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Kinetochore structure and spindle assembly checkpoint signaling in the budding yeast, *Saccharomyces Cerevisiae*

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1. ABSTRACT

The Spindle Assembly Checkpoint (SAC) delays the onset of anaphase until every chromosome is properly bioriented at the spindle equator. Mutations in SAC genes have been found in tumors and compromised SAC function can increase the incidence of some carcinomas in mice, providing further links between cancer etiology, chromosome segregation defects and aneuploidy. Here we review recent developments in our understanding of SAC control with particular emphasis on the role of the kinetochore, the nature of the tension sensing mechanism and the possibility that the SAC encompasses more than just stabilization of securin and/or cyclin-B via inhibition of the APC/C to delay anaphase initiation. Our primary emphasis is on the SAC in the budding yeast *Saccharomyces cerevisiae*. However, relevant findings in other cells are also discussed to highlight the generally conserved nature of SAC signaling mechanisms.

2. INTRODUCTION: OVERVIEW OF THE SPINDLE ASSEMBLY CHECKPOINT

In eukaryotic prophase, paired centrosomes separate allowing microtubule (MT) asters to interact to form an amphiaster, first described in the late 1800s. Nuclear envelope break down then enables dynamic kinetochore microtubules (KMTs) to capture the newly condensed chromosomes. Biorientation and congression of every chromosome forms the equatorial (metaphase) plate, a state of mechanical equilibrium that triggers the onset of anaphase, defined cytologically as sister chromatid separation and chromosome segregation. In *S. cerevisiae*, the nuclear envelope remains intact throughout the cell cycle. Moreover, spindle assembly is largely uncoupled from mitosis because it is initiated simultaneously with bud emergence and DNA replication at the G1/S-phase transition (1). Identical to higher eukaryotes, however, the yeast Spindle Assembly Checkpoint (SAC) controls

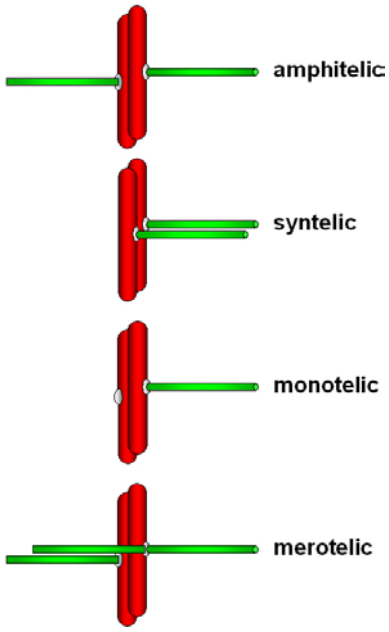

Figure 1. Chromosome Orientation on the Mitotic Spindle. Microtubule (MT) attachment to the kinetochores of sister chromatids must occur in an amphitelic arrangement (top) to achieve biorientation that generates the appropriate tension between the kinetochores to silence the spindle checkpoint. Other orientations (as diagramed) are possible, however, raising the need for error correction mechanisms. In budding yeast, merotelic attachment is unlikely to occur because each kinetochore has a single MT binding site.

anaphase initiation in response to chromosome biorientation. Conservation of this checkpoint mechanism from yeast to humans highlights its importance for precise genome transmission.

Flawless chromosome segregation, whether in yeast or higher eukaryotes, relies on the precise function of the mitotic apparatus, serving to capture and segregate the chromosomes. But, chromosome capture is inherently imprecise (Figure 1). Physical attributes of the chromosomes and of the mitotic spindle do favor a bioriented arrangement at the metaphase plate, for example because kinetochores (Ks) of mitotic chromosomes face away from each other (unlike the position of sister Ks during the first meiotic division) and MT plus ends are stabilized upon capture by Ks. However, the lateral surfaces of MTs can also interact with Ks, which makes possible the connection of a K to both spindle poles simultaneously (merotelic attachment) and the connection of both sister Ks to one pole (syntelic attachment). Problems of aberrant attachment are compounded in higher eukaryotes because each K possesses many MT binding sites. Prometaphase of mitosis is therefore rather variable in length, as all, or most, of these errors in attachment need to be fixed before anaphase. In *S. cerevisiae*, each K has a single binding site for the plus end of a MT, meaning that merotelic attachments are quite unlikely to occur (2-5); but, even in this simplified system, syntelic attachments must be corrected before anaphase and mechanisms must be in place that determine when the error correction phase has been completed.

Orderly progression of the cell cycle is controlled by checkpoints; signaling pathways that establish the dependence of one process upon completion of another (6). In the case of the SAC, sister chromatid separation does not have a physical dependence on chromosome biorientation and so biochemical pathways (collectively, the SAC) have evolved to enforce the dependency relationship. As a cellular control, the SAC was probably first observed in video time-lapse movies of plant cell mitosis by Andrew Bajer, who noticed that anaphase seems to "wait" until the last chromosome reaches the plate (7). Four decades later, after the discovery of microtubules and their chromosomal attachment site, the kinetochore, elegant phenomenological studies were able to accurately define the SAC as a cellular response that monitors biorientation of chromosomes on the mitotic spindle and prevents the initiation of anaphase until the last chromosome congresses to the equatorial plate (8, 9). The term spindle "assembly" checkpoint is a partial misnomer, being derived from the hypothesis of Murray and Kirschner that proper assembly of the mitotic spindle is "checked" before continued progression through mitosis becomes licensed (10). Subsequent genetic screens in *S. cerevisiae* revealed components of the SAC pathway based on the phenotype of continued cell division in the absence of a spindle (11, 12).

These genetic screens and subsequent studies in yeast revealed that Mad1, Mad2, Mad3 (BubR1 in metazoans), Bub1, Bub3 and Mps1 are essential for SAC activation (11-13). Orthologs of each of these have been identified in all eukaryotes studied (14-17) and heterozygous mice lacking some of the SAC genes suffer increased frequencies of colon and lung carcinoma (18). Reduced SAC function inevitably results in aneuploidy (18), that may contribute to the etiology of some tumors, though the evidence favors the idea that cancer cells generally rely on the SAC for survival, indicating that cancer therapies might do well to inhibit SAC function (18-20). Indeed, in most higher eukaryotes the SAC is essential for viability and requires proteins such as CENP-E, p31, ZW10, ROD and Zwilch, that are not present in yeasts (16). Given the arguments made above, that the SAC couples anaphase with a stochastic process, chromosome-KMT attachment, that takes varied times for completion from cell to cell, it seems reasonable that the SAC is essential and that mice homozygous for SAC genes are early embryonic lethal (18). However, yeasts lacking SAC genes are viable and *Drosophila* lacking a component that is essential for the SAC, Mad2, are viable and fertile (21). In these cases, either chromosome capture or error correction mechanisms must be efficient enough to allow proper biorientation of all chromosomes before the cell is able to induce anaphase. In other words, the fidelity of chromosome segregation relies on relative timing and error correction rather than checkpoint control.

Despite these differences, the known consequences of SAC activation across Eukarota are

strikingly unified in their ability to block anaphase onset by inhibiting the activity of the Anaphase Promoting Complex/Cyclosome (APC/C) which is an E3-ubiquitin ligase that was biochemically isolated over a decade ago (22, 23). The term Cyclosome denoted that it has "important roles in cell cycle regulation" (24), but later the name Anaphase Promoting Complex became commonplace reflecting the fact that some of its components had yeast orthologs (*CDC27* and *CDC16*) essential for anaphase onset (25). The APC/C consists of about 11 core components that contain the active site and presumably form a scaffold that increases the processivity of ubiquitin chain addition to substrates. In addition, the APC/C requires its so-called specificity factors, Cdc20 and Cdh1/Hct1, that can bind directly to the APC/C and to the substrates. Cdc20 is the only known target of the SAC. The SAC inhibition of Cdc20 blocks APC/C-mediated ubiquitination of specific proteins that act as anaphase inhibitors. A key anaphase inhibitor, named Pds1/Securin, keeps dormant an endopeptidase called Esp1/Separase that has several mitotic targets including the chromosomal cohesion factor, Rad21/Mcd1/Scc1. Together with other cohesins, Rad21 forms a ring-like structure that, at least in *S. cerevisiae*, is important for sister chromatid cohesion. Thus, through degradation of the anaphase inhibitor Pds1, cohesin is removed from chromosomes by Esp1/Separase and their segregation to the spindle poles is irreversibly initiated. The SAC is therefore thought to maintain the fidelity of inheritance by simply inhibiting Cdc20 until biorientation of every pair of sister chromatids has been achieved.

In this review we attempt to describe the mechanisms of the SAC; how it is activated at unattached Ks, then silenced upon chromosome biorientation, and what are the targets of the SAC that enforce anaphase delay. We also discuss models for how a tension defect might be translated to an occupancy defect, and how tension could be sensed by a molecular "Tensiometer". Fundamentally, the SAC appears to be primarily responsive to the status of K-MT interactions. Thus, we begin our discussion with an overview of yeast K structure, which will hopefully provide a framework for considering SAC signal transduction in the context of current information regarding the molecular basis of K-MT attachment.

3. OVERVIEW OF YEAST KINETOCHORE STRUCTURE

Ks are large multi-protein complexes that enable chromosomes to attach, biorient and segregate on the mitotic spindle. They assemble specifically at a single unique centromeric (*CEN*) region on each chromosome and function to capture spindle MTs and mediate chromosome movement. More is currently known about the structure of the *S. cerevisiae* K than that of any other organism. In addition to tractable genetics, this is in large part due to the fact that the K has proven surprisingly amenable to biochemical analysis, allowing the identification of at least six discrete K sub-complexes comprising ~65 different proteins. It can be argued that the constituent parts of the yeast K are now largely in hand, and this progress is detailed in several comprehensive reviews (26-29). Here we present a more abbreviated overview that touches on some current topics, especially regarding the molecular basis of K-MT attachment.

3.1. Functional aspects of K-MT interactions

It is currently not feasible to directly visualize the sequence of events that lead to stable K attachment to the spindle in *S. cerevisiae*, and so details of this process are inferred from observations on the much larger spindles of metazoan cells. In these cases, as chromosomes align on the spindle, Ks frequently initially attach to the side of a MT and translocate towards the spindle pole (30, 31). These lateral attachments eventually transition into more stable end-on attachments in which the plus ends of the MTs appear to be directly embedded in the outer K plate (for a review see 32). MT plus ends are the site at which the assembly and disassembly reactions characteristic of dynamic instability occur, thus positioning the K to influence and utilize MT assembly dynamics for chromosome movement. The actual number of MTs connected to the K varies between different types of cells, ranging from one MT per K in *S. cerevisiae* to 20-25 MTs per K in human cells (estimates can go as high as 109 for the large K fibers found in the endosperm of the blood lily *Haemanthus*, see 33). In the case of *S. cerevisiae*, the value for K-MTs has been derived indirectly by counting the number of MTs that appear to connect with chromosomes in three-dimensional reconstructions of the spindle (34). The uncertainties regarding how yeast chromosomes connect to the spindle are worth mentioning, at least in passing, because a recent report has argued that the transition between lateral and end-on attachments in yeast can be blocked by a specific mutation in the Dam1 K protein (35). Surprisingly, this has relatively little effect on cell cycle progression or the accuracy of chromosome segregation, suggesting there may be an unappreciated flexibility in K-spindle attachments that can satisfy SAC surveillance and support chromosome segregation. Be this as it may, end-on K-MT attachments appear to be the configuration that is typically associated with chromosome biorientation and anaphase chromosome movements. The end on coupling between Ks and MTs allows several remarkable forms of regulation to occur, which are briefly summarized below.

1) K-MT interactions transmit or generate force for chromosome movement. There appear to be two primary mechanisms by which Ks mediate chromosome movement. First, Ks provide binding sites for motor proteins that translocate along MT tracks or influence MT assembly (these are not specifically covered here; see 31, 36-38). Second, Ks function as coupling devices that keep chromosomes tethered to MTs as they grow or shrink in length. It is fascinating that Ks remain stably connected to K-MTs while still allowing tubulin subunits to associate and dissociate from the plus end of the polymer, and structural models for how this might be achieved are discussed below.

2) Ks directly influence MT assembly dynamics. It has long been observed that attachment stabilizes KMTs

from depolymerization, indicating a role for the K in controlling KMT assembly (39-41). This regulation may be quite sophisticated. For example, after chromatid pairs achieve bipolar attachments to the spindle they undergo oscillatory movements that are thought to contribute to chromosome congression on the metaphase plate (42-44). It has been proposed that the switch between poleward and anti-poleward movement is controlled, at least in part, by a tension-dependent mechanism that allows the K to increase the rescue frequency of disassembling KMTs (2, 45).

3) Ks enhance sister chromatid cohesion. A third function that has been described for Ks, at least in *S. cerevisiae*, is to specify the preferential deposition of the cohesin complex within an extended peri-*CEN* chromatin domain (46-49). While the mechanism by which the K promotes cohesion is not yet known, recent evidence suggests the K-dependent component of cohesion does in fact contribute directly to chromosome segregation accuracy (49). It will be of interest to determine whether this aspect of K function is conserved in metazoans.

4) Finally, Ks are sites for assessing the quality of chromosome-spindle attachments. As has already been mentioned, one way in which this is thought to occur is that Ks that are not connected to spindle MTs (unoccupied Ks) present or retain binding sites for SAC components. However, the quality of chromosome-spindle attachments is also ensured by a K-directed error correction mechanism(s) that disconnects mal-oriented chromosomes (such as the syntelically and merotelically attached chromosomes depicted in Figure 1) from the spindle. The relationship between error correction and SAC activation is discussed in Section V.

3.2. K Structure and assembly-the inner CEN/K complex

K assembly in *S. cerevisiae* is specified by an ~125 bp *CEN* DNA locus that is sufficient for all aspects of high-fidelity chromosome segregation (50). The *CEN* is composed of three sequence motifs called CDE I, II and III (51). CDE I and III contain recognition sites for sequence-specific DNA binding proteins (52, 53) while CDE II is an ~80 bp A/T rich sequence element that probably also makes contacts with *CEN*-proximal K proteins (Ndc10-54, Cse4-55 or Mif2-56, 57) within the overall architecture of the K complex. In particular, assembly of the CBF3 complex onto the CDE III *CEN* element represents a key early step in the formation of the K. The role of the CBF3-CDE III interaction in specifying the site of K assembly would appear to contrast with that of other eukaryotes, in which *CEN*s often contain long tracts of repetitive sequence elements and specific *cis*-acting determinants of *CEN* identity have not been identified (see 26 and 58 for reviews). In these cases the *CEN* locus appears to be specified by an epigenetically propagated chromatin structure consisting of nucleosomes containing the conserved histone H3 variant CENP-A. CENP-A-containing nucleosomes are a defining feature of centric chromatin in all organisms that have been examined (see 59 and 60 for reviews), and the *S. cerevisiae CEN* is also characterized by at least one CENP-A (called Cse4 in yeast) nucleosome (55, 61, 62). The mechanism controlling CENP-A nucleosome deposition at centric regions and how these nucleosomes are organized within the *CEN*/K complex are active areas of investigation. A consensus view (63), supported by initial chromatin immuno-precipitation (ChIP) experiments (55), is that the budding yeast *CEN* CDE II element is wound around a single Cse4 nucleosome centered directly over the *CEN* locus. A copy number of two GFP-tagged Cse4 molecules (one nucleosome) per K was recently used as a reference point to calculate reasonable stoichiometries for other GFP-tagged K proteins by quantitative fluorescence microscopy (64). However, it is important to note that Cse4 has recently been detected by ChIP in a region of at least 20 kB around *CEN*s (65). Thus, the distribution of Cse4 within *CEN* chromatin may not yet be completely settled (for an alternative viewpoint, see 54).

The relative contributions of CBF3 and Cse4 to further K assembly are also not yet clear. The interaction between CBF3 and CDE III is required for all aspects of *CEN*/K assembly that have been examined (57, 66-74), including Cse4 localization to *CEN* chromatin (75). In contrast, existing temperature sensitive alleles have produced somewhat confusing results with respect to the role of Cse4 in K assembly and function (76, 77). However, recent experiments using a degron allele of Cse4 have shown that the *de novo* recruitment of several K factors, including CBF3 components, to a conditional *CEN* is reduced, but not completely eliminated, following Cse4 depletion (78). Thus, CBF3 and Cse4 appear to make inter-dependent contributions to early K assembly, an interpretation in keeping with the observation that CDE III by itself is not sufficient for chromosome segregation activity (79, 80). Very recently, three studies have provided additional insight into how CBF3 might promote Cse4 nucleosome assembly (81-83). The Scm3 protein was initially isolated as a dosage suppressor of *cse4* mutants (84), and, remarkably, the current data suggests Scm3 actually assembles with Cse4 into chromatin, effectively replacing histones H2A and H2B within the core Cse4 nucleosome particle. Scm3 was further shown to interact with Ndc10, potentially providing a link between CBF3 and Cse4 that could direct Cse4 nucleosome deposition. Beyond these studies, what is needed at this point is to define subsequent interactions that allow the CBF3/Cse4 chromatin platform to template further K assembly. Atomic force microscopy has shown that CBF3 induces a kink in *CEN* DNA (85). In the context of the Cse4 nucleosome this may produce a larger bend to form a 20 nm wide "C loop" that positions the K facing outward from the chromosome-a geometry predicted to facilitate interactions with spindle MTs (86).

3.3. K Structure and assembly-connecting chromosomes to the spindle

None of the proteins comprising the *S. cerevisiae* inner *CEN*/K complex bind directly to MTs, indicating the connection between the K and the spindle must be mediated by other factors. To identify these factors, a powerful combination of yeast genetics and affinity purification methods has been employed in a concerted

effort to dissect the molecular basis of yeast K-spindle attachment, leading to the identification of a large number of additional K-associated proteins. When purified from protein extracts, the majority of these partition as components of one of several soluble complexes that are believed to represent discrete sub-assemblies of K structure (for recent review, see 29). The names of these complexes have not yet been standardized, and are given here as the Ctf19/COMA complex, Ndc80/Hec1 complex, the Mtw1/MIND complex, the Spc105/KNL-1 complex, and the Dam1/DASH complex. With the important exception of Dam1/DASH (87, 88), until very recently none of these complexes have been demonstrated to possess intrinsic MT-binding activity. They have therefore been proposed to function as linker complexes that provide a bridged interaction between inner K components and the factors that actually bind MTs (63). Another essential yeast K protein that may function as a linker is Mif2. Mif2 is the homologue of CENP-C, a component of the inner plate of the vertebrate K that is required for virtually all aspects of K assembly (56, 89). Two non-exclusive models for Mif2 function have been proposed. In one, Mif2 mediates protein-protein interactions that facilitate assembly of *CEN* DNA into Cse4 nucleosomes (56). A second view is that Mif2 functions at the interface of the inner K as an adaptor for other linkers. This latter interpretation is suggested by the observation that Mif2 interacts with Cse4 (62, 90) and also co-purifies as a sub-stoichiometric component of Mtw1/MIND (62).

Of the proposed linker complexes, the highly conserved Ndc80/Hec1, Mtw1/MIND and Spc105/KNL-1 complexes have received the most recent attention. Functional studies in fungal, invertebrate and vertebrate cells indicate the proteins that comprise these complexes all play essential roles in forming robust K-MT interactions (Figure 2) (reviewed in 28, 29). In particular, mutations affecting the yeast Ndc80/Hec1 complex result in a severe chromosome detachment defect (68, 69, 91, 92). Yeast Ndc80/Hec1, Mtw1/MIND and Spc105/KNL-1 fractionate from protein extracts as three separate complexes (93). However, TAP tag affinity purification of Mtw1/MIND results in the isolation of both Ndc80/Hec1 and Spc105/KNL-1 components, indicating these complexes are likely to interact *in vivo* (73). Similarly, components of Mtw1/MIND, Ndc80/Hec1 and Spc105/KNL-1 co-purify in *C. elegans* and human cells, and have been suggested to form a larger K assembly called the KMN network (for KNL-1, Mtw1 and Ndc80, see 94, 95). There is a hierarchy of interactions within KMN such that Spc105/KNL-1 and Mtw1/MIND create a binding site for Ndc80/Hec1 in the outer K plate (96). This may be analogous to the situation in yeast where at least some mutant alleles affecting Mtw1/MIND reduce Ndc80/Hec1 complex *CEN* localization as evaluated by ChIP (62, 74, 93). Thus, current evidence points towards Ndc80/Hec1, Mtw1/MIND and Spc105/KNL-1 forming a core architectural motif within the Ks of widely divergent organisms (97).

The *C. elegans* KMN network has recently been reconstituted using bacterially produced proteins. In a key finding, biochemical analysis of these *in vitro* complexes revealed that both Spc105/KNL-1 and the Ndc80/Nuf2 heterodimer of the Ndc80/Hec1 complex co-sediment with stabilized MTs (96). The binding of Ndc80/Nuf2 and Spc105/KNL-1 to MTs was not particularly strong, but a synergistic increase in affinity was observed when KNL-1 and Ndc80/Hec1 were combined in the context of the KMN network. Visualization of purified Ndc80/Hec1 by rotary shadowing electron microscopy has previously shown that this complex is shaped as a 570Å helical rod with globular regions at either end (98). Remarkably, negative stained preparations of Ndc80/Hec1-decorated MTs revealed the Ndc80/Hec1 rods projected from the polymer surface at a fixed angle with regular polarity, much like bristles on a brush (96). Using fragments of the *S. cerevisiae* Ndc80 and Nuf2 proteins, a second group has shown that Ndc80/Hec1 MT binding activity resides in the globular head region of the Ndc80/Nuf2 dimer (99). This was accompanied by a crystal structure showing the Ndc80 globular region folds into a structure resembling a known MT-binding domain. Based on these observations, it is tempting to think of the Ndc80/Hec1-MT interaction in terms of the K "sleeve" that has been proposed, on theoretical grounds, to mediate the end-on coupling between MTs and the K (100). In the sleeve model, the K engages the sides of the MT plus end through a large number of low affinity interactions, leaving the protofilaments tips available for tubulin subunits to come on and off the polymer. Biased diffusion would allow the K to track with the MT during cycles of growth and shrinkage.

Obviously, determining whether Ndc80/Hec1 and/or Spc105/KNL-1 form this type of K-MT interface *in vivo* will require further study, and theoretical expectations may be overly simplistic. Along these lines, a recent electron tomography study of K structure in PtK1 cells showed that, in the absence of bound MTs, the outer K plate consisted of a network of long, parallel fibers that may represent the extended helical regions of Ndc80/Hec1 and Spc105/KNL-1 complexes (101). In Ks with end-on attachments, this network reorganized such that the fibers extended outward to contact the embedded MTs. One interpretation of this study might be that, rather than consisting of pre-formed "sleeves", Ks tailor sleeve-like connections that are appropriate to the occasion, depending on the placement and geometry of K-MT encounters.

In *S. cerevisiae*, another K factor with MT binding activity is the Dam1/DASH complex. All ten subunits of this complex are essential, and conditional loss-of-function mutations display defects in chromosome-spindle attachments (67, 70-72, 87, 88, 102, 103). Recombinant Dam1 protein can bind to MTs as efficiently as the entire complex, suggesting the MT binding activity may reside solely with the Dam1 subunit (87, 88). Furthermore, the association of Dam1/DASH with the K (as evaluated by ChIP of *CEN* DNA) is MT-dependent, indicating K-MT interactions are a pre-requisite for Dam1/DASH localization (72). But by far the insight into Dam1/DASH that has attracted the most attention is the finding that the reconstituted complex is capable of oligomerizing to form rings and helical gyres around MTs *in vitro* (104-107). This discovery was greeted with

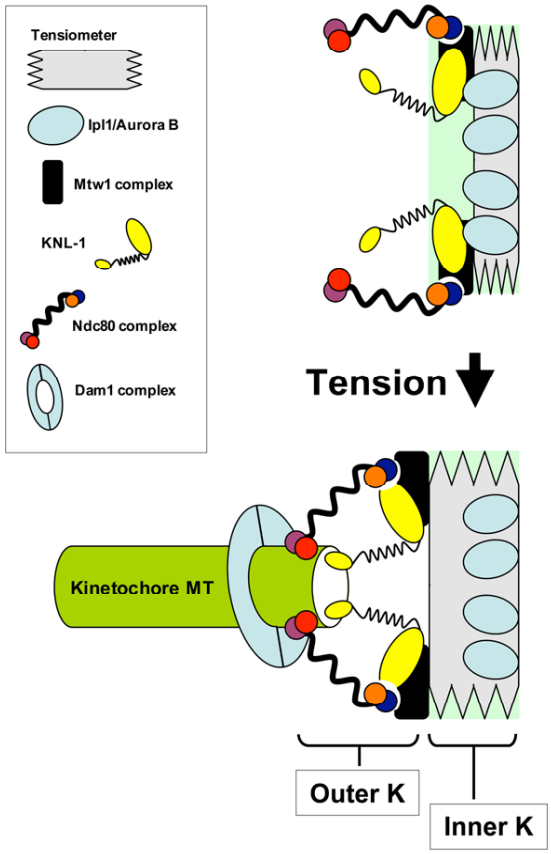

Figure 2. Microtubule Capture and Error Correction. Microtubule (MT) capture is achieved by interaction of a kinetochore microtubule (KMT) with KNL-1 and Ndc80 complexes. Ipl1/Aurora B-dependent phosphorylation of kinetochore (K) components limits these interactions. Under the proper conditions of tension, i.e. when a K-K pair is bioriented (only one of the pair is shown in the cartoon), the tension-sensitive apparatus (Tensiometer) physically separates Ipl1/Aurora B from its substrates, promoting stable kinetochore-KMT interaction.

considerable excitement, as such structures immediately suggest a mechanism for harnessing MT disassembly to power chromosome motion. As MTs plus ends disassemble, the protofilaments curl outward as they release mechanical strain locked within the polymer lattice, and this movement has been shown to be capable of generating force on a coupled object (108). By encircling MTs, Dam1/DASH rings could therefore conceivably form a highly processive coupling device that would allow this "conformational wave" to slide the K over the surface of a disassembling MT (109). In a beautiful set of experiments, two groups have shown that Dam1/DASH does in fact appear to be capable of harnessing MT assembly dynamics to move chromosomes, at least *in vitro* (109, 110). To focus on one of these papers, Dam1/DASH complexes coupled to a microbead cargo were combined with MTs undergoing dynamic instability (110). In a Dam1-dependent fashion, the coated beads were observed to connect with the MTs and track processively for several microns along both growing and shrinking MTs ends. The trans-locating beads could continue to move even when a pulling force of up to 3 pN was applied using an optical trap. At greater tensile forces, the beads could be displaced from the plus end and pulled freely back and forth along the shaft of the MT. These movements are consistent with Dam1/DASH forming rings that can slide along MTs and be used to direct chromosome movement.

While these studies are compelling, there are two caveats to the ring model. First, although Dam1/DASH is critically required for chromosome segregation in *S. cerevisiae*, the components of this complex are not obviously conserved outside of fungi. The corresponding fission yeast proteins are not essential for cell growth, but are required for high-fidelity chromosome segregation (111, 112). Second, MT-encircling rings have so far not been observed from *in vivo* studies of K ultra-structure (see 113 for a discussion of this topic). However, since *S. cerevisiae* Ks have not been visualized at this level of resolution, the question of whether rings form on *S. cerevisiae* Ks-where Dam1/DASH is actually present-is open. Given the current state of knowledge, what then is one to make of the relative contributions of the MT binding activities of the Ndc80/Hec1 and Dam1/DASH complexes? One speculative idea is that a K-spindle interface comprised of a single KMT might impose an elevated requirement for multiple reinforcing attachment mechanisms. In particular, Dam1/DASH rings could contribute greatly to the ability of *S. cerevisiae* Ks to track processively with its singlet MT during cycles of assembly/disassembly. To pursue the analogy of sleeves and rings, a K-MT interface comprised of a single MT may require the MT to be fitted with a well-formed sleeve that has a cuff at the end. A schematic view of how different K complexes may participate in forming the K-MT interface is depicted in Figure 2; readers are referred to (29) and (16) for more detailed representations of yeast and vertebrate *CEN*/K structure. Our rapidly increasing knowledge of the K-MT interface is likely to bring deeper insights into fundamental questions regarding the K and SAC signaling. For example, at a structural level, what distinguishes an unoccupied K from an occupied one? Furthermore, what components of the K-MT interface are targeted by error correction mechanisms to release mal-oriented chromosomes from the spindle? These questions and other related issues are explored below.

4. SAC ACTIVATION BY UNATTACHED KINETOCHORES

The elaborate systems that dock MTs at Ks serve to establish biorientation and simultaneously affect K structure in ways that turn off SAC signaling. Presumably this is possible because the SAC is generated by complexes that are either integral to K structure or are transient K components. From the original genetic screens and subsequent studies performed in yeast, Mad1, Mad2, Mad3 (BubR1 in metazoans), Bub1, Bub3 and Mps1 are all present at Ks. That each of these are essential for SAC activation (11-13) suggested a linear pathway, requiring all of the components for its function, but it has been challenging to define the SAC in these terms. As mentioned

above the SAC seems to be essential in the metazoans that have been examined (except *Drosophila*) but is dispensable in yeasts, though in human cells, depletion of Bub1 does not completely eliminate the SAC, indicating only a partial requirement (114). Historically, cytological studies in larger cells accompanied the yeast genetic approaches, revealing the localization of Mad and Bub proteins at the K outer plate or corona during checkpoint activation, followed by their release upon K-MT connections becoming established (14, 115-118). K localization appeared coincident with nuclear envelope breakdown, perhaps defining the moment that the SAC is needed. Striking studies then revealed that destroying the last remaining K (using laser light) rapidly inactivated the SAC (8). Earlier genetic studies in yeast in which centromeric DNA mutations or mutations affecting K function led to mitotic arrest (119-121) had led to the idea that K-MT interactions are needed to permit anaphase onset. Together with the laser experiments, these studies led to the hypothesis that K-MT attachments are monitored, that Ks are the sites from which the SAC signal is generated, and led to the assumption that amplification and diffusion of the signal is necessary to inhibit APC/C throughout the cell.

Based mainly on fluorescence recovery after photo-bleaching (FRAP) experiments, Mad1, Bub1 and about 50% of Mad2 appear to be stably associated with unattached Ks, whereas the remaining Mad2, as well as BubR1 (the yeast ortholog of Mad3), have very short half lives at Ks (122-124). Together, these data led to the idea of a checkpoint scaffold at the K upon which active checkpoint complexes are generated and then released into the cytosol. Exactly how the active complex (often called the mitotic checkpoint complex; MCC) is formed is a current focus in the literature (16, 125). In the models presented, stably K-associated Mad1-Mad2 complexes serve to catalyze formation of active checkpoint complexes in which Mad2 is bound to the APC/C specificity factor Cdc20. Inactivation of Cdc20 (and/or APC/C) by the MCC throughout the cell would, in theory, then preclude anaphase onset. However, these models also need to take into account the results of cell fusion studies which indicated that the checkpoint signal is not freely diffusible (126). These models also do not yet go as far as to explain the formation of the complete MCC which contains Mad2, Mad3 (BubR1), Bub3 and Cdc20. To add further complexity there appear to be other modes of APC/C inhibition, including Mad-dependent control of Cdc20 protein levels (127), inhibition of Cdc20 through phosphorylation by Bub1 (128, 129) and direct binding of Mad3 to Cdc20 with higher affinity than Cdc20 substrates, which would then out-compete the substrates so that they cannot reach the APC/C (130). A general theme appears to be that the SAC uses a multi-pronged approach in its assault on anaphase progression, and similarly, once metaphase plate formation is achieved, multiple mechanisms collaborate to rapidly and irreversibly trigger sister chromatid separation and chromosome segregation.

Genetic studies in yeast indicate that Mps1 kinase likely acts as an upstream activator of the SAC, or at least Mps1 function permits SAC checkpoint signaling (131, 132). Overexpression of Mps1 in *S. cerevisiae* leads to robust metaphase arrest that depends on each of the other SAC genes. Mps1 phosphorylates Mad1 in yeast (132) though the consequences of this are not clear. In metazoan cells, Mps1 cycles on and off Ks (124, 133) and it functions in the recruitment of other SAC components to Ks including Mad1, Mad2, and CENP-E (134). This may not be its sole SAC activity since the accumulation of SAC components at metazoan Ks is partly dispensable for SAC signaling and because Mps1 over-production in *S. cerevisiae* induces metaphase arrest in the absence of Ks (see below). Nevertheless, Mps1 is likely to modify substrates at Ks to promote SAC signaling. At least in yeast, Mps1 has been additionally implicated in proper spindle assembly and serves some function, probably at the Ks, to allow proper spacing of sister Ks in metaphase (135). The relevant substrates for these functions are not known.

Similar to Mps1, Bub1 kinase is important for recruiting SAC components to the outer K, but, Bub1 is also needed for the localization of components to the inner K (14, 136-141). Bub1 and Bub3 form a complex (14, 142, 143) that localizes to Ks in yeast in the absence of spindle damage, when Mad1 and Mad2 are not present at Ks (144), and the Bub1-Bub3 interaction may mutually allow their binding to Ks (14). This might form part of a platform for recruitment of other components such as Mad1 and Mad2. Metazoan Bub1 is relatively stable at unattached Ks or in the absence of tension (124, 145) and is important not only for recruiting SAC components but also other regulators such as the Chromosome Passenger Complex proteins (CPCs) and Shugoshin (Sgo1), that localize to the inner centromere region (discussed further in 16, 138). Less is known about the dynamics of Bub3 at Ks. While the kinase activity of Bub1 is needed for the localization of the CPCs and Sgo1 to the inner centromere region (138), kinase-inactive Bub1 is generally sufficient for recruitment of SAC proteins to Ks (139-141) (except in the case of Mps1, see 141). These data are consistent with soluble Bub1 being sufficient for localizing CPCs and Sgo1 to the inner centromere region (138) whereas Bub1 at the outer-K perhaps physically recruits the SAC components. Together with CPCs and the kinase activities of Mps1 and Aurora B/Ipl1 (146), Bub1 likely organizes K structure, maintaining the inner centromere region (138) and producing a landing-pad for SAC proteins in the outer-K.

Depletion of Bub1 from human cells results in biorientation or alignment defects, leading to a prolonged prometaphase before anaphase onset in the presence of unaligned chromosomes (114). Addition of spindle poisons led to a robust SAC arrest (unlike the case of *bub1* yeast mutants that completely lack the SAC). It seems plausible that Bub1, through multiple activities including its interaction with Bub3, orchestrates aspects of K-MT interaction as well as assembly of checkpoint competent Ks. Studies in yeast have similarly described SAC-independent functions of the Bub1-Bub3 complex needed for accurate chromosome segregation and importantly these data genetically separate this from the SAC function of Bub1 (147). It is curious that Bub1-depletion does not completely abolish the SAC in human cells (114), but that

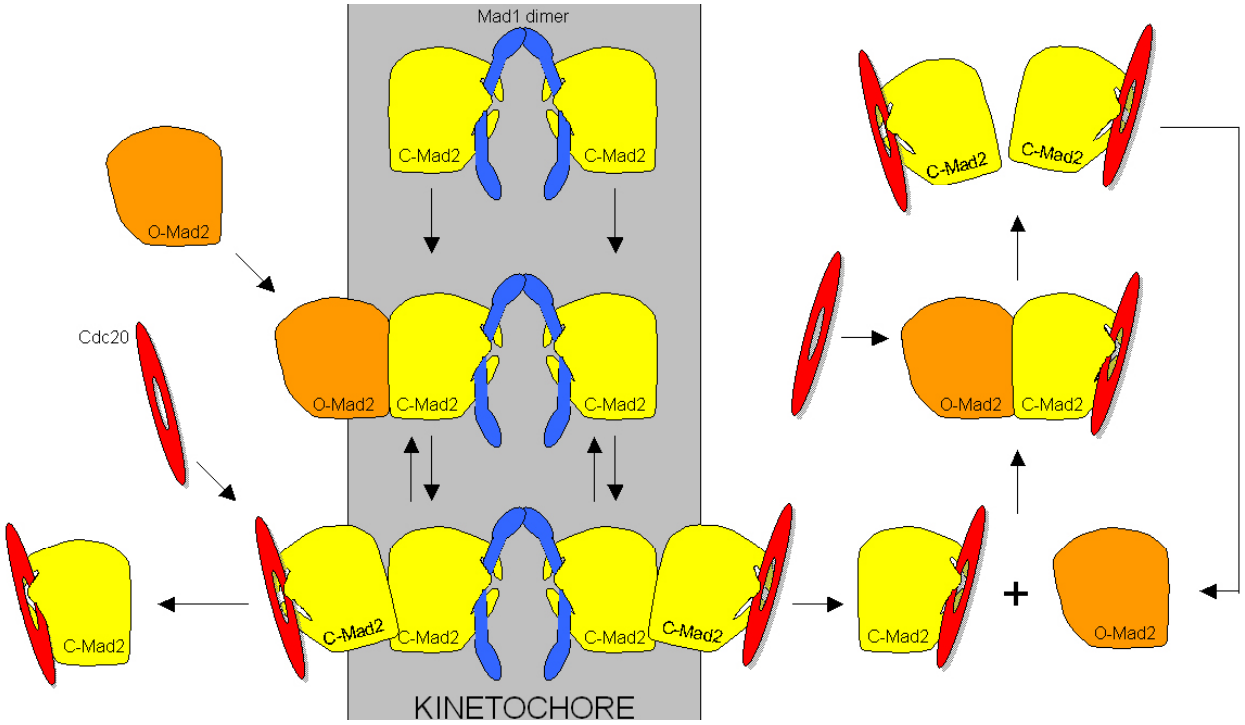

Figure 3. Mad2 Activation at the Kinetochore. Upon binding to its ligands (Mad1 or Cdc20) Mad2 undergoes a conformational change that has been described as a conversion from the open form (O-Mad2) to a closed form (C-Mad2). This is rather misleading because in O-Mad2 the ligand binding site is actually obscured, or closed. At unattached kinetochores (Ks) Mad1-C-Mad2 complexes are relatively stably associated (Note that Mad1 exists as a homodimer). O-Mad2 (also called N1-Mad2) from the soluble pool joins the complex and upon association with Cdc20, and most likely coincident with release from the Mad1-C-Mad2 complex, adopts the C-Mad2 (N2'-Mad2) conformation. Presumably, in the transient Mad2-C-Mad2 dimer, Mad2 will have an un-obscured ligand binding site, but this structure (N2-Mad2) has not been solved. C-Mad2-Cdc20 could form a trimer with another soluble Mad2 molecule which would then perhaps allow the exponential formation of C-Mad2-Cdc20 away from the K.

Bub1 seems to become critical for robust inhibition of anaphase when small numbers of chromosomes are unattached or are not bioriented. For now, a detailed description of how factors like Mps1 and Bub1 contribute to the production of checkpoint competent Ks is lacking, but it is clear that a crucial step is the tethering of stable Mad1-Mad2 complexes to the K.

After Mad1-Mad2 complexes are recruited to unattached Ks, activated SAC complexes that can inhibit APC/C must be produced (see Figure 3). Recent discoveries indicate that conformational activation of Mad2 at Ks leads to Cdc20 binding (125, 148-152). Models have emerged, based on FRAP and structural data, that describe rapid cycling of Mad2 and Cdc20 on and off Ks (123, 124, 153) and a Mad2 conformation change (from O-Mad2 to C-Mad2) upon formation of a C-Mad2-Cdc20 complex (148-150). Mad2 is in the C-Mad2 conformation in the pool of relatively stable Mad1-Mad2 at unattached Ks (151, 154). This stable complex binds O-Mad2 to form a trimeric complex (148-151) which is then hypothesized to bind Cdc20 to produce two dimers: Mad1-C-Mad2 (which stays at the K) plus C-Mad2-Cdc20, which dissociates. Theoretically, the large excess of O-Mad2 in cells (155) would allow propagation of C-Mad2-Cdc20 complex formation. Since C-Mad2-Cdc20 can also bind O-Mad2, it is possible that addition of another Cdc20 molecule to this trimeric complex could then produce two C-Mad2-Cdc20 dimers, leading to exponential amplification of the SAC signal independently of Ks.

Once C-Mad2-Cdc20 complexes are generated, MCC formation must occur producing the Mad2-Mad3(BubR1)-Bub3-Cdc20 complex (156-160). This is assumed to be critical because MCC is about three orders of magnitude more active in inhibiting APC/C *in vitro* than Mad2 alone (157). Presumably, K-activated Mad2-Cdc20 complexes recruit Mad3(BubR1) and Bub3. BubR1 and Mad2 can bind Cdc20 at the same time and occupation of each binding site may collaborate to inhibit APC/C within the MCC (161-163). Some evidence indicates that BubR1 only binds its Cdc20 site once Mad2 is bound to Cdc20 (160), consistent with a step-wise formation of MCC. Similarly, binding of Mad3 to Cdc20 is at least stimulated by Mad2 in yeast (130). BubR1 kinase function is activated upon binding CENP-E at unattached Ks (164, 165) and this activity seems to be important when a small number of Ks are unattached (166). But, the relevant substrates of BubR1

are unclear and any relevant mechanism is not conserved in yeast as Mad3 lacks a kinase domain. Phosphorylation of BubR1 by Aurora B (Ipl1) has been implicated in SAC signaling because this modification appears important for localization of BubR1 to Ks but whether this modification affects MCC formation directly is not known. Similarly, yeast Ipl1 can phosphorylate Mad3 *in vitro* and mutation of two of these serine residues (S303 and S337) to alanine results in a defect in SAC signaling when proper K-K tension is abolished (167). How these phosphorylations might affect MCC formation or Mad3 localization has not yet been studied. Except for the requirement for Bub1 for recruitment of Bub3 to Ks (136), what factors contribute to Bub3 joining the MCC have not been determined, though it seems that Bub1 and Bub3 are required for localization of BubR1 to Ks (14). Although important studies are beginning to tease out the molecular details of MCC formation, we appear to be some way off from a satisfying description of either MCC formation or the network of phospho-regulated interactions required to convert the K into a platform for converting O-Mad2 to C-Mad2-Cdc20.

Once formed, MCC binds to the APC/C (114, 130, 157, 162, 168), stimulated both by Aurora B (Ipl1) and Bub1 (114). How MCC may inhibit APC/C directly is not understood. By extension from the mechanism of Emi1 action, a pseudo-substrate inhibitor of APC/C in S-phase cells, the MCC might possess one domain that allows APC/C binding and another domain capable of direct inhibition of APC/C ubiquitin ligase activity (169). By binding to the APC/C, the MCC could also preclude binding of active Cdc20 to APC/C. Quite likely such mechanisms are additive in preventing degradation of APC/C substrates.

Several other mechanisms also contribute to APC/C inhibition. As well as stimulating MCC interaction with APC/C, Bub1 phosphorylation of Cdc20 promotes its ability to inhibit APC/C (perhaps as part of the MCC) (128, 129, 170). In *S. cerevisiae*, Cdc20 itself is de-stabilized upon SAC activation. Cdc20 mRNA remains constant but the Cdc20 protein level is reduced in an APC/C-dependent and Mad3-dependent manner (127, 130). Mad2 binding to Cdc20 is also required (127). Furthermore, *S. cerevisiae* Mad3 can bind to Cdc20 as a pseudo-substrate inhibitor, i.e. it can compete with substrates for binding Cdc20. Therefore, blocking Cdc20-substrate interaction is part of the SAC mechanism (130) and may even be more critical in some cells than Cdc20 degradation or direct inhibition of APC/C through MCC binding (171).

Much effort is currently being put into the mapping of SAC activity to Ks and K sub-complexes and most of the evidence supports a strict requirement of Ks for SAC signaling. Yeast Ndc10 is an inner-K protein that binds *CEN* DNA as part of the CBF3, centromere-binding factor 3, complex (53, 54, 66, 172) and which is essential for K assembly (68, 69, 75, 173, 174). At the restrictive temperature, *ncd10-1* mutants completely fail to assemble Ks and therefore lack any K-MT interactions (66). The lack of attachment of chromosomes to the spindle may have been expected to persistently activate the SAC, but in fact no SAC response is mounted in such yeast cells (66), providing the first genetic evidence that functional Ks are required to generate the SAC signal. Similarly, *ndc10-1* mutants fail to respond to nocodazole (66, 158, 175-177). If temperature sensitive Ndc10-1 is inactivated after cell cycle arrest in the presence of microtubule poisons, the SAC fails (178), indicating that Ks are required for establishment and maintenance of the SAC signal. This argues that catalytic production of MCCs by Ks must be sustained to keep the SAC turned on. Other, more subtle defects in K structure that weaken but do not abolish K-MT interaction, can also result in reduced efficiency of SAC signaling (179) although the majority of K mutants produce a robust SAC-dependent (and Ipl1-dependent) delay in Pds1 degradation. Overall, these genetic analyses mirror the classic phenomenological studies described above, in which laser-ablation of the last remaining K in mammalian cells resulted in rapid SAC bypass (8).

While these data are robust, other evidence indicates that MCC formation does not strictly depend on Ks and that in some instances SAC signals are generated independently of K-mediated MCC production. Surprisingly, yeast Ndc10 is not required for Bub3-Cdc20, Mad2-Mad3 or Mad2-Cdc20 complex formation (176, 180), events that ought only to occur if MCCs are being generated on Ks. In these cases, however, the SAC is clearly non-functional as the Ndc10 loss of function prevents K assembly (180). In HeLa cells, MCC is present in interphase as well as in mitosis (157). The ability to form MCCs might therefore not equate with SAC activation or proficiency. Depletion of Ndc80 complex components from mammalian cells results in defective K-MT attachment and robust activation of the SAC, and yet key components of the SAC, Mps1, Mad1 and Mad2, are barely detectable at these unattached Ks (181, 182). Mad2 was nonetheless essential for SAC signaling in this situation (182) and experiments which appear to have achieved more complete inactivation of Ks through Ndc80 complex depletion resulted in SAC deficiency (183). This may indicate that only a small (barely detectable) amount of MCC generated at Ks is needed for a robust SAC response, or, a SAC signal could be generated at Ks without requiring MCC formation at Ks. This signal might act by promoting MCC-APC/C interaction, or some other mode of Mad1-Mad2 or Mad3(BubR1)-dependent APC/C inhibition, away from Ks. This case assumes generation of MCCs (or partial MCC complexes) on structures other than Ks or in the cytosol. Based on the genetic experiments, the K requirement for SAC signaling in yeast seems to be absolute and, in fact, ndc80 mutants that activate the SAC do localize Mad2 to Ks, whereas other alleles that are SAC defective fail to recruit Mad2 (144). Even so, unlike in the absence of a spindle, ncd10-1 yeast do arrest before anaphase upon Mps1 over-production (176, 180), perhaps suggesting that an excess of Mps1 may be capable of forcing K-independent SAC signaling.

Overall, we are left with the conclusion that MCC formation, whether at Ks or elsewhere in the cell, is

not the whole story. Other mechanisms are at play that promote the ability of MCC to inhibit anaphase, either by amplifying MCC production, or through some other means, such as modifying its localization or APC/C inhibitory properties. Lastly, we bring to the readers attention genetic evidence in both mammalian and yeast systems that there are control systems acting independently of the APC/C to regulate anaphase initiation (184-186). These additional controls are largely ill-defined at present, and so are not elaborated upon herein, but nonetheless it will be important to define these cellular controls to gain a complete understanding of the mechanisms that control chromosome segregation.

5. SAC SILENCING

Exactly how the checkpoint signal generated by unattached kinetochores is turned off (checkpoint silencing) is not known, though several factors must collaborate because the transition into anaphase is rapid and irreversible. Of note is that release of cohesion freeing the sister chromatids, and consequently releasing inter-K tension, does not reactivate the SAC.

In higher eukaryotes, CENP-E dependent inactivation of BubR1 kinase activity occurs (164) upon binding of MTs to CENP-E (165). (In yeast, Kar3 might replace CENP-E; discussed in 16). It is not clear how Bub1 kinase is turned off in yeast, but in metazoans, Bub1 releases from Ks that are occupied by MTs and have correct tension (124, 145). Though speculative at this point in time, this may affect K structure such that biorientation is stably maintained and SAC signaling is down-regulated at the K (perhaps making that particular K-K pair less sensitive to changes in tension during metaphase oscillations). Elaborating from the ideas discussed above, removal from Ks of factors such as Bub1 and Mps1 could have a fundamental effect on K structure, resulting in Ks that are unable to mount SAC signals.

Existing checkpoint proteins seem to be removed from Ks by dynein-dependent traffic to contribute to silencing. Evidence suggests Mad1-Mad2, RZZ, Mps1, CENP-F, BubR1, and Cdc20 are moved away from Ks along MTs and to the spindle poles upon K-MT binding (187-190). It is an interesting question whether this traffic to the poles just physically separates the SAC components, or whether these traffic events are, in addition, taking complexes to the poles where they might serve a positive function in the initiation of anaphase.

Mad2 is phosphorylated upon SAC silencing and this seems to perturb its interaction with Mad1 (191). Other dephosphorylation events, or removal of phospho-proteins, must also occur since 3F3/2 epitopes are removed from attached Ks. The identity of all of these phospho-proteins is probably not known, nor do we have a full understanding of the relevant kinases and phosphatases.

Physically dismantling the K-dependent SAC apparatus no doubt helps to halt production of MCCs and likely contributes to silencing in other ways, but in addition, existing MCCs have to be dealt with. $p31^{comet}$, originally identified as a Mad2-binding protein in human cells (192), is hypothesized to halt amplification of MCCs (templated from Mad1-C-Mad2 and C-Mad2-Cdc20) by competing with O-Mad2 for binding (154, 192-194). Consistent with a requirement for SAC silencing, $p31^{comet}$ depletion delays mitotic exit in human cells following reversal of nocodazole arrest (192, 193). In yeast cells, $p31^{comet}$ and CENP-E are not present, but a recent report indicates that Mps1 is degraded upon SAC silencing which likely plays a large role in effecting the irreversibility of anaphase initiation (195).

Lastly, APC/C must be activated. It can be argued that this requires activation of another signaling pathway, essentially producing a "Go Anaphase!" signal, in addition to the inhibitory SAC signal being diminished. For example, the levels of free Cdc20 have to be somehow restored because within MCC, Cdc20 is inactive (130). These mechanisms must first explain how the energy is provided for Mad2 and Cdc20 to dissociate, because C-Mad2-Cdc20 is a stable complex. Recent mammalian studies provided evidence that APC/C-Cdc20 itself, stimulated by the E2 enzyme UbcH10, multi-ubiquitinates Cdc20 which then drives separation of Cdc20 from Mad2 and BubR1 (196, 197). Cdc20 seems also to become unstable during this process, although its degradation likely follows its release from MCC. Importantly, $p31^{comet}$ was observed to stimulate the ability of UbcH10 to promote Cdc20 ubiquitination. Other work identified a de-ubiquitinating enzyme, USP44, which counteracts UbcH10-dependent ubiquitination of Cdc20 during mitosis (197, 198). USP44 is required for SAC arrest and its protein levels are increased in mitosis and then drop upon mitotic exit. Depletion of USP44 results in reduced levels of Mad2-associated APC/C-Cdc20 and an increased level of Cdc20 ubiquitination. Strikingly, co-depletion of USP44 and UbcH10 rescued the SAC defect seen after USP44 depletion alone. These data indicate that USP44 restrains anaphase onset by limiting Cdc20 ubiquitination.

The mitotic functions of USP44 are likely to be more complex than merely serving as an SAC component, since depletion of USP44 causes early mitotic defects in chromosome alignment as well as defects at cytokinesis. However, these studies have provided a convenient explanation for the rapid switch mechanism that triggers anaphase onset. In metaphase, ubiquitination of Cdc20 within MCCs is limited by USP44 activity and $p31^{comet}$ inactivity. Upon SAC silencing, MCC production wanes and Go-Anaphase! signals are triggered that encompass a block to USP44 activity (by a yet unknown mechanism) and activation of $p31^{comet}$. Cdc20 ubiquitination then predominates, releasing Cdc20 from MCCs. Since this Cdc20 appears to be unstable, it is still a requirement that cells somehow rescue APC/C-Cdc20 function. Also of interest is that nocodazole-arrested cells possess ubiquitinated Cdc20, indicating that this pre-anaphase arrest is a state of delicate balance (196, 198). Perhaps this can explain why SAC arrest is transient, giving way to c-anaphase after a certain number of hours that varies

between different cell lines. Upon activation, APC/C promotes securin and cyclin-B degradation, but as argued in Sections 7 and 8, these may not be the sole proteolysis events necessary and sufficient for anaphase initiation.

6. SAC ACTIVATION BY LACK OF TENSION-THE TENSIOMETER

6.1. Uses for tension within the spindle

As chromatids biorient on the spindle, cohesive linkages resist the pulling of sister Ks towards opposing spindle poles. This places the structural elements that link the chromosomes to the spindle-KMTs, the K itself, and intervening *CEN* chromatin-under mechanical tension. As a fundamental parameter of spindle mechanics, tension is thought to provide an inward-directed force that restricts spindle extension prior to anaphase. However, tension also appears to regulate at least two additional aspects of spindle dynamics.

First, tension is thought to provide a basis for distinguishing correctly oriented K-spindle attachments. The most direct evidence for this comes from the pioneering work of Nicklas and colleagues. In grasshopper spermatocytes, syntelically attached bivalents (in these meiotic cells the load bearing linkages between paired homologues are provided by chiasmata rather than by chromatid cohesion) have been observed to have a higher probability of detaching from the spindle than correctly bioriented homologues. However, syntelic attachments can be stabilized if an artificial source of tension is applied using a micromanipulation needle (199). Also, dissipating tension by mechanically detaching one of the two Ks on a bioriented bivalent was observed to lead to a reduction in the number of MTs connected to the remaining K. Occupancy could then be restored by pulling on the detached chromosome (200). The important implication of these findings is that tension controls an error correction mechanism within the spindle. Correctly tensed K-spindle interactions are relatively stable and persist, while mis-directed interactions that fail to produce tension are comparatively unstable. By eliminating un-tensed K-MT interactions, this error correction mechanism provides additional opportunities for chromatids to reorient until they align in a correct, tension-producing configuration.

In addition to controlling the stability of K-MT attachments, a second role for tension may be to control the dynamics of correctly oriented KMTs. As mentioned in Section 3.1, after chromatids biorient KMTs undergo cycles of plus end shrinkage and growth that cause the attached chromosomes to congress to a stable position on the metaphase spindle (42, reviewed in 45). In budding yeast, congression can be accurately simulated using models in which tension controls the switch between KMT disassembly and rescue (2). Specifically, periods of high tension (as when sister Ks are drawn apart) correspond with an increase in the probability of KMT rescue, which causes chromosomes to start moving towards the spindle equator. Conversely, as tension dissipates the catastrophe frequency for KMTs increases, allowing a resumption of pole-ward movement. Thus, while tension stabilizes K-MT attachment, there also appears to be a governing mechanism that ensures the amount of tension exerted upon paired Ks is kept within defined limits. In the remainder of this section, we focus on the nature of the error correction mechanism that de-stabilizes inadequately tensed K-MT attachments and how its activity is connected to the SAC. We then consider in more detail how tension is generated and assessed within the spindle.

6.2. Tension, chromatid-spindle attachment, and SAC signaling

The early work of Rieder and colleagues (9; see Section 4) not only provided the first accurate description of the SAC, but also provided evidence that the SAC might be capable of responding to altered inter-K tension as well as altered K occupancy. These workers added low levels of taxol to cells once bipolar chromosome alignments had been achieved. Under these conditions, K attachment was not obviously perturbed. However, oscillatory movements ceased and many cells displayed a prolonged delay in metaphase. Since Ks appeared to remain occupied, it was suggested that the effect of taxol was to perturb KMT dynamics in a manner that lead to a reduction in tension, and it was this defect that was responsible for signaling the SAC. Micromanipulation experiments in the grasshopper spermatocyte system have provided additional evidence to support this view (201, 202). During the first meiotic division, one of the three sex chromosomes occasionally fails to pair properly, resulting in a situation where the unpaired chromosome can only connect to a single spindle pole and therefore cannot be placed under tension. Cells that form these monopolar attachments delay anaphase entry for considerable periods of time. However, restoring tension by pulling on the unpaired chromosome is sufficient to both stabilize attachment and trigger anaphase.

Analogous experiments to address whether a lack of tension can activate the SAC in budding yeast have been performed using mutations that prevent chromosomes from forming bipolar attachments to the spindle. In meiosis, deletion of *SPO11* prevents chromosomes from being placed under tension because homologous chromosomes cannot be linked by recombination. These mutant cells go through a defective MI division in which the spindle extends and homologues segregate to spindle poles (203). Normally, spindle extension signifies that Pds1 has been degraded, triggering progression into anaphase. However, in *spo11* mutants Pds1 levels remain elevated in an SAC-dependent manner. Since Ks remain connected to spindle poles during defective spindle extension, it was argued that the SAC is activated by the absence of tension (although see discussion below). A similar relationship between tension and SAC activation has been suggested for mitotic cells depleted for the Cdc6 protein, which leads to an inability to initiate DNA replication (204). Unreplicated chromosomes fail to generate tension because they can only connect to a single spindle pole. Once again, the SAC responds by delaying Pds1 degradation (204), but as there is no inward force to prevent spindle extension cells

undergo a "reductional" anaphase in which unreplicated chromosomes segregate randomly to spindle poles (205).

Collectively, these experiments reveal that the SAC is capable of responding to a broad range of lesions within the spindle, including those that would appear to be primarily characterized by a reduction or complete absence of tension. However, because the stability of K-MT attachments is also influenced by tension, determining what the SAC is actually monitoring under different experimental situations becomes a complicated issue (this topic is specifically addressed in 206). For example, the absence of tension on grasshopper spermatocyte chromosomes reduces the number of MTs attached to Ks (200), potentially creating some unoccupied MT binding sites that might elicit SAC activation. Similarly, taxol treatment has been shown to create a broader distribution in the number of K-MT attachments (207), suggesting that in each cell there may exist a few Ks that could be perceived by the SAC control mechanisms introduced in Section 4 as being relatively unoccupied. Even in budding yeast, where there is only a single KMT it is difficult to completely rule out the possibility that the SAC responds primarily to a defect in attachment versus a defect in tension. The above examples show that chromosomes remain tethered to spindle poles via KMTs when the SAC is induced to respond in the absence of tension. But the nature of these attachments and the reason that they persist despite the absence of tension are currently unclear. Ultimately, the issue is whether there are two separate monitoring activities associated with the SAC, one responding to occupancy and one responding to tension. Alternatively, the SAC may monitor only attachment, and improperly tensed Ks would need to first be at least partially uncoupled from the spindle through error correction mechanisms before they could trigger the SAC. This issue will probably only be completely resolved once the underlying molecular mechanisms that activate the SAC have been revealed.

6.3. Aurora B kinases as regulators of the SAC

In budding yeast, the Ipl1 protein kinase has emerged as a key regulator of the SAC response to tension deficient K-spindle attachments. The vertebrate homologue of Ipl1, Aurora B, functions as the catalytic subunit of the CPC (Chromosome Passenger protein Complex) that includes the inner *CEN* (INCENP) and Survivin proteins, as well as other less conserved subunits (reviewed in 208-210). Similarly, Ipl1 has been shown to complex with Sli15 and Bir1, the yeast homologues of INCENP and Survivin (211-213). The Ipl1 and Aurora B passenger complexes have been implicated in numerous aspects of mitosis, including chromosome condensation, chromatid cohesion, K-MT attachment, spindle dynamics in late mitosis, and cytokinesis (see 208-210). In keeping with these multiple functions, the Aurora B passenger complex undergoes a distinctive series of movements throughout mitosis that are thought to enable the kinase to contact spatially separated substrates in a defined temporal order (208-210, 214). In particular, during prometaphase and metaphase Aurora B accumulates within inner *CEN* chromatin and then transfers onto the central spindle shortly after the onset of anaphase. In a seemingly related fashion, Ipl1 associates with the elongating spindle in anaphase cells (211, 212, 215-218). Due to the small size of yeast chromosomes, however, the localization of the Ipl1 passenger complex prior to anaphase has been harder to define. Immuno-localization studies have revealed that in pre-anaphase cells Ipl1 is distributed throughout the nucleus in a pattern that parallels chromosomal DNA (215). GFP tagging (68, 211, 216, 217)(which may only reveal where the kinase is most abundant) and chromosome spreading analysis (216, 219), however, indicate that is a sub-population of Ipl1 that preferentially associates with either the K or a sub-adjacent chromatin region. The uncertainty regarding whether Ipl1 functions directly at the K or within *CEN* chromatin during the time period in which bipolar attachments are formed is worth mentioning because, as discussed below, this issue is likely to have a bearing on how Ipl1 monitors tension and modulates K-spindle attachments.

A role for Ipl1 at the K was initially suggested because *ipl1* mutants display an unusual chromosome segregation defect in which both sister chromatids segregate to the same spindle pole (220-222). Further analysis suggested that this segregation pattern reflects an underlying role for Ipl1 in allowing chromatids to biorient efficiently rather than a requirement for Ipl1 to promote sister chromatid separation (211, 215, 223). In an elegant analysis, Tanaka and co-workers (216) then provided evidence that *ipl1* mutants fail in biorientation because they cannot disconnect improperly tensed K-MT attachments. They showed (and see also 215) that *ipl1* cells displayed a bias for both chromatids to move to the maternally inherited spindle pole, and that this bias could be alleviated by simply disrupting pre-existing chromatid-spindle connections with nocodazole. They reasoned such a bias might arise due to a delay in the ability of the newly duplicated pole to nucleate MTs and thus connect to Ks following DNA replication (224, 225). Such a situation could impose a requirement to actively reorient K-MT connections towards the newly duplicated pole. In agreement with this prediction, un-replicated chromosomes in *cdc6* mutants were found to connect to either the maternal or daughter spindle pole with similar frequencies, while chromosomes were attached exclusively to the pre-existing pole in *cdc6 ipl1* and *cdc6 sli15* mutants (216). These findings indicated that budding yeast are pre-disposed to form syntelic attachments and that Ipl1 is required to uncouple these aberrant connections in order for biorientation to proceed. Thus, these findings strongly implicated Ipl1 as a component of the error correction mechanism responsible for destabilizing improperly tensed chromatid-spindle attachments.

Importantly, the activity of Ipl1 in detaching un-tensed Ks appears to be tightly coupled to the ability of the SAC to detect attachment errors that are primarily characterized by the absence of tension. This was first suggested by the observation that the accumulation of syntelic attachments in *ipl1* cells did not appear to trigger cell cycle arrest (215). Furthermore, SAC activation (as monitored by Pds1 stabilization) in replication-defective *cdc6* mutants and cohesin-defective *mcd1* mutants was shown to require Ipl1 activity, conditions under which the

SAC is likely to be signaled by reduced bipolar tension (219). Conversely, *ipl1* mutants are proficient for SAC arrest following spindle disassembly with nocodazole-in this case chromatids are not tensed but Ks are detached from the spindle as a direct consequence of KMT depolymerization. Based on these observations, Ipl1 appears to be required for a sub-set of SAC responses in which Ks exhibit some form of attachment but are not placed under proper tension. Ipl1 has therefore been considered to formally define a tension sensitive branch of the SAC. Subsequent studies have examined whether the SAC response to genetic lesions within the K also requires Ipl1. Surprisingly, as first shown for mutations affecting the Mtw1 K protein (90), a large number of different K mutants activate the SAC in an Ipl1-dependent fashion (226). Furthermore, by several criteria, inactivating Ipl1 restores some level of K-MT attachment to K-defective strains (226). This is true even for K mutants, such as those affecting the Ndc80/Hec1 complex, that exhibit strong detachment defects. Thus, the apparent severity of many previously characterized K mutants is, at least in part, a reflection of Ipl1 K-MT uncoupling activity. These observations do not rule out a direct role for Ipl1 in signaling the SAC; for example, one recent paper has indicated that Ipl1 may promote SAC arrest by directly phosphorylating Mad3 (167). However, the bulk of the current data are most simply interpreted in terms of a model in which the roles of Ipl1 in error correction and signaling the SAC are linked. That is, Ipl1 promotes SAC signaling by generating unattached Ks, or at least acts to amplify weakly activating signals SAC signals by modulating K-MT interactions.

Aurora B function in other types of cells has been studied through a variety of approaches, including RNAi knockdown or dominant interference with Aurora B/CPC components (227-233) and through the use of hesperadin (234) or ZM447439 (146), two chemical inhibitors of Aurora B. In general, these studies have revealed that perturbations to Aurora B produce phenotypes similar to those observed in *ipl1* mutants, including an accumulation of chromosome alignment errors such as syntelic and merotelic attachments and defects in chromosome congression and segregation. Aurora B-depleted cells are also defective in activating the SAC in response to treatments-such as taxol-that reduce tension, but are proficient for SAC activation when unoccupied Ks are produced directly using nocodazole (146, 229, 232, 234, 235). Consistent with a failure to generate unattached Ks, Aurora B checkpoint defects are associated with reduced binding of SAC proteins such as BubR1 and Mad2 to Ks (146, 232, 236). Thus, roles for Aurora B in coupling error correction to SAC signaling appear to be generally conserved.

An additional, but related, function for Aurora B in vertebrate cells is in the correction of merotelic attachments. These alignment errors are detectable in at least 30% of prometaphase PtK1 cells (237, 238). In ~1% of cells, merotelic attachments persist into anaphase, producing lagging chromosomes where the misaligned K is pulled towards both spindle poles. Merotelic attachments do not appear to activate the SAC, probably because most of them only have a few mis-aligned KMTs and are therefore placed under significant levels of bipolar tension. Nonetheless, the majority of merotelic attachments are resolved prior to anaphase through Aurora B-dependent error correction mechanisms (239). This is all the more remarkable because Aurora B appears to be capable of specifically targeting only those KMTs that are oriented towards the incorrect pole. Given the prevalence of merotelic attachments and their ability to evade the SAC, this aspect of Aurora B error correction activity may be particularly important in preventing chromosome segregation errors that produce aneuploid cells.

6.4. Ipl1/Aurora B substrates

An important question concerns the identity of Ipl1 substrates that mediate K detachment from the spindle. A number of Ipl1 targets within the K have been uncovered using *in vitro* kinase assays and by mapping native phosphorylation sites, including inner (Ndc10-215, 240, Mif2-62), linker (Ndc80-213), and outer (Dam1-72, 212, 213, Spc34-213, Ask1-213) K proteins. Any combination of these could presumably be negatively regulated by Ipl1 to effect detachment, and one set of recent experiments has begun to address this systematically (241). Currently, Ipl1 substrates within the Dam1/DASH complex have received the most attention. Mutations abolishing phosphorylation at three Ipl1 sites in Dam1 and a single site in Spc34 were shown to produce chromosome segregation defects similar to *ipl1* mutants (213). Mutations at these sites also display genetic interactions with *IPL1*; perhaps most strikingly, certain combinations of phospho-mimetic mutations suppress *ipl1* phenotypes. These observations strongly suggest Dam1/DASH is an important Ipl1 target controlling detachment. However, a recent study has shown that, similar to other K mutants, eliminating Ipl1 phosphorylation on Dam1/Spc34 actually leads to SAC activation in an Ipl1-dependent fashion (226). In addition, these mutations can be shown to activate Ipl1 K detachment activity. Thus, Ipl1 presumably targets additional sites within either Dam1/DASH or other components to promote K detachment and checkpoint activation.

A likely candidate for such an additional factor is the Ndc80/Hec1 complex. In concert with the demonstration that purified *C. elegans* Ndc80/Hec1 could bind MTs *in vitro*, it was also shown that phosphorylation of Ndc80 by recombinant Ipl1 could antagonize this binding activity (96). Similarly, it was recently demonstrated that injection of an antibody to the N-terminus of Ndc80 into vertebrate cells resulted in a stabilization of K-MT interactions that is similar to what is observed following down-regulation of Aurora B, possibly because the antibody blocks Ndc80 phosphorylation by Aurora B (242). In support of this interpretation, over-production of N-terminal non-phosphorylatable mutants of Ndc80 also displayed Aurora B-defective phenotypes. In yeast, phospho-mimetic mutations at Ipl1 sites in Dam1 have been shown to reduce binding to Ndc80, and it was mentioned that Dam1 and Ndc80 phospho-mimetic mutant combinations produce a severe growth defect (241).

However, to our knowledge it has not yet been reported whether mutations that eliminate Ipl1 phosphorylation sites on both Dam1 and Ndc80 synergize to produce a more complete Ipl1-deficient phenotype (i.e., stabilization of syntelic attachments).

In vertebrate cells, an important downstream target of Aurora B in correcting merotelic attachments, and possibly other alignment errors, is mitotic centromere associated kinesin (MCAK), a member of the Kin I family of kinesin MT depolymerases (243). Like Aurora B, MCAK is required for the repair of merotelic and syntelic attachments (244, 245). Both proteins co-localize to inner *CEN* chromatin prior to biorientation (245-247) and a recent study has shown that Aurora B is required for MCAK enrichment at merotelic attachments that persist into metaphase (248). Thus, at least part of the role of Aurora B in destabilizing merotelic attachments may be exerted by actively recruiting (or retaining) MCAK depolymerase activity. MCAK has been shown to be an Aurora B substrate, but, counter to the predictions of a model in which Aurora B works through MCAK, Aurora B phospho-regulation antagonizes MCAK depolymerase activity, at least *in vitro* (249-251). However, the ratio of phosphorylated to unphosphorylated MCAK at merotelically attached *CEN*s does appear to change in a manner that would be predicted to increase the likelihood of MT depolymerization (248). Thus, the repair of merotelic attachments is likely to contain an additional regulatory step that promotes MCAK activity.

6.5. Sgo proteins as Ipl1/Aurora B regulators

Another area of recent interest concerns roles for the Shugoshin (Sgo) family of proteins in promoting Ipl1/Aurora B function. Sgo proteins have been primarily characterized as factors required to maintain sister chromatid cohesion at peri*CEN* regions (reviewed in 252). To summarize this data, during meiosis I in all organisms that have been examined, cohesion is selectively retained at *CEN*s because Sgo1 protects cohesin complexes from Polo-like kinase (Plk) phosphorylation at sites that potentiate separase cleavage. In vertebrate cells, cohesion at *CEN*s is also preferentially maintained in an Sgo1-dependent manner during mitosis. Current evidence suggests Sgo1 blocks cohesion dissolution in both meiotic and mitotic cells, at least in part, by recruiting the PP2A phosphatase to *CEN* chromatin, where it is thought to promote the dephosphorylation of cohesin subunits (65, 253). Alternatively, PP2A may counteract phosphorylation of other Plk1 substrates to maintain Sgo1 binding sites at *CEN*s (254).

Unlike vertebrate cells, Sgo1 in budding yeast is not required to protect *CEN* cohesion during mitosis. Instead, mutations in Sgo1 were recovered in a screen to identify SAC components necessary for cell cycle arrest in response to weakened chromatid cohesion (255). Like *ipl1* mutants, *sgo1* mutants were found to be proficient for SAC arrest in response to nocodazole treatment, but were unable to form correct bipolar attachments once the MT depolymerizing agent was removed. Thus, Sgo1 appears to promote both chromatid reorientation and SAC signaling in response to some types of tensioning defects. However, a recent study has shown that *sgo1* mutants are proficient for activating the tension branch of the SAC in response to K mutations (226); therefore, the role of Sgo1 in activating the SAC in budding yeast would appear to be more restricted than that of Ipl1. Work by Salic *et al.* (256) has shown that vertebrate Sgo1 binds strongly to MTs *in vitro*, and Sgo1 depletion results in spindle defects that are consistent with errors in chromosome alignment. These observations initially suggested Sgo1 might be connected to error correction/SAC signaling by forming contacts between chromosomes and KMTs that would allow it to function as a tension sensor.

In addition to a tension sensor role, several recent papers have provided evidence for a different scenario in which Sgo proteins function to localize Aurora B/CPCs within *CEN* chromatin. Unlike budding yeast, fission yeast and vertebrate cells contain two Sgo proteins, Sgo1 and Sgo2 (253, 257, 258). Sgo2 is not involved in maintaining cohesion but, in an analogous fashion to Sgo1 in budding yeast, Sgo2 is required to promote bipolar K-MT connections and to signal SAC arrest in response to cohesion defects (259, 260). A potentially key insight is that these phenotypes appear to correspond with a significant reduction in the recruitment of the Passenger proteins Aurora B, INCENP and Survivin to *CEN* chromatin. In vertebrate cells, down-regulation of Sgo2 by RNAi also leads to aberrant chromosome-spindle attachments (261). In this case, however, the localization requirements for Aurora B and Sgo2 appear to be reversed, with Aurora B being required for proper localization of Sgo2, and Sgo2 in turn being required for localization of MCAK. As introduced in Section 4, another important player in this series of interactions is the Bub1 protein kinase, which in vertebrate cells appears to play an initiating role in the recruitment of Sgo1, Sgo2 and the Aurora B/passenger complex (138). In budding yeast, a recent study has shown that Sgo1 is required to recruit Ipl1 to *CEN*/K regions during meiosis I (262), but it is not yet clear whether a similar dependency exists in mitotic cells. Thus, at a current level of resolution, these observations suggest a complex web of mutually reinforcing, phospho-regulated interactions that are required for optimal Aurora B activity. One possible way to explain the relative roles of Ipl1 and Sgo1 in activating the SAC is that by facilitating Ipl1/Aurora B recruitment, Sgo1 locally increases Ipl1 activity to a level necessary to respond to weakly tensed attachments, such as those resulting from partially reduced sister chromatid cohesion. On the other hand, attachment defects characterized by the complete absence of tension (for example, syntelic attachments) would not require this same amplification to promote SAC signaling.

To summarize this section, a considerable body of evidence supports a model in which the critical role for Ipl1/Aurora B in controlling the SAC is to convert tension-deficient chromatid-spindle attachments into unoccupied Ks. Importantly, downstream targets and upstream regulators of Ipl1/Aurora B are beginning to emerge that will help define the workings of error correction pathways that respond to tension. In particular, the structural basis of

"detachment" is an interesting and open question. For example, as mentioned above (Section 6.2), the detachment function of Ipl1 is activated in *cdc6* mutants, yet chromatids remain sufficiently connected to segregate with spindle poles during reductional anaphase spindle movements. Furthermore, since Ipl1 is activated by numerous K mutations, it is puzzling that different K alleles do not display a more uniform detachment defect. To explain this, one speculative possibility is that Ipl1 only uncouples a subset of attachment points within the K-MT interface, or that lateral attachments are not affected by Ipl1 activity. Alternatively, detachment may be complete but reattachment occurs rapidly due to counter-acting phosphatases or a high density of spindle MTs. In either scenario, the operational parameters for detachment are presumably to create a binding site for Mad2 and/or other SAC components for a sufficient period of time to block APC/C activity, while at the same time allowing reorientation to proceed quickly.

6.6. Tension assessment and tensiometers

This overview of how the SAC is activated by tension defects leads to a critical issue, namely the manner in which Aurora B is coupled to tension. As discussed above, insights into this facet of Aurora B regulation are beginning to emerge. As a direction for future studies, we suggest there are two main areas in which a clearer understanding would facilitate analysis of tension assessment mechanisms. First, we need to understand how the structural elements that link bioriented Ks to the spindle deform into a configuration that registers tension. The requirement for some sort of tensile apparatus within *CEN* chromatin or the K-MT interface is intuitively obvious, but the mechanical basis for constructing such an apparatus is likely to be complex. For example, bioriented chromatids are perceived as being continuously tensed, even though they undergo oscillatory movements that would appear to transiently dissipate tension. From a regulatory standpoint, how is this achieved? Second, tension assessment requires a tensiometer, which we define here as a mechanism that responds to a particular mechanical read-out of tension by converting this information into a specific form of regulation. Given the two principal uses for tension introduced in Section 6.1, we therefore suggest that there are likely to be at least two types of tensiometers-one controlling Aurora B activity or access to substrates and a second controlling chromosome oscillatory movements.

6.7. *CEN* chromatin dynamics and tension

The most obvious candidate for a deformable tensile element within the spindle is *CEN* chromatin, an idea that is to varying degrees implicitly or explicitly integrated into many models of spindle dynamics. Microscopic studies in a number of organisms have shown that the chromatin sub-adjacent to the K stretches during biorientation, sometimes quite extensively. In diatoms, for example, K-associated fibers are pulled out from the chromosomes and stretched until they nearly span the entire distance between the spindle poles (263). The first indication that *CEN*s could actually behave in an elastic fashion, however, came from the work of Shelby *et al.* (264), who used GFP tagged CENP-B to visualize centric α-satellite repeats in human cells. This analysis revealed inner *CEN* chromatin underwent dynamic cycles of extension and relaxation as chromosomes established bipolar connections and were placed under tension. More recently, an analogous phenomenon (variously called *CEN* breathing, *CEN* splitting or precocious *CEN* separation) has been observed in yeast using GFP chromosome tagging techniques or by visualizing Cse4-GFP (43, 44, 265-267). From these studies, it is now quite clear that, prior to anaphase onset, the single MT attached to each K in budding yeast can generate sufficient traction to pull chromatids apart at *CEN* proximal regions, resulting in sister Ks being drawn into two uniform clusters located between the equator and poles of the metaphase spindle. The separated *CEN* regions then typically oscillate as two discrete foci that rarely cross the spindle equator, although they occasionally re-associate and split again. The foci also quickly ($t_{1/2}$ ~ 7 min) rejoin when the spindle is disassembled, revealing separated *CEN* regions remain under tension and are coupled by an elastic restorative force (268).

Several features of yeast *CEN* dynamics merit further comment, as they may have a bearing on the mechanochemical processes controlling tension. Progressively increasing the distance with which GFP chromosome tags are placed from the *CEN* has revealed that tags ~10 kB from the *CEN* undergo a high frequency of separation, while tags integrated at more *CEN*-distal regions remain tightly cohered (43, 44). Thus, there appears to be a boundary beyond which further un-zippering of the chromatids does not occur. In yeast, cohesin complexes are preferentially deposited over an ~50 kB *CEN*-flanking region through a little understood, K-dependent, loading pathway (46-48, 269). This enrichment appears to help define *CEN* separation boundaries, as cohesin is required to restrict *CEN* splitting (266). In a seemingly paradoxical manner, however, cohesion must also be locally released to allow *CEN* splitting to occur, and at present it is unclear how this is achieved. Although *CEN* splitting does not require separase, a recent study has shown that the application of bipolar tension does lead to a decrease in *CEN* cohesin density (49). This does not appear to be accompanied by a corresponding increase in cohesin at more *CEN*-distal regions, suggesting cohesin rings do not slide away from the point of K attachment. An alternative fate for *CEN* proximal cohesin complexes has recently been suggested by microscopic studies indicating some cohesin may encircle separated *CEN* chromatin regions (86). This observation has led to the intriguing hypothesis that some fraction of cohesin may reorganize during biorientation to form intra-strand cohesive linkages that stabilize separated chromatid fibers in a folded back hairpin configuration (Figure 4).

Another interesting aspect of yeast *CEN* dynamics concerns the extent of chromatin stretching during biorientation. Since both the amount of *CEN* chromatin that separates and the distance to which Ks are drawn apart are known, it can be estimated that separated *CEN* fibers exhibit a packing ratio of 10 to 20 relative to B-form DNA (43). This implies

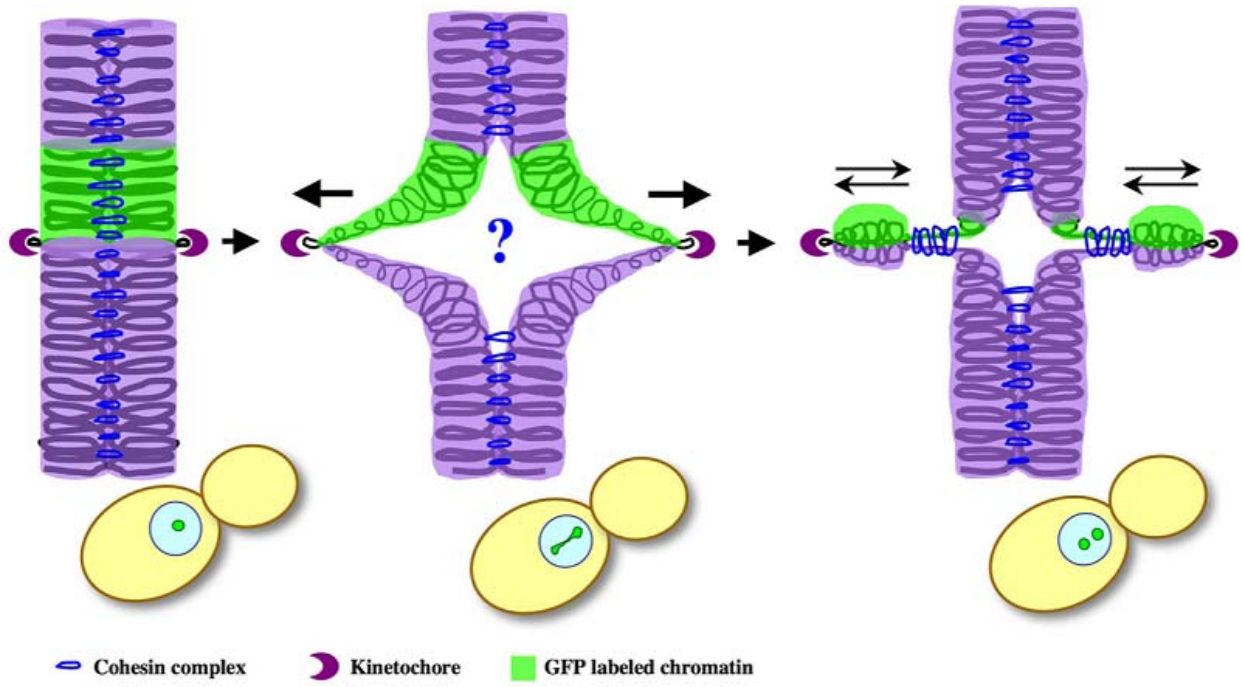

Figure 4. Hypothetical Depiction of *CEN* Chromatin Dynamics during Biorientation. As sister chromatids biorient towards opposing spindle poles, the K and *CEN* proximal chromatin come under tension. GFP labeled *CEN* chromatin can be observed to transiently stretch into a filamentous configuration. *CEN* separation in response to spindle traction is presumed to reflect release of cohesion through an unknown mechanism (question mark), and some cohesin complexes may reorganize to form intra-strand C-loop linkages. In theory, this could allow separated *CEN* regions to exhibit the re-compaction that accompanies successful biorientation without rejoining. The separated chromatin foci could then behave as distinct mechanical elements during chromatid oscillatory movements.

that, on average, separated *CEN* regions unravel beyond the level of compaction expected for the 30-nM fiber (packing ratio ~40). In fact, it has been suggested that the distension of *CEN* chromatin may be of sufficient magnitude to actually displace nucleosomes from portions of the chromatid fiber (44, 270). While this has not been demonstrated directly, chromatin-pulling experiments indicate nucleosomes dissociate from DNA at a force of around 20 pN (271), and, if chromatin is pulled in protein extracts containing ATP, nucleosome disassembly can be achieved at forces as low as 2 pN (272). These values are well within the estimated range of force generated by depolymerizing MTs (108) or the stall force of Ks translocating towards spindle poles (273). A direct relationship between chromatin packaging and *CEN* stretching has recently been demonstrated by transiently repressing histone H3 and H4 expression, resulting in an ~ two-fold reduction in nucleosome density (274) Under these conditions, *CEN*s undergo even further deformation, corresponding with an overall increase in the length of the metaphase spindle. These results indicate chromatin packaging is one of the main factors controlling the elastic resistance of *CEN* fibers to deformation. The change in spindle length is also telling, as it implies that the extent of *CEN* stretching is proportional to the magnitude of the inward directed tensile force that restricts pre-anaphase spindle extension.

A final component to yeast *CEN* dynamics that has received relatively little comment is that early in the process of establishing bipolar connections GFP tagged *CEN* chromatin deforms into a linear extended configuration, as would be expected for a uniformly tensed DNA segment (44). However, the labeled chromatin subsequently reorganizes to form two GFP foci that, although still separated and under tension, appear to have undergone a spatially limited compaction (43, 44, 265). It is intriguing that the stretched chromatin relaxes in this fashion without contracting more uniformly. These observations seem to imply that the chromatin deformation associated with *CEN* tensioning may not be uniformly distributed along the chromatid fiber. Figure 4 depicts a speculative scenario for how this might occur. In this view, once bipolar connections are established sister Ks are rapidly pulled towards opposite spindle poles. The resulting stretch of *CEN* chromatin, and thus induction of tension, could be limited by at least three processes. One is the breaking (or regulated disassembly) of cohesive connections, some of which may reform as intra-strand C-loop linkages. A second is nucleosome displacement, which would tend to dissipate tension by increasing the resting length of the separated chromatid fibers. A third would be a switch in the mode of K-MT attachment (for example, from lateral to end-on attachment), or a switch in KMT dynamics that reduces the pulling force. As tension

dissipated through any of these mechanisms, formation of the C-loop could conceivably prevent the separated regions from recoiling uniformly. In theory, this could produce distinct tensile elements that, in essence, functioned as a linked pair of springs.

Obviously, the tenets of this model remain to be supported or refuted by further experimentation. The main intent is to begin to rationalize how different read-outs of tension could be compartmentalized within the spindle, potentially allowing chromatids to be perceived as being continually tensed while oscillating back and forth along the spindle axis. In summary, the cohesive properties of peri*CEN* chromatin appear to be calibrated to strike an appropriate balance between chromatid separation and stretching in response to spindle traction. The elastic resistance of separated chromatid fibers to stretching may in turn define the "spring constant" that controls how much tension is generated within the spindle. It is also possible that tension is stored in a sophisticated fashion to regulate different aspects of spindle dynamics.

6.9. Tensiometers

How might a tensiometer "read" tension and convey regulatory information? The original proposal of McIntosh (275) was that *CEN*s would contain tension responsive enzymes that produce a diffusible inhibitor of anaphase. Placing chromatids under bipolar tension would inactivate these enzymes, and after the last chromosome bioriented anaphase would ensue once the inhibitor decayed below a threshold concentration. From a current standpoint, the inhibitor would appear to be MCC complexes, and in the case of syntelic and possibly other tension deficient attachments this inhibitor may be produced indirectly through the activity of Ipl1/Aurora B in creating unoccupied Ks. We are thus left with the issues of how Ipl1/Aurora B is coupled to tension and only targets those attachments that are incorrectly tensed. One elegant model has been proposed in which inner *CEN* chromatin is the source of a relatively steep gradient for active Aurora B (216, 239, 276; this model is incorporated into the error correction diagram shown in Figure 2). Correctly tensed K-MT attachments are stabilized because Aurora B substrates within the K are simply physically displaced from the kinase by *CEN* stretching. Conversely, attachments that do not generate stretching are more susceptible to the uncoupling activity of the enzyme. The actual tensiometer device in this case would consist of the factors required to localize and activate Aurora B within inner *CEN* chromatin (e.g. other passenger components, Sgo proteins, Bub1; see Section 6.5).

One attractive feature of this model is that Aurora B does not need to be silenced to allow biorientation to occur; thus, if problems arise error correction mechanisms do not need to be reactivated. In addition, this model can also explain how Aurora B might selectively target those KMTs on merotelically-attached chromosomes that are oriented towards the wrong pole. Assuming sufficient flexibility within the K, portions of the K plate that are oriented towards the correct pole will be pulled away from Aurora B, while portions that are pulled in both directions are more likely to be subjected to detachment (239, 276). One caveat is that while this model fits with the localization of Aurora B in vertebrate cells, it is less clear whether it applies to budding yeast given the difficulties in defining the location of active Ipl1 during biorientation. In particular, Ipl1 might be predicted to bind at the junction between separated and unseparated *CEN* regions (the base of the C-loop; Figure 4), which arguably is the closest equivalent to inner *CEN* chromatin in yeast.

A different tensiometer model has recently been proposed based on the work of Sandall *et al.* (277). It has previously been shown that beads coupled to *CEN* DNA will bind to MTs *in vitro* when incubated in a yeast cell extract (215, 240, 278-280). This interaction requires the inner K CBF3 complex as well as other proteins in the lysate, and these investigators devised a biochemical fractionation to identify these factors. Surprisingly, rather than one of the K components currently thought to mediate MT binding-such as Dam1/DASH or Ndc80/Hec1- they isolated Bir1 using this strategy. Furthermore, they were able to show that a purified complex consisting of Bir1 and Sli15 appeared to be the only extract components required to link CBF3 to MTs in this assay. As Sli15 has been shown to bind MTs *in vitro* (212) and Bir1 interacts with the Ndc10 subunit of CBF3 (281), Bir1/Sli15 complexes possess binding activities that could mediate such a direct linkage. Importantly, the authors found that deleting the MT-binding domain of Sli15 resulted in an *ipl1*-like phenotype in which both sister chromatids segregated to the same spindle pole. Thus, the ability of Sli15 to bind MTs is not necessary for Ks to attach to the spindle, but apparently is required for Ipl1 to resolve syntelic attachments.

These results (277) suggested a model in which the Bir1/Sli15 passenger complex could form a tension .sensitive coupling device for regulating Ipl1. In this view, in the absence of tension the Bir1/Sli15 complex would be in a conformation that allowed Ipl1 to bind to the IN box domain of Sli15, thereby directing Ipl1 to inappropriate K-spindle connections. Conversely, once tension was applied this binding site would no longer be available, effectively stabilizing K attachment. This model is not necessarily incompatible with the "displacement" model discussed above, and in fact both tensiometer mechanisms could work in tandem. For example, the Ipl1 targeting mechanism identified by Sandall *et al.* (277) could be primarily involved in recruiting active Ipl1 onto Ks that were not sufficiently displaced from a "source" of the kinase within *CEN* chromatin. This recruitment might be critical for optimally positioning Ipl1 to phosphorylate K substrates that lead to detachment.

Another way in which the K-MT interface might function as a tensiometer is by regulating KMT dynamics during chromosome oscillatory movements (42, 282, 283). As introduced in Section 6.1, chromosome congression can be simulated using computer models in which increasing tension promotes KMT rescue, allowing oscillating chromosomes to resume movement towards the equator of the spindle (2). It has been proposed that the K might influence KMT polymerization reactions through a purely physical effect whereby, with increasing tension, the

proposed K sleeve encircling the MT was pulled back towards the plus end tip (2, 45). This could have the effect of preventing further outward curling of MT protofilaments, thus increasing the probability of KMT rescue. As with other tensiometers, this deformation of the K sleeve would presumably be mechanically linked to developing tension within the *CEN* chromatin spring. If so, there must be some sort of differential regulation to ensure that the reduction in tension associated with the switch to anti-poleward movement did not trigger Ipl1 to detach the K. This could presumably be achieved through several strategies. 1) Ipl1 could be inactivated on bioriented chromosomes. 2) Tension may not dissipate sufficiently during oscillatory movements to trigger Ipl1. 3) The duration of anti-poleward movement may be too short to effectively activate Ipl1. 4) The amplitude of oscillatory movements may ensure Ks remain sufficiently distant from the source of active Ipl1 such that catastrophes are likely to occur before detachment. These considerations highlight some of the complexities associated not just with tension assessment but also with coordinating the different uses of tension within the spindle.

7. SAC TARGETS: INHIBITORS OF ANAPHASE

Above we have discussed K structure, the tension-sensitive properties of Ks that facilitate error correction and the means by which unattached Ks generate a "Wait Anaphase!" signal. Next, we turn our attention to the APC/C substrates that must be degraded to permit anaphase onset. The known mechanism of the SAC is to monitor biorientation of chromosomes and establish inhibitory MCCs that bind or phosphorylate Cdc20 in order to inhibit the APC/C. Pds1/Securin is the key (and only known) anaphase inhibitor in *S. cerevisiae* (284, 285). But, another target of the APC/C, in all eukaryotes, that is degraded at the time of anaphase onset, is cyclin-B. Much debate has centered on whether or not cyclin-B degradation is necessary for sister chromatid separation (286-289). Strong evidence indicates that, at least *in vitro*, Separase can be kept inactive through phosphorylation and direct binding by the mitotic kinase Cdk1/cyclin-B complex (287, 288). Other reports appeared consistent with this because they indicated that at near-to physiological levels, stable cyclin-B could block separation of sister chromatids and arrest mammalian cells in metaphase (290). Moreover, 1.5-2 fold higher levels of cyclin-B than the amount normally present in mitotic cells can inhibit Separase (reviewed in 291). However, this was clearly not the case in yeast, where over-production of stable B-type cyclin freely permitted anaphase progression and resulted in telophase arrest (292). The apparent difference between yeast and higher cells now appears to have been resolved by a recent report that suggested stable cyclin-B does not prevent sister chromatid separation, but instead prevents chromosome segregation in human cells (289). These processes were cleverly distinguished by expressing stable cyclin-B in cells lacking the Kid chromokinesin motor which normally produces polar ejection forces (known as polar wind) that may contribute to chromosome congression in prometaphase (293) and, during metaphase, keep chromosomes correctly oriented in the vicinity of the spindle equator (294). With Kid, the stable cyclin-B appeared to result in metaphase arrest, based on time-lapse analysis of cells expressing GFP-tagged histone to visualize chromosomes (i.e. the chromosomes remained at the metaphase plate) (289). However, in the absence of Kid, those chromosomes segregated to the spindle poles, revealing that the stable cyclin was most likely not blocking separation of sister chromatids, but rather was preventing their segregation. The implication is that cyclin-B needs to be degraded upon anaphase onset to turn off the polar wind and allow anaphase A chromosome movements to the spindle poles. Threonine 463 of Kid has been shown to be a cdc2-cyclin B target site during mitosis in mammalian cells, and the behavior of a T463A mutant Kid is consistent with phosphorylation of that site being important for Kid-mediated polar ejection forces (295). Thus, Kid could be inactivated in anaphase due to dephosphorylation of T463 following cyclin B degradation. However, because Kid is itself degraded upon anaphase onset in an APC/C dependent manner, it will be important to determine if cyclin B degradation is a necessary pre-requisite for Kid degradation.

If cyclin B does not control sister chromatid separation at the metaphase-anaphase transition, then does Pds1/Securin alone perform this task? In yeast cells lacking Pds1, some ability to retain sister chromatid association remains, although it is clearly less robust than in wild type cells (284). Similarly in mammalian cells lacking Securin, c-mitosis arrest can be transiently achieved (296). Securin-/- human cells have been reported to have an increased rate of chromosome loss (296), but the reported massive chromosomal instability of Securin-/- cells (296) appears to have been overstated because more recent studies indicate that, at best, this instability is temporary, lasting maybe 8-12 cell cycles, before the Securin-/- cells become chromosomally stable and perform anaphase normally (297). These data are made all the more remarkable by the observation that the chromosomally stable Securin-/- cells have dramatically reduced levels of Rad21 cleavage in the presence of nocodazole despite Separase apparently being active (based on the presence of the auto-cleaved form of Separase) (297). These results also indicate that active Separase is not sufficient for chromatid separation because despite Separase autocleavage the cells do not lose sister chromatid cohesion prematurely (297). Together with the fact that Securin-/- mice are viable and that cells derived from these animals show normal spindle checkpoint activation in the absence of a spindle (i.e. sister chromatids remain cohered at their centromeres in Securin-/- cells even after a 24 hour treatment with colcemid, see 298), we are led to conclude that a mechanism independent of Securin can regulate anaphase accurately. Because the above experiments revealed cyclin-B is unable to prevent sister chromatid separation at physiological levels, this mechanism is likely to be as yet unknown (289). Thus, a growing number of observations strongly suggest that a Securin/Separase regulatory circuit controlling sister chromatid cohesion is insufficient to explain SAC cell cycle arrest at the metaphase to anaphase transition (185). First, that Cdk/cyclin-B does not function to prevent sister chromatid separation; second, that Securin is dispensable

for highly regulated mitotic progression, even in the context of a whole organism; and third, that Separase activity does not appear to correlate with sister chromatid separation.

8. ADDITIONAL SAC CIRCUITRY

The above experiments indicate that Securin cannot be the sole SAC target and that additional mechanisms must inhibit anaphase. If this hypothesis bears out, then presumably the alternative mechanisms would inhibit Esp1/Separase. However, this, even, is not necessarily this case. Recent data have revealed a possible differential control of sister chromatid cohesion in yeast by Esp1-dependent and independent mechanisms. Cdc55 is the B regulatory subunit of protein phosphatase 2A (PP2A) in *S. cerevisiae*; its deletion does not result in inviability, but cells lose cohesion at centromeres and along chromosome arms in the presence of either nocodazole or telomere damage (in *cdc13-1* mutants) (299). In the nocodazole experiment, loss of cohesion was accompanied by a slight decrease in Pds1 levels and cleavage of a minor pool of cohesin Mcd1, though it was not determined whether these biochemical events were required for the observed loss of cohesion in cells. Moreover, loss of cohesion in *cdc55* null cells was at least partly independent of Esp1 in the presence of nocodazole and also occurred in cells that became arrested in G2/M by the induction of non-degradable Pds1. Altogether, these studies indicate that there are alternative mechanisms through which cohesion can be dissolved that do not rely exclusively on Esp1. Cdc55 might function to inhibit such activities, or might instead enhance the activity of factors that promote cohesion independently of the Pds1/Esp1 relationship. In either case, the Cdc55-related cohesion function may be targeted by SAC pathways that are yet to be described.

The above arguments indicate that there are mechanisms that can regulate anaphase onset independently of Pds1/Esp1 regulation. While our current understanding does not completely rule out cooperation between Cdk-cyclin and Pds1/Securin to inhibit Esp1/Separase as the sole mechanism of anaphase control, it perhaps would not be entirely unexpected if Separase modulation represents only one anaphase regulatory mechanism. In an exclusive model, where cohesin removal from chromosomes by Separase at anaphase onset is paramount, Separase ought to remain inactive during the rest of the cell cycle. It might even have been expected that Separase would be absent for most of the cell cycle. But, in yeast for example, Esp1 is present during the entire cell cycle (300). Moreover, roles for Separase before the metaphase to anaphase transition have been described (301, 302). In mammalian cells in particular, Separase activity is needed for timely initiation of mitosis (301, 302), and this might be linked to evidence that a lack of Separase causes defects in chromosome formation (301), presumably reflecting a role in S-phase. Separase also appears to function in proper spindle morphogenesis as human cells lacking Separase have prometaphase defects (301, 302), increased monopolar spindles (302), fail to properly resolve sister chromatids in prometaphase (301) and are apparently unable to disassemble nucleoli at the correct time in prophase (301). In yeast, defects in spindle elongation and stability are observed (300, 303).

In summary, the goal of the SAC is to block sister separation. A key SAC function is regulation of the APC/C^{Cdc20}-Pds1-Esp1 circuit. But there may be additional pathways required to maintain cohesion during extended arrest periods and the SAC might need to promote their activity through a completely independent circuitry to prevent sister separation.

9. TOPO II AND THE SAC

We were motivated to write this review in part because of accumulating evidence that, while still fragmentary, points towards DNA topoisomerase II (topo II or Top2 in yeast) as a novel factor influencing SAC signaling. Photobleaching studies have shown that topo II is remarkably dynamic inside the nucleus, exchanging rapidly between chromosomally bound and unbound pools (304, 305). Superimposed on this mobility, there also appear to be cell cycle regulated mechanisms that direct topo II to specific chromosomal regions. In particular, live and fixed cell studies have shown that topo II preferentially accumulates around vertebrate *CEN*s during mitosis (304-308). This enrichment is accompanied by robust topo II activity within *CEN* chromatin (309, 310). Preferential topo II cleavage sites have also been mapped onto *CEN* DNA (311, 312).

Although the manner in which topo II accumulates at *CEN*s is not well understood, current evidence suggests that SUMO modification may be one regulatory mechanism that is involved. In both yeast and vertebrates, SUMO conjugated forms of topo II accumulate specifically during mitosis (133, 268). In *Xenopus* extracts, blocking SUMO conjugation increases the fraction of topo II retained on mitotic chromosomes (133). In addition, in mammalian cells RNAi knockdown of PIASγ, the SUMO ligase for topo II and other mitotic substrates (313), reduces topo II enrichment at *CEN*s (308). From these results, SUMO modification may play a role in promoting topo II turnover on chromosomes, thereby facilitating *CEN* recruitment. Whether Top2 SUMO modification serves a similar function in yeast is currently less certain. A recent ChIP experiment has shown that a Top2-SUMO fusion protein exhibits robust cross-linking to *CEN* DNA (314). However, eliminating Top2 SUMO modification using the *top2-SNM* allele (in which SUMO acceptor lysines are replaced with non-conjugatable arginines) does not obviously reduce Top2 ChIP to *CEN*s (J.B., *unpub. obs*).

A complete analysis of the functional significance of topo II enrichment at *CEN*s is beyond the scope of this review (the reader is referred to 315 for an analysis of this topic). To cite a few relevant observations, one early study showed that treating cells with high concentrations of ICRF-193, a topo II inhibitor that traps the enzyme on DNA in the closed clamp conformation, blocks topo II accumulation at *CEN*s and causes inner *CEN* chromatin to become less organized (306). Furthermore, in *Xenopus* extracts and mammalian cultured cells, treatments

that interfere with topo II SUMO modification prevent chromatids from disjoining efficiently (308, 313). Finally, in both yeast and vertebrate cells topo II is required for sister separation when cohesin function is perturbed (316-318). Perhaps the simplest interpretation of these observations is that catenation between sister chromatids is modulated by topo II at *CEN* regions as a means to reinforce cohesin-based chromatid linkages. However, the relationship between topo II and cohesion may be more complex. For example, chromosome topology could influence the distribution or stability of cohesin complexes. It has also been suggested that, as a dimeric DNA binding protein, topo II could play a structural role in linking sister chromatids in a manner analogous to cohesin (319). Despite the uncertainties surrounding how topo II functions within *CEN* chromatin, it has become clear that topo II disruption activates checkpoint pathways that overlap extensively, if are not identical to, the SAC. In mammalian cells, perturbing topo II during mitosis through a number of means-including topo II poisons, which stabilize the topo II-DNA cleavable complex, catalytic inhibitors such as ICRF-193, and topo II depletion using RNAi-all cause cells to delay progression through mitosis at the metaphase-anaphase transition (reviewed in 320). This topo II-responsive metaphase checkpoint has uniformly been shown to be Mad2-dependent, and can also be overridden by treatment with the Aurora B inhibitor ZM447439 (308). In budding yeast, commonly used *top2* alleles such as *top2-4* do not activate pre-anaphase checkpoint mechanisms (321). However, a new group of *top2* mutants has been identified that are distinct from *top2-4* in that they transiently block anaphase spindle extension (322). This delay is necessary to prevent a dramatic chromosome segregation defect and requires all SAC components that have been examined, including both Mad2 and Ipl1.

Why would inhibition of topo II appear to activate the SAC? Two competing models have been proposed. In the first, DNA breaks in the vicinity of *CEN*s produce "wounded" Ks that experience difficulties in attaching to the spindle (323). In contrast, the second model posits the existence of a Mad2-dependent checkpoint that is operationally distinct from the SAC (324). This checkpoint monitors catenation between sister chromatids (as originally proposed in 325) and restrains anaphase until entanglements fall below a threshold level. A fundamental distinction between these models is whether the signal that activates the topo II-responsive checkpoint is generated through unoccupied Ks. Perhaps not surprisingly there is conflicting data on this point. Based on photon counting studies, it has been reported that Mad2 and Bub1 are not enriched at Ks during ICRF-193-induced metaphase arrest (324). In contrast, a different group reported that at least one K per cell retained Mad2 staining using this same inhibitor (323). In budding yeast, it should be possible to address this issue unambiguously using the *ndc10-1* mutant. As described in Section 4, *ndc10-1* cells are unable to assembly Ks and are completely defective for SAC signaling. It will therefore be informative to examine whether the topo II-responsive checkpoint is abolished in an *ndc10-1* mutant background.

In our view, the requirement for Ipl1/Aurora B in activating the topo II-responsive checkpoint suggests an alternative interpretation that encompasses elements of both these models. We propose that, either by resolving catenation between sister chromatids, by influencing the distribution/stability of cohesin linkages, or by modulating other aspects of *CEN* topology, topo II functions as a determinant of the tensile properties of *CEN* chromatin (Figure 5). That topo II can in fact alter tension is supported by experiments in which cohesin was depleted from either yeast or mammalian cells, resulting in defective biorientation and activation of the SAC (317, 326). These defects could be corrected by down-regulating topo II activity, which was interpreted as evidence that catenation was sufficient to restore tension and silence SAC signaling. By extension, in the presence of functional cohesin linkages *CEN*s might be prone to separate less efficiently following topo II inhibition, and this could be construed by Ipl1/Aurora B-dependent surveillance mechanisms as a deficit of tension; for example, because Ks were not sufficiently displaced from active Ipl1/Aurora B (see Section 6.8). In potential support of this idea, one study found that the metaphase plate appeared less organized in ICRF-193-treated cells, with a more variable distance between sister Ks than in untreated controls (318). But again there is conflicting data, as a different study failed to observe this difference (324).

If the above arguments are correct, it is puzzling that yeast *top2-4* mutants do not activate topo II-responsive checkpoints even though they exhibit a lethal defect in de-catenating sister chromatids. It is therefore necessary to assume that other aspects of Top2 function beside catenate resolution influence checkpoint signaling. In this context, recent data from one of our labs indicates *top2-4* and *top2-SNM* mutants share a similar phenotype in that *CEN* chromatin is prone to stretch extensively when placed under tension. Chromatids biorient correctly, but Ks are pulled further apart and pre-anaphase spindle length is extended (J. B., *unpub. obs*.). Such an increase in K-K separation would not be expected if sister *CEN*s were simply catenated together more tightly. Intriguingly, *CEN* hyper-stretching is accompanied by defects in a sub-set of Ipl1-dependent checkpoint responses (J. B., *unpub. obs*.). As discussed in Section 6.3, mutations affecting Mtw1 exhibit weakened K-MT attachments, activating Ipl1 to detach Ks from the spindle and elicit SAC arrest (90). In *mtw1top2-4* and *mtw1top2-SNM* cells, however, chromatid attachments are restored and cells progress through the metaphase to anaphase transition more rapidly. Thus, in budding yeast it would appear that different *top2* mutants either activate an Ipl1-dependent checkpoint response, or, conversely, potentiate Ipl1 assessment of certain tension defects. To explain this, we hypothesize that a correct calibration between *CEN* separation and stretching during biorientation is influenced by at least two distinct aspects of *CEN* topology (Figure 5). As discussed above, modulation of catenation may provide a means to control the extent of *CEN* separation. But placing Ks under tension could also be accompanied by torsional/winding problems along individual *CEN* chromatid fibers. Conceivably, accumulation of this type of strain in some *top2* mutants

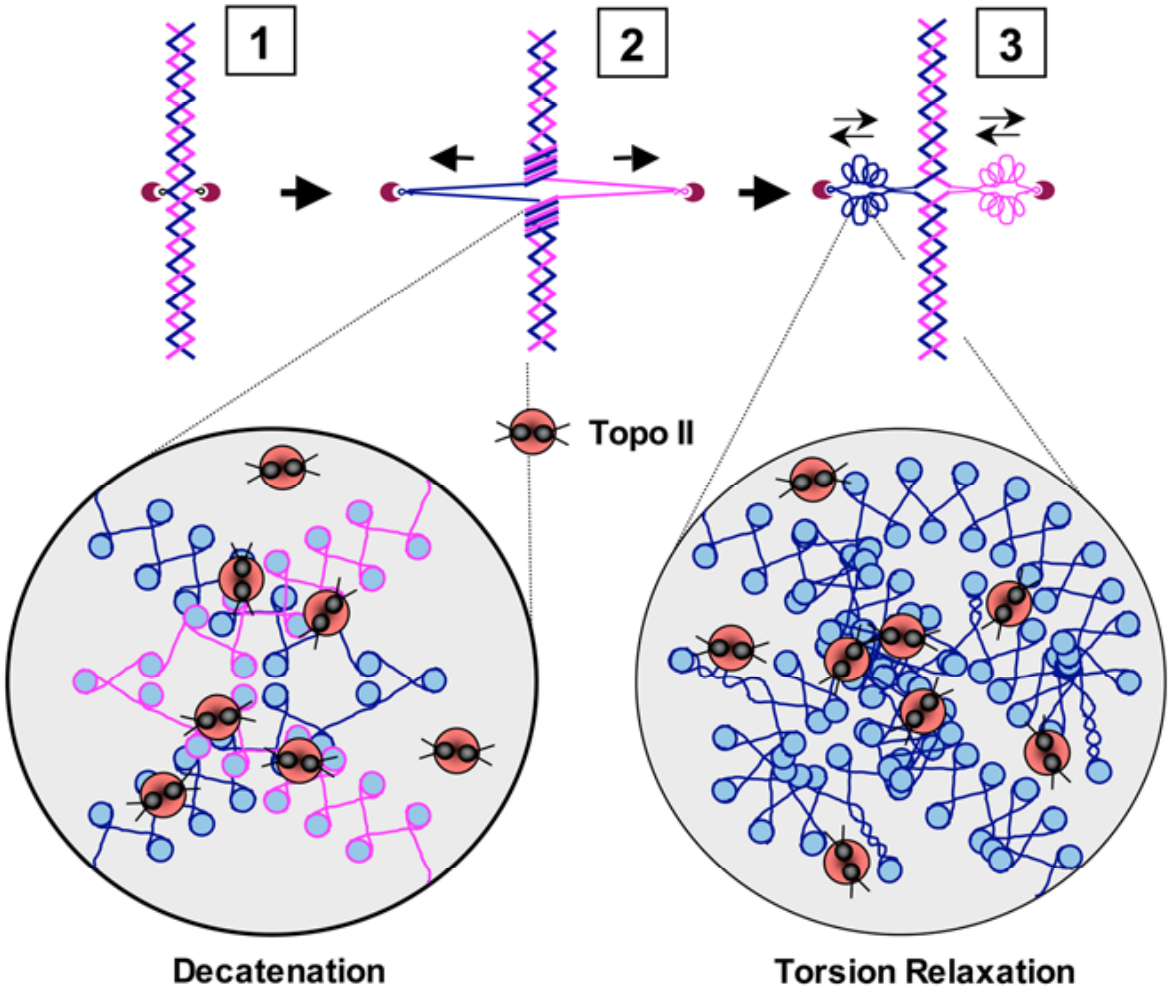

Figure 5. Possible Enzymatic Roles for Topo II within *CEN* Chromatin. 1) It is possible that catenates remain between mitotic sister chromatids at *CEN* regions, although the extent of such intertwining is unclear. For simplicity, cohesin linkages are not depicted. 2) *CEN* separation during biorientation may induce localized chromatid over-winding. The decatenation activity of topo II may allow *CEN*s to separate appropriately (inset). 3) The apparent re-compaction of separated *CEN* chromatin following biorientation suggests a localized relaxation of stretched chromatid fibers. Topo II may act to relieve torsional stress so recompaction/rewinding can occur efficiently (inset). By influencing the extent of *CEN* separation/stretching, it is proposed that topo II impacts how tension is generated within *CEN* chromatin. This may in turn affect tension surveillance mechanisms and SACsignaling.

might reduce the tendency of stretched *CEN* chromatin to undergo elastic recoil, allowing even weakened K-MT attachments to distend *CEN* chromatin into a configuration that registered tension (for example because the K could be more easily stretched away from a source of active Ipl1/Aurora B). Different topo II mutations or inhibitors might be expected to differentially affect these aspects of *CEN* topology, leading to complex effects on Ipl1/Aurora B signaling. It is our hope that a more comprehensive analysis of K-K tension and Ipl1/Aurora B activity following perturbations to topo II will provide a means to unravel this possible connection between chromatin mechanics and tensiometer function.

10. SUMMARY AND FUTURE DIRECTIONS

As a characteristic of eukaryotic cells undergoing mitosis, the SAC was described as early as the 1950s based on video microscopy of *Haemanthus* chromosome movements. Since that time we have gained detailed insight into the molecular events that delay sister chromatid separation and segregation until each and every chromosome is properly bioriented at the metaphase plate. The success of this research field can be largely attributed to the combined powerful genetic approaches possible in simple eukaryotes and the accurate descriptive (often phenomenological) work of microscopists studying larger eukaryotic cells. Both approaches have been instrumental

and yet important questions remain, several of which we will briefly summarize here in closing.

In considering K structure, a first issue is that there likely is a pre-biorientation K and a post-biorientation K that exhibit intrinsic structural differences. These differences influence SAC signaling, presumably at least in part by controlling the binding of SAC factors (such as Mad2) regulating MCC production. Understanding the molecular basis of these structural transitions and how they are influenced by different modes of KMT attachment and the action of error correction mechanisms is an important remaining challenge. Ultimately, attainment of a proper MT-K attachment state, or the tension generated upon successful biorientation, are likely events to promote this re-organization of the K. A second emerging issue is that tension/attachment assessment mechanisms appear to be coupled to a network of phospho-regulated interactions that are currently conceived as creating "binding sites" for regulators such as Ipl1/Aurora B within *CEN* chromatin. Further molecular elaboration of what a chromatin-binding site actually means will likewise be important for understanding error correction mechanisms and how they interface with SAC signaling.

Historically, understanding the mechanisms by which checkpoints delay cell cycle progression has proven to be a powerful approach for dissecting the molecular basis of cell cycle transitions. This may prove especially true for the SAC, as the evidence outlined in this review suggests we have arrived at a point where the mechanism(s) of anaphase restraint controlled by the SAC appear to be only partly understood. In yeast, Pds1 clearly plays a significant role, but in higher cells Securin is dispensable even in the context of a whole organism (in mice). Other, more fragmentary, evidence hints that Separase may not be the sole activity responsible for anaphase initiation. It will therefore be important to gain an understanding of the additional regulatory circuits that contribute to the inhibition of anaphase by the SAC and to probe further into the mechanisms that lead to sister chromatid separation.

Especially in higher eukaryotes, there must be multiple steps in preparing sister chromatids to separate efficiently. These may vary in prominence and order of release between different types of cells. Thus, sister chromatid separation could be differentially orchestrated, both from a temporal standpoint and regionally along chromosomes so that, as biorientation ensues, there remain a limited number of separation events that could be completed rapidly to trigger a sharp and irreversible transition to chromatid disjunction and segregation. Depending on when and how attachment/tensioning problems arise, the SAC could conceivably target multiple steps in the separation process as "brake pedals" to maintain sister chromatid association. For the small chromosomes in yeast, retaining/releasing cohesin may be particularly important, and thus the Securin/Separase circuit is especially critical in this organism. In other cells, where cohesion appears to be dissolved in a more incremental and regionally specified fashion, perhaps the SAC needs to interface with other processes as well to maintain optimal attachment. In this case, a deeper understanding of the pressure points that are targeted by the SAC to block sister chromatid disjunction should lead us to a complete understanding of how the metaphase to anaphase transition is achieved.

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