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Linked In: Formation and Regulation of Microtubule Attachments During Chromosome Segregation

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Abstract

Accurate segregation of the replicated genome during cell division depends on dynamic attachments formed between chromosomes and the microtubule polymers of the spindle. Here we review recent advances in mechanistic analysis of microtubule attachment formation and regulation.

Introduction

Accurate genome distribution during cell division requires dynamic attachments between *kinetochores*, proteinaceous structures assembled on the centromeric regions of chromosomes, and spindle microtubules. Kinetochores harness the forces generated by microtubule dynamics to drive chromosome segregation and ensure chromosome biorientation—the state where sister chromatids are attached to microtubules from opposite spindle poles. Here we review recent advances in mechanistic analysis of kinetochore-microtubule attachment formation and regulation. Due to space constraints, we do not discuss the chromatin-proximal features important for building microtubule attachment sites [1–3].

Current Views of the Kinetochore-Microtubule Interface

EM tomography, super-resolution imaging, EM of purified kinetochore complexes, and atomic structures of kinetochore proteins are providing increasingly detailed views of the kinetochore-microtubule interface. EM tomography of cultured cells has revealed an amorphous interface between plus ends of spindle microtubules and the kinetochore (Fig. 1A) [4,5]. The primary feature revealed by this approach is flared protofilaments at attached plus ends, which appear to connect to chromatin by slender fibrils, whose molecular composition is unclear [4]. The curvature of the protofilament flaring is distinct for kinetochore microtubules, compared to non-kinetochore spindle microtubules, consistent with a special coupling interface. EM tomography across multiple organisms has revealed similar flared protofilaments at kinetochore-attached plus ends [6].

Complementing the EM analysis in vivo is super-resolution imaging with probes to specific kinetochore proteins—this approach has generated positional maps of individual proteins and revealed broad conservation of the architecture and composition of the kinetochore-

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microtubule interface[7–10]. There has been some controversy about the number of specific molecules per microtubule attachment site measured by fluorescence microscopy—earlier work suggested approximately 8 core microtubule-binding Ndc80 complexes (see below) per kinetochore microtubule but recent work has suggested that this number may actually ~20 [11]—additional work is needed to definitively address this important question. Multivalency of attachment complexes suggested by these value ranges is now widely accepted as being critical for generating a dynamic interface with spindle microtubules although the detailed physical mechanism remains an active topic of investigation.

In addition to the EM and superresolution imaging, atomic resolution views are now available for a significant number of kinetochore parts, including the key microtubule binding complexes and the components linking these complexes to the chromatin [1,2,12]. A specific domain, referred to as the RWD domain, has emerged as a common element of functionally distinct kinetochore proteins, potentially reflecting their common origin (Fig. 1B) [13–17]. While we are still some way from placing atomic structures into the picture of an intact kinetochore, the possibility of doing so has been greatly advanced by the purification of native kinetochore-like particles from budding yeast [18,19]. EM of these particles has revealed a central hub surrounded by globular domains (Fig.1C). In the presence of microtubules these particles adopt different conformations consistent with multivalent attachments being formed by the globular domains (Fig.1C). Fitting molecular views of well-studied components into the various elements observed in these striking images is an important future goal.

Though much of the work in the past decade has focused on the kinetochore itself, earlier studies showed that the kinetochore fiber, comprised of multiple stable microtubules that extend from kinetochore to the spindle pole, is highly organized (Fig.1A)[20]. Recent work suggests that the vesicle coat protein clathrin contributes to this organization, providing a mitotic function distinct from its well-studied interphase role [21–23]. Other proteins whose primary studied role is outside of mitosis, such as the Y-complex of nucleoporins and the actin-nucleating formins, also target to and function at kinetochores, highlighting an emerging importance of “moonlighting” activities in chromosome segregation [24,25].

Force Generation at the Kinetochore

Genetic analysis in multiple organisms has cemented the much-debated view that microtubule dynamics, rather than motor activity, is the primary driver of chromosome movement [26]. In support of this, *in vitro* experiments estimate that a single depolymerizing microtubule can generate 30–65pN force [27]. In classic experiments performed in grasshopper spermatocytes, the force needed to stall a chromosome in motion was estimated to be ~ 700 piconewtons (pN) [28]. A recent study that revisited this issue in the same experimental system using an optical trap has suggested that the stall force may be 100 times less than what was originally measured [29]. New force measurement experiments are urgently needed in vivo to resolve this large discrepancy. Regardless, the coupling interface between the kinetochore and dynamic microtubules must transduce sufficient force to the chromatin to drive chromosome movement.

The kinetochore-like particles purified from budding yeast are providing important new insights into the biophysical properties of kinetochore-microtubule interactions. Optical trapping of beads coated with these particles has revealed a catch bond‘-like force-dependent stabilization of attachments [18]. This finding suggests a first-principles model for selective stabilization of bi-oriented attachments that are being pulled towards opposite spindle poles. Tension-based modulation of microtubule dynamics has also been documented during metaphase oscillations in vertebrate kinetochores [30]. Though the

identity of molecular enforcers of tension-dependent stabilization remains unclear, super-resolution imaging has shown that kinetochore conformation/organization is altered in response to microtubule dynamics [9,31,32]. In addition, recent work analyzing vertebrate kinetochore structure during metaphase oscillations has shown that the kinetochore is pliant and undergoes compression while moving poleward, potentially due to differentially positioned active and passive force-generating microtubule attachment sites [33]. As oscillations are not a universal feature of attached chromosomes, potentially the passive site positioned further out from the chromatin represents a conserved coupling point. Determining the molecular basis for force-dependent attachment stabilization and the dynamic conformational changes observed within the kinetochore are challenging but important avenues to explore in the future.

New Insights into the Primary Conserved Mediator of Kinetochore-Microtubule Interactions: The Ndc80 Complex

The 4-subunit Ndc80 complex is the primary mediator of dynamic attachments at the kinetochore [34,35]. The microtubule-binding activity of the complex resides in heterodimers of Ndc80 and Nuf2 subunits whose N termini fold into calponin homology (CH) domains. Given its central importance in chromosome segregation and ease of reconstitution, a number of structural and biophysical studies have been conducted on the Ndc80 complex. Early work revealed that microtubule-binding activity resides in the CH domains of Ndc80 and Nuf2 and in the basic N-terminal tail of Ndc80, predicted to be unstructured and targeted for phosphorylation by Aurora B kinase [36]. High resolution cryo-EM of Ndc80 complex-decorated microtubules revealed that the Ndc80 CH domain is in direct contact with the microtubule lattice (reviewed in [12]) [37,38]. Consistent with this, disruption of the interface residues on the Ndc80 CH domain abrogate microtubule binding in vivo [39,40]. A recent higher resolution cryo-EM analysis of the Ndc80-microtubule interface map points to a more complex multimodal interaction with additional points of contact involving the tail and the Nuf2 CH domain [41]. However, the precise roles of the N-terminal tail and of the Nuf2 CH domain in vivo are unclear. Tail deletion of Ndc80 in budding yeast does not affect viability whereas in human cells a similar deletion prevents kinetochore fiber formation [42–44]. Mutations in the Nuf2 CH domain appear to cause only mild defects in cultured human cells even though microtubule binding is impaired by these mutations to the same extent as Ndc80 CH domain mutations in vitro [39]. One clue into the origin of differing outcomes of similar Ndc80 complex perturbations in different species has come from biophysical experiments—e.g., unlike the budding yeast Ndc80 complex, human Ndc80 complex by itself stabilizes microtubule ends by promoting rescue [45–47]. Developing a unified conceptual framework for the mechanistic contributions of the N-terminal Ndc80 tail and the Nuf2 CH domain function is essential given the central role of the Ndc80 complex at the kinetochore.

Cooperators of the Ndc80 Complex: Different Flavors, Different Mechanisms?

An emerging theme in recent years is that the Ndc80 complex needs cooperators to generate efficient coupling of the kinetochore to dynamic microtubule ends. Surprisingly, the cooperators appear to be distinct in different species, a feature that is somewhat disconcerting and the explanation for which remains unclear. All of the cooperators described to date are characterized by their dependency on the Ndc80 complex for kinetochore localization. To date, these cooperators include bona fide dynamic end couplers like the Dam1 and Ska complexes[48–50], microtubule dynamics modulators like XMAP215 [51,52], and, somewhat surprisingly, the DNA replication factor Cdt1 [53]. The

yeast Dam1 complex, which oligomerizes into rings/spirals encircling the microtubule lattice, is the best characterized Ndc80 cooperator [48,49,54–56]. However, a direct physical connection between the Dam1 and Ndc80 complexes has not been observed. Though the precise mechanism behind this cooperativity is still being investigated, a recent in vitro study suggests that the concerted action of a fibrillar element and a ring-based coupler provides the ideal coupling geometry for transducing force generated by depolymerization of a microtubule end [57].

Although metazoans lack the Dam1 complex, the 3-subunit Ska complex, which does not exhibit primary sequence similarity to Dam1 complex subunits, is emerging as a functional counterpart [58]. The Ska complex is a microtubule end coupler similar to Dam1 and can bind curved protofilament rings that mimic depolymerizing ends[50]. Structural work indicates that the Ska complex forms a W-shaped dimer with a winged helix motif, commonly found in DNA-binding proteins, imparting microtubule-binding activity to the outer arms [59]]. Similar to Dam1, Ska does not exhibit a direct interaction with the Ndc80 complex but enhances Ndc80 microtubule binding and its ability to track depolymerizing ends [50]. In vivo, kinetochore-microtubule interactions are compromised in Ska-inhibited human cancer cells but the complex is also implicated in controlling anaphase onset and is dispensable for viability in chicken cells [58,60,61]. Further work is needed to integrate the in vitro and in vivo analysis of Ska complex – Ndc80 complex cooperation.

Cdt1 in mammalian cells and fission yeast XMAP215 family members Dis1/TOG1 and Alp14 have also emerged as Ndc80 complex cooperators [51–53]. Although both Cdt1 and Dis1 are proposed to associate with the NDC80 loop, a short region that breaks the NDC80 coiled coil, different mechanisms have been proposed for how they stabilize Ndc80-mediated attachments. Based on super resolution microscopy, Cdt1 has been suggested to stabilize an extended confirmation of Ndc80 and enhance its microtubule binding. Dis1/Alp14 are members of the well-studied XMAP215 family of microtubule dynamics regulators and presumably a locally enriched pool at the kinetochore stabilizes bound microtubules [62]. In vitro studies, similar to those performed for Dam1 and Ska complexes mixed with Ndc80 complexes, will be important to understand the precise means by which Cdt1 and the XMAP215 family proteins cooperate with the Ndc80 complex. More importantly, the reason for the diversity of Ndc80 cooperators, despite the conservation of the Ndc80 complex, needs to be addressed.

Regulation of Kinetochore-Microtubule Attachments: From Mechanisms to Origins of Chromosomal Instability

During prometaphase, kinetochores initially interact laterally with the microtubule lattice [63]. These initial lateral interactions accelerate microtubule capture and help the kinetochore achieve the proper orientation to form stable end-coupled attachments that generate tension [64,65]. The formation and stability of end-coupled attachments are tightly regulated since incorrect attachments lead to lagging chromosomes and segregation errors. Multiple studies suggest that precise regulation of kinetochore-microtubule attachments involves interplay between kinases and phosphatases that control kinetochore composition and microtubule-binding properties of their kinetochore substrates.

The best-studied regulator of kinetochore-microtubule attachment stability is the Aurora B kinase, which is proposed to be the primary tension sensor at the kinetochore. Aurora B kinase promotes turnover of microtubule attachments by directly altering the microtubule binding properties of its substrates and regulating the recruitment of multiple proteins to the kinetochore [66–69]. Biophysical assays are providing valuable insight into the mechanism by which Aurora B kinase-mediated phosphorylation promotes kinetochore-microtubule

turnover. Experiments employing phosphomimetic substitutions suggest that Aurora B-mediated phosphorylation does not simply detach the Ndc80 complex or yeast kinetochore-like particles from a microtubule; instead, phosphorylation reduces tip stabilization leading to disassembly of the bound microtubules and subsequent detachment [46,47].

Aurora B is enriched at the inner centromere region inbetween the sister kinetochores (Fig. 1A). The localization of the kinase between sister kinetochores, the compliance of centromeric chromatin, and the detrimental effects of forced localization of the kinase to the kinetochore led to a model in which attachment stability was a function of substrate proximity to the kinase at the centromere [70]. In this model, tension stabilized attachments by spatially displacing substrates from the influence of the kinase. This model has been challenged by analysis of a mutant that is defective in localizing Aurora B to centromeres in budding yeast, which surprisingly nonetheless exhibited normal tension-sensitive regulation of attachments [71]; earlier work perturbing one of the two known mechanisms involved in Aurora B centromere targeting in chicken cells suggest that this also may be true to some extent in vertebrates [72]. Thus, the mechanism by which Aurora B discriminates between correctly bioriented and incorrectly attached kinetochores remains an open question. Protein phosphatase 1 (PP1) has been known for a long time to genetically oppose Aurora B in budding yeast [73]. Both the outer kinetochore protein Knl1 (Spc105 in budding yeast) and the motor protein CENP-E harbor PP1 docking sites that are themselves Aurora B targets [74,75]. Progressive recruitment of PP1 following microtubule attachment to the kinetochore, by a mechanism that remains unclear, was proposed to stabilize bi-oriented microtubule attachments [74,76]. However, at least in budding yeast, the importance of PP1 to attachment stabilization appears to be limited [77]. Thus, the precise interplay between attachment, tension, Aurora B and PP1 remain unclear. We speculate that discrimination of correctly bioriented (under tension) versus incorrect (tensionless) attachments is intrinsic to the kinetochore and requires activated Aurora B. This speculation is based on the observation that bioriented kinetochores bound to dynamic microtubules are in a very different structural state compared to taxol-treated non-dynamic kinetochores [9]; this structural transition may control susceptibility of the kinetochore-microtubule interface to the action of active Aurora B. Inner centromere-targeted Aurora B likely has roles that are also important in segregation, e.g. in protecting against merotely and in centromeric cohesion [71,78,79] that may explain the widely conserved localization pattern.

In recent years, the mitotic kinase Plk1 has emerged as an additional major regulator of kinetochore-microtubule attachments [80,81]. Plk1, unlike Aurora B, has many distinct roles preceding anaphase (as well as post-anaphase functions), which makes it difficult to study and explains the greater attention that has been paid to Aurora B. Plk1 is enriched on kinetochores before biorientation, is reduced following microtubule attachment, and has multiple kinetochore substrates, identified in proteomic studies [82–84]. An appealing mechanism proposed for how Plk1 stabilizes attachments is via phosphorylation of BubR1, a dual function pseudokinase involved in checkpoint signaling and chromosome segregation [85]. Phosphorylation of a specific region in BubR1 by Plk1 promotes interaction with the PP2A-B56a phosphatase that stabilizes kinetochore-microtubule attachments, potentially via counteraction of Aurora B [86,87]. Consistent with this, a recent study reported that tethering constitutively Plk1 to the kinetochore stabilized kinetochore-bound microtubules [88]. However, as Plk1 has multiple targets enriched at kinetochores, which include stabilizers and destabilizers of microtubules, a more complex view of how Plk1 controls kinetochore-microtubule interactions is already emerging [89–92]. One challenge limiting analysis of Plk1 has been an inability to precisely perturb its localization to the kinetochore. Kinetochore recruitment of Plk1 is thought to involve Cdk1-, Aurora B- and Plk1-dependent priming of multiple kinetochore proteins [93–95]. Nonetheless, it is our opinion that the primary Plk1 docking site at the kinetochore remains to be identified.

In addition to the local regulation of attachment stability at the kinetochore-microtubule interface, global control of kinetochore-microtubule turnover has emerged as an important contributor to error-free segregation. In mammalian cultured cells, kinetochore microtubules switch from a dynamic state in prometaphase to a more stable state in metaphase; this change is dependent on degradation of Cyclin A during prometaphase [96] and is reminiscent of the well-known metaphase-anaphase transition, when there is additional stabilization of attachments following degradation of Cyclin B [97]. Thus Cyclin A appears to create a less stable kinetochore-microtubule interface during prometaphase, presumably to facilitate error correction. The mechanism by which Cyclin A-Cdk1 globally controls kinetochore-bound microtubule dynamics will be important to elucidate to integrate this new finding into the prior studies focused on control at individual kinetochores. Potentially contributing to the prometaphase-metaphase and metaphase-anaphase transitions in attachment stability is the Astrin-SKAP microtubule-binding complex that is recruited to kinetochores following chromosome bi-orientation [98–100].

Studies in human cells have also revealed important roles for motor proteins in kinetochore microtubule stability. The kinesin-8 family member Kif18, a processive plus end motor that suppresses end dynamics, confines kinetochores to the middle of the spindle by limiting the extent of dynamic transitions [101–104]. The kinesin-13 family of depolymerases, enriched between sister kinetochores, reduce the likelihood of merotelic attachments, where a single kinetochore connects to opposite spindle poles [105]. The chromokinesins Kif4 and Kid, concentrated on chromosome arms, regulate chromosome positioning by altering inter-kinetochore tension in a position-dependent manner within the spindle [104]; chromokinesins also likely move chromosomes to form an equatorial ring in prometaphase prior to establishment of kinetochore-microtubule attachments [64,106]. Finally the kinesin-7 family motor, CENP-E, concentrated on the outer kinetochore, increases the stability of attachments [107]. The combination of an N-terminal plus end motor activity, a long flexible interrupted coiled-coil tether, and a C-terminal non-motor microtubule-binding domain enable CENP-E to bidirectionally track growing and depolymerizing microtubule plus ends, a striking property that likely underlies its role at the kinetochore [107]. Overall motors play important roles in restricting kinetochore position and maintaining attachment stability.

Proper regulation of kinetochore microtubule attachments is important because misattachments lead to unequal distribution of the genome. A majority of solid tumor cells are aneuploid and exhibit elevated rates of chromosome missegregation [108]. Many cancer cells appear to have hyperstable kinetochore-microtubule attachments that underlie increased rates of missegregation [109,110]; in addition, cancer cells may be less efficient in error correction [68]. Remarkably, reducing kinetochore-microtubule attachment stability in cancer cell lines by overexpression of a kinesin-13 family microtubule depolymerase reduces chromosome missegregation [111]. While these studies highlight the importance of precise control of kinetochore-microtubule dynamics in preventing chromosomal instability, the genetic/epigenetic basis underlying loss of this control in cancer cells remains unclear and is important to elucidate.

Interplay Between Attachment Formation and Checkpoint Signaling

The mechanical events at the kinetochore are coordinated with checkpoint signaling, which ensures that the separation of all chromosomes only occurs after the last chromosome has attached to the spindle [112]. Silencing of the spindle assembly checkpoint is coordinated with microtubule attachment, which leads to dynein motor-based stripping of checkpoint proteins from the kinetochore in metazoans [113,114] Recent work suggests that the key event in this silencing mechanism is removal of the dynein adaptor protein Spindly [115]. In

the absence of Spindly, but not in the presence of a Spindly mutant that fails to recruit dynein, the checkpoint is still silenced following attachment; as dynein is not ubiquitously present at kinetochores across species, the presence of a dynein-independent but microtubule attachment-dependent silencing pathway is perhaps not surprising. Two other components implicated in silencing are protein phosphatase I, whose recruitment by Knl1/Spc105 is important for checkpoint silencing in fungi and worms [77,116,117]. As constitutive tethering of PP1 to budding yeast kinetochores does not prevent checkpoint activation [77], whether PP1 responds to microtubule attachment is unclear. Microtubule binding by the PP1 docking protein KNL-1 has been proposed to contribute to checkpoint silencing in *C. elegans*, although this contribution appears to be genetically parallel to PP1 docking [117]. Thus, while microtubule attachment must be relayed to control checkpoint signaling at the kinetochore, the underlying molecular mechanisms remain to be clarified in future work. A major challenge here is our lack of understanding of how the checkpoint is activated at the kinetochore. As progress is made on this topic, understanding how attachment silences the checkpoint will become more feasible, potentially through the use of in vitro systems such as the kinetochore-like particles from budding yeast.

Perspective

The cataloguing of the majority of kinetochore proteins, genetic/RNAi analysis in multiple model organisms and in cultured mammalian cells, in vitro reconstitutions of complexes, biophysical assays with purified components/complexes and native assemblies, and structural approaches are cumulatively building a detailed picture of how kinetochore-microtubule interactions are formed and regulated. Still, many challenges lie ahead, notably resolving contradictions between in vitro and cellular studies, and elucidation of the complex kinase-phosphatase activity fluxes that coordinate different events at the kinetochore but are themselves under mechanical control. In addition, surprises are likely to emerge from work in different biological contexts, such as stem cell divisions that exhibit asymmetric chromatid segregation [118,119] and meiosis I, where homologues rather than sisters segregate [120,121]. Thus, the many outstanding questions and emerging new areas will keep the field occupied for some time to come.

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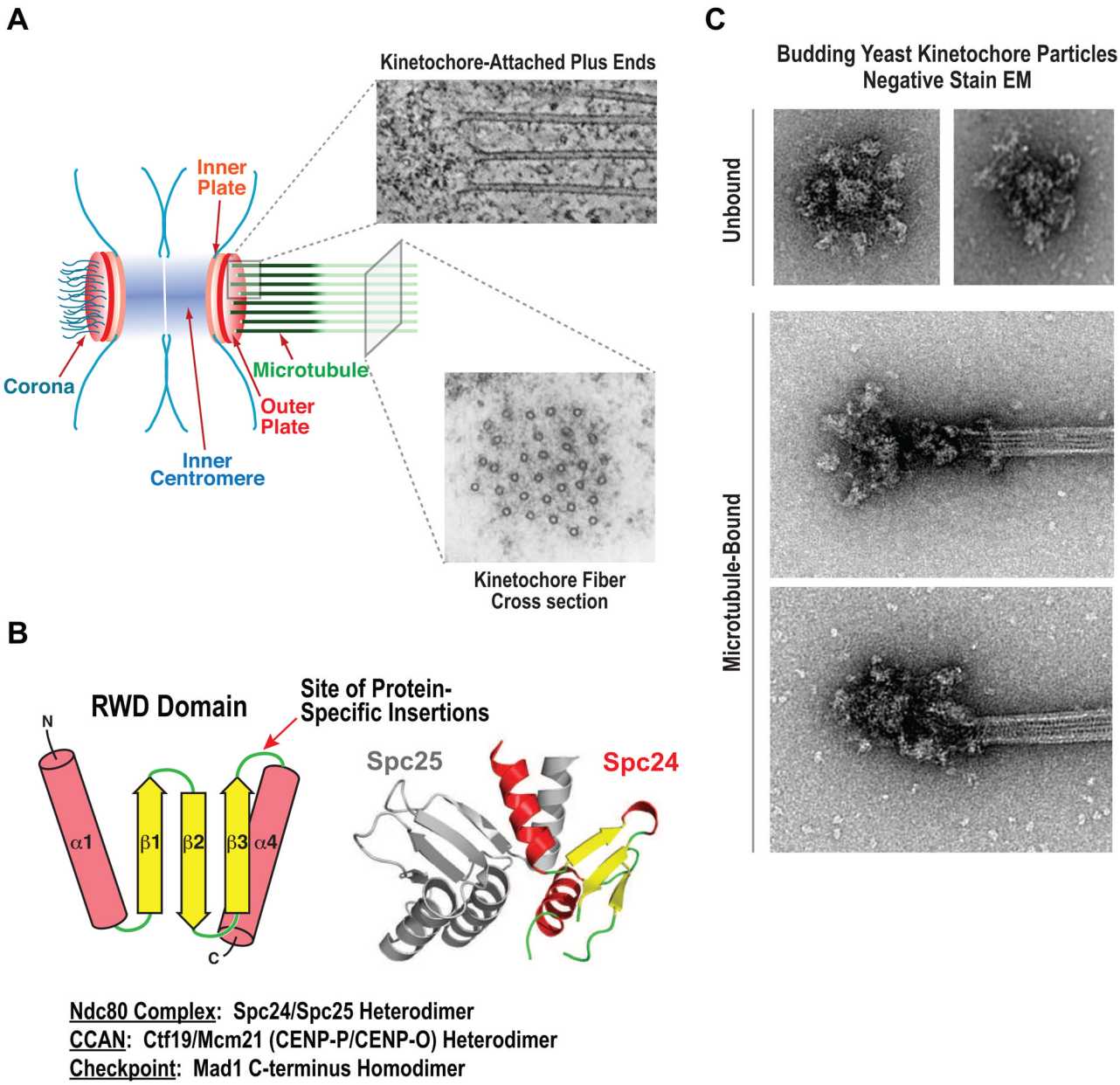

Figure 1. Different Resolution Views of Kinetochore Architecture

(A) A slice from an EM tomogram of plus ends embedded in the outer kinetochore (*top*; courtesy of R. McIntosh) and a thin cross-sectional view of a cold-stable kinetochore fiber (courtesy of C. Rieder; ref. 20). The schematic on the left highlights a plate-like architecture at the kinetochore evident in older EM studies whose existence has come under debate following the development of new EM preservation methods [see ref. 5]. **(B)** Schematic of the RWD domain that recurs in multiple kinetochore proteins. The domain is always present in 2 copies – either as a heterodimer or as a homodimer. The structure of the Spc24/25 heterodimer from the Ndc80 complex, the first kinetochore components found to harbor this domain, is shown on the right (PDB 2FTX); Spc24 has a minimal RWD domain—the other kinetochore protein listed below harbor different insertions in the loop indicated by the arrow. **(C)** Negative stain EM images of purified native yeast kinetochore-like particles.

Images of particles on their own and bound to microtubule ends are shown (courtesy of S. Biggins; ref 19].