Loss of RPGR glutamylation underlies the pathogenic mechanism of retinal dystrophy caused by *TTLL5* mutations

Xun Sun[a,1], James H. Park[b,1], Jessica Gumerson[a], Zhijian Wu[a], Anand Swaroop[a], Haohua Qian[c], Antonina Roll-Mecak[b,2], and Tiansen Li[a,2]

[a]Neurobiology Neurodegeneration & Repair Laboratory (N-NRL), National Eye Institute, Bethesda, MD 20892; [b]Cell Biology and Biophysics Unit, National Institute of Neurological Disorders and Stroke, Bethesda, MD 20892; and [c]Visual Function Core, National Eye Institute, Bethesda, MD 20892

Edited by Jeremy Nathans, Johns Hopkins University, Baltimore, MD, and approved April 8, 2016 (received for review November 27, 2015)

Mutations in the X-linked *retinitis pigmentosa GTPase regulator* (*RPGR*) gene are a major cause of retinitis pigmentosa, a blinding retinal disease resulting from photoreceptor degeneration. A photoreceptor specific ORF15 variant of RPGR (RPGR^{ORF15}), carrying multiple Glu-Gly tandem repeats and a C-terminal basic domain of unknown function, localizes to the connecting cilium where it is thought to regulate cargo trafficking. Here we show that tubulin tyrosine ligase like-5 (TTLL5) glutamylates RPGR^{ORF15} in its Glu-Gly–rich repetitive region containing motifs homologous to the α-tubulin C-terminal tail. The RPGR^{ORF15} C-terminal basic domain binds to the noncatalytic cofactor interaction domain unique to TTLL5 among TTLL family glutamylases and targets TTLL5 to glutamylate RPGR. Only TTLL5 and not other TTLL family glutamylases interacts with RPGR^{ORF15} when expressed transiently in cells. Consistent with this, a *Ttll5* mutant mouse displays a complete loss of RPGR glutamylation without marked changes in tubulin glutamylation levels. The *Ttll5* mutant mouse develops slow photoreceptor degeneration with early mislocalization of cone opsins, features resembling those of *Rpgr*-null mice. Moreover TTLL5 disease mutants that cause human retinal dystrophy show impaired glutamylation of RPGR^{ORF15}. Thus, RPGR^{ORF15} is a novel glutamylation substrate, and this posttranslational modification is critical for its function in photoreceptors. Our study uncovers the pathogenic mechanism whereby absence of RPGR^{ORF15} glutamylation leads to retinal pathology in patients with *TTLL5* gene mutations and connects these two genes into a common disease pathway.

cilia | polyglutamylation | retinitis pigmentosa | tubulin tyrosine ligase-like | RPGR

Inherited forms of retinal dystrophies, clinically known as retinitis pigmentosa (RP), are degenerative conditions affecting photoreceptor cells and are an important cause of blindness (1). Autosomal dominant, recessive, and X-linked forms of the disease are well documented. The disease etiology is highly heterogeneous, with mutations in more than 200 genes with diverse functions shown to underlie various forms of retinal dystrophy (https://sph.uth.edu/retnet/sum-dis.htm). Among these, mutations in the gene encoding retinitis pigmentosa GTPase regulator (RPGR) are a frequent cause, accounting for more than 70% of X-linked RP and up to 20% of all RP cases (2–5). The disease associated with RPGR is severe and impacts central vision important for visual acuity (6). The high disease incidence and associated central visual handicap make *RPGR* one of the most clinically important RP genes and the focus of a large number of studies aimed at understanding its disease mechanism and the development of effective therapies (7).

RPGR is expressed in a complex pattern, with both default (*RPGR^{default}* or constitutive) and ORF15 (*RPGR^{ORF15}*) variants having been described. *RPGR^{default}* spans exons 1–19 (hence also referred to as *RPGR^{ex1-19}*) and shares the first 14 exons with *RPGR^{ORF15}* (8, 9). Exons 1–10 code for an RCC1 (regulator of chromatin condensation 1)-like domain, suggestive of a role in regulating the activity of small GTPases. *RPGR^{ORF15}* terminates in a large alternative ORF15 exon, characterized by a purine-rich, highly repetitive sequence coding for multiple Glu-Gly repeats followed by a C-terminal tail region rich in basic amino acid residues (basic domain) with unknown function. Although both variants are ciliary proteins, RPGR^{default} is the predominant form in a broad range of ciliated tissues (10), whereas RPGR^{ORF15} is found primarily in the connecting cilia of photoreceptor cells (10, 11). The connecting cilium is structurally analogous to the transition zone of primary or motile cilia and links the biosynthetic inner segment to the photo sensing outer segment and serves as a gateway for protein trafficking between these cellular compartments. Although RPGR^{default} has yet to be linked to any human disease, genetic studies (8, 12) have established an essential role for RPGR^{ORF15} in photoreceptor function and survival. The ORF15 exon is a mutation hotspot in *RPGR*, with mutations identified in this region in up to 60% of patients with X-linked RP (8, 13, 14). Notably, in-frame deletions or insertions that alter the length of this region are well tolerated, as are missense changes (2, 15–19). However, frame shift mutations that lead to loss of the C-terminal basic domain are always disease causing, underscoring the functional importance of this domain (7).

To explore *RPGR* gene function and disease mechanism, we developed the first mouse model, to our knowledge, carrying a null mutation by ablation of both *RPGR* isoforms (11). *Rpgr*-null mice manifest a slowly progressive retinal degeneration characterized by early cone opsin mislocalization in cell bodies and synapses and reduced levels of rhodopsin in rods that are

Significance

Mutations affecting two unrelated genes, *retinitis pigmentosa GTPase regulator* (*RPGR*) and *tubulin tyrosine ligase like 5* (*TTLL5*), lead to photoreceptor degeneration and blindness in humans. We find that RPGR function in photoreceptor cilia requires glutamylation by TTLL5. Glutamylation is a poorly understood posttranslational modification that consists of the addition of glutamates to target proteins. Moreover, we find that mice lacking RPGR or TTLL5 exhibit similar phenotypes characterized by photoreceptor degeneration and opsin mislocalization. Our work identifies a novel essential regulator of RPGR and demonstrates that disease-causing mutations in these two genes share a common pathogenic pathway in humans.

Author contributions: X.S., J.H.P., A.R.-M. and T.L. designed research; X.S., J.H.P., J.G., Z.W., A.S., H.Q., and T.L. performed research; X.S., J.H.P., J.G., A.R.-M., and T.L. analyzed data; and A.R.-M. and T.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]X.S. and J.H.P. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: antonina@mail.nih.gov or tiansen.li@nih.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1523201113/-/DCSupplemental.

detectable at weaning (11). However, significant loss of photoreceptor cells is only evident after 12–16 mo of age. Thus, the early opsin mislocalization appears to reflect a primary defect rather than the result of secondary changes following cell degeneration.

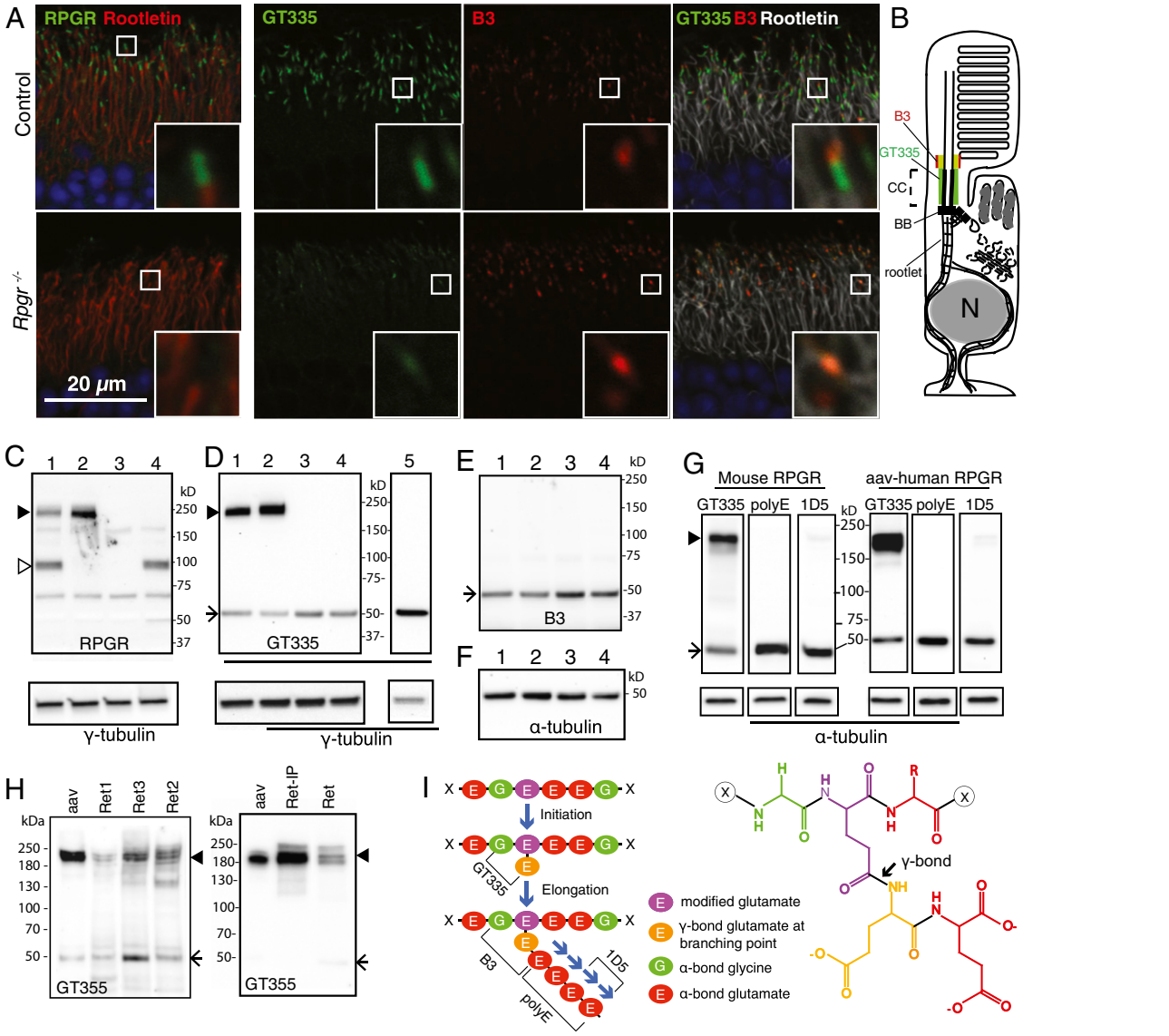


Fig. 1. RPGR^{ORF15}, but not RPGR^{default}, is glutamylated in vivo. (*A*) Immunofluorescence staining of retinal cryosections. (*Left*) RPGR (green) localizes to the connecting cilia as indicated by its position just distal to the ciliary rootlet (red) in the control retina but is absent in the *RPGR*$^{-/-}$ retina. (*Right*) GT335 (green) and B3 (red) antibodies both stain the connecting cilia in the control; GT335 signal is greatly diminished in the *RPGR*$^{-/-}$ retina, whereas B3 is maintained or slightly enhanced. GT335 is a monoclonal antibody that detects monoglutamylation; B3 detects longer glutamate chains on α-tubulin. *Insets* show images at higher magnification. (*B*) Schematic representation of a photoreceptor cell. CC, connecting cilia; BB, basal bodies. (*C* and *D*) Immunoblotting of mouse tissue extracts with antibodies indicated at the bottom of each blot. (*Lower*) Same blots probed with γ-tubulin as a loading control. Lane 1, WT retina; 2, RPGR^{default} KO (lacking RPGR^{default} only) retina; 3, RPGR KO (lacking both RPGR^{default} and RPGR^{ORF15} isoforms) retina; 4, rd9 (lacking RPGR^{ORF15}) retina; 5, WT brain. (*C*) An RPGR antibody detected both RPGR^{ORF15} (solid arrowhead) and RPGR^{default} (open arrowhead) in the WT retina. RPGR^{ORF15} was retained (solid arrowhead) in the *RPGR*^{default} *KO* retina but was lost in the full *RPGR KO* and the rd9 retinas. (*D*) GT335 reacts with RPGR^{ORF15} (arrowhead) but not with RPGR^{default}. Glutamylated tubulin bands (arrow) show comparable intensities in all samples. Brain expresses only RPGR^{default} and hence does not have the higher-molecular-weight RPGR^{ORF15} band (lane 5). (*E*) The B3 antibody shows similar signal intensity in all lanes. (*F*) α-Tubulin expression levels are comparable in all samples, indicating that tubulin glutamylation is similar in all samples. (*G*) RPGR^{ORF15} is predominantly monoglutamylated. (*Upper*) Retinal lysates from a WT mouse or an *Rpgr*-null mouse expressing human RPGR^{ORF15} (through AAV gene transduction) were run in triplicate and probed with the GT335, polyE, and 1D5 antibodies. Glutamylated mouse and human RPGR^{ORF15} is readily detected with GT335, which recognizes branched glutamate side chains. PolyE, which reacts with the side chains of three or more glutamates, did not detect RPGR. 1D5, which reacts with the chains of two or more glutamates, detects trace amounts of RPGR on prolonged exposure. (*H*) RPGR^{ORF15} from human photoreceptors is glutamylated. (*Left*) Three donor retinal lysates (Ret 1, 2, and 3) were run together with recombinant human RPGR^{ORF15} expressed in mouse retina (aav, lane 1) on an immunoblot probed with GT335. Bands corresponding to the position of RPGR were detected (arrowhead). (*Right*) Following immunoprecipitation with an RPGR antibody, the bound fraction was enriched for the GT335-reactive band (Ret-IP). Ret is lysate before IP (5% input). Note that the tubulin band (arrow) was present in the lysate (Ret) but was lost in the eluate (Ret-IP). Separate blot probed with an RPGR antibody confirmed enrichment of RPGR in the eluate and reduction in the flow-through fraction. (*I*) (*Left*) Schematic diagram of glutamylation. The sequence in the diagram can be found in RPGR and the α-tubulin C-terminal tail but does not denote an exact, experimentally mapped modification site. Epitopes for GT335, B3, polyE, and 1D5 antibodies are shown. (*Right*) Chemical structure of two postranslationally added glutamates (the first one connected to the main chain of the target protein through an isopeptide bond).

The disease course of $Rpgr^{-/-}$ mice is slow considering the human disease with *RPGR* mutations is severe, which initially generated some debate as to the cause for this discrepancy. Subsequent studies in independent KO mouse lines found a similar slowly progressing disease, indicating that species differences likely account for the disparate disease severity. A naturally occurring *rd9* mouse, carrying a frame shift mutation in the ORF15 exon that disrupts $RPGR^{ORF15}$ but leaves $RPGR^{default}$ intact, manifests a virtually identical phenotype (20). $RPGR^{ORF15}$ was shown to interact with a number of transition zone proteins essential for ciliary maintenance highlighting a central role for $RPGR^{ORF15}$ in this subcellular compartment. It is generally thought that $RPGR^{ORF15}$ plays a role in regulating protein trafficking through the connecting cilium (11, 21, 22); however, the molecular mechanisms of $RPGR^{ORF15}$ function are not understood.

A distinguishing feature of the ciliary transition zone is the myriad of posttranslational modifications present on axonemal microtubules that set them apart from other microtubule populations in the cell (23–25). The majority of these modifications concentrate on the disordered and highly negatively charged C-terminal tails of tubulin (25). One such abundant modification is glutamylation, the addition of glutamates (one or multiple) to internal glutamates on the tubulin tails. It is catalyzed by glutamylases belonging to the tubulin tyrosine ligase like (TTLL) family (25, 26), many of which localize to cilia (26). Tubulin glutamylation can modulate the affinity of microtubule binding proteins for the microtubule (27–29). Both hyper- and hypoglutamylation disrupt ciliary functions (30–32). Recently, a member of the TTLL family, TTLL5, was reported to underlie a form of human retinal dystrophy with an early and prominent cone involvement (33). Intriguingly, mice with targeted disruption of the *Ttll5* gene showed changes in tubulin glutamylation levels only in sperm, but no phenotype related to retinal function was reported (34). Thus, the pathogenic process leading from a gene defect in TTLL5 to retinal dystrophy remained unexplained.

In this study, we identify $RPGR^{ORF15}$, the RPGR variant predominantly expressed in photoreceptors, as a novel glutamylation substrate. We show that $RPGR^{ORF15}$ recruits TTLL5 via its C-terminal basic domain and is glutamylated specifically by TTLL5 in a region that shares glutamylation consensus motifs with the α-tubulin C-terminal tail. Loss of TTLL5 abolishes $RPGR^{ORF15}$ glutamylation with no detectable effect on tubulin glutamylation levels, and results in a cellular pathology that phenocopies that of *Rpgr null* animals. Our findings demonstrate that $RPGR^{ORF15}$ is the physiological substrate of the TTLL5 glutamylase and connect the recently reported TTLL5-associated retinal dystrophy pathophysiology to RPGR dysfunction, integrating it into the large family of RPGR-related retinal dystrophies.

Results

$RPGR^{ORF15}$ Is Glutamylated in Its Glu-Gly–Rich Repetitive Region. Axonemal microtubules are glutamylated. The monoclonal antibody GT335 is widely used to detect this modification (35). In the retina, GT335 prominently stains the connecting cilia of photoreceptors (Fig. 1*A*, *Upper*) and primary cilia in the inner retina. It is generally believed that GT335 stains cilia by binding to glutamylated tubulin. However, in the $Rpgr^{-/-}$ mouse retina, we found that the GT335 signal in photoreceptor connecting cilia was greatly diminished (Fig. 1*A*, *Lower*), whereas staining in other parts of the retina was unchanged. Because the GT335 antibody does not recognizes glutamate branches exclusively on tubulin, we also tested the retinas with the B3 monoclonal antibody known to be specific for polyglutamylated α-tubulin (36, 37) (the antigenic epitopes of glutamylation specific antibodies are illustrated in Fig. 1*I*). The B3 antibody gives comparable if not elevated signal intensity in the $Rpgr^{-/-}$ mouse retina (Fig. 1*A*). These findings suggest that GT335 stains primarily $RPGR^{ORF15}$ at the photoreceptor connecting cilia. To confirm this finding, we performed immunoblotting analyses of retinal lysates from WT and *Rpgr* mutant retinas (Fig. 1*C*–E). An RPGR antibody detects two major variants in the WT retina: $RPGR^{ORF15}$ and $RPGR^{default}$, that migrate at molecular weights of ∼200 kDa (Fig. 1*C*, lane 1, solid arrowhead) and 100 kDa (open arrowhead), respectively. As expected neither variant was present in the $Rpgr^{-/-}$ retina (lane 3). An $RPGR^{default}$-specific KO mouse had only the $RPGR^{ORF15}$ variant (lane 2), whereas the *rd9* mouse had only the $RPGR^{default}$ variant (lane 4). When the same samples were probed with GT335, only those that retained the $RPGR^{ORF15}$ variant showed a band at the higher molecular weight (Fig. 1*D*, lanes 1 and 2). Similarly, mouse brains, which do not express the ORF15 variant, did not show a high-molecular-weight band (lane 5). Notably, neither GT335 nor B3 (Fig. 1*E*) detected a decrease in tubulin glutamylation in mice lacking the $RPGR^{ORF15}$ variant. Moreover, the α-tubulin signal was comparable among all lanes (Fig. 1*F*). Thus, tubulin expression and glutamylation levels were unchanged in *Rpgr* mutant retinas. These results indicate that the GT335 staining at the photoreceptor connecting cilia can be attributed primarily to RPGR, and that $RPGR^{ORF15}$ and not $RPGR^{default}$ is glutamylated in photoreceptors.

The GT335 antibody reacts with the branched glutamate that is first added to an internal glutamate in the sequence of the protein being modified (Fig. 1*I*) and thus does not distinguish between monoglutamylation (addition of one glutamate) or polyglutamylation (addition of longer chains of glutamate residues) (38). Therefore, it was not clear whether $RPGR^{ORF15}$ is mono- or polyglutamylated. To resolve this question, we performed immunoblotting analysis with additional antibodies: 1D5 (also known as ID5), which recognizes glutamate chains longer than two (39), and polyE, which recognizes C-terminally located glutamate chains of four or more residues (26). In the WT mouse retina, polyE did not react with $RPGR^{ORF15}$, whereas 1D5 detected only trace amounts (Fig. 1*G*, *Left*), indicating RPGR is primarily monoglutamylated. We further analyzed recombinant human $RPGR^{ORF15}$ expressed in the $Rpgr^{-/-}$ mouse retina via AAV (adeno-associated virus)-mediated gene transduction, and found nearly identical results (Fig. 1*G*, *Right*). These data indicate that $RPGR^{ORF15}$ is primarily monoglutamylated regardless of species of origin. To rule out the possibility that the mouse photoreceptor environment was responsible for glutamylation of recombinant human $RPGR^{ORF15}$, we examined lysate of donor human retinas directly. Three independent donor retina extracts (Ret1, 2, and 3) showed GT335-reactive bands that comigrated with recombinant human $RPGR^{ORF15}$ (aav) by immunoblotting (Fig. 1*H*, *Left*). Following immunoprecipitation with an RPGR antibody, GT335-reactive RPGR was highly enriched in the bound fraction (Ret-IP) (Fig. 1*H*, *Right*), confirming that native human $RPGR^{ORF15}$ is glutamylated.

$RPGR^{ORF15}$ Interacts with TTLL5 via Its Basic Domain and Is Glutamylated by TTLL5. The $RPGR^{ORF15}$ sequence diverges from the default variant, which is not glutamylated, at its C-terminal half. The C-terminal half of $RPGR^{ORF15}$ consists of a Glu-Gly rich repeat region followed by a C-terminal basic domain (Fig. 2*A*). Sequence analysis of the repeat region identified 11 glutamate rich consensus motifs (GEEEG) homologous to the α-tubulin C-terminal tail (Fig. 2*A*). The number of these motifs depends on the mouse strain: 18 were found in C57BL6 and 22 in the 129/Sv strain. Thus, the repetitive region is a natural candidate for glutamylation. The basic domain next to the repetitive region is highly conserved among vertebrates, but it was not known whether it has any role in $RPGR^{ORF15}$ glutamylation. To explore this question we examined data from a yeast two-hybrid protein interaction screen in which the basic domain from human $RPGR^{ORF15}$ was used as bait

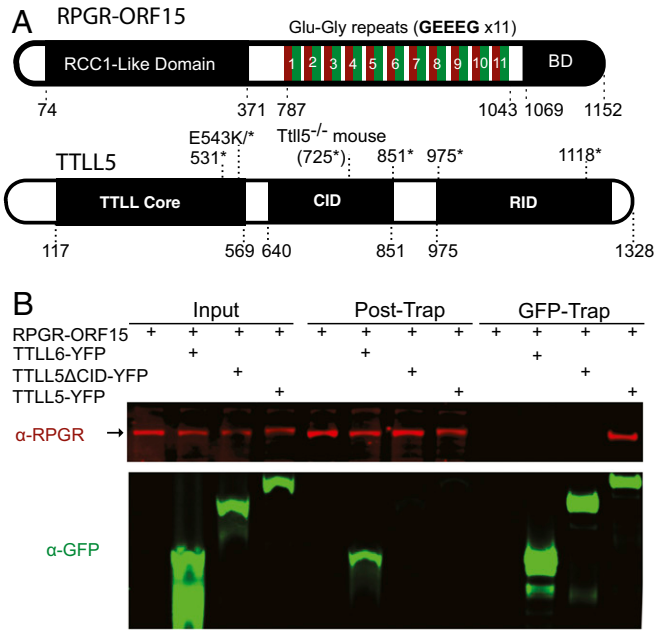


Fig. 2. RPGR^{ORF15} interacts with TTLL5 via its C-terminal basic domain (BD). (*A*) Diagrams showing the domain organization of RPGR^{ORF15} and TTLL5. The RCC1-like domain is shared among all RPGR variants, whereas the Glu-Gly–rich region and the basic C-terminal domain are unique to RPGR^{ORF15}. Human RPGR^{ORF15} possesses 11 tandem GEEEG repeats, whereas mouse RPGR^{ORF15} possesses 18. TTLL5 is comprised of a core tubulin tyrosine ligase-like (TTLL) domain, a cofactor interaction domain (CID), and a receptor interaction domain (RID). Point and deletion mutations relevant to this study (see later sections) are denoted on the diagrams. (*B*) Confirmation of the physical interaction between RPGR^{ORF15} and TTLL5 by GFP-trap precipitation and immunoblotting of transiently transfected HEK293 cell lysates. RPGR^{ORF15} was detected with a polyclonal antibody (red; *Upper*). All TTLL constructs were YFP-fusions. TTLL5, TTLL5 ΔCID, and TTLL6 (*Lower*) were detected with a GFP antibody (green; *Lower*). Input, posttrap (unbound), and GFP-Trap (bound) fractions were analyzed. In the bound (GFP-Trap) fractions, TTLL5, TTLL5 ΔCID, and TTLL6 proteins were efficiently recovered. RPGR was recovered only together with TTLL5. GFP-Trap results were reproduced in more than four independent trials.

(Fig. S1). Among candidate proteins identified as potential interaction partners, one group represented TTLL5, a glutamylase of the TTLL superfamily. Sequence analysis indicated that the region of TTLL5 that interacted with RPGR^{ORF15} overlapped with the cofactor interaction domain (CID) thought to bind transcription activators (34). To verify a physical interaction between RPGR^{ORF15} and TTLL5, we carried out pull-down assays using lysates of transiently transfected HEK 293 cells. Recombinant RPGR^{ORF15} was coexpressed with YFP-tagged full-length TTLL5 or TTLL5 missing the CID. Full-length YFP-tagged TTLL6 and TTLL7 (glutamylases also belonging to the TTLL superfamily) were used as negative controls. Full-length TTLL5, but not TTLL6 or TTLL7, was able to pull down RPGR^{ORF15} after cell lysates were put through a GFP-Trap (Fig. 2*B* and Fig. S2*A*). TTLL5 ΔCID also failed to form a stable complex with RPGR^{ORF15} as clearly indicated by lack of RPGR^{ORF15} recovery in the GFP-Trap (Fig. 2*B* and Fig. S2*B*). Full-length TTLL5 not only bound to but also glutamylated RPGR^{ORF15}, as indicated by the colabeling of the RPGR^{ORF15} band by GT335 (Fig. S2*B*) and an upward shift in its apparent molecular weight detectable on a longer gel run (Fig. S2*B*, white arrowheads). Both TTLL5 and TTLL5 ΔCID also produced a number of high-molecular-weight, GT335-positive proteins. These bands are unrelated to RPGR as they did not react with RPGR antibodies and also appeared in samples not transfected with RPGR. This observation is consistent with a previous study that detected similar unknown high molecular substrates for TTLL5 in cultured human cell lines (26). Together, our yeast two-hybrid and cellular assays indicate that RPGR^{ORF15} interacts specifically with TTLL5 and that the CID of TTLL5 and the C-terminal basic domain of RPGR^{ORF15} are critical for a specific interaction between these proteins.

RPGR^{ORF15} Is the Physiological Substrate for TTLL5 in the Retina. We next investigated the relationship between RPGR^{ORF15} and TTLL5 in vivo. Like RPGR^{ORF15}, TTLL5 was also reported to localize in the vicinity of the photoreceptor connecting cilium (33). A mouse line carrying a targeted disruption of the *Ttll5* gene was reported to be viable with no overt retinal phenotype (34). We examined the state of RPGR^{ORF15} glutamylation in the retina of this mutant mouse. Immunostaining of cryo-sections illustrated that glutamylation of ciliary tubulin was not diminished nor was RPGR expression altered on loss of TTLL5 (Fig. 3*A*). In contrast, GT335 signal was nearly absent (Fig. 3*A*). Higher-magnification views revealed two domains of GT335 signal: a strong one that overlapped with RPGR in the transition zone and a weak one that extended distally and represents axonemal microtubules. In *Ttll5* mutant photoreceptors, GT335 signal in the transition zone was absent but the portion attributable to glutamylated tubulin remained. These data indicate that RPGR^{ORF15} glutamylation is abolished, whereas tubulin glutamylation is not visibly affected in the absence of TTLL5. This interpretation was further corroborated by immunoblotting analysis. Loss of TTLL5 had no effect on the expression levels of RPGR (Fig. 3*B*) nor did it affect tubulin glutamylation (Fig. 3 *C* and *D*). However, glutamylation of RPGR^{ORF15} was abolished as indicated by the absence of GT335 staining (Fig. 3*C*). In heterozygous *Ttll5* retina, the glutamylation level of RPGR^{ORF15} was indistinguishable from that of WT (Fig. 3*B*), indicating that the enzyme level is not limiting. These data indicate that RPGR^{ORF15} is the major physiological substrate for TTLL5 in the retina. This conclusion is reinforced by our analysis of nucleophosmin, a protein that was reported to be glutamylated in HeLa cells (40) and in *Xenopus* oocytes (41). It interacts with the C-terminal basic domain of RPGR^{ORF15} (42) and thus could be in close proximity to RPGR^{ORF15} in photoreceptors. However, immunoblotting (Fig. 3*E*) analysis demonstrates that nucleophosmin is not glutamylated in the murine retina. These findings rule out nucleophosmin as a possible mediator of pathogenesis in *TTLL5*-related retinal dystrophy. Because both RPGR^{ORF15} and TTLL5 localize at or near the connecting cilia of photoreceptors, we next asked whether RPGR^{ORF15} is modified in this subcellular compartment or at an earlier step of protein biosynthesis. RPGR^{ORF15} is known to be anchored by RPGRIP1 in the connecting cilia, and in *Rpgrip1* KO mice, RPGR^{ORF15} is expressed at normal levels but fails to localize to the connecting cilia (43). By immunoblotting with GT335, we found that RPGR^{ORF15} glutamylation was greatly diminished in the *Rpgrip1* mutant retinas compared with the WT controls (Fig. 3*F*). This finding suggests that RPGR^{ORF15} is glutamylated by TTLL5 primarily at the connecting cilia.

***Ttll5*$^{-/-}$ Mice Develop a Retinal Phenotype Similar to *Rpgr*$^{-/-}$ Mice.** Although the initial study of the *Ttll5*$^{-/-}$ mice did not find an overt retinal defect based on histology (34), we thought it likely that using additional assays and extending the observation period to older ages might reveal a late-onset, slowly progressive form of photoreceptor degeneration, in line with the phenotypes observed in the *Rpgr*-null mice. Indeed, immunofluorescence analysis at a young age (postnatal 20–40 d) found ectopic localization of S- and M-cone opsin in the cell body (Fig. 4*A*). Both rhodopsin and cone opsins mislocalized at older ages (Fig. S3). Glial fibrillary acidic protein (GFAP), a general indicator of photoreceptor stress and degeneration,

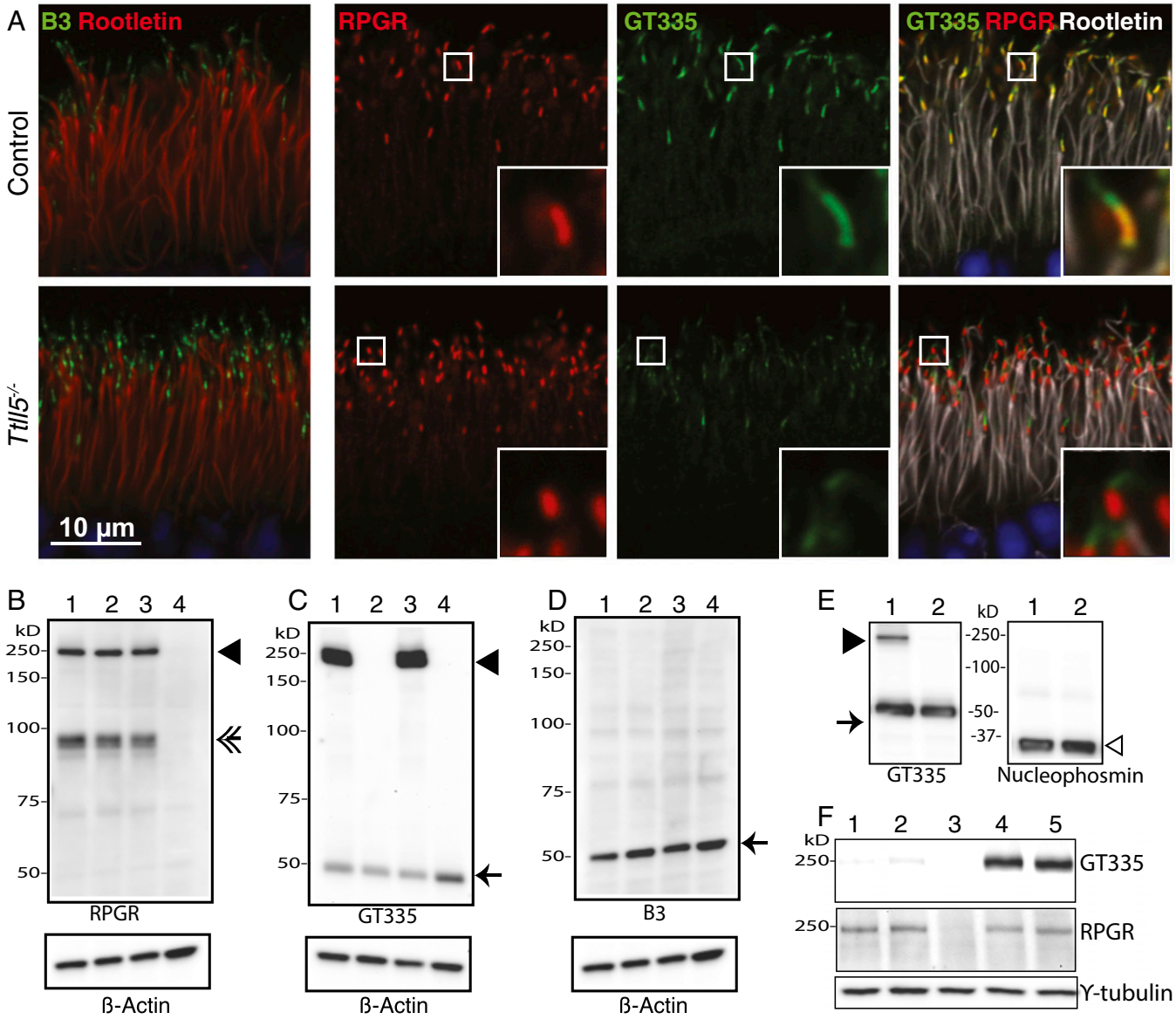


Fig. 3. RPGR^{ORF15} is glutamylated by TTLL5 in vivo. (*A*) Analysis of WT and *Ttll5* mutant retinal sections by immunofluorescence. Polyglutamylation of ciliary tubulin (indicated by staining with the B3 antibody) is not diminished on TTLL5 loss (green; leftmost panels), nor is RPGR itself (red; second column). However, GT335 signal is greatly diminished on TTLL5 loss. Zoomed in images in *Insets* reveal two domains of GT335 signal: one overlapping with RPGR and the other extending more distally. The GT335 signal in the distal domain, which corresponds to glutamylated tubulin, is unaffected by the *Ttll5* mutation. (*B–D*) Immunoblotting analysis of *Ttll5*$^{+/-}$ (lane 1), *Ttll5*$^{-/-}$ (lane 2), WT (lane 3), and *Rpgr*$^{-/-}$ (lane 4) mouse retinal lysates. *Ttll5*$^{+/-}$, *Ttll5*$^{-/-}$ and WT mice were littermates. Antibodies used for immunoblotting are marked under each panel. RPGR^{ORF15} (solid arrowhead) and RPGR^{default} (double arrow) expression was unchanged in the *Ttll5*$^{-/-}$ mutant (*B*). Glutamylation of RPGR^{ORF15} is abolished in the *Ttll5*$^{-/-}$ retina (lane 2 of *C*), whereas tubulin glutamylation (arrow) remains constant. The B3 antibody, which recognizes polyglutamylated tubulin specifically, shows comparable signal intensity among all samples (*D*). (*Lower*) Blots reprobed for β-actin as loading controls (*B–D*). Similar results were obtained from two independent experiments. (*E*) Nucleophosmin is not glutamylated. Mouse retinal lysates probed with GT335 show signal for tubulin (arrow) in both WT (1) and *Ttll5*$^{-/-}$ mutant (2) samples, and RPGR^{ORF15} (solid arrowhead) only in the WT sample. No GT335-reactive bands at the molecular weight of nucleophosmin (~33 kDa) were detected even after prolonged exposure (*Left*). Nucleophosmin (open arrowhead) was readily detected in both samples (*Right*). (*F*) Glutamylation of RPGR^{ORF15} is greatly diminished in the *Rpgrip1*$^{-/-}$ retina. (*Top*) GT335 immunoblot of retinal extracts from *Rpgrip1*$^{-/-}$ retinas (lanes 1 and 2) at age postnatal day 20 and age-matched WT controls (lanes 4 and 5). Four mice of each genotype were analyzed in this experiment. Lane 3: *Rpgr*$^{-/-}$ as a negative control. (*Middle*) Immunoblot of the same retinal lysates probed with an RPGR^{ORF15} C-terminal antibody. (*Bottom*) A γ-tubulin immunoblot is shown as a loading control.

is also up-regulated in *Ttll5*$^{-/-}$ retinas at older ages (Fig. 4*B*). Early cone opsin mislocalization is also a distinguishing feature of *Rpgr*$^{-/-}$ mice (Fig. 4*A*) at the same age (11). A second distinguishing feature in *Rpgr*$^{-/-}$ mice is a change of fundus color from orange red to a metallic gray from an early age, which may reflect reduced rhodopsin content in the outer segments (11). This feature was also observed in the *Ttll5*$^{-/-}$ mice (Fig. S4).

Electroretinograms (ERGs) that measure photoreceptor function were recorded at 4 and 20–22 mo of age. The dark-adapted ERG reflects primarily rod function at lower stimulus intensities and mixed rod and cone function at high stimulus intensities, whereas light-adapted ERG reflects cone function. At the younger age, rod and cone ERG waveforms and amplitudes were comparable between the control and mutant mice, with the exception of the rod a-wave amplitude at the highest

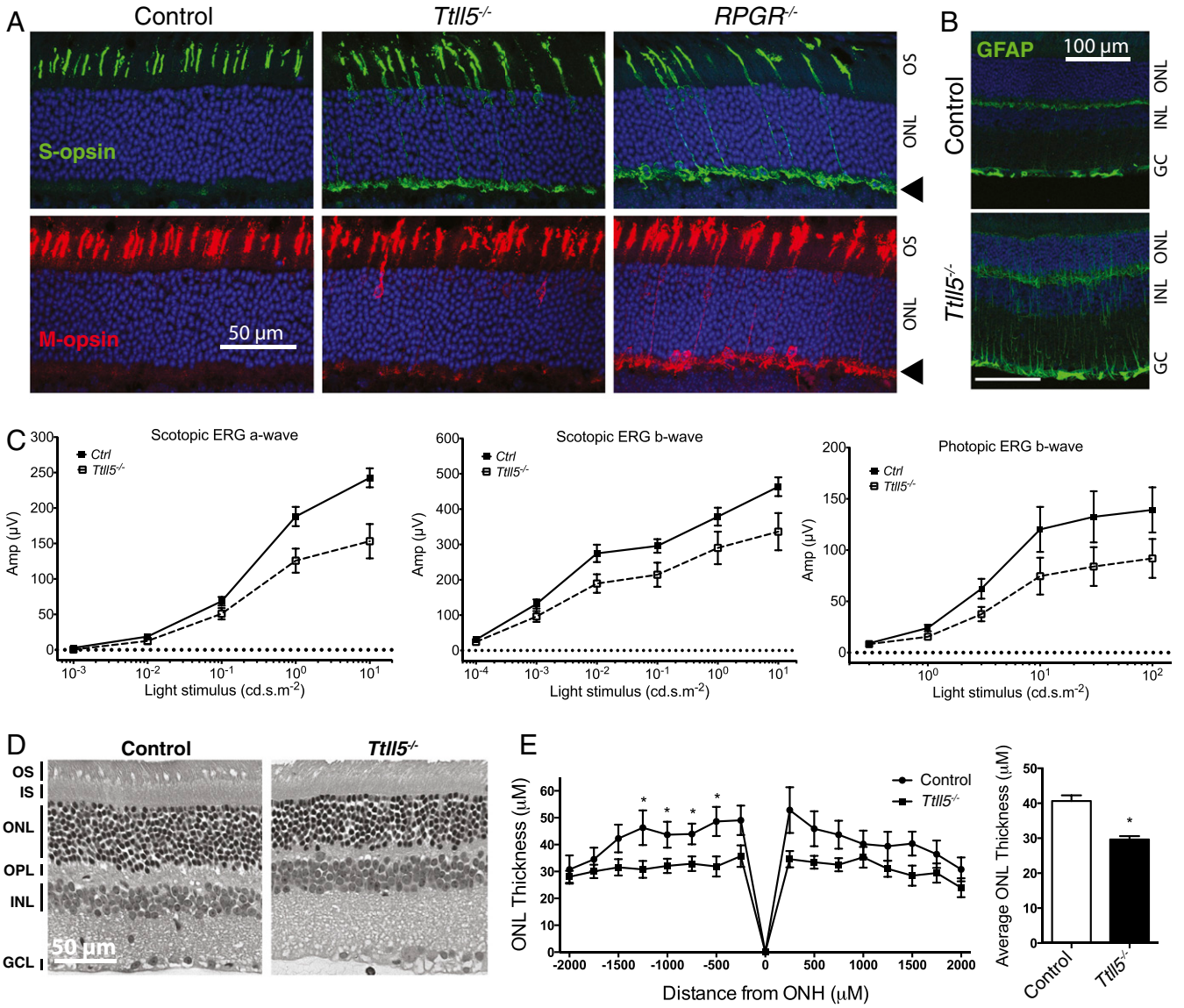


Fig. 4. *Ttll5*$^{-/-}$ mice develop late-onset, slowly progressive photoreceptor degeneration that phenocopies *Rpgr*$^{-/-}$ mice. (*A*) Immunofluorescence analysis of cone opsins at a young age (20–40 d). Comparison with the control shows both the S- and M-opsins mislocalized to the cell body and the synaptic layer (arrowhead) in the *Ttll5*$^{-/-}$ and *Rpgr*$^{-/-}$ retinas. (*B*) GFAP is up-regulated in the *Ttll5*$^{-/-}$ retinas at older ages (20–22 mo old). (*C*) ERG stimulus intensity–amplitude functions in aged mice. ERG amplitudes in *Ttll5*$^{-/-}$ mice are significantly lower than control mice of this age (20–22 mo; $P < 0.0001$ for scotopic ERG a- and b- waves; $P = 0.0013$ for photopic ERG b-wave in two-way ANOVA). (*D*) Light microscopy of retina sections show mild thinning of the photoreceptor layers. (*E*) Measurements of ONL thickness at different positions from the optic nerve head (ONH) to the far peripheral retinas (*Left*) shows thinning of ONL in the *Ttll5*$^{-/-}$ mice ($n = 11$) compared with the controls ($n = 9$). Overall, the mean ONL thickness (*Right*) in *Ttll5*$^{-/-}$ retinas was significantly reduced compared with controls ($P < 0.05$). Mice were 20–22 mo old in *D* and *E*.

stimulus intensity where the mutant mice showed a borderline decline (Fig. S5). At older ages, both rod and cone ERG amplitudes declined significantly (Fig. 4*C*). Morphometric measurements of plastic embedded retinal sections also showed a significant thinning of the photoreceptor layer in aged animals (Fig. 4 *D* and *E*). The overall disease course and accompanying features of *Ttll5*$^{-/-}$ mice thus appear to phenocopy those of *Rpgr*$^{-/-}$ mice.

Sperm from *Ttll5*$^{-/-}$ mice has defective axonemes (34). Specifically doublet 4 is missing from the 9+2 microtubule arrays. Given the prominent function of TTLL5 in photoreceptors, we asked if loss of TTLL5 might have caused structural defects in photoreceptor connecting cilia. We performed transmission electron microscopy of cross sections through the connecting cilia of WT and mutant photoreceptors (Fig. S6). Unlike sperm tails, which are motile and therefore have a central microtubule pair, the connecting cilia have a 9+0 configuration. Examination of the electron micrographs found no apparent microtubule defects in the mutant, at least not at the level of the connecting cilia.

RPGR Glutamylation in Vivo Requires Both the Glu-Rich Repetitive Region and the Basic Domain. We postulated that binding of the basic domain of RPGR^{ORF15} to the CID recruits TTLL5 to glutamylate the RPGR^{ORF15} Glu-Gly repeat region at multiple consensus sites along its length. To test this hypothesis in vivo, we packaged a series of human RPGR^{ORF15} deletion constructs (Fig. 5*A*) into an AAV vector under the control of a photoreceptor-specific promoter and injected them into the subretinal space of *Rpgr*$^{-/-}$ mice. Retinal lysates expressing recombinant RPGR^{ORF15} were subjected to immunoblot analysis. Recombinant proteins

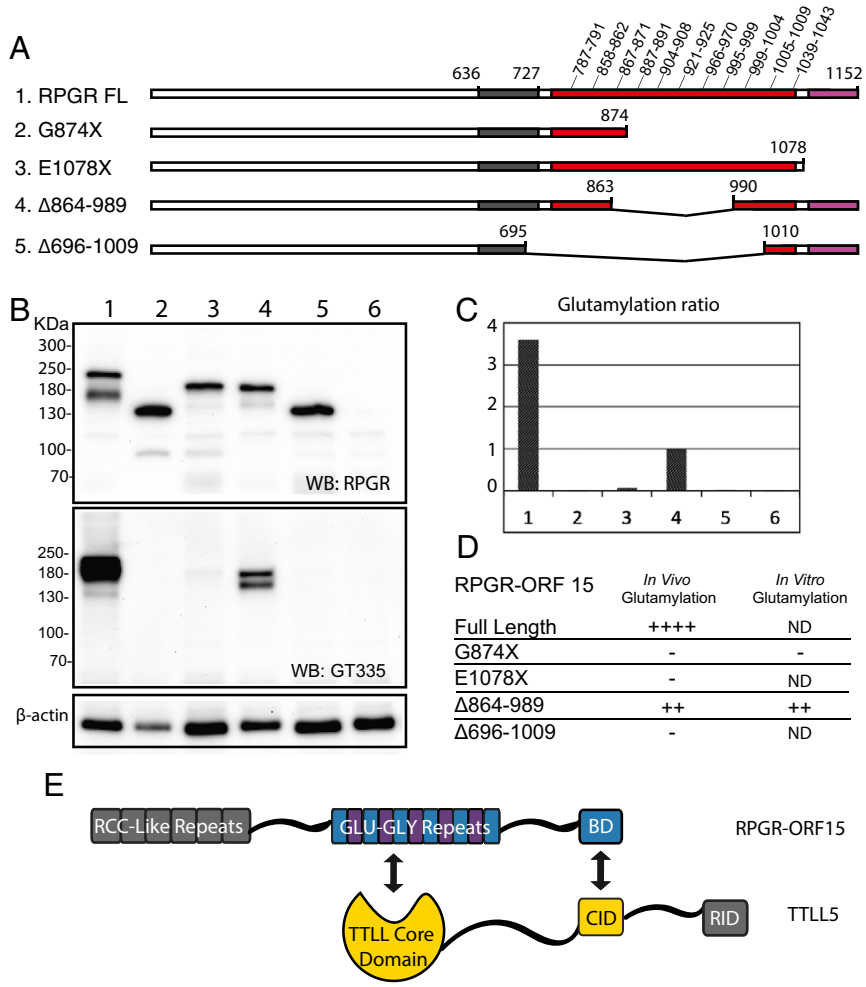
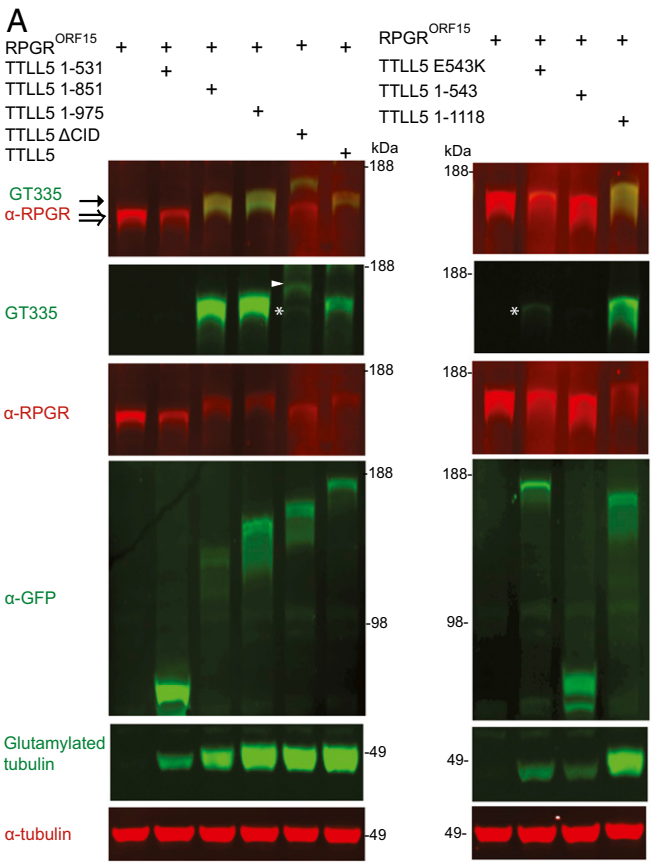


Fig. 5. RPGR glutamylation in vivo requires both the C-terminal basic domain and the Glu-Gly–rich region. (*A*) Diagrams of human *RPGR*^{ORF15} expression constructs packaged into AAV vectors. The Glu-Gly–rich region is marked in red and the C-terminal basic domain in magenta. The position of glutamylation consensus motifs is shown in the schematic for the full-length (FL) construct. (*B*) Immunoblots of retinal extracts from *Rpgr*$^{-/-}$ mice injected with RPGR expression constructs. Lanes 1–5 match the construct numbers shown in *A*, and lane 6 is an uninjected control. Full-length RPGR and to a lesser extent RPGRΔ864–989 are glutamylated as indicated by detection with GT335 (*Middle*). Probing with an RPGR antibody shows expression levels for recombinant RPGR (*Top*). Reprobing blots with β-actin provides a loading control (*Bottom*). (*C*) Quantification of glutamylation levels by densitometry after normalizing for RPGR levels, with sample 4 level set arbitrarily at 1. These results are summarized in *D*. ND, not determined. Similar results were obtained from two independent experiments. (*E*) Schematic diagram illustrating mapped interactions between RPGR and TTLL5.

were expressed at comparable levels (Fig. 5*B*, *Top*). GT335 immunoblotting (Fig. 5*B*, *Middle*), indicated that full-length RPGR^{ORF15} was strongly glutamylated (lane 1), whereas constructs missing the basic domain showed no detectible glutamylation (G874X and E1078X, lanes 2 and 3). Δ864–989, which lacks more than one third of the Glu-Gly repeat region and 6 of the 11 glutamylation consensus motifs, but retains the basic domain, is moderately glutamylated (lane 4). Δ696–1009, which retains a single Glu-Gly motif (the last one next to the basic domain), showed no detectable glutamylation (lane 5). Interestingly, a recent study tested the therapeutic efficacy of RPGR^{ORF15} deletion constructs Δ864–989 (ORF15-L) and Δ696–1009 (ORF15-S) and showed that the former was functional but the latter was not (17). Two of the constructs were also tested in cultured cells with similar results (Fig. 5*D*). The in vivo and in vitro data are quantified and summarized in Fig. 5 *C* and *D*, respectively. The in vivo experiments in photoreceptors suggest that RPGR^{ORF15} is glutamylated at multiple sites, and partial elimination of the consensus sites appears to result in a matching decrease in glutamylation levels. These data confirm the requirement for the C-terminal basic domain for RPGR^{ORF15} glutamylation by TTLL5.

Impact of *TTLL5* Disease Mutants on RPGR^{ORF15} Glutamylation. To define critical domains required for glutamylation of RPGR^{ORF15} and to explore the pathogenicity of mutant alleles, we next examined a series of deletion mutants of TTLL5 for their ability to modify RPGR^{ORF15}, as well as three disease-causing mutations by cotransfection in 293 cells. Five disease-causing *TTLL5* alleles were identified in patients with retinal dystrophy, all of which are recessive (33), indicating that they are loss-of-function alleles. Previous studies showed that the N-terminal 569 residues in TTLL5 are part of the TTLL glutamylase core domain and are required for glutamylase activity of TTLL5 (26, 44). An early frame-shift mutant L134Rfs results in an early truncation of the TTLL core and was presumed a null. The E529Vfs*2 mutant is also truncated well within the TTLL5 core domain. We therefore focused on the three remaining disease-causing mutants, E543K, E543* (1-543), and W1118* (1-1118), and analyzed these together with a battery of domain truncation mutants. Consistent with the core domain organization of TTLL5, TTLL5 truncated at residue



B

	RPGR^{ORF15} Glutamylation	Tubulin Glutamylation	Expression Level
TTLL5	+	++	++
TTLL5 1-531	-	+	+++
TTLL5 ΔCID	+/-	++	++
TTLL5 1-851	+	++	+
TTLL5 1-975	+	++	+++
TTLL5 E543K	+/-	+	++
TTLL5 1-1118 (W1118*)	+	++	++
TTLL5 1-543 (E543*)	-	+	+++

Fig. 6. Effect of TTLL5 domain deletion and disease mutations on RPGR^{ORF15} glutamylation in transient cotransfection assays. (*A*) Coexpression of RPGR^{ORF15} and WT TTLL5 in HEK293 cells leads to RPGR^{ORF15} glutamylation as detected by GT335 staining. An early truncation mutant TTLL5 1–531 shows no activity toward RPGR^{ORF15}. Deletion of the CID or mutation of a conserved residue E543K in the TTLL core dramatically reduce RPGR^{ORF15} glutamylation as indicated by only residual GT335 staining (denoted by a star). Truncation downstream from CID generally preserves TTLL5 activity toward RPGR^{ORF15} in the in vitro assay. White arrowhead indicates the unknown high-molecular-weight protein that is also gluatmylated. Note an upward shift in molecular weight of RPGR^{ORF15} on glutamylation as denoted by hollow (unmodified) and solid (modified) arrows, consistent with the glutamate addition at multiple Glu-Gly–rich motifs. Expression of YFP-TTLL5 constructs was confirmed with a GFP antibody. This experiment is representative for more than three independent cotransfections. (*B*) Summary table of RPGR-ORF15 glutamylation by mutant TTLL5 constructs in heterologous cells (disease-causing mutations are shown in blue).

531 shows no detectable glutamylation activity, whereas TTLL5 truncated at residues 851 or 975 has robust activity (Fig. 6*A*, *Left*). In line with our finding that the CID (640-851) of TTLL5 mediates binding to RPGR, TTLL5 ΔCID only produced a trace amount of glutamylated RPGR, even in a transient overexpression assay system while retaining full glutamylase activity toward tubulin (Fig. 6*A*, *Left*). Deletion mutants truncating at codons 851 and 975 that retain the TTLL5 catalytic core and the RPGR binding CID are fully active toward both tubulin and RPGR (Fig. 6*A*, *Left*). These data show that the core TTLL domain (1-569) is required for glutamylase activity (for any substrate), but the CID (640-851) is additionally required for efficient RPGR^{ORF15} glutamylation. High-molecular-weight protein(s) unrelated to RPGR^{ORF15} were also glutamylated and migrated as a collection of bands or smear (arrowhead), as reported in a previous study (26). TTLL5 ΔCID produced a band (white arrowhead, Fig. 6*A*) migrating just above RPGR^{ORF15} along with a higher-molecular-weight smear, whereas TTLL5 produced only higher molecular smears. These bands are unrelated to RPGR^{ORF15} and are also present in cells transfected only with TTLL5 or TTLL5ΔCID (Fig. S2*B*). The disease-causing charge-reversal mutant E543K and the catalytic core truncation mutant E543* show greatly diminished tubulin glutamylation (Fig. 6*A*, *Right*). The E543K mutant, which retains the RPGR adaptor CID produced trace amounts of glutamylated RPGR (indicated by an asterisk), whereas E543* showed no detectable RPGR glutamylation. E543K and E543* thus behave as strong hypomorphic or null alleles. A third disease-causing mutant, W1118* that retains an intact TTLL glutamylase core and the RPGR binding CID, displays full activity toward both tubulin and RPGR^{ORF15} in vitro (Fig. 6*A*, *Right*). Interestingly, all three mutants truncated downstream of the CID (1-851, 1-975, W1118*) glutamylated RPGR efficiently but not the unknown high-molecular-weight targets (Fig. 6), suggesting that the C-terminal tail may target TTLL5 to these other substates. Taken together, the results of these cotransfection assays (Fig. 6*B*) confirm the critical role of the CID in recruiting TTLL5 to modify RPGR. Because the E543X disease mutant behaves as a null, it is highly likely that the early frame-shift mutant L134Rfs would also behave as a null. Thus, the majority of disease-causing mutants are defective for RPGR glutamylation. The only exception is the W1118* mutant. We hypothesize that the C-terminal domain lost in the W1118* allele may have in vivo roles such as protein trafficking or regulatory activities that could not be tested in a heterologous cell culture system. Deciphering the pathogenicity of this allele will require future in vivo studies.

Discussion

Here we report the unexpected discovery that RPGR^{ORF15}, the functional variant of RPGR in photoreceptor cilia, is glutamylated by the TTLL family glutamylase TTLL5 and that *Ttll5*$^{-/-}$ mice phenocopy the *Rpgr*$^{-/-}$ retinal degeneration phenotype. The significance of our findings is threefold. First, they resolve at the molecular level the pathophysiology of *TTLL5* gene mutations that cause retinal dystrophy and connect the *RPGR* and *TTLL5* disease mutations into a common pathway. In the absence of TTLL5, no glutamylation occurs on RPGR^{ORF15}, leading to compromised if not abolished function of RPGR^{ORF15} in photoreceptors and cellular defects that resemble loss of RPGR^{ORF15}. Second, TTLL5 appears to be highly selective for RPGR^{ORF15}, with the CID unique to TTLL5 acting as an adapter to engage the C-terminal basic domain of RPGR^{ORF15}. Other TTLL family members do not compensate for TTLL5 in RPGR^{ORF15} glutamylation in vivo, consistent with our in vitro transfection experiments that show that neither TTLL6 nor TTLL7 glutamylates RPGR^{ORF15}. The high degree of selectivity of TTLL5 for RPGR^{ORF15} in vivo is likely enhanced by their close proximity at the same subcellular compartment. RPGR^{ORF15} is recruited through its RCC1-like domain by RPGRIP1 (43) to the connecting cilia, whereas TTLL5 localizes to the ciliary base (33). Subsequent engagement of RPGR^{ORF15} with TTLL5 and glutamylation occur primarily at this subcellular compartment. Our study further supports the idea that some TTLL family members, despite their name, act on nontubulin substrates and use accessory domains outside the TTLL core for their recognition. It is possible

that TTLL5 also modifies tubulin in vivo as it does in vitro consistent with the axonemal defects observed in the sperm of *Ttll5*$^{-/-}$ mice (34), although such defects were not observed in our analysis of the retina. Third, our data shed light on the long-standing question regarding the roles of the Glu-Gly–rich region and the conserved C-terminal basic domain in RPGR^{ORF15}. RPGR^{ORF15} binds TTLL5 with its basic domain, bringing TTLL5 in close proximity to glutamylation sites along the Glu-Gly–rich repetitive region of RPGR ^{ORF15}. This interaction likely forms the basis for the recruitment of TTLL5 to RPGR^{ORF15}.

Five disease-causing mutant alleles were found in a recent study that identified *TTLL5* as a disease gene for human retinal dystrophy (33). We examined three of these disease mutations and demonstrate that two reduce TTLL5 glutamylase activity to near background levels. Three alleles prematurely truncate the TTLL glutamylase catalytic core (including E543* examined in this study) and are apparent nulls. One missense mutation affects the TTLL core catalytic domain (E543K) and severely impairs glutamylation activity both toward RPGR^{ORF15}, as well as tubulin. The W1118* mutant glutamylates RPGR^{ORF15} efficiently in vitro (Fig. 6), consistent with our domain mapping experiments showing that the TTLL core and CID are required for robust RPGR^{ORF15} glutamylation. Interestingly, the W1118X mutant truncates the receptor interacting domain (RID). These observations indicate that the RID may have a yet unidentified but important role in photoreceptors.

The disease resulting from *TTLL5* mutations has prominent cone photoreceptor involvement and is clinically characterized as cone-rod dystrophy or cone dystrophy, although rods are also involved in some of these patients (33). This phenotype would seem somewhat dissimilar to the majority of RP patients with *RPGR* mutations. However, clinical manifestations of RP are known to be highly variable, and *RPGR* mutations are also known to cause disease with early cone involvement (6, 8, 45–48). Moreover, it is possible that the greater involvement of cones in *TTLL5* patients could be due to a selection bias because the study was initiated on patients diagnosed with cone or cone-rod dystrophies. If so, future genetic screens among patients with typical RP may yet turn up cases that are caused by *TTLL5* mutations. Comparison of the *Rpgr*$^{-/-}$ and *Ttll5*$^{-/-}$ mouse models revealed several similarities, including early cone involvement and overall long-term disease progression. Therefore, in our view, the *Ttll5*$^{-/-}$ mouse model phenocopies the *Rpgr*$^{-/-}$ mice, and the disease in both models can be attributed to a nonfunctional RPGR^{ORF15}. Finally our working model does not exclude the possibility that *TTLL5* mutations lead to a strongly hypomorphic rather than a null RPGR^{ORF15}; RPGR^{ORF15} might be glutamylated at an extremely low level by another enzyme or the nonglutamylated protein may still retain very low levels of function. This scenario would be consistent with patients carrying *TTLL5* mutations manifesting a somewhat milder disease than patients with *RPGR* mutations.

RPGR^{ORF15} appears to be glutamylated at multiple consensus sites along the length of its repetitive region, but seems to be primarily monoglutamylated, i.e., each internal glutamylation site has a single postranslationally added glutamate as opposed to longer glutamate chains. This observation is consistent with the enzymatic properties of TTLL5, which was shown to have higher activity initiating glutamate chains than elongating them (26). Because elongating glutamylases also localize to the cilium, e.g., TTLL6 (49, 50), it is possible that the primarily monoglutamylated RPGR species is a result of a balance between elongating glutamylases and carboxypeptidases (CCPs) that shorten the glutamate chains (31). It is not clear how glutamylation of the repetitive region of RPGR^{ORF15} functionalizes it in photoreceptor cells. The similar phenotypes found in *Ttll5*$^{-/-}$ and *Rpgr*$^{-/-}$ mice strongly argue for the critical role of glutamylation for this functionalization. The posttranslational addition of negatively charged glutamates can act as an electrostatic tuner to modulate the binding affinity of the modified protein for interacting partners (28). Such a mechanism has been shown for microtubule associated proteins (27, 29) and is likely relevant for histone chaperones Nap1 (51), which are also known to be glutamylated in vivo. Interestingly, RPGR^{ORF15} function is preserved as long as the protein is still able to be glutamylated, even after substantially lowering glutamate content by shortening the Glu-rich region by a third (Fig. 5), which substantially lowers glutamate content (17, 18). These observations suggest that it is the branched glutamate structure added posttranslationally by TTLL5 that is required for RPGR^{ORF15} function. These branched glutamates could act as signals for the recruitment of key regulatory factors to RPGR^{ORF15}. Past attempts at identifying an RPGR interactome used recombinant nonglutamylated forms of RPGR^{ORF15} and proved unproductive. Future studies could benefit from our findings by incorporating glutamylated forms of RPGR^{ORF15} as affinity ligands in search of novel binding partners, which may yield fresh insight into RPGR functions.

Materials and Methods

All animal care and procedures were approved by the Animal Care and Use Committees at the National Institutes of Health. Mice were used in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology. Normal human donor eyes were obtained from the National Disease Research Interchange (NDRI), in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. Immunofluorescence staining of RPGR was performed on fresh frozen mouse eyes without prior fixation as prior fixation restricts antibody access to the ciliary transition zone. Retinal sections were than postfixed with 1% formaldehyde for 5 min before continuing with blocking and antibody staining. For yeast two-hybrid protein interaction screen, human RPGR^{ORF15} C-terminal basic domain (BD) was used as bait. Further details are in SI Materials and Methods including experimental procedures and reagents.

ACKNOWLEDGMENTS. We thank Dr. Stoney Simons, Jr. (National Institute of Diabetes and Digestive and Kidney Diseases) for transferring the *Ttll5*$^{-/-}$ mice to A.R.-M., Dr. Robert Fariss [National Eye Institute (NEI)] for help with confocal microscopy, Dr. Mones Abu-Asab (NEI) for help with TEM, Dr. Suresh Sharma (NEI) for advice on statistics, Suja Hiriyanna (NEI) and Dr. Wenhan Yu (NEI) for AAV vector production, Yide Mi (NEI) for colony management, Megan Kopera (NEI) and Hideko Takahashi (NEI) for rederivation of *Ttll5* mice to the C57BL/6J background, Drs. Heather Narver and Brian Wilgenburg [National Institute of Neurological Disorders and Stroke (NINDS)] for veterinarian service and *Ttll5* mouse colony management, and Arturo Rivera (NEI) for help with genotyping. This work was supported by the intramural programs of the NEI and NINDS (to T.L. and A.R.-M., respectively).

1. Berson EL (1993) Retinitis pigmentosa. The Friedenwald Lecture. *Invest Ophthalmol Vis Sci* 34(5):1659–1676.
2. Bader I, et al. (2003) X-linked retinitis pigmentosa: RPGR mutations in most families with definite X linkage and clustering of mutations in a short sequence stretch of exon ORF15. *Invest Ophthalmol Vis Sci* 44(4):1458–1463.
3. Pelletier V, et al. (2007) Comprehensive survey of mutations in RP2 and RPGR in patients affected with distinct retinal dystrophies: Genotype-phenotype correlations and impact on genetic counseling. *Hum Mutat* 28(1):81–91.
4. Branham K, et al. (2012) Mutations in RPGR and RP2 account for 15% of males with simplex retinal degenerative disease. *Invest Ophthalmol Vis Sci* 53(13):8232–8237.
5. Churchill JD, et al. (2013) Mutations in the X-linked retinitis pigmentosa genes RPGR and RP2 found in 8.5% of families with a provisional diagnosis of autosomal dominant retinitis pigmentosa. *Invest Ophthalmol Vis Sci* 54(2):1411–1416.
6. Sandberg MA, Rosner B, Weigel-DiFranco C, Dryja TP, Berson EL (2007) Disease course of patients with X-linked retinitis pigmentosa due to RPGR gene mutations. *Invest Ophthalmol Vis Sci* 48(3):1298–1304.
7. Megaw RD, Soares DC, Wright AF (2015) RPGR: Its role in photoreceptor physiology, human disease, and future therapies. *Exp Eye Res* 138:32–41.
8. Vervoort R, et al. (2000) Mutational hot spot within a new RPGR exon in X-linked retinitis pigmentosa. *Nat Genet* 25(4):462–466.
9. Yan D, et al. (1998) Biochemical characterization and subcellular localization of the mouse retinitis pigmentosa GTPase regulator (mRpgr). *J Biol Chem* 273(31):19656–19663.
10. Hong DH, et al. (2003) RPGR isoforms in photoreceptor connecting cilia and the transitional zone of motile cilia. *Invest Ophthalmol Vis Sci* 44(6):2413–2421.
11. Hong DH, et al. (2000) A retinitis pigmentosa GTPase regulator (RPGR)-deficient mouse model for X-linked retinitis pigmentosa (RP3). *Proc Natl Acad Sci USA* 97(7):3649–3654.

12. Vervoort R, Wright AF (2002) Mutations of RPGR in X-linked retinitis pigmentosa (RP3). *Hum Mutat* 19(5):486–500.
13. Breuer DK, et al. (2002) A comprehensive mutation analysis of RP2 and RPGR in a North American cohort of families with X-linked retinitis pigmentosa. *Am J Hum Genet* 70(6):1545–1554.
14. Sharon D, Sandberg MA, Rabe VW, Stillberger M, Dryja TP, Berson EL (2003) RP2 and RPGR mutations and clinical correlations in patients with X-linked retinitis pigmentosa. *Am J Hum Genet* 73(5):1131–1146.
15. Jacobi FK, Karra D, Broghammer M, Blin N, Pusch CM (2005) Mutational risk in highly repetitive exon ORF15 of the RPGR multidisease gene is not associated with haplotype background. *Int J Mol Med* 16(6):1175–1178.
16. Karra D, Jacobi FK, Broghammer M, Blin N, Pusch CM (2006) Population haplotypes of exon ORF15 of the retinitis pigmentosa GTPase regulator gene in Germany : Implications for screening for inherited retinal disorders. *Mol Diagn Ther* 10(2):115–123.
17. Pawlyk BS, et al. (2016) Photoreceptor rescue by an abbreviated human RPGR gene in a murine model of X-linked retinitis pigmentosa. *Gene Ther* 23(2):196–204.
18. Hong DH, Pawlyk BS, Adamian M, Sandberg MA, Li T (2005) A single, abbreviated RPGR-ORF15 variant reconstitutes RPGR function in vivo. *Invest Ophthalmol Vis Sci* 46(2):435–441.
19. Deng WT, et al. (2015) Stability and safety of an AAV vector for treating RPGR-ORF15 X-linked retinitis pigmentosa. *Hum Gene Ther* 26(9):593–602.
20. Thompson DA, et al. (2012) Rd9 is a naturally occurring mouse model of a common form of retinitis pigmentosa caused by mutations in RPGR-ORF15. *PLoS One* 7(5):e35865.
21. Hong DH, Li T (2002) Complex expression pattern of RPGR reveals a role for purine-rich exonic splicing enhancers. *Invest Ophthalmol Vis Sci* 43(11):3373–3382.
22. Hong DH, Yue G, Adamian M, Li T (2001) Retinitis pigmentosa GTPase regulator (RPGRr)-interacting protein is stably associated with the photoreceptor ciliary axoneme and anchors RPGR to the connecting cilium. *J Biol Chem* 276(15):12091–12099.
23. Song Y, Brady ST (2015) Post-translational modifications of tubulin: Pathways to functional diversity of microtubules. *Trends Cell Biol* 25(3):125–136.
24. Janke C, Rogowski K, van Dijk J (2008) Polyglutamylation: A fine-regulator of protein function? 'Protein Modifications: beyond the usual suspects' review series. *EMBO Rep* 9(7):636–641.
25. Garnham CP, Roll-Mecak A (2012) The chemical complexity of cellular microtubules: Tubulin post-translational modification enzymes and their roles in tuning microtubule functions. *Cytoskeleton (Hoboken)* 69(7):442–463.
26. van Dijk J, et al. (2007) A targeted multienzyme mechanism for selective microtubule polyglutamylation. *Mol Cell* 26(3):437–448.
27. Bonnet C, et al. (2001) Differential binding regulation of microtubule-associated proteins MAP1A, MAP1B, and MAP2 by tubulin polyglutamylation. *J Biol Chem* 276(16):12839–12848.
28. Roll-Mecak A (2015) Intrinsically disordered tubulin tails: Complex tuners of microtubule functions? *Semin Cell Dev Biol* 37:11–19.
29. Valenstein ML, Roll-Mecak A (2016) Graded control of microtubule severing by tubulin glutamylation. *Cell* 164(5):911–921.
30. Ikegami K, et al. (2007) Loss of alpha-tubulin polyglutamylation in ROSA22 mice is associated with abnormal targeting of KIF1A and modulated synaptic function. *Proc Natl Acad Sci USA* 104(9):3213–3218.
31. Rogowski K, et al. (2010) A family of protein-deglutamylating enzymes associated with neurodegeneration. *Cell* 143(4):564–578.
32. Ikegami K, Sato S, Nakamura K, Ostrowski LE, Setou M (2010) Tubulin polyglutamylation is essential for airway ciliary function through the regulation of beating asymmetry. *Proc Natl Acad Sci USA* 107(23):10490–10495.
33. Sergouniotis PI, et al.; UCL-Exomes Consortium (2014) Biallelic variants in TTLL5, encoding a tubulin glutamylase, cause retinal dystrophy. *Am J Hum Genet* 94(5):760–769.
34. Lee GS, et al. (2013) Disruption of Ttll5/stamp gene (tubulin tyrosine ligase-like protein 5/SRC-1 and TIF2-associated modulatory protein gene) in male mice causes sperm malformation and infertility. *J Biol Chem* 288(21):15167–15180.
35. Wolff A, et al. (1992) Distribution of glutamylated alpha and beta-tubulin in mouse tissues using a specific monoclonal antibody, GT335. *Eur J Cell Biol* 59(2):425–432.
36. Gagnon C, et al. (1996) The polyglutamylated lateral chain of alpha-tubulin plays a key role in flagellar motility. *J Cell Sci* 109(Pt 6):1545–1553.
37. Kann ML, Soues S, Levilliers N, Fouquet JP (2003) Glutamylated tubulin: Diversity of expression and distribution of isoforms. *Cell Motil Cytoskeleton* 55(1):14–25.
38. Magiera MM, Janke C (2013) Investigating tubulin posttranslational modifications with specific antibodies. *Methods Cell Biol* 115:247–267.
39. Rüdiger AH, Rüdiger M, Wehland J, Weber K (1999) Monoclonal antibody ID5: Epitope characterization and minimal requirements for the recognition of polyglutamylated alpha- and beta-tubulin. *Eur J Cell Biol* 78(1):15–20.
40. van Dijk J, et al. (2008) Polyglutamylation is a post-translational modification with a broad range of substrates. *J Biol Chem* 283(7):3915–3922.
41. Onikubo T, et al. (2015) Developmentally regulated post-translational modification of nucleoplasmin controls histone sequestration and deposition [published online ahead of print March 11, 2015]. *Cell Rep* doi:10.1016/j.cetrep.2015.02.038.
42. Shu X, et al. (2005) RPGR ORF15 isoform co-localizes with RPGRIP1 at centrioles and basal bodies and interacts with nucleophosmin. *Hum Mol Genet* 14(9):1183–1197.
43. Zhao Y, et al. (2003) The retinitis pigmentosa GTPase regulator (RPGR)- interacting protein: Subserving RPGR function and participating in disk morphogenesis. *Proc Natl Acad Sci USA* 100(7):3965–3970.
44. Garnham CP, et al. (2015) Multivalent microtubule recognition by tubulin tyrosine ligase-like family glutamylases. *Cell* 161(5):1112–1123.
45. Zahid S, et al. (2013) Phenotypic conservation in patients with X-linked retinitis pigmentosa caused by RPGR mutations. *JAMA Ophthalmol* 131(8):1016–1025.
46. Ebenezer ND, et al. (2005) Identification of novel RPGR ORF15 mutations in X-linked progressive cone-rod dystrophy (XLCORD) families. *Invest Ophthalmol Vis Sci* 46(6): 1891–1898.
47. Yang Z, et al. (2002) Mutations in the RPGR gene cause X-linked cone dystrophy. *Hum Mol Genet* 11(5):605–611.
48. Thiadens AA, et al. (2011) Clinical course of cone dystrophy caused by mutations in the RPGR gene. *Graefes Arch Clin Exp Ophthalmol* 249(10):1527–1535.
49. Lee JE, et al. (2012) CEP41 is mutated in Joubert syndrome and is required for tubulin glutamylation at the cilium. *Nat Genet* 44(2):193–199.
50. Pathak N, Austin CA, Drummond IA (2011) Tubulin tyrosine ligase-like genes ttll3 and ttll6 maintain zebrafish cilia structure and motility. *J Biol Chem* 286(13):11685–11695.
51. Miller KE, Heald R (2015) Glutamylation of Nap1 modulates histone H1 dynamics and chromosome condensation in Xenopus. *J Cell Biol* 209(2):211–220.
52. Mehalow AK, et al. (2003) CRB1 is essential for external limiting membrane integrity and photoreceptor morphogenesis in the mammalian retina. *Hum Mol Genet* 12(17): 2179–2189.
53. Mattapallil MJ, et al. (2012) The Rd8 mutation of the Crb1 gene is present in vendor lines of C57BL/6N mice and embryonic stem cells, and confounds ocular induced mutant phenotypes. *Invest Ophthalmol Vis Sci* 53(6):2921–2927.
54. Yang J, et al. (2002) Rootletin, a novel coiled-coil protein, is a structural component of the ciliary rootlet. *J Cell Biol* 159(3):431–440.
55. Wu Z, et al. (2015) A long-term efficacy study of gene replacement therapy for RPGR-associated retinal degeneration. *Hum Mol Genet* 24(14):3956–3970.
56. Khani SC, et al. (2007) AAV-mediated expression targeting of rod and cone photoreceptors with a human rhodopsin kinase promoter. *Invest Ophthalmol Vis Sci* 48(9): 3954–3961.