Published in final edited form as:
Curr Opin Struct Biol. 2011 October ; 21(5): 661–669. doi:10.1016/j.sbi.2011.07.009.

Visualizing kinetochore architecture

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Abstract

Kinetochores are large macromolecular assemblies that link chromosomes to spindle microtubules during mitosis. Here we review recent advances in the study of core microtubule-binding kinetochore complexes using electron microcopy methods in vitro and nanometer-accuracy fluorescence microscopy in vivo. We synthesize these findings in novel three-dimensional models of both the budding yeast and vertebrate kinetochore in different stages of mitosis. There is a growing consensus that kinetochores are highly dynamic, supra-molecular machines that undergo dramatic structural rearrangements in response to microtubule capture and spindle forces during mitosis.

Introduction

The kinetochore is a complex protein network that assembles on centromeric chromatin and connects chromosomes with the spindle microtubule (MTs) that segregate them during mitosis (reviewed in [1–3]). This intricate machinery plays many crucial roles, including microtubule capture, chromosome alignment, enforcement of the spindle assembly check point (SAC), and ultimately chromosome segregation between daughter cells. This last function is particularly intriguing, as chromosome movement during anaphase does not require motor activity [4]; depolymerizing ends of MTs constitute the primary force-generating element.

Early electron microscopy (EM) studies described kinetochores as multilayered disk structures, although distinct layers are not as apparent using modern sample preparation techniques[5]. Nevertheless, the notion of an overall layered geometry has been validated by subsequent studies showing protein complexes arranged along the inner-outer kinetochore axis. The components of the constitutive centromere-associated network (CCAN) are located close to the centromere, while the core complexes of the microtubule-interacting KMN network, Knl-1-Blinkin/Zwint (Spc105-Kre28 in budding yeast), Mis12 (Mtw1 in yeast) and Ndc80, which assemble at the kinetochore in prometaphase, are positioned further outwards. Additional outer components interact more transiently with kinetochores, including MT motors, plus-end binding proteins and the SAC machinery[1–3,6].

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In spite of sequence divergence among components, the overall subunit composition and architecture of kinetochores is largely conserved, suggesting that common biophysical principles may underlie kinetochore function across species. Here we highlight structural information that has emerged from two recent parallel approaches: direct imaging of reconstituted kinetochore complexes using EM, and quantitative visualization of kinetochore components in vivo by fluorescence microscopy. We focus on the most studied microtubule-binding components: the KMN network and the fungal Dam1 complex.

The KMN Network

The identification of the KMN network as the primary conserved kinetochore-microtubule interface[7] lead to a flurry of biochemical and structural studies of its three sub-complexes. The Knl-1 complex has proven recalcitrant to reconstitution or purification. *C. elegans* and budding yeast complexes bind MTs with only moderate affinity [7–9] and only in *C. elegans*[7] is the complex required for interaction between Mis12 and Ndc80 [10–12]. Its most critical function may be as a scaffold, as it acts to localize both the PP1 phosphatase (opposing the Aurora B kinase [13]) and elements of the SAC [14,15] to a specific region of the kinetochore. Structural studies will play an important part in dissecting the function of this universally conserved complex.

Architecture of the Mis12 Complex

EM has revealed a similar architecture for the Mis12 complex in budding yeast [11,12] and humans [10], despite their low sequence similarity. The complex is elongated (~22 nm long), with globular regions at the ends that are significantly larger in yeast. The thin central region probably contains coiled-coils[12]. Labeling studies in yeast[11], indicate that all its constituents (Mtw1p, Dsn1p, Nnf1p and Nsl1p) contribute to the major globular head, with only Dsn1p and Nnf1p contributing to the rest. EM of the human Mis12 suggests four consecutive globular domains along its length, with cross-linking data indicating that Nsl1 spans most of the complex length[10]. Both studies suggest that Nnf1 is localized most distal from the major head.

CENP-C, which directly binds centromeric chromatin containing histone H3 variant CENP-A (Cse4p in yeast) [16,17], makes a direct interaction with the Mis12 subunit Nnf1 via a short N-terminal segment in both humans and drosophila [18,19]. EM analysis revealed that CENP-C elicited a conformational change in the human Mis12 complex[18], from a primarily bent conformation to a straight one. The physiological significance of this transition is as yet unclear, but Mis12 could be biased towards an inactive conformation until properly localized to the kinetochore by CENP-C[18]. Interestingly, the yeast ortholog of CENP-C, Mif2p, does not interact with the Mtw1 complex in vitro, while the 4-protein inner kinetochore COMA complex binds it robustly[12].

Ndc80

The Ndc80 complex is a heterotetramer of Ndc80, Nuf2, Spc24, and Spc25. Each component has a globular domain and a coiled-coil domain, forming heterodimers of Ndc80-Nuf2 and Spc24-25 that then tetramerize. The resulting complex is an elongated, dumbbell-like molecule approximately 57nm in length[20,21]. The Ndc80-Nuf2 coiled-coil features a break half way down its length, resulting in a hinge point that makes the molecule highly flexible[22], a feature that is conserved[18]. The Ndc80-Nuf2 globular head binds microtubules[7,23–25], and the Spc24-25 head tethers the complex to the kinetochore by binding the Mis12 complex[7,23].

Ndc80 interacts with Mis12 via Spc24-25. EM analysis showed that Spc24-25 binds at the tip of the head domain of the human Mis12[10], and images of full-length Ndc80 complex bound to Mis12 in both yeast[11] and humans[18] showed a highly elongated structure (~80nm), suggesting that the two complexes add their lengths in tandem. The yeast Mis12-Ndc80 appeared to interact at an angle, forming a V-like structure[11], while this interface was linear in the human complex[18].

Visualization of microtubule-kinetochore interactions

A significant advance in understanding how kinetochores bind microtubules has come with cryo-EM analysis of microtubule-bound Ndc80 complexes from *C. elegans*[26] and subsequently, at higher resolution, for the human complex [27]. Both studies concluded that the complex contacts microtubules at the interface between tubulin monomers via the Ndc80 subunit's calponin-homology (CH) domain, with the Nuf2 CH domain distal from the microtubule surface. Docking of crystallographic model of the Ndc80-Nuf2 heterodimer [25] into the human cryo-EM reconstruction [27] proved predictive at the single amino-acid level; single point mutations on the microtubule-binding surface of the Ndc80 CH domain (which had already been predicted and tested *in vitro* based on the crystal model alone [25]) were devastating in both the human[28,29] and yeast systems (S. Westermann, personal communication), while changes in the Nuf2 CH resulted in a relatively mild phenotype [29]. Thus, the binding interface identified is both physiologically relevant and conserved.

An additional site of microtubule interaction is Ndc80's enigmatic, unstructured amino-terminal tail domain. Universally present, but variable in sequence and length, this region is critical for tubulin binding in vitro[24,25]. When fully extended, the 80 amino-acid tail of human Ndc80 would be approximately 12nm long (Fig. 1). Biochemistry [7,23,30,31] and mutagenesis [28–30,32,33] support the prevailing model of the tail as a primary site of Aurora B phospho-regulated microtubule-Ndc80 interaction (with a less crucial role in yeast [34,35]). We found that the tail was critical for the formation of Ndc80 clusters on the MT surface *in vitro*, which could be modulated by phosphomimetic mutations at Aurora B sites[27]. It remains to be determined if Ndc80 clustering plays a role *in vivo*, but this is one plausible explanation for the importance of the Ndc80 tail and the Nuf2 CH domain[27,29].

In addition to acting as a direct physical link between kinetochores and microtubules, the Ndc80 complex plays a critical role as a protein-interaction hub (reviewed in [1–3]). Recently, Ndc80's hinge region, previously of unknown functional significance, was found to be necessary for the recruitment of two other microtubule-binding factors: the Dis1/TOG microtubule polymerase in fission yeast [36] (through a direct physical interaction), and the Dam1 (or DASH) complex in budding yeast [37] (probably indirectly, although Ndc80 and Dam1 are known to physically associate [38,39], possibly via another part of the Ndc80 complex[27], discussed later). Structural characterization of the Ndc80 complex together with other microtubule-binding factors and microtubules will be critical for understanding the multimodal and interconnected kinetochore-microtubule interface.

The yeast Dam1 kinetochore complex

The fungal Dam1 complex is essential for spindle function in budding yeast, which has a single microtubule attachment per kinetochore [40,41]. It is involved in microtubule-kinetochore attachment and is a major target of the checkpoint kinase Ipl1 (yeast Aurora B homolog) [31,42,43]. Reconstitution of this ten-subunit complex in bacteria[44] opened the door for structural and biophysical studies of the interaction of the Dam1 complex with microtubules. EM showed the microtubule-induced assembly of the complex into rings and spirals [44,45]. A ring structure seems an ideal coupler for the energy released during microtubule depolymerization by protofilament peeling [4]. Several labs have now described

the movement of Dam1 complexes on microtubule depolymerizing ends [46], both for rings and smaller oligomers, although with different degrees of processivity [47,48]. None of the complex's subunits bind nucleotide; thus, its directed motion is derived from microtubule depolymerization.

No atomic structures for any of the Dam1 complex subunits exist, nor any detectable homologs at the primary sequence level. EM reconstructions showed the Dam1 complex to be a 150 Å rod, with a central enlargement from which several densities emanate [49]. Dam1 spirals bound to microtubules have been characterized by cryo-EM using both helical [49] and single-particle reconstruction approaches [50]. More recently the cryo-EM reconstruction of bona fide rings, arguably a more physiological assembly form, has also been reported [51]. The ring contains sixteen copies of the complex surrounding a thirteen protofilament microtubule, a symmetry mismatch that requires flexible elements for interaction, likely in both tubulin [51] and the Dam1 complex [52].

Putting it all together: in vivo studies of kinetochore architecture

The Delta assay developed by Salmon and colleagues measures the average separation (Delta) along the inner-outer kinetochore axis between two proteins in a kinetochore labeled with two different fluorophores, often with an accuracy better than 5 nm [53,54]. This methodology has been employed to probe the architecture of the budding yeast kinetochore, attached to a single microtubule [53], and the human kinetochore containing multiple MT attachments [54]. Combining the information from these average 1-dimensional in vivo maps, with the 2- and 3-dimensional data derived from EM studies of isolated complexes in vitro, and quantitative assignments of complex copy number [55–57], we have attempted to build 3-dimensional models of single microtubule attachment sites bound to depolymerizing microtubules. The peeling protofilaments provide important steric restraints on the position of kinetochore components; therefore, measuring the position of the microtubule end in the kinetochore accurately would help inform future modeling efforts.

3D model of the budding yeast kinetochore

Joglekar and coworkers carried out Delta studies of the budding yeast kinetochore, both at metaphase and the end of anaphase[53]. As expected, the kinetochore is arranged with the inner kinetochore COMA complex closest to the centromeric histone, and the microtubule-binding head of the Ndc80 complex and the Dam1 complex at the most outer position of the structural kinetochore components tested. Figure 2A shows a model attempting to combine the metaphase data with in vitro structural information. Generally there is excellent agreement between the position of components in the model and their measured positions in vivo. The measured length for the Ndc80 complex was 55nm, suggesting full extension along the microtubule axis. However, if the complex binds the microtubule via its Ndc80 CH domain, its projected length along the microtubule axis would be shorter, as the interaction requires the first coiled-coil segment to protrude from the microtubule at ~45° [27]. Given this requirement, the length of the first coiled-coil segment positions Ndc80's hinge so that the second coiled-coil segment would be engaged by peeling protofilaments, causing the complex to bend. The fluorophore fused to Ndc80 was at the end of the flexible 100 amino-acid N-terminal tail, and thus this region could be several nanometers away from the head domain (Fig. 1), which could be a potential explanation for this discrepancy.

In the kinetochore configuration displayed, it is necessary that Ndc80's coiled-coil be placed underneath the Dam1 ring in order to maximize its extension, as was suggested by Joglekar *et al*. This arrangement argues against a proposed interaction between the Ndc80's hinge region and the ordered regions of the Dam1 complex [37], although there may be flexible elements in Dam1 that span the ~10nm distance necessary. Instead, existing data favors a

direct interaction between the top of Ndc80's CH domain and Dam1, as suggested based on conservation [27].

We modeled the Mtw1 complex as a 22nm rod based on EM analysis, a simplification of its structure, which has been observed to vary in length and conformation [11][58][18]. The Delta analysis measured a length of ~10nm, suggesting that the complex is tilted away from the microtubule axis. Given the point centromere of budding yeast[3], we have modeled this as a tilt inwards towards the Cse4 nucleosome. The Delta analysis suggested a gap between the COMA and Mtw1 complexes (which might be artifactual since the positions of fluorophores may not precisely mark the ends of the complex[11]), while biochemistry supports a direct interaction. We have modeled an average 42° tilt that spans the measured distance between Spc24-25 and the COMA complex. The resulting geometry for Ndc80-Mtw1 interaction is consistent with the bent interface observed *in vitro*[11].

We positioned Spc105's C-terminus for reference, but a lack of structural information prevents detailed modeling. Notably, the Delta data suggests that its N-terminus would be near the microtubule peels in our model.

We have also constructed a model based on Joglekar *et al.*'s anaphase data (Figure 2B)[53]. They found a compression of the Ndc80 complex and an inwards movement of the Dam1 complex. The COMA complex appeared largely unchanged, and the Mis12 complex only slightly compressed or at an increased tilt. In our model, as also previously proposed by Joglekar *et al.*, Ndc80's coiled-coil is now outside the Dam1 complex, putting the hinge in a reasonable position for interaction, although the Ndc80 CH domain is still close to the ring. For simplicity, Dam1 is shown as a ring despite *in vivo* data supporting too few copies of the complex to support ring formation during late anaphase[55]; a similar geometry could exist with smaller oligomers.

The two depicted models may seem mutually exclusive, as it is seems unlikely that the Ndc80 coiled-coil could switch from being inside to outside the Dam1 complex during the metaphase to anaphase transition. However, such a transition could be envisioned following ring breakdown and post-translational modification of interfaces. We hope that our two models will stimulate thinking about yeast kinetochore architecture, as they predict specific distances between kinetochore components in 3D that could be tested *in vivo*.

3D model of the vertebrate kinetochore

While the KMN copy number per microtubule attachment in vertebrates is very similar to that in budding yeast (about eight)[59], inner kinetochore components are approximately stoichiometric to the KMN, and thus are present in higher number than seen in yeast[55], in agreement with the larger vertebrate inner kinetochore (Figure 3) [54]. Based on these findings, Salmon and coworkers have proposed the existence of six to nine attachment modules of fibrous character in vertebrate cells per kinetochore microtubule, each containing a copy of each of the constitutive components, and extending along the inner-outer kinetochore axis.

Wan and colleagues utilized the Delta assay to characterize human metaphase kinetochores[54]. Figure 3 synthesizes their findings with our observations that the Ndc80 complex forms clusters along protofilaments and that the interface between the complex and Mis12 is straight, rather than bent[27]. Since the vertebrate centromere structure is not well established, the restraints provided by the Delta data are looser. Modeling two Ndc80 clusters that are one tubulin dimer away from the depolymerizing MT end gives an average length of the complex that matches the measured dimensions [54].

Wan *et al*. found that the projection of the Mis12 complex along the microtubule axis is quite small, ~10nm[54], which is difficult to reconcile with the *in vitro* observation that the Ndc80 complex and Mis12 complex add their lengths in series[18]. It is possible that the labeling antibodies do not bind epitopes at the ends of the Mis12 complex, or that *in vivo* additional factors lead to a bending of the Mis12-Ndc80 interface. The authors also measured the positions of the N-terminus and a middle region of Knl1. In our model these regions once again fall in the area of microtubule peels.

Because in metaphase the instabilities of sister kinetochore pairs give rise to oscillatory stretching of the corresponding centromeric chromatin, Wan and coworkers were also able to measure the stiffness of protein linkages at the kinetochore. With the exception of the inner kinetochore proteins CENP-A and CENP-C, all linkages were stiff. Interestingly, a recent EM labeling study has found that CCAN components are stretched in the presence of tension, whereas outer-kinetochore components are not[60].

Alternative models of the vertebrate kinetochore organization have been proposed based on different interpretations of electron tomographic data. In one model the KMN forms a network that reshapes to accommodate the arrival and engagement of a spindle microtubule[61]. In the alternative model, extended fibrils bind to the inner surface of peeling protofilaments[62]. In our models extended coiled-coil domains are quite tightly packed, generally within 5–10 nm, which could give the appearance of a meshwork when visualized by tomography. Although more data is needed, significant features of both models are likely to be correct and even compatible.

Concluding remarks

Microscopy methods that allow direct visualization of kinetochores components in vivo and in vitro are giving us an increasingly rich picture of the macromolecular architecture of kinetochores and the structural transitions that occur during mitosis. The structure of centromeric chromatin, the presence and dynamics of microtubules, and spindle forces all contribute to kinetochore organization and re-organization; uncovering the underlying mechanics will be critical to developing an integrated model of kinetochore function.

Highlights

- Here we review recent advances in the study of microtubule-binding kinetochore complexes, concentrating on studies using electron microscopy methods in vitro and nanometer-accuracy fluorescence microscopy in vivo.
- Putting together existing experimental data, we have generated three-dimensional models, both of the vertebrate and budding yeast kinetochores.

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*** Of special interest**

**** Of outstanding interest**

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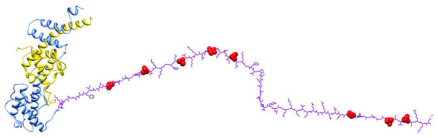

Figure 1. Model the N-terminus of human Ndc80

The microtubule-binding head of human Ndc80 (PDB 2VE7), which lacked the N-terminal 80 amino acids, is shown in ribbon diagram (Ndc80 in blue, Nuf2 in gold), with an added model of the N-terminus shown in an extended conformation in stick format in magenta. Kink points correspond to the positions of prolines. Aurora B phosphorylation sites are shown in space-filling representation in red. The N-terminus model was built with PyMOL; the figure was generated with UCSF Chimera.

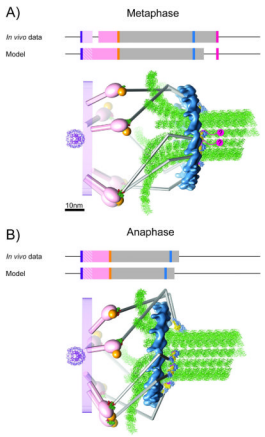

Figure 2. 3D models of the budding yeast kinetochore bound to a depolymerizing MT

Three dimensional models of kinetochore components were assembled to fit the Delta data of Joglekar *et al.* for A) metaphase and B) anaphase/telophase. The microtubule model is shown in green ribbon representation and was generated by docking the electron crystallographic structure of taxol-bound tubulin (PDB 1JFF) into the cryo-EM reconstruction of human Ndc80 bound to the microtubule (EMDB 5223); protofilament peels were generated from the structure of RB3-stathmin colchicine bound tubulin (PDB 1SA0). The yeast nucleosome (PDB 1ID3) is shown as a ribbon model in dark purple, the region of inner kinetochore complexes (such as the COMA complex) localization is shown as a light purple transparent cylinder. The Mtw1 complex is modeled as a rotationally symmetric rigid rod with dimensions based on 2D single-particle analysis and is displayed in pink. The position of the C-terminus of Spc105 is shown as an orange sphere. The globular domains of the human Ndc80 complex (PDB 2VE7) are shown in surface representation and are colored as follows: Ndc80, light blue, Nuf2, gold, Spc24, green, Spc25, red. The attachment point of Ndc80's unstructured N-terminus is shown in magenta, and a possible extension of this protein region along the microtubule in the metaphase case is illustrated. The Ndc80-Nuf2 globular heads were oriented on the microtubule surface by docking into the cryo-EM map. The coiled-coil segments (grey) were modeled as rigid rods based on the average lengths reported by Wang *et al.* and the width of an idealized dimeric coiled-coil. The shorter segment was oriented with the truncated coiled-coil of the crystallized construct, which was found in two different conformers in the asymmetric unit of the crystal lattice. The more bent conformation was used in the metaphase model (A), while the straighter conformation was used in the anaphase model (B). The hinge point between the two coiled-coil segments was treated as a freely-rotatable interface, in accordance with the observations of Wang *et al.* The models and the figure were made with UCSF Chimera.

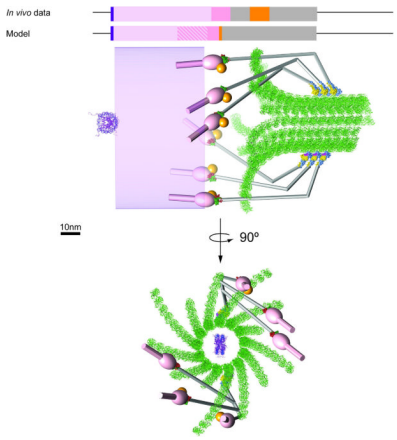

Figure 3. 3D model of the vertebrate kinetochore bound to a depolymerizing MT

Analogously to Figure 2, structures were assembled to fit the Delta data of Wan *et al.* The high-resolution structure of the vertebrate nucleosome is shown (PDB 1KX5). The probable position of the C-terminus of Knl1 is shown for reference based on *in vitro* data, although it was not probed in the Delta study. The position of Ndc80's shorter coiled-coil segment was once again modeled based on the orientations observed in the crystal for the two outer molecules in each cluster; an intermediate position was generated for the central molecule.