CEP290 alleles in mice disrupt tissue-specific cilia biogenesis and recapitulate features of syndromic ciliopathies

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Abstract

Distinct mutations in the centrosomal-cilia protein CEP290 lead to diverse clinical findings in syndromic ciliopathies. We show that CEP290 localizes to the transition zone in ciliated cells, precisely to the region of Y-linkers between central microtubules and plasma membrane. To create models of CEP290-associated ciliopathy syndromes, we generated Cep290ko/ko and Cep290gt/gt mice that produce no or a truncated CEP290 protein, respectively. Cep290ko/ko mice exhibit early vision loss and die from hydrocephalus. Retinal photoreceptors in Cep290ko/ko mice lack connecting cilia, and ciliated ventricular ependyma fails to mature. The minority of Cep290ko/ko mice that escape hydrocephalus demonstrate progressive kidney pathology. Cep290gt/gt mice die at mid-gestation, and the occasional Cep290gt/gt mouse that survives shows hydrocephalus and severely cystic kidneys. Partial loss of CEP290-interacting ciliopathy protein MKKS mitigates lethality and renal pathology in Cep290gt/gt mice. Our studies demonstrate domain-specific functions of CEP290 and provide novel therapeutic paradigms for ciliopathies.

Introduction

Defects in primary cilia biogenesis or associated functions can lead to pleiotropic phenotypes in syndromic diseases, collectively termed ‘ciliopathies’ (1,2). Recent studies have begun to unravel complex genotype–phenotype relationships in ciliopathies that are caused by mutations in dozens of different genes (3–5). Affected tissues include the retina (6), brain (7) and kidney (8), as well as abnormalities at the organismal level including situs inversus (9) and obesity (10). Unique clusters of symptoms have acquired clinical diagnoses such as Joubert (11,12), Meckel–Gruber (13) and Bardet–Biedl syndrome (BBS) and nephronophthisis (14).

Retinal degeneration is a highly penetrant component in most syndromic ciliopathies; one type of non-syndromic ciliopathy is Leber congenital amaurosis (LCA) that includes childhood blindness caused by retinal dysfunction (15,16).

Pioneering studies in Chlamydomonas first linked intraflagellar transport with genes related to sensory ciliopathy phenotypes in mammals (17). Although the cilia in Chlamydomonas are motile, in contrast to the sensory cilia of vertebrates, basic genetic principles of microtubule organization and cilia function appear to be highly conserved (18). The sensory or primary cilium plays an important developmental role (3,19), serving as a signaling center.
(20,21) and mechanosensor (22). In sensory tissues, cilia are modified to mediate specific functions such as establishment of the stereocilia bundles, which transmit sound wave frequency and intensity in the cochlea, transduction of photons by photoreceptors, and ligand–receptor interactions in the olfactory epithelium. Some of the physiological complexity of cilia function arises from the diversity of signaling pathways active within the cilium, and the fact that different signaling pathways might operate in specific tissues. For example, Shh and canonical Wnt signaling, non-canonical Wnt/planar cell polarity and calcium signaling/flow are associated with organogenesis and/or physiological functions in multiple tissues (9,22–30).

A fundamental question is how cilia dysfunction affects such a broad spectrum of tissues/organs and results in wide range of pathologies. One explanation is that cilia contribute to disparate functions in individual organs (31). One ciliopathy protein may exert distinct impact on specific ciliated cells by virtue of alternate interactors, different signaling pathways or unique and tissue-specific functions. Among dozens of proteins contributing to major ciliopathies, a prominent hub is CEP290 (15), which is shown to localize to the ciliary transition zone and has numerous interacting partners (32–34). CEP290 has been a target of intense scrutiny since its discovery as the causal gene for Joubert syndrome in humans (35,36) and congenital blindness in rd16 mice (37). Mutations in CEP290 are now shown to be a frequent cause of LCA (38) and diverse syndromic ciliopathies (39).

As CEP290 mutations cause BBS and a range of other syndromic phenotypes (15), CEP290 is expected to exert important connectivity to the BBSome and many ciliary proteins (40). Indeed, CEP290 interacts with CEP72 and PCM-1 for ciliary localization of Rb8a to promote ciliogenesis (42), a function antagonized by Cep290rd16 (37). The domain deleted in Cep290rd16 mouse is not null and produces low levels of a shorter CEP290 protein (37). The domain deleted in Cep290rd16 interacts with Mkks (Bbs6), another ciliopathy protein that causes BBS and is part of the chaperonin complex (48–50). Interestingly, decreasing Mkks expression in the Cep290d16 mouse leads to partial rescue of photoreceptor structure and function (33).

CEP290 is expected to have diverse functions in mammalian ciliated cells/tissues as suggested by its extensive interaction network and association with numerous clinical phenotypes (15,39). To understand CEP290 function and its role in human syndromic ciliopathies, we generated null (Cep290ko) and truncated (Cep290δ77) alleles of Cep290 in mice. Our findings suggest that CEP290 is critical for formation of the ciliary transition zone in several tissues, including photoreceptors and ventricular ependyma. Dominant effects of a truncated Cep290 protein are detected in kidney and in viability, both of which are partially rescued by decreasing Mkks. Our studies further elucidate the complex actions of CEP290 in cilia and provide insights into the mechanism of pathology in human CEP290-associated ciliopathies, particularly Joubert and Meckel–Gruber syndromes.

Results

CEP290 localizes to the transition zone in cilia, and specifically to the Y-linker region of photoreceptor connecting cilium

Using both immunolocalization and biochemical approaches, CEP290 had been localized to the basal body (44,51), transition zone (33,37,52) and pericentriolar satellites (41,53). To clarify and validate CEP290 localization, we performed immunostaining in a number of ciliated mouse tissues (Fig. 1). In choroid plexus, we identified CEP290 at the apical surface, distal to rootletin, in an array corresponding to the base of the multiple cilia (Fig. 1A), which in these cells transition from motile to sensory function in the neonatal period (54,55). CEP290 also localized to the base of motile cilia in the ventricular ependymal lining and in trachea between rootletin and acetylated alpha-tubulin, a marker of the ciliary axoneme (Fig. 1B and C). In the kidney, CEP290 was detected at the base of the primary cilia (Fig. 1D). In photoreceptors, CEP290 is in a location consistent with the connecting cilium (Fig. 1E).

We confirmed the localization of CEP290 in the connecting cilium of adult wild-type photoreceptors using immuno-electron microscopy. In longitudinal sections, CEP290 immunogold particles were present in the transition zone region and not in the basal body (Fig. 1F). In cross-sections of the cilium, we could more precisely localize CEP290 to the region of the Y-linkers, between the central 9+0 ring of microtubules and the plasma membrane (Fig. 1G and H).

Phenotype of Cep290-ko mice parallels Joubert syndrome in humans

The Cep290 gene spans 85 kb on mouse Chromosome 10 and includes 54 exons (Fig. 2A). To create a null allele, Cep290 exons 1–4 were replaced in embryonic stem (ES) cells with a β-gal-neoCas-sette by homologous recombination. Southern blot analysis of ES cell clones showed appropriate targeting (Fig. 2B), and PCR genotyping could discriminate the wild-type (295 bp) and knockout alleles (391 bp) (Fig. 2C). No CEP290 protein was detectable in the knockout retina by immunoblotting (Fig. 2D).

About 80% of the Cep290ko mice died between birth and weaning (Supplementary Material, Table S1) likely due to lethal hydrocephalus (clinical ventriculomegaly) that became apparent at 2–3 weeks of age, as manifested by a domed head (asterisk, Fig. 3A). Subclinical ventriculomegaly was observed in mutant pups at P3 (data not shown). The Cep290ko mice that did not develop overt hydrocephalus by 1 month of age survived with normal fertility and lifespan for as long as 2 years. We noticed that a mixed genetic background of C57BL/6 and 129/SvJ resulted in higher survival than either pure inbred background, in which no homozygous knockout mice survived beyond the N2 or N3 backcross generation (data not shown). A higher number of Cep290ko mice survived on the C57BL/6 background than on 129/SvJ (Supplementary Material, Table S2); indeed, we were unable to propagate the mutation on the 129/SvJ line beyond N3 backcross generation. The Cep290ko mice developed early signs of retinal pathology, including blood vessel attenuation and pigmented changes of the fundus by 2–3 months of age (data not shown). As predicted from both human CEP290 mutations and the Cep290d16 mouse, both scotopic and photopic
ERGs showed complete lack of response by 3–4 weeks of age (Fig. 3B). Similar to the Cep290rd16/rd16 mutant (33,46), profound degenerative changes were evident at Postnatal day (P)14, including a thinner outer nuclear layer (ONL), no outer segments and severely shortened inner segments; most photoreceptors were lost by P28 (Fig. 3C). To assess the cerebral pathology and cause of the domed head, we performed high-resolution MRI imaging of brains after fixation (Fig. 3D and E). Massive expansion of the lateral ventricles in Cep290ko/ko mice caused severe hydrocephalus, visible in both frontal/coronal and sagittal axes, with cortical thinning and distortion of the cerebellum and brain stem (red lines, Fig. 3E). Subclinical ventriculomegaly was also noticeable in an asymptomatic P14 Cep290ko/ko mouse (blue arrows, Fig. 3E). The kidney phenotype, featured in many Cep290-mediated ciliopathies (56,57), was slowly progressive. Kidneys appeared slightly pale and enlarged in a young adult (Fig. 3F), and, cysts were observed in Cep290ko/ko mice after 12 months of age (Fig. 3G). A relatively mild renal phenotype is consistent with Joubert syndrome (58), in which only 2% of cases exhibit renal cysts at an average age of 8 years (59).

CEP290 is required for photoreceptor cilium and outer segment biogenesis

Vision loss prompted us to closely examine the photoreceptors (Fig. 4). Using antibodies to acetylated tubulin and rootletin, markers of the ciliary axoneme and ciliary rootlet, respectively, we observed defects in inner and outer segment formation in P14 Cep290ko/ko retina (Fig. 4A). While inner segments were rudimentary, no evidence of outer segment formation was detected, demonstrating a more severe phenotype compared with Cep290rd16/rd16 retina (37). Electron microscopy studies (Fig. 4B–F) revealed complete lack of outer segments and connecting cilia in P14 Cep290ko/ko retina (red circles, Fig. 4B), although basal bodies were intact (blue circles, Fig. 4B). The 9+0 microtubule ring was...
Cep290 is essential for maturation of ventricular ependymal cilia

In ventricular cells of the Cep290ko/ko brain, primary cilia do form but show progressive developmental defects (Fig. 5A). At P0, the primary cilia, as identified by acetylated tubulin, were longer compared with control (Fig. 5B). Motile cilia emerged later and were fewer in number, but appeared longer. In addition, the surface area of each cell, outlined by ZO-1, was smaller than in wild-type cells (Fig. 5C). Scanning electron microscopy images of the ventricular surface showed the transition from single primary cilia to tufts of multiple motile cilia from P0 to P5 in the wild-type brain; yet, in the Cep290ko/ko, single primary cilia continued to grow longer with possibly delayed transition into fewer but longer tufts of cilia (Fig. 5D).

We used transmission electron microscopy to define cilogenesis defects in ventricular ependyma (Fig. 6). In P0 wild-type lateral ventricles, a single primary cilium is present per epithelial cell (Fig. 6A), and the deuterosome, a nucleation structure for biogenesis of multiple basal bodies, was occasionally visible (Fig. 6A, P0 right panel; white arrow). A docked primary ciliary vesicle surrounding the emerging primary cilium was detected in wild-type ependyma at P0 (Fig. 6A, dotted circle and red arrows). In Cep290ko/ko ependyma, however, though cilia began to form by P0, no enclosure around the cilium (the ciliary vesicle) was detected and the plasma membrane constrictors did not generate a bubble-like structure for eventual cilia emergence (Fig. 6B, red arrows). By P3, the deuterosome is surrounded by nascent basal bodies in the wild-type (Fig. 6C, left panel; white arrow) and multiple cilia emerged through the ciliary pocket (Fig. 6C, middle panel; dotted circle), which surrounded emerging ciliary tufts oriented in a similar direction (Fig. 6C, red arrows). In P3 Cep290ko/ko ependyma, few nascent basal bodies were seen congregating around the deuterosome (Fig. 6D, left panel, white arrow), and

distinct from the basal body as in wild-type photoreceptors (green circles; Fig. 4C), but failed to dock at the apical plasma membrane of the inner segment. By comparison, Cep290ko/ko retina developed both basal bodies and connecting cilia (blue and red circles, respectively, Fig. 4C), even though photoreceptors degenerated rapidly thereafter. Instead of the elongated disks of normal outer segments, the Cep290ko/ko photoreceptors developed small, round membrane vesicles between the inner segments and the microvilli of the retinal pigment epithelium (Fig. 4D). In contrast, Cep290rd16/rd16 photoreceptors had rudimentary outer segments (Fig. 4E). A cross-section view of the connecting cilia region revealed 9+0 microtubule ring in the cytoplasm of the Cep290ko/ko photoreceptors, whereas photoreceptors in Cep290ko/ko retina developed a membrane-bound connecting cilium with abnormal morphology (irregular membrane and microtubule ring structure; Fig. 4E). Thus, full-length CEP290 protein is essential for connecting cilium development.

To gain further insights, we immunostained P13 Cep290ko/ko retina with several antibodies against photoreceptor proteins, including CEP290 and RPGR, a ciliary protein that interacts with CEP290 (37) and mutations in which are a major cause of inherited retinal degeneration (60). We compared the data with the same markers in Cep290d16/d16 retina and RPgrko retina (Supplementary Material, Fig. S1). CEP290 was normally expressed in the connecting cilium of RPgrko photoreceptors, significantly reduced but still present in Cep290d16/d16, and undetectable in Cep290ko/ko retina (Supplementary Material, Fig. S1A–C). The photoreceptor organization as identified by rootletin/CROCC, which marks the ciliary rootlets in the inner segments, was noticeable in the Cep290ko/ko retina. RPGR immunostaining was absent in the RPgrko, partly detected in Cep290d16, and minimal in the Cep290ko/ko retina (Supplementary Material, Fig. S1B). RPGRIP1, another cilia protein that interacts with both CEP290 and RPGR and is associated with retinal degeneration (15), was present in the RPgrko but absent in Cep290 mutant alleles (Supplementary Material, Fig. S1C). These results suggest that Cep290 may act downstream (epistatic) to RPGR and RPGRIP1 and reaffirms its central role in photoreceptor development.
Figure 3. Inactivation of the Cep290 gene in mice leads to features of Joubert syndrome. (A) Postnatal (P)21 day Cep290\textsuperscript{ko/ko} mouse shows stunted growth and hydrocephalus (enlarged head, asterisk) compared with normal littermate. (B) Dark-adapted (scotopic) and photopic ERG recordings in Cep290\textsuperscript{ko/ko} mice around 3 weeks of age show complete lack of response under both conditions. Wild-type littermates show normal responses. (C) H&E images of control and Cep290\textsuperscript{ko/ko} retina at P14 and P28. Lower panels are enlargements of areas indicated in upper panels. At P14, note lack of outer segment formation. At P28, Cep290\textsuperscript{ko/ko} mice exhibit rapid loss of photoreceptors with only a single row of photoreceptors (likely cones) remaining, similar to Cep290\textsuperscript{rd16/rd16} (46). Original magnification 40×. (D and E). Brains of P14 and P28 wild-type and Cep290\textsuperscript{ko/ko} mice viewed by high-resolution (50 µm), T1 weighted 3D-MRI imaging. Note enlarged cerebral ventricles in the mutant (asterisks in D and E), resulting in severe thinning of the cerebral cortex. Slightly enlarged lateral ventricles in the normal-appearing P14 Cep290\textsuperscript{ko/ko} pup (blue arrows in E) reveal both that cortical thinning is secondary to hydrocephalus, and that subclinical ventriculomegaly occurs in asymptomatic knockout mice. Lower row of panels in E, sagittal views of the cerebellum from same brains shown above. Note secondary distortion of the brainstem from the enlarged ventricles. (F) Whole kidneys from adult wild-type (left) and Cep290\textsuperscript{ko/ko} (right) mice. Note slight pallor and enlargement of the knockout. (G) In each panel, cortex is positioned at the top and medulla at the bottom of the image. At 18 months of age, Cep290\textsuperscript{ko/ko} kidney shows moderate cystic tubular pathology. Scale bar = 50 µm.
Figure 4. Cep290 is required for photoreceptor ciliogenesis and outer segment morphogenesis. (A) P16 wild type and Cep290ko/ko retina labeled with acetylated α-tubulin (red) and rootletin (green, left panels) or RKIP (green, right panels). (B) EM cross-section through connecting cilia region in wild-type and Cep290ko/ko P14 retina showing isolated, membrane-bound cilia among inner and outer segment profiles. Cilia are circled in red; basal bodies in blue. In the Cep290ko/ko retina, although basal bodies form, no distinct cilia are identified. Instead, the circular 9+0 microtubule structures remain within inner segment cytoplasm and fail to extend (green circles). Thus, outer segments are likewise absent. (C) EM cross-sections through P10 Cep290rd16/rd16 photoreceptors showing connecting cilia (red) and basal bodies (blue). (D) EM longitudinal sections at P14 show connecting cilia and OS in wild type; lack of OS in Cep290ko/ko. Instead, vesicular membranes (ves) form between the RPE microvilli (mv) and the inner segments. (E) Comparison of longitudinal EM sections of P14 wild-type and Cep290rd16/rd16 photoreceptors showing rudimentary development of the outer segments in Cep290rd16/rd16 mice, a phenotype intermediate between wild type and Cep290ko/ko. (F) EM cross-sections through the connecting cilium of P14 wild type, Cep290ko/ko and Cep290rd16/rd16 photoreceptors showing a clear, isolated membrane around the connecting cilium in wild type; failure of the microtubule ring to extend from the cytoplasm in Cep290ko/ko, and malformed (oval) connecting cilia in Cep290rd16/rd16.
the cilia that emerged directly from the plasma membrane were misoriented in the absence of the ciliary pocket (Fig. 6D, red arrows). At P5, multiple cilia emerging from each wild-type cell were oriented in the same direction, exiting from the apical surface into the ventricle (Fig. 6A, P5). While each wild-type ventricular ependymal cell had a large number of cilia at P5 (Fig. 6E), the Cep290ko/ko cells revealed only a few misoriented cilia (Fig. 6F) that seemed to exit individually rather than together through the ciliary pocket (Fig. 6F). We then imaged cross-sections of P5 cilia to examine the internal structure of the axoneme. As expected, the normal 9+2 pattern of microtubule doublets was apparent in the wild type (Fig. 6G). In contrast, the Cep290ko/ko cilia had multiple abnormalities, including multiple or no central microtubules and disturbances in the number, order and structure of the outside ring of microtubules (Fig. 6H). These findings suggest a critical role of CEP290 during the emergence of ventricular ependymal cilia.

A truncation allele of Cep290 exhibits a more severe phenotype

In humans, distinct CEP290 mutations (missense, nonsense, internal deletion or frame-shift) result in varying ciliopathy phenotypes. Interestingly, the Cep290rd16/rd16 mice have a sensory cilia-restricted phenotype, and while the Cep290ko/ko does manifest as a ciliopathy, the kidney phenotype appears milder than some
human CEP290 syndromes. To expand the phenotypic spectrum, we obtained a Cep290 gene-trap (hereafter referred to as Cep290gt) ES cell line and generated mice which produce a 116 kDa protein including the N-terminal part of CEP290 at Glu1036 (encoded by the first 25 out of 55 Cep290 exons) fused to β-galactosidase (Fig. 7A and B). As shown by X-gal staining, the CEP290 protein is widely expressed (Fig. 7C). A vast majority of Cep290gt/gt embryos die between E12 and E14 (Supplementary Material, Table S3, and data not shown); however, except for pallor, mutant embryos that survive appeared grossly normal even until E17 (Fig. 7D). By

Figure 6. Defects in ventricular ependymal ciliary pocket formation and cilia orientation. (A and B) Cilia formation at P0 in wild type (A) and Cep290ko/ko (B) ventricular ependyma. Note prominent ciliary pocket in the normal cell (red circle and red arrows), and presence of a deuterosome (white arrow). In the Cep290ko/ko, the ciliary pocket either does not form or forms incompletely. (C) At P3 in wild type, basal bodies congregate around the deuterosome (white arrow) and cilia (red arrows) exit through the ciliary pocket (red circle). (D) In the knockout at P3, deuterosomes are present but basal bodies fail to congregate around them, and cilia emerge single and directly from the cell body rather than from a ciliary pocket. Moreover, when multiple cilia emerge from the same cell, they are not all oriented in the same direction. At lower power (far right), microvilli are seen to protrude but very few cilia. (E,F) Note the difference between numbers of basal bodies and emerging cilia in wild type (E) and Cep290ko/ko (F) ependyma. (G,H) Cross-sections of wild type and knockout cilia showing 9+2 microtubule configuration in wild type (G), and various defects in the knockout (H).
Figure 7. Truncation allele of Cep290 has gain of function phenotype with severe kidney disease and mid-gestation lethality. (A) Diagram of mouse Cep290 gene showing insertion site of the pGT0xfT2 gene trap vector in intron 25. (B) Immunoblot using anti-β-galactosidase antibody, showing band of expected size (≈116 kDa) for the CEP290-β-galactosidase fusion protein in the gt/+ retina. Renal levels of CEP290 expression are lower and invisible at this level of detection. GAPDH loading control is shown below. (C) Expression of CEP290 revealed by X-gal staining of tissue expressing β-galactosidase from Cep290-lacZ fusion construct. Expression is observed in epithelial tissues, including choroid plexus (enlargement shown below), ventricular ependyma containing tufts of motile cilia, airways (enlargement below), ureter (upper panel), renal tubules (lower panel), cerebellum and mid-gestation embryos. (D) Cep290<sup>gt/</sup> embryos at E13, E17; hepatic pallor is a primary visible feature. (E) P19 kidneys; rare Cep290<sup>gt/</sup> survivor with massive cystic kidney. (F) H&E kidney staining, P19 wild type and P3, P19 Cep290<sup>gt/</sup> pups, showing progressive cystic kidneys eliminating renal parenchyma (upper panels, 10×) resulting in cellular infiltrate (lower panels, 40×). (G) H&E stained P10 and P18–19 retinal sections, wild type and Cep290<sup>gt/</sup>. Note lack of outer limiting membrane (white arrow) and OS; pyknotic nuclei/thinning of the ONL, indicating rapid cell death in Cep290<sup>gt/</sup>. Original magnifications: P10 upper, 40×; P10 lower and P18–19, 60×. (H) TEM images of P14 wildtype and Cep290<sup>gt/rd16</sup> compound heterozygous retinas in longitudinal (upper) and cross sections (lower). Cross-sections of connecting cilia, red circles; basal body-cilia transition, blue circles. Abbreviations: OS, outer segments; IS, inner segments; ONL, outer nuclear layer; INL, inner nuclear layer; CC, connecting cilia.
2–3 weeks of age, the rare surviving Cep290<sup>gt/gt</sup> mice had massively dilated, pale kidneys (Fig. 7E), characterized by loss of tubules, cellular infiltrate and massive cysts replacing most of the renal cortex and outer medulla (Fig. 7F). Thus, the truncation allele (gt) causes a Meckel syndrome like phenotype. The retina of Cep290<sup>gt/gt</sup> mice showed clear abnormalities at earlier ages compared with Cep290<sup>ko/ko</sup> or Cep290<sup>1616/1616</sup> (Fig. 7G,H); for example, apparent over-proliferation of the ONL was observed by P10, along with loss of the outer limiting membrane (Fig. 7G).

**Loss of Mkks rescues the Cep290<sup>gt/gt</sup> phenotype but accentuates the Cep290<sup>ko/ko</sup> phenotype**

We previously reported mitigation of the Cep290<sup>1616/1616</sup> phenotype by allelic loss of Mkks (33). Thus, we tested whether removal of one or both alleles of Mkks would influence the severity of Cep290<sup>ko</sup> and Cep290<sup>gt</sup> alleles. Surprisingly, when combined with Mkks<sup>ko/ko</sup> alleles, we observed a partial rescue of the Cep290<sup>gt/gt</sup> embryonic lethality (Fig. 8A, Supplementary Material, Table S4) and renal pathology (Fig. 8B). In contrast, Cep290<sup>ko/ko</sup>, Mkks<sup>ko/ko</sup> embryos died before E11, as no double homoyzogotes were recovered at or after this stage (Supplementary Material, Tables S4 and S5). The Cep290<sup>ko/ko</sup>, Mkks<sup>ko/+</sup> mice survived for 2–3 weeks and exhibited severe hydrocephalus and faster retinal degeneration than Cep290<sup>ko/ko</sup> mice (Fig. 8C).

**Modeling of a CEP290 oligomeric structure in the connecting cilia**

Based on the requirement of CEP290 in photoreceptor and ventricular ependyma ciliogenesis, we sought to predict CEP290 tertiary structure to gain further insight into its possible mechanism of action at the transition zone (Supplementary Material, Fig. S2). Domain prediction of both mouse and human CEP290 identified extensive coiled-coils throughout the protein, almost to the exclusion of other domains (Supplementary Material, Fig. S2A). Only a few other cilia proteins share such a domain structure. To explore the tertiary structure of CEP290, we used GlobProt 2 (version 2.3, http://globplot.embl.de) and identified six coiled-coil domains covering the full length of the protein, interspersed with five small peptide regions exhibiting structural disorder (Supplementary Material, Fig. S2B; Table S6), which indicate five nodes that might maintain molecular flexibility of an otherwise rigid, rod-like molecule (Supplementary Material, Fig. S2C). The Multicoil 2 program (http://groups.csail.mit.edu/cb/multicoil/cgi-bin/multicoil.cgi) predicted the location of coiled-coil regions and suggested that CEP290 has the propensity to form oligomeric structures; the possible dimer, trimer and tetramer models of the DSD showed predicted coiled-coil diameters of 2.3, 2.7 and 2.9 nm, respectively (Supplementary Material, Fig. S2F and G). A monomer would have a predicted diameter of ~1.4 nm. The entire protein is predicted to have a maximum theoretical length of ~348 nm, thus forming a long, thin rod-like molecule.

In contrast, the truncated CEP290 protein of 116 kDa had a relatively high propensity to form a dimeric coiled-coil structure compared with that of the full-length trimeric protein (Supplementary Material, Fig. S2F). The structures of full-length and truncated CEP290 are interrupted by the coiled-coil vimentin-like structure (residues 696–752), which showed 29% identity to vimentin protein sequence as demonstrated by Phyre2 and SMART. The rest of the truncated CEP290 protein revealed 13% sequence identity to tropomyosin with 12 coiled-coil heptad motifs (HxxHxCxH) confirming the coiled-coil conformation. The 116 kDa protein is shortened by 58% and predicted to have a maximum length of 145 nm.

**Discussion**

Loss of CEP290 results in widespread ciliary defects in multiple tissues and accompanying disease phenotypes consistent with...
human ciliopathies. Interestingly, the CEP290 defects manifest as excessively longer primary cilia in the ventricular lining and other cell types rather than the absence of cilia as in many other ciliary gene mutants. Motile cilia in the ventricular lining are delayed in emergence and fewer in numbers but are also longer in the adult mutants than in the controls. In the photoreceptors, however, outer segment morphogenesis fails completely, and the connecting cilia are often not docked at the apical inner segment membrane. Given that the outer segment is a modified sensory cilium and the connecting cilium is analogous to the transition zone of primary cilia, we show that ciliogenesis in photoreceptors is dramatically compromised in the Cep290 null mutant. We hypothesize that either photoreceptors are particularly sensitive to loss of CEP290, or that CEP290 has additional specialized functions in this cell type, probably due to the exceptionally large amount of biomolecules trafficked through the connecting cilium. In concordance, CEP290 expression is far greater in photoreceptors compared with any other cell type. A formal possibility, yet to be excluded, is that ciliogenesis does occur at one point, but rapid failure of outer segment morphogenesis is so damaging to the cell that we are observing a late outcome. We also note that the excessively long primary and motile cilia are unlikely to function normally. These intriguing possibilities require further exploration in future studies.

The pleiotropic human disease phenotypes caused by CEP290 mutations and a large number of its interacting proteins suggest multiple functions for this large, coiled-coil domain protein. Previous studies have suggested a role for CEP290 as a ciliary gatekeeper (32,61) and identified both microtubule binding and auto-inhibitory domains (62). The dramatically longer cilia that we have observed here could be the result of unchecked entry of biomolecules into the cilia, consistent with the putative gating role for CEP290 at the transition zone. Alternately, the longer cilia could indicate that CEP290 is a sensor for regulating cilia length via a feedback mechanism from the tip. Signaling processes through the primary cilia are dependent on a segregated ciliary compartment that maintains a distinct pattern of biomolecule distribution from the remainder of the cell. Additional points from our study shed further light on the function of CEP290 in mammalian ciliogenesis and its role in ciliopathies, helping us define the role of CEP290 in space and time: (i) in terms of ciliogenesis, developmental stage and aging; (ii) in terms of tissue-specificity, region of the cilium and protein domains responsible for various functions. Moreover, our findings assist in correlating the range of phenotypic manifestations of human CEP290 mutations.

At what point in the process of ciliogenesis does CEP290 act? In the unicellular flagellate Chlamydomonas, CEP290 has been shown to tether transition zone microtubules to the membrane (32). We now show that transition zone formation essentially depends on CEP290, as the absence of this protein in photoreceptors results in failure of the connecting cilium to extend from the plasma membrane, although basal bodies and distal appendages are present. Docking of ciliary vesicles with the mother centriole (basal body) at its anchor point to the plasma membrane involves CEP164 (63) and CCDC41 (64). The mother cilium connects to the plasma membrane via distal appendages, structures whose formation is dependent on CEP123, ODF1 (65) and CC2D2A (66). Both Rab8a (63) and PCM1 have been implicated in these processes, and both interact with CEP290 (42), as do CEP123 (65) and CC2D2A (51). This suggests that CEP290 is required closely downstream of CEP164, CCDC41, Cep123, ODF1 and CC2D2A, but likely upstream of RPGR and RPGRIP1, as our results indicate; epistasis could thus explain why in general CEP290-mediated retinal degeneration is more severe than that caused by RPGR or RPGRIP1 mutations. Recent evidence shows that RPGR and RPGRIP1 have reciprocal requirements for proper ciliary localization and function (67,68).

Multiple mutations in human CEP290-based disease lead to a variety of conditions and symptoms (39), suggesting complex genetic regulation and tissue- and domain-specific functions of this large protein. Both N and C termini contain dimerization domains, and binding sites for numerous interactors are spread throughout the protein (15,39,43,69). We have now studied three alleles of Cep290: Cep290dt1 (hypo morph), Cep290m (null) and Cep290d (dominant). Tissue-specific requirements for CEP290 are evident by varying effects of its loss in different organs. Photoreceptors are highly sensitive to the absence of CEP290 (70), resulting in complete absence of cilia formation and rapid death of the cells in all three alleles (present results and 37). In Cep290d, only sensory organs are affected (33,37,47), whereas the Cep290d exhibits more severe systemic manifestations. To our initial surprise, the Cep290 null allele generated by removing exons 1–4 in mouse does not result in overt or serious kidney dysfunction; however, the fact that the Cep290 mouse does exhibit severe cystic renal disease validates a role for CEP290 in kidney function. Pronephros morphogenesis utilizes Wnt signaling (71), and loss of canonical Wnt signaling is associated with development of nephropathosis in adult mice (23). A disruption of this pathway occurs in renal cysts but not retina of a ciliopathy mouse model (72). These results, in combination with our findings, suggest that loss of Cep290 function does not directly disrupt Wnt signaling, but that a dominant or novel function of the Cep290d N-terminal fragment may do so.

While our manuscript was in preparation, another report described investigations on Cep290d mice, generated independently by using the ES-clone we also employed (73). This report further supports the concept that Wnt signaling is not directly disrupted in Cep290-related pathology and finds that Hedgehog (Hh) signaling may be the pathway through which kidney development is disrupted in the Cep290d mice; however, a direct relationship between Hh and CEP290 remains to be shown. The newly discovered BBS-Shh negative regulator LZTFL1/BBS17 may provide a link (9,74). Based on the negative regulation of BBSome ciliary trafficking and Shh signaling by LZTFL1 (9,74), the rescue we observe with Cep290d in combination with reduced Mkks could be mediated by a similar mechanism. The fact that both truncation alleles Cep290d and Cep290d show rescue, lends support to this hypothesis and provides insights into diversity of phenotypes observed in human CEP290 mutations.

Human mutations in CEP290 result in a broad spectrum of ciliopathies, from non-syndromic congenital vision loss (LCA) to nephropathosis, Joubert syndrome and embryonic lethal Meckel–Gruber syndrome (39). A review of CEP290 base mutations (http://medgen.ugent.be/cep290base/overview.php) shows that LCA can be caused by mutations throughout the protein, whereas Joubert syndrome results from nonsense mutations in the C-terminal half of the protein, and many Meckel–Gruber mutations are nonsense mutations within the first half of the protein. A mutation causing nephropathosis, in contrast, results from a single residue deletion in the last exon (exon 54). With the exception of LCA, a rough correlation exists between severity of disease phenotype and mutation position within Cep290. Similar findings occur with mouse Cep290d and Cep290d alleles; our findings with the Cep290d suggest that some mutations leading to severe phenotypes in human, such as Meckel–Gruber syndrome, may in fact be gain-of-function alleles. However, a construct similar to the truncated protein in Cep290d mice was found to rescue vision...
in a zebrafish model of cep290 vision loss (75), indicating differences among species.

The specific cilia defects observed in photoreceptors and ventricular ependymal cilia provide deeper understanding of various spatial and temporal components of the cilium, including the ciliary pockets, diffusion barrier and transition zone formation. The striking absence of the ciliary pocket in Cep290rd16 ependyma raises the question of the normal significance of this structure. The ciliary pocket has been suggested to orient primary cilia for directional sensing (76), as we see with the highly misoriented cilia in the absence of the ciliary pocket in the Cep290ko-ko. That CEP290 is required for development of the ciliary pocket provides another potential link between ciliogenesis and planar cell polarity (77), as has been demonstrated in airway epithelia (78). The transition from a single primary to motile cilia has likewise been demonstrated in airway epithelia (79).

Our data show that CEP290 is essential for integrity of the transition zone, and that it localizes to the region of the Y-linkers, which connect the microtubule ring with the plasma membrane (80). While not known to constitute a component of the Y-connectors, CEP290 has been demonstrated to connect the plasma membrane and the ciliary microtubules (62), although evidence conflicts as to whether CEP290 binds microtubules directly or through an interaction protein such as CEP162, a critical component of transition zone assembly (81). Recent evidence supports the existence of a ciliary pore complex, analogous to the nuclear pore complex, acting as a diffusion barrier at the transition zone (82,83). CEP290, perhaps in connection with the Y-linkers, could form part of this barrier. The precise relationship between CEP290 and the Y-linkers remains to be determined.

Plausible limits for the structural and functional position of CEP290 within the transition zone can be reached by combining these results with the immunoEM results (see Fig. 1F and G) and previously determined dimensions, also confirmed here, of individual microtubules (25 nm), ciliary microtubule rings (∼150 nm diameter) and the photoreceptor connecting cilium (250 nm width by 1500 nm length) (Fig. 9). These numerical constraints suggest that CEP290 (either singly or as an oligomer) may be located vertically along the length of the transition zone in the region of the Y-linkers, between the plasma membrane and the microtubule ring. This is the region of intraglellar transport, through which proteins traversing the length of the cilium must pass (84). In line with our modeling predictions, the defects in connecting cilium formation in the Cep290 mutants begin to make sense. In the absence of CEP290, formation of the photoreceptor transition zone/connecting cilium is prevented, suggesting that CEP290 may act to facilitate ciliary vesicle docking with the plasma membrane, thus initiating transition zone formation. CEP290-ADSD, the truncated protein found in the Cep290dic mutant, is able to mediate vesicle docking and transition zone formation; however, the connecting cilium/transition zone is of aberrant shape, suggesting incomplete or defective function of CEP290 in providing structural support and/or trafficking of essential interacting proteins into appropriate orientation. Thus, at least two related functions for CEP290 can be deduced from these data: initiation of transition zone formation and structural integrity of the transition zone at the cilial base.

Modifier genes have been shown to alter the phenotypic severity of various ciliopathy-causing mutations (39,85–88). A third and more specific molecular role for CEP290 as a regulator of BBSome function can be postulated from previous studies and from the different interactions of Cep290 alleles with Mkks. Ciliogenesis is impaired in mice with each of the three Cep290 mutant alleles. While background difference may provide a partial explanation, differential rescue of the three Cep290 mutants by Mkks loss is intriguing. Truncated forms of CEP290 are expressed in Cep290dic and in Cep290fl, both of which show ciliary rescue with loss of Mkks (present results and (33)). In contrast, no protein is detected in the Cep290ko, which exhibits a more severe phenotype in combination with Mkksko. Mkks is part of a complex with BBS10 and BBS12 involved in BBSome assembly (50). Yeast two-hybrid studies suggest an interaction of CEP290 with several components of the BBSome (Helen L. May-Simera, unpublished data), required for transporting membrane proteins to the cilium (89,90). In addition to its interaction with Mkks (33), CEP290 interacts with BBS4, likewise required for BBSome assembly (40,41). The BBSome is intact in Bbs4ko mice (91) but disrupted in Mkksko mice (50). Compared with Cep290dic/dic, Mkksko mice exhibit a more severe phenotype (40), whereas Cep290dic/dic, Mkksko mice show rescue (33). Thus, similar to LZTFL1/BBS17, a cytoplasmic negative regulator of BBSome ciliary trafficking mentioned above (74), our present findings suggest that CEP290 may be a negative regulator of BBSome function at the transition zone. Our studies have implications for designing therapies to treat ciliopathies, in that inhibiting BBSome function may, at least in some CEP290-mediated ciliopathies, result in mitigation of phenotypic severity.

Materials and Methods

Generation of Cep290 knockout (Cep290ko) mice

Murine centrosomal protein 290 (Cep290) gene, as currently annotated, spans 85.37 kb on chr10 with a total of 53 exons. The genomic organization of the locus is highly conserved between mouse and human. To inactivate the Cep290 gene creating a null allele in mouse, exons 1–4 of the gene were replaced in ES cells with a ß-gal-neo cassette by homologous recombination (92) as shown in Figure 2. Briefly, a gene targeting vector of replacement type was constructed from a BAC clone isogenic to the SV129 mouse genome and carrying the Cep290 locus through ET recombination or recombineering (93). The bacteria Diphtheria toxin A (DTA) coding sequence was included in the targeting vector driven by PKG promoter as negative selection (94). Linearized targeting vector was electroporated into the R1 ES cells (95). Homologous recombination events were identified by genomic Southern, after digest with AvrII, using a 519 bp probe upstream of exon 1 and outside the left targeting arm, generating bands of 10 and 13 kb for wild type and mutant, respectively. Correctly recombined ES clones were microinjected into blastocysts of C57BL/6J to make chimeras, and germline transmitted F1 mice from clones A2 and B1 were obtained by crossing high percentage male chimeras with wild-type C57BL/6J mice (92). The presence of the targeted allele was confirmed by long genomic PCR. The null or knockout allele will be referred to as the ko allele in this paper. Genotyping primers are common forward, CATTATCGTA TAGCCGTGTGTCAGGGGACT; wild type reverse, ATCTGCTAATC TTCCTGCGGTGGCAGGTCT; knockout reverse, TGTGCTGCAAGG CGATTAAGTTGGGTAACG. 

Generation of Cep290 genetrap (Cep290gt) mice

Cep290gt mice were generated from an ES gene trap cell line, CC0582 (SIGTR), made by the Sanger Center. Sequencing identified the chromosome 10 breakpoint in Cep290 intron 25, 3.2 kb downstream of exon 25. The breakpoint sequence is as follows: intron 25: AATGTGGAAGGCAATGGAAAACGGATAATGGGAGAGTGTGG- pGT0xT2 beta-geo vector sequence: GTTCAGGTTTGAGCGATTAAGTTGGGTAACG.
CAGGCCAGGCCTCTCCCGTGGTCTCGCCCT. These ES cells were injected into C57BL/6 blastocysts to generate Cep290Gt(CC0582)Wtsi mice (here referred to as Cep290gt mice). Mice were backcrossed onto both C57BL/6J and 129/SvJ lines without significant differences in observed phenotype. Note that this is the same allele as described in ref. (80), although they calculate it as integrating into intron 23; according to the publically available sequence of the murine Cep290 gene, the forward primer site used for integration site mapping (F4 in reference 80) currently maps to intron 25.

Mouse breeding and maintenance

All experiments conform to an animal study protocol reviewed and approved by the NEI Animal Care and Use Committee. Animals were housed under standard light/dark conditions and given regular chow ad libitum. For Cep290 ko, agouti founder males carrying the ko allele were backcrossed to wild-type C57BL/6J or 129/SvJ mice for up to six generations to obtain mice >98% pure on each background. Both F1 and N4–6 backcross heterozygous knockout mice were intercrossed to generate homozygotes on either a mixed C57BL/6J–129/SvJ or pure strain. Viable F2 and F3 homozygotes were used to maintain a homozygous colony, while N4F1 (for 129/SvJ) or N6F1 (for C57BL/6J) crosses were used to generate homozygotes on either individual strain. For Cep290 gt, the same mating strategy was used to generate heterozygous animals. With rare exceptions, Cep290gt/gt animals did not survive on either genetic background (compare with findings in ref. (80), which likewise found embryonic lethality for the same allele on C57BL/6 background).

ERG and fundoscopy

Mice were examined by fundus photography and by electroretinogram according to standard procedures (33).
Histology, immunolocalization and confocal microscopy

Primary antibodies: rabbit and rat anti-CEP290 (33); mouse anti-acetylated alpha-tubulin, Sigma; chicken anti-rootletin, T. Li; anti-RKIP, gift of H. Khanna (69); anti-RPG, T. Li; adenylate cyclase, T. Li; rabbit anti-ZO1, Invitrogen/Zymed; rabbit anti-RNA polymerase II, Abcam; mouse anti-GT335, Sigma secondary antibodies were used were Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647 and gold-conjugated anti-rabbit. Imaging: Olympus FV1000 laser scanning confocal microscope, 63× oil immersion objective.

Transmission and scanning electron microscopy

For TEM, whole eyes were prepared, sectioned and imaged using standard fixation, embedding and scanning procedures. For SEM, samples were fixed for 2 h at room temperature in EM fix (2.5% glutaraldehyde, 4% paraformaldehyde, 10 mM CaCl_2 in 0.1 M HEPES). They were then coated with 1% tannic acid and 1% osmium tetroxide, after which they were dehydrated through an ethanol series and critical point dried. Images were taken using an S-4800 field emission scanning electron microscope and morphometric analyses were carried out.

High-resolution MRI imaging of mouse brain

Wild type and Cep290 ko mice on both mixed and pure genetic backgrounds were deeply anesthetized with ketamine/xylazine IP and perfused with cold PBS followed by 4% paraformaldehyde containing 1:500 Magnemist MRI contrast agent. Following postfix in the same solution, the head was placed in a 15 mm NMR tube (Wilmad Lab Glass, Buena, NJ) and submerged in Fomblin (Ausimont, NJ, USA), a fluorinated non-hydrogenated liquid, to minimize magnetic susceptibility difference which may result in image artifacts between the tissue and surrounding (air) and to eliminate intense water signal which would otherwise affect tissue contrast. The head sample was then centered in a 15 mm radio frequency antenna. The brain MRI experiments were performed, as described (96,97), on a vertical bore 14 T scanner operating on a Bruker Avance platform (Bruker Biospin Inc., Billerica, MA). Three mutually perpendicular scout images of the excised brain were acquired [repetition time (TR)/echo time (TE) = 100/6 ms, magnetization 30°, in-plane resolution = 200 µm, slice thickness = 1 mm]. These images were used to obtain the optimum volumetric field of view that encompasses the whole brain for the subsequent 3-D scan. A T1 weighted gradient echo image sequence (TR/TE = 100/6.4 ms, number of averages = 8, field of view = 25.6 × 25.6 × 16 mm) was used to achieve an isotropic resolution of 50 µm in a total scan time of ~23 h (Supplementary Material, Table S3).

Bioinformatic analysis

Protein sequence information on centrosomal protein of 290 kDa (CEP290) from human and mice (acc.# O15078 and Q6A078, respectively) was obtained from the Uniprot database (http://www.uniprot.org/). Structurally disordered regions were determined for protein sequences using the intrinsic protein disorder, domain and globularity prediction program GlobProt 2, version 2.3 (http://globplot.embl.de/). In addition, protein sequence fragments in coiled-coil conformation were predicted using simple modular research architecture tool, SMART (http://smart.embl-heidelberg.de/). Propensities to form coiled-coil dimers and trimers were evaluated using the Multicoil program (http://groups.csail.mit.edu/cb/multicoil/cgi-bin/multicoil.cgi). Finally, the identification of coiled-coil structures in structural domains and molecular modeling of dimeric, trimeric and tetrameric structures of DSD domains were performed using the protein homology/analogy recognition Phyre2 engine v. 2.0 (http://www.sbg.bio.ic.ac.uk/phyre2/). Information on similar protein atomic structures from the RCSB Protein Data Bank (http://www.rcsb.org/pdb) was used to model possible oligomeric structures of DSD domain.

Supplementary Material

Supplementary Material is available at HMG online.

Authors’ contributions

R.R., T.L. and A.S. conceived the study, designed the experiments, analyzed the data and wrote the manuscript. R.A.R., E.A.Y., H.L. M-S., A.N.H., K.P. and N.G. performed the experiments and analyzed the corresponding data. Y.V.S. and B.W. did structural analysis. M.D. and J.M. performed MRI imaging and analyzed the data. L.D. generated the knockout mice. R.A.R., E.A.Y. and R.N.F. performed confocal or electron microscopy and analyzed the data.

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References


70. den Hollander, A.I., Koenekoop, R.K., Yzer, S., Lopez, I., Arends, M.L., Voesenek, K.E., Zonneveld, M.N., Strom, T.M.,


