Report

Control of Centriole Length by CPAP and CP110

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Summary

Centrioles function as the major components of centrosomes, which organize microtubule (MT) arrays in proliferating cells, and as basal bodies for primary cilia formation in quiescent cells. Centrioles and basal bodies are structurally similar, barrel-shaped organelles composed of MTs. In proliferating cells, two new centrioles, termed procentrioles, form during the S phase of the cell cycle in close proximity to the proximal ends of the two preexisting parental centrioles, often at a near-orthogonal angle [1]. Considerable progress has been made toward understanding the biogenesis of centrioles, but the mechanisms that determine their lengths remain unknown. Here we show that overexpression of the centriolar protein CPAP in human cells enhances the accumulation of centriolar tubulin, leading to centrioles of strikingly increased length. Consistent with earlier work [2], we also find that elongated MT structures can be induced by depletion of the distal end-capping protein CP110 from centrioles. Importantly, though, these structures differ from genuine primary cilia. We thus propose that CPAP and CP110 play antagonistic roles in determining the extent of tubulin addition during centriole elongation, thereby controlling the length of newly formed centrioles.

Results and Discussion

Recent work in protists, invertebrates, and vertebrates reveals an evolutionarily conserved pathway for the formation of centrioles and basal bodies [3–5]. For example, in the nematode *Caenorhabditis elegans*, several gene products critical for centriole biogenesis, notably the coiled-coil proteins SAS-5, SAS-6, and SAS-4, have been shown to assemble sequentially in response to the activation of a protein kinase, ZYG-1 [6–12]. Similarly, PLK4/SAK (a putative functional homolog of ZYG-1) has been identified as a key regulator of centriole duplication in both human cells and *Drosophila* [13, 14], and homologs of nematode SAS-4 and SAS-6 are essential for centriole biogenesis in all organisms examined [7, 11, 15–20]. Procentriole formation in human cells also requires CP110, Cep135, and γ-tubulin [17, 21, 22]. Procentrioles then elongate throughout S and G2 phase and reach a relatively constant length of approximately 0.4–0.5 μm in a typical human cell [1]. Here we report that two centriolar proteins, CPAP and CP110, contribute to regulate the length of centrioles in human cells. CPAP, the putative SAS-4 homolog [19, 20], is of considerable medical interest because homozygous mutations in the corresponding gene (*CENPJ*) cause primary recessive microcephaly [23, 24]. CPAP binds microtubules (MTs) [20, 25] and associates with both parental centrioles and nascent procentrioles [17], whereas its depletion compromises centrosome integrity and leads to the formation of multipolar spindles [26]. CP110 was originally identified as a Cdk2 substrate involved in centriole duplication [21]. It localizes specifically to the distal tips of both parental and nascent centrioles, suggesting a capping function during centriole biogenesis [17]. Interestingly, CP110 is removed specifically from the mature basal body during ciliogenesis, and its depletion from proliferating cells results in the formation of MT extensions reminiscent of ciliary axonemes [2] (see also Figure S5A available online). The latter observation has been interpreted to suggest that CP110 serves to suppress a default pathway of ciliogenesis [2, 27].

To study the role of CPAP in centriole assembly, myc-CPAP was transiently overexpressed in U2OS and hTERT-RPE1 cells or inducibly expressed in U2OS cells under control of a tetracycline-inducible promoter. To favor the visualization of centriolar MTs, which are stabilized by polyglutamylation and acetylation, the bulk of the cytoplasmic tubulin was extracted by combined cold and detergent treatment. Staining of cells with antibodies against α-tubulin and CP110 revealed that elevated levels of CPAP caused the formation of strikingly elongated centriolar structures (Figures 1A and 1C; see also Figure S1A). The length of these structures increased with time of induction and eventually surpassed 1 μm, or two to three times the normal length of centrioles (Figure 1E). Elongated structures carried myc-CPAP spreading over their lengths (Figure 1B), whereas C-Nap1 was present only at the proximal ends [28] and CP110 only at the distal ends [17], as predicted for genuine centrioles (Figure 1C). Western blotting demonstrated an increase in myc-CPAP levels over time of induction (Figure 1D). A rough estimate based on quantification of chemiluminescence suggests a 20- to 40-fold increase of CPAP over endogenous levels, whereas CP110 levels were unchanged. Immunofluorescence analysis showed that the bulk of exogenous CPAP was cytoplasmic (data not shown). Interestingly, a significant recruitment of α-tubulin to centrioles could already be detected at short induction times,

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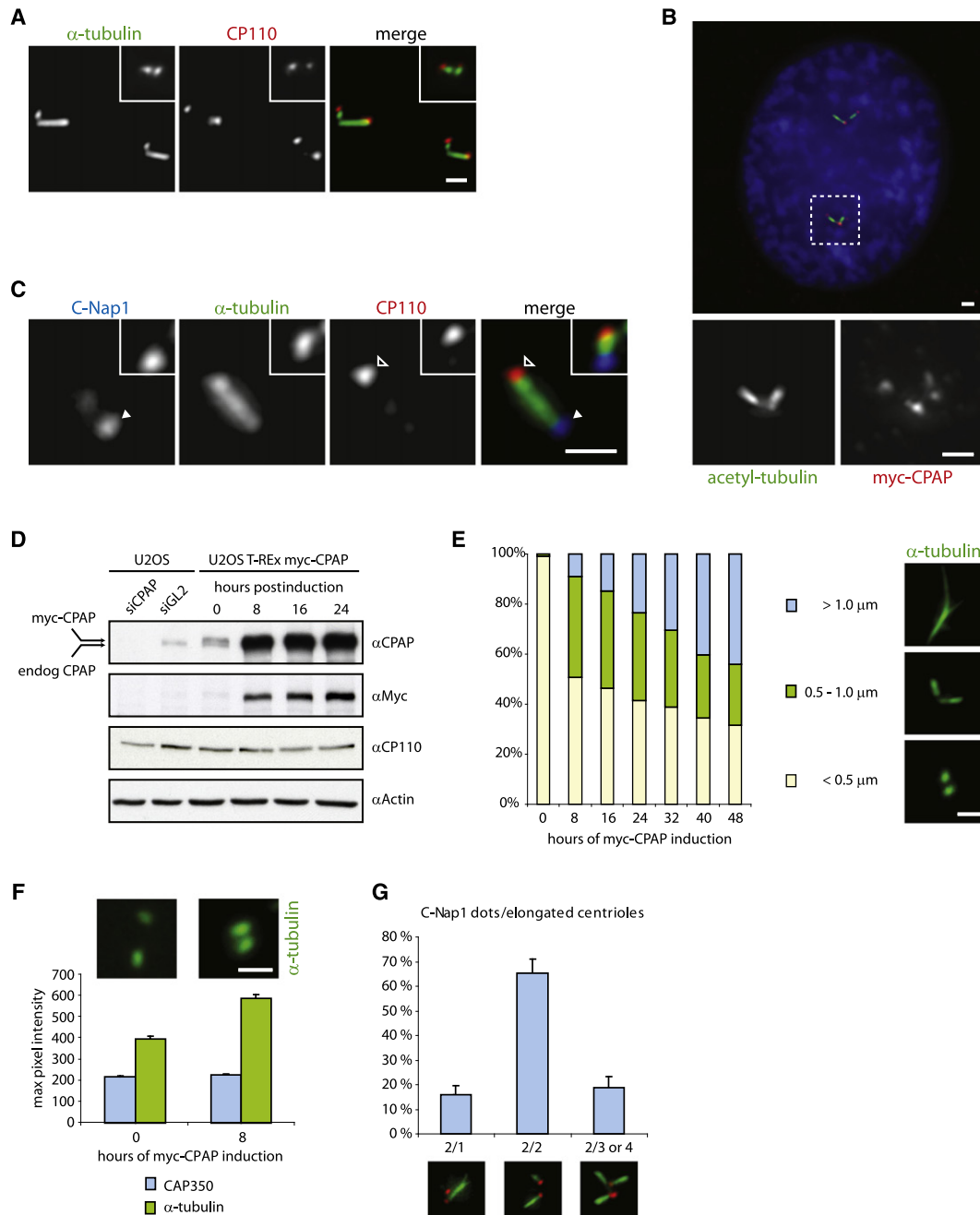
Figure 1. CPAP Overexpression Leads to Centriole Elongation

(A) Full length myc-CPAP was transiently expressed for 72 hr in asynchronously growing U2OS cells, and centrioles were stained with antibodies against α-tubulin (green) and CP110 (red). The insets show a pair of normal-size G2 phase centrioles for comparison.

(B) myc-CPAP expression was induced in a U2OS T-REx cell line, and the association of myc-CPAP with elongated centrioles was visualized in a prophase cell counterstained for acetylated tubulin and DNA. Lower panels show magnifications of the boxed area.

(C) Visualization of an elongated centriole after induction of myc-CPAP expression (as in B) by staining with antibodies against C-Nap1 (blue; filled arrowhead), CP110 (red; open arrowhead), and α-tubulin (green). Insets show a normal-size centriole for comparison.

(D) myc-CPAP was induced for 0–24 hr, and cell lysates were probed by western blotting with the indicated antibodies. Actin was monitored as a loading control. Lysates from U2OS cells treated for 48 hr with GL2 or CPAP siRNA were analyzed in parallel.

(E) CPAP was induced for 0–48 hr before cells were stained with anti-α-tubulin antibodies, and the lengths of centriolar extensions were measured. Centriolar structures were classified into three categories according to their length (<0.5 μm, 0.5–1.0 μm, and >1 μm) as illustrated by representative fluorescence images (right); results are shown in the histogram. Results shown are from three independent experiments (n = 50).

(F) Histogram showing maximal pixel intensity of α-tubulin- and CAP350 (control)-stained centrioles after induction of myc-CPAP for 0 or 8 hr. Insets show representative fluorescence images of α-tubulin staining. Scale bars represent 1 μm in (A), (B), (E), and (F) and 500 nm in (C).

(G) myc-CPAP expression was induced for 24 hr, and cells were stained with antibodies against α-tubulin and C-Nap1. The histogram shows the ratio between the number of C-Nap1 dots and the number of elongated centrioles present in each cell, and the fluorescence images underneath show representative examples of cells counted (C-Nap1 red, α-tubulin green). Results shown in (F) and (G) are from three independent experiments (n = 100); error bars indicate standard error of the mean (SEM).
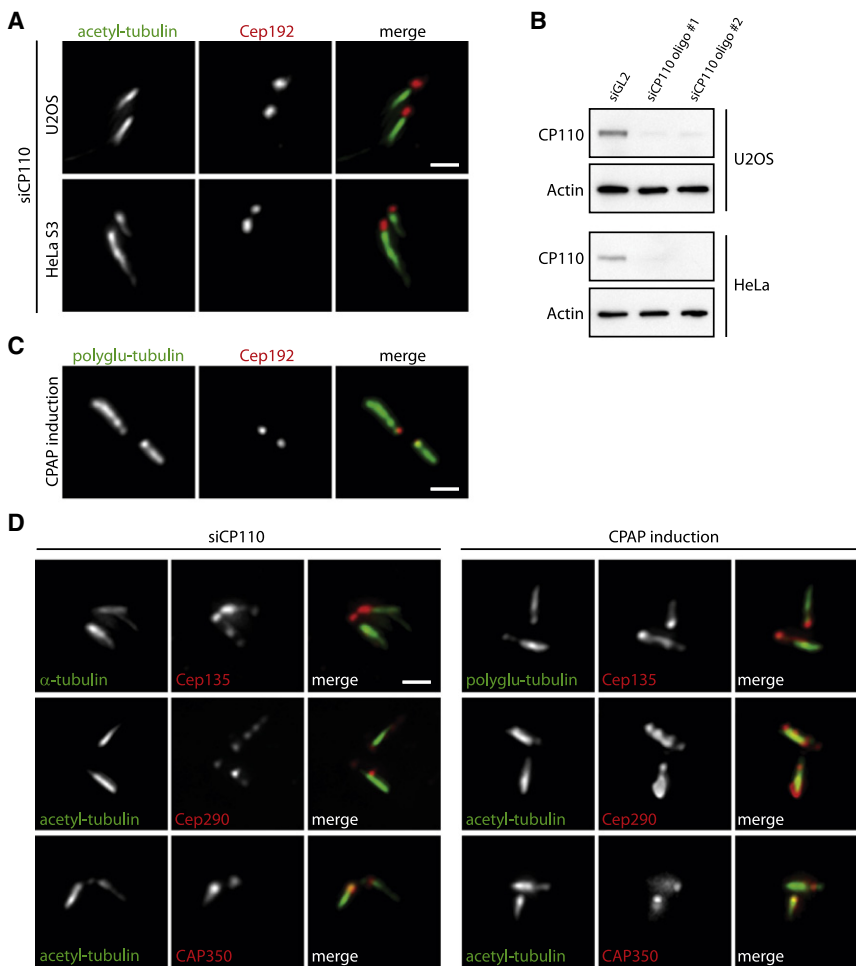
Figure 2. Comparison of Centriolar Extensions Generated by CP110 Depletion or CPAP Overexpression

(A) CP110 was depleted by siRNA treatment of U2OS and HeLa S3 cells for 72 hr before centrioles were stained with antibodies against acetylated tubulin (green) and Cep192 (red).

(B) Western blots showing CP110 levels in U2OS and HeLa S3 cells (compared to actin) after 48 hr of treatment with control GL2 or two different CP110-specific siRNA oligonucleotides.

(C and D) Following CPAP induction in U2OS T-REx cells (C, right panel of D) or CP110 depletion (left panel of D), elongated centrioles were stained with the indicated antibodies. All scale bars represent 1 μm.

the elongated centrioles produced by CPAP overexpression (Figure 2C). This prompted us to compare the two structures in more detail. Therefore, we determined the localizations of various centriolar proteins on the microtubular structures induced by either CP110 depletion or CPAP overexpression (Figure 2D). Elongated structures were visualized by costaining with GT335 antibody, which recognizes polyglutamylated tubulin, or by staining with antibodies against α-tubulin or acetylated tubulin. In contrast to Cep192 (Figures 2A and 2C) and Plk4 (data not shown), which were confined to the expected ends of all structures, the proteins CAP350, Cep135, and Cep290 additionally spread over the elongated structures, being particularly visible in the case of CPAP overexpression (Figure 2D). Interestingly, both types of microtubular extensions were stabilized by acetylation and polyglutamylation (Figures 2A, 2C, and 2D), consistent with their resistance to cold treatment and detergent extraction.

Having shown that both mature parental centrioles and procentrioles are competent to elongate in response to either CPAP overexpression or CP110 depletion, we asked whether the positions of subdistal or distal appendages were affected by centriole elongation. Cells overexpressing CPAP or depleted of CP110 were stained with antibodies against ninein and Cep164, markers of subdistal and distal appendages, respectively [29, 30]. As shown by immunofluorescence as well as immunoelectron microscopy, the distances between the proximal ends of centrioles and appendages were unchanged when comparing elongated centrioles with control centrioles (Figures 3A–3C). Considering that CP110 associates early with nascent procentrioles and then stays associated with the distal tips of elongating centrioles [17], these results suggest that under conditions of CPAP-induced elongation, tubulin insertion into the growing centriolar cylinder occurs within a relatively narrow region located between appendages and a CP110 cap. Overall, many of the elongated centriolar structures formed in response to CPAP overexpression appeared to represent compact cylinders (Figures 1A and 1C; see also Figure 4B). The longest structures, however, frequently exhibited splayed MTs, whose distal ends were invariably decorated by CP110 (Figures 3D and 3E). This

before any obvious centriole elongation became apparent (Figure 1F). CPAP overexpression did not cause a detectable increase in centriole numbers, nor did it significantly affect cell-cycle distribution or spindle bipolarity (Figures S2A–S2C).

To determine whether parental centrioles, procentrioles, or both are competent to elongate in response to excess CPAP, we counted the number of elongated centrioles relative to the number of C-Nap1 dots per cell. After a 24 hr induction of CPAP expression in asynchronously growing cells, most cells showed a 2:2 ratio between C-Nap1 dots and elongated centrioles, but about 15%–20% of cells showed a 2:3 or 2:4 ratio (Figure 1G). Because only parental centrioles stain positively for C-Nap1 [28], this latter population must represent G2 cells in which parental centrioles as well as new procentrioles are elongated, demonstrating that both mature centrioles and procentrioles are elongation competent. In further support of this conclusion, the induction of CPAP expression in cells transfected with Plk4 resulted in the formation of flower-like structures in which the parental centriole as well as several of the newly formed (engaged) procentrioles were clearly elongated (Figure S3).

Recently, it has been reported that depletion of the centriolar protein CP110 promotes the formation of primary cilia in proliferating U2OS cells [2]. We had independently observed that depletion of CP110 causes microtubular extensions from the distal ends of centrioles in both U2OS and HeLa S3 cells (Figures 2A and 2B), but rather than interpreting these structures as primary cilia, we were intrigued by their similarity to
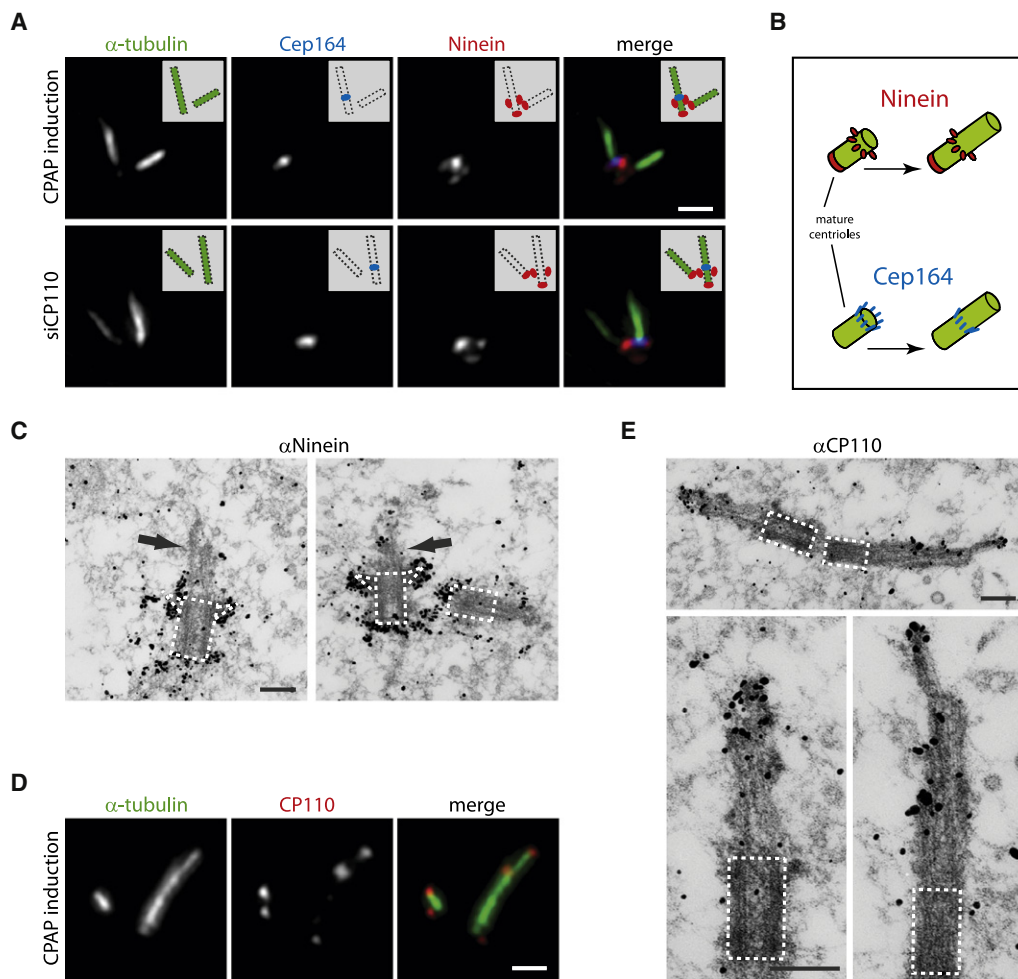
Figure 3. Appendage Positioning and CP110 Decoration on Elongated Centrioles

(A) Elongation does not affect positioning of distal and subdistal appendages. After induction of myc-CPAP expression or CP110 depletion, pairs of elongated parent and progeny centrioles were stained with antibodies against α-tubulin (green), Cep164 (blue), and ninein (red). Insets show corresponding drawings to facilitate data interpretation.

(B) Schematic illustrating the unchanged position of distal and subdistal appendages on elongated mature centrioles.

(C) Pre-embedding immunoelectron microscopy performed after 24 hr of CPAP induction. Subdistal appendages were visualized with anti-ninein antibodies, followed by Nanogold-labeled secondary antibodies. Dashed white lines mark the normal sizes of centrioles and the positions of subdistal appendages on mature centrioles, and arrows point to extensions.

(D) CP110 decorates the distal ends of elongated centriolar microtubules (MTs). Staining of centrioles after CPAP overexpression with anti-α-tubulin and anti-CP110 antibodies shows two parental centrioles of differing length and a newly growing procentriole at each of their proximal ends (hence presumably representing an S phase cell).

(E) Pre-embedding immunoelectron microscopy visualizes CP110 at two disengaged centrioles after CPAP induction for 24 hr. The bottom images show 2-fold magnifications of the two centrioles. Dashed white lines mark the normal sizes of centrioles. Scale bars represent 1 μm in (A) and (D) and 250 nm in (C) and (E).

suggests that although CPAP overexpression does not always result in a homogenous extension of centriolar walls, each of the MT extensions is recognized by the distal end-capping protein CP110.

To compare the structures induced by CPAP overexpression or CP110 depletion to bona fide primary cilia, we searched for proteins that would associate differentially with the different structures (Figure 4A). We found that centrin-3 readily decorated the extended structures formed in U2OS cells by either CPAP overexpression or CP110 depletion, but the same protein was confined to the basal bodies when primary cilia formation was induced by serum starvation of hTERT-RPE1 cells (left columns in Figure 4A). Conversely, the intraflagellar transport protein Polaris/IFT88 [31] was detectable on genuine cilia but

not on the microtubular extensions seen in myc-CPAP-overexpressing cells or cells depleted of CP110 (central columns in Figure 4A). Finally, CP110 was conspicuously absent from the basal body underlying the single primary cilium in serum-starved hTERT-RPE1 cells (right columns in Figure 4A; see also Figure S5A), consistent with previous results [2]. In contrast, it decorated the distal tips of the two elongated centrioles that were frequently seen in cells overexpressing CPAP (right columns in Figure 4A). Similarly, Cep97, the interaction partner of CP110 [2], was removed selectively from the ciliated basal body but persisted on both centrioles upon CPAP-induced centriole elongation (Figures S5B and S5C).

The three microtubular structures were also compared by transmission electron microscopy. The structures seen after
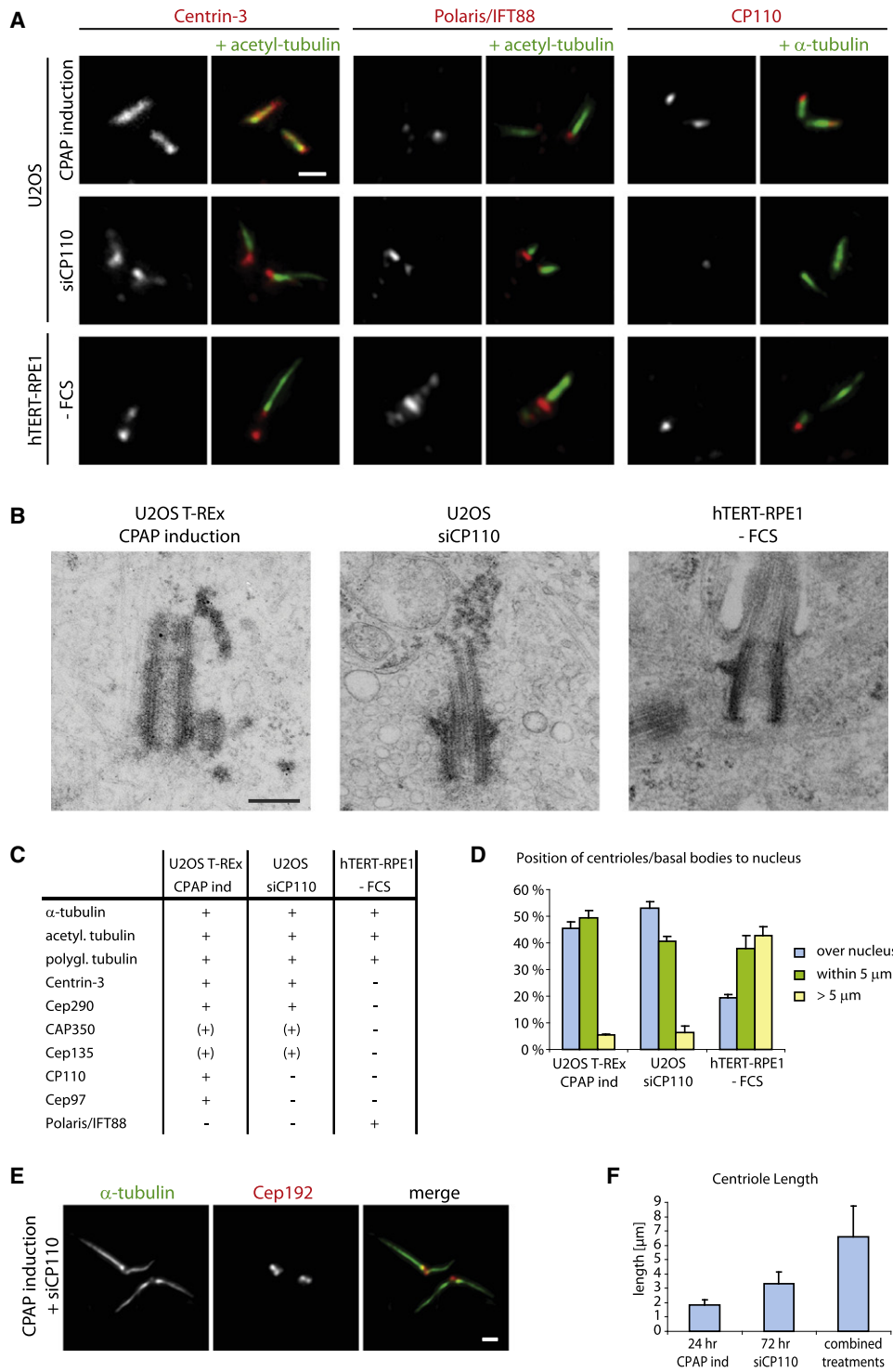
Figure 4. Structures Generated by CPAP Overexpression or CP110 Depletion versus Primary Cilia

(A) Via immunofluorescence staining with the indicated antibodies, the centriolar extensions produced in U2OS cells by either CPAP overexpression (upper row) or CP110 depletion (center row) were compared with primary cilia formed in quiescent hTERT-RPE1 cells (bottom row).

(B) Centriolar extensions produced in U2OS cells by CPAP overexpression (left) or CP110 depletion (middle) were compared with primary cilia formed in quiescent hTERT-RPE1 cells (right) by transmission electron microscopy.

(C) Table comparing the localization of various centriolar and ciliary markers on centriolar structures produced in U2OS cells by CPAP overexpression or CP110 depletion and on primary cilia in hTERT-RPE1 cells [+, protein localizes to extended MT structures; (+), positive localization detectable on some but not all structures; −, protein not found on extended structures].

(D) Histogram comparing the distance between centrioles/basal bodies and the nucleus after overexpression of CPAP in U2OS cells, CP110 depletion in U2OS cells, or induction of ciliogenesis in hTERT-RPE1 cells. Results shown are from three independent experiments (n = 100); error bars indicate SEM.

overexpression of CPAP often resembled genuine centrioles of extended length (Figure 4B; see also Figure S4A), but centrioles showing partial extensions of their cylindrical wall could also be seen. Similar partially extended microtubular structures were commonly seen in response to CP110 depletion, but these often protruded distally from a centriole of normal length (Figure 4B; Figure S4B). In contrast, primary cilia were characterized by the presence of membranous sheaths surrounding the axonemal MTs and a clear structural transition between the basal body and the cilium (Figure 4B). Thus, the structures induced by overexpression of CPAP or depletion of CP110 resemble each other, but both can be readily distinguished from genuine primary cilia (as summarized in Figure 4C), implying that the removal of CP110 from basal bodies is most likely required but not sufficient for ciliogenesis. In further support of this conclusion, we note that elongated centrioles and microtubular structures produced by CPAP overexpression or CP110 depletion were generally located in close proximity to the nucleus, whereas most of the basal bodies giving rise to primary cilia in quiescent cells had migrated to the plasma membrane (Figure 4D).

To further address the relationship between CPAP and CP110, we first asked whether depletion of CP110 would synergize with CPAP overexpression. Although combined treatment resulted in significant cell death (data not shown), surviving cells exhibited exceptionally long MT structures emanating from centrioles (Figures 4E and 4F). Conversely, overexpression of CP110 together with induction of CPAP suppressed CPAP-induced centriole elongation (Figure S6A). Thus, we conclude that CPAP and CP110 exert opposite effects on centriole length (Figure S6B). Furthermore, elegant data by Dynlacht and coworkers show that the removal of CP110 from the distal tip of the mature centriole is required for the formation of a primary cilium [2, 32], implying that CP110 also acts as a suppressor of ciliogenesis (Figure S6B).

In conclusion, our data have implications for two important areas. First, they address the question of how the length of centrioles is controlled during centriole biogenesis. We have shown that CPAP promotes the extension of the centriolar cylinder, presumably via its ability to recruit tubulin to the nascent structure [8, 25], echoing the function of SAS-4 in *C. elegans* [8, 11]. Similar conclusions have been reached independently by Gönczy and coworkers in this issue of *Current Biology* [33] and by Tang and coworkers [34]. In the future, it will be interesting to examine how CPAP functionally interacts with POC1, a WD40 domain protein recently implicated in centriole length control [35]. We have further shown that CP110 acts as a capping protein to limit centriole extension. How the activities of these two proteins are equilibrated so that each centriole reaches a defined length requires further study. Second, our results bear on the question of whether ciliogenesis represents a default pathway. Our data are consistent with previous data indicating that removal of CP110 from the distal tip of the basal body is necessary for the formation of a primary cilium [2, 32], but at least for U2OS cells, they lend no support for the idea that removal of CP110 is sufficient to trigger ciliogenesis [2, 27]. We conclude that CPAP and CP110 are both needed for the formation of cylindrical centrioles of defined length. We propose that CPAP functions as a scaffold for tubulin addition, whereas CP110 acts as a distal end-capping protein, so that the two proteins play opposite roles in the control of centriole length.

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(E) CP110 depletion and CPAP overexpression synergize to produce extraordinarily long MT extensions. U2OS cells were depleted of CP110 for 24 hr before CPAP was induced for 24 hr, and centriolar structures were stained with antibodies against α-tubulin (green) and Cep192 (red). Scale bars represent 1 μm in (A) and (E) and 250 nm in (B).
(F) Histogram illustrating the maximal length of centriolar MTs observed in U2OS cells after CPAP induction for 24 hr, CP110 depletion for 72 hr, and combined treatment (48 hr CP110 siRNA followed by 24 hr CPAP induction). Error bars indicate ± standard deviation (n = 25).

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