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The Microtubule Binding Properties of CENP-E's C-terminus and CENP-F

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Abstract

CENP-E (Centromere Protein E) and CENP-F, also known as mitotin, are large, multi-functional proteins associated with the outer kinetochore. CENP-E features a well-characterized kinesin motor domain at its N-terminus and a second microtubule-binding domain at its C-terminus of unknown function. CENP-F is important for the formation of proper kinetochore-microtubule attachment and, like CENP-E, contains two microtubule-binding domains at its termini. While the importance of these proteins is known, the details of their interactions with microtubules have not yet been investigated. We have biochemically and structurally characterized the microtubule-binding properties of the amino- and carboxyl-terminal domains of CENP-F as well as the carboxyl-terminal (non-kinesin) domain of CENP-E. CENP-E's C-terminus and CENP-F's N-terminus bind microtubules with similar affinity to the well-characterized Ndc80 complex, while CENP-F's C-terminus shows much lower affinity. Electron microscopy analysis reveals that all of these domains engage the microtubule surface in a disordered manner, suggesting that these factors have no favored binding geometry and may allow for initial side-on attachments early in mitosis.

Keywords

kinetochore; mitosis; MAPS; coiled-coil; electron microscopy

Introduction

The kinetochore constitutes the region of chromosome-microtubule interaction. It is responsible for the alignment of chromosomes along the metaphase plate and the segregation of sister chromatids into daughter cells during anaphase [1,2]. A major component of the kinetochore essential for microtubule attachments is the highly conserved

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KMN-network, consisting of the KNL-1 complex, the Mis12 complex, and the NDC80 complex [3]. The Dam1 complex in yeast and the Ska complex in higher eukaryotes, both of which cooperate with the KMN network [4,5,6], are additionally required for the formation of robust kinetochore-microtubule attachments. However, in metazoans two additional outer kinetochore proteins, CENP-E (Centromere Protein E) and CENP-F, also known as mitotin, have also been shown to be critical for this process [7,8,9,10].

CENP-E is the better characterized of these two proteins. It was discovered over two decades ago when it was shown to have an essential role in progression from metaphase to anaphase [11]. This 340 kDa coiled-coil protein accumulates during G2, associates with kinetochores during M-phase, and is degraded shortly thereafter [12]. It was later found to regulate the spindle assembly checkpoint, which causes cell cycle arrest when kinetochores do not form stable interactions with microtubules [13], by binding and silencing Bub1, an important member of this checkpoint [14]. When CENP-E is depleted in vivo, cells lack robust microtubule-kinetochore interaction [8]. Although in these cases the number of kinetochore-microtubule attachments is normal, the kinetochores are unable to produce sufficient force to generate proper tension between sister kinetochores [7].

CENP-E contains three distinct domains (Fig. 1A): a well-characterized N-terminal kinesin domain [15,16], a central coiled-coil domain, and a C-terminal domain that is required for kinetochore localization [17] and possesses microtubule-binding activity [18], which we refer to here as 'EC'. The plus-end directed motion of the kinesin domain has been implicated in chromosome congression [19], while the role of the EC domain is not entirely clear. One model of CENP-E's function invokes an intramolecular interaction between the kinesin and EC domains, where in EC inhibits the motor activity of the kinesin domain until phosphorylated by MPS1 or CDK1-cyclin B early in mitosis [20]. An opposing model does not invoke any intramolecular interaction, focusing on CENP-E's kinesin domain providing the power necessary to congress chromosomes during prometaphase, while the rest of the protein acts as a tether to kinetochores [16]. Neither of these models has considered the role of EC in microtubule interaction.

CENP-F was also discovered about two decades ago [21]. It is a large 350 kDa coiled-coil protein that remains at low levels during S phase but accumulates during G2 in the nucleus and nuclear envelope, followed by relocalization to kinetochores at the G2/M transition [10]. CENP-F remains associated with kinetochores until anaphase, when it localizes to the spindle midzone and degrades thereafter [22,23]. Several reports [9,24,25,26] have shown that CENP-F is important for kinetochore-microtubule attachment. The depletion of CENP-F in various cell types results in an extended mitotic delay and decreased tension between microtubule-associated kinetochores [26]. CENP-F is also required for proper operation of the spindle assembly checkpoint, as its depletion results in a failure to retain spindle checkpoint proteins BubR1 and Mad1 at kinetochores [24]. Clinically, CENP-F is used as a proliferation marker in several cancers, including lung cancer, breast cancer, node-negative breast cancer, Non-Hodgkin lymphoma, and Mantle Cell lymphoma [27]. Its expression has a positive correlation with tumor size in node-negative breast cancer [28].

CENP-F has been reported to contain two microtubule-binding domains, one at each terminus of the protein, of unknown function (Fig. 1A) [29]. We refer to the domain at the N-terminus as 'FN', and that at the C-terminus as 'FC'. CENP-F's primary function has remained elusive, though one study proposed that CENP-F plays a role in microtubule nucleation at the centrosome, inconsistent with its kinetochore localization [30]. CENP-E has been shown to be closely associated with the activity of CENP-F, and has been implicated as a potential interacting partner [17,31].

Despite their discovery two decades ago, the structures and microtubule-binding properties of CENP-F's two MT-binding domains and CENP-E's EC domain have not been well-characterized. We have employed both a biochemical approach and electron microscopy to characterize the interaction of these domains with microtubules in vitro.

Results

The CENP microtubule-binding domains form elongated dimers

In order to characterize the potential contributions of EC, FN, and FC to kinetochore-microtubule interactions, we heterologously expressed and purified the three domains (Fig. 1B). BLAST searches suggested that none of the domains possesses significant sequence homology to other known protein sequences (data not shown). We therefore focused our initial analysis on the gross structural characteristics of the CENP domains. Circular dichroism (CD) spectra of both EC and FN are consistent with folded domains composed primarily of α -helices (Fig. 1C). The FC spectrum, on the other hand, is not consistent with secondary structural content, suggesting that this segment does not form an autonomously folding domain. We next performed analytical gel filtration to investigate the hydrodynamic properties of the domains (Fig. 2A). All three of the domains studied eluted from the column after the void, but much earlier than expected for globular proteins of comparable size. FN and EC, which are of similar predicted size (~40 kDa) eluted at almost exactly the same retention volume, while FC, which is smaller (~20 kDa), eluted later, yet still before a very large globular standard (Thyroglobulin, 660 kDa).

The anomalously large Stokes' radii required to give rise to these elution profiles are consistent with the domains possessing elongated structures, forming very large oligomers, or simply forming heterogenous, non-specific soluble aggregates. To distinguish between these possibilities, we performed Multi-Angle Light Scattering (MALS) experiments to obtain shape-independent estimates of the molecular weight of species present in each CENP domain sample. The molecular weights measured are consistent with each domain forming a mono-disperse homo-dimer (Table 1). However, due to the low working protein concentrations necessitated by the relative insolubility of the domains and the consequent weak scattering, the uncertainty for the measurements ranges from 31–47%, preventing us from distinguishing dimers from monomers or trimers conclusively. However, we can confidently exclude the formation of very large oligomers or polydisperse soluble aggregates. We therefore conclude that the early elution in our SEC experiments results from the domains having an elongated conformation, similar to other kinetochore components such as the Ndc80[32] and Mis12 complexes [33,34,35]. The EC domain likely contributes to full length CENP-E's observed structure: a highly flexible, ~230 nm elongated dimer whose shape has been attributed to extensive coiled-coil regions. [16], Also consistent with our observations, dimerization of CENP-F has been suggested [22], likely occurring via predicted coiled-coils. Furthermore, super-resolution fluorescence microscopy measurements suggest that both molecules adopt an extended conformation in vivo [36].

We performed coiled-coil analysis of the sequences of the three isolated MT-binding domains, as extensive coiled-coil content would be consistent with their elongated shapes [37]. Interestingly, FN and EC present a very similar pattern of predicted coiled-coil content at their N-terminal ends: a long coiled-coil of ~125 amino acids and a shorter coiled-coil of 31–45 amino acids, separated by a 30–35 amino acid linker (Fig. 2B). In combination with their nearly identical gel filtration profiles, sizes, and similar CD spectra, this supports a potential structural similarity between these two domains, despite the lack of any sequence similarity. FC, on the other hand, contains no predicted coiled-coils, consistent with the lack of helical content inferred from its CD spectrum. This isolated domain nevertheless has the propensity to dimerize and take on an elongated conformation.

One model of CENP-E function invokes an intramolecular interaction between the N-terminal kinesin domain and EC, although no interaction between the domains was detected when they were not part of the same polypeptide [20]. Since CENP-F also contains two microtubule-binding domains at its termini, we incubated FN and FC together before performing gel filtration to test for complex formation in trans, which would support a model in which the molecule formed a closed loop. The chromatogram showed two peaks at identical positions to the domains run individually, suggesting no interaction between the two termini (Fig. 2A).

The CENP-E C-terminus and CENP-F N-terminus bind MTs with high affinity

The Ndc80 complex, a member of the conserved KMN network, has been identified as a primary high-affinity microtubule binding site at the kinetochore [3,38]. We performed co-sedimentation assays with the three CENP domains in order to determine the affinity of the domains for microtubules (Fig. 3), comparing them to an engineered version of the Ndc80 complex (Ndc80 bonsai) which retains full affinity for microtubules [39] to investigate the relative contributions the CENP domains could make to kinetochore-microtubule interactions. The dissociation constant we calculate for Ndc80 bonsai is 260 nM, approximately 6-fold higher than the previously reported value, which may be the result of differences in the buffer conditions (substitution of 5% sucrose for BSA as a stabilization agent in the absence of salt). Remarkably, under identical conditions CENP-E's C-terminal (EC) domain binds microtubules with even higher affinity than Ndc80 bonsai (Fig. 3B), suggesting that this domain could make a substantial contribution to kinetochore-microtubule interactions in vivo.

CENP-F's domains bind microtubules with lower affinity than Ndc80 or CENP-E's C-terminus, with the FN construct showing 3-fold lower affinity than the Ndc80 complex and FC almost 10-fold lower. Nevertheless, FN has sub-micromolar binding affinity. Furthermore, both FC and EC have been reported to be post-translationally modified by farnesylation [40], which may increase the microtubule-binding affinity of CENP-F [41].

Given the two domains CENP-F domains are physically connected under physiological conditions, we hypothesized that FN and FC might cooperate in microtubule binding. To test this idea, we incubated FN and FC with microtubules in the same reaction under conditions of limiting microtubule binding sites, and compared the fraction bound to that of each domain alone under equivalent conditions (Fig. 4). Both domains show a substantial decrease in binding when co-incubated, with the effect being more pronounced for FN. This suggests that CENP-F's N- and C-terminus do not interact with each other in the context of the microtubule. It also suggests that the two domains share an at least partially overlapping binding site on the microtubule surface, and, in the context of the full complex, that they likely interact with adjacent but different tubulin subunits.

The CENP domains form flexible interactions with the microtubule surface

To investigate the structural details of the interaction of CENP domains with the MT surface, we initially used negative-stain electron microscopy. We examined paclitaxel-stabilized microtubules after incubation with each of the three protein constructs (Fig. 5). The FN and EC samples showed pronounced stain-excluded density adjacent to the microtubule surface when compared to control microtubules, consistent with a direct, high affinity interaction (Fig. 5). Samples containing FC showed very patchy instances of stain excluded density (Fig. 5C, arrows), which we interpret as rare instances of microtubule binding, consistent with the lower affinity determined by our co-sedimentation assay. However, given the small size of the construct and its unstructured nature the interaction is difficult to visualize.

By visual inspection, binding of EC and FN to the microtubule surface appears disordered, with no obvious fixed position of the domains with respect to the tubulin lattice. To quantitatively verify this observation, we examined paclitaxel-stabilized microtubules in the presence and absence of the two high-affinity CENP MT binding domains using cryo-electron microscopy. Once again, microtubule binding by the two constructs was apparent by visual inspection (Fig. 6A, top). Consistent with both domains forming a disordered interaction with the microtubule, power spectra calculated from individual microtubules decorated with the CENP domains did not show the presence of 80Å layer-lines (Fig. 6A, bottom), as would be expected for a MAP which binds a specific site on the tubulin heterodimer. However, this does not rule out an ordered interaction with a tubulin monomer repeat, as we have previously reported for the Ndc80 complex [42].

To investigate this possibility, we calculated reference-free class averages from extracted microtubule segments (Fig. 6B). Alignment of the segments is expected to be driven by the high signal contributed by the regular microtubule lattice [43]: consistently, we observe well-resolved tubulin density in all of the class averages. If a MAP forms a regular interaction with the microtubule surface with a fixed orientation, density for the MAP would also appear in the class averages, as we have previously reported for the Ndc80 complex [42]. However, if a MAP does not bind with a fixed orientation, no well-defined density would appear when the random binding orientations present in different segments are averaged together. Class averages from the FN and EC bound microtubules contain no significant extra density when compared with averages calculated from microtubules alone (Fig. 6B), supporting our assertion that both of these domains form a flexible interaction with the microtubule surface.

The CENP-E C-terminus promotes tubulin polymerization

When observing EC incubated with paclitaxel microtubules, single microtubules decorated with EC were extremely rare, as the domain demonstrated an extreme propensity to bundle microtubules (Fig. 7A), in line with a proposed role in microtubule cross-linking in vivo [18]. This activity may play a role in providing additional microtubule substrates for its motor domain, which may jump between several bundled microtubules due to its low processivity [44]. Alternatively, bundling may aid the kinesin domain in promoting plus-end elongation of multiple microtubules [45]. In agreement with this proposal we observed sheet-like structures, as have previously been observed at the plus ends of growing microtubules [46,47], and small microtubule fragments (Fig. 7B), suggesting that EC promoted additional tubulin polymerization upon addition to paclitaxel-stabilized microtubules.

To test this biochemically, we performed microtubule co-sedimentation assays that showed increased tubulin pelleting in the presence of EC, both for dynamic and paclitaxel-stabilized microtubules (Fig. 8A,B). These results clearly indicate that EC has a positive effect on tubulin assembly and suggest that the EC domain may also play a role in microtubule elongation. Neither FN nor FC produced extensive MT bundling, suggesting that CENP-F does not cross-link microtubules in vivo. To directly examine the effect of EC on tubulin dynamics, we carried out a microtubule polymerization assay (Fig. 8C). While tubulin alone shows a moderate increase in signal over time, due to self-assembly, the addition of EC dramatically enhances the kinetics of polymerization, eliminating the lag phase. The total turbidity signal increases substantially as well, presumably due to extensive microtubule bundling, as we observed in our EM experiments. We therefore conclude that EC has a positive impact on tubulin polymerization.

Robust CENP binding does not require tubulin's C-terminus

The unstructured acidic C-terminus of tubulin, or E-hook, contributes to most if not all interactions of MTs with their associated factors. Given the extended character of this tubulin tail, factors that bind exclusively via this moiety, without engaging the globular domain of tubulin, are generally flexibly attached to the MT lattice. In order to further characterize the nature of the microtubule interaction with the CENP domains, we cleaved the C-terminus of tubulin from polymerized microtubules using the protease subtilisin, and assayed the effect on CENP binding (Fig. 9A). As previously reported [18], and despite the disordered nature of the interaction we observed, EC binding is relatively insensitive to subtilisin treatment, showing only a 20% reduction in binding to cleaved microtubules (Fig. 9B,C). Interestingly, FN is also almost completely insensitive, demonstrating a similar ~15% reduction in binding. These results suggest that the flexible character of the EC and FN interaction with microtubules is likely due to flexible elements in these domains themselves, and once again highlights the similarity between these two microtubule-interaction modules.

FC, on the other hand, was substantially affected by subtilisin treatment of MTs: it showed nearly a 60% reduction in binding, suggesting that the E-hook of tubulin contributes substantially, although not exclusively, to the MT binding of FC (Fig. 9B,C). This result helps explain our observation that FC was able to out compete FN for microtubule binding sites (Fig. 4B) despite its lower microtubule-binding affinity. One explanation for FC's ability to retain more microtubule-binding capacity when there is competition for the globular domain site is that FC recognizes a composite binding site formed by the tubulin E-hook and the tubulin globular domain, whereas FN only recognizes the site on the tubulin globular domain.

Discussion

Although EC and CENP-F's terminal domains have been recognized as direct microtubule-binding factors for some time, the importance of this activity relative to the other roles of these large, multipurpose proteins in generating robust kinetochore-microtubule attachments has remained unknown. Our finding that both EC and FN bind microtubules with comparable affinity to the Ndc80 complex, the primary microtubule-binding arm of the KMN network, suggests that these domains could make a substantial contribution to microtubule engagement by metazoan kinetochores. In particular, these results suggest that CENP-F depletion phenotypes may not solely be explained by the resultant inability of dynein to localize to kinetochores through the Nde1 pathway [48].

Several lines of evidence highlight the similarities between EC and FN despite the absence of any detectable sequence homology. Structurally, they are very similar in size, show an almost identical elution profile in our analytical gel filtration experiments, possess very similar CD spectra, and display a similar pattern of predicted coiled-coil content at their N-terminal ends. Additionally, their microtubule binding properties are strikingly similar: both have submicromolar affinity for microtubules and form a disordered interaction with the microtubule surface despite the relatively minor contribution of the disordered tubulin E hooks to binding. Cumulatively, these results suggest that these two domains may belong to a class of microtubule-associated proteins (MAPs) that have evolved to make flexible but strong connections to microtubules without competing with traditional MAPs for E hook binding sites. Further studies will be required to investigate if the two domains possess similar three-dimensional structures and to probe their ability to bind microtubules in the presence of other MAPs.

CENP-F and CENP-E are two of the earliest factors to localize to kinetochores that are not constitutively associated with centromeres [10,17]. Immuno-EM and high-resolution fluorescence studies have demonstrated that both are localized to the outermost kinetochore layer (i.e. furthest away from the inner centromere) [22,36,49], consistent with them being in the fibrous corona region, along with other microtubule-binding factors such as dynein [50], earlier in mitosis [51]. It is thus tempting to speculate that CENP-E's C-terminus and CENP-F are involved in making side-on kinetochore-microtubule attachments early in mitosis, facilitating chromosome congression, a process in which the CENP-E kinesin domain has previously been implicated [19]. The absence of a preferred microtubule-binding geometry as well as the ability to bind in the presence of traditional MAPs would be well suited for this role. Investigating the phenotypic consequences of CENP proteins with specific lesions in their MT-binding domains in vivo would serve to test this hypothesis.

Materials and Methods

Cloning

The constructs presented in this study consist of amino acids 2305-2663 of CENP-E (EC) and amino acids 1-385 (FN) and 3023-3210 (FC) of CENP-F (all human sequences). An optimized coding sequence for EC was synthesized (GENEART). The coding sequences for FN and FC were amplified using standard PCR protocols. BamHI and XhoI restriction sites were engineered at the 5' and 3' ends, respectively. EC and FC were cloned into a pGEX-2RBS vector, which encodes an N-terminal GST tag and prescission protease cleavage site. FN was cloned using identical protocols, except that NdeI and XhoI restriction sites were used for insertion into a pETM-14 vector, which encodes an N-terminal polyhistidine tag and HRV 3C protease cleavage site.

Purification of EC and FC

NDC80 bonsai was purified as previously described [39]. EC and FC were expressed in BL21(DE3)-RIL-CodonPlus cells purchased from the QB3 Macrolab at UC Berkeley (<http://qb3.berkeley.edu/qb3/macrolab/>). Each construct was induced at an OD₆₀₀ of 0.4–0.6 for 16 hours at 16°C with 500 µM IPTG (Isopropyl β-D-1-thiogalactopyranoside) in Terrific Broth. Cells were harvested by centrifugation followed by resuspension in lysis buffer: 50 mM Tris-Cl pH 7.5, 300 mM NaCl, 1 mM DTT, and 1 mM EDTA, containing protease inhibitor tablets (Roche). Cells were lysed via sonication and lysates were cleared by ultracentrifugation in a Beckman Ti45 rotor for 45 minutes at 30,000 RPM. Lysates were then applied to Glutathione SepharoseFastFlow resin (1ml beads per liter culture) pre-rinsed in lysis buffer for 2 hours. After binding, beads were collected via centrifugation at 3,000 RPM for 3 minutes followed by 2 washes with lysis buffer and 2 washes with elution buffer (same as lysis buffer but containing 150 mM NaCl). The resuspended beads were incubated with 30 µl of PreScission protease for 16 hours at 4°C to cleave the fusion protein from its GST tag, which remained bound to the beads. The eluate was desalted into ion exchange buffer with a PD-10 desalting column (GE Healthcare). The buffer used for CENP-E C-term contained 25 mM HEPES pH 7.5, 2mM DTT, and 1mM EDTA; the buffer for CENP-F C-term contained 50mM Tris pH8.0, 10mM NaCl, 1 mM DTT, and 1mM EDTA. The samples were then applied to a monoS 5/50 GL cation exchange column and eluted with a linear gradient from 10 to 500 mM NaCl. Appropriate fractions were pooled, concentrated, and flash frozen in 50 µl aliquots.

Purification of CENP-F N-term

Cells harboring the FN expression construct were grown and harvested as described above. Cells were resuspended in 40ml lysis buffer (50 mM Tris-Cl pH 8.75, 300 mM NaCl, 1 mM DTT, 10 mM imidazole) containing EDTA-free protease inhibitor tablets (Roche) and

sonicated and clarified as described above. Lysates were applied to His-select Nickel Affinity beads (Sigma) for 2 hours at 4°C. Beads were then washed 4 times with 40 ml wash buffer containing 20 mM imidazole and eluted 4 times with 500 µl elution buffer containing 250 mM imidazole. Samples were then dialyzed overnight into a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and HRV 3C protease (EMD Biosciences) to cleave the His tag, and desalted with a PD-10 desalting column (GE Healthcare) into an ion exchange buffer containing 25 mM HEPES pH 7.5, 2mM DTT, and 1mM EDTA. Finally, samples were purified by cation exchange as above, followed by gel filtration on a Superdex 200 10/30 GL column. Appropriate fractions were pooled, concentrated, and flash frozen in 50 µl aliquots.

Sample preparation for biochemical experiments and EM

Microtubule binding proteins were thawed rapidly followed by storage on ice, then desalted into the indicated buffers using Zebra Spin Desalting Column (Pierce). To remove aggregates, proteins were clarified in an ultracentrifuge (Beckman TLA100 rotor) for 20 minutes at 40,000 RPM at 4°C. Protein concentration was determined using A₂₈₀ or with the Coomassie Plus Protein Assay Reagent (Pierce) with the use of BSA as a standard reference.

Circular dichroism

Purified protein samples were prepared as described above in a buffer containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, and 1mM DTT. Circular dichroism (CD) measurements were performed at 4 °C on an Aviv 62DS spectrometer using a 0.5 cm path length cuvette. Final protein concentrations measured using A₂₈₀ were 0.19 mg/ml, 0.32 mg/ml, and 0.078 mg/ml for EC, FC and FN respectively. Spectra were collected in 1 nm steps from 260 nm to 200 nm, averaging each point for 5 seconds.

Multi-angle light scattering

Purified protein samples were prepared as described above for CD experiments. Samples were then concentrated by ultrafiltration using a 10 kDa molecular weight cutoff Amicon® (Millipore) concentrator, and re-clarified to remove aggregates as described above. Final protein concentrations measured using A₂₈₀ were 0.23 mg/ml, 0.63 mg/ml and 0.26 mg/ml for EC, FC and FN respectively. 100 µl of the sample was injected onto a Superdex 200 PC 2.3/30 column (GE Healthcare) equilibrated in the same buffer at room temperature. The column was coupled to an 18-angle light scattering detector (DAWN EOS) and refractive index detector (Optilab T-rEX, Wyatt Technology). Data were collected at a flow rate of 0.05 ml/min and analyzed using the program ASTRA to determine the molecular weight and polydispersity. Bovine serum albumin (BSA) injected at 2.1 mg/ml was used to confirm the molecular weight determination. The calculated BSA molecular mass was 63.6 kD (4% uncertainty), indicating an absolute error of 4 % from the expected value of 66.5 kD.

Analytical Gel Filtration

Samples were prepared as described above for CD experiments and run on a Superose 6 PC 3.2/30 column (GE Healthcare) at 4°C in the same buffer. Reference proteins were run under identical conditions. These included Thryoglobulin at 5 mg/ml, Aldolase at 7 mg/ml, Ovalbumin at 8 mg/ml, and Ribonuclease A at 5 mg/ml (GE Healthcare).

Paclitaxel-stabilized Microtubule Preparation

Soluble tubulin was obtained from bovine brain (Cytoskeleton) at 10 mg/ml. Aliquots were thawed and polymerized in BRB80 buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 1 mM MgCl₂) supplemented with 10% glycerol and 1 mM GTP at 37°C for 15 minutes, followed by addition of 160 µM paclitaxel and an additional 60 minutes of incubation at 37°C. For

biochemical experiments, paclitaxel microtubules were first pelleted in a tabletop microcentrifuge for 20 minutes at room temperature at 17,000 RCF. The pellet was then resuspended in binding buffer (BRB80, 5% sucrose, 1 mM DTT) containing 160 μ M paclitaxel. For EM experiments, EM buffer (BRB80, .05% NP-40, 1 mM DTT) replaced binding buffer. Microtubules were diluted in the presence of 20 μ M paclitaxel for all subsequent experiments.

Microtubule co-sedimentation assays

Subtilisin-cleaved microtubules were generated using paclitaxel-stabilized microtubules digested with 1.5 μ M subtilisin A (Calbiochem) in binding buffer for 20 minutes at 37°C. PMSF (phenylmethanesulfonylfluoride) was added at 2mM to stop the reaction. A parallel mock reaction that substituted subtilisin with binding buffer was also treated with PMSF. Reactions were spun in a tabletop centrifuge at 17,000 RCF at room temperature and resuspended in binding buffer containing 20 μ M paclitaxel.

When performing the assay, paclitaxel microtubules or subtilisin/mock cleaved microtubules were incubated at appropriate concentrations with the experimental protein at room temperature for 20 minutes in a 50 μ l reaction supplemented with 20 μ M paclitaxel in binding buffer. For CENP-F domains, reactions were layered on a 100 μ l cushion consisting of 50% glycerol, BRB80, 20 μ M paclitaxel, and 1mM DTT. Reactions were then ultracentrifuged (Beckman TLA 100 rotor) at 90,000 RPM for 10 minutes at 25°C. For experiments with CENP-E C-term, no cushion was used, and reactions were spun in a tabletop centrifuge at 17,000 RCF for 20 minutes. Supernatant and pellet fractions were separated and precipitated overnight in 90% ethanol, followed by analysis with SDS-PAGE using NuPAGENovex 10% Bis-Tris 1.0 mm gels (Life Technologies). Gels were stained with Flamingo Fluorescent Gel Stain (Bio-Rad) and visualized with either a Typhoon Trio (GE Healthcare) or a GelDoc EZ System (Bio-Rad). Protein bands were quantified using ImageJ software (National Institutes of Health, USA).

Binding Curves

Data indicating percent protein bound to microtubules was determined by dividing the intensity of the pellet fraction by the sum of the supernatant and pellet. These data, excluding controls with no tubulin, were fit to the following equation:

$$\text{Complex formed} = \frac{(B_t + K_d + x) - \sqrt{(B_t + K_d + x)^2 - (4B_t x)}}{2}$$

Where B_t is the maximum fractional complex of protein bound to tubulin, K_d is the dissociation constant, and x is the concentration of tubulin dimer. Curve fitting was performed using GraphPad Prism, version 5.0 (GraphPad software, Inc.)

Microtubule and EC co-sedimentation assays using dynamic microtubules

Tubulin was polymerized in BRB80, 10% glycerol, 1 mM DTT and 1 mM GTP for 25 minutes at 37 °C. Steady state microtubules were added to pre-warmed binding buffer supplemented with 1 mM GTP for a final concentration of 10 μ M tubulin and 4 μ M EC. Samples were incubated at 37 °C for 20 min and were spun in a tabletop centrifuge at 17,000 RCF for 20 minutes. Supernatant and pellet were separated and brought up to equal volumes in gel loading buffer and were directly run on gels.

Microtubule polymerization measurements by turbidity

EC protein samples were prepared as described above in the same binding buffer used for the co-sedimentation assays, supplemented with GTP (BRB80, 5% sucrose, 1 mM DTT and 1 mM GTP). Reactions were assembled on ice containing 25 μ M tubulin and 2 μ M in the above buffer and placed in pre-cooled quartz cuvettes with a 1 cm path length. The total reaction volume was 70 μ l. Turbidity measurements at 350 nm were taken using a DU 800 spectrophotometer equipped with a temperature controller unit (Beckman Coulter) set to 37 °C. Measurements were taken every 10 seconds, with a one second integration time for 45 minutes.

Negative Stain Electron Microscopy

Samples used for EM were prepared as described for biochemical experiments, with the exception of EM buffer replacing binding buffer. Samples were prepared on continuous carbon grids and stained with 2% uranyl acetate. Images were collected at 49,000x nominal magnification and approximately 1 μ m underfocus with a Tecnai 12 microscope operating at 120 kV on a 4k $\times$ 4k CMOS camera (TVIPS).

Cryo-electron Microscopy and Image Processing

Samples for cryo-EM were prepared as previously described [42]. Briefly, 2 μ l of 2 μ M paclitaxel stabilized microtubules in EM buffer supplement with 20 μ M paclitaxel were applied to glow-discharged C-flat grids (Protochips) in the humidity chamber of a Vitrobot (Maastich instruments). 4 μ l of EC or FN in EM buffer was applied to the specimen for one minute, then excess buffer was removed. An additional 4 μ l of the MT binding domain was applied for another minute, followed by removal of excess buffer. The specimen was then blotted and plunged into ethane slush for vitrification.

Electron micrographs were recorded on a 4k $\times$ 4k Gatan Ultrascan CCD camera semi-automatically with the Leginon software package [52] under low-dose conditions (20 e⁻/Å²) using an FEI Tecnai 20 microscope operating at 120 kV with a nominal magnification of 80,000x.

Image processing was carried out using the Appion software package [53]. The defocus of each image was estimated with CTFFIND3 [54]. Using software from the EMAN[55] and XMIPP[56] processing packages, segments were extracted from phase-flipped images along the length of each microtubule with a step size of 80Å, corresponding to the length of a tubulin dimer, and subsequently normalized, binned four-fold, and masked.

Reference free-classification was performed using a topology-representing network approach [43,57] alternating with multi-reference alignment with IMAGIC [58]. A final round of classification using IMAGIC Multiple Statistical Analysis (MSA) was performed without further alignment. The number of microtubule segments were as follows for each condition: paclitaxel microtubules alone, 1,327, EC-bound microtubules, 834, FN-bound microtubules, 2,611 segments. Due to the propensity of EC to bundle microtubules, only a limited number of isolated segment images could be obtained.

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References

1. Santaguida S, Musacchio A. The life and miracles of kinetochores. *EMBO J.* 2009; 28:2511–2531. [PubMed: 19629042]
2. Cheeseman IM, Desai A. Molecular architecture of the kinetochore-microtubule interface. *Nat Rev Mol Cell Biol.* 2008; 9:33–46. [PubMed: 18097444]
3. Cheeseman IM, Chappie JS, Wilson-Kubalek EM, Desai A. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell.* 2006; 127:983–997. [PubMed: 17129783]
4. Gaitanos TN, Santamaria A, Jeyaprakash AA, Wang B, Conti E, et al. Stable kinetochore-microtubule interactions depend on the Ska complex and its new component Ska3/C13Orf3. *EMBO J.* 2009; 28:1442–1452. [PubMed: 19360002]
5. Lampert F, Hornung P, Westermann S. The Dam1 complex confers microtubule plus end-tracking activity to the Ndc80 kinetochore complex. *J Cell Biol.* 2010; 189:641–649. [PubMed: 20479465]
6. Tien JF, Umbreit NT, Gestaut DR, Franck AD, Cooper J, et al. Cooperation of the Dam1 and Ndc80 kinetochore complexes enhances microtubule coupling and is regulated by aurora B. *J Cell Biol.* 2010; 189:713–723. [PubMed: 20479468]
7. McEwen BF, Chan GK, Zubrowski B, Savoian MS, Sauer MT, et al. CENP-E is essential for reliable bioriented spindle attachment, but chromosome alignment can be achieved via redundant mechanisms in mammalian cells. *Mol Biol Cell.* 2001; 12:2776–2789. [PubMed: 11553716]
8. Putkey FR, Cramer T, Morphew MK, Silk AD, Johnson RS, et al. Unstable kinetochore-microtubule capture and chromosomal instability following deletion of CENP-E. *Dev Cell.* 2002; 3:351–365. [PubMed: 12361599]
9. Holt SV, Vergnolle MA, Hussein D, Wozniak MJ, Allan VJ, et al. Silencing Cenp-F weakens centromeric cohesion, prevents chromosome alignment and activates the spindle checkpoint. *J Cell Sci.* 2005; 118:4889–4900. [PubMed: 16219694]
10. Liao H, Winkfein RJ, Mack G, Rattner JB, Yen TJ. CENP-F is a protein of the nuclear matrix that assembles onto kinetochores at late G2 and is rapidly degraded after mitosis. *J Cell Biol.* 1995; 130:507–518. [PubMed: 7542657]
11. Yen TJ, Compton DA, Wise D, Zinkowski RP, Brinkley BR, et al. CENP-E, a novel human centromere-associated protein required for progression from metaphase to anaphase. *EMBO J.* 1991; 10:1245–1254. [PubMed: 2022189]
12. Yen TJ, Li G, Schaar BT, Szilak I, Cleveland DW. CENP-E is a putative kinetochore motor that accumulates just before mitosis. *Nature.* 1992; 359:536–539. [PubMed: 1406971]
13. Rieder CL, Cole RW, Khodjakov A, Sluder G. The checkpoint delaying anaphase in response to chromosome mono orientation is mediated by an inhibitory signal produced by unattached kinetochores. *J Cell Biol.* 1995; 130:941–948. [PubMed: 7642709]
14. Mao Y, Desai A, Cleveland DW. Microtubule capture by CENP-E silences BubR1-dependent mitotic checkpoint signaling. *J Cell Biol.* 2005; 170:873–880. [PubMed: 16144904]
15. Garcia-Saez I, Yen T, Wade RH, Kozielski F. Crystal structure of the motor domain of the human kinetochore protein CENP-E. *J Mol Biol.* 2004; 340:1107–1116. [PubMed: 15236970]
16. Kim Y, Heuser JE, Waterman CM, Cleveland DW. CENP-E combines a slow, processive motor and a flexible coiled coil to produce an essential motile kinetochore tether. *J Cell Biol.* 2008; 181:411–419. [PubMed: 18443223]
17. Chan GK, Schaar BT, Yen TJ. Characterization of the kinetochore binding domain of CENP-E reveals interactions with the kinetochore proteins CENP-F and hBUBR1. *J Cell Biol.* 1998; 143:49–63. [PubMed: 9763420]
18. Liao H, Li G, Yen TJ. Mitotic regulation of microtubule cross-linking activity of CENP-E kinetochore protein. *Science.* 1994; 265:394–398. [PubMed: 8023161]
19. Kapoor TM, Lampson MA, Hergert P, Cameron L, Cimini D, et al. Chromosomes can congress to the metaphase plate before biorientation. *Science.* 2006; 311:388–391. [PubMed: 16424343]

20. Espeut J, Gaussen A, Bieling P, Morin V, Prieto S, et al. Phosphorylation relieves auto inhibition of the kinetochore motor Cenp-E. *Mol Cell*. 2008; 29:637–643. [PubMed: 18342609]
21. Rattner JB, Rao A, Fritzler MJ, Valencia DW, Yen TJ. CENP-F is a .ca 400 kDa kinetochore protein that exhibits a cell-cycle dependent localization. *Cell Motil Cytoskeleton*. 1993; 26:214–226. [PubMed: 7904902]
22. Zhu X, Chang KH, He D, Mancini MA, Brinkley WR, et al. The C terminus of mitotin is essential for its nuclear localization, centromere/kinetochore targeting, and dimerization. *J Biol Chem*. 1995; 270:19545–19550. [PubMed: 7642639]
23. Gurden MD, Holland AJ, van Zon W, Tighe A, Vergnolle MA, et al. Cdc20 is required for the post-anaphase, KEN-dependent degradation of centromere protein F. *J Cell Sci*. 2010; 123:321–330. [PubMed: 20053638]
24. Laoukili J, Kooistra MR, Bras A, Kauw J, Kerkhoven RM, et al. FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat Cell Biol*. 2005; 7:126–136. [PubMed: 15654331]
25. Yang Z, Guo J, Chen Q, Ding C, Du J, et al. Silencing mitotin induces misaligned chromosomes, premature chromosome decondensation before anaphase onset, and mitotic cell death. *Mol Cell Biol*. 2005; 25:4062–4074. [PubMed: 15870278]
26. Bomont P, Maddox P, Shah JV, Desai AB, Cleveland DW. Unstable microtubule capture at kinetochores depleted of the centromere-associated protein CENP-F. *EMBO J*. 2005; 24:3927–3939. [PubMed: 16252009]
27. Varis A, Salmela AL, Kallio MJ. Cenp-F (mitotin) is more than a mitotic marker. *Chromosoma*. 2006; 115:288–295. [PubMed: 16565862]
28. Clark GM, Allred DC, Hilsenbeck SG, Chamness GC, Osborne CK, et al. Mitotin (a new proliferation marker) correlates with clinical outcome in node-negative breast cancer. *Cancer Res*. 1997; 57:5505–5508. [PubMed: 9407959]
29. Feng J, Huang H, Yen TJ. CENP-F is a novel microtubule-binding protein that is essential for kinetochore attachments and affects the duration of the mitotic checkpoint delay. *Chromosoma*. 2006; 115:320–329. [PubMed: 16601978]
30. Moynihan KL, Pooley R, Miller PM, Kaverina I, Bader DM. Murine CENP-F regulates centrosomal microtubule nucleation and interacts with Hook2 at the centrosome. *Mol Biol Cell*. 2009; 20:4790–4803. [PubMed: 19793914]
31. Yao X, Abrieu A, Zheng Y, Sullivan KF, Cleveland DW. CENP-E forms a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint. *Nat Cell Biol*. 2000; 2:484–491. [PubMed: 10934468]
32. Ciferri C, De Luca J, Monzani S, Ferrari KJ, Ristic D, et al. Architecture of the human ndc80-hec1 complex, a critical constituent of the outer kinetochore. *J Biol Chem*. 2005; 280:29088–29095. [PubMed: 15961401]
33. Petrovic A, Pasqualato S, Dube P, Krenn V, Santaguida S, et al. The MIS12 complex is a protein interaction hub for outer kinetochore assembly. *J Cell Biol*. 2010; 190:835–852. [PubMed: 20819937]
34. Maskell DP, Hu XW, Singleton MR. Molecular architecture and assembly of the yeast kinetochore MIND complex. *J Cell Biol*. 2010; 190:823–834. [PubMed: 20819936]
35. Hornung P, Maier M, Alushin GM, Lander GC, Nogales E, et al. Molecular architecture and connectivity of the budding yeast Mtw1 kinetochore complex. *J Mol Biol*. 2011; 405:548–559. [PubMed: 21075115]
36. Wan X, O'Quinn RP, Pierce HL, Joglekar AP, Gall WE, et al. Protein architecture of the human kinetochore microtubule attachment site. *Cell*. 2009; 137:672–684. [PubMed: 19450515]
37. Delorenzi M, Speed T. An HMM model for coiled-coil domains and a comparison with PSSM-based predictions. *Bioinformatics*. 2002; 18:617–625. [PubMed: 12016059]
38. DeLuca JG, Gall WE, Ciferri C, Cimini D, Musacchio A, et al. Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. *Cell*. 2006; 127:969–982. [PubMed: 17129782]
39. Ciferri C, Pasqualato S, Screpanti E, Varetto G, Santaguida S, et al. Implications for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell*. 2008; 133:427–439. [PubMed: 18455984]

40. Ashar HR, James L, Gray K, Carr D, Black S, et al. Farnesyl transferase inhibitors block the farnesylation of CENP-E and CENP-F and alter the association of CENP-E with the microtubules. *J Biol Chem.* 2000; 275:30451–30457. [PubMed: 10852915]
41. Hussein D, Taylor SS. Farnesylation of Cenp-F is required for G2/M progression and degradation after mitosis. *J Cell Sci.* 2002; 115:3403–3414. [PubMed: 12154071]
42. Alushin GM, Ramey VH, Pasqualato S, Ball DA, Grigorieff N, et al. The Ndc80 kinetochore complex forms oligomeric arrays along microtubules. *Nature.* 2010; 467:805–810. [PubMed: 20944740]
43. Ramey VH, Wang HW, Nogales E. Ab initio reconstruction of helical samples with heterogeneity, disorder and coexisting symmetries. *J Struct Biol.* 2009; 167:97–105. [PubMed: 19447181]
44. Kim Y, Holland AJ, Lan W, Cleveland DW. Aurora kinases and protein phosphatase 1 mediate chromosome congression through regulation of CENP-E. *Cell.* 2010; 142:444–455. [PubMed: 20691903]
45. Sardar HS, Luczak VG, Lopez MM, Lister BC, Gilbert SP. Mitotic kinesin CENP-E promotes microtubule plus-end elongation. *Curr Biol.* 2010; 20:1648–1653. [PubMed: 20797864]
46. Chretien D, Fuller SD, Karsenti E. Structure of growing microtubule ends: two-dimensional sheets close into tubes at variable rates. *J Cell Biol.* 1995; 129:1311–1328. [PubMed: 7775577]
47. Vitre B, Coquelle FM, Heichette C, Garnier C, Chretien D, et al. EB1 regulates microtubule dynamics and tubulin sheet closure in vitro. *Nat Cell Biol.* 2008; 10:415–421. [PubMed: 18364701]
48. Vergnolle MA, Taylor SS. Cenp-F links kinetochores to Ndel1/Nde1/Lis1/dynein microtubule motor complexes. *Curr Biol.* 2007; 17:1173–1179. [PubMed: 17600710]
49. Yao X, Anderson KL, Cleveland DW. The microtubule-dependent motor centromere-associated protein E (CENP-E) is an integral component of kinetochore corona fibers that link centromeres to spindle microtubules. *J Cell Biol.* 1997; 139:435–447. [PubMed: 9334346]
50. Wordeman L, Steuer ER, Sheetz MP, Mitchison T. Chemical subdomains within the kinetochore domain of isolated CHO mitotic chromosomes. *J Cell Biol.* 1991; 114:285–294. [PubMed: 1830054]
51. Howell BJ, McEwen BF, Canman JC, Hoffman DB, Farrar EM, et al. Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *J Cell Biol.* 2001; 155:1159–1172. [PubMed: 11756470]
52. Suloway C, Pulokas J, Fellmann D, Cheng A, Guerra F, et al. Automated molecular microscopy: the new Legimon system. *J Struct Biol.* 2005; 151:41–60. [PubMed: 15890530]
53. Lander GC, Stagg SM, Voss NR, Cheng A, Fellmann D, et al. Appion: an integrated, database-driven pipeline to facilitate EM image processing. *J Struct Biol.* 2009; 166:95–102. [PubMed: 19263523]
54. Mindell JA, Grigorieff N. Accurate determination of local defocus and specimen tilt in electron microscopy. *J Struct Biol.* 2003; 142:334–347. [PubMed: 12781660]
55. Ludtke SJ, Baldwin PR, Chiu W. EMAN: semiautomated software for high resolution single-particle reconstructions. *J Struct Biol.* 1999; 128:82–97. [PubMed: 10600563]
56. Sorzano CO, Marabini R, Velazquez-Muriel J, Bilbao-Castro JR, Scheres SH, et al. XMIPP: a new generation of an open-source image processing package for electron microscopy. *J Struct Biol.* 2004; 148:194–204. [PubMed: 15477099]
57. Ogura T, Iwasaki K, Sato C. Topology representing network enables highly accurate classification of protein images taken by cryo electron-microscope without masking. *J Struct Biol.* 2003; 143:185–200. [PubMed: 14572474]
58. van Heel M, Harauz G, Orlova EV, Schmidt R, Schatz M. A new generation of the IMAGIC image processing system. *J Struct Biol.* 1996; 116:17–24. [PubMed: 8742718]

Highlights

- CENP-E's C- and CENP-F's N-terminus bind microtubules with similar, high affinity
- CENP-E's C- and CENP-F's N-terminus interact in a flexible manner with microtubules
- CENP-E's C- and CENP-F's N-terminus do not require tubulin's C-terminus for binding

Figure 1. The non-motor microtubule-binding domains of CENP-E and CENP-F

A) Schematic diagram depicting full length CENP-E and CENP-F. Green, red, and blue regions indicate the domains used in this study and their corresponding amino acid segments. Note that the length of central coiled-coil regions is not to scale. **B)** SDS-PAGE of the microtubule-binding domains used in this study. EC and FC preparations both contain minor proteolytic degradation products or contaminants that could not be eliminated through multiple rounds of purification. EC, FN, and FC are 83%, 94%, and 84% of their expected size, respectively. **C)** Circular Dichroism (CD) spectra of the three CENP domains. Both EC and FN contain α -helices, while FC is largely unstructured.

Figure 2. The CENP domains are elongated oligomers

A) Analytical gel filtration chromatograms of CENP domains (left) and corresponding SDS-PAGE of peak fractions (right). The first dashed line indicates the position of the void; the second indicates the elution peak of thyroglobulin, a 660 kDa globular standard run under identical conditions. EC, 2.7 μ M, FN, 4 μ M, FC, 4 μ M. B) Predicted coiled-coil content of the CENP domains. Black lines indicate regions of at least 50% coiled-coil probability, calculated with MARCOIL [37]. FC has no predicted coiled-coils.

Figure 3. EC and FN bind microtubules with high affinity

A) SDS-PAGE of co-sedimentation assays for determining dissociation constants. S, supernatant, P, pellet. All microtubule-binding proteins, 0.5 μM . B) Quantification of A. Error bars represent mean $\pm$ s.d., $n = 3$

Figure 4. The two termini of CENP-F do not interact on the microtubule surface

A) SDS-PAGE of co-sedimentation assays of FN and FC incubated with microtubules individually (left) and in combination (right). S, supernatant, P, pellet. FN, 0.5 μ M, FC, 0.5 μ M, tubulin, 1 μ M. B) Quantification of A. Errors represent mean $\pm$ s.d., $n = 3$

Figure 5. Negative stain EM confirms a direct interaction between CENP domains and Microtubules

Negative stain TEM images of A) EC B) FN C) FC bound to paclitaxel-stabilized microtubules. D) Negative stain TEM image of paclitaxel-stabilized microtubules visualized in the absence of any additional proteins. Insets show zoomed-in images of individual microtubules. All experiments, tubulin, 2.5 μ M, CENP domain, 5 μ M. Bars, 25 nm.

Figure 6. EC and FN form disordered interactions with the microtubule surface

A) Top: Cryo-EM images of paclitaxel-stabilized microtubules in the presence and absence of the indicated CENP domains. Additional density is visible along the microtubule surface in the presence of both EC and FN, consistent with robust microtubule binding by both domains. Bottom: Power spectra calculated from individual microtubules do not show additional layer lines in the presence of CENP domains. B) Representative reference-free class averages calculated from each sample. No additional density is present in the averages calculated from EC and FN-bound microtubules, suggesting that both domains form disordered interactions with the microtubule surface. Bars, 25 nm.

Figure 7. EC bundles microtubules and promotes formation of sheets

Negative stain TEM images of EC incubated with paclitaxel-stabilized microtubules showing A) extensive bundling and B) sheet-like structures. Tubulin 2.5 μM , EC 0.5 μM . Bars, 25 nm.

Figure 8. EC promotes tubulin polymerization

A) SDS-PAGE of co-sedimentation assays with paclitaxel-stabilized ($1\mu\text{M}$ tubulin with $0.5\mu\text{M}$ EC) or dynamic microtubules ($2.5\mu\text{M}$ tubulin with $0.5\mu\text{M}$ EC), showing additional tubulin sedimentation in the presence of EC. S, supernatant, P, pellet. B) Quantification of A showing fraction in pellet. Error bars represent mean $\pm$ s.d., $n = 4$. C) Microtubule polymerization assay showing a dramatic increase in turbidity when tubulin is polymerized in the presence of EC. Inset is a zoomed in version for visualization of the tubulin control signal.

Figure 9. The tubulin E hook makes a minor contribution to EC and FN microtubule binding
 A) Schematic diagram depicting subtilisin treatment of microtubules, which cleaves the E hook (red) from the tubulin globular domain (green). B) SDS-PAGE of co-sedimentation assays with cleaved and mock-cleaved microtubules and the indicated CENP domains. S, supernatant, P, pellet. EC, 0.5 μ M with 0.125 μ M tubulin, FN, 1 μ M with 1 μ M tubulin, FC, 1 μ M with 3 μ M tubulin. Tubulin concentrations were chosen for each CENP domain where it would be 50% bound without subtilisin cleavage. C) Quantification of B. Error bars represent the propagated error between mock and cleaved experiments, $n = 3$.

Table 1

Molecular Masses of CENP Domains Measured by Multi-Angle Light Scattering

	Measured molecular mass (kDa)	Expected dimer molecular mass (kDa)
EC	96.9 ± 44.6	82.3
FN	99.5 ± 34.8	89.2
FC	45.3 ± 15.4	40.1