Chapter 9

How are Dynamic Microtubules Stably Tethered to Human Chromosomes?

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Abstract

During cell division, microtubules capture and pull chromosomes apart into two equal sets. Without the establishment of proper chromosome-microtubule attachment, microtubules cannot impart the pulling forces needed to separate sister chromatid pairs. How are chromosomes captured along microtubule walls? How is the attachment of chromosomes to dynamic microtubule-ends achieved and monitored? We discuss these key questions by considering the roles of kinetochore-bound microtubule regulating proteins and also the complex regulatory loops of kinases and phosphatases that control chromosome-microtubule attachment and ensure the accurate segregation of chromosomes.

Keywords: chromosome segregation, microtubules, mitosis, kinetochore, mitotic spindle

1. Introduction

When a human cell prepares to divide, its microtubule cytoskeletal network disassembles and reassembles to form a bipolar structure—the mitotic spindle. Microtubules of the mitotic spindle capture chromosomes and segregate the DNA into two equal sets (**Figure 1**). To ensure accurate segregation, the proper attachment between the chromosome and microtubule is important. Chromosome-microtubule attachment is facilitated through a submicron-sized macromolecular structure called the kinetochore. The kinetochore appears as a three-layered structure in electron microscopy: an outer layer that contacts microtubules, a middle layer

or interzone and an inner plate assembled on centromeric chromatin. In humans, multiple microtubules engage with the kinetochore, and kinetochore-bound microtubules are bundled into k-fibers. Understanding how microtubules are tethered to kinetochores is not only fascinating but also clinically important. We could exploit some of the redundant regulatory mechanisms in chromosome-microtubule attachment to specifically target properties of cancer cells displaying chromosomal instability (reviewed in Ref. [1]).

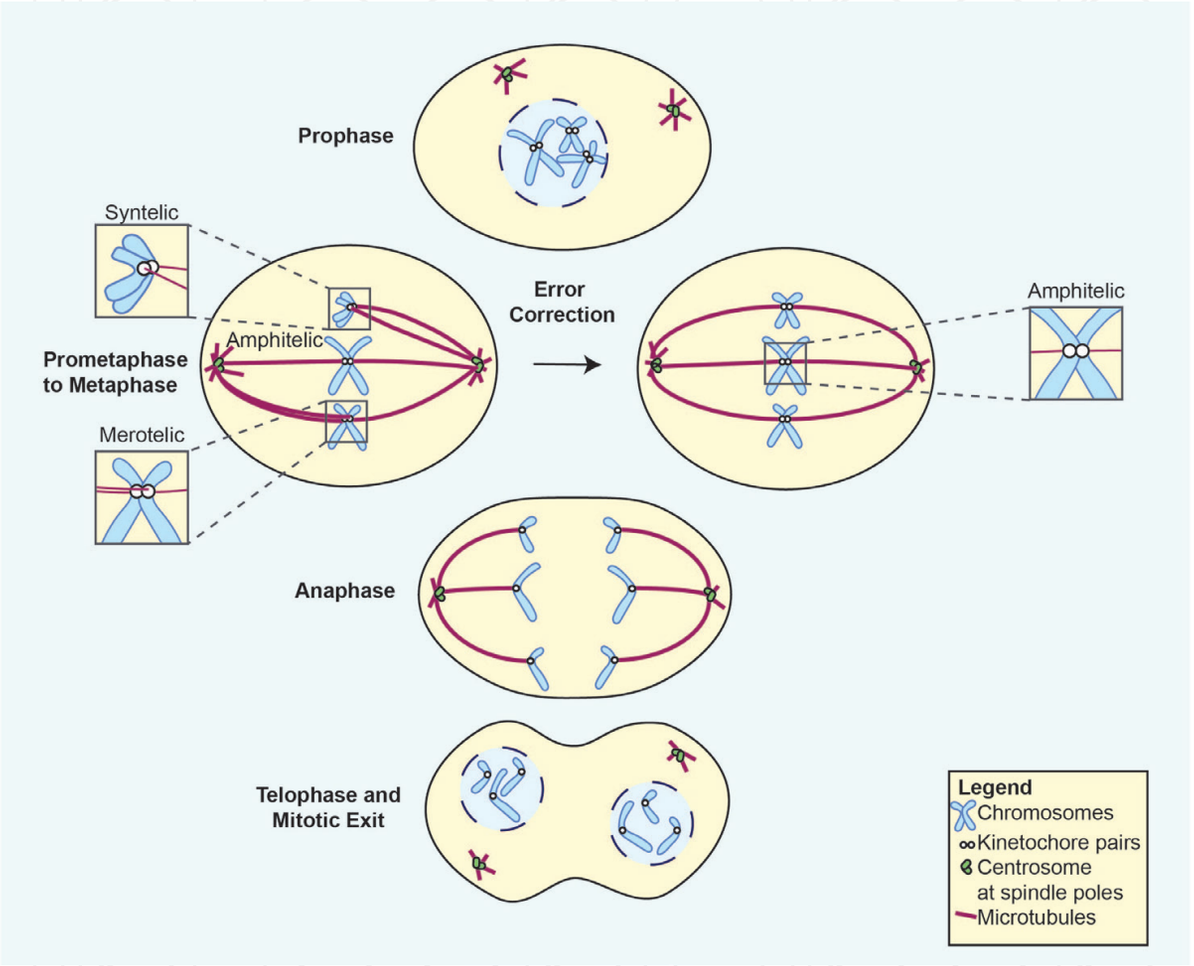

Figure 1. Proper kinetochore-microtubule attachment is needed for the accurate segregation of chromosomes: in prophase, the nuclear envelope breaks down and the kinetochore is poised for microtubule capture. Attachments between microtubules from opposing spindle poles to chromosomes must be bioriented: one kinetochore of a pair is tethered to one spindle pole and the other sister kinetochore of the pair is attached to the opposing pole. Errors in biorientation—syntelic or merotelic attachments—are resolved through the error correction process during prometaphase and metaphase stages. Only after all the kinetochores have achieved biorientation, cells initiate anaphase and pull sister kinetochores apart into two equal sets.

Human kinetochores become available for capture by microtubules soon after nuclear envelope breakdown, when chromosomes are first exposed to the cytoplasm. Kinetochores are initially captured along the lateral walls of microtubules and then tethered to microtubule-ends [2–4], by a multi-step process called end-on conversion. Such a change in the *plane* of kinetochore-microtubule attachment is an important event. Only when kinetochores are tethered to the ends of microtubules, the growth and shrinkage of microtubules can be translated into

pushing or pulling forces that move chromosomes. In addition to the proper *plane* of kinetochore-microtubule attachment, proper *orientation* of the attachment is also crucial: one sister kinetochore of a pair must be attached to one spindle pole and the other sister kinetochore of the pair must be attached to the opposing spindle pole. This *orientation* of chromosome-microtubule attachment is called biorientation or an amphitelic attachment (**Figure 1**). When *all* kinetochores are attached in an amphitelic fashion, the cell initiates anaphase, allowing sister chromatids to be synchronously pulled apart into two daughter sets.

To ensure that cells do not prematurely initiate anaphase, the status of kinetochore-microtubule (KT-MT) attachment is continuously monitored by a complex, evolutionarily conserved Spindle Assembly Checkpoint (referred to as SAC) mechanism. Whether the SAC monitors the physical geometry of KT-MT attachment or the outcome of the correct attachment geometry (intra or inter-kinetochore tension) is not fully resolved (reviewed in Refs. [5, 6]).

In this chapter, we focus on events that occur at the kinetochore-microtubule interface. First, we introduce the key molecular components that form the kinetochore-microtubule interface, with a focus on microtubule-associated proteins that bridge the kinetochore and microtubule polymer. Second, we discuss how the plane and orientation of kinetochore-microtubule interaction is correctly established. Finally, we review our current knowledge of phosphorylation-dephosphorylation cycles that control KT-MT attachments and in turn ensure the accurate segregation of chromosomes.

2. The kinetochore-microtubule interface

Over 100 proteins are now known to form the human kinetochore (reviewed in Ref. [7]). The major constituent at the outer kinetochore surface that is responsible for the connection between microtubules and chromosomes is the 10-subunit KMN network, made up of Knl1, Mis12, and Ndc80 complexes. Each of these complexes are composed of proteins, which are evolutionarily conserved from yeasts to humans: the Knl1 complex consists of Zwint1 and KNL1 (hSpc105), the Mis12 complex consists of Mis12, Dsn1, Nnf1 and Nsl1, and the Ndc80 complex consists of Ndc80 (HEC1), Nuf2, Spc24, and Spc25 (reviewed in Ref. [7]). Positioned at the outer face of the KMN complex is the Ndc80-Nuf2 complex, which makes direct contact with microtubules [8, 9].

The KMN network of proteins is recruited to the kinetochore in early mitosis [10–13]. KMN recruitment is mediated by the multi-subunit CCAN—Constitutive Centromere Associated Network—that forms the core structure of centromeres (specialized chromatin regions) [14]. A recent biochemical reconstitution study showed that the KMN network and a seven-subunit CCAN complex, the CHIKMLN complex, along with CENP-A (Histone H3 variant) bound nucleosomes, is sufficient to bridge the DNA and microtubules [15]. Thus, the KMN network bridges the inner centromeric DNA-bound proteins with the microtubule polymer. In addition, the KMN network of proteins acts as a platform for several proteins that monitor and control kinetochore-microtubule attachment status: regulators of the spindle assembly checkpoint (Mad1, Mad2, Bub1, Bub3, BubR1, RZZ complex, Mps1, Aurora-B), microtubule

associated nonmotor proteins (for example, CLASP, CLIP170, EB1, APC, ch-TOG, SKA complex, CENP-F and Astrin-SKAP complex), and motor proteins (CENP-E, Dynein/Dynactin complex) are all recruited in a KMN-dependent fashion [12, 13, 16–23]. Therefore, the KMN network is considered as an essential core component of the kinetochore.

3. Mechanisms that ensure proper chromosome-microtubule attachment

3.1. The plane of KT-MT attachment and the end-on conversion process

During early mitosis, the outer kinetochore surface appears expanded [2], and this expansion facilitates the initial interaction between the kinetochore and microtubule wall (lateral attachment). In addition, kinetochore-derived microtubules can interact with spindle-pole-derived microtubules, facilitating kinetochore attachment onto lateral walls of microtubules [24]. The steps involving the conversion of lateral attachments to end-on attachments take place during prometaphase and are collectively termed the end-on conversion process. This multi-step end-on conversion process happens similarly in humans and in yeast, where it was first described (reviewed in Ref. [25]), with a few differences. The process in human cells involves: (a) initial interaction of a kinetochore with the lateral surface of microtubules; (b) transport of the kinetochore toward centrosomes or spindle equator; (c) physical change in the plane of kinetochore interaction, from microtubule-wall to microtubule-end; (d) selective destabilization of laterally engaging kinetochore-microtubules; (e) transient stabilization of end-on attached microtubules; and (f) maintenance of stable end-on attachments after the biorientation of sister kinetochores (**Figure 2**).

The initial attachment between the kinetochore and microtubules, which occurs on the lateral microtubule walls, is an immature one. Laterally attached kinetochores can undergo movement toward and away from the spindle pole; these movements are motor dependent and microtubule-dynamics independent [26–28]. Laterally attached chromosomes should be transported away from the spindle pole and congressed at the spindle equator. This antipoleward transport is, in part, due to an ejection force that pushes chromosomes outward from the spindle pole [29]. Antipoleward forces needed to transport laterally attached kinetochores can also be facilitated by (i) electrostatic repulsion between negatively charged chromosome arms and neighboring negatively charged astral microtubule-ends [30] and (ii) kinetochore or chromosome-associated kinesin motors (reviewed in Ref. [31]).

A well-understood example is the kinetochore-bound kinesin motor protein, CENP-E [32], which facilitates the tethering of kinetochores to the lateral wall of microtubules and slides chromosomes towards the microtubule plus-end [26], providing antipoleward drag force. The CENP-E motor is brought to the kinetochore by a core kinetochore protein, CENP-Q [33] (part of the CENP-O/MCM21R complex [34]). CENP-E facilitates end-on conversion in at least two ways: first, CENP-E prolongs the lifetime of laterally attached kinetochores and thus enables efficient end-on conversion [4]. Second, by transporting kinetochores to the spindle equator, CENP-E ensures that kinetochores are not trapped behind the spindles poles, which would make biorientation geometrically impossible and lead to futile end-on conversion cycles.

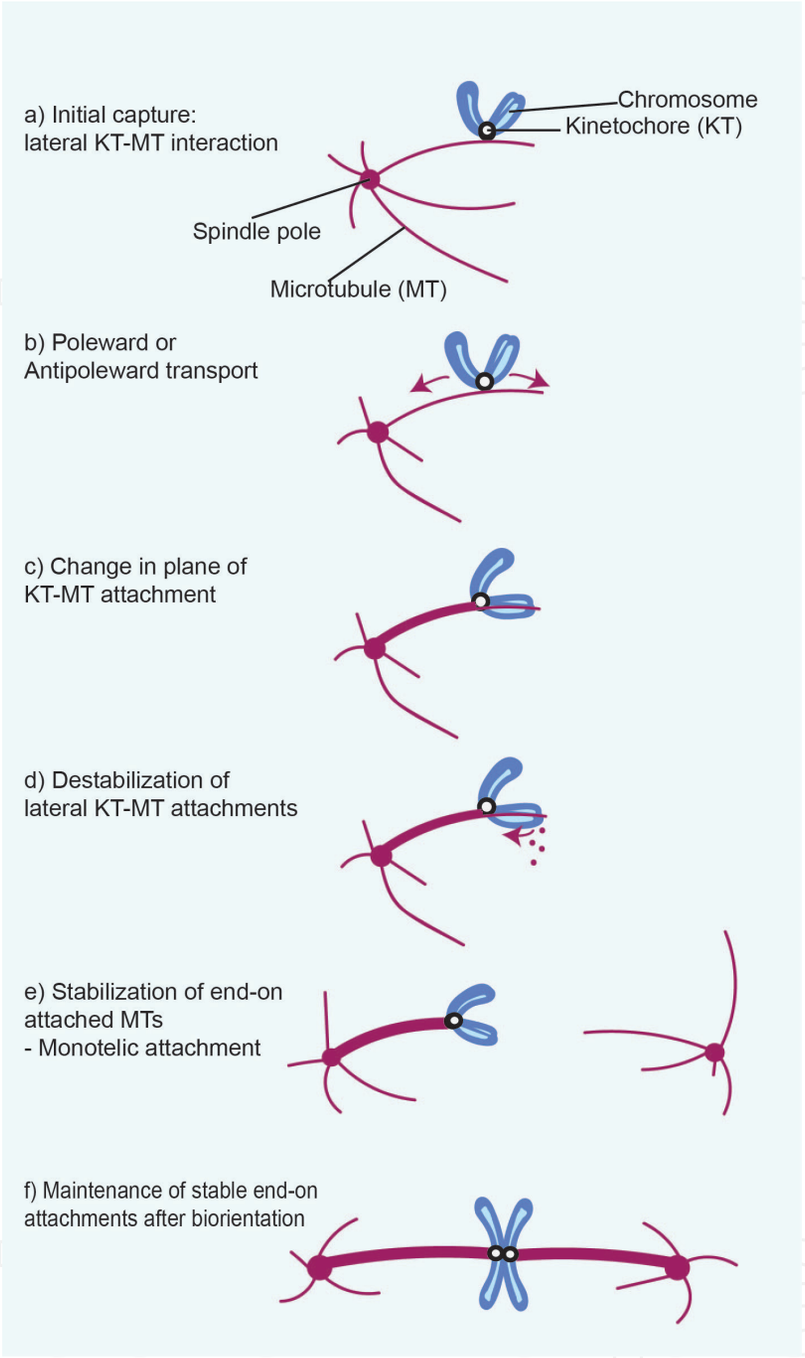

Figure 2. The end-on conversion process: Kinetochores are first captured along lateral walls of microtubules, then are tethered to the ends of microtubules, through multiple steps guided by the kinetochore and microtubule-associated proteins. The end-on conversion process enables the formation of mature end-on tethered kinetochores. In parallel, the error correction process ensures that monotelic attachments are converted into amphitelic attachments (KT-MT, kinetochore-microtubule).

Following end-on conversion, mechanisms that actively remove laterally interacting microtubules are crucial for the maintenance of end-on attachments. The microtubule depolymerizing kinesin MCAK, needed for proper kinetochore-microtubule attachments (reviewed in Ref. [35]), removes the ends of laterally interacting ends of microtubules and aids in the maintenance of a mature end-on attached kinetochore [4].

Whether microtubule wall *versus* end interactions are dominantly controlled by distinct sets of proteins in human cells remains unknown. It is clear that several Microtubule-Associated Proteins (MAPs) are recruited selectively to the immature kinetochore-microtubule interface in prometaphase, but not the mature kinetochore-microtubule interface in metaphase [36, 37]. How MAPs recognize and switch between mature and immature kinetochore states is an exciting unexplored area, which may shed light on the molecular basis underpinning the change in the plane of kinetochore-microtubule interaction.

3.2. Proteins that distinguish ends and walls of microtubules

To develop possible paradigms for how kinetochore proteins may distinguish microtubule walls *versus* microtubule-ends, we will first look at how microtubule associated proteins distinguish microtubule walls from ends.

EB1 differentiates the plus end from the rest of the microtubule wall because of its high affinity for GTP-tubulin, which is enriched at the growing end of microtubules [38–40]. Electrostatic interaction between the C-terminal tail of EB1 and microtubule walls contributes to targeting EB1 to the plus end of microtubules [41]. In addition, EB1 preferentially associates with tubulins along the lateral surface of microtubules, facilitating closure of the open protofilaments and in turn promoting the growth of microtubule ends [42]. Thus, EB1 is believed to directly identify the structural features and chemical signals of the growing end of microtubules.

At the kinetochore-microtubule interface of a mature microtubule-end tethered kinetochore, at least six well-established microtubule-binding proteins are present: Ndc80/HEC1 of the KMN network, SKAP of the Astrin-SKAP complex, the microtubule stabilizer CENP-F and microtubule polymerizers, chTOG, CLASP and the SKA complex. Ndc80 recognizes both the tubulin intradimer and interdimer interface and forms oligomeric arrays along microtubule walls [9, 43]. Similarly, SKAP recognizes intra-tubulin sites [18], enabling a direct link for the Astrin-SKAP complex between the kinetochore and microtubule wall. In addition, SKAP directly interacts with EB1 through its S-X-I-P motif [44] and could potentially link the kinetochore specifically to the growing microtubule-end.

The microtubule-end binding protein chTOG1 interacts directly with Ndc80 [23]. chTOG1 has two TOG domains and could use one to bind to the microtubule end, and the other to present an $\alpha\beta$-tubulin protein for addition to the microtubule-end, thereby promoting microtubule assembly (reviewed in [45]). Similarly, the TOG domains in CLASP1 can interact with tubulin dimers and regulate microtubule rescue (reviewed in Ref. [46]). Although the TOG domains of both chTOG1 and CLASPs bind conserved $\alpha\beta$-tubulin interacting sites, chTOG1 can directly associate with microtubules and CLASPs can be brought to microtubule ends *via* EB1's S-X-I-P motif (reviewed in [16]). In contrast, CENP-F is thought to have a strong binding to depolymerizing microtubules, as one of its two microtubule-binding domains binds strongly to bent microtubules [47–49]. In summary, the kinetochore-microtubule interface recruits a variety of MAPs that can selectively bind to either microtubule walls or ends. Regulated recruitment of kinetochore-bound MAPs can be a fundamentally important way to direct microtubule dynamics and stability.

3.3. Biorientation and the error correction process

The end-on conversion and the error correction process have nonoverlapping tasks. The end-on conversion process facilitates the maturation of kinetochore-microtubule attachment by converting immature lateral attachments into end-on attachments. In contrast, the error correction process acts on kinetochores that are attached to microtubules emanating from incorrect spindle poles. For instance, an erroneous syntelic attachment occurs when both sister chromatids are attached to microtubules from the same spindle pole; an erroneous merotelic attachment occurs when one sister chromatid is attached to microtubules from both spindle poles (**Figure 1**). These erroneous attachments are primarily resolved by an evolutionarily conserved mitotic kinase, Aurora B and to some extent by other kinases; Mps1, Chk1 and Haspin kinases (reviewed in Refs. [50, 51]). When attachments are immature (lateral) or incorrect (nonbiorientated), proteins of the spindle assembly checkpoint are recruited to the kinetochore, generating an inhibitory signal that delays anaphase onset. Our current understanding of how the spindle assembly checkpoint senses attachment status and ensures amphitelic attachments is briefly discussed below.

3.4. The spindle assembly checkpoint (SAC) monitors chromosome-microtubule attachment status

The establishment of amphitelic kinetochore-microtubule attachment (biorientation; see Section 3) is crucial for the cell to accurately segregate its chromosomes in anaphase. For this reason, the status of attachment has to be continuously checked at the kinetochore-microtubule interface by SAC proteins.

The SAC was discovered through genetic screens in yeast, which revealed the majority of the spindle checkpoint proteins: Mad1, Mad2, Bub1, and Bub3 [52, 53]. Other checkpoint proteins Mps1 and BubR1 were later identified [54, 55]. While the above six proteins are generally agreed as evolutionarily conserved SAC proteins from yeasts to humans, several additional proteins have been implicated in monitoring attachment status in human cells (reviewed in Refs. [56, 57]).

In general, two models have been proposed to explain how kinetochores ensure amphitelic attachments (reviewed in Refs. [58, 59]). In the first model—the tension model—only when sister kinetochores are pulled in opposing directions, is there either inter-kinetochore or intra-kinetochore stretching established, which separate the enzymes at the inner or outer kinetochore from their substrates at outer or inner kinetochore, respectively. This tension model is elegant in that it relies on microtubule-pulling induced spatial separation of enzyme-substrate interactions [60]. However, it cannot fully explain how sister kinetochores are allowed to biorientate in the first place, if nonbiorientated attachments are continuously detached.

An alternative model questions the tension model and emphasizes the "stability" of attachment, *per se*, as the sensor for the checkpoint. The attachment stability model relies on evidence for direct competition between microtubule occupancy and checkpoint protein recruitment at the outer kinetochore [61–63].

Because both tensions at kinetochore and microtubule attachment stability depend on each other [64], it is likely the two mechanisms feed into each other to relay signals to the SAC.

The SAC can also monitor the plane of kinetochore-microtubule attachment. The spindle checkpoint proteins Mad2, Mad1, and Mps1 are all present on immature lateral kinetochores [4, 61], but at reduced levels compared to a completely detached kinetochore [61]. The significance of this quantitative difference in checkpoint protein recruitment extent is not fully understood. One possibility, apart from influencing checkpoint signaling strength, could be that different levels of the checkpoint proteins, particularly kinases, facilitate distinct stages of KT-MT attachment.

4. Phosphoregulation of kinetochore-microtubule attachment status

A kinetochore can remain attached, or actively detach, from microtubules tethered to it. The affinity of a kinetochore for microtubules is finely tuned by phosphorylation-dephosphorylation events. Over the years, a number of kinases and phosphatases have been identified as important players in establishing attachment, geometry stabilization and error correction. This section details the role and regulation of principal kinases and phosphatases that monitor and control kinetochore-microtubule attachments through phosphorylation-dephosphorylation cycles.

4.1. Kinases that control kinetochore-microtubule attachments

Several mitotic kinases are recruited to the kinetochore, which, through phosphorylation, dynamically control the localization and function of other kinetochore and microtubule-associated proteins. We have chosen to discuss a few major kinases to illustrate the role of phosphorylation in monitoring and regulating kinetochore-microtubule attachment status (for a detailed review of kinases, we refer to Ref. [36]).

4.1.1. Aurora B kinase

Aurora B is a major regulator of kinetochore-microtubule interaction; it has a dual role in sensing biorientation errors and maintaining the SAC (reviewed in Refs. [50, 65]). Aurora B forms a complex along with INCENP, Survivin and Borealin, termed the Chromosome Passenger Complex or CPC [66–68]. Until anaphase, the CPC is mainly targeted to the inner centromeric region of kinetochores; recently, CPC subpools and active Aurora B has been shown to localise to the outer kinetochore and on microtubules close to the kinetochore-microtubule interface [19, 69–72].

Aurora B forms a gradient of phosphorylation at kinetochores, which suggests its mechanism of action on substrates to be diffusive [60, 73]. To influence kinetochore-microtubule attachment, Aurora-B phosphorylates various members of the KMN network, including Ndc80, that directly interact with the microtubule [74–76]. Recent studies on the establishment of mature end-on attachments in budding yeast indicate a role for Aurora-B in phosphorylating the microtubule associated protein complex, Dam1, and thus promoting lateral kinetochores [77]. Whether Aurora-B similarly controls the plane of kinetochore-microtubule attachment in other models is not known.

4.1.2. Mps1 kinase

Mps1 is an upstream regulator of the SAC. It is recruited to the kinetochore *via* the microtubule-binding domain of the Ndc80 complex, allowing the kinase to "sense" kinetochore-microtubule attachment status [22, 61–63]. Once at kinetochores, Mps1 phosphorylates KNL1 on its repeated MELT motif allowing the recruitment of the Bub1-Bub3-BubR1 module, further strengthening the checkpoint signal (reviewed in Ref. [78]). Dynamic localization of Mps1 kinase is essential for proper kinetochore-microtubule attachment [79]. Thus, although Mps1 is primarily responsible for SAC initiation, it indirectly facilitates the formation of kinetochore-microtubule attachments.

4.1.3. Plk1 and Cdk1

Plk1 and Cdk1 are major mitotic kinases responsible for dramatic cellular reorganization during cell division and are recruited to kinetochores in early mitosis [80–82]. Phosphoproteomic studies revealed Cdk1's ability to phosphorylate a high number of substrates involved in chromosome segregation [83, 84]. In line with its broad range of substrates, Cdk1 controls several crucial events for chromosome segregation, including the building of the spindle, assembly of kinetochore and functioning of the SAC [85–89]. Like Aurora B, Cdk1 is able to modulate microtubule dynamics by acting directly on microtubule-end associated proteins (for example, EB2 [90]). Inactivation of Cdk1 ultimately stabilizes kinetochore-microtubule attachments in anaphase [91] and this is crucial for the proper segregation of chromosomes.

Plk1 activity is essential for prometaphase to metaphase transition in human cells [92, 93]. Phosphoproteomic studies show several substrates of Plk1 among spindle and kinetochore proteins [94–96]. Through phosphorylation, Plk1 directly controls the function of several microtubule-associated proteins: Plk1-mediated phosphorylation promotes the activity of the microtubule depolymerizing kinesin, MCAK [97]. Plk1-mediated phosphorylation can also enhance the association of CLIP-170 with Caesin Kinase 2 and is needed for timely formation of kinetochore-microtubule attachment [98]. To initiate and maintain SAC signal, Plk1 acts on multiple checkpoint components: first, Plk1 acts in synergy with Mps1 kinase by phosphorylating the MELT repeats on KNL1 (reviewed in Ref. [99]). In addition, Plk1 phosphorylates Sds22 to recruit PP1 (Protein Phosphatase 1, introduced in Section 4.2) to the outer kinetochore [100]. Plk1 binds directly to Bub1 kinase to help sustain the SAC signal [101, 102]. In conclusion, Plk1 simultaneously regulates both kinetochore-microtubule interaction and checkpoint signaling.

4.1.4. Bub1 kinase

Bub1, as part of a complex including Bub3 and Mad3/BubR1, is recruited to kinetochores through KNL1 MELT repeats of the KMN network (reviewed in Ref. [99]). In addition to serving as a platform for recruiting Plk1 onto KNL1 [102], Bub1 kinase safeguards sister chromatid cohesion through the Sgo1/PP2A-B56 pathway [103, 104]. Bub1 is also required for the kinetochore recruitment of the RZZ checkpoint protein complex (composed of Rod, Zwilch and Zw10 proteins) [105]. Thus, Bub1 kinase acts as a massive scaffold protein for SAC signaling initiation—a role in addition to its enzymatic activity in sustaining SAC signaling.

4.2. Mitotic phosphatases that influence kinetochore-microtubule attachments

4.2.1. PP1

Protein phosphatase PP1 controls both normal mitotic timing and chromosome segregation accuracy (reviewed in Ref. [106–108]). During mitosis, PP1 localizes specifically at the kinetochore, in addition to spindle microtubules and the cell cortex [109]. PP1 docking proteins share a consensus motif (RVxF motif [110]): KNL1, CENP-E, RepoMan, Sds22 and SKA complex all recruit PP1 to the kinetochore during different stated of kinetochore-microtubule attachment [70, 111–115]. PP1 at the outer-kinetochore is thought to counteract Aurora B for both silencing of SAC and congressing chromosomes (reviewed in [59, 65]). Whether recruiting PP1 to multiple sites of the kinetochore is simply to enable network robustness or to manage a step-wise control of KT-MT attachments is unclear.

4.2.2. PP2A

Protein phosphatase PP2A is targeted to several regions of the kinetochore through its regulatory subunit B56, which is essential for maintaining stable kinetochore-microtubule attachments [116]. At the inner centromere, PP2A is recruited through Sgo1 and Sgo2, where it prevents untimely sister chromatid separation [104, 117, 118]. At the outer kinetochore PP2A directly interacts with BubR1; thus associating with the KMN network, through Knl1 [85, 119–121]. BubR1-bound PP2A-B56 counteracts Aurora B's role in destabilizing kinetochore-microtubule attachments; Sgo1-bound PP2A-B56 is also involved in this role, suggesting a degree of redundancy [85, 116, 119, 120, 122]. PP2A-B56 activity, when bound to Sgo1, is negatively regulated by Bod1 protein and the presence of the latter is important for sister chromatid cohesion [123]. By bringing PP2A to the centromeric region, Sgo1 that is sensitive to intra/inter-kinetochore tension can directly help sense different kinetochore-microtubule attachment states [124]. The various forms of B56 associated with PP2A (positioned at inner centromere and outer kinetochore) are thought to fine-tune the otherwise broad gradient-like action of Aurora-B kinase.

4.2.3. PP4

Protein phosphatase PP4 is a new addition to the list of kinetochore regulating phosphatases important for kinetochore assembly, SAC maintenance, and chromosome congression [125]; its recruitment mode and targets are less clear.

4.3. Higher order regulatory networks controlling KT-MT attachment

4.3.1. Kinetochore-bound kinases form a network to self-regulate localization and activation

Kinetochore-bound kinases are part of a very complex network in which they modulate one another's localization and enzymatic activity [126]. For a detailed overview on the topic, we refer readers to Refs. [59, 65]. Here, we use Aurora-B as an example to illustrate the complex control network that kinetochore-bound kinases are exposed to (**Figure 3**).

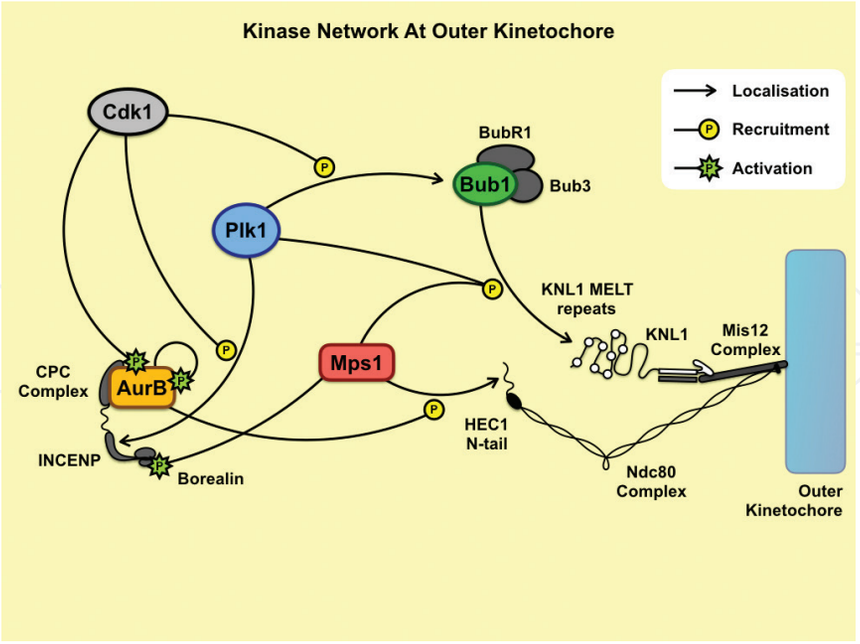

Figure 3. Phospho states are used to monitor and control microtubule attachment status: several kinases act on the KMN network at the outer kinetochore. Recruitment and activation of kinases are finely controlled through a network of phosphorylation-dephosphorylation cycles, allowing rapid monitor and dynamic control of attachment status. Phosphorylation controlled events (recruitment and activation) alone are shown here.

The centromeric enrichment of the CPC complex can be influenced by Cdk1, which phosphorylates CPC components Survivin in fission yeast and Borealin in human cells [127]. Mps1 can also promote rapid accumulation of Aurora B at the centromere [128]. Aurora B-mediated phosphorylation of the HEC1 N-terminus tail allows the recruitment of the checkpoint kinase Mps1 to kinetochores, which, together with Plk1, phosphorylates KNL1 MELT repeats to recruit other checkpoint proteins including Bub1, Bub3, BubR1 and Mad1, Mad2 and Cdc20 (reviewed in Ref. [65]). Moreover, Aurora B modulates its own activity by phosphorylating *in trans* both its kinase domain and INCENP, which results in an enhanced catalytic activity and an increased concentration of the kinase at kinetochores (reviewed in Refs. [50, 65]). In subcellular areas where Aurora-B is highly enriched, with the aid of the positive feedback loop, Aurora-B can thus overcome counteraction by phosphatases and establish areas of high activity [129]. However, when Aurora-B gradient lowers in intensity, many other kinases are able to finely tweak Aurora-B activity locally.

4.3.2. Regulatory loops linking phosphatases as a network

Unlike kinases that usually recognize specific consensus motifs, phosphatases are promiscuous with respect to their substrates. The presence of multiple binding sites for phosphatases at kinetochores could therefore allow precise, spatially restricted counteraction of kinase activity. Moreover, B55 and B56 regulatory subunits bear a highly conserved RVxF motif—a docking domain for PP1—and this puts PP2A and PP1 regulation into a complex loop [130]. In addition, the two phosphatases PP2A and PP1 are part of a KNL1-based feedback loop, which controls both their kinetochore localization and, in turn counteracts Aurora B

and Mps1 kinases for silencing the SAC [131, 132]. These complex regulatory loops among phosphatases, together with the loops between kinases, have made it very difficult to dissect upstream and downstream players influencing KT-MT attachment status.

In this final section, we present an overarching model of the key regulatory mechanisms that kinetochores employ to control microtubule interaction, through phosphorylation-dephosphorylation cycles. Kinases and phosphatases are present within the kinetochore scaffold and also the kinetochore-microtubule interface. Kinases regulate one another's activity and localization by building a regulatory network (**Figure 3**), in addition to their direct action on proteins, responsible for kinetochore-microtubule attachment. This regulatory network, coupled to phosphatase-mediated counteraction, is essential to temporally and spatially modulate kinetochore-microtubule attachment. As a general tendency, phosphorylation is associated with lower affinity of the outer kinetochore substrates (e.g., HEC1 and KNL1) to the microtubule, leading to a sustained SAC signal. Nevertheless, kinases and phosphatases can also directly stimulate or silence the checkpoint, respectively, by controlling the dynamic localization of other SAC proteins. Thus, a self-regulating turnover of proteins allows the fine-tuning of the SAC signal in close relationship to the state of kinetochore-microtubule attachments.

5. Concluding remarks and future prospects

Exploring the molecular basis for how cells tether chromosomes to dynamic microtubules has highlighted the importance of phosphorylation-dependent dynamic changes in recruitment and activation of kinetochore and microtubule-associated proteins. In general, these studies provide a conceptual framework for how bulky structures may be precisely moved within the cell. They also shed light on complex regulatory networks that operate at the microtubule end, explaining how cells harness energy stored within the microtubule cytoskeleton.

Our understanding of how microtubules capture and tether onto chromosomes has expanded through the identification of kinetochore proteins which assemble onto the chromatin, the characterization of protein interactions between the outer face of the kinetochore and the microtubule lattice, and last but not the least, the elucidation of how kinetochore-microtubule attachments control the recruitment of checkpoint kinases and phosphatases that finally silence the signal that delays anaphase onset. There is still work needed to understand how kinetochores distinguish between the walls and ends of microtubules; the precise molecular steps that allow separated sister kinetochores to latch onto a disassembling microtubule, without retriggering the checkpoint; whether the feedback loops used by the checkpoint to monitor attachment status can also be used to influence attachment status; and finally, how generally applicable are the mitotic mechanisms to human meiotic systems, where chromosomes remain stably tethered to microtubules for years.

By exploring redundant and nonredundant molecular steps of the highly conserved chromosome segregation process, we progress toward exploiting the mechanism for therapeutic purposes, either to develop biomarkers or drug targets to tackle chromosomal instability in cancers.

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