Phospho-regulated auto-inhibition of Cnn controls microtubule nucleation during cell division

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Abstract

γ-tubulin-ring-complexes (γ-TuRCs) nucleate microtubules. They are recruited to centrosomes in dividing cells via binding to N-terminal CM1 domains within γ-TuRC-tethering proteins, including Drosophila Cnn. Binding promotes microtubule nucleation and is restricted to centrosomes, but the mechanism regulating binding remains unknown. Here we identify an extreme N-terminal “CM1 auto-inhibition” (CAI) domain within the centrosomal isoform of Cnn (Cnn-C) that inhibits γ-TuRC binding. Cnn-C is phosphorylated at centrosomes and we find that phospho-mimicking sites within the CAI domain helps relieve auto-inhibition. In contrast, the testes-specific mitochondrial Cnn-T isoform lacks the CAI domain and can bind strongly to cytosolic γ-TuRCs. Ubiquitously expressing a version of Cnn-C lacking the CAI domain leads to major cell division defects, which appears to be due to ectopic cytosolic microtubule nucleation. We propose that the CAI domain folds back to sterically inhibit the CM1 domain, and that this auto-inhibition is relieved by phosphorylation that occurs specifically at centrosomes.
Microtubules are polarised polymers of tubulin necessary for cell function (Akhmanova and Steinmetz, 2015). They provide structural support, pulling and pushing forces, and tracks for the transport of intracellular cargo. Microtubules are arranged into specialised arrays that are crucial for cell function, such as the mitotic spindle or the polarised arrays within neurons. Correct assembly of these arrays relies in part on the spatiotemporal regulation of microtubule formation, and this is achieved by restricting microtubule formation and organisation to specific microtubule organising centres (MTOCs), such as the centrosome during mitosis or Golgi apparatus in motile fibroblasts (Tillery et al., 2018; Sanchez and Feldman, 2016; Petry and Vale, 2015).

The common link between most MTOCs is the presence of multi-protein γ-tubulin ring complexes (γ-TuRCs) (Teixidó-Travesa et al., 2012; Tovey and Conduit, 2018; Lin et al., 2014a; Farache et al., 2018), which template and catalyse the kinetically unfavourable process of microtubule nucleation (Kollman et al., 2011). γ-TuRCs are recruited to MTOCs by γ-TuRC-tethering proteins, that directly link γ-TuRCs to the MTOC (Tovey and Conduit, 2018). γ-TuRCs contain 14 γ-tubulin molecules held in a single-turn helical shape by laterally associating γ-tubulin complex proteins (GCPs) (Kollman et al., 2011). The γ-tubulin molecules bind directly to α/β-tubulin dimers to promote new microtubule assembly (Thawani et al., 2020). γ-TuRCs have a low activity within the cytosol but are thought to be “activated” after recruitment to MTOCs (Tovey and Conduit, 2018; Farache et al., 2018). In this model, the controlled recruitment and activation of γ-TuRCs enables the spatiotemporal control of microtubule nucleation and array formation. Consistent with this model, recent structural studies have shown that γ-TuRCs purified from the cytosol of HeLa cells and Xenopus eggs are in a semi-open conformation, in which the γ-tubulin molecules do not perfectly match the geometry of a 13 protofilament microtubule (Consolati et al., 2020; Liu et al., 2019; Wieczorek et al., 2019). A conformational change into a fully closed ring that perfectly matches the geometry of a microtubule is expected to increase the nucleation capacity of the γ-TuRC (Liu et al., 2019; Consolati et al., 2020). This agrees with studies in budding yeast, where a conformational change is observed and artificial closure of the ring increases microtubule nucleation capacity (Kollman et al., 2015).

How activation via an open-to-closed conformation change occurs is currently unclear, but various factors have been reported to increase the capacity for γ-TuRCs to nucleate microtubules. The nucleation capacity of γ-TuRCs purified from Xenopus egg extract is
dramatically increased by the addition of the TOG domain protein XMAP215 (Thawani et al., 2020). TOG domain family members mediate α/β-tubulin addition via their TOG domains (Nithianantham et al., 2018), bind directly to γ-tubulin, and function in microtubule nucleation in vivo (Thawani et al., 2018; Flor-Parra et al., 2018; Gunzelmann et al., 2018). Single molecule experiments combined with modelling suggest that XMAP215 indirectly promotes the open-to-closed conformation change of purified γ-TuRCs by increasing the chance of protofilament formation, where the lateral contacts between protofilaments are predicted to force the closure of the γ-TuRC (Thawani et al., 2020). While this is an attractive model, evidence suggests that γ-TuRC activation can occur in different ways and may be context specific. Phosphorylation of γ-TuRCs by Aurora A around mitotic chromatin increases the activity of γ-TuRCs (Scrofani et al., 2015; Pinyol et al., 2013), as does the addition of NME7 kinase in vitro (Liu et al., 2014). γ-TuRC activity is also increased after binding of the Augmin complex (Tariq et al., 2020), which occurs on the side of pre-existing microtubules such as within the mitotic spindle (Goshima et al., 2008; Uehara et al., 2009; Zhu et al., 2008; Lawo et al., 2009). Another potential γ-TuRC activator is the Centrosomin Moti 1 (CM1) domain, which is conserved in γ-TuRC-tethering proteins across Eukaryotes (Sawin et al., 2004; Lin et al., 2014b). Addition of protein fragments containing part of the CM1 domain, known as γ-TuNA, dramatically increases the nucleation capacity of γ-TuRCs purified from human cells (Choi et al., 2010; Muroyama et al., 2016), although the degree of this activity change is much lower when using γ-TuRCs purified from Xenopus eggs (Liu et al., 2019; Thawani et al., 2020). While there is evidence that CM1-domain binding does not alter the semi-open conformation of purified γ-TuRCs (Wieczorek et al., 2019; Liu et al., 2019), expression of γ-TuNA within human cells leads to the ectopic nucleation of microtubules throughout the cytosol, and this effect is abolished by the introduction of mutations that prevent CM1 domain binding to γ-TuRCs or by the depletion of γ-TuRC components (Choi et al., 2010; Hanafusa et al., 2015; Cota et al., 2017). Similar effects are seen in fission yeast, where expression of fragments of Mto1 containing the CM1 domain results in cytosolic microtubule nucleation (Lynch et al., 2014), and in Xenopus egg extracts containing activated Ran which, when supplemented with CM1-domain fragments, show dramatically increased random microtubule aster formation (Liu et al., 2019). Thus, while CM1-domain binding may not directly alter γ-TuRC conformation in vitro, there is strong evidence that it induces an activity change in various contexts in vivo. Given that CM1-domain binding appears to increase γ-TuRC activity, this binding must be spatiotemporally controlled under normal conditions. Indeed, results from numerous mass spectrometry experiments on purified γ-TuRCs have shown that γ-TuRCs do not associate
with CM1-domain proteins within the cytosol (Choi et al., 2010; Oegema et al., 1999; Hutchins et al., 2010; Teixidó-Travesa et al., 2012; Thawani et al., 2018; Consolati et al., 2019; Liu et al., 2019; Wieczorek et al., 2019). Rather, CM1 domain proteins are thought to bind γ-TuRCs only at MTOCs, such as the centrosome during mitosis. This presumably helps to ensure that γ-TuRCs do not become active within the cytosol, but how CM1-domain binding is controlled still remains unknown.

In this paper, we investigate how the binding between the Drosophila CM1-domain protein Centrosomin (Cnn) and γ-TuRCs is regulated. Cnn is the only CM1-domain protein in Drosophila but is a multi-isoform gene with all isoforms containing the CM1 domain (Eisman et al., 2009). The centrosomal isoform (Cnn-C) has a dual role, both in recruiting γ-TuRCs to centrosomes (Zhang and Megraw, 2007; Conduit et al., 2014b) and in forming a centrosome-scaffold that supports mitotic PCM assembly (Conduit et al., 2014a; Feng et al., 2017). Phosphorylation of a central PReM domain by Polo kinase drives the oligomerisation of Cnn-C molecules via interactions between the PReM and C-terminal CM2 domain to promote formation of the centrosome-like scaffold, and this occurs specifically at centrosomes (Conduit et al., 2014a; Feng et al., 2017). A testes-specific Cnn-T isoform, which has mitochondrial localisation domains instead of the PReM and CM2 domains, recruits γ-TuRCs to mitochondria in sperm cells (Chen et al., 2017). Cnn-T also has a different, shorter, N-terminal region upstream of the CM1 domain. We previously showed that N-terminal fragments of Cnn-T could immunoprecipitate γ-TuRCs more readily than the equivalent fragments of Cnn-C (Tovey et al., 2018). Here we confirm this result using an in vivo recruitment assay and show that the failure of Cnn-C to bind γ-TuRCs is due to the N-terminal extension region absent from Cnn-T. Truncating this N-terminal extension allows Cnn-C to bind γ-TuRCs, as does the introduction of phospho-mimetic mutations into a conserved helix within the N-terminal extension. Mimicking just one of these sites, a conserved Polo kinase site, also increases binding, suggesting that Polo kinase regulates Cnn-C-to-γ-TuRC binding as well as Cnn-C scaffolding. Collectively, our data suggests that centrosome-specific phosphorylation of Cnn-C’s N-terminal extension by Polo kinase helps relieve Cnn-C auto-inhibition to ensure γ-TuRC binding specifically at centrosomes. This regulation is important, as expression of a modified form of Cnn-C, where the auto-inhibited N-terminal region of Cnn-C was exchanged for the unregulated γ-TuRC-binding N-terminal region of Cnn-T, results in major cell division defects, apparently caused by ectopic cytosolic microtubule nucleation.
Results

The N-terminal regions of Cnn isoforms differ in their ability to bind γ-TuRCs

We previously published evidence that different isoforms of Cnn bind γ-TuRCs within the cytosolic extracts of *Drosophila* embryos with different affinities (Tovey et al., 2018). We found that bacterially purified MBP-tagged N-terminal fragments of Cnn-T (MBP-Cnn-T-N) could immunoprecipitate cytosolic γ-tubulin with a much higher affinity than the equivalent fragments of Cnn-C (MBP-Cnn-C-N). Both isoforms share a short sequence just proximal to the CM1 domain (residues 78-97 in Cnn-C), but differ in their extreme N-terminal region, which is 77 residues long in Cnn-C but only 19 residues long in Cnn-T (dark blue and red, respectively, in Figure 1A). While it is possible that the difference in binding between the MBP-Cnn-C-N and MBP-Cnn-T-N fragments could have been an artefact induced by bacterial purification, we had hypothesised that the larger extreme N-terminal region of Cnn-C may fold back and auto-inhibit the CM1 domain, restricting its ability to bind γ-TuRCs. To address this directly, and to confirm the in vitro binding results, we developed an in vivo γ-TuRC recruitment assay based on mRNA injection into *Drosophila* eggs. Using this assay, we could quickly and easily manipulate the sequence of Cnn (without having to generate transgenic lines) and, importantly, the folding of Cnn proteins would occur in a natural environment. The assay is based on a previous experiment where we had injected mRNA encoding a GFP-tagged phosho-mimetic form of Cnn into unfertilised *Drosophila* eggs to produce centrosome-like scaffolds spontaneously throughout the cytosol. The phosho-mimetic mutations are placed within the central PReM domain of Cnn-C (Cnn-C-PReMm) and drive the oligomerisation of GFP-Cnn-C molecules into the GFP-labelled scaffold structures (Conduit et al., 2014a) (Figure 1B; Figure S1). We realised that we could use these scaffolds to assess the ability of Cnn-C, and any modified forms of Cnn-C (Figure 1B), to bind and recruit γ-TuRCs by examining the recruitment of fluorescently-tagged γ-TuRC proteins to the scaffolds.

We first compared scaffolds formed from GFP-Cnn-C-PReMm to scaffolds where the extreme N-terminal region of Cnn-C-PReMm was either exchanged with the extreme N-terminal region of Cnn-T (GFP-Cnn-T-PReMm) or was removed (GFP-Cnn-C\textsuperscript{1-77}-PReMm). For simplicity we refer to these as Cnn-C, Cnn-T, and Cnn-C\textsuperscript{1-77} scaffolds, respectively, regardless of the fluorescent tag used. In order to assess recruitment of γ-tubulin complexes to the scaffolds, we endogenously tagged the maternal form of γ-tubulin with mCherry (γ-tubulin-37C-mCherry) and injected unfertilised eggs laid by these mothers (we refer to γ-tubulin complexes rather than γ-TuRCs, as it is possible that Cnn can bind and recruit γ-TuSCs as well as γ-TuRCs). Initial visual observations suggested that γ-tubulin-37C-mCherry associated more
readily with Cnn-T and Cnn-Cα1-77 scaffolds than with Cnn-C scaffolds (Figure 1C-E). We measured the GFP (Cnn) and mCherry (γ-tubulin-37C) fluorescence signals at multiple scaffolds of different sizes and from different eggs and generated linear lines of best-fit (Figure 1F; see Methods for details). The slopes of these lines (S) are an estimation of the relative binding affinity between the different Cnn constructs and γ-tubulin complexes. S values for the various Cnn scaffolds differed significantly overall (p<0.001, F_{2,354}=1645) and also in pairwise comparisons: the S value for Cnn-T scaffolds (0.55) was ~26-fold higher than the S value for Cnn-C scaffolds (0.02) (p<0.001, t_{350}=−57) (Figure 1F), indicating that the N-terminal region of Cnn-T binds γ-tubulin complexes with ~26-fold higher affinity than the N-terminal region of Cnn-C, and confirming the results from our previous in vitro binding experiments (Tovey et al., 2018). Importantly, the S value for Cnn-Cα1-77 scaffolds (0.17) was ~8-fold higher than the S value for Cnn-C scaffolds (p<0.001, t_{357}=36) (Figure 1F), showing that removing the extreme N-terminal region of Cnn-C increases binding affinity to γ-tubulin complexes by ~8 fold. This therefore confirms that the extreme N-terminal region of Cnn-C inhibits binding to γ-tubulin complexes. Consistent with this, we also found that MBP-tagged N-terminal fragments of Cnn-Cα1-77 (MBP-Cnn-C-Nα1-77) could immunoprecipitate γ-tubulin from embryo extracts more efficiently than N-terminal fragments of Cnn-C (Figure 1G). Comparing the band intensities (normalised to the γ-tubulin band in the MBP-Cnn-T-N IP lane, see Methods) of γ-tubulin across three experiments showed that in this assay the truncated MBP-Cnn-Cα1-77-N form bound nearly as well as MBP-Cnn-T-N (average normalised value of 0.90, Figure 1H). Thus, while it is known that the CM1 domain is required for binding γ-tubulin complexes, we find that the extreme N-terminal region of Cnn-C inhibits binding to γ-tubulin complexes; we therefore name this region the “CM1 auto-inhibition” (CAI) domain.

Intriguingly, the in vivo assay also showed that the S value for Cnn-Cα1-77 scaffolds was ~3.3-fold lower than that of Cnn-T scaffolds (p<0.001, t_{357}=−21) (Figure 1F), suggesting that the extreme N-terminal region of Cnn-T (red in Figure 1A) promotes binding to γ-tubulin complexes.

To test whether Cnn scaffolds recruit other γ-TuRC components, we assessed the ability of mKATE-tagged Cnn-T scaffolds to recruit the γ-TuRC-specific components Grip75, Grip128, and Grip163 (homologues of GCP4, 5 and 6, respectively) that we had endogenously tagged with GFP (Figure 2). We again quantified the fluorescence signals at multiple scaffolds across embryos and generated S values. We found that all S values for Cnn-T scaffolds were significantly higher than the respective S values for Cnn-C scaffolds: fold change of 7.4 for
Grip75-sfGFP (p<0.001, t_{36}=−28) (Figure 2C), 6.7 for Grip128-sfGFP (p<0.001, t_{34}=−37) (Figure 2F), and 2.2 for Grip163-sfGFP eggs (p<0.001, t_{55}=−9) (Figure 2I). The lower fold changes as compared to γ-tubulin37C-mCherry could be a consequence of the lower signal-to-noise ratios for Grip75-sfGFP, Grip128-sfGFP and Grip163-sfGFP due to their lower stoichiometry within γ-TuRCs. Alternatively, the lower fold changes could be caused by a proportion of γ-tubulin complexes bound by Cnn-T that do not contain Grip75, Grip128, or Grip163, which would be the case if Cnn could bind and recruit both γ-TuSCs and γ-TuRCs.

Nevertheless, a combination of western blotting and mass spectrometry showed that bacterially purified MBP-Cnn-T-N fragments could co-immunoprecipitate the γ-TuRC components γ-tubulin, Grip71, Grip75, Grip84, Grip91, Grip128, Grip163 (Figure S2). We therefore conclude that the Cnn-T N-terminus can bind γ-tubulin complexes containing γ-TuRC-specific proteins.

Cnn-T scaffolds organise microtubules more robustly than Cnn-C scaffolds

If Cnn-T scaffolds can recruit functional γ-tubulin complexes, the scaffolds should be able to nucleate and organise microtubules. We had previously shown that Cnn-C scaffolds could organise microtubule asters, but only when these scaffolds were large (Conduit et al., 2014a).

To test whether microtubules are organised more robustly by Cnn-T scaffolds (which recruit more γ-tubulin complexes than Cnn-C scaffolds) we formed scaffolds within eggs expressing the microtubule binding protein Jupiter-mCherry (Figure 3A,B). We used a blind analysis to quantify the propensity of these Cnn scaffolds to organise microtubules by categorising eggs into those where the scaffolds organised “strong”, “medium”, “weak”, or no microtubule asters. We also included a “tubulin overlay” category, where the Jupiter-mCherry signal did not extend beyond the GFP scaffold signal. For simplicity, we refer to eggs containing Cnn-C and Cnn-T scaffolds as Cnn-C and Cnn-T eggs, respectively. Consistent with the increased recruitment of γ-tubulin complexes to Cnn-T scaffolds, we found that a higher proportion of Cnn-T eggs (43.8%) than Cnn-C eggs (11.5%) contained scaffolds that organised strong or medium microtubule asters (Figure 3C). Moreover, while 69.2% of Cnn-C eggs contained scaffolds that did not organise any visible microtubule asters, none of the Cnn-T eggs fell into this category (Figure 3C). Based on these differences, we conclude that the γ-tubulin complexes recruited to Cnn scaffolds are able to nucleate microtubules, at least to some extent. The ability of some Cnn-C scaffolds to organise microtubules is not unexpected, as these scaffolds can still recruit low levels of γ-tubulin complexes (Figure 1C,F).
Filming the scaffolds through time revealed that scaffolds could merge and could also be quite mobile, especially those that had microtubules emanating from just one side (Video 1). In these instances, the microtubules appeared to push the scaffolds through the cytosol. Most intriguingly, we could observe events where spindle-like structures formed between adjacent Cnn-T scaffolds (Figure 3D). This was unexpected, but suggested that the microtubules organised by the scaffolds are dynamic and can be regulated by motor proteins. We managed to film the formation of these transient spindle-like structures (Video 2; Video 3), and in one example multiple spindle-like structures formed simultaneously in close proximity and were organised by a nearby group of coalescing scaffolds into a flower-like arrangement; the spindles then quickly disappeared (Video 3). On some occasions, we could observe giant Cnn-T scaffolds that organised large bundles of microtubules (Video 4). One of these giant scaffolds rotated, dragging its associated microtubule bundles, indicating that the microtubules were robustly anchored to the scaffolds, presumably via linkage by γ-tubulin complexes. In summary, we conclude that Cnn-T scaffolds can recruit γ-tubulin complexes that are capable of nucleating and anchoring microtubules.

Phosphorylation of a conserved helix within the CAI domain, including a conserved Polo kinase site, helps relieve auto-inhibition

The data above shows that the extreme N-terminal CAI domain of Cnn-C inhibits binding to γ-tubulin complexes. Given the similar length of the CAI and CM1 domains, we speculated that the CAI domain may fold back over and inhibit the CM1 domain (Figure 4A). Given that Cnn-C recruits γ-TuRCs to centrosomes (Zhang and Megraw, 2007; Conduit et al., 2014b) and that Cnn-C is phosphorylated specifically at centrosomes and not within the cytosol (Conduit et al., 2014a), we reasoned that CAI domain inhibition could be relieved by phosphorylation, with negatively charged phosphate groups driving the separation of the CAI and CM1 domains (Figure 4A). We therefore used sequence alignments and secondary structure predictions of amino acids 1 to ~255 of Cnn-C from various Drosophila species to search bioinformatically for potential phosphorylation sites (Figure S3). We identified three putative phosphorylation “patches” based on a high concentration of often conserved serine and threonine residues (Figure 4A; Figure S3). Patch 1 (P1) and Patch 2 (P2) lie within the CAI domain and so are absent from Cnn-T; Patch 3 (P3) lies within the CM1 domain and so is conserved between all Cnn isoforms. P1 also represented the only predicted structured region within the CAI domain, corresponding to an α-helix (Figure S3).
We purified MBP-tagged N-terminal fragments of Cnn-C containing phosphomimetic mutations in all serine and threonine residues within either P1 (MBP-Cnn-C-NP1), P2 (MBP-Cnn-C-NP2), P3 (MBP-Cnn-C-NP3), or in all three patches (MBP-Cnn-C-NP1-3). We then assessed how much γ-tubulin associated with these fragments compared with the original MBP-Cnn-C-N (low binding) and MBP-Cnn-T-N (high binding) fragments when performing anti-MBP immunoprecipitation. We found that MBP-Cnn-C-NP1 bound more γ-tubulin than MBP-Cnn-C-N, but less than MBP-Cnn-T-N (Figure 4B). MBP-Cnn-C-NP2 also bound more γ-tubulin than MBP-Cnn-C-N, although not as much as MBP-Cnn-C-NP1 (Figure 4B). In contrast to P1 and P2, introducing mimetic mutations into P3 appeared to impede γ-TuRC binding.

Firstly, MBP-Cnn-C-NP3 bound less γ-tubulin compared to MBP-Cnn-C-N, and secondly MBP-Cnn-C-NP1-3 bound less γ-tubulin compared to MBP-Cnn-C-NP1 (Figure 4B), suggesting that the phosphomimetic insertions in P3 counteracted the positive effect of the phosphomimetic insertions in P1 and P2. Quantifying γ-tubulin band intensities across 5 experiments showed that while MBP-Cnn-C-N immunoprecipitated ~18% of γ-tubulin compared to MBP-Cnn-T-N, MBP-Cnn-C-NP1 immunoprecipitated ~44% of γ-tubulin (Figure 4D). Thus, the data suggest that phosphorylation of one or more sites within P1, and to a lesser extent P2, helps relieve CAI domain-mediated auto-inhibition of Cnn-C’s CM1 domain to allow the CM1 domain to bind to γ-tubulin complexes.

Given that mimicking sites within P1 gave the strongest effect, we wanted to narrow down which site or sites within P1 were important for relieving autoinhibition. There are six serine and threonine residues within P1 (S21, S22, T27, T31, T33, S34) (Figure 4A; Figure S3). T27 is a putative Polo site based on the surrounding residues that match the LΦE/D/N/Q-X-S/T-Φ consensus motif of Plk1 (the human homologue of Polo) (Santamaria et al., 2011). Moreover, a previous study that mutated T27 to alanine within Cnn-C constructs reported microtubule defects at centrosomes in Drosophila embryos (Eisman et al., 2015). We therefore generated three different phospho-mimetic fragments, where either the proximal three residues (S21, S22, T27) were mimicked (MBP-Cnn-C-NP1α), the distal three residues (T31, T33, S34) were mimicked (MBP-Cnn-C-NP1β), or only the predicted Polo site was mimicked (MBP-Cnn-C-NT27). We found that MBP-Cnn-C-NP1α and MBP-Cnn-C-NT27 bound more γ-tubulin than MBP-Cnn-C-N, but that this was not true of MBP-Cnn-C-NP1β (Figure 4C). Quantification of γ-tubulin band intensities from 5 experimental repeats indicated that MBP-Cnn-C-NT27 immunoprecipitated ~34% of γ-tubulin compared to MBP-Cnn-T-N, which is more than for MBP-Cnn-C-N (~18%) although less than when all sites within P1 are mimicked (~44%). This data suggests that phosphorylation of multiple residues within P1, including the predicted Polo site at T27, helps
to relieve CAI domain inhibition to allow binding to γ-tubulin complexes. This is consistent with
the microtubule defects previously observed at centrosomes in T27A mutant Drosophila
embryos (Eisman et al., 2015).

**Ubiquitous expression of Cnn-C containing the high binding-affinity Cnn-T N-terminal
region has a dominant negative effect and leads to fertility defects**

We next wanted to test whether Cnn-C auto-inhibition is important for cell and developmental
fidelity in Drosophila, as previous experiments in human and fission yeast cells have shown
that expression of CM1 domain fragments leads to ectopic cytosolic microtubule nucleation
(Choi et al., 2010; Lynch et al., 2014; Hanafusa et al., 2015; Cota et al., 2017). We generated
a transgenic fly line by random insertion of a ubiquitously-driven untagged Cnn-C construct in
which its N-terminal region had been replaced with the N-terminal region of Cnn-T (pUbq-Cnn-
C T) (Figure 5A). We decided to use the N-terminal region of Cnn-T, rather than simply
removing the CAI domain or introducing phospho-mimetic mutations, as our in vivo and in vitro
assays had shown that this would allow the strongest and most consistent binding. As a control
we generated a line ubiquitously expressing untagged wild-type Cnn-C (pUbq-Cnn-C), whose
binding to cytosolic γ-tubulin complexes should be restricted by the CAI domain.

Consistent with the prediction that pUbq-Cnn-C T would bind and activate cytosolic γ-tubulin
complexes, we found it difficult to generate a viable pUbq-Cnn-C T line. We quickly discovered
that the pUbq-Cnn-C T stock that we had generated was difficult to maintain and combine with
other alleles, presumably due to toxic effects caused by pUbq-Cnn-C T expression. For
example, we were unable to generate a stock where pUbq-Cnn-C T was expressed in a cnn
mutant background. Thus, all of the following experiments were performed with the pUbq
constructs expressed in the presence of endogenous Cnn.

We first tested the fertility rates of males and females bred at 25°C, comparing them to the
pUbq-Cnn-C control stock. We quantified the hatching rate of embryos that were generated
when pUbq-Cnn-C or pUbq-Cnn-C T males or females were crossed to w118 “wild-type” flies.
Young (0-1 week old) pUbq-Cnn-C T females produced embryos of which only 55% hatched,
compared to an average hatching rate of ~85% for embryos from young pUbq-Cnn-C females
(Figure 5B; p<0.001). Hatching rates for older (1-2 week old) females of both genotypes were
similar to the younger females (52.2% for pUbq-Cnn-C T, 74.8% for pUbq-Cnn-C) and were
also significantly different from each other (Figure 5B; p<0.001). An even larger reduction in
hatching rate between pUbq-Cnn-C and pUbq-Cnn-C T flies was observed when crossing
males. When young (0-1 week old) pUbq-Cnn-C*T males were crossed to wild-type females only 40.3% of embryos hatched, compared to 84.7% when crossing pUbq-Cnn-C males (Figure 5B; p<0.001). When older (1-2 week old) pUbq-Cnn-C*T males were crossed to wild-type females only 9.9% of embryos hatched, compared to 90.5% for pUbq-Cnn-C males (Figure 5B; p<0.001). Thus, the ability of both males and females to generate progeny is reduced when ubiquitously expressing Cnn-C*T compared to Cnn-C, and males are more strongly affected.

Given this difference between females and males, we tested whether the expression of the two different pUbq constructs varies within female and male germlines. We used two different Cnn antibodies to probe western blots for Cnn protein: one raised against the N-terminal half of Cnn-C (aa1-660), the other against the C-terminal half (aa661-1147). The N-terminal antibody recognises fewer unspecific bands on western blots, but its ability to bind Cnn-C*T could be affected by the differences in the extreme N-terminal region of Cnn-T. Western blotting extracts from wild-type, pUbq-Cnn-C, and pUbq-Cnn-C*T embryos or testes with either the N-terminal or C-terminal Cnn-C antibody showed that each extract contained a Cnn-C band at ~150kDa (Figure 5C). This band represents the endogenous form of Cnn-C in both the wild-type and pUbq-Cnn-C*T extracts (black arrowheads, Figure 5C), but is a combination of endogenous Cnn-C and pUbq-driven Cnn-C in the pUbq-Cnn-C extract (blue arrowhead, Figure 5C), explaining why this band is much stronger in the pUbq-Cnn-C extract lanes. The lower band in the pUbq-Cnn-C*T extract lanes is of an appropriate size for the slightly smaller Cnn-C*T protein (red arrowheads, Figure 5C). The C-terminal antibody generated a weaker, non-specific band of similar size to the Cnn-C*T band, which could be observed in the wild-type and pUbq-Cnn-C lanes (asterisks). The unspecific nature of this band was highlighted by its absence on membranes probed with the N-terminal Cnn antibody. Nevertheless, to confirm that the lower bands in the pUbq-Cnn-C*T lanes do indeed represent Cnn-C*T, we generated an isoform-specific antibody raised against a short peptide within the isoform-specific extreme N-terminal exon of Cnn-T (anti-Cnn-TN). This antibody recognised a single band of the expected size only in the pUbq-Cnn-C*T extract lanes (Figure 5C), thus confirming the identity of the Cnn-C*T band.

Importantly, there was a clear difference in the relative levels of pUbq-Cnn-C*T between embryo and testes extracts. In the embryo extracts, the pUbq-Cnn-C*T band was much weaker than the endogenous Cnn-C band, which is unusual for pUbq-driven Cnn constructs (P. Conduit unpublished observations); presumably the intensity of the pUbq-Cnn-C*T band
observed when using the C-terminal antibody would have been even lower were it not for the overlapping unspecific band recognised by this antibody. Indeed, the relative band intensity of pUbq-Cnn-C\(^T\) is much lower when using the N-terminal antibody, although this could also reflect a difference in the ability of this antibody to recognise the modified N-terminal region of pUbq-Cnn-C\(^T\). In contrast to embryo extracts, the pUbq-Cnn-C\(^T\) band was of a similar intensity to, if not higher than, the endogenous Cnn-C band in the testes extracts. We therefore conclude that, relative to endogenous Cnn-C, pUbq-Cnn-C\(^T\) is weakly expressed within the maternal germline but is expressed to levels similar to endogenous Cnn within the testes. While other factors could be involved, such as cell-specific effects of CM1 domain binding on \(\gamma\)-tubulin complexes, differences in expression levels of pUbq-Cnn-C\(^T\) between cells could explain the difference in the ability of male and female flies to generate progeny. Consistent with this, it was easier to maintain the pUbq-Cnn-C\(^T\) stock at lower temperatures (where expression is likely reduced).

**pUbq-Cnn-C\(^T\) binds to cytosolic \(\gamma\)-tubulin complexes with a higher affinity than pUbq-Cnn-C**

We performed IP experiments to confirm that pUbq-Cnn-C\(^T\) binds \(\gamma\)-TuRCs more efficiently than pUbq-Cnn-C, which would presumably account for the toxic effects. We tried using beads coated with the anti-Cnn\(^{TN}\) antibody but found that it did not pull down any protein (data not shown), presumably as this antibody was raised against a peptide antigen and recognises only denatured pUbq-Cnn-C\(^T\) on western blots. We therefore used beads coupled to Cnn-C C-terminal antibodies, which should recognise both pUbq-Cnn-C and pUbq-Cnn-C\(^T\) equally well. In order to compare the amount of \(\gamma\)-tubulin bound by each type of Cnn molecule, we adjusted gel loading to reflect the differences in the expression levels of pUbq-Cnn-C and pUbq-Cnn-C\(^T\) within embryos. We found that more \(\gamma\)-tubulin was immunoprecipitated with pUbq-Cnn-C\(^T\) than with pUbq-Cnn-C, as expected (Figure 5D, right panels).

**Mis-regulation of binding to \(\gamma\)-tubulin complexes results in ectopic microtubule nucleation and defects during cell division**

The failure to generate normal numbers of progeny suggested that ectopic binding of Cnn to \(\gamma\)-tubulin complexes leads to cellular defects during germline or embryo development. We therefore carried out immunostainings to examine directly any potential defects. We first fixed and stained oocytes for markers of polarity, where specific microtubule arrangements are required to establish and maintain polarity (Bastock and Johnston, 2008). In wild-type oocytes, the nucleus is positioned in the dorsal corner from stage 8 to 10 and Staufen protein localises...
in the centre of the oocyte at stage 8 and then at the posterior in stage 9 and 10. This was true of all pUbq-Cnn-C (n=35, stage 8; n=35, stage 9; n=30, stage 10) and all pUbq-Cnn-C\(^T\) (n=40, stage 8; n=50, stage 9; n=40, stage 10) oocytes (Figure S4A,B). Gurken protein is normally positioned close to the nucleus in the dorsal corner of the oocyte and its mis-positioning or its absence results in abnormal dorsal appendages that protrude from the surface of the egg. Once again, we found that Gurken protein was localised normally in all pUbq-Cnn-C (n=30) and all pUbq-Cnn-C\(^T\) (n=35) stage 9 oocytes (Figure S4C), and the dorsal appendages were normal on all pUbq-Cnn-C (n=724) and all pUbq-Cnn-C\(^T\) (n=488) eggs. We therefore conclude that there are no severe microtubule organisation defects in pUbq-Cnn-C\(^T\) oocytes and that polarity is normally established.

We next stained early embryos laid by either wild-type, pUbq-Cnn-C, or pUbq-Cnn-C\(^T\) females for DNA, microtubules, and \(\gamma\)-tubulin. Prior to cellularisation, embryos go through 13 rounds of rapid and near-synchronous mitotic divisions within a syncytium. Centrosomes are constantly in a “mature” state and centrosomal microtubules help coordinate rearrangements of the actin network that in turn ensures the correct spacing of nuclei and microtubule-based spindles. In theory, ectopic nucleation of cytosolic microtubules could interfere with this process, as well as spindle formation. We therefore performed a blind analysis of pUbq-Cnn-C or pUbq-Cnn-C\(^T\) embryos categorising each embryo into those with severe, moderate, mild, or no defects, depending on both the breadth of defects across the embryo and how severe individual defects appeared. We found that a higher percentage of both pUbq-Cnn-C and pUbq-Cnn-C\(^T\) embryos fell into the severe and moderate categories compared to wild-type embryos, but that a higher proportion of pUbq-Cnn-C\(^T\) embryos than pUbq-Cnn-C embryos fell into the severe category (Figure 5E). Broadly, the categorisation reflects the observed hatching rates in Figure 5B. Cytosolic non-centrosomal microtubules appeared to be present within \(\sim\)10.8% of pUbq-Cnn-C\(^T\) embryos, but this was very similar in pUbq-Cnn-C embryos (\(\sim\)9.6%). Nevertheless, this suggests that ectopic binding of \(\gamma\)-tubulin complexes by Cnn (either due to the presence of unregulated Cnn-C\(^T\) or due to overexpression of Cnn-C) can lead to ectopic microtubule nucleation in at least a fraction of embryos.

To directly test whether Cnn binding to \(\gamma\)-tubulin complexes could initiate ectopic cytosolic microtubule nucleation within embryos, we injected mRNA encoding the N-terminal region of Cnn-T tagged with GFP (GFP-Cnn-T-N) into unfertilised eggs expressing the microtubule marker Jupiter-mCherry. We injected into eggs rather than fertilised embryos to avoid complications caused by centrosomes and mitotic figures that would have been present in
developing embryos. Strikingly, live imaging revealed the presence of highly dynamic microtubules throughout the cytosol within eggs injected with GFP-Cnn-T-N mRNA that were not observed within eggs injected with water (Video 5). Thus, it appears that binding of Cnn to γ-tubulin complexes can stimulate ectopic microtubule nucleation within these eggs/embryos, similar to how binding of the CM1 domain can stimulate ectopic cytosolic microtubule nucleation within human cells (Choi et al., 2010; Cota et al., 2017; Hanafusa et al., 2015) and fission yeast (Lynch et al., 2014).

We next examined testes from pUbq-Cnn-C$^T$ males, which were less able to generate progeny than pUbq-Cnn-C$^T$ females. Production of sperm involves a series of mitotic and meiotic cell divisions followed by sperm elongation within the testes (Fabian and Brill, 2012). Mutations in $cnn$ or γ-TuRC genes lead to defects during the two meiotic divisions (Vogt et al., 2006; Li et al., 1998). These defects can be quickly revealed by using phase contrast microscopy to examine round spermatids, which are the cells produced immediately after the meiotic divisions. Round spermatids exist as interconnected cysts of 64 cells with each cell normally containing a single round phase-light nucleus and a single round phase-dark mitochondrial derivative (nebenkern) of similar size (Figure 5G). Problems in spindle formation can lead to mis-segregation of chromosomes during meiosis (karyokinesis defects), and this can result in nuclei of variable size between different cells within the same cyst and, when major problems occur, to cells containing multiple or no nuclei. Cytokinesis defects can also result in cells containing the wrong number of nuclei. Nebenkerns assemble from mitochondria that are also equally distributed by the spindle during cell division, and so defects in spindle formation and cytokinesis also lead to variations in nebekenkern size and number. We therefore quantified and compared the variability in nuclear size within cysts (as a marker of karyokinesis defects) and the nucleus:nebenkern ratio (as a marker of major karyokinesis defects and/or cytokinesis defects). We found that the variability in nuclear size was larger within pUbq-Cnn-C$^T$ testes compared to pUbq-Cnn-C testes (Figure 5H). Moreover, while the nucleus:nebenkern ratio was always close to 1 in pUbq-Cnn-C testes, it varied between 0.5 and 1.75 in pUbq-Cnn-C$^T$ testes (Figure 5H). Thus, major karyokinesis and cytokinesis defects exist within pUbq-Cnn-C$^T$ testes.

To examine this more directly, we fixed and stained the testes for DNA, microtubules, and the centriole marker Asterless (As$^l$). Consistent with the phase contrast imaging of round spermatids, we observed examples of major karyokinesis and cytokinesis defects during meiosis in pUbq-Cnn-C$^T$ testes (Figure 5I; Figure S5). For example, the middle panel of Figure
5I shows a pUbq-Cnn-C T cell in telophase of meiosis I where cleavage furrow ingress had failed or was delayed and where both sets of daughter chromosomes had ended up on the same side of the cell, rather than being partitioned between the two future daughter cells. The right panel shows an example of a cell in metaphase of meiosis II that contained two spindles (instead of one) with one centriole at each pole. Presumably this cell had failed cytokinesis after meiosis I. This cell also contained ectopic clusters of microtubules within the cytosol away from the centrosomes, suggesting that microtubules had been nucleation randomly within the cytosol. These ectopic clusters of microtubules were observed in other pUbq-Cnn-C T spermatocytes at different cell cycle stages (Figure S5). We conclude that expressing pUbq-Cnn-C T, which can bind γ-tubulin complexes away from centrosomes, leads to major defects during male meiosis due to ectopic nucleation of microtubules within the cytosol.
Discussion

We propose a molecular model for the spatiotemporal regulation of γ-tubulin complex binding by Cnn-C during cell division in Drosophila. In this model, Cnn-C is prevented from binding γ-tubulin complexes within the cytosol by its extreme N-terminal region that we name the CM1-autoinhibition (CAI) domain. We propose that the CAI domain folds back to sterically inhibit the CM1 domain, and that this inhibition is relieved only once Cnn-C is recruited to centrosomes and phosphorylated at sites within the CAI domain, including the predicted Polo site T^{27}. This mechanism explains why Cnn-C does not normally bind γ-Tubulin complexes within the cytosol, which can lead to ectopic microtubule nucleation and major defects during cell division.

In contrast to Cnn-C, we show that the testes-specific isoform of Cnn, Cnn-T, binds cytosolic γ-tubulin complexes with high affinity. We now show that this difference is largely due to the presence of the CAI domain at the extreme N-terminus of Cnn-C, which is absent from Cnn-T due to differential exon splicing. Removal of the CAI domain from Cnn-C increases binding to cytosolic γ-TuRCs. Moreover, phospho-mimicking sites in a conserved α-helix within the CAI domain, including the putative Polo kinase site at T^{27}, also allows increased binding to γ-tubulin complexes, presumably by relieving the auto-inhibition to some degree. We previously showed that Cnn-C is phosphorylated only at centrosomes during cell division with Polo phosphorylating sites within the central PReM domain to drive Cnn oligomerisation and centrosome scaffold assembly (Conduit et al., 2014a; Feng et al., 2017). Our new data presented here suggests that Polo also phosphorylates the CAI domain, presumably specifically at centrosomes, to spatially regulate binding to γ-tubulin complexes. Coupling of centrosome scaffolding and γ-tubulin complex binding via Polo phosphorylation provides an elegant way for the cell to control proper spindle formation during cell division.

While phospho mimicking residues within P1 quantifiably increased binding to γ-tubulin complexes, binding was less efficient than with N-terminal fragments of Cnn-T. There are several possibilities for this. Cnn-C may never bind as strongly to γ-tubulin complexes as Cnn-T, perhaps because other proteins can support recruitment of γ-tubulin complexes to the centrosome. It is possible that a very particular combination of phosphorylation events within P1, or P1 and P2, must occur, and that we have not yet tried those combinations. Perhaps the phospho-mimetic mutations do not fully mimic natural phosphorylation events, which is anecdotally common. Alternatively, other regulatory events, possibly dependent on other proteins, may need to occur. These events could help the unfolding of the CAI domain or they...
could mediate the binding of the CAI domain to γ-tubulin complexes after unfolding occurs. We note that phospho-mimicking either all sites within P1, or T27 alone, did not result in increased recruitment of γ-tubulin to Cnn scaffolds in the majority of eggs in our in vivo recruitment assay (data not shown). It is therefore possible that in the presence of full length Cnn-C that is oligomerising into scaffolds, other regulatory events become even more important. Nevertheless, our data still support a model in which phosphorylation of residues within P1, including T27, help to relieve CAI-domain mediated auto-inhibition.

While T27 has not been identified in phospo-mapping mass spectrometry (Dinkel et al., 2010; Zhai et al., 2008; Conduit et al., 2014a; Bodenmiller et al., 2008), it has previously been identified bioinformatically as a potential Polo kinase site (Eisman et al., 2015). In their study, the authors mutated T27 and an adjacent putative Polo docking site (S22) to prevent putative phosphorylation and found defects in microtubule organisation at centrosomes in Drosophila syncytial embryos (Eisman et al., 2015), consistent with our model. While we have demonstrated that phospho-mimicking T27 is sufficient to increase binding to γ-tubulin complexes, we have so far been unable to phosphorylate T27 in vitro using recombinant human Plk1 kinase. We suspect that this could be because other phosphorylation events must first occur, such as phosphorylation of S22 within the putative Polo docking site, or that human Plk1 cannot replace fly Polo in these in vitro assays.

It will be interesting to see whether the CAI domain is functionally conserved through evolution. While sequence similarity outside the CM1 domain is very low, CM1-domain proteins from different species tend to contain extended N-terminal regions (Lin et al., 2014b), and the lengths of these regions can vary between isoforms, similar to the length differences between Cnn-C and Cnn-T. A study analysing budding yeast Spc110 showed that phosphorylation of Cdk1 and Mps1 phosphorylation sites in the region proximal to the CM1 domain promoted binding to γ-TuSCs and subsequent oligomerisation of γ-TuSCs in vitro (Lin et al., 2014b), although a later study questioned the effect of these phosphorylation sites (Lyon et al., 2016). The binding between human CDK5RAP2 and γ-TuRCs also appears to be regulated by phosphorylation. It was shown that depletion of LLRK1 kinase, which functions downstream of Plk1, reduced the ability of N-terminal fragments of CDK5RAP2 (aa51-200) to co-immunoprecipitate γ-tubulin from HEK293 cells, and also reduced the ability of these fragments to promote ectopic microtubule nucleation within the cytosol (which is dependent on γ-TuRC binding) (Hanafusa et al., 2015). In contrast to Drosophila Cnn-C, however, the phosphorylation site identified as being important for γ-TuRC binding is located downstream.
of the CM1 domain (S140). Nevertheless, it remains possible that this downstream region, in
place of, or in combination with, the region upstream of the CM1 domain, could fold back over
the CM1 domain and function in an equivalent manner to the CAI domain within Drosophila
Cnn. Clearly, CDK5RAP2 must somehow be prevented from binding cytosolic γ-TuRCs, and
this will have to be addressed in future.

While we have focussed on how the binding between Cnn-C and γ-tubulin complexes is
regulated during cell division, our data also highlights differences in how binding is regulated
between cell types and MTOCs. We have shown both here and previously (Tovey et al., 2018)
that the testes specific Cnn-T isoform, which lacks the CAI domain, binds efficiently to γ-tubulin
complexes in the apparent absence of any upstream regulatory events. Cnn-T is expressed
primarily within developing sperm cells and isoform-specific C-terminal exons mediate its
recruitment to mitochondria, where it binds and recruits γ-tubulin complexes (Chen et al.,
2017). The mitochondrial surface is very different from mature centrosomes, which
concentrate a selection of kinases, including Polo. It therefore seems appropriate that Cnn-T
isoforms splice out the exons that comprise the CAI domain to ensure that phosphorylation is
not required for γ-tubulin complex binding. Presumably, binding and potential activation of γ-
tubulin complexes within the shrinking cytosol of developing sperm cells is not detrimental to
sperm development (and may even be important for amplifying cytoplasmic microtubules),
unlike in dividing cells where our data shows that spindle formation and cytokinesis are clearly
perturbed.

Intriguingly, our data suggest that Cnn-T binds γ-tubulin complexes with a higher affinity than
a truncated version of Cnn-C lacking the inhibitory CAI domain. This shows that the Cnn-T
specific extreme N-terminal exon (red in Figure 1A) promotes binding. It also suggests that
binding within sperm cells might be stronger than binding at centrosomes within dividing cells.
Our preferred interpretation, however, is that the CAI domain, once unfolded from the CM1
domain, may promote γ-tubulin complex binding once its inhibitory role has been relieved,
possibly by direct binding or by binding to other proteins that support γ-tubulin complex
binding. This is consistent with observations in budding yeast, where removal of SPC110’s N-
terminal region up to the CM1 domain reduces its binding affinity to γ-TuSCs (Lyon et al.,
2016).

There is currently some debate as to how the binding of CM1-domain proteins influences the
activity of γ-tubulin complexes. There is good evidence from different labs that expression of
human N-terminal CDK5RAP2 fragments containing either a part of or the whole CM1 domain results in random microtubule nucleation throughout the cytosol (Choi et al., 2010; Muroyama et al., 2016; Hanafusa et al., 2015). This effect is dependent on the ability of these fragments to bind γ-tubulin complexes within the cytosol (Choi et al., 2010) and on LRKK1 kinase, which facilitates binding (Hanafusa et al., 2015). Thus, binding of cytosolic γ-tubulin complexes by CDK5RAP2 appears to promote the ability of the γ-tubulin complexes to nucleate microtubules. This is also true in fission yeast, where a truncated form of Mto1 lacking MTOC localisation domains but containing the CM1 domain formed puncta within the cytosol that nucleated microtubules (Lynch et al., 2014). In Drosophila, ectopic expression of Cnn-T in cultured fly cells converts mitochondria to MTOCs capable of nucleating microtubules (Chen et al., 2017). Based on these studies, the CM1 domain has been regarded as an activator of γ-tubulin complexes. Two recent papers, however, reported only a small increase in microtubule nucleation from purified human or Xenopus γ-TuRCs after addition of CM1-domain fragments (Liu et al., 2019; Thawani et al., 2020). Moreover, recent cryo-EM data shows no evidence for structural changes when purified human or Xenopus γ-TuRCs are bound by CM1-domain fragments (Wieczorek et al., 2019; Liu et al., 2019). Nevertheless, one of these studies did show that adding CM1-domain fragments to purified γ-TuRCs within Xenopus egg extracts supplemented with activated Ran resulted in a dramatic increase in microtubule aster formation (Liu et al., 2019), suggesting that specific conditions may be required for the activating ability of the CM1 domain. Taken together, the data from different reports suggest that binding of the CM1 domain can increase the propensity of γ-tubulin complexes to nucleate microtubules, but how this occurs and whether it is context specific remains unclear.

The idea that CM1-domain binding may promote γ-tubulin complex activity in a context-specific manner is consistent with our in vivo data showing that ectopic binding of cytosolic γ-TuRCs by Cnn leads to major defects during male meiosis but apparently to less severe defects in embryos and no obvious polarity defects in oocytes. These differences, however, could be due to the observed differences in expression levels of pUbq-Cnn-C at between cell types. They may also be due to variation in the ability of different cell types to cope with increased cytosolic microtubule nucleation. For example, signals that control cleavage plane positioning and furrow ingression during cytokinesis are mediated by direct contacts between both astral microtubules and central spindle microtubules and the cell cortex (D’Avino et al., 2015). It is easy to imagine how ectopically nucleated microtubules, that could interact at random with the cell cortex, could interfere with cytokinesis of spermatocytes during...
Drosophila male meiosis. Oocytes that already contain a large number of microtubules throughout the cytosol and early embryos where nuclei divide within a syncytium may be less severely affected.

In summary, we have revealed molecular details that help explain how microtubule nucleation is spatially controlled to ensure fidelity of cell division and development. Our work encourages future research to investigate how binding of γ-tubulin complexes by CM1-domain proteins is regulated in other species and how this binding leads to microtubule nucleation.
**Materials and Methods**

**DNA cloning**
5-alpha competent *E. coli* cells (high efficiency, NEB) were used for bacterial transformations.
DNA fragments were purified using QIAquick Gel Extraction kits (Qiagen); plasmid purification was performed using QIAprep Spin Miniprep kits (Qiagen). Phusion high-fidelity PCR master mix with HF buffer (ThermoFisher Scientific) was used for PCRs.

**Transgenic Drosophila lines**
All endogenously-tagged lines were made using CRISPR combined with homologous recombination, by combining the presence of a homology-repair vector containing the desired insert with the appropriate guide RNAs and Cas9. The γ-tubulin37C-mCherry and Grip128-sfGFP alleles were generated by inDroso. For γ-tubulin37C-mCherry, eggs from nos-Cas9 expressing females were co-injected with a plasmid encoding the expression of dual guides targeting each side of the 3'UTR, TACACATATCAAGATACATG and CCCAGATCGATTATCCCCAG, and a plasmid containing a SSSS-mCherry-3'UTR-LoxP-3xP3-dsRED-Lox P cassette flanked by homology arms (the multi-serine insert acts as a flexible linker). After screening for dsRED, the selection marker was excised by Cre recombination. For Grip128-sfGFP, eggs from nos-Cas9 expressing females were co-injected with a plasmid encoding the expression of a single guide containing the target sequence ATGGGGCACACTGGAGTTGA and a pBluescript plasmid containing sfGFP and linker sequence (4X GlyGlySer) flanked on either side by 1.5kb of DNA homologous to the genomic locus surrounding the 3’ end of the coding region. The homology vector was made within the lab (and sent to InDroso) by HiFi assembly (NEB) of PCR fragments generated from genomic DNA prepared from nos-Cas9 flies (using MicroLYSIS, Microzone) and a vector containing the sfGFP tag (DGRC, 1314). Screening for the insert was performed with the following primers: AGGAAGATGCGAACACGT and GTACAGCTCATCCATGCCC.

The Grip75-sfGFP and Grip163-sfGFP lines were made within the lab following a similar approach to that used previously (Tovey et al., 2018; Mukherjee et al., 2020). Flies expressing a single guide RNA containing the target sequence CAAAAACATCGTATTCTAG or ACCACTATTACAAGGTATCT for Grip75-sfGFP or Grip163-sfGFP, respectively, were crossed to nos-Cas9 expressing females and the resulting embryos were injected with homology vectors by the Department of Genetics Fly Facility, Cambridge, UK. The homology vectors comprised a pBluescript plasmid containing sfGFP and linker sequence (4X GlyGlySer).
GlyGlySer) flanked on either side by 1.5kb of DNA homologous to the genomic locus surrounding the 3' end of the appropriate coding region. The homology vectors were made as for Grip128-sfGFP. F1 and F2 males were screened by PCR using the following primers: for Grip75-sfGFP: GAGAAGTTTGCGCATATGACCC and AGCAGCACCATGTGATCGCGC; for Grip163-sfGFP: AGTCGCAGTCCTTTATTGTGG and AGCAGCACCATGTGATCGCGC.

pUbq-Cnn-C and pUbq-Cnn-C\(^{\dagger}\) were made from a pDONR-Cnn-C vector (gift from Jordan Raff). To generate a Cnn-T-specific N-terminal region of Cnn, an appropriate DNA fragment (made by Genewiz, based on the FlyBase sequence of Cnn-T) was synthesised and amplified by PCR and used to replace the N-terminal region of Cnn in a pDONR-Cnn-C vector cut with Xmal. The pDONR-Cnn-C and newly made pDONR-Cnn-T vectors were then inserted into a pUbq transformation vector (gift from Jordan Raff) by Gateway cloning (ThermoFisher Scientific). All DNA vectors were injected into embryos by the Department of Genetics Fly Facility, Cambridge, UK.

**Recombinant protein expression and purification**

Fragments of Cnn-C-N and Cnn-T-N were amplified from the pDONR-Cnn-C and pDONR-Cnn-T vectors described above by PCR and inserted into a pDEST-HisMBP (Addgene, #11085) vector by Gateway cloning (Thermo Fisher Scientific). Proteins were expressed in *E. coli* (BL21-DE3) and purified using affinity chromatography. MBP-tagged fragments were purified by gravity flow through amylose resin (New England Biolabs) and step elution in maltose. The concentration of each fraction was determined on a Nanodrop and peak fractions were diluted 1:1 with glycerol and stored at -20°C.

Phosphomimetic fragments were created by modifying the pDONR-Cnn-C-N entry clone. The backbone was linearised by PCR or by digestion, omitting the phospho-patch to be replaced, and phosphomimetic patches in which all S/T residues were swapped for D/E residues, respectively, were synthesised by PCR using two overlapping primers and were inserted by NEB HiFi Assembly. The entry clones were checked by restriction enzyme digest and sequencing before being inserted into the pDEST-HisMBP destination vector via a Gateway reaction.

Truncated fragments of Cnn-C were made by modification of the pDONR-Cnn-C-N entry clone. The N-terminal region was removed by a Quikchange reaction (Agilent), and the
resulting shortened fragment was inserted into the pDEST-HisMBP destination vector via a Gateway reaction.

pRNA vectors were made by modification of the pDONR-Cnn-C-PReM\textsuperscript{p} vector containing phospho-mimetic mutations in the PReM domain (Conduit et al., 2014a). N-terminal variants were introduced by restriction digests (SspI-HF and AatII) of pDONR-Cnn-C, pDONR-Cnn-T, and Cnn-C-PReM\textsuperscript{p} entry clones. Fragments were combined as necessary by NEB HiFi assembly to create new pDONR vectors, which were inserted into a pRNA-GFP or pRNA-mKate destination vector (Conduit et al., 2014a) via a Gateway reaction. The Cnn-T-N fragment was inserted directly into pRNA-GFP destination vectors via Gateway cloning.

**Primers**

|                | Forward primer                                | Reverse primer                                |
|----------------|-----------------------------------------------|-----------------------------------------------|
| Cnn-C-N fragment | GGGGACAAGTTTGTACAAAAAAA GCAGGCTTAATGGGACCAGTCTA AACAGGTTTGC | GGGGACCACCTTTGTACAAGAAAACCTGGGTCTATAGGCGCTCGGCC AAC |
| Cnn-T-N fragment | GGGGACAAGTTTGTACAAAAAAA GCAGGCTTAATGAATAGTAATC GAACGTGCCTTCG | GGGGACCACCTTTGTACAAAGGAAG CTGGGTCTATAGGCGCTCGGCC AAC |
| Cnn-C-N\textsuperscript{P1} insert | GCAGGACTATTGCGGCGACGG CAATGGTACCTGTGCAAGACGAC TGGAAGGAAATCGGATTAATGG AGGAGGTGG | GCAGGACCTTTCTGTGGATTTTGC GCAGGCCATTTCTCTCCAGGAA GTCCTCCACCTCTCAATTTA CAGTATCC |
| Cnn-C-N\textsuperscript{P2} insert | CCTGCAGCAAACTAGCGCGAGGC ACTGGACAAAGACATAGACGAC GAGGCCGGGAGCGCCTGCAA GATGCTG | GCCTGGAAGGTGCTGGAAACGTG GCAGGCCATTTCTCTCCAGGAA GTCCTCCACCTCTCAATTTA CAGTATCC |
| Cnn-C-N\textsuperscript{P3} insert | GGGTCAGCGCGGCGGGTCCCAGGC AGACGACGACGAGGAAGACTTA GACACACAGTCATCGATGCCA AGATCGAAATCGC | CTTTGAAGACATCCATCTTTTACA TCGACCTCTTCTCCTAAGTGTC GCAGGCCATTTCTCTCCAGGAA GATTTTGAGATGCGATCGATGA GC |
| Cnn-C-N\textsuperscript{P1a} insert | GCAGGACTATTGCGGCGACGG CAATGGTACCTGTGCAAGACGAC TGGAAGGAAATCGGATTAATGG AGGAGGTGG | GCAGGACCTTTCTGTGGATTTTGC GCAGGCCATTTCTCTCCAGGAA GATTTTGAGATGCGATCGATGA GC |
| Cnn-C-N<sup>i1b</sup> insert | GCGGGACTATTGCAGGCGACGGGCAATGGTACCTGTGCTGATCGTCCTTGAAGGAAAATCACCTTAATTGG | GGACCCTTCTGTGATTTCGGCGGCGCCATTCTCCTCAATTTAGTATTGCTCCCTTC | Cnn-C-N<sup>i27</sup> insert | GCGGGACTATTGCAGGCGACGGGCAATGGTACCTGTGCTGATCGTCCTTGAAGGAAAATCACCTTAATTGG | GGACCCTTCTGTGATTTCGGCGGCGCCATTCTCCTCAATTTAGTATTGCTCCCTTC | Cnn-C-N<sup>i51-77</sup> insert | GCCAATTTGTACAAAAAAGCAAGCTTAATGGGCGAATTGTTACAGTTTACGAGTTTGGCAGTTTCCTTC | GGAACGTCERRAAAAAGCAGCCTGCTTTTTGTACAAAAAGCAAGCTTAATGGGCGAATTGTTACAGTTTACGAGTTTGGCAGTTTCCTTC |
|-------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------|-------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------|-------------------------------|---------------------------------------------------------------------------|-----------------------------------------------------------------|

**Immunoprecipitation**

1g/ml of embryos were homogenised with a hand-pestle in homogenisation buffer containing 50 mM HEPES, pH7.6, 1mM MgCl<sub>2</sub>, 1 mM EGTA, 50 mM KCl supplemented with PMSF 1:100, Protease Inhibitor Cocktail (1:100, Sigma Aldrich) and DTT (1M, 1:1000). Extracts were clarified by centrifugation twice for 15 minutes at 16,000 rcf at 4°C.

For the MBP-Cnn fragment IPs, 30 μl magnetic ProteinA dynabeads (Life Technologies) coupled to anti-MBP antibodies (gift from Jordan Raff) were incubated with an excess of purified MBP-Cnn fragments and rotated for 1 hour at 4°C. Unbound fragments were washed off in PBST, and the saturated beads were resuspended in 100 μl embryo extract and rotated at 4°C overnight. Beads were washed 5 times for 1 min each in PBST, boiled in 2x sample buffer, and separated from the sample using a magnet. Samples were analysed by western blotting as described below.

For the Grip-GFP IPs, 20 μl high-capacity ProteinA beads (Abcam) coupled to anti-MBP antibodies (gift from Jordan Raff) were incubated with an excess of purified MBP-Cnn fragments and rotated at 4°C for 1 hour. Unbound fragments were washed off in PBST and the saturated beads were resuspended in 65 μl embryo extract and rotated at 4°C overnight. Beads were washed 5 times for 1 min each in PBST, boiled in 2x sample buffer, and separated from the sample by centrifugation. Samples were analysed by western blotting as described below.
For the IPs from pUbq-Cnn-C and pUbq-Cnn-C\(^T\) embryo extract, 50 µl magnetic ProteinA dynabeads (Life Technologies) coupled to anti-Cnn (C-terminal) antibodies (gift from Jordan Raff) were rotated at 4°C for 1 hour. Unbound fragments were washed off in PBST and the saturated beads were resuspended in 100 µl embryo extract and rotated at 4°C overnight. Beads were washed 5 times for 1 min each in PBST, boiled in 2x sample buffer, and separated from the sample using a magnet. Samples were analysed by western blotting as described below.

**Electrophoresis and western blotting**

Samples were run on 4-20% TGX Precast Gels (BioRad) (except Figure 5C and D, in which samples were run on 7.5% TGX Precast gels (BioRad)), alongside 5µl Precision Plus WesternC Standard markers (BioRad). For western blotting, semi-dry blotting was carried out using TransBlot Turbo 0.2µm nitrocellulose membrane transfer packs (BioRad), and a TransBlot Turbo transfer system running at 1.3A, up to 25V, for 7 minutes (BioRad mixed molecular weight pre-set programme). Membranes were stained with Ponceau, washed, first with distilled water then with milk solution (PSBT + 4% milk powder), and then blocked in milk solution for 1 hour at room temperature. Sections of blots were incubated with primary antibodies as indicated in figures (antibodies found in table). Blots were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-sheep secondary antibodies (1:2000 in PSBT + 4% milk powder, ImmunoReagents) as appropriate for 45 mins at room temperature, washed in PSBT for 3 times for 15 mins each, and then incubated with ECL substrate (BioRad ECL Clarity or ThermoFisher SuperSignal West Femto Max) for 5 minutes. Membranes were imaged using a Kodak Image Station 4000R.

**Mass spectrometry**

Samples were run 1 cm into the lanes of 4-20% TGX Precast Gels (BioRad). Gels were rinsed in dH\(_2\)O and lanes were excised using a clean razor blade and cut into 1mm\(^2\) pieces on a fresh glass slide, destained if necessary, reduced (DTT) and alkylated (iodoacetamide) and subjected to enzymatic digestion with sequencing grade trypsin (Promega, Madison, WI, USA) overnight at 37°C. After digestion, the supernatant was pipetted into a sample vial and loaded onto an autosampler for automated LC-MS/MS analysis.

**LC-MS/MS.** All LC-MS/MS experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific Inc, Waltham, MA, USA) system and a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). Separation
of peptides was performed by reverse-phase chromatography at a flow rate of 300 nL/min using a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2 μm particle size, 100Å pore size, 75 μm i.d. x 50cm length). Peptides were initially loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5 μm particle size, 100Å pore size, 300 μm i.d. x 5mm length) from the Ultimate 3000 autosampler with 0.1% formic acid for 3 minutes at a flow rate of 10 μL/min. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1% formic acid and solvent B was 80% acetonitrile, 20% water + 0.1% formic acid. The linear gradient employed was 2-40% B in 30 minutes. Further wash and equilibration steps gave a total run time of 60 minutes.

The LC eluant was sprayed into the mass spectrometer by means of an Easy-Spray source (Thermo Fisher Scientific Inc.). All m/z values of eluting ions were measured in an Orbitrap mass analyzer, set at a resolution of 70000 and was scanned between m/z 380-1500. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD, NCE:25%) in the HCD collision cell and measurement of the resulting fragment ions was performed in the Orbitrap analyser, set at a resolution of 17500. Singly charged ions and ions with unassigned charge states were excluded from being selected for MS/MS and a dynamic exclusion window of 20 seconds was employed.

**Database searching.** Post-run, all MS/MS data were converted to mgf files and the files were then submitted to the Mascot search algorithm (Matrix Science, London UK, version 2.6.0) and searched against the Uniprot Drosophila_melanogaster_20180813 database (23297 sequences; 16110808 residues) and common contaminant sequences containing non-specific proteins such as keratins and trypsin (123 sequences; 40594 residues). Variable modifications of oxidation (M), deamidation (NQ) and phosphorylation (S,T and Y) were applied as well a fixed modification of carbamidomethyl (C). The peptide and fragment mass tolerances were set to 20ppm and 0.1 Da, respectively. A significance threshold value of p<0.05 and a peptide cut-off score of 20 were also applied.

**Antibodies**

Primary antibodies used in the study are indicated in the table below. For western blotting, primary and secondary antibodies were diluted in PBST + 4% milk; primary antibodies were diluted at concentrations indicated in the table; secondary antibodies were diluted at 1:2000.
For immunostaining, primary and secondary antibodies were diluted in PBS + 0.1% Triton (PBT) + 5% BSA; primary antibodies were diluted at concentrations indicated in the table; secondary antibodies (AlexaFluor 488, 561, or 633 conjugated secondary antibodies (ThermoFisher)) were diluted at 1:1000 for testes and 1:1500 for embryos. DNA was stained with Hoechst (Life Technologies, 33342) or DAPI.

| Antibody                  | WB Concentration | IF Concentration | Source                 |
|---------------------------|------------------|------------------|------------------------|
| α-Tubulin mouse monoclonal| -                | 1:1000           | Sigma Aldrich, DM1a    |
| Asl (N-terminal) guinea pig polyclonal | 1:1000           | 1:1000           | Gift from Jordan Raff  |
| Cnn (N-terminal) rabbit monoclonal | 1:1000           | 1:1000           | Gift from Jordan Raff  |
| Cnn (C-terminal) sheep polyclonal | 1:1000           | -                | Gift from Jordan Raff  |
| Cnn-TN^Rabbit polyclonal | 1:500            | -                | This study             |
| γ-Tubulin mouse monoclonal| 1:500            | 1:500            | Sigma Aldrich, GTU-88  |
| γ-Tubulin rabbit polyclonal| -                | 1:500            | Sigma Aldrich, T5192   |
| GFP mouse monoclonal      | 1:250 or 1:500   | 1:250 or 1:500   | Roche, 11814460001    |
| Grip71 rabbit polyclonal  | 1:100            | 1:100            | CRB (crb2005268)      |
| MBP rabbit polyclonal     | 1:3000           | -                | Gift from Jordan Raff  |
| Phospho-histone H3 rabbit polyclonal | -                | 1:500            | Abcam, AB5176         |
| Staufen Mouse monoclonal  | -                | 1:100            | Santa Cruz dN-16      |
| Gurken Mouse monoclonal   | -                | 1:200            | DSHB 1D12             |
| Lamin Dm0                 | -                | 1:30             | DSHB 84.12            |

**Immunostaining**

Testes were dissected in PBS, fixed in 4% paraformaldehyde for 30 minutes, washed 3x 5 minutes in PBS and incubated in 45% and then 60% acetic acid before being squashed onto slides and flash-frozen in liquid nitrogen. Coverslips were removed and samples were post-fixed in methanol at -20°C, washed 3x 15 minutes in PBS + 0.1% Triton (PBST), then
incubated overnight in a humid chamber at 4°C with primary antibodies diluted in PBST + 5% BSA + 0.02% azide. Slides were washed 3x 5 minutes in PBST and then incubated for 2 hours at room temperature with Alexa-Fluor secondary antibodies (ThermoFisher) (all 1:1000 in PBST + 5% BSA + 0.02% azide). Slides were washed 3x 15 minutes in PBST, 10 minutes in PBST with Hoechst, and then 5 minutes in PBST. 10 μl of mounting medium (85% glycerol in H2O + 2.5% N-propyl-galate) was placed on top of the tissue and a coverslip was gently lowered and sealed with nail varnish.

Embryos were collected within 2-3 hours of laying and were dechorionated in 60% bleach for 2 minutes. Vitelline membranes were punctured with a combination heptane and methanol + 3% EGTA (0.5M) before three washes in neat methanol. Embryos were fixed in methanol at 4°C for at least 24 hours before rehydrating. Embryos were rehydrated by washing 3x 20 mins in PBST, then blocked in PBST + 5% BSA for 1 hour, followed by overnight incubation in primary antibodies in PBST + 5% BSA at 4°C. Embryos were washed 3x 20 mins in PBST at room temperature, then incubated for 2 hours at room temperature with Alexa-Fluor secondary antibodies (ThermoFisher) (all 1:1500 in PBST + 5% BSA). Finally, embryos were washed 3x 20 mins in PBST at room temperature before being mounted in Vectashield containing DAPI (VectorLabs).

Oocytes were dissected from 2-day-old females. For Staufen and Gurken detection, 10 to 15 ovaries were fixed with PBS buffer containing 4% paraformaldehyde and 0.1% Triton X-100, washed three times for 5 mins in PBST (PBS+0.1% Triton X-100) and blocked in PBST containing 1% BSA. Incubation with the primary antibodies (anti-Staufen, Santa Cruz; anti-Gurken 1D12, DSHB) was performed overnight at room temperature or 4°C for Staufen and Gurken labelling, respectively, in PBT (PBS containing 0.1% BSA and 0.1% Tween 20). The ovaries were then briefly washed three times and three times for 30 min each in BBT and incubated for 2 hours at room temperature in Alexa-conjugated secondary antibodies. The ovaries were then washed 3x for 15 min each time in PBST, dissected, and mounted in Citifluor (Electron Microscopy Science).

**Phase contrast imaging of round spermatids**

For analysis of round spermatids under phase contrast, testes were dissected in PBS, transferred to a 50μl droplet of PBS on a slide, cut open midway along the testes and, under observation, gently squashed under a coverslip using blotting paper.
RNA preparation and injection

pRNA vectors containing the appropriate cDNA were generated using Gateway cloning of PCR amplified cDNA and either a pRNA-GFP or a pRNA-mKate backbone. pRNA vectors were linearised with Ascl, precipitated using EDTA, sodium acetate, and ethanol, then resuspended in RNase-free water. mRNA was generated from these pRNA vectors in vitro using a T3 mMESSAGE mMACHINE kit (ThermoFisher) and was then purified using an RNasy MinElute Cleanup kit (Qiagen). Freshly-laid early embryos, or unfertilised eggs, were collected from apple juice plates within 15-30 minutes of laying and were dechorionated on double-sided sticky tape. Embryos/eggs were lined up on heptane glue to keep them in place during injections and imaging. Embryos were dried at 25°C for 5 mins and covered with immersion oil (Voltalef). RNA was injected into embryos/eggs using needles made from borosilicate glass capillary tubes, at a final concentration of ~2 μg/μl. Embryos/unfertilised eggs were left for 1-2 hours before imaging to allow for translation of the mRNA.

Microscopy

Confocal imaging of Cnn scaffolds organising microtubules and of fixed embryo and testes samples was carried out on an Olympus FV3000 scanning inverted confocal system run by FV-OSR software using a x60 1.4NA silicone immersion lens (UPLSAPO60xSilicone) or x30 0.95NA silicone immersion lens (UPLSAPO30xSilicone). Confocal imaging of oocytes was carried out on a Zeiss LSM700 confocal microscope. Epifluorescence microscopy of Cnn scaffolds recruiting γ-TuRC components and phase contrast microscopy of round spermatids was performed on a Leica DM IL LED inverted microscope controlled by μManager software and coupled to a RetigaR1 monochrome camera (QImaging) and a CoolLED pE-300 Ultra light source using a 63X 1.3NA oil objective or a 40X 0.55NA air objective, respectively.

Fertility tests

Cages that were sealed with apple juice agar plates with a spot of dried yeast paste were set up at 25°C containing ~50 newly-hatched test flies (e.g. pUbq-Cnn-C/-C+) and ~50 newly-hatched wild-type males or virgin females. The apple juice agar plates were exchanged with fresh plates 2-4 times a day, and the removed plates were kept at 25°C for at least 25 hours before the proportion of hatched eggs was calculated.

Image analysis

All images were processed using Fiji (ImageJ). Maximum intensity Z-plane projections were used to quantify the intensity of Cnn and γ-TuRC components at Cnn scaffolds, and intensities
of each channel at each scaffold were corrected for cytoplasmic background intensity. Background intensity subtraction was also performed for quantification of western blot band intensities. Within each experiment, the intensities of the γ-tubulin IP bands were normalised to the intensity of the γ-tubulin band in the MBP-Cnn-T-N IP. Images of Cnn scaffolds organising microtubules within eggs and of stained pUbq-Cnn-C or pUbq-Cnn-CT embryos were analysed blind to allow unbiased categorisation.

**Statistical analysis**

Most statistical analysis and all graph production were performed using GraphPad Prism 7 or 8. Analysis of Cnn scaffolds was carried out in the R programming language (https://www.r-project.org/) using the emmeans package. The fluorescent signals at Cnn scaffolds were collected from multiple embryos but included measurements from multiple scaffolds from individual embryos. For linear regression analysis we therefore needed to use a mixed effects model to take account of the ‘repeated’ measurements from individual embryos. The raw data was not Normally distributed and was log transformed prior to performing the linear regression analysis. An iterative process was used to explore the best model, which revealed that a straight-line relationship best fitted the raw data and that regression lines should pass through the origin (0;0). This could have been expected given the biological prediction that an increase in Cnn intensity should result in a linear increase in the intensity of a γ-TuRC component. For the final model, we transformed the data by taking the log of the γ-tubulin/Cnn ratio. The graphs plotted for scaffold data are presented with raw data points with an overlay of the line of best fit calculated by the linear mixed effects model. In the case where more than two lines were being compared, Tukey’s Test was used to compare the pairwise differences.

**Bioinformatics**

Protein alignments were produced using JalView. Secondary structure predictions were performed using JPred 4.

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**Author Contributions**

PTC and CAT designed the study and wrote the manuscript. CAT carried out cloning for all
experiments and performed the *in vitro* recruitment assays, fertility tests, embryo analysis, and
round spermatid analysis. CT performed the majority of mRNA injection experiments and
analysed the scaffold data. AE helped establish the mRNA assay. AG performed the oocyte
analysis and analysed the data. FB and AG generated the γ-tubulin-mCherry fly line. MDR
prepared bacterial cultures and assisted with protein purification.

**Declaration of Interests**

The authors declare no competing interests.

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**Figure Legends**

**Figure 1**

**The extreme N-terminal region of Cnn-C inhibits binding to γ-tubulin complexes.** (A) Diagram of the centrosomal Cnn (Cnn-C) and testes-specific Cnn (Cnn-T) isoforms that exist in vivo. (B) Diagram of artificial Cnn proteins with differing N-terminal regions that were used to form Cnn scaffolds (induced by phospho-mimetic mutations in the PReM domain (beige)) via mRNA injection into unfertilised eggs. (C-E) Fluorescence images of unfertilised eggs expressing γ-tubulin37C-mCherry that were injected with mRNA encoding the different types of artificial Cnn protein, as indicated. Insets show representative examples of individual scaffolds. (F) Graph showing fluorescence intensity measurements of γ-tubulin37C-mCherry and GFP-Cnn at Cnn scaffolds of different types (a.u., arbitrary units). Linear lines of best fit are shown. (G,H) Western blot (G) and quantification of γ-tubulin bands (H) showing immunoprecipitation of γ-tubulin from embryo extracts by bacterially-purified, MBP-tagged Cnn-C-N, Cnn-T-N and Cnn-C<sup>41-77</sup>-N fragments. The blot was probed with anti-MBP and anti-γ-tubulin antibodies, as indicated. Band intensities were normalised within each of 3 experimental repeats to the γ-tubulin band in the respective MBP-Cnn-T-N IP.

**Figure 2**

**Multiple γ-TuRC-specific proteins are recruited to Cnn-T scaffolds.** Fluorescence images (A,B,D,E,G,H) and graphs (C,F,I) showing recruitment of Grip75-sfGFP (A-C), Grip128-sfGFP (D-F), or Grip163-sfGFP (G-I) to either mKATE-Cnn-C or mKATE-Cnn-T scaffolds. Insets in (A,B,D,E,G,H) show representative examples of individual scaffolds. Fluorescence values in (C,F,I) are arbitrary units (a.u.). Linear lines of best fit are shown. In each case, the mKATE-Cnn-T scaffolds recruited more of the sfGFP-tagged Grip protein than the mKATE-Cnn-C scaffolds.

**Figure 3**

**Cnn-T scaffolds organise microtubules more robustly than Cnn-C scaffolds.** (A-C) Fluorescence images (A,B) of Cnn-C scaffolds (A) or Cnn-T scaffolds (B) formed within eggs expression the microtubule marker Jupiter-mCherry. (C) Bar-graph showing quantification of a blind categorisation of eggs containing Cnn-C or Cnn-T scaffolds with regard to the ability of the scaffolds within the eggs to organise microtubule asters. The results show that Cnn-T scaffolds tend to organise microtubule asters more readily than Cnn-C scaffolds. (D)
Fluorescence images showing that adjacent Cnn-T scaffolds can organise spindle-like structures.

**Figure 4**

Phosphorylation of the CAI domain relieves auto-inhibition to allow binding to γ-tubulin complexes. (A) A hypothesis of how the CM1 domain (purple) might be sterically inhibited by folding back of the CAI domain (dark blue). Putative phospho-patches (containing high concentrations of Serine and Threonine residues) are indicated in yellow. The amino acid sequence of each putative phospho-patch is displayed with S and T residues highlighted in orange and the proportion of S and T residues within each patch indicated in brackets. Potential Polo kinase sites (green), Cdk1 sites (blue), and kinase landing sites (black) are indicated. (B-D) Western blots (B,C) and quantification of γ-tubulin bands (D) showing the immunoprecipitation of γ-tubulin from embryo extracts by bacterially-purified MBP-tagged N-terminal fragments of Cnn, as indicated. The blot was probed with anti-MBP and anti-γ-tubulin antibodies, as indicated. Band intensities were normalised within each experiment to the γ-tubulin band in the respective MBP-Cnn-T-N IP, as in Figure 1G. On average, the band intensities of γ-tubulin from the MBP-Cnn-C-N, MBP-Cnn-C-NP1, and MBP-Cnn-C-NT27 IPs were ~18%, 44%, and 34%, respectively, in comparison to the average band intensity of γ-tubulin from the MBP-Cnn-T-N IP. Horizontal lines represent the mean.

**Figure 5**

Mis-expression of the Cnn-T N-terminus results in cell division defects. (A) Diagram of normal Cnn-C and chimeric Cnn-CT in which the unique N-terminus of Cnn-C (dark blue) is replaced by the unique N-terminus of Cnn-T (red). (B) Graph showing quantification of hatching rate of progeny from crosses of pUbq-Cnn-C or pUbq-Cnn-CT males and females to wild-type flies, as indicated. The mean proportion of embryos that hatched is shown, and error bars show 95% confidence intervals. (C) Western blots of protein extracts from embryos and testes of wild-type (WT), pUbq-Cnn-C, and pUbq-Cnn-CT flies as indicated. Blots were probed with anti-γ-tubulin, anti-Cnn-C (N-term), anti-Cnn-C (C-term), and anti-Cnn-TN antibodies as indicated. Endogenous Cnn-C is indicated by a black arrowhead; combined endogenous and pUbq-driven Cnn-C is indicated by a blue arrowhead in the pUbq-Cnn-C lanes. Cnn-CT is indicated by a red arrowhead; non-specific bands of approximately the same size as Cnn-CT are marked with asterisks. (D) Western blot showing results of anti-Cnn immunoprecipitation from embryo extracts expressing pUbq-Cnn-C or pUbq-Cnn-CT as indicated. Red arrowhead indicates Cnn-CT. Note that, given the low expression of pUbq-Cnn-CT within embryos, gel
loading of the IP lanes was adjusted to try to better balance the amount of Cnn protein. (E)

Graph showing quantification of defects in fixed embryos. Embryos were classified into
categories (during a blind analysis) based on whether they were perceived to be normal, or to
display severe, moderate or mild defects, based both on how widespread defects were and
the severity of individual defects. (F) Fluorescent images show either syncytial pUbq-Cnn-C
embryos (top panels) or pUbq-Cnn-C\textsuperscript{T} embryos (bottom panels) in either S-phase (left panels)
or M-phase (right panels). Images were selected to show extreme examples i.e. normal S-
phase and M-phase in pUbq-Cnn-C embryos and strongly perturbed S-phase, including the
presence of cytosolic microtubules, and M-phase in pUbq-Cnn-C\textsuperscript{T} embryos. (G) Phase
contrast images showing round spermatids from testes of flies expressing pUbq-Cnn-C or
pUbq-Cnn-C\textsuperscript{T}. Alterations in nucleus: nebenkern ratio (normally 1:1, asterisks in right panel)
and size (normally approximately equal) indicate defects in cytokinesis and karyokinesis. (H)
Graph showing quantification of the nucleus:nebenkern ratio (left panel) and variance in
nuclear diameter (right panel) in pUbq-Cnn-C and pUbq-Cnn-C\textsuperscript{T} testes. Error bars show
standard error of the mean. (I) Fluorescence images showing cytokinesis and karyokinesis
defects during meiosis in pUbq-Cnn-C\textsuperscript{T} testes. Microtubules (green, \textalpha-tubulin), centrosomes
(pink, Asterless), and DNA (blue) are marked. Cytosolic microtubules not associated with
centrosomes can be observed (arrows), suggesting ectopic cytosolic microtubule nucleation.
Supplementary Figure Legends

Figure S1

Diagrams of different Cnn constructs (omitting the tags) used in this study. (A) Diagram showing full-length Cnn constructs without modifications to the PReM domain. Cnn-C is the major centrosomal isoform in Drosophila. Cnn-T is the testes-specific isoform in Drosophila. Cnn-C\textsuperscript{T} represents an artificial form of Cnn-C in which the N-terminal region of Cnn-C (dark blue) has been replaced with the N-terminal region of Cnn-T (red). (B) Diagram showing Cnn constructs containing phospho-mimetic mutations in their PReM domain to drive scaffold formation \textit{in vivo}. The constructs differ only in their N-termini: Cnn-C\textsubscript{PReM}\textsuperscript{m} and Cnn-C\textsubscript{T}\textsubscript{PReM}\textsuperscript{m} each contain the Cnn-C or Cnn-T unique N-terminal region, respectively. Cnn-C\textsubscript{∆1-77} is a version of Cnn-C lacking its specific N-terminal extension region (the CAI domain). (C) Diagram showing bacterially-purified N-terminal fragments of different Cnn types. Each fragment contains the CM1 domain (purple); in addition, Cnn-C-N and Cnn-T-N contain unique N-terminal regions (dark blue and red respectively).

Figure S2

Bacterially-purified MBP-Cnn-T-N fragments immunoprecipitate \(\gamma\)-Tubulin Ring Complexes. (A) Western blot showing results of anti-MBP immunoprecipitation from embryo extracts expressing GFP-tagged Grip proteins (homologues of GCP4,5,6), either supplemented (+) or not supplemented (-) with MBP-Cnn-T-N, as indicated. Blots were probed with anti-GFP, anti-Grip71 and anti-\(\gamma\)-tubulin antibodies as indicated. When using MBP-Cnn-T-N, \(\gamma\)-tubulin and Grip71, as well as Grip75, 128, or 163, are co-immunoprecipitated. (B) Mass spectrometry results from IPs with MBP-Cnn-T-N showing the presence of various \(\gamma\)-TuRC components. Note that Mzt1 is not expressed within embryos. Results of a control experiment on Grip75-GFP embryo extract not supplemented with any MBP-Cnn-T-N fragment are also shown. Numbers indicate emPAI scores as a proxy for protein abundance. Grip84 (A) and Grip84 (E) represent two different isoforms of Grip84 (promoters 1 and 2 respectively).

Figure S3

Protein alignment of N-terminal regions of Cnn-C proteins from different \textit{Drosophila} species. The alignment was carried out in JalView keeping \textit{D. melanogaster} at the top with the closest related species in order below. Only the N-terminal regions of the proteins were used in the alignment (~1-255aa). Potential phosphorylation patches are highlighted in yellow,
with the proportion of S/T residues present in the *Drosophila melanogaster* sequence indicated in brackets. The CM1 domain is highlighted in purple. Red boxes and green arrows indicate α-helices and β-sheets based on predictions from JPred.

**Figure S4**
**Polarity is established normally in pUbq-Cnn-C{sup}T oocytes. (A-B)** Fluorescent images show localisation of Staufen protein in oocytes expressing pUbq-Cnn-C (A) or pUbq-Cnn-C{sup}T (B) at stages 8, 9 and 10, as indicated. (C) Fluorescent images show localisation of Gurken protein in oocytes expressing pUbq-Cnn-C or pUbq-Cnn-C{sup}T at stage 9. Localisation of Staufen and Gurken are not perturbed in oocytes expressing pUbq-Cnn-C{sup}T indicating that there are no major defects in microtubule organisation.

**Figure S5**
**Major spermatocyte defects are observed within testes from pUbq-Cnn-C{sup}T flies**
Fluorescence images showing cytokinesis and karyokinesis defects during meiosis in pUbq-Cnn-C{sup}T testes. Microtubules (green, γ-tubulin), centrosomes (pink, Asterless), and DNA (blue) are marked.

**Supplementary Videos**

**Video 1**
**Cnn-T scaffolds organise microtubule asters and can be mobile.** Movie showing Cnn-T scaffolds (green) organising microtubule asters (marked with Jupiter-mCherry (magenta)). A mobile scaffold (lower left) with an asymmetric microtubule aster can be seen moving through the cytosol.

**Video 2**
**Transient spindle-like structures can form between Cnn scaffolds.** Movie showing the formation and disappearance of a transient spindle-like structure between adjacent Cnn-T scaffolds (green). Microtubules are marked with Jupiter-mCherry (magenta).

**Video 3**
**Spindle-like structures organised by Cnn scaffolds can form in synchrony.** Movie showing the synchronous formation and disappearance of a multi-polar spindle-like array of microtubules that is subsequently organised by a nearby group of coalescing Cnn scaffolds (green). Microtubules are marked with Jupiter-mCherry (magenta)
**Video 4**

**Microtubules are robustly anchored to Cnn scaffolds.** Movie showing rare giant Cnn-T scaffolds (green). One scaffold can be seen rotating and dragging the microtubules, indicating that the microtubules are robustly attached to the scaffold, presumably via γ-TuRCs. Microtubules are marked with Jupiter-mCherry (magenta).

**Video 5**

**Expression of GFP-Cnn-T-N leads to the formation of dynamic microtubules within the cytosol of unfertilised eggs.** Left panel shows a movie displaying dynamic cytoplasmic microtubules formed upon injection of mRNA encoding GFP-Cnn-T-N into unfertilised eggs. Right panel shows a movie of an unfertilised egg injected with water alone (control). Microtubules are marked with Jupiter-mCherry (magenta).
Figure 1

A True Cnn isoforms

| Cnn-C isoform | Cnn-T isoform |
|---------------|---------------|
| N | Cnn-C | Cnn-T |
| 1 | CM1 | CM1 |

B Artificial Cnn constructs

| Cnn-C-PReM | Cnn-T-PReM | Cnn-C∆1-77-PReM |
|-------------|-------------|------------------|
| N | Cnn-C | Cnn-T | Cnn-C∆1-77 |
| 1 | CM1 | CM1 | CM1 |

induce scaffold formation spontaneously within cytosol

Cnn-C-PReM (Cnn-C scaffolds)  γ-tub37C-mCherry  merge

Cnn-T-PReM (Cnn-T scaffolds)  γ-tub37C-mCherry  merge

Cnn-C∆1-77-PReM (Cnn-C∆1-77 scaffolds)  γ-tub37C-mCherry  merge

Cnn-T-PReM (Cnn-T scaffolds)  γ-tub37C-mCherry  merge

Cnn-C-PReM (Cnn-C scaffolds)  γ-tub37C-mCherry  merge

F  γ-Tubulin37C-mCherry (a.u.)

G IP:

H relative γ-tubulin band intensity

IP:

MBP-Cnn-C-N  MBP-Cnn-T-N  MBP-Cnn-C∆1-77-N

anti-MBP

anti-γTub
**Figure 2**

(A) Cnn-C scaffolds, Grip75-GFP, merge

(B) Cnn-T scaffolds, Grip75-GFP, merge

(C) Grip75

(D) Cnn-C scaffolds, Grip128-GFP, merge

(E) Cnn-T scaffolds, Grip128-GFP, merge

(F) Grip128

(G) Cnn-C scaffolds, Grip163-GFP, merge

(H) Cnn-T scaffolds, Grip163-GFP, merge

(I) Grip163
Figure 3

A

B

C

D

Jupiter-mCherry
Cnn-T scaffolds

Cnn-C scaffolds

Cnn-T scaffolds

Cnn-C scaffolds

5 μm

5 μm

strong aster
medium aster
weak aster

tubulin overlay

no aster

0 20 40 60 80 100
% eggs
Figure 4

A

Phosphorylation in P1, P2 or P3 at centrosome relieves auto-inhibition?

Patch 1 (6/14)

Polo site?
Polo binding?

CM1 P3

Patch 2 (7/23)

CM1 domain?
Polo site?
Polo site?

Skiddagal

Patch 3 (7/23)

Cdk1 site?
Polo site?
Polo site?

CM1

B

IP:

anti-MBP
anti-γTub

C

IP:

anti-MBP
anti-γTub

D

relative γ-tubulin band intensity

| Sample       | Relative Intensity |
|--------------|-------------------|
| Cnn-C-N      | 0.1               |
| Cnn-T-N      | 0.2               |
| Cnn-C-NV1    | 0.3               |
| Cnn-C-NV2    | 0.4               |
| Cnn-C-NV3    | 0.5               |
| Cnn-C-NV1t   | 0.6               |
| Cnn-C-NV2t   | 0.7               |
| Cnn-C-NV3t   | 0.8               |

E

MBP-Cnn-C-NMBP-Cnn-T-NMBP-Cnn-C-N

P1

MBP-Cnn-C-N

P2

MBP-Cnn-C-N

P3

MBP-Cnn-C-N

P1-3

IP:

anti-MBP
anti-γTub

F

Cnn-C-NCnn-T-N

Cnn-C-N

T27

0.0

0.2

0.4

0.6

0.8

1.0

Relative γ-tubulin band intensity

| Sample       | Relative Intensity |
|--------------|-------------------|
| Cnn-C-N      | 0.1               |
| Cnn-T-N      | 0.2               |
| Cnn-C-NV1    | 0.3               |
| Cnn-C-NV2    | 0.4               |
| Cnn-C-NV3    | 0.5               |
| Cnn-C-NV1t   | 0.6               |
| Cnn-C-NV2t   | 0.7               |
| Cnn-C-NV3t   | 0.8               |
Figure 5

A

B

C

D

E

F

G

H

I

**Cnn-CT**

**Cnn-C**

| Females | Males |
|---------|-------|
| 0-1 week old | 0-1 week old |
| 1-2 week old | 1-2 week old |
| % hatching |

**embryos**

**testes**

| WT | pUbq Cnn-C | pUbq Cnn-CT | WT | pUbq Cnn-C | pUbq Cnn-CT |
|----|------------|-------------|----|------------|-------------|
| anti-Cnn-C (N-term) | | | | | |
| 150 kDa |
| anti-α-tub |
| anti-Cnn-C (C-term) |
| 150 kDa |
| anti-α-tub |
| anti-Cnn-TN |
| 150 kDa |
| anti-α-tub |

**D**

**Inputs**

**IP: anti-Cnn**

**anti-Cnn**

**anti-γ-Tub**

**E**

**% Embryos**

**F**

**pUbq-Cnn-C**

**pUbq-Cnn-CT**

**pUbq-Cnn-C**

**pUbq-Cnn-CT**

**anti-Cnn**

**anti-γ-Tub**

**G**

**Cnn-C**

**Cnn-C**

**H**

**Nucleus/Neighboring Ratio**

**I**

**pUbq-Cnn-C (control)**

**pUbq-Cnn-C**

**pUbq-Cnn-C**

**pUbq-Cnn-CT**

**successful cytokinesis**

**cytokinesis errors**

**cytokinesis failure**

**cytosolic microtubule nucleation?**

**meiotic DNA microtubules**

**centerosomes**
**Figure S2**

**A**

MBP-Cnn-T-N:

| Input | - | + |
|-------|---|---|

- anti-GFP
- anti-Grip-71
- anti-γ-tub

**B**

| UniProt entry | Protein       | G75, no fragment | G75 + Cnn-T-N | G128 + Cnn-T-N | G163 + Cnn-T-N |
|---------------|---------------|------------------|---------------|---------------|---------------|
| A0A0B4K6Z9    | Cnn           | -                | 4.50          | 15.63         | 13.03         |
| M9PDN9        | ytubulin 37C  | 0.45             | 24.45         | 63.16         | 39.41         |
| P29257        | ytubulin 23C  | -                | 1.04          | 1.90          | 1.43          |
| E1JJQ3        | Grip 84       | -                | 1.76          | 8.14          | 4.76          |
| Q8NOW7        | Grip 84       | -                | 1.54          | 7.44          | -             |
| Q6XKP8        | Grip 91       | -                | 1.74          | 4.45          | 3.54          |
| Q9W0K7        | Grip 75       | 0.07             | 0.88          | 3.57          | 2.54          |
| Q8VXU8        | Grip 128      | -                | 0.08          | 0.40          | 0.16          |
| Q8T5B3        | Grip 163      | -                | 0.24          | 1.09          | 0.24          |
| Q8VJ57        | Grip 71       | -                | 0.30          | 1.87          | 0.93          |
| X2JCP8        | Actin         | 40.63            | 17.87         | 22.66         | 32.21         |
Figure S4

A

| pUbq-Cnn-C | stage 8 | stage 9 | stage 10 |
|-----------|---------|---------|---------|
| lamin Dm0 |         |         |         |
| Staufen   |         |         |         |

B

| pUbq-Cnn-C\textsuperscript{T} | stage 8 | stage 9 | stage 10 |
|-------------------------------|---------|---------|---------|
| lamin Dm0                     |         |         |         |
| Staufen                       |         |         |         |

C

| pUbq-Cnn-C | pUbq-Cnn-C\textsuperscript{T} |
|------------|-------------------------------|
| stage 9    | stage 9                       |
| Gurken     | Gurken                        |
examples of spermatocytes within testes of pUbq-Cnn-C\textsuperscript{T} flies

- meiotic DNA
- centrosomes
- microtubules

Figure S5

- displaced DNA
- mis-segregated centrosome
- cytokinesis failure

10\textmu m