In-frame deletion in a novel centrosomal/ciliary protein CEP290/NPHP6 perturbs its interaction with RPGR and results in early-onset retinal degeneration in the rd16 mouse

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Centrosome- and cilia-associated proteins play crucial roles in establishing polarity and regulating intracellular transport in post-mitotic cells. Using genetic mapping and positional candidate strategy, we have identified an in-frame deletion in a novel centrosomal protein CEP290 (also called NPHP6), leading to early-onset retinal degeneration in a newly identified mouse mutant, rd16. We demonstrate that CEP290 localizes primarily to centrosomes of dividing cells and to the connecting cilium of retinal photoreceptors. We show that, in the retina, CEP290 associates with several microtubule-based transport proteins including RPGR, which is mutated in ~15% of patients with retinitis pigmentosa. A truncated CEP290 protein (ΔCEP290) is detected in the rd16 retina, but in considerably reduced amounts; however, the mutant protein exhibits stronger association with specific RPGR isoform(s). Immunogold labeling studies demonstrate the redistribution of RPGR and of phototransduction proteins in the photoreceptors of rd16 retina. Our findings suggest a critical function for CEP290 in ciliary transport and provide insights into the mechanism of early-onset photoreceptor degeneration.

INTRODUCTION

The centrosome is a highly specialized organelle consisting of a pair of centrioles surrounded by amorphous proteinaceous matrix, the pericentriolar material (PCM) (1). It serves as the primary microtubule organizing center of the cell by orchestrating nucleation, anchoring, and release of microtubules during cell division and chromosome segregation (2). In post-mitotic cells, such as photoreceptors, one of the centrioles (basal body) migrates to the base of the cell

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membrane, recruits intraflagellar transport proteins and microtubule motors and nucleates the assembly of primary cilia (3). Owing to the involvement of primary cilia in diverse cellular processes, mutations in centrosomal/ciliary proteins result in human disorders with a wide phenotypic spectrum, including randomization of body symmetry, obesity, cystic kidney diseases and retinal degeneration (4).

The photoreceptors are highly polar neurons with a distinct inner and an outer segment; the outer segment is a specialized sensory cilium linked to the inner segment by the connecting cilium. The photoreceptor polarity is established by unique distribution of phototransduction proteins to the outer segments from their site of synthesis in the inner segment (5). Approximately 10% of the outer segments are turned over each day, with new discs being formed proximally and shed distally. The proteins destined for outer segments are believed to dock at the basal bodies to be transported distally via the connectingcilium (6,7). Perturbations in the intersegmental cilary transport are associated with retinal degeneration (7,8).

Animal models of spontaneous retinal degeneration provide insights into pathological mechanism(s) of disease progression and help in designing therapeutic strategies. Moreover, identification of disease-associated genes in mice has led to the discovery of corresponding genes that cause retinal degeneration in humans. For example, identification of retinal degeneration 1 (rd1), retinal degeneration 7 (rd7) and retinal degeneration slow (rds) mouse models led to the identification of β-phosphodiesterase, photoreceptor-specific nuclear receptor and peripherin-rds mutations, respectively, which are associated with inherited retinopathies (9–14). Additional retinal mutants have now been described by screening genetically diverse but inbred strains of mice at The Jackson Laboratory (15).

Here, we report the identification and characterization of the rd16 mouse, which exhibits early-onset retinal degeneration with autosomal recessive inheritance. We show that the rd16 mouse carries an in-frame deletion in a novel centrosomal protein, CEP290. The CEP290 protein also localizes to the connectingcilium of photoreceptors and associates with several ciliary and centrosomal proteins, including RPGR. In the rd16 retina, we observe altered interaction of RPGR and mutant CEP290 and a redistribution of RPGR and phototransduction proteins. Our findings suggest that CEP290 plays an important role in protein trafficking and shed light on the pathways leading to photoreceptor degeneration, a major cause of inherited blindness in developed countries.

RESULTS

Clinical and histological examination of the rd16 mouse

The phenotype of homozygote rd16 mice can be distinguished from wild-type (WT) animals by the appearance of white retinal vessels at 1 month and large pigment patches at 2 months of age (Fig. 1A). Electroretinograms under dark- and light-adapted conditions indicate a considerable deterioration of rod and cone functions in the rd16 homozygotes compared with the WT as early as postnatal (P) day 18 (Fig. 1B). Light microscopy of the rd16/rd16 retina shows degeneration of outer segments and reduction in the thickness of the outer nuclear layer as early as postnatal day 19 and progresses with age. Little or no change was observed in other cellular layers (Fig. 1C).

Cep290 is mutated in the rd16 mouse

By linkage analysis of back-crossed mice, we mapped the causative gene defect in rd16 to chromosome 10 in the genomic region flanked by D10Mit244 (99.4 M) and D10Nds2 (105 M) (Fig. 2A and B). In silico analysis of the critical region revealed over 30 putative expressed sequences, which were then examined for differential expression in mouse photoreceptors using gene expression profiles generated by us (16) or others (17). We found that the expression of one of the hypothetical genes, BC004690, was increased nearly 3-fold during rod maturation (P2–P6). Its expression was dramatically reduced in the Ctx−/− mice in which photoreceptors fail to develop (18) and in the rodless, cone-enriched retina of Nrl−/− mice (19) (data not shown). Real-time PCR analysis using primer pair F1–R1 derived from BC004690 (Supplementary Material, Table S1) validated the gene-profiling data (Fig. 2C and D).

Further in silico analysis revealed that BC004690 is part of the mouse Cep290 gene (exons 27–48), which encodes a protein similar to human centrosomal protein CEP290 (20). Given that mutations in certain centrosomal proteins may result in retinal degeneration owing to ciliary dysfunction in photoreceptors (4), we screened the Cep290 gene for possible mutations in the rd16 mouse. Initial RT-PCR analysis using the F1–R1 primer pair did not amplify any product (data not shown); yet another primer set (F2–R2; Supplementary Material, Table S1) validated the gene-profiling data (Fig. 2C and D).

Domain composition of CEP290

The Cep290 gene, spanning over 85 kb and 52 exons, encodes a putative protein of 2472 amino acids (apparent molecular weight 290 kDa). To investigate the domain structure of CEP290, we scanned the MotifScan and SMART protein databases (www.expasy.org) and identified at least nine coiled-coil domains and a C-terminal myosin-tail homology domain, which provides a structural backbone to the myosin motor (Fig. 2H). Moreover, CEP290 exhibits significant similarity to SMC (structural maintenance of chromosomes) chromosomal segregation ATPases (21), six RepA/Rep+ protein motifs KID, glycine-rich ATP/GTP-binding site motif (P-loop) involved in the binding of motor proteins to the nucleotides and the transforming acidic coiled-coil (TACC) domain involved in microtubule organization by centrosomal proteins. A majority of the myosin-tail homology region is deleted in rd16 mouse (see Fig. 2H and blue region in
Figure 1. Examination of the rd16 mouse retina. (A) Fundus photographs of WT C57BL/6J mouse and the rd16 homozygote mutants (rd16/rd16) demonstrating retinal degeneration at 1 month of age and at 2 months. (B) ERG responses of WT and mutant (rd16/rd16) mice under dark- (SCOTOPIC) and light- (PHOTOPIC) adapted conditions. Arrows indicate the A-wave and arrowheads the B-wave. (C) Histology of retina of WT and rd16 homozygotes mice at indicated ages. RPE, retinal pigment epithelium; OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.
Supplementary Material, Fig. S1). CLUSTALW analysis shows strong evolutionary conservation of the CEP290 protein, with orthologs in *Danio rerio* and *Anopheles gambiae* (Supplementary Material, Fig. S1).

**Expression and localization of CEP290 in mouse retina**

A monoclonal antibody, 3G4 (22), against CEP290 recognized a band at ~290 kDa in protein extracts from different tissues of WT mice as well as in COS1 cells transfected with a full-length myc-tagged CEP290 construct (data not shown). We also generated a polyclonal antibody against two peptides corresponding to the mouse CEP290 protein; this antibody also recognized the CEP290 protein in transfected COS-1 cells (Fig. 3A). Immunoblot analysis revealed a fainter band of faster mobility (ΔCEP290) in retinal extracts from the rd16 mouse compared with the 290 kDa band in WT (Fig. 3B). Additional bands of low molecular mass are also...
observed in bovine retina extracts (our unpublished data). On the basis of this and in silico analysis, we suggest that these bands represent alternately spliced isoforms of CEP290.

We then investigated the localization of CEP290 in mouse retina by immunofluorescence and immunogold microscopy. CEP290 is localized primarily to the connecting cilium of mouse photoreceptors, although some labeling is detected in the inner segments (Fig. 3C; Supplementary Material, Fig. S2). Connecting cilium staining of CEP290 was also observed in dissociated rod photoreceptors of mouse retina, as determined by co-localization with acetylated alpha-tubulin (data not shown).

**CEP290 localizes to centrosomes in a dynein-independent manner**

Immunocytochemical analysis using the CEP290 antibody revealed that CEP290 co-localizes with the centrosomal and pericentriolar matrix markers gamma-tubulin and PCM1 (2) at the centrosomes of mouse kidney inner medullary collecting duct (IMCD-3) (Fig. 3D). Co-localization with PCM1 is reminiscent of the staining pattern of BBS4, a ciliary/centrosomal protein involved in microtubule dynamics (23). We also find consistent co-labeling of CEP290 with gamma-tubulin through different stages of cell cycle (Fig. 3E).
We next queried how CEP290 is recruited or assembled at the centrosomes. Previous studies have shown that microtubule depolymerization using nocodazole does not alter centrosomal localization of CEP290 (20). Given that a number of centrosomal proteins, including RPGR-ORF15 and PCM1, are anchored at the centrosomes via the functional dynein–dynactin molecular motor, whereas others such as γ-tubulin and BBS6 are not (24,25), we examined whether localization of CEP290 depends on dynein–dynactin motor by overexpressing the p50-dynamitin subunit of the dynactin complex (26). Like γ-tubulin, the localization of CEP290 at centrosomes is not altered in cells overexpressing p50-dynamitin (Fig. 3F). Our data suggest that functional microtubule motor or polymerized microtubules are not necessary to maintain CEP290 at the centrosomes; however, we cannot rule out their requirement for the recruitment of newly synthesized CEP290 to the centrosomes.

CEP290 associates with RPGR in mammalian retina

Given that RPGR, a ciliary/centrosomal protein (27–29) mutations in which are detected in retinitis pigmentosa (30–32), interacts with centrosomal disease-associated proteins (29,33–35), we hypothesized that CEP290 may also associate with RPGR and its interacting proteins and participate in common functional pathways. The ORF15CP antibody against the retina-enriched RPGR-ORF15 isoform(s) (28,29,34) was able to precipitate very low amounts of CEP290 from WT mouse retinal extracts (Fig. 4A). Reverse co-immunoprecipitation using the 3G4 antibody detected RPGR-ORF15 upon immunoblotting (Fig. 4B). Notably, yeast two-hybrid experiments do not appear to reveal a direct interaction of CEP290 with RPGR (data not shown).

We next performed co-immunoprecipitation experiments using rd16 retinal extracts. RPGR-ORF15 could recruit over 50 times higher levels of the ΔCEP290 protein from rd16 retina compared with the WT protein (Fig. 4A). Reverse immunoprecipitation pulled down a few, but not all, RPGR-ORF15 isoforms from the rd16 retina (Fig. 4B). Consistent with this, the endogenous CEP290 co-localizes with RPGR-ORF15 in IMCD-3 cells (Fig. 4C) and dissociated mouse rod photoreceptors (data not shown).

CEP290 is part of selected centrosomal and microtubule-associated protein complex(es)

To evaluate whether CEP290 and ΔCEP290 are part of multi-protein complex(es) with other centrosomal and microtubule-associated motor assemblies, some of which may also overlap with RPGR-ORF15-containing complexes, we performed additional co-immunoprecipitation experiments using mouse or bovine retinal extracts. Our data show that CEP290 is present in complex with RPGR-interacting protein 1 (RPGRIP1), dynactin subunits p150Glued and p50-dynamitin, kinesin subunit KIF3A, kinesin-associated protein (KAP3), γ-tubulin, PCM1, centrin, pericentrin and ninein, but not with nucleophosmin (NPM), or Nephrocystin-5 (NPHP5) (Fig. 4D and E). Dynexin subunits are not detectable probably because of the low abundance or instability of the dynein–dynactin interaction. As RPGR-ORF15, CEP290 also interacts with SMC1 and 3. Varying degree of association with SMC proteins and p50-dynamitin may be due to relative abundance of the proteins. CEP290 is not associated with RP1, another ciliary protein mutated in retinopathies (36) (Fig. 4D). Similar results are obtained with rd16 as well as bovine retinal extracts (data not shown). No immunoreactive bands are detected when normal IgG is used for IP. Notably, RPGR-ORF15 interacts with NPM (28) and NPHP5 (34) but not with centrin and pericentrin (29). Our results therefore indicate that CEP290 and RPGR may perform multiple overlapping yet distinct microtubule-based transport functions in the retina.

Perturbed localization of RPGR and opsin in the rd16 retina

We next examined whether increased association of ΔCEP290 affects the localization of RPGR-ORF15 in the rd16 retina. ImmunoEM experiments revealed that RPGR-ORF15 aggregates in the inner segments of P12 rd16 retina, indicating a trafficking defect, whereas, as shown previously (29), the axoneme and basal bodies in photoreceptors of normal retinas are strongly labeled with the ORF15CP antibody (Fig. 5A–C). However, we did not observe any obvious structural defects in the connecting cilium of the rd16 retina.

Given the involvement of RPGR-ORF15 in regulating intracellular trafficking in photoreceptors (29,37), we sought to examine the effect of CEP290 mutation and/or RPGR mislocalization on the trafficking of phototransduction proteins in the retina. Immunogold EM and immunofluorescence analyses revealed redistribution of rhodopsin and arrestin throughout the plasma membrane of rd16 photoreceptors when compared with the normal outer segment localization in WT photoreceptors (Fig. 5D–F).

DISCUSSION

Photoreceptor degeneration is associated with a number of syndromic diseases, such as Senior–Loken syndrome and Bardet–Biedl syndrome (BBS) (4,34), as well as with non-syndromic inherited retinal degenerative diseases. Over 100 genetic loci for inherited retinal degenerations have been identified (see RetNet: http://www.sph.uth.tmc.edu/Retnet/). Mouse models of retinal degeneration provide candidate genes for human retinopathies and offer critical insights into disease pathogenesis. Here, we report that early-onset retinal degeneration in the rd16 mouse is associated with an in-frame deletion in a novel centrosomal protein CEP290. Our data suggest that CEP290 regulates intracellular protein trafficking and that perturbation of its function leads to mis-localization of ciliary and phototransduction proteins resulting in photoreceptor degeneration.

The defects observed in the rd16 retina are consistent with a role of CEP290 in ciliary transport processes. Because the association of ΔCEP290 with microtubule motors (dynein and kinesin-II) remains unaltered, we conclude that abnormal transport of RPGR and opsin in rd16 is not a direct effect of impaired or defective association of ΔCEP290 with the motor proteins. An intriguing finding in this study is that the reduced
amount of intact ΔCEP290 is able to associate with considerably higher amounts of specific RPGR-ORF15 isoforms. Such higher association may recruit RPGR from the connecting cilium and redistribute it in the inner segments. Consistent with the involvement of RPGR in intracellular protein trafficking, we observe redistribution of phototransduction proteins, which are potential ‘cargo’ proteins (8) being transported via the connecting cilium to the outer segments, in the rd16 retina. However, we do not rule out the possibility of a direct association of CEP290 (or ΔCEP290) with rhodopsin and/or arrestin. Attempts to directly identify associations of cargo proteins with the ciliary transport assemblies have been unsuccessful so far perhaps because of weak or transient interactions (29,38,39).

Our findings suggest that misrouting of phototransduction proteins is the underlying cause of photoreceptor degeneration in the rd16 retina. Such redistribution of opsins in photoreceptor inner segments owing to impaired ciliary transport has been detected previously in Kif3a conditional mutants, Tg737orpk, Bbs2−/− and Bbs4−/− mice (7,40–42) as well as some other...
types of retinal degenerations (e.g. rd1, rds/peripherin and opsin mutants, Q334ter and P347S, but not P23H) (43,44). The mislocalization of opsins is possibly due to redistribution of RPGR-ORF15 to the inner segments. The support for this hypothesis comes from a previous observation that the connecting cilium staining of RPGR is impaired in the Rpgrip1<sup>−/−</sup> mice, which also develop early-onset photoreceptor degeneration owing to abrogated opsin trafficking (45). Notably, the
**Materials and Methods**

**Animal Studies**

The mice were bred and maintained in standardized conditions at The Jackson Laboratory and Kellogg Eye Center. The use of mice was approved by the Institutional Animal Care and Use Committee. The rd16 mouse was discovered in strain BXD-24/Ty at about F140 generation and the mutation was performed as described (29). The rabbit polyclonal CEP290 antibody was generated (Invitrogen) against the mouse sequence 5′SKRLKQQQYRAENQ530 and 2457SEHS-EDGESPHSPPIY2472. Rabbit polyclonal antibodies to RGR, RGRIP1 and NPHP5 have been described previously (29,34). Antibodies against acetylated α-tubulin, γ-tubulin, p50-dynamitin, SMC1 and SMC3 were purchased from Chemicon (Temecula, CA). Mouse anti-p150Glued antibody was obtained from BD Transduction Labs (San Jose, CA); anti-KIF3A, anti-KAP3, anti-centrin and anti-pericentrin antibodies were obtained from Sigma and anti-ninein was from BioLegend (San Diego, CA). Anti-RP1 antibody was generously provided by Dr. Eric A. Pierce, anti-NPM antibody by Dr Alan F. Wright and anti-PCM1 by Dr A. Merdes.

**Cell Culture and Immunolocalization**

Kidney m-IMCD-3 cells (American Type Culture Collection, Manassas, VA; CRL 2123) were grown in six well plates and transfected with p50-dynamitin expression construct (a gift from Dr Trina A. Schroer) using Fugene-6 reagent (Roche). Experimental details about immunocytochemistry and immunogold EM procedures are as described (29). Immunofluorescence microscopy of retinal sections for rhodopsin and arrestin was performed using published procedures (50). For immunolabeling of CEP290, eyes were fixed in methanol, and sections were labeled with 3G4, followed by goat anti-mouse conjugated to AlexaFluor 488.

**Supplementary Material**

Supplementary Material is available at HMG Online.

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