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## Retinitis Pigmentosa GTPase Regulator (RPGR) protein isoforms in mammalian retina:

insights into X-linked Retinitis Pigmentosa and associated ciliopathies

Shirley He<sup>1</sup>, Sunil K. Parapuram<sup>1</sup>, Toby W. Hurd<sup>2</sup>, Babak Behnam<sup>1</sup>, Ben Margolis<sup>2</sup>, Anand Swaroop<sup>1,3</sup>, and Hemant Khanna<sup>1,\*</sup>

<sup>1</sup>Department of Ophthalmology and Visual Sciences, University of Michigan, Ann Arbor, MI-48105

<sup>2</sup>Department of Internal Medicine, University of Michigan, Ann Arbor, MI-48105

<sup>3</sup>Department of Human Genetics, University of Michigan, Ann Arbor, MI-48105

### Abstract

Mutations in the cilia-centrosomal protein Retinitis Pigmentosa GTPase Regulator (RPGR) are a frequent cause of retinal degeneration. The *RPGR* gene undergoes complex alternative splicing and encodes multiple protein isoforms. To elucidate the function of major RPGR isoforms (RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup>), we have generated isoform-specific antibodies and examined their expression and localization in the retina. Using sucrose-gradient centrifugation, immunofluorescence and co-immunoprecipitation methods, we show that RPGR isoforms localize to distinct sub-cellular compartments in mammalian photoreceptors and associate with a number of cilia-centrosomal proteins. The RCC1-like domain of RPGR, which is present in all major RPGR isoforms, is sufficient to target it to the cilia and centrosomes in cultured cells. Our findings indicate that multiple isoforms of RPGR may perform overlapping yet somewhat distinct transport-related functions in photoreceptors.

### Keywords

Cilia; Centrosomes; Photoreceptor; Ciliary transport; Rod Outer Segments

### INTRODUCTION

X-linked retinitis pigmentosa (XLRP) is a clinically and genetically heterogeneous disorder, characterized by early-onset visual symptoms, with night-blindness generally in the first decade and rapid progression towards blindness by age 40 (Bird, 1975; Fishman et al., 1988). Some XLRP patients also exhibit abnormal sperm phenotype or hearing defects (Hunter et al., 1988; Iannaccone et al., 2003; Zito et al., 2003). XLRP accounts for 10-20% of inherited retinal dystrophies (Breuer et al., 2002; Fishman, 1978; Jay, 1982). To date, six RP loci have been mapped on the X-chromosome: *RP2*, *RP3*, *RP6*, *RP23*, *RP24* and *RP34* (Fujita et al., 1996; Gieser et al., 1998; Mears et al., 2000; Meindl et al., 1996; Melamud et al., 2006; Schwahn et

\*Correspondence should be addressed to: Hemant Khanna, Ph.D., Department of Ophthalmology and Visual Sciences, W. K. Kellogg Eye Center, 1000 Wall Street, Ann Arbor, MI 48105, Ph: (734) 615-4354; Fax: (734) 647-0228, E. mail: hkhanna@umich.edu

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al., 1998; Shu et al., 2006; Wright et al., 1991) (<http://www.sph.uth.tmc.edu/Retnet>). The genes for *RP3* and *RP2* disease are Retinitis Pigmentosa GTPase Regulator (*RPGR*) and *RP2*, respectively (Meindl et al., 1996; Roepman et al., 1996; Schwahn et al., 1998).

Mutations in *RPGR* account for a majority of XLRP and over 25% of simplex RP (Breuer et al., 2002; Sharon et al., 2000; Shu et al., 2006; Vervoort and Wright, 2002). Some of the patients with *RPGR* mutations exhibit a syndromic phenotype, including respiratory tract infections, hearing loss, and primary cilia dyskinesia (Iannaccone et al., 2003; Koenekoop et al., 2003; Moore et al., 2006; van Dorp et al., 1992; Zito et al., 2003). RT-PCR studies have demonstrated complex alternative splicing patterns of the *RPGR* gene, with over 20 different variant mRNAs (Ferreira, 2005; Hong and Li, 2002; Kirschner et al., 1999; Neidhardt et al., 2007; Yan et al., 1998). All protein isoforms are predicted to include an amino-terminal domain (RCC1-like domain; RLD; encoded by exons 2-11) homologous to Regulator of Chromosome Condensation 1 (RCC1), which is a guanine nucleotide exchange factor for Ran-GTPase involved in nucleo-cytoplasmic transport (Meindl et al., 1996; Renault et al., 1999). However, no GTPase binding or activity has yet been associated with *RPGR* isoforms.

Two widely-expressed isoforms of *RPGR* are: *RPGR*<sup>ex 1-19</sup> (derived from exons 1-19, encoding a protein of 815 amino acids), which is detected in all cell types examined; and *RPGR*<sup>ORF15</sup> (exons 1 - part of intron 15), which has been associated with primary cilia (Khanna et al., 2005; Kirschner et al., 1999; Shu et al., 2005; Vervoort et al., 2000; Yan et al., 1998). Interestingly, mutations in exons 1-14 account for less than 25% of XLRP (Buraczynska et al., 1997; Fujita et al., 1997; Sharon et al., 2000). An additional 50-60% of XLRP patients reveal mutations in the terminal exon ORF15 of the *RPGR*<sup>ORF15</sup> isoform (Shu et al., 2006; Vervoort et al., 2000), which includes a C-terminal acidic domain rich in Glu-Gly repeats (EEEEGE repeat in mouse, EEEGEGEGE repeat in human) (Vervoort et al., 2000) and undergoes additional alternative splicing due to the presence of purine-rich exonic splicing enhancers (Hong and Li, 2002).

*RPGR* interacts directly with PDE6- $\delta$ , *RPGR*-interacting protein 1 (*RPGRIP1*), Structural Maintenance of Chromosomes (*SMC*) 1, *SMC3*, and nucleophosmin (Boylan and Wright, 2000; Hong et al., 2001; Khanna et al., 2005; Linari et al., 1999; Roepman et al., 2000; Shu et al., 2005). *RPGR* can be immunoprecipitated from retinal extracts with selected ciliary and microtubule-associated proteins, including motor proteins and intraflagellar transport polypeptide IFT88 (Khanna et al., 2005). *RPGR* also interacts with nephrocystin (*NPHP*) family of ciliary disease proteins, *NPHP5* and *CEP290/NPHP6*; mutations in these are associated with Senior-Loken Syndrome (*NPHP5*), Joubert Syndrome, Leber congenital amaurosis, and Meckel Syndrome (*CEP290/NPHP6*) (Baala et al., 2007; Brancati et al., 2007; Chang et al., 2006; den Hollander et al., 2006; Perrault et al., 2007; Sayer et al., 2006; Valente et al., 2006). All patients with *NPHP5* or *NPHP6/CEP290* mutations reveal a retinal disease phenotype. These observations point to a key role of *RPGR* in photoreceptor ciliary transport.

A *Rpgr*-knockout (*Rpgr*-ko) mouse, generated by deleting exons 4-6, exhibits a relatively slow retinal degeneration with apparent mis-localization of cone opsins (Hong et al., 2000). Expression of truncated *RPGR*<sup>ORF15</sup>-isoforms can be detected in the retina of this mutant mouse (Khanna et al., 2005). Notably, a truncated version of *RPGR*<sup>ORF15</sup> can act as a dominant gain of function mutant or rescue the phenotype of *Rpgr*-ko mouse (Hong et al., 2004; Hong et al., 2005). Two spontaneous canine mutants of *RPGR*<sup>ORF15</sup> - X-linked Progressive Retinal Atrophy (XLRA) 1 and XLRA2 - harbor different frameshift mutations and exhibit slow or rapid photoreceptor degeneration, respectively (Zhang et al., 2002). Different ORF15 mutations can therefore result in distinct phenotypes, probably depending upon the degree of alternative splicing and loss-of-function of *RPGR*.

RPGR was first localized to the connecting cilium of mammalian photoreceptors and to the transition zone of motile cilia (Hong et al., 2003). Further studies reported species-specific localization of RPGR to the connecting cilium (mouse) or photoreceptor outer segments (human and bovine) (Mavlyutov et al., 2002). By immunogold microscopy, we demonstrated RPGR<sup>ORF15</sup> isoforms predominantly in the cilia and basal bodies of human and mouse photoreceptors; however, additional staining in the outer and inner segments is detectable (Khanna et al., 2005). RPGR is also expressed in motile cilia of mouse trachea, human and monkey cochlea, and localizes to centrosomes and primary cilia of renal epithelial cells (Hong et al., 2003; Iannaccone et al., 2003; Khanna et al., 2005; Shu et al., 2005).

A clearer understanding of sub-cellular localization and physiological relevance of RPGR isoforms is critical for elucidating the mechanism of retinal disease caused by RPGR defects. Here, we show that RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> isoforms are present as isotypes, which localize to distinct subcellular compartments in the retinal photoreceptors. We also demonstrate that, like RPGR<sup>ORF15</sup>, RPGR<sup>1-19</sup> also associates with microtubule-based protein assemblies and ciliary components in the retina. We propose that distinct RPGR isoforms mediate overlapping functions in regulating photoreceptor protein transport by associating with discrete macromolecular complexes.

## MATERIALS AND METHODS

All animal experiments were performed according to the protocols approved by the University Committee on Use and Care of Animals (UCUCA).

### Antibodies and constructs

We generated and characterized several isoform-specific antibodies against RPGR (Figure 1A). The ORF15<sup>CP</sup> and ORF15<sup>C2</sup> antibodies were raised against carboxyl-terminal epitopes, <sup>1100</sup>HKTYQKKSVTNTQNGKE<sup>1117</sup> and <sup>1097</sup>YGKHKTQKKS<sup>1107</sup>, respectively, of the human RPGR-ORF15 protein. The CT-15 antibody was generated against the peptide sequence <sup>1131</sup>KNGPSGSKKFWNNILPHYLELK<sup>1152</sup> of human RPGR<sup>ORF15</sup>. Antibody common to both RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> isoforms, GR-P1 was raised against the peptide sequence <sup>18</sup>GKSKFAENNPQKFWFK<sup>33</sup> encoded by exon 1 of human RPGR. The RPGR-E19 antibody was against amino acid sequence <sup>982</sup>NLQDSTTPNMEGKS<sup>995</sup>, encoded by RPGR exon 19 (Invitrogen). Characterization of the ORF15<sup>CP</sup>, GR-P1, CT-15 and ORF15<sup>C2</sup> antibodies has been described (Khanna et al., 2005; Otto et al., 2005; Shu et al., 2005; Yan et al., 1998). Antibodies against acetylated  $\alpha$ -tubulin, SMC1, SMC3,  $\gamma$ -tubulin, and p50-dynamitin were procured from Chemicon (Temeculla, CA); anti-p150<sup>Glued</sup> and anti-KIF3A were obtained from BD Transduction Labs (San Jose, CA) and Sigma, respectively. Anti-RP1 antibody was a generous gift of Dr. Eric A. Pierce; Anti-IFT88 (Polaris) was provided by Dr. Bradley Yoder and anti-RPGRIP1 and ORF15<sup>C2</sup> antibodies were a gift of Dr. Alan Wright. To generate the FLAG-RLD construct, PCR was performed to amplify the region of human RPGR encoding residues 34 to 372 of RPGR. This amplicon was ligated in-frame into the HindIII and BamHI sites of p3xFLAG-CMV-10 (Sigma).

### Retinal fractionation

Frozen bovine retinas were thawed in 5 volumes (w/v) of a buffer containing 100 mM Tris-HCl buffer (pH 7.4), 0.25 M sucrose, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol and Complete protease inhibitor cocktail (Roche), and homogenized using a Dounce homogenizer. Homogenates were fractionated by differential centrifugation. The membrane/nuclei (800 × g, 10 min) and microsomal (100,000 × g, 60 min) fractions were washed with and suspended in the buffer described above. The supernatant obtained after centrifugation at 100,000 × g for 60 min was used as the cytosol fraction.

Cilia-enriched fractions were prepared according to (Fleischman, 1982), with minor modifications. Briefly, frozen bovine retinas were thawed in Buffer A (10 mM Pipes, 5mM MgCl<sub>2</sub>, pH.7.0) containing 50% sucrose (w/w) with 0.1mM of PMSF and protease inhibitors and swirled on ice for 30 min. RIS-ROS were separated from the remainder of the retina by centrifuge at 900 × g for 10 min, followed by centrifugation for 1 hr at 13000 × g. Crude RIS-ROS were collected from the top of the sucrose, injected at the bottom of a linear sucrose gradient (25-45%) in Buffer A, and centrifuged for 1 hr at 13000 × g. Purified RIS-ROS were collected from the top half of the gradient. The purified RIS-ROS were diluted with Buffer A, followed by centrifugation for 1 hr on a cushion of Buffer A with 50% (w/w) sucrose and collected at the interface. The fraction was extracted for 1 hour by adding an equal volume of Buffer A/1mM DTT/2% Triton X-100/protease inhibitors. The detergent soluble and insoluble materials were separated by centrifugation for 1 hr at 13000 × g, and the supernatant was saved as soluble fraction. Insoluble cilia-enriched fractions were suspended in Buffer A/1mM DTT/2% Triton X-100/protease inhibitors and sonicated.

### Retinal dissociation, Cell Culture, and Immunolocalization

Mouse retina was fixed in Histochoice (Amersco, Inc.) according to manufacturer's instructions. The sections were blocked in 0.5% Triton X-100 and 5% bovine serum albumin in PBS and labeled with ORF15<sup>CP</sup>, RPGR-E19, or acetylated  $\alpha$ -tubulin antibodies followed by fluorescent-labeled secondary antibodies.

For retinal dissociation, adult mouse retina was cut into small pieces and incubated for 10 min in phosphate-buffered saline (PBS) containing papain activated with L-cysteine. The pieces of retina were then gently triturated with a pipette and a sample checked under microscope for identifying single cells. Serum (10%)-containing DMEM was added to stop the reaction. Dissociated photoreceptors were put on slides and fixed with 4% paraformaldehyde. After 30 min, slides were washed with PBS and used for immunocytochemistry.

HeLa and MDCK II cells were propagated in DMEM plus 10%FBS, supplemented with Penicillin, Streptomycin and L-Glutamine. For cell synchronization, HeLa cells were growth-arrested by incubating with serum-free DMEM for 48 hours and fixed for immunocytochemistry. MDCK II cells stably expressing FLAG-RLD were selected in media containing 600  $\mu$ g/ml G418 (Invitrogen). For immunostaining cilia, MDCKII and FLAG-RLD MDCKII cells were seeded onto transwell filters (Corning) and grown for 7 days post-confluence. Cells were fixed in 4% PFA/PBS followed by permeabilization in 0.1% TritonX100. Cells were blocked and immuno-stained in 2% goat serum/PBS with indicated antibodies. Alexa-labeled secondary antibodies were obtained from Invitrogen. Cells were mounted using ProLong Gold reagent (Invitrogen).

### Co-immunoprecipitation (Co-IP) assay

The details of co-IP experiments have been described earlier (Khanna et al., 2005).

## RESULTS

### Characterization of the isoform-specific RPGR antibodies

Our previous studies using GR-P1 (Khanna et al., 2005; Yan et al., 1998), CT-15, ORF15<sup>CP</sup> (Khanna et al., 2005) and ORF15<sup>C2</sup> (Shu et al., 2005) have demonstrated the existence of multiple isotypes of RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> isoforms in mammalian retina. Whereas GR-P1 and HR1 antibodies detected immunoreactive bands at 90 and 250 kDa, CT-15, ORF15<sup>CP</sup>, and ORF15<sup>C2</sup> detected multiple bands in the molecular mass range of 100-250 kDa (data not shown; (Khanna et al., 2005; Otto et al., 2005; Shu et al., 2005). To specifically analyze the RPGR<sup>1-19</sup> isoform, we generated the RPGR-E19 antibody against an epitope in exon 19.

The RPGR-E19 antibody recognizes Xpress-tagged *Rpgr* exon 16-19-derived protein in transiently-transfected COS-7 cells (Figure 1B), and RPGR<sup>Ex1-19</sup> isoforms in mouse retina (Figure 1C). Additional protein bands of higher molecular weight probably indicate post-translational modifications or alternative isoforms. Pre-immune serum did not detect a signal.

For subsequent studies, we selected ORF15<sup>CP</sup> and RPGR-E19 antibodies to differentiate between the two primary RPGR isoforms. Pre-incubation of the antibodies with specific peptide but not non-specific peptide eliminated the immuno-reactive signal for ORF15<sup>CP</sup> antibody in our immunoblots analyses (data not shown; (Otto et al., 2005)).

### RPGR isoforms in different species

We examined the expression of different RPGR<sup>ORF15</sup> and RPGR<sup>1-19</sup> isoforms in human, bovine, and mouse retinas. The ORF15<sup>CP</sup> antibody detected bands at 100, 120, and 140 kDa (Figure 2; labeled as isoforms RPGR<sup>ORF15</sup>-1, 2, & 3, respectively). Higher molecular weight bands of 240-250 kDa (RPGR<sup>ORF15</sup>-4 & 5) were also observed in the retinal homogenates. The expected apparent molecular weight of the full-length RPGR<sup>ORF15</sup> isoform is ~140 kDa (Vervoort et al., 2000); however, due to the highly acidic carboxyl-terminal region, it may migrate at an aberrant rate. The expression of RPGR<sup>ORF15</sup>-3 isoform is not consistently detected in these experiments, indicating that this isoform is either unstable, expressed at very low levels, or post-translationally modified. Isoforms RPGR<sup>ORF15</sup>-1 & 2 may represent proteolytically processed fragments of RPGR<sup>ORF15</sup>.

The RPGR-E19 antibody detected bands of 90 (RPGR<sup>1-19</sup>-1), 110 (RPGR<sup>1-19</sup>-2), 140 (RPGR<sup>1-19</sup>-3), 150 (RPGR<sup>1-19</sup>-4), and 160 kDa (RPGR<sup>1-19</sup>-5), while the expected molecular mass of the RPGR<sup>1-19</sup> isoform is ~90 kDa (Yan et al., 1998). The expression of RPGR<sup>1-19</sup>-2 was not consistently detectable in retinal homogenates; however, upon fractionation it is enriched in the cytosolic compartment (see Figure 3). Some of the bands observed with these antibodies may represent post-translationally modified isotypes or alternatively spliced isoforms. Notably, less expression of both RPGR isoforms is observed in mouse as compared to human and bovine retinal extracts.

### Compartmentalization of RPGR isoforms in bovine retina

We next examined the sub-cellular distribution of RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> isoforms in bovine retina. Sucrose-gradient fractionation, followed by immunoblot analysis, revealed the presence of RPGR<sup>ORF15</sup>-4 & 5 isoforms in the nuclear and ciliary compartments and of RPGR<sup>ORF15</sup>-2 in the cytosolic fraction. RPGR<sup>ORF15</sup>-4 and RPGR<sup>ORF15</sup>-5 were detected in considerably lower amounts in microsomes and excluded from the rod outer segment (ROS) fraction (Figure 3).

Analysis of retinal fractions shows that the RPGR<sup>1-19</sup>-5 isoform is distributed in all fractions except soluble ROS and ciliary compartments. RPGR<sup>1-19</sup>-3 does not seem to be enriched in any of the fractions tested, whereas RPGR<sup>1-19</sup>-1 is enriched in the cytosolic and microsomal fractions. We also observe the enrichment of a 250 kDa RPGR-E19 immuno-reactive band (RPGR<sup>1-19</sup>-5a) in the ciliary fraction (Figure 3).

### Localization of RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> in mouse retina

To validate the fractionation data, we performed immunohistochemistry of mouse retinal sections using the RPGR-E19 and ORF15<sup>CP</sup> antibodies. As shown in Figure 4A and B, RPGR<sup>ORF15</sup> and RPGR<sup>1-19</sup> localize to the inner segments of photoreceptors, with punctate staining in the outer and inner plexiform layers of the retina. Labeling at the junction between the inner and outer segment indicates ciliary localization. We confirmed this finding by co-staining with acetylated  $\alpha$ -tubulin, a ciliary marker. Consistent with this, staining of dissociated

photoreceptors of mouse retina also detected inner segment/connecting cilium staining for the RPGR<sup>ORF15</sup> and RPGR<sup>1-19</sup> isoforms (Figure 4C; data not shown).

### Cell-cycle dependent localization of RPGR to centrosomes

We previously demonstrated the localization of RPGR to centrosomes in cultured cells (Shu et al., 2005). Given the importance of centrosomes in regulating cell division and in recruiting components of microtubule-nucleation machinery (Badano et al., 2005; Doxsey et al., 2005), we performed a more detailed analysis of the localization of RPGR during different stages of cell division using synchronized He La cells. Immunocytochemical studies demonstrated that endogenous RPGR<sup>ORF15</sup> exhibits cell cycle-dependent localization in the nucleus and centrosomes (Figure 5A). During prophase, RPGR localization is predominant in the nucleus, whereas in other stages, it co-localizes with  $\gamma$ -tubulin at the centrosomes (spindle poles). We also detected RPGR at the midbody in telophase (cytokinesis; Figure 5B), a feature shared by several other core centrosomal proteins (Tsvetkov et al., 2003). In addition to centrosomes and midbody, staining of other cellular structures is also detected for RPGR<sup>ORF15</sup>.

### RPGR-RLD is sufficient for basal body/ciliary localization of RPGR

Given that disease-associated mutations in the RPGR-RLD are predicted to affect its function and that all RPGR isoforms contain this domain (Shu et al., 2006), we tested the possibility that the RLD is sufficient for localization to the functionally relevant compartments, basal bodies and cilia. Hence, we generated MDCKII cells stably expressing FLAG-tagged RPGR-RLD. Remarkably, the RLD localizes to the basal bodies/centrioles in polarized non-ciliated MDCK cells (Figure 5C; a-d), whereas after ciliogenesis it traffics to the primary cilia (Figure 5C; e-h). These results suggest that the RLD can assist in basal body/ciliary localization of RPGR and may partially explain the predominant localization of RPGR<sup>ORF15</sup> and RPGR<sup>1-19</sup> to the connecting cilium of photoreceptors. However, evidence for the requirement of RLD in the localization of RPGR to basal bodies and cilia merits further investigation.

### RPGR<sup>1-19</sup> interacting proteins in the retina

RPGR<sup>ORF15</sup> is shown to be a component of the multiprotein complexes involved in regulating the intraphotoreceptor protein transport (Khanna et al., 2005). To investigate functional overlap between the RPGR<sup>ORF15</sup> and RPGR<sup>1-19</sup> isoforms, we looked at the interaction of RPGR<sup>1-19</sup> with other proteins involved in intracellular trafficking. Immunoprecipitation (IP) of RPGR<sup>1-19</sup> from bovine retinal extract followed by immunoblotting revealed the presence of RPGRIP1, CEP290/NPHP6, SMC1,  $\gamma$ -tubulin, kinesin motor subunits (KIF3A and KAP3), and dynein-dynactin subunits (p150<sup>Glued</sup>, dynein intermediate chain (DIC), and p50-dynamitin) (Figure 6A). Reverse IP experiments demonstrated that RPGRIP1, CEP290/NPHP6, SMC1, p150<sup>Glued</sup>, p50-dynamitin, KIF3A, and KAP3 can pull-down RPGR<sup>1-19</sup> but SMC3,  $\gamma$ -tubulin, and DIC did not, likely due to relatively low abundance of RPGR<sup>1-19</sup> in these multi-protein complexes (Figure 6B). We did not detect association of RPGR<sup>1-19</sup> with RP1, Nephrocystin-5 (NPHP5), and IFT88 (Polaris). IP with the pre-immune serum did not detect a signal. Notably, RPGR<sup>ORF15</sup> interacts with NPHP5 and IFT88 (Khanna et al., 2005). Taken together, these results indicate that different RPGR isoforms may perform overlapping yet distinct roles in regulating photoreceptor protein trafficking.

## DISCUSSION

A majority of mammalian genes produce multiple RNA and protein isoforms, generating extensive diversity of cellular functions (Kapranov et al., 2007). Complex splicing patterns results in a large degree of heterogeneity of RPGR expression. Therefore, it is imperative to dissect the different protein isoforms of RPGR in the retina. We show that RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> are detected as multiple immunoreactive bands in mammalian retina and localize

to distinct subcellular compartments, likely contributing to the reported disparate localization of RPGR. Notably, these isoforms associate with different sets of proteins involved in microtubule transport in the retina.

Different isoforms generated by the RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> splice variants can occur due to post-translational modifications (such as phosphorylation, prenylation, or proteolytic processing) or additional splicing, as demonstrated with exon ORF15 (Hong and Li, 2002; Yan et al., 1998). Proteolytic processing has been demonstrated for RPGRIP1 and the different products differentially localize to subcellular compartments (Lu and Ferreira, 2005; Lu et al., 2005). Proteolysis of ciliary proteins, such as polycystin-1 and Gli transcription factors serves as a mechanism of transducing extracellular signals to cell interior (Haycraft et al., 2005; Low et al., 2006; Singla and Reiter, 2006). We previously demonstrated that nuclear proteins SMC1 and SMC3 localize to the photoreceptor connecting cilium and interact with RPGR-RLD (Khanna et al., 2005). Given that RPGR isoforms can be detected in the nuclear fractions, we hypothesize that RPGR undergoes proteolytic processing or other post-translational modifications, resulting in association with SMC1/3 complex and transport to the nucleus as a macromolecular complex.

As demonstrated earlier for RPGRIP1 (Lu and Ferreira, 2005), expression of multiple RPGR isoforms in the retina and their differential distribution merits the consideration of isoform ratios. It is plausible that some RPGR isoforms are enriched in specific species and cell-types, resulting in apparent differences in localization and functional compensation. Given that over-expression of a truncated RPGR<sup>ORF15</sup> isoform can result in a dominant gain-of-function effect (Hong et al., 2004) and that a splice-site mutation upstream exon 9a of RPGR, which is associated with RP, leads to high levels of exon 9a-containing transcripts (Neidhardt et al., 2007), abnormal enrichment of some isoforms over others may drastically change the RPGR isoform ratio and result in retinopathy.

Microsomes contain fragmented endoplasmic reticulum and are enriched in cytoskeletal proteins, metabolic enzymes, and transmembrane moieties (Han et al., 2001). We had previously demonstrated that RPGR<sup>1-19</sup> is prenylated at the carboxyl-terminus, a modification that assists in membrane association (Yan et al., 1998). Although the RPGR<sup>ORF15</sup> isoform(s) do not contain the isoprenylation site (CTIL) encoded by exon 19 (Yan et al., 1998), RPGR<sup>ORF15</sup> may interact with other proteins in order to localize to microsomes.

The association of RPGR with midbody and centrosomes indicates its involvement in regulating cell division. Midbody marks the intercellular bridge during the formation of cleavage furrow at telophase (Tsvetkov et al., 2003). It is possible that RPGR is involved in the assembly of proteins involved in the formation of the furrow by regulating microtubule-based membrane trafficking, a phenomenon critical for normal cytokinesis (Tsvetkov et al., 2003). Support of this hypothesis comes from a previous report demonstrating the localization of another cilia-centrosomal disease protein BBS6 at midbody (Kim et al., 2005). The precise role of RPGR in the midbody and centrosomes during cell division warrants additional studies.

In postmitotic cells, such as photoreceptors, the mother centriole of the centrosomes migrates to the apical membrane of cells and nucleates the assembly of cilia (Badano et al., 2005). A predominant ciliary localization of both RPGR<sup>ORF15</sup> and RPGR<sup>1-19</sup> isoform may be due to the ability of the RCC1-like domain of RPGR to localize to the primary cilia. The photoreceptor connecting cilium serves as a conduit for bidirectional inter-segmental transport of protein complexes along the microtubule network (Besharse et al., 2003; Young and Droz, 1968). The cargo vesicles undergo polarized post-Golgi trafficking and dock at the basal bodies to be transported along the connecting cilium. This vectorial transport depends upon the activity of small GTPases, including Rab8 (Deretic et al., 1995; Moritz et al., 2001). Localization of



RPGR<sup>1-19</sup> isoform to the Golgi (Yan et al., 1998) and other compartments in the inner segment (present study) may indicate a possible involvement of RPGR in post-Golgi sorting of cargo-containing vesicles for delivery to the base of the cilium. We previously showed that the carboxyl-terminal amino acids CTIL are involved in the localization of RPGR<sup>1-19</sup> to Golgi compartments in transfected cells (Yan et al., 1998). Given the involvement of Golgi in processing membrane proteins destined to the cilium (Cai et al., 1999) and the localization of IFT20 to Golgi (Follit et al., 2006), we postulate that RPGR<sup>1-19</sup> may be involved in sorting of membrane versus soluble proteins for transport towards the connecting cilium. Owing to a lack of disease-associated mutations in exons 16-19 of RPGR<sup>1-19</sup> isoform, this domain is either dispensable for RPGR function or mutations in this domain may lead to a lethal phenotype.

Protein trafficking via the connecting cilium is important for outer segment disc morphogenesis and renewal (Besharse et al., 2003; Young and Droz, 1968). Though basic components associated with the transport have been discovered (Rosenbaum, 2002; Rosenbaum et al., 1999), the mechanisms of cargo sorting and assembly of protein complexes, and their regulation by signaling pathways have not been elucidated. Unlike RPGR<sup>ORF15</sup>, RPGR<sup>1-19</sup> isoforms did not exhibit an association with ciliary proteins NPHP5 and IFT88 in the retina. We and others have shown that RPGR isoforms localize to the proximal region of photoreceptor connecting cilium, called the transition zone, and to basal bodies, which may act as a selection barrier for the proteins being transported distally to the axoneme and outer segments (Liu et al., 2007; Singla and Reiter, 2006). Moreover, RPGR<sup>ORF15</sup> and RPGR<sup>1-19</sup> do not associate with RP1, which localizes to the distal axoneme of photoreceptors (Liu et al., 2004). Taken together, we predict that different RPGR isoforms facilitate the assembly, selection, and transport of multiprotein cargo complexes by interacting with distinct ciliary - basal body - centrosome (CBC) proteins, and that RPGR function is necessary for maintaining efficient inter-segmental transport. Further studies will be necessary to understand the composition of the CBC macromolecular complexes in the retina and the connections made by distinct RPGR isoforms within these transport particles.

We propose that diversity of RPGR isoforms is a critical determinant of its function. However, the potential of functional redundancy between RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup> isoforms in the retina requires additional investigations aiming at delineation of the role of individual RPGR isoforms. It will also be necessary to differentiate between hypomorphic, loss-, and gain-of-function RPGR alleles to delineate the pathogenesis of photoreceptor degeneration.

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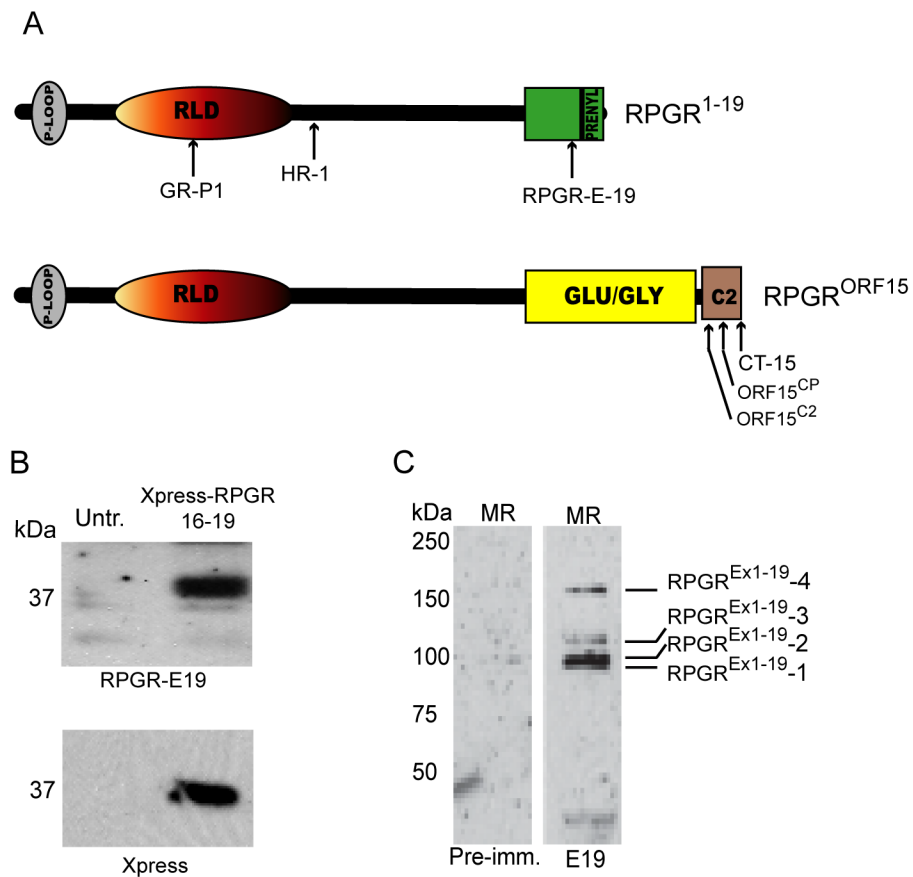
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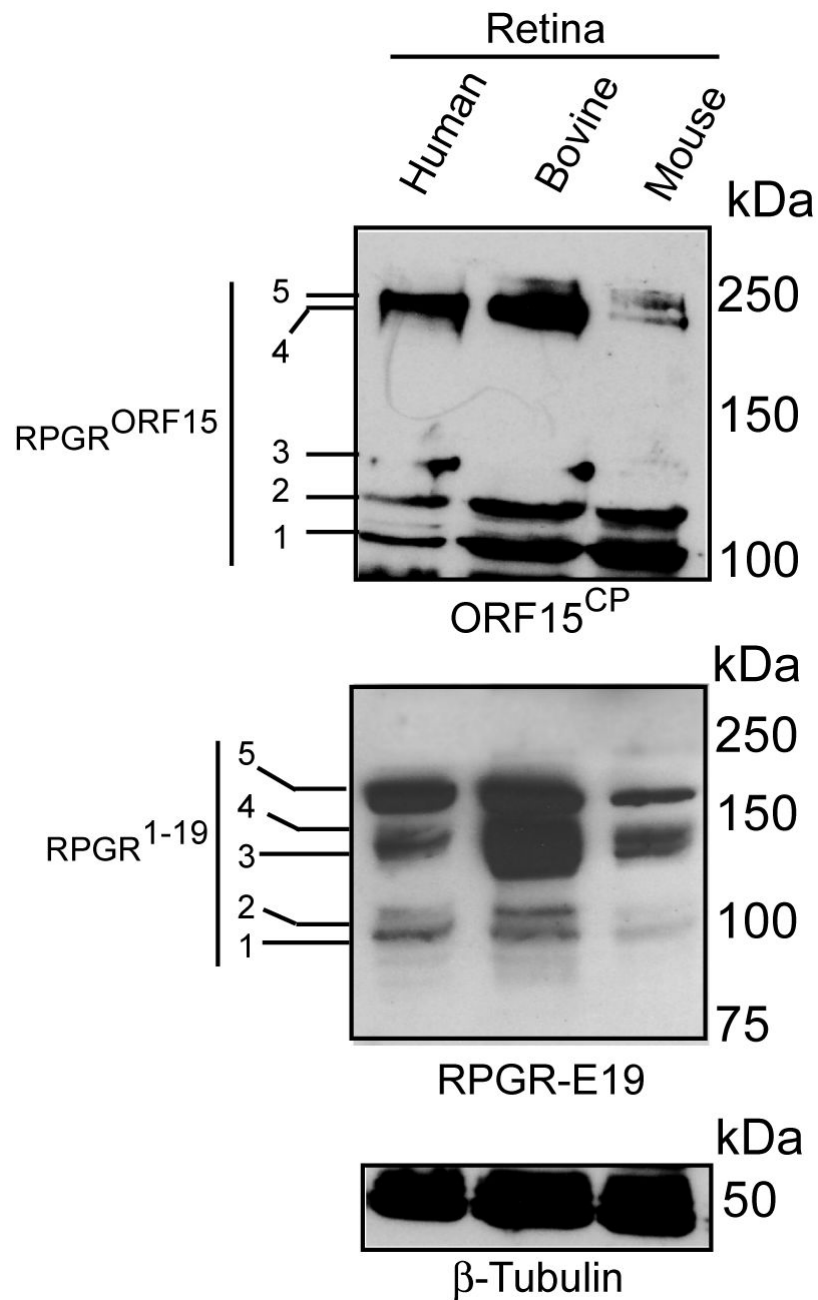
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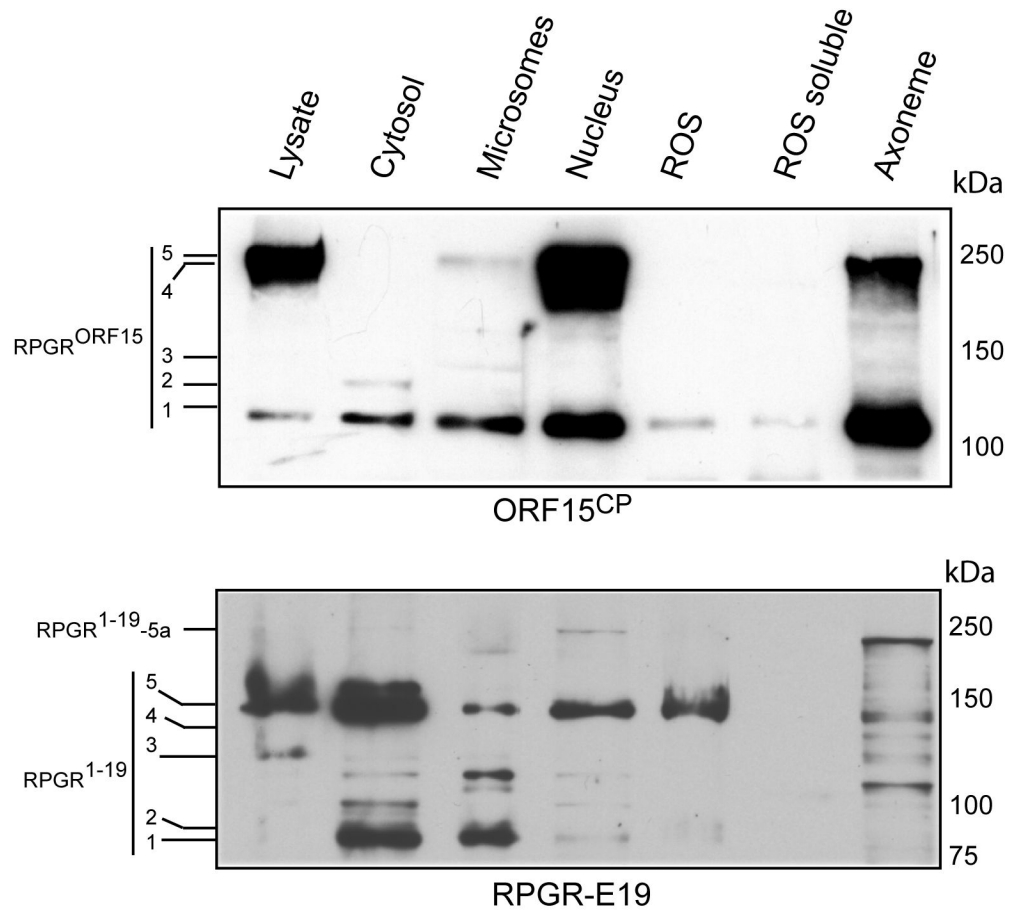


**Figure 1.**

**A.** Schematic representation of the major RPGR protein isoforms (constitutive RPGR<sup>1-19</sup> and RPGR<sup>ORF15</sup>) with distinct predicted domains. RPGR antibodies against peptides from different parts of the protein are represented by arrows. P-loop: ATP-GTP binding region; RLD: RCC1-like domain; Glu/Gly: Glutamic acid and Glycine rich domain; Prenyl: C-terminal isoprenylation site; C2: C-terminal basic domain. **B.** COS-7 cells were transiently transfected with pcDNA4A-Ex16-19. The cell extracts were analyzed by SDS-PAGE and IB using anti-Xpress or RPGR-E19 antibodies. **C:** Immunoblots of mouse retinal extract using pre-immune or RPGR-E19 anti-serum. Specific immunoreactive bands are indicated.



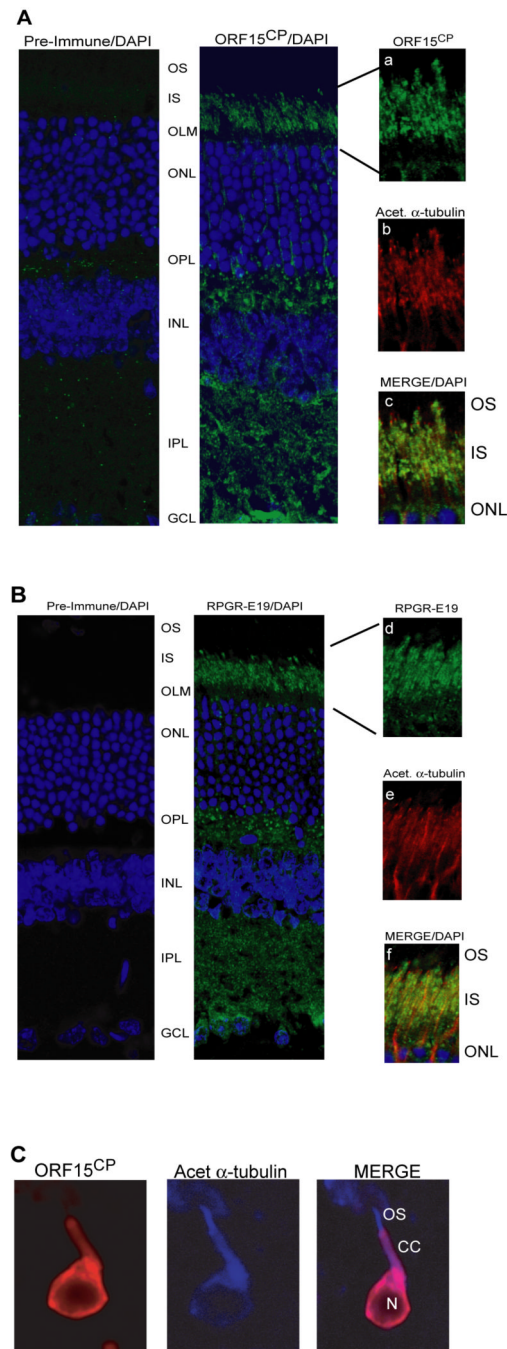
**Figure 2.** Immunoblot analysis of human, bovine, and mouse retinal homogenates using the ORF15<sup>CP</sup> and RPGR-E19 antibodies. Equal amount of protein (100  $\mu$ g) was analyzed by SDS-PAGE followed by immunoblotting using indicated antibodies.  $\beta$ -tubulin was used to assess loading control. Molecular mass markers are shown on the right and individual immunoreactive bands for different RPGR isoforms are depicted on the left.



**Figure 3.**

Bovine retinas were subjected to sucrose gradient centrifugation followed by immunoblotting using the ORF15<sup>CP</sup> and RPGR-E19 antibodies. Equal amount of protein (50  $\mu$ g) was analyzed by SDS-PAGE and immunoblotting. Purity of fractions was determined by enrichment of marker proteins (data not shown). Protein amount control was not used as different fractions may have enrichment of distinct proteins, including  $\beta$ -tubulin. Different RPGR isoforms are identified on the left and the molecular mass markers (in kDa) are denoted on the right.

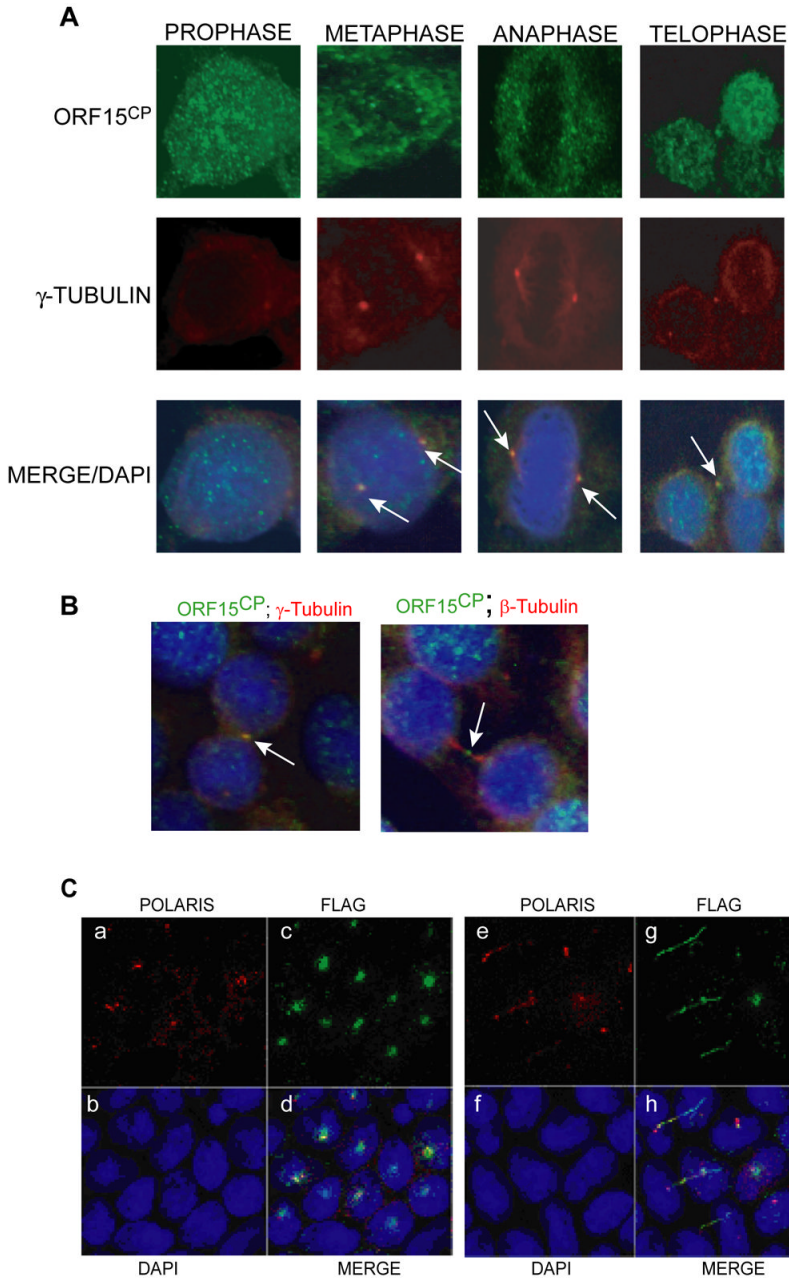




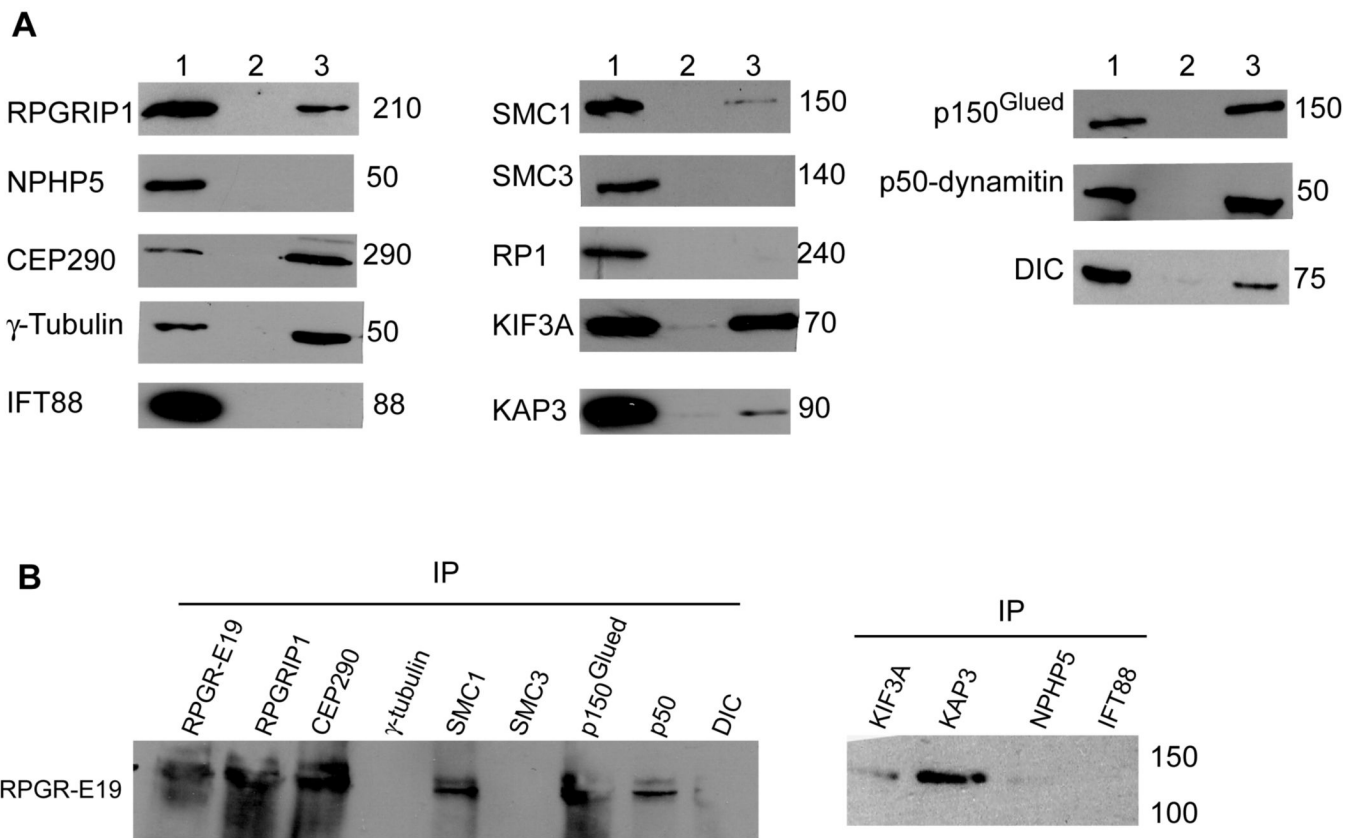
**Figure 4.**

Localization of RPGR isoforms in mouse retina. Immunohistochemical analysis of cryosections of adult wild-type mouse retina was performed using ORF15<sup>CP</sup> (**A**) or RPGR-E19 (**B**) antibodies (green). Enlarged image of the inner segment/connecting cilium indicates co-localization with acetylated (Acet.)  $\alpha$ -tubulin (red) (Merge; yellow). ORF15<sup>CP</sup>-specific staining is also observed in the Inner segment and proximal outer segment (**A**). DAPI was used to stain the nuclei (blue). OS: Outer segments; CC: connecting cilium; IS: inner segments; ONL: Outer nuclear layer; OLM: outer limiting membrane; OPL: outer plexiform layer; INL: Inner nuclear layer; GCL: ganglion cell layer. **C**. Immunocytochemistry using dissociated GFP-tagged rod photoreceptors from *Nrl*-GFP mouse retina (Akimoto et al., 2006).

ORF15<sup>CP</sup> antibody (red) co-localizes partially with acetylated  $\alpha$ -tubulin (blue) (Merge; pink) at the proximal connecting cilium (CC) and not in outer segment (OS). N: nucleus.



**Figure 5.**  
**A and B:** Cell cycle dependent localization of RPGR<sup>ORF15</sup>: Synchronized HeLa cells were fixed and analyzed by immunocytochemistry using ORF15<sup>CP</sup> (green) or  $\gamma$ -tubulin (red) antibodies. RPGR<sup>ORF15</sup> is detected in the nuclei at prophase and co-localizes with  $\gamma$ -tubulin at the centrosomes during cell division (**A**; arrows). Staining for other cellular structures is also detected with the ORF15<sup>CP</sup> antibody. DAPI was used to stain the nuclei (blue). RPGR<sup>ORF15</sup> also localizes to the midbody at telophase, depicted by co-localization with  $\gamma$ -tubulin and  $\beta$ -tubulin (**B**). **C.** Non-ciliated (a-d) and ciliated (e-h) MDCK cells stably expressing FLAG-tagged RPGR-RLD were stained with anti-FLAG (green) or anti-polaris (IFT88; red) antibodies. Nuclei were stained with DAPI. Merge image shows basal body (d) and ciliary (h) localization of RPGR-RLD prior to and after ciliogenesis, respectively.

**Figure 6.**

Co-immunoprecipitation (IP) of bovine retinal homogenates (200  $\mu$ g) using RPGR-E19 (A) was performed followed by SDS-PAGE and immunoblotting using indicated antibodies. Input represents 20% of the protein used for IP. Lanes are: 1, Input; 2, IP with pre-immune serum; 3, IP with RPGR-E19. B. Reverse IP using indicated antibodies was performed and proteins analyzed by SDS-PAGE and immunoblotting using the RPGR-E19 antibody.