Changes in Kinetochore Structure and Molecular Composition in Response to Mis-attachment

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Abstract

Each mitotic chromosome is constituted by two sister chromatids whose correct segregation to the daughter cells is ensured by amphitelic attachment, in which the two sister kinetochores (KTs) are attached to microtubules (MTs) from opposite mitotic spindle poles. KT mis-attachments can occur in early mitosis and cause chromosome mis-segregation and aneuploidy if not corrected. These mis-attachments include monotelic (one attached and one unattached sister KT), syntelic (both sister KTs attached to the same spindle pole), and merotelic (a single KT attached to MTs from opposite spindle poles) attachments. A biochemical pathway named the Spindle Assembly Checkpoint (SAC) is responsible for delaying anaphase onset to allow correction of KT mis-attachments. SAC activation is believed to occur due to KT localization of certain SAC proteins and/or lack of tension, but only monotelic attachment has been proven to activate the SAC. To determine if and how other KT mis-attachments may activate the SAC, we studied how molecular composition and structure of the KT changes in response to different types of attachments. Our data suggest that monotelic attachment is the only type of attachment that can induce a SAC response thanks to the accumulation of the SAC protein Mad2 at the KT. Our data also indicate that structural changes of the KT, measured as intra- or inter-KT stretching, do not directly induce a SAC response. Instead, our findings suggest decreased KT stretching, especially in inter-KT stretching of syntelic chromosomes, may play a key role in bringing MCAK and other KT substrates closer to Aurora B kinase for rapid and efficient correction of KT mis-attachments.

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Chapter 1: Literature Review

Mitosis

Equal partitioning of the genome is achieved through a process called mitosis, during which cells equally distribute the replicated chromosomes between the two daughter cells. Mitosis occurs during the M phase of the cell cycle. M phase follows a number of characteristic cell cycle phases, including G1, S, and G2, which together constitute interphase, and during which the cell prepares to divide by growing in size (G1) and replicating its DNA (S) (Blow and Tanaka, 2005; Howard, 1951; Smith and Martin, 1973; Wittmann et al., 2001). In eukaryotic cells, mitosis can be divided into five distinct stages: prophase, prometaphase, metaphase, anaphase, and telophase. Mitosis is followed by cytokinesis, which leads to cytoplasm division, and thus completes cell division (Rieder and Khodjakov, 2003; Wittmann et al., 2001).

In prophase, the chromatin condenses so that individual chromosomes become discernible (Figure 1.1A). Condensins are thought to take part in initiating the process of condensation by promoting DNA coiling with the energy of ATP hydrolysis (Hirano et al., 2001). Each chromosome is composed of two identical sister chromatids held together by cohesin complexes, which are required for maintenance of cohesion during the first part of mitosis. A portion of cohesin is retained between sister chromatids in the centromeric region until the onset of anaphase while the bulk is dissociated from the chromosome arms in early mitosis (Blow and Tanaka, 2005; Waizenegger et al., 2000). Prophase is also marked by a change in microtubule (MT) dynamics, which leads to the disassembly of the interphase microtubules (MTs) (long and not very dynamic), and the assembly of mitotic MTs (short and highly dynamic). The centrosomes, which are replicated during S phase and serve as MT-organizing centers in animal

cells, move apart during prophase, and an aster starts to form around each duplicated centrosome (Karsenti and Vernos, 2001; Zhai et al., 1996). The separation of centrosomes initiates the formation of the mitotic spindle outside the nucleus.

The breakdown of the nuclear envelope (not occurring in lower eukaryotes) marks the beginning of prometaphase (Figure 1.1B). MTs have access to the nuclear region immediately after nuclear envelope disassembly, and can search the nuclear space by undergoing polymerization/depolymerization cycles, until they encounter a kinetochore (KT), a specialized protein structure that mediates chromosome-spindle attachment. Once attached to a KT, MTs turn into a stable state, and no longer undergo catastrophe (i.e., switch from growing to shortening). A MT can initially establish a lateral interaction with a KT, but it will be rapidly converted to an end-on attachment, the type of attachment responsible for regulating chromosome movement (Hayden et al., 1990; Tanaka et al., 2005; Tanaka and Desai, 2008). Because of the stochastic nature of KT-MT encounters, most chromosomes initially become mono-oriented, with one KT bound to MTs and its sister KT unattached, and move to the pole to which they are attached (Rieder and Alexander, 1990; Rieder and Salmon, 1998). Once the unattached sister KT binds MTs from the opposite spindle pole, the chromosome is said to be bi-oriented, and moves to the spindle equator (Rieder and Salmon, 1998).

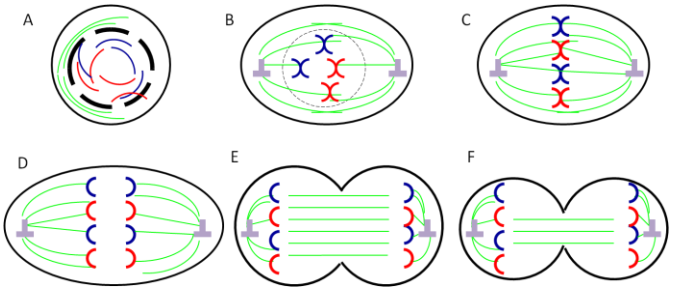

Figure 1.1. Six stages of cell division.
A: prophase; B: prometaphase; C: metaphase; D: anaphase; E: telophase; F: cytokinesis. DNA is shown in blue and red, MTs in green.

The metaphase stage of mitosis is achieved when all the chromosomes have congressed to the spindle equator (Figure 1.1C). This congressional movement is thought to depend on both the force exerted by the kinetochore microtubules (kMTs) and those exerted by other MTs on the chromosome arms, and referred to as polar ejection forces (Maddox et al., 2002). The chromosomes aligned at the spindle equator form the so-called metaphase plate. Chromosomes at the metaphase plate exhibit continuous oscillations back and forth about the spindle equator (Matos et al., 2009; Skibbens et al., 1993).

Anaphase onset is marked by the abrupt and synchronous splitting of the sister chromatids, which is due to the sudden degradation of the cohesin complexes between the sister chromatids (Blow and Tanaka, 2005; Uhlmann, 2003; Waizenegger et al., 2000). During anaphase, the two sister chromatids, now daughter chromosomes, move to opposite spindle poles as their respective kMTs shorten. This process is also referred to as anaphase A, to distinguish it from anaphase B, in which the spindle elongates, thus moving the two groups of segregating chromosomes further apart (Figure 1.1D). In many organisms, anaphase A and B occur nearly simultaneously.

By telophase, the last stage of mitosis, the chromosomes have reached the spindle pole regions, and start to decondense. Meanwhile, the nuclear envelope starts to reassemble around the decondensing chromosomes to form two daughter interphase nuclei (Figure 1.1E). Cytokinesis normally starts during the later stages of mitosis (i.e., late anaphase/telophase), when a cleavage furrow containing an acto-myosin contractile ring forms and pinches the cell membrane of the dividing cell in a region that normally corresponds to the spindle equator (Figure 1.1F). The process of cytokinesis divides the cytoplasm in two, thus completing cell division.

The Mitotic Spindle

The mitotic spindle is a bipolar array of antiparallel MTs assembled during mitosis. The bipolarity of a mitotic spindle is crucial for its function to direct chromosome congression and segregation during mitosis. At the onset of mitosis, the duplicated centrosomes nucleate two asters of MTs, which move around the nucleus in prophase. The MTs emanate from the asters, and grow both toward the cell cortex and toward the chromosomes, which become accessible to the MTs after nuclear envelope breakdown. As the interaction between MTs and chromosomes is established, the conformation of the mitotic spindle becomes (Karsenti and Vernos, 2001; Zhai et al., 1996). By metaphase, the mitotic spindle appears as a symmetrical radial array of MTs.

MTs are hollow cylinders that assemble from α-/β-tubulin heterodimers, and exhibit structural and functional polarity. *In vitro*, MT polymerization occurs at higher rates at one end (defined as the plus end) compared to the other (minus end), and in steady-state conditions. MTs incorporate tubulin subunits at the plus end and release them at the minus end, without net growth, in a process referred to as treadmilling (Margolis and Wilson, 1981). Within a MT, the α-tubulin is exposed at the minus end, while the β-tubulin is exposed at the plus end (Gadde and Heald, 2004; Heidemann and McIntosh, 1980; Mountain and Compton, 2000), and in the mitotic spindle, MTs are oriented with their minus end anchored to the centrosome (MTOC) and the plus end away from the centrosome (Gadde and Heald, 2004; Mazia, 1984). The centrosomes constitute the poles of the mitotic spindle, and play a critical role in mitotic spindle assembly. Each centrosome contains a pair of perpendicularly arranged centrioles, which are surrounded by an amorphous mass, known as pericentriolar material. Each centriole is composed of nine triplets of

MTs. A third type of tubulin, γ-tubulin, is exclusively found at centrosomes. By binding one or more proteins, it can form γ-tubulin ring complex (γ-TuRC), a ring-like structure which is involved in nucleating MTs at the centrosome (Wiese and Zheng, 2006; Zheng et al., 1995).

Three classes of MTs are defined within a mitotic spindle based on their distinct structures and roles in mitotic cells. These are astral, interpolar, and kMTs (Figure 1.2) (Karsenti and Vernos, 2001). Astral MTs are oriented with their plus end away from the chromosomes and toward the cell cortex, with which they can interact via motor proteins. This interaction with the cell cortex plays a role in orienting the spindle within the cell (Gadde and Heald, 2004; Mountain and Compton, 2000). Interpolar MTs overlap in an antiparallel way in the spindle midzone. The interaction of interpolar MTs with minus and plus end-directed motor proteins contributes to maintaining mitotic spindle structure (Odde, 2005). Finally, kMTs are those MTs whose plus end interacts with a KT. In most cell types, each KT can bind multiple MTs, which form a MT bundle named kinetochore-fiber (K-fiber) (Figure 1.2). kMTs are mainly responsible for directing chromosome movement during mitosis.

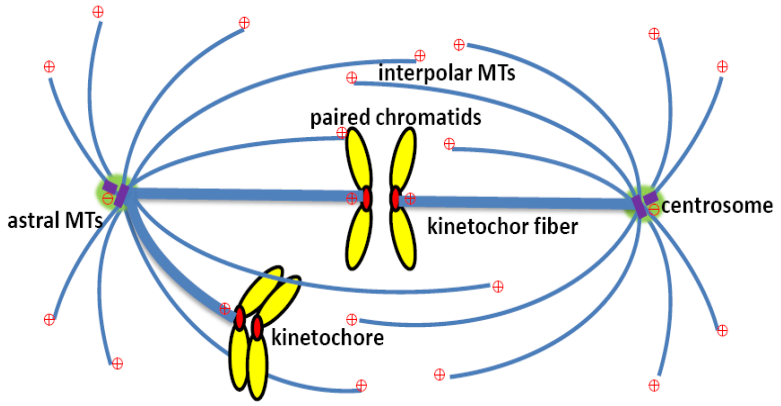

Figure 1.2. Diagrammatic representation of the mitotic spindle and the three classes of MTs. MTs extend from the centrosome (minus end) and grow towards cell cortex (plus end) or chromosomes (plus end). Astral MTs extend away from the spindle poles. Interpolar MTs can overlap with MTs from the opposite poles and are important for maintaining spindle structure. Multiple kMTs bind each KT to form a k-fiber, which connects the KT to the spindle pole.

MTs switch rapidly between phases of growth and shrinkage, a process termed dynamic instability (Kirschner and Mitchison, 1986; O'Connell and Khodjakov, 2007; van der Vaart et al., 2009). At the onset of mitosis, the half-life of MTs decreases compared with that of interphase MTs. Many studies in the field of mitosis have focused on how the dynamic assembly and disassembly of MTs leads to the formation of a stable bipolar mitotic spindle. The "search and capture" model proposes that MTs grow from the centrosomes in all directions. KT-MT attachment occurs by random encounter, and spindle assembly is complete when all KTs have established MT attachments (Hyman and Mitchison, 1990; Mitchison and Kirschner, 1985). The "search and capture" model has been validated in experimental systems in various organisms (Gadde and Heald, 2004; Mitchison and Kirschner, 1985). However, it presents an inefficient mechanism due to the passive and random capture (Wollman et al., 2005), and mathematical modeling has shown that spindle assembly could not be completed within physiological times if "search and capture" were the only mechanism in action. An alternative model proposes that MTs can nucleate from KTs and/or chromatin (Wadsworth and Khodjakov, 2004). These chromatin-nucleated MTs become stabilized thanks to a RanGTP concentration gradient (Kalab and Heald, 2008; Nachury et al., 2001; Wiese et al., 2001). Motor proteins associated with chromosome arms take part in sorting these MTs into a bipolar array that surrounds chromosomes (Cai et al., 2009). It is reasonable to assume that both "search and capture" and RanGTP stabilization of chromatin-nucleated MTs contribute to spindle assembly in mitotic cells (Wollman *et al.*, 2005; O'Connell and Khodjakov, 2007; Kalab and Heald, 2008).

The Kinetochore

The KT is a protein complex that assembles on the centromere region of each chromatid during mitosis. The centromere region refers to the primary constriction of condensed chromosomes and always contains some repetitive DNA sequences. A variety of molecular components recruited to KTs facilitate chromosome segregation by connecting each chromatid to the mitotic spindle upon mitotic entry, and promoting poleward chromosome movement in anaphase, when the sister chromatids are pulled towards opposite spindle poles while maintaining stable KT-MT attachment.

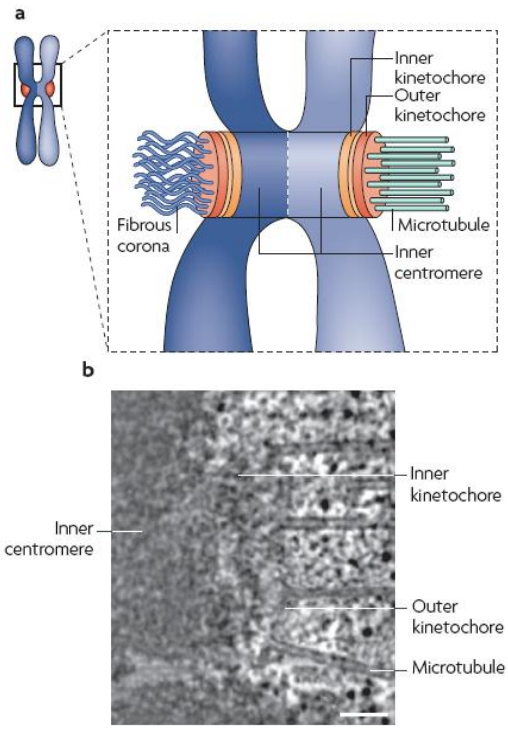

Figure 1.3. [used with permission] Structure of a vertebrate KT. a. Diagrammatic representation of the KT trilaminar structure and fibrous corona; b. Electron micrograph of a KT with bound MTs (Cheeseman and Desai, 2008).

The ultrastructure of a KT is revealed by electron microscopy in vertebrates as a trilaminar stack of plates (Figure 1.3). The outer plate (or outer KT) contains mainly MT-interacting proteins. In the absence of MT attachment, a meshwork of fibers, referred to as fibrous corona, extends outward from the outer plate. The inner plate (or inner KT) comprises centromeric chromatin associated with constitutive KT proteins. The middle zone between the outer and inner plate is less dense and its protein composition is not clear (Chan et al., 2005). More than 80 KT proteins have been identified (Figure 1.4), and they play critical roles in KT specification and assembly,

binding with spindle MTs, monitoring KT attachment and tension, and providing the driving forces for chromosome movement (Cheeseman and Desai, 2008).

Molecular composition and function of the vertebrate inner KT

The inner KT serves as a platform for the assembly of the outer portions of the KT. CENP-A (Centromeric Protein A), CENP-B, and CENP-C, which constitutively localize at the inner KT, were the first three KT proteins to be identified using human autoantibodies (Earnshaw and Rothfield, 1985), and they (particularly CENP-A) are believed to specify the site of KT assembly. In humans, CENP-B protein appears to bind the CENP-B box in a sequence-specific manner. CENP-B box is a 17-bp motif within the 171-bp tandem repeat sequence, known as α-satellite DNA, and was found at the centromere of human chromosomes (Earnshaw and Rothfield, 1985). Some studies have suggested that both α-satellite DNA and CENP-B boxes are required for *de novo* centromere formation (Ohzeki et al., 2002). However, in most eukaryotes centromeric loci can be stably maintained in the absence of CENP-B, CENP-B box, or α–satellite DNA, indicating that the site of KT assembly and maintenance are primarily controlled by epigenetic rather than sequence-based mechanisms (Allshire and Karpen, 2008; Black and Bassett, 2008; Karpen and Allshire, 1997). CENP-A is a histone H3 variant only found at the centromere. It serves as the fundamental determinant of KT identity and is targeted to the centromere through a 15-residue sequence known as CATD (CENP-A targeting domain) (Sullivan et al., 1994). The $H3^{CATD}$ chimera not only specifies the centromere localization but also mediates the recruitment of additional KT proteins (Black et al., 2007; Black et al., 2004). Recent studies have also shown a key role of CENP-C in directing KT assembly (Cheeseman et al., 2004; Desai et al., 2003). Indicative of such a role is the fact that CENP-C interacts with 13 other proteins, including

CENP-H, CENP-I, CENP-K-U, to constitute a network of proteins proximal to the CENP-A nucleosome. Although these proteins, referred to as constitutive centromere-associated network (CCAN), do not affect the association of CENP-A with the centromere after CENP-A deposition, the CENP-H/I/K subclass, recruited by the CENP T/W sub-complex, and CENP-M/N may help target and/or stabilize new CENP-A (Foltz et al., 2006; Izuta et al., 2006; Okada et al., 2006). Indeed, a recent study establishes CENP-N as the promoter and stabilizer of centromere assembly due to its direct recognition of CENP-A nucleosomes (Carroll et al., 2009). Another group of proteins localize in proximity of the inner KT, but are not integral part of the KT itself. Rather, they are believed to localize at the inner centromere, in the inter-KT region (i.e., between the two sister KTs). These proteins comprise the chromosomal passenger complex (CPC), which includes Borealin, Bir1/Survivin, Aurora B kinase (AurB), Sli15/INCENP (inner centromere protein), and mitotic centromere-associated kinesin (MCAK), and are mainly responsible for regulating the stability of KT-MT attachments (Musacchio and Salmon, 2007; Sandall et al., 2006; Vader et al., 2006). Sli15/INCENP-Bir1/Survivin may also couple KTs with MTs, given that INCENP contains a MT-binding site in its C-terminal region (Sandall et al., 2006). The localization and activity of AurB require the three CPC regulatory subunits (Carmena et al., 2009). For example, Borealin is suggested to promote local clustering that leads to AurB auto-activation at the centromere (Kelly et al., 2007; Sessa et al., 2005). INCENP binds to AurB and increases its basal activation (Bishop and Schumacher, 2002; Sessa et al., 2005). Besides, INCENP functions as a tension sensor through its interaction with MTs and relays the mechanical state of KT-MT attachments into local control of Ipl1 kinase (yeast homolog of AurB) activity (Sandall et al., 2006). By phosphorylating its substrates such as MCAK (a MT depolymerase at the plus end), the Ndc80 complex (a core protein complex localized at the outer

KT), and the Dam1 complex (a ten-subunit MT-binding protein complex necessary for end-on attachment in yeast) (Cheeseman et al., 2002), AurB contributes to reducing the binding affinity of KTs for MTs in vitro (Cheeseman et al., 2006) and serves to correct KT-MT interactions until bipolar attachment is achieved(Cimini et al., 2006; DeLuca et al., 2006). In addition, cells expressing a CPC mutant compromised the mitotic arrest but were still able to create unattached KTs, a characteristic of AurB to correct non-bipolar attachments (Vader et al., 2007). Therefore, AurB was suggested to influence the efficiency of anaphase progression independent from its MT destabilizing activity because the AurB-dependent destabilization of non-bipolar attachments could be uncoupled from its ability to induce mitotic arrest (Vader et al., 2008). MCAK, a member of the kinesin-13 family, is also important in coupling MT dynamics with sister KT motility. Indeed, recruitment of additional MCAK increased the chromosome speed, the oscillation amplitude, and the coordination between sister KTs (Joglekar et al., 2010). To conclude, the CPC complex plays a critical role in ensuring correct chromosome orientation and alignment. Another inner KT protein, Shugoshin (Sgo/MEI-S332), is responsible for maintaining centromeric cohesion until metaphase. For example, mammalian shugoshin (Sgo1) is a centromeric protein that is localized at the inner KT from G2 phase to metaphase (Wang et al., 2006). Interestingly, a truncated version of Sgo1 (sSgo1) has been shown to localize to centrosomes and spindle poles, and has a role in the maintenance of spindle integrity (Dai, 2009; Macy et al., 2009; Wang et al., 2008). Sgo2, another shugoshin-like protein, is also required for the centromeric protection of cohesion, but in germ cells. AurB-phosphorylated Sgo2 can recruit MCAK and PP2A to centromeres, thus contributing to both centromeric protection and attachment correction (Tanno et al., 2010).

Molecular composition and function of the vertebrate outer KT

The outer KT contains a number of key protein complexes that play a critical role in the assembly of the site interacting with kMTs, known as core attachment site. The evidence collected to date indicates that the KNL1-Mis12-Ndc80 complex (KMN network) plays a crucial role in establishing the core attachment site by combining two closely apposed low-affinity MT-binding sites, that of the Ndc80 complex and that of KNL1 (Cheeseman et al., 2006). The Mis12 complex is composed of four subunits, Mis12, Nnf1, Nsl1, and Dsn1, and is believed to contribute to KT-MT attachment by connecting the KNL1 and the Ndc80 complex portions of the KNL-Mis12-Ndc80 network (Cheeseman et al., 2006). The study of conformational change of the Mis12 complex, the linker of KNL1 arm and Ndc80 arm, in response to taxol (a MT-stabilizing drug) shows that one end of the complex (Nnf1) shifts towards the centromere while the other end (Dsn1) does not (Wan et al., 2009). This finding suggests a delicate model in which Dsn1 connects the KNL1 arm while Nnf1 is connected to Ndc80 arm through Nsl1 and is oriented inward. Therefore, Mis12 functions to connect KNL1 arm and Ndc80 arm in a way to couple force generation with molecular structure. Finally, the

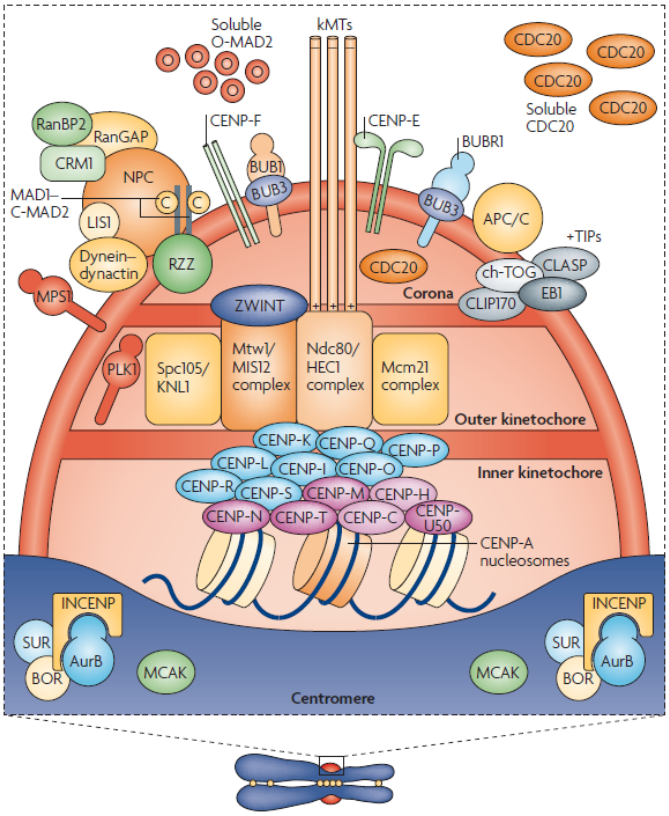

Figure 1.4. [used with permission] Diagrammatic representation of KT associated proteins. The figure shows the classes of proteins associated with each KT subregion (Musacchio and Salmon, 2007).

Mis12 complex has been proposed to serve as a protein interaction hub for outer KT assembly (Petrovic et al., 2010). The rod-like Ndc80 complex is composed of four protein subunits, Ndc80, Nuf2, Spc24 and Spc25 (Wilson-Kubalek et al., 2008). The globular regions of Spc24 and Spc25 form one end and those of Nuf2 and Ndc80 form the other (Miller et al., 2008). In *C. elegans*, Spc24 and Spc25 associate with Mis12, and the interaction of KNL1 and Mis12 complex generates a binding site for the Ndc80 complex (Cheeseman et al., 2006). The Ndc80 and Nuf2 subunits contain a pair of globular, calponin-homology (CH) domains that contribute to high-affinity MT binding (Wei et al., 2007). Besides, KNL1 may also contain a MT-binding region, but the boundaries of the region are still unknown (Cheeseman et al., 2006). A high-resolution map of the KT reveals that the Ndc80 arm moves inward toward the inner KT component CENP-I relative to the KNL1 arm upon treatment with taxol, as the KNL1 arm maintains a relatively constant distance from CENP-I (Wan et al., 2009). In this way, the intra-KT stretching (distance between inner KT and outer KT within a KT) is reduced. In conclusion, connecting the low-affinity binding sites of Nuf2/Ndc80 and KNL1 within the KMN network synergizes the overall MT binding activity. However, Ndc80 appears to be the main substrate for AurB-regulated kMT dynamics (DeLuca et al., 2006). In addition, the outer KT-localized Hec1 (homologue of Ndc80 in vertebrates) also plays a critical rule in controlling dynamic behavior of kMTs through the AurB-dependent phosphorylation of its N terminus (DeLuca et al., 2005; DeLuca et al., 2006).

Molecular composition and function of the fibrous corona

The outermost portion of the KT is the “fibrous corona” (Figure 1.3). This region, which constitutes the KT-MT interface, is occupied by a number of proteins with different functions. These include motor proteins like dynein and CENP-E, non-motor proteins like CENP-F and

dynein-interacting proteins (NDE1 and NDEL1), and many proteins (such as Mad1, Mad2, Bub1, BubR1, Bub3, Cdc20, and the RZZ complex) involved in mitotic checkpoint signaling. The motor proteins found at the fibrous corona are thought to play a key role in the initial steps of KT attachments and chromosome congression. Indeed, both CENP-E (Cai et al., 2009; Kapoor et al., 2006) and dynein (Gassmann et al., 2008; Varma et al., 2008) play an important role in establishment of the initial KT-MT lateral interaction, which precedes the formation of stable end-on attachments. The Rod-ZW10-Zwilch (RZZ) complex functions to recruit the dynein/dynactin complex to the KT (Karess, 2005). However, this pathway seems to be more important for mitotic checkpoint signaling (discussed in next section) than for KT attachment (Gassmann et al., 2008). Finally, a number of MT-binding proteins are also found at the fibrous corona. For example, cytoplasmic linker protein (CLIP)-associating protein (CLASP), MAP215 (chTOG), and CLIP170 are three major MT-associated non-motor proteins that promote the polymerization of MTs at the KT interface (Maiato et al., 2003; Maiato et al., 2005; Tanenbaum et al., 2006). EB1, a MT-plus-end tracking protein, is also found in the corona region, where it is believed to bind to the MT lattice to stabilize MTs (Sandblad et al., 2006; Tanaka and Desai, 2008). APC (Adenomatous Polyposis Coli) is another protein that is associated with MT plus ends, thus regulating MT dynamics. Studies of APC/EB1 found APC to be a substrate for Bub1/BubR1 kinases in vitro. By directly interacting with APC/EB1, BubR1 is able to regulate the position of chromosomes at the metaphase plate and the establishment of stable KT-MT attachments (Logarinho and Bousbaa, 2008). Recent studies also reveal the possible association of the Ska (Ska1, Ska2 and Ska3) complex (Hanisch et al., 2006) with MTs at the KT interface in metazoan (Daum et al., 2009; Gaitanos et al., 2009; Theis et al., 2009; Welburn et al., 2009). Indeed, the Ska complex has been proposed as a functional homolog of the yeast Dam1 ring

complex, tracking shortening MT plus ends and slowing down the rate of disassembly in vitro (Guimaraes and Deluca, 2009), and it is responsible for maintaining stable end-on attachments and coupling MT dynamics with chromosome movement (Asbury et al., 2006; Franck et al., 2007; Tanaka et al., 2007; Westermann et al., 2006).

Temporal sequence of KT assembly and disassembly in vertebrates

Studies in various model systems have shown that KT assembly is a temporally-ordered and cooperative event, and that KT assembly is hierarchical, but not linearly-ordered. The constitutive CENP-A and its associated proteins (CCAN) are found at the KT throughout the cell cycle (Figure 1.5). New CENP-A is loaded at the interphase-mitosis transition in a Mis18- and CENP-C-dependent manner (Erhardt et al., 2008; Fujita et al., 2007; Hayashi et al., 2004; Hemmerich et al., 2008; Maddox et al., 2007; Schuh et al., 2007). The enrichment in CENP-A and CCAN facilitates the recruitment of Mis12 complex in G1 phase (Figure 1.5). Specifically, CENP-C is required to recruit subunits of the Mis12 complex to interphase KTs (Milks et al., 2009). The stabilization of the Mis12 complex, in turn,

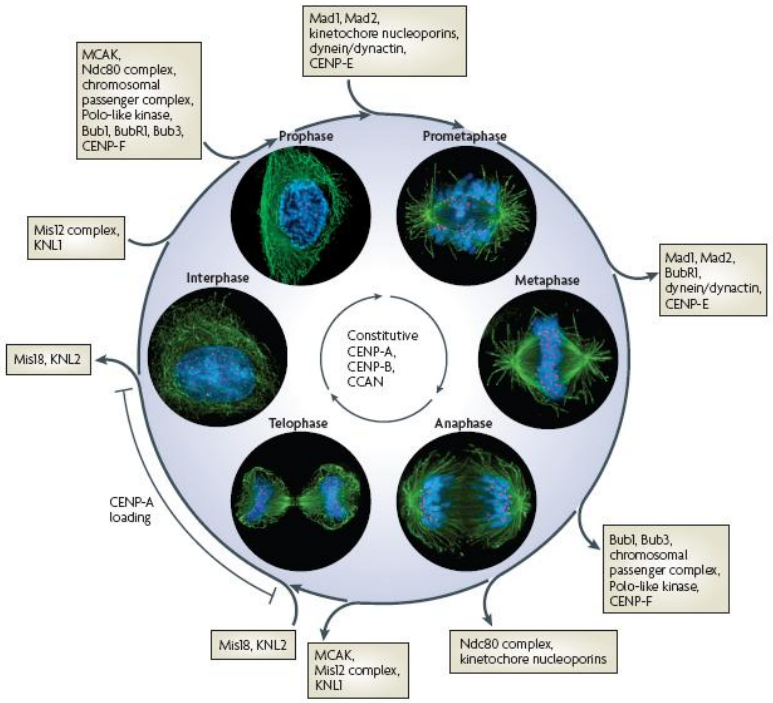

Figure 1.5. [used with permission] Schematic representation of temporally ordered KT assembly and disassembly (Cheeseman and Desai, 2008).

is a prerequisite for incorporation of multiple proteins into the KT (Cheeseman and Desai, 2008). Indeed, inhibition of the Mis12 complex produces a decrease in recruitment of multiple proteins to KTs and affects KT assembly in yeast, *C. elegans*, *Drosophila*, and vertebrates (Cheeseman et al., 2004; Goshima et al., 2003; Kline et al., 2006; Obuse et al., 2004). In vertebrates, the subsequent recruitment of CENP-H/I/K is required to localize Ndc80 complex to the outer KT, and several checkpoint proteins such as Bub1 (required for KT localization of MCAK and BubR1), BubR1 and Bub3 as well as CENP-F to the fibrous corona during late G2 phase and prophase (Figure 1.5) (Hori et al., 2003; Liu et al., 2003; Liu et al., 2006; Okada et al., 2006). The inner centromere proteins are also recruited in prophase (Figure 1.5). AurB is then localized and activated by INCENP, Borealin, and Survivin (Klein et al., 2006; Vader et al., 2006). AurB is required to localize Bub1 and BubR1 to the outer KT (Ditchfield et al., 2003). CENP-E and multiple checkpoint proteins such as Mad1 and Mad2 are recruited to KTs after nuclear envelope breakdown (Figure 1.5). Some of these proteins such as Mad2, BubR1, and CENP-E are removed from the KT at metaphase after the establishment of KT-MT attachments. The level of checkpoint proteins such as Bub1, Bub3, Plk1 and CENP-F decreases at the metaphase-anaphase transition (Figure 1.5). By telophase the level of MCAK, Mis12 complex and KNL1 is greatly reduced and cells exit mitosis at which time Mis18 and KNL2 promote loading of new CENP-A (Figure 1.5) (Cheeseman and Desai, 2008).

The Spindle Assembly Checkpoint

To ensure correct segregation of sister chromatids towards the opposite poles, anaphase onset must not occur until all chromosomes have aligned at the metaphase plate. This is ensured by the

mitotic checkpoint, or spindle assembly checkpoint (SAC), a biochemical pathway involving a myriad of proteins. The SAC monitors KT-MT attachment and/or tension and generates a "wait-anaphase" signal if these requirements (attachment and tension) are not satisfied. In a study published in 1994, Rieder et al. found that PtK1 cells entered anaphase about 23 minutes after the last KT attached to the spindle, suggesting that vertebrate cells possess a metaphase-anaphase checkpoint control that monitors not only sister KT attachment to the spindle but also the increase in tension between sister KTs or between KTs and their associated MTs (Rieder et al., 1994). In a subsequent study, Rieder et al. used laser ablation to destroy the unattached KT of the last mono-oriented (one sister KT bound to MTs and one unattached) chromosome, and concluded that the inhibitory signal for metaphase-anaphase transition was generated at or near the unattached KT (Rieder et al., 1995). Thus, by detecting single unattached KTs or some kinds of improper attachments, the SAC can help prevent unequal distribution of genetic material during cell division.

Upon mitotic entry, sister chromatids are bound along their length by a multi-subunit protein complex called cohesin. Most cohesin is removed by metaphase, except for that at the centromere, which maintains cohesion between sister chromatids until anaphase onset (Waizenegger et al., 2000). The removal of these last cohesin molecules depends on degradation by the protease separase. Prior to anaphase, however, separase, is maintained inactive by binding of its inhibitory subunit, securin. Once all KTs are bi-oriented and attached to kMTs, the anaphase promoting complex/cyclosome (APC/C), activated by Cdc20, polyubiquitinates both cyclin B and securin. The polyubiquitination of these two proteins leads to their degradation by the 26S proteasome (Musacchio and Salmon, 2007). As a result of securin degradation, separase

is released and proteolyzes centromeric cohesin, thus leading to sister chromatid separation. Therefore, anaphase onset is triggered by the interaction of APC/C with Cdc20. The SAC functions by inhibiting formation of the Cdc20-APC/C complex, thus preventing precocious degradation of cohesin until all KTs establish bipolar attachment with spindle MTs and all chromosomes are aligned at the metaphase plate (Fang et al., 1998).

The role of the SAC in determining anaphase onset relies on the ability of numerous SAC proteins to interact with unattached KTs. Core components of the SAC include six evolutionary conserved proteins, Bub1, Bub3, Mad1, Mad2, BubR1 (Mad3 in yeast), and Mps1 (multipolar spindle-1) (Hardwick et al., 1996; Musacchio and Salmon, 2007). Additional proteins, including the plus-end directed kinesin motor CENP-E (Mao et al., 2005), the minus-end directed motor dynein, dynein-interacting proteins such as dynactin (Howell et al., 2001; Tai et al., 2002), CLIP170 and LIS1 (Tai et al., 2002), the RZZ (ROD-ZW10-ZWILCH) complex (Karess, 2005; Lu et al., 2009), $p31^{comet}$ (Mapelli et al., 2006; Xia et al., 2004). CDK1-cyclin B (D'Angiolella et al., 2003), Plk1 (polo-like kinase 1) (van Vugt and Medema, 2005), AurB (Hauf et al., 2003; Murata-Hori et al., 2002), and MAPK (mitogen-activated protein kinase), can indirectly affect the SAC, but do not participate to SAC signaling.

Recent studies suggested that the SAC might be maintained active by the sequestration of Cdc20 into a mitotic checkpoint complex (MCC) containing three core SAC proteins, Mad2, BubR1/Mad3 and Bub3, as well as Cdc20 (Musacchio and Salmon, 2007). Studies about the formation of MCC proposed a Mad2 template model. In this model, Mad2 is present in two conformations, closed-Mad2 (C-Mad2) and open-Mad2 (O-Mad2). Mad1-C-Mad2 complexes

are deposited at the KT by $p31^{comet}$ (Vink et al., 2006; Xia et al., 2004). O-Mad2 is then recruited at the KT by this Mad1-C-Mad2 complex, and converted into C-Mad2, which can bind Cdc20, generating C-Mad2-Cdc20 complex, and possibly MCC by binding of other SAC proteins. The C-Mad2 within the C-Mad2-Cdc20 complex was also proposed to function as a cytoplasmic template for conversion of cytoplasmic O-Mad2 into Cdc20-bound C-Mad2 (De Antoni et al., 2005). This would lead to amplification of the SAC signal, thus explaining how even a single unattached KT can sustain mitotic arrest. All SAC proteins concentrate at KTs during prometaphase, indicating that KTs act as a catalytic platform to accelerate the production of the MCC (Howell et al., 2000; Howell et al., 2001; Kallio et al., 2002; Shah et al., 2004; Vink et al., 2006). However, the contribution of KTs to MCC formation remains controversial based on a number of studies. For example, mature KTs only exist in mitosis, but the MCC might exist throughout the cell cycle (Sudakin et al., 2001). Consistent with the finding above, the MCC persisted in SAC-inactive *S. cerevisiae Ndc10* mutants whose KT assembly was defective (Gillett et al., 2004).

Studies aimed at determining the exact nature of the defect(s) detected by the SAC have indicated that both lack of attachment (Rieder et al., 1995) (Figure 1.6A) and lack of tension (Jang et al., 1995; Li and Nicklas, 1995) at the KT can be detected, although the role of tension in checkpoint signaling is still controversial (Khodjakov and Rieder, 2009; King and Nicklas, 2000; Nicklas and Ward, 1994). The attachment hypothesis is supported by the evidence that some SAC proteins are immediately removed from KTs once MT attachment is established. For example, Mad2 localizes to unattached KTs in prometaphase but its level is highly reduced at metaphase KTs (Chen et al., 1996; Li and Benezra, 1996), when their multiple MT-binding sites

are completely occupied. Consistently, Mad2 is detected in nocodazole-treated cells with no KT-MT attachments (Chen et al., 1996; Waters et al., 1998), but it is not recruited at KTs in cells treated with taxol, which reduces tension, but does not induce MT detachment (Waters et al., 1998). Therefore, Mad2 is thought to be a molecular marker for KTs with lower or no MT occupancy. Other studies have pointed out that the SAC may also detect lack of tension at the KT. For example, taxol (a MT drug that stabilizes MTs rather than depolymerizing them) treatment results in mitotic arrest, loss of KT tension (as detected by reduced inter-KT distance), and accumulation of certain markers at KTs. Such tension markers include BubR1, Bub1, and the phosphoepitope detected by the 3F3/2 antibody (Gorbsky and Ricketts, 1993; Maresca and Salmon, 2009; Nicklas et al., 1995). Sgo1 has also been suggested to act as a tension sensor (Indjeian et al., 2005). Some researchers have proposed AurB as an integral component of the SAC, but this idea is not widely accepted. In the absence of tension, AurB can access and phosphorylate Ndc80 complex and MCAK, which are involved in stabilizing KT-MT binding. By regulating Ndc80 and MCAK phosphorylation, AurB directly affects the binding affinity of KTs to MTs, and can generate unattached KTs/attachment sites that can recruit *bona fide* SAC proteins such as Mad1 and Mad2, thus eliciting SAC signaling

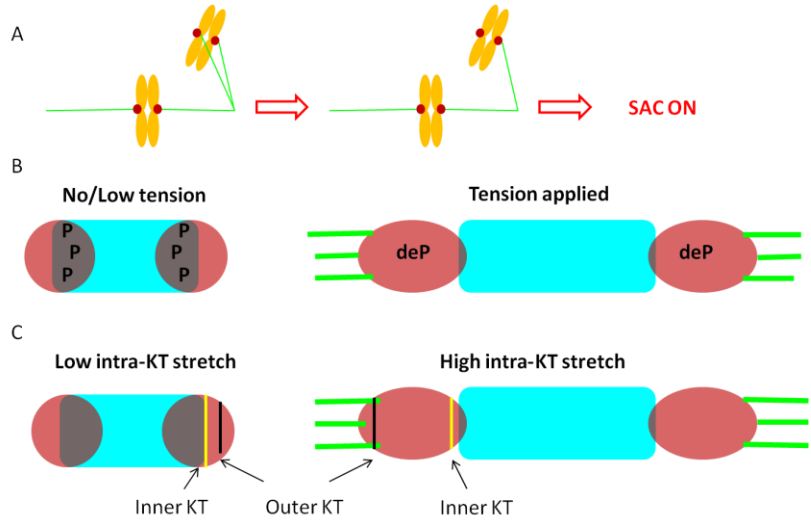

Figure 1.6. Schematic representations of attachment- Vs. tension-sensitive SAC. A. Correcting mis-attached KTs creates unattached KTs, activating the SAC; B. Tension separates AurB and its substrates, thus stabilizing KT-MT attachments; C. The SAC is satisfied in the presence of intra-KT stretch.

(Pinsky and Biggins, 2005; Pinsky et al., 2006). Moreover, AurB activity appears to be high at unattached/ tensionless KTs (Liu et al., 2009). These observations have led to the idea that AurB is directly involved in SAC control (DeLuca et al., 2006; Knowlton et al., 2006; Santaguida and Musacchio, 2009). As shown in figure 1.6B, when KTs are stretched (i.e., under tension), AurB is unable to access its substrates, and this results in dephosphorylation of its substrates, which stabilizes KT-MT attachments. Studies in yeast suggested that that the CPC components Bir1-Sli15 link centromeres to MTs in a manner that allows Sli15 to locally activate Ipl1 when core attachment sites are not under tension (Sandall et al., 2006). Whereas KT tension has been traditionally measured as separation between sister KTs (inter-KT stretch), two recent studies (Maresca and Salmon, 2009; Uchida et al., 2009) suggested that intra-, rather than inter-, KT stretching might be the actual tension-signaling mechanism (Figure 1.6C). However, this would not explain how the inner centromere CPC components may sense the reduced tension.

Some investigators have suggested the separation of the SAC into two signaling branches, one depending on the attachment and the other on the tension status of the KT (Zhou et al., 2002). However, previous studies have also pointed out a possible interdependence between tension and attachment (Nicklas et al., 2001). Indeed, whereas tension is known to stabilize KT-MT attachment, loss of tension could lead to destabilization and detachment of kMTs, thus making it difficult to separate tension from attachment. On the other hand, studies in budding yeast have attempted to resolve this controversy. Deletion of a cohesin subunit results in mitotic cells possessing only single chromatids, and each yeast chromatid possess a single MT attachment site (Shonn et al., 2000). Thus, these KTs exhibit full occupancy, yet they lack tension because they lack a sister KT (Pinsky and Biggins, 2005; Shonn et al., 2000). Interestingly, these cells

exhibit a SAC-dependent mitotic arrest (Pinsky and Biggins, 2005). Maresca and Salmon have recently proposed a model that explains how tension defects could be translated into a wait-anaphase signal. They proposed that low intra-KT stretching promotes Mad1-Mad2 binding at the KT by positioning multiple low-affinity MT binding sites near each other. In this case, Mad1-Mad2 is phosphorylated by KT- and centromere-localized kinases, and this increases its binding affinity to the KT, amplifying the SAC signal. However, the affinity of Mad1-Mad2 for the KT is reduced when the KT is stretched because the binding sites are repositioned and Mad1-Mad2 is dephosphorylated. As a result, the SAC is satisfied upon the increase in the intra-KT stretching (Maresca and Salmon, 2010; Wan et al., 2009). In conclusion, although this topic is still controversial, both attachment and tension seem to be important for SAC signaling.

Upon the establishment of bipolar KT attachment, the SAC is turned off to allow entry into anaphase. Different mechanisms appear to contribute to SAC silencing. For example, Mad1-Mad2 is stripped from attached KTs via a dynein-mediated poleward movement (Howell et al., 2001). $p31^{comet}$ also seems to contribute to removal of Mad1-Mad2 from the KT (Vink et al., 2006; Xia et al., 2004). The regulation of BubR1 activity, instead, appears to rely on CENP-E binding to MTs. The interaction between CENP-E and the attached kMTs inactivates BubR1, resulting in the release of Mad2 and Mad1, together with Rod and ZW10, from the outer KT. Therefore, the dissociation of Mad2 from the attached KT leads to SAC silencing (Mao et al., 2005). Mps1 is ubiquitylated by the activated APC/C (Palframan et al., 2006) upon anaphase onset. Finally, dephosphorylation events dependent on protein phosphatase 1 have been recently shown to contribute to SAC silencing (Pinsky et al., 2009; Vanoosthuyse and Hardwick, 2009). A diagram of SAC signaling pathway is shown in figure 1.7.

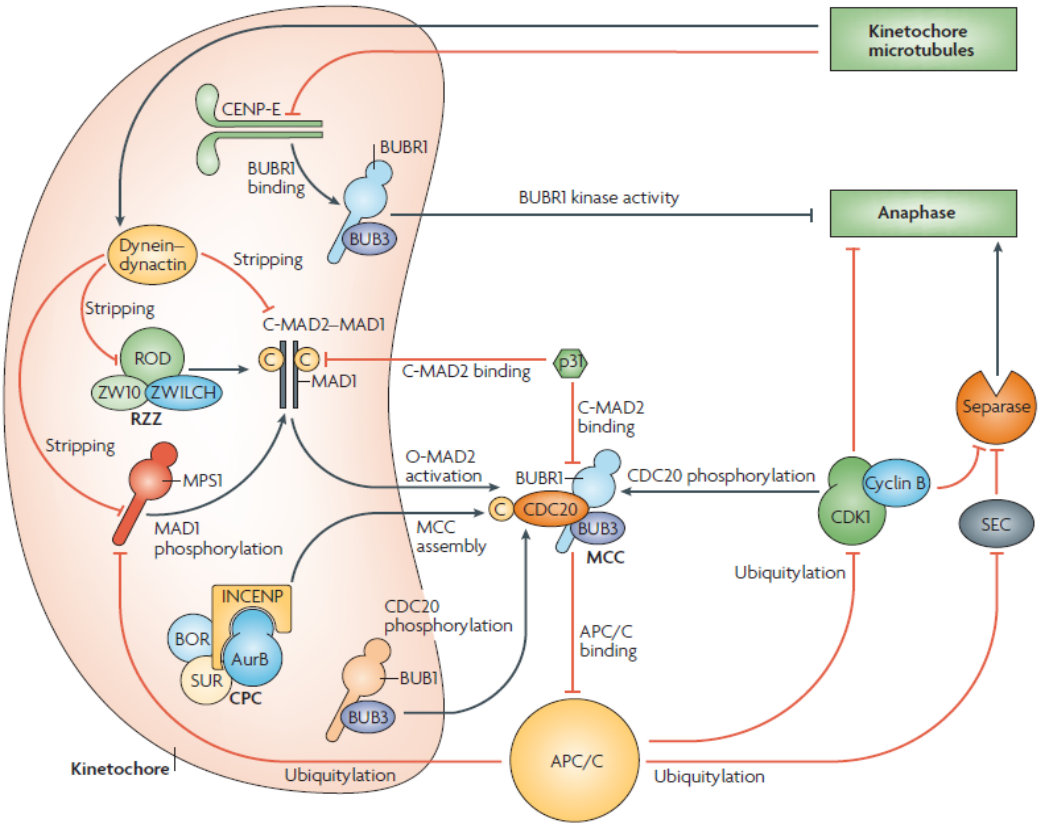

Figure 1.7. [used with permission] The protein network and signaling pathway of the SAC (Musacchio and Salmon, 2007).

KT mis-attachments

For accurate separation of chromosomes during mitosis, sister KTs must bind MTs from opposite spindle poles, a type of KT orientation termed amphitelic attachment (Figure 1.8A). However, other types of KT attachments can be established in early mitosis. These KT attachments, including monotelic, syntelic, and merotelic attachment, if not corrected before anaphase onset, will result in chromosome mis-segregation and lead to aneuploidy (Figure 1.8).

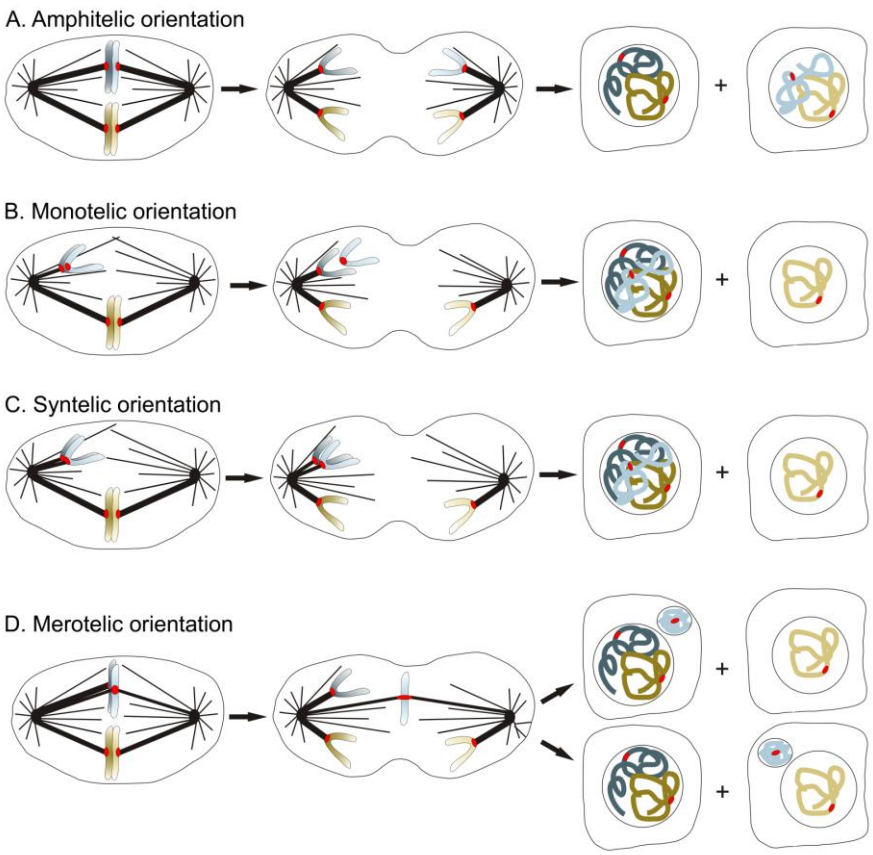

Figure 1.8. [used with permission] Types of KT attachments. Left column, KT attachment in metaphase. Middle column, anaphase in the presence of different types of KT attachment. Right column, daughter cells (Cimini, 2008).

Monotelic attachment occurs when one sister KT becomes attached to MTs from one spindle pole but the other remains unattached [Figure 1.8B; (Cimini, 2008)]. Monotelic orientation is a common event during the early stages of mitosis. In fact, each chromosome initially establishes monotelic attachment, until the sister KT binds MTs from the opposite spindle pole, which will lead to amphitelic attachment and chromosome congression to the metaphase plate. Monotelically oriented chromosomes are positioned close to the pole to which they are attached (Figure 1.8B, left). If cells progress into anaphase in the presence of monotelic attachment, the sister chromatids separate, but will be inevitably included into the same daughter cell due to their proximity to one spindle pole (Figure 1.8B, middle). Therefore, monotelic attachment could give

rise to aneuploid daughter cells (i.e., cells possessing an incorrect chromosome number; Figure 1.8B, right), one of which would be trisomic (one extra chromosome) and one monosomic (one missing chromosome). However, monotelic attachment is detected by the SAC, which will delay anaphase onset until the chromosome has achieved amphitelic attachment.

Syntelic attachment is achieved when both sister KTs bind MTs from the same spindle pole [Figure 1.8C; (Cimini and Degrassi, 2005)]. Like monotelic chromosomes, syntelically oriented chromosomes localize close to the spindle pole to which they are attached (Figure 1.8C, left). As a result, the syntelically oriented sister chromatids will also be included into the same daughter cell if the cell progresses through mitosis in the presence of syntelic attachment (Figure 1.8C, middle). As a consequence, in this case a trisomic and a monosomic daughter cell would arise (Figure 1.8C, right). It is not clear whether syntelic KT orientation is detected by the SAC (Maiato et al., 2004; Rieder and Maiato, 2004). However, although anaphase onset in the presence of syntelic chromosomes can occur in AurB-inhibited cells (Lampson et al., 2004), syntelic attachments are rarely observed in untreated cells. This suggests that in unchallenged cells either syntelic attachments rarely occur, or they are readily converted into different types of attachment, possibly via AurB-dependent mechanisms (Cimini, 2008).

Merotelic attachment only occurs in organisms whose KTs bind multiple MTs, and is established when a single KT becomes attached to MTs from both spindle poles (Figure 1.8D). This type of KT attachment occurs frequently in early mitosis (Cimini et al., 2003) and it is not detected by the SAC (Cimini et al., 2004; Cimini et al., 2002). Before anaphase onset, merotelically attached chromosomes are not localized close to either spindle pole, but align at the metaphase plate

[Figure 1.8D, left; (Cimini et al., 2004; Cimini et al., 2003)]. Although pre-anaphase correction mechanisms for merotelic attachments exist (Cimini et al., 2003; Cimini et al., 2006; DeLuca et al., 2006), cells progress into anaphase without significant delay (Cimini et al., 2004; Cimini et al., 2002). Thus, a fraction of merotelic KTs persist throughout mitosis. In anaphase, the movement and positioning of a merotelic KT (and chromosome) depends on the relative sizes of the two bundles of MTs attached to the two spindle poles (Cimini et al., 2004). If the two bundles are different in size, the chromosome will move towards the pole to which the thicker bundle is attached, without losing attachment to either of the MT bundles, which therefore elongate rather than shorten during anaphase to compensate for anaphase B spindle elongation (Cimini et al., 2004). If the two MT bundles are similar in size, the merotelic KT will lag behind at the spindle equator during anaphase [Figure 1.8D, middle; (Cimini, 2008; Cimini et al., 2004; Salmon et al., 2005)]. Consistent with this, it is known that a fraction of merotelically oriented chromosomes induces anaphase lagging chromosomes (Cimini et al., 2001). When the cleavage furrow ingresses during cytokinesis, these lagging chromosomes are included in either one of the two daughter cells, leading to aneuploidy about 50% of the time (Cimini and Degrassi, 2005). Finally, upon mitotic exit, lagging chromosomes become micronuclei, separated from the main nucleus [Figure 1.8D, right; (Cimini and Degrassi, 2005; Cimini et al., 2002)].

Chapter 2: Introduction

The behavior of the SAC in response to KT-MT mis-attachments

Accurate chromosome segregation during mitosis is critical to organism development and maintenance. The sister chromatids of each replicated chromosome must be segregated to the two daughter cells during mitosis, and this is achieved thanks to the interaction of the two sister KTs with MTs from opposite poles of the mitotic spindle. This type of KT-MT interaction is referred to as amphitelic attachment, and it is the only one that ensures accurate chromosome segregation. Other types of attachments can occur in early mitosis and cause chromosome mis-segregation and aneuploidy if not corrected before anaphase onset (Cimini and Degrassi, 2005). These mis-attachments include (i) monotelic attachments, in which one sister KT is bound to MTs and the other is unattached; (ii) syntelic attachments, in which the two sister KTs are attached to MTs from the same spindle pole; and (iii) merotelic attachments, in which a single KT is attached to MTs from both spindle poles. A biochemical pathway named SAC is responsible for detecting mis-attached chromosomes and delay anaphase onset until those mis-attachments have been corrected (Musacchio and Salmon, 2007). However, only monotelic attachments have been clearly shown to sustain SAC signaling (Rieder et al., 1995). This is believed to occur thanks to the accumulation of SAC proteins (e.g., Mad1, Mad2, etc.) at the unattached KT of monotelic chromosomes (Cheeseman and Desai, 2008; Chen et al., 1996; Li and Benezra, 1996; Waters et al., 1998). Conversely, all the evidence available to date suggests that merotelic attachments are not detected by the SAC (Cimini et al., 2004; Cimini et al., 2002; Khodjakov et al., 1997; Wise and Brinkley, 1997), although a previous study in *Xenopus leavis* cells showed accumulation of certain proteins involved in MT turnover (and hence mis-attachment correction), such as AurB and MCAK, at the centromere of chromosomes with one

merotelic KT (Knowlton et al., 2006). Finally, syntelic attachments have not been extensively studied and whether the SAC can detect such mis-attachments is currently a matter of debate (Maiato et al., 2004; Rieder and Maiato, 2004).

Attachment Vs. Tension in SAC signaling

An unanswered question about SAC signaling is whether the SAC can only detect lack of attachment or also lack of tension (Khodjakov and Rieder, 2009; King and Nicklas, 2000; Nicklas et al., 2001; Pinsky and Biggins, 2005). Several studies suggested that lack of tension at the KT might be detected by the SAC (Jang et al., 1995; Li and Nicklas, 1995). If that is the case, then syntelic KTs should maintain the SAC active, because they do not experience the inter-KT tension that normally develops upon amphitelic attachment. However, many researchers believe that the SAC can only detect lack of attachment and not lack of tension (Khodjakov and Rieder, 2009; Nezi and Musacchio, 2009). Thus, syntelic attachments would not be detetcted by the SAC. However, the fact that syntelic attachments are rarely seen in cells progressing through mitosis (Hauf et al., 2003) suggests that they might be efficiently corrected. A new hypothesis has recently emerged (Liu et al., 2009; Nezi and Musacchio, 2009) to explain how certain KT mis-attachments (merotelic and syntelic) can be corrected despite the inability of the SAC to detect them. This hypothesis states that, although the SAC cannot detect the lack of tension at syntelic (or merotelic) KTs, this lack of tension causes the MT attachment sites on these KTs to be proximal to the inner centromere, where AurB is enriched, and this will promote AurB-dependent detachment of kMTs. This hypothesis is based on recent work showing that proximity of Hec1 to the inner centromere will lead to its phosphorylation by AurB (Liu et al., 2009), and

AurB-dependent Hec1 phosphorylation is known to result in increased kMT turnover (DeLuca et al., 2006).

But how can tension be measured? It is widely documented that unattached chromosomes exhibit minimal inter-KT stretching, but as chromosomes bind MTs and establish amphitelic attachment the inter-KT stretching increases, reaching a maximum for chromosomes aligned at the metaphase plate (Musacchio and Hardwick, 2002). Based on this, one could argue that syntelic KTs may be detected by the SAC because of a reduced inter-KT stretching. Although traditionally the inter-KT stretching (distance between sister KTs) has been used as a measure of KT tension, two recent studies (Maresca and Salmon, 2009; Uchida et al., 2009) suggested that what may be important for SAC signaling is the intra-KT stretching (measured as the distance between an inner and an outer KT marker) rather than inter-KT stretching (the distance between two sister KTs). This raises the possibility that syntelic attachments may be detected by the SAC due to a reduction of intra-KT stretching in the syntelic KTs, leading some to propose that reduced intra-KT stretching in one or both of the syntelic KTs may result in both attachment correction and wait-anaphase (SAC) signaling (Maresca and Salmon, 2010).

Rationale and Hypothesis

Many SAC proteins are known to localize at the KT in early mitosis (Logarinho et al., 2004; Vigneron et al., 2004), and this localization is believed to play a key role in SAC signaling (Zhou et al., 2002). Most studies, however, have focused exclusively on the unattached KT of monotelic chromosomes. To gain a better understanding on whether and how the SAC may detect various types of mis-attachments (in particular syntelic), we systematically studied how

the molecular composition and structure of the KT changes in response to different types of attachments. Our hypothesis is that if syntelic attachments are detected by the SAC, then syntelic KTs should exhibit the same molecular composition (localization of SAC proteins) and structure (inter- and intra-KT stretching) displayed by unattached KTs, which are known to trigger a SAC response. Alternatively, syntelic attachments may not be detected by the SAC, but may be efficiently corrected, in which case syntelic KTs would be expected to exhibit features similar to those of merotelic KTs, many of which are formed in early mitosis, but corrected before anaphase onset without triggering a SAC response. Specifically, syntelic KTs may exhibit similar stretching and/or accumulation of proteins involved in mis-attachment correction, as previously shown for merotelic attachments (Knowlton et al., 2006). To test these different possibilities, the following markers were quantified at KTs with different types of attachments in PtK1 cells: Mad2 (marker for partial or lack of KT-MT attachment) (Waters et al., 1998), BubR1 (previously proposed to participate in the tension-sensing portion of SAC signaling) (Logarinho and Bousbaa, 2008; Logarinho et al., 2004), 3F3/2 phosphoepitope (known to accumulate as a result of low KT tension) (Logarinho et al., 2004; Nicklas et al., 1995), MCAK (involved in correction of mis-attachments) (Knowlton et al., 2006), and AurB (involved in correction of mis-attachments) (Biggins and Murray, 2001; Cimini et al., 2006; Tanaka et al., 2002). Furthermore, we measured and compared intra- and inter-KT stretching of KTs/chromosomes with different types of attachment.

Chapter 3: Materials and Methods

Cell culture and drug treatment

PtK1 cells (American Type Culture Collection, Rockville, MD) were cultured in HAM F-12 media (Invitrogen, Carlsbad, CA) supplemented with 0.5% sodium pyruvate (Fisher Scientific, Pittsburg, PA), 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a 37℃, 5% CO_2, humidified incubator. For experiments, cells were grown on coverslips up to ~70% confluency, and then treated for 2 hours with 2 μM of the Eg5 inhibitor S-trityl-L-cysteine (STLC) to increase the number of syntelic and monotelic attachments (Kapoor et al., 2000). To increase the number of merotelic attachments, cells were arrested in 2 μM STLC for 2 hours, washed out of the drug, and then fixed after a 40-minute incubation in drug-free media (Silkworth, Nardi, and Cimini, unpublished). DMSO (Fisher Scientific, Pittsburg, PA) was added in control cultures used to image amphitelic attachments.

Antibodies

Antibodies used in this study included: rabbit anti-Mad2 (from Dr. E. D. Salmon, The University of North Carolina at Chapel Hill); mouse anti-3F3/2 (Boston Biologicals, Wellesley, MA); rabbit anti-BubR1 (from Dr. T. Yen, University of Pennsylvania); rabbit anti-AurB (Abcam, Cambridge, MA); rabbit anti-MCAK (from Dr. R. Ohi, Vanderbilt University); rabbit anti-INCENP (from Dr. Aaron Straight, Stanford University); mouse anti-Hec1 (Abcam, Cambridge, MA); human anti-ACA (anti-centromere antigen) (Antibodies Inc., Davis, CA); mouse anti-DM1α (anti-α-tubulin) (Sigma-Aldrich Corp, St. Louis, MO); Alexa-488 goat anti-mouse (Invitrogen, Carlsbad, CA); Alexa-488 goat anti-rabbit (Invitrogen, Carlsbad, CA); X-

Rhodamine goat anti-human (Jackson Immunoresearch Laboratories Inc., West Grove, PA); Cy5 goat anti-mouse (Abcam, Cambridge, MA); Cy5 goat anti-rabbit (Invitrogen, Carlsbad, CA).

The antibodies were diluted for immunostaining as follows: anti-Mad2, 1:200; 3F3/2, 1:250; anti-BubR1, 1:1000; anti-AurB, 1:200; anti-MCAK, 1:200; anti-INCENP, 1:1250; anti-Hec1, 1:500; ACA, 1:100; DM1α, 1:500; Alexa-488 goat anti-mouse, 1:400; Alexa-488 goat anti-rabbit, 1:400; X-Rhodamine goat anti-human, 1:100; Cy5 goat anti-mouse, 1:100; Cy5 goat anti-rabbit, 1:100.

Immunostaining

For BubR1, INCENP, and AurB staining, cells were first fixed in freshly prepared 4% formaldehyde (Fisher Scientific, Pittsburg, PA) for 20 minutes and then permeabilized in 0.5% Triton X-100 (Fisher Scientific, Pittsburg, PA Fisher) in 1XPHEM buffer [60mM Pipes (Fisher Scientific, Pittsburg, PA), 25mM HEPES (Fisher Scientific, Pittsburg, PA), 10mM EGTA, 2mM $MgSO_4$ (Fisher Scientific, Pittsburg, PA), pH 7.0] at room temperature for 10 minutes. For MCAK staining, cells were fixed as described above, but permeabilized in 0.1%Triton X-100 in 1XPHEM buffer. For Mad2 staining, cells were first lysed in freshly prepared 0.5% Triton X-100 in 1XPHEM buffer for 5 minutes and then fixed in freshly prepared 4% formaldehyde in 1XPHEM for 20 minutes at room temperature. For Hec1 staining, cells were prefixed in freshly prepared 4% formaldehyde for 5 seconds before a 5-minute lysis in 0.5% Triton-X and 20-minute fixation in 4% formaldehyde. For 3F3/2 staining, cells were processed as for Hec1 staining, but the lysis buffer consisted of 0.5% Chaps (VWR, Radnor, PA) in 1XPHEM to which 100nM microcystin LR (VWR, Radnor, PA) was added. Cells were then rinsed three times for 5

minutes in 1XPBS with 0.05% Tween-20 (PBST) (Fisher Scientific, Pittsburg, PA) and blocked for 1 hour at room temperature in 10% boiled goat serum (BGS) (Jackson Immunoresearch Laboratories, West Grove, PA). Primary antibodies were diluted into 1XPHEM with 5% BGS, and cells were incubated overnight at 4℃. After four 5-minute PBST washes, cells were incubated with secondary antibodies in 5% BGS for 45 minutes at room temperature. After four 5-minute PBST washes, cells were counterstained with DAPI (Sigma-Aldrich Corp, St. Louis, MO) for 5 minutes, then rinsed three times with PBST for 5 minutes, and mounted on microscope slides with an anti-fading solution containing 90% glycerol, 10% Tris buffer, and 0.5–1% n-propyl galate.

Confocal microscopy and image acquisition

Immunofluorescently labeled cells were imaged with a swept field confocal unit (Prairie Technologies) attached to a Nikon Eclipse TE2000-U microscope. Images were obtained with a cooled CCD digital camera, using a 100X / 1.4NA Plan-Apochromatic phase-contrast objective lens. Digital images were acquired by Elements image processing software. Z-series optical sections through each cell were obtained at 0.6-μm steps.

Data analysis

Identification of different types of attachments. Merged images of the α-tubulin and ACA fluorescence were used to identify the different types of KT attachments. First, maximum intensity projections of the two fluorescence channels were generated and merged. The merged image was viewed for identification of possible examples of various attachments. To ascertain the nature of the attachment, the original Z-stacks were then merged and a few selected focal

planes above and below the KT/KT pair of interest were viewed by scrolling up and down. This was done to make sure that a KT that appeared unattached was not attached to a MT bundle going off the focal plane at an angle or that a KT that appeared attached was not simply overlapping with a MT bundle running past it on an adjacent focal plane. Once the KTs of interest were identified, the original single channel images were used for measurements and quantifications.

Fluorescence intensity measurements. Fluorescence intensity of KT proteins, minus background, was quantified using Elements image processing software, using an adaptation of the method used by Hoffman et al. (Hoffman et al., 2001). Briefly, computer-generated circular regions with a diameter of 0.9 μm and 1.2 μm were centered over each KT on the appropriate focal plane and the total integrated fluorescent counts were obtained for each region (Figure 3.1A). These data were exported to Microsoft Excel using the Elements Export function. Background fluorescence was calculated by subtracting the fluorescence intensity of the inner circle from the fluorescence intensity of the outer circle and then was scaled in proportion to the area of inner circle [(Fb/Ai=(Fo-Fi)/(Ao-Ai)]. The integrated fluorescence of the KT was calculated by subtracting the background fluorescence from the fluorescence intensity of the inner circle (Fs=Fi-Fb). The relative fluorescence intensity of the protein of

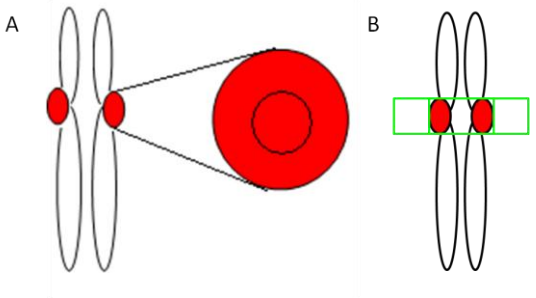

Figure 3.1. Diagram of measurement of fluorescence intensities of the signal (Fs).
A Schematic representation of circular measurement; B Schematic representation of rectangular measurement
Fs=Fi-Fb, where Fb=Fo-Fi
Fb: Fluoresence intensity of background
Fo: Fluorescence intensity of the outer circle/rectangle
Fi: Fluorescence intensity of the inner circle/rectangle

interest was calculated as a fraction of the ACA fluorescence intensity. ANOVA statistical tests used to compare each type of KT attachment to all the others. For INCENP and MCAK, in addition to the quantification of the KT-localized protein (performed as described above), a second method was used to quantify the total protein (i.e., KT- plus inner centromere-localized). To this aim, two rectangles were centered over each KT pair. The inner rectangle was set to cover over 90% of the KT pair and the outer rectangle was set to be twice the inner one in length (we chose not to change the width to prevent the inclusion of adjacent sister KT pairs, Figure 3.1B). The relative fluorescence intensity of protein of interest on chromosomes with different types of attachments was calculated as described above for KT-localized proteins, and the data were compared using ANOVA statistical tests.

Inter- and intra-KT stretching measurements. Fluorescence intensity profiles were generated automatically by the Elements software. The intra-KT distance was measured as the distance between the centroids of the X-Rhodamine labeled ACA and the Alexa-488 labeled Hec1 fluorescent signals within a single KT using linear pixel regions. The intra-KT distance for each KT was measured at a single focal plane because a KT was not expected to span over two focal planes. The inter-KT distance was measured as the distance between the two centroids of the X-rhodamine labeled ACA within a sister

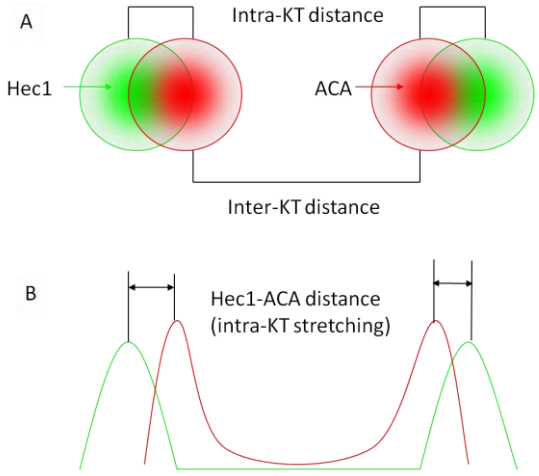

Figure 3.2. Schematic of intra-KT stretching measurement. A Projection of the fluorescent signals showing the centroids; B Fluorescence intensity profiles of the two signals. The intra-KT stretching corresponds to the distance between the peak of Hec1 intensity and ACA intensity.

KT pair (Figure 3.2). The measurements were exported and recorded into Microsoft Excel spreadsheets and ANOVA statistical tests were used to compare each type of KT/chromosome attachment to all the others.

Chapter 4: Results

Attachment-dependent changes in inter- and intra-KT stretching.

Centromere/KT tension has long been thought to play a role in SAC signaling (Jang et al., 1995; Li and Nicklas, 1995) and/or correction of KT mis-attachments (Gorbsky, 2004; Lampson et al., 2004; Nezi and Musacchio, 2009). For many years, tension was estimated by measuring centromere stretching, or inter-KT distance (i.e., the distance between the two sister KTs). However, two recent studies (Maresca and Salmon, 2009; Uchida et al., 2009) suggested that intra-KT stretching may play a more important role than inter-KT stretching in SAC activity. To determine whether and how syntelic KTs may be detected by the SAC, we compared intra- and inter-KT stretching of syntelic KTs/chromosomes to intra- and inter-KT stretching of chromosomes with other types of attachments. Different types of attachment (Figure 4.1) were identified and analyzed as described in Materials and Methods. We found that the intra-KT stretching in both unattached KTs (n=89) and merotelic KTs (n=66) was significantly lower

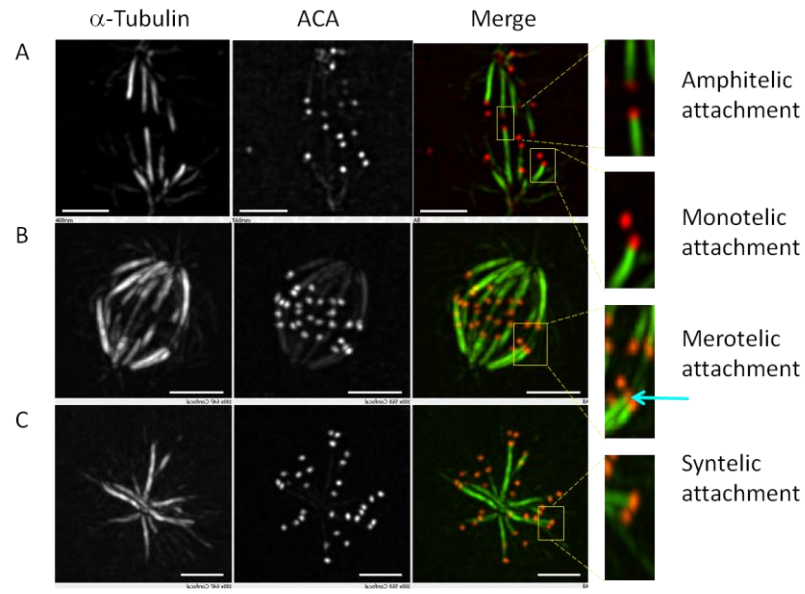

Figure 4.1. Examples of different types of KT mis-attachments in PtK1 cells.
The four columns of images represent, from left to right, single fluorescence channel images of α-tubulin and ACA staining, merged images of selected focal planes, and enlargements (5X for the first two arrows and 3X for the last two arrows) of the KT pair of interest, respectively. Examples of amphitelic and monotelic attachments are displayed in A, and examples of merotelic and syntelic attachments are displayed in B and C, respectively. Arrow, merotelic KT. Scale bar = 5μm.

compared with that in amphitelic KTs (n=237) (Figure 4.2E). This is not surprising because the force exerted on these KTs is either eliminated (unattached) or exerted in a direction that is at an angle rather than perpendicular to the KT (merotelically attached). We also found that both intra-KT and inter-KT stretching in syntelic KTs or chromosomes (n=69) were lower (ANOVA, $P<0.05$) compared to those in amphitelic KTs (n=474) or chromosomes (n=237). Finally, there was no difference in inter-KT stretching between amphitelic (n=237) and merotelic (n=66) chromosomes, whereas monotelic chromosomes (n=89) exhibited a slight decrease in inter-KT stretching (ANOVA, $P<0.05$) compared to amphitelic chromosomes (Figure 4.2D). We then

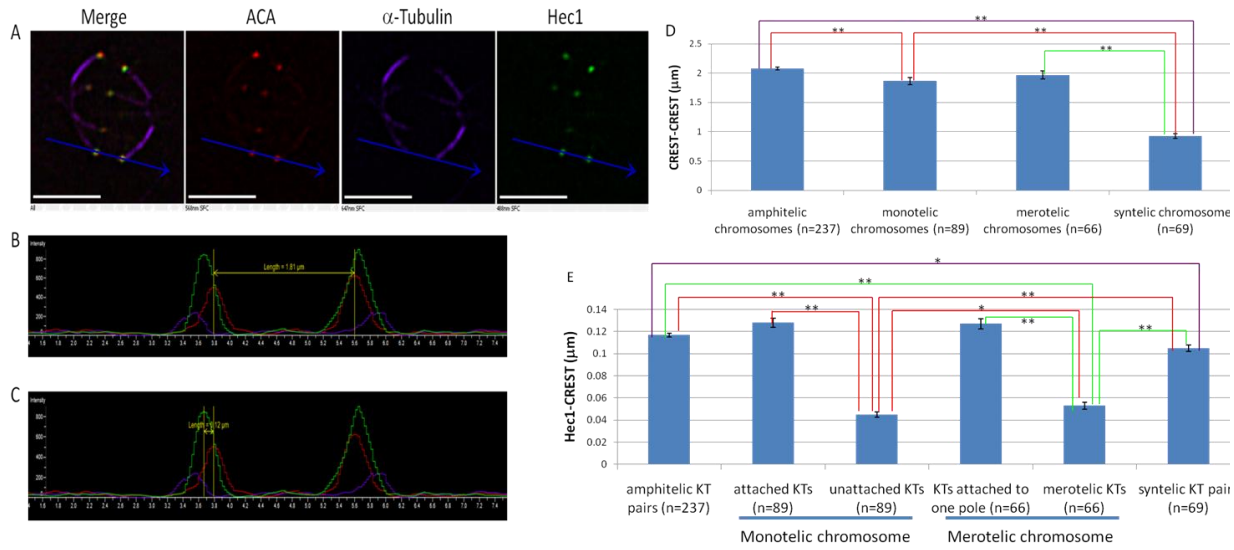

Figure 4.2. Changes in intra- and inter-KT stretching in response to different types of attachments. A. Representative fluorescent images of single focal plane of a PtK1 cell immunostained for inner KT proteins (ACA, red), Hec1 (green), and α-tubulin (purple). The blue arrow was traced so that it overlapped with the inter-KT axis of an amphitelic KT pair. The intensity profiles for the three fluorescence channels are shown in B and C. The yellow marks in B illustrate how the inter-KT stretching was measured, whereas those in C show how intra-KT stretching measurements were performed. Quantification of inter- and intra-KT stretching are shown in D and E, respectively. Error bars represent standard errors of the mean. *: ANOVA, $P<0.05$; **: ANOVA, $P<0.01$

determined the difference in KT stretching between mis-attached and amphitelically attached chromosomes. There was a substantial decrease in intra-KT stretching in response to monotelic (61.5%) and merotelic (54.7%) attachments. But the decrease in inter-KT stretching was much

less: 10.3% for monotely, 5.3% for merotely. Conversely, in syntelic attachments the decrease in intra-KT stretching (10.3%) was less pronounced than that in inter-KT stretching (55.6%).

Quantification of Mad2 at attached, unattached, and mis-attached KTs.

Mad2 plays a key role in SAC signaling (Hardwick et al., 1996; Musacchio and Salmon, 2007) and has been shown to accumulate at unattached KTs (Chen et al., 1996; Howell et al., 2000). It is not known whether Mad2 localizes at mis-attached (i.e., merotelic and syntelic) KTs before anaphase onset. However, because Mad2 displacement from the KT is believed to depend on MT attachment, merotelic KTs are not expected to accumulate Mad2 as a result of their attachment to spindle MTs. Although syntelic KTs are also attached to spindle MTs, it has been proposed that they may be not fully occupied due to the decreased tension, which would destabilize MT attachment (Kapoor et al., 2000; King and Nicklas, 2000; Nicklas et al., 2001). This may lead to accumulation of moderate levels of Mad2 on syntelic KTs. To test this prediction, we quantified Mad2 levels at KTs with different types of attachments (Figure 4.3A, D). As expected, the unattached KT of a monotelic chromosome accumulated significantly more (ANOVA, $P<0.01$) Mad2 (n=79, Mad2:CREST=1.07) than its attached sister (n=79, Mad2:CREST=0.43). Conversely, the comparison of Mad2 fluorescence intensity among merotelic (n=38, Mad2:CREST=0.43), syntelic (n=53, Mad2:CREST=0.38), and amphitelic KTs (n=206, Mad2:CREST=0.39) showed no significant difference (Figure 4.3D), indicating that Mad2 is not recruited to KTs as long as they are attached to MTs. Our data also suggest that both merotelic and syntelic KTs may be fully occupied (i.e., all their MT attachment sites bound to MTs) or that the number of unoccupied sites is not enough to recruit detectable levels of Mad2.

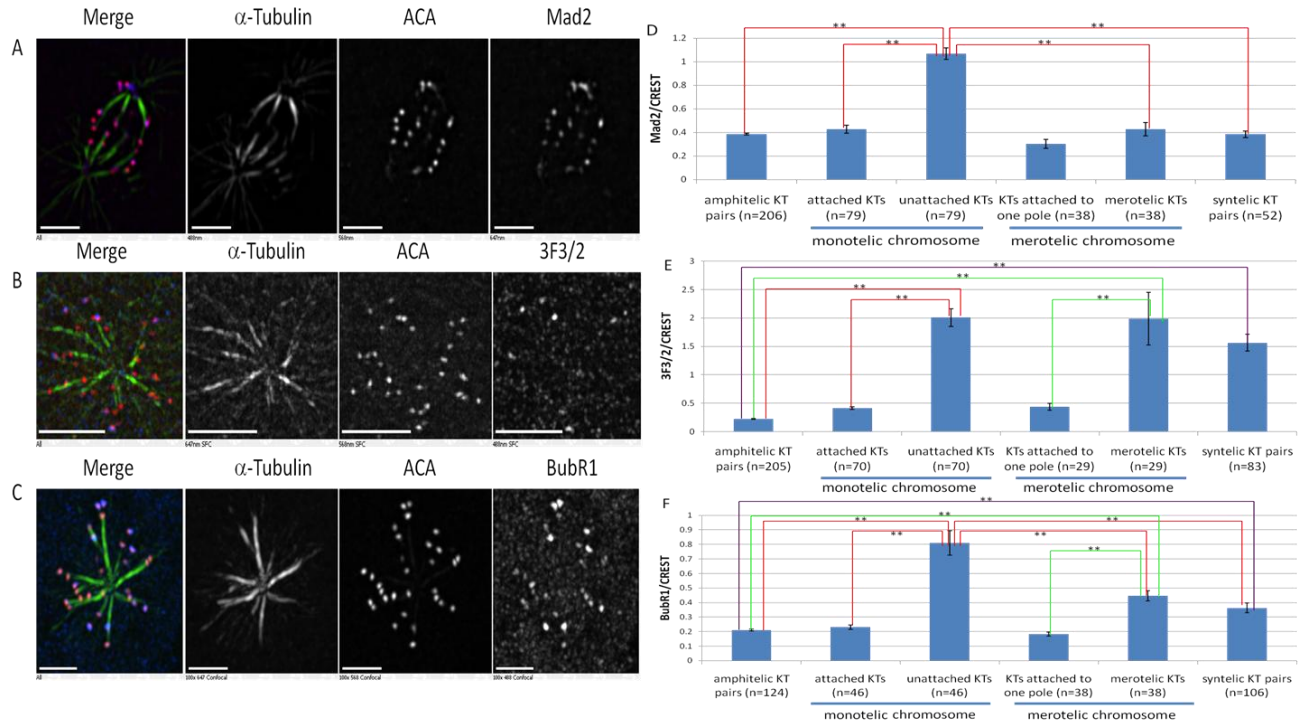

Figure 4.3. Quantification of Mad2, 3F3/2 phosphoepitope, and BubR1 at KTs with different attachments. Representative fluorescent images of PtK1 cells immunostained for Mad2 (A), 3F3/2 phosphoepitope (B), and BubR1 (C). *Scale bar*, 5μm. D-F. Quantification of fluorescence intensity of Mad2 (D), 3F3/2 (E), and BubR1 (F). Error bars represent standard errors. **: ANOVA, P<0.01

Quantification of 3F3/2 phosphoepitope and BubR1 at attached, unattached, and mis-attached KTs.

Previous studies have shown that the accumulation of certain proteins at the KT may depend on the levels of tension (Logarinho et al., 2004; Pinsky and Biggins, 2005). Two such molecules are the SAC protein BubR1 (Logarinho et al., 2004; Shannon et al., 2002; Skoufias et al., 2001) and the phosphoepitope recognized by the 3F3/2 antibody (Nicklas et al., 1995). We thus decided to measure the amount of these two components at attached, unattached, and mis-attached KTs (Figure 4.3B, E). We found that the 3F3/2 phosphoepitope accumulated on the unattached KTs (n=70, 3F3/2:CREST=2.00) of monotelic chromosomes compared to both their attached sister KTs (3F3/2:CREST=0.41; ANOVA, P<0.01) and to amphitelic KTs (n=206, 3F3/2:CREST=0.22; ANOVA, P<0.01) (Figure 4.3E). 3F3/2 phosphoepitope was also higher at

merotelic KTs (n=29, 3F3/2:CREST=1.98), compared to both their sister KTs attached to one spindle pole (3F3/2:CREST=0.43; ANOVA, P<0.01) and to amphitelic KTs (ANOVA, P<0.01). Finally, syntelic KTs showed significantly higher levels of 3F3/2 phosphoepitope (n=83, 3F3/2:CREST=1.56) compared to amphitelic KTs (ANOVA, P<0.01). These observations suggest that 3F3/2 phosphoepitope is accumulated on unattached and erroneously attached (i.e., merotelic and syntelic) KTs. The enrichment of 3F3/2 phosphoepitope on mis-attached KTs indicates that these KTs are under low tension.

The levels of BubR1 exhibited a pattern that somewhat resembled that of 3F3/2 phosphoepitope (Figure 4.3C, F). Indeed, unattached KTs of monotelic chromosomes displayed higher levels of BubR1 (n=46, BubR1:CREST=0.81) than both their attached sister KTs (BubR1:CREST=0.23, ANOVA, P<0.01) and amphitelic KTs (n=124, BubR1:CREST=0.21; ANOVA, P<0.01). Similarly, BubR1 was enriched on merotelic KTs (n=38, BubR1:CREST=0.45) compared to both their sister KTs attached to one spindle pole (BubR1:CREST=0.18; ANOVA, P<0.01) and amphitelic KTs (ANOVA, P<0.01) (Figure 4.3F). Finally, syntelic KTs (n=107, BubR1:CREST=0.36) recruited more BubR1 than did amphitelic KTs (ANOVA, P<0.01). These findings revealed that BubR1 was enriched on unattached, merotelic, and syntelic KTs. In summary, both the 3F3/2 phosphoepitope and BubR1 accumulated at unattached and mis-attached (merotelic and syntelic) compared to normally attached KTs. However, BubR1 accumulated at significantly higher levels on unattached KTs compared to both merotelic and syntelic KTs.

Quantification of AurB, INCENP, and MCAK at attached, unattached, and mis-attached KTs.

Correction of KT mis-attachments is believed to largely depend on AurB activity (Hauf et al., 2003; Lampson et al., 2004; Tanaka et al., 2002). AurB promotes correction of KT mis-attachment by inducing kMT turnover (Cimini et al., 2006), which occurs as a result of AurB-dependent phosphorylation of the outer KT protein Hec1 (DeLuca et al., 2006). AurB and its binding partner INCENP localize along the chromosome arms in early mitosis, but relocalize to the inner centromere during prometaphase. Substantial experimental evidence supports a model for AurB-dependent correction of KT mis-attachments in which the mis-attached (e.g., merotelic or syntelic) KTs are under reduced tension (thus exhibiting modest stretching), and therefore more proximal to the region (inner centromere) of high AurB concentration (Cimini et al., 2003; Liu et al., 2009). This would promote rapid turnover of kMTs, and thus correction of mis-attachments. INCENP is a binding partner of AurB that is important for AurB localization at the centromere (Bishop and Schumacher, 2002). Because none of the currently available antibodies against AurB recognizes the centromere-bound AurB in PtK1 cells, we quantified INCENP as an indirect measure of centromeric AurB. Another protein that is believed to contribute to correction of KT mis-attachments is MCAK (Mitotic Centromere-Associated Kinesin). Reduced levels of MCAK result in a dramatic increase in KT mis-attachments and anaphase lagging chromosomes (Kline-Smith et al., 2004). One study in Xenopus tissue culture cells found that both AurB and MCAK were increased at the centromeres of chromosomes with merotelic attachment (Knowlton et al., 2006). Thus, it appears that correction of KT mis-attachments may be promoted in two different ways: (i) by the proximity of AurB substrates (e.g., Hec1) to the AurB-enriched centromeric region (spatial component); (ii) by a preferential localization of AurB and MCAK at the centromere of chromosomes with mis-attached KTs (concentration

component). To account for both the spatial and the concentration component, we quantified the fluorescence intensity of AurB, INCENP, and MCAK in two ways: 1. By quantifying the amount overlapping with the KT (spatial component) as detected by CREST staining (same quantification method as that used for quantification of Mad2, 3F3/2 phosphoepitope, and BubR1). 2. By quantifying the amount of protein in a rectangular region including the sister KTs and the centromere (concentration component).

Our data showed that AurB was enriched at unattached KTs (n=60, AurB: CREST=0.26) compared to their attached sister KTs (AurB:CREST=0.08) (ANOVA, $P<0.01$) (Figure 4.4A, D). AurB was also recruited to merotelic KTs (n=22, AurB: CREST=0.24), but not to their sister KTs attached to one spindle pole (AurB: CREST=0.10) (ANOVA, $P<0.01$) (Figure 4.4D). Besides, AurB was more enriched at syntelic KTs (n=76, AurB: CREST=0.16) than at amphitelic KTs (n=134, AurB: CREST=0.10) (ANOVA, $P<0.01$) (Figure 4.4D). Thus, high levels of AurB are present at unattached, merotelic, and syntelic KTs. Quantification of KT-localized (spatial component) INCENP showed that it was present at higher levels on unattached KTs (n=51, INCENP/CREST=0.11) compared to their attached sister KTs (INCENP:CREST=0.07, ANOVA, $P<0.05$) (Figure 4.4B, E). INCENP was also enriched on syntelic KTs (n=40, INCENP:CREST=0.08) compared to amphitelic KTs (n=45, INCENP:CREST=0.04, ANOVA, $P<0.01$) (Figure 4.4E). However, INCENP showed similar levels on merotelic KTs (n=31, INCENP:CREST =0.06) compared to their sister KTs (INCENP:CREST =0.06) and to amphitelic KTs (ANOVA, $P>0.05$) (Figure 4.4E).

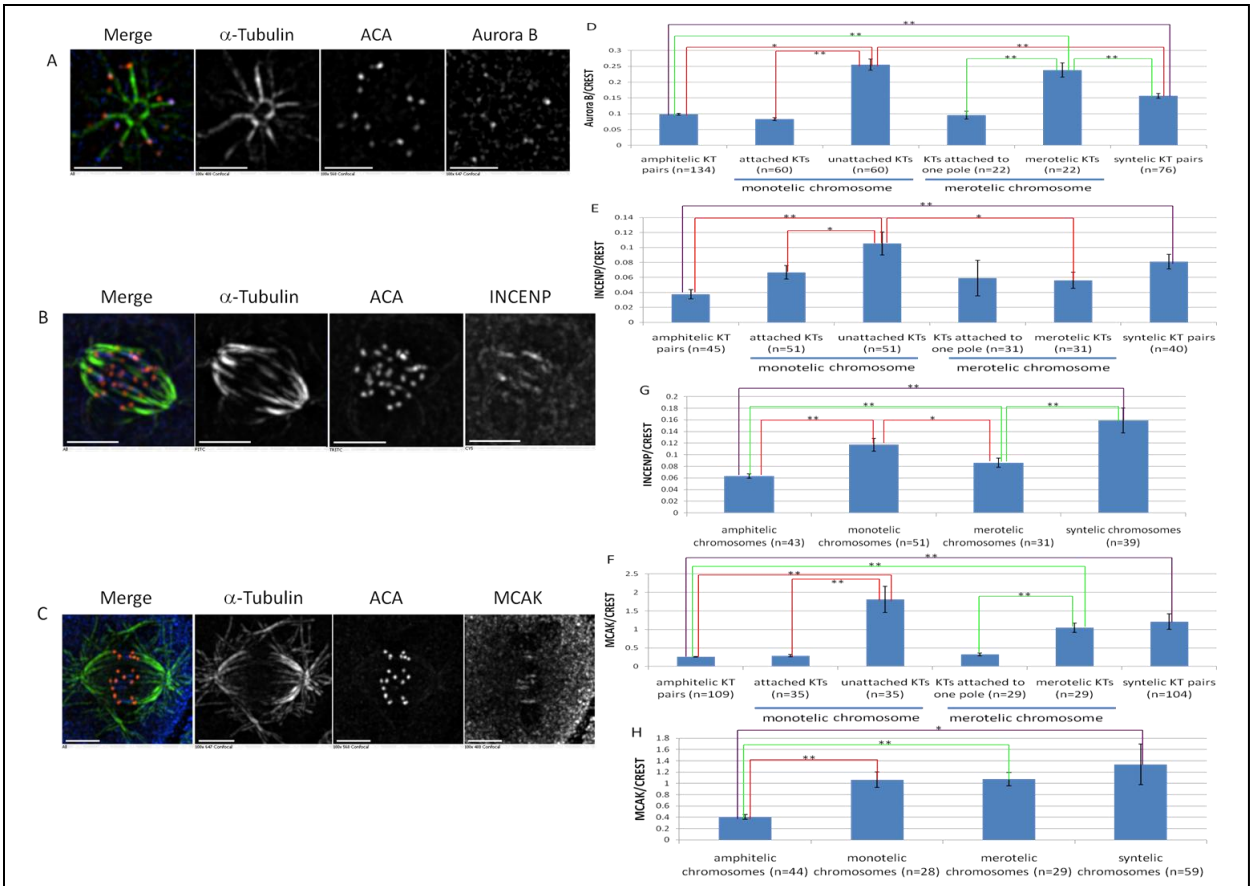

Figure 4.4. Quantification of AurB, INCENP, and MCAK at KTs with different attachments. Representative fluorescent images of PtK1 cells immunostained for MCAK (A), INCENP (B), and AurB (C). *Scale bar*, 5μm. D-F. Quantification of fluorescence intensity of Aurora B (D), INCENP (E), and MCAK (F) at KTs with different attachments. G-H. Quantification of fluorescence intensity of centromere –associated INCENP (G) and MCAK (H). Error bars represent standard errors. *: ANOVA, P<0.01; **: ANOVA, P<0.05

Quantification of the "concentration" component indicated that INCENP was present at higher levels at the centromere of chromosomes with mis-attached KTs (Figure 4.4G). Indeed, INCENP showed a 2-fold increase on monotelic chromosomes (n=51, INCENP:CREST =0.12), a 2.5-fold increase on syntelic chromosomes (n=39, INCENP:CREST =0.16), and 1.5-fold increase on chromosomes with one merotelic KT (n=31, INCENP:CREST =0.09) compared to amphitelic chromosomes (n=43, INCENP:CREST =0.06) (ANOVA, P<0.01 in all cases).

Quantification of the "spatial" component (KT-localized) of MCAK indicated that it was enriched at unattached KTs (n=35, MCAK:CREST=1.81) compared to the attached KTs

(MCAK:CREST=0.29) (ANOVA, P<0.01) of monotelic chromosomes (Figure 4.4C, F). MCAK was also enriched at merotelic KTs (n=29, MCAK:CREST=1.04) compared to their sister KTs (MCAK:CREST=0.32, ANOVA, P<0.01) attached to only one spindle pole (Figure 4.4F). Finally, MCAK was present at significantly higher levels on syntelic KTs (n=104, MCAK:CREST=1.21) than on amphitelic KTs (n=109, MCAK:CREST=0.26, ANOVA, P<0.01) (Figure 4.4F). Quantification of the "concentration" component (centromere-associated) showed that MCAK was present at high levels at the centromere of mis-attached chromosomes (Figure 4.4H). Indeed, the amount of MCAK at the centromere of both monotelic (n=28, MCAK:CREST=1.07) and merotelic chromosomes (n=29, MCAK:CREST=1.08) was 2.5-fold that found at the centromere of amphitelic chromosomes (n=44, MCAK:CREST=0.41). MCAK was present at even higher levels (3.27-fold compared to amphitelic) at the centromeres of syntelic chromosomes (n=59, MCAK:CREST=1.34) (ANOVA, P<0.01 in all cases).

	Intra-KT stretching	Mad2	3F3/2	BubR1	INCENP	MCAK	AurB
Unattached KTs	↓ ↓	↑	↑ ↑	↑ ↑	↑	↑ ↑	↑ ↑
Syntelic KTs	↓	=	↑	↑	↑ ↑	↑ ↑	↑
Merotelic KTs	↓ ↓	=	↑ ↑	↑ ↑	=	↑ ↑	↑

Table 4.1. Summary of the changes in KT structure and molecular composition in response to mis-attachments. Amphitelic KTs are set as control.

	Inter-KT stretching	INCENP	MCAK
Monotelic attachments	↓	↑ ↑	↑ ↑
Syntelic attachments	↓ ↓	↑ ↑	↑ ↑
Merotelic attachments	=	↑ ↑	↑ ↑

Table 4.2. Summary of the changes in KT structure and molecular composition in response to mis-attachments. Amphitelic attachment is set as control.

Chapter 5: Discussion

Accurate mitotic chromosome segregation is ensured by amphitelic KT attachment. However, different types of attachments, including monotelic, merotelic, and syntelic, can and do occur in early mitosis. All these attachments are efficiently, although not always completely, converted into amphitelic attachments before anaphase onset. In some cases, the SAC induces a delay in anaphase onset to allow for correction. The SAC is known to respond to a signal generated by unattached KTs (Rieder et al., 1995; Rieder et al., 1994). It has also been suggested that the SAC may respond to lack of tension (Jang et al., 1995; Li and Nicklas, 1995), which for many years was estimated by measuring the distance between the two sister KTs (inter-KT distance) (Waters et al., 1996). Two recent studies (Maresca and Salmon, 2009; Uchida et al., 2009) suggested that intra- rather than inter-KT distance may play a major role in SAC signaling and in the consequent mitotic delay (Maresca and Salmon, 2010). Here, we investigated what factors may be important for detection and/or correction of syntelic attachments. In order to do this, we also characterized KTs with other types of attachments for which the SAC response and/or the efficiency of correction are well characterized.

Intra-KT stretching per se does not appear to be the key factor in the response to syntelic attachment.

We found that the intra-KT distance of syntelic KTs is reduced compared to amphitelic KTs. However, the intra-KT distance is also reduced, and even more so, in merotelic KTs and in the unattached KT of monotelic chromosomes. Whereas there is substantial evidence that unattached KTs induce a SAC-dependent mitotic delay (Rieder et al., 1995; Rieder et al., 1994; Waters et al., 1998), merotelic attachments do not appear to cause a mitotic delay (Cimini et al., 2004; Cimini

et al., 2002; Khodjakov et al., 1997), and they can persist throughout mitosis (Cimini et al., 2001). Thus, it appears that the association between intra-KT stretching and SAC response is not causal. This is not surprising if one considers the finding of Uchida et al. (Uchida et al., 2009) that amphitelic KTs oscillate between high and low intra-KT stretching. One would imagine that only a permanent structural change could cause a biochemical response. Given that intra-KT stretching cycles between high and low, it seems unlikely that this alone may cause the SAC-dependent mitotic delay in response of monotelic attachment.

We also found that inter-KT stretching is reduced for both monotelic and syntelic chromosomes compared to amphitelic ones, but not for chromosomes in which one sister is merotelically attached. This may suggest that the inter-KT stretching is important for the mitotic delay induced by unattached KTs. However, Maresca and Salmon (Maresca and Salmon, 2009) could, under certain conditions, reduce the inter-KT stretching without delaying anaphase onset. Thus, we conclude that neither intra- nor inter-KT stretching is a direct cause of mitotic delay in response to KT mis-attachments. However, the reduced inter-KT stretching may play a key role in rapid correction of syntelic attachments (see below).

What causes the reduced intra-KT distance in unattached, merotelic, and syntelic KTs?

We propose that intra-KT stretching is due to opposing forces applied on individual KTs. These forces are the pulling force from the attached MTs and an opposing force dependent on the anchoring of the KT to the underlying centromeric chromatin (Figure 5.1A). Based on this assumption, it is not surprising that unattached KTs exhibit reduced intra-KT stretching. Indeed,

the lack of MT-dependent pulling forces would result in lack of stretching (Figure 5.1B). For syntelic KTs, the intra-KT stretching is also reduced compared to amphitelic KTs, but to a lesser extent. This may be because syntelic KTs are subject to pulling forces exerted by the attached MTs. However, the pulling forces exerted on the two sister KTs are directed in the same direction, as opposed to amphitelic sister KTs, in which the forces are directed in opposite directions (Figure 5.1C). Whereas it seems more likely that the opposing MT-dependent forces applied on the sister KTs contribute to inter-KT stretching rather than to intra-KT stretching, they may also provide a minor contribution to the intra-KT stretching. If that is the case, the opposing pulling forces in amphitelic sister KTs may result in a slightly larger stretching. This conclusion, of course, implies that the forces are transmitted through the centromeric chromatin, an assumption that to date has no clear experimental evidence. The reason why intra-KT stretching is highly reduced in

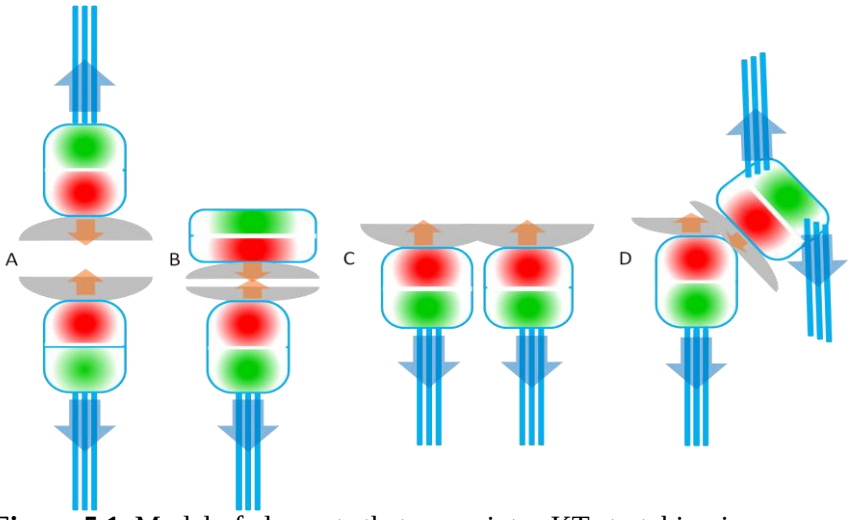

Figure 5.1. Model of elements that cause intra-KT stretching in response to different attachments.
Intra-KT stretching is represented by the distance between the outer (green) and inner (red) KT, The chromatin underlying each KT is shown in grey. Intra-KT stretching is mainly due to two opposing forces applied on individual KTs. These forces are the pulling force from the attached MTs and an opposing force dependent on the anchoring of the KT to the underlying centromeric chromatin. The pulling forces from the MTs bound to the sister KT may also provide a minor contribution. Amphitelic KTs (A) are subject to all these forces, and thus the intra-KT stretching is maximal. Unattached KTs (B) do not experience the MT-dependent pulling forces, and thus exhibit the lowest stretching. Syntelic KTs (C) do not experience the pulling forces from the MTs bound to the sister KT, and thus their intra-KT stretching is slightly smaller than the stretching in amphitelic KTs. For merotelic KTs (D) the pulling forces from the bound MTs pull the KT at an angle rather than perpendicularly, and this result in a significantly smaller stretching. See text for further details.

merotelic KTs is less obvious. However, it may be explained by the fact that for merotelic attachments the bound MTs pull the KT at an angle rather than perpendicularly. Thus, merotelic KTs lack the MT-dependent pulling forces oriented perpendicularly to the KT that would cause a significant increase in intra-KT stretching (Figure 5.1D).

Kinetochore attachment and accumulation of Mad2, 3F3/2, and BubR1.

We found high levels of Mad2 only at unattached KTs, whereas mis-attached (merotelic or syntelic) KTs possessed levels of Mad2 comparable to those found at amphitelic KTs. This finding indicates, in agreement with previous studies (Waters et al., 1998; Yang et al., 2009), that the Mad2-dependent SAC response is only triggered by lack of attachment. Our finding further suggests that merotelic and syntelic KTs are fully attached.

Conversely, we found that both the 3F3/2 phosphoepitope and BubR1 were present at high levels at unattached as well as at mis-attached (merotelic and syntelic) KTs compared to amphitelic KTs. Because cells with merotelic attachments do not exhibit a delay of anaphase onset (Cimini et al., 2001; Kline-Smith et al., 2004), this finding may indicate that the 3F3/2 phosphoepitope and BubR1 do not play a significant role in the SAC response. Nevertheless, most cells are believed to establish at least one merotelic attachment in early mitosis (Cimini et al., 2003; Silkworth et al., 2009), so it is possible that the average duration of mitosis includes a 3F3/2- and/or BubR1-dependent delay. But because nearly all cells would exhibit this kind of delay, it is impossible to separate the contribution of merotelic and syntelic attachments and the contribution of 3F3/2 and BubR1 to the mitotic delay. It is also worth noting that although BubR1 levels are high at both unattached and mis-attached KTs, the levels at unattached KTs are the highest when

compared to merotelic and syntelic KTs. Thus, one possibility is that there is a threshold for the amount of BubR1 necessary to cause a significant mitotic delay.

Aurora B, INCENP, and MCAK at unattached and mis-attached KTs.

We found high levels of Aurora B kinase (AurB) at unattached, merotelic, and syntelic KTs. However, we were only able to detect AurB at the outer KT and could not detect centromeric AurB. A recent study showed that there are, indeed, two pools of AurB, one at the centromere and one at the outer KT, and the outer KT pool appears to be the active, phosphorylated version of the protein (DeLuca et al., 2011). It has been suggested that the distance between the AurB substrates at the outer KT and the centromeric AurB may play a key role in correction of KT mis-attachments. However, the antibody used in this study and many others commercially available do not recognize the centromeric AurB in PtK1 cells (our tests and DeLuca, personal communication), and thus we estimated the amount of centromere-bound AurB by quantifying its binding partner INCENP. We found that INCENP is high at unattached, merotelic, and syntelic KTs. We also observed high levels of the centromeric INCENP at monotelic, merotelic, and syntelic chromosomes. The same pattern, more at the KT and more at the centromere, was observed for MCAK. However, for INCENP and MCAK the KT-associated population did not appear as a separate pool. Instead, it simply appeared to be portion of the centromeric pool overlapping with the KT, whose amount varied depending on the inter-KT stretch. Indeed, the levels of KT-associated MCAK and INCENP were highest for monotelic and syntelic chromosomes, which exhibited the lowest inter-KT stretching. However, there was also a difference between the unattached and the attached sister of monotelic chromosomes and between merotelic KTs and their sisters. This observation suggests that intra-KT stretching may

play a role in removal of these proteins from specific sub-regions of the centromere. In other words, upon attachment, the protein would leave the centromeric region closest to the attached KT, but not the region closest to the unattached or merotelically attached KT.

How do cells respond to different types of attachments?

Taken together, our data suggest that monotelic attachments are the only ones that can trigger a SAC-dependent mitotic delay, and that Mad2 is the major molecular player in SAC signaling. Whereas intra-KT stretching is significantly reduced in unattached KTs, the same levels of intra-KT stretching are observed for merotelic KTs, which do not induce a SAC-dependent mitotic delay, thus suggesting the intra-KT stretching *per se* is not a key factor in SAC signaling. Inter-KT stretching does not appear to play a key role in SAC signaling either (Maresca and Salmon, 2009; Uchida et al., 2009). However, it may play a role in promoting correction of KT mis-attachment. Indeed, syntelic chromosomes exhibit the lowest inter-KT stretching and the largest KT-associated amounts of those proteins (AurB, MCAK) that have been shown to play a role in correction of KT mis-attachments. This may lead to rapid and efficient correction of syntelic attachments. That syntelic attachments are not detected by the SAC is not surprising given that conditions that induce an increase in syntelic attachments do not necessarily induce a mitotic arrest (Yang et al., 2009).

In figure 5.2, we illustrate how inter-KT stretching contributes to AurB-dependent error correction. Upon amphitelic attachment (Figure 5.2A), the sister KTs are pulled away from the region of high AurB concentration. Although there appears to be an outer KT-localized subpopulation of AurB, amphitelic attachment is also believed to lead to Protein Phosphatase 1

(PP1) recruitment (Liu et al., 2010), so that AurB-dependent phosphorylation would be counteracted by PP1-dependent dephosphorylation. In the case of monotelic orientation (Figure 5.2B), the unattached sister KT is expected to move closer to the region of high AurB concentration, which is confirmed by our quantification of KT-localized INCENP. However, in the case of monotelic attachment the SAC is maintained active. It is also worth noting that, although reduced, the inter-KT stretching in monotelic chromosomes is significantly larger than in syntelic chromosomes. Thus, binding of just one or few MTs may be enough to move the KT out of the region of high AurB, and possibly induce centromeric AurB removal (see above), for the attachments to become rapidly stable. For syntelic chromosomes (Figure 5.2C), the inter-KT stretching is largely reduced,

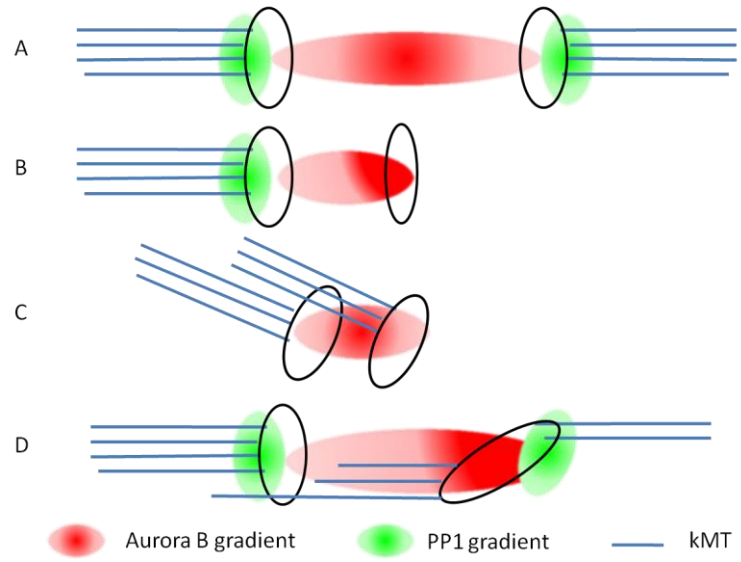

Figure 5.2. Model illustrating how inter-KT stretching contributes to AurB-dependent error correction. Upon amphitelic attachment (A), the sister KTs are pulled away from the region of high AurB concentration. Amphitelic attachment is also believed to lead to Protein Phosphatase 1 (PP1) recruitment, so that residual phosphorylation due to the low AurB gradient or to the low levels of kinetochore-localized AurB (not depicted here) would be counteracted by PP1-dependent dephosphorylation. In the case of monotelic orientation (B), the unattached sister KT is expected to move closer to the region of high AurB concentration. However, in the case of monotelic attachment the SAC is maintained active. For syntelic chromosomes (C), the inter-KT stretching is largely reduced, which would bring both sister KTs in close proximity with the region of high AurB concentration. This may lead to rapid turnover of kMTs bound to syntelic KTs, and thus to prompt correction of this type of attachment. Merotelic attachment (D) does not result in significant reduction of the inter-KT stretching compared to amphitelic attachment. However, merotelic KTs frequently exhibit a lateral stretch, which would bring the portion of the KT bound to the incorrect pole closer to the high AurB region compared to the rest of the KT. This could lead to preferential detachment of the incorrectly attached MTs. See text for further details.

which would bring both sister KTs in close proximity with the region of high AurB concentration. This may lead to rapid turnover of kMTs bound to syntelic KTs, and thus to prompt correction of this type of attachment. Merotelic attachment (Figure 5.2D) does not result in significant reduction of the inter-KT stretching compared to amphitelic attachment. As a result, the merotelic KT as a whole is not expected to move close to the region of high AurB. However, merotelic KTs frequently exhibit a lateral stretch, which would bring the portion of the KT bound to the incorrect pole closer to the high AurB region compared to the rest of the KT. This could lead to preferential detachment of the incorrectly attached MTs. It will be interesting to determine whether PP1 localizes at syntelic and merotelic KTs to determine its possible contribution to kMT turnover versus stabilization.

Chapter 6: Summary and future direction

In summary, the data shown here indicate that monotelic attachment is the only type of attachment that can induce a SAC response. The unattached KT of monotelic chromosomes retains significantly higher level of SAC protein, Mad2, than its sister KT or amphitelic KTs. Neither merotelic KTs nor syntelic KTs accumulate Mad2, strongly suggesting that these KTs are fully occupied by MTs and cannot induce a SAC response. However, our data indicate that both syntelic and merotelic KTs are under less tension because they show higher level of 3F3/2 phosphoepitope and BubR1. Our data also suggest that neither intra- nor inter-KT stretching is a direct cause of SAC activation. But together with the MCAK, INCENP, and AurB data, our findings suggest that the decrease in KT stretching may contribute to bringing specific KT substrates closer to AurB for rapid and efficient correction of KT mis-attachments. Furthermore, decreased stretching also appears to result in retention of the error correction complex at the centromere, which would thus result in efficient correction of merotelic and syntelic attachments.

A question that remains to be addressed is how tension regulates preferential turnover of incorrectly attached MTs. It is possible, for instance, that the phosphorylated form of certain proteins (e.g., AurB substrates) may not persist at the same location as the unphosphorylated form, as is the case for MCAK (Knowlton et al., 2006). Therefore, it will be interesting to map the localization of active AurB, its substrates, its regulators [e.g., ICIS (Ohi et al., 2003)], and its binding partners (e.g., INCENP) within a KT on a nanometer scale under changing KT attachment conditions. Other unanswered questions include if/how the AurB regulators/partners directly respond to tension. For example, it was recently proposed that INCENP may function as a tension-sensor (Sandall et al., 2006), but it is still largely unknown how INCENP and MTs interact and how this interaction can be translated into an increase in AurB activity. Structural

studies may be necessary to gain insight into this issue. Finally, because of its proposed role in counteracting AurB activity, it will be important to determine whether and how PP1 localizes at syntelic and merotelic KTs, and how its localization is regulated by tension at the level of individual KTs. The combination of all these pieces of knowledge will be critical to gain complete understanding of how the equilibrium between kMT turnover and stabilization is achieved. Because cancer cells are prone to form KT mis-attachments (Ganem et al., 2009; Silkworth et al., 2009; Thompson and Compton, 2008) and exhibit defective error correction (Bakhoum et al., 2009), understanding the mechanisms behind SAC activation and error correction might yield new insights into the mechanisms of chromosomal instability in cancer cells.

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