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Human microcephaly protein CEP135 binds to hSAS-6 and CPAP, and is required for centriole assembly

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 09 November 2012

Thank you for submitting your manuscript on human CEP135 for consideration by The EMBO Journal. It has now been assessed by three expert referees, whose comments are copied below. As you will see, all referees consider your results important in principle, but they also raise a number of points that would need to be addressed before publication. These points, which for the most part appear to be well taken, include both experimental and writing/discussion issues.

Should you be able to adequately answer the reviewers' queries during the course of a revision, then we should be happy to consider this work further for publication in The EMBO Journal. I would therefore like to invite you to revise the manuscript in response to the referees' comments and criticisms. Please keep in mind that it is our policy to allow only a single round of major revision and that it is therefore important to carefully respond to all points at this stage. When preparing your letter of response to the referees' comments, please also remember that this will form part of the Review Process File, and will therefore be available online to the community. Finally, please make sure to carefully revise the manuscript reference list, both in terms of format and completeness, before resubmission.

We generally allow three months as standard revision time, and it is our policy that competing manuscripts published during this period will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider this work. I look forward to your revision.

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

In this paper Lin and colleagues report functional analyses of human CEP135, which is a centriole protein homologous to the Chlamydomonas cartwheel component Bld10p. They show that CEP135 is involved in centriole duplication mediated by hSAS-6, STIL, and Plk4, and in centriole elongation mediated by CPAP; specifically, CEP135 directly interacts with hSAS-6, CPAP and microtubules, and is necessary for the centriole localization of CPAP and STIL. From these results, the authors propose that the CEP135 protein connects the cartwheel and the centriolar microtubules.

This manuscript thus reports new findings on the CEP135 function in the centriole assembly, a process that has recently been shown to involve multiple proteins. A study of CEP135/Bld10 in the canonical centrioles of humans is timely, because diverse functions of Bld10 have been suggested in non-canonical centrioles of fly. The data reported in this paper are of high quality; however, I feel that the results need to be more adequately discussed in the context of previous studies. If the author could provide adequate discussions, I would recommend this paper for publication in the EMBO Journal.

In particular, I would like the authors to consider the following points.

1. The authors show that CEP135 directly bind to CPAP and hSAS-6 and suggest that these three proteins form a complex in vivo. On the other hand, the authors' group previously reported that STIL directly binds to CPAP and suggested that these proteins plus hSAS-6 form a complex (Tang et al., 2011). How are these two complexes with overlapping components related to each other? If the four proteins form a complex, where is STIL located in the schematic diagram (Fig. 9)?

2. It seems odd to me that the authors do not mention a Bld10 truncation experiment performed in Chlamydomonas (Hiraki et al., 2007), which showed that substantial portions of the N- and C-terminal regions of Bld10 are dispensable, while extensive truncations cause detachment of the cartwheel spoke from the triplet microtubules. The latter result led Hiraki et al. to suggest that Bld10 connects the cartwheel to the triplets. At least some mention of this suggestion must be made.

3. The authors nicely determined the minimal regions of CEP135 necessary for its interaction with hSAS-6, CPAP, and microtubules, as well as the minimal regions of hSAS-6 necessary for its interaction with CEP135. However, the authors do not mention these results in Discussion. The finding that hSAS-6 binds to CEP135 through its C-terminal region is in excellent agreement with the previous observation that the C-terminus of SAS-6 is located at the distal part of the cartwheel spoke (van Breugel et al., 2011) where the Bld10 is also localized (Hiraki et al., 2007). This also should be mentioned.

Minor points

1. The sites of interaction between hSAS-6 and CEP135 are not precisely indicated in Fig. 2A. The hSAS-6 figure shows as if the entire coiled coil region of the protein would interact with CEP135. However, the minimal region the authors determined spans from the middle of the coiled coil domain to the middle of the C-terminal non-coiled coil domain. The figure should be corrected to incorporate the finding.

2. The authors use "polymerized microtubules" to mean microtubules. However, it is clear that "microtubule" is polymerized tubulin and there is no need to use "polymerized microtubules".

3. I would suggest that the title of Figure 4 in the legend "CEP135 maintains proper architecture of nine-fold symmetry of human centriole" be changed to something like "CEP135 maintains proper length of human centriole". This is because the absence of some triplets from the cross section images of the centriole shown in Fig. 4B implies that some triplets are short, not that the centriole's nine-fold symmetry is disorganized. Mention that these images are similar to the images of Bld10-

depleted basal bodies reported by Jerka-Dziadosz et al. (2011).

4. Page 12, Line 6
"SAS5" should be "SAS-5".

5. Page 10, Line 10; Page 16, Line 7; Fig. 9B, middle
"hSAS6" should be "hSAS-6".

Referee #2 (Remarks to the Author):

The manuscript "Human microcephaly protein CEP135 binds to hSAS-6 and CPAP, and is required for centriole assembly" by Lin et al addresses the role of CEP135 in centriole assembly.
This is a nice and important manuscript that deserves to be published in EMBO journal. However, I have some concerns that should be addressed prior publication.

1-A major claim of the manuscript is the interaction of CEP135 with SAS-6.
In figure 2 the authors should draw a diagram of the interaction regions between SAS6 and CEP135 summarizing their results as they do in Figure 8B for CPAP. If the region of interaction in SAS6 is 307-586, then it is not clear for me why 307-657 would interact so little. Moreover, if 146-536 and 307-586 interact with CEP135, why does 307-536 not interact? Is there a domain that inhibits the interaction? It is most confusing. Moreover the direct interaction is very weak- Figure 2F, as the band is much weaker than what exists in the input (diluted 1/20)- so I am a bit worried that this interaction is really very weak and this should be discussed.
The authors do point mutations in CEP135 in the SAS6 binding region to reduce its ability to form a coiled-coil. However, this reduction in the ability to form a coiled coil (coiled-coil index Figure 5A) does not correlate with the loss of ability to bind SAS6 (Sup Fig 2). Perhaps it would be better to eliminate the other mutants from the manuscript and just keep L588P (as a point mutation that abolishes SAS6 binding) and not make any statement regarding coiled-coil structure.
Another major claim is that SAS6 is not recruited by CEP135 (Figure S1); since it was shown in other species that bld10 might stabilize SAS6 at centrioles this is an important point. It could be that there is a permanent pool of each protein that does not depend on the other and a dynamic pool that does. The authors should investigate the recruitment of SAS6 using FRAP or perhaps tone a little bit down their conclusions.

2- A major claim of the manuscript is the interaction of CEP135/Bld10 with the microtubules
The authors should say that interaction between the N-terminus of CEP135 and tubulin has been already shown in Drosophila in Carvalho-Santos et al, 2012.
Is the overexpression of the N-terminus stabilizing the microtubules in Figure 3E? Does full length also bind the microtubules in cells? Does it stabilize the microtubules in cells or is this particular of this N-terminus domain?

3- A major claim of the manuscript is the interaction of CEP135/Bld10 with CPAP (Figure 8).
Again here I am a bit confused. There seem to be two regions that are essential (together) for binding- 50-190 and 415-460- perhaps this should be further discussed.

4-Are all these molecules -SAS6, CEP135 and CPAP- in a complex, if accessed by gel filtration? Do the authors have an idea if some of the interactions compete/synergise with each other?

5-Since bld10/CEP135 is supposed to be important for cartwheel assembly: is the cartwheel structure missing after CEP135 RNAi (Figure 4).

Minor comments:
-The authors should guide the readers with arrows in their blots.
-In the discussion the authors claim that CEP135 binds the A-tubule- I would just write microtubules in general as there is no evidence so far that it is the A.
-In their cartoons of the molecules it would be useful to map domains that are known or conserved regions that have been characterized.

Referee #3 (Remarks to the Author):

This ms is addressing the important question of how the SAS-6/Bld12-based cartwheel elements can intereract with microtubule blades growing in an orthogonal direction, to scaffold pro-centrioles
From a series of evidences accumulated in several model systems, the two obvious candidates for participating in this key step are CEP135/Bld10, a microcephaly protein in the human species, and CPAP/CENPJ, another human microcephaly protein, which has homology with SAS4, and has been first studied and further characterized previously by the same group.
Using several well controlled approaches including depletion experiments by siRNA, immunoprecipation or GST-pull down, of endogenous or tagged-versions of full length or partial protein constructs as well as of in vitro translated proteins, and confirming the results by yeast two-hybrid experiments, this work convincingly demonstrated and mapped a direct interaction between SAS-6 and the C-term of CEP135, a domain whose truncation has been associated with human primary microcephaly and is demonstrated in the present work to perturb centriole duplication while it does not preclude SAS6 recruitment to the pro-centriole.
It also demonstrates a direct interaction of the N-term of CEP135 with microtubules in vitro, wheras SAS-6 does not bind to MTs, supporting a model in which CEP135 acts as a linker between the cartwheel elements with the MT wall of the centriole. The abnormal centrioles observed upon depletion of CEP135, in agreement with results reported in other models like Paramecium or Chlamydomonas, also support the model.
That a CEP135 mutation that disrupted its interaction with SAS-6 inhibited centriole duplication, and that depletion of CEP135 not only led to the development of abnormal centriole structures, but also blocked PLK4 and STIL mediated centriole amplification, are also consistent with the model.

Altogether, this work is solid and timely and will be of interest for the field. I would recommand publication in the EMBO J after the few concerns listed below are addressed.

Main concerns :
- How does centriole elongation is taking place with respect to the formation of the doublet/triplets of MTs shown of the parental centriole on fig 9A is not discussed. CPAP over-expression has been shown by several groups including this group to promote abnormal elongation of the centriole wall microtubules rather than the controlled elongation of the centriole organelle as a whole. In their introduction, the authors state that centrioles 'start to elongate and increase their length' during S and G2 phase (a reference would be useful at this point). There are indeed some evidence in the litterature indicating that specific proteins are required for the elongation phase, and not for the nucleating phase (Azimzadeh et al., J Cell Biol 2009),. Could this important issue be addressed ?

- It would be very useful to have a immunoelectron localization of CPAP in the present work, as the precise localization of CPAP is not clearly known. If we trust the localizations reported by Kleylein-Sohn et al. (Dev Cell 2007, fig 6), although it is sometimes difficult to distinghish the proximal end of the parental centriole from the PLK4-induced pro-centrioles, there is clearly a different localization for CPAP, apparently more proximal, and for CEP135, distributed all along the centriole wall. This has two implications : first, CPAP could be limited to the proximal end, a possibility which could fit with the interaction of CPAP with the gamma-tubulin complex reported previously by the same group, as well as with its interaction with CEP152 reported by Cizmecioglu et al (J Cell Biol 2010) ; second, CEP135 function cannot be limited to connecting the central hub protein SAS6 to the outer microtubules. This is also suggested by the EM figure presented in fig4B after siCEP135, which shows a defect at the distal end of outer centriolar MTs, calling for other partners to be identified and localized in the more distal part of the centriole walls. This could also agree with the mild phenotype of CEP135 depletion observed in Drosophila in which centrioles are short and apparently lack the distal part as well as many otherwise conserved centriole proteins. Although this is not the main focus of this ms, it should be addressed in the discussion to help the reader to have a more comprehensive overview on the role of CEP135.

- Fig 9A : the pro-centriole lumen is cartooned with a significantly smaller diameter than that of the parental centriole. Is there any cryptic reason for that, or is it an error ? Similarly, the walls of the pro-centriole bud are shown as triplets. Is it that clear that the C-tubule is set in place before centriole elongation is taking place during S/G2 phase ?

- In fig 9B , CPAP is shown lying along the seam of A-tubule ? Is this on purpose, suggesting a

specific interaction of CPAP with the microtubule lattice ? The protein is also given a specific and rather complex shape. Is this on purpose? Where would be the PN2-3 domain ?

Minor point :
- The litterature survey could be better treated. Most of the references are all very recent as if the centrosome field would be an emergent field. One example : the Introduction section starts with four references between 2010 and 2012 to indicate that ´ centriole duplication involves the growth of a procentriole next to the proximal end of the parental centriole ª !

1st Revision - authors' response 29 January 2013

We appreciate the reviewers' thoughtful reviews and constructive comments. Our revised manuscript is attached, and our point-by-point responses to the reviewers' comments are given below. The corrected portions are underlined below and in the revised manuscript.

Referee #1
1) We report here that CEP135 directly interacts with CPAP and hSAS-6. Our previous study showed that STIL directly binds to CPAP and indirectly forms a complex with hSAS-6 (EMBO J. 30, 4790-4804, 2011). How are these two complexes with overlapping components related to each other? There are two possibilities. First, at least two CPAP-containing complexes are present in the cytosol, each of which may function at different hierarchical levels during procentriole formation. Another possibility is that at least four proteins (i.e., STIL, CPAP, CEP135, and hSAS-6 plus other potential partners) form a complex within the cells. These two possibilities may not be mutually exclusive. Currently, we do not yet know which alternative holds true, nor do we understand how the STIL-CPAP complex participates in procentriole formation. Future biochemical and functional characterizations of these complexes may resolve this puzzle. We now include this in the discussion section of the revised manuscript (page 17).

2) As the reviewer suggested, we now include Hiraki's findings in the Introduction section of the revised manuscript (page 4), as follows:

The studies from Bld10 truncation experiments and immunoelectron microscopy in *Chlamydomonas* revealed that the middle region of Bld10 is essential for centriole formation and large deletions of either the N-terminus or the C-terminus that interfere with this region cause detachment of the cartwheel spoke from the triplet microtubules, suggesting that Bld10 may connect the cartwheel to the triplets (Hiraki et al., 2007).

3) As suggested by the reviewer, we now mention the interaction between hSAS-6 and CEP135 and discussed its possible role in the Discussion section of the revised manuscript (page 16), as follows:

One possible scenario is that CEP135 directly binds to a region close to the C-terminus of hSAS-6, and this interaction is essential for cartwheel stabilization and procentriole formation. This notion is supported by previous reports showing that the C-terminus of *Chlamydomonas* SAS-6 is located at the distal region of the centriolar cartwheel spoke (van Breugel et al., 2011), where the Bld10 is also localized (Hiraki et al., 2007).

Minor points:
1) We have corrected Figure 2A as suggested by the reviewer.

2). We have replaced "polymerized microtubules" with "microtubules" as suggested by the reviewer.

3). We have revised the title of Figure 4 (page 25) and now mention the findings of Jerka-Dziadosz et al. (2010) and Mottier-Pavie and Megraw (2009) in the Discussion section of the revised manuscript (page 18), as follows:

Page 25:

Figure 4. CEP135 is required to build a correct centriole structure and maintain the proper length of the human centriole.

Page 18:
In addition to showing that CEP135 serves to link the central hub protein, hSAS-6, to the outer microtubules, our current study demonstrated that CEP135 depletion produced shorter centrioles (Fig. 4C). Similar results were also observed in siBld10-treated *Paramecium* (Jerka-Dziadosz et al, 2010) and in *Drosophila bld10/*CEP135 mutants (Mottier-Pavie and Megraw, 2009), implying that CEP135 may have a second function: that of maintaining proper centriole length in human cells.

4 and 5) In the revised manuscript, we have corrected SAS5 to SAS-5 (page 12) and hSAS6 to hSAS-6 (page 10, page 18).

Referee #2
1a) We have diagrammed the regions through which hSAS-6 and CEP135 interact, and now summarize the results in Figure 2D. To resolve the reviewer's questions regarding whether a direct interaction (strong *vs.* weak) is present between CEP135 and hSAS-6, we performed a yeast two-hybrid liquid assay (Supplementary Figure S1A) that allowed us to quantify the interaction strengths between various truncated fragments of hSAS-6 (pray) and CEP135 (416-1140) (bait). Consistent with our co-IP experiments, the yeast two-hybrid assay (Supplementary Fig. S1A) showed a strong interaction between CEP135 and hSAS-6 (amino acids 307-586 and 307-657). Unexpectedly, we also observed relatively high beta-galactosidase activity in the hSAS-6 (307-536)-CEP135 (416-1140) interacting pair, suggesting a direct interaction between these two truncated proteins (Supplementary Fig. S1A). A careful examination of our original co-IP blot (Fig. 2D) revealed that a single broad band had been detected from the co-IP complex by ectopically expressed hSAS-6-myc (307-536). Because, hSAS-6-(307-536) is similar in size to a non-specific band (marked as "#" in Figure 2D) detected by immunoblotting using the anti-myc antibody. This led us to initially conclude that there was no interaction between hSAS-6 (307-536) and CEP135 in the original manuscript. However, we repeated the co-IP experiments using a different batch of secondary antibody and found that the anti-CEP135 antibody could co-precipitate hSAS-6-myc (307-536) with CEP135 (Supplementary Fig. S1B). We thus conclude that the C-terminus of CEP135 (residues 416-1140) directly interacts with hSAS-6 (residues 307-536).

We have added the following paragraph to the revised manuscript (page 7).

Unexpectedly, our fine interaction mapping using a yeast two-hybrid assay further narrowed down the CEP135-interacting region to residues 307-536 in hSAS-6 (Supplementary Fig. S1A). Further co-IP experiments demonstrated that the anti-CEP135 antibody could co-precipitate hSAS-6-myc (307-536) with CEP135 (Supplementary Fig. S1B). We did not initially observe a distinguishable co-IP band in Figure 2D (307-536), because hSAS-6-myc (307-536) is similar in size to a non-specific band (marked by a "#" in the figure) detected by the anti-myc antibody. We thus conclude that residues 307-536 comprise the minimum region through which hSAS-6 interacts with CEP135.

1b) As the reviewer suggested, we have deleted the results obtained from the other two mutants from the revised manuscript.

1c) Indeed, our immunofluorescent analysis revealed a very weak decrease of the centriolar hSAS-6 signals in CEP135-depleted cells (Supplementary Fig. S2B in the revised manuscript). We have toned down our conclusions, and now state that CEP135 depletion appears to have little or no effect on the recruitment of hSAS-6 to the centriole (page 10).

2a) We now mention the finding that fly CEP135/Bld10 interacts with microtubules (Carvalho and Santos, 2012) in the revised manuscript (page 9), as follow:

Consistent with this finding, the N-terminus of *Drosophila* Bld10/CEP135 was previously reported to directly interact with MTs (Carvalho-Santos et al., 2012).

2b) We ectopically expressed myc-tagged CEP135 constructs (full-length CEP135 and CEP135-1-190) in U2OS cells and found that the overexpressed N-terminal CEP135 fragment (1-190; Fig. 3E-i), but not the full-length CEP135 (Fig. 3E-iii), bound to microtubules in cells (in the revised

manuscript). However, the binding of CEP135 (1-190) to microtubules was significantly disrupted in the presence of nocodazole (a microtubule-destabilizing drug), suggesting that nocodazole may destabilize the CEP135 (1-190)-bound microtubules (Fig. 3E-ii). Unexpectedly, overexpressed full-length human CEP135 formed aggregates in cells and appeared not to interact with cytoplasmic MTs (Fig. 3E-iii). The reason for this is currently not known. One possibility is that the N-terminal-most domain (residues 1-190) that binds to microtubules is folded and buried inside the intact CEP135 molecule, blocking its binding to MTs. Taken together, our results define the N-terminal 190-residues of CEP135 as directly binding to MTs. Future three-dimension structural analysis of intact CEP135 molecules may resolve this question. We have included these new results (Figure 3E) in the revised manuscript (pages 9-10).

3) Our co-IP experiments (Fig. 8C) suggested that two short regions (residues 50-190 and 415-460) in the N-terminal domain of CEP135 appear to be essential for binding with CPAP, as truncated CEP135 fragments lacking either region lost their ability to bind CPAP. Intriguingly, the most N-terminal domain (residues 1-190) of CEP135 also binds to MTs (Fig. 3). In the future, it will be interesting to determine how CEP135 interacts with CPAP to promote centriolar microtubule assembly. We have included this discussion in the revised manuscript (page 15).

4) Gel-filtration-based isolation of the endogenous hSAS-6-CEP135-CPAP complex would be time-consuming and tedious, and would require a huge amount of cells. Our co-IP experiment showed that all three molecules (hSAS-6, CEP135 and CPAP) are present together in a complex (Fig. 8B). This hSAS-6-CEP135-CPAP protein complex was also independently demonstrated by a GST pulldown assays using recombinant full-length GST-hSAS-6 or GST-CPAP (895-1338) (Supplementary Fig. S4A). We mapped the specific interacting domains in CEP135 and CPAP, and have provided these new results in the revised manuscript (Fig. 8D, Supplementary Fig. S4A, S4C, and S4D). Furthermore, it is interesting to note that CPAP can directly interact with STIL and also indirectly forms a complex with hSAS-6 (Tang et al., 2011). How are these two complexes (hSAS-6/CEP135/CPAP and hSAS-6/STIL/CPAP) with overlapping components related to each other? For an answer to this question, please see our response to the first point of Reviewer 1.

5) The cartwheel has been successfully visualized by conventional electron microscopy of resin-embedded samples in *Tetrahymena*, *Paramecium*, and *Chlamydomonas,* but not in human cells *(*P. Conczy*, Nat. Cell Biol. 2012*). To date, technical difficulties have barred us from examining the cartwheel structure in resin-embedded siCEP135-treated human cells.

Minor points:

1) As suggested by the reviewer, we have added arrows to some of the blots.
2) Figure 9 shows a hypothetical model. We believe that CEP135 interacts with the A-tubule based on the following observations: (1) Studies in *Chlamydomonas* and *Paramecilum* (reviewed by Azimzadeh and Marshall, 2010) and X-ray crystallographic analyses (Kitagawa et al., 2011; van Breugel et al., 2011) have yielded a structural model of the SAS-6/Bld12-based cartwheel in which the emanating spokes terminate in a pinhead structure that attaches the A-tubule of the microtubule triplet, (2) CEP135 directly binds to CPAP, and both interact with microtubules (this report), (3) Cryo-electron tomographic analysis revealed that the proximal end of the A-tubule is capped by a conical structure that resembles gamma-TuRC (Guichard et al., 2010) and CPAP was reported to be associated with the gamma-tubulin complex (Hung et al., 2000).
3) We now include a schematic summary of the established interactions of hSAS-6, CEP135, microtubules, and CPAP, and their corresponding structural and functional domains (Fig. 9C).

Referee #3
1a) We now discuss how a centriole elongates with respect to the formation of MT doublet/triplets in the discussion section of the revised manuscript (page 15), as follows:

In most vertebrate cells, each triplet contains an A-microtubule, which is assembled first during centriole assembly and is the only complete microtubule with 13 protofilaments. The B- and C-microtubules are incomplete microtubules that are assembled later. A recent study using cryo-electron microscopy showed that the proximal end of the A-microtubule in a nascent human procentriole is capped by a structure similar to the gamma-tubulin ring complex (Guichard et al., 2010), a known MT nucleator in animal cells. In contrast, the incomplete B- and C-microtubules are

never capped at their proximal ends, and their appear to undergo bidirectional growth along the A- or B-microtubules, respectively (Guichard et al., 2010).

1b) Centrioles start to elongate and increase their length during S and G2 phase. We now include an appropriate reference (Kuriyama and Borisy, 1981) (page 3).
1c) As the reviewer suggested, we now mention that several specific proteins, including hPOC5 (Azimzadeh et al., 2009) and Ofd1 (Singla et al., 2010), are known to be required for the elongation phase during centriole duplication (page 3), as follows:

Further studies revealed that CPAP is required for the initiation of procentriole assembly and its subsequent elongation (Kohlmaier et al, 2009; Schmidt et al, 2009; Tang et al., 2009), while hPOC5 (Azimzadeh et al., 2009) and Ofd1 (Singla et al., 2010) appear to be essential for building the distal ends of centrioles.

2a) Our antibody against CPAP is sensitive to glutaraldehyde fixation, which prevents us from analyzing the localization of CPAP by immuno-EM. A study by Kleylein-Sohn et al., (2007) showed that CPAP was concentrated within the proximal lumen of both parental centrioles and procentrioles, and recent studies from super-resolution imaging of centrosomes are consistent with this notion (Fu and Glover, 2012; Lawo et al., 2012).
2b) We thank the reviewer for this helpful suggestion. In addition to indicating that CEP135 links that bridges the central hub protein, hSAS-6, to the outer MTs, our current study also suggests that CEP135 may have a second function: that of maintaining the proper centriole length in human cells. We have included this in the discussion section of the revised manuscript (page 18), as follows:

In addition to suggesting that CEP135 links the central hub protein, hSAS-6, to the outer MTs, our study demonstrated that CEP135 depletion produced shorter centrioles (Fig. 4C). Similar results were also observed in siBld10-treated *Paramecium* (Jerka-Dziadosz et al, 2010) and in *Drosophila bld10/*CEP135 mutants (Mottier-Pavie and Megraw, 2009). Together these findings suggest that CEP135 may have a second function: that of maintaining proper centriole lengths in human cells.

3) We have corrected the errors in Figure 9A of the revised manuscript. The B- and C-tubules appear to start assembling at different positions along the A- and B-tubules, respectively, via a gamma-TuRC-independent mechanism. Thereafter, they grow bidirectionally to reach their final lengths (Azimzadeh and Marshall, 2010). It is difficult for us to show dynamic growth of B- and C-tubules in Figure 9A, which represents the overall growth pattern of a centriole.

4) Figure 9B is a hypothetical model. CPAP has been demonstrated to directly interact with tubulin-dimers via its PN2-3 domain (Hung et al., 2004), and with microtubules via its A5N domain (Hsu et al., 2008). This finding implies that CPAP may bind to the microtubule lattice. The molecular details of these interactions are not yet known. In the revised manuscript, we provide new evidence (Fig. 8D and Supplementary Fig. S4C, S4D) showing that the C-terminal domain of CPAP directly interacts with the CEP135 N-terminus. We also include a schematic summary of the established interactions of hSAS-6, CEP135, microtubules, and CPAP, and their corresponding structural and functional domains (Fig. 9C).

Minor points:
1). In the Introduction section of the revised manuscript (page 3), we have added a paragraph that describes the discovery of several key centrosomal proteins essential for centriole duplication, as follows:

Recent studies have identified several key centrosome-associated proteins (ZYG-1/PLK4, SAS-5/Ana2/STIL, SAS-6, and SAS-4/CPAP) that are essential for centriole duplication in worms (O'Connell et al., 2001; Kirkham et al., 2003; Leidel and Gonczy, 2003; Leidel et al., 2005; Delattre et al., 2006; Pelletier et al., 2006), flies (Bettencourt-Dias et al., 2005; Peel et al., 2007; Rodrigues-Martins et al., 2007), and mammals (Kleylein-Sohn et al., 2007).

We hope that the revised manuscript is acceptable and that the changes we have made constitute an appropriate response to the reviewers. Thank you for your kind consideration.

Sincerely yours,

Tang K. Tang, Ph. D.
Professor and Distinguished Research Fellow
Inst. of Biomedical Sciences, Academia Sinica
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Acceptance letter 18 February 2013

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by two of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Thank you again for your contribution to The EMBO Journal and congratulations on a successful publication. Please consider us again in the future for your most exciting work!

You shall shortly receive a formal letter of acceptance, containing information and instructions for further proceedings at this point.

Referee #1

(Remarks to the Author)
I found that the authors have satisfactorily answered all of my questions and comments, and adequately revised the manuscript. The authors seem to have also adequately answered the criticism that the other reviewers made. I thus recommend publication of this paper in the EMBO Journal.

Referee #2

(Remarks to the Author)
I have now read the revised version of "Human microcephaly protein CEP135 binds to hSAS-6 and CPAP, and is required for centriole assembly" and the author¥s answer to referees and consider the manuscript is ready for publication.

