Centromere proteins and chromosome inheritance: a complex affair

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Centromeres and the associated kinetochores are involved in essential aspects of chromosome transmission. Recent advances have included the identification and understanding of proteins that have a pivotal role in centromere structure, kinetochore formation, and the coordination of chromosome inheritance with the cell cycle in several organisms. A picture is beginning to emerge of the centromere–kinetechore as a complex and dynamic structure with conservation of function at the protein level across diverse species.

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Abbreviations

APC	anaphase-promoting complex
BUB	budding uninhibited by benzimidazole
CENP	centromere protein
CPC	checkpoint protein complex
CREST	calcinosis, Raynaud's phenomenon, esophageal dismotility, sclerodactyly, telangiectasia
INCENP	inner centromere protein
MAD	mitotic arrest-deficient
M→A	metaphase to anaphase
MT	microtubule

Introduction

Faithful chromosome transmission during mitosis and meiosis is essential for an organism's normal development and for the inheritance of genetic traits. Aberrant chromosome transmission results in aneuploidy, classically defined as deviations from the normal chromosome complement [1]. Aneuploidy has catastrophic consequences for public health. In humans, 45% of spontaneous abortions and 7% of all conceptions exhibit aneuploidy [2], and aneuploidy causes common birth defects (e.g. Down, Klinefelter and Turner syndromes). Aneuploidy is also tightly correlated with almost all types of cancer: there are >84,000 documented cases of abnormal karyotypes associated with human neoplastic disorders [3]. Despite the high frequency and importance of aneuploidy, we are only beginning to understand the causes of aberrant chromosome transmission.

The centromere is essential for the passage of chromosomes to daughter cells during cell division. It was defined over a century ago as a cytogenetic entity, the primary chromosome constriction that serves as the site of spindle attachment to the chromosome. More detailed analyses have demonstrated that the centromere contains centromeric DNA associated with an exquisite and dramatic proteinaceous structure, the kinetochore, which in turn interacts with the spindle microtubules (MTs). In most eukaryotes there must be one (and only one) centromere per chromosome; this stable structure is comprised of *cis*-acting DNA sequences and *trans*-acting proteins. Several recent reviews (e.g. [4,5]) discuss the surprising plasticity of the *cis*-acting elements required for centromere function in different species. There is enormous diversity in centromere DNA composition both between and within species; kinetochores can fail to form despite the presence of centromeric DNA, and non-centromeric DNA appears capable of forming active kinetochores. The paradoxical behaviors of centromeric DNA — stability and plasticity — led to the idea that centromere structure and function may be dictated by an epigenetic, self-propagating mechanism that requires imprinting of DNA or *trans*-acting protein constituents [6–8].

The *trans*-acting centromere proteins can be divided into two broad classes. First, there are architectural proteins that contribute to the structure of the centromere and centromeric chromatin; these proteins are responsible for kinetochore formation and may regulate other functions exhibited by the broader centromeric region (i.e. condensation and sister chromatid cohesion). Second, there are kinetochore proteins that function to promote MT capture, chromosome congression, resolution of sister chromatid cohesion, movement of sister chromatids to opposite poles, and coordinating chromosome segregation with the cell cycle.

Here, we review recent advances in our understanding of how centromere proteins facilitate and coordinate chromosome transmission through mitosis. Three general themes are presented. First, the diversity in primary DNA sequence is accompanied by poor homology in primary DNA-binding proteins across species but, as these structural elements segue into the functional components (i.e. motor and checkpoint proteins), a high degree of sequence and functional conservation becomes evident. Second, chromosome transmission and coordination with the cell cycle is a complicated and dynamic process: there are many transient protein–centromere interactions and a division of labor is utilized within protein networks to accomplish the broad range of functions undertaken by the centromere. Third, kinetochores are central for the assembly of checkpoint protein complexes (here abbreviated as CPCs) that interact with the anaphase-promoting complex (APC) to coordinate chromosome transmission with the cell cycle.

Centromere structural proteins

In *Saccharomyces cerevisiae*, unlike higher eukaryotes, the centromere contains specific primary DNA sequences that are conserved among all 17 chromosomes [6]. Many structural proteins were identified from mutational analyses and centromere DNA binding assays and the functions and interactions of *S. cerevisiae* centromere proteins are the best characterized at this time (Table 1). Recent elegant studies [9,10,11••] have revealed that the CBF3 complex (p110, p64, p58, and p23), Cse4p (a histone H3 variant similar to mammalian CENP-A, see below), Mif2p (similar to mammalian CENP-C, see below) and Cbf1p develop the architecture of the *S. cerevisiae* centromere by interacting with the DNA. Future analysis will focus on the three-dimensional structures produced by these DNA–protein interactions.

If, in higher eukaryotes, primary DNA sequence is neither necessary nor sufficient for centromere function (reviewed in [4,5,7,8,12]), then how is the spindle-attachment site defined? This question can be separated into two parts. First, how is 'centromere identity' determined; how is one region 'marked' as the site where kinetochore formation will stably occur through generations? Current hypotheses postulate that higher-order structure of the DNA, centromere-specific chromatin proteins (e.g. CENP-A, see below), protein modifications (e.g. histone acetylation [13••]), replication timing [14] or some combination of these factors may determine centromere identity. Second, how is the primary kinetochore layer built on top of the 'marked' DNA? Exactly which proteins build the kinetochore is unclear and because centromeric DNA differs across and even within species, the marking or structural proteins that bind this DNA may also be different.

Four candidates for structural proteins in mammals — CENP-A, CENP-B, CENP-C and CENP-G — were fortuitously identified using autoimmune antisera [15,16•]. All are present at the centromere throughout the cell cycle and could mark the site through divisions or they could promote kinetochore formation. What roles might these proteins play at the centromere? CENP-A, a histone H3 variant, may be an epigenetic mark for the centromere [17,18]. CENP-B binds centromere-associated DNA *in vitro* and promotes nucleosome positioning [19] but localization data and recent mutational analyses indicate that CENP-B is not absolutely required for centromere function [15,20,21]. Disruption of the CENP-C gene is a lethal event [22•] associated with gross mitotic abnormalities [22•,23,24] and CENP-C may function both in creating the primary kinetochore layer and in monitoring its proper formation [23–27]. CENP-G is the newest member of the constitutive centromere proteins and, although its role is undefined, one attractive possibility is that it plays a role similar to CENP-B. Is CENP-A the mark for the centromere? What proteins, if any, compensate for the loss of CENP-B? Is CENP-C a signaling molecule and how does it perform its functions? The mammalian centromere protein story is not complete but the tools to answer these questions now exist. For example, targeted mislocalization of CENP-A will address whether this protein can 'mark' the site for centromere formation. In addition, the further characterization of CENP-G and the identification of the structural centromere proteins in CENP-B knockout mice should help determine how the kinetochore is built.

Although several kinetochore proteins have been identified in *S. cerevisiae* and mammals, our knowledge of these proteins in *Schizosaccharomyces pombe* and *Drosophila melanogaster* is less satisfying. A number of *S. pombe* centromere-associated proteins have been identified in screens on the basis of minichromosome loss and gene silencing [28,29] but their exact roles in determining centromere identity or kinetochore formation are unknown (Table 1). To date there is only one candidate for a structural centromere protein in *D. melanogaster*. Can the *S. cerevisiae* and mammalian proteins be used to identify centromere proteins in *S. pombe*, *D. melanogaster* and other species? Homologs of some mammalian constitutive centromere proteins — CENP-A, CENP-B, CENP-C, see above — do exist in *S. cerevisiae* and *S. pombe*, but weak homology makes them difficult to identify via sequence alone. Thus, in many cases, proteins that are structurally non-homologous may be functionally analogous. Given the recent advances in our understanding of centromere structure and function in *S. pombe* and *D. melanogaster* [13••,30], both systems would benefit greatly from newly designed screens and biochemical approaches to identify centromere proteins.

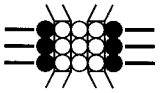
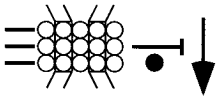
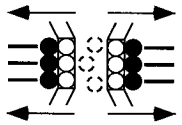
What other proteins might help define the centromere? Correlative data suggest that some proteins containing chromodomains may be involved in assembling the kinetochore [31]. They are associated with centric heterochromatin in a variety of organisms (*S. pombe*, Swi6p [32]; *D. melanogaster*, HP1 [33]; *Mus musculus*, M31 and M33 [34,35]) and mutational analyses link them to chromosome inheritance (*S. pombe*, Swi6p and Clr4p [29,32,36]; *D. melanogaster*, HP1 [37]). The possibility that chromodomain proteins build the primary kinetochore layer is particularly intriguing; they have been implicated in the cellular memory of epigenetic states [13••,31] and could perhaps mark the site for centromere formation through generations. Whether or not these proteins are involved in defining the centromere is unclear, and other factors must be involved in at least some species (e.g. no chromodomain proteins have been found at the *S. cerevisiae* centromere). Overall, it appears that a wide range of DNA-binding proteins create a centromere structure upon which a conserved layer of motor and signaling proteins is then built.

Microtubule capture and kinetochore congression

As the chromosomes condense at the onset of mitosis, the proteinaceous kinetochore nucleates at the site of the centromere. Kintechores initially interact with MTs laterally and rapidly move polewards, and remain in the proximity of a pole until bipolar attachments are made via end-on MT–kinetochore connections. Once bipolar spindles are established, kinetochores move platewards — congressing

Table 1

Proteins with a role in centromere function

Function	*S. cerevisiae*[a]	*S. pombe*[b]	*D. melanogaster*[c]	*X. laevis*[d]	*M. musculus*[e]	*H. sapiens*[f]	Reference
Structure 	Cse4p, Cbf1p p110(Ndc10p) p64(Cep3p) p58(Ctf13p) p23(Skp1p)* Mif2p	Swi6p	PROD	–	mCENP-A‡ mCENP-B‡ mCENP-C‡	hCENP-A hCENP-B hCENP-C	[a][6,9,10,11••] [b][101] [c][102] [e][20,21,22•,103] [f][15,16•,17]
Sister chromatid cohesion 	Scc1p/Mcd1p* SMCs*	Mis6p	Mei-S332 ORD	SMCs*			[a][39,98] [b][104] [c][105,106] [d][39] [e][107]
Microtubule capture and congression 	Cbf5p*(?)	Chp1p Swi6p	dZW10 dDynein dDynactin ROD	xKCM1 xCENP-E	#MCAK	hCENP-E CLIP-170 hDynein hDynactin	[a][108] [b][55,101] [c][46•,109] [d][53•,110] [e][107] [f][45,49,51,52•,54,72•]
M→A checkpoint 	scBUB1, scBUB2 scBUB3, scMAD1 scMAD2, scMAD3 Cdc20p, Cdc27p	spBUB1 spMAD1† spMAD2 Slp1p	dFZY(?) dFZR(?) dBUB1 dBUB3	xMAD1 xMAD2 xFZY(?)	mBUB1 Tsg24	hBUB1, hBUBR1§ hBUB3, hMAD1,2 p55CDC/ hCDC20 hZW10, ERK 3F3/2 epitope	[a][58,64,68,70,73••] [b][65•,76•,85•] [c][80,82,126] [d][81,85•] [e][66•,67•,93••] [f][63•,67•,72•,74,78••,84•,86•,88,109,111]
Resolution and segregation 	Pds1p Esp1p Ase1p(?) Clb2p	Cut1p(?) Cut2p(?)	Pimples(?) Three rows(?) dZW10, dDynein, dDynactin, ROD	–	#MCAK	hCENP-E	[a][98,112,113] [b][99] [c][46•,114,115] [e][107] [f][72•]
Unknown	NSP1p/ Sth1p(?) Spt4p(?)	Rik1p, Clr4p Abp1p/Cbp1p Cbh1p	HP1(?) POLO MODULO(?)	–	M31 M33 Ikaros Helios	CENP-D CENP-F CENP-G PcG(?)	[a][116,117] [b][36,101,118,119] [c][37,120,121] [e][34,35,122,123] [f][15,16•,124,125]

We have compiled proteins with centromere function in *S. cerevisiae*, *S. pombe*, *D. melanogaster*, *X. laevis*, *M. musculus* and *H. sapiens*. The black circles in each function panel highlight the class of proteins represented in the rows. The 'Unknown' row contains proteins that may have a centromere function but the precise role is ambiguous. Proteins marked '(?)' have not been precisely localized to the kinetochore. We have generally cited recent references that are discussed in the text; original references can be found within the papers we cite. Proteins with older citations or unknown functions are generally not discussed in the text. The resolution of cytology in *S. cerevisiae* and *S. pombe* is generally insufficient to localize the checkpoint proteins to centromeres but genetic studies demonstrate that they have centromere function (see text). *The function of these proteins is not limited to the centromere. Other proteins listed in the table may have functions that are not limited to the centromere although the precise non-centromeric role is unclear. For example, some checkpoint proteins also localize to the spindle poles in mammalian cells [78••,86•]. †The spMAD1 protein has not been characterized but its presence is implied by the gene, GenBank/EMBL/DDBJ accession no. Z95620 [85•]. ‡The *M. musculus* CENP proteins have yet to be characterized but their presence is inferred from sequence homology. §hBUBR1 and hMAD3 may be the same protein [67•]. #MCAK in CHO cells. Although the table presents what is currently known, it also illustrates gaps in our knowledge about each function in each organism. Abbreviations: BUB, budding uninhibited by benzimidazole; cdc, cell division cycle; CENP, centromere protein; CLIP, cytoplasmic linker protein; ERK, extracellular signal-regulated kinase (also known as MAP kinase); HP1, heterochromatin protein 1; KCM, kinesin central motor; MAD, mitotic arrest deficient; ORD, orientaion disrupter; PcG, Polycomb group; PROD, proliferation disrupter; ROD, rough deal; SMC, structural maintenance of chromosome.

the entire genome at the spindle equator — and await anaphase [38••]. Concomitant with this arrangement is the requirement for polar ejection forces (i.e. 'polar wind') and the tight apposition of sister chromatids via sister chromatid cohesion (see recent reviews [39–41] and Biggins and Murray, this issue [pp 230–236]).

Prometaphase and anaphase chromosome movements are mediated by interactions with MTs the minus-ends of which lie at the poles. MTs interact with bulk chromatin through DNA-binding proteins (chromokinesins) that are thought to be responsible for generating the polar wind, which acts largely on chromosome arms. In addition, a subset of MTs interact with the kinetochore complex. After a kinetochore coalesces during prometaphase, it must capture and bundle kinetochore MTs, direct poleward and plateward movement (congression), and ultimately generate the poleward forces that partition chromosomes to daughter nuclei. These activities seem quite independent, yet all can be explained by the actions of MT-binding motor proteins, factors known to associate with these motors, and MT assembly and disassembly. Recent work has suggested that the first steps of kinetochore–MT association are dependent on non-movement properties of molecular motors.

Motors and associated factors are found at the maturing kinetochore, in regions that are seen to interact with growing spindle MTs. Dynein, a plus-end-directed molecular motor, is found at the outermost layer of the kinetochore, the fibrous corona [42,43]. The localization of dynein overlaps with that of dynactin, a multisubunit complex thought to mediate the dynein–cargo interaction [44,45]. The presence of these factors at the site of MT contact is dependent on ZW10 and ROD [46•], which have been localized to the outer plate. These two proteins interact genetically and physically (R Karess, M Goldberg, personal communication), and may serve as a scaffold upon which dynein and dynactin rest [46•]. However, the dynamic MT 'streaming' of ZW10 throughout spindle assembly [47] allows for functions of ZW10 beyond that of kinetochore motor localization. The observation that *ZW10* mutants have no chromosome phenotype until anaphase [48] suggests that the activities of dynein and dynactin at the early kinetochore are redundant with other factors. At least two other plus-end-directed molecular motors, MCAK (XKCM1 in *Xenopus laevis*) and CENP-E [49–51], are present at the kinetochore. The role of CENP-E, recently reviewed by Rieder and Salmon [38••], is a motor protein required for chromosome congression and segregation. Cells immunodepleted for CENP-E fail to assemble bipolar metaphase-aligned chromosomes, suggesting that CENP-E activity plays a role at prometaphase [52•,53•]. It is expected that these motor activities — CENP-E, MCAK/XKCM1, dynein — are redundant or overlapping and that any one is dispensable without losing all the chromosome movements during normal mitosis [52•,53•].

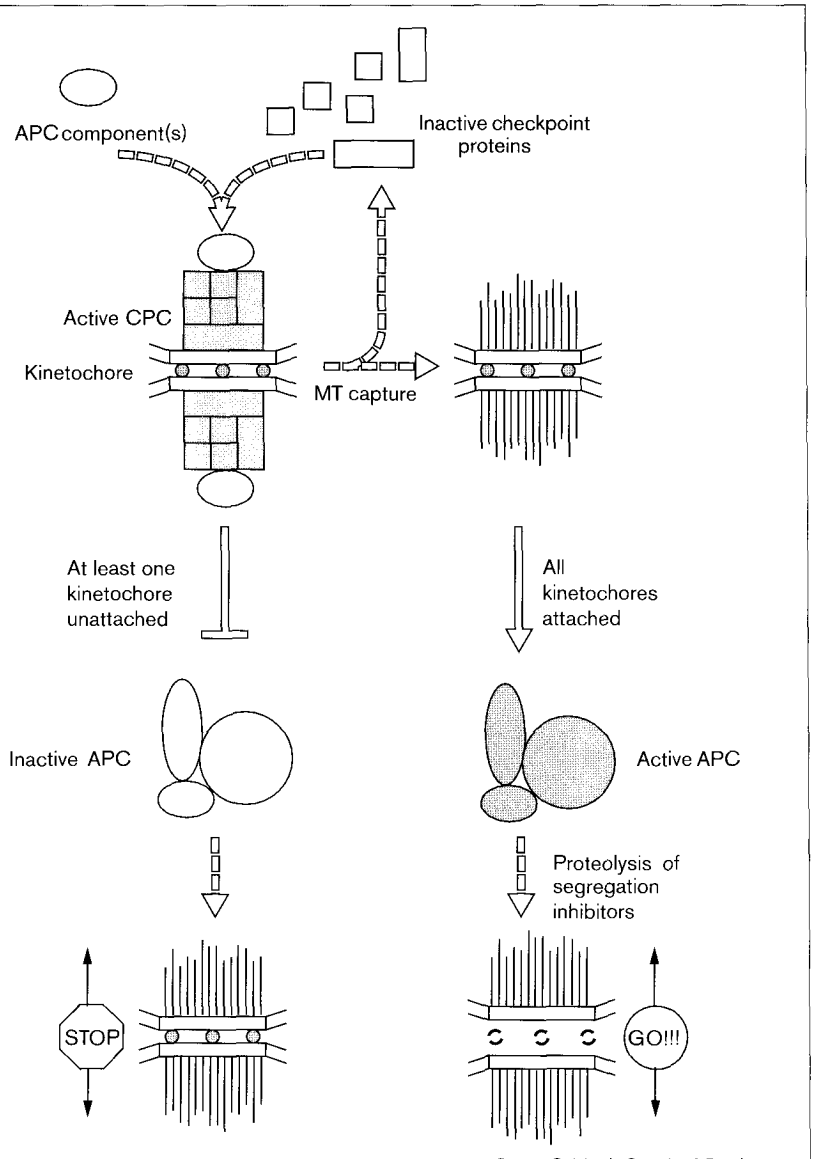
MT binding to motors in the prometaphase kinetochore is facilitated by the presence of MT-associated fidelity factors. CLIP-170, a vertebrate factor, is located at the kinetochore until metaphase. Originally characterized as an MT-endocytic vesicle interaction factor, its overexpression leads to a delay in anaphase onset [54]. CLIP-170 is proposed to act as a fidelity factor, increasing the binding of MTs to the motors in the corona and outer plate of the kinetochore. A similar phenomenon may be occurring in *S. pombe*. Chp1p, an *S. pombe* chromodomain-containing protein, interacts genetically and pharmacologically with tubulin [55] and *chp1* mutants display a delay in anaphase onset. *chp1* mutations and CLIP-170 overexpression may exert their effects at the prometaphase kinetochore by interfering with the fidelity of MT capture but it is as yet unclear if the defects are caused by either direct or indirect interactions.

MT-based motors in combination with motor-cargo fidelity factors appear to be responsible for initial MT binding and kinetochore-mediated congression. Although the initial binding involves factors like dynactin, CLIP-170, and Chp1p, epitopes for these proteins are lost in fixed metaphase figures, suggesting that these may be dispensable once bipolar end-on binding of MTs to the kinetochores is established [46•,54]. The prometaphase role for motor proteins during MT capture has been interpreted as stabilization of the MT end, rather than catalytic movement [38••]. This hypothesis has received support from the recent observation that the ratio of motor proteins (Kar3p:Kip2p in *S. cerevisiae*) at the spindle poles affects the number of polymerized MTs [56], suggesting that motors not only move along MTs but also affect MT dynamics.

Recent results and previous analyses lead to a working model for the role of motors and other factors in MT capture and congression. A growing spindle MT encounters a kinetochore fibrous corona, rich in MT-binding motors (e.g. dynein and CENP-E). The motors bind polymerized GDP–tubulin and move the kinetochore in a retrograde direction until they approach the pole, where they are perhaps kept at bay by polar wind. The monopolar kinetochore stalls until it binds polymerized GTP–tubulin [57] from the other pole, which acts as a stabilizing end-binding protein. The frequency and strength of interactions between the motors and the polar MTs are affected by the presence of fidelity and crosslinking factors (e.g. CLIP-170, Chp1p, and dynactin). Once the initial interaction with the MT ends are made, more stable interactions between the ends and outer-plate motor proteins (e.g. dynein, MCAK, and CENP-E) are made. The factors involved in initial binding may be either lost or dispensable, while remaining kinetochore motors and the tension created by the polar wind carry the chromosomes to proper metaphase alignment. Whether congression occurs either as a result of motor-based movement or a product of MT growth and decay is a contested issue. Once congressed at the plate, the kinetochores signal the cell-cycle machinery via resident attachment and/or tension-sensing mechanisms and await the signal for anaphase progression (see below).

This model suggests that specific structures are present within the MT-free and MT-bound kinetochores. Complexes could vary in membership between prometaphase and metaphase, and between MT-free and MT-bound kinetochores. As many of these proteins may be redundant or supported by similar activities (e.g. the

Figure 1



Kinetochore proteins and the M→A checkpoint. A model is presented that can account for the transition from the 'wait anaphase' (STOP) to the 'go anaphase' (GO!!!) response after the last kinetochore has attached to MTs. Open shapes represent inactive states and shaded shapes represent active states. Unattached kinetochores act as centers for the assembly of CPCs from prometaphase through to metaphase. CPCs comprise targeting proteins like MAD1 or BUB3 (larger rectangle) that are required for the localization of other MAD and BUB proteins (square boxes) and effector proteins like Cdc20p/Slp1p/p55CDC (smaller rectangle) to unattached kinetochores. Further, components of the APC (Tsg24 and Cdc27p) have been localized to kinetochores (elipse). CPCs are formed while there are unattached kinetochores, resulting in an inactive APC and a halt in the M→A transition. A single unattached kinetochore is capable of completely inhibiting the APC. CPCs would no longer be created once all the kinetchores become attached to MTs. This may be due to a kinase-dependent signal from MT attachment/tension-sensing protein complexes. This results in an active APC that is free to induce progression into anaphase. It is likely that this is an over simplistic view of M→A control and that other feedback loops are involved. Nonetheless, with the enormous progress already made, the foundations are now in place to build a deeper understanding of these processes.

overlap of CENP-E and dynein and MCAK), experiments investigating necessity and sufficiency are difficult to interpret. Ongoing immuno-electron-microscopy studies and immunodepletion or mutation analyses will contribute to our understanding of which factors are present and necessary for faithful MT–kinetochore interaction.

Kinetochores and the metaphase→anaphase checkpoint

MT–kinetochore associations are stochastic. Cells possess a metaphase to anaphase (M→A) or spindle checkpoint that monitors the attachment of MTs to kinetochores and alignment of chromosomes at the metaphase plate. The checkpoint guards against aneuploidy by delaying progress into anaphase (chromosome segregation) until all the kinetochores are attached to MTs and proper chromosome congression has occurred. The signal that everything is ready for M→A progression involves communication between checkpoint protein complexes (CPCs) and the anaphase-promoting complex (APC). The checkpoint can be dramatically demonstrated in yeast by treating dividing cells with drugs that cause depolymerization of MTs and M→A arrest. Hoyt *et al.* [58] and Li and Murray [59] utilized this property to screen for genes in *S. cerevisiae* that prevent arrest. These screens were groundbreaking because they uncovered the *scBUB1*, *2*, and *3* (budding uninhibited by benzimidazole) and *scMAD1*, *2*, and *3* (mitotic-arrest-deficient) genes. Remarkable live-time, micromanipulation and laser-ablation studies later demonstrated that checkpoint function resides at kinetochores and that a single unattached kinetochore can delay progression into anaphase [60–62].

The BUB and MAD checkpoint proteins appear functionally conserved

1998 was a very exciting year for studies of CPCs, the APC and the M→A checkpoint. Homologs of *scBUB*s and *scMAD*s have recently been identified in diverged species (Table 1). A combination of homology searches, powerful yeast genetics and mammalian cytology are beginning to uncover functional relationships. *hBUB1* mutations have recently been shown to be associated with aneuploidy in two colorectal cancer cell lines, indicating that faithful checkpoint protein function may be required for genome stability in humans [63•]. Several BUB and MAD proteins from diverse species have been shown to be required for M→A cell cycle arrest when spindles or kinetochores are disrupted [63•,64,65•–67•,68–70] and there is interdependence between the BUB and MAD family of proteins [64,71]. For example, phosphorylation of MAD1 depends on BUB1, BUB3 and MAD2, but not BUB2 and MAD3. There is evidence that interdependence is caused by at least one linear relationship and/or interactions within complexes (see below). BUB1 and BUB3 act upstream of MAD1 and MAD2, and MAD3 and BUB2 act downstream of the above.

Checkpoint proteins can form complexes

BUB1 has three conserved domains: an amino-terminal MT-binding domain, a carboxy-terminal serine/threonine kinase domain and a BUB3-binding domain [63•,67•]. hBUB3 was recently cloned and shown to interact with mBUB1 and be required for localization of hBUBR1 to unattached kinetochores [67•]. hBUBR1 and hMAD3 have been proposed to be the same protein [67•]. Chan *et al.* [72•] recently demonstrated that hCENP-E interacts with hBUBR1 and localizes to kinetochores in mammalian cells. These studies hint that motor/kinase protein complexes are resident at the MT–kinetochore interface and that these complexes may sense and signal attachment/tension, possibly via a kinase phosphorylation cascade.

Recent studies demonstrate conclusively that MAD proteins form CPCs that include at least one regulator of the APC (Figure 1). scMAD1, 2 and 3 form a ternary complex that binds the cell-cycle regulator Cdc20p [73••] which is required to activate APC-mediated proteolysis [74,75]. The scMAD protein complex may prevent the M→A transition by inhibiting Cdc20p function until all the kinetochores are attached to MTs. Similar interactions have been described in *S. pombe* [76•] and mammalian cells [77•,78••] and a further link between CPCs and cell-cycle regulation was established with the description of checkpoint protein/APC interactions [74,77•,79•]. *Fizzy* (*dFZY*) and *fizzy-related* (*dFZR*) in *D. melanogaster* and *xFZY* in *X. laevis* are members of the same family as Cdc20p, Slp1p and p55CDC, and are involved in cell-cycle regulation [80–82]. Regulators of dFZY, dFZR and xFZY have yet to be identified and it is unclear whether they function at kinetochores (Table 1). Numerous WD (tryptophan/aspartic acid) repeats may facilitate CPC formation [67•,75,81–83].

Further studies are required to understand exactly how checkpoint proteins regulate the APC. Hyperphosphorylation of MAD1 is important, although not essential, for checkpoint function, and phosphorylation has been implicated in the regulation of the APC [74]. The precise role(s) of phosphorylation in checkpoint signaling needs to be determined. Nonetheless, the studies described above provide evidence for a signal transduction mechanism that links CPCs and the APC.

Checkpoint proteins associate with unattached kinetochores and disassociate from attached kinetochores

xMAD1 and xMAD2 interact in *X. laevis* egg extracts and localize to unattached kinetochores [85•]. xMAD2 localization is dependent upon xMAD1 but not vice versa, implying that xMAD1 targets xMAD2 to unattached kinetochores. mBUB1, hBUB3, xMAD1, xMAD2, hMAD2 and p55CDC associate with unattached kinetochores at prophase and prometaphase and with kinetochores that are late to congress at metaphase [66•,67•,77•,78••,85•,86•]. Generally, the signal diminishes at kinetochores when all the chromosomes have congressed at metaphase (Figure 1). MAD2 localizes to all the kinetochores in cells with depolymerized MTs and at the unattached kinetochore on mono-orientated chromosomes. A monoclonal antibody (3F3/2) that recognizes phosphoepitopes also localizes to unattached kinetochores and exhibits similar differential localization as MAD2 on mono-orientated chromosomes [60,84•,87]. Phosphorylation of the 3F3/2 epitope requires ERK (extracellular signal-regulated kinase) which exhibits similar localization with 3F3/2 in PtK1 cells [88].

In vitro and *in vivo* micromanipulation and 3F3/2 phosphoepitope-tag experiments demonstrate that the checkpoint is sensitive to differential tension between kinetochore pairs and that phosphorylation of kinetochore proteins is associated with unattached kinetochores [84•,89]. Cells do not proceed into anaphase until tension is generated across all the centromeres, suggesting that localization of kinetochore-checkpoint proteins may be tension sensitive; however, cells may use more than one mechanism to monitor chromosome congression [61]. Recently, Waters *et al.* [90•], showed that although MAD2 normally localizes to unattached kinetochores it does not localize to attached kinetochores that have tension reduced by taxol treatment. Therefore, the association of at least one checkpoint protein appears to be insensitive to tension. It is likely that there will be as yet unidentified kinases involved in these signaling processes. Recently, Biggins *et al.* [91] identified the Ipl1p kinase which phosphorylates Ndc10p, a structural protein in *S. cerevisiae*, and appears to be involved in regulating MT attachment. Further studies are required to determine the precise signaling processes involved between MT attachment/tension and the checkpoint. However, it is plausible to suggest that some checkpoint proteins monitor tension/attachment (e.g. BUB1 and BUB3) and other checkpoint proteins (e.g. MADs) transduce the 'unattached

kinetochore' signal to the APC and cell cycle apparatus. Perhaps localization of BUBR1/MAD3 to unattached kinetochores via interactions with BUB3 provides a link between the attachment/tension-sensing complexes and the signal transduction complexes. Conservation of function, as demonstrated by Nicklas *et al.* [84•] for one kinetochore kinase, may help identify proteins involved in these signaling processes.

Some CPC/APC interactions occur at kinetochores

Several recent studies describe CPC formation and how CPC/APC interactions might occur [71,77•,78••,85•,86•,92]. The most favored model is one in which CPCs are assembled at unattached kinetochores and, once assembled, CPCs inhibit the APC, remote from kinetochores. However, Tsg24, a component of the murine APC, was recently localized to kinetochores in mammalian cells [93••]. Tsg24 is related to components of the APC in other organisms including *S. cerevisiae* [94], *S. pombe* [95], *A. nidulans* [96] and *X. laevis* [96]. This raises the possibility that Tsg24 homologs and perhaps other APC components are localized to kinetochores (Figure 1). Indeed, microinjection of antibodies for Cdc27p, a component of the *S. cerevisiae* APC, localize to kinetochores in PtK1 cells and induce metaphase arrest (G Gorbsky, personal communication).

Exactly how a single unattached kinetochore can delay anaphase onset remains unclear. It is unlikely that simple sequestration of APC components to unattached kinetochores is the sole mechanism for M→A inhibition because a single unattached kinetochore would have to recruit all of the APC components in the cell. Further, live studies of fused PtK1 cells with four sets of MTs (two spindle pairs) in a common cytoplasm demonstrate that the inhibitory signal does not diffuse between attached and unattached spindle pairs [97]. Localization and immunoprecipitation studies with other CPC and APC components are required to address what CPC/APC interactions occur and where. These questions will surely be an exciting focus in the near future.

Once the criteria for progression from metaphase to anaphase are fulfilled, the activated APC targets downstream proteins involved in maintaining sister chromatid cohesion (Table 1). Pds1p in *S. cerevisiae* and Cut2p in *S. pombe* are targets for APC-mediated ubiquitination and proteolysis [98,99]. Destruction of these proteins leads to rapid resolution of sister chromatid cohesion and separation of chromatids to opposite poles driven by kinetochore/MT-bound motors (reviewed in [38••]). Finally, centromeres may act as a 'processing site' for other cell-cycle functions, such as the delivery of INCENPs to the cleavage midzone at anaphase [100]. And so, even towards the end of mitosis, there is still plenty of action at the kinetochore.

Conclusions

Centromere/kinetochore assembly, chromosome movement and coordination with the cell cycle are complex and dynamic processes. Research published in the past year demonstrates that there are many transient protein–centromere interactions and that protein networks accomplish different centromere functions. Defining centromere identity and architecture can be thought of as a two-step process. First, the site of kinetochore formation through divisions must be 'marked' and second, the primary kinetochore layer must be built on top of that mark. Putative marking and structural proteins do exist in some organisms and tools now exist to test various models. The further identification and characterization of centromere-defining proteins will advance our understanding of this basic and ancient biological activity. The proteins involved in the outer kinetochore layer, MT capture and chromosome congression are highly conserved across diverse species, in comparison to centromere structural proteins. We now have identified many of the motor proteins, and proteins that serve as a 'scaffold' that links the motors to the kinetochore. Finally, a number of checkpoint proteins have been identified across diverse species and their functions are evolutionary conserved. Kinetochores appear to be central for the assembly of CPCs. Many checkpoint proteins have been shown to associate with unattached kinetochores and they disassociate once the kinetochores become attached to MTs. A plausible route for signaling MT–kinetochore attachment to the cell cycle has been identified with the association of checkpoint proteins and proteins involved in APC regulation. Still, we are only beginning to scratch the surface. Novel genetic screens targeted at identifying transmission-defective mutants and utilization of expanding genome databases should help fill in the gaps, and provide a clearer, more complete picture of centromere–kinetochore protein structure and regulation.

Note added in proof

The identification and characterization of dBUB1 and dBUB3 have recently been described by Basu *et al.* [126]. The localization of dBUB3 is affected when the gene encoding dBUB1 is disrupted. This dependency is similar to that described in human cells (see main text and [67•]) which further confirms the conservation of function observations.

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A yeast two-hybrid screen demonstrates an interaction between the checkpoint proteins MAD1, MAD2, MAD3 and the cell-cycle regulator Cdc20p. MAD2 and MAD3 interact directly with Cdc20p whereas the MAD1 interaction with Cdc20p is indirect. A series of mutant strain combinations demonstrated that MAD1 and MAD2 interact directly and they bind MAD3 via MAD2. Although MAD2 and MAD3 interact directly with Cdc20p, this interaction is MAD1 dependent. The significance of this work is that it demonstrates the presence of a CPC and provides a link between checkpoint proteins and the cell cycle arrest apparatus.

74. Fang G, Yu H, Kirschner MW: **Direct binding of CDC20 protein family members activates the anaphase- promoting complex in mitosis and G1.** *Mol Cell* 1998, **2**:163-171.

75. Visintin R, Prinz S, Amon A: **CDC20 and CDH1: a family of substrate-specific activators of APC- dependent proteolysis.** *Science* 1997, **278**:460-463.

76. Kim SH, Lin DP, Matsumoto S, Kitazono A, Matsumoto T: **Fission**
• **yeast Slp1: an effector of the Mad2-dependent spindle checkpoint.** *Science* 1998, **279**:1045-1047.

A yeast two-hybrid system demonstrates that spMAD2 interacts with Slp1p. A 29 amino acid domain in Slp1p is important for this interaction; mutations within this region abolish the MAD2–Slp1p interaction and prevent the arrest that would normally be induced by overexpression of spMAD2. This work establishes a link between a checkpoint protein and the cell cycle arrest apparatus in *S. pombe*.

77. Fang G, Yu H, Kirschner MW: **The checkpoint protein MAD2 and**
• **the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation.** *Genes Dev* 1998, **12**:1871-1883.

Recombinant hMAD2 exists as a monomer and a tetramer. Only tetramers were capable of inducing cell-cycle arrest after injection into *Xenopus* embryos. *In vitro*, the monomeric form of hMAD2 associates with hCDC20 but does not appear to associate with the APC and has no effect on APC activity. The tetrameric form of hMAD2 associates with hCDC20 and the APC to form a ternary complex *in vitro* and, when formed, the APC is inactive. This study suggests that it is not simply the association of hMAD2 with hCDC20 that inhibits the APC. They present the novel idea that structural changes with hMAD2 may also be involved in the signal transduction process that is required for checkpoint activation.

78. Kallio M, Weinstein J, Daum JR, Burke DJ, Gorbsky GJ: **Mammalian**
•• **p55CDC mediates association of the spindle checkpoint protein Mad2 with the cyclosome/anaphase-promoting complex, and is involved in regulating anaphase onset and late mitotic events.** *J Cell Biol* 1998, **141**:1393-1406.

Anti-p55CDC antibodies exhibit diffuse staining at interphase. Punctate staining was first observed at kinetochores at late prophase which colocalized with CREST antibodies. The kinetochore labeling was less intense at anaphase and disappeared at telophase. p55CDC–GFP fusions exhibited more intense labeling at kinetochores that had not aligned at the metaphase plate. Sequential anti-p55CDC immunoprecipitations from mitotic HeLa cells contained MAD2 and the Cdc27p component of the APC. The MAD2–Cdc27p association was p55CDC dependent. p55CDC and MAD2 appear to interact directly. Injection of p55CDC antibodies into PtK1 and HeLa cells caused several unusual mitotic events which suggest that p55CDC has multiple roles or is involved in a process that branches into various functions within mitotic mammalian cells. As well as a nice localization study, this paper demonstrates a connection between a CPC and the APC.

79. Wassmann K, Benezra R: **Mad2 transiently associates with an**
• **APC/p55Cdc complex during mitosis.** *Proc Natl Acad Sci USA* 1998, **95**:11193-11198.

Coimmunoprecipitations demonstrate that MAD2 associates with hyperphosphorylated Cdc27p in HeLa cells either in the presence or absence of nocodazole. This association is cell-cycle dependent: they associate at the start of mitosis and disassociate at the end of mitosis. Further coimmunoprecipitation experiments in nocodazole-arrested cells demonstrated that p55CDC associates with MAD2, and that Cdc27p associates with p55CDC. Immunodepletion with anti-MAD2 caused a significant reduction in the association of Cdc27p and p55CDC, thereby confirming the presence of a ternary complex. Some Cdc27p–p55CDC complexes exist without MAD2 associations.

80. Dawson IA, Roth S, Artavanis-Tsakonas S: **The *Drosophila* cell cycle gene *fizzy* is required for normal degradation of cyclins A and B during mitosis and has homology to the *CDC20* gene of *Saccharomyces cerevisiae*.** *J Cell Biol* 1995, **129**:725-737.

81. Lorca T, Castro A, Martinez AM, Vigneron S, Morin N, Sigrist S, Lehner C, Doree M, Labbe JC: **Fizzy is required for activation of the APC/cyclosome in *Xenopus* egg extracts.** *EMBO J* 1998, **17**:3565-3575.

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83. Matsumoto T: **A fission yeast homolog of CDC20/p55CDC/Fizzy is required for recovery from DNA damage and genetically interacts with p34cdc2.** *Mol Cell Biol* 1997, **17**:742-750.

84. Nicklas RB, Campbell MS, Ward SC, Gorbsky GJ: **Tension-sensitive**
• **kinetochore phosphorylation *in vitro*.** *J Cell Sci* 1998, **111**:3189-3196.

This paper extends previous *in vivo* studies by demonstrating that kinetochore phosphorylation is dependent upon tension across insect spermatocyte kinetochores *in vitro*. Relaxed kinetochores exhibit more intense staining with 3F3/2 than kinetochores with micromanipulated tension forces. A kinetochore kinase from HeLa cells is capable of phosphorylating insect kinetochores, demonstrating conservation of sensing mechanisms. This system will provide another method for investigating tension sensing/checkpoint signalling mechanisms.

85. Chen RH, Shevchenko A, Mann M, Murray AW: **Spindle checkpoint**
• **protein Xmad1 recruits Xmad2 to unattached kinetochores.** *J Cell Biol* 1998, **143**:283-295.

When xMAD2 is immunodepleted from extracts, the M→A checkpoint is inactivated. The addition of recombinant xMAD2 to normal levels did not restore the checkpoint, indicating that immunodepletion removed some other factor(s) involved in checkpoint activation. Anti-xMAD2 immunoprecipitations have an 85 kDa protein (p85) strongly associated with xMAD2. Mass spectrometry generated eight peptides that were used to isolate *Xenopus* cDNA clones and identify an open reading frame. Sequence alignments and protein structure predictions with scMAD1, hMAD1 and spMAD1 illustrated that the p85 protein complexed with xMAD2 is xMAD1. *Xenopus* extracts fail to achieve mitotic arrest after injection of anti-MAD1 antibodies indicating that xMAD1 is also involved in the checkpoint. Localization studies demonstrate that both xMAD1 and xMAD2 have dynamic localization patterns and localize to kinetochores that have not congressed to the metaphase plate.

86. Gorbsky GJ, Chen RH, Murray AW: **Microinjection of antibody to**
• **Mad2 protein into mammalian cells in mitosis induces premature anaphase.** *J Cell Biol* 1998, **141**:1193-1205.
Immunofluorescence using anti-MAD2 antibodies demonstrate dynamic localization of MAD2 during the cell cycle. At interphase MAD2 localization is nuclear and cytoplasmic, at prophase the nuclear staining is less diffuse, and at early prometaphase MAD2 is at spindle poles, kinetochores and exhibits granular staining in the cytoplasm. At late prometaphase, MAD2 localization is weak on aligned kinetochores but still prominent on unaligned kinetochores. MAD2 is absent from all kinetochores when all the chromosomes become aligned at metaphase. Microinjection of anti-MAD2 antibody into Ptk1 cells caused early resolution of sister chromatid cohesion and the cells entered into anaphase prematurely. An identical result was observed in human foreskin keratinocytes demonstrating that the arrest is not specific to inbred lines. Therefore MAD2 appears to be necessary for the cell-cycle checkpoint in mammalian cells even in the absence of MT-destabilizing drugs.

87. Campbell MS, Gorbsky GJ: **Microinjection of mitotic cells with the 3F3/2 anti-phosphoepitope antibody delays the onset of anaphase.** *J Cell Biol* 1995, **129**:1195-1204.

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89. Li X, Nicklas RB: **Tension-sensitive kinetochore phosphorylation and the chromosome distribution checkpoint in praying mantid spermatocytes.** *J Cell Sci* 1997, **110**:537-545.

90. Waters JC, Chen RH, Murray AW, Salmon ED: **Localization of Mad2**
• **to kinetochores depends on microtubule attachment, not tension.** *J Cell Biol* 1998, **141**:1181-1191.
MAD2 is localized to unattached kinetochores up until late metaphase when it is lost from attached kinetochores. The authors used the localization of MAD2 to address whether a facet of the M→A checkpoint is to monitor attachment of MTs to the kinetochore or to detect tension across the kinetochore. 'Double-dot' labeling of Ptk1 kinetochores using CREST serum demonstrated that cells treated with taxol (a MT-stabilizing drug) had less space between kinetochores than cells that were not treated with taxol, demonstrating that there is tension across attached kinetochores. Taxol-treated cells with kinetochores attached to MTs did not bind MAD2 even though there was reduced tension. This demonstrates that MAD2 localization in these cells is determined by MT attachment to kinetochores and not tension at the kinetochore. Kinetochores with the fewest MTs had the most MAD2.

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•• **anaphase-promoting complex is a centromere-associated protein in mammalian cells.** *Mol Cell Biol* 1998, **18**:468-476.
Immunolocalization studies demonstrate that anti-Tsg24 antibodies localization is cell-type dependent. In CHO cells, Tsg24 is absent from kinetochores at interphase and anaphase and colocalize with CREST sera from prophase to early anaphase. Western-blot studies demonstrated that the transient localization of Tsg24 in CHO cells was not caused by transient expression. Contrary to CHO cells, Tsg24 is present at kinetochores throughout mitosis, including late anaphase in two murine cell lines, Swiss-3T3 and L cells. They propose that the transient vs. constitutive localizations could be caused by masking of epitopes in CHO cells. This is a very important paper because it opens the possibility that the APC or components of the APC associate with checkpoint protein complexes at kinetochores.

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