Early steps in primary cilia assembly require EHD1/EHD3-dependent ciliary vesicle formation

Quanlong Lu1,8, Christine Insinna1,8, Carolyn Ott2, Jimmy Stauffer4, Petra A. Pintado5, Juliati Rahajeng4,5, Ulrich Baxa6, Vijay Wallia1, Adrian Cuenca1, Yoo-Soon Hwang1, Ira O. Daar1, Susana Lopes6, Jennifer Lippincott-Schwartz2, Peter K. Jackson7, Steve Caplan4,5 and Christopher J. Westlake1,9

Membrane association with mother centriole (M-centriole) distal appendages is critical for ciliogenesis initiation. How the Rab GTPase Rab11–Rab8 cascade functions in early ciliary membrane assembly is unknown. Here, we show that the membrane shaping proteins EHD1 and EHD3, in association with the Rab11–Rab8 cascade, function in early ciliogenesis. EHD1 and EHD3 localize to preciliary membranes and the ciliary pocket. EHD-dependent membrane tubulation is essential for ciliary vesicle formation from smaller distal appendage vesicles (DAVs). Importantly, this step functions in M-centriole to basal body transformation and recruitment of transition zone proteins and IFT20. SNAP29, a SNARE membrane fusion regulator and EHD1-binding protein, is also required for DAV-mediated ciliary vesicle assembly. Interestingly, only after ciliary vesicle assembly is Rab8 activated for ciliary growth. Our studies uncover molecular mechanisms informing a previously uncharacterized ciliogenesis step, whereby EHD1 and EHD3 reorganize the M-centriole and associated DAVs before coordinated ciliary membrane and axoneme growth.

Primary cilia play essential roles in signal transduction, and defects in cilia formation or function cause ciliopathies1,2. Cilia form at the distal end of the mother centriole (M-centriole) through recruitment of preciliary membranes, intraflagellar transport (IFT) machinery and transition zone (TZ) components to enable microtubule-based axonemal assembly. Association of preciliary membranes with M-centriole distal appendages is needed for basal body formation and ciliogenesis progression3–11. The membrane trafficking regulator Rab small GTPases and in particular, the Rab11–Rab8 cascade are required for ciliary membrane formation during ciliogenesis12–17. In this cascade, Rabin8 (also known as Rab3ip), the guanine nucleotide exchange factor for Rab8, binds to Rab11 and is delivered to the centrosome on vesicles to activate Rab8 to promote ciliary membrane assembly15.

Over 50 years ago, Sergei Sorokin proposed a model whereby intracellular membranes organize at the distal end of the M-centriole before axoneme formation. A large ciliary vesicle (CV) assembles, reorganizes to form a sheath around the extending axoneme and later fuses with the plasma membrane10,18. The requirement for the Rab11–Rab8 cascade in ciliogenesis provides a molecular explanation for these early ciliogenesis steps but is poorly understood. Furthermore, how ciliary membrane assembly is coordinated with other early ciliogenesis processes including establishment of the basal body, IFT recruitment, TZ assembly and axoneme growth is largely unknown.

The Eps15 homology domain (EHD) family of proteins, composed of EHD1–4, is associated with Rab11 and Rab8 membranes and regulates endosomal membrane trafficking19. EHDs are characterized by an ATP-binding G-domain, a central coiled-coil domain and a carboxy-terminal EH domain, which interacts with asparagine-proline-phenylalanine (NPF) motif containing proteins20. EHD1 and EHD3 exhibit 87% amino-acid identity, whereas EHD2 and EHD4 are less than 74% identical to EHD1. EHD1 and EHD3 regulate Rab11 endosome recycling compartment (ERC) trafficking and bind to Rab11fip2, a Rab11 effector21. Furthermore, EHD1 and EHD3 bind to the Rab8 effector MICAL-L1 and affect membrane tubulovesicle formation and scission19,22–25. EHD1 also associates with the membrane fusion regulator SNAP29 (ref. 26). Here, we investigated EHD protein involvement in ciliary membrane biogenesis. Using

1Laboratory of Cell and Developmental Signaling, National Cancer Institute—Frederick, Frederick, Maryland 21702, USA. 2Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892, USA. 3CEDOC, Faculdade de Ciências Médicas, Universidade Nova de Lisboa, 1169-056 Lisboa, Portugal. 4Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA. 5The Fred and Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA. 6ATRF, NCI-Frederick National Laboratory, Frederick, Maryland 21701, USA. 7Baxter Laboratory for Stem Cell Biology, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305, USA.
8These authors contributed equally to this work.
9Correspondence should be addressed to C.J.W. (e-mail: chris.westlake@nih.gov)

Received 7 February 2014; accepted 13 January 2015; published online 16 February 2015; corrected after print 12 March 2015; DOI: 10.1038/ncb3109

© 2015 Macmillan Publishers Limited. All rights reserved
advanced microscopy imaging approaches, we dynamically observed the recruitment of proteins essential for early ciliogenesis processes. Moreover, depletion of EHD proteins sheds light on a previously uncharacterized but required step in ciliogenesis. Our data suggest a model in which EHD1 and EHD3 coordinate critical steps at the onset of ciliogenesis.

RESULTS

EHD1 and EHD3 function in ciliogenesis and localize to the ciliary pocket membrane

Because EHD1 and EHD3 have been linked to both Rab11 and Rab8 membrane compartments, we tested their role in ciliogenesis. Short interfering RNA (siRNA)-mediated knockdown of EHD1, but not EHD2–4, impaired ciliation in hTERT-RPE (RPE) cells (Fig. 1a,b and Supplementary Fig. 1a–c). Importantly, an siRNA resistant form (Res) of GFP–EHD1 or GFP–EHD3 but not GFP (green fluorescent protein), GFP–EHD2 or GFP–EHD4 rescued ciliation (Fig. 1c and Supplementary Fig. 1d–f), suggesting that EHD1 and EHD3 function in ciliogenesis. Interestingly, only GFP–EHD1 and GFP–EHD3 were detected in the proximal ciliary region (Fig. 1d and Supplementary Fig. 1d). Endogenous EHD1 was also detected in the proximal ciliary region in 30 ± 5% (±s.d.) of cells (n = 162, pooled from three experiments) and did not completely overlap with ciliary Smo–tRFP, where Smo is Smoothened and tRFP is red fluorescent protein TagRFP, or GFP–Rab8a (Supplementary Fig. 1g,h). Overexpressed GFP–EHD3 co-localizes with EHD1 in this region (Supplementary Fig. 1g). Immunoblotting of RPE cell lysates revealed that EHD1 levels were more than five times higher than those of EHD3 (Fig. 1e), indicating that EHD3 may be dispensable for RPE cell ciliation. In IMCD3 cells, EHD1 and EHD3 had similar expression levels (Fig. 1e) and were both required for ciliogenesis (Fig. 1f,g). GFP-fused EHD1 and EHD3 were detected in the proximal ciliary region in 8 ± 2% (±s.d., n = 164, pooled from two experiments) and 8 ± 4% (±s.d., n = 152, pooled from two experiments) respectively (Supplementary Fig. 1i).

We investigated EHD protein ciliary localization using correlative light and electron microscopy (CLEM), immuno-electron microscopy (EM) and super-resolution structured illumination microscopy (SIM), and demonstrated that EHD1 and EHD3 are primarily localized to the ciliary pocket membrane, unlike Rab8 and Smo, largely present in the ciliary membrane (Fig. 1h–j and Supplementary Fig. 1j,k). Furthermore, EHD1 localization did not overlap with Inversin (Supplementary Fig. 1l), a proximal intra-ciliary marker. The localization of EHD1 and EHD3 in IMCD3 cells is consistent with lower levels of ciliary pockets reported in these cells when compared with RPE cells, which could point to different ciliogenesis mechanisms. Together, our results indicate that EHD1 and EHD3 are important for ciliogenesis and localize to the ciliary pocket membrane.

Requirements for Ehd1 and Ehd3 in ciliogenesis during development in zebrafish

We investigated the ciliogenesis function of EHD proteins in zebrafish embryos. Zebrafish have five ehd homologues: ehd1a, ehd1b, ehd2a, ehd2b and ehd3. ehd1a and 1b (89% identity) are closely related to human EHD1 (85% and 87% respectively), and ehd3 is 93% identical to human EHD3. Combination of ehd1(a+b) morpholino oligonucleotides (MOs) gave synergistic effects, whereas the triple knockdown with ehd3 MO had 70 ± 6% lethality (±s.d., n > 150, from three experiments). As was observed in mammalian cells, hEHD1 and hEHD3 rescued phenotypes observed in ehd3 and ehd1 morphants, unlike hEHD4 (Fig. 2b,d,f and Supplementary Fig. 2a). Histological and EM analysis in ehd1 and ehd3 morphants revealed a failure to form photoreceptor cilia/outer segments (Fig. 2b,c), suggesting that both proteins are important for photoreceptor ciliogenesis, similar to IMCD3 cell requirements. However, in other tissues, requirements for Ehd proteins varied. Ehd1, but not Ehd3, was required for kinocilium formation in otic vesicles (Fig. 2d,e). Similarly, Ehd1 was essential for neuromast ciliation, whereas ehd3 morphants showed a partial reduction in kinocilia (Fig. 2f and Supplementary Fig. 2b). Consistent with these results, Ehd1 and Ehd3 were expressed in photoreceptors and neuromasts, whereas only Ehd1 and not Ehd3 was expressed in otic vesicles during embryonic development (Fig. 2f and Supplementary Fig. 2c–e). In these tissues, Ehd1 and Ehd3 localized to puncta throughout the cell, with some protein detected at or close to the ciliary base. Together, these results indicate that Ehd1 and Ehd3 have overlapping, albeit tissue-specific, functions in ciliogenesis in zebrafish embryos.

EHD1 localizes to preciliary membranes but is dispensable for their centrosomal trafficking

Next, we investigated EHD1 association with Rab11-dependent preciliary vesicle transport of Rabin8. EHD1 co-localizes with GFP–Rabin8 vesicles following serum starvation, but not in the presence of serum (Fig. 3a). Moreover, in contrast to Rab11 requirements, depletion of EHD1 did not affect GFP–Rabin8 centrosomal accumulation (Fig. 3b), suggesting that EHD1 is associated with preciliary vesicles but is not required for their centrosomal trafficking. We carried out live imaging on GFP–EHD1 and tRFP–Rab8a expressing RPE cells and determined that EHD1 and Rab8 associate with developing cilia (Fig. 3c and Supplementary Fig. 3 and Supplementary Video 1). Remarkably, GFP–EHD1 was detected at a site in the cell that later accumulated tRFP–Rab8a and extended ciliary structures, suggesting that EHD1 accumulates at M-centriole-associated membranes before Rab8. Additionally, Smo–tRFP, a ciliary receptor marking early cilium membrane structures, localized to developing cilia about the same time as GFP–EHD1 (Fig. 3d and Supplementary Video 2) and before GFPRab8 (Fig. 3e and Supplementary Video 3), suggesting that EHD1 and Smo occupy the same preciliary vesicles. Similarly, the constitutive active SmoM2 mutant localized to the M-centriole following serum starvation, and its trafficking was not affected by EHD1 or Rab8 depletion (Fig. 3f), indicating that SmoM2 M-centriole transport is independent of these proteins. Furthermore, SmoM2–GFP and EHD1 co-localize at the centrosome in the absence of Rab8 (Fig. 3g). Together, these results indicate that EHD1 functions upstream of Rab8 in ciliogenesis.

EHD1 is required for small distal appendage vesicle assembly into the CV, whereas Rab8 functions in CV extension

RPE cells use an intracellular ciliogenesis pathway with formation of the CV as a critical step. To investigate EHD1 function in this pathway, we carried out EM studies on serum-starved EHD1-depleted cells (Fig. 4a). Strikingly, the majority (68%) of the non-ciliated cells had small ∼40–60 nm diameter vesicles associated with the
Figure 1 EHD1 and EHD3 function in ciliogenesis and localize to the ciliary pocket membrane. (a) Western analysis of EHD proteins from 72 h siRNA treated RPE cells (siRNA no 1 from Supplementary Table 1). (b) Ciliation quantification in 72 h siRNA treated RPE cells (EHD1 siRNA no 2, EHD2 siRNA no 1, EHD3 siRNA no 1, EHD4 siRNA no 1), with 24 h serum starvation, followed by staining with acetylated α-tubulin (α-tub) and pericentrin (PCNT) antibodies. Means ± s.e.m. from n=3 independent experiments are shown (>100 cells per treatment). Two tailed t-test analysis compared with control siRNA. (c) Quantification of ciliation in RPE cells siRNA treated for 6 h, followed by transfection with plasmids encoding ResGFP–EHD proteins or GFP. At 48 h post-transfection, cells were serum starved and stained as in b. Means ± s.e.m. from n=3 independent experiments are shown (>100 cells per condition). Two tailed t-test analysis compared with control siRNA or Ehd(1+3)mus siRNA. (f) Western analysis of EHD protein depletion in IMCD3 cells treated with siRNA for 72 h. (g) Quantification of ciliation in IMCD3 cells treated with siRNAs for 72 h, and where indicated rescued with human GFP–EHD proteins and stained as in a-c. Means ± s.e.m. from n=3 independent experiments are shown (>300 cells per condition). Two tailed t-test analysis compared with control siRNA or Ehd1+3mus siRNA. (h) Immuno-electron micrograph of RPE cells expressing GFP–EHD1 stained with anti-GFP antibody. 10 nm gold particles accumulate on the ciliary pocket (CP) membrane. Insets: magnifications of regions 1 and 2. Ax, axoneme; BB, basal body. Scale bar, 500 nm. (i,j) SIM imaging of 24 h serum starved RPE cells transiently expressing Smo–tRFP stained with anti-EHD1 antibody (i) or transiently expressing GFP–EHD3 (j). Middle panels show fluorescence profile plots and lower panels show orthogonal views of the dotted line region. Representative image of more than ten cilia. Scale bar, 500 nm. ** P < 0.0001, * P < 0.05. Uncropped blots are shown in Supplementary Fig. 6. Statistics source data for b,c and g can be found in Supplementary Table 2.
Figure 2 ehd1 and ehd3 regulate ciliogenesis in zebrafish. (a) Western analysis and quantification of Ehd1 and Ehd3 protein expression in 3 dpf (days post-fertilization) MO injected embryos. Means ± s.e.m. from n=3 independent experiments (lysates with 50 embryos per condition) are shown. (b) Quantification of photoreceptor outer segment (OS) number from histological sections of 3 dpf retinae stained with toluidine blue. Means ± s.e.m. from n=3 independent experiments are shown (>100 photoreceptors per condition). ehd1 MO + EHD1 from n=2 independent experiments (>100 photoreceptors), two tailed t-test analysis, compared with uninjected. (c) Representative electron micrographs from n=3 independent experiments (one or two fish per condition) showing two photoreceptor cells in control, ehd1 and ehd3 morphants at 3 dpf. IS, inner segment. Scale bar, 500 nm. (d) Quantification of otic vesicles with kinocilia in 2 dpf Tg(arl13b–GFP) embryos uninjected or injected with ehd1 or ehd3 MO. Uninjected, n=18, ehd1 MO, n=13, ehd1 MO + hEHD1, n=6; ehd1 MO + hEHD3, n=4; ehd3 MO + hEHD1, n=10. (e) Representative images of otic vesicles described in d with insets showing sensory patches fixed and stained with anti-α-tub antibody and phalloidin (Phal.). Note that ehd1 morphants lack kinocilia. Scale bar, 10 μm. (f) Quantification as in d of neuromasts with normal cilia in 2 dpf embryos stained with α-tub and phalloidin antibodies. Uninjected, n=20; ehd1 MO, n=10; ehd1 MO + hEHD1, n=23; ehd1 MO + hEHD3, n=27; ehd1 MO + hEHD4, n=22; ehd3 MO, n=16; ehd3 MO + hEHD3, n=41; ehd3 MO + hEHD1, n=12; ehd3 MO + hEHD4, n=16. Note that ehd3 morphants present a partial phenotype with reduced number of kinocilia (quantification in Supplementary Fig. 2b). Pooled data across three independent experiments are shown in d and f. (g) Representative images of neuromasts as described in f. Scale bar, 10 μm. (h) Representative images of photoreceptors in retinae of 3 dpf embryos, anterior cristae of otic vesicles and neuromasts stained with anti-Ehd1 or anti-Ehd3 and anti-rhodopsin or anti-α-tub antibodies showing punctate accumulation of proteins in the cytosol and around the base of cilia in these organelles. Organs were imaged with the same fluorescence microscopy settings, which show that Ehd3 levels were low in the otic vesicles. Scale bar, 10 μm. DAPI, 4′,6-diamidino-2-phenylindole. Uncropped images of blots are shown in Supplementary Fig. 6. Statistics source data for a and b are found in Supplementary Table 2.
Figure 3 EHD1 localizes to preciliary membranes and the developing cilia.

(a) Representative images of GFP–Rabin8 stably expressed in RPE cells grown in the presence or absence (1 h) of serum, and stained with antibodies to EHD1 and centrosomal marker γ-tubulin. Scale bar, 1 μm. (b) Quantification of RPE GFP–Rabin8 cells treated with siRNA for 72 h and imaged live by epifluorescence microscopy for GFP–Rabin8 centrosomal accumulation 1 h after serum starvation. Means ± s.e.m. from n=3 independent experiments are shown (total number of cells counted in all experiments: control siRNA, 198 cells; Rab11a+8b siRNA, 318 cells; EHD1 siRNA, 139 cells). Two tailed t-test analysis, compared with control siRNA. (c) RPE GFP–EHD1 cells transiently expressing tRFP–Rab8a were serum starved for 1 h and imaged live by spinning disc confocal (SDC) microscopy. Images are maximum intensity projections of z-stacks. (d) RPE GFP–EHD1 cells transiently expressing Smo–tRFP were imaged as in c. Arrows show accumulation of EHD1 and Smo over time in developing cilia. (e) RPE GFP–Rab8a cells transiently expressing Smo–tRFP were imaged as in c. Images are single xy-planes. Note that c–e are representative image series of more than ten cilium assembly events observed. Scale bars c–e, 2 μm. (f) RNA interference (RNAi) of EHD1 or Rab8a+8b in stably expressing RPE SmoM2–GFP cell line. Left: Cells were stained with cilium marker (AcCtub) and centrosome marker (PCNT). Right: Quantification of SmoM2–GFP centrosomal accumulation. Means ± s.d. are pooled data from n=9 areas imaged in three independent experiments (total number of cells counted in all experiments: control siRNA (+ ser), 123; control siRNA (− serum), 179; Rab8a+8b siRNA, 172; EHD1 siRNA no 1, 241). Two tailed t-test analysis, compared with control siRNA (− serum). Scale bar, 1 μm. P < 0.0001. Statistics source data for b and f can be found in Supplementary Table 2.
cells, 63% of Rab8 siRNA-treated unciliated cells had CV structures, whereas only 28% had DAVs (Fig. 4a,b and Supplementary Fig. 4a). Combined with our live imaging studies, these results suggest that EHD1 and Rab8 regulate different steps in ciliogenesis, with EHD1 important for CV formation and Rab8 functioning in CV extension.

SIM imaging has been used to resolve the structure of CEP164, a distal appendage protein forming a ring ~300 nm in diameter at the distal end of the M-centriole29. We carried out SIM on SmoM2–GFP expressing RPE cells to attempt to resolve preciliary membrane assembly steps at the M-centriole distal end (Fig. 4c–e). In serum-starved EHD1-depleted cells, SmoM2–GFP did not co-localize with the CEP164 ring, although SmoM2–GFP and Rab11 partially co-localized on pericentriolar vesicles (Fig. 4c). However, in serum-starved EHD1-depleted cells, SmoM2–GFP vesicles were detected at the CEP164 ring and partially co-localized with Rab11 both at the distal appendages and in the pericentriolar region (Fig. 4d). From our EM studies, the SmoM2–GFP vesicles are probably DAVs. In contrast, in Rab8-depleted cells SIM showed a single large structure at the M-centriole distal end, which is probably a CV (Fig. 4e). On the basis of our previous work13 examining ciliogenesis initiation following serum withdrawal, we looked for DAVs similar to those observed in EHD1-depleted cells using SIM (Fig. 4d,f). Following 3 h starvation, we observed Smo–GFP in the pericentriolar region and on DAVs that co-localized with EHD1, supporting an association of EHD1 with preciliary vesicles and DAVs (Fig. 4f).

Because intracellular ciliogenesis has been observed in mouse photoreceptors30, we tested Ehd1 and Ehd3 ciliogenesis function in zebrafish photoreceptors. Consistent with our human cell data, in ehd1 and ehd3 double morphants, DAVs were frequently observed on the M-centriole distal appendages, whereas in rab8a morphants, the majority of non-ciliated M-centrioles had CVs (Fig. 4g,h and Supplementary Fig. 4b). Interestingly, Ehd proteins were detected near the centrioles before outer segment formation at 50 hpf (hours post-fertilization) (Fig. 4i). Together, our results in human RPE cells and zebrafish photoreceptors support a model wherein EHD1 is recruited on preciliary vesicles to the M-centriole to function in CV assembly from DAVs, whereas Rab8 is associated with post-CV ciliogenesis processes.

EHD1-dependent CV formation is required for recruitment of TZ proteins and IFT20

To study the relationship between DAV docking, CV assembly steps and other ciliogenesis processes, we examined recruitment of TZ proteins and IFT proteins. We discovered that EHD1 was required for the recruitment of TZ proteins CEP290, RPGRIP1L, TMEM67 and B9D2–GFP to the M-centriole distal region, whereas Rab8 seemed to be dispensable (Fig. 5a–d). We further confirmed that preciliary membranes accumulated at the M-centriole before TZ protein recruitment by live cell imaging of B9D2–GFP and Smo–tRFP (Fig. 5e), and another ciliary receptor 5-HT6–tRFP (Supplementary Fig. 5). B9D2–GFP also localized to the developing cilium before Rab8a (Fig. 5f). IFT20, a protein known to localize to the Golgi, centrosome and cilia, is thought to be important for CV formation63. Similarly to TZ proteins, IFT20 accumulation at the M-centriole was strongly blocked in EHD1-depleted cells, but not Rab8-depleted cells (Fig. 5g). Furthermore, IFT20–GFP accumulated at the M-centriole after Smo–tRFP recruitment (Fig. 5h and Supplementary Video 4), but before Rab8 localization (Fig. 5i and Supplementary Video 5). IFT20 basal body enrichment coincided with B9D2 recruitment (Fig. 5j). Therefore, our studies suggest that EHD1-dependent CV assembly function is required before IFT20 and TZ protein recruitment, whereas Rab8 targets to the CV following these steps.

EHD1 is required for CP110 loss from the distal end of the M-centriole

Because CP110 loss from the M-centriole is required for basal body formation and precedes axoneme growth32, we examined its relationship with EHD1-dependent CV assembly. Rab8 seems to be dispensable for CP110 loss, with ~70% of cells having only daughter centriolar CP110. In contrast, in EHD1-depleted cells 80% of cells had CP110 localized to both centrioles (Fig. 6a,b). To further demonstrate requirements for DAV membranes in CP110 loss during ciliogenesis, we investigated this process in live cells using preciliary membrane markers. We observed 5-HT6–tRFP membranes accumulating at the M-centriole following the loss of GFP–CP110 a few minutes later (Fig. 6c). Thus, these results indicate that CP110 loss from the M-centriole occurs after DAV docking to the M-centriole and before CV assembly.

EHD1 membrane tubulation function is required for CV formation

Because EHD1-dependent CV assembly is critical for ciliogenesis initiation, we wanted to determine how EHD1 regulates DAV reorganization. We examined the ability of specific EHD1 loss-of-function mutations to rescue ciliation in RPE cells depleted of endogenous EHD1. EH domain mutants specifically affecting either NPF-substrate binding and membrane recruitment (W485A) or tubulo-vesicular (K483E) membrane functions failed to rescue when compared with the wild-type siRNA resistant protein33,34 (Fig. 7a–c). Importantly, neither mutant protein accumulated significantly in the ciliary pocket (Fig. 7d). Remarkably, using SIM we found that the GFP–K483E mutant localized to structures near the distal appendages when endogenous EHD1 was depleted following starvation (Fig. 7e). Furthermore, CP110 removal failed in K483E expressing cells (Fig. 7f). Together, these results indicate that the EHD1 K483 membrane tubulation function is critical for DAV assembly into the CV and M-centriole reorganization.

EHD1 is required for the fusion of DAVs into the CV

On the basis of our findings for EHD1 and EHD3 functioning in CV assembly from DAVs, we predicted that the SNARE membrane fusion machinery would play a critical role in early ciliogenesis. Because the SNARE protein SNAP29 directly interacts with EHD1 and EHD3 (ref. 26), we tested its function in ciliogenesis. Consistent with the EHD–SNAP29 interaction, SNAP29 co-localized with EHD1 in the ciliary pocket and was required for ciliogenesis in RPE cells (Fig. 8a–c). Depletion of SNAP29 also did not affect GFP–Rabin8 preciliary trafficking, or SmoM2–GFP recruitment to the M-centriole (Fig. 8d,e). As was observed with EHD1, SNAP29 co-localized with SmoM2–GFP on DAV-like membranes in 3 h starved cells (Fig. 8f,g). Interestingly, SNAP29 failed to co-localize with SmoM2–GFP at the M-centriole distal end following EHD1 depletion (24 h starved) when compared with 3 h starved control-siRNA-treated cells, whereas
Figure 4 EHD1 functions in CV formation upstream of Rab8. (a) Representative electron micrographs of RPE cell M-centrioles treated with siRNA for 72 h, with serum starvation (−serum) for the last 24 h. Scale bar, 200 nm. (b) Quantification of M-centriole non-cilium distal appendage structures from a. Pooled data from two to four independent experiments (total number of cells counted in all experiments: control siRNA + serum, 48 cells; EHD1 siRNA, 44 cells; Rab8a+8b siRNA, 51 cells). DA, non-membrane-associated distal appendages. (c-e) SIM images of RPE SmoM2–GFP cells treated with siRNA as in a and stained with Rab11 and CEP164 antibodies. The arrow marks the orthogonal view in the lower panels and corresponds to fluorescence profile plots. (c) Representative images from five random serum-fed cells showing no centriolar SmoM2–GFP accumulation. (d) Five out of eight cells (63%) showed SmoM2–GFP-positive DAV-like structures. (e) Five out of nine cells (56%) showed CV-like structures. Note that 37.5% of EHD1 siRNA-treated cells showed CV-like structures, whereas 22% of Rab8 siRNA depleted cells had DAV-like structures. Profile plots, CEP164 values (c) and SmoM2–GFP values (d) were normalized by a factor of two. Scale bars, 500 nm. (f) 3 h serum-starved RPE Smo–GFP cells stained with anti-CEP164 and anti-EHD1 antibodies and imaged by SIM. Seven out of 17 cells (41%) showed distal appendage Smo–GFP co-localization with EHD1. The arrow marks the orthogonal view in the lower panels and corresponds to fluorescence profile plots. Scale bar, 500 nm. (g) Representative electron micrographs of M-centrioles from uninjected zebrafish photoreceptors at 50 hpf, and 3 dpf photoreceptors injected with ehd1 and ehd3 or rab8sp MOs. ehd1 and ehd3 MOs were co-injected to maximize depletion of both proteins. Because of high lethality resulting from ehd1 and ehd3 MOs, only viable embryos with small eyes were analysed. Scale bar, 250 nm. (h) Quantification of M-centriole distal appendage structures observed in electron micrographs described in g. Averages from pooled data across three independent experiments (>25 photoreceptors per treatment in total across the experiments). (i) Representative images of M-centrioles in zebrafish photoreceptors at 50 hpf stained with anti-Ehd1 or Ehd3 and anti-γ-tubulin antibodies and imaged by SDC microscopy. IS, inner segment. Scale bar, 10 μm.
Figure 5 TZ proteins and IFT20 are recruited after DAV reorganization and before Rab8-dependent ciliary membrane extension. (a) Quantification of CEP290 centrosome levels as previously described in RPE cells treated with siRNA for 72 h, and serum starved for the final 24 h, followed by staining with CEP164 and CEP290 antibodies. Means ± s.e.m. from n = 3 independent experiments are shown (total number of cells from all experiments: control siRNA, 127 cells; Rab8a+8b siRNA, 110 cells; EHD1 siRNA no 1, 143. (b,c) Quantification of RPGRIP1L (b) and TMEM67 (c)–positive M-centrioles–basal bodies in RPE cells treated as in a, stained with γ-tubulin and RPGRIP1L or TMEM67 antibodies. Means ± s.d. are pooled data from three independent experiments with (b) n = 15 and (e) n = 6 areas imaged (total number of cells counted in all experiments: b, control siRNA, 288; Rab8a+8b siRNA, 323; EHD1 siRNA no 1, 218; c, control siRNA, 131; Rab8a+8b siRNA, 138; EHD1 siRNA no 1, 117). (d) Right: RPE B9D2–GFP cells were treated with siRNA as described (a), serum starved for 24 h and stained with α-tub and PCNT antibodies. Scale bars, 2 μm. Left: Quantification cells with B9D2–GFP-positive M-centrioles–basal bodies. Means ± s.d. are pooled data from three independent experiments with n = 7 areas imaged (total number of cells counted in all experiments: control siRNA, 134; Rab8a+8b siRNA, 171; EHD1 siRNA no 1, 149). Two tailed t-test analyses, compared with control siRNA or between Rab8a+8b siRNA and EHD1 siRNA in a–d. (e,f) RPE B9D2–GFP cells transiently expressing IFT20–Smo (e) or IFT20–Rab8a (f) were serum starved for 1 h and imaged live by SDC microscopy. Images show single xy–planes from z–stacks. (g) RPE GFP–Centrin1 cells treated with siRNA as described in a and stained for IFT20 and α-tub. Quantification of IFT20 fluorescence intensity at the M-centrioles–basal bodies in cells from the left panel. Means ± s.e.m. from n = 3 independent experiments are shown (total number of cells counted in all experiments: control siRNA, 183; Rab8a+8b siRNA, 142; EHD1 siRNA no 1, 127). Two tailed t-test analysis, compared with control siRNA. (h–j) RPE cells transiently expressing IFT20–GFP and Smo–trRFP (h), RPE GFP–Rab8a cells transiently expressing IFT20–tRFP (i) and RPE B9D2–GFP cells transiently expressing IFT20–tRFP (j) were imaged and analysed as described in e. Scale bars, 2 μm. Note that e, f and h–j are representative image series of more than five ciliation assembly events observed. ∗∗ P < 0.01, ∗∗∗ P < 0.001. Statistics source data for a–d and g can be found in Supplementary Table 2.
Figure 6  EHD1-dependent CV formation stimulates CP110 loss from the distal end of the M-centriole. (a) RPE SmoM2–GFP cells treated with siRNA for 72 h, grown in serum or serum starved for the last 24 h, and stained with anti-CP110 and anti-γ-tubulin antibodies. Scale bar, 2 μm. (b) Quantification of serum-starved RPE cells described in a showing CP110 localization on the mother and daughter centrioles (two dots) or only the daughter centriole (one dot). Note that we disregarded cells with more than two dots in our quantification. Means ± s.d. are pooled data from three independent experiments with n = 6 areas imaged (total number of cells counted in all experiments: control siRNA, 184; Rab8a + Bb siRNA, 161; EHD1 siRNA no 1, 157). Two tailed t-test analysis, compared with control siRNA, **P < 0.0001. Statistics source data can be found in Supplementary Table 2. (c) RPE cells transiently co-expressing GFP–CP110 and 5-HT6–tRFP, serum starved for 1 h and imaged live over time by SDC microscopy as in Fig 5c. Representative images of maximum intensity projections of z-stack imaging series from five cilium assembly events observed. White and blue arrows indicate CP110-positive centrioles and 5-HT6 vesicles respectively. For presentation purposes images were smoothed with a Gaussian blur filter. Scale bar, 2 μm.

SNAP29 was dispensable for EHD1 recruitment to early ciliary membranes (Fig. 8g). Given these findings and the requirement for EHD1 and EHD3 tubulation function in CV assembly, we theorized that EHD proteins recruit SNAP29 to DAVs where the SNARE functions in fusion of tubulated DAVs.

DISCUSSION

In the intracellular ciliogenesis pathway, the ciliary membrane is thought to arise from a CV that is reshaped into a membrane sheath around the developing axoneme. However, the molecular details governing this process remained unresolved. On the basis of Rabin8 preciliary vesicle transport observed following cues to ciliate15, we reasoned that the recruitment of smaller preciliary vesicles to the M-centriole would be followed by fusion into a larger CV. Here, we describe a role for EHD1 and EHD3, regulators of Rab compartments, in early primary cilium assembly. We find that EHD1 and EHD3 are indispensable for CV formation from DAVs and orchestrate other ciliogenesis events, whereas Rab8 functions downstream in CV extension. In addition, we show that the EHD interacting protein SNAP29, a SNARE membrane fusion protein, regulates CV assembly.

A role for EHD1 and EHD3 in CV formation

EHD1–/– mice were reported to have ocular defects, which could be associated with defective photoreceptor cilia formation35, as we have observed in zebrafish. These mice also had higher rates of embryonic lethality, suggesting an essential role of EHD1. Unlike EHD1-null mice, EHD3-knockout mice had no discernible pathology36. Our studies in human cells and zebrafish suggest that EHD1 and EHD3 show functional overlap in cilium formation, probably owing to their ability to heterodimerize37. Importantly, EHD1 and EHD3 share the same binding partners through interaction through NPF motif–EH domain binding21,26,28. Our results are also consistent with previous studies showing that EHD proteins have tissue-specific and redundant roles39.
Figure 7 EHD1 tubulation function is required for DAV assembly into the CV. (a) Domain structure of EHD1 and loss of function mutations. (b) Immunoblot analysis of ResGFP–EHD1 wild-type (WT), K483E and W485A proteins stably expressed in RPE cells 72 h after transfection with control siRNA or EHD1 siRNA no 1. Endogenous and GFP–EHD protein expression levels were detected using anti-EHD1 antibody. Note that the EHD1 antibody also recognizes endogenous EHD4 as indicated. Uncropped images of blots are shown in Supplementary Fig. 6. (c) Quantification of cilia in cells treated with EHD1 siRNA no 1 as described in b and serum starved for the last 24 h. Means ± s.d. are pooled data from three independent experiments with n=8 areas imaged (total number of cells from all experiments: ResGFP–EHD1, 293; ResGFP–EHD1 K483E, 269; ResGFP–EHD1 W485A, 263). Two tailed t-tests, compared with wild type. (d) Cell lines described in b were serum starved for 24 h and stained with α-tub antibody to mark the cilia. Scale bar, 2 μm. (e) Representative SIM image of RPE cells expressing the siRNA resistant GFP–K483E mutant transfected with EHD1 siRNA as in c and stained with CEP164 antibody. Nine out of 15 cells (60%) imaged expressing the K483E showed DAV-like structures. The arrow marks the orthogonal view (bottom panels) and corresponds to fluorescence profile plots. Scale bar, 500 nm. (f) Quantification of CP110 localization on the mother and daughter centrioles in RPE cells treated as in c and stained as described in Fig. 6b. Means ± s.d. are pooled data from three independent experiments with n=6 areas imaged (total number of cells from all experiments: control siRNA, 148; EHD1 siRNA no 1, 114). Two tailed t-tests, compared with control siRNA. ***P < 0.0001. Statistics source data for c,f can be found in Supplementary Table 2.

The connection between EHD1 and EHD3 and the Rab11–Rab8 cascade is supported by our findings that these proteins co-localize with both Rab proteins in ciliary-associated membrane compartments. Although our data do not exclude the possibility that endocytic recycling dysfunction might affect ciliogenesis indirectly, EHD1 co-localized with Rab11, Rab8 and Smo on preciliary vesicles and DAVs, and with Rab8 on the growing ciliary membrane. EHD1 is presumably transported to the centrosome by means of preciliary vesicles, probably originating from the ERC (ref. 15), which subsequently associate with distal appendages and fuse to form the CV. In non-ciliating cells, a Rab11–ERC interaction at the M-centriole has been previously described18. EHD1 and EHD3 function in tubular membrane regulation of the ERC, although in HeLa cells EHD1 seems to function as a vesiculator of tubular recycling endosomes24. Our finding that GFP–EHD1 K483E fails to rescue ciliation and yet still localizes to DAVs suggests that tubulo-vesicular functioning of EHD proteins is important for CV formation. Thus we hypothesize that the EHD1– and EHD3–membrane remodelling function is responsible.
for bringing DAV membranes into close proximity to promote SNAP29-dependent fusion during CV assembly. Our discovery that SNAP29 localizes to the ciliary pocket and functions in ciliogenesis in RPE cells is also interesting, as SNAP29 mutations are responsible for CEDNIK syndrome and SNAP29 knockout mice are embryonic lethal. CEDNIK patients do not seem to have classic ciliopathy, but this is something that cannot be ruled out at this time. In addition to SNAP29, other SNAREs would be expected to function in membrane docking.
fusion during the CV assembly stage. Given the observed importance of intracellular membrane assembly during ciliogenesis, investigation of SNARE protein function in CV assembly and ciliary progression merits further investigation.

**Preciliary membrane reorganization and ciliogenesis initiation**

Failure to dock membranes to the distal appendages prevents CP110 loss from the M-centriole needed for basal body formation and ciliogenesis progression. Our work further clarifies the molecular mechanism of this critical ciliogenesis initiation step by demonstrating that M-centriole modifications are initiated following docking of DAVs to the M-centriole. To our knowledge, EHD1 and EHD3 are the first direct membrane-associated proteins whose function affects M-centriole reorganization. How EHD1-dependent DAV assembly into the CV affects CP110 loss is not clear, but there are provactive possible mechanisms. CP110 loss from the M-centriole is associated with ubiquitylation. One possibility is that preciliary membranes and/or EHD proteins recruit components of the ubiquitylation machinery to the M-centriole. Alternatively, changes in the membrane accompanying DAV to CV formation could help establish the basal body.

The orchestration of DAV reorganization by EHD proteins is also a prerequisite step for TZ proteins and IFT20 recruitment, which is consistent with other reports showing that, without membrane association at the M-centriole, TZ and IFT components fail to accumulate. Likewise, Rab8 recruitment to the developing cilia seems to be different from that of IFT20 and TZ. Importantly, our work suggests that a mechanism may be present to control the timing of Rab8 localization to the CV. Could this be a checkpoint to ensure proper membrane association with the growing axone. Hence, only following IFT20 and TZ protein recruitment does coordinated axone and ciliary membrane growth occur.

**EHD1 membrane shaping and the ciliary pocket**

A surprising finding was the localization of EHD1 and SNAP29 to the ciliary pocket membrane. The ciliary pocket is a site for TGF-β signalling and its proximity to the ciliary membrane suggests it is important for ciliary trafficking. The ciliary pocket membrane resembles a tubular structure surrounding the proximal part of the cilium, which much like a vesicle or tubule has a positive curvature membrane facing the cytosol, a preferential site for EHD protein binding. In contrast, the intracellular face of the ciliary membrane has a negative curvature. Our work suggests that EHD1 and EHD3 are excluded from the ciliary membrane, possibly by the TZ established before CV reshaping and axone growth. We can speculate that TZ gating of EHD proteins is important for establishment of negative curvature after the CV stage to enable close association with the axone and the developing ciliary membrane. In mature cilia, EHD proteins could also be important for ciliary pocket maintenance. Finally, whether EHD1 and EHD3 are important for signalling associated with the ciliary pocket or the cilium remains an open question.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

We thank K. Nagashima and A. Kamata for help with IEM and EM, C. Lamont for data analysis, S. Lockett for help with SIM imaging, S. Specht for help with cell culture, A. Peden for SNAP29 antibodies, J. Golderring for Rab11 antibodies, Z. Sun for the transgenic line and S. Burgess for assistance raising zebrafish embryos. We are grateful to J. Donaldson for critical reading of this manuscript. This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research. This project has also been funded in part with federal funds from the Frederick National Laboratory for Cancer Research, National Institutes of Health, under contract HHSN26120080001E.

**AUTHOR CONTRIBUTIONS**

Q.L. and C.I. carried out most experiments with help from C.I.W. (RNAi, fluorescence imaging and PCR with reverse transcription), C.O. (SIM imaging). P.A.P., S.L., I.O.D. and Y-S.H. (in situ expression in zebrafish), U.B. (EM and CLEM), V.W. (immunoblotting), T.S. (zebrafish MO experiments) and A.C. (cell line generation and RNAi rescue experiments). S.C. and J.R. provided reagents. S.C., C.O., J.L-S., and P.K.J. discussed the results and commented on the manuscript. C.J.W. and C.I. wrote the paper with suggestions from Q.L. and C.O. C.J.W. and C.I. conceived and designed the research.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at www.nature.com/dofinder/10.1038/ncb3109

Reprints and permissions information is available online at www.nature.com/reprints

1. Greitz, S. C. & Anderson, K. V. The primary cilium: a signalling centre during vertebrate development. Nat. Rev. Genet. 11, 331–344 (2010).
2. Hildebrandt, F., Benzing, T. & Katsanis, N. Ciliopathies. N. Engl. J. Med. 364, 1533–1543 (2011).
3. Graser, S. et al. Cep164, a novel centriole appendage protein required for primary cilia formation. J. Cell Biol. 179, 321–330 (2007).
4. Ishikawa, H., Kubo, A., Tsukita, S. & Tsukita, S. ODF2-deficient mother centrioles lack distal/subdistal appendages and the ability to generate primary cilia. Nat. Cell Biol. 7, 517–524 (2005).
5. Schmidt, K. N. et al. Cep164 mediates vesicular docking to the mother centriole during early steps of ciliogenesis. J. Cell Biol. 199, 1083–1101 (2012).
6. Joo, K. et al. CCO41 is required for ciliary vesicle docking to the mother centriole. Proc. Natl Acad. Sci. USA 110, 5987–5992 (2013).
7. Sillibourne, J. E. et al. Primary ciliogenesis requires the distal appendage component Cep123. Biol. Open 2, 535–545 (2013).
8. Tanos, B. E. et al. Centriole distal appendages promote membrane docking, leading to cilia initiation. Genes Dev. 27, 163–168 (2013).
9. Kobayashi, T., Kim, S., Lin, Y. C., Inoue, T. & Dynlacht, B. D. The CP110-interacting proteins Talpid3 and Cep290 play overlapping and distinct roles in cilia assembly. J. Cell Biol. 204, 215–229 (2014).
10. Sorokin, S. Centrioles and the formation of rudimentary cilia by fibroblasts and smooth muscle cells. J. Cell Biol. 15, 363–377 (1962).
11. Ye, X., Zeng, H., Ning, G., Reiter, F. & Liu, A. C2c3d is critical for centriolar distal appendage assembly and ciliary vesicle docking in mammals. Proc. Natl Acad. Sci. USA 111, 2164–2169 (2014).
12. Nachury, M. V. et al. A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. Cell 129, 1201–1213 (2007).
13. Yoshimura, S., Egner, J., Fuchs, E., Haas, A. K. & Barr, F. A. Functional dissection of Rab GTPases involved in primary cilia formation. J. Cell Biol. 178, 363–369 (2007).
14. Knodler, A. et al. Coordination of Rab8 and Rab11 in primary ciliogenesis. Proc. Natl Acad. Sci. USA 107, 6346–6351 (2010).
15. Westlake, C. J. et al. Primary cilia membrane assembly is initiated by Rab11 and transport protein particle II (TRAPPII) complex-dependent trafficking of Rab8 to the centrosome. Proc. Natl Acad. Sci. USA 108, 2759–2764 (2011).
16. Salo, T. et al. Rab8a and Rab8b are essential for multiple apical transport pathways but insufficient for ciliogenesis. J. Cell Sci. 127, 422–431 (2014).
17. Bryant, D. M. et al. A molecular network for de novo generation of the apical surface and lumen. Nat. Cell Biol. 12, 1035–1045 (2010).
18. Sorokin, S. P. Reconstructions of centriole formation and ciliogenesis in mammalian lungs. J. Cell Sci. 3, 207–230 (1968).
19. Zhang, J., Naslavsky, N. & Caplan, S. Rab and EHDs: alternate modes for traffic control. Biosci. Rep. 32, 17–23 (2012).
20. Naslavsky, N. & Caplan, S. EHD proteins: key conductors of endocytic transport. Trends Cell Biol. 21, 122–131 (2011).
21. Naslavsky, N., Rahajeng, J., Sharma, M., Jovic, M. & Caplan, S. Interactions between EHD proteins and Rab11-FIP2: a role for EHD3 in early endosomal transport. Mol. Biol. Cell 17, 163–177 (2006).
22. Roland, J. T., Kenworthy, A. K., Peranen, J., Caplan, S. & Goldenring, J. R. Myosin Vb interacts with Rab8a on a tubular network containing EHD1 and EHD3. Mol. Biol. Cell 18, 2828–2837 (2007).
ARTICLES

23. Sharma, M., Giridharan, S. S., Rahajeng, J., Naslavsky, N. & Caplan, S. MICAL-L1 links EHD1 to tubular recycling endosomes and regulates receptor recycling. Mol. Biol. Cell 20, 5181–5194 (2009).

24. Cai, B. et al. Differential roles of C-terminal Eps15 homology domain proteins as vesiculators and tubulators of recycling endosomes. J. Biol. Chem. 288, 30172–30180 (2013).

25. Giridharan, S. S., Cai, B., Vitale, N., Naslavsky, N. & Caplan, S. Cooperation of MICAL-L1, syndapin2, and phosphatidic acid in tubular recycling endosome biogenesis. Mol. Biol. Cell 24, 1776–1790 (2013).

26. Rotem-Yehudar, R., Galperin, E. & Horowitz, M. Association of insulin-like growth factor 1 receptor with EHD1 and SNAP29. J. Biol. Chem. 276, 33054–33060 (2001).

27. Shiba, D. et al. Localization of Inv in a distinctive intracellular compartment requires the C-terminal ninein-homolog-containing region. J. Cell Sci. 122, 44–54 (2009).

28. Molla-Herman, A. et al. The ciliary pocket: an endocytic membrane domain at the base of primary and motile cilia. J. Cell Sci. 123, 1785–1795 (2010).

29. Sonnen, K. F., Schermelleh, L., Leonhardt, H. & Nigg, E. A. 3D-structured illumination microscopy provides novel insight into architecture of human centrosomes. Biol. Open 1, 965–976 (2012).

30. Sedmak, T. & Wolfrum, U. Intraflagellar transport proteins in ciliogenesis of photoreceptor cells. Biol. Cell 103, 449–466 (2011).

31. Follit, J. A., Tuft, R. A., Fogarty, K. E. & Pazour, G. J. The intraflagellar transport protein IFT20 is associated with the Golgi complex and is required for cilia assembly. Mol. Biol. Cell 17, 3781–3792 (2006).

32. Spektor, A., Tsang, W. Y., Khoo, D. & Dynlacht, B. D. Cep97 and CP110 suppress a growth factor 1 receptor with EHD1 and SNAP29. J. Biol. Chem. 282, 16612–16622 (2007).

33. Naslavsky, N., Boehm, M., Backlund, P. S. Jr & Caplan, S. Rabenosyn-5 and EHD1 co-localize in the C-terminal ninein-homolog-containing region. J. Cell Sci. 123, 1785–1795 (2010).

34. Naslavsky, N., Rahajeng, J., Chenavas, S., Sorgen, P. L. & Caplan, S. EHD1 and Eps15 interact with phosphatidylinositols via their Eps15 homology domains. J. Biol. Chem. 282, 16612–16622 (2007).

35. Rainey, M. A. et al. The endocytic recycling regulator EHD1 is essential for spermatogenesis and male fertility in mice. BMC Dev. Biol. 10, 37 (2010).

36. George, M. et al. Renal thrombotic microangiopathy in mice with combined deletion of endocytic recycling regulators EHD3 and EHD4. PLoS ONE 6, e17838 (2011).

37. Galperin, E. et al. EHD3: a protein that resides in recycling tubular and vesicular membrane structures and interacts with EHD1. Traffic 3, 575–589 (2002).

38. De Beer, T. et al. Molecular mechanism of NPF recognition by EH domains. Nat. Struct. Biol. 7, 1018–1022 (2000).

39. George, M. et al. Shared as well as distinct roles of EHD proteins revealed by biochemical and functional comparisons in mammalian cells and C. elegans. BMC Cell Biol. 8, 3 (2007).

40. Hehly, H., Chen, C. T., Powers, C. M., Liu, H. L. & Doxsey, S. The centrosome regulates the Rab11-dependent recycling endosome pathway at appendages of the mother centriole. Curr. Biol. 22, 1944–1950 (2012).

41. Sprecher, E. et al. A mutation in SNAP29, coding for a SNARE protein involved in intracellular trafficking, causes a novel neurocutaneous syndrome characterized by cerebral dysgenesis, neuropathy, ichthyosis, and palmoplantar keratoderma. Am. J. Hum. Genet. 77, 242–251 (2005).

42. Fuchs-Telem, D. et al. CEDNIK syndrome results from loss-of-function mutations in SNAP29. Br. J. Dermatol. 164, 610–616 (2011).

43. Tsang, W. Y. et al. CP110 suppresses primary cilia formation through its interaction with CEP290, a protein deficient in human ciliary disease. Dev. Cell 15, 187–197 (2008).

44. Li, J. et al. USP33 regulates centrosome biogenesis via deubiquitination of the centriolar protein CP110. Nature 495, 259–259 (2013).

45. CajaneK, L. & Nigg, E. A. Cep164 triggers ciliogenesis by recruiting Tau tubulin kinase 2 to the mother centriole. Proc. Natl Acad. Sci. USA 111, E2841–E2850 (2014).

46. Goetz, S. C., Liem, K. F. Jr & Anderson, K. V. The spinocerebellar ataxia-associated gene Tau tubulin kinase 2 controls the initiation of ciliogenesis. Cell 151, 847–858 (2012).

47. Clement, C. A. et al. TGF-beta signaling is associated with endocytosis at the pocket region of the primary cilium. Cell Rep. 3, 1806–1814 (2013).

48. Klinger, M. et al. The novel centriolar satellite protein SSX2IP targets Cep290 to the ciliary transition zone. Mol. Biol. Cell 25, 495–507 (2014).
For whole-mount zebrafish cilium studies, embryos were fixed in Dent’s fixative images from two or more areas using a solution for 1 h at RT or overnight at 4 °C. Processed for immunostaining and indirect immunofluorescence as described, quantification, cells were serum starved for 24 h, unless otherwise stated, fixed and immunofluorescence and time-lapse microscopy. For whole-mount zebrafish cilium studies, embryos were fixed in Dent’s fixative images from two or more areas using a solution for 1 h at RT or overnight at 4 °C. Processed for immunostaining and indirect immunofluorescence as described, quantification, cells were serum starved for 24 h, unless otherwise stated, fixed and immunofluorescence and time-lapse microscopy.

Immunofluorescence and time-lapse microscopy. For RPE and IMDM3 ciliation quantification, cells were serum starved for 24 h, unless otherwise stated, fixed and processed for immunostaining and indirect immunofluorescence as described. Briefly, cells were fixed in 4% paraformaldehyde for 10 min or cold methanol for 5 min and blocked with 1% BSA in PBS 0.1% Triton X-100 or BSA solution alone, respectively, for 10 min following by incubation with primary antibody in blocking solution for 1 h at RT or overnight at 4 °C. Fluorophore-conjugated secondary antibodies were incubated for 1 h at RT. Precillary membranes, cilia, TZ proteins, IFT20 and CPI10 were imaged in more than six fields or as a montage of four to six images from two or more areas using a x40 1.4 numerical aperture (NA) or x63 1.3 NA objective, unless otherwise indicated. Imaging was carried out using a Zeiss Axio Scan. Z1 inverted epifluorescence microscope equipped with a CoolSNAP HQ2 camera. Images were analysed using the Slidebook software. Immunocytochemistry on cryosections of 3 dpf zebrafish retinae was carried out as previously described.

Time-lapse imaging of RFP and GFP fusions was carried out using the Marivanas SDC. The environmental chamber containing the XYZ automatic stage (ASI) was set at 37 °C and 3% CO2. For SDC time-lapse experiments, image acquisition of z-stacks (10x0105cm z-stack with 500 nm step size) was carried out every 5–20 min using an Evolve 512 electron multiplying charge coupled device camera and ×40 1.4 NA (325 nm/pixel) or ×63 1.3 NA (206 nm/pixel) objective and Slidebook software. For all zebrafish studies, image acquisition (35 μm z-stacks with 1 μm step size) was carried out using a ×40 1.4 NA or ×63 1.3 NA objective lens. Processing of images and fluorescence intensity measurements were carried out using either ImageJ or Slidebook software. GFP–Rabni8 vesicle centrosome localization was detected by time-lapse epifluorescence microscopy and analysed as described using a HQ2 digital camera (Photometrics).

Structural illumination microscopy (SIM). Cells were grown on no 1.5 cover glasses (Zeiss). After immunofluorescence processing, cover glasses were mounted onto glass slides with VECTASHIELD (Vector) and sealed with nail polish. Five phases and three rotations of 3D SIM images were captured using a Zeiss or Nikon N-SIM electron multiplying charge coupled device camera and ×40 1.4 NA (325 nm/pixel) or ×63 1.3 NA (206 nm/pixel) objective and an electron multiplying charge coupled device camera (Andor DU-897). The pixel size of both cameras is 16 μm. The raw data pixel size is 80 nm with the Zeiss SIM using a ×63 objective and 60 nm with the Nikon SIM using a ×100 objective. Raw images captured by the Nikon SIM microscope in Figs 1l, 1j, 4f, 7e and 8a, are 14 bit and raw images captured by the Zeiss SIM microscope in Fig. 4c–e and Supplementary Fig. 1k,l are 16 bit. Laser power and exposure time were optimized to use a large portion of the camera’s dynamic range while minimizing bleaching.

To avoid reconstruction artefacts we have done the following: during the sample preparation antibody dilutions, coverslip thickness and mounting media conditions were optimized to give the best signal to noise ratio; samples were checked by wide-field or confocal microscopy before SIM imaging to check the integrity of the sample; SIM images were acquired using the largest possible portion of the dynamic range provided by the camera and images were reconstructed using the Zeiss Zen software and Nikon NIS-Elements software, with Wiener filter settings to reduce image noise. The estimated resolutions after reconstruction were the following: Zeiss channel 488, ×110 nm lateral and ×350–400 nm axial; Zeiss channel 561, ~130 nm lateral and ×500–600 nm axial; Nikon channel 488, ×130 nm lateral and ×400 nm axial; Nikon channel 561, ×140 nm lateral and ×400–600 nm axial; Nikon channel 640, ~160 nm lateral and ×400–600 nm axial. Histograms of reconstructed images were adjusted in the Nikon NIS-Elements or Zeiss Zen software and tiffs cropped in Imagej. Intensity profile plots were created in the NIS-Elements or the Zen software by drawing lines across the structure on the reconstructed images (non-histogram adjusted) and by exporting the values into Excel and GraphPad Prism 6 for Macintosh OS.

Transmission electron microscopy and histology. Electron microscopy processing in RPE cells and zebrafish embryos were carried out as previously described. Briefly, cells or zebrafish embryos were fixed in a solution of 2% glutaraldehyde with or without 4% paraformaldehyde in 0.1 M sodium cacodylate buffer followed by post-fixation with 1% osmium tetroxide and 1% uranyl acetate. After dehydration in graded ethanol, cells were embedded in EMBed 812 (Electron Microscopy Sciences) and 80 nm sections were cut using a Leica EM UC7 microtome. Electron micrographs were acquired using a transmission electron microscope (Hitachi 7650 TEM). For photoreceptor cilium counting from histological analysis, semi-thin sections (250 nm) cut from samples embedded for TEM analysis were stained with toluidine blue. Photoreceptor outer segments with intact inner segments were counted (>100). For immuno-EM, RPE cell lines expressing GFP–EHD1 were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M sodium cacodylate buffer. After dehydration in graded ethanol, cells were embedded in LR White Embedding Resin, Cells were cut on a Leica Ultracut microtome and the sections were collected on 100 nm gold particles for 1 h in R.T. For CLEM, cells were plated on gridded-glass bottom dishes (MatTek). Fluorescence and DIC images of fixed cells were taken with a Zeiss fluorescence microscope. The position of cells was recorded using grid numbers on cover glasses. After imaging, cells were...
Morpholino knockdown. Fish care and husbandry were carried out in compliance with NIH guidelines for animal care. Zebrafish used here were Tg-5 and Tg(arl13b–GFP) lines (a gift from Z. Sun, Yale University School of Medicine). Knockdowns for each gene were carried out using the following translation blocking morpholinos obtained from GENETOOLS: ehd1a 5'-CTGAACATGGTGGACGTTACACGAC-3', ehd1b 5'-ATCTTTGTTAGACCACTGAACATT-3' and ehd3 5'-CATCGGTACCCACCTGAAAAT-3'. The splice-blocking MOs have been previously described. Injections were done using a microinjector PLI-90 (Harvard Apparatus). For rescue experiments, the full-length sequences of human EHD1, EHD3 and EHD4 were cloned into PCS2+. Vectors and messenger RNAs were transcribed using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's instructions. Embryos were co-injected with 250 ng of morpholinos and 300 pg nl⁻¹ of capped mRNAs at the one-cell stage.

Expression analysis. TaqMan gene expression probes for EHD1–4 were purchased from Applied Biosystems and quantitative PCR with reverse transcription was performed as specified in the figure legends, and two group comparisons were done with an unpaired two tailed Student t-test. A value of *P < 0.05 was considered as statistically significant. All P values were indicated on graphs as follows: **P < 0.01, ***P < 0.001. Exact P values can be found in the Statistics Source Data Sheet (Supplementary Table 2).

Experiments were reproducibly carried out as follows: one experiment (Fig. 1h and Supplementary Fig. 2d), two independent experiments (Figs 1j, 2h, 3a, 4i, 5e and 7e and Supplementary Figs 1l, 2e, 4a and 5), three independent experiments (Figs 1d, 2e, 4b and Supplementary Figs 1g, 2f and 4c), two to four independent experiments (Fig. 4a and Supplementary Fig. 4a) and more than three independent experiments (Figs 1i, 3e, 7d and 7f, Supplementary Figs 1h, j and 3 and Supplementary Videos 1–5).

47. Kee, K. M. & Desai, A. A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. Sci. STKE 2005, pl1 (2005).
48. Gray, D. C. et al. pHUSH: a single vector system for conditional gene expression. BMC Biotechnol. 7, 61 (2007).
49. Murone, M., Rosenthal, A. & de Sauvage, F. J. Sonic hedgehog signaling by the patched-smoothened receptor complex. Curr. Biol. 9, 76–84 (1999).
50. Wright, K. J. et al. An ARL3-UNC119-RP2 GTPase cycle targets myristoylated NPHP3 to the primary cilium. Genes Dev. 25, 2347–2362 (2011).
51. Jaiswal, B. S. et al. Combined targeting of BRAF and CRAF or BRAF and PI3K effector pathways is required for efficacy in NRAS mutant tumors. PLoS ONE 4, e5717 (2009).
52. Insinna, C., Pathak, N., Perkins, B., Drummond, I. & Besharse, J. C. The homodimeric kinase Kif17 is essential for vertebrate photoreceptor sensory outer segment development. Dev. Biol. 316, 160–170 (2008).
53. Malicki, J., Avanesov, A., Li, J., Yuan, S. & Sun, Z. Analysis of cilia structure and function in zebrafish. Methods Cell Biol. 101, 39–74 (2011).
54. Lu, Q. et al. Chromatin-bound NLS proteins recruit membrane vesicles and nucleoporins for nuclear envelope assembly via importin-σ. Cell Res. 22, 1562–1575 (2012).
55. Omori, Y. et al. Elipsa is an early determinant of ciliogenesis that links the IFT particle to membrane-associated small GTPase Rab8. Nat. Cell Biol. 10, 437–444 (2008).
56. Thisse, C. & Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protoc. 3, 59–69 (2008).
Early steps in primary cilium assembly require EHD1/EHD3-dependent ciliary vesicle formation

Quanlong Lu, Christine Insinna, Carolyn Ott, Jimmy Stauffer, Petra A. Pintado, Juliati Rahajeng, Ulrich Baxa, Vijay Walia, Adrian Cuenca, Yoo-Seok Hwang, Ira O. Daar, Susana Lopes, Jennifer Lippincott-Schwartz, Peter K. Jackson, Steve Caplan & Christopher J. Westlake

Nat. Cell Biol. 17, 228–240 (2015); published online 16 February 2015; corrected after print 12 March 2015

In the version of this Article originally published Fig. 3g was incorrectly labelled. The top right panel should have been labelled ‘EHD1’ in red and the entire of 3g should have been labelled ‘Rab8a+8b siRNA’ in black. The corrected image is shown below and is corrected in all online versions of the Article.

![Image of corrected figure 3g](image-url)
Correction notice:

_Nat. Cell Biol._ http://dx.doi.org/10.1038/ncb3128 (2015)

Kinetochore–microtubule error correction is driven by differentially regulated interaction modes

Maria Kalantzaki, Etsushi Kitamura, Tongli Zhang, Akihisa Mino, Béla Novák and Tomoyuki U. Tanaka

In the version of this Supplementary file originally posted online, the caption of Supplementary Fig. 2 was incorrectly cited. The penultimate sentence should have read 'Note that the KT on CEN3 before being caught on the lateral surface of a spindle MT (a MT extending from a spindle pole) often generates short MTs that are thought to facilitate a subsequent KT encounter with a spindle MT \(^5\); such short KT-derived MTs were found similarly in the wild-type control, Dam1ΔCclv, Ndc80ΔN and double deletion (our unpublished result).’ This has been corrected 11 March 2015.
Supplementary Figure 1 KTs show detachment from the spindle, followed by recapture, when Dam1 C-terminus and Ndc80 N-tail are deleted. NDC80\textsuperscript{+} DAM1\textsuperscript{+} (T9659), ndc80ΔN (T9298), dam1-TEVsites (T9258), ndc80ΔN dam1-TEVsites (T11454) cells with P\textsubscript{GAL}-TEV (except for T9298) MTW1-4\texttimes\texttimes mCherry Venus-TUB1 P\textsubscript{MET3}-CDC20 were treated as in Fig 1e, except that images were acquired every 30 sec. A representative cell with Ndc80ΔN plus Dam1ΔCclv (0 min: start of image acquisition) is shown here. The graphs show percentages of cells that showed KT detachment from the spindle (usually followed by reattachment); n= 33, 38, 34 and 35 cells were analysed (from left to right). Experiments were performed twice (statistics source data are shown in Supplementary Table 2) and a representative experiment is shown here. p-values (two tailed) were obtained by Fisher’s exact test.
Supplementary Figure 2 Mutations at the Ndc80 calponin-homology domain lead to a defect in the lateral KT–MT attachment. $P_{\text{CUP1-ubi-DHFR-ndc80}}$ (ndc80-td) $P_{\text{GAL-CEN3-tetOs}}$ Tetr-GFP YFP-TUB1 $P_{\text{MET3}}$-CDC20 cells with NDC80+ (T10069), ndc80-CH-K6A (T7427) [expressed from NDC80 promoter] or no additional NDC80 construct (T7428), inserted at his3 locus, were treated with α factor at 25°C in methionine-dropout medium with raffinose, galactose and 2% CuSO$_4$. After 3 h, cells were released to YPA medium with raffinose, galactose (to inactivate $P_{\text{GAL-CEN3}}$) and methionine (to deplete Cdc20) at 35°C (to degrade Ndc80-td). After 20 min, cells were transferred to synthetic complete medium with glucose (to re-activate $P_{\text{GAL-CEN3}}$) and methionine, and images were acquired every 5 min (start of image acquisition; 0 min) at 35°C. In the ndc80-CH-K6A mutant, six lysines were replaced with alanines within the calponin-homology domain.

Representative images of T10069 and T7427 cells at 20 min (left) and the percentage of cells (n = 20–30 cells were analysed at each time point) with CEN3 that is unattached to MTs (right). The above result and Fig 2 suggest the Ndc80 calponin-homology domain, but not the Dam1 C-terminus or the Ndc80 N-tail, is required for the initial lateral KT-MT attachment. Note that the KT on CEN3 before being caught on the lateral surface of a spindle MT (a MT extending from a spindle pole) often generates short MTs (a MT extending from a spindle pole) often generates short MTs that are thought to facilitate a subsequent KT encounter with a spindle MT$^{57}$; such short KT-derived MTs were found similarly in the wild-type control, Dam1ΔCclv, Ndc80ΔN and double deletion (our unpublished result). Once CEN3 is loaded on the lattice of a spindle MT, CEN3 showed sliding along this MT towards a spindle pole$^2$; this sliding was also found similarly in wild-type control and the deletion mutants (our unpublished result).
Supplementary Figure 3 Dam1 and Stu2 interact physically in a two-hybrid assay and this interaction is abolished with Dam1ΔC and with Dam1C-4D[AurB]. (a) Stu2 protein and its C-terminus and N-terminus deletions are shown in the diagram. These deletions were used in b and c. (b, c) b shows that Dam1 and Stu2 interact physically in a two-hybrid assay and this interaction requires the Dam1 C-terminus. c shows that Dam1–Stu2 interaction in a two-hybrid assay is abolished with Dam1C-4D[AurB], i.e. with phospho-mimicking mutations of the Dam1 C-terminus at Aurora B sites. Duo1 is a component of the Dam1 complex and serves as a control. Ras and Raf were also used as controls. AD and BD were as in Fig 3g. (d) Phospho-mimicking mutants of the Dam1 C-terminus at Aurora B sites are defective in assisting Stu2 in rescuing a MT. Graph shows percentage of Stu2 transport events along a MT, leading, or not leading, to MT rescue, as in Fig 4b. DAM1+ (T11596) and dam1C-4D[AurB] dam1-aid (T11595) cells with TIR GAL-CEN3-tetOs TetR-3xFp STU2-4×mCherry GFP-TUB1 MET3-CDC20 were treated as in Fig 6a–g. n = 13 Stu2 transport events were analysed in each of T11595 and T11596. p-values (two tailed) were obtained by Fisher's exact test. Data represent one out of two independent experiments.
Supplementary Figure 4 Phospho-mimicking mutants of the Dam1 C-terminus at Mps1 sites do not show KT detachment from the spindle. We tested whether phospho-mimicking Dam1 mutants at Mps1 sites (dam1C-4D[Mps1]; T217, S218, S221 and S232 replaced with aspartates) show phenotypes similar to those of dam1C-4D[AurB], dam1C-4D[Mps1](T11680) and dam1C-8D[AurB+Mps1](T11642) cells with dam1-aid TIR P_{GAL}-CEN3-tetOs TetR-3xCFP GFP-TUB1 P_{MET3}-CDC20 were treated and analysed as in Fig 5 c and d. The results of T9530 and T11326 in Fig 5 c and d are shown again for comparison. n= 65 and 59 cells were analysed for T11680 and T11642, respectively. Experiments were performed twice (statistics source data are shown in Supplementary Table 2) and a representative experiment is shown here. p-values (two tailed) were obtained by Fisher’s exact test. In contrast to dam1C-4D[AurB], dam1C-4D[Mps1] did not significantly increase the level of bi-orientation defects or CEN3 detachment from the spindle. Furthermore, the combination of phospho-mimicking mutations at both Aurora B and Mps1 sites (dam1C-8D[AurB+Mps1]) did not exacerbate the defects found in dam1C-4D[AurB]. Thus Mps1 phosphorylation of the Dam1 C-terminus may not suppress the function of this domain and may not contribute to error correction. Consistently, non-phosphorylatable mutants of Dam1 at these Mps1 sites did not show a defect in bi-orientation (our unpublished result).
Supplementary Figure 5 Non-phosphorylatable mutants of the Ndc80 N-tail and Dam1 C-terminus at Aurora B sites show normal lateral KT–MT interaction and slower bi-orientation establishment. Cells in Fig 8b were treated as in Fig 6 a–g; i.e., CEN3 under the GAL promoter was inactivated upon release from α factor treatment and then reactivated during metaphase arrest. Percentage of cells at each step of KT–MT interaction is shown (as in Fig 2c–f). Note that, in these cells, we did not observe any CEN3 detachment from a MT or from the spindle after CEN3 was caught on the MT or on the spindle. n = 21 cells were analysed in each strain. Data represent one out of two independent experiments.
Original uncropped western blots

For Figure 1c

For Figure 5a

Supplementary Figure 6: Full scans of western blots (for Figs 1c and 5a). Positions of protein size markers are shown at left of each blot.
| Strain | Genotype |
|--------|----------|
| T7427  | MATa PCUP1-ubi-DHFR-ndc80::kanMX PGAL-UBR1::HIS3 his3::ndc80::HIS3 PGAL-CEN3-tetOs::URA3 leu2::TetR-GFP::LEU2 trp1::YFP-TUB1::TRP1 PMET3-CDC20::TRP1 | |
### Supplementary Table 2 Statistics source data.

Tables show statistics source data. All p values were two-tailed, and calculated in comparison with wild-type controls (shown in the first row of each table) unless otherwise stated. Data from Experiment 1 (Exp1) are shown in each corresponding figure.