CEP162 is an axoneme-recognition protein promoting ciliary transition zone assembly at the cilia base

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The transition zone is a specialized compartment found at the base of cilia, adjacent to the centriole distal end, where axonemal microtubules are heavily crosslinked to the surrounding membrane to form a barrier that gates the ciliary compartment. A number of ciliopathy molecules have been found to associate with the transition zone, but factors that directly recognize axonemal microtubules to specify transition zone assembly at the cilia base remain unclear. Here, through quantitative centrosome proteomics, we identify an axoneme-associated protein, CEP162 (KIAA1009), tethered specifically at centriole distal ends to promote transition zone assembly. CEP162 interacts with core transition zone components, and mediates their association with microtubules. Loss of CEP162 arrests ciliogenesis at the stage of transition zone assembly. Abolishing its centriolar tethering, however, allows CEP162 to stay on the growing end of the axoneme and ectopically assemble transition zone components at cilia tips. This generates extra-long cilia with strikingly swollen tips that actively release ciliary contents into the extracellular environment. CEP162 is thus an axoneme-recognition protein pre-tethered at centriole distal ends before ciliogenesis to promote and restrict transition zone formation specifically at the cilia base.

The primary cilium is a membrane-bound, microtubule-based sensory organelle, composed of 9 doublet microtubules, the axoneme, nucleated directly from the distal end of centrioles or basal bodies1,2. Primary cilia can sense a wide range of signals in the extracellular surroundings, and thus critically regulate the physiology of cells during proliferation and morphogenesis3.

In vertebrate cells, cilia biogenesis follows a series of stereotyped steps1,2,4. Through the distal end, centrioles first interact with small membrane vesicles of unclear specificity, mediated by a set of accessory structures known as the distal appendages5,6. This interaction facilitates the nucleation of short doublet microtubules from the centriole distal end5. Nascent doublet microtubules adjacent to the centriole distal end are heavily crosslinked to the surrounding membrane to form a specialized compartment described as the transition zone (ref. 7). microtubule-to-membrane connections in the transition zone can be seen under electron microscopy as multiple rows of Y-shaped linkers7 that form a unique organization termed the ciliary necklace7. After transition zone formation, the development of a full-length, mature axoneme (or cilium) is supported and maintained by the intraflagellar transport (IFT) machinery8,9.

The transition zone has been shown to form a barrier or part of the ciliary gate that works together with the septin ring10, nucleoporins11 and probably distal appendages12 to regulate selective targeting and sorting of proteins to and from the ciliary compartment13,14. Several multiprotein complexes, including NPHP1-4-8 (refs 15,16), MKS/B9 (refs 13,15,17) and CEP290/NPHP5 (ref. 15) complexes have been found to associate with the transition zone, and many of them are ciliopathy molecules that critically regulate not only cilia biogenesis but also the activity of cilia as a sensory organelle15-20. Although a large number of transition zone components have been discovered19, how ciliary microtubules (or axonemes) are recognized to form the transition zone, and how transition zone assembly is limited to the cilia base during axoneme elongation remain largely unclear.

RESULTS

Identification of the centriole-distal-end protein CEP162 that binds microtubules

The centriole distal end marks the base of cilia, and is immediately adjacent to the transition zone. To identify factors that promote and/or specify transition zone formation at the cilia base, we screened
**Figure 1** Identification of CEP162 as a microtubule-binding protein localized at centriole distal ends. (a) Schematic outline of the SILAC-based proteomic analysis carried out to quantitatively dissect the centrosome proteome (see Supplementary Methods for details). Core centriolar proteins localizing to both mother and newly formed daughter centrioles give a higher H/L ratio (6:2) than mother-centriole-specific proteins (1:1), a.a., amino acid. (b) CEP162 is present at centrioles throughout the cell cycle. RPE1 cells in different cell-cycle stages were processed for immunofluorescence microscopy with the indicated antibodies. Magnified centrioles are shown in the insets. Nuclei were visualized with DAPI (blue). (c) CEP162 (red) localizes to the region between mother and newly formed daughter centrioles giving a higher H/L ratio. (d) A microtubule-pelleting assay. CEP162 was probed by anti-CEP162 antibodies; pelleted microtubules were detected by Coomassie blue staining. S, supernatant; P, pellet. Samples from both fractions were probed with the antibodies indicated. ERK and γ-tubulin were used as negative and positive controls for microtubule binding respectively. (e) Coomassie blue stains of GST–CEP162 and GST–CEP164 fusion proteins purified from E. coli. (f) Domain analyses of CEP162 for its microtubule binding and centriolar localization (CEN). (g,h) The binding of CEP162 to centrioles and to microtubules are separable. RPE-1 cells expressing HA-tagged fragments of CEP162 as indicated were stained with the antibodies indicated. Magnified centrioles are shown in the insets. DNA (DAPI, blue). (i) Endogenous CEP162 binds to microtubules. U2OS cell extracts were incubated with increasing amounts of taxol-stabilized microtubules and then centrifuged (258,000 g for 10 min) to form supernatant (SUP) and pellet fractions. Samples from both fractions were probed with the antibodies indicated. Magnified centrioles were visualized with DAPI (blue). (j) Uncropped images of all immunoblots are presented in Supplementary Fig. S1.
Figure 2 CEP162 is required for ciliogenesis. (a–c) RPE1 cells transfected with control or CEP162 siRNA for 96 h were stained with the antibodies indicated. (d) Effects of CEP162 depletion on cilia formation in proliferating (left) or G0-arrested (right) cells were quantified from 3 or 5 independent experiments as indicated. Error bars represent standard deviation (s.d.). The significance (two-tailed t-test) is indicated, *P < 0.05, **P < 0.001. (e) Cilia defects in RPE1 cells were rescued by expressing a HA-tagged, RNAi-resistant form of CEP162 (CEP162R). Error bars indicate standard deviation (s.d.). The significance (two-tailed t-test) is indicated, *P < 0.05, **P < 0.001, N = 4 individual experiments. The protein level of CEP162 in different experimental conditions is shown. Uncropped images of immunoblots are presented in Supplementary Fig. S1. (f) In zebrafish embryos, whole-mount RNA in situ hybridization revealed that cep162 mRNA is maternally loaded at the 1-cell stage and then ubiquitously expressed at the 50% epiboly stage and 8 somite stage (ss). At 24 h post-fertilization (hpf), Cep162 is highly expressed in the head region. A control cep162 sense probe was used to determine probe specificity. (g,h) Morphology of zebrafish embryos at 2 days post-fertilization injected with either control or Cep162 MOs at the 1-cell stage. Cep162-MO-injected embryos showed body axis defects, hydrocephalus (arrow) and altered left–right asymmetric heart looping. (i) PCR with reverse transcription showed that levels of correctly spliced cep162 mRNA were significantly reduced in Cep162 knockdown embryos. Loading control: actin. (j) Cep162 MO embryos showed an altered left–right asymmetric heart looping. Transgenic Tg(cmlc2:GFP) embryos that express GFP specifically in the heart (red arrows) were used to visualize heart looping. In control embryos the heart looped to the right, whereas Cep162 MO knockdown often resulted in reversed or no heart looping. Human CEP162 mRNA partially rescued these heart looping defects. Data were collected from 4 independent experiments (N = 4). The significance (one-way analysis of variance (ANOVA) and post-hoc test) is indicated, *P < 0.05. (k) Left–right asymmetry defects were observed using RNA in situ hybridization analysis of spaw expression, which is normally detected in left lateral plate mesoderm at 14 h post-fertilization, but was often found to be bilateral or right-sided in Cep162-MO-injected embryos. Co-injecting Cep162 MOs with human CEP162 mRNA partially rescued spaw defects. Data were collected from 7 independent experiments (N = 7). The significance (one-way ANOVA and post-hoc test) is indicated, *P < 0.05. L, left; R, right. Total cell counts are shown in the figures (as n). Sample size for each independent experiment: n > 50 (d), n > 25 (e), n > 15 (j,k).
accessory structures such as the pericentriolar material (PCM) and appendages\(^1\) (Fig. 1a), an increase in the number of unmodified centrioles within a centrosome would not change the total amount of accessory proteins in that centrosome. However, an increase in centriolar proteins would occur proportionally. Such differences can be quantitatively detected and analysed by SILAC (stable isotope labelling by amino acids in cell culture) mass spectrometry\(^2\), and this technique has recently been used to successfully isolate distal appendage proteins\(^6\) associating with modified centrioles (Fig. 1a; 1:1 ratio). Here, the same proteomics tool was used to specifically identify core centriolar proteins present equally in both modified and unmodified centrioles (Fig. 1a; 6:2 ratio). Candidate core centriolar proteins were further examined for their subcellular localization. Nine centriole-distal-end proteins were identified so far, including six previously described proteins: CP110, CEP97, CEP76, OFD1, CEP290 and SDCCAG8 (refs 23–27), and three largely uncharacterized proteins: MPP9, KIAA1009 (or QN1) and CCHCR1 (refs 28,29). Among the uncharacterized, KIAA1009 exhibits microtubule-binding activity (Fig. 1), and thus was further examined.

KIAA1009, now renamed CEP162, was found in organisms ranging from trypanosomes to vertebrates. It localizes to the distal end of centrioles throughout the cell cycle (Fig. 1b), co-localizing with the known distal-end marker CP110 (ref. 23) but not with the proximal-end markers C-Nap1 (ref. 30) or SAS6 (Fig. 1c). During ciliogenesis, the CEP162 signal became disc-shaped, and was found at the cilia base surrounded by the distal appendage protein CEP164 (ref. 31; Fig. 1d), suggesting an intimate association with the axoneme. Consistently, CEP162 was found on spindle microtubules in mitosis (Fig. 1e), suggesting that it may possess microtubule-binding activity. Full-length CEP162 (FL; amino acids 1–1,403) contains 3 coiled-coil (CC) stretches in the carboxy-terminal region: CC1 (amino acids 617–906), CC2 (amino acids 957–1,121) and CC3 (amino acids 1,167–1,386; Fig. 1f). Deletion of CC3 (ΔCC3 or tNC1C2) abolished centriole localization without affecting microtubule association (Fig. 1f,h). Conversely, a fragment containing CC2 and CC3 (C2C3) localized to centrioles at distal ends, but failed to associate with microtubules (Fig. 1f,g), indicating that the two targeting activities of CEP162 are separable. To determine whether CEP162 binds directly to microtubules, a microtubule-pelleting assay was used. Endogenous CEP162 in cell lysates (Fig. 1i) or a microtubule-binding fragment of CEP162 (tNC1C2) purified from Escherichia coli (Fig. 1j), but not a control fragment (CEP162\(^{1–227}\)), could be co-pelleted with pure, taxol-stabilized microtubules (Fig. 1k), demonstrating that CEP162 binds directly to microtubules. Furthermore, overexpression of CEP162\(^{tNC1C2}\) in cells induced a large number of microtubule bundles that were insensitive to cold treatment (Fig. 1l).

**CEP162 is required for ciliogenesis**

To determine the function of CEP162, the protein was depleted from cells by RNA-mediated interference (RNAi; Figs 2 and 3). Unlike in a previous report\(^9\), neither mitosis nor centriole duplication was affected by CEP162 depletion (Fig. 2a,b). However, in the absence of CEP162, ciliogenesis was specifically disrupted in retinal pigment epithelial (RPE1) cells (Fig. 2c–e). In vivo studies using zebrafish indicated that the zebrafish CEP162 homologue is maternally supplied and left–right asymmetry defects (Fig. 2g–k), which could be partially rescued by expressing an RNAi-resistant form of CEP162 (Fig. 2j,k).

**Loss of CEP162 arrests ciliogenesis at the stage of transition zone assembly**

Interestingly, the acquisition of distal appendages (Fig. 3a), the removal of CP110 from the mature centriole\(^3\) (Fig. 3b) and the localization of a known centriole-distal-end protein OFD1 (ref. 24; Fig. 3c) all occurred normally in the absence of CEP162. Moreover, several non-membrane-bound transition zone components including IFT88, TCTN1 and CC2D2A were recruited normally to CEP162-deficient centrioles (Fig. 3d–f), indicating that although cilia fail to form, some early steps of ciliogenesis can proceed without CEP162.

Electron microscopy was used to examine the ciliogenesis defect in more detail (Fig. 3g). In wild-type cells, more than 88% of mature centrioles (30 out of 34) in G0/G1-arrested cells, judged by the appearance of appendages, efficiently dock to vesicle membranes and support the formation of axoneme or ciliary microtubules known to organize the transition zone (Fig. 3g, left). Note that by establishing connections alongside the ciliary microtubules to form the transition zone, the membrane vesicle becomes severely deformed to embrace the elongated axoneme (Fig. 3g, left; schematic 1), whereas the contact between distal appendages and membranes remains at the base of the cilium (red in schematic 1). In contrast, in CEP162-depleted cells, a small membrane vesicle with an unaltered or slightly flattened shape was often seen linked peripherally through the distal appendage to centriole distal ends (22 out of 49 mature centrioles), where the outgrowth of a short ciliary bud was evident, but no signs of membrane crosslinking alongside microtubules were apparent (Fig. 3g, middle and right; schematic 2). These observations suggest that loss of CEP162 does not affect centriole-to-membrane docking mediated by distal appendages, but instead arrests ciliogenesis at the stage of transition zone assembly.

To confirm the absence of an intact transition zone at CEP162-deficient centrioles, the localization of other transition zone components was examined. The transition zone houses several multiprotein complexes including NPHP1–4–8 (refs 15,16), MKS/B9 (refs 13,15,17) and CEP290/NPHP5 (ref. 15) complexes. Intriguingly, on G0/G1 arrest, NPHP8 (or RPGRIP1L) was efficiently recruited to wild-type centrioles before cilia formed, but failed to do so in the absence of CEP162 (Fig. 3h). Consistently, CEP162-deficient centrioles failed to recruit NPHP1 (Fig. 3i), but had no problem to recruit IFT88 (Fig. 3i,d), indicating that targeting of the NPHP1–4–8 complex to the transition zone depends on CEP162. Moreover, whereas CEP162 is not required for the localization of TCTN1 and CC2D2A (members of MKS/B9 complex; Fig. 3e,f), CEP162 depletion severely disrupted the recruitment of TCTN2 and TMEM67 (Fig. 3j,k), two components of the MKS/B9 complex that contain the transmembrane domain\(^15,17,19\). Note that the stability or protein level of transition zone components examined here is not affected by CEP162 depletion (Fig. 3i), and that these transition zone deficiencies are consistent with the electron microscopy results, revealing a lack of intact transition zone structures or membranes. Together, our results indicate that the microtubule-binding protein CEP162 plays a critical role in the assembly or maintenance of the ciliary transition zone.
Figure 3 Loss of CEP162 blocks ciliogenesis at the stage of transition zone assembly. (a-f) RPE1 cells transfected with control or CEP162 siRNA followed by serum starvation for 48 h were stained with the antibodies indicated. Only G1 cells that contain two unduplicated centrioles were scored. In some controls (b, d-f), both ciliated and non-ciliated G1 cells are shown. Note that the removal of CP110 (b), or the recruitment of IFT88 (d), TCTN1 (e) and CC2D2A (f), occur at wild-type centrioles before cilia formed, as well as at CEP162-deficient centrioles where cilia failed to form. Data were collected from 3 independent experiments. The percentage of cells showing the presence or absence of indicated markers at centrioles is indicated (mean ± s.d.). No significant differences were detected between control and CEP162-depleted cells. (g) Transmission electron micrographs of basal bodies in RPE1 cells transiently transfected with control (left) or CEP162 (middle and right) siRNA, followed by 48 h serum starvation for cilia formation. Serial sections of a basal body (right) in a CEP162-knockdown cell are shown in addition to the single-sectioned image (middle). Arrows indicate small membrane vesicles connected to CEP162-deficient centrioles through the distal appendage. Schematic diagrams summarizing the phenotype are shown. Grey links, membrane-to-microtubule connections; red, membrane-to-appendage connections. (h-k) Control or CEP162-depleted RPE1 cells as described in d–f were examined for the presence of indicated transition zone components (red). Data were collected from 3 independent experiments (N = 3), and mean% ± s.d. ± s.d. is shown. The significance (two-tailed t-test) is indicated, *P < 0.05, **P < 0.001. (l) The protein levels of indicated transition zone components were examined in control or CEP162-depleted RPE1 cells by western blotting. Uncropped images of immunoblots are presented in Supplementary Fig. S1. Total cell counts are shown in the figures as n. More than 40 cells were analysed for each independent experiment. Supplementary Table S1 presents the source data for the graphs.
CEP162 recognizes ciliary axonemes at the tip

We reasoned that CEP162 recognizes and/or stabilizes ciliary microtubules at centriole distal ends to promote transition zone formation. To explore whether CEP162 interacts with ciliary microtubules, CEP162ΔC3 and CEP162ΔNC1C2, which are both defective in centriole tethering but retain microtubule-binding activity, or CEP162ΔC3C, which lacks microtubule-binding activity, was inducibly expressed in wild-type RPE1 cells (Fig. 4a–d). Strikingly, exogenous CEP162ΔC3 (Fig. 4a) or CEP162ΔNC1C2 (Fig. 4b), but not the centriole-tethering domain CEP162ΔC3C (Fig. 4c), strongly labelled the tips of elongated cilia, whereas endogenous CEP162 remained tethered at the cilia base (Fig. 4e). Weak labelling of CEP162ΔNC1C2 along the axoneme was also noted (Fig. 4b). Accumulation of CEP162 at cilia tips may reflect an indirect consequence of a trafficking defect in the IFT pathway, particularly the retrograde transport\(^6\), caused by overexpression of truncated CEP162. To investigate this idea, we examined the cilia length, as most IFT defects are known to either eliminate or shorten the cilium\(^4\). Intriguingly, the length of cilia modified by CEP162ΔC3ΔC3 was greatly enhanced rather than reduced (Fig. 4f).

Moreover, higher fractions of cells with cilia were seen in proliferating populations (Fig. 4g), suggesting that overexpression of CEP162ΔC3ΔC3 does not compromise IFT activity in cilia assembly. Rather, this result suggests that CEP162 recognizes and stabilizes axonemes at tips, but that this is normally restricted to distal ends of centrioles, where nascent doublet microtubules grow and form the transition zone.

To explore whether purified CEP162 can bind directly to the tip of membrane-extracted axonemes, wild-type RPE1 cells cultivated to form cilia were extracted with detergent-containing buffer (0.1% Triton X-100 in phosphate buffered saline). This treatment shortened the IFT machinery off the axoneme (Fig. 4i), leaving short axonemes marked with acetylated α-tubulin in nearly every ciliated cell (>80%). These membrane/IFT-extracted cilia remained attached to cells, and were incubated with different concentrations of purified CEP162...
Figure 5 CEP162 interacts with CEP290 and mediates its association with microtubules. (a) Endogenous CEP162 or CEP290 in HEK293T cell extracts was immunoprecipitated (IP), and co-precipitated proteins were probed with the indicated antibodies by western blotting. Uncropped images of immunoblots are presented in Supplementary Fig. S1. (b) Mapping the CEP290-binding domain of CEP162. HEK293T cells expressing various Flag-tagged fragments of CEP162 were subjected to immunoprecipitations and western blotting with antibodies as indicated. Uncropped images of immunoblots are presented in Supplementary Fig. S1. (c) RPE1 cells at different cell-cycle stages were processed for immunofluorescence microscopy with anti-CEP290 (red), anti-CEP162 (green) and anti-centrin (blue) antibodies. Magnified centrioles are shown in the insets. DNA (DAPI, blue). (d) Overexpression of CEP162 induced microtubule bundles recruiting endogenous CEP290. U2OS cells transiently expressing HA-tagged CEP162 induced microtubule bundles. NIH3T3 cells transiently expressing HA-tagged CEP162 were stained with antibodies against acetylated-tubulin (green), CEP162 (red) and CEP290 (blue). (e) Overexpression of CEP162 induced microtubule bundles recruiting endogenous CEP290. U2OS cells transiently expressing HA-tagged CEP162 or HA-tagged CEP162 were stained with antibodies against CEP290 (red), CEP290 (green), CEP290 (red) and DAPI (DNA, blue). (f) Spindle association of CEP290 with human CEP162 or GFP-tagged mouse CEP290 were stained with antibodies against α-tubulin (red), CEP290 (HA, green) or CEP290 (GFP, green). (g) Centriolar localization of CEP290 and CEP162 are independent of each other. RPE1 cells were transfected with control, CEP162 or CEP290 siRNA for 72 h were stained with the indicated antibodies against acetylated-tubulin (green), CEP162 (red) and CEP290 (blue). DNA (DAPI, blue).

CEP162 interacts with CEP290 and mediates its association with microtubules

To determine whether CEP162 bridges transition zone components to the axoneme, we examined its relationship with CEP290. CEP290 forms a complex with multiple ciliopathy molecules, including NPHP5 (ref. 15), CC2D2A, TCTN1, MKS1 (ref. 17), and mediates the membrane-to-microtubule connection in the transition zone (ref. 20). However, whether CEP290 associates directly or indirectly with ciliary microtubules is unclear. Immunoprecipitation of endogenous CEP162 pulled down endogenous CEP290 (Fig. 5a), and vice versa, indicating that CEP162 and CEP290 are present in the same protein complex. Further analyses revealed that the amino-terminal region of CEP162
that contains CC1 and CC2 is required to pull down endogenous CEP290 (Fig. 5b), the same region where the axoneme-binding activity resides. Endogenous CEP290 localized to the distal ends of centrioles throughout the cell cycle (Fig. 5c), and to mitotic spindles during mitosis (Fig. 5d, top), similar to CEP162. Intriguingly, CEP162 localizes to spindle microtubules in the absence of CEP290 (Fig. 5d, middle), but CEP290 requires CEP162 to associate with microtubules (Fig. 5d, bottom). Furthermore, overexpression of CEP162 induced microtubule bundles that recruited endogenous CEP290 (Fig. 5e), whereas overexpression of CEP290 failed to decorate microtubules or induce microtubule bundles (Fig. 5f), demonstrating that CEP290 is not a microtubule-binding protein but associates with microtubules through CEP162. Interestingly, however, the localization of CEP162 and CEP290 at centriole distal ends is independent of each other (Fig. 5g), explaining the recruitment of Cdc2a and TCTN1 to centrioles in the absence of CEP162 (Fig. 3e,f), and further suggesting that CEP290 may serve as an anchor that recruits other transition zone components to the vicinity of the transition zone, whereas CEP162 antibodies against CEP290 (a), TCTN1 (b), TMEM67 (c) or RPGRIP1L (d) in red, acetylated-tubulin (AcTu, green) and HA (blue). Nuclei were visualized with DAPI (blue). (e) Centriole-bound components, centrin (top) and CEP164 (bottom), remained localized at the cilia base in CEP162ΔCC3-expressing cells.

**Figure 6** Untethered CEP162 promotes ectopic recruitment of transition zone components to cilia tips. (a–d) Ectopic recruitment of transition zone components through CEP162ΔCC3 and CEP162ΔNC1C2, HA- or GFP-tagged CEP162ΔCC3 or CEP162ΔNC1C2 was expressed in RPE1 cells followed by serum starvation for 48 h and staining with DAPI (blue). (e) Ectopic recruitment is specific to transition zone components, as antibodies against CEP290 (a), TCTN1 (b), TMEM67 (c) or RPGRIP1L (d) in red, acetylated-tubulin (AcTu, green) and HA (blue). Nuclei were visualized with DAPI (blue). (e) Centriole-bound components, centrin (top) and CEP164 (bottom), remained localized at the cilia base in CEP162ΔCC3-expressing cells.

**CEP162 promotes ectopic assembly of transition zone components at cilia tips**

To characterize further the relationship between CEP162 and transition zone assembly, we examined whether CEP162 can promote ectopic assembly of transition zone components. Strikingly, in cells expressing CEP162ΔCC3 or CEP162ΔNC1C2, several endogenous transition zone components including CEP290, TCTN1, TMEM67 and NPHS8/RPGRIP1L, which are normally seen at the base of cilia, could now be found at cilia tips marked by CEP162 (Fig. 6a–d). This ectopic recruitment is specific to transition zone components, as other centrosomal proteins, including CEP164 and centrin, remained localized at the cilia base under the same conditions (Fig. 6e). Thus, transition zone components are targeted to an ectopic ciliary site through non-tethered CEP162, revealing CEP162 as a critical promoter for transition zone assembly.

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Figure 7 Cilia tips modified by CEP162 swell exceedingly and discharge ciliary contents. (a) RPE1 cells expressing GFP-tagged CEP162tNC1C2 were traced by time-lapse fluorescence microscopy. Images were taken at 2-min time intervals. Existing cilia tips are marked by arrows, and newly formed tips are marked with arrowheads. (b–e) HA- or GFP-tagged CEP162ΔCC3 or CEP162tNC1C2 was conditionally expressed in RPE1 cells followed by serum starvation for 48 h. Cells were stained with antibodies against CEP290 (b), RPGRIP1L (c), IFT88 (d), Arl13b (e) in red, acetylated-tubulin (green) and HA (blue). DNA was stained with DAPI. Arrows indicate the CEP162-positive structures released from cilia tips. (f) Correlative light and scanning electron microscopy images of control cells (left), or RPE1 cells expressing GFP-tagged CEP162ΔCC3 (green) marked cilia tips (middle and right). Primary cilia in control cells (left) were labelled with acetylated-tubulin (green). Arrows indicate cilia tips. Merged images are shown at a low scanning electron microscopy (SEM) magnification. (g) Correlative light and scanning electron micrographs of the swollen structure released from cilia tips in RPE1 cells expressing GFP-tagged CEP162ΔCC3. Released structures from cilia tips were marked with GFP (green). Merged images are shown at a low scanning electron microscopy magnification.

The consequence of having a transition zone-like structure at cilia tips was further examined. RPE1 cells expressing GFP-tagged CEP162tNC1C2 were imaged by time-lapse fluorescence microscopy. The GFP–CEP162 signal was seen first at the cilia tip as expected, but surprisingly, it became detached from the tip, and released into the extracellular environment (Fig. 7a and Supplementary Video S1). In a few cases, violent bursts of cilia tips were observed (Supplementary Video S2). Similar phenotypes were also seen in cells expressing CEP162ΔCC3. The detached, CEP162-positive structures, scattering amongst cells, were also positive for the transition zone marker CEP290 (Fig. 7b) or RPGRIP1L (Fig. 7c), IFT marker IFT88 (Fig. 7d) and ciliary membrane marker Arl13b (Fig. 7e), indicating that CEP162-modified
cilia are actively discharging ciliary contents from their tips. Correlative light and scanning electron microscopy revealed that CEP162-modified tips are severely swollen (Fig. 7f), forming a blister-like configuration. The swelling is probably a consequence of excess accumulation of proteins at tips, including CEP162, and other ciliary materials (for example, transition zone components, IFT and Arl13b). We do not understand how these ciliary materials are excessively trapped there, although it is consistent with the idea that some form of a barrier is built, separating the tip from the rest of the ciliary compartment. The bulging structures released from cilia tips differed in size, and were irregularly shaped (Fig. 7g), suggesting that they are membranous structures randomly breaking away from the exceedingly enlarged tip. However, the GFP signal was retained over time in some of these detached structures (Supplementary Video S1), rather than being diluted or dispersed, an indication of intact membrane-coated structures. Note that secretion-like activities have been reported to occur at the flagella tip in Chlamydomonas. Together, these observations demonstrate that the spatial restriction of transition zone assembly to the cilia base is critical for proper cilia structure and function, and that one important role of centriole-distal-end proteins is to provide such spatial cues.

**DISCUSSION**

In cycling cells, the transition zone is formed transiently with most of its components recruited from the cytoplasm. However, a few transition zone factors are pre-tethered at the assembly site, that is, the distal end of centrioles, before ciliogenesis initiates, potentially serving as a promoter or anchor that drives transition zone assembly. Our results reveal CEP162 as a critical promoter as: it localizes to distal ends of centrioles, where nascent ciliary microtubules form; it binds directly to ciliary microtubules; CEP162 interacts with the core transition zone component CEP290, and mediates its association with (ciliary) microtubules; loss of CEP162 blocks transition zone assembly; and gain of CEP162 at cilia tips promotes ectopic assembly of transition zone components. CEP290, which localizes to centriole distal ends in a manner that is independent of CEP162, and anchors its associated components to the vicinity of ciliary microtubules, also plays a critical role in transition zone formation.

Most of centriolar components involved in ciliogenesis are concentrated at the distal portion of cilia. Some of these factors, for example CEP162 and CEP290, are present at every centriole regardless of the age, but others, such as the appendages, associate only with mature cilia. A systematic method that can differentially identify these two distinct classes of distal-end-associated molecules would greatly facilitate the study of cilia biogenesis. Here we have established a quantitative proteomic approach to map proteins associated with specific structural elements of the centrosome (Fig. 1a). This has allowed us to identify a list of molecules present at distal ends of all centrioles, and in a separate study, carefully characterize the molecular composition and function of the distal appendages. The centriole distal end seems to play several critical roles in ciliogenesis: it interacts with the membrane vesicles, which leads to docking of cilia to the plasma membrane; it is the site where ciliary microtubules grow; and it is immediately adjacent to the site of transition zone assembly. It will be interesting to determine whether the centriole-distal-end components identified here are involved in any of these processes.

**METHODS**

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

Note: Supplementary Information is available in the online version of the paper.

**ACKNOWLEDGEMENTS**

We are grateful to K. Anderson and her laboratory at Memorial Sloan-Kettering Cancer Center, USA, for reagents and antibodies. We thank L. Gunther, G. Perumal and F. Macaluso at the Analytical Imaging Center of Albert Einstein College of Medicine for assistance with transmission and scanning electron microscopy; K. Uryu at Rockefeller University and N. Lampe at MSKCC for assisting with the usage of electron microscopes; F. Foley at SUNY Upstate and D. Gutiérrez at MSKCC for technical assistance and zebrafish management; and A. Hall, Z. Bao and C. Haynes at MSKCC for comments on the manuscript. This work was supported by the National Institutes of Health grants HL095690 to J.D.A. and GM088233 to M-F.B.T.

**AUTHOR CONTRIBUTIONS**

W-J.W. and M-F.B.T. designed experiments and analysed data. W-J.W. performed most of the experiments. W-J.W., R.S. and J.M.A. did the quantitative centrosome proteomics. W-J.W. and R.S. prepared purified centrosomes. J.M.A. carried out SILAC mass spectrometry and analysed the data. The zebrafish works were carried out by H.G.T. and J.D.M. M.G.G. helped with initial zebrafish experiments. G.S.P., F.P.M. and W-J.W. performed the correlative light and scanning electron microscopy. M-F.B.T. and W-J.W. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at www.nature.com/doifinder/10.1038/ncmb1739

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METHODS

Quantitative centrosome proteomics by SILAC mass spectrometry. The following two types of centrosome were isolated from S-phase-arrested cells: wild-type centrosomes containing one modified centriole that is engaged with one unmodified centriole, and Plk4-induced rosette centrosomes containing one modified centriole engaged with 4 to 6 unmodified centrioles. In both samples, only modified centrioles carry the accessory structure (that is, PCM and appendages). Thus, the amount of any accessory protein associated only with modified centrioles should be the same between rosette and wild-type centrosomes, showing a 1:1 ratio in the SILAC analysis (Fig. 1a). Importantly, non-specific contaminants co-purified with centrosomes are also present equally. Conversely, higher amounts of proteins containing the epitope were excised and digested overnight at pH 8.0 with modified sequencing-grade trypsin (Promega). Peptide mixtures were eluted and each gel section was analysed separately by micropipet liquid chromatography with tandem mass spectrometry using suroose gradient centrifugation as described previously. Centrosomes or SILAC-labelled protein mixtures were run by SDS-PAGE and 12 gel sections were excised and digested overnight at pH 8.0 with modified sequencing-grade trypsin (Promega). Peptide mixtures were eluted and each gel section was analysed separately by micropipet liquid chromatography with tandem mass spectrometry using the EASY-nLC nanoflow HPLC (Thermo Fisher Scientific) with a 75-μm inner-diameter × 15-cm-length PicoFrit capillary column (New Objective) self-packed with 5 μm Magic C18 res (Michrom Bioresources) coupled to a hybrid LTQ Orbitrap XL-ETD mass spectrometer (Thermo Fisher Scientific). Tandem mass spectrometry fragmentation spectra were searched for protein identification using the Andromeda search engine (http://maxquant.org/) against the reversed and concatenated IPI_HUMAN protein database (v3.87). One unique peptide was required for high-confidence protein identifications and a minimum ratio of two peptides (one unique and one razor) were required for SILAC ratio determination. Normalized SILAC ratios (H/L) were used for subsequent analysis.

Cloning and plasmids. The CEP162 cDNA was obtained from Open Biosystems. To generate HA-tagged CEP162, full-length (1-1,403) or CEP162 fragments containing 1-1,121, 300-1,121, 617-1,121, 300-956, 617-1,403, 957-1,403 and 1243-1,403 amino acid residues were cloned into pcDNA3-HA vector. Human CEP162 fragments containing 1-1,121 and 300-1,121 residues were also cloned into pEGFP-C1 to generate EGFP-CEP162 proteins. All HA- and GFP-tagged fragments were also subcloned into the pLVX-Tight-Puro vector (Clontech) so that protein expression can be controlled under a tetracycline-inducible promoter. To generate GST-CEP162 proteins, CEP162 fragments containing 1-227, 1-121 and 300-1,121 residues were cloned into pGEX-4T1, pEGFP-mCEP90 was obtained from J. Gleeson at UCSD, USA (Addgene plasmid #27379; ref. 36).

Cell culture. HeLa, NIH3T3 and U2OS cells were cultured in DMEM medium with 10% FBS and 1% penicillin/streptomycin. Human telomerase-immortalized retinal pigment epithelial cells (hTERT-RPE1, RPE1) were cultured in DME/F-12 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Stable clones of RPE1 or HeLa cells expressing Plk4, CEP164 or CEP162 deletion mutants from the tetracycline-inducible promoter were obtained through in vivo gene delivery using the lentiviral vector pLVX-Tight-Puro vector (Clontech). Transient transfections of plasmids into U2OS cells were performed using Lipofectamine 2000 (Life Technologies).

RNAi and expression of RNAi-resistant hCEP162. Synthetic short interfering RNA (siRNA) oligonucleotides were obtained from Open Biosystems. Transient transfection of siRNA oligonucleotides into RPE1 cells was performed using RNAiMAX (Life Technologies). The 21-nucleotide siRNA sequences targeting CEP162 corresponded to 5'-GGTGGACAGTGTTAGCTTACTAT-3', 5'-CAACCATAGTATGCTCGATT-3' and 5'-GATGGACAGTGTTAGCTTACTAT-3'. The control siRNA was from Silencer Select Negative Control No. 1 (Life Technologies). The RNAi-resistant construct (CEP1622) was made by introducing nucleotide changes in the target regions without changing the corresponding amino acids using site-directed mutagenesis (QuickChange; Agilent Technologies). The following primers were used: 5'-GTTCCTGAGCTCAACATGCTCAG-3' and 5'-TAATGTTGCAATCCACTCGGAGGAG-3'. To generate an inducible expression system, stable clones of RPE1 cells expressing CEP1622 from the tetracycline-inducible promoter were obtained through in vivo gene delivery.

Immunofluorescence and time-lapse microscopy. Cells were fixed with methanol at −20 °C for 5 min or 4% paraformaldehyde at room temperature for 15 min. Cells were blocked with 3% bovine serum albumin (w/v) with 0.1% Triton X-100 in PBS before incubation with the primary antibody. Secondary antibodies were from Molecular Probes and were diluted 1:500. DNA was visualized using 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Fluorescent images were acquired on an upright microscope (Axio imager; Carl Zeiss) equipped with ×100 oil objectives, NA of 1.4, and a camera (ORCA ER; Hamamatsu Photonics). For time-lapse fluorescence experiments, GFP-tagged CEP162 and CEP1642 were induced in hTERT-RPE1 cells and cells were imaged using a Zeiss Axiosvert microscope configured with a ×65 objective, a motorized temperature-controlled stage, an environmental chamber and a CO2 enrichment system (Zeiss). Images were acquired and processed by an electron-multiplying CCD (charge-coupled device) camera (Hamamatsu Photonics) and axiovision software (Zeiss).

Antibodies. A rat polyclonal antibody against human CEP162 was produced using affinity-purified GST-CEP162221 as the antigen, and used at a 1:5,000 dilution. A rabbit polyclonal antibody against human C-Nap1 was produced as described previously. Other antibodies used in this study include anti-CEP162 (HPA030170; 1:2,000 dilution), CC2D2A (HPA044141; 1:200 dilution), RPRGPI (HPA039405; 1:200 dilution), ODF1 (HPA031103; 1:200 dilution), acetylated-tubulin (clone 6-11B1-1, T7451; 1:2,000 dilution), Flag (clone M2, F3165; 1:5,000 dilution) and α-tubulin (clone DM1A, T0262; 1:2,000 dilution for immunofluorescence microscopy; 1:5,000 dilution for immunoblotting) (Sigma-Aldrich); anti-HA.11 (clone 16B12, MMS-101P; 1:1,000 dilution for both immunofluorescence microscopy and immunoblotting; Covance); anti-CP110 (12780-1-AP; 1:200 dilution), IFT88 (13967-1-AP; 1:500 dilution), TCTN1 (15004-1-AP; 1:200 dilution), TCTN2 (17053-1-AP; 1:200 dilution) and TME67 (13975-1-AP; 1:100 dilution) (Proteintech); anti-centrin3 (clone 3E6, H00001070-M01; 1:200 dilution) and CEP164 (4535002; 13,000 dilution) (Novus Biologicals); anti-CEP290 (A301-698A; 1:2,000; Bethesda Laboratories); anti-α-tubulin (MCA77G; 1:1,000 dilution; AbDserotec); anti-GFP-FITC (600-102-215; Rockland); anti-NPHP1 (nephrocystin-20, sc20024; 1:300 dilution); γ-tubulin (Tu30; sc-51715; 1:500 dilution) and hSAS-6 (clone 91.390.21, sc-81413; 1:200) (Santa Cruz Biotechnology).

Immunoblotting and immunoprecipitation experiments. Cells were lysed in NP-40 lysis buffer (50 mM Tris–HCl at pH 8.0, 150 mM NaCl and 1% NP40) with protease inhibitors for 30 min at 4 °C. After centrifugation, cell lysates were resolved by SDS–PAGE and analysed by immunoblotting using different antibodies as indicated. For immunoprecipitation, cell lysates were incubated with rat anti-CEP162, rabbit anti-CEP290 or control IgG. The immunoprecipitates were resuspended in SDS sample buffer (50 mM Tris–HCl at pH 6.8, 2% SDS, 10% glycerol and 0.1% bromophenol blue) containing 2-mercaptoethanol, and processed for immunoblotting.

Microtubule co-sedimentation. Purified taxol-stabilized microtubules were purchased from Cytoskeleton and diluted to a final concentration of 2 μg ml−1 in taxol-containing BRB80 buffer (80 mM Pipes, pH 6.8, 1 mM MgCl2, 1 mM EGTA including protease inhibitors, 1 mM GTP, 1 mM diothiohetoile and 20 μM taxol). Taxol-stabilized microtubules were mixed with CEP162 fragments affinity-purified from E. coli, or with cell extracts (1.5 mg protein) prepared from U2OS cells in BRB80 buffer, and pre-spun at 70,000 r.p.m. for 10 min with a TLN 100 rotor (Beckman) to remove protein aggregates and short microtubules. After 30 min of incubation at 30 °C, microtubules in samples were spun through a 1.5 ml cushion of 1 mM GTP, protease inhibitors, 1 mM MgCl2 and 20 μM taxol, at 70,000 r.p.m. for 10 min in a TLS-55 rotor (Beckman). Both supernatants and pellets were collected and analysed.

Zebrafish. Wild-type AB zebrafish (Danio rerio) and transgenic Tg(sox17:GFP) (ref. 37) and Tg(elt2:GFP) (ref. 38) were obtained from the Zebrafish International Resource Center (ZIRC). Embryos were staged as described previously. For whole-mount in situ RNA hybridization, PCR-amplified zebrafish cep162 cDNA was cloned into the PC2S+ vector. cep162 and southpaw (spaw) cDNA constructs were used to generate in vitro-synthesized RNA probes labelled with
digoxigenin (Roche DIG RNA labelling kit). In situ RNA hybridizations were performed as described previously.61

For embryo injections, reverse transcription and PCR, antisense MOs were obtained from Gene Tools. Two nanograms of a splice-site blocking MO designed to disrupt splicing of cep162 (5'-CGGGCCCACACGACCTCACCCATTTG-3') was injected between the 1–2-cell stages to allow the MO to be distributed to all embryonic cells. Cep162 MO was co-injected with 4 ng p53 MO (5'-CGGGCAATTGCTTTTCAGAAATTTG-3') to avoid nonspecific MO effects.62 For analysis of cep162 expression by PCR with reverse transcription, total mRNA was extracted from 8-somite-stage embryos using Trizol and a cDNA library was generated using the iScript cDNA synthesis kit (Bio-Rad). Primers (Cep162-F: 5'-atggctcatagactgaccaaagagg-3' and Cep162-R: 5'-AATCCGGATGTTCCTCCTGTAAG-3') were used to amplify the N-terminus of Cep162 to determine the efficacy of knockdown.

Transmission electron microscopy. Cells grown on coverslips made of Aclar film (Electron Microscopy Sciences) were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde with 0.1% tannic acid in 0.1 M sodium cacodylate buffer at room temperature for 30 min, post-fixed in 1% OsO4 in sodium cacodylate buffer for 30 min on ice, dehydrated in a graded series of ethanol, infiltrated with EPON812 resin (Electron Microscopy Sciences) and then embedded in the resin. Serial sections (~90-nm thickness) were cut on a microtome (Ultracut UC6; Leica) and stained with 1% uranyl acetate as well as 1% lead citrate. Samples were examined on a JOEL transmission electron microscope.

Correlative light and scanning electron microscopy. Cells were grown on square 22 mm coverslips, and fixed for 30 min at room temperature with 4% paraformaldehyde, 2% glutaraldehyde, buffered with 0.1 M sodium cacodylate followed by 0.1% sodium borohydride to quench glutaraldehyde autofluorescence. Cells were then processed for immunofluorescence microscopy (as above). Following fluorescent staining, the cells were dehydrated through a graded ethanol series to 95% ethanol to limit quenching of florescence that occurs when exposed to paraformaldehyde, 2% glutaraldehyde, buffered with 0.1 M sodium cacodylate, and a cDNA library was generated using the iScript cDNA synthesis kit (Bio-Rad). Primers (Cep162-F: 5'-atggctcatagactgaccaaagagg-3' and Cep162-R: 5'-AATCCGGATGTTCCTCCTGTAAG-3') were used to amplify the N-terminus of Cep162 to determine the efficacy of knockdown.

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Figure S1  Full images of immunoblots used in figures. Regions of interest are boxed.
Supplementary Table and Video Legends

Table S1 Summary of sample sizes and means analyzed in each independent experiment.

Supplementary Video S1 Ciliary contents are actively released from cilia tips modified with CEP162. RPE1 cells expressing GFP-tagged CEP162\textsuperscript{N1C1C2} were serum-starved for 24hr, and imaged by time-lapse fluorescence microscopy, using 2 min as the time interval. Images shown in movies were generated from maximum intensity projections of a z-stack.

Supplementary Video S2. Bursts of cilia tips modified by CEP162. Images were collected and processed from RPE1 cells expressing GFP-tagged CEP162\textsuperscript{N1C1C2} as described in Supplemental video S1.