A CEP104-CSPP1 Complex Is Required for Formation of Primary Cilia Competent in Hedgehog Signaling

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Graphical Abstract

Highlights
- cep104-depleted zebrafish display shortened KV cilia and defective brain development
- CEP104 interacts with CSPP1 at the tip of the primary cilium to regulate cilia length
- CEP104 or CSPP1 loss in human cells leads to defective Hedgehog signaling
- Impaired signaling is linked to reduction of ciliary SMO but not ARL13B or INPP5E

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In Brief
Deleterious mutations in CEP104 or CSPP1 cause Joubert syndrome, a ciliopathy causing an underdeveloped mid- and/or hindbrain. Frikstad et al. show that loss of cep104 in zebrafish leads to defective brain development and that CEP104 interacts with CSPP1 at the tip of the primary cilium to regulate axoneme length and Hedgehog signaling competence.

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SUMMARY

CEP104 is an evolutionarily conserved centrosomal and ciliary tip protein. CEP104 loss-of-function mutations are reported in patients with Joubert syndrome, but their function in the etiology of ciliopathies is poorly understood. Here, we show that cep104 silencing in zebrafish causes cilia-related manifestations: shortened cilia in Kupffer’s vesicle, heart laterality, and cranial nerve development defects. We show that another Joubert syndrome-associated cilia tip protein, CSPP1, interacts with CEP104 at microtubules for the regulation of axoneme length. We demonstrate in human telomerase reverse transcriptase-immortalized retinal pigment epithelium (hTERT-RPE1) cells that ciliary translocation of Smoothened in response to Hedgehog pathway stimulation is both CEP104 and CSPP1 dependent. However, CEP104 is not required for the ciliary recruitment of CSPP1, indicating that an intra-ciliary CEP104-CSPP1 complex controls axoneme length and Hedgehog signaling competence. Our in vivo and in vitro analyses of CEP104 define its interaction with CSPP1 as a requirement for the formation of Hedgehog signaling-competent cilia, defects that underlie Joubert syndrome.

INTRODUCTION

The primary cilium is a signaling organelle formed by a confined microtubule (MT)-based cell membrane protrusion that originates from the modified mother centriole of the centrosome (basal body). Generation of a signaling-competent primary cilium from the centrosome is a multi-step process. It is initiated by the re-organization of the distal end of the mother centriole and the recruitment of pre-ciliary membrane, and completed by docking to the cell membrane and maturation of the ciliary axoneme and membrane. A highly specialized region at the base of the MT axoneme, the transition zone (TZ), regulates the exchange of membrane-bound and soluble cytosolic factors with the cell body (Reiter et al., 2012). Ciliary compartmentalization is further regulated by the intraflagellar transport system (IFT), which mediates anterograde (base to tip) with IFT-B cargo via kinesin-2 and retrograde (tip to base) with IFT-A complex-bound cargo via dynein 2 motors (Taschner and Lorentzen, 2016). Kinesin-2 motors and the IFT-B sub-complex are known to promote ciliogenesis via the anterograde ciliary transport of soluble axonemal cargoes such as tubulin (Kozminski et al., 1995; Bhogaraju et al., 2013). Emerging evidence has implicated IFT-A also in the ciliary entrance of specific G protein-coupled receptors via Tubby family adaptor proteins (Mukhopadhyay et al., 2010; Pal et al., 2016), and IFT-B complex members and dynein 2 motors were shown to promote the ciliary export of specific membrane proteins, such as the Sonic hedgehog (SHH) signaling pathway receptors Patched1 and Smoothened (Keady et al., 2012; Eguether et al., 2014), via the Bardet-Biedl syndrome protein complex (BBSome), an IFT cargo adaptor (Lechtreck, 2015; Nachury, 2018). The restricted ciliary expression of receptor molecules allows cell surface area-independent sensitivity to surrounding ligands and orientation-dependent signal reception within a tissue context (Mahjoub, 2013). Several key pathways in vertebrate development and tissue homeostasis depend on primary cilia, including Hedgehog (Hh), WNT, transforming growth factor β (TGF-β) and platelet-derived growth factor
receptor α (PDGFR-α) signaling (Bangs and Anderson, 2017; Christensen et al., 2017; May-Simera and Kelley, 2012). Structural and/or functional ciliary defects conferred by inherited mutations in ciliary and/or centrosomal protein-encoding genes are acknowledged as a leading cause of developmental disorders and degenerative diseases, collectively called ciliopathies (Waters and Beales, 2011; Reiter and Leroux, 2017). Affected individuals typically present with multi-system pathologies of the brain and/or neurological system, eye, kidney, skeleton, and other organs relying on ciliary signaling and function.

Joubert syndrome (JBTS) is a rare autosomal recessive ciliopathy classified by a characteristic mal-development of the mid- and/or hindbrain (manifesting as a “molar tooth sign” on brain MRI). Besides developmental delay, ataxia, and intellectual disabilities, retinal dystrophy and cystic kidney disease (nephronophthisis, NPHP) frequently co-occur (Romani et al., 2013). To date, JBTS-causing mutations have been identified in 35 genes (JBTS1–JBTS35; MIM Phenotypic series MIM: PS213300), partially overlapping with related ciliopathies such as Meckel-Gruber syndrome (MKS), Senior-Løken syndrome (SLSN), BBS, and NPHP (Sang et al., 2011). The majority of JBTS genes have been tied to the regulation of the HH signaling pathway and function of the TZ (Garcia-Gonzalo et al., 2011; Yang et al., 2015; Chih et al., 2011). A subset of four JBTS proteins localizes to the ciliary tip, and these proteins have opposing effects on ciliary structure: cells depleted of KIF7 (JBTS12) or KIAA0556 (JBTS26) have extended axonemes (He et al., 2014; Dafinger et al., 2011; Sanders et al., 2015), as opposed to cells with reduced CSPP1 (JBTS21) or CEP104 (JBTS25), which manifest shortened or absent axonemes (Patzke et al., 2010; Akizu et al., 2014; Shaheen et al., 2014; Tuz et al., 2014; Satish Tammana et al., 2013; Jiang et al., 2012). Of note, the cilia phenotype of CEP104 mutation carriers has not yet been reported, nor has genetic silencing yet been interrogated in vertebrate development, and CEP104 has not previously been shown to physically interact with components of the JBTS protein network. To study the effect of cep104 depletion in Danio rerio (zebrafish), we injected morpholino oligonucleotides targeting the single ortholog cep104 at the translation site (ceph104 translation blocking morpholino oligonucleotide [ATG MO]) and a splice junction (ceph104 splice MO). Morphant zebrafish at 48 h post fertilization (hpf) displayed cardiac phenotypes, mild tail curvature, and microphthalmia (Figures 1A–1C and S2A–S1C). The combined injection of cep104 ATG MO and cep104 splicing MO potentiated the severity of morphant phenotypes (Figure 1D). RT-PCR and western blotting of whole zebrafish MO potentiated the severity of morphant phenotypes (Figure S2F). In contrast, analysis of Kupffer’s vesicle, a ciliated organelle important for left-right axis formation, showed a ciliary defect, with a reduction in ciliary length (Figures 1F and 1G), which was rescued by the co-administration of CEP104 mRNA. In addition to pericardial edema, which was not directly related to laterality defects, cardiac defects included abnormal cardiac looping, with reversed or no looping seen in 55% of morphants, which was also rescued by co-injection with CEP104 mRNA (Figures 1H and 1I). Most relevant in regard to JBTS, characteristic developmental defects were observed within the brains of cep104 zebrafish morphants. The transgenic zebrafish line, islet1-GFP, allows visualization of the cranial motor neurons. In morphant embryos, islet1-GFP positivity was disrupted with the loss of the overall neuronal structure and with a specific, recurrent loss of oculomotor neurons. Notably, the degree of cranial nerve defect did not necessarily correlate with the severity in body structure abnormalities, indicating that the neuronal phenotype is not secondary to a more general developmental defect. This specific phenotype was rescued by co-administration of CEP104 mRNA (Figures 1J and 1K). Additional analysis of the F0 populations of cep104 crisprants confirmed the specificity of the gross morphological changes, as well as the heart looping, Kupffer’s vesicle cilia, and cranial nerve defects seen in cep104 morphants (Figures S2G–S2Q). Severe crisprants and morphants showed some yolk sac abnormalities, which are likely to be linked to the pericardial edema. These data reveal that cep104 knockdown phenotypes are highly consistent with a ciliopathy syndrome and suggest a role for cep104 in cilia formation within Kupffer’s vesicle, as well as development of the heart and cranial nerves in zebrafish.
Identification of Microtubule-Associated CEP104-CSPP-L Complexes

A key to understanding the underlying mechanism of the zebrafish phenotype is to place CEP104 within known ciliopathy-associated protein networks. We identified an interaction of CEP104 with the JBTS protein CSPP-L (the larger and predominantly expressed isoform of CSPP1) in a bi-directional yeast two-hybrid screen of an arrayed cDNA panel encoding 163 ciliary proteins or protein fragments (Table S1; Figures S3A–S3D). This finding was supported by BirA(R118G)-CEP104 proximity labeling studies in Hek293 cells (Al-Jassar et al., 2017; Gupta et al., 2015), as well as co-fractionation of endogenous proteins in hTERT-RPE1 and Hek293T cells during sucrose gradient centrifugation and size exclusion chromatography (Figures S3E and S3F). We validated the interaction of CSPP-L and CEP104 in reciprocal co-immunoprecipitation experiments using EGFP-tagged CEP104 and CSPP-L full-length or truncated constructs (Figures 2A–2C). The localization pattern of the N-terminally fluorescent protein-tagged CEP104 to the distal end of centrioles and the cilia axoneme, and to some extent MT plus ends, closely resembles that reported for endogenous CEP104 (Figures S3G and S3H; Satish Tammana et al., 2013; Jiang et al., 2012). CSPP-L and known CEP104-interacting proteins CEP97, CP110, and CEP290 co-purified with EGFP-CEP104.

Figure 1. cep104 Knockdown in Zebrafish Embryos Leads to Ciliopathy Phenotypes

(A–C) 48 hpf morphant zebrafish display mild and severe pericardial edema and cardiac defects (*) following cep104 knockdown and additional phenotypes in severe morphants of mild tail curvature and microphthalmia, with a quantified reduction in area expressed as a ratio to control embryos of 0.45 (p < 0.0001, unpaired t test, n = 39 versus 28 control).

(D) Percentage of zebrafish displaying phenotypes following injection of cep104 splice MO and translation blocking morpholino cep104 ATG MO alone or in combination (control n = 98, cep104 splice MO n = 166, cep104 ATG MO n = 95, cep104 splice MO + cep104 ATG MO n = 77).

(E) Western blotting (WB) of cep104 at 48 hpf in zebrafish un.injected and injected with cep104 ATG MO and cep104 splice MO.

(F) IFM of cilia and cell junctions (a-acetylated tubulin, red) in Kupffer’s vesicle (KV; atypical protein kinase C [aPKC], green) at the 10-somite stage in control and cep104 knockdown embryos.

(G) Dot plots of the length of cilia in KV in control, cep104 splice MO knockdown, and cep104 splice MO and CEP104 mRNA co-injected zebrafish embryos (ANOVA with Tukey post hoc test, *p < 0.05).

(H) 48 hpf cmlc2:GFP zebrafish treated with cep104 splice MO show changes to heart looping at 48 hpf, which is rescued by co-injection with CEP104 mRNA.

(I) Percentage of embryos displaying heart looping phenotypes following injection of cep104 splice MO and co-injection with CEP104 mRNA (**p < 0.0001, *p = 0.0208, chi-square test of independence; control n = 186; cep104 splicing MO n = 132; cep104 splicing MO + CEP104 mRNA n = 130).

(J and K) cep104 knockdown in 48 hpf islet-1:GFP transgenic fish leads to cranial nerve defects, rescued by co-injection with CEP104 mRNA. Co-injection with CEP104 mRNA produces a partial rescue of phenotypes (**p < 0.0001, *p = 0.0010, chi-square test of independence; control n = 200; cep104 splicing MO n = 80; cep104 splicing MO + CEP104 mRNA n = 120).
Analysis of truncation mutants identified the N-terminal 200 amino acid domain of CEP104 as being essential for interaction with CSPP-L (Figures 2A and 2B). Conversely, CSPP-L-EGFP specifically co-purified with endogenous CEP104 and CEP97, CP110, and the earlier identified CEP-L interaction partners CEP290 and PCM1 (Figure 2C; Patzke et al., 2005, 2012; Shearer et al., 2015). The co-purification of CSPP-L with CEP104, CEP97, and CP110 was strictly dependent on the C-terminal 379 amino acid of CSPP-L. CSPP-L truncates failed to co-purify CEP290 or PCM1, which may indicate the requirement of a distinct tertiary fold of CSPP-L for stable interaction with these centriolar satellite proteins. In line with subcellular localization data, PCM1 co-purified only with CSPP-L-EGFP but not EGFP-CEP104 (Figures 2A, 2C, S5A, and S5B). CSPP-L may thus participate in distinct sub-complexes. Figures 2D and 2E summarize the biochemical data and superimpose protein partner interacting regions of CSPP-L and CEP104 on their functional (Patzke et al., 2005, 2006, 2010, 2012; Jiang et al., 2012; Hauge et al., 2007) and predicted domain architectures (Hildebrand et al., 2009; Meier and Söding, 2015). Bioinformatic analysis of CSPP-L did not identify regions of significant structural homology to functionally annotated proteins. In contrast, the N-terminal galactose-binding-like domain of CEP104 (amino acids [aa] 1–156) is predicted to share close structural homology to the HH signaling pathway regulating IFT-B complex protein IFT25 (Keady et al., 2012), and the central domain (aa439–658) is homologous to a single ch-TDO domain of proteins involved in MT plus end dynamics (Al-Jassar et al., 2017; Al-Bassam and Chang, 2011; Akhmanova and Steinmetz, 2015).

We noticed earlier that the localization of CSPP-L to MTs is spatiotemporally restricted to the cilium axoneme and mitotic MTs and otherwise generally confined to centrosomes and centriolar satellites (Patzke et al., 2010; Shearer et al., 2018). Hence, the interaction of endogenous CSPP-L with MTs may depend on post-translational modification and/or require a secondary factor. Overexpressed CSPP-L, however, decorates cytoplasmic MTs (Patzke et al., 2006, 2010). Co-expression of mCherry-CEP104 with GLAP3-CSPP-L or its truncated variants CSPP-L(294–842)-EGFP or CSPP-L(842–1221)-EGFP (described in Patzke et al., 2006) in non-ciliated hTERT-RPE1 cells revealed the recruitment of mCherry-CEP104 to MTs (Figures 2F–2H, S3I, and S3J). CSPP-L may thus support the localization of CEP104 to MTs in the cilium to facilitate axoneme formation and/or stabilization. GLAP3-CSPP-L and mCherry-CEP104 partially co-localized at the cilary tip in transient hTERT-RPE1 transfectants (Figure 2I).

Ciliary Localization of CSPP-L and CEP104
The IFM analyses described above supported an MT-associated function of CSPP-L and CEP104 at the cilium axoneme and/or tip, but they were limited by the transient overexpression of CEP104 and CEP104 fusion proteins. To refine the ciliary localization at a higher resolution, we resolved the localization of endogenous CSPP-L by IFM and electron microscopy on multiciliated mouse trachea epithelia cells and of N-terminal monomeric NeonGreen fluorescent protein fusions of CSPP-L (mNG-CSPP-L) and CEP104 (mNG-CEP104) by 3D-superresolution immunofluorescence microscopy (3D-SIM) in transformed hTERT-RPE1 cells (Figures 3 and 4). CSPP-L localizes predominantly to the very end of axonemal MTs near the capping structure (Figures 3A and 3B) and to the membrane proximal end of the transition fibers of motile cilia in mouse trachea cells (Figure 3A). The axone end localization is more proximal to the cilium tip than that of the apical membrane-singlet MT linker protein Sentan (Figure 3C) (Kubo et al., 2008). CSPP-L staining along outer and central axonemal MTs was occasionally observed (Figure 3A). In addition, staining of electron-dense particles, tentative centriolar satellites, was noticed (Figure 3A). Notably, MT end localization was not seen in the axonemes of mouse sperm flagella (Figure S4A) or the cytoplasmic MTs of hTERT-RPE1 cells (Figure S4B). mNG-CSPP-L closely resembled the localization pattern of endogenous CSPP-L in hTERT-RPE1 cells, including centriolar satellite localization (Figures 3D, 3E, and S4C; Patzke et al., 2010). mNG-CSPP-L is partially co-localized with the central dot of γ-tubulin (centriole lumen; Lawo et al., 2012) of both centrioles. It extends from the mother centriole through the TZ into the cilia lumen to peak in intensity at the ARL13B encased tip (Figure S4D), distal to the antibody-stained golgulated MT axoneme. mNG-CEP104 localizes to the cilium tip and the distal end of the daughter centriole (Figures 4 and S4E). The cilia tip localization with respect to ARL13B (Figures 4A and 4B) and glutamylated tubulin (Figures 4C and 4D) is highly reminiscent of CSPP-L, and partial co-localization of CSPP-L and mNG-CEP104 is evident (Figure 4C). mNG-CEP104 partially co-localizes with CP110 at the distal end of
the daughter centriole (Figure 4E). Fixation conditions largely inhibit centriolar staining of glutamylated tubulin. We conclude that interaction data, subcellular localization, and sequence analyses collectively support the notion that centrosome and cilia tip proteins CEP104 and CSPP-L interact, with a potential role in cilia axoneme MT regulation.

**CSPP1 and CEP104 Are Dispensable for Early ARL13B Recruitment but Critical for Axoneme Length Regulation**

Based on the above and earlier reports on the requirement of Recruitment but Critical for Axoneme Length Regulation that CEP104 and CSPP-L cooperate in cilia axoneme formation. We therefore targeted CSPP1 and CEP104 in hTERT-RPE1 cells using CRISPR-Cas9 nickase (Ran et al., 2013). Guide RNAs (gRNAs) were designed to target CSPP1 at the first common exon of CSPP and CSPP-L splice isoforms (Figures S5A–S5C) and CEP104 within exon 2, just after the translational start codon (Figures S5D–S5F). Individual clones devoid of the expression of full-length CSPP-L (Figure 5A) or CEP104 (Figure 5B), respectively, were identified and characterized.

The CSPP-L-deficient clone was determined to be a compound heterozygote by allele-specific sequencing of the exon of CSPP and CSPP-L splice isoforms (Figures S5A–S5C). One of the compound heterozygotes revealed ciliary localization for both proteins (Figures 5H, S6I, and S6J). In contrast to cep104 mutant levels. Nonetheless, IFM analysis of mNG-CEP104 expressing cells did not rescue the cilia phenotype at statistical significance. This is likely due to a dominant negative effect of the N-terminally truncated 81 kDa CEP104 protein (Srour et al., 2015).

We next compared WT, CSPP1+/−, and CEP104mut cells in cell-cycle progression assays, response to serum starvation, and cilia formation capability (Figures S5D and S6). A total of 69% ± 5% of WT hTERT-RPE1 cells generated a cilium upon serum withdrawal, while only 32% ± 8% of CSPP1+/− and 48% ± 9% of CEP104mut cells developed cilia (Figure 5D). No significant differences in cell-cycle progression were evident between asynchronously growing mutant and WT hTERT-RPE1 cells, and all three cell lines arrested in G0/G1 phase in response to 48 h of serum starvation (Figures S6A and S6B). Furthermore, hTERT-RPE1 mutants and WT cells showed an indistinguishable IFM pattern of acetylated tubulin, α-tubulin, and EB3 (Figures S4F and S4G). These results excluded cell-cycle progression defects and gross alterations in cytoplasmic MT organization as possible causes for defective ciliogenesis. Notably, ~40% of CSPP1+/− and CEP104mut cells that failed axoneme formation (glutamylated tubulin) depicted the loss of CP110 from the mother centriole (Figure 5E; similar results obtained with CEP97, data not shown) and ~20% recruited ARL13B to the pre-ciliary vesicle (Figure 5F), indicating that cilia formation was impaired at an axoneme-forming permissive stage. Axoneme length in CSPP1+/− (1.5 ± 0.05 μm) and CEP104mut (1.9 ± 0.06 μm) cells was significantly reduced compared to WT cells (2.9 ± 0.05 μm) (Figure 5G), suggesting a cilia-specific defect in MT organization. Cilia length was rescued in CSPP1+/− cells by mNG-CSPP-L (Figure S6E). In contrast to cep104 rescue experiments in zebrafish, the expression of mNG-CEP104 in CEP104mut cells did not rescue the cilia phenotype at statistical significance. This is likely due to a dominant negative effect of the N-terminally truncated CEP104 proteins expressed in these cells (Figures 5B and S6F).

The antagonizing effects of EF1α-promotor-driven NeonGreen fusion proteins cannot be excluded as both fusion proteins are overexpressed compared to endogenous levels. Nonetheless, IFM analysis of mNG-CSPP-L expressing CEP104mut and mNG-CEP104 expressing CSPP1+/− cells revealed ciliary localization for both proteins (Figures 5H, 5I, S6E, and S6F). Hence, intra-ciliary interaction of CEP104-CSPP-L is required to achieve regular axoneme length.
Figure 4. Localization of mNG-CEP104 and CSPP-L at Primary Cilia

(A–D) 3D-SIM IFM of hTERT-RPE1 cells expressing mNG-CEP104 (green) and co-stained for centrosomal marker γ-tubulin (white) and cilia membrane marker ARL13B (red). (A and B) Glutamylated tubulin (white) and CSPP-L (red) (C) or glutamylated tubulin (white) and CP110 (red) (D). Scale bars in magnified areas, 500 nm. mNG-CEP104 localizes to the capping complex of the daughter centriole (A, B, and D) and co-localizes with CSPP-L at the cilia tip (C). Low cilia tip signal intensity of mNG-CEP104 compared to daughter centriole localization is observed in all of the cells. Axoneme staining of CSPP-L is fixation condition dependent (Patzke et al., 2010; Hua and Ferland, 2017) and not resolved.
Depletion of CEP104 in CSPP1<sup>−/−</sup> cells strongly diminished cilia formation (Figure S6G), further supporting this hypothesis. These data suggest that neither CEPT104 nor CSPP1 are strictly essential for the initial stages of cilia formation but that their intra-ciliary interaction is critical for regular axoneme elongation or maintenance.

**CSPP1<sup>−/−</sup> and CEPT104mut Cilia Are Defective in SMO Translocation in Response to Hh Pathway Activation**

The cilia phenotypes in CSPP1<sup>−/−</sup> and CEPT104mut cells are in agreement with reported cilia aberrations in CSPP1 and CEPT104 mRNA targeting small interfering RNA (siRNA) transfectants (Patize et al., 2010; Jiang et al., 2012; Satish Tammana et al., 2013). In contrast to CSPP1, a cilia phenotype in CEPT104 mutated JBTS patients has not yet been reported. CSPP1-mutated JBTS patient fibroblasts are deficient in SHH-induced GLI1 expression, as determined by bulk analysis (Tuz et al., 2014; Shaheen et al., 2014). To discriminate whether the reported SHH sensitivity defect could be attributed to reduced cilia numbers or defective cilia function and whether CEPT104mut cells share pathway impairment, we investigated the efficacy of Smoothened (SMO) translocation to cilia in response to SHH pathway activation by soluble SHH-ligand (ShhN) conditioned medium or 100 nM Smoothed agonist (SAG) treatment (Figure 6). Cells were serum starved for 48 h to promote cilia formation and then stimulated for 24 h before fixation and assessment of SMO and ARL13B by IFM. Semiquantitative assessment revealed that CSPP1<sup>−/−</sup> and particularly CEPT104mut cells had significantly decreased SMO translocation to primary cilia in response to ShhN stimulation compared to WT hTERT-RPE1 cells (Figure 6A). Similarly, the quantitative assessment of median cilary fluorescence intensities of ARL13B and SMO in SAG-treated cells revealed a strong dependence of cilary SMO accumulation on CEPT104 (30% of WT SMO intensity) and to a lesser extent on CSPP1 (70% of WT SMO intensity) integrity (Figure 6B). In contrast, median cilary ARL13B intensities were indistinguishable between cell lines. Likewise, cilare membrane localization of the ARL13B-dependent Hh pathway modulator INPP5E (Figure 6C) (Humbert et al., 2012; Garcia-Gonzalo et al., 2015; Chávez et al., 2015) and the ARL13B regulatory TZ proteins CBY1 (Figure S6J) and AH1 (Figure S6K) [Lee et al., 2014] were not decreased in CSPP1<sup>−/−</sup> or CEPT104mut cells. Finally, the localization pattern of IFT88 indicated CEPT104 and CSPP1 independent ciliary entry and tip localization of the IFT-B core complex (Figure S6L).

We conclude that the cilia tip protein CEPT104 is a critical factor for Hh signaling in hTERT-RPE1 cells. These data support the hypothesis that defective ciliary Hh signaling causes the perturbed heart and cranial nerve development observed in cep104 morphant zebrafish embryos.

**DISCUSSION**

The disturbance of physical interaction networks between proteins encoded by disease genes of a distinct ciliopathy is likely to explain the observed genetic heterogeneity and account for certain genetic overlap between phenotypically related ciliopathies, such as JBTS, NPHP, MKS, or BBS (Sang et al., 2011; Nachury et al., 2007). In the case of JBTS, 35 disease loci have been identified to date; almost all of the affected proteins are exclusively connected to the TZ and the regulation of Hh signaling. CEPT104 is an exception to this understanding, by (1) being localized to the daughter cilie and the ciliary tip, but not the mother cilie or the TZ of the cilie and/or basal body entity (Jiang et al., 2012; Satish Tammana et al., 2013), and (2) having an undetermined role in Hh signaling or interaction with other JBTS proteins. The identified interaction with CSPPL-L (Figure 2) and the cep104 zebrafish ciliopathy phenotypes (Figures 1 and S7) reported here resolve this apparent discrepancy and, in context with reported mutations in KIF7 (alias JBTS12) and KIAA0556 (alias JBTS26), contribute evidence to the expansion of the JBTS network to the ciliary tip compartment (Dafinger et al., 2011; He et al., 2014; Sanders et al., 2015).

The combined interaction data and cilia analysis in CEPT104mut and CSPP1<sup>−/−</sup> cells identify a requirement for CEPT104/CSPP-L interplay to form Hh signaling-competent cilia (Figure 7). Mutual independence for ciliary localization and the severe cilie deficiency phenotype in co-depleted cells suggest that the intra-ciliary interaction of CSPP-L with CEPT104 is essential for attaining normal cilie stature. The inhibition of cilie genesis after CP110/CEP97 release and the formation of shortened, ARL13B-positive cilie in CSPP1<sup>−/−</sup> cells are in concordance with reported phenotypes in CSPP1 JBTS patient fibroblasts (Akizu et al., 2014; Shaheen et al., 2014; Tuz et al., 2014; Figures 1 and 5) and imply that CSPP-L is not strictly required for cilie formation until axoneme elongation.

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**Figure 5. Intra-ciliary Interaction of CEPT104 and CSPP-L Is Critical for Axoneme Length**

(A) CSPP1<sup>−/−</sup> hTERT-RPE1 cells are negative for the expression of CSPP-L full-length or truncated CSPP1 proteins, as determined by immunoblotting with N-terminal and C-terminal domain-specific CSPP-L antibodies and compared to γ-tubulin (loading control).

(B and C) CEPT104mut hTERT-RPE1 cells express N-termiaally truncated CEPT104 proteins at strongly decreased expression levels compared to WT hTERT-RPE1 cells by use of alternative start codons (B). The prominent truncated CEPT104 protein of 81 kDa, lacking the N-terminal 203 aa does not co-purify with the C-terminal CSPP-L domain expressed in hTERT-RPE1 transfectants (C).

(D) CSPP1<sup>−/−</sup> and CEPT104mut hTERT-RPE1 cells form primary cilie at lower frequency (error bars depict SEM of 3 experiments; n = 150 cells; t test; **p < 0.05). (E and F) A total of 40% of CSPP1<sup>−/−</sup> and 34% of CEPT104mut hTERT-RPE1 cells without detectable glutamylated axoneme have licensed mother cilare (i.e., single CP110 signal) compared to 50% in WT hTERT-RPE1 cells (E). Independent of genotype, ~20% of non-ciliated cells show ARL13B signal at the mother cilie centriole, indicative of pre-ciliary vesicle formation (F). Error bars depict SEM of 4 experiments (n = 150 cells; t test; ***p < 0.005).

(G) Cilia in CSPP1<sup>−/−</sup> and CEPT104mut hTERT-RPE1 cells have decreased axoneme length (center lines in boxplots show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by dots; crosses represent sample means; n = 262, 102, 158 sample points; t test; ***p < 0.005).

(H and I) 3D-SIM IFM of CEPT104mut hTERT-RPE1 cells stably expressing mNG-CSPP-L (H) and CSPP1<sup>−/−</sup> hTERT-RPE1 cells stably expressing mNG-CEPT104 (I). mNG-CSPP-L and mNG-CEPT104 show no gross localization defects to primary cilie (ARL13B, red) or the centrosome (γ-tubulin, white).
CEP104 binds via its N-terminal domain to the C-terminal domain of CSPP-L, which is dispensable for the localization of CSPP-L to MTs but critical for the regulation of the effect of CSPP-L on MT organization (Patzke et al., 2006, 2010). In analogy to CEP97-mediated CEP104 localization to cytoplasmic MT plus ends (Jiang et al., 2012), CEP104 and CSPP-L may mutually enhance MT stabilization at the ciliary tip compartment. CSPP-L-dependent CEP104 stabilization at the axoneme lattice

Figure 6. Deficient Ciliary SMO Accumulation in CEP104mut and CSPP1−/− hTERT-RPE1 Cells in Response to Hh Pathway Activation

(A) Assessment of ciliary SMO translocation in response to Hh signaling pathway stimulation by addition of ShhN conditioned medium. Cells were serum starved for 48 h in 2 mL DMEM/F12 before replacement of 1 mL with ShhN conditioned or control DMEM containing 2% serum and further incubation for 24 h. CSPP1−/− and CEP104mut hTERT-RPE1 show decreased SMO (a-SMO; red) accumulation to the primary cilium (a-ARL13B, green). Ciliary SMO levels were scored by inspection and classified in absent, weak, or strong subgroups. Error bars in bar graph depict SEMs of 3 independent experiments, n = 150 per treatment and cell line; t test; *p < 0.05 and ***p < 0.001).

(B) Quantitative assessment of median fluorescence intensities of ciliary ARL13B (a-ARL13B, green) and SMO (a-SMO, red) in serum-starved and SAG-stimulated WT (n = 60), CSPP1−/− (n = 66), and CEP104mut (n = 52) hTERT-RPE1 cells (t test; **p < 0.01 and ***p < 0.001), and determination of SMO expression in total cell lysates of SAG-treated cell lines by immunoblotting.

(C) Quantitative assessment of ciliary INPP5E by IFM in SAG-stimulated and non-stimulated serum-starved WT, CSPP1−/−, and CEP104mut hTERT-RPE1 cells (a-INPP5E, green; a-SMO, red) (n > 30 in each treatment group; t test; *p < 0.05 and ***p ≤ 0.001). Center lines in boxplots show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by dots. Scale bars, 1 μm.
in the tip region could enhance CEP104-mediated tubulin addition at MT plus ends, as suggested by Al-Jassar et al. (2017) and supported by the severe ciliation defect of CEP104 in depleted CSPP1/C0/C0/C0 cells. Speculatively, the CSPP-L/CEP104 complex hence opposes KIF7 and KIAA0556 mediated axoneme growth restriction at the ciliary tip (He et al., 2014; Sanders et al., 2015). Future studies are warranted, including advanced in vitro and live cell imaging analyses, to determine the detailed effect of CSPP-L/CEP104 complexes on isolated MTs and to elucidate their potential dynamic behavior during cilia formation and maintenance.

The positive role of CEP104 in cilia formation and requirement for ciliary SMO accumulation in hTERT-RPE1 cells resembles the requirement of cep104 in vivo, in which shortened cilia in Kupffer’s vesicle of cep104 morphant and crispant zebrafish embryos were observed. Ciliary morphology was regained by co-administration of human CEP104 mRNA (Figures 1 and S2). Likewise, the loss of cep104 manifested in the mal-development of the heart and the cranial nerves, the latter being highly reminiscent of the defective development of the mid- and/or hindbrain characteristic of JBTS. Accumulating evidence supports the hypothesis that defective ciliary Hh signaling is the underlying cause of the phenotype associated with JBTS (Hynes et al., 2014; Roosing et al., 2015; Aguilar et al., 2012). Bulk analysis of CSPP1 JBTS patient fibroblasts showed reduced Hh signaling pathway activity sensitivity and/or responsiveness (reduced Hh-induced GLI1 mRNA expression [Shaheen et al., 2014]). Cardiac developmental defects in cep104 zebrafish morphants may be a consequence of laterality defects. Lost Shh sensitivity may have a contributing effect, since highly similar ventricle size and orientation defects are seen in smo-deficient or cyclopamine-treated (an Hh antagonist) embryos (Thomas et al., 2005). A role for cilia in cardiac development is well established (Li et al., 2015). Congenital heart defects are evident in JBTS and NPHP patients (Koefoed et al., 2014; San Agustin et al., 2016), but they are not reported for rare CEP104-mutated JBTS patients (Srour et al., 2015). Comparable to our loss-of-function studies, these three CEP104 patients carry either nonsense or splice-site mutations. However, it is known that dependent on the JBTS model system, Hh signaling may be increased, as shown in CEP290 patient fibroblasts (Shimada et al., 2017), or decreased, as in murine models of Cep290 knockdown (Hynes et al., 2014). It cannot be excluded that other CEP104 mutations, including gain-of-function mutations, may cause embryonic lethality in humans, but CEP104 mutations have not been observed in MKS embryos thus far.

Functional analysis of our hTERT-RPE1 cell lines determined that CSPP-L and, in particular, CEP104 mutant cilia are Hh pathway compromised in a manner that is distinct from the ciliary INPP5E regulating pathway found targeted in JBTS (Humbert et al., 2012; Bielas et al., 2009; Jacoby et al., 2009; Thomas et al., 2014; Cantagrel et al., 2008; Ferland et al., 2004; Slaats et al., 2016). Our analysis of ShhN or SAG-induced SMO translocation to the cilia compartment revealed a significantly stronger impairment in CEP104mut than in CSPP1/C0/C0/C0 cells (Figure 6), suggesting that CSPP-L may potentiate the capability of CEP104 at the tip compartment in regulating ciliary SMO turnover in response to Hh pathway stimulation. This is different from PDE6D, ARL13B, and INPP5E compromising JBTS-related mutants, which recruit SMO (Humbert et al., 2012; Garcia-Gonzalo et al., 2015; Chávez et al., 2015; Larkins et al., 2011). Of note, Tuz et al. (2014) observed reduced ciliary ARL13B levels in CSPP1 JBTS patient fibroblasts. This is not recapitulated in cardiac development.
in our quantitative analysis of ARL13B and INPP5E in hTERT-RPE1 cells (Figure 6) and may thus be either cell type dependent or mutation specific. 3D-SIM revealed that mNG-CSPP-L extends from the central lumen of the mother centriole into the cilia compartment (Figures 3D and 3E), a localization pattern that is partially supported by fixation/antibody-dependent immunoelectron microscopy (immuno-EM) (Figure 3A) and IFM (Patzke et al., 2010; Hua and Ferland, 2017). We and others have shown previously that CSPP-L is required for the localization of the larger retinitis pigmentosa guanosine triphosphatase (GTPase) regulator-interacting protein 1-like (RPGRIP1-LIKE) at the TZ (Patzke et al., 2010; Shaheen et al., 2014), which is an Hh signaling pathway modulator in mouse embryonic fibroblasts (Vierkotten et al., 2007). A contribution of a TZ defect to the reduced SMO translocation phenotype can thus not be fully excluded in CSPP1/C0 hTERT-RPE1 cells. In contrast, CSPP-L is present at the cilia of CEP104mut hTERT-RPE1 cells, which is unlikely to bear a TZ defect.

Inferring from the flagellar defects in the FAP256 mutants in Chlamydomonas (Satish Tammana et al., 2013) and Tetrahymanea (Louka et al., 2018), structural aberrations at the tip of the primary cilia of CEP104mut cells are likely to be expected. However, KIF7/C0 mouse embryonic fibroblasts (MEFs) do gain ciliary SMO in response to Hh pathway stimulation (He et al., 2014), suggesting that structural defects at the cilia tip may not affect the ciliary accumulation of SMO per se. Peripheral IFT-B sub-complex IFT25/IFT27 defective MEFs show a complementary phenotype and accumulate SMO in cilium, even in the absence of pathway stimulation, indicating SMO regulation at the export level (Eguether et al., 2014; Keady et al., 2012). Under the presumption of a similar balance existing in hTERT-RPE1 cells, ciliary export of SMO may be favored in Hh-unstimulated WT, CEP104mut, and likely also CSPP1/C0 hTERT-RPE1 cells. One may hypothesize that CEP104, beyond its architectural function, could interfere via its IFT25-homologous N-terminal domain with IFT25/IFT27 at the cilia tip to regulate ciliary residence of SMO in response to pathway activation (Keady et al., 2012; Eguether et al., 2014; Huet et al., 2014; Bhogaraju et al., 2011; Milenkovic et al., 2015). In support of this idea, we have detected an interaction between recombiant CEP104 and IFT27 in preliminary experiments (Figure S7), but the validation and functional analyses warrant a study in their own right.

To conclude, we demonstrate in vitro and in vivo that ciliary complex formation of CEP104 and CSPP-L is essential for Hh signal-competent tip compartment and/or axoneme formation, indicating abrogation of this process as the underlying molecular defect in JBTS resulting from CEP104 mutations. The localization pattern of CSPP-L defines an interesting link between the centriolar satellite and the MT axoneme compartment. Further investigations on the dynamic regulation of cilia tip and centriolar satellite protein networks may thus provide a future avenue of ciliopathy research.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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- **METHOD DETAILS**
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  - Protein extraction and immunoblotting on zebrfish samples
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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.07.025.

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.
REFERENCES


transition zone to promote Sonic hedgehog signalling. Nat. Commun. 8, 14177.


### KEY RESOURCES TABLE

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### Chemicals, Peptides, and Recombinant Proteins

- **Smoothen Agonist (SAG)**: Cayman Chemicals | Cat#1914
- **Prolong Gold**: Life Technologies | Cat#P36930
- **bisBenzimide H 33258 (Hoechst 33258)**: Sigma-Aldrich | Cat#14530

### Critical Commercial Assays

- **GFP-trap_MA**: Chromotek | Cat#gtma-20

### Experimental Models: Cell Lines

- **Human: hTERT-RPE1**: ATCC | Cat#CRL-4000; RRID: CVCL_4388
- **Human: hTERT-RPE1 CSPP1 --/--**: This Paper | N/A
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**Experimental Models: Organisms/Strains**

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LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and request for reagents should be directed to and will be fulfilled by the Lead Contact, Sebastian Patzke (sebastip@rr-research.no).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish husbandry

All zebrafish procedures were performed under Home Office UK license regulations. We used the zebrafish golden strain, AB strain, the transgenic islet1:GFP strain (Tg(islet1::GFP) which expresses GFP in cranial motor neurons under the control of islet1 promoter (Higashijima et al., 2000) and the transgenic strain (Tg(cmlc2::GFP), expressing the GFP gene under the control of the cmlc2 promoter (Huang et al., 2003).

METHOD DETAILS

Cell culture and genetic manipulation of hTERT-RPE1 cells

hTERT-RPE1 cells (ATCC #CRL-4000) were maintained in DMEM-F12 medium (Life Technologies, Carlsbad, CA, US) supplemented with 10% Fetal Calf Serum (Life Technologies) and Penicillin/Streptomycin (Sigma-Aldrich, St.Louis, MO, US) in a humidified environment at 37°C and 5% CO2. For ciliogenesis assays 7x10^4 cells/30 mm well were seeded on coverslips 24h prior to serum withdrawal by two washes with 2 mL pre-warmed serum-free DMEM/F12, and further incubated for 48h. Hh-pathway stimulations were performed on serum starved cells (48h) by addition of 100 nM SAG (Cayman chemical, Ann Arbor, MI, US) at 100 nM (f.c.) or replacement of 1ml medium with ShhN conditioned DMEM (Life Technologies) including 2% Fetal Calf Serum derived from sterile filtered culture supernatants of Hek293T (Clonetech #632180, Takara Bio Europe, Saint-Germain-en-Laye, FR) cells transfected with a ShhN expression plasmid described earlier in (Zeng et al., 2001). Control cells were mock treated with DMSO or Hek293T culture supernatant, respectively. For genetic targeting of CSPP1 and CEP104 loci hTERT-RPE1 cells were transfected in at 70% confluence in 10 cm culture dishes with custom made single vectors (DNA2.0, Newark, CA, US) expressing CAS9-D10A (Nickase), Paprika-RFP, and two gene specific gRNA sequences (see Figures S2 and S3 for targeting sequence details). Single transfected cells were isolated by flow cytometry assisted cell sorting on Paprika-RFP expression, individually expanded and 24 monoclonal colonies characterized for target gene and protein expression. Genomic Target regions from selected clones were amplified by PCR on genomic DNA using gene specific primers (see Figure S5). PCR products were sub-cloned into pGEM-T vector (Promega) and ten individual clones analyzed by Sanger sequencing using SP6 and T7 directed primers, respectively. Flow cytometry assisted cell sorting and cell cycle analysis was performed as described in (Dale Rein et al., 2015).

Third-generation lentivirus was generated using procedures and plasmids as previously described (Campeau et al., 2009). Briefly, tagged fusions of transgenes were generated as Gateway ENTRY plasmids using standard molecular biology techniques. From
these vectors, lentiviral transfer vectors were generated by Gateway LR recombination into lentiviral destination vectors (Gateway-enabled vectors derived from pCDH-EF1a-MCS-IRE-PURO (SystemBiosciences)). VSV-G pseudotyped lentiviral particles were packaged using a third-generation packaging system (Addgene plasmids #12251, 12253, 12259). Cells were then transduced with low virus titers (multiplicity of infection < 1) and stable expressing populations were generated by antibiotic selection. Detailed cloning procedures are available from the authors.

**Plasmids, antibodies and reagents**

Plasmids for genetic targeting were acquired from DNA2.0 (Newark, CA, US)). pmCherry-CEP104, pEGFP-CEP104 and pEGFP-CEP104-truncates were described earlier (Jiang et al., 2012). pDEST-GLAP3 was acquired from Addgene (Cambridge, MA, US; Torres et al., 2009). Plasmid for expression of soluble Hedgehog ligand (ShhN; (Zeng et al., 2001)) was obtained from B. K. Yoder (Department of Cell Biology, University of Alabama, USA), the plasmid for expression of mCherry-ARL13B from K. J. Verhey (University of Michigan, USA), and plasmids for IFT25/IFT27 expression from Esben Lorentzen (University of Aarhus, Denmark). CSPP-L and CSPP-L truncate expression plasmids were described earlier (Patzke et al., 2010). All plasmid transfections were performed using Lipofectamine3000 (Life Technologies) according to the protocol from the manufacturer. Detailed information on antibodies are provided in the STAR Methods. Directly Atto488-fluorophore conjugated probes for GFP detection and anti-GFP conjugated paramagnetic beads for immunoprecipitations were from Chromotek (Chromotek GmbH, Munich, DE).

**Immunoprecipitations and immunoblotting**

Preparation of cell lysates, gel electrophoresis, blotting and immuno-detection was performed as described earlier (Sternemalm et al., 2015). For immunoprecipitation cells were washed thrice in Phosphate buffered saline (PBS; Sigma-Aldrich) and then lysed on ice in cold lysis buffer (50 mM HEPES, pH 7, 150 mM NaCl, 5 mM EDTA, pH 8, 0.1% NP-40, 10% glycerol) supplemented with phosphatase inhibitor cocktails II and III (Sigma-Aldrich) and complete™ protease inhibitor (Roche Diagnostics, Basel, Switzerland). Lysates were collected using a cell scraper, transferred to reaction tubes, left on ice for 20 min for solubilization and then centrifuged at 20,000g for 15 min. Clarified supernatants were transferred to new reaction tubes for immunoprecipitation using GFP-trap paramagnetic beads (Chromotek GmbH) at 4°C for 2h on a spinning wheel. Beads were washed twice in 500 µl lysis buffer and transferred to a new reaction tube for a third wash. Purified proteins were released from beads and denatured in 40 µl SDS-sample loading buffer and 5 min incubation at 95°C.

**Immunofluorescence and live cell microscopy**

Cells were grown on heat-sterilized cover glasses (No.1014; Glaswarenfabrik Karl Hecht GmbH & Co KG, Sondheim/Rhön, DE), fixed for 15 min in 1% neutral buffered formalin solution at room temperature prior to post-fixation in methanol (−20°C). Cells were rehydrated for IFM staining by three consecutive washes in PBS and blocked and permeabilized for 30 min in PBS-AT (PBS containing 5% wt/vol Bovine serum albumin (BSA) and 0.1% vol/vol Triton X-100). Cells were stained with primary antibodies for 2h at room temperature, washed thrice in PBS, and stained with secondary antibodies for 45 min. All antibody incubations were performed in PBS-AT. Cells were washed thrice in PBS, counterstained for DNA (Hoechst 33258 in PBS, Sigma), washed briefly in distilled water, dried and mounted on object glasses using Prolong Gold (Life Technologies). Fluorescence images were acquired using appropriate optical filters on a multi-fluorescent bead calibrated Axiolmager Z1 ApoTome microscope system (Carl Zeiss, Jena, DE) equipped with a 100x or a 63x lens (both PlanApo N.A.1.4) and an AxioCam MRm camera. To display the entire cell volume, images are presented as maximal projections of z stacks using Axiovision 4.8.2 (Carl Zeiss).

3D-SIM imaging was performed using a Deltavision OMX V4 microscope (GE Healthcare, Little Chalfont, UK) equipped with three water-cooled PCO.edge sCMOS cameras, 405 nm, 488 nm, 568 nm and 642 nm laserlines and a 60x 1.42NA Plan-Apochromat lens (Olympus, Tokyo, JP). z stacks covering the whole cell, with sections spaced 0.125 mm apart, were recorded. For each z section, 15 raw images (three rotations with five phases each) were acquired and the final super-resolution images were reconstructed using softWoRx software (GE Healthcare).

Images for quantitative IFM imaging were acquired on a multi-fluorescent sub-micron beads calibrated CellObserver microscope system (Carl Zeiss) equipped with a 40 ×/1.3 PlanApo Phase 3 lens and an AxioCam MRm camera. Images were acquired with constant exposure times at 10 random positions per coverslip and in seven optical sections at 0.5 μm distance, centered around focal planes for cilia. Focal planes were identified by glutamylated tubulin or ARL13B labeling as cilia reference, respectively, using a contrast based autofocus routine (AxioVision 4.8.2). Image analysis was performed in Fiji/ImageJ (Schindelin et al., 2012). SUM projections of individual channels were background corrected using a 5px rolling circle algorithm and cilia segmented in cilia reference channels by signal intensity and morphological thresholds to create cilia masks. Fluorescence signal intensities under each mask were measured in all channels and median signal intensities determined.

All statistical analysis was performed using t test analysis tool in SigmaPlot v12.5 (Systat, Inc., San Jose, CA,US) and boxplots created in BoxplotR (Spitzer et al., 2014).

For live cell microscopy cells were grown in 35-mm ibiTreat μ-culture dishes (Ibidi, Munich, DE) and imaged using a CellObserver microscope system (Carl Zeiss) equipped with a 40 ×/1.3 PlanApo Phase 3 lens, a Hamamatsu ORCA-Flash4.0 v3 camera, a temperature controlled XL-chamber, a temperature, humidity and CO₂ controlled stage incubator, a motorized coded X,Y-stage, a Definite Focus system and a HXP120 Metal-Halide illumination unit.
Post-embedding immunogold electron microscopy

Small pieces (about 2 mm²) of mouse trachea were fixed in MT-buffer (30 mM HEPES, 5 mM Na-EGTA, 15 mM KCl, pH 7.0) containing 3.5% formaldehyde for 2–3 h at 4°C. After two brief washes with MT-buffer the tissue was dehydrated to 100% ethanol (30% and 50% ethanol on ice; then 70%, 95%, 100% ethanol at −20°C, 15 min each). Infiltration of the samples with LR Gold resin (London Resin Company, Reading, GB) was performed at −20°C according to the following scheme: LR Gold/ethanol (1:1) for 2 h, LR Gold/ethanol (3:1) for 4 h, LR Gold containing 0.4% benzil for 36 h (with several changes of the medium). Polymerization was performed under fluorescent light for 48 h at −20°C. Ultrathin sections (60-80 nm) were cut with a diamond knife (type ultra 35°; Diatom, Biel, CH) on a EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, DE) and mounted on pioiloform-coated, single-slot gilded copper grids (Service Science, Munich, DE). For immunolabeling, the sections were blocked for 1-2 h at room temperature with blocking buffer (2% BSA, 0.1% fish gelatin and 0.05% Tween 20 in PBS; pH 7.4) and incubated in anti-CSPPL antibody (polyclonal, rabbit, diluted 1:200 or 1:1000 in blocking buffer) overnight at 4°C. Grids were washed 3-5 times with PBS containing 0.15% BSA-c (Aurion, Wageningen, NL) for 10 min each and incubated for 1.5 h with 15-nm gold particles conjugated to goat anti-rabbit IgGs (British Bio-cell, Cardiff, GB) diluted 1:30 in blocking buffer. Grids were washed 3-5 times with PBS containing 0.15% BSA-c for 10 min each, fixed for 8 min in 1% glutaraldehyde in PBS and washed three times with 5 min each in distilled water. After immunolabeling, the sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a JEM-2100 transmission electron microscope (JEOL, Tokyo, JP) operated at 80 kV. Micrographs were taken using a 4,080 × 4,080 pixels charge-coupled device camera (UltraScan 4000, Gatan, Pleasanton, CA, US) and Gatan Digital Micrograph software (version 1.70.16). Image brightness and contrast were adjusted using Adobe Photoshop 8.0.1.

Direct yeast two-hybrid interaction assay, sucrose density fractionation and size exclusion chromatography

The direct interaction between CSPPL and other ciliary proteins was tested using a GAL4-based yeast two-hybrid system, with yeast strain PJ69-4A and PJ69-4x, using general procedures for yeast mating described previously (Letteboer and Roepman, 2008). In brief, a construct encoding full-length CSPPL, fused to either a DNA-binding domain (GAL4-BD), or to a transcription activating domain (GAL4-AD) were used to screen a gridded library of cDNA clones, expressing different ciliary and/or ciliopathy-associated proteins, fused to GAL4-AD or GAL4-BD, respectively. The direct interaction between baits and preys induced the activation of the reporter genes, resulting in the growth of yeast colonies on selective media (deficient of Leu, Trp, His, and Ade) and induction of α-galactosidase and β-galactosidase colorimetric reactions. Positive clones were subsequently validated by co-transformation of the cognate plasmids, and growth selection on media lacking His, Leu, and Trp, supplemented with 10 mM 3-AT. For density fractionation 200 μl total cell lysate was loaded onto a continuous 10%–60% sucrose gradient prepared by mixing 60 and 10% sucrose solutions (5.5 mL of each) using a Biocomp Gradient Master (BioComp Instruments, Fredericton, CA) and centrifuged at 100.000×g for 16 h at 4°C in a SW-40Ti rotor (Beckman-Coulter, Pasadena, CA, US). Procedures for size exclusion chromatography have been published previously (Schou et al., 2017).

Zebrafish genetic manipulation

All zebrafish procedures were performed under Home Office UK license regulations. Zygotes were collected from natural spawning and placed in Petri dishes of E3 medium (Westerfield, 2000). Zebrafish embryos were collected and raised at 28.5°C and staged in somite stage and hpf according to standard criteria (Kimmel et al., 1995). Antisense morpholino oligonucleotides (MOs) were designed (Gene Tools, Philomath, Oregon, US) to target zebrafish cep104 (XP_003199125.2) as follows: Intron-Exon splice MO: 5'-TGACAAAACCTACACACAATAGAT-3'; translation blocking (cep104 ATG MO): 5'-CACCCTTTGA CAACTGTGGCATGTG-3'. Stock MOs in RNase free water were diluted with 0.05% phenol red in Danieau buffer (Nasevicius and Ekker, 2000) to produce the solution for injection. Escalating doses of each MO were tested for phenotypic effects. Embryos were injected with 0.8 pmol/embryo of cep104 splicing MO or 0.8 pmol/embryo of cep104 ATG MO at 1- to 2-cell stage. For combined knockdown experiments, 0.8 pmol/embryo of each MO was used. For rescue experiments, morpholino was co-injected with 250 pg/embryo of CEP104 mRNA.

Protein extraction and immunoblotting on zebrafish samples

Whole protein extracts were obtained from 48 hpf de-yolked zebrafish embryos by mechanical desegregation of the embryos into Laemmli sample buffer (Laemmli, 1970). Samples were incubated at 98°C for 5 min and resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (Thermo Fisher). The membranes were incubated with the following antibodies: mouse monoclonal IgG2a anti-CEP 104 (XP_003199125.2) as follows: Intron-Exon splice MO: 5'-TGACAAAACCTACACACAATAGAT-3'; translation blocking (cep104 ATG MO): 5'-CACCCTTTGA CAACTGTGGCATGTG-3'. Stock MOs in RNase free water were diluted with 0.05% phenol red in Danieau buffer (Nasevicius and Ekker, 2000) to produce the solution for injection. Escalating doses of each MO were tested for phenotypic effects. Embryos were injected with 0.8 pmol/embryo of cep104 splicing MO or 0.8 pmol/embryo of cep104 ATG MO at 1- to 2-cell stage. For combined knockdown experiments, 0.8 pmol/embryo of each MO was used. For rescue experiments, morpholino was co-injected with 250 pg/embryo of CEP104 mRNA.

Kupffer’s vesicle imaging

Uninjected and cep104 MO injected zebrafish embryos were fixed at the 10 somite stage, using 4% paraformaldehyde in PBS at 4°C overnight. To permeabilise, embryos were washed in ddH2O then pre-chilled acetone (−20°C) for 7 min. Embryos were washed in ddH2O and blocked in 5% BSA, with 1% DMSO and 0.1% Tween. For cilia staining, embryos were incubated in primary antibody (mouse anti-acetylated tubulin antibody, 1:500, Sigma T6793) overnight at 4°C and detected using a donkey anti-mouse.
AlexaFluor594 conjugated secondary antibody (1:300, Life Technologies). For identification of KV epithelium, antibodies directed toward aPKC were used (rabbit anti-aPKC (1:500, SCBT) detected with goat anti-rabbit Alexa Fluor 488 conjugated secondary antibody (1:300, Life Technologies). Embryos were washed into PBS, mounted and imaged using an Axio Imager Z1 fluorescence microscope (Zeiss).

**Zebrafish husbandry and genetic manipulation**
The cep104 sgRNA was designed using https://www.crisprscan.org/ to target the following region of cep104 gene: 5'-GGTGGGCGAACGGTTGGGC-3'. sgRNA and Cas9 protein (NEB) were solubilized with 300 mM KCl and diluted in 0.05% phenol red in RNase free water at final concentration of respectively 300ng/ul and 4uM and were injected into 1-cell stage embryos. Zebrafish were anaesthetized with Tricaine solution and phenotyped at 48 hpf. Images were captured using a fluorescent stereomicroscope (Leica MZ16F). sgRNA was synthesized using a cloning-free, oligo-based method (Varshney et al., 2016). In vitro transcription was carried out using MEGAscript T7 transcription kit (Thermo Fisher). sgRNA was then purified with mirVana Isolation Kit (Thermo Fisher). To check the specificity and efficiency of genome editing, 11 embryos from a sample population of F0 animals were genotyped by amplifying surrounding region of targeted cep104 sequence, using gene-specific primer pairs (5'-TTGGCAAGTCAATGTCTTCTTT-3' and 5'-GCTGATGGTAGACTGCGAGT-3'). Amplification product from each F0 crispant embryos was then sequenced and compared to amplification product from uninjected embryos to screen for mutations.

**Zebrafish RNA isolation and RT-PCR**
Total RNA was isolated from single zebrafish embryos at 48 hpf. RNA was used for each experimental group in reverse transcription (RT) reactions. Superscript VILO cDNA synthesis kit (Life Technologies) was used for RT. PCR using gene-specific primer pairs (5'-ATGCCAAAAAGCTGATGGTC-3 and 5'-ACCCAACAGCATCAACATGA-3) was performed to identify splice products following cep104 splice MO injection.

**Pronephros imaging**
For pronephros imaging, uninjected and cep104 MO or CRISPR/Cas9- injected embryos were fixed at 72 hpf with 4% paraformaldehyde in PBS at 4°C overnight. To permeabilise embryos they were washed in ddH2O then pre-chilled acetone (−20°C) for 7 min. Embryos were washed in ddH2O, treated with collagenase A (Roche) at 1mg/ml in PBS-Tween 0.1% for 30’ at room temperature and blocked in 5% horse serum in PBS-Tween 0.1% for 1 h at room temperature. For cilial staining, embryos were incubated in primary antibody (mouse anti-acetylated tubulin antibody, 1:500, Sigma T6793) overnight at 4°C and detected using a donkey anti-mouse AlexaFluor594 conjugated secondary antibody (1:400, Life Technologies). Embryos were washed into PBS and imaged using confocal microscopy (A1R Confocal, Nikon).

**Evolutionary and comparative structure analysis**
Putative CEP104 and CSPP1 orthologs were identified using a combination of reciprocal best BLASTP and iterative BLASTP as simple BLAST searches. Protein sequences were used to query the non-redundant predicted proteomes of flagellate and non-flagellate organisms, chosen to represent a wide evolutionary spread of eukaryotes. Searches were carried out at eggNOG4.5 (Huerta-Cepas et al., 2016), NCBI (https://www.ncbi.nlm.nih.gov/) or JGI (https://www.jgi.doe.gov/) depending on the organism. Comparative structure analysis was carried out at HH Pred (Hildebrand et al., 2009).

**QUANTIFICATION AND STATISTICAL ANALYSIS**
Statistical analysis were performed using SigmaPlotv12.5 (SysStat). Statistical details of experiments are stated in the legends of figures displaying the respective data, including the statistical tests used, the number of replicates and number of investigated cells/fish, measures of precision and definitions of significance.