Length measurement mechanisms that control chromosome alignment

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Abstract

During metaphase, chromosomes are aligned in a fascinating lineup at the equatorial plane of the spindle, to ensure synchronous poleward movement of chromatids in anaphase and proper nuclear reformation at the end of mitosis. Chromosome alignment relies on microtubules, several types of motor proteins and numerous other microtubule-associated and regulatory proteins. Because of the multitude of players involved, the mechanisms of chromosome alignment are still under debate. In this Review, we discuss the current models of alignment based on poleward pulling forces exerted onto sister kinetochores by kinetochore microtubules, which show length-dependent dynamics and undergo poleward flux, and polar ejection forces that push the chromosome arms away from the pole. We link these models with the recent ideas based on mechanical coupling between bridging and kinetochore microtubules, where sliding of bridging microtubules promotes overlap length-dependent sliding of k-fibers and thus the alignment of sister kinetochores at the spindle equator. Finally, we discuss theoretical models of forces acting on chromosomes in metaphase.

Introduction

Essential to the reliable genome inheritance is the mitotic spindle, which drives physical separation of a complete set of genetic material into two equal parts destined to the two daughter cells. This self-assembled macromolecular machine is built from microtubules and microtubule-associated proteins (MAPs) (Pavin and Tolic, 2016; Petry, 2016; Prosser and Pelletier, 2017). Microtubules of the spindle can be divided into three main groups based on their position and function. Kinetochore microtubules bind the kinetochore, a protein complex at the centromere of each chromosome, and form a kinetochore fiber (k-fiber). Overlap microtubules grow from the opposite spindle halves and overlap in the middle. In numerous cell types and organisms they link sister k-fibers like a bridge, which is why they are called bridging fibers (Tolic, 2018). Astral microtubules extend from the spindle pole towards the cell periphery and contact the cell cortex.

Before chromosome segregation in anaphase, chromosomes are neatly aligned at the spindle equator (Figure 1 A,B), undertaking different pathways to get there (Cai et al., 2009; Kapoor et al., 2006; Maiato et al., 2017; Walczak et al., 2010). This process, termed chromosome congression, coincides with chromosome biorientation, i.e., formation of stable attachments of sister kinetochores to microtubules that emanate from the opposite spindle poles (Figure 1C). Proper kinetochore-microtubule attachments are monitored by the spindle assembly checkpoint and required for correct chromosome segregation (Gregan et al., 2011; Musacchio and Salmon, 2007). Similarly, the alignment of chromosomes at the spindle equator is important for mitotic fidelity because it promotes synchronous anaphase poleward movement of chromatids and proper telophase nuclear reformation (Figure 1D) (Fonseca et al., 2019; Matos et al., 2009).

Central to chromosome positioning within the spindle are microtubules, polar polymeric structures with their plus ends being more dynamic and undergoing dynamic instability characterized by persistent periods of growth and shrinkage (Desai and Mitchison, 1997; Howard and Hyman, 2003). *In vitro* experiments have demonstrated that growing or shrinking microtubules can generate pushing or pulling forces, respectively (Dogterom and Yurke, 1997; Grishchuk et al., 2005), suggesting that these forces drive chromosome positioning on the spindle.

More than 100 proteins are involved in chromosome alignment and for many of them the mechanisms are not known (Maiato et al., 2017). Thus, chromosome alignment is a complex process that is still not fully understood. In this Review, we present the prevailing view and recently introduced concepts of how the forces that align the chromosomes are generated and regulated. We discuss how the alignment is achieved through the regulation of the dynamics of microtubule plus ends at kinetochores and minus ends at the spindle pole, polar ejection forces (PEFs) arising through interactions between spindle microtubules and the chromosome arms, and mechanical coupling between kinetochore and bridging microtubules.

Box 1. Glossary of terms.

Mitosis – cellular process in which duplicated chromosomes are separated into two nuclei

Spindle – microtubule-based bipolar apparatus that separates duplicated chromosomes

Kinetochore – protein complex assembled on the centromere of each chromosome that mediates attachment between the chromosomes and the microtubules

Microtubule – polymer formed by the polymerization of a dimer of α- and β-tubulin into protofilaments that form a hollow tube

Kinetochore microtubule – microtubule that attaches to a kinetochore

Kinetochore fiber (k-fiber) – parallel bundle formed by kinetochore microtubules associated with the same kinetochore

Bridging microtubule – non-kinetochore microtubule acting as a constituent of the bridging fiber

Bridging fiber – antiparallel bundle formed by bridging microtubules associated with a pair of sister k-fibers

Centrosome – cellular structure that serves as main microtubule organizing center

Spindle pole – part of the spindle consisting of centrosomes and various non-motor and motor proteins responsible for nucleation of microtubules and focusing of microtubule minus ends

Microtubule plus and minus end – end with the β-subunits and α-subunits of the αβ tubulin dimers exposed, respectively; plus end grows faster

Microtubule dynamics – stochastic conversion between phases of growth and shrinkage at the microtubule plus end

Microtubule catastrophe – transition from microtubule growth to shrinkage

Microtubule rescue – transition from microtubule shrinkage to growth

Microtubule-associated proteins (MAPs) – proteins that bind to microtubules, involved in regulation of their dynamics and organization

Motor proteins – enzymes that convert chemical into mechanical energy, thus generating force and movement along cytoskeletal filaments

Microtubule crosslinkers – non-motor MAPs that interconnect adjacent microtubules

Chromosome congression – process of chromosome movement toward the spindle equator which leads to the formation of the metaphase plate

Chromosome biorientation – establishment of attachments between sister kinetochores and microtubules extending from the opposite spindle poles

Poleward flux – conveyor belt-like movement in which spindle microtubules are being transported towards the spindle pole, accompanied by depolymerization at the minus end and polymerization at the plus end

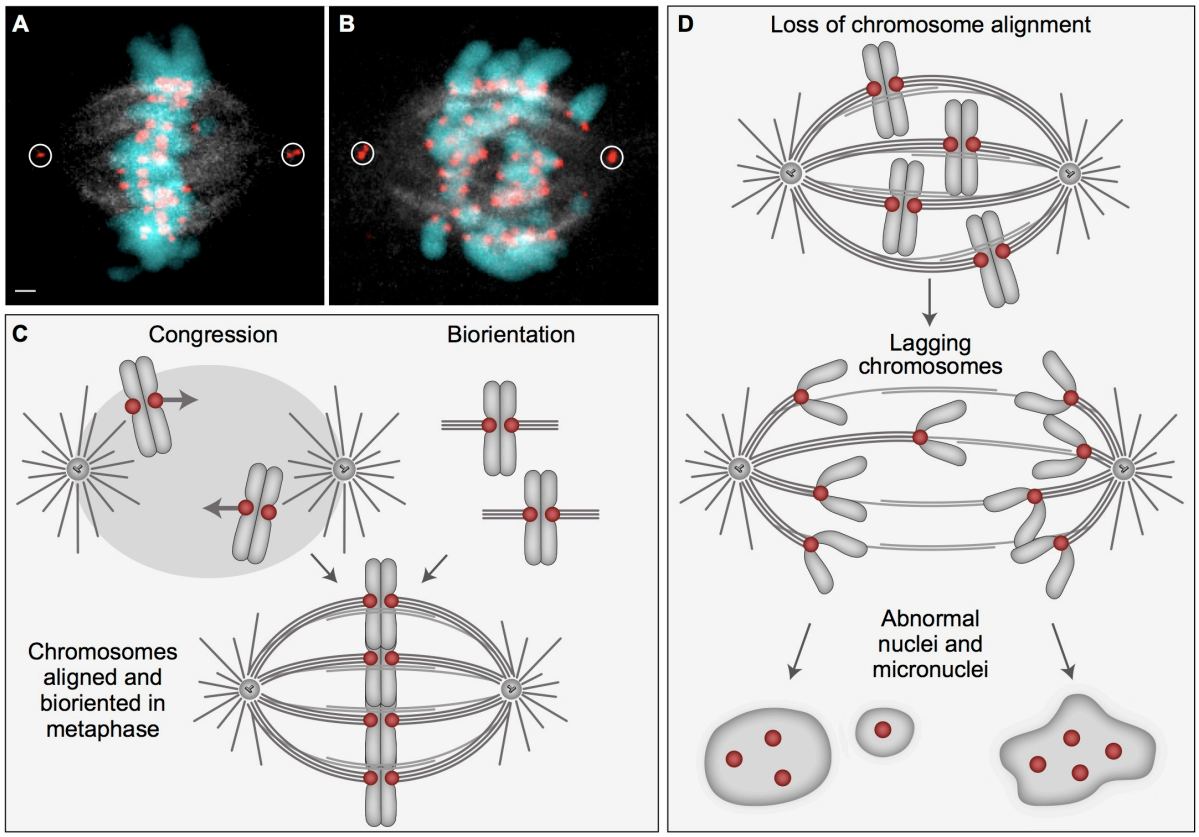

Figure 1. Chromosome alignment and its biological relevance.
(A) Spindle in a human retinal pigment epithelial 1 (RPE1) cell with aligned chromosomes (cyan) at the equatorial plane during metaphase. Kinetochores and centrin are shown in red and PRC1-labeled bridging fibers in grey. White circles indicate centrosomes. Scale bar, 1 µm. (B) Example of a spindle with misaligned chromosomes due to co-depletion of Kif18A/kinesin-8 and Kif4A/kinesin-4; legend as in (A). (C) During spindle assembly chromosomes congress to the spindle equator and become bioriented, meaning that their kinetochores (red) are attached to microtubules (grey lines) extending from the opposite spindle poles (grey spheres). (D) Loss of chromosome alignment leads to asynchronous poleward movements of chromatids in anaphase and impaired nuclear formation in telophase. For all figures, please find a more detailed discussion and references in the text.

Physical Mechanisms that Can Center the Chromosomes

In order to position something in the central part of an object such as the spindle, there needs to be a mechanism that measures length. How can the spindle measure length to position the chromosomes in the midplane? Three classes of mechanisms that sense the length exist, based on microtubule length-dependent pushing forces, pulling forces, and microtubule dynamics.

Microtubule Length-dependent Pushing Forces

The idea that chromosomes experience pushing forces within the spindle has a long history. More than 80 years ago, Darlington hypothesized that chromosomes move towards the spindle equator because they are repelled by the poles due to electric charges (Darlington, 1937). Darlington's initial idea about the existence of repulsive forces and their ability to help

center the chromosomes on the spindle was right, though the origin of forces was later shown to be mechanical and microtubule-dependent (Rieder et al., 1986).

Microtubule pushing forces depend on the distance from the centrosome for three reasons. First, because most of the microtubules grow from the centrosome, their density is high close to the centrosome, and many of them reach a unit area situated close to the centrosome, producing a high pushing force (Figure 2A, left aster). Far away from the pole, microtubules are rare and few of them reach large distances, generating a smaller pushing force per unit area. In mathematical terms, the microtubule density decreases with the distance from the centrosome, d, as $1/d^2$ for an ideal isotropic aster (Figure 2A, left graph) (Campas and Sens, 2006).

Second, the length distribution of microtubules is roughly exponential, with many short microtubules and few long ones (Figure 2A, middle aster and graph). Such a distribution is a consequence of microtubule dynamic instability (Dogterom and Leibler, 1993; Mitchison and Kirschner, 1984) and has been observed in electron tomography images of spindles in *C. elegans* embryos (Redemann et al., 2017). The exponential length distribution amplifies the microtubule density effect described above, resulting in an even larger difference in the number of microtubules reaching shorter and longer distances.

The third effect is based on microtubule buckling (Figure 2A, right aster). If the pushing force exerted by a growing microtubule exceeds a critical force, the microtubule buckles under its own compression. The critical force for buckling, also called the Euler force, depends on microtubule length, L, as $1/L^2$ (Figure 2A, right graph) (Howard, 2001). Therefore, a shorter microtubule has a larger Euler force and thus produces a stronger push than a long one.

When all three effects are put together, a chromosome that is displaced towards one spindle pole has more microtubules extending from the nearer than the farther pole, pushing it away. Moreover, the Euler force of the microtubules extending from the nearer pole is higher. Due to the higher number of microtubules and their higher force, the chromosome will be pushed away from the nearer pole towards the spindle center (Figure 2A, bottom).

Microtubule Length-dependent Pulling Forces

In contrast to the microtubule pushing forces within the spindle, for which it is widely accepted that they depend on microtubule length and the distance from the centrosome, the length-dependence of the pulling forces within the spindle is controversial. In pioneering works on spindle forces, Ostergren proposed that a longer traction fiber of a displaced chromosome generates a stronger pulling force toward the more remote pole, causing the stabilization of chromosome positioning at the equatorial plate (Ostergren, 1950), but at that time the microtubules were not yet discovered and these concepts were not developed further.

The discovery of the dynamic instability of microtubules led to the idea that the events on the plus and minus ends of kinetochore microtubules regulate the pulling forces on the kinetochore (Mitchison et al., 1986; Mitchison and Salmon, 1992). Thus, the field has focused on the forces generated at microtubule ends, whereas pulling forces exerted along the microtubule length were largely neglected.

Kinetochore microtubules are not isolated within the spindle, but are laterally attached to non-kinetochore microtubules (Kajtez et al., 2016; O'Toole et al., 2020). Motor proteins

may bind within the overlaps of kinetochore and non-kinetochore microtubules and longer overlaps accumulate more motors, consequently exerting larger forces (Figure 2B, left). The total force on the chromosome is then directed towards the spindle center and proportional to the difference in the length of the overlap on either side (Figure 2B, right). This centering mechanism was recently proposed for spindles in human cells (Risteski et al., 2021).

Microtubule Length-dependent Regulation of Microtubule Dynamics

The pulling force generated by the depolymerizing plus end of a microtubule does not depend on microtubule length and thus cannot center the chromosome, but motor proteins can "measure" microtubule length and make microtubule dynamics length-dependent. Such length-dependent mechanisms are achieved by motors that bind along the microtubule lattice and walk towards the microtubule plus end. Thus, the longer the microtubule, the more motors accumulate at its plus end. This effect known as the antenna model has been shown for kinesin-8 (Mayr et al., 2007; Varga et al., 2006; Varga et al., 2009) and kinesin-4 (Bieling et al., 2010). For the antenna model to be efficient, the motors must walk faster than the microtubule growth in order to reach the plus end, and must be also highly processive, i.e., walk for a large distance along the microtubule without detachment. If the motors showing this behavior are regulators of microtubule dynamics, then the dynamics will be regulated in a length-dependent manner. For example, due to the kinesin-8 Kip3, long stabilized microtubules *in vitro* depolymerize faster than short microtubules (Varga et al., 2006). Similarly, the dynamics of individual microtubules in the fission yeast *S. pombe* during interphase is regulated in a length dependent manner, though a different feature is affected, namely the catastrophe rate of long microtubules is higher than that of short ones (Tischer et al., 2009). Finally, dynamic microtubules *in vitro* become less dynamic and spend more time in a pausing state when the human kinesin-8 Kif18A accumulates at their plus end (Du et al., 2010; Stumpff et al., 2012).

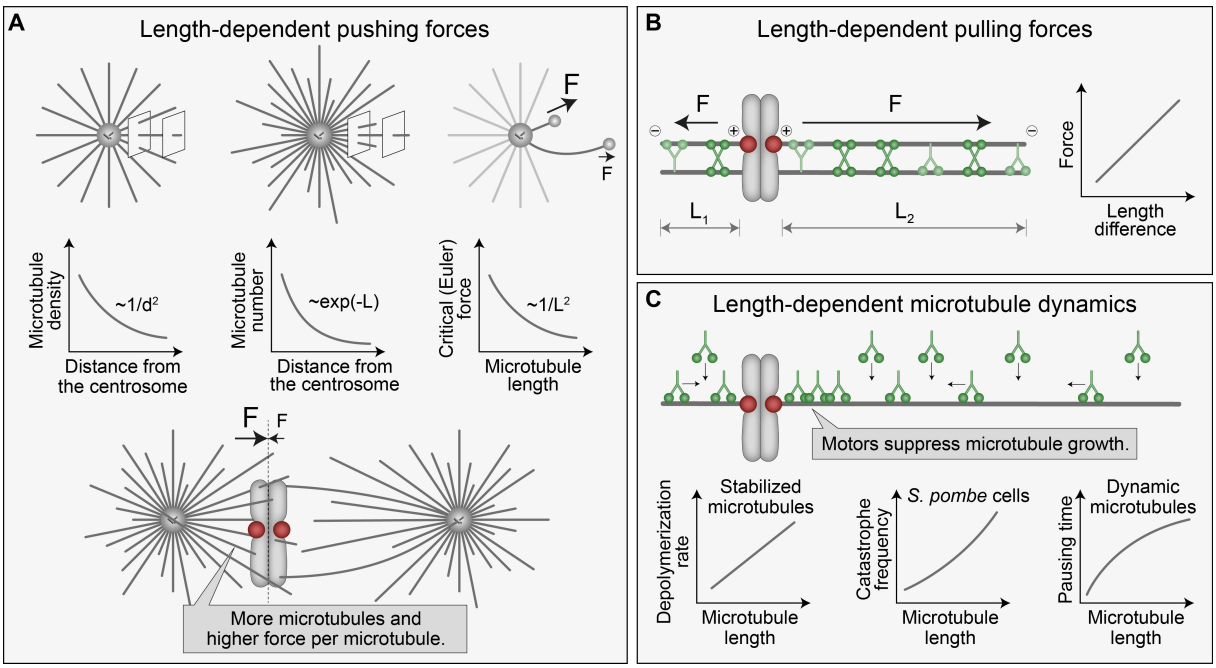

Figure 2. Principles of length measurements within the spindle.
(A) Pushing forces exerted by growing microtubules decrease with an increasing distance from the centrosome because microtubule density decreases due to aster geometry (left aster and graph), a roughly exponential distribution of microtubule lengths due to microtubule dynamics (middle aster and graph), and a decreasing critical (Euler) force, *F*, at which the microtubule buckles (right aster and graph). A displaced chromosome is contacted by more microtubules from the nearer pole, and they can exert a higher force per microtubule than the long ones extending from the other spindle half, resulting in a net force towards the spindle center (bottom). (B) Pulling forces, *F*, exerted by motor proteins (tetrameric, dark green and/or dimeric, light green) attached along the k-fibers depend on the length of the overlap between the k-fiber and non-kinetochore microtubules, *L*. The net force is proportional to the difference in the overlap length on either side (graph at the right). (C) Motor proteins (green) that walk towards the microtubule plus end with a low detachment rate accumulate there in a microtubule length-dependent manner. If these motors are modulators of microtubule dynamics, then microtubule depolymerization rate, catastrophe frequency, or the fraction of time that the microtubule spends in a pausing state depend on the microtubule length (graphs).

Molecular Mechanisms that Generate and Regulate Pulling Forces on Kinetochores

Kinetochore Microtubules Pull on Kinetochores

Back in 1980's the laser ablation experiments on prometaphase or metaphase mitotic spindles revealed the existence of poleward pulling force on chromosomes exerted by k-fibers. By ablating proximal sister kinetochore, it was shown that chromosome moves towards the spindle pole to which the distal, non-ablated kinetochore is oriented (McNeill and Berns, 1981). Similarly, ablation of chromosome arms of mono-oriented chromosomes causes the kinetochore to move towards the proximal spindle pole (Rieder et al., 1986). These experiments have demonstrated that k-fibers exert pulling forces on kinetochores.

Mitotic chromosomes are elastic and upon biorientation their centromeres become stretched by kinetochore microtubules pulling on both sister kinetochores (Claussen et al., 1994; Pickett-Heaps et al., 1982). This pulling generates opposing forces that tend to return the centromere to its non-stretched configuration. In line with this, inter-kinetochore distance between bioriented sister kinetochores is larger than of the mono-oriented kinetochores, suggesting increased tension when both sister kinetochores are attached to opposing spindle poles (Waters et al., 1996).

Even though attached to kinetochores, kinetochore microtubule plus ends remain dynamic yet with slower tubulin turnover compared to non-kinetochore microtubules (Zhai et al., 1995). Due to the mechanical coupling between kinetochore microtubules and kinetochores, dynamic instability of kinetochore microtubules contributes to the oscillatory motion of the chromosomes along the spindle axis (Rieder and Salmon, 1994; Skibbens et al., 1993). These abrupt changes between poleward and anti-poleward movement of chromosomes, termed directional instability, are less prominent during chromosome congression in prometaphase when chromosomes have directional persistence towards the spindle equator (Skibbens et al., 1993). However, once aligned, the oscillatory movements of the chromosomes are confined to a narrow region in the central part of the spindle.

Molecular Mechanisms that Regulate the Dynamics and Length of Kinetochore Microtubules

How are the length measurement mechanisms and the forces that control chromosome alignment realized in cells at the molecular level? Motor proteins can make microtubule dynamics length dependent, which has a centering effect on chromosomes, the principles of which are described above (Figure 2C). This centering mechanism works well due to the precise regulation of microtubules dynamics achieved by a large number of motor proteins and other microtubule-associated proteins.

Microtubule plus ends, responsible for pulling forces on kinetochores, are a hub for multiple microtubule regulators (Figure 3, box 1). One of the most important regulators is kinesin-8, which promotes microtubule catastrophe in budding yeast (Gupta et al., 2006), increase catastrophe frequency in fission yeast (Tischer et al., 2009; West et al., 2001) and promotes microtubule destabilization in *Drosophila* (Goshima and Vale, 2003). Similar activity was observed for the human homolog Kif18A (Mayr et al., 2007), although more recent studies indicate it suppresses microtubule dynamics rather than induces microtubule depolymerization (Du et al., 2010; Stumpff et al., 2012). Accordingly, depletion of Kif18A results in increased spindle length and loss of inter-kinetochore tension (Mayr et al., 2007; Stumpff et al., 2008). With its role at kinetochore microtubule plus ends, kinesin-8 is required for proper mitotic chromosome movement and alignment (Gandhi et al., 2004; Garcia et al., 2002; Goshima and Vale, 2003; Klemm et al., 2018; Mayr et al., 2007; Stumpff et al., 2008; Stumpff et al., 2012; Wargacki et al., 2010; West et al., 2002; Zhu et al., 2005). Quantitative tracking of kinetochore positioning upon Kif18A depletion indicated that Kif18A limits kinetochore movements around the spindle equator by affecting the frequency of kinetochore directional switches and by decreasing the velocity of kinetochore movements (Stumpff et al., 2008), although another study reported the opposite effect on kinetochore velocity (Mayr et al., 2007).

Similarly to kinesin-8, Kif4A/kinesin-4 inhibits microtubule plus end growth (Bieling et al., 2010). Accordingly, depletion of Kif4A leads to elongation of microtubule overlap regions in anaphase (Hu et al., 2011; Kurasawa et al., 2004; Zhu and Jiang, 2005) and to elongation of overlaps of bridging microtubules in metaphase (Jagrić et al., 2020). As a chromokinesin, Kif4A is implicated in the regulation of polar ejection forces (PEFs) by suppressing the dynamics of microtubules contacting chromosome arms (Stumpff et al., 2012; Wandke et al., 2012).

Among plus end-tracking proteins, several are implicated in affecting kinetochore alignment. MCAK/Kif2C/Kinesin-13 is a microtubule depolymerase, which localizes on centromeres and kinetochores, thus being a strong candidate for force generation involved in chromosome movements and positioning (Figure 3, box 1) (Hunter et al., 2003; Wordeman and Mitchison, 1995). MCAK is a motor which diffuses along the microtubule lattice without directional bias, i.e., it targets and destabilizes both microtubule ends *in vitro* (Helenius et al., 2006). Depletion of MCAK leads to chromosome alignment defects, decrease of chromosome oscillation speed and directional coordination between sister kinetochores, without affecting the period of oscillations (Jaqaman et al., 2010; Kline-Smith et al., 2004; Wordeman et al., 2007). Based on these results and the observation that MCAK preferentially accumulates on

the leading sister kinetochore, i.e., the one moving towards its associated pole, it was suggested that MCAK sets the velocity of chromosome oscillations together with Kif18A. In this model, MCAK depolymerizes microtubules within the k-fiber of the leading kinetochore, whereas Kif18A promotes depolymerization at the trailing kinetochore, i.e., the one moving away from its associated pole, thereby providing resistance to sister pair movement (Jaqaman et al., 2010; Kline-Smith et al., 2004).

Upon microtubule attachment, cytoplasmic linker-associated proteins (CLASPs) remain localized at kinetochore-microtubule interface (Figure 3, box 1) (Maiato et al., 2003; Mimori-Kiyosue et al., 2006; Pereira et al., 2006). With their redundant roles in promoting microtubule rescue and suppressing microtubule catastrophe, without affecting the overall microtubule polymerization rate, CLASPs act as microtubule stabilizers (Al-Bassam and Chang, 2011; Al-Bassam et al., 2010). By stabilizing kinetochore microtubules, CLASPs increase tension on kinetochores, and decrease both oscillations and microtubule growth (Mimori-Kiyosue et al., 2006). Thus, CLASPs help keep the kinetochores in tight alignment at the spindle equator.

Contrary to the highly dynamic plus ends that interact with kinetochores, minus ends are mostly anchored at the microtubule nucleation sites, i.e., centrosomes or microtubule lattice in the case of augmin-dependent microtubule nucleation (Akhmanova and Steinmetz, 2019). The key player involved in the minus end dynamics is Kif2A/kinesin-13, which depolymerizes microtubules (Figure 3, box 2) (Ganem et al., 2005; Rogers et al., 2004).

Dynamics at the microtubule ends underlie a process termed poleward flux, defined as a continuous translocation of tubulin subunits in the direction of the minus end (Mitchison, 1989). Even though the molecular mechanisms responsible for this process are not yet fully elucidated, two main models have been suggested. One model proposes that flux is driven by kinesin-13 mediated depolymerization at spindle poles with simultaneous CLASP-mediated polymerization at kinetochore microtubule plus-ends (Ganem et al., 2005; Girao et al., 2020; Maiato et al., 2005). A different model explains the origin of poleward flux as a response to sliding of antiparallel interpolar microtubules, which is transmitted to kinetochore microtubules due to their coupling mediated by different crosslinking molecules (Brust-Mascher et al., 2009; Miyamoto et al., 2004). Recently, it was proposed that poleward flux is driven by Kif4A/kinesin-4 on chromosome arms, and that the distribution of poleward flux across the spindle is achieved by coupling of non-kinetochore and kinetochore microtubules (Steblyanko et al., 2020). Microtubule flux has been implicated in regulation of spindle length, correction of erroneous kinetochore-microtubule attachments and equalization of forces at kinetochores prior to segregation (Ganem et al., 2005; Matos et al., 2009; Rogers et al., 2004).

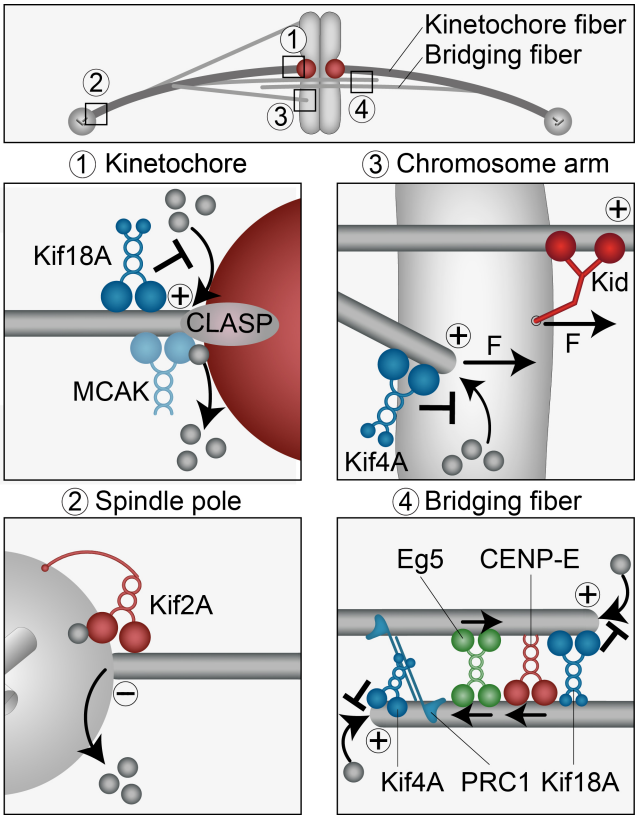

Figure 3. Molecular players involved in kinetochore alignment.
A chromosome with k-fibers (dark grey) and non-kinetochore microtubules (bridging microtubules overlapping in the middle and other microtubules interacting with chromosome arms, all in light grey) is sketched at the top with boxes marking the enlarged areas shown below. Box 1: At the kinetochore, Kif18A suppresses microtubule dynamics, CLASP promotes microtubule polymerization and MCAK promotes depolymerization. Box 2: At the spindle pole, Kif2A promotes microtubule depolymerization. Box 3: At the chromosome arm, chromokinesins generate polar ejection forces. Kid moves the chromosome along the microtubule and Kif4A suppresses microtubule dynamics. Growing microtubules also generate polar ejection forces as they push into the chromosome. Box 4: Within the bridging fiber, Eg5 and CENP-E slide the antiparallel microtubules apart. Kif18A and Kif4A, which interacts with the crosslinker PRC1, suppress the dynamics of microtubule plus ends, thereby controlling the length of antiparallel overlaps.

Polar Ejection Forces Act on Chromosome Arms

The existence of polar ejection forces (PEFs), generated by microtubules that push the chromosomes away from the pole, was first demonstrated by laser ablation of chromosome arms on chromosomes in monopolar and bipolar spindles, which resulted in transport of the created acentric chromosome fragments away from the pole (Rieder et al., 1986; Rieder and Salmon, 1994). PEFs originate from interactions between non-kinetochore microtubules and chromosome arms, with anti-poleward forces being generated by microtubule polymerization against chromosome arms, or by activity of chromokinesins, proteins that bind to both microtubules and chromosomes (Figure 3, box 3) (Ault et al., 1991; Bajer et al., 1982; Brouhard and Hunt, 2005; McIntosh et al., 2002; Rieder et al., 1986). It was shown that PEFs

exerted by individual microtubules on metaphase chromosomes are consistent with forces generated by polymerizing microtubules pushing against chromosomes or by individual kinesin motors (Marshall et al., 2001). However, chromokinesins contribute to PEFs to a larger extent than the pushing forces of polymerizing microtubules, given that a larger fraction of acentric chromosome fragments is able to congress to the spindle equator when chromokinesins are present (Barisic et al., 2014).

Among chromokinesins, generation of PEFs primarily depends on Kid/kinesin-10 activity to move chromosomes toward the microtubule plus ends, a conclusion based on the experiments showing that Kid is involved in chromosome alignment (Antonio et al., 2000; Funabiki and Murray, 2000), chromosome oscillation, and chromosome arm orientation (Levesque and Compton, 2001; Wandke et al., 2012). By directly suppressing dynamics of microtubule plus ends, chromokinesin Kif4A/kinesin-4 independently contributes to PEF modulation (Figure 3, box 3) (Levesque and Compton, 2001; Stumpff et al., 2012; Wandke et al., 2012).

Within the spindle, PEFs were shown to depend on the surface area of chromosome arms available for interaction with microtubules. The underlying experiment showed that laser ablation of a larger portion of a chromosome arm allows the kinetochore-containing chromosome fragment to move further away from the equator, evident in the increase of its oscillation amplitude (Ke et al., 2009). Similarly, stronger PEFs acting on peripheral chromosomes due to their large size in comparison with central chromosomes were proposed to cause the more extensive oscillations of central versus peripheral chromosomes (Civelekoglu-Scholey et al., 2013).

Furthermore, PEFs were hypothesized to depend on microtubule density, meaning that PEFs should increase towards the spindle pole due to an increase in microtubule density (Figure 2A). The precise spatial distribution of PEFs across the spindle was determined experimentally based on the relationship between reduction in chromosome size after laser ablation and increased oscillation amplitude, yielding a force map in which PEFs increase most rapidly near the equator and flatten towards the poles (Ke et al., 2009). These experiments led to a model where PEFs limit the extent of oscillations by exerting tension on the leading kinetochore during its movement away from the equator, thus inducing microtubule rescue and chromosome reversal (Ke et al., 2009). Accordingly, elevated PEFs achieved by overexpression of Kid stabilized synthelic kinetochore-microtubule attachments, i.e., those where both sister kinetochores are attached to microtubules from the same spindle pole, through higher tension exerted on kinetochores and by preventing chromosomes to move closer to the poles where error correction takes place (Cane et al., 2013). Altogether, by operating in concert with the mechanisms of length-dependent modulation of microtubule dynamics, PEFs contribute to the positioning of chromosomes at the spindle equator by promoting reversal in their movement as the chromosomes approach the pole.

Forces coming from mechanical coupling of k-fibers and bridging fibers

First electron microscopy studies of the mitotic spindle in Ptk1 and grasshopper cells proposed that interpolar microtubules contribute to the structural integrity of the spindle and

provide mechanical support for the forces exerted on chromosomes (Mastronarde et al., 1993; McDonald et al., 1992; Nicklas et al., 1982). Indeed, recent findings show that sister k-fibers are physically linked with an antiparallel interpolar microtubule bundle, termed bridging fiber (Kajtez et al., 2016; Pavin and Tolic, 2016; Tolic, 2018). These fibers have been observed also in electron microscopy images of human cells (Nixon et al., 2017; O'Toole et al., 2020; Yu et al., 2019), where the majority of their minus ends are seen as incorporated at the walls of proximal k-fibers, while their plus ends fan out and comingle with nearby k-fibers (O'Toole et al., 2020). Accordingly, bridging microtubules are mainly nucleated in an augmin-dependent manner (Manenica et al., 2020) with NuMa-mediated crosslinking of parallel regions between bridging and kinetochore microtubules (Elting et al., 2017; Risteski et al., 2021).

Bridging microtubules are linked together by the protein regulator of cytokinesis 1 (PRC1) that crosslinks antiparallel microtubules, and several motor proteins that slide microtubules or regulate microtubule dynamics are found within the bridging fiber (Figure 3, box 4), including Eg5 (Kajtez et al., 2016; Mann and Wadsworth, 2018), CENP-E (Steblyanko et al., 2020), Kif4A, Kif18A, and MKLP1 (Jagrić et al., 2020). Eg5 is likely the main microtubule slider given that its inactivation during metaphase results in spindle shortening and collapse (Gayek and Ohi, 2014). The reduced poleward flux velocity of the bridging microtubules observed after CENP-E depletion suggests that this motor also has a role in sliding of bridging microtubules (Risteski et al., 2021). The overlap length of the antiparallel microtubules within the bridging fiber is regulated by Kif4A and Kif18A localized at their plus ends (Jagrić et al., 2020). PRC1 stabilizes the overlaps probably together with MKLP1, Eg5, and other crosslinkers.

Bridging fibers, by spanning the gap and acting as a bridge between sister k-fibers, balance the tensile forces at kinetochores (Kajtez et al., 2016) and restrict extensive stretching of the centromere (Suresh et al., 2020). This mechanical support for k-fibers extends up to ~2 μm laterally from each sister kinetochore (Kajtez et al., 2016; Suresh et al., 2020), and is defined as an overlap region selectively marked by the microtubule crosslinker PRC1 (Kajtez et al., 2016; Polak et al., 2017).

Since PRC1-labeled bridging fibers show one-to-one association with a pair of sister k-fibers (Polak et al., 2017), this could give rise to flux-dependent equalization of tension at kinetochores (Matos et al., 2009) and a closed-loop force network independent of centrosomes (Pereira and Maiato, 2012). Indeed, it was shown that bridging microtubules slide apart and serve as a platform for force generation that underlies microtubule poleward flux (Risteski et al., 2021). Upon loss of k-fibers in the spindle bridging fibers undergo similar rates of poleward flux, suggesting that bridging fiber flux is independent of k-fibers. Interestingly, poleward flux of k-fibers is slower than that of bridging fibers, indicating that the coupling between bridging and k-fibers is not rigid but allows for sliding. This sliding opens a new perspective on the physical mechanisms of chromosome positioning, where forces are generated within the overlaps between bridging and k-fibers. Such forces belong to the class of length-dependent pulling forces, which have a centering effect as described above (Figure 2B).

Typical amplitude of chromosome oscillations in human cells is about 1.2 μm (Stumpff et al., 2008), which lies within the PRC1-labeled overlap region. Interestingly, upon

acute PRC1 removal by an optogenetic approach kinetochores are found to extrude out of the narrow region in the central part of the spindle, suggesting that bridging fibers have a role in buffering chromosome movements within this region. As PRC1 removal results in elongated overlaps of antiparallel microtubules, this suggests that chromosome centering is achieved by overlap length-dependent forces transmitted to the associated k-fibers (Jagrić et al., 2020).

To explain this, imagine a bioriented chromosome positioned away from the spindle equator (Figure 4A). The kinetochore facing the nearer pole has a shorter k-fiber than its sister that faces the farther pole, implying a shorter and longer overlap with the bridging fiber, respectively. As the length of the overlap determines the strength of the coupling, the friction force arising due to the sliding of bridging microtubules is higher for longer overlaps, leading to higher poleward flux velocity of the longer k-fiber and the net force in the direction towards the spindle equator. This was corroborated by experiments in which the spindles and thus the k-fibers were elongated, creating longer overlap regions with the bridging fibers, which increased the velocity of the k-fiber poleward flux (Risteski et al., 2021).

By following the same rationale, shorter and longer k-fiber have shorter and longer antiparallel overlap with the bridging fiber, respectively (Figure 4B). Here, more motor proteins that slide the microtubules apart accumulate in the longer overlap, which leads to a higher force sliding the k-fiber along the bridging fiber. Thus, the net force on both k-fibers is towards the spindle equator. Experiments in which the bridging fiber overlap regions were elongated, thus creating also longer overlap regions with k-fibers, and resulting in faster k-fiber flux support this idea (Risteski et al., 2021).

The centering efficiency depends on the relative asymmetry of the chromosome position within the overlap. This means that the same displacement of the chromosome implies a larger relative asymmetry and thus better centering when the overlap is short in comparison with longer overlaps. Accordingly, spindles in treatments which result in longer overlap regions of bridging fiber, e.g., depletion of Kif18A, exhibit chromosome misalignment (Jagrić et al., 2020; Risteski et al., 2021).

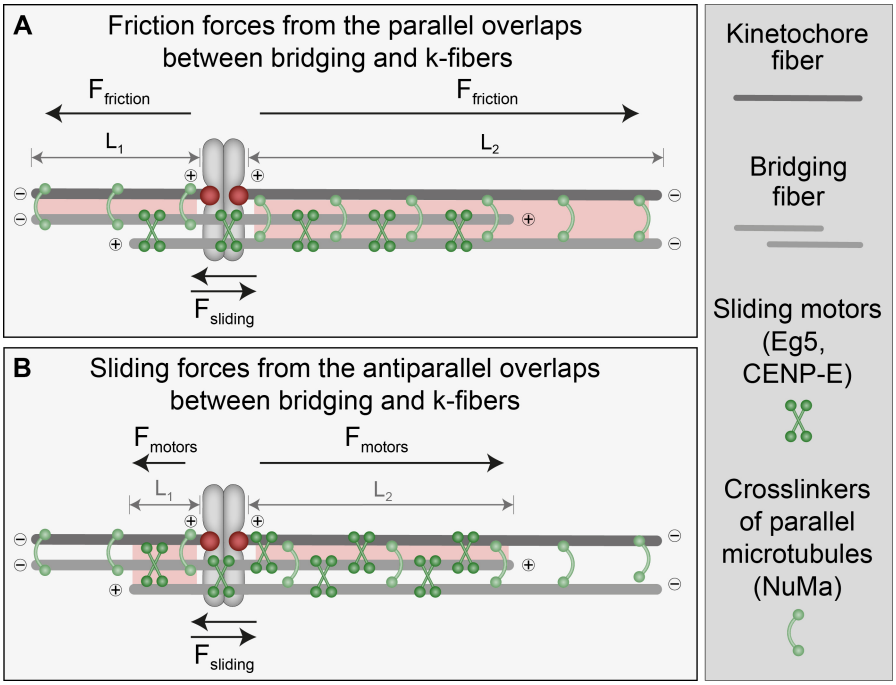

Figure 4. Forces arising from mechanical coupling of k-fibers and bridging fibers center the chromosomes.
(A) A displaced chromosome has a longer overlap between the k-fiber extending towards the distal pole and the parallel bridging microtubules coming from the same pole, L_2, than the k-fiber extending towards the proximal pole and the bridging microtubules on that side, L_1. The friction force, $F_{friction}$, is generated by the sliding force of bridging microtubules, $F_{sliding}$, that is transmitted to the k-fibers in the overlaps between parallel bridging and kinetochore microtubules (areas shaded in red) crosslinked by NuMa (green C-shaped pictograms). These friction forces are larger for longer overlaps, leading to a net force towards the spindle center. (B) A displaced chromosome also has a longer antiparallel overlap between the k-fiber extending towards the distal pole and the bridging microtubules coming from the opposite pole, L_2, than the k-fiber extending towards the proximal pole and the bridging microtubules from the opposite side, L_1. The sliding force generated by motors (green X-shaped pictograms) within these overlaps (areas shaded in red), F_{motors}, is larger for longer overlaps, leading to a net force towards the spindle center.

Comparison of Mechanisms for Chromosome Alignment in Yeast and Mammalian Spindles

With their plus ends, 15-25 microtubules attach to kinetochores and make up k-fibers in human cells (McEwen et al., 1997; Wendell et al., 1993). Unlike human spindles, in which only few direct connections between spindle poles and kinetochores exist (O'Toole et al., 2020), spindles in budding yeast contain a single and in fission yeast around three kinetochore microtubules which emanate from the spindle pole body and directly attach to kinetochores (Ding et al., 1993; O'Toole et al., 1999; Winey et al., 1995). Furthermore, in lower eukaryotes microtubules that overlap in the central part of the spindle emanate from the spindle pole body (Ding et al., 1993; Winey et al., 1995). This is not the case in human spindles where the majority of overlapping microtubule minus ends are incorporated in the k-fiber lattice, mediated by augmin-dependent nucleation, which is not observed in yeasts (Kamasaki et al., 2013; O'Toole et al., 2020).

Yeast spindles do not show poleward flux (Mallavarapu et al., 1999), which may be related to the fact that fission yeast lacks kinesin-13 (Wood et al., 2002), though this kinesin family is present in budding yeast (Tytell and Sorger, 2006). Yeasts also lack chromokinesins responsible for polar ejection forces (Wood et al., 2002). Thus, chromosome positioning in yeasts is exclusively dependent on microtubule polymerization and depolymerization at kinetochores (Maddox et al., 2000; Mallavarapu et al., 1999). When a pair of kinetochores is displaced towards one spindle pole, it is crucial that the longer kinetochore microtubules undergo catastrophe and start to shrink, to bring the kinetochores back to the spindle center (Figure 5A). Catastrophe is mainly regulated by kinesin-8 that accumulates on longer microtubules, as described above, thereby preventing excessive growth of trailing microtubules and excessive movements of kinetochores away from the spindle center.

In higher eukaryotes, beside microtubule dynamics at kinetochores, microtubules undergo poleward flux and depolymerization at the minus end close to the spindle pole (Mitchison et al., 1986; Mitchison and Salmon, 1992). Measurements in RPE1 cells, which

undergo fast flux (Dudka et al., 2018), show that kinetochore microtubule plus ends are predominantly growing or pausing, suggesting that microtubule depolymerization at the plus ends plays a minor role in kinetochore movements (Risteski et al., 2021). In agreement with this difference in microtubule dynamics in yeast and human cells, yeast kinesin-8 promotes depolymerization or catastrophe (Gupta et al., 2006; Tischer et al., 2009; Varga et al., 2006), whereas human kinesin-8 suppresses microtubule dynamics (Du et al., 2010; Stumpff et al., 2012).

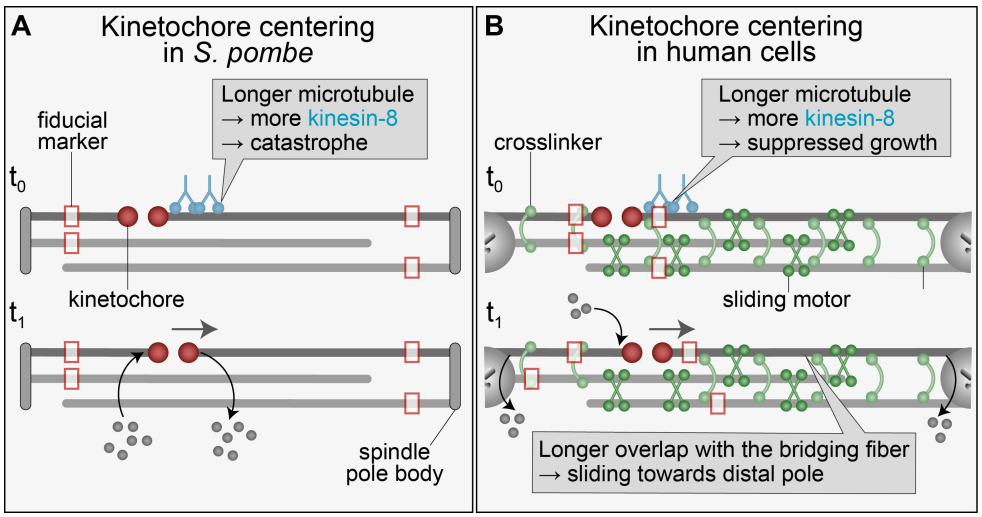

Figure 5. Mechanisms of kinetochore centering in yeast and human spindles.
(A) In fission yeast, spindle microtubules do not undergo poleward flux (red squares denote fiduciary marks on the microtubules). When kinetochores (red circles) move towards one pole, the longer kinetochore microtubules on the trailing side accumulate more kinesin-8 motors (blue), which induce their catastrophe and subsequent shrinkage, thereby bringing the kinetochores back towards the spindle center. (B) In human spindles, microtubules undergo poleward flux (note the movement of red squares between times t_0 and t_1). Motors that slide bridging microtubules (green X-shaped pictograms) together with microtubule depolymerization at the minus end drive the flux, which is transmitted to k-fibers via crosslinkers (NuMa, green C-shaped pictograms). As the crosslinkers allow for sliding between microtubules, k-fiber flux is generally slower than the bridging microtubule flux (note that the red squares on the k-fiber move less from time t_0 to t_1 than those on the bridging fiber). Because the longer k-fiber has a longer overlap with the bridging fiber, the force on the longer k-fiber is higher (see Figure 4) resulting in a higher flux velocity (note that the red square on the right k-fiber moves more from time t_0 to t_1 than the one on the left k-fiber). Thus, the net movement of the k-fibers and kinetochores is towards the spindle center. In addition, kinesin-8 motors (blue) accumulate on the longer k-fiber and suppress its growth, promoting kinetochore centering.

Theoretical Models of Chromosome Alignment

Experiments on the metaphase spindle revealed what forces are most relevant for chromosome positioning at the metaphase plate. These forces are orchestrated in such a way that point towards the metaphase plate, resembling the principal mechanism for chromosome

alignment, termed centering mechanism. To work efficiently, these forces are also large enough to move chromosome throughout the cell.

To answer how a combination of the known forces can drive chromosome movement in higher eukaryotic cells, several theoretical models were proposed. In an elegant computational model of the force balance on chromosomes, the major centering force is described by a phenomenological function that represents the polar ejection force (Joglekar and Hunt, 2002). The authors find that this centering force, working together with microtubule dynamic instability, can explain the experimentally observed chromosome movement. In a computational model for chromosome movement in *Drosophila* embryos, the main forces are collective motor forces, microtubule polymerization and depolymerization, and a polar ejection force as the major centering force (Civelekoglu-Scholey et al., 2006). To explain a difference in the movement between central and peripheral chromosomes in PtK1 cells, polar ejection forces are described by introducing two phenomenological functions: a shallow function for polar ejection forces on central chromosomes and a steep function for peripheral chromosomes (Civelekoglu-Scholey et al., 2013).

A novel centering mechanism that relies on the interaction between bridging microtubules and k-fibers was recently proposed (Risteski et al., 2021). The model describes lateral interactions of kinetochore microtubules and bridging microtubules. Pulling forces exerted by k-fibers are proportional to the length of their overlap with bridging microtubules, generating larger forces by longer k-fibers. To make this mechanism function, velocities of the poleward flux of bridging microtubules should be larger than those of kinetochore microtubules, as shown experimentally (Risteski et al., 2021).

In lower eukaryotic cells, the major centering mechanism differs from those in higher eukaryotes. In early theoretical studies the major centering mechanism relies on a chemical gradient with a maximum in the middle of the spindle, which regulates microtubule catastrophe frequency (Gardner et al., 2005; Sprague et al., 2003). Such spatially regulated microtubule catastrophe, together with force-dependent rescue, reproduces the observed chromosome movement. For chromosome congression, a mechanism which relies on length-dependent suppression of microtubule polymerization governed by kinesin-5 motors was proposed (Gardner et al., 2008). A centering mechanism that relies on length-dependent forces can also explain chromosome congression and chromosome movement. This was shown in a model with a phenomenological parameter in the force-velocity relationship, which depends on microtubule length (Mary et al., 2015). A centering mechanism based on length-dependent microtubule catastrophe regulated by kinesin-8 motors can also explain the chromosome movements (Gergely et al., 2016). Finally, a model that describes the dynamics of kinesin-8 motors shows a length-dependent accumulation of these motors at growing microtubules, which promote microtubule catastrophe of longer microtubules, keeping kinetochores predominantly under tension and supporting their centering (Klemm et al., 2018). Taken together, the studies describing yeast spindles have shown that length-dependent regulation of kinetochore microtubule dynamics by kinesin-8 motors is crucial for kinetochore centering. Experiments have shown that kinesin-8 plays an important role also in human spindles (Mayr et al., 2007; Risteski et al., 2021; Stumpff et al., 2008; Stumpff et al., 2012), and we expect that future theoretical studies will show how the activity of this motor works together with other mechanisms to position the kinetochores at the spindle midplane.

Conclusions and Outlook

Chromosome alignment at the metaphase plate is the most eye-catching image of mitosis, with biological relevance for proper chromosome segregation and nuclear reformation. Yet, the mechanisms driving alignment are still under debate due to the large number of players and processes involved. We have here discussed the generally accepted mechanisms based on the regulation of microtubule plus end dynamics by motors, notably kinesin-8, which can "measure" microtubule length and suppress excessive microtubule growth and thus also the excessive kinetochore movements away from the spindle midplane. Polar ejection forces arising through interactions between spindle microtubules and the chromosome arms also promote chromosome alignment because they are highest close to the pole and decay towards the equator.

A recently introduced concept based on mechanical coupling between kinetochore and bridging microtubules provides a new perspective on chromosome alignment. In this model, bridging microtubules slide apart and this sliding is transmitted to kinetochore microtubules. The longer the overlaps between kinetochore and bridging microtubules, the larger the forces, resulting in a net force towards the spindle center. We propose that this mechanism based on length-dependent relative sliding of kinetochore along bridging microtubules works together with the length-dependent regulation of microtubule dynamics and polar ejection forces to ensure alignment of kinetochores at the equatorial plane of the spindle in metaphase. The centering mechanism based on sliding opens an attractive new avenue of research on the molecular players involved in the sliding of bridging fibers, regulation of their plus and minus ends, and their coupling with k-fibers. It is tempting to imagine that this mechanism also works in prometaphase during chromosome congression to promote chromosome movement from polar regions of the spindle towards the equator, which will be an exciting topic for future studies.

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