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Kinetochore structure and function

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The vertebrate kinetochore is a complex structure that specifies the attachments between the chromosomes and microtubules of the spindle and is thus essential for accurate chromosome segregation. Kinetochores are assembled on centromeric chromatin through complex pathways that are coordinated with the cell cycle. In the light of recent discoveries on how proteins assemble onto kinetochores and interact with each other, we review these findings in this article (which is part of the *Chromosome Segregation and Aneuploidy* series), and discuss their implications for the current mitotic checkpoint models – the template model and the two-step model. The template model proposes that Mad1–Mad2 at kinetochores acts as a template to change the conformation of another binding molecule of Mad2. This templated change in conformation is postulated as a mechanism for the amplification of the 'anaphase wait' signal. The two-step model proposes that the mitotic checkpoint complex (MCC) is the kinetochore-independent anaphase inhibitor, and the role of the unaligned kinetochore is to sensitize the anaphase-promoting complex/cyclosome (APC/C) to MCC-mediated inhibition.

The ability of chromosomes to achieve spindle bi-orientation is key to accurate chromosome segregation. At the onset of mitosis, rapidly growing and shrinking microtubules (MTs) probe the cytoplasm in search of kinetochores, which are macromolecular complexes assembled on opposite sides of the centromere that mediate interactions between the chromosomes and MTs. Once the sister kinetochores are attached to MTs from opposite poles, the chromosome undergoes a series of oscillatory motions that guide the chromosome towards the spindle equator. These oscillatory motions are dictated by the coordinated growth and shrinkage of the kinetochore MTs. Chromosome motility relies on the unique ability of the kinetochore to interact with the highly dynamic ends of MTs. This differs from other MT-based transport events that rely on interactions with a relatively static lattice of the MTs.

The stochastic nature by which kinetochores encounter MTs is an error-prone process that can result in non-productive connections [1]. The kinetochore is therefore a highly complex machine that does not merely bind and affect the dynamics of its attached MTs but possesses quality-control mechanisms that detect and correct defective or nonproductive interactions. Mechanistic insights into these remarkable activities will only be achieved by identifying and characterizing the molecular components of kinetochores in a wide variety of species.

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General organization of the kinetochore

The vertebrate kinetochore, as seen by transmission electron microscopy, appears as a trilaminar stack of plates that is situated on opposite sides of the centromeric heterochromatin of the mitotic chromosome [2]. In the absence of MT attachments, a meshwork of fibers, termed the fibrous corona, can be seen to extend from the surface of the outer plate [3]. The fibrous corona and the outer plate contain the majority of the known MT-interacting proteins (CENP-E, dynein, see Table 1 and Figure 1) [4] as well as checkpoint proteins (Bub1, BubR1, Bub3, Mad1, Mad2, Mps1, Nuf2, HEC1, Zwint-1, ZW10, Roughdeal; see Tables 2 and 3) [5] that monitor the integrity of kinetochore attachments. The protein composition of the middle zone is not known. The inner plate is immediately adjacent to the centromere but also comprises centromeric chromatin that is specified by the presence of the histone H3 variant CENP-A [6] (Figure 2).

Kinetochore assembly

From a morphological standpoint, the vertebrate kinetochore appears to be vastly more complex than the kinetochore of budding yeast. At the molecular level, the kinetochore compositions of all organisms studied to date share a surprisingly large number of proteins (Figure 2). One explanation that is commonly used to explain the apparent discrepancy between the morphological and molecular data is that the kinetochores of metazoans are assembled from repeated subunits [7], where each repeat might reflect the unit module of the yeast kinetochore. This is indirectly supported by the observation that there are multiple copies of proteins at the human kinetochore as opposed to what is likely to be a single copy at yeast kinetochores (some yeast proteins are dimers or tetramers, based on purification of pre-existing subcomplexes [8]). With the increase in copy number, mechanisms must also be in place to assemble these modules into supramolecular complexes that form the trilaminar plates. Additional mechanisms might be also required to coordinate the multiple repeats folding into the highly condensed three-dimensional structure. How the higher organization is achieved is unclear, but it might be

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Table 1. Kinetochore MT-binding proteins[a]

MT binding proteins	Localization	Knockout, knockdown, dominant-negative or overexpression phenotypes	Proposed role in mitosis	Refs
CENP-E, member of kinesin-7 family	Fibrous corona, outer KT	Antibody microinjection: mitotic arrest; defective chromosome alignment and MT attachment; unable to convert monopolar attached chromosomes to bipolar attached chromosomes; reduced KT tension; spindle pole fragmentation. Overexpression of motorless CENP-E: defective chromosome alignment.	CENP-E interacts with BubR1 at KTs and activates BubR1 kinase activity in the absence of MTs *in vitro*. CENP-E is proposed to be the MT sensor that relates KT MT occupancy or tension status to the mitotic checkpoint.	[4,12, 81]
		siRNA: mitotic arrest; defective chromosome alignment and reduced stability of KT MTs; reduced KT tension; spindle pole fragmentation.		[82]
		Immunodepletion from *Xenopus* extracts: defective mitotic checkpoint; failure to recruit Mad1 and Mad2.		[83]
		Mouse knockout: embryonic lethal, conditional knockout MEFs: defective mitotic checkpoint; defective chromosome alignment; reduced stability of KT MTs; reduced BubR1 kinase activity; reduced recruitment of Mad1 and Mad2.		[84]
MCAK/ XKCM1, member of kinesin 13 family	Centromere	Lagging chromosomes; long spindles; KT–MT attachment errors; reduced KT tension Overexpression of MCAK: mitotic arrest, lack of KT MTs.	Regulates spindle MT dynamics by catalyzing the depolymerization of MTs. Activity of MCAK is modulated by the tension-sensitive aurora B kinase and the PP1 gamma phosphatase.	[33,85]
Dynein/ dynactin	KT	siRNA knockdown of cytoplasmic DHC: mitotic arrest.		[86]
		Inhibition of dynein/dynactin by dominant-negative p150-CC1 or 70.1 anti-dynein antibody: elongation of MT in spindle assembled in *Xenopus* egg extracts.	Dynein/dynactin is required for targeting Kif2a, a MT-depolymerizing KinI kinesin, to the spindle pole.	[87]
		Microinjection of p50 dynamitin or 70.1 anti-dynein antibody: prevented Mad2 depletion from KTs; reduced KT tension; mitotic arrest at metaphase	Dynein/dynactin is required for the inactivation of the mitotic checkpoint by transporting Mad2 poleward and away from KT after metaphase alignment.	[46]
		Overexpression of p50 dynamitin: defect in chromosome alignment, distorted mitotic spindle		[88]
CLIP170	KT, plus-ends of MTs	Overexpression of dominant-negative CLIP170: delay in mitosis; displacement of endogenous CLIP170	CLIP170 is a+TIPs and is proposed to function in the establishment of metaphase chromosome alignment and as an anti-catastrophe factor.	[33]
Lis1	KT, plus ends of MTs	Microinjection of antibody: defects in chromosome alignment; delay in mitotic progression; defects in chromosome segregation. Overexpression of Lis1: defects in chromosome alignment; defects in spindle orientation.	Lis1 mediates CLIP-170–dynein interactions and coordinates dynein cargo-binding and motor activities.	[89]
CLASP1	Fibrous corona, plus-ends of MTs	Microinjection of antibody: spindle collapse; defective chromosome attachment; defective MT dynamics at KTs Overexpression of dominant-negative CLASP1: spindle collapse; MT bundling.	CLASP1 regulates MT dynamics at KTs	[33]
Adenomatous polyposis coli (APC)	KT, plus-ends of MTs	Immunodepletion from *Xenopus* egg extracts: aberrant spindle formation	APC functions to mediate KT–plus-end-MT attachment. APC is found to interact with the Bub1 mitotic checkpoint kinase and might functionally link to the mitotic checkpoint.	[33]
		Mutant APC C-terminal truncation in mouse ES cells or overexpression of APC C-terminal 253 amino acids: weakened KT–MT interaction; defective chromosome segregation; reduced KT tension; defective mitotic checkpoint.		
EB1	KT, plus-ends of MTs		EB1 binds APC, CLASP1 and 2, and dynein/ dynactin. EB1 might modulate KT MT polymerization and/or attachment	[90]
Sgo1	KT, inner centromere[b]	siRNA knockdown and microinjection of antibody: premature segregation of sister chromatid; defective KT-MT attachment; prolonged mitotic delay; reduced KT tension; reduced KT MT dynamics.	Sgo1 might be involved in the establishment of sister centromere cohesion. Sgo1 might link sister centromere cohesion with MT interactions at KT.	[91]

[a]Abbreviations: ES, embryonic stem; KT, kinetochore; +TIPs, plus-end-tracking proteins; MEF, mouse embryonic fibroblast; MT, microtubule.
[b]It is currently unresolved whether human Sgo1 localizes to outer kinetochore, inner centromere or both.

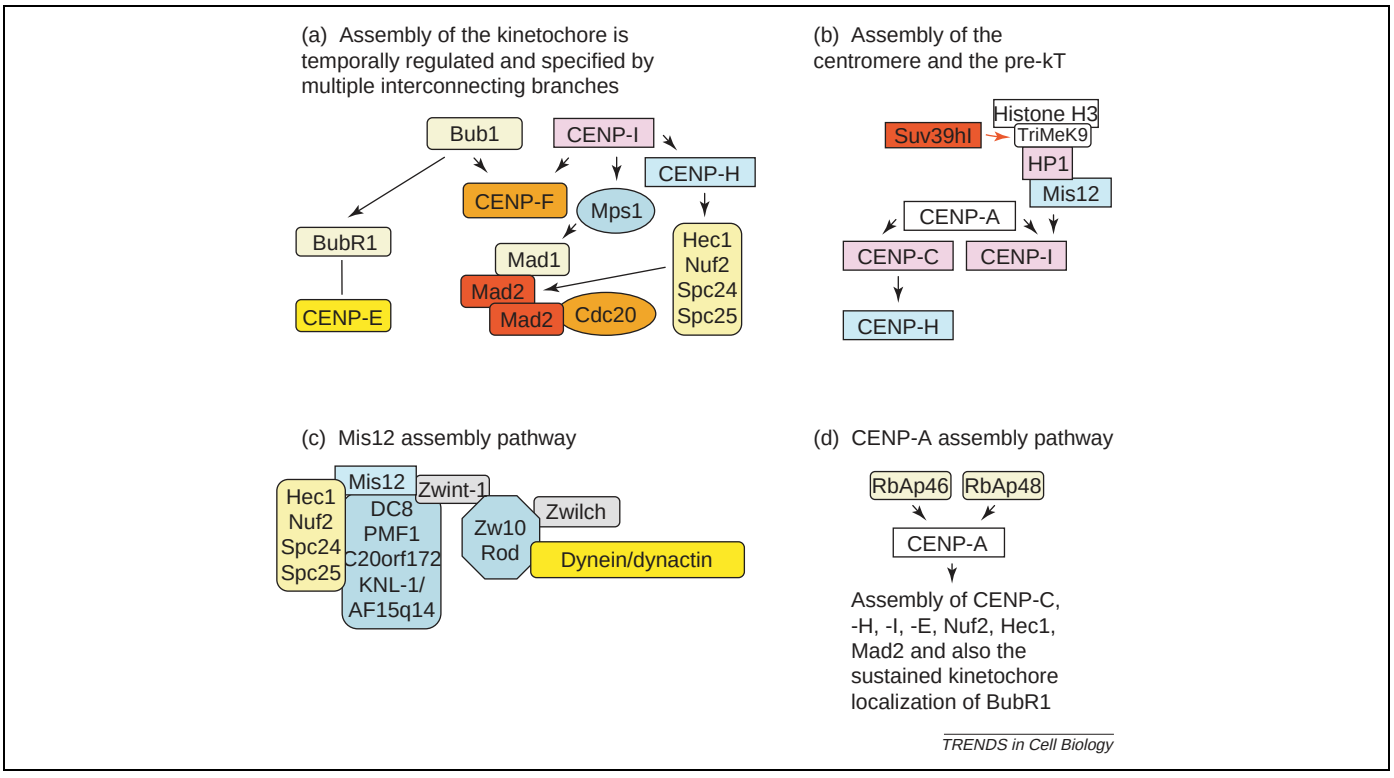


Figure 1. Kinetochores are assembled from multiple pathways that are temporally controlled. **(a)** Multi-branched assembly pathway that links inner kinetochore proteins CENP-H and CENP-I with outer kinetochore proteins CENP-E, CENP-F, HEC1/Ndc80 complex, Mad1 and Mad2. The arrows indicate assembly dependencies. Lines depict the order of appearance at the kinetochore. **(b)** Mis12 and CENP-A specify two major assembly branches. The trimethyl-lysine 9 histone H3 (triMeK9H3) modification recruits binding of HP1 to the centromere, and HP1 presumably recruits the binding of Mis12. The red arrow indicates the dependency of the trimethyl lysine 9 modification of histone H3 on the suv39h1 methyltransferase. **(c)** The Mis12 assembly pathway. Mis12 co-immunoprecipitates with Zwint-1, which is required for the kinetochore localization of Zw10. Zw10 and Rod are required for the recruitment of the motor protein dynein/dynactin to the kinetochore. Mis12 also interacts with DC8, PMF1, C20orf172, KNL-1/AF15q14/KIAA1570 and the Ndc80 complex. **(d)** CENP-A assembly at the kinetochore is dependent on RbAp46 and RbAp48, proteins that are involved in histone deacetylation.

specified by proteins that are unique among metazoans [93].

Molecular evidence supporting a kinetochore assembly pathway came from studies in HeLa cells that showed that there was a temporal order in which proteins appeared at kinetochores. Some proteins, such as CENP-F [9], an outer kinetochore protein, appeared at kinetochores between late G2 and prophase, whereas others, such as CENP-E and dynein, did not appear until after nuclear envelope breakdown (CENP-E and dynein are MT motors, see Table 1). Indeed, closer examination has revealed the following temporal pattern of localization for the following proteins: hBub1→CENP-F→hBubR1→CENP-E [5,10] (Figure 1a). Bub1 and BubR1, homologs of budding yeast mitotic checkpoint proteins Bub1 and Mad3 (Figure 2c), are mitotic checkpoint kinases that assemble on kinetochores during the late G2 phase of the cell cycle [5]. The temporal order in which proteins appear at kinetochores reflects, in part, the hierarchical relationship among these proteins. For instance, Bub1 in human cells has been shown to be required for the assembly of BubR1, CENP-F, CENP-E and Mad2 [11]. However, these proteins do not exhibit a linear relationship, as exemplified by the finding that the localization of Mad2 does not depend on CENP-E [12], and the localization of CENP-E does not depend on CENP-F (T.J. Yen, unpublished). hBub1 must therefore specify multiple branches of assembly that direct the assembly of CENP-F, hBubR1 and CENP-E (Figure 1a).

There are examples where the assembly of a protein depends on multiple branches. For example, the localization of CENP-F to kinetochores not only depends on hBub1 but also on CENP-I/hMis6 [13], a constitutive kinetochore protein (Figure 1a). As an aside, CENP-I/hMis6 also specifies a separate branch that is required for the sequential assembly of hNuf2/Ndc80, Mps1, Mad1 and Mad2 [13,14] (Figure 1a; T.J. Yen unpublished). As hBub1 assembly does not depend on CENP-I/hMis6, the localization of CENP-F depends on two separate pathways. The significance of this is unclear but might reflect a way to coordinate the different pathways so that no single assembly pathway outpaces the other.

Recent efforts have shown that CENP-A and hMis12 define two major branches of the assembly pathway in human cells (Figure 1b,c). Mis12 was identified in the fission yeast *Schizosaccharomyces pombe* as part of a collection of temperature-sensitive (ts) minichromosome instability (*mis*) [15] mutants. Vertebrate orthologs of Mis12 have been identified that exhibit limited sequence similarity with *S. pombe* Mis12 [16]. Although the phenotype of cells depleted of hMis12 is very similar to that seen for cells depleted of CENP-A, these proteins do not appear to depend on each other for their localization at kinetochores [16], as was the case in fission yeast [17]. Thus, hMis12 represents a centromere/kinetochore assembly pathway that is independent of CENP-A.

Table 2. Kinetochore mitotic checkpoint proteins[a,b]

Budding yeast	Vertebrates	Localization and roles in mitosis
Bub1	Bub1	Mitotic checkpoint kinase [33] Localizes to KTs
Bub3	Bub3	Binds to Bub1 and BubR1 Localizes to KTs [33] Part of the MCC complex [70]
Mad1	Mad1	Required for kinetochore assembly of Mad2 Localizes to nuclear pores and unattached KTs [47]
Mad2	Mad2	Binds to Cdc20 Part of the MCC complex [70] Localizes to nuclear pores and unattached KTs [47]
Mad3	BubR1	BubR1, localizes to KTs [44] Mitotic checkpoint kinase [44] Part of the MCC complex [70]
MPS1	MPS1	Mitotic checkpoint kinase [33] Localizes to nuclear pores, centrosomes and KTs [48]

[a]The Bub and Mad mitotic checkpoint proteins were originally identified in budding yeast and are evolutionarily conserved in vertebrates.
[b]Abbreviations: KT, kinetochore; MCC, mitotic checkpoint complex.

The deposition of CENP-A was assumed to be the crucial step in establishing centromere identities in all species. The mechanism by which newly replicated centromere DNA becomes incorporated into CENP-A-containing chromatin remains an open question. In the budding yeast *Saccharomyces cerevisiae*, simultaneous deletion of the chromatin assembly factor subunit *cac1* and histone regulatory gene *hir* caused mislocalization of its CENP-A protein, Cse4p [18]. In *S. pombe*, CENP-A localization depends on Mis6, Ams2 and the recently discovered Mis15, Mis16, Mis17 and Mis18 proteins [17,19,20]. The Mis16–Mis18 complex seems to sit at the top of the hierarchy and is responsible for the recruitment of SpCENP-A to centromeres. Centromeres of *mis16* and *mis18* mutants exhibit increased levels of acetylated histone H3 and H4 [20]. The human Mis16 orthologs, RbAp46 and RbAp48, are part the NuRD multisubunit complex that has deacetylase and nucleosome-remodeling activities [21]. These are the first proteins reported to be responsible for CENP-A binding to centromeres in human cells and thus represent an important advance in our understanding of the epigenetic mechanism that specifies centromere identity (Figure 1d). Although it is clear that neither Mis16 nor RbAp46/48 acts specifically on centromeric chromatin, specificity might come through their interactions with Mis18 and its orthologs [20].

Recently, separate groups have identified a complex of proteins that copurifies with hMis12 [22,23]. These include hPMF1, DC8, C20orf172, KIAA1570, HEC1 (highly expressed in cancer, human ortholog of yeast Ndc80 [24]), hNuf2, hSpc24, hSpc25, HP1α, HP1γ and hZwint-1 [23] (Figure 1c). HP1 proteins are heterochromatin-binding proteins that bind to the tri-methylated lysine 9 of histone H3 (triMe3K9H3) [25,26]. The histone methyltransferase suv39h1 modifies lysine 9 of histone H3 at pericentric heterochromatin [27] (Figure 2a). Simultaneous knockdown of HP1α and HP1γ reduced the level of Mis12 at kinetochores [23]. A proposed assembly pathway involves the epigenetic modification of histone H3 at pericentric heterochromatin, and the recruitment of HP1, which in turn, specifies the assembly of Mis12 (Figure 1b). Mis12 is then required for assembly of other kinetochore proteins such as hZwint-1 and the hZW10/RoughDeal (Rod) complex (Figure 1c), which is at the outer kinetochore [23]. However, recent examination of human and *Drosophila* centromeric chromatin failed to find heterochromatin-specific histone H3 modification (di- or tri-methylation of lysine 9), leaving open the question regarding where exactly Mis12 and the associated proteins bind to centromeres [28].

Microtubule binding

Kinetochores of metazoans contain the molecular motor proteins CENP-E [29], dynein/dynactin [30,31] and KinI kinesin MCAK [32] (see Table 1). In addition, the kinetochore also contains MT-binding proteins including CLIP170, CLASP/Orbit, EB1, Sgo1 and APC (adenomatous polyposis coli). These can be further separated into those whose localization at kinetochores depends on MTs (APC and EB1) and those that bind to kinetochores independent of MTs (CLIP170, CLASP, hSgo1). The functions of these proteins were recently reviewed [33,34], and we have summarized this information in Table 1 and Figure 2.

Aside from these MT-binding proteins, the evolutionarily conserved Nuf2–Ndc80–Spc–Spc25 complex is also essential for kinetochore attachments [35]. How this complex specifies microtubule attachments is unclear, but it might play an indirect role in organizing the supramolecular structure of the kinetochore [36]. The coordination among different MT-binding proteins is likely to be an important factor in specifying chromosome alignment. In vertebrates, where the number of MTs is of the order of 20 per kinetochore [12], the dynamics of

Table 3. Kinetochore mitotic checkpoint proteins[a]

Drosophila	Vertebrates	Localization and roles in mitosis
ZW10 [92]	HZW10 [92]	Localizes to KTs [93] Essential component of the mitotic checkpoint [93] Required for the recruitment of dynein/dynactin [93,94] Interacts with p50 dynamitin directly [94]
Roughdeal (Rod) [95]	hRod [96]	Localizes to KTs [93] Essential component of the mitotic checkpoint [93] Required for the recruitment of dynein/dynactin [93,96]
No homolog	hZwint-1 [97]	Localizes to KTs [97] Required for the mitotic checkpoint [98] Interacts with ZW10 [97], Mis12 [23]

[a]Kinetochore mitotic checkpoint proteins in the ZW10–Rod complex have no known homologs in budding yeast (point kinetochores) and might reflect the increased complexities of compound kinetochores and mitotic checkpoint regulatory mechanism(s) in metazoans.

individual MTs might be averaged out so that the chromosomes display a much smaller range of oscillations that function to position the chromosomes midway between the poles. By extension, this might explain a long-standing paradox regarding why a bipolar-attached chromosome would move towards the pole that has the fewest MT attachments: the dynamics of multiple microtubules bound at the lagging kinetochore might just dampen each other's activities.

Future analysis of these MT-binding proteins will also have to move beyond the traditional 'search and capture' paradigm. A new mechanism for bipolar attachment has recently been described [37] whereby MTs nucleated from kinetochores become incorporated into the spindle [37]. The capture of the minus-ends of these kinetochore fibers by astral MTs and eventual incorporation into the spindle pole is mediated by dynein [38]. These kinetochore fibers have been observed to grow through plus-end-biased polymerization at kinetochores in *Drosophila* S2 cells. It is hypothesized that short MTs formed in the vicinity (probably because of a high Ran–GTP concentration at kinetochores [39]) are captured by the kinetochores. Alternatively, MT growth might be initiated by proteins at the kinetochore.

The role of checkpoint proteins at kinetochores

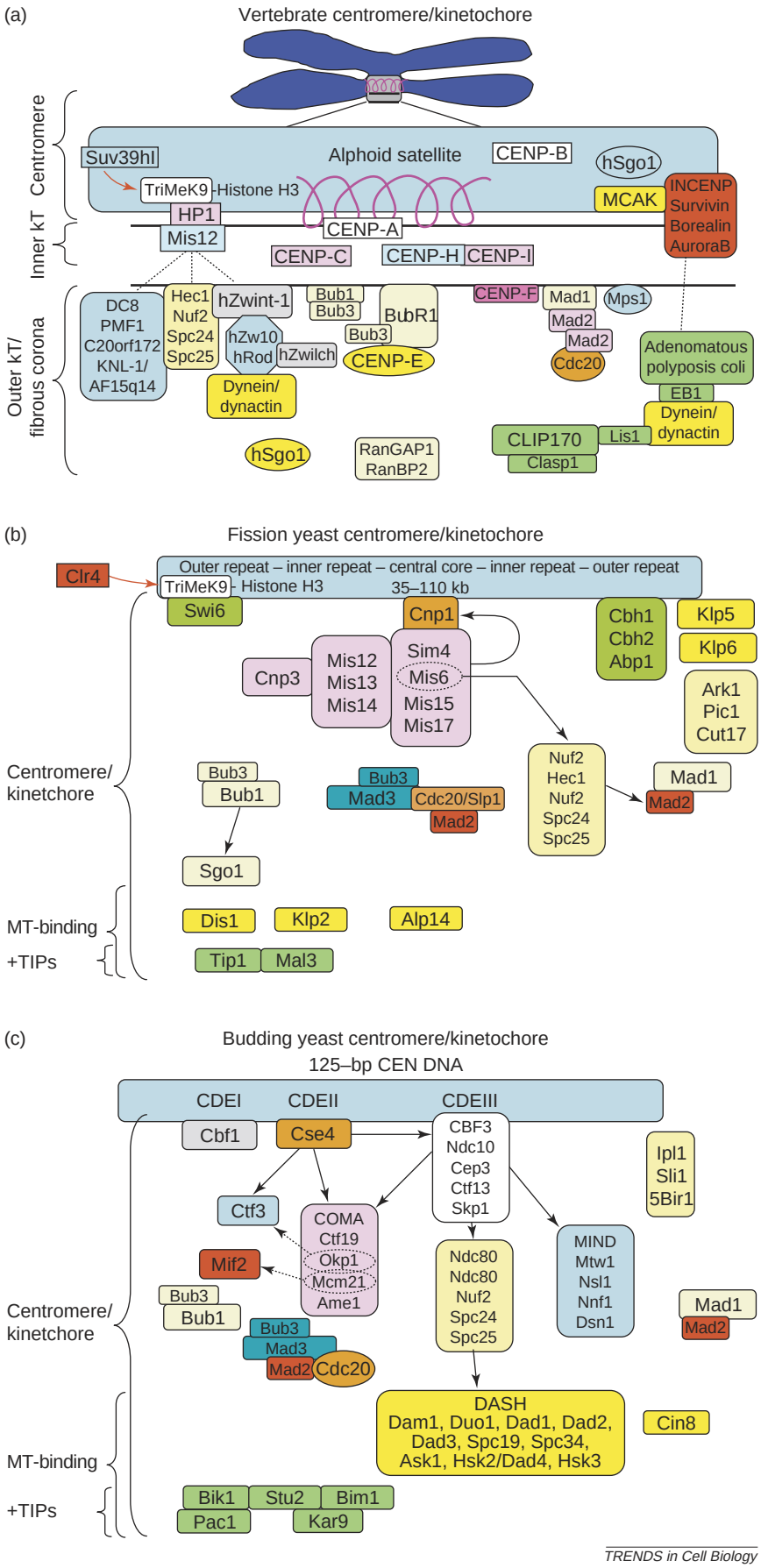
The mechanical interactions between the kinetochore and the spindle are monitored by Mad1, Mad2, Mad3, Bub1, Bub3 and Mps1, an evolutionarily conserved group of checkpoint proteins first identified in budding yeast [33,40]. Rae1 is an evolutionarily conserved protein that is structurally related to Bub3. Both budding and fission yeast Rae1 are important for mRNA export [41,42], but mammalian Rae1 has been reported to associate with hBub1 and contribute to the spindle checkpoint [43]. In higher eukaryotes, BubR1 is probably the Mad3 homolog, but it has evolved to contain a kinase domain [40]. BubR1 is localized on the outer plate of the kinetochore, where it is postulated to act as a mechanosensor that monitors the activity of CENP-E [44]. *In vitro* studies have shown that BubR1 kinase activity is stimulated when it interacts with CENP-E [45]. This suggests that the checkpoint activity of BubR1 might be regulated *in vivo* by conformational changes in CENP-E when it interacts with MTs. Similarly, Mad1 and Mad2 also respond to mechanical input as they are displaced from kinetochores with MT attachments [46]. The molecular basis by which MT attachment displaces Mad1 and Mad2 from the kinetochore is unknown. An intriguing explanation might come from the unexpected interaction between proteins of the kinetochore and the nuclear pore complex (NPC). Mad1, Mad2 and Mps1 are localized to the nucleoplasmic face of the NPC during interphase [47–49], whereas a constellation of NPC proteins localizes to kinetochores during mitosis [49,50]. Nup358/RanBP2 and RanGAP1 stand out as their localization at kinetochores is MT dependent and inversely related to Mad1 and Mad2 [39,51]. One intriguing model is that MTs deliver Nup358/RanBP2 and RanGAP1 to the kinetochore, and these proteins compete with Mad1 and Mad2 for common binding sites.

In vertebrate cells, all of the mitotic checkpoint proteins are associated with kinetochores at the onset of mitosis and persist there until the metaphase-to-anaphase transition [40]. In budding yeast, Bub1 and Bub3 are detected at kinetochores during normal mitosis. However, Mad1 and Mad2 are only detected at kinetochores upon activation of the mitotic checkpoint [52]. The discrepancy with the vertebrate kinetochores might be resolved if MT attachments occur more rapidly in yeast. Mad1 and Mad2 are therefore only detectable if the kinetochores are unoccupied by MTs for a prolonged period. This situation is not dissimilar to that of vertebrates as the mitotic checkpoint proteins and MT motor proteins CENP-E and cytoplasmic dynein accumulate to higher levels at unattached kinetochores [33]. Upon MT attachment, Mad2, Mad1, BubR1, Bub1, CENP-E, dynein/dynactin and Bub3 are released, to varying degrees, from the kinetochore [33]. This is partially due to dynein-dependent transport of some kinetochore proteins (Mad2 [53], CENP-E [46], Zw10 [54] and Rod [55]) off the kinetochore via the spindle MTs and also through direct release of proteins from the kinetochore into the cytoplasm (see below) [56,57]. The release of mitotic checkpoint proteins upon MT attachment at kinetochores has been proposed as a possible mechanism for silencing the mitotic checkpoint [46].

The role of checkpoint proteins was recently expanded with the discovery that hBubR1 is also required for kinetochore MT attachments [58]. Cells depleted of hBubR1 are unable to maintain stable kinetochore attachments. This defect can be reversed by inhibition of the Aurora B kinase (presumably by stabilizing attachments) [58]. It is unlikely that hBubR1 is directly responsible for MT attachments, but hBubR1 is more likely to regulate the activity of a MT-binding protein. CENP-E might be a target, given its association with hBubR1. Other proteins are probably involved as the BubR1 defect is inconsistent with the mere loss of CENP-E function. The contribution of BubR1 to kinetochore attachments might be analogous to how hBub1 is thought to play a role in attachments. The attachment defects seen in cells depleted of hBub1 are likely to arise from the combined loss of CENP-E, CENP-F and hBubR1 from kinetochores [11].

Checkpoint mechanisms

How defective kinetochore–microtubule attachment is detected remains an open question, but we are in a better position to explain how the checkpoint proteins effect inhibition of the anaphase-promoting complex/cyclosome (APC/C), the E3 ubiquitin ligase that drives progression through mitosis. An earlier model for how Mad2 contributes towards the checkpoint signaling pathway was proposed based on the *in vivo* dynamic properties of Mad2 as well as its ability to inhibit the APC/C *in vitro*. Using an assay where the APC/C activity was dependent on an exogenous supply of Cdc20, Mad2 was shown to bind to Cdc20 and prevent it from activating the APC [40,59]. Structural studies have shown that recombinant Mad2 can adopt an open form, O-Mad2 (also known as N1), and a closed form, C-Mad2 (also known as N2) [60,61]. The closed form is more potent in inhibiting the APC/C *in vitro*



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because it has a higher affinity than the open form for Cdc20. These observations, along with other *in vitro* data, led Luo *at al.* to propose the Mad2 'Exchange model'. In this model, kinetochore-bound Mad1 is thought to promote the conformational switch from O-Mad2 to C-Mad2 [60]. As the Mad2-binding sites in Mad1 and Cdc20 are very similar, Cdc20 is envisioned to compete with Mad1 for C-Mad2. As long as there are unattached kinetochores, sufficient amounts of C-Mad2 are thought to be generated to tie up the cellular pool of Cdc20 and block its ability to recruit substrates to the APC/C. An inherent problem with this model is that the affinities between Mad1–Mad2 and Cdc20–Mad2 might be too high to account for the rapid exchange of Mad2 between Mad1 and Cdc20. Furthermore, De Antoni *et al.* recently showed that Mad2ΔC, a mutant that cannot directly bind Mad1 alone, can still bind the Mad1–Mad2 complex, which counters the notion that Mad1 is a chaperone for Mad2–Cdc20 binding [62].

Template model

A new 'Template model' has recently been proposed whereby Mad1 and C-Mad2 form a stable complex that recruits O-Mad2 that is present in the cytosol. Upon binding to the Mad1–C-Mad2 complex, O-Mad2 is converted to C-Mad2, which is released from the complex upon binding to Cdc20. This model has its roots in an earlier idea that recombinant Mad2 can form homo-oligomers that were more potent at sequestering Cdc20 and blocking APC/C activity [63]. This oligomer theory was dispelled by the $Mad2_{R133A}$ mutant, which cannot oligomerize *in vitro* but retains its abilities to bind Cdc20 and Mad1 and mediates mitotic arrest when overexpressed along with Mad1 in HeLa cells [64]. In the latest report, the same group showed that a preformed Mad1–C-Mad2 complex can recruit another molecule of O-Mad2 through Mad2 dimerization. This was verified through the use of various Mad2 mutants. The Mad2ΔC mutant was found to bind the Mad1–Mad2 complex even though loss of its C-terminal 10 residues rendered it unable to stably bind Mad1 and Cdc20. Thus, Mad2ΔC must be associated with the complex by binding to Mad2. The $Mad2_{R133E\text{-}Q134A}$ mutant is incapable of forming oligomers [62] and was found to be unable to bind to the Mad1–Mad2 complex. When the Alexa Fluor 488-labeled $Mad2_{R133E\text{-}Q134A}$ mutant was injected into PtK1 cells, it failed to localize to unattached kinetochores. By contrast, the Mad2ΔC mutant retained its ability to bind to kinetochores despite losing its ability to bind Mad1 (and Cdc20). Functionally, neither of the Mad2 mutants retained checkpoint activity *in vivo* [61,65–68]. These results, combined with the *in vitro* data, strongly suggest that Mad2 dimerization is likely to occur at kinetochores and is an important feature of its checkpoint activity.

By extension, the initial C-Mad2–Cdc20 complex that is released from the kinetochore is speculated to further catalyze the conversion of the cytosolic pool of O-Mad2 into the inhibitory C-Mad2. This proposed prion-like activity of Mad2 provides a mechanism that exponentially amplifies the signal that was initiated at the unattached kinetochore. Silencing of this pathway is believed to rely on p31comet (CMT2), a protein that promotes mitotic exit by selective binding to C-Mad2 and release of Cdc20 to activate the APC/C [69]. For both the Exchange and Template models, unattached kinetochores are proposed to convert the cytosolic pool of Mad2 into a form that can sequester Cdc20, preventing activation of the APC/C. While this model fits the *in vitro* data, the existence of alternative conformers of native Mad2 remains to be demonstrated. Regardless, the mechanism by which the kinetochore generates the 'wait anaphase' signal is likely to be more complex as it must take into account the roles of other checkpoint proteins.

The Two-step model

An alternative view proposes that kinetochores might not have to directly generate the inhibitor of the APC/C

Figure 2. Comparison of the molecular organization of kinetochores between vertebrates and fungi. **(a)** The vertebrate kinetochore/centromere. Schematic representation of the spatial distribution of kinetochore proteins and their relationships between one another. Proteins that physically interact with each other are depicted as shapes that contact each other or dotted-line linkages. The coil at the centromere depicts the proposed helical path organization of the chromatin fibre, where CENP-A-bearing nucleosomes are exposed as repeat subunits at the inner plate of the kinetochore. The red arrow indicates an enzymatic relationship, rather than structural dependency, between the trimethyl-lysine 9 modification of histone H3 and the suv39h1 methyltransferase. All protein complexes with their subunits are depicted as a single shape. The Ndc80 complex consists of Hec1, Spc24, Spc25 and Nuf2. The Mis12-interacting proteins, DC8, PMF1, C20orf172 and KNL-1/AF15q14, are cartooned also as a single shape. The inner centromere Aurora B complex, consisting of Aurora B, INCENP, survivin, and Borealin/Dasra, is illustrated as a red shape. The nuclear pore proteins RanGAP1 and RanBP2 are cartooned as a single shape. The yellow shapes indicate kinetochore MT-binding proteins, which include the kinetochore MT motors CENP-E, dynein/dynactin and MCAK. The green shapes indicate plus-end MT-binding proteins. BubR1 is not depicted as interacting with Cdc20 as there is currently no evidence that these proteins interact at the kinetochore or their kinetochore localizations are interdependent. **(b)** The fission yeast kinetochore/centromere. The assembly dependencies of centromere and kinetochore proteins are depicted diagrammatically. The red arrow indicates the dependency of the trimethyl-lysine 9 modification of histone H3 on the Clr4 methyltransferase. Assembly dependencies are indicated by arrows, and physical interactions by overlap of the shapes. Cnp1, the *Schizosaccharomyces pombe* CENP-A homolog, is depicted as binding the central core of the fission yeast centromere DNA. The three *S. pombe* CENP-B homologs, Cbh1, Cbh2 and Abp1, bind the outer repeat of the centromeric DNA. The Mis6 complex consists of Sim4 (= CENP-H), Mis6 (= CENP-I), Mis15 and Mis17. Unlike budding yeast and higher eukaryotes, the Mis6 complex is required for the assembly of CENP-A in *S. pombe*. The interaction between Mis6 and Nuf2 is indicated by the dotted shape of Mis6 and the arrow connecting to the Nuf2 complex. The fission yeast Aurora B complex consists of Ark1 (= Aurora B), Pic1 (= INCENP) and Cut17 (= survivin). The yellow shapes indicate the kinetochore MT-binding proteins Dis1, Alp14 and Klp2. Dis1 and Alp14 are the *S. pombe* homologs of the vertebrate MT-binding protein XMAP215/chTOG. Klp2 is a kinesin kar3 family member that localizes to the centromere. Klp5 and Klp6 belong to the kinesin-8 (formerly known as 'kinI') family and are probably the MCAK homologs. Tip1= CLIP170 and Mal3= EB1 **(c)** The budding yeast kinetochore/centromere. The budding yeast centromere consists of 125 base pairs of CEN DNA. The assembly dependencies of centromere and kinetochore proteins are depicted diagrammatically. Direct interactions are indicated by overlaps and/or touching of the cartoon shapes. The CBF3 complex, consisting of Ndc10, Cep3, Ctf13 and Skp1, is cartooned as a single shape that binds directly to the CDEIII element of the CEN DNA. Cse4 is the budding yeast homolog of CENP-A and binds the CDEII element of the CEN DNA. Cbf1 is the putative CENP-B homolog and binds the CDEI element of the CEN DNA. Ctf3 is the homolog of Mis6/CENP-I. The MT-binding DASH complex is cartooned as a single shape and has no known homolog in higher eukaryotes. Cin8 is a kinesin-related protein. The +TIPs, Bik1 (= CLIP170), Stu2 (= ChTOG), Bim1 (= EB1), Pac1 (= Lis1) and Kar9 (= APC) are cartooned as green shapes. The MIND complex consists of Mtw1 (= Mis12), Nsl1 (= Dc8/Mis14), Nnf1 (= PMF1) and Dsn1 (=c20orf172/Mis13). The COMA complex, consisting of Ctf19, Okp1, Mcm21 and Ame1, is depicted as a single complex. Mcm21 interacts directly with Mif2, the CENP-C homolog, as indicated by the dotted arrow. The Aurora B complex consists of Ipl1 (= Aurora B), Sli15 (= INCENP) and Bir1 (= survivin). Note: = sign denotes vertebrate homologs.

(Mad2-sequestering Cdc20). Instead, the 'wait anaphase' signal sensitizes the APC/C to inhibition by a factor that acts independently of kinetochores. This Two-step model (kinetochore-dependent and -independent) was proposed based on the discovery of the mitotic checkpoint complex (MCC) [70]. The MCC was identified in a biochemical screen for factors in HeLa cells that can inhibit the APC/C. The MCC consists of BubR1, Bub3, Cdc20 and Mad2 in near-equal stoichiometry and is several orders of magnitude more potent at inhibiting the APC/C than the recombinant Mad2 that was used in all other studies. Unexpectedly, MCC was found to be present and active even in interphase cells, when the mature kinetochore has not yet assembled. Thus, MCC formation and activity does not depend on kinetochores. Importantly, MCC was found to selectively inhibit APC/C that was purified from mitotic cells. This contrasts with other studies that showed that recombinant Mad2 and BubR1 were only effective against interphase, but not mitotic, APC/C [71].

The existence of the MCC during interphase provides the cell with a rapid mechanism to inhibit the APC/C when cells enter mitosis. By necessity, the affinity between MCC and APC/C cannot be very high as the inhibition must be reversible in order for cells to exit mitosis. The role of the kinetochore is to provide a signal that maintains the interaction between the MCC and the APC/C. This was supported by reconstitution experiments that suggested that kinetochores might act on the APC/C to sensitize it to prolonged inhibition by the MCC [70]. This possibility is consistent with the recent finding that subunits of the APC/C are preferentially associated with kinetochores [72–74]. While these observations suggest that the APC/C might be directly modified by the kinetochore, it will be important to examine the dynamics of its interactions.

Evidence supporting the existence of a kinetochore-independent inhibitor of the APC/C has come from several studies. As discussed previously, the assembly of the checkpoint proteins Mad1, Mad2 and Mps1, but not BubR1 and Bub1, depends on CENP-I and HEC1 (Figure 1b). Interestingly, cells whose kinetochores were selectively depleted of these proteins were able to delay mitosis in the presence of unaligned chromosomes. Importantly, the delay was still dependent on Mad2 despite the fact that its level at kinetochores had been reduced 10 to 20 fold [13,75]. This result is consistent with the presence of a kinetochore-independent inhibitor of the APC/C, such as the MCC. Additional evidence for a two-step model came from a detailed comparison of the mitotic timing of HeLa cells that were depleted of various checkpoint proteins [76]. This study showed that selective depletion of Bub1, Bub3 and Mad1 checkpoint proteins from kinetochores did not significantly alter the time cells spent in mitosis. Mitotic timing (from nuclear envelope breakdown to anaphase A) was accelerated (from ~25 min to 12 min) only when either BubR1 or Mad2 was directly depleted from the cell. This finding also confirmed earlier reports that showed direct inhibition of BubR1 and Mad2 by antibody injections caused cells to accelerate through mitosis [66]. This study concluded that BubR1 and Mad2 act independently of kinetochores to establish the time cells spend in mitosis, regardless of the status of chromosome alignment. The role of unattached kinetochores is to lengthen this time, perhaps by sensitizing the APC/C to prolonged inhibition by BubR1 and Mad2 (MCC) or by generating an additional pool of inhibitor (Mad2).

The MCC appears to be evolutionarily conserved as it has been identified in budding yeast [77], fission yeast [78] and in *Xenopus* [79]. Interestingly, formation of the yeast MCC was found also to be independent of the kinetochore [80]. By contrast, the formation of the MCC in *Xenopus* egg extracts depends on kinetochores [79]. The reason for this discrepancy is unclear but might be due to inherent differences in the mechanisms that control the somatic and embryonic cell cycles. It will be important to test whether these complexes are competent to inhibit the APC/C that is purified from mitotic cells.

Concluding remarks

The past few years have witnessed an explosion in the number of newly identified kinetochore proteins in yeast and other organisms (Figure 2). The increase in the complexity, in terms of size and function, of the vertebrate kinetochore likely arose in response to the expansion of the genome as well as the transition from a closed to an open mitosis. Interestingly, these changes have not required drastic alterations in kinetochore architecture but rather appear to have evolved by accessorizing the primordial kinetochore. The finding that many proteins are conserved suggests a common core that, in vertebrates, has been expanded to accommodate newly evolved proteins that are essential for accurate chromosome segregation. This expansion appears to have been achieved in part by simply replicating the core module to generate a string of modules that is organized into a cytologically visible structure. This concept is rooted in a model described by Brinkley and colleagues nearly 15 years ago when they presented evidence that mammalian kinetochores might be assembled from a repeat structure [7]. This idea might actually be extended to explain the curious observation that kinetochores in budding yeast appear to be clustered into a single focus rather than dispersed. If yeast kinetochores are indeed physically linked to one another, their interactions might form the foundations for how complex kinetochores evolved.

The list of kinetochore proteins that have been identified is impressive. The question that naturally arises has to be: how many more proteins are left to be discovered? The answer is obviously not known, but this question should stimulate efforts to reconstitute kinetochore assembly *in vitro*. While this is a daunting task, identifying proteins that physically interact with each other is an important first step. The ability to reconstitute and analyze subcomplexes *in vitro*, whose interactions are validated *in vivo,* would represent a major step forwards towards realization of this goal [8]. These efforts might also be of practical use as they might provide *in vitro* assays to screen for novel inhibitors of kinetochore assembly. Chemical inhibitors should provide new tools and expand our capabilities to investigate the dynamic

properties of kinetochores as well as being utilized as novel drugs that specifically inhibit mitosis.

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