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Mechanisms of force generation by end-on kinetochore-microtubule attachments

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Generation of motile force is one of the main functions of the eukaryotic kinetochore during cell division. In recent years, the KMN network of proteins (Ndc80 complex, Mis12 complex, and KNL-1 complex) has emerged as a highly conserved core microtubule-binding complex at the kinetochore. It plays a major role in coupling force generation to microtubule plus-end polymerization and depolymerization. In this review, we discuss current theoretical mechanisms of force generation, and then focus on emerging information about mechanistic contributions from the Ndc80 complex in eukaryotes and the microtubule-binding Dam1/DASH complex from fungi. New information has also become available from super-resolution light microscopy on the protein architecture of the kinetochore-microtubule attachment site in both budding yeast and humans, which provides further insight into the mechanism of force generation. We briefly discuss potential contributions of motors, other microtubule-associated proteins, and microtubule depolymerases. Using the above evidence, we present speculative models of force generation at the kinetochore.

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Introduction

The kinetochore is a unique motor essential to the segregation of chromosomes during cell division. It is a macromolecular protein complex that assembles on a specialized locus on each DNA molecule, known as the centromere. It attaches to the plus-ends of one or more spindle microtubules (MTs) forming kinetochore-microtubules (kMTs), and tightly couples chromosome movement to kMT polymerization and depolymerization. More than 40 different proteins are necessary for kMT formation and persistent attachment (see Ref. [1•] for an in-depth review of kinetochore composition, organization, and regulation). Many of these proteins are conserved in all eukaryotes.

The goal of this review is to discuss the biophysical mechanisms of force generation coupled to MT polymerization and depolymerization, and their relevance to the kinetochore. An exciting confluence of results from diverse fields has brought us closer to understanding this problem. Investigations utilizing structural biology, single molecule biophysics, high-resolution microscopy, and cell biology seek to examine two fundamental aspects of kinetochore function: the nature and characteristics of the force generation mechanism, and the processes that control transitions between polymerization and depolymerization of kMT plus-ends. We first describe the principles of force generation at the MT plus-end. We review available biophysical data on the kinetochore-MT attachment and then results from cell biology for the kinetochore complexes necessary for end-on attachment: the Ndc80 complex and the Dam1/DASH complex (in fungi), are emphasized. These two complexes have been studied extensively *in vitro*. We summarize these results, and discuss their implications *in vivo*. We then examine new data on the protein architecture of the kinetochore and its predictions in force generation mechanisms. We also briefly examine the general contributions of motors, depolymerases, and MT associated proteins (MAPs) to controlling force generation and stable maintenance of the kinetochore-MT attachment. Kinetochore functions in attachment error correction and in the spindle assembly checkpoint are also vital for accurate segregation. These topics have been reviewed elsewhere [2,3•].

Theoretical mechanisms of force generation at the plus-end

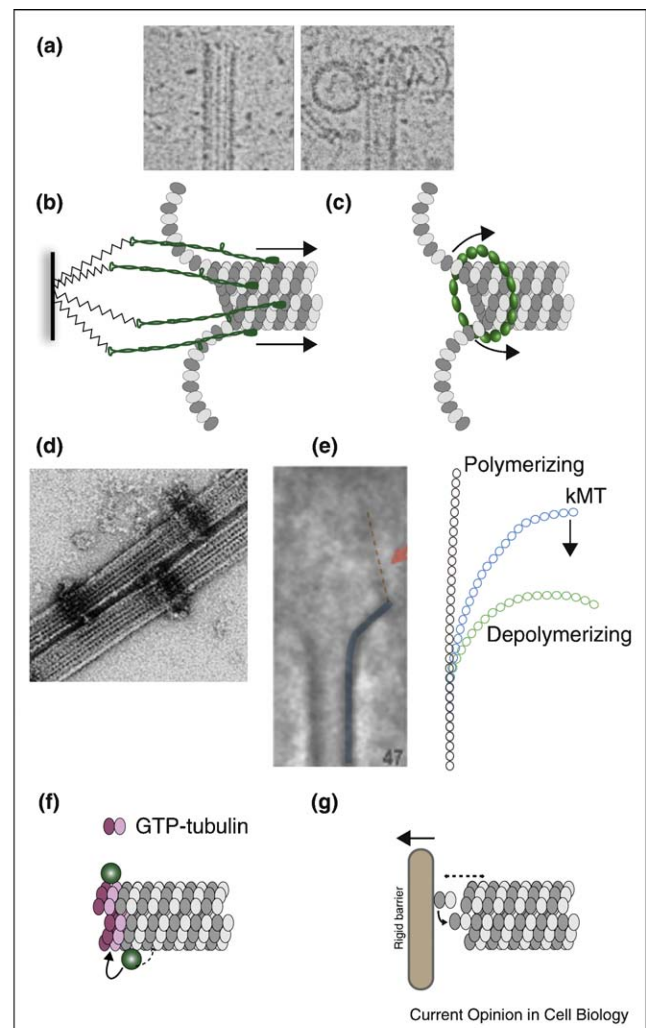
Discovery of polymeric structure of MTs, which grows and shrinks in length by adding or losing subunits only from its two ends, prompted inquiries into general MT end-based force generation mechanisms [4•]. Details of these mechanisms depend on the nature of kinetochore-MT attachment, and several molecular arrangements were proposed for force generation at the kinetochore that is coupled with MT disassembly (reviewed in Ref. [5]). During growth, a MT plus-end adds GTP–tubulin dimers, which have a relatively straight conformation along the long axis. GTP bound to β-tubulin is hydrolyzed after incorporation in the MT lattice, but with some time delay. GDP–tubulin dimers have a bent conformation, but within the MT lattice relaxation to this state

is not allowed because of lateral interactions. The energy of GTP hydrolysis is stored in the lattice as a strain instead, which manifests itself in the flared geometry of depolymerizing plus-ends (Figure 1a) [6]. A depolymerizing plus-end thus provides two modes of force generation. The MT lattice immediately behind the plus-end provides regular array of tubulin dimers as attachment sites. Alternatively, the strain energy associated with bending of GDP–tubulin dimers during MT disassembly can be converted into work. A polymerizing plus-end can also generate forces. The mechanisms include differential binding energies of a kinetochore protein for GTP–tubulin and GDP–tubulin or thermal ratcheting against a rigid barrier.

The first class of models for MT disassembly coupled force generation uses the MT lattice as an array of binding sites (Figure 1b). These models propose that the kinetochore-MT attachment is achieved by multiple, relatively weak linkages that are dispersed over a stretch of MT lattice behind the plus-end. The weak linkages break and reform under the influence of thermal fluctuations allowing the attachment site to diffuse over the MT lattice. Loss of dimers at the plus-end creates a moving boundary and biases the diffusion of the coupler toward the minus end of the MT (hence the term 'biased diffusion'). For this general mechanism, persistence of attachment is an important characteristic, as the reliance on multiple, weak linkages near a shortening plus-end makes the kinetochore susceptible to complete detachment. The persistence of attachment depends on the rate of MT depolymerization, energy of individual linkages, and the distribution of these linkages over the MT lattice. To avoid complete detachment, linkages must be established away from the plus-end. Therefore, explicit mechanisms that bias the formation of linkages away from the plus-end or alternatively, low probability of detachment from the shortening end are important for biased-diffusion models. The inside-out curved protofilament geometry at the disassembling plus-end may bias movement of the binding proteins away from the plus-end. The maximum amount of force generated by biased diffusion depends on the energy of individual interactions and the number of such linkages. For example, the sleeve mechanism as originally proposed by Hill can generate up to 13 pN of force, although the persistence of attachment diminishes with higher forces [7,8].

The second class of models can be termed as 'forced-walk' mechanisms. These models rely on the peeling of protofilaments at depolymerizing plus-ends [6,9]. A force coupler that resists the outward bending of protofilaments at the MT plus-end effectively transforms the bending of individual GDP–tubulin dimers as they lose their lateral lattice attachments into a power stroke (Figure 1c). Explicit mechanical and mechanochemical models of MT depolymerization [10,11] enabled characterization of

Figure 1



(**a**) Morphology of polymerizing and depolymerizing MT plus-ends *in vitro*. Disassembling MT plus-ends allows two mechanisms of force generation coupled to the disassembly. (**b**) Biased-diffusion models — proteins weakly bound to the MT lattice diffuse along the lattice in the absence of external force. However, the shortening plus-end acts as a moving boundary and biases the diffusive movement toward the minus end. (**c**) A forced-walk model — a ring around the lattice serves as a barrier to the relaxation of curling protofilaments. Continuous outwards curling pushes the ring toward the minus end. Two types of forced-walk couplers based on EM and *in vitro* data have been proposed (**d** and **e**). (**d**) Dam1/DASH rings *in vitro* act as force couplers that convert the strain energy efficiently into a pushing force or work. (**e**) Morphology of kMT plus-ends observed in ultrathin sections. Averaging over many protofilaments reveals the presence of fibrillar structures (orange) of unknown identity that bind on the inside of the curling protofilament (blue). kMT protofilaments (blue) display an intermediate curvature that may be indicative of incomplete relaxation of strain. If these fibrils bound to each protofilament are responsible for this curvature, then they can convert the strain into a minus-end directed force. (**f** and **g**) Two mechanisms can generate force coupled to MT polymerization. (**f**) A MT-binding protein (green circle) with higher affinity for GTP–tubulin than GDP–tubulin can track a growing plus-end. (**f**) A plus-end growing against a rigid barrier generates force by thermal ratchet mechanism.

two types of forced-walk models that are relevant to force generation at the kinetochore: rings that encircle the MT lattice just behind a flared plus-end (Figure 1d) and fibrils that bind at the top of a curling protofilament (Figure 1e) [12••,13]. By design, ring couplers can track a flared plus-end indefinitely. Theoretically, the energy of GTP hydrolysis is stored as strain energy in the MT lattice, and it is available for performing work [14]. An optimally designed ring (an oligomeric Dam1/DASH ring, see below) acts as a highly efficient coupler converting up to 70% of the strain energy into work generating 40–60 pN of force per MT [13,14].

Growing MT plus-ends can also provide force generation via two different modes. The first mechanism depends on the presence of GTP–tubulin dimers at the MT plus-end. A kinetochore protein that binds preferentially to GTP–tubulin over GDP–tubulin is biased in its thermal movement over the MT lattice toward the plus-end (Figure 1f). The second mechanism is commonly known as 'thermal ratcheting', wherein the plus-end is made to grow against an impenetrable barrier (Figure 1g). Bacterial plasmid segregation machinery involving *parC* and *parM* provides an excellent example of thermal ratchets. The free energy that can be converted into work comes in the form of chemical potential for GTP–tubulin addition at the plus-end [15]. Physiological values predict forces as high as 3 pN per dimer added at plus-end, which was confirmed by *in vitro* force measurements [16].

Biophysical characterization of the kinetochore *in vivo*

The *in vivo* mechanism of kinetochore force generation remains uncharacterized largely owing to a dearth of *in vivo* biophysical data. There are models based on microtubule-motors, on non-motor mechanisms involving microtubule depolymerization-dependent mechanisms as discussed above and more conceptual mechanisms involving hypothetical long range electrostatic forces [17]. Current lack of quantitative information on the molecular interactions with the MT plus-end however, necessitates major assumptions regarding MT plus-end behavior at the kinetochore to force these models to mimic observed chromosome dynamics [7,18,19].

Some biophysical characteristics of the kinetochore-MT attachment are well-characterized. MT-depolymerization coupled kinetochore movement occurs *in vitro* in the absence of ATP, demonstrating that the core kinetochore attachment machinery acts as a passive motor [9,20]. Kinetochores tethered to the spindle pole by kMTs move toward and away from the spindle pole with similar speeds, which suggests that the speed of movement does not crucially depend on the opposing force. Direct measurement of force generated by a kinetochore in anaphase was obtained by the classic experiments conducted by Nicklas in grasshopper spermatocytes [21]. These experiments estimated the *attachment* force per kMT to be >10 pN. However, it should be noted that poleward kMT flux (poleward movement of MTs coupled with depolymerization at the minus-end) is a significant component of kinetochore motility in meiotic systems including grasshopper spermatocytes. The measured rate of poleward flux is similar to the speed of chromosome movement implying that the kMTs must *polymerize* at the plus-end even as the kinetochore is moving poleward [22]. The force measured in the Nicklas experiments indicates the maximum forces that can be *transmitted* by the kinetochore machinery including motors and MAPs. MT-depolymerization coupled force generated by the core kinetochore machinery then remains an unknown quantity. Thus, persistent attachment and force generation may involve two different mechanisms.

The morphology of kMT plus-ends could potentially provide valuable information regarding the correlation between kMT state and the direction of kinetochore movement, and point to mechanisms that modulate plus-end polymerization dynamics. For example, sister kinetochore pairs in HeLa cells can oscillate about the spindle equator with excursions of ~2 μm in either direction. During such oscillations, kMTs attached to the leading kinetochore must lose tubulin subunits (depolymerize), whereas its trailing sister kinetochore must gain subunits (polymerize). Surprisingly, the morphology of kMT plus-ends determined in ultrastructural studies failed to find significant differences in protofilament curvature of kMT populations at leading and trailing kinetochores [12••,23]. These studies found that both kinetochores in a sister kinetochore pair have ~70% of the kMT plus-ends with a flared geometry suggesting that they probably depolymerize (Figure 1e), whereas the remaining plus-ends have a blunt morphology suggesting that they polymerize. This finding implies that the kMTs can on average add and lose subunits without detectable changes in plus-end structure, and that mechanochemical transitions from one state to the other may be fluid and reversible. Comparison of protofilament morphology reveals that the curvature of kMT protofilaments is significantly less than the curvature at the ends of unattached, depolymerizing MT protofilaments (Figure 1e). This observation implies that the release of lattice strain is incomplete and possibly opposed by kinetochore proteins. Indeed, this study finds that fibrils of unknown identity consistently attach to a specific region of the protofilaments, where such fibrils can impart a minus-end directed force to the kinetochore (Figure 1e). It is important to establish what fraction of the strain energy in these protofilaments is converted into useful work, or if the observed geometry is a consequence of strong modulation of kMT dynamics [24]. Finally, polymerization force by kMTs is thought to be small, since the centromere of a bioriented chromosome is rarely compressed [25].

Cell biology: Ndc80 and Dam1/DASH complex

Ndc80 and Dam1/DASH complexes have emerged as the principle MT-binding kinetochore proteins that organize end-on kinetochore-MT attachment. The crucial role of Ndc80 in vertebrate cells is vividly demonstrated by the observation that the deletion of an 80 amino acid long N-terminal tail of Ndc80 molecule completely abrogates end-on kinetochore-MT attachments [26••,27,28••]. Although Dam1/DASH has been discovered only in fungi, its ability to form rings around a MT lattice *in vitro* and preferential binding to GTP–tubulin set it apart from a number of other MAPs studied so far. KNL-1/ Blinkin/AF15q14 is another MT-binding conserved kinetochore protein that may be as important for MT attachment [29•,30•]. So far, its ability to act as a force generator has not been investigated in detail.

The Ndc80 complex is the principle MT-attachment site at the kinetochore in all eukaryotes. It also organizes key components of the kinetochore-based spindle assembly checkpoint machinery [31]. Structural studies of the Ndc80 complex reveal that it is a 56 nm long rod-like molecule, with globular domains at either end of the rod [32,33••]. The Ndc80/Nuf2 subunits contain a pair of globular, calponin-homology domains (CH-domains) that are involved in MT attachment [27]. As mentioned above, the Ndc80 subunit (Hec1 in vertebrates) also carries an N-terminal unstructured tail with nine positively charged residues that primarily mediates MT-binding through electrostatic interactions with the negatively charged, C-terminal tail of β-tubulin [26••,28••]. *In vitro* binding studies have shown that the Ndc80 complex binds the sides of microtubules by its N-terminal end and the rod domain extends toward the microtubule plus-end at a 30–45° angle. At low concentrations, binding is cooperative by mechanisms that are not understood [32]. Surprisingly, this tail is not essential in budding yeast [34]. The CH-domains are thought to participate in MT-binding and these domains are also required for activating the spindle assembly checkpoint [26••,28••]. Initial structural studies and point mutations in amino acids suggested that the adjacent CH-domains of Ndc80 and Nuf2 (the 'heel' of the foot-like conformation of the N-terminal end) both make contacts with the microtubule lattice [32]. However, the binding geometry is uncertain since subsequent cryo-EM studies reveal the presence of alternating strong and weak densities protruding from tubulin monomers, with the heavier density (probably the 'toe' of the CH-domain of Ndc80 with the CH-domain of Nuf2 situated behind) binding in the ridge between two tubulin heterodimers [35••]. Structural analysis and EM images of isolated Ndc80 complexes also show that the alpha helical coiled-coil rod domain of the Ndc80/Nuf2 dimer contains a loop in the Ndc80 polypeptide that produces a flexible domain about 16 nm behind the globular CH-domains [36]. The crucial role of Ndc80 in regulating kMT attachment and in plus-end coupled force generation was first demonstrated by antibody injection experiments that disrupt Ndc80 phosphorylation [37]. This perturbation led to a strong suppression of kMT plus-end dynamics and turnover at bioriented chromosomes. kMT depolymerization at the minus-end by the flux machinery led to hyper-stretched centromeres and short kMTs. A study of kinetochore composition using GFP-fusion proteins and *in vivo* fluorescence microscopy found that there are 7–8 molecules of Ndc80 per MT in budding and fission yeast [38•,39]. In light of the crucial function of the Ndc80 complex, this number plays an important role in determining the properties of the kinetochore as a force generator.

The discovery that oligomeric Dam1/DASH complex forms rings around the MT lattice *in vitro* generated a lot of excitement [40,41]. This heterodecameric MAP has been found only in fungi so far, and in budding yeast, it is necessary for kinetochore-MT attachment and spindle integrity [42]. *In vitro* studies of the Dam1/DASH rings revealed that each ring contains 16–23 monomers (Figure 1f). It has an inside diameter of ∼35 nm and thus there is a gap between it and the 25 nm outside diameter microtubule. Interactions occur through Dam1/DASH protein projections that extend to the MT lattice [40,43]. The data that indirectly support the existence of rings comes from fluorescence measurements of the number of copies of the complex per kinetochore on average [39]. Although the Dam1/DASH rings have been well-characterized *in vitro*, such structures have not been discovered *in vivo*. Dam1/DASH is loaded onto the kinetochore only after kinetochore-MT attachment is established [44,45]. Dam1/DASH complex molecules assemble into rings autonomously. Once assembled, these rings dissociate from the MT mostly when they encounter a depolymerizing end *in vitro* [46•]. These observations raise important issues. What process/factors regulate the assembly of rings *in vivo* to ensure that each kinetochore acquires one ring? *In vivo* observation of Dam1/DASH recruitment during the retrieval of unattached kinetochores in budding yeast cells shows that the kinetochore gradually acquires Dam1/DASH molecules after it establishes end-on attachment [45]. These observations lead us to two alternative scenarios for the possible recruitment of Dam1/DASH rings to the kinetochore: either end-on kinetochore-MT attachment is necessary for ring formation or a monitoring process distributes rings evenly. Finally, Dam1/DASH may also be important for establishing kinetochore coupling with growing kMTs [47]. Although Dam1/DASH is essential for survival in budding yeast, it is dispensable in fission yeast. The fission yeast kinetochore recruits a much smaller number of Dam1/DASH complex molecules, and this number is insufficient to form even one Dam1/DASH ring per kinetochore [38•]. Interestingly, the fission yeast kinetochore supports 2–3 MT attachments as opposed to the single attachment in budding yeast.

Both Ndc80 and Dam1/DASH are regulatory targets of mitotic kinases. In vertebrates, Aurora B kinase suppresses the binding affinity of Ndc80 mainly through the phosphorylation of positively charged residues in the N-terminal tail [26••,27,28••,33••,37]. Phosphorylation is cell cycle dependent, and may be antagonized by the PP1 phosphatase. This phosphorylation is essential in the correction of erroneous kMT attachments in prometaphase cells (reviewed in Refs. [3•,48]). Ipl1/Aurora B also targets several subunits of the Dam1/DASH. *In vitro*, phosphorylation affects the ability of Dam1/DASH to form rings and to associate with the Ndc80 complex [49].

In vitro interaction of Ndc80 and Dam1/DASH with a MT

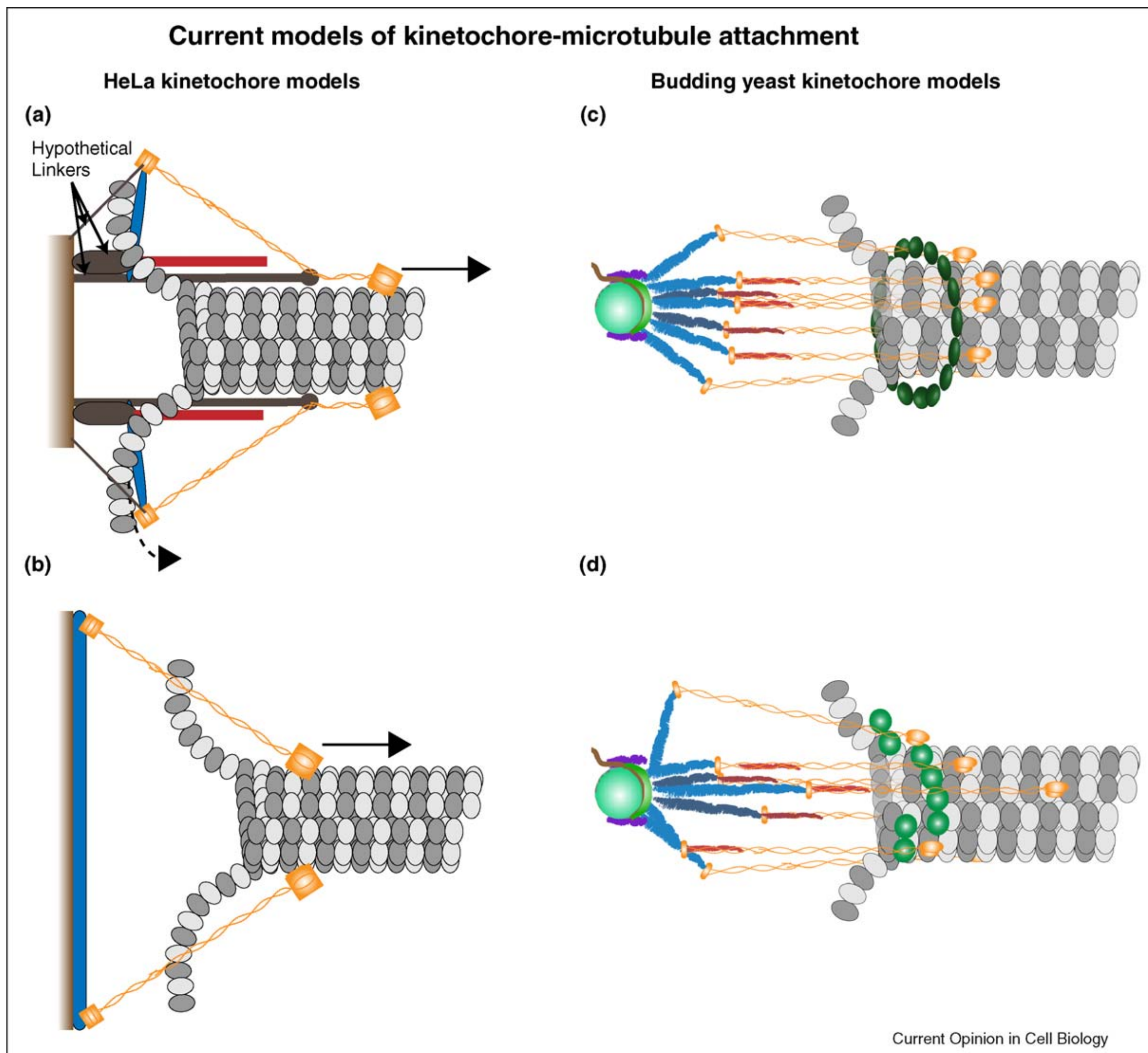
Characteristics of Ndc80 complex interaction with a MT at the single molecule level were recently examined *in vitro* using bacterially reconstituted protein (no phosphorylation) [50••]. On the basis of the residence times of Ndc80 complex molecules on stabilized MTs, this study found that the Ndc80 complex binds to the MT lattice with a relatively low affinity. Single Ndc80 molecules also diffuse along the MT lattice, although they cannot track a depolymerizing end. Instead, the low affinity necessitates an interlinked assemblage of at least 16 molecules for persistent depolymerization coupled movement with a lower diffusion coefficient. Beads coated with Ndc80 sustain depolymerization-driven movement against opposing forces as high as 3 pN. Tip-tracking behavior of Ndc80-coated microbeads is not surprising, as the relatively large diameter of the bead allows the Ncd80 molecules to attach over a significant length of the MT (~250 nm assuming a 40 nm long interaction zone between a 440 nm diameter bead surface and the MT lattice). Such motion has been seen for beads coated with a high enough number of a MT-binding protein [51,52]. The relatively low affinity of the Ndc80 molecule to the MT is also important, as it will allow diffusion of the molecule over the lattice instead of stable binding in one position. Together with the requirement of multiple Ndc80 molecules for tip-tracking ability, these observations lend support to biased-diffusion type of mechanism for force generation *in vitro*. It should be stressed that the geometry of binding between the Ndc80 complex aggregates and the MT tip, which is crucial for reaching a definitive conclusion regarding its role in the mechanism of force generation at the kinetochore, was not characterized in this assay. An important issue to address is whether MT-binding proteins like the Dam1/DASH complex or the Ska complex (see below) enhance the affinity of the Ndc80 complex to the MT lattice, since the Ndc80 complex and not the above MAPs are directly linked to the inner kinetochore.

The Dam1/DASH complex exhibits complex behavior *in vitro* owing to the two distinct oligomeric configurations: patches and bracelets, and rings containing 16–23 monomers. Grishchuk *et al.* showed that the Dam1/DASH ring binding to the lattice is highly stable, and the strong binding allows very little thermal diffusion of the rings over the lattice [46•]. This observation implies that rings must be moved over the lattice by an active mechanism significantly stronger than random thermal forces. In the *in vitro* assay, this pushing force comes from curling protofilaments on a depolymerizing plus-end, a forced-walk type of mechanism. Dam1/DASH rings harness a large portion of the strain energy to develop as much as 3–5 pN of force per protofilament (40–60 pN per MT) [14]. The study also found that oligomeric Dam1/DASH patches support tip-tracking behavior. Furthermore, these assemblies can act as force couplers, although the forces generated are lower on average. The diffusion coefficient of these assemblies depends inversely on the number of monomers. This observation is consistent with concurrent studies demonstrating that rings are not necessary for persistent tip-tracking behavior of the Dam1/DASH molecules [53•]. Interestingly, beads coated with Dam1/DASH complex also track growing plus-ends [54]. Such beads resist forces of up to 3 pN directed in the direction of growth. By contrast, a much lower force toward the minus-end is needed to dislodge the bead from the plus-end. Finally, tension applied to the MT plus-end through beads coated with the Dam1/DASH complex increases the rescue rate and also suppresses the rate of catastrophe [55•], a key feature of the kinetochore that has been proposed by cell biological observations and mathematical simulations [18,56]. This effect is negated by Aurora B phosphorylation. In conclusion, the Dam1/DASH complex can generate forces via both modes of MT disassembly coupled force generation.

Protein architecture of the kinetochore

Both Ndc80 and Dam1/DASH operate in the context of the kinetochore as a macromolecular machine. Two recent studies made significant advances toward understanding the protein architecture of the kinetochore in yeast and in fixed HeLa cells [57••,58••]. Using super-resolution microscopy, these studies measured separations between a reference point on the kinetochore and known kinetochore proteins to assemble a map of kinetochore protein localization along the kinetochore-MT attachment axis. Significant similarities in the positions of kinetochore proteins emerge from the two maps, suggesting a high degree of conservation. There are significant differences as well. The length of the Ndc80 complex was measured to be 46 nm in metaphase in HeLa cells. This length is shorter than the 55 nm length of isolated Ndc80 complexes measured by structural studies [33••]. Although the axis of a MT-bound Ndc80 molecule is tilted by 33° with respect to the MT axis *in vitro*, Wan *et al.* envisioned Ndc80 molecules as constitutively bent at the hinge region to account for forces acting along the MT axis (Figure 2a). Force along the kMT axis is transmitted by a hypothetical linker

Figure 2



Protein architecture and force generation in HeLa and yeast kinetochores. (**a**, **b**) Proposed models for force generation at the vertebrate kinetochore. These schematics show the two possible arrangements of the Ndc80 complex binding to the MT lattice in HeLa kinetochores. (a) This model is deduced from distance measurement data obtained by Wan *et al.* (b) This model is based on the tilted orientation of the Ndc80 bound to MT lattice observed *in vitro*. (**c**, **d**) Kinetochore architecture obtained from live budding yeast cells. Of particular interest are the positions of Dam1/DASH complex (green ellipsoids) and Ndc80 complex (orange fibrils). (c) This version depicts the simplest arrangement representing average distances. Other arrangements are also possible. (d) This cartoon depicts a proposed model for persistent kMT attachment through the Ndc80 complex. The Mtw1 complex (blue fibrils) is assumed to attain various orientations with respect to the kinetochore-MT axis and allow the Ndc80 complex (orange fibrils) to reach significant distances downstream from the kMT plus-end. Color key: NDC80 — ORANGE, KNL-1/Spc105 — RED, Mis12/MTW1 — BLUE, Dam1/DASH — GREEN.

protein that attaches the hinge region of the Ndc80 complex to the inner centromere. Furthermore, they proposed that force from the curling protofilaments may also push the kinetochore complexes that link the Ndc80 complex to the centromere toward the minus-end (Figure 2a). It is also possible that the measured length is smaller because of a small number of unattached Ndc80 molecules that lower the average length owing to their random orientation. However, unattached Ndc80 molecules can activate the spindle assembly checkpoint, leading the authors to propose the bent Ndc80 molecule. Models of kinetochore architecture based on straight but tilted Ndc80 complex molecules require a rigid underlying foundation that resists the inwardly directed forces arising from the angle of the transmitted force (Figure 2b) [1•].

Budding yeast measurements reveal a simpler architecture. The 55 nm long Ndc80 complex binds to the kMT with its axis aligned with the MT axis, the alignment

arising due to the force generated (Figure 2c). Alignment of all Ndc80 molecules in metaphase raises two possibilities. The alignment arises because all Ndc80 molecules are constrained in this configuration even in the absence of kMT attachment. Alternatively, all eight Ndc80 molecules in a kinetochore mostly remain attached to the kMT. Interestingly, anaphase measurements in budding yeast revealed that the length of the Ndc80 molecule reduces to ~39 nm, which probably results from the bending of Ndc80 molecules in the hinge region. The Dam1/DASH complex localizes ~10 nm behind the Ndc80 head domains. At this location, a Dam1/DASH complex ring would encircle the kMT lattice as well as the coiled-coil domain of the Ndc80 complex. The 35 nm inner diameter of the Dam1/DASH ring makes this formally possible. This depiction of the kinetochore also suggests that the formation of the Dam1/DASH ring must follow the stable binding of a majority of the Ndc80 molecules to the kMT. Alternatively, the Dam1/DASH complex does not form rings *in vivo*, and instead contributes function as oligomeric patches or incomplete rings bound to the kinetochore.

Location of the MT plus-end within the kinetochore is crucial for elucidating the mechanism of force generation. In Ptk1 cells, the end of a cold-stable fiber of kMTs extends ~60 nm beyond the MT-binding domain of the Ndc80 complex. Although this position probably marks the farthest that kMT plus-ends penetrate into the kinetochore, the measurement suggests that the Ndc80 complex binds the kMT lattice under the experimental conditions. The location of the Dam1/DASH complex within the yeast kinetochore implies that the kMT must extend beyond the MT-binding domains of the Ndc80 complex. For the Ndc80 complex to act as a forced-walk coupler, the position at which it binds to a curling protofilament decides the direction of the force being generated. Binding at the top of the ram's horn is necessary for a minus-end directed force. If the kMT plus-end indeed extends beyond the MT-binding domains of the Ndc80 complex, action of the Ndc80 complex as a forced-walk coupler becomes difficult to envision.

Help in kMT attachment may also come in *C. elegans* from kinetochore protein KNL-1, which binds MTs *in vitro* [30•]. Its fission yeast homolog Spc7 also binds MTs when overexpressed [59]. In HeLa cells, Blinkin/AF15q14 RNAi leads to the abrogation of kinetochore-MT attachment, although this phenotype may also arise from significant perturbation of kinetochore architecture [29•]. The location of this protein ~30–40 nm inside the MT-binding head domains of the Ndc80 complex could provide another potential set of MT-binding sites. This arrangement of MT interactions sites distributed over 40 nm is well-suited for persistent attachment using biased diffusion. More studies are important to establish how KNL-1 and its homologs in vertebrates and fungi bind MTs *in vivo*, location of the MT-binding domain within the protein and within the kinetochore, and its role in force generation.

The observed architecture of the budding yeast kinetochore suggests a biased-diffusion mechanism for Ndc80 complex-mediated kinetochore motility (Figure 2d). The Ndc80 complex connects to the inner kinetochore via a filamentous Mtw1 complex (blue bars in Figure 2c, d). Such a mechanism may be possible if one assumes that this complex is free to orient itself along the kMT axis, the *in vitro* length of this complex (up to 40 nm) combined with the 56 nm length of the Ndc80 complex can allow the MT-binding Ndc80 domains to reach significant distances (~100 nm) behind the MT plus-end. Such a model must invoke molecular strain or some form of position-dependent regulation that reduces the ability of the Ndc80 complex to bind close to the centromere, instead promoting binding to regions of the kMT lattice that are further away. Under this scenario, the role of the Dam1/DASH complex becomes completely dependent on the Ndc80 complex. Furthermore, Dam1/DASH molecules cannot form rings, but instead act as oligomeric patches to assist in MT-binding, and possibly influence the polymerization dynamics of the kMT.

Contributions of motors, MT depolymerases, and MAPs

Motors, depolymerases, and MAPs are important regulators of MT polymerization dynamics *in vivo*. Assigning specific roles to each of these proteins at the kinetochore proves difficult for two reasons. First, many of these proteins have global and often overlapping roles in the regulation of MT polymerization dynamics in the mitotic spindle. Observed effects on kinetochore function may be non-specific effects that arise from perturbations in MT polymerization dynamics or spindle structure. Second, spindle architectures possess different features and complexities in the various model organisms studied. Although many motors and MAPs are conserved, some of these proteins assume prominent, but system-specific roles, to maximize chromosome segregation accuracy. We therefore describe the general role of motors, depolymerases, and MAPs in HeLa cells, and highlight only a few intriguing results here.

Motors generate an auxiliary force at the kinetochore. Dynein and CENP-E provide prominent examples. The ability of some motors to depolymerize a MT plus-end or to induce catastrophe at a growing plus-end can significantly improve the persistence of kinetochore force generation coupled to kMT depolymerization. This function is especially important in chromosome segregation in anaphase, as in the case of the Kinesin-13 family proteins [60]. The ability of depolymerases to switch kMT plus-ends from growing to shrinking state also allows them to control coordination of motion of sister kinetochores. Both these

functions are intriguingly on display in recent studies of Kinesin-8 and Kinesin-13 depolymerases [61•,62•]. Despite similar depolymerase activities, overexpression or depletion of these motors from the kinetochore has the opposite effects on the characteristics of chromosome oscillations in HeLa cells. Recruitment of additional MCAK to the centromeres increases chromosome speed, oscillation amplitude, and also increases the coordination between sister kinetochores. On the contrary, overexpression of Kif18A decreases the rate of chromosome movement as well as the amplitude of oscillations. These seemingly antagonistic functions probably arise owing to the mode of recruitment of these proteins at the kinetochore and their location with respect to the plus-end. MCAK is bound to the interior of the kinetochore and in front of kMT plus-end, whereas Kif18A uses its motor activity to travel along the lattice to reach and act on kMT plus-ends. The location of MCAK within the kinetochore likely provides a built-in negative feedback to the location of kMT plus-ends, whereas Kif18A recruitment by the kMTs represents a constant influence on the plus-ends. MAPs yield an antagonistic influence on the state of kMT plus-ends, battling with the depolymerases to promote plus-end growth. A number of MAPs including EB1, CLIP170, XMAP215, CLASP, and Ska1/RAMA are important for kinetochore function [63–67]. The Ska1 complex has garnered attention in the recent year owing to its role in maintaining stable end-on attachments at the kinetochore (see Ref. [68•] for a review). It localizes to the kinetochores during mitosis, and depletion of multiple Ska1 subunits severely reduces the stability of kMT attachments. It can oligomerize *in vitro*, and it is subject to phosphoregulation. Furthermore, Ska1 complex coated beads were shown to track shortening plus-ends persistently and slow down the rate of disassembly in a manner reminiscent of the activity of the Dam1/DASH complex [69]. A more recent study demonstrated that the Ska1 complex is also involved in the satisfaction of the spindle assembly checkpoint and for maintaining sister chromatid cohesion [70]. More studies are necessary to establish the role of this complex in force generation at the kinetochore.

The influence of motors and MAPs on kinetochore activity demonstrates that the leading and trailing kinetochores in a sister kinetochore pair must have distinct kMT populations, even if such populations cannot be discerned from their morphology apparent in ultrastructural studies. Indeed, EB1, which recognizes and tracks with polymerizing plus-ends, is seen at much higher concentrations at a trailing kinetochore where there is net kMT growth (polymerization) in comparison with its leading sister kinetochore where there is net kMT shrinkage (depolymerization) [71].

Conclusions and outlook

The kinetochore is a sophisticated macromolecular machine that has two crucial functions in chromosome segregation: it must establish end-on attachments that enable the dividing cell to distinguish and selectively stabilize chromosome bi-orientation, and it must generate forces to move and segregate chromosomes in anaphase. Although we compare and contrast two different mechanisms of MT-depolymerization coupled force generation, both mechanisms may contribute to kinetochore force generation *in vivo*. A complete understanding of force generation and MT dynamics control mechanisms will require concerted efforts to obtain *in vivo* biophysical measurements of the kinetochore-MT attachment, and *in vitro* reconstitution of the entire kinetochore. Biophysical properties of interest are: magnitude of force generated, dynamics of the coupling between the kMT plus-end and the MT-binding proteins, and so on. The fluid control over the kMT plus-end dynamics exerted by the kinetochore is a crucial aspect of its function. Understanding the mechanochemical events that allow a MT to polymerize and depolymerize will greatly facilitate elucidation of the kinetochore-MT interaction. Establishing the interfaces between different kinetochore protein complexes is a key objective of structural and biochemical studies of the kinetochore. Much more is understood about the protein architecture and mechanisms of attachment and force generation at budding yeast kinetochores than for higher eukaryotes. There is significant conservation in the core attachment site proteins, such as the KMN network, but so far, only the functions of the Ndc80 complex appear highly conserved, and much needs to be learned about the similarities and differences in the homologs of KNL-1 as well as other microtubule binding proteins important for attachment like the Ska1 complex. Functional contributions of motors and MAPs are essential for maximizing the accuracy of chromosome segregation. Single molecule studies of these proteins will greatly aid in assigning specific functions to these proteins *in vivo*. Finally, regulation of kinetochore activity by mitotic kinases and spindle assembly checkpoint machinery is a crucial factor in the accurate segregation of all the chromosomes in a cell. Exciting progress has been reported on this front recently [72]. Concerted efforts across multiple disciplines in the coming years will ultimately provide an integrative model of kinetochore function and regulation for accurate chromosome segregation.

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