RPGR1-19 and RPGRORF15 in COS7 cells. As shown in Fig. 5, the mutations, D1114G and ΔE1279 in RPGRIP1α1 neither affected its co-localization with RPGR1-19 or RPGRORF15, nor did they cause aggregation of mutant RPGRIP1α1 upon co-expression with either of the RPGR isoforms.

Cell-type and multivalent-dependent expression of disease mutations in RPGRIP1α1 and RPGR isoforms

The previous observations support that i) disease-causing mutations in either the RID of RPGRIP1α1 or RHD of RPGR isoforms impair the coupling of these domains in yeast cells (Lu et al., 2005) (Fig. 2), ii) the selective subcellular localization and suppression of aggregation of RPGRIP1α1 in COS7 cells critically depend on the co-expression of RPGR1-19 or RPGRORF15 (Fig. 3), and iii) yet disease-mutations, when singly expressed, apparently caused no disturbances in the subcellular co-localization of any mutant protein construct with its wild-type partner in COS7 cells (Figs 4, 5). Collectively, these observations together with the genetic data of tissue- and species-specific effects of mutations affecting components of the RPGRIP1 interactome led us to hypothesize that the interaction between RPGRIP1α1 and RPGR1-19 or RPGRORF15 is multivalent and aided by putative accessory protein(s) inherent

Fig. 5. Co-expression analyses of disease mutations in YFP-RPGRIP1α1 with wild-type RFP-RPGR1-19 or RFP-RPGRORF15 in COS7 cells. YFP-RPGRIP1α1 with disease-associated mutations (D1114G or EA1279) in its RID co-localizes with wild-type RFP-RPGR1-19 and RFP-RPGRORF15 isoforms and without forming intracellular deposits in COS7 cells. Images shown are representative of cells examined. Scale bar, 25 μm.
to COS7 cells that compensate for structural disturbances and mutational loads in RPGRIP1α₁, RPGR₁₋₁₉ or RPGRORF₁₅. Hence, a mutation in RPGRIP1α₁, RPGR₁₋₁₉ or RPGRORF₁₅ alone is not sufficient to uncouple completely and subcellularly RPGRIP1α₁ from each of the RPGR isoforms or their tethered assembly complex. However, we reasoned that an increase of the mutational load in the RPGRIP1α₁ complex by the concomitant expression of mutations in RPGRIP1α₁ and RPGR₁₋₁₉ or RPGRORF₁₅ might have a synergistic effect on disrupting the interaction between RPGRIP1α₁ and each of the RPGR isoforms and overcome any compensatory mechanisms caused by multivalent or combinatorial interactions between two or more components of the RPGRIP1 interactome. Indeed, co-expression of D1114G in RID of RPGRIP1α₁ and G173R in the RHD of either RPGR₁₋₁₉ (Fig. 6A–C) or RPGRORF₁₅ (Fig. 6D–F) led to widespread uncoupling of mutant RPGRIP1α₁ from either mutant.

Fig. 6. Co-expression analyses of disease mutations in YFP-RPGRIP1α₁ and RFP-RPGR₁₋₁₉ or RFP-RPGRORF₁₅ in COS7 cells. The G173R mutation in RFP-RPGR₁₋₁₉ (A–C) or RFP-RPGRORF₁₅ (D–F) co-expressed with the D1114G mutation in RID of YFP-RPGRIP1α₁ cause the subcellular uncoupling (delocalization) of mutant RFP-RPGR₁₋₁₉ or RFP-RPGRORF₁₅ from mutant YFP-RPGRIP1α₁ without grossly affecting the subcellular distribution pattern intrinsic to mutant RFP-RPGR₁₋₁₉ or RFP-RPGRORF₁₅ and the formation of intracellular deposits of mutant YFP-RPGRIP1α₁. Pictures in (B) and (E) are enlarged views of boxed areas in (A) and (D) depicting the delocalization of mutant RFP-RPGR₁₋₁₉ or RFP-RPGRORF₁₅ from mutant YFP-RPGRIP1α₁. (C) and (F) are plots of two-channel fluorescent intensities along the line scans of images (B) and (E), respectively. Scale bars, 13.3 μm; 2.6 μm for inset pictures. In fluorescent plots of (E) and (F), the distance of 56 units = 1 μm. Images shown are representative of cells examined.
RPGR isoform, even though the overall subcellular distribution of these proteins still remained largely unaffected and there were still a small fraction of cells (≤20%) with co-localization of the mutant constructs (data not shown). Although some clustering of mutant RPGRIP1\textsubscript{\textalpha} and RPGR\textsubscript{1–19} containing vesicles could be observed (Fig. 6), such attenuated clustering was clearly distinct from that of large and profuse aggregates of RPGRIP1\textsubscript{\textalpha} when it was expressed alone (Fig. 3C) or co-expressed with RFP alone (supplementary material Fig. S8A).

To support the notion further that the coupling of RPGR isoforms with RPGRIP1\textsubscript{\textalpha} was dependent on other unknown compensatory factor(s) with cell-type selective expression that contribute to the subcellular tethering, targeting and cell-type-dependent expression of mutations in components of the RPGRIP1\textsubscript{\textalpha} interactome, we probed the RPGR-dependent targeting of RPGRIP1\textsubscript{\textalpha} in two other distinct cell lines, 661W (cone photoreceptor line) and Hep3B (human hepatoma cell line). Like with COS7 cells, co-expression of RPGRIP1\textsubscript{\textalpha} with the RFP empty vector in 661W cells led to the formation of very discrete RPGRIP1\textsubscript{\textalpha} aggregates with pan-intracellular dispersion (supplementary material Fig. S8B), although self-aggregates of RPGRIP1\textsubscript{\textalpha} in 661W cells were always smaller than those in COS7 cells (supplementary material Fig. S8A,B). Remarkably and unlike COS7 cells, co-expression of G173R in RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} with wild-type or mutant RPGRIP1\textsubscript{\textalpha} in 661W cells suffices to promote the formation of intracellular aggregates of RPGRIP1\textsubscript{\textalpha} throughout all cells examined and its disassociation from either RPGR isoform, whereas co-expression of mutant RPGRIP1\textsubscript{\textalpha} with either wild-type RPGR isoform had no effect on their subcellular co-localizations (Fig. 7). Unlike COS7 cells, we could not identify any cells with co-localization of G173R in RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} with wild-type or mutant RPGRIP1\textsubscript{\textalpha}.

Even though the transfection efficiency is decreased the formation of the RPGRIP1-RPGR complexes, we performed quantitative analyses of triplicate reactions of similar co-transfections. Quantitative immunoprecipitations reactions shown in Fig. 8A. Quantitative analyses of co-immunoprecipitates of lysates of ~6 million cells transfected with wild-type and mutant RPGRIP1\textsubscript{\textalpha}, RPGR isoforms and endogenously expressed NPHP4, NPHP5 and SDCCAG8. As shown in Fig. 8A, immunoprecipitation of wild-type RPGR\textsubscript{1–19} and RPGR\textsubscript{ORF15} co-precipitates RPGRIP1\textsubscript{\textalpha}, but the association of RPGRIP1\textsubscript{\textalpha} with RPGR\textsubscript{ORF15} appears more robust than with RPGR\textsubscript{1–19}, an effect that may be caused by the stronger up-regulation of the expression of wild-type and mutant RPGRIP1\textsubscript{\textalpha} with RPGR\textsubscript{ORF15} than RPGR\textsubscript{1–19} (e.g. lanes 4, 6, 8, 10 vs lanes 3, 5, 7, 9). Mutations in either member of the complex significantly decreased the formation of the RPGRIP1\textsubscript{\textalpha}-RPGR complex, whereas the co-expression of D1114G in RPGRIP1\textsubscript{\textalpha} and G173R in either RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} strongly suppresses the tethering of the RPGRIP1\textsubscript{\textalpha}-RPGR complex. Interestingly, the G173R in RPGR\textsubscript{1–19} led to a decrease of its association with NPHP4 only when co-expressed with wild-type RPGRIP1\textsubscript{\textalpha} (e.g. lane 5 vs lane 3), whereas the G173R in RPGR\textsubscript{ORF15} led to such decrease only upon co-expression with mutant RPGRIP1\textsubscript{\textalpha} (lane 10 vs lanes 8 and 4). Finally, NPHP5 and SDCCAG8 were never tethered to the RPGRIP1 complex under any conditions (Fig. 8A).

To validate further the qualitative effects of mutations in the tethering of the RPGRIP1-RPGR complexes, we performed quantitative analyses of triplicate reactions of similar co-immunoprecipitations reactions shown in Fig. 8A. Quantitative analyses of co-immunoprecipitates of lysates of ~6 million cells transfected with wild-type and mutant RPGRIP1\textsubscript{\textalpha} and RPGR isoforms showed that double mutations in RPGRIP1\textsubscript{\textalpha} and either RPGR isoform had the strongest effect in uncoupling the tethering of the RPGRIP1-RPGR complex (Fig. 8B). In addition, mutations in either RPGR isoform had a stronger effect than mutations singly in RPGRIP1\textsubscript{\textalpha}. Hence, these observations independently validate the widespread subcellular uncoupling and physical untethering between RPGRIP1\textsubscript{\textalpha} and RPGR\textsubscript{1–19} or RPGR\textsubscript{ORF15} across cultures of co-transfected COS 7
Fig. 7. Mutations singly in RFP-RPGR1–19 or RFP-RPGRORF15 but not YFP-RPGRIP1α cause dissociation of YFP-RPGRIP1α1 from RFP-RPGR isoforms and the clustering of YFP-RPGRIP1α1 in a cone photoreceptor cell line (661W). Wild-type RFP-RPGR1–19 or RFP-RPGRORF15 co-localize with wild-type YFP-RPGRIP1α1 in 661W cells. The D1114G mutation alone in YFP-RPGRIP1α1 had no effect on its co-localization with wild-type RFP-RPGR1–19 or RFP-RPGRORF15, whereas the G173R mutation in RFP-RPGR1–19 or RFP-RPGRORF15 is sufficient to delocalize wild-type YFP-RPGRIP1α1 from either mutant RFP-RPGR isoform and promote the clustering of YFP-RPGRIP1α1 throughout the cytosol. Images shown are representative of cells examined. Scale bar, 26 μm.
cells when mutations are harbored concomitantly by both partners (Fig. 6). On the other hand, single mutations in either RPGR isoform or RPGRIP1α also weaken the physical tethering between these partners in COS 7 cells (Fig. 8B), even though uncoupling at subcellular level is not apparent (Figs 4, 5).

The RPGRORF15 isoform selectively protects the RID of RPGRIP1α from proteolytic processing

We have previously shown that RPGRIP1α undergoes limited proteolytic processing of its N-terminal end and nuclear-cytosolic partitioning of its N- and C-terminal domains when expressed alone and at low levels (Lu et al., 2005). In this study, we never

Fig. 8. Effects of disease mutations on the physical tethering of RFP-RPGR isoforms with YFP-RPGRIP1α, and limited proteolysis of YFP-RPGRIP1α.

(A) Qualitative analyses of co-immunoprecipitation assays with extracts of COS7 cells (~6 x 10^6) co-transfected with wild-type or mutant RFP-RPGR isoforms and YFP-RPGRIP1α to examine the effects of disease mutations on the physical association of RPGR isoforms and other partners with RPGRIP1α. Mutations singly in RPGR isoforms or RPGRIP1α weaken their physical association, whereas the presence of mutations in both RPGR isoforms and RPGRIP1α suppresses the physical association between these. The ectopically expressed wild-type and mutant RPGRIP1-RPGR complex also co-precipitate endogenous NPHP4, but not endogenous NPHP5 and SDCCAG8. Note that compared to RPGR1-19, RPGRORF15 enhances the expression levels of RPGRIP1α.

(B) Quantitative analyses of co-immunoprecipitation assays on extracts of ~6 x 10^6 COS7 cells co-transfected with wild-type and mutant RFP-RPGR isoforms and YFP-RPGRIP1α. Independent co-immunoprecipitation assays in triplicate (n=3) with mutant constructs were normalized against the amount of wild-type YFP-RPGRIP1α co-precipitated by either wild-type RFP-RPGR1-19 (black bars) or RFP-RPGRORF15 (gray bars). Double disease mutations in RPGR1-19 or RPGRORF15 and RPGRIP1α cause maximal untethering of these partners, whereas the G173R mutation singly in either RPGR1-19 or RPGRORF15 typically has a stronger effect than the D1114G mutation singly in RPGRIP1α. Data represent the mean ± S.D., n=3; * comparison of mutant constructs’ group with wild-type; #, comparison between single and double mutant constructs; *,# P<0.05 (Mann-Whitney test).

(C) The lack of C-terminal proteolytic processing of YFP-RPGRIP1α upon co-expression with RFP-RPGRORF15, but not RFP-RPGR1-19, supports RPGRORF15 protects RPGRIP1α from proteolytic cleavage, whereas mutations in RPGRORF15 destroy such protection (middle panel). Note that expression of YFP-RPGRIP1α alone also suppresses its limited proteolysis because of the formation of self-aggregates of YFP-RPGRIP1α in cell culture. In comparison to the wild type protein, G173R in RFP-RPGRORF15, but not RFP-RPGR1-19, induce a significant upward electrophoretic mobility shift and a reduction of its expression level (upper panel). Lower panel is an immunoblot with anti-Hsc70 (loading control).
observed the nuclear-cytosolic partitioning of RPGRIP1a1 in any cell line either upon its expression under a standard expression vector, which caused the sequestration of RPGRIP1a1 in profuse self-aggregates/deposits, or when co-expressed with RPGR1-19 or RPGRORF15 (Figs 3–7, supplementary Fig. S10). Hence, we examined whether the RPGR isoforms and mutations therein played a role in the limited proteolytic processing of RPGRIP1a1 upon co-expression of these in cell culture. We found that RPGRIP1a1 had a much lower susceptibility to undergo cleavage of its C-terminal domain (RID) when expressed alone or under co-expression with RPGRORF15 than when co-expressed with RPGR1-19 (Fig. 8C). This protection was impaired by the G173R and E230fs (and E463X) mutations in RPGRORF15, although the former mutation promoted also a decrease of the levels of mutant RPGRORF15 compared to the wild-type RPGRORF15, while no remarkable changes were observed between the wild-type and G173R mutation in RPGR1-19 (Fig. 8C). Notably, the G173R selectively reduces also the electrophoretic mobility of RPGRORF15 (as observed also in the co-immunoprecipitation assays; Fig. 8A) but not of RPGR1-19 (Fig. 8C). The reason for such change in electrophoretic mobility of mutant RPGRORF15 needs further investigation, but it may result from stable changes in secondary conformation, post-translation modification or both. Hence, these results support that self-aggregation of RPGRIP1a1, or its co-expression with the clinically relevant RPGR ORF15 isoform selectively protects the RID of RPGRIP1a1 from limited proteolytic cleavage, whereas the RPGR1-19 isoform or disease mutations in RPGRORF15 impair such protection.

Pre-existing RPGRIP1a1 deposits are cleared, recruited to and dispersed through the cytosolic compartment upon expression of RPGRORF15. Although it remains unknown whether disease-mutations in RPGR or RPGRIP1 promote deposits of RPGRIP1 in photoreceptor neurons, the propensity for formation of intracellular deposits by RPGRIP1a1 in the absence of RPGR expression across all cells examined in culture is a pathological feature, which is hallmark to many neurodegenerative diseases (Douglas and Dillin, 2010). Hence, we investigated whether RPGRORF15 was competent to promote the dispersion of long-lived and pre-existing intracellular deposits of RPGRIP1a1 formed as result of the absence of RPGRORF15 expression and then redirect RPGRIP1a1 to the RPGR-dependent targeting pathway after RPGRORF15 expression. To this effect, we carried out sequential transfection assays, in which COS7 cells were transfected first with RPGRIP1a1, cultured until the formation of visible intracellular deposits of RPGRIP1a1 and then the singly RPGRIP1a1 transfected cells underwent a second transfection with RPGRORF15 alone. The non-dividing cells were then monitored for about eighteen hours by time-lapse and live-cell imaging microscopy. As shown in Fig. 9 and supplementary material Movie 1, RPGRORF15 promoted the dispersion of deposits of RPGRIP1a1 as soon as after ~13 hr of RPGRORF15 transfection followed by strong bursts of widespread colocalization of RPGRORF15 with RPGRIP1a1. Strikingly, we also observed that the apparent phagocytosis at 1350 min of an atrophic cell, which was present since the beginning of the recording and singly expressed intracellular deposits of RPGRIP1a1, by a cell co-expressing RPGRORF15 and RPGRIP1a1, led rapidly to the redistribution and colocalization of RPGRIP1a1 with RPGRORF15. Hence, RPGRORF15 is capable of promoting the break-up of preformed intracellular deposits of RPGRIP1a1 followed by its recruitment and colocalization with RPGRORF15 regardless of the intracellular origin of the RPGRIP1a1 deposits.

To validate further that RPGRORF15 breaks-up pre-existing intracellular deposits of RPGRIP1a1, as observed by the time-lapse microscopy of live cells, we carried out in parallel time-course and biochemical subcellular fractionations of cytosolic and non-cytosolic fractions of cells expressing singly YFP-RPGRIP1a1 followed by co-expression of RFP-RPGRORF15 exactly under the same conditions described for live-cell imaging. Immunoblots of such subcellular fractions were performed at various time points (e.g. 0, 6 and 20 hours) after RPGRORF15 transfection (Fig. 9B, upper panel). The relative ratios of non-cytosolic to cytosolic of RPGRIP1a1 were then calculated upon their normalization to markers of the respective fractions from quadrupled independent experiments (Fig. 9B, lower panel). As shown in Fig. 9B, the majority of RPGRIP1a1 is present in the non-cytosolic fraction soon after RPGRORF15 transfection, whereas RPGRIP1a1 presence in the non-cytosolic fraction is strongly decreased after 20 hours of RPGRORF15 transfection. This is also reflected by a ~30-fold decrease of non-cytosolic to cytosolic ratios of RPGRIP1a1 between subcellular fractions at 6 and 20 hours post-transfection with RPGRORF15 (Fig. 9B). Hence, pre-existing intracellular deposits of RPGRIP1a1 accumulate in the non-cytosolic fraction, whereas they are shifted to the soluble cytosolic fraction upon co-expression of RPGRORF15 where they co-localize dynamically with RPGRIP1a1.

**Discussion**

This study uncovers novel structural, molecular and subcellular facets underpinning the functional interplay between two major RPGR isoforms, RPGR1-19 and RPGRORF15, and RPGRIP1a1, and the differential effects of disease mutations affecting RPGR isoforms and RPGRIP1 in the subcellular targeting of RPGRIP1 among distinct cell types. In addition, our study underscores the shared role of RPGR in the pathogenesis of etiologically distinct diseases, such as XIRP3 and LCA.

The modeling of RHD of RPGR and disease mutations therein to the RCC1 structure and the mutation analyses of the interaction between RID of RPGRIP1a1 and RHD of RPGR isoforms, provide novel insights into the interplay between critical residues and domains of RPGR isoforms and RPGRIP1a1. First, it is surprising that the G173R mutation confers the strongest impairment to the interaction between RPGRIP1a1 and RPGR, because this highly conserved residue in RCC1 is important to the formation of the structure of the β-wedge loop and hence to its interaction with Ran GTPase and triggering the nucleotide exchange reaction in the GTPase (Renault et al., 2001). This observation with the prediction that β-wedge loop is distorted in RPGR, because of the absence of two residues at F146 and R147 positions of RCC1, support that the structural integrity of this loop is critical not just to its interaction with a GTPase, but also to its association with RPGRIP1a1, either directly or indirectly and possibly via an evolutionary conserved GTPase. The other distinct feature of the G173R mutation is that it causes a prominent electrophoretic mobility shift in RPGRORF15, but not RPGR1-19, and its decreased stability that is reflected by the much lower expression level of the mutant versus the wild-type protein.
Fig. 9. Temporal dynamics of the dispersion of pre-existing intracellular deposits of YFP-RPGRIP1α1 upon expression of RFP-RPGR_ORF15 in COS7 cells. (A) Pre-existing YFP-RPGRIP1α1 deposits (arrows) were monitored by time-lapse imaging after subsequent expression of RFP-RPGR_ORF15. Time points represent still snapshots of a focused optical slice along the Z-axis captured from a time-lapse sequence and featuring significant events. Image capturing began 5 hours (300 min) after transfection of RFP-RPGR_ORF15 in COS7 cells expressing already YFP-RPGRIP1α1 for 16 hours. RFP-RPGR_ORF15 co-localizes with YFP-RPGRIP1α1 deposits (300–690 min). Then, aggregates of YFP-RPGRIP1α1 begin to diffuse and bursts of strong co-localization of YFP-RPGRIP1α1 with RFP-RPGR_ORF15 are visible during the rest of the 18.5 hour time-lapse experiment (810–1410 min). Note that an atrophic cell (arrowhead at 810, 1320 and 1350 min) with prominent YFP-RPGRIP1α1 deposits becomes phagocytized by another cell co-expressing RFP-RPGR_ORF15 and YFP-RPGRIP1α1 at 1350 min (e.g. arrowhead pointing to a restricted green area within the cell in yellow of the overlay image). This event leads to another strong burst of dispersed co-localization signal between YFP-RPGRIP1α1 and RFP-RPGR_ORF15 throughout the cell at 1410 min. All live images captured during the time-lapse imaging experiment were converted into a movie, which is presented in supplementary material Movie 1. Scale bar, 20 μm. (B) Time-course and biochemical partitioning of intracellular deposits of YFP-RPGRIP1α1 from non-cytosolic to cytosolic fractions upon expression of RFP-RPGR_ORF15 in COS7 cells with pre-existing aggregates of YFP-RPGRIP1α1. Non-cytosolic and cytosolic fractions of 1×10⁶ COS7 cells expressing YFP-RPGRIP1α1 for 16 hours and subsequently transfected with RFP-RPGR_ORF15 were analyzed at 0, 6 and 20 hours after transfection by quantitative (upper panel) and qualitative (lower panel) immunoblot analyses. The levels of YFP-RPGRIP1α1 decrease and increase in the non-cytosolic and cytosolic fractions, respectively, after 20 hr of expression of RFP-RPGR_ORF15. The ratio of non-cytosolic (NC) to cytosolic (C) of normalized levels of YFP-RPGRIP1α1 strongly decreases by ~30-fold after 20 hours of expression of RFP-RPGR_ORF15 (lower panel). Results shown represent the mean ± S.D. (n=4). *, comparison of 20 to 6 and 0 hours; ns (non-significant), comparison of 6 to 0 hours; P<0.05 is considered significant (Mann-Whitney test). A.U., arbitrary units.
Hence, the multifunctional effects of the G173R mutation may explain the unique clinical syndromic expressivity and penetrance of this mutation (Iannaccone et al., 2003; Iannaccone et al., 2004; Zito et al., 2003). Second, the data support that the RID of RPGRIP1Δ1 embraces the RHD of RPGR in a multivalent manner. For example, all class III mutations in the exposed loops and located throughout multiple blade motifs of the RHD of RPGR have a weaker effect than the G173R mutation in impairing the interaction of RHD of RPGR with RID of RPGRIP1Δ1. Likewise, N-RPGR with the truncation, Q236X, exhibits still some binding activity toward the RID of RPGRIP1Δ1 and such activity extends beyond the conserved RHD as supported by the partial loss of binding activity caused by class IV mutations, such as G43R and G43E. These data support that the RID physically interacts with RHD of RPGR around its propeller-like shaft and provides support to the notion that such interaction is required to stabilize or chaperone the propeller-like shaft of the RHD of RPGR that otherwise may compromise the multivalent interaction of RPGR with other unknown factors, such as a putative GTPase. The notion of a multivalent interface in RHD of RPGR gains further support also from genetic and physiological studies of mice with an in-frame deletion of exon 4 of Rpgr and structure-function studies on RCC1 combined with our modeling analyses of RHD of RPGR. Mice with exon 4 deleted present very mild and cell context-dependent phenotypes between cone and rod photoreceptors (Brunner et al., 2010). On the other hand, the top and bottom surfaces of the shaft of RCC1, whose residues or structural regions in RPGR do not interface with RPGRIP1 or any other putative partner, associate, respectively, with Ran GTPase and the chromatin-associated proteins, H2A and H2B histones (Nemergut et al., 2001). Third, class II mutations affecting intra-blade interactions of RHD indirectly impair its interaction with RID likely by destabilizing the predicted rigid structure of the RCC1-like propeller scaffold. Likewise, class VI mutations, which are unique to ORF15, gain further support also from genetic and physiological studies of mice with an in-frame deletion of exon 4 of Rpgr and structure-function studies on RCC1 combined with our modeling analyses of RHD of RPGR.

Our studies provide also multiple lines of evidence to support that RPGR1-19 and RPGR ORF15 play distinct and highly specific roles in the targeting and localization of RPGRIP1Δ1, whereas RPGRIP1Δ1 has no effect on the targeting and localization of either RPGR isoform to the ER network or pan-intracellular cytosolic distribution. These evidence are reflected by data showing: i) disease-causing mutations within and between distinct domains of RPGR isoforms and with known allelic heterogeneity differentially affect the strength of the direct interaction between RPGR and RPGRIP1 in yeast two-hybrid assays that directly correlate with the penetrance of non-syndromic and syndromic visual phenotypes; ii) RPGR1-19 or RPGR ORF15 are biochemically partitioned to membrane and cytosolic fractions, respectively; iii) RPGR1-19 or RPGR ORF15 determine specifically and distinctively the subcellular localization of RPGRIP1Δ1 and the RFP tags have no selective effect on the distinct subcellular localization of RPGRIP1Δ1; instead, the unique C-terminal domains of the RPGR isoforms are determinant to the subcellular localization of RPGRIP1Δ1; and iv) RPGR specifically reverses the formation of newly and pre-existing deposits (self-aggregates) of RPGRIP1Δ1 affecting its differential and RPGR-dependent partitioning between the cytosolic and non-cytosolic fractions; v) the expression of the mutation load in RPGR and RPGRIP1 are highly cell type-dependent with the photoreceptor cell line (661W) presenting the complete delocalization of RPGR from RPGRIP1Δ1 singly by the G173R mutation in RPGR among the three distinct cell lines employed, even though the G173R mutation alone weakened the tethering of RPGR to RPGRIP1Δ1, but not their co-localization, in COS7 cells; hence, the 661W cells phenocopy the pathophysiological expressivity of mutations in RPGR observed in the human. It is noteworthy that the break-up and dispersion of deposits of RPGRIP1Δ1 were always observed regardless of the co-expression of wild-type or mutant RPGR isoforms. This observation together with the apparent lack of an effect of the D1114G mutation in RPGRIP1 when singly expressed with either wild-type RPGR isoform in any cell line provides additional support that there are other critical accessory factor(s) expressed...
differentially among the three distinct cell types employed in this work and they are required for the plastic tethering or remodeling of the RPGRIP1 interactome. NPHP4 is clearly one of such accessory factors, because of its direct interaction with RPGRIP1 in a manner that is independent of the interaction of RPGRIP1 with the RPGR isoforms (e.g. Fig. 8A, lane 2). Other accessory components of the RPGRIP1 interactome may form higher order complexes, such as NPHP5, NPHP6 and SDCCAG8 (Chang et al., 2006; Otto et al., 2010; Otto et al., 2005), even though we found no evidence in these studies of their direct participation in the RPGRIP1-RPGR-NPHP4 complex.

The RPGR$_{1,19}$ isoform is reported to co-localize with α-mannosidase II in the Golgi apparatus (Yan et al., 1998). However, our data does not support such observation, because the anti-α-mannosidase II antibody employed by Yan et al. (Yan et al., 1998) produces in our hands non-specific staining (data not shown) and other Golgi markers, including a different anti-α-mannosidase II antibody, do not colocalize with RPGR$_{1,19}$. Instead, RPGR$_{1,19}$ distribution overlaps extensively with the ER network. On the other hand, constructs expressing only selective domains of RPGR$_{ORF15}$ were reported to present centriolar and basal body localizations (Shu et al., 2005). This observation is also contradictory to the data herein shown. This is most likely explained by the differential pan-intracellular distribution of the whole RPGR$_{ORF15}$ protein used in our studies versus the off-target localization of isolated domains of RPGR$_{ORF15}$ to centrioles and basal bodies or potential over-expression of partial constructs (Shu et al., 2005).

Our data supporting the determinant effect of RPGR isoforms in the subcellular localization of RPGRIP1$_{x1}$ is in apparent contradiction with reports of transgenic mice with endogenous expression of RPGRIP1 lacking the C2 and RID, but still retaining its upstream domains (Rpgrip$_{1tm1Tili}$) (Won et al., 2009; Zhao et al., 2003), and mice lacking the expression of Rpgr (Hong et al., 2000). Rpgrip$_{1tm1Tili}$ photoreceptors are reported to lack the localization of RPGR at the connecting cilium (Zhao et al., 2003), whereas the localization of RPGRIP1$_{x1}$ at the cilium of photoreceptors was not affected in Rpgr$^{-/-}$ (deletion of exons 4–6) (Hong et al., 2000). Likewise, mice with in-frame deletion of exon 4 of Rpgr present change in the localization neither of RPGRIP1$_{x1}$ nor of RPGR$_{ORF15}$ at the connecting cilium (Brunner et al., 2010). Since none of the transgenic mice reported apparently abolishes completely the expression of RPGR isoforms, such as of RPGR$_{ORF15}$, its expression may still suffice to the proper intracellular targeting of RPGRIP1$_{x1}$. Hence, the lack of RPGR localization to the cilium of Rpgrip$_{1tm1Tili}$ is surprising within the context of the work herein reported. Alternatively, the data suggest that other limiting factors, such as NPHP4, play combinatorial or compensatory roles in RPGR targeting, since RPGR associates with endogenous NPHP4 independently of the presence of disease mutations in RPGR (Fig. 8A). Regardless, recent work from our laboratory that the absence of RPGRIP1 expression in RPGRIP1$^/-$ mice promotes profound and differential physiological effects in the ciliary targeting of proteins between photoreceptor and kidney cells that are thought to partake in high-order assembly complexes with RPGRIP1 (PA Ferreira, N Tserentsoolod, A Saha, H Patil, Y Hao and M Webb, unpublished observations).

Collectively, a model emerges from our study whereby the presence of factors intrinsically unique to distinct cell types contribute determinately to the RPGR-dependent targeting of RPGRIP1$_{x1}$ or tethering of its complex (Fig. 10). In this regard, mutations either in RPGR of RPGRIP1$_{x1}$ are sufficient to uncouple these partners in yeast cells, because they lack mammalian or cell-type selective accessory factors required to modulate RPGR interaction with RPGRIP1$_{x1}$. In contrast, a single mutation in RHD of RPGR$_{1,19}$ or RPGR$_{ORF15}$, but not in RID of RPGRIP1$_{x1}$, suffices to promote the subcellular uncoupling of these proteins only in the 661W cone photoreceptor cell line. A disease mutation in RHD of RPGR$_{1,19}$ or RPGR$_{ORF15}$ alone, or RID of RPGRIP1$_{x1}$ alone, does not cause the subcellular uncoupling of RPGR from RPGRIP1$_{x1}$ in COS7 cells, but each alone impairs partially the physical tethering of RPGR with RPGRIP1$_{x1}$ and with the double mutations having a maximal effect in delocalizing subcellularly and un tethering RPGR from RPGRIP1$_{x1}$ in COS7 cells. Finally, Hep3B present additional compensatory factors to overcome concomitant mutations in any RPGR isoform and RPGRIP1$_{x1}$. Collectively, the ratio and interplay of yet unknown cell-type specific factors among cell types, and whose functions are differentially affected by the location of mutations throughout either RPGR or RPGRIP1$_{x1}$, may explain the strong difference in disease expression and progression between XIRP3 and LCA and other syndromic retinal phenotypes, such as the ocular-renal Senior-Løcken syndrome. Finally, our data support that complementary deficits in RPGR$_{1,19}$ or RPGR$_{ORF15}$ caused by mutations in their shared RHD domain may impair cumulatively the subcellular targeting of RPGRIP1$_{x1}$ and underlie the pathogenesis of XIRP3 even though human mutations were never found in the unique C-terminal domain of RPGR$_{1,19}$. However, it is possible that the RPGR$_{ORF15}$ isoform alone suffices to carry the subcellular targeting of RPGRIP1$_{x1}$ essential to cell function, since mutations in its unique ORF15 domain present clinical expression.

Our data demonstrate also that the C-terminal RPGR-interacting domain (RID) of RPGRIP1$_{x1}$ is protected from limited proteolysis by two independent mechanisms: RPGRIP1$_{x1}$ self-aggregation, when it is expressed alone, or upon its interaction with RPGR$_{ORF15}$, but not RPGR$_{1,19}$, when co-expressed. Regardless, either mechanism likely results in the sequestration of the RID of RPGRIP1$_{x1}$, thus protecting it from proteolysis, a biochemical feature which is compromised by the G173R mutation in RPGR$_{ORF15}$. Finally, the bursts of co-localization between RPGR$_{ORF15}$ and RPGRIP1$_{x1}$ captured by time-lapse microscopy support that their coupling is highly dynamic. Further, the role of RPGR$_{ORF15}$ in these coupling processes is crucial to suppress the formation of intracellular deposits of RPGRIP1$_{x1}$ or promote the mobilization and cytosolic dispersion of pre-existing RPGRIP1$_{x1}$ aggregates. Thus, this study defines a novel and essential RPGR- and RPGRIP1-dependent trafficking pathway with intrinsic structural and functional plasticity. The plasticity of this pathway is cell-context-dependent and to such effect other cell-type selective factors of the RPGRIP1$_{x1}$ interactome are likely to contribute critically to the spatial and temporal targeting of preassembled complexes from the ER network that are determinant to the morphogenesis of the outer segments of photoreceptors (Won et al., 2009). The impairment of components of the RPGRIP1$_{x1}$ interactome and the cell-context-dependent plasticity of its components provide a rationale for variations in the expressivity and penetrance of disease processes affecting
selectively photoreceptor neurons, epithelial tubular cells, or both.

Materials and Methods

Molecular Modeling

All molecular modeling was conducted using a stochastic global energy optimization procedure in Internal Coordinate Mechanics (ICM) using the ICM-Pro package version 3.7 (MolSoft LLC, San Diego, CA) (Abagyan et al., 2010; Abagyan et al., 1994). An alignment was generated between RPGR and RCC1 using an adaptation of the Needleman and Wunsch algorithm (Abagyan and Batalov, 1997; Needleman and Wunsch, 1970). The initial RPGR model was built based on the alignment and threading the sequence of RPGR onto the template crystal structure of RCC1 (PDB code: 1a12 subunit A (Renault et al., 1998)). The model was refined by globally optimizing the side-chains and annealing the backbone. The iterative refinement procedure contains three main steps: (i) random sampling of the dihedral angles according to the biased probability Monte Carlo method (Abagyan and Totrov, 1994), (ii) a local minimization step, (iii) the Metropolis criterion (Metropolis et al., 1953) is then used to accept or reject a conformation. Loop regions (residues 216 to 247 and 268 to 285) were modeled by searching a database of loop fragments and identifying loop templates with matching ends and homologous sequence followed by refinement. The final model was selected based on an ICM calculated energy profile (Abagyan and Totrov, 1994; Maiorov and Abagyan, 1998). ICM was used to calculate the electrostatic potential using the boundary element solution of the Poisson equation (Totrov and Abagyan, 2001). The electrostatic potential was projected onto the molecular surface using a color scale from red to blue based on calculated values of +/-5 kcal/electron units (+5 blue –5 red).

Cloning and mutagenesis

Cloning of human RPGR1–19, N-RPGR (N-terminal half of RPGR1–19), RPGR-C (C-terminal half of RPGR1–19) and bovine wild-type and mutant RPGRIP1c (Genbank Acc#: AF227258, bRPGRIP1) were previously described (Lu et al., 2005; Roepman et al., 2000; Roepman et al., 2000b). The ORFs of these clones were placed in a pDEST mammalian expression vectors (Invitrogen) when applicable. The cloning of RPGR ORF15 comprised a multistep process. The ORF15 sequence was isolated by a 3-step PCR procedure from genomic DNA isolated from blood of a healthy and young female volunteer. First two partially overlapping amplicons of ~400 bp and ~1.7 kb comprising the 5' and 3'-ends of ORF15 were isolated by PCR from human RPGR cDNA and genomic DNA, respectively. Then, the two amplicons were combined and mixed with primers complementary to the 5'- and 3'-ends of the ~400 bp and ~1.7 kb amplicons and a 2.1 kb amplipon was isolated by PCR. The 2.1 kb product comprises the sequences of the 400 bp and ~1.7 kb comprising the 5' and 3'-ends of ORF15 were sequenced by PCR from human RPGR cDNA and genomic DNA, respectively. Then, the two amplicons were combined and mixed with primers complementary to the 5'- and 3'-ends of the ~400 bp and ~1.7 kb amplicons and a 2.1 kb amplipon was isolated by PCR. The 2.1 kb product was subcloned into TOPO XL with the PCR cloning kit (Invitrogen). The ligation product contained the PpuMI at the 5'-end that was used to ligate into the N-RPGR sequence with the PpuMI site at its 3'-end in a pBD vector. The full-length RPGRORF15 clone obtained was used as template for PCR followed by subcloning into pDEST Directional TOPO and pDEST vectors (Invitrogen). Site-directed mutagenesis of RPGR isoforms was carried out by PCR with one pair of complementary primers containing the point mutation(s) and another pair flanking upstream and downstream the mutated primers. For frame shift mutations, primers were synthesized with the frame-shift mutations and incorporated into amplicons by employing wild-type constructs as templates. Deletion mutagenesis was carried out with pairs of primers against domains of interest. PCR products, mutations and clones were confirmed by DNA sequencing.

Yeast two-hybrid assays

Quantitative interactions between RPGR constructs and RID of RPGRIP1c21 were quantified by liquid growth assays in selective SD-dropout media without Leu, Trp and His as described in detail previously (Roepman et al., 2000; Roepman et al., 2000b). Briefly, the maximum specific growth ($\frac{\text{max}}{\text{max}}$) was determined by calculating $\mu_{\text{max}}=\frac{\ln(x_t)-\ln(x_0)}{t}$, where $x_t$ is the OD$_{600}$ of the culture at time $t$, $x_0$ is the OD$_{600}$ at time 0 and $t$ is the time between $x_0$ and $x_t$. The growth of three independent clones and three samples of each clone were averaged and the standard deviations calculated. Serial-dilution growth assays were carried out in SD-dropout agar plates without Leu, Trp and His. Two-tailed t-test statistical analysis was performed; $P<0.05$ was defined as significant.

Co-immunoprecipitations and Western blot analysis

For immunoprecipitation and immunoblot analysis, six million cells were harvested after 36 hours of transfection and reaching 100% confluency, washed with 1 x PBS and lysed in NP-40 immunoprecipitation buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, mini-complete protease inhibitor tablet (Roche) in 1 x PBS) after 30 minutes of incubation at 4°C, the cell lysates were passed through 21 gauge syringes and centrifuged for 20 minutes at 10,000 x g, 4°C. The supernatant was collected and used for co-immunoprecipitation assays. The NP-40

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**Fig. 10. Model of cell-context-dependent effects of XRIP3 and LCA mutations in the tethering of RPGR-RPGRIP1 complex.** In yeast cells, the lack of mammalian compensatory factors complementing the tethering of RPGR with RPGRIP1 causes the uncoupling of the RHD of either RPGR isoform (RPGR1–19 or RPGRORF15) from the RID of RPGRIP1 upon mutations affecting either domain of these proteins. In the 661W photoreceptor cell line, there is at least an accessory X factor, whose role compensates for the loss-of-function caused by the D1114G mutation in RPGRIP1, but not for the loss-of-function of G173R mutation in the RHD of either RPGR isoform. In COS7 cells, the association between RPGR isoforms and RPGRIP1 depends on two accessory factors, X and Y, whose roles compensate for the loss of function in either RPGR isoform or RPGRIP1, but not both. In Hep2B liver cells, concomitant mutations in RPGR and RPGRIP1 do not cause the uncoupling of these, because of the expression of a third (Z) accessory and tethering factor, which compensates for functional deficits in RPGR and RPGRIP1. Hence, differences in expression of accessory and compensatory cell-type selective factors of the RPGRIP1 interactome may underlie the clinical and subcellular expression of the mutational load affecting components of the RPGRIP1 interactome with the retina, kidney or both. Legend: red bar, G173R substitution in RHD of RPGR isoforms causing XRIP3 disease; blue bar, D1114G substitution in RID of RPGRIP1 causing LCA disease. Mutant complex refers to mutations scenarios in RPGR, RPGRIP1 or both, in various cell types. Outcome refers to the effect of mutations in RPGR, RPGRIP1 or both, in the untethering of components of the RPGRIP1 interactome in various cell types.
cell extracts of transiently transfected COS-7 cells were adjusted to 1 μg/ml in a 500 μl immunoprecipitation reaction assay and pre-cleared with non-immune IgG and protein-A/G agarose (Santa Cruz Biotechnology) for 1 hour at 4°C. Immunoprecipitations were performed with 2 μg rabbit-DsRed antibody (Clontech, Mountain View, CA) and 20 μl of 50% protein A/G agarose beads at 4°C for 8 hours. Beads were washed three times with NP-40 buffer and proteins were eluted with Laemmli buffer. Immunoprecipitated complexes were resolved on 7.5% SDS-PAGE and blotted on PVDF membrane. Membranes were probed with mouse-Ig-L (125 ng/ml) (Clontech, Mountain View, CA) antibodies against YFP, Ab938 against RID of RPGR1P1 (Roepman et al., 2000a), anti-NP-H4 (Roepman et al., 2005), anti-NP-H5 (Novus Biologicals, Littleton, CO) and anti-SDC-CAG8 (Protein Tech Group, Inc. Chicago, IL) (Otto et al., 2010). Then blots were incubated with horseradish peroxidase conjugated secondary antibody (25 ng/ml). The immunoreactive tagged proteins were visualized by incubating with enhanced chemiluminescence reagent according to ECL plus kit (Pierce) and exposed to X-ray Hyperfilm (Amersham Biosciences). Quantitative immunoprecipitation assays were performed by scanning immunoblots, integrated density values (idv) of immunoprecipitated and non-saturating bands were calculated with Metamorph v7.7 (Molecular Devices, Sunnyvale, CA) and corrected against the background of the same immunoprecipitated lanes and then compared with band values of each immunoprecipitation reaction with mutant constructs were normalized against the control reactions with wild-type constructs.

Cell culture, transfections and immunocytochemistry
COS-7 (ATCC), 661W (al-Ubaidi et al., 1992) and Hep3B2.1–7 (ATCC) cells were maintained in DMEM (Invitrogen, Carlsbad, CA, cat:no.: 11995) supplemented with 10% fetal (COS-7) or bovine (661W, Hep3B) calf serum (10%FBS), 100 units/ml penicillin (100 U/ml), 100 μg/ml streptomycin (100 μg/ml), and antibiotics (Invitrogen, Carlsbad, CA, cat.no.:15070063) at 37°C in a humidified 5% CO2 atmosphere. Upon 70% confluency, cells were transiently transfected with Lipofectamine-2000 (Invitrogen) and 500 ng of YFP-RPGR1P12, YFP-RPGR1P12 (D1114G), mRFP-RPGR1P12, mRFP-RPGR1P15, mRFP-RPGR1P15 (G173R), mRFP-RPGR1P15 (G173R), mRFP or YFP constructs. The mammalian expression and pDEST vectors were used to cloned in a N-terminal mRFP tag, pDEST-733 (mRFP-tag vector), or N-terminal-YFP tag, pDEST-733Y (YFP-tag vector), were used to subclone in-frame all ORFs of RPGR and RPGR1P1 constructs previously cloned to a pENTR vector (Invitrogen). Transfection efficiencies obtained with Cos-7 and Hep3B2.1–7, and 661W cells were ~50% and 5%, respectively.

Immunocytochemistry of cell cultures was performed about 24 hours after transfection by fixing cells at 70% confluency with 3.7% formaldehyde in DMEM for 10 minutes at 37°C, then wash twice with 1× PBS followed by sequential incubation with primary and Alexa Flour-conjugated secondary antibodies (Invitrogen) in 0.1 Triton X-100 and 1× PBS buffer. Cells were counterstained with DAPI and mounted with Fluoromount-G (Southern Biotech) prior to visualization. The primary antibodies were the following: anti-mannose-6-phosphate receptor was a gift of Nancy M. Dovan (Medical College of Wisconsin) anti-giantin (cat. no.: ab24586, Abcam, Cambridge, MA), anti-mannosidase II (clone 53FC3, cat. no.: MMS-110R, Convance Princeton, NJ [data not shown] and cat. no.: A122277, Abcam, Cambridge, MA) and anti-calreticulin (cat. no.: PA-3900, Thermo Scientific/Affinity Bioreagents, Rockford, IL), anti-KDEL (cat. no.: SPA-827, Enzo Life Sciences/Stein Research to Prevent Blindness Professor). Other antibodies were described previously. The authors have no financial conflict of interest.

Subcellular fractionation of cultured and transfected COS7 cells
Subcellular fractionation of RFP-RPGR1–19, RFP-RPGR1P15 or RFP alone was performed with six million COS7 cells singly transfected with 3 μg of RFP-RPGR1–19, RFP-RPGR1P15 or RFP alone in pDEST-733 vector. Thirty-six hours after transfection, cytotoxic, membrane, nuclear and cytoskeletal subcellular fractionations were collected with the Qproteome Cell Compartment kit exactly as described by the manufacturer instructions (Qiagen). All subcellular fractions were solubilized in SDS-sampler buffer, resolved by SDS-PAGE and immunoblots with antibodies against RFP and standard markers unique to each subcellular fraction were performed as described previously. Anti-GAPDH, anti-RanBP2/Nup358 and anti-acetylated-α-tubulin were from Santa Cruz Biotechnology, Inc., Covance and Sigma, respectively. Other antibodies were described previously.

For the time-course subcellular fractionation of intracellular deposits/aggregates of YFP-RPGR1P12 and its soluble form, 1 million cells were transfected with 500 ng of YFP-RPGR1P12 in pdest-733Y vector, cultured for 16 hours followed by co-transfection with 500 ng of YFP-RPGR1P15 and Nucleofector Kit R, Program W-001 (Lonza, Cologne, Germany) exactly as described in the live-cell imaging section of Material and Methods. Cytosolic and non-cytosolic fractions of co-transfected cells were prepared with the Qproteome Cell Compartment kit (Qiagen) at 0, 6 and 20 hrs post-co-transfection by lysing hypotonically transfected cells with CE1 buffer, collecting the supernatant (cytosolic fraction) and solubilizing the pellet in CE2 buffer for 30 minutes (non-cytosolic fraction). Cytosolic and non-cytosolic fractions were further solubilized in SDS-sample buffer before loading onto SDS-PAGE. Immunoblots were carried out as previously described. The partitioning of YFP-RPGR1P12 between the cytosolic and non-cytosolic fractions was quantified from immunoblots of independent experiments, the integrated density values (idv) of scanned bands were determined, YFP-RPGR1P12 in cytosolic and non-cytosolic fractions was normalized to GAPDH and RPGR1P1 expression, respectively, followed by the calculation of the ratios of the normalized levels of non-cytosolic to cytosolic YFP-RPGR1P12. Mann-Whitney statistical analysis was performed; P<0.05 was defined as significant.

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Disclosure
The authors have no financial conflict of interest.

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