Molecular basis for CPAP-tubulin interaction in controlling centriolar and ciliary length

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Centrioles and cilia are microtubule-based structures, whose precise formation requires controlled cytoplasmic tubulin incorporation. How cytoplasmic tubulin is recognized for centriolar/ciliary-microtubule construction remains poorly understood. Centrosomal-P4.1-associated-protein (CPAP) binds tubulin via its PN2-3 domain. Here, we show that a C-terminal loop-helix in PN2-3 targets β-tubulin at the microtubule outer surface, while an N-terminal helical motif caps microtubule’s α-β surface of β-tubulin. Through this, PN2-3 forms a high-affinity complex with GTP-tubulin, crucial for defining numbers and lengths of centriolar/ciliary-microtubules. Surprisingly, two distinct mutations in PN2-3 exhibit opposite effects on centriolar/ciliary-microtubule lengths. CPAPF375A, with strongly reduced tubulin interaction, causes shorter centrioles and cilia exhibiting doublet- instead of triplet-microtubules. CPAPEE343RR that unmasks the β-tubulin polymerization surface displays slightly reduced tubulin-binding affinity inducing over-elongation of newly forming centriolar/ciliary-microtubules by enhanced dynamic release of its bound tubulin. Thus CPAP regulates delivery of its bound-tubulin to define the size of microtubule-based cellular structures using a ‘clutch-like’ mechanism.
Centrioles are microtubule-based eukaryotic structures that build centrosomes and cilia, which are required for accurate cell division and cellular signaling.\(^1\) Centrioles have highly conserved architecture displaying defined numbers and lengths of microtubules. Tubulin heterodimers (hereafter tubulin) are the building blocks of centriolar- and ciliary-microtubules. During ciliogenesis, intraflagellar transport (IFT) machineries mediate the transport of ciliary building blocks of tubulin from the cytoplasmic ciliary base to the tip.\(^2\) However, it remains poorly understood how a fraction of tubulin is selected from its large cytoplasmic pool and the mechanisms that operate to deliver these specialized tubulin to construct defined lengths of centriolar- and ciliary-microtubules. Since ciliogenesis from a centriole that resides within a centrosome, it is conceivable that a centrosomal protein that can directly interact with cytoplasmic tubulin could play a role in selective regulation of tubulin incorporation during centriolar- and ciliary-microtubule construction. Among the centrosomal proteins, CPAP and its ortholog Sas-4 plays roles in centriolar- and ciliary-microtubule elongation.\(^9\)–\(^14\) Data have shown that these functions require CPAP-tubulin interaction. Specifically, a CPAP/Sas-4 mutant that does not bind tubulin caused shortening of centrioles and primary cilium.\(^9\)–\(^11\) These data suggest the possibility that CPAP could play a role in delivering its bound tubulin to the site of centriole assembly and/or building centriolar-microtubules.

Free tubulin is ubiquitously present in high amounts in cells in contrast to CPAP, which has lower expression levels.\(^9\)\(^\text{2}\)\(^,\text{15,16}\) It is known that CPAP binds its conserved PN2-3 domain (amino acids 319–394) sequesters free tubulin into a non-polymerizable 1:1 complex.\(^17,18\) Thus at equilibrium, the amount of CPAP bound cytoplasmic tubulin that is unavailable for polymerization would only be a small fraction of the free tubulin. This suggests that the small fraction of CPAP-bound tubulin is required in certain CPAP-specific processes that are regulated in a spatiotemporal manner such as centriolar- and ciliary-microtubule size control. While recent studies have substantially improved our understanding on the significance of CPAP-tubulin interaction in centriole biogenesis and pericentriolar material recruitment, its significance in centriolar- and ciliary-microtubule construction remains unclear.\(^9\)\(^\text{11}\)–\(^\text{14}\) Thus, understanding the molecular basis of PN2-3-tubulin interaction is crucial in dissecting how cytoplasmic tubulin is sequestered by CPAP for the controlled delivery to define centriolar- and ciliary-microtubule lengths in cells.

In this study, we therefore investigated the structural basis of CPAP-tubulin interaction and identified that CPAP via its conserved PN2-3 domain forms a high affinity complex with GTP-tubulin and prevents it from polymerization. Our functional studies in human cells and flies then identified that, through the different facets of its PN2-3 domain, CPAP defines centriolar- and ciliary-microtubule lengths, firstly, through the sufficient binding of cytoplasmic tubulin and secondly by regulated release of its bound tubulin to define centriolar- and ciliary-microtubule lengths.

**Results**

**Molecular basis for PN2-3-tubulin interaction.** The PN2-3 domain, at the N-terminus of CPAP, is conserved from invertebrates to vertebrates (Fig. 1a). Using isothermal titration calorimetry (ITC), we determined a 25.6 nM binding affinity between PN2-3 and tubulin (Fig. 1b), and found PN2-3C (PN2-3’s C-terminus, aa 372–394) is sufficient to bind tubulin (Fig. 1c). By adopting a DARPin strategy, in which DARPin molecule didn’t affect binding of PN2-3C to tubulin, we solved the 2.1 Å crystal structure of bovine tubulin bound to PN2-3C (ref. 19) (Fig. 1d, Table 1 and Supplementary Fig. 1). In this structure, a loop-helix motif spanning residues 372–386 of PN2-3C targets an acidic microtubule outer surface on β-tubulin, although the last eight C-terminal residues (aa 387–394) were not traceable in the density map (Fig. 1e). A GTP molecule is identified in the β-tubulin nucleotide-binding pocket despite tubulin exhibiting a curved conformation (Fig. 1e and Supplementary Fig. 2). Recognition specificity arises from a number of water-mediated and direct interchain hydrogen bonds, and hydrophobic contacts between β-tubulin and PN2-3C (Fig. 2a). Key interacting residues within PN2-3C were then confirmed by structure-guided mutagenesis and ITC (Fig. 2b). Notably, F375A caused a drastic 109-fold drop in affinity from 25.6 nM to 2.8 μM, indicating its significance in forming a high-affinity CPAP-tubulin complex. We observed a perfect shape complementarity between the crouched PN2-3C loop-helix and its binding groove along the vertical axis for tubulin polymerization (Fig. 2c).

The N-terminal helical region of PN2-3 (PN2-3N, aa 323–361) alone has weak tubulin binding affinity (K_D = 68.0 μM) (Fig. 1c). However, together with PN2-3C, it contributes a ~14-fold increased binding affinity (K_D = 25.6 nM) (Fig. 1b). As the PN2-3C-tubulin co-crystal structure could not be resolved, we used cross-linking mass spectrometry (CL-MS) to identify PN2-3C contacts with tubulin (Fig. 2c and Supplementary Fig. 3). CL-MS analysis indicates that PN2-3C wraps around the microtubule α-β-tubulin interface and makes contacts at the β-tubulin’s microtubule-lumen surface, as a majority of cross-linking peaks were identified between PN2-3-N-terminal amine and lysine 372 in proximity to the β-tubulin M-loop (Fig. 2d). Such a binding mode is further supported by competition nuclear magnetic resonance spectroscopy (NMR) titration, where the weakly binding PN2-3N region can be displaced by the high affinity ligand vinblastine, which is known to bind to the microtubule α-β-tubulin interface, but not by colchicine, which binds to the β-α interface within a tubulin dimer (Fig. 2e and Supplementary Fig. 4). Arginine substitutions of the conserved PN2-3C’s E343 and E344 (hereafter EE343RR) caused a 8-fold reduced interaction (K_D = 194.2 nM) (Fig. 2b).

We then compared the NMR spectra of wild type and mutant (PN2-3F375A and PN2-3EE343RR) PN2-3 fragments, which exhibited numerous chemical shift differences between PN2-3WT and PN2-3EE343RR. This suggests a potential disruption of the helix, which extends from residues F338-E368 (Supplementary Fig. 4c). On the other hand, we noticed similar spectra of PN2-3WT and the PN2-3F375A, which is further confirmed by the comparison of PN2-3F375A and PN2-3EE343RR spectra. Upon addition of unlabeled tubulin at equimolar ratio to 11N-labeled PN2-3WT, PN2-3F375A and PN2-3EE343RR mutants, we observed line broadening/disappearance of the peaks, which is suggestive of binding. Exchange broadening can become obvious in low affinity (μM K_D) binding events. Accordingly, we observed that line broadening increases from PN2-3WT to PN2-3EE343RR and is the highest with the PN2-3F375A mutant, as the affinity for tubulin decreases in the same order agreeing with our ITC titrations (Figs 1b and 2b).

Together, our structural and mutagenesis data indicate that PN2-3 wraps around β-tubulin like a necklace with its PN2-3N covering the microtubule α-β and lumen surfaces of β-tubulin and its PN2-3C occluding the β-tubulin microtubule outer surface (Fig. 2c,d and Supplementary Fig. 3d). This unique binding mode suggests that PN2-3 could prevent the polymerization of its bound tubulin (Supplementary Fig. 3e).

PN2-3 prevents its bound tubulin from polymerization. To test if PN2-3 could bind tubulin and prevent the bound tubulin from polymerization, we performed microtubule-pelleting assays using...
free tubulin and PN2-3 variants (Supplementary Fig. 5a). Tubulin at a high concentration self-polymerizes into microtubules. While polymerized tubulin could be pelleted by centrifugation, free tubulin remains in solution in the supernatant. When we included PN2-3 variants in this assay, we found that in contrast to PN2-3 WT, PN2-3F375A did not prevent tubulin polymerization (Supplementary Fig. 5b). This could be due to the presence of F375, which is required for the formation of high affinity CPAP-tubulin complex (Fig. 2b). In addition, F375-containing PN2-3C domain binds the β-tubulin at the microtubule outer surface, which might allosterically disrupt microtubule formation by inducing or stabilizing a curved tubulin dimer (Supplementary Fig. 2). Supporting this notion, PN2-3C alone was sufficient to prevent polymerization of in vitro, the ability of PN2-3 to bind tubulin and prevent it from polymerization is F375-dependent (Supplementary Fig. 6b).

Figure 1 | Molecular basis for PN2-3-tubulin interaction. (a) Domain architecture of CPAP and sequence conservation analysis of PN2-3N and PN2-3C. Arrowheads mark E343-E344 (purple) and F375 (blue). (b) ITC titration and fitting curve of wild type (WT) PN2-3 with tubulin. (c) Overlay of ITC fitting curves of PN2-3N (green) and PN2-3C (magenta) titrated into tubulin. (d) Ribbon view of PN2-3C-GTP-tubulin-DARPin complex. Free tubulin and PN2-3 variants (Supplementary Fig. 5a). Tubulin at a high concentration self-polymerizes into microtubules. While polymerized tubulin could be pelleted by centrifugation, free tubulin remains in solution in the supernatant. When we included PN2-3 variants in this assay, we found that in contrast to PN2-3 WT, PN2-3F375A did not prevent tubulin polymerization (Supplementary Fig. 5b). This could be due to the presence of F375, which is required for the formation of high affinity CPAP-tubulin complex (Fig. 2b). In addition, F375-containing PN2-3C domain binds the β-tubulin at the microtubule outer surface, which might allosterically disrupt microtubule formation by inducing or stabilizing a curved tubulin dimer (Supplementary Fig. 2). Supporting this notion, PN2-3C alone was sufficient to prevent polymerization of in vitro, the ability of PN2-3 to bind tubulin and prevent it from polymerization is F375-dependent (Supplementary Fig. 6b).

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green fluorescent protein (GFP)-tagged PN2-3 variants in HeLa cells. In contrast to PN2-3 WT, overexpression of PN2-3F375A did not cause the collapse of cytoplasmic microtubules indicating that in vitro, the ability of PN2-3 to bind tubulin and prevent it from polymerization is F375-dependent (Supplementary Fig. 6b).

CPAP-tubulin regulates centriole and cilium lengths. First, we tested the functional significance of CPAP’s F375-tubulin interaction in centrosome biogenesis. To test this, we established stable HeLa lines expressing RNAi-resistant GFP-tagged CPAP, and CPAPF375A using bacterial artificial chromosome recombineering (BACs). This system allows expression of gene products under their own endogenous promoters. As described previously, treating cells with CPAP-specific siRNA depleted endogenous protein, retaining the RNAi-resistant CPAP (Supplementary Fig. 7a). Expressing wild type siRNA-resistant CPAP completely rescued the effects of siRNA-mediated CPAP depletion as cells proliferated without centrosome duplication defects. In contrast, RNAi-resistant CPAPF375A failed to rescue the centrosome duplication phenotype caused by wild type CPAP depletion. Specifically, when cultured for prolonged periods of time, we noticed a proportion of cells displaying less than two centrosomes reaffirming previous findings that CPAP-tubulin interaction is
required for centrosome duplication in cultured human cells\textsuperscript{11,12} (Supplementary Fig. 7b).

Since, depleting endogenous CPAP perturbs centrosome duplication, we took an alternative approach to study the role of CPAP-tubulin interaction in regulating centriolar- and ciliary-microtubule lengths. We overexpressed GFP-tagged CPAP\textsuperscript{WT} and CPAP\textsuperscript{F375A} in RPE1 cells using lentiviral-transduction. We analyzed ciliated cells and determined daughter centrioles from mother centrioles by immunostaining CP110, a centrosomal protein that localizes to the distal end of daughter centrioles providing a cap-like structure\textsuperscript{14,22}. CP110 predominately localizes to the daughter centriole, which does not template the formation of cilium (Fig. 3a). Compared to CPAP\textsuperscript{WT} expression, CPAP\textsuperscript{F375A}, which fails to efficiently bind tubulin, resulted in shorter centrioles. These data suggest the need of a high-affinity protein that localizes to the distal end of daughter centrioles

We then expressed CPAP\textsuperscript{EE343RR} that perturbs PN2-3's N-terminal helix, which caps the microtubule lumen surface of β-tubulin and possibly unmask the β-tubulin surface for polymerization (Fig. 2d)\textsuperscript{23,24}. To our surprise, CPAP\textsuperscript{EE343RR} expression caused overly long mother and daughter centrioles. In contrast to most of the CPAP\textsuperscript{F375A} centrioles, which were shorter in length, we could readily distinguish and measure the length centrioles induced by CPAP\textsuperscript{EE343RR}. The finding that CPAP\textsuperscript{EE343RR} causes longer daughter centrioles, suggests that CPAP\textsuperscript{EE343RR} can affect newly forming microtubule-based structures (Fig. 3a-ii and Supplementary Fig. 8b).

Next, we analyzed the significance of CPAP-tubulin interaction in ciliogenesis. Although CPAP\textsuperscript{F375A} expressing cells could assemble cilia, they were shorter in length. Serial sectioning-electron microscopy (EM) identified that they appear structurally normal until the transition zone where they displayed abnormal axonemes with distorted doublet-microtubules (Fig. 3b,c,e and Supplementary Fig. 9). On the other hand, expressing CPAP\textsuperscript{EE343RR} resulted in structurally normal but overly long cilia also causing ~10% of cells to be biciliated (Fig. 3d,e, Supplementary Figs 9 and 10a). EM analyses revealed that biciliated cells contained four centrioles, in which two of them template cilium formation consistently displaying centriolar-appendages, indicating that they are either the mother and/or matured daughter centriole. This data suggests that CPAP\textsuperscript{EE343RR} not only favors centriolar-microtubule elongation but also promotes daughter centrioles to form cilia (Fig. 3d,e and Supplementary Figs 9 and 10b,c).

To exclude that overly long cilia in cells expressing CPA- pE343RR is due to defective ciliary disassembly, we synchronized RPE-1 cells expressing doxycycline inducible CPAP\textsuperscript{EE343RR} at G0 by serum starvation for 96 h (ref. 25). The doxycycline inducible system prevented cells from forming long cilia caused by constitutive expression of CPAP\textsuperscript{EE343RR}. Upon serum stimulation and doxycycline induction, the percentages of cells expressing CPAP\textsuperscript{WT} and CPAP\textsuperscript{EE343RR} re-entering cell cycle concurrent with cilium disassembly did not differ. This excluded the involvement of defective ciliary disassembly in causing long cilia (Supplementary Fig. 11).

**CPAP via PN2-3 controls dynamic release of its bound tubulin.** Based on the above findings (Figs 1–3), we speculated that in the cytoplasm, CPAP binds GTP-tubulin forming a high-affinity cytoplasmic complex and prevents the bound-tubulin from spontaneous polymerization until targeted to centriolar- and ciliary-microtubule elongation sites during the early phase of cilium/cilium formation. The experiment that supports this idea is the finding of shorter centriolar- and ciliary-microtubules upon CPAP\textsuperscript{F375A} expression, a variant with strongly reduced tubulin affinity and unmasking bound-tubulin release (Fig. 4b,c and Supplementary Movies 1–3).

To test PN2-3's bound-tubulin releasing ability in vitro, we performed microtubule-end tracking assays using GMPPCP (a non-hydrolysable GTP analog) stabilized microtubules and PN2-3 variants (Fig. 4a-c). PN2-3, which can bind tubulin dimers at high affinity but not microtubules, has a microtubule-destabilizing activity primarily harbored in PN2-3\textsubscript{C} (Supplementary Fig. 5)\textsuperscript{17,18}. Thus, centriolar-CPAP could be an atypical centriolar-microtubule builder, regulating microtubule growth to define centriolar lengths. This is demonstrated by the overly long centrioles observed upon CPAP\textsuperscript{EE343RR} expression, a variant with reduced tubulin affinity and unmasking bound-tubulin surface making it available for tubulin polymerization (Fig. 3 and Supplementary Fig. 12). If this were true, CPAP\textsuperscript{EE343RR} would exhibit an enhanced release of its bound-tubulin via a ‘clutch-like’ mechanism so as to favor microtubule growth at the onset of centriolar-microtubule assembly (Supplementary Fig. 12a).

**Table 1 | Data collection and refinement statistics.**

| Data collection | DARPin-Tubulin-PN2-3C |
|-----------------|-----------------------|
| **Space group** | P2₁                  |
| **Cell dimensions** (Å) | 73.9, 91.1, 83.3 |
| **α, β, γ** (°) | 90, 97, 90 |
| **Wavelength (Å)** | 0.9792 |
| **Resolution (Å)** | 50.2-2.1 (2.17-2.10)* |
| **R<sub>merge</sub> (%)** | 12.8 (89.9) |
| **Completeness (%)** | 99.1 (98.2) |
| **Redundancy** | 4.6 (4.6) |

**Refinement (F > 0)**

| Resolution (Å) | 50-2.1 |
| No. reflections | 63,551 |
| R<sub>work/R<sub>free</sub></sub> (%) | 17.8/22.2 |
| No. atoms | 8021 |
| Protein | 66 |
| Ligand | 597 |
| Water | 21.5 |
| B-factors (Å²) | 597 |
| Protein | 35.3 |
| Ligand | 16.8 (2.5) |
| Water | 0.01 |
| r.m.s. deviations | 1.26 |

Values in parentheses are for highest-resolution shell.

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To directly analyze CPAP in elongating centrioles of living cells, we expressed inducible CPAPWT and CPAPEE343RR in RPE1 cells. Real-time measurements reveal CPAPEE343RR to have faster centriole elongation rates of \( \sim 0.014 \mu\text{m min}^{-1} \) (CPAPWT = 0.0017 \( \mu\text{m min}^{-1} \)) reaching up to 3.7 \( \mu\text{m} \) in centriolar length. This suggests that CPAP regulates microtubule

![Figure 2](image)
Figure 3 | Effects of CPAP-tubulin interaction on centriolar- and ciliary-microtubule formation in human cells. CPAPEE343RR expression but not CPAP or CPAPF375A (green) causes overly long daughter centriolar- (a) and ciliary-microtubules (b). The centrosomal protein CP110 (red) caps the distal end of daughter centrioles in (a), which does not template cillum formation. Schematic is given at right. Acetylated-α-tubulin (magenta) in (a) or Arl13b (red) in (d) labels cilia. A bar diagram at top right quantifies percentage of cells and their centriolar lengths (n = 103 for WT, n = 100 for CPAPF375A and n = 97 for CPAPEE343RR), ANOVA, **p < 0.001. Error bars represent ± s.e.m. Number of experiments (N) = 3. Note that the exact lengths of CPAPF375A centrioles could sometimes not be measured due to their small size. EM-micrographs of cilia in cells expressing the respective CPAP variants are given at the sides of light microscopy images. Arrows mark centriolar-appendages. (c) Serial section-EM of centriole and cillum from cells expressing CPAPWT or CPAPF375A. Arrows mark the abnormally organized ciliary microtubule doublets (n = 32). Scale bar 100 nm. (d) CPAPEE343RR expression causes biciliation (i and i’). Serial sectioning-EM showing single- and double-cilia (arrowheads and red/yellow dotted lines). Red arrows mark centriolar-appendages. In biciliated cells, mother and/or matured daughter centrioles template the formation of cilia. M, mother centriole. D, daughter centriole. Scale bar 500 nm. (e) Ciliary-length quantifications. ANOVA, ***p < 0.0001, n > 250. The error bars represent ± s.e.m. Number of experiments (N) = 3. Scale bar 1 μm.
Figure 4 | Microtubule end-tracking and live-imaging measurements of centriolar growth rates. (a) Schematic view of PN2-3 and PN2-3_ex constructs. (b) Kymographs and averaged depolymerization velocities of PN2-3WT, PN2-3F375A, and PN2-3EE343RR during microtubule end-tracking assay. (c) Snapshots of microtubule end-tracking assays of PN2-3WT (i), PN2-3F375A (ii), and PN2-3EE343RR (iii) at different time points. (d) Expression of CPAPEE343RR-GFP but not CPAPWT-GFP causes a rapid centriolar-elongation that is distinct enough to measure in contrast to CPAPWT expression. Arrowheads mark elongating (at 145th and 170th mins) and separating (at 420th mins) centrioles. Centriolar-growth rate curve is given (n = 57, CPAPEE343RR; 50, CPAPWT). The error bars represent ± s.d. Number of experiments (N) = 3. (e) Kymographs and averaged polymerization velocities of PN2-3WT_ex, PN2-3F375A_ex, and PN2-3EE343RR_ex during microtubule polymerization experiments.
growth for defined centriolar-microtubule lengths (Fig. 4c, Supplementary Fig. 12b and Supplementary Movies 4–7). Consistently, our fixed experiments also revealed that CPA-PEE343RR caused a faster rate of ciliary-microtubule growth (Supplementary Fig. 12c). It thus appears that CPAP regulates the growth of microtubule-based structures through controlled release of its bound tubulin and its ability to function as a centriolar-microtubule builder, both of which occur at the site of centriole assembly.

Extended PN2-3 promotes microtubule polymerization in vitro.

In order to prove the microtubule polymerization activity of CPAP in vitro, we recombinitely prepared an extended CPAP frame spanning from PN2-3 to a C-terminal coiled coil (CC) motif (aa 121–1060, PN2-3EX; Fig. 4a) using mammalian cell expression system and conducted microtubule polymerization assays (Fig. 4e and Supplementary Movies 8–11). In an optimized microtubule growth condition in the presence of 15 μM GTP-tubulin, we could observe background microtubule growth at an average rate of ~0.0106 μm s⁻¹ without PN2-3EX. After adding 1 μM wild type PN2-3EX, the microtubule growth rate was increased to ~0.014 μm s⁻¹, suggesting a role of PN2-3EX in promoting microtubule polymerization. Interestingly, PN2-3EX caused slight inhibition of microtubule polymerization with a growth rate of ~0.0081 μm s⁻¹. In conclusion, with our live imaging experiments of CAPAPEE343RR, PN2-3EX displayed an enhanced microtubule growth with a rate of ~0.018 μm s⁻¹. In addition, GFP-tagged EB1 (refs 26,27) was used to trace the plus end of growing microtubule. We observed active and dynamic plus end microtubule growth in the presence of PN2-3EX, especially in its EE343RR mutant form. These data suggest wild type and EE343RR mutant PN2-3EX may localize at the plus end during the process of microtubule polymerization (Fig. 4e and Supplementary Movies 8–11). PN2-3EX contains a tubulin-binding PN2-3 motif²⁸, a microtubule-binding A5N motif²⁹ and a dimerization CC motif²⁹ (Fig. 4a), which likely constitute as a key element for the observed microtubule polymerization activity. In summary, our in vitro microtubule growth studies support a critical role of CPAP in regulating centriolar and ciliary length.

Sas-4-tubulin controls centriole and ciliary lengths in vivo.

We then confirmed whether the Sas-4-tubulin interaction (the fly counterpart of CPAP) has a conserved function in controlling centriolar and ciliary length in vivo. To do this, we generated transgenic Drosophila expressing endogenous levels of GFP-tagged Sas-4WT, Sas-4F112A (corresponding to CPAH³⁷⁵A) and Sas-4EE78RR (corresponding to CPAH³⁷⁵A) in Sas-4 null flies (Supplementary Fig. 12d). In contrast to Sas-4WT and Sas-4EE78RR, Sas-4F112A failed to rescue the uncoordination and sterile phenotypes of null flies indicating that Sas-4F112A interaction is required to form functional sensory cilia and sperm flagella⁹,³⁰.

However, Sas-4F112A flies displayed correct centrosome numbers in spermatagonia indicating that Sas-4-tubulin interaction in flies is dispensable for centrosome duplication (Fig. 5a). This allowed us to specifically study the significance of Sas-4-tubulin interaction in centriole length determination in vivo. Analyzing mature spermatocytes of Sas-4F112A flies, we found centrioles with significantly reduced lengths. On the other hand, Sas-4EE78RR flies displayed overly elongated centrioles, consistent with observations in human cells (Fig. 5b,d). Examining Sas-4's localization at the elongating centrioles of spermatocytes, we consistently found a concentrated proximal localization of Sas-4WT. In contrast, Sas-4EE78RR that causes elongated centrioles displayed an extended localization along the length of its overly elongated centrioles, coherent with our in vitro experiments suggesting that CPAP binds at the plus end of microtubule during polymerization (Figs 4e and 5b,c).

We then performed EM analyzes of Sas-4F112A centrioles to analyze centriolar architecture when Sas-4–tubulin interaction is significantly perturbed. In contrast to Sas-4WT and Sas-4EE78RR, the spermatogonium and spermatocyte centrioles of Sas-4F112A mostly contained doublet instead of triplet centriolar-microtubules (Fig. 6a,b). This finding indicated that Sas-4F112A-tubulin interaction is not required for nucleating centriolar-microtubules per se but for the formation of third centriolar-microtubules suggesting insufficient tubulin delivery by perturbed Sas-4-tubulin interaction. Indeed, γ-TuRC has been suggested to nucleate the A-microtubule that subsequently templates B- and C-microtubule elongation.³¹

When analyzing primary cilia of spermocytes, we identified that flies expressing Sas-4EE78RR resulted in long cilia. In contrast, spermatocyte cells expressing Sas-4F112A displayed shorter cilia (Fig. 6c). Furthermore, EM analyzes revealed that Sas-4F112A cilia displayed incomplete and distorted microtubules (Fig. 6e). These findings indicate that Sas-4-tubulin interaction is required to form structurally stable and defined lengths of ciliary microtubules. Taken together, these studies indicate that via interacting with tubulin, CPAP/Sas-4 proteins have a conserved function to define centriolar- and ciliary-microtubule lengths.

Discussion

In cells, how a fraction of specialized tubulin from a large cytoplasmic pool is specifically recognized and licensed to form defined numbers and lengths of centriolar- and ciliary-microtubules is poorly understood. In this work, we have undertaken an integrated approach to dissect the conserved function of CPAP-tubulin interaction, which represents a crucial step in understanding centriolar- and ciliary-microtubule length control. Our results support a model in which CPAP/Sas-4 through the different facets of its PN2-3 domain, defines centriolar- and ciliary-microtubule lengths. Our structural studies reveal that PN2-3 embraces a ‘necklace-like’ mode for tubulin recognition, in which, CPAP via its PN2-3N targets the lumen surface of β-tubulin, and PN2-3C-loop-helix motif occupies the β-tubulin microtubule outer surface (Fig. 2d). This unique binding mode practically approves the formation of a high-affinity CPAP-GTP-tubulin complex and simultaneously prevents the polymerization of its bound GTP-tubulin, which is reactive, unstable, and ready for microtubule build-up once released. Noticeably, the last eight C-terminal residues of PN2-3C were not visible in the electron density map. However, these residues together with F385, seem to play important roles in microtubule depolymerization, as the PN2-3C variant with truncation of its last ten residues, almost failed to depolymerize GTP-stabilized microtubules, when compared with full length PN2-3C (Supplementary Fig. 6a).

To our knowledge, CPAP is the first centrosomal protein shown to form a high-affinity complex with tubulin, making it unavailable for polymerization. Although, we do not yet fully understand the mechanisms by which how the CPAP-bound tubulin is released at the sites of centriole assembly, it appears that controlled delivery of tubulin is essential for constructing defined numbers and lengths of centriolar- and ciliary-microtubules (Figs 3, 5 and 6). One of the potential mechanisms that could facilitate tubulin release at the site of centriole assembly is perhaps uncapping of CPAP’s PN2-3N from the β-tubulin surface. Thus, the cleared tubulin surface is then
Figure 5 | Effects of Sas-4-tubulin interaction on centriolar- and ciliary-microtubule formation in Drosophila. (a) Sas-4-tubulin interaction is dispensable for centrosome duplication in spermatogonium cells. Neither Sas-4 variant prevents centrosome duplication as numerically normal numbers of centrosomes were found in Drosophila expressing GFP tagged Sas-4WT, Sas-4F112A, or Sas-4EE78RR in Sas-4 null flies. Quantification is shown at top right. Centrosomes are co-labeled with Asl (red) (n ≥ 70), ANOVA, ***P < 0.0001. Error bars represent ± s.e.m. Number of experiments (N) = 3. Scale bar, 1 μm. (b) Mature but not early or intermediate spermatocyte centrioles of Drosophila expressing Sas-4EE78RR (green) display long centrioles. Asl (red) labels the elongating centrioles. Scale bar 1 μm (insets 0.5 μm). (c) Sas-4 localization in mature spermatocyte centrioles. In contrast to Sas-4WT (control) and Sas-4F112A, Sas-4EE78RR displays an extended localization all along the centriole length. Length measurements are given at bottom. ANOVA, ***P < 0.0001, (n > 120). The error bars represent ± s.e.m. Scale bar 1 μm. (d) Longitudinal EM-sections of mature spermatocytes expressing Sas-4WT (control), Sas-4F112A and Sas-4EE78RR. Sas-4EE78RR expression causes long centrioles. In contrast, Sas-4F112A fails to elongate centrioles. Scale bar 500 nm.
available for polymerization into micrometer scale centriolar-microtubules. This is supported by our experiments with CPAP-EE343RR / Sas-4-EE78RR and CPAP PN2-3EXEE343RR variants with reduced tubulin affinity and unmasking of the β-tubulin surface causing overly long centriolar-microtubules (Figs 3d, 4c, 5b and 6d).

**Figure 6** | Effects of Sas-4-tubulin interaction on centriolar and ciliary architecture in vivo. EM-micrographs displaying centrioles of spermatogonium. Scale bar 100 nm. (a) and matured spermatocyte cells (b). In contrast to Sas-4WT and Sas-4EE78RR, Sas-4F112A centrioles contain doublet- instead of triplet-microtubules and aberrant ciliary microtubules (arrowheads and cartoon). (c) Primary cilia of matured spermatocytes expressing Sas-4WT, Sas-4F112A and Sas-4EE78RR. Compared to Sas-4WT, Sas-4EE78RR spermatocytes display long cilia (arrows). In contrast, Sas-4F112A spermatocytes form cilia with reduced lengths. Asl (red) labels elongating centrioles and acetylated α-tubulin (green and arrows) labels primary cilia. Bar diagrams at right show the ciliary length measurements (n = 40). ANOVA, ***P<0.0001. Error bars represent ± s.e.m. Scale bar, 1 μm. Number of experiments (N) = 3. Scale bar, 1 μm (d) Hypothetical model depicts how Sas-4EE78RR differs from Sas-4WT in promoting centriolar microtubule growth. Various domains of Sas-4 are shown. Red dots mark the mutations studied at the N- and C-terminus of PN2-3. Microtubule-binding domain (MBD) and TCP domain that are C-terminus to PN2-3 are shown.
The finding that CPAP\textsuperscript{E3E34RR} causes overly long daughter centrioles and enhances ciliary lengths without affecting mother centrioles suggests that CPAP specifically acts on growing microtubule-based structures. Although, these experiments indirectly suggest that CPAP could function as a centriolar-microtubule builder, currently we are unable to dissect centriole-specific CPAP functions. Thus, it is unknown how CPAP spatiotemporally exerts both its tubulin binding and polymerization activities. Since CPAP is a cell cycle regulated protein, it is likely that cell cycle-dependent posttranslational modifications and its interaction partners including centriolar-microtubule length determining factors are required to modulate CPAP’s tubulin binding and polymerization activities\textsuperscript{7–11,14,32,33}. As centriolar-microtubules markedly differ from cytoplasmic microtubules, experiments employing tools that allow inducible expression of centriole-specific CPAP or its microtubule bound atomic structure are additionally required to solve the puzzle of how CPAP acts on centriolar-microtubules.

CPAP\textsuperscript{E3E34RR} expression causes enhanced ciliogenesis. This finding is strikingly similar to phenotypes observed when Kif24, a centriolar kinase, is depleted\textsuperscript{33}. Interestingly, Kif24 specifically acts on mother centrosomes and possesses microtubule-depolymerizing activity. Thus, it is tempting to speculate that an enhanced expression of CPAP\textsuperscript{E3E34RR} could counteract endogenous Kif24 to promote ciliogenesis. Taken together, several questions remain, requiring future studies.

Although, our \textit{in vitro} experiments suggest a possible role for CPAP functioning as an atypical centriolar-microtubule builder, our \textit{in vivo} experiments in flies seem to support Sas-4’s role mainly as a regulator of tubulin release at the onset of centriolar-microtubule elongation. The experiment that highlights this aspect is the proximal localization of endogenous Sas-4 at the elongating spermatocyte centrioles (Fig. 5b)\textsuperscript{34,35}. This finding supports an idea that Sas-4 has a tendency to release tubulin to microtubule elongation. The experiment that highlights this aspect is the proximal localization of endogenous Sas-4 at the elongating spermatocyte centrioles (Fig. 5b)\textsuperscript{34,35}. This finding supports an idea that Sas-4 has a tendency to release tubulin to microtubule-based structures. Although these experiments strongly suggest an idea that Sas-4 has a tendency to release tubulin to microtubule-based structures. Although these experiments strongly suggest an idea that Sas-4 has a tendency to release tubulin to microtubule-based structures.

How does CPAP/Sas-4 play a role in ciliary microtubule growth control? It is known that during ciliogenesis, IFT machineries transport ciliary building blocks of tubulin from the cytoplasmic ciliary base to the tip\textsuperscript{6–8}. As CPAP/Sas-4 mainly localizes to the ciliary base, it rules out a direct role for CPAP in ciliary microtubule elongation at the ciliary tip. Thus, CPAP/Sas-4 likely controls cilia growth indirectly by regulating the available pool of unpolymerized soluble tubulin at the ciliary base. This diffusible tubulin is then concentrated and transported by intraglial transport proteins for cilia elongation\textsuperscript{6–8}. If this is true, there could be an interaction between CPAP-tubulin complex and a subset of IFT particles. Future experiments revealing these molecular interactions will help in linking cytoplasmic tubulin recognition and their subsequent transport within the cilium by IFTs.

As free tubulin is ubiquitously present in cells, this raises the possibility of forming increased numbers and lengths of centriolar and ciliary structures. However, cells have strict mechanisms to prevent this. One such mechanism is CPAP/Sas-4–tubulin in the cytoplasm. Under physiological conditions, CPAP/Sas-4 has lower expression levels in contrast to free tubulins in cells\textsuperscript{15}.

Thus, at equilibrium, the amount of cytoplasmic tubulin bound by CPAP/Sas-4 making it unavailable for polymerization would only be a small fraction of the free tubulin pool. Therefore, it makes sense that CPAP/Sas-4 has a dedicated function in binding limited amounts of cytoplasmic tubulin and licenses them for centriolar- and ciliary-microtubules, which is then spatiotemporally regulated for constructing defined numbers and lengths of centriolar and ciliary structures.

**Methods**

**Protein and peptide preparation.** The cDNA encoding human CPAP (UniProtKB: Q9HC77) PN2-3 domain (aa 319-394) was cloned into a modified pRSFDuet vector with an N-terminal 10xHis tag followed by a PreScission\textsuperscript{TM} protease cleavage site. Recombinant wild type and mutant PN2-3 proteins were expressed in \textit{E. coli} BL21 (DE3). After overnight induction by 0.2 mM isopropyl β-D-thiogalactoside (IPTG) at 20 °C in LB medium, cells were harvested and suspended in buffer: 20 mM Tris, 200 mM NaCl, pH 8.0. Then cells were lysed with an Emulsiflex C3 (Avestin) high-pressure homogenizer. After centrifugation at 32,000 × g, the supernatant was applied to a HiTrap Heparin HP and Superdex 200 columns (GE Healthcare). Both wild type and mutant PN2-3 proteins were concentrated to 1 mM in 1xBRB80 buffer (80 mM PIPES-K, 1 mM MgCl2, 1 mM EGTA, pH 6.8), and stored at −80 °C for future use.

Human CPAP PN2-3-\textit{xG} (aa 121-1060) was cloned into the pMLink vector (gifted from Yigong Shi’s lab) with an N-terminal 2xStrep-Flag tag, and point mutations were further introduced following the same method described above. Recombinant wild type and mutant PN2-3-\textit{xG} proteins were expressed in HEK 293F cells (Invitrogen), which were transfected with plasmids expressing in \textit{E. coli} BL21 (DE3). After overnight induction by 0.2 mM IPTG at 20 °C, cell lysis was conducted by sonication on ice, and the suspension was centrifuged at 38,000 × g for 1 h to remove any cell debris. The supernatant was incubated with Strep-Tactin Superflow (IBA BioTAGnology) beads for 30 min at 4 °C. The resin was washed three times, each with 10 ml lysis buffer, and wild type or mutant PN2-3-\textit{xG} proteins were finally eluted with lysis buffer supplied with 10 mM di-Desthiobiotin (Sigma).

The codon-optimized DARPin cDNA was assembled from a dozen oligos following a reported method\textsuperscript{26}, and then cloned into the pRSFDuet vector (New England Biolabs) with an N-terminal 10xHis tag. Recombinant DARPin-tubulin complex was reconstituted by mixing DARPin with purified tubulin in the molar ratio of 1.2:1 (PN2-3C to DARPin-tubulin) in 1xBRB80 buffer supplied with 10 mM NaCl, 200 mM NaCl, pH 8.0, and protease inhibitor cocktails (Amresco). DARPin-tubulin-PN2-3C complex was reconstituted by mixing PN2-3C with DARPin-tubulin complex in the molar ratio of 1.2:1 (PN2-3C to DARPin-tubulin) in 1xBRB80 buffer at 20 °C. After cell harvest and lysis, the 6xHis DARPin was purified to homogeneity over successive HisTrap, anion exchange Q, and Superdex 200 columns (GE Healthcare). The protein was concentrated to 1 mM in 1xBRB80 buffer and stored at −80 °C for future use.

Recombinant GFP-tagged fly EB1 protein was expressed in \textit{E. coli} BL21 (DE3) and purified following the method reported previously\textsuperscript{26} with minimal modifications.

Peptides of PN2-3-\textit{xG} (aa 323-361), PN2-3-C (aa 372-394), and other PN2-3-\textit{xG} variants were synthesized in >95% purity by SciLight Biotechnology.

**Complex reconstitution and crystallization.** Porcine/bovine brain tubulin was purified by modified procedures of two cycles of polymerization and depolymerization\textsuperscript{27}. The purified tubulin was stored at −80 °C in 1xBRB80 buffer until use.

DARPIn-tubulin complex was assembled by mixing DARPin and tubulin in a 1:1.5 molar ratio (DARPIn to tubulin). The sample was incubated at 4 °C for 1 h and then loaded onto a pre-equilibrated Superdex 200 column (GE Healthcare) using 1xBRB80 buffer. Fractions responding to DARPin-tubulin complex were pooled and concentrated for future use.

DARPIn-tubulin-PN2-3-C complex was reconstituted by mixing PN2-3-C, with DARPin-tubulin complex in the molar ratio of 1:21 (PN2-3-C to DARPin-tubulin) at a total concentration of 20 mg ml\textsuperscript{−1}. After incubation at 4 °C for 1 h, crystallization screen was performed using an Art Robbins Gryphon crystallization robot by mixing equal volumes of DARPin-tubulin-PN2-3-C complex with different screening conditions from the commercial crystallization kits. Crystals appeared three days after tray set-up under 9 °C from the Hampton Research PEG/Ion crystallization screen kit. Manual crystallization and optimization were then conducted via the sitting-drop vapor diffusion method, and diffraction quality crystals were finally obtained under the reservoir condition of 0.2 M potassium sodium tartrate, 20% (v/v) polyethylene glycol 3,000, and 4% polypropylene glycol P 400.
Data collection and structure determination. Crystals were briefly soaked in a cryo-protectant composed of reservoir solution supplemented with 20% glycerol, and were flash-frozen for data collection at 100 K. Data collection was performed at beamline BL71U at the Shanghai Synchrotron Radiation Facility under the wavelength of 0.9792 Å. Diffraction data were indexed, integrated and merged using the HKL2000 software package (http://www.hkl-crax.com/).

The structure of the N-terminal-tubulin-PN2-3 was solved by the molecular replacement using MOLREP43,49 with the published DARPin-tubulin structure (PDB code: 4DRX) as search model. Structure refinement was performed using PHENIX50 with iterative manual model building using COOT41. In the final structure, PN2-3 residues 372–386 were modelled. The last eight residues (387–394) are invisible due to flexibility. Data collection and structure refinement statistics were shown in Table 1. All structural figures were prepared using PYMOL (http://www.pymol.org/).

Isothermal titration calorimetry. Calorimetric experiments were conducted at 15 °C with a MicroCal iTC200 instrument (GE Healthcare). All proteins and peptides, including tubulin, wild type and mutant PN2-3 proteins/peptides, were dialyzed against the 1 × BRB80 buffer (80 mM Pipes-K, 1 mM MgCl₂, 1 mM EGTA, pH 6.8) prior to titration. Concentration of tubulin and PN2-3 proteins were determined by their respective absorbance at 280 nm. Peptides were quantified by weighing on a large scale and further confirmed and adjusted by their respective absorbance at UV₂₈₀ (14). Acquired calorimetric titration data were analyzed using Origin 7.0 (GE Healthcare) using the ‘One Set of Binding Sites’ fitting model. In order to avoid microtubule formation, tubulin sample was kept under low temperature (<15 °C) and low concentration (<15 mM) during wild type and mutant PN2-3 titrations. Specifically, for ITC curves in Fig. 1d and Supplementary Fig. 3c, a sample cell tubulin concentration of 9 μM was used with resultant titration Cₐ values ranging from 3.2 (PN2-3²R²⁵) to 360 (PN2-3²W). For ITC titrations in Supplementary Fig. 1, a tubulin or DARPin-tubulin concentration of 36 μM was used to ensure reaction titration value (0.5–10) and measurable heat signal.

Microtubule pelleting assays. For microtubule co-sedimentation pelleting assays in Supplementary Figs 6 and 7, tubulin polymerization was first conducted at 37 °C under accession code 5EIB. The data that support the findings of this study are available from the corresponding author upon request.

Data availability. Coordinates have been deposited at the Protein Data Bank under accession code 5EIB. The data that support the findings of this study are available from the corresponding author upon request.

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Author contributions

H.L. and J.G. conceived and supervised this study; X.Z., A.R., J.G. and H.L. designed the experiments; X.Z. and S.Z. performed the X-ray crystallography, cross-linking mass spectrometry, microtubule pelleting assays, ITC and TIRF studies under the guidance of H.L.; A.R. and L.M.G. performed the cell culture, western blot, transgenic flies, immunofluorescence and light microscopy with help from A.M. and A.W.; K.S. and M.S. performed NMR spectroscopy; M.G., M.R., and G.C. performed electron microscopy; S.F. and H.D. performed mass spectrometry; W.L. and G.O. helped with TIRF study; P.W., A.P., I.P. and A.A.H. provided expertise in molecular biology and critical feedback; X.Z., A.R., J.G. and H.L. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Additional information

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