The function of lateral attachments in stable chromosome biorientation and in silencing the mitotic checkpoint Literature thesis

by Jolien van Hooff, supervised by prof. dr. Geert Kops

Proper cell division relies on equal distribution of sister chromatids over daughter cells. This process is executed by a bipolar mitotic spindle, which via microtubule plus ends connects kinetochores of both sister chromatids to opposite poles. Various control systems have evolved to ensure equal segregation, such as destabilization of erroneous kinetochore-microtubule attachments. How these destabilizing actions are eventually repressed to allow for establishment of correct attachments has not yet been completely elucidated. Here, I will discuss a model that explains how destabilization of kinetochore-microtubule attachments can be overcome. In this model, attachments of kinetochores to the sides of microtubules play a crucial role. Properties of such lateral attachments will be examined here, including their effects on stable kinetochore-microtubule attachments, the generation of force on the chromosomes and mitotic progression.

During mitosis, duplicated chromosomes forming sister chromatids are being separated and translocated to opposite ends of the cell by the machinery of the mitotic spindle. This bipolar spindle consists of two oppositely located centrosomes from which microtubules extend to the chromosomes that are aligned on the spindle equator. Microtubules from the opposite poles are to attach to each of the two sister chromatids via kinetochores, multiprotein complexes assembled on centromeres (see Box 1 for a summary of the kinetochore composition). Cleavage of cohesin, the protein multimer that holds the sister chromatids together, combined with spindle pulling forces, separates the sister chromatids. Subsequent depolymerization of the spindle microtubules carries them to the opposite centrosomes. The need for a daughter cell to obtain one copy of each chromosome after cell division demands the process of chromosome segregation to be strictly regulated. Errors might occur if kinetochores lack microtubule attachments, but also if a single kinetochore has attachments to microtubules of both poles (merotelic attachment) or if both sister kinetochores are attached to the same pole (syntelic attachment). Two control mechanisms have been proposed to monitor errors and postpone sister chromatid separation until these errors are corrected. First, unattached kinetochores produce a signal that inhibits the progression of mitosis by preventing cohesin cleavage, a regulatory mechanism known as the mitotic checkpoint or the spindle assembly checkpoint (reviewed in Lara-Gonzalez, Westhorpe, Taylor 2012; Musacchio and Salmon 2007; see also Box 2). Second, erroneous attachments, for example the ones in which both sister kinetochores are connected to the same spindle pole, are destabilized by phosphorylations of kinetochore proteins involved in microtubule binding (Welburn et al. 2010). An indicator of incorrect attachment is a lack of tension on the centromeres: proper bipolar microtubule attachments produce tension as a result of cohesin which opposes the bidirectional poleward forces, incorrect attachments would not. The absence of tension induces

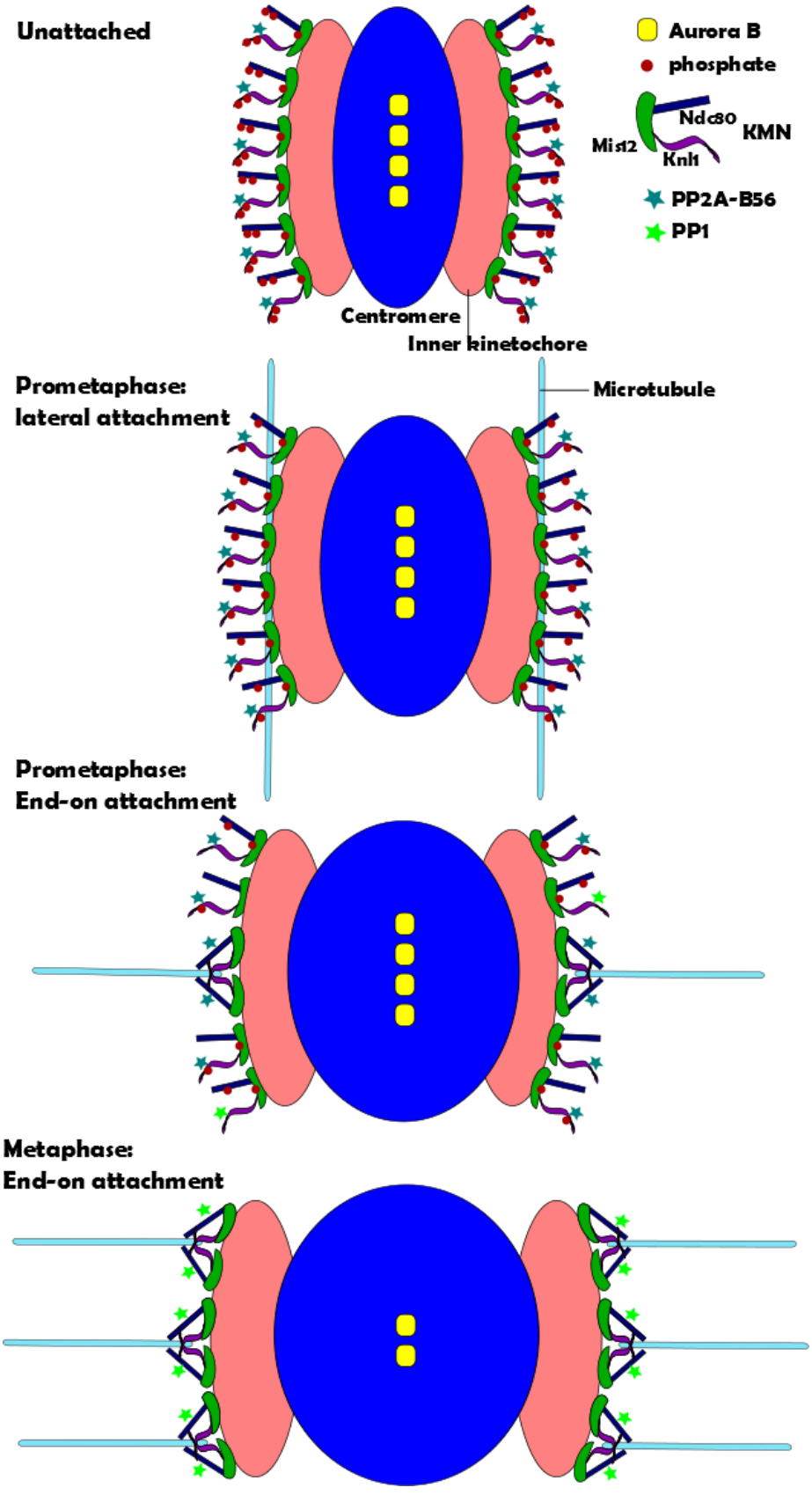


Figure 1. Model suggested by Foley and Kapoor (2013). The outer kinetochore microtubule binding site of the KMN network is phosphorylated by Aurora B when the distance of Aurora B to its targets is small, which is the case at unattached kinetochores. These phosphorylations hinder sustainable microtubule-kinetochore attachments. During prometaphase, lateral attachments, which do not require the KMN, increase the distance between Aurora B and its targets. Phosphorylation levels decrease due to phosphatase PP2A-B56. Attachments mediated by the KMN become more and more stable, and are fully stabilized by PP1 phosphatase.

destabilizing phosphorylations carried out by the kinase Aurora B, a protein part of the chromosomal passenger complex residing between the two sister kinetochores (Ditchfield et al. 2003; Hauf et al. 2003; Tanaka et al. 2002). This suggested error correction mechanism however implies a positive feedback loop that initially unattached kinetochores need to circumvent in order to acquire stabilized microtubule attachments. After all, unattached kinetochores do not generate any tension and will therefore be highly phosphorylated. Foley and Kapoor (2013) proposed the following model for the

Box 1: Composition of the kinetochore

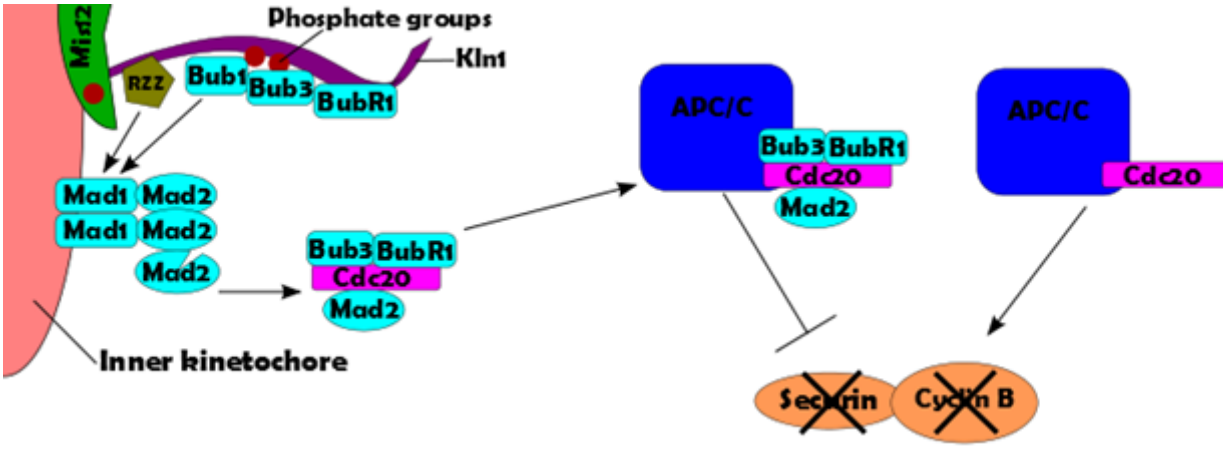
The kinetochore consists of a large number of proteins (approximately 80 have been defined in humans) that physically link chromosomes to mitotic spindle microtubules (reviewed in Cheeseman and Desai 2008; Lampert and Westermann 2011). The kinetochore is built on the centromeres of chromosomes which are defined by the presence of nucleosomes containing a centromere-specific variant of histone H3, termed CENP-A (Van Hooser et al. 2001). Various other CENPs form a layer on top of CENP-A nucleosomes, which makes up the constitutive centromere-associated network (CCAN, e.g. Foltz et al. 2006). Remarkably, most CCAN genes have not been detected yet in invertebrate animals and in plants. Throughout the cell cycle, CCAN proteins are located at the centromere. With the onset of the M-phase, the elements important for microtubule binding are assembled onto the centromere. The KMN network is the main site for microtubule attachment. The KMN network consists of the Mis12 complex, the Ndc80 complex and Knl1 (Varma and Salmon 2012). In this, Mis12 is thought to link the CCAN with the microtubule interacting components Knl1, Ndc80 and Nuf2. In addition, microtubule motor proteins dynein (via Zwint, the RZZ complex and Spindly) and CENP-E are localized to the kinetochore at prometaphase. Furthermore, CENP-F, the SKA complex (metazoa) and the Dam1 complex (yeast) are putative microtubule-interacting factors at the kinetochore, predicted to play a role in tracking depolymerizing microtubules (Gaitanos et al. 2009; Tien et al. 2010; Vergnolle and Taylor 2007).

formation of stable kinetochore-microtubule association (Figure 1). Starting from the unattached kinetochores, the centromere-localized Aurora B kinase is in close proximity to the outer kinetochore and is therefore able to phosphorylate the KMN network, the core microtubule-binding site of the kinetochore (Cheeseman et al. 2006, see also Box 1). The highly phosphorylated KMN network has a low affinity for microtubules. During prometaphase, the kinetochores associate with the sides of microtubules. In contrast to end-on attachments, these lateral interactions occur independently of the KMN network. The lateral interactions produce a small amount of tension that is just sufficient to cause stretching of the centromere. As a result, Aurora B becomes spatially separated from its outer kinetochore targets, after which dephosphorylations by outer kinetochore-localized phosphatase PP2A-B56 can take place freely (Foley, Maldonado, Kapoor 2011). End-on attachments of microtubules with the KMN network are allowed and stabilized, creating even more tension and diminishing Aurora B activity. Full microtubule occupancy of the kinetochore is established and PP2A-B56 is removed from the kinetochore. Low level Aurora B phosphorylation of KMN subunit Knl1 enables Knl1 to recruit phosphatase PP1. PP1 ensures the sustained unphosphorylated state of the outer kinetochore and thus of stabilized kinetochore-microtubule attachments (Liu et al. 2010). This model relies on several important assumptions regarding the microtubule-binding capacities of non-KMN kinetochore constituents, the need for lateral attachments prior to end-on attachments and synchronization of mitotic checkpoint activity with the lateral to end-on attachment conversion. Here, I will discuss the role of lateral attachments in the establishment of the metaphase plate, mainly focusing on metazoan and mammalian systems. In addition, I will assess whether the proposed model can be integrated with current knowledge of mitotic checkpoint activation and silencing.

Box 2: Components and functioning of the mitotic checkpoint

The spindle assembly checkpoint or mitotic checkpoint is the conserved surveillance system of mitosis, controlling the attachment of kinetochores to the spindle. It is comprised of various proteins that are being localized to unattached kinetochores. At this site, the checkpoint proteins produce a signal that inhibits the progression of mitosis (Musacchio and Salmon 2007). In summary, kinetochore-bound Mad1 forms a dimer with Mad2 that enables the conversion of unbound Mad2 from an open form (O-Mad2) to a closed form (C-Mad2). C-Mad2 is able to associate with Cdc20, and this complex engages with Bub3/BubR1. The resulting tetrameric complex is referred to as the mitotic checkpoint complex (MCC). Free Cdc20 is a cofactor of the anaphase-promoting complex/cyclosome (APC/C), which degrades securin (an inhibitor of separase, the enzyme which cleaves cohesins) and cyclin B (an important regulator of mitosis). The MCC complex inhibits the APC/C by interacting with it. As long as there is sufficient MCC to block APC/C, securin and cyclin B will continue to hold sister chromatids together and to inhibit mitotic progression, respectively (see figure). The protein kinase Mps1 seems to play a central role in the kinetochore recruitment of mitotic checkpoint components (Hewitt et al. 2010; London et al. 2012). At unattached kinetochores, Mps1 phosphorylates the KMN network member Knl1. Knl1 binds the checkpoint proteins Bub1 and Bub3 and, indirectly, is responsible for kinetochore binding of Rod-ZW10-Zwilch (RZZ) and BubR1/Mad3. RZZ and Bub1 cooperate to recruit a Mad1/Mad2 heterodimer to the unattached kinetochore, an essential step in generating soluble C-Mad2 and thereby MCC.

The architecture of the spindle assembly checkpoint meets certain specific requirements regarding its sensitivity. First, as it has been shown in mammalian cells that a single unattached kinetochore can produce a signal strong enough to delay mitotic exit, a strong amplification of the signal is required. One of the suggested mechanisms for signal amplification is additional conversion of Mad2 by the Cdc20-Mad2 complex in the cytosol, thereby producing more C-Mad2 able to bind Cdc20 (Musacchio and Salmon 2007). Second, it needs to be highly responsive, so the mitosis inhibition signal is quickly switched off and on upon kinetochore-microtubule attachment or detachment, respectively. Probably, multiple processes contribute to silencing of the mitotic checkpoint by eliminating the presence of mitotic checkpoint proteins at the kinetochore if microtubules are attached (reviewed in Kops and Shah 2012). The stripping of mitotic checkpoint elements by the kinetochore-localized motor protein dynein appears to be important, as is diminishing Mps1 levels at the kinetochore by reduced activity of Aurora B. Also, the reversal of Mps1 phosphorylations by phosphatase PP1 and the disassembly of the MCC by p31comet have been suggested to contribute to mitotic checkpoint silencing (Kops and Shah 2012). The existence of complementary and additional silencing pathways might aid in quickly responding to microtubule attachment. However, given the robust clearance of the checkpoint proteins from the kinetochore upon microtubule attachment, an outstanding question is if and how the checkpoint is reactivated in case of a loss of attachment.



Lateral attachments during prometaphase

Proper anaphase chromosome replacement to the spindle poles depends on attachment of the plus ends of spindle microtubules to the kinetochores. End-on attachments of microtubules to kinetochores form so-called K-fibers. Earlier in mitosis, side-on attachments of kinetochores to microtubules occur. These so-called lateral attachments offer a possible solution to overcome the destabilizing actions of Aurora B, a kinase that probably inhibits direct constitution of stable, end-on attachments to the outer kinetochore KMN network. If this is true, lateral attachments are an important prerequisite for proper mitosis, preceding the formation of end-on attachment. It indeed has long been recognized that just after breakdown of the nuclear envelope, initial contacts between microtubules and kinetochores consist of kinetochores binding to the lateral walls of the microtubules in various organisms (Alexander and Rieder 1991; Kitajima, Ohsugi, Ellenberg 2011; Magidson et al. 2011). Since microtubule sides provide a much larger surface area than microtubule tips, there is a higher probability for a kinetochore to first interact with the sides. Laterally captured by a microtubule, chromosomes are being translocated towards one of the spindle poles by microtubule motor protein dynein and its cofactor dynactin (Li et al. 2007; Vorozhko et al. 2008; Yang et al. 2007). Here, the lateral attachment is being converted into an end-on attachment connecting one or both kinetochores to the nearest spindle pole. After this, chromosomes need to congress to the metaphase plate (aka the spindle equator) a process aided by CENP-E, a plus end-directed motor of the kinesin-7 subfamily (Kapoor et al. 2006; Wood et al. 1997; Yang et al. 2007). By then, most chromosomes are bioriented. Although Foley and Kapoor (2013) state that the KMN network is not involved in these lateral interactions, it has also been suggested that the KMN network itself directly interacts with the sides of microtubules in yeast (Tanaka et al. 2005).

Examining the model drafted by Foley and Kapoor entails questioning A) if stable end-on attachments can be established without initial lateral interactions and B) whether lateral attachments are capable of generating interkinetochore tension. In the following sections, I will attempt to answer both questions by zooming in on the formation and nature of lateral attachments and the molecular factors that are involved in this process.

Molecular factors in lateral attachments

If lateral attachments are required for the formation of end-on attachments, this should be reflected by the protein mediators of lateral attachments. What are the consequences of inhibiting the proteins involved in lateral attachments, namely dynein and CENP-E? Interestingly, recruitment of both motor proteins to the kinetochore seems to be partially dependent on Aurora B (Chan et al. 2009; Ditchfield et al. 2003; Kim et al. 2010). So although Aurora B inhibits the formation of stable end-on attachments, it might actually help in the formation of lateral attachments.

Dynein-mediated lateral attachments assist in K-fiber formation

The role of dynein in microtubule-kinetochore binding was first indicated by the observation that its levels at the kinetochore

are high during the initial capture of kinetochores by microtubules during prometaphase and decline upon end-on attachment (Howell et al. 2001). Experimental inhibition of dynein is not straightforward, since this motor protein plays an important role in spindle organization (Echeverri et al. 1996; Merdes et al. 2000; Vaisberg, Koonce, McIntosh 1993). Various methods were developed to nevertheless study the function of kinetochore-associated dynein specifically (Table 1). Of particular relevance here is the function of dynein in the formation of lateral attachments and the importance of these lateral attachments for the establishment of stable end-coupled attachments.

ZW10 is part of the RZZ complex which recruits dynein to the kinetochore. Inhibiting dynein in human cells by silencing ZW10 results in reduced bioriented attachment and thereby less efficient congression and alignment (Li et al. 2007; Yang et al. 2007). It should be noted here that metaphase alignment is often seen as a readout for biorientation, although alternative routes to alignment have been revealed (Cai et al. 2009; Kapoor et al. 2006). Li and colleagues proposed that kinetochore-bound dynein generates a lateral pulling force on the chromosome during prometaphase, which brings this chromosome to one of the spindle pole. Positioned closer to a spindle pole, the laterally attached kinetochore will be captured more easily by the microtubule plus ends extending from this pole. If the chromosome subsequently turns perpendicular to the pole axis, this might also facilitate end-on capture of the other kinetochore by microtubules from the opposite spindle pole . This could also explain the observed depletion effects on fully aligned chromosomes, where interkinetochore tension is decreased and where fewer stable K-fibers are observed: since dynein is targeted specifically to unattached kinetochores, it is not expected to be directly responsible for tension and K-fiber stability on already attached and aligned chromosomes. Instead, the lateral attachments carried out by dynein might assist in the formation of stable attachments earlier, in prometaphase. In addition, a delay of anaphase onset was observed in ZW10 depleted cells. Apparently ZW10 depletion did not completely impair the mitotic checkpoint, although the RZZ complex is important for mitotic checkpoint activation (Box 2, Basto, Gomes, Karess 2000; Kops et al. 2005). The observed delay might be caused by a lack of stable attachments to silence the mitotic checkpoint. By inhibiting the recruitment of dynein to the kinetochore, RZZ depletion might abolish the coupling of kinetochores to microtubule lattices and hinder stable-end on capture. Alternatively, the mitotic delay could reflect the role of dynein in the silencing process because dynein has been shown to remove mitotic checkpoint proteins from the kinetochore (Howell et al. 2001; Wojcik et al. 2001). In *C. elegans*, depletion of RZZ subunits Rod-1 and Zwilch did not prevent normal congression, although a delay was observed (Gassmann et al. 2008). Moreover, a higher frequency of lagging chromatin during anaphase was reported in RZZ inhibited cells, which could indicate the occurrence of merotelic attachments. Both phenotypes were ascribed to a lack of dynein/dynactin at the kinetochore. Dynein would, in the view of the authors, accelerate the formation and increase the fidelity of end-on attachments by its role in lateral interactions (Gassmann et al. 2008). The discovery of another kinetochore dynein recruiter called Spindly allowed for a

Table 1. A summary of experiments concentrating on the consequences of inactivating kinetochore dynein.

Method of interference	Organism	Results: Kt-Mt attachment	Results: mitotic checkpoint activation & silencing	References
ZW10 RNAi	Human	Delayed chromosome congression Chromosome misalignment Monooriented/unattached chromosomes Reduction of stable K-fibers in metaphase Reduced interkinetochore tension on fully congressed chromosomes	ZW10 probably impairs mitotic checkpoint activation; No abolishment of mitotic checkpoint activity; Delay during prometaphase and metaphase	Li et al. (2007), Yang et al. (2007)
Rod-1 and Zwilch RNAi	C. elegans	Increase in anaphase lagging chromatin No effects on chromosome congression and alignment	Abolishment of mitotic checkpoint activity	Gassmann et al. (2008)
Spindly RNAi	Human	Chromosome misalignment and delay in alignment Aligned chromosomes: reduced interkinetochore tension versus no effects (Chan and Gassman, resp.) K-fiber stability: partial impairment versus no effects (Chan and Gassmann, resp.) Increase in spindle length	Delay during prometaphase Persistent mitotic checkpoint activation on unattached kinetochores Normal silencing upon attachment (based on Mad2 presence)	Chan et al. (2009), Gassmann et al. (2010)
Spindly RNAi	C. elegans	No congression of chromosomes Large increase in anaphase lagging chromatin	Abolishment of mitotic checkpoint activity	Gassmann et al. (2008)
Spindly mutant: dynein recruitment motif	Human	Stable attachment and biorientation Interkinetochore tension unaffected Intrakinetochore stretch unaffected	Delay during metaphase Retention of Mad1 and Mad2 on properly attached and aligned kinetochores	Gassmann et al. (2010)
p50-Dynamitin overexpression/microinjection	Human	Chromosome misalignment Reduced interkinetochore tension	Impairment of mitotic checkpoint silencing Mad2 retention on attached kinetochores	Li et al. (2007), Chan et al. (2009), Howell et al. (2001)
Dynamitin microinjection	Xenopus laevis	In vitro: moderate impairment of microtubule binding to chromosomes No defects in metaphase alignment		Vorozhko et al. (2008)
Dynein tail fragment expression	Human	Lateral attachments still observed Reduction of bioriented chromosomes Reduction of stable K-fibers Reduced interkinetochore tension on aligned chromosomes	Normal recruitment of mitotic checkpoint proteins BubR1 and Mad1 Retention of Mad1 and BubR1 on the kinetochores of aligned chromosomes	Varma et al. (2008)

functional analysis of kinetochore dynein without disturbing the activation of the mitotic checkpoint checkpoint (Chan et al. 2009; Gassmann et al. 2010). Depletion of human Spindly caused chromosome misalignments and a delay in congression and anaphase onset. While Chan et al. (2009) reported a reduction in interkinetochore tension and a partial impairment of K-fiber stability on aligned chromosomes, this was not observed by Gassmann et al. (2010). Furthermore, it is doubtful whether the alignment defects observed in Spindly-depleted cells are the sole result of a lack of kinetochore dynein: a single amino acid Spindly mutant, incapable of recruiting dynein, rescued the alignment defects in Spindly-depleted cells (Gassmann et al. 2010). Direct inactivation of dynein is also possible, for example by injecting or overexpressing dynamitin. In *Xenopus* cells, dynamitin injection did not lead to any defects in metaphase alignments (Vorozhko et al. 2008). This suggests that Ndc80-based stable attachments are not dependent on dynein-mediated lateral attachments. Vorozhko et al. also demonstrated in vitro that inhibition of dynein decreases but does not abolish kinetochore-microtubule attachment. In yet another study, dynein motor function was inhibited in human cells by expression of a dynein tail fragment, which replaces the motor domain (Varma et al. 2008). This intervention reduces the number of bioriented chromosomes, increases the number of unattached and mono-oriented chromosomes and destabilizes kinetochore microtubules. Interestingly, lateral attachments were still observed in the dynein-motor depleted cells. Perhaps this is caused by the type of treatment; dynein itself is at the kinetochore and might still function to bridge the kinetochores to the sides of microtubules. Apparently, it is the impaired motor function that affected the formation of stable and bioriented end-on attachments, which might be dispensable for initial lateral attachment.

The above described studies do not agree on the role kinetochore-associated dynein plays in the establishment of end-on attachments, as indicated by the different results of dynein inhibition in congression, metaphase alignment, K-fiber stability and interkinetochore tension (Table 1). Focusing on human cells, it is likely that kinetochore dynein aids in end-on attachment formation, but is not strictly required. Hence it can be concluded that dynein-mediated lateral attachments are not as essential as proposed (Foley and Kapoor 2013). Another option is to interpret the model of Foley and Kapoor in a less restrictive way. Accordingly, lateral attachment facilitate in alleviating Aurora B's positive feedback loop by generating tension. Although it is not entirely clear how, dynein-mediated lateral attachments might put tension on the centromere, for example during poleward movement, and as such allow plus ends of other microtubules to engage end-on interactions with the kinetochore(s) (Figure 2A). Alternatively, the role of dynein's motor function could lie in pulling the chromosome to a spindle pole and positioning the sister kinetochores for easy capture by microtubule plus ends extending from one or both poles (Figure 2B). Recently, another role of dynein in stable attachments has been suggested. The RZZ complex was demonstrated to inhibit the Ndc80 complex from associating with microtubules. This suppression was removed by the recruitment of dynein to the kinetochore (Cheerambathur et al. 2013). Although it is not elucidated whether dynein itself or dynein-mediated lateral attachments enables the Ndc80

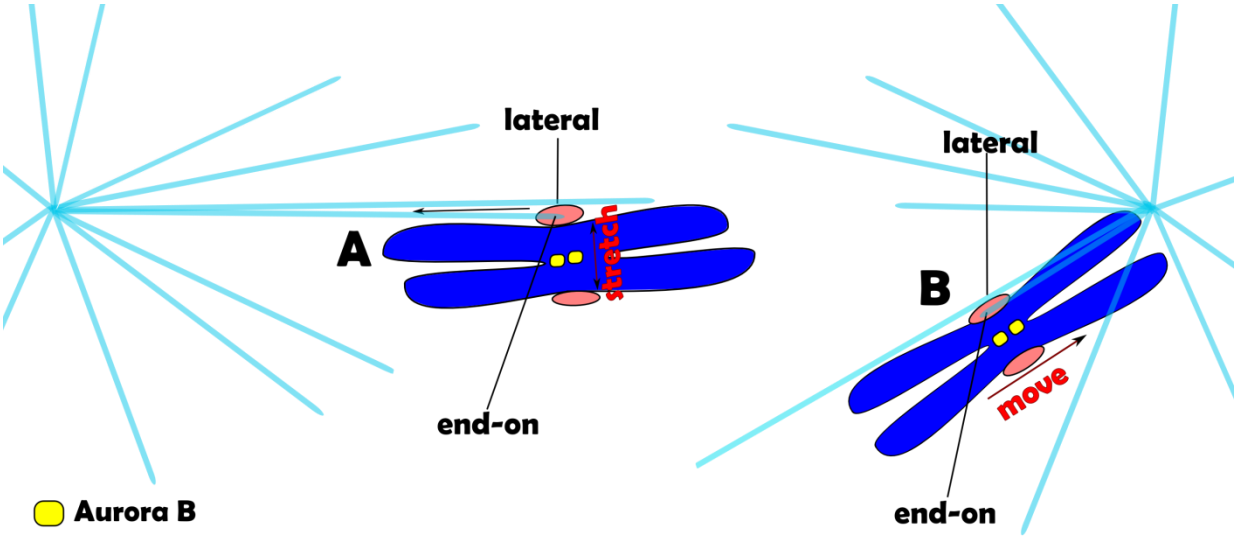


Figure 2. The role of dynein-mediated lateral attachments in the formation of end-on attachments. By pulling the kinetochore towards the spindle pole, dynein might increase the interkinetochore tension, thereby reducing Aurora B phosphorylations on the outer kinetochore and allowing for end-on attachments (A). Alternatively, dynein-mediated transport to the spindle pole could bring the kinetochores in closer proximity to microtubule plus ends, thereby increasing the capture probability (B).

complex to bind to microtubules, it suggests that there is an Aurora B-independent manner to destabilize and stabilize kinetochore-microtubule interactions localized to the kinetochore itself.

CENP-E assists in K-fiber formation via lateral attachments or via conversion

Perhaps the lateral attachments described by Foley and Kapoor are not carried out by dynein, but by CENP-E. The plus end-directed motor CENP-E is associated with kinetochores from prometaphase through anaphase (Brown, Wood, Cleveland 1996; Wood et al. 1997). As suggested by its direction of movement, CENP-E is not required for fast poleward movement of chromosomes, but is involved in chromosome congression towards the metaphase plate (Putkey et al. 2002; Wood et al. 1997). Disruption of CENP-E function yields a higher incidence of cells with some mono-oriented chromosomes close to one of the spindle poles, apparently unable to arrive at the spindle equator (Mao, Desai, Cleveland 2005; McEwen et al. 2001; Putkey et al. 2002; Schaar et al. 1997). Here, I will focus on the proposed roles of CENP-E-mediated lateral attachments in the establishments of stable tip attachments of microtubules to kinetochores. McEwen et al. (2001) demonstrated in HeLa cells that both on aligned and unaligned chromosomes the number of microtubules bound per kinetochore was decreased if CENP-E was blocked. The effect was most dramatic for unaligned chromosomes, where many kinetochores lacked attachment and no bioriented chromosomes were found. In a study in mouse cell lines, removal of CENP-E also led to a decrease in microtubules bound to kinetochores, even when they were fully aligned (Putkey et al. 2002). Various studies tried to reveal how CENP-E contributes to microtubule attachment. Focusing on the chromosomes stuck in a position close to one of the spindle poles, at least two alternative explanations for their situation can be put forward. First, these chromosomes might not

be able to establish stable biorientation and therefore cannot achieve alignment. It has generally been assumed that chromosome congression requires biorientation, which would fit with this explanation (McEwen et al. 1997; Murray and Mitchison 1994). This assumption has been undermined by the observation that human cells lacking an essential KMN network component are still able to congress to the metaphase plate due to the motor action of CENP-E (Cai et al. 2009). Therefore, the alignment failure is maybe not related to bipolar attachment, but to a lack of action of CENP-E itself. By visualizing individual chromosome movements, Kapoor and colleagues (2006) established that the relationship between biorientation and congression is not universal: although some chromosomes were bi-attached in a tip-on manner, others only showed K-fibers attached to the trailing kinetochore. The leading kinetochore was laterally attached to K-fiber microtubules of an already aligned chromosome. CENP-E is the motor protein responsible this lateral attachments and for transport of the chromosome towards the spindle equator. In search of the function of lateral attachments mediated by CENP-E, it is particularly interesting that this lateral attachment is not required for the formation of a mature K-fiber on the kinetochore closest to the spindle pole. It is however likely to play a role in subsequent biorientation, which occurs when the chromosomes approaches the spindle equator. By what mechanism does CENP-E lateral attachments facilitate bioriented end-on attachment? Again similar to dynein, CENP-E could, by the formation of lateral attachments increase tension between kinetochores (Figure 3A). This tension might downregulate Aurora B phosphorylations and allow for successive establishment of end-on attachments. Rather than the attachments themselves, the motor protein pulling force on the chromosome directed to the microtubule plus ends might be a source of interkinetochore tension, considering the attachment of the other sister kinetochore to the nearest spindle pole. Such counteracting forces seem to contrast the CENP-E-mediated attachments to dynein-mediated ones. As a matter of fact, an increase in interkinetochore distance has been observed in the congressing chromosomes that are end-on attached on the trailing kinetochore and laterally attached on the leading kinetochore (Kapoor et al. 2006). Alternatively, while the chromosome moves to the spindle equator the density of microtubules emerging from the opposite spindle increases, amplifying the chance of the chromosome to become captured (Figure 3B). Both explanations do not necessarily need to contradict each other; the two processes - tension increase and positioning - might have additive effects on the realization of stable tip attachments. CENP-E depletion does not generally make chromosomes stuck to a spindle pole; most chromosomes still achieve complete alignment and biorientation during metaphase. Nevertheless, these aligned chromosomes experience a lower degree of mature microtubules attached to them (McEwen et al. 2001; Putkey et al. 2002). It is unclear if these bioriented chromosomes under normal conditions become laterally attached, therefore I cannot exclude that the absence of CENP-E-mediated lateral attachments is affecting K-fiber density on aligned kinetochores. Alternatively, CENP-E has a second role next to the establishment of lateral connections and translocation. Various studies indeed demonstrated that CENP-E functions in the conversion to stable end-on attachments of kinetochores to dynamic

microtubule tips (Gudimchuk et al. 2013). Similar to dynein, I conclude that CENP-E-mediated lateral attachments are not strictly required for the formation of end-on attachments. Nevertheless, they do probably assist in forming end-on attachments of pole-proximal chromosomes while moving them in the direction of the metaphase plate. Maybe CENP-E-mediated lateral attachments in wild-type conditions also aid in the formation of stable end-on attachments of aligned chromosomes. Furthermore, I cannot exclude that CENP-E and dynein cooperate in establishing lateral attachments that contribute to efficient formation of stable end-coupled attachment. Although both motor proteins seem to operate in distinct stages of prometaphase, it would be interesting to study the effects of co-depleting both motor proteins on the number and stability of K-fibers.

Lateral attachments probably do not generate force on aligned chromosomes

The previous sections pointed out that both dynein- and CENP-E-mediated lateral attachments may facilitate the formation of end-on attachments. It is however not known whether they do so by increasing tension on the centromeres, as proposed by Foley and Kapoor. Therefore, it is crucial to find out whether lateral attachments can produce tension at all. At first glance, it does not seem very likely that just dynein- or CENP-E-mediated lateral attachments are able to generate tension: they are involved in pole- and anti-pole- directed chromosome movement, respectively. Especially poleward movement is not expected to experience resistance from another force, except for perhaps some friction. If both motor proteins however operate simultaneously on the same chromosome, this chromosome might come under tension. An answer to the question whether lateral attachment are able to produce tension might come from experiments that depleted the core microtubule binding site. This intervention restricts kinetochore-microtubule attachments to lateral ones. In two studies, the lab of Trisha Davis revealed the spindle phenotypes of the yeast kinetochore mutant

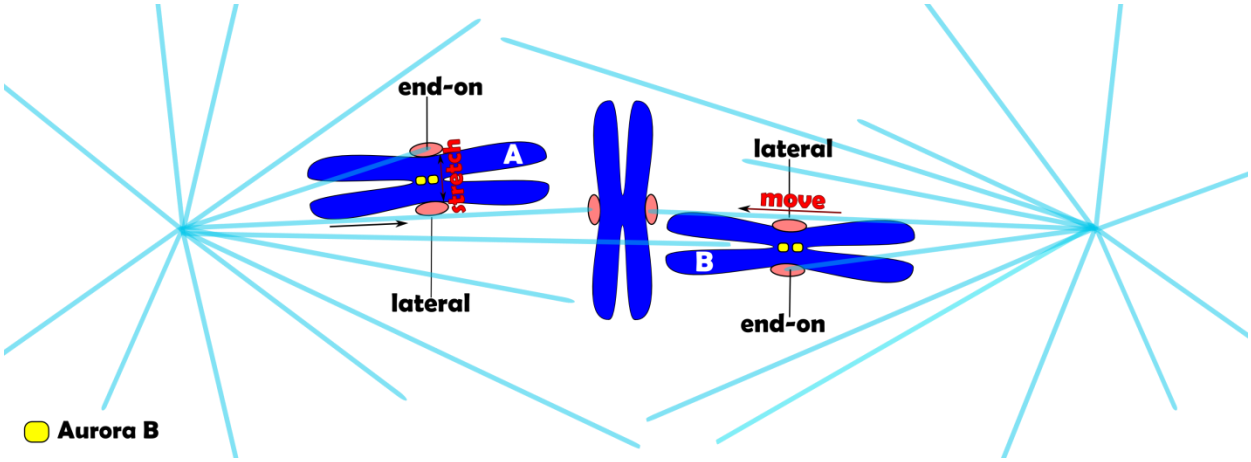


Figure 2. Kapoor et al. showed that monoattached chromosomes can congress to the metaphase plate by lateral attachment to microtubule K-fibers of aligned chromosomes (2006). Such lateral attachments are mediated by CENP-E and may facilitate in end-on attachment of the laterally attached kinetochore by increasing centromere stretch (A) or by translocating the chromosome to the equator, where more microtubules extending from the opposite pole can be encountered (B).

Dam1-765 (Shimogawa et al. 2006; Shimogawa et al. 2010). This mutant contains an amino acid substitution in the Dam1 gene, which is encoding Dam1 complex components. The Dam1 complex, of which no metazoan equivalents have been detected so far, is responsible for tracking microtubule dynamics at their plus ends (Westermann et al. 2006). The mutation involves a Mps1 phosphorylation site. Preventing Mps1 phosphorylation specifically at this site yields a narrower mitotic spindle in which kinetochores were not attached to the microtubule plus ends but to their sides (Shimogawa et al. 2006). The lateral nature of these attachments was deduced from fluorescence images revealing that microtubules extended further than their attached chromosomes. The mean distance between kinetochore clusters, which were marked by Nuf2 (a Ndc80 complex component), was rather increased than decreased compared to the wild-type, suggesting that indeed lateral attachments are able to produce tension. However, it is likely that the interkinetochore tension observed in this mutant is caused simply by biorientation. The occurrence of biorientation in this mutant was demonstrated for example by the unaffected levels of chromosome loss (Shimogawa et al. 2006; Shimogawa et al. 2010). Since it is not explained how biorientation is established by these lateral attachments, it does not provide cues on whether wild-type lateral attachments might be able to produce tension by some form of biorientation as well. Depletion of KMN network constituents offers another option to study the properties of lateral attachments. Disruption of Nuf2 in human cells eliminates K-fiber formation but allows for lateral attachments (DeLuca et al. 2005). In these cells, interkinetochore distances are smaller (Cai et al. 2009). In fact, the interkinetochore distances were comparable to cells treated with nocodazole, suggesting that lateral attachments do not produce any tension at all. In *Drosophila* cells, depletion of KMN elements also results in the formation of lateral attachments only (Feijão et al. 2013). Under these conditions again a significant decrease is observed in interkinetochore distance. Furthermore, colchicine treatment resulting in microtubule depolymerization did not further decrease the interkinetochore distance, which also here suggests that the microtubules attached to the chromosomes in untreated conditions do not generate any force. In a study which was not based on disrupting KMN or Dam1, tension was measured in chromosomes attached in an end-on manner at the trailing kinetochore and laterally on the leading kinetochore (Kapoor et al. 2006). This heterogenic attachment conformation was demonstrated to generate tension on the chromosome.

Nevertheless, currently available studies contradict that lateral attachments by themselves are sufficient for a small increase in interkinetochore tension, as measured in a pseudo-metaphase state (Cai et al. 2009; DeLuca et al. 2005; Feijão et al. 2013). At this stage, probably most dynein has disappeared from the kinetochore and CENP-E only is responsible for the attachment (Cai et al. 2009). Could it be that earlier in mitosis, during prometaphase, both dynein and CENP-E attach to either a single kinetochore or both kinetochore sisters? Although both motor proteins are assumed to play roles in different stages of prometaphase, maybe counteraction should not be ruled out, given also the question of how their spatiotemporal separation is regulated. A model in which counteraction of both kinetochore motor proteins acts in the formation of end-on

attachments has been proposed before (Mao, Varma, Vallee 2010). For this reason, it would be interesting to examine the tension on prometaphase chromosomes in conjunction with dynein/CENP-E activity on the kinetochore.

Given that currently no convincing evidence exists for force generation by lateral attachments only, how could lateral attachments otherwise aid in the formation of stable tip-on attachments? And how can the positive feedback loop consisting of tension and high K-fiber stability be entered? First, one should realize that end-on attachments are not irreversible. Instead, microtubules are attaching and detaching with a frequency related to the interkinetochore tension. The total numbers of microtubule ends bound to kinetochores also depends on the abundance of polar microtubules in the vicinity of the chromosomes. Both minus and plus end-directed lateral attachments transport microtubules to microtubule-rich regions, thereby increasing the amount of microtubules that (initially unstably) attach to the kinetochore. The more microtubules attach, the more tension is produced and stabilization of attachments commences. In addition, chromosomes do not all have the same initial location after nuclear envelope breackdown. As a result, chromosomes vary in their need for repositioning to and within the spindle.

Not interkinetochore distance, but intrakinetochore distance?

In the model proposed by Foley and Kapoor (2013), a major task for lateral attachments lies in increasing the distance between the inner centromere, where Aurora B is localized, and the outer kinetochore, which harbours Aurora B targets in the KMN network. Although lateral attachments may not increase interkinetochore distance, they might have a second option to directly increase the distance between Aurora B and the KMN network. Increasing the width of the kinetochore itself could after all also help to move Aurora B away from its outer kinetochore targets. In 2009, two independent studies reported that changes in intrakinetochore stretch are not necessarily coupled to the level of interkinetochore stretch (Maresca and Salmon 2009; Uchida et al. 2009). Maresca and Salmon demonstrated in *Drosophila* S2 cells that when the intrakinetochore distance is reduced by a high concentration taxol treatment, mitotic progression is slowed down. This is not the case when interkinetochore distance is reduced. While this study just demonstrates a mitotic delay, Uchida et al. reveal that a reduction in intrakinetochore stretch is associated with prolonged mitotic checkpoint activity (Uchida et al. 2009). Low level nocodazole treatment specifically decreases intrakinetochore stretch. Interestingly, the authors report varying levels of stretch between both sister. This might point to conformational changes responsible for stretching, rather than tension. If deformation is also a means to increase the distance between Aurora B and its targets, it is more likely that one-sided (lateral) attachments are capable of this. In fact, it has been demonstrated that mono-oriented, end-on attached chromosomes are subject to increased intrakinetochore stretch on the attached kinetochore (Uchida et al. 2009). It is unclear whether kinetochores that are attached to the microtubule lattice are also stretched. Of particular relevance here is the

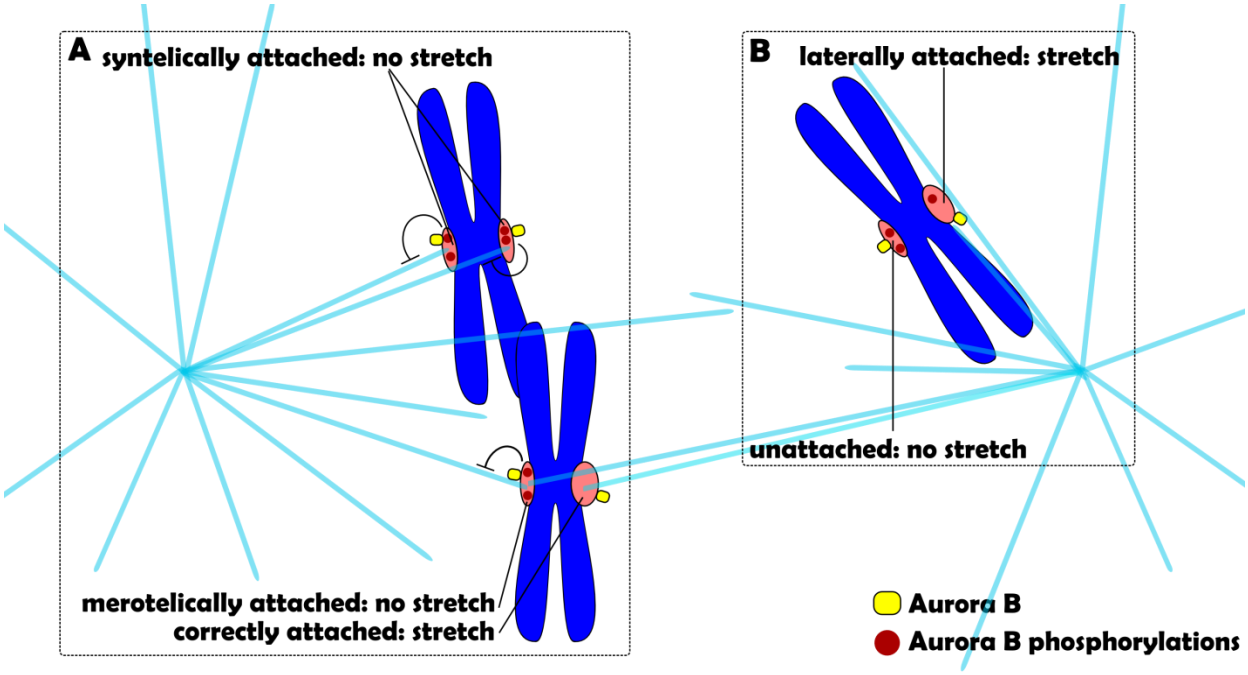


Figure 4. Aurora B might respond to intrakinetochore stretch, eventually while being localized on the kinetochore as depicted here, by phosphorylating kinetochore targets if intrakinetochore stretch is low. The level of intrakinetochore stretch might be dependent on the type of end-on attachment at that kinetochore: syntelic and merotelic attachments may not increase intrakinetochore stretch to the same level as correct attachments (A). Furthermore, lateral attachments might also increase intrakinetochore stretch, thereby decreasing the level of KMN network phosphorylations and allowing for end-on attachment (B).

association between intrakinetochore stretch and the formation of end-on attachments. Microtubule attachments are probably not affected when intrakinetochore stretch is decreased during metaphase, but the use of microtubule poisons makes these results difficult to interpret (Maresca and Salmon 2009; Uchida et al. 2009). It would be interesting to study microtubule attachment process during prometaphase, especially since metaphase alignment is delayed in cells with decreased intrakinetochore stretch (Uchida et al. 2009). Is this delay caused by less efficient end-on attachment formation in prometaphase resulting from Aurora B activity? It must however be pointed out that the absolute variation in interkinetochore stretch is much larger than in intrakinetochore stretch: on average 220 nm versus 37 nm in *Drosophila*, respectively (Maresca and Salmon 2009). In terms of making a substantial contribution to moving inner centromere-localized Aurora B away from the outer kinetochore, interkinetochore stretch is therefore a more obvious candidate. On the other hand, the exact localization of functional Aurora B is topic of debate. Phosphorlyated Aurora B has for example been shown to be concentrated at the kinetochore in absence of PP1 (Posch et al. 2010). Recently, in budding yeast Aurora B was shown to fulfill its function in the formation of properly bioriented chromosomes without being localized in the inner centromere (Campbell and Desai 2013). Instead, it was able to sense and respond to incorrect orientation while being localized to spinde microtubules. This could also mean that Aurora B selectively responds to the attachment status of a single kinetochore, for example by sensing its conformational changes after correct or incorrect microtubule binding (Wan et al. 2009). The level of intrakinetochore stretch as a result of

conformational change could be dependent on the nature of the attachment; syntelic or merotelic attachments might have reduced comformational changes compared to amphitelic attachment (figure 4A). Lateral attachments might also induce a kinetochore reorganization and stretch (figure 4B). The increased intrakinetochore stretch could help in dampening Aurora B phosphorylation activity and thereby pave the way for kinetochores to attach to microtubule plus ends.

The mitotic checkpoint checkpoint is not completely silenced during lateral attachment

Although maybe not by interkinetochore tension, lateral attachments likely contribute to preparing the chromosomes for sister chromatid separation. Mitotic exit should thus be postponed until all lateral attachments are converted into end-on attachments, the load-bearing attachments able to carry each sister chromatid to the opposite pole. This requires the mitotic checkpoint to still be active during lateral interactions (see Box 2 for a summary of the mitotic checkpoint). Whether laterally attached kinetochores silence the checkpoint is however topic of debate. Interestingly, both dynein and CENP-E motor proteins have been linked to checkpoint silencing, thereby suggesting that the kinetochore-microtubule attachments these proteins establish could dampen checkpoint signaling (Howell et al. 2001; Mao, Desai, Cleveland 2005; Wojcik et al. 2001). As discussed before, depletion of dynein results in a mitotic delay (Li et al. 2007; Yang et al. 2007), but it is not clear if this is due to a lack of mitotic checkpoint silencing by dynein or to reduced kinetochore-microtubule attachment. The presence of Mad2 on lateral kinetochores but not on end-on kinetochores suggests the checkpoint is still active on laterally attached kinetochores (Shrestha and Draviam 2013). Furthermore, partial depletion of Ncd80 by RNAi and thereby inhibition of end-on attachments delays mitosis in a Mad2-dependent manner, although lateral attachments were observed in this assay (Zhang et al. 2012). The opposite has however also been observed: depletion of Hec1 (the human variant of Ncd80) and Nuf2 (the direct binding partner of Hec1) resulted in a reduction of checkpoint proteins Mad1 and Mad2 at kinetochores during prometaphase, which could be countered by treatment with microtubule depolymerizing reagent nocodazole (DeLuca et al. 2003). The authors suggested that both checkpoint proteins are carried away by motor proteins present through lateral and/or transient unstable attachments, although insufficient to allow for mitotic exit (DeLuca et al. 2003). Apparently motor proteins dynein and CENP-E localize to the kinetochore, also in the absence of Ncd80 complex (DeLuca et al. 2005). Studies involving deletions in the KMN network should be interpreted with caution in the light of mitotic checkpoint silencing; various KMN components have been shown to be essential for establishment of a functional mitotic checkpoint (Ciferri, Musacchio, Petrovic 2007). Deleting these components could therefore abolish the mitotic checkpoint altogether rather than demonstrating lateral-attachment induced silencing. Interpretation of such results coming from KMN mutants demand that A) lateral attachments have been observed and B) there is a functional mitotic checkpoint, for example demonstrated by nocodazole treatment. *Drosophila* cell cultures depleted

of Ndc80 and Spc105 fulfill the first (although Spc105 mutants had very unstable lateral attachments), but not the latter requirement (Feijão et al. 2013). Depleting the Mis12 complex in the same cells however meets both requirements. In these cells, a mitotic delay is observed, so the mitotic checkpoint does not seem to be satisfied by the lateral attachments (Feijão et al. 2013). In yeast, mutant *Dam1-765* is not able to form stable end-on attachments, but nevertheless progresses through the cell cycle without prolonged checkpoint activation (Shimogawa et al. 2010). As discussed previously, although no end-on attachments were observed in this mutant, the chromosomes still biorient and segregate without increased levels of chromosome loss (Shimogawa et al. 2010). Possibly, some alternative form (perhaps yeast-specific) of attachments is being displayed here. The nature of the alternative attachments responsible for checkpoint silencing in this mutant need to be further examined to solve this issue. Altogether, most evidence seems to indicate some, but incomplete, silencing of mitotic checkpoint by lateral attachments (Figure 4). Despite clearance of checkpoint proteins by for example dynein, recruitment and production of MCC might still be ongoing when a kinetochore is laterally attached. This could be related to incomplete inhibition of Aurora B activity, as Aurora B plays a role in establishing and/or maintaining the checkpoint in various ways, maybe even as a key regulator (Ditchfield et al. 2003; Famulski and Chan 2007; Saurin et al. 2011).

Destabilization of end-on attachment during chromosome movement along microtubules

Interpreting dynein- and CENP-E-mediated lateral attachments from the perspective of the Foley and Kapoor model has given some

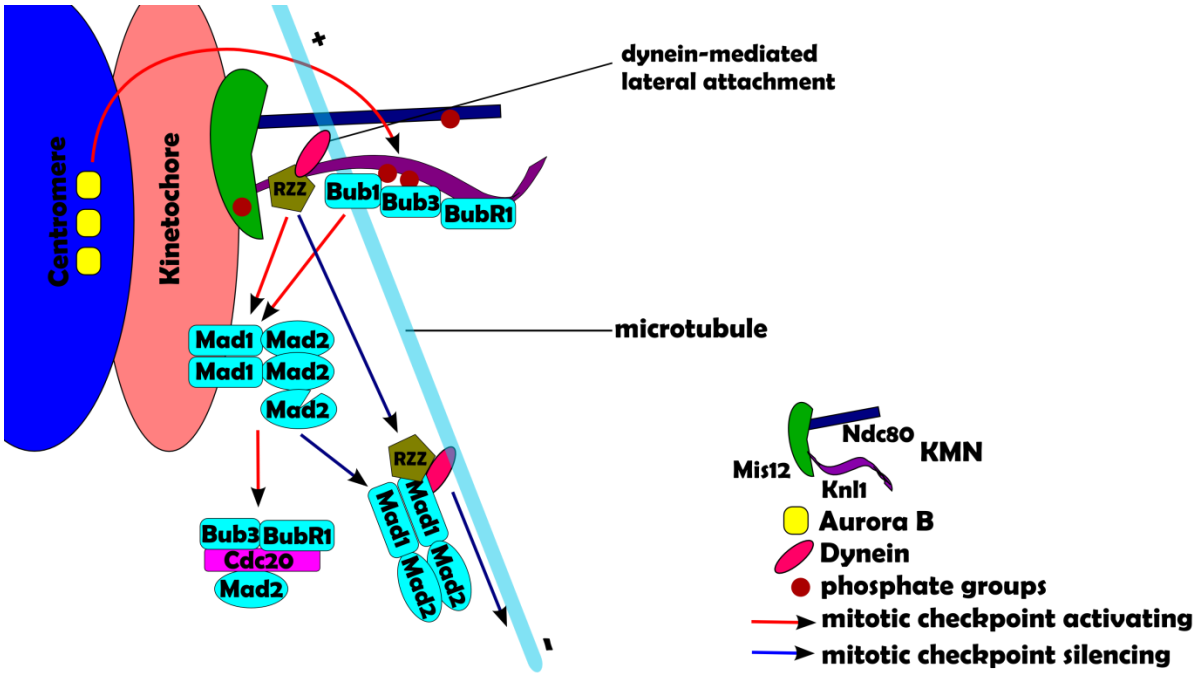


Figure 5. Partial silencing of the mitotic checkpoint by lateral attachments. In this example, dynein-mediated lateral attachments cause removal of checkpoint proteins from the kinetochore by dynein itself. The production of MCC is however not stopped, due to for example the continuing indirect recruitment of checkpoint proteins via protein kinases Aurora B and Mps1.

important insights. First, the lateral attachments these motor proteins establish do not seem as essential for end-on formation as the model suggested. Such lateral attachments nevertheless contribute to the formation of end-on attachments, as indicated by chromosome congression, the numbers of K-fibers and interkinetochore distance. The mechanism by which lateral attachments facilitate end-on attachment remains obscure; tension does not seem to be produced by lateral attachments, so maybe they simply function in transporting the chromosome to an area with high microtubule density. If this is the case, the level of Aurora B phosphorylation on the kinetochore is not directly decreased upon lateral attachment. As pointed out, the discussed experiments do not necessarily reject the possibility that lateral attachments are generating tension during prometaphase. It would therefore be interesting to study if lateral attachments affect tension and phosphorylation levels during prometaphase chromosome movements. Coupling the molecular processes like phosphorylation and mitotic checkpoint signaling to chromosome location might also give insights into alternative routes of attachment regulation, such as Aurora A phosphorylations taking place on kinetochores close to the spindle pole (Kim et al. 2010). Furthermore, the possibility that lateral attachments affect Aurora B activity by stretching the kinetochore should be further explored.

A second important insight is that the facilitating role of lateral attachments in the establishment end-on attachments seems to be reflected by the way they affect the mitotic checkpoint. As lateral attachments are not sufficient for proper mitosis, the mitotic checkpoint is still active on laterally attached chromosomes. The mitotic checkpoint activity is however reduced compared to completely unattached kinetochores. Since laterally attached kinetochores are more likely to quickly become attached to microtubule plus-ends, the reduced mitotic checkpoint signaling coming from laterally attached kinetochores might not impose any problems. In fact, it might aid in rapidly switching off the mitotic checkpoint when the end-on attachments are established.

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Checks and balances of cell division (layman's summary)

Prior to cell division, a mother cell duplicates all chromosomes into so-called 'sister chromatids' that will subsequently be divided over the two daughter cells. The sister chromatids stick together until the mother cell enters mitosis, the phase in which they are pulled apart towards opposite ends of the mother cell. After segregation of the chromosomes, the cell itself is split into two daughter cells. In order to produce two healthy cells, it is of vital importance that no errors occur during the segregation of the sister chromatids. Errors could result in daughter cells with abnormal chromosome numbers, a genetic aberration frequently observed in tumors. To ensure equal chromosome segregation, two control systems have been evolved. The first system is called the mitotic checkpoint. The mitotic checkpoint makes sure that all sister chromatids are attached to the mitotic spindle, an apparatus made up of thick strings that pull the sister chromatids apart. Until all sister chromatids are attached, the mitotic checkpoint delays their segregation by preventing breakdown of the protein cohesin, the 'glue' that holds the sister chromatids together. The second surveillance system is not focused on the attachments of sister chromatids themselves, but on their *correct* attachments. This means that both sister chromatids should be attached, via the mitotic spindle, to the opposite ends of the cell. This second system destabilizes those erroneous attachments that connect both sister chromatids to the same end of the cell. Due to this destabilization, the mitotic checkpoint will continue to work until proper attachments are established. Destabilization of erroneous attachments is based on tension: if the still connected sister chromatids are attached to opposite ends, they will be under tension. If they are attached to the same end of the cell, they will not experience any tension. Lack of tension is the key for destabilization of the attachments. The workflow of this tension-based system

however imposes a problem: because unattached chromosomes do not experience any tension, every single first attachment will be destabilized (see figure below). How can attachments then be achieved at all? In this literature study, a model is discussed that possibly explains how this problem can be overcome. The model was drafted by Foley and Kapoor (2013) and is based on the observation that there is a second way by which chromosomes can attach to the mitotic spindle, called lateral attachments ("to the side"). Foley and Kapoor think that lateral attachments are able to produce tension and also are immune to destabilization (see figure). The model states that after the lateral attachments generated tension on the chromosomes, normal attachments (so-called "end-on" attachments) are not destabilized anymore, unless they are incorrect. Based on currently available literature, it does not seem very likely that these lateral attachments produce tension. Nevertheless, most experiments indicate that lateral attachments still play a role in the establishment of normal end-on attachments, but by other means than tension. Another question is whether lateral attachments are sufficient to satisfy the first surveillance system, the mitotic checkpoint. Does the mitotic checkpoint consider lateral attachments to be good enough for cell cycle progression? Or is the checkpoint still delaying the separation of the sister chromatids if they are laterally attached? Most studies in this field suggest that lateral attachments do not completely switch off the 'delay' signal of the mitotic checkpoint. However, lateral attachments probably do decrease the magnitude of the signal, so if normal attachments succeed the lateral attachments, the mitotic checkpoint will be switched off quickly since it was already somewhat faded out. This safeguards equal chromosome segregation without losing too much time.