Multimodal microtubule binding by the Ndc80 kinetochore complex

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The Ndc80 complex is a key site of kinetochore-microtubule attachment during cell division. The human complex engages microtubules with a globular ‘head’ formed by tandem calponin-homology domains and an 80-amino-acid unstructured ‘tail’ that contains sites of phosphoregulation by the Aurora B kinase. Using biochemical, cell biological and electron microscopy analyses, we dissected the roles of the tail in binding of microtubules and mediation of cooperative interactions between Ndc80 complexes. Two segments of the tail that contain Aurora B phosphorylation sites become ordered at interfaces; one with tubulin and the second with an adjacent Ndc80 head on the microtubule surface, forming interactions that are disrupted by phosphorylation. We propose a model in which Ndc80’s interaction with either growing or shrinking microtubule ends can be tuned by the phosphorylation state of its tail.

The Ndc80 complex, a member of the conserved KMN (Knl1 complex, Mis12 complex, Ndc80 complex) network1, has been identified as a primary site of kinetochore-microtubule attachment2–5 and a necessary factor for the proper operation of the spindle-assembly checkpoint6–8. The complex is a heterotetramer of Ndc80 (also known as HEC1 in humans9), Nuf2, Spc24 and Spc25, each of which features a globular head domain and a coiled-coil stalk that mediates dimerization into subcomplexes: Ndc80 with Nuf2 and Spc24 with Spc25 (refs. 10,11). The tetramerization interface is formed by the dimerized coiled coils, resulting in an elongated, dumbbell architecture10–12. The Ndc80–Nuf2 coiled coil features a break halfway along its length that imparts flexibility by acting as a molecular hinge12, and which may also be a binding site for other factors13. The Spc24–Spc25 head, which has a unique fold14, mediates kinetochore association by direct interaction with the Mis12 linker complex15,16. The Ndc80–Nuf2 head is formed by the tandem association of calponin homology (CH) domains contributed by each protein17,18 and mediates microtubule binding17,18.

In addition to being dependent on the presence of the Ndc80–Nuf2 head, microtubule association in vitro and in vivo depends on the disordered N-terminal tail of the Ndc80 protein19,20. The tail can be phosphorylated on multiple residues by Aurora B kinase1,5,21, which reduces the affinity of the Ndc80 complex for microtubules in vitro1,18 and destabilizes improper kinetochore-microtubule attachments in vivo5,19,20,22. Although an unstructured, Aurora B–regulated tail is a conserved feature of the Ndc80 protein in all eukaryotes, the region is highly divergent in sequence and length, making functionally relevant features difficult to identify by bioinformatic methods.

We therefore set out to experimentally characterize in detail the role of this key 80-amino-acid region in the function of the human Ndc80 complex. We previously reported a subnanometer resolution cryo-EM reconstruction of an engineered version of the human Ndc80 complex featuring a shortened, rigid central coiled-coil rod domain, known as ‘bonsai’18, bound to the microtubule23, revealing tail-dependent oligomerization of the complex on the microtubule surface and details of the interaction between the Ndc80 CH domain and tubulin’s globular domains. Here we extend our analysis of the Ndc80 tail, delineating in detail its interactions with tubulin and the Ndc80–Nuf2 globular head when bound to the microtubule. Using biochemical, structural and cell-biological methods, we dissect the physiological role of specific Aurora B phosphorylation sites and provide support for a model in which dynamic phosphorylation of the Ndc80 complex by Aurora B can support directed chromosome motions during mitosis24 in addition to promoting proper attachment.

RESULTS

Dissection of Ndc80’s N-terminal tail

The primary sequence of the Ndc80 N-terminal tail comprises two distinct regions of Aurora B phosphorylation sites separated by a proline-rich linker (Fig. 1a). We refer to the cluster of phosphorylation sites between amino acids 41 and 80 as ‘zone 1’ and those between amino acids 1 and 20 as ‘zone 2’. We previously found that the tail has a role in the formation of phosphorylated Ndc80 complex clusters along microtubule protofilaments in vitro23, suggesting that this segment mediates protein-protein interactions between Ndc80 complex molecules in addition to contacting tubulin. To probe these two activities of the tail, we removed the tail from the context of the entire complex and fused it to glutathione S-transferase (GST) for primary structure-function analysis. All GST–Ndc80 tail peptides

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Identification of the microtubule-binding region

We first performed microtubule cosedimentation assays as described\(^{10,23}\). As had been reported previously for Ndc80 residues 1–80 in the absence of GST\(^{20}\), we found that the full-length tail (GST-Ndc80\(_{1–80}\)) exhibited significant interaction \((P = 8 \times 10^{-6}, t\text{-test})\) with microtubules independently of the CH domains of Ndc80 and Nuf2, when compared to the negative control of GST alone, which we found to not interact with microtubules (Fig. 1c,d). This interaction was due to residues 41–80, as both GST-Ndc80\(_{41–80}\) and GST-Ndc80\(_{21–80}\) bound microtubules as well as the full-length tail (GST-Ndc80\(_{1–80}\)) did in our assay, whereas GST-Ndc80\(_{1–40}\) and GST-Ndc80\(_{1–20}\) exhibited negligible microtubule-binding activity. GST-Ndc80\(_{41–60}\) exhibited weaker but still significant \((P = 0.008, t\text{-test})\) microtubule-binding activity, but GST-Ndc80\(_{61–80}\) did not, suggesting that residues from both of these regions are necessary for full binding.

We therefore made finer truncations of the region between Ndc80 residues 41 and 80 in 3-amino-acid steps and identified residues 47–68 as the minimal tubulin-binding region of the tail (Supplementary Fig. 2a–d), corresponding very closely to zone 1 of Aurora B phosphorylation sites. This is the most conserved region of the Ndc80 tail, with residues 49–61 exhibiting modest but detectable conservation in all eukaryotes except nematodes and insects and the Aurora B phosphorylation site at Ser55 exhibiting the greatest conservation (Supplementary Fig. 2e). Most species have prolines adjacent to this region (Fig. 1a and Supplementary Fig. 2e), suggesting that the architecture of the tail may be conserved although the sequence is not.

Phosphoregulated interaction between Ndc80 complexes

We next investigated the ability of the tail to interact with the Ndc80 complex in trans. Using GST pull-down assays, we could not detect an interaction between the tail and the Ndc80 complex (Supplementary Fig. 3), in agreement with the observation that the Ndc80 complex does not form clusters in the absence of microtubules\(^{23}\). We therefore turned to an indirect assay: we mixed microtubules with a bonsai complex lacking the tail (bonsai Ndc80\(_{1–80}\)) alone control. (d) SDS-PAGE of microtubule cosedimentation assays with GST–Ndc80 tail constructs. Tubulin, 3 µM, GST tails, 1 µM. (e) SDS-PAGE of microtubule cosedimentation assays with bonsai Ndc80\(_{1–80}\) in the presence of GST–Ndc80 tail constructs. Tubulin, 3 µM, bonsai Ndc80\(_{1–80}\), 0.5 µM, GST tails, 1 µM.
We next investigated whether the two zones of Aurora B phosphorylation sites differently regulated tail–tubulin binding and tail–Ndc80 complex microtubule binding, and by extension Ndc80 complex–microtubule binding and cluster formation, respectively. Nine Aurora B phosphorylation sites (Ser4, Ser5, Ser8, Ser15, Ser44, Thr49, Ser55, Ser62 and Ser69) have been reported in the Ndc80 tail19 (Fig. 1a), but only seven of these sites (Ser5, Ser8, Ser15, Ser44, Ser55, Ser62 and Ser69) had been previously unambiguously verified in an in vitro Aurora B phosphorylation assay using the wild-type bonsai as a substrate18 (Fig. 1a). In a recent study investigating human Ndc80 (HEC1) phosphorylation by Aurora B in vivo, Ser4 and Thr49 were not probed24. We therefore conservatively focused on the in vitro–verified phosphorylation sites. It is likely that Ser4 and Thr49 have a role in vivo as both fall inside the two Aurora B phosphorylation zones we identified.

We made phosphomimetic serine to aspartic acid mutations in both zone 1 (Ndc80 55D,58D,61D,64D, which we refer to as ‘3D’) and zone 2 (Ndc80 54D,57D,62D,69D, which we refer to as ‘4D’), individually and in combination (which we refer to as ‘7D’). All phosphomimetic mutation-containing GST–Ndc80 tail peptides exhibited significantly reduced (P < 0.015, t-test) binding relative to wild-type GST–Ndc80 1–80 (Fig. 2a, b). The tail with phosphomimetic mutations in zone 2 (GST–Ndc80 1–80 3D) exhibited a modest (20%) reduction in microtubule binding, whereas tail containing phosphomimetic mutations in zone 1 (GST–Ndc80 1–80 4D) and both zone 1 and zone 2 (GST–Ndc80 1–80 7D) exhibited severe reductions (60% and 80%, respectively). These results demonstrate that phosphorylation of zone 1 has a stronger negative impact on microtubule binding than phosphorylation of zone 2, consistent with our finding that residues in zone 1 directly interact with tubulin and supporting a functional segregation between the two Aurora B phosphorylation zones.

Interplay between the N-terminal tail and CH domains

We next examined the same phosphomimetic mutation–containing constructs in the context of the Ndc80 bonsai complex (Fig. 2c, d). All constructs exhibited a significant reduction (P < 0.05, t-test) in microtubule binding relative to the wild-type, although the effect of phosphomimetic mutations was more modest, presumably owing to the contribution of the Ndc80–Nuf2 head domain to microtubule binding. Indeed, we found that the bonsai Ndc80 A1–40 complex retained a tail-independent residual microtubule binding activity, as has been previously reported17,18.

As was the case with the GST tail peptides, phosphomimetic mutations in both zone 1 and zone 2 in combination produced the greatest reduction in microtubule binding (70%; Fig. 2c, d). However, the bonsai construct with phosphomimetic mutations in zone 2 (bonsai Ndc80 3D) exhibited a slightly greater reduction in microtubule binding (35%) than that with mutations in zone 1 (bonsai Ndc80 4D, 20%), the reverse of what we observed for the GST–Ndc80 tail peptides. As residues in zone 2 do not directly interact with tubulin, these results suggest that the zone 2 mediates interactions between Ndc80 complex molecules that dominate apparent microtubule binding. Consistent with this observation, an Ndc80 complex lacking the first 40 amino acids of the tail (bonsai Ndc80 A1–40) also exhibited significantly reduced (P = 2.0 × 10−4, t-test) binding compared to the wild-type (20%), even though these amino acids do not interact with tubulin. This construct nevertheless bound microtubules more robustly than the bonsai Ndc80 3D construct, suggesting that phosphorylation of zone 2 negatively regulates the interaction between Ndc80 complex molecules.

Although our co-sedimentation assays report on the relative contributions of elements of the Ndc80 complex to microtubule binding, they do not address the underlying mechanism. To directly investigate the effects of mutations on cooperative microtubule binding, we turned to electron tomography to visualize and quantify Ndc80 complex clusters along protofilaments23 (Fig. 3). We found that both the bonsai Ndc80 4D and bonsai Ndc80 3D mutants were significantly impaired (P < 2.5 × 10−7, t-test) in clustering relative to wild-type bonsai23 (Fig. 3b, c), with a most probable cluster size of 2 and 1, respectively, distributions that were statistically indistinguishable.
from each other ($P = 0.82$, t-test; see Supplementary Table 1 for pair-wise statistical comparisons between all of the cluster distributions in Fig. 3). Both could form significantly larger ($P < 0.0015$, t-test) clusters than the bonsai Ndc80 7D mutant\(^2\), or a complex completely lacking the tail\(^2\) (bonsai Ndc80\(_{\Delta1–80}\)). These results suggest that the region of the tail that directly interacts with tubulin may also contribute to Ndc80 complex–Ndc80 complex binding in the context of a cluster. The bonsai Ndc80\(_{\Delta1–40}\) mutant clustered robustly, with a distribution of cluster sizes similar to that of the wild-type bonsai (Fig. 3b), consistent with our observation of higher microtubule-binding affinity for this mutant. This result supports our model that phosphorylation of zone 2 results in repulsion between Ndc80 complex molecules, even though this region is not strictly required for clustering.

To investigate the role of the two Aurora B phosphorylation zones in vivo, we used RNA interference (RNAi)-mediated knockdown and replacement protocol\(^2\) in HeLa cells to assay kinetochore function during the first mitosis in cells that contained mutated Ndc80 proteins with phosphomimetic mutation–containing tails (Fig. 4). As has previously been reported\(^2\), we found that wild-type Ndc80 (Ndc80–GFP) rescued the RNAi knockdown phenotype, as indicated by a well-organized metaphase plate, whereas Ndc80 with a N-terminal tail containing phosphomimetic mutations in both zone 1 and zone 2 (Ndc80 7D–GFP) resulted in a disorganized plate, consisting mostly of unaligned chromosomes. Phosphomimetic mutants in both zone 1 (Ndc80 4D–GFP) and zone 2 (Ndc80 3D–GFP) individually produced intermediate phenotypes; most cells had chromosomes that were poorly aligned or unaligned in both cases. These results demonstrate that zone 1, which is involved in Ndc80 complex–tubulin interactions, and zone 2, which is involved in Ndc80 complex–Ndc80 complex interactions, are both critical for Ndc80 complex function in vivo.

Delineating the path of the N-terminal tail
To structurally characterize tail–tubulin and tail–Ndc80 complex interactions, we turned to cryo-electron microscopy (cryo-EM) analysis (Fig. 5). Using a new multimodel refinement strategy (Supplementary Fig. 4a), we generated an improved reconstruction of wild-type bonsai bound to a microtubule (Fig. 5a,c and Supplementary Movie 1) with a resolution of 7.9 Å (based on the Fourier shell

Figure 3 Zone 2 of Aurora B phosphorylation sites negatively regulates Ndc80 complex clustering. (a) Representative slices from tomographic reconstructions of microtubules decorated with substoichiometric amounts of the indicated constructs. The positions of bonsai molecules are indicated by black lines; all reconstructions featured both clusters and single molecules. Tubulin, 2.5 µM; bonsai constructs, 3.3 µM. Scale bar, 25 nm. (b) Cluster quantification of a. N, number of reconstructed microtubules, n, number of bonsai molecules counted. (c) Summary of cluster distributions for all bonsai constructs tested. Circles indicate mode(s); lines indicate range. Asterisks indicate data sets from ref. 23.

Figure 4 Both Aurora B phosphorylation zones regulate kinetochore–microtubule interactions in vivo. (a) Representative immunofluorescence images of prometaphase or metaphase HeLa cells depleted of endogenous Ndc80 and expressing GFP-tagged rescue constructs stained for tubulin, GFP and ACA. Note that nonrescued cells were not imaged for GFP. Scale bars, 5 µm. (b) Quantification of the chromosome alignment phenotypes for metaphase or prometaphase cells. ‘Aligned’ indicates a tight metaphase plate, ‘mostly aligned’ indicates only 1–3 pairs of kinetochores off the metaphase plate, ‘poorly aligned’ indicates greater than 3 pairs of kinetochores off the metaphase plate, ‘unaligned’ indicates no visible metaphase plate.
The density is branched, which has three potential explanations. The first is that our map represents a mixture of states in this region because the E hooks of α- and β-tubulin, which are indistinguishable in our reconstruction and thus averaged together, actually adopt different conformations. The second possibility is that one of the two branches corresponds to a portion of the N-terminal tail engaging the E hook (part of density 2). The third and most intriguing explanation is that the branch corresponds to polyglutamylation of the E hook.28, a post-translational modification that is enriched in brain29 (the source of the tubulin used in our experiments) and the mitotic spindle.30 Assuming an extended polypeptide conformation, Glu445, the site of glutamylation28, would be near the branch point. If binding of the Ndc80 complex to microtubules were sensitive to the glutamylation state of tubulin, it would allow for an additional mechanism to regulate the interaction between kinetochores and microtubules. This hypothesis could be tested by measuring the affinity of Ndc80 complex for de glutamylated microtubules, an experiment currently rendered difficult by a lack of purified de glutamylating enzymes.31

The E-hook contact site on the CH domain of Nuf2 corresponds to a positively charged patch (Fig. 5e) previously identified as being important for microtubule binding in vitro.32 In vivo multiple charge-reversal substitutions in this patch are required to generate a deleterious phenotype,33 consistent with an electrostatic interaction between Nuf2’s positive patch and the negatively charged E hook. The contact with density 2 corresponds to a direct interaction between the Ndc80 tail and the E hook that had been previously suggested based on biochemical studies.19,20 Based on the difference map analysis of the bonsai Ndc80 4D and bonsai Ndc80 1–40 mutants (Supplementary Fig. 5), to structurally interrogate the positions of zone 1 and zone 2, respectively. Difference map analysis (Fig. 5b) showed distinct peaks of density present in the wild-type complex that are not present in maps of each of the two mutants. The bonsai Ndc80 1–40 difference density mapped to the location of density 2 in the high-resolution wild-type reconstruction, suggesting that some of the residues in the Ndc80 1–40 region, which contains zone 2 of Aurora B phosphorylation sites, make a major contribution to this density. The bonsai Ndc80 4D difference density largely mapped to the area between densities 1 and 2 and the base of density 2, consistent with a localization of zone 1 residues (41–67) to this area.

### Tubulin E hook interacts with both Ndc80 tail and Nuf2

An examination of the high-resolution wild-type reconstruction at lower threshold (Fig. 5c and Supplementary Movie 2) revealed additional density extending from the C-terminal end of the docked crystal structure of tubulin; by parsimony we attribute this density to an ordered portion of the tubulin C terminus, or ‘E hook’.24 This density connects to both density 2, and to the CH domain of Nuf2 within an Ndc80 complex molecule whose toe (the region of the Ndc80 CH domain which contacts the globular portion of tubulin27) is bound to a laterally adjacent protofilament.
Figure 6 Models of Ndc80 complex interacting with dynamic microtubule ends. (a) Cartoon of fully dephosphorylated Ndc80 complex molecules incorporated into a tight cluster on the surface of a dynamic microtubule, colored as in Figure 1. The interactions formed by zones 1 and 2 of the Ndc80 tail are numbered, and tubulin E hook–Nuf2 CH interactions are depicted as red circles. For simplicity only interactions within the Ndc80 complex cluster are shown. In this configuration, the cluster can promote both tubulin longitudinal contacts along protofilaments, through its toe–tubulin interactions, and lateral contacts between protofilaments, by interacting with tubulin E hooks from a neighboring protofilament. This network of interactions has a cumulative effect on microtubule stability. (b) Cartoon of Ndc80 complex phosphorylated in zone 2 but not zone 1. We envision that in this state the complex will remain attached to the microtubule surface but in the context of smaller and looser clusters. In some cases a weak interaction between Ndc80 complexes may still be maintained via zone 1 (top, left), whereas in other cases all cooperative interactions may be absent (bottom). This intermediate phosphorylation state may enhance the mobility of the complex, enabling kinetochore tracking on depolymerizing microtubule ends by a biased diffusion mechanism.

that under conditions of limiting microtubule binding sites, the tail cannot effectively compete with the wild-type bonsai complex despite being in excess (2:1 GST–Ndc80 tail:bonsai; Supplementary Fig. 6). Collectively our results support a three-way interaction between the tail, the Ndc80–Nuf2 globular head and the microtubule that is substantially stronger than the binary tail–microtubule interaction. This model is in agreement with our finding that the presence of the full-length tail increases binding of the bonsai Ndc80 complex (Fig. 1c,e).

Although these results provide a rich description of Ndc80 complex’s microtubule-binding inside a cluster, the mechanism by which the complex engages the microtubule before cluster formation or at the edge of a cluster remains unknown, and is an important area for future research.

DISCUSSION

The existence of two different zones of Aurora B phosphorylation sites, one of which regulates both Ndc80 complex–tubulin and Ndc80 complex–Ndc80 complex interactions (zone 1), the other of which negatively regulates Ndc80 complex–Ndc80 complex interactions (zone 2), suggests that there are multiple mechanisms by which Aurora B can regulate the Ndc80 complex–microtubule interface. In our reconstruction of the wild-type Ndc80 bonsai bound to the microtubule, residues in zone 2 are located on the periphery of the cluster and are more accessible, whereas residues in zone 1 are buried in the cluster. Thus, we speculate that residues in zone 2 may still be susceptible to phosphorylation by Aurora B once a cluster has formed, whereas residues in zone 1 may be protected from rephosphorylation. This model is consistent with the recent report that all Aurora B phosphorylation sites in the tail are highly phosphorylated in early stages of cell division, after which phosphorylation sites in zone 2 remain phosphorylated at an intermediate level while phosphorylation sites in zone 1 are dephosphorylated34,25. Our finding that phosphorylation in zone 2 negatively regulates cluster formation suggests that the cell may dynamically regulate the degree of Ndc80 complex clustering throughout mitosis, even after microtubule attachment has occurred.

In our model the Ndc80 complex begins prometaphase with zones 1 and 2 maximally phosphorylated at an unattached kinetochore. We propose that once proper attachment is achieved the tail is dephosphorylated and Ndc80 complex clusters are formed (Fig. 6a). Given the recent revised estimate that there are ~20 copies of the Ndc80 complex per kinetochore microtubule35, we believe that the high local concentration of the complex on the microtubule surface makes clustering likely in vivo. Zone 1 would become buried upon cluster formation and inaccessible for rephosphorylation, whereas zone 2 would remain available as an Aurora B substrate, thereby acting as a cluster clutch. During metaphase, aligned chromosomes undergo oscillatory motions across the metaphase plate with a period of tens of seconds34,35. It was recently found that cells expressing a version of the Ndc80 complex that could not be phosphorylated by Aurora B did not support chromosome oscillations24, so we thus speculate that zone 2 may be dynamically phosphorylated and dephosphorylated during oscillations.

If clusters were strongly disrupted, it is possible that zone 1 would also become re-accessible for phosphorylation by Aurora B, although this may not happen on a physiologically relevant time scale. Others have found that phospohespecific antibodies to Aurora B phosphorylation sites in the Ndc80 tail still reacted with the kinetochores of chromosomes that had not yet achieved biorientation upon nocodazole treatment to depolymerize microtubules24. Unexpectedly, however, antibodies to zone 1 did not react with the kinetochores of chromosomes that had achieved bi-orientation, even though kinetochore microtubules were no longer present and Aurora B activity was not downregulated, whereas antibodies to zone 2 exhibited some reactivity. It is tempting to speculate that as a bi-oriented kinetochore–microtubule attachment matures, additional factors sequester zone 1 of the Ndc80 tail, either through direct interaction with this region or by stabilizing Ndc80 complex clusters independently from microtubules. It appears that zone 2 still remains somewhat accessible. The detailed kinetics of microtubule-bound Ndc80 complex phosphorylation by Aurora B is an important subject for future studies in vivo and in vitro, as is the potential role of the Ndc80 tail in mediating interactions between kinetochore complexes.

Our high-resolution cryo-EM reconstruction corresponds to the fully dephosphorylated state (Fig. 6a). We previously found that the dephosphorylated form of the Ndc80 complex strongly enhanced microtubule stability against depolymerization by cold, promoting straight microtubule tubulin polymers23. Our improved cryo-EM reconstruction provides a richer mechanistic explanation for effect: in addition to promoting a straight tubulin conformation by wedging its toe into both intradimer and interdimer longitudinal interfaces, where protofilaments bend during microtubule depolymerization, and by oligomerizing along protofilaments, with the consequent stabilization of longitudinal contacts, the complex also engages a neighboring protofilament via the C terminus of tubulin, stabilizing...
lateral contacts. Thus, the dephosphorylated complex is optimized, via a three-component mechanism, to promote microtubule stability and polymerization.

We envision zone 2 could be rephosphorylated, loosening or disrupting Ndc80 complex clusters and making the complex more mobile on the microtubule surface and thus capable of tracking depolymerizing ends via a biased diffusion mechanism (Fig. 6b). The presence of other microtubule-binding inner kinetochore components, such as the Ska complex, which cooperates with the KMN network, kinesins and plus-tip tracking proteins, are also likely to alter the architecture of the kinetochore–microtubule attachment site to promote microtubule growth under certain conditions and enable processive tracking along depolymerizing microtubule ends in others.

METHODS

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

Accession codes. Reconstructed volumes are deposited in the EMDataBank with identifiers EMD-5489 (improved reconstruction of the Ndc80 complex–microtubule interface), EMD-5490 (wild-type bosaln), EMD-5491 (bosaln Ndc80 4D), EMD-5492 (bosaln Ndc80Δ1–40) and EMD-5493 (microtubule alone).

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

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AUTHOR CONTRIBUTIONS

G.M.A. and E.N. designed research. G.M.A. and V.M. purified proteins and performed microtubule-binding assays. G.M.A. carried out electron microscopy experiments and image processing. D.M. and J.T. performed cell biology experiments and generated new constructs. G.M.A. and E.N. wrote the paper. G.M.A., V.M., D.M., J.T., P.S. and E.N. contributed to data analysis and editing of the manuscript.

COMPETING FINANCIAL INTERESTS

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ONLINE METHODS

Expression cloning. All constructs were generated using standard molecular biology methods. The bonsai Ndc80 4D and bonsai Ndc80 3D constructs were generated as previously described for the bonsai Ndc80 7D construct.23 GST–Ndc80 tail constructs were generated by ligation-independent cloning42 in the 2B7 vector, which 5′ to 3′ encodes a polyhistidine tag, GST and a Tobacco Etch virus (TEV) protease cleavage sequence, followed by the expression cassette.

Protein purification. All constructs were expressed in BL21-CodonPlus-(DE3)-RIL cells. Bonsai constructs were purified as described.18 GST tail constructs were induced at OD600 0.4–0.6 for 4 h at 37 °C with 400 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) in Luria broth. Cells were collected by centrifugation, washed once with PBS and flash-frozen in liquid nitrogen. Cell pellets were resuspended in 50 mM Tris–Cl pH 8.1, 1 mM dithiothreitol (DTT), 300 mM NaCl and 10 mM imidazole with protease inhibitor cocktail tablets (Roche) and lysed by sonic disruption. Lysates were clarified by centrifugation at 17,640 relative centripetal force, then applied to His-Select nickel affinity resin (Sigma), followed by washing and elution with buffer supplemented with 20 mM NaCl and 250 mM imidazole, respectively. By using a limiting amount of affinity resin, a homogenous purification was achieved in a single step. Proteins were desalted into storage buffer (50 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM DTT and 1 mM EDTA) and flash-frozen in liquid nitrogen.

Microtubule cosedimentation assays. Microtubules and binding proteins were prepared as described23 in binding buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM MgCl2, 1 mM DTT and 5% sucrose), supplemented with 20 µM taxol as appropriate. Cosedimentation assays were performed as described.18,23: briefly, binding proteins were mixed with microtubules in binding buffer supplemented with 20 µM taxol in a 50-µl reaction volume. After a 20 min incubation at 25 °C, the reactions were layered on to a 50% glycerol cushion in 80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM MgCl2, 1 mM DTT and 25 °C. Supernatant and pellet fractions were collected, precipitated with 90% ethanol, then analyzed by SDS-PAGE and densitometry. Statistical analyses were performed with Microsoft Excel. For all cosedimentation assays, n represents technical replicates.

Electron tomography. Negatively stained samples were prepared as described23 using 2.5 µM tubulin and a single application of 3.3 µM bonsai. Tilt series were collected from −65 to 65 degrees with a Tecnai F20 operating at 120 kV using the Leginon software package43. Images were recorded on a Gatan Ultrascan 4K × 4K CCD camera between 1.2 and 2.5 µm underfocus with a dose of 20 electrons/Å² at a nominal magnification of ×80,000.

Image processing. The power spectra of images were manually inspected for quality: those featuring obvious drift were excluded. The defocus of the images was estimated with CTFIND3 (ref. 47). Microtubule segments were selected using BOXER48, extracted, phase-flipped with EMA18 and normalized with XMIPP49. The data were then subjected to reference-free classification using IMAGIC50 and a topology-representing network algorithm51 as described.23,52. Segments that contributed to averages that did not feature well-ordered Ndc80 decoration were excluded from subsequent processing.

Remaining segments were then processed using a multireference implementation of the IHRSR method53 in the EMAN2 (ref. 54) and SPARX55 processing packages, using naked 13- and 14-protofilament microtubule reconstructions as starting references to avoid model bias. The refined 14-protofilament reconstructions, which generally incorporated more segments and were of higher resolution, were selected for subsequent analysis. The four reconstructions for difference-map calculation featured between 1,600–2,700 segments, corresponding to 67,200–113,400 asymmetric units.

Initial alignments for the high-resolution wild-type Ndc80 reconstruction were performed in this manner. The 14-protofilament reconstruction was further refined using FREALIGN56 v8.9, which implements helical processing. As the multireference approach was used to sort the different symmetry groups, rather than reference-free classification, a larger proportion of the data set could be aligned and incorporated into the reconstruction (5,268 segments, corresponding to ~220,000 asymmetric units). A negative B-factor of 1,000 Å² was applied with a peak at 8.3 Å and high-resolution cutoff of 7.6 Å, which showed features that matched those of docked crystal structures well. All rigid-body docking of protein crystal structures (tubulin: PDB 1JFF57; bonsai Ndc8031–40: PDB 2VE7 ref. 18), and preparation of molecular graphics illustrations was performed with University of California San Francisco (UCSF) Chimera58.

Differences map analysis. To minimize artifacts caused by differences in data collection or image processing, all reconstructions used for difference map analysis were performed identically. When comparing reconstructions of the bonsai Ndc80 4D and bonsai Ndc8031–40 reconstructions with the wild-type bonsai reconstruction it was apparent that the mutants were decorating the microtubule substoichiometrically (Supplementary Fig. 5). This interfered with the direct calculation of amplitude scaled difference maps, as the signal from the microtubule outweighed that of the bound Ndc80 bonsai in the mutants at low resolution but was equivalent in the wild-type, where stoichiometric binding was observed. Thus, we generated a reconstruction of a naked taxol–stabilized microtubule, which we presubtracted from the three bonsai reconstructions without amplitude scaling using the SPIDER software package59 after rigid-body docking of the reconstructions using the wild-type bonsai as the reference in UCSF Chimera.

We subsequently calculated amplitude-scaled difference maps with DIFFMAP (http://emlab.roose2.brandeis.edu/diffmap), which largely showed positive differences along the expected path of the Ndc80 tail and minimal artifacts when filtered to 12 Å resolution. A single minor peak was observed between microtubule protofilaments in the case of the wild-type bonsai minus bonsai Ndc80 4D difference map (Fig. 3b). We also found that applying a soft mask to the edges of the segmented volume and padding by two dimensions before difference calculation with

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DIFFMAP reduced edge artifacts. No amplitude scaling was performed before difference map calculation, which we also found to introduce artifacts.

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