Mitotic spindle assembly:

May the force be with you

Roy van Heesbeen

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Cover: Mitosis Artwork made by Laura van Bruggen. Mitosis is the process in which a cell divides its chromosomes into two new identical daughter cells and is essential for growth, reproduction and maintenance of tissues for all living organisms. Some of the essential processes driving mitosis are studied in more detail in this thesis.
In addition, each chapter contains a photograph of a mountain passes climbed by bike by the author of this thesis. Climbing some of these mountains in sometimes extreme circumstances requires a lot of stamina and persistence, similar to the hurdles that one encounters during the PhD track. Reaching the top gives a lot of satisfaction, comparable to some of the joyful moments in science, like getting your paper published and completing the final thesis.

Mitotic spindle assembly:
May the force be with you

Mitotische spoel formatie: moge de kracht met u zijn

(met een samenvatting in het Nederlands)

Proefschrift

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door

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te Waalwijk

Promotor: prof. dr. R.H. Medema

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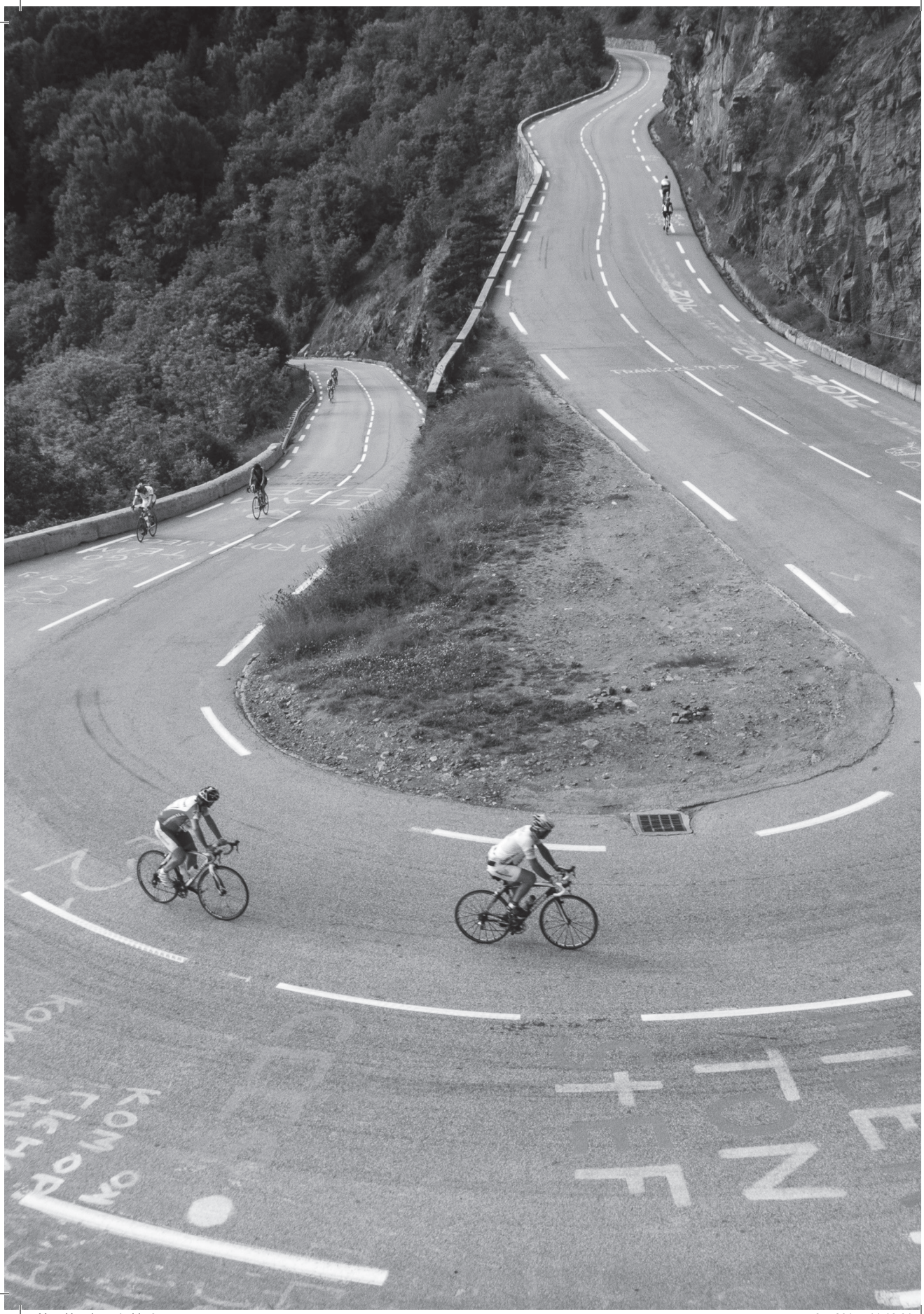

Chapter 1

General Introduction

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The cell cycle

Every human being consists of trillions of cells that all descend from a single fertilized oocyte. This extraordinary number of cells can only be reached by multiple rounds of cell division of a preexisting cell into two identical daughter cells. The series of events, required for cell division is known as the cell cycle (Figure 1). Cell division is not only required for the development of an organism, but also for its reproduction and the maintenance and regeneration of tissues in the body; while most cells in an adult body are in an quiescent state, about 300 billion cells are newly produced every day.
The cell cycle consists of four phases, in addition to a resting phase that is referred to as G_0. The first phase of the cell cycle, G_1 or Gap 1 phase, starts when quiescent or G_0 cells are triggered by external signals to divide. During this G_1 phase, the cell grows in size. The duration of this phase is highly variable and depends on many different factors. In G_1, the cell can increase the number of organelles, including ribosomes and mitochondria, such that the respective daughter cells not only inherit a complete copy of the genome, but also sufficient organelles to function. After the G_1 phase, cells enter S (synthesis) phase. During this phase, a complete replicate of each chromosome is made during a process called DNA replication. Precise replication of the DNA is essential to ensure genome integrity through multiple generations of cell division and to prevent genetic abnormalities that can lead to cell death or disease. After S phase, the cells enter a second, short growth phase, known as G_2 or Gap 2 phase. In this phase, cells prepare for the actual act of cell division by upregulating expression of mitotic regulators and ensure that the genome is intact and fully replicated. During the final phase of about one hour, known as mitosis, the cells physically divide in two identical daughter cells. This phase will be explained in more detail below.

Molecular control of the cell cycle

The molecular events driving the cell cycle are highly conserved in eukaryotic cells and involve two classes of proteins called cyclins and cyclin-dependent kinases (Cdks). These proteins ensure that the cell cycle occurs in a directional and irreversible manner. While the levels of Cdks remain largely constant during the cell cycle, the cyclins accumulate during the different cell cycle phases and form a complex with Cdks. The cyclin-Cdk complexes promote the progression to the next cell cycle phase through phosphorylation of target proteins, hereby inducing essential programs for each cell cycle phase, including transcription, DNA replication, and morphological changes during mitosis. D and E-type cyclins accumulate sequentially as the cells progresses through G_1 phase. A-type cyclins accumulate from S phase until mitosis and B-type cyclins accumulate during G_2 phase, peak during mitosis and rapidly disappear upon exit from mitosis.

Mitosis

The most dramatic events of the cell cycle take place during mitosis. During this phase, the duplicated chromosomes are segregated from each other in a carefully controlled manner to end up in the newly formed daughter cells. The transport of chromosomes is mediated by a highly dynamic structure known as the mitotic spindle. The formation of this microtubule-based structure will be discussed in more detail below. Mitosis is a complex process and similar to the cell cycle, divided into phases that

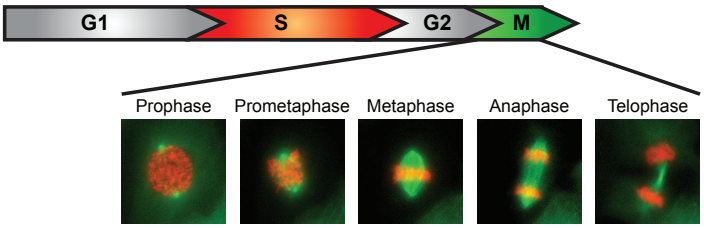

Figure 1. The cell cycle and the phases of mitosis.

Schematic model of the cell cycle. Each cycle starts with G1 phase, followed by a synthesis phase (S), in which the DNA is replicated. During G2, the cell prepares itself for division. The last phase of the cell cycle is mitosis (M). During this phase, the DNA is divided over two new identical daughter cells.
Mitosis itself is a complex phase and highly regulated. The microscopic images display the different phases of mitosis. DNA is shown in red, microtubules from the mitotic spindle are shown in green.

are executed in an irreversible manner. The different phases are mainly based on visible morphological changes that the chromosomes undergo during mitosis (Figure 1).
The first phase of mitosis is **prophase**. During this phase, the duplicated chromosomes, tightly held together by a ring-shaped protein complex called cohesin (reviewed in[1]), start to condense due to the increased activity of B-type cyclin-Cdk1 complexes. The condensation of chromosomes is mediated by the condensin complex (reviewed in[2]) and together with cohesin, this results in the classical X-shaped morphology of mitotic chromosomes. Another important process during prophase is the separation of the centrosomes. The centrosomes are the main microtubule-organizing center in cells, which form the spindle poles during mitosis. Similar to chromosomes, the centrosome is duplicated in S phase, hereby giving rise to a mother and daughter centrosome. The centrosomes stay close together until the onset of mitosis and move to opposite sites of the nucleus in prophase. This step is important for bipolarization and assembly of the mitotic spindle. The molecular details of centrosome function and separation will be explained in more detail below.
After prophase, the cell enters **prometaphase**. This phase is initiated by the full activation of B-type cyclin-Cdk1 complexes, triggering the dramatic morphological changes of the cell that occur during mitosis. Upon entry into prometaphase, the cell starts to round up and the nuclear envelope breaks down. Simultaneously, the microtubule cytoskeleton changes in its morphology and dynamics, to be able to build the mitotic spindle. During prometaphase, the condensed chromosomes interact with the microtubules from the mitotic spindle and the chromosomes migrate towards the equator of the cells. The interactions between microtubules and chromosomes are mediated via a specialized protein platform, known as the kinetochore, which is assembled on the centromeric region of each chromosome. The molecular details of this process will be explained in more detail below.
Metaphase is achieved when all chromosomes are correctly attached to the mitotic spindle and lined up along the so-called metaphase plate. After full alignment of all chromosomes, the anaphase promoting complex / cyclosome (APC/C) becomes activated. This protein complex marks B-type cyclins for destruction and initiates **anaphase** by inducing cleavage of the cohesion rings, hereby triggering the physical separation of the sister chromatids. The mitotic spindle subsequently drags the separated sister chromatids towards the spindle poles. After this process, the mitotic spindle elongates further and the cell stretches into an oval-like form.
After the completion of anaphase, the cell enters **telophase**. During this final stage, the separated chromosomes decondense and the nuclear envelope reforms around the segregated chromosomes.

In addition, the mitotic spindle starts to disassemble and cytokinesis is initiated. During this step, abscission of the cell membrane takes place and two new daughter cells arise.

The mitotic spindle

Microtubule structure and behavior during mitosis

As mentioned above, the mitotic spindle plays a key role in chromosome segregation. This structure is build up of a bipolar array of microtubules and many microtubule-associated proteins (MAPs). Microtubules are polymers that form the main component of the mitotic spindle and their behavior is essential for proper function of the mitotic spindle. The building blocks of the microtubule polymer are dimers of two highly conserved proteins known as α- and β-tubulin. The tubulin dimers are stacked into large, linear protofilaments that are aligned in parallel to form the microtubule. The microtubule ends are structurally distinct from each other and we discriminate plus and minus ends of microtubules. The minus ends of microtubules are usually stable, non-dynamic and embedded into the centrosomes. In contrast, the plus ends of microtubules are highly dynamic and are continuously growing and shrinking due to the addition and removal of tubulin dimers. This process is driven by hydrolysis of GTP by β-tubulin and the conversion between a rapid shrinking state (catastrophe) and rapid growing state (rescue) is known as dynamic instability (reviewed in[3]).

The mitotic spindle consists of three different populations of microtubules (reviewed in[4]). The first population, kinetochore-microtubules, are usually parallel bundles of microtubules that are embedded with their minus end in the centrosome and attach end-on with their plus ends to the outer region of the kinetochore. The number of microtubules in a kinetochore bundle varies depending on the organisms and cell type. Kinetochore-microtubules play an essential role in positioning of the chromosomes at the metaphase plate and the segregation of the chromosomes during anaphase. Kinetochore-microtubules bundles are very stable by themselves. However, they continuously depolymerize at their minus ends and incorporate new subunits at the plus ends, attached to the kinetochore. This process is known as microtubule flux and is driven by MAPs that localize to centrosomes and kinetochores. When the loss of subunits at the minus ends equals the addition of subunits at the plus ends, the kinetochore-microtubule length remains constant. During anaphase, the removal of subunits at the minus ends exceeds the addition of subunits at the plus end, hereby moving the chromosome poleward.

The second population of microtubules are known as interpolar microtubules. This population of microtubules originate from the spindle poles and invade the opposite aster. Interpolar microtubules from opposite poles will interact in an antiparallel fashion near the midzone due to the action of MAPs, including the motor protein Eg5. They have been shown to play an essential role in mediating the bipolarity of the spindle and are the main component of the central spindle during anaphase (reviewed in[5]). The molecular details how interpolar microtubules participate in spindle bipolarization are discussed below in more detail.

The third population of microtubules are astral microtubules. This population of microtubules extend away from the centrosomes towards the cell cortex, where their plus tips can interact with cortical MAPs. While they seem not be required for chromosome segregation, they do play a key role in positioning and orientation of the mitotic spindle many different cell types (reviewed in [6]).

Centrosome function during mitosis

The centrosomes play a key role during mitosis in organizing the microtubules of the mitotic spindle (reviewed in [7]). Although the mitotic spindle can form relatively normal without centrosomes, centrosomes are thought to enhance the robustness of mitotic spindle assembly[8]. Aberrant centrosome numbers can lead to severe defects in mitotic spindle assembly and errors in chromosome segregation[9,10]. In addition, centrosome amplification can lead to tumor progression[11].

The centrosomes are composed of an organized matrix of pericentriolar material that embeds a pair of centrioles[12,13]. The centrioles are positioned orthogonally to each other and are composed of a short cylindrical array of microtubules. The exact function of the centrioles in centrosome function is still poorly understood, but they play a key role in the recruitment of ϒ-tubulin ring complexes (ϒ-TuRC,[14]). This complex plays an essential role in microtubule nucleation (reviewed in[15]). At the onset of mitosis, large numbers of ϒ-TuRC are recruited to the centrosomes by the action of mitotic kinases, including Aurora A and Plk1 (a process known as centrosome maturation (reviewed in[16]). The ϒ-TuRC acts as a seed for microtubule outgrowth by stabilizing and capping minus ends of microtubules, hereby preventing depolymerization from this end. The highly dynamic plus end of the microtubule extends away from the centrosome in order to search and capture kinetochores and other components of the mitotic spindle. Due to its function in focusing microtubule minus ends, centrosomes help to maintain the integrity of the spindle poles (reviewed in[17]). In addition, centrosomes play an essential role in spindle positioning and orientation by embedding and nucleating astral microtubules (reviewed in[6]).

Assembly of the mitotic spindle

Centrosome separation in prophase

As mentioned above, one of the first steps in mitotic spindle assembly is the separation of the two centrosomes to opposite sides of the nucleus. After duplication in S phase, the duplicated centrosomes remain in close proximity of each other during G_2 phase due to the connection of a cohesive link between the two centrosomes (reviewed in[18]). Two proteins have been identified as components of this cohesive link: C-Nap1 and rootletin[19,20]. Depletion of C-Nap1 or rootletin results in premature centrosome separation, indicating that these proteins help to maintain the centrosomes in close proximity[21]. Both proteins are phosphorylated in late G_2 phase / prophase by the NIMA-related kinase Nek2A, hereby triggering their displacement from centrosomes and allowing centrosome separation[22]. Several other factors have been implicated in maintenance of centrosome cohesion, including Cep68, Cep215, and β-catenin[23,24], but the exact contribution of these factors remains to be determined.

After removal of the cohesive linker in prophase, the centrosomes move apart to opposite sides of the nucleus. This process is known as prophase centrosome separation (reviewed in[25]). Multiple factors have been implicated in this process, including microtubule motors, actin, and microtubule pushing forces (Figure 2,[25]).

One of most intensively studied proteins involved in centrosome separation is the microtubule motor protein Eg5 (kinesin-5,[26]). Eg5 forms a unique, tetrameric configuration, with two motor domains on both sides of its long axis[27], hereby allowing it to crosslink and slide microtubules in an antiparallel direction (Figure 2,[28]). During prophase, Eg5 localizes to centrosomes and microtubules[29], making it an attractive candidate to promote centrosome separation. Indeed, inhibiting Eg5 function in mammalian cells blocks prophase centrosome separation[30-32]. Although Eg5 is highly conserved from mammals to yeast, inhibition of Eg5 homologs in *C. elegans* and *Drosophila* does not affect prophase centrosome separation[33,34], indicating that Eg5-indepedent pathways for prophase centrosome separation exist. In

addition, it has been shown in mammalian cells that the centrosomes move independently from each other during prophase[32,35], which would be unlikely a consequence of Eg5-mediated forces.
Another factor involved in prophase centrosome separation is the minus-end-directed motor dynein. Dynein consists out of multiple subunits and is structurally distinct to kinesins[36,37]. Dynein is involved in many cellular processes, including vesicle and organelle transport, microtubule organization and cilia function (reviewed in[37]). Indeed, dynein plays a complex role during mitosis and is involved in multiple mitotic processes[36]. Dynein has been shown to contribute to prophase centrosome separation in *C. elegans* and *Drosophila,* and recently also in mammalian cells[32,38,39]. The molecular details how dynein promotes prophase centrosome separation differ depending on the organism.
Dynein has been shown to localize to the cortex and this pool of dynein can pull on astral microtubules using its minus-end-directed motility to position the mitotic spindle during mitosis[6]. A study in *Drosophila* embryos indicated that this pool of dynein is also responsible for separation of the centrosomes during prophase[40]. However, there are some caveats with this model, since it requires an asymmetric distribution of dynein on the cortex[41], for which evidence is still lacking. In addition, pulling forces from the cortex would rather pull the centrosomes away form the nuclear envelope, instead of moving them along the nuclear envelope, suggesting that additional dynein-mediated centrosome separation pathways exist.
In addition of the cortex, dynein also localizes to the nuclear envelope and this pool is recruited in late G_2 phase, just before the initiation of centrosome separation[38,42,43]. It has been shown that this pool of dynein is required to keep the centrosomes in close proximity of the nucleus[42,43], and to support nuclear envelope breakdown[44]. In addition, a model was proposed in which specifically this pool of dynein drives prophase centrosome separation in *C. elegans* embryos[38]. Follow-up studies in human cells showed that this pool of dynein indeed supports prophase centrosome separation (Figure 2, [32,45]). These studies made use of Eg5-independent cells (EICs), in which dynein is main driver of prophase centrosome separation[32]. Prophase centrosome separation was blocked in these cells when dynein was specifically removed from the nuclear envelope, while leaving other pools of dynein intact [32].
Besides Eg5 and dynein, other pathways have been proposed to contribute to prophase centrosome separation, including the actin cytoskeleton and forces generated by individual microtubules growing from one centrosome, pushing on the opposing centrosome[41,46]. However, experimental evidence for a direct role of these pathways in prophase centrosome separation is still lacking.
Based on the current literature regarding prophase centrosome separation, it is clear that multiple pathways contribute to this process and the importance of individual pathways differs per cell type and organism.

Centrosome separation and mitotic spindle assembly in prometaphase

The most dramatic events in mitotic spindle assembly occur after nuclear envelope breakdown. Many different pathways contribute to robust mitotic spindle assembly in prometaphase and several of these pathways will be discussed below. The two major steps in mitotic spindle assembly are first, the construction of a bipolar array of microtubules that surround the duplicated chromosomes and second, attachment of the chromosomes to the bipolar array of microtubules in a correct manner (biorientation). An important feature of mitotic spindle components is their ability to self-organize. This requires complex interactions between microtubules and MAPs[47]. The contribution of several important MAPs for mitotic spindle assembly will be discussed below in more detail.

Similar to prophase centrosome separation, Eg5 plays a central role in the separation of centrosomes and mitotic spindle assembly in prometaphase. Eg5 is thought to act on interpolar microtubules

and with the exception of *C. elegans* and *Dictostelium*[48,49], depletion or inhibition of Eg5 prevents bipolarization of the mitotic spindle and results in the formation of monopolar spindles, in which the two centrosomes remain in close proximity with chromosomes scattered around them[29,50-52]. As mentioned above, the unique tetrameric configuration of Eg5 allows it to crosslink and organize spindle microtubules in an antiparallel configuration[27,28,53]. Due to the plus-end-directed motility, Eg5 motors move towards the plus-ends of microtubules and slide the minus-ends outward, hereby providing a key step in the construction of a bipolar array of microtubules (Figure 2).
Recently, a second plus-end-directed motor was identified to cooperate with Eg5 to drive mitotic spindle bipolarity. Kif15 (Kinesin-12/Hklp2) was shown in *Xenopus* egg extracts to localize on the mitotic spindle by binding to a cofactor, Tpx2[54]. Inhibition of Kif15 in *Xenopus* egg extracts using inhibitory antibodies, blocked mitotic spindle formation[54]. In addition, studies in *C. elegans* and human cell lines also showed a role for Kif15 in mitotic spindle assembly (Figure 2,[55-58]). Although not essential for mitotic spindle assembly in human cells, loss of Kif15 sensitized cells to Eg5 inhibitors[57-59]. Interestingly, overexpression of Kif15 allows normal mitotic spindle assembly in the complete absence of Eg5 activity[57], indicating that Kif15 can produce outward-directed sliding forces on spindle poles that drive bipolarization of the mitotic spindle. In addition, Eg5-independent cells, require Kif15 for assembly of a bipolar mitotic spindle[32,60]. A comprehensive review regarding Kif15's function in mitotic spindle formation is described in chapter 5 of this thesis.

A consequence of continuous antiparallel microtubule sliding by Eg5 and Kif15 would eventually be loss of the antiparallel overlap between the two spindle halves. In order to build a functional bipolar mitotic spindle, minus-end-directed motors have been shown to counteract the activity of Eg5, by sliding antiparallel microtubules inward (Figure 2,[31,33,59,61-65]). While Eg5 is the dominant outward-sliding force in nearly all organism tested, the contribution of antagonistic motors seems to differ per organism. The minus-end-directed motor kinesin-14 has been shown to counteract Eg5 in several experimental systems, including yeast[61], *Drosophila*[33] and human cells[62]. In addition to kinesin-14, dynein has also been shown to counteract Eg5 activity during prometaphase in different experimental system, and seems to be major Eg5-counteracting force in human cells[31,59,63-66]. While Eg5 is able to crosslink microtubules due to its tetrameric configuration, kinesin-14 is a dimeric motor and harbors a second microtubule binding domain in its C-terminus, hereby allowing it to crosslink and slide microtubules in an opposing direction of Eg5[67-69]. The mechanism by which dynein crosslinks and slides antiparallel microtubules is still under debate. Dynein has been shown to bind multiple cofactors, from which several have been shown to have microtubule binding affinity, including CLIP-170, NuMa, and dynactin[70-72]. Some of these factors might act together with dynein to crosslink microtubules, but a recent study showed that the two motor domains of dynein can simultaneously bind two microtubules, and slide these microtubules apart without the requirement of any cofactor[65].
Besides antagonizing outward directed forces, minus-end-directed motors also play an important role in focusing and anchoring microtubule minus-ends to the centrosomes, hereby giving rise to the characteristic diamond shape of the mitotic spindle (Figure 2,[69,73-75]). Although centrosomes contribute to the formation of tightly focused poles, spindle microtubules also become focused in the absence of centrosomes[76]. Minus-end-directed motors are thought to promote spindle pole focusing by two different mechanisms. First, they counteract outward-directed forces from Eg5 and Kif15[59]. Second, they bind to microtubule minus-ends via MAPs and subsequently transport these microtubules towards the minus-end of another microtubule[66,75,77]. In addition to motors, microtubule crosslinking proteins such as NuMa, Asp, and Tpx2, also promote spindle pole focusing[70,78,79].

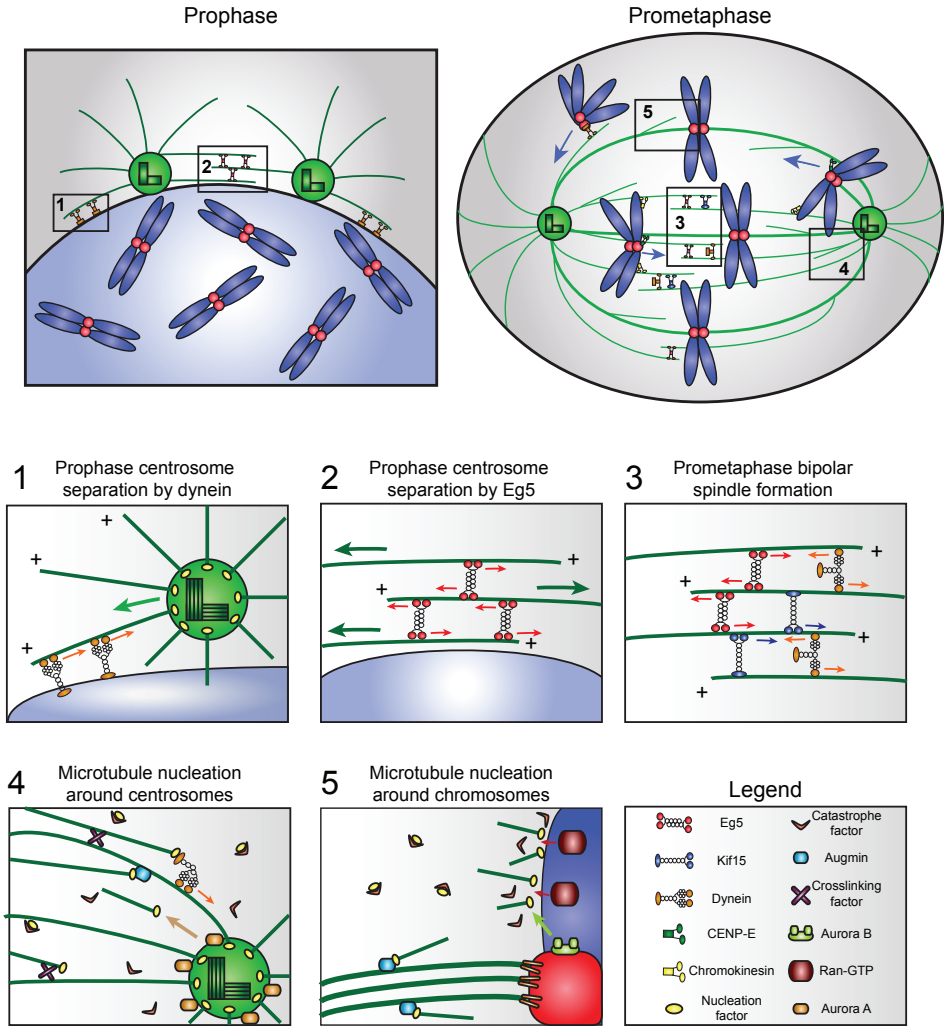

Figure 2. Pathways contributing to mitotic spindle assembly.

Upper left image illustrates the first steps in mitotic spindle assembly during prophase. During this phase, the centrosomes are separated to opposite sites of the nucleus by the action of different motor proteins. **Inset 1** depicts dynein-mediated centrosome separation. Nuclear envelope-associated dynein can pull on centrosomal microtubules, hereby moving the centrosome along the nuclear envelope. **Inset 2** depicts Eg5-mediated centrosome separation. Eg5 crosslinks antiparallel microtubules in the spindle midzone and subsequently slide them apart, hereby driving centrosome separation.

Upper right image illustrates mitotic spindle assembly pathways during prometaphase. **Inset 3** depicts the major force-generating motor proteins that control bipolar spindle formation. Eg5 and Kif15 generate outward-directed forces. Dynein acts antagonistically by sliding antiparallel microtubules inward. The balanced activity of these motors is important for assembly and maintenance of a robust bipolar mitotic spindle. **Inset 4** depicts the nucleation and stabilization of microtubules near the centrosomes. Factors like γ-tubulin and Augmin are recruited and activated at and near centrosomes to promote microtubule nucleation and stabilization upon mitotic entry. Aurora A plays a critical role in this process. In addition, centrosomes and microtubule-crosslinking factors like NuMa and dynein also play an important role in focusing microtubule minus ends to maintain proper mitotic spindle structure and function. **Inset 5** depicts nucleation and stabilization of microtubules near chromosomes. A local Ran-GTP gradient in the vicinity of chromosomes triggers the release of spindle assembly factors from importins. The released factors support mitotic spindle formation near chromosomes.

Besides centrosomal microtubule nucleation, also non-centrosomal microtubule nucleation pathways exist and these pathways have been shown to play an important role in mitotic spindle assembly in a variety of different systems. One of these pathways is the Ran-GTP pathway (Figure 2). Ran is a member of the small GTPases that act as regulatory switches in various cellular processes[80]. Experiments in *Xenopus* egg extracts showed that the presence of the guanine-nucleotide exchange factor (GEF) RCC1 on chromosomes produces a local gradient of active Ran-GTP in the vicinity of mitotic chromosomes which stimulates microtubule nucleation and organization[81,82]. Ran-GTP is thought to promote mitotic spindle assembly by releasing substrates from importins, thereby enabling them to perform their function in mitotic spindle assembly. Several important spindle assembly factors that are targets of Ran-GTP have been identified, including Tpx2[83], HURP[84], NuMa[85], and TOGp[86].

Another important non-centrosomal pathway for microtubule nucleation and mitotic spindle assembly is nucleation from existing microtubules within the spindle. This pathway relies on the eight-subunit Augmin complex (Figure 2[87]). The Augmin complex binds laterally along existing microtubules and recruits the γ-TuRC to stimulate nucleation of new microtubules[87-89]. The exact molecular details how Augmin functions are still not fully understood, but *in vitro* experiments indicate that its function depends on Ran-GTP and TPX2[89]. Interestingly, microtubules nucleated by Augmin grow at a low branch angle and with the same polarity of as the existing microtubule[89], hereby making Augmin an efficient factor to promote amplification and maintenance of spindle organization and for the formation of robust parallel bundles of microtubules, like kinetochore-microtubules. In addition, Augmin also plays a crucial role in the assembly of the central spindle during anaphase, by nucleating and organizing the dense network of interpolar microtubules[90].

Besides microtubule nucleation, chromosomes and centrosomes also promote mitotic spindle assembly by stimulating microtubule stabilization (Figure 2[91]). This is mediated in large extent by Aurora kinases. Higher eukaryotes have three Aurora family members and two of them, Aurora A and Aurora B, regulate many essential processes during mitosis[92]. While Aurora A mainly localizes to centrosomes and spindle microtubules, Aurora B is part of the chromosomal passenger complex (CPC), that localizes to centromeric chromatin[92]. The specific localization of both kinases is thought to create a spatial signaling gradient of active kinase around centrosomes and chromosomes that can either inhibit or activate proteins by phosphorylation[92]. Both Aurora A and B have been shown to inactivate microtubule catastrophe factors like MCAK, Kif18b, and OP18/Stathmin[93-95], hereby promoting microtubule growth in the vicinity of centrosomes and chromosomes.

Attachment of chromosomes to the mitotic spindle by kinetochores

The construction of the bipolar mitotic spindle is followed by the attachment of the sister chromatids to microtubules of opposing spindle poles. Immediately after nuclear envelope breakdown, the condensed chromosomes undergo different patterns of movement, including poleward movements or movements away from spindle poles. Eventually all chromosomes have to be correctly aligned at the spindle equator and this collective movement process is known as chromosome congression. As mentioned previously, the attachment of chromosomes to microtubules of the spindle is mediated by the kinetochore. The kinetochore is a specialized multiprotein complex that is assembled on the centromeric region of each sister chromatid (Figure 3,[96]). The kinetochore is assembled on nucleosomes that contain a centromere-specific variant of histone H3, CENP-A[96-98] and a nucleosome-like structure, composed of CENP-T-W-S-X[99-101]. The kinetochore consists of a stable inner kinetochore structure, known as the constitutive centromere-associated (CCAN) network, that is directly assembled on CENP-A and CENP-T-containing nucleosomes[100,102]. In addition, it includes an outer plate making direct contact with the microtubules of the mitotic spindle[103]. The microtubule-binding interface on

the kinetochore is formed by the KNL-1, Mis12 and NCD80 complexes that together form the KMN network[103]. The Mis12 complex consists of Dsn1, Nnf1, Nsl1 and Mis12[104], and is important for the integrity of the outer kinetochore by binding directly to the KNL-1 and NDC80 complex and linking both complexes to the inner kinetochore[105,106]. KNL-1 can interact directly with microtubules and is essential for the recruitment of different components of the spindle assembly checkpoint (SAC). This checkpoint monitors the attachment of the kinetochore with microtubules and prevents the onset of anaphase until all of the chromosomes are correctly attached to the mitotic spindle[103,107-111]. The Ndc80 complex consists of four proteins, Spc24, Spc25, Hec1 and Nuf2[112]. Ndc80 directly interacts with microtubules via Hec1 and Nuf2 and serves as the main microtubule-binding platform of the KMN network[113-116]. In addition to Ndc80, the Ska complex, composed of Ska1-3 is also involved in microtubule capture[117-119]. This complex directly binds to microtubules and the Ndc80 complex. Similar to its proposed functional homolog Dam1 in yeast, the Ska complex allows kinetochores to track depolymerizing microtubules[120-122].

Before chromosomes attain stable kinetochore-microtubule attachments from opposing spindle poles in prometaphase, intermediate attachment states are formed (Figure 4). Attachments usually start with the formation of a monotelic attachment, in which only one kinetochore of a sister chromatid pair is attached. When the second kinetochore subsequently attaches to microtubules from the opposing pole, biorientation or amphitelic attachment is achieved (Figure 4), which is a prerequisite for correct segregation of the sister chromatids during anaphase. However, the second attachment is not always correct and sister kinetochores can attach to microtubules from the same spindle pole. This status is known as a synthelic attachment (Figure 4). In addition, a single kinetochore can attach to microtubules from both spindle poles and this status is known as a merotelic attachment (Figure 4). Incorrect attachment presents a danger to the cells, since they can result in unequal segregation of chromosomes, resulting in aneuploidy. Formation of incorrect attachments is prevented to some degree by the back-to-back geometry of sister kinetochores. In addition, incorrect attachments can be sensed by the error-correction machinery, which can destabilize them and hereby allowing the cell to correct the errors (discussed below in more detail). In contrast, bioriented attachments come under tension due to the pulling of microtubules, leading to stabilization of correct kinetochore-microtubule attachments.

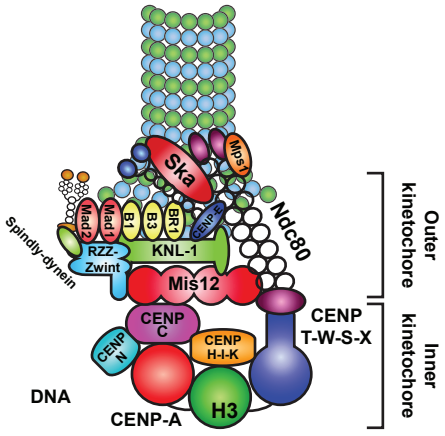

Figure 3. Molecular structure and organization of the kinetochore.

The kinetochore is built on centromeric chromatin that contains CENP-A and CENP-T/W/S/X nucleosomes. The CCAN network is assembled on top of CENP-A and CENP-T/W/S/X nucleosomes and forms the inner-kinetochore. On top of the CCAN network, the mitosis-specific outer-kinetochore is assembled, that forms the core microtubule attachment site. The outer kinetochore is formed of KNL-1, Mis12 and Ndc80 (KMN-network) and associated proteins, including components of the SAC.

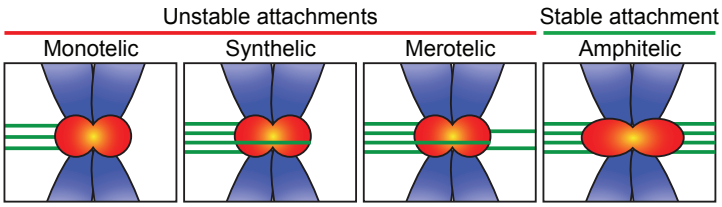

Figure 4. Kinetochore-microtubule interaction states.

Schematic representation of different kinetochore-microtubule interactions. The first three panels represent unstable attachment states. In a monotelic attachment only one kinetochore is attached to microtubules and the other kinetochore is unattached. In a synthelic attachment both kinetochores are attached to microtubules from a single spindle pole. In a merotelic attachment one kinetochore is correctly attached to microtubules from a single pole and the other kinetochore is attached to microtubules from both spindle poles. Synthelic and merotelic attachments do not activate the SAC and require the error correction machinery in order to be resolved and to reactivate the SAC. The last panel represents an amphitelic attachment status, in which the sister kinetochores are attached to microtubules from opposing spindle poles. Only this attachment state will become fully stabilized due to the generation of sufficient tension across the kinetochore pairs.

Chromosome congression

Besides the attachment to the ends of microtubules discussed above, kinetochores can also make lateral attachments, in which a kinetochores binds to the side of microtubules. Lateral attachments are achieved by the action of the two kinetochore-based motors, CENP-E and dynein, that both play an important role during chromosome congression[123-127]. In addition, motors localizing to chromosome arms (chromokinesins) also bind microtubules, hereby contributing to chromosome congression[127-130]. The exact pathways of chromosome capture and congression that eventually leads to the establishment of stable amphitelic attachments is still not fully understood. Recent studies showed that upon establishment of lateral attachments, chromosomes are transported towards the spindle poles by the minus-end-directed activity of dynein[124,125,131,132]. During this process, establishment of premature end-on attachments are prevented, likely by the action of Aurora kinases[125], and the Rod1-Zwilch-Zw10 (RZZ) complex[133]. This latter kinetochore-bound complex exists only in metazoans and targets spindly and dynein to kinetochores[134-136]. Although no direct interaction between Spindly and dynein has been reported, removal of Spindly displaces dynein from kinetochores[134,137,138], indicating that spindly links dynein to the RZZ complex. Interestingly, Zw10 depletion in cells results, besides a SAC defect (discussed below in more detail), in only a mild chromosome congression defects[132] while both Spindly and dynein are removed from kinetochores in this condition. Depletion of Spindly itself causes a much stronger chromosome congression defect that mimics depletion of the Ndc80 complex, since no stable end-on microtubule attachments are formed[134,135,137]. Strikingly, co-depletion of Spindly and RZZ in *C. elegans* and human cells, rescues the attachment defects of Spindly-depleted cells[133,135,138]. This suggests that that RZZ-spindly-dynein next to poleward chromosome transport, prevents the formation of premature end-on attachments and spindly might act as a regulatory switch in this process. Indeed, data from *C. elegans* indicate that the RZZ complex inhibits Ndc80, by binding to the tail of Ndc80 and the relief of this inhibition is dependent on spindly[133]. Thus, Spindly likely acts as a switch, that mediates the transition from lateral to end-on attachments when chromosomes are in close proximity of the spindle poles. Near the pole, kinetochores can form either monotelic attachments or they congress in the absence of end-on attachments[139,140]. Congression to the spindle equator is driven by CENP-E, by using existing stable spindle microtubules as its track[125,127,141]. How this functional shift between dynein and CENP-E is regulated is still unclear, but involves the

activation of CENP-E by Aurora kinases near spindle poles[142]. During this process, forces mediated by chromokinesins help to correctly orient the arms of chromosomes and to force the chromosomes back the spindle equator[125,127,139,143]. Chromosomes at the spindle equator also display oscillatory movements and this behavior is observed for both attached as well as unattached chromosomes[144]. These movements, termed directional instability, are thought to support the formation of amphitelic attachments, as wells as preventing overstretching of attached kinetochore pairs. Important players in this process are chromokinesins and the microtubule-depolymerizing kinesins MCAK and Kif18a, which both localize to kinetochores[143,145,146]. Together, the current data indicates that chromosome congression is a complex, multistep process. The exact contribution of each factor to this process is not fully clear. Redundancy between different pathways and additional mitotic functions for some of the factors, like dynein, make it difficult to study this process. Further studies are required to get a full understanding of the regulation of all of the individual factors that control the different steps of chromosome congression.

Error-correction by Aurora B

Although the multiple pathways mediating bipolar spindle assembly and chromosome congression support robust and quick formation of stable amphitelic attachments[139], the formation of erroneous attachments cannot be completely prevented. As mentioned previously, these erroneous attachments mostly include merotelic and synthelic attachments and importantly, these types of incorrect attachment do not actively induce the SAC to stop further mitotic progression (discussed below in more detail). In order to correct erroneous attachments, the error correction machinery has evolved[147]. The central player of this machinery is the kinase Aurora B. As mentioned above, Aurora B localizes to centromeric chromatin and forms together with INCENP, Borealin, and Survivin the chromosomal passenger complex (CPC). Depletion of any of the CPC components disrupts the complex and results in the chromosome alignment defects due to high numbers of synthelic attachments[148]. Correct attachment creates a poleward force by polymerization and depolymerization of kinetochore-microtubules. These forces generate tension on sister kinetochores with amphitelic attachments, resulting in stretch of the kinetochore itself as well as the chromatin structures between them, a phenomenon that has been observed in nearly all organisms[149,150]. An important feature of incorrectly attached kinetochores is that they are not under tension, and outer kinetochore components remain in close proximity of the CPC[151].

Aurora B can phosphorylate multiple targets at the outer kinetochore, including the Ndc80, KLN1, Mis12, and Ska complexes. These phosphorylations result in decreased affinity of the respective complexes for microtubules, leading to an overall destabilization of the erroneous attachments[113,152,153]. The phosphorylation of these substrates has been shown to depend on the distance between the CPC and the outer kinetochore[151], and this led to the model that error-correction by Aurora B is driven by lack of kinetochore tension. In addition, formation of kinetochore-microtubule attachments also recruits a number of phosphatases to outer kinetochores that antagonize the activity of Aurora B [154-158]. The recruitment of these phosphatases allows the rapid de-phosphorylation of kinetochore substrates after spatial displacement of Aurora B, hereby further stabilizing kinetochore-microtubule interactions. Although kinetochore tension is an attractive model to explain how Aurora B drives error-correction, recent studies in yeast indicate that Aurora B can also function tension-independent [159]. This indicates that Aurora B might function via multiple mechanisms and it needs to be addressed if these mechanisms exist in higher eukaryotes as well.

The Spindle assembly checkpoint

Equal segregation of sister chromatids is an essential aspect of a successful mitosis, thus anaphase onset should only occur when all sister chromatids formed stable, amphitelic attachments. Premature anaphase onset can lead to chromosome missegregation and aneuploidy, which is associated with cancer, aging and birth defects[160]. The spindle assembly checkpoint (SAC) has evolved to safeguard the attachment status of the kinetochores (Figure 5,[161]). Studies in yeast identified this evolutionary conserved checkpoint, in which genes (Mad1, Mad2, Mad3, Bub1, Bub2 and Bub3) were described that upon depletion, failed to arrest in mitosis after treatment with microtubules poisons[162,163]. Later studies in human cells showed that a single unattached chromosome was sufficient to block anaphase onset for longer periods of time, until it was correctly attached to the mitotic spindle[164]. The molecular components of the SAC are all recruited to kinetochores during mitosis, suggesting that the kinetochore acts as the main platform for the SAC, hereby spatially linking microtubule attachment formation to SAC signaling. However, the molecular details how every SAC component is recruited to and functions at kinetochore are still not fully clear.

Anaphase onset is controlled by a highly conserved multisubunit E3 ubiquitin ligase known as the anaphase promoting complex or cyclosome (APC/C). Upon activation, the APC/C targets the essential mitotic regulator cyclin B and the cohesion protector securin for proteasomal destruction[165,166]. In order to become activated during mitosis, the APC/C requires binding of its cofactor Cdc20, which is inactivated by the mitotic checkpoint complex (MCC,[167]). This complex is composed of the SAC components Mad2, BubR1 (Mad3 in yeast), Bub3, and Cdc20 itself[167].

With the exception of *C. elegans*, the kinetochore-localized kinase Mps1 is thought to be the central regulator of the SAC[168]. Inhibition or depletion of Mps1 results in premature anaphase onset and massive chromosome missegregation and cells fail to arrest in mitosis upon treatment with microtubule poisons[168-170]. Overexpression of Mps1 in yeast, or artificially tethering Mps1 to kinetochores in human cells, results in a metaphase arrest, confirming that Mps1 plays a key role in the SAC[171,172]. Furthermore, Mps1 has been shown to promote error-correction by stimulating Aurora B activity[169]. It is currently not fully clear how recruitment of Mps1 to kinetochores is regulated. Interestingly, Mps1 had been shown to bind the microtubule-binding domain of Hec1 in yeast and human, hereby creating a direct link between kinetochore-microtubule attachment status and SAC signaling[173,174].

The essential function of Mps1 depends on its ability to recruit SAC components to the kinetochore, which is a key requirement for the formation of the MCC. An important step in the formation of this complex is the activation of Mad2 from an inactive “open” form (O-Mad2) into an active “closed” form (C-Mad2) that can bind to Cdc20[175,176]. Subsequent studies indicated that the kinetochore is the main site for this conversion, and soluble O-Mad2 is activated at this location by a stably kinetochore-bound C-Mad2[177,178]. This conversion depends on the SAC component Mad1, which is the kinetochore receptor for C-Mad2[179] and is recruited to the kinetochore through the action of Mps1[168,170]. After binding of Mad2, Cdc20 undergoes a conformational change that allows it to interact with BubR1 and Bub3 [180], but where this interaction exactly takes place and if kinetochores are required for this second step is still under debate. In addition to kinetochore-driven MCC formation, recent studies indicate that nuclear pores in interphase can also function as scaffold for MCC formation, hereby controlling a minimum length of time a cell will spend in mitosis[181,182].

In addition to Mad1 and Mad2, Mps1 also regulates the recruitment of Bub1, BubR1 and Bub3 to kinetochores[109,183]. Mps1 directly phosphorylates the MELT and SHT motifs of KNL-1, that are required for the recruitment of Bub1-Bub3 dimers[110,111,184-186]. Bub1 and BubR1 are highly similar at the sequence level[187], but make different contributions to the SAC. BubR1, in complex with Bub3, is

together with Mad2 and Cdc20, part of the MCC and is essential for inhibition of the APC/C[167]. Bub1 is not part of the MCC, but forms a complex with Bub3 that is required for the kinetochore recruitment of BubR1-Bub3 dimers[188]. Studies in yeast and *C. elegans* showed that Bub1 also recruits Mad1-Mad2 to unattached kinetochores[189-191]. In addition, Bub1 phosphorylates histone H2A at the centromere, hereby stimulating centromeric recruitment of the CPC[192]. Centromeric accumulation of the CPC in turn stimulates kinetochore-recruitment and activation of Mps1, hereby creating a positive feedback loop between Mps1 and Aurora B[169,193,194].

In addition to SAC signaling, Bub1, BubR1 and Bub3 are also important for chromosome alignment. In complex with Bub3, Bub1 recruits BubR1 to kinetochores and BubR1 in turn recruits the phosphatase PP2A-B56, which de-phosphorylates kinetochore substrates in order to counteract Aurora B activity[155,156,195]. In addition, Bub1 stimulates centromere accumulation of the CPC to prevent premature stabilization of erroneous kinetochore-microtubule attachments[192,196].

In contrast to yeast and *C. elegans*, the role of Bub1 in recruiting Mad1 to unattached kinetochores in mammalian cells is still under debate[197,198]. The RZZ complex has also been implicated in kinetochore recruitment of Mad1 and Mad2 in metazoans, and depletion of the RZZ results in a defective SAC response[199-201]. Kinetochore localization of the RZZ complex is mediated by KNL-1 and Zwint[199,202], and depends on Aurora B and Mps1 signaling[203-205], hereby linking Mps1 to the recruitment of Mad1 in higher organisms. How Mad1 binds to the RZZ complex and if Bub1 stimulates the recruitment of Mad1 to the RZZ complex is currently not clear.

After attachment of all kinetochores to the mitotic spindle, the SAC is rapidly inactivated and inhibition of the APC/C by the MCC is relieved. SAC silencing is a multistep process and starts with the recruitment of phosphatases to kinetochores upon attachment of microtubules. These phosphatases, including PP1 antagonize the action of kinases like Mps1 and Aurora B, hereby stripping of SAC components from KNL-1[154,158,206,207]. An additional pathway has been described that removes Mad1 and Mad2 from kinetochores in higher eukaryotes. This pathway involves kinetochore-bound dynein and Spindly. Upon attachment of microtubules, dynein transports Mad1 and Mad2 along kinetochore fibers, to the spindle poles[208-213]. Aurora B might directly regulate stripping of SAC proteins by dynein. The RZZ recruitment factor Zwint needs to be phosphorylated by Aurora B in order to stably bind to the kinetochore and expression of a phosphomimetic Zwint mutant for Aurora B sites has been shown to cause an delay in metaphase[203]. Interestingly, this mode of regulation also links dynein-mediated stripping of SAC proteins to biorientation since tension spatially separates Aurora B from outer kinetochore substrates[151].

SAC silencing does not only take place at kinetochores. The MCC itself is also destabilized in mammalian cells by p31^{comet}, which is structurally related to Mad2[214]. Depletion of p31^{comet} results in a metaphase arrest and overexpression overrides the SAC in cells treated with spindle poisons[215-217]. p31^{comet} has been shown to specifically bind C-Mad2[216,217], and is thought to extract C-Mad2 from the MCC that is incorporated in the APC/C[217]. How p31^{comet} activity is controlled during SAC-silencing is currently not fully understood. In addition to p31^{comet}, the APC/C itself also promotes MCC dissociation by ubiquitination of Cdc20[218,219]. Ubiquitination of Cdc20 destabilizes its interaction with Mad2, resulting in disassembly of the MCC[219]. It remains to be determined how these pathways crosstalk to each other to promote irreversible and fast mitotic exit.

Outline of this thesis

The research described in this thesis is focused on multiple pathways required for assembly of a bipolar mitotic spindle. Proper assembly of a bipolar mitotic spindle is essential for the generation of stable kinetochore-microtubule attachments and correct segregation of the sister chromatids. Defects in

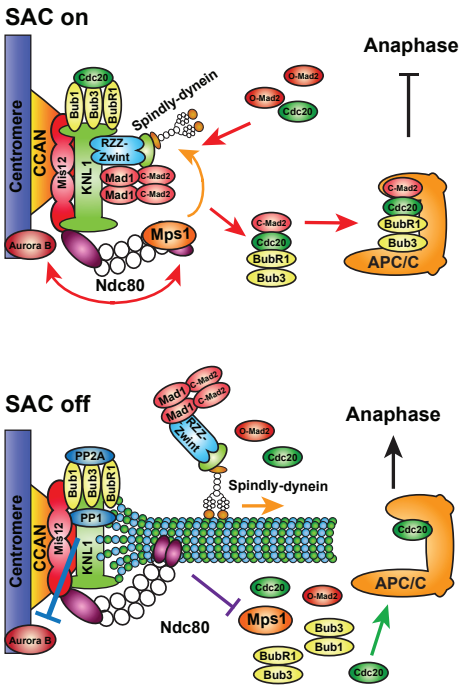

Figure 5. The spindle assembly checkpoint.

Upper panel depicts an unattached kinetochore, actively generating a SAC signal. MPS1 is thought to bind the microtubule-binding domain of Ndc80 in an Aurora B-dependent manner, hereby connecting microtubule-attachment status to SAC signaling. Mps1 in turn phosphorylates KNL-1, which serves as a docking platform for Bub1, Bub3, BubR1, Mad1, Mad2 and the RZZ complex. The SAC components catalyze the formation of the MCC at the kinetochore that prevents anaphase onset by inhibiting the APC/C. The lower panel depicts silencing of the SAC signal upon microtubule attachment. Attachment of microtubules displaces Mps1 from kinetochores. In concert, dynein-mediated stripping displaces most SAC components from kinetochores, hereby stopping the further formation of the MCC. The recruitment of phosphatases, including PP1 and PP2A in turn counteracts Aurora B and Mps1 to stabilize the attachment.

pathways controlling mitotic spindle assembly can give rise to chromosome segregation defects and as a consequence, chromosomal instability, which is a hallmark of cancer cells. Microtubule-targeting drugs like the vinca alkaloids and taxanes, have been shown to be effective anti-cancer drugs[220]. They are thought to induce specific cytotoxic effect on cancer cells due to the induction of a mitotic delay[221]. However, in addition to targeting mitotically active cells, microtubule poisons also cause neurotoxicity due to perturbation in microtubule dynamics in general and also drug resistance is commonly observed in patients treated with microtubule poisons[220]. Due to these observations, novel targets to perturb mitotic progression are currently considered in the treatment of cancer, including kinases and motor proteins involved in mitotic spindle assembly[221].

One of the promising targets for anti-cancer therapy is the mitotic kinesin Eg5. Inhibition of Eg5 results in a mitotic arrest due to the formation of monopolar spindles. In **chapter 2**, we describe the generation of human cancer cells that can grow in the complete absence of Eg5. By studying mitotic spindle assembly in these Eg5-independent cells (EICs), we uncovered a novel pathway for prophase centrosome separation. This pathway depends on the nuclear envelope associated pool of dynein and enables cells to assemble bipolar spindles in the absence of Eg5 activity. In **chapter 3**, we performed a genome-wide siRNA screen in EICs to uncover essential components for Eg5-independent bipolar spindle formation that might promote resistance to treatment with Eg5 inhibitors. In this screen, we found that the mitotic kinase Aurora A and two mitotic kinesins MCAK and Kif18b are essential for bipolarization of the mitotic spindle in EICs and in cells with reduced Eg5 activity. In **chapter 4**, we show that in human cells, three mitotic motors act together to produce the right force balance for correct assembly of a bipolar mitotic spindle and chromosome segregation. Eg5 and Kif15 are in human cells the main outward force-generating kinesins, and their activity is antagonized by dynein. While loss of Eg5 and Kif15 blocks bipolar spindle formation, we show that excessive force generation

by these motors in the absence of dynein, results in the formation aberrant bipolar spindles with splayed spindle poles and unaligned chromosomes. In **chapter 5**, we review the function of Kif15 in different organisms and discuss if Kif15 might be a useful target for anti-cancer therapy. In **chapter 6 and 7**, we show that human HAP1 cells can survive without a functional spindle assembly checkpoint (SAC). Due to the haploid nature of these cells, we used SAC-deficient HAP1 cells for a genetic screen to identify novel factors involved in chromosome congression. We found that loss of the condensin II complex, the RZZ complex and Bub1 are synthetic lethal with SAC-deficiency. We confirmed that these factors delay chromosome congression. In addition, we show that cells lacking the RZZ complex or Bub1, which both are thought to be essential SAC components, have a functional SAC response that is essential for the survival of these cells. Finally, in **chapter 8**, we summarize the results described in this thesis and propose future research to investigate several remaining questions.

Chapter 2

Nuclear envelope-associated dynein drives prophase centrosome separation and enables Eg5-independent bipolar spindle formation

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Abstract

The microtubule motor protein kinesin-5 (Eg5) provides an outward force on centrosomes, which drives bipolar spindle assembly. Acute inhibition of Eg5 blocks centrosome separation and causes mitotic arrest in human cells, making Eg5 an attractive target for anti-cancer therapy. Using *in vitro* directed evolution, we show that human cells treated with Eg5 inhibitors can rapidly acquire the ability to divide in the complete absence of Eg5 activity. We have used these Eg5-independent cells to study alternative mechanisms of centrosome separation. We uncovered a pathway involving nuclear envelope (NE)-associated dynein that drives centrosome separation in prophase. This NE-dynein pathway is essential for bipolar spindle assembly in the absence of Eg5, but also functions in the presence of full Eg5 activity, where it pulls individual centrosomes along the NE and acts in concert with Eg5-dependent outward pushing forces to coordinate prophase centrosome separation. Together, these results reveal how the forces are produced to drive prophase centrosome separation and identify a novel mechanism of resistance to kinesin-5 inhibitors.

Introduction

Successful chromosome segregation in mitosis requires the formation of a bipolar spindle. In mammalian cells, spindle organisation is to a large extent dominated by the centrosomes. In prophase, centrosomes move to opposite sides of the nucleus along the NE[25]. After nuclear envelope breakdown (NEB), microtubules interact with chromosomes and the bipolar spindle is formed[222]. A key player driving centrosome separation and bipolar spindle assembly is the microtubule motor protein kinesin-5 (Eg5 in humans). Eg5 has a unique tetrameric configuration, which allows it to crosslink and slide microtubules apart[27,28]. In this way, Eg5 is thought to push centrosomes apart, thereby promoting bipolar spindle formation. This function of Eg5 is conserved from yeast to humans[26,223], and inhibition of Eg5 activity was shown to inhibit centrosome separation in prophase[30,31,224-226] and block bipolar spindle assembly in prometaphase[27,29,30,52,227]. Consequently, inhibition of Eg5 arrests cells in mitosis with a monopolar spindle[26] and results in cell death[228,229]. Because of this essential role of Eg5 in bipolar spindle assembly, much attention has focussed on Eg5 as a drug target for cancer therapy.

While Eg5 is clearly a key player in bipolar spindle assembly, recent studies identified a second kinesin, kinesin-12 (known as Kif15/Hklp2 in humans), which acts together with Eg5 in bipolar spindle assembly[57,58]. Normally, kinesin-12 activity is not sufficient for bipolar spindle formation, as acute inhibition of Eg5 results in monopolar spindles. Nonetheless, the existence of such redundant pathways for bipolar spindle assembly has major implications, not only for our understanding of the mechanism of spindle assembly, but also for the use of Eg5 inhibitors as anti-cancer agents.

To address whether redundant pathways can take over the functions of Eg5, we asked if human cells could be established that bypass the need for Eg5 in spindle assembly. To this end, we used an *in vitro* "directed evolution" approach to obtain human cells that can grow in the complete absence of Eg5 activity. Characterization of these Eg5-Independent Cells (EICs) reveals that centrosome separation occurs relatively normal, both in prophase and in prometaphase. We show that bipolar spindle assembly in EICs depends on kinesin-12 in prometaphase, but that prophase centrosome separation does not. Rather, we show that a pathway involving dynein drives prophase centrosome separation in EICs and find that this pathway is essential for Eg5-independent bipolar spindle assembly. Surprisingly, the NE-associated pool of dynein, rather than the well-studied cortical pool of dynein, is required for Eg5-independent prophase centrosome separation. Finally, we show that in the parental cells, where Eg5 is fully active, NE-associated dynein acts in concert with Eg5 to coordinate prophase centrosome separation. Thus, our data have uncovered a pathway of centrosome separation in human cells that is driven by NE-associated dynein and may play an important role in the resistance to Eg5 inhibitors.

Results

Generation and characterization of cells that can divide independently of Eg5

In an attempt to generate human cells that grow independently of Eg5, we treated HeLa cells for several weeks with increasing concentrations of the Eg5 inhibitor S-trityl-L-cysteine (STLC,[230]. Using this method, we generated three different EIC clones that can grow in the presence of a high dose (20 μM) of STLC, sufficient to fully inhibit Eg5 activity[231]. Colony formation assays confirmed that proliferation was efficiently blocked upon STLC treatment in parental HeLa cells (hereafter referred to as parental cells), while the newly derived EICs survived in the presence of STLC (Fig.1A). Further analysis of EICs indicated that the majority of cells in all three EIC clones were able to assemble a bipolar spindle (Fig.1B,C) (EICs were always cultured in the presence of 20 μM STLC unless stated

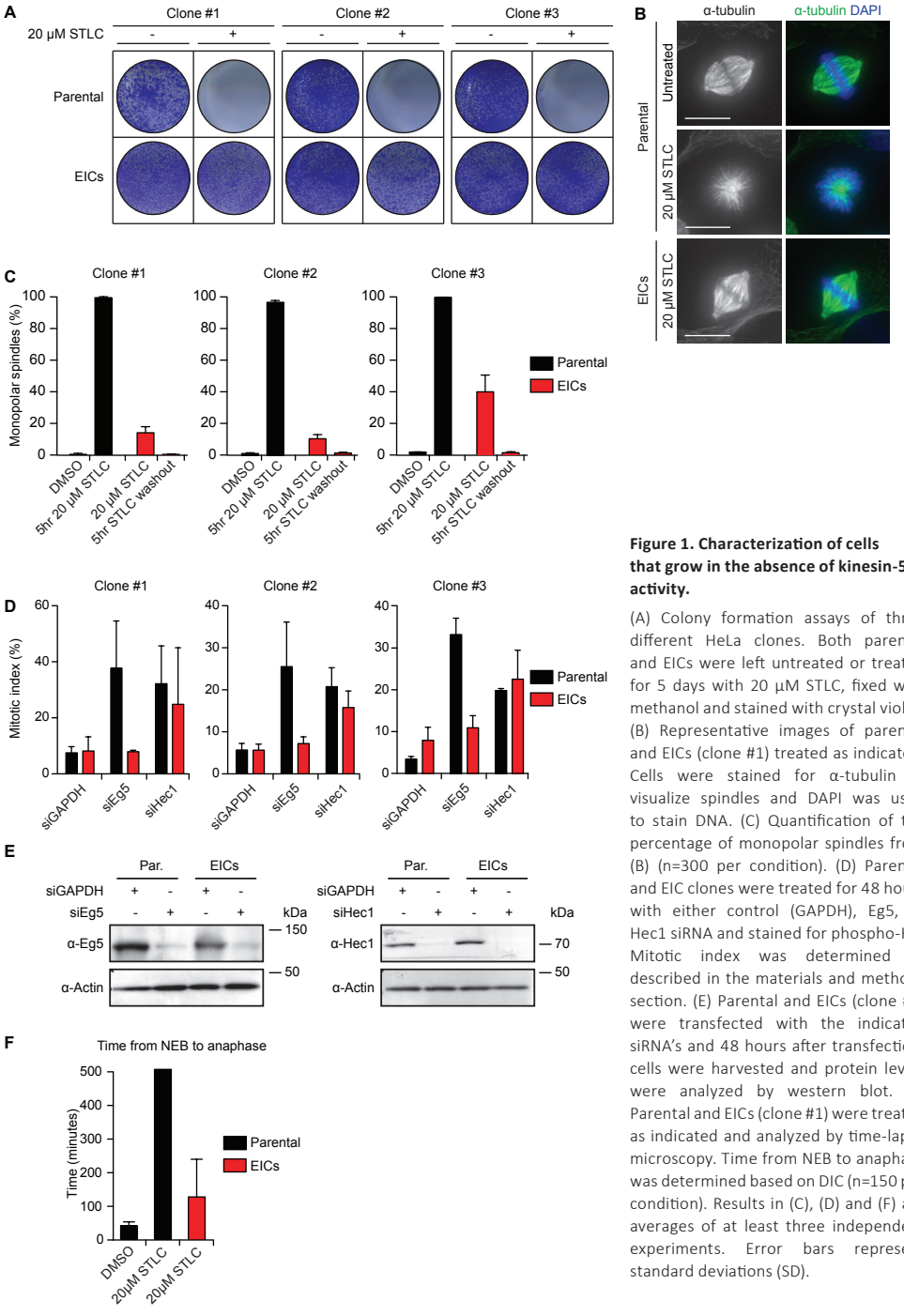

Figure 1. Characterization of cells that grow in the absence of kinesin-5 activity.

(A) Colony formation assays of three different HeLa clones. Both parental and EICs were left untreated or treated for 5 days with 20 μM STLC, fixed with methanol and stained with crystal violet. (B) Representative images of parental and EICs (clone #1) treated as indicated. Cells were stained for α-tubulin to visualize spindles and DAPI was used to stain DNA. (C) Quantification of the percentage of monopolar spindles from (B) (n=300 per condition). (D) Parental and EIC clones were treated for 48 hours with either control (GAPDH), Eg5, or Hec1 siRNA and stained for phospho-H3. Mitotic index was determined as described in the materials and methods section. (E) Parental and EICs (clone #1) were transfected with the indicated siRNA's and 48 hours after transfection, cells were harvested and protein levels were analyzed by western blot. (F) Parental and EICs (clone #1) were treated as indicated and analyzed by time-lapse microscopy. Time from NEB to anaphase was determined based on DIC (n=150 per condition). Results in (C), (D) and (F) are averages of at least three independent experiments. Error bars represent standard deviations (SD).

otherwise). To confirm that EICs acquired resistance to STLC by bypassing Eg5 function, rather than via mutations in Eg5 or upregulation of multi-drug resistance genes, we depleted Eg5 from both parental and EICs by siRNA. Knockdown of Eg5 in parental cells resulted in a dramatic increase of the mitotic index, while it did not affect EICs (Fig.1D,E), demonstrating that EICs are truly Eg5-independent. As a control, kinetochore disruption by Hec1 depletion increased the mitotic index similarly in both cell lines, indicating that the EICs are not impaired in the ability to maintain a mitotic arrest (Fig.1D). While EICs can form bipolar spindles, mitotic timing was increased and they proliferated slightly slower than parental cells (Fig.1F and data not shown). Together, these results show that cells can be generated that form a bipolar spindle and proliferate in the absence of Eg5 activity, indicating that redundant pathways can take over all essential functions of Eg5.

Kinesin-12 is essential for bipolar spindle assembly in EICs

Recently, we and others showed that the plus-end-directed motor kinesin-12 (Kif15/Hklp2 in humans) cooperates with Eg5 in bipolar spindle assembly[57,58]. We therefore tested whether kinesin-12 is required for Eg5-independent bipolar spindle assembly in the EICs. Indeed, depletion of kinesin-12 resulted in a dramatic increase in the percentage of monopolar spindles in all three clones of EICs, while it had no effect on parental cells (Fig.2A). Thus, kinesin-12 becomes essential for bipolar spindle assembly in human cells that divide independent of Eg5. We therefore tested if Eg5-independent growth of EICs is due to kinesin-12 overexpression. Interestingly, although clone #1 and #3 do not upregulate kinesin-12, clone #2 showed a clear upregulation in kinesin-12 protein levels (Fig.2B). Thus, upregulation of kinesin-12 protein levels may contribute to Eg5-independent cell growth, but additional mechanisms must exist.

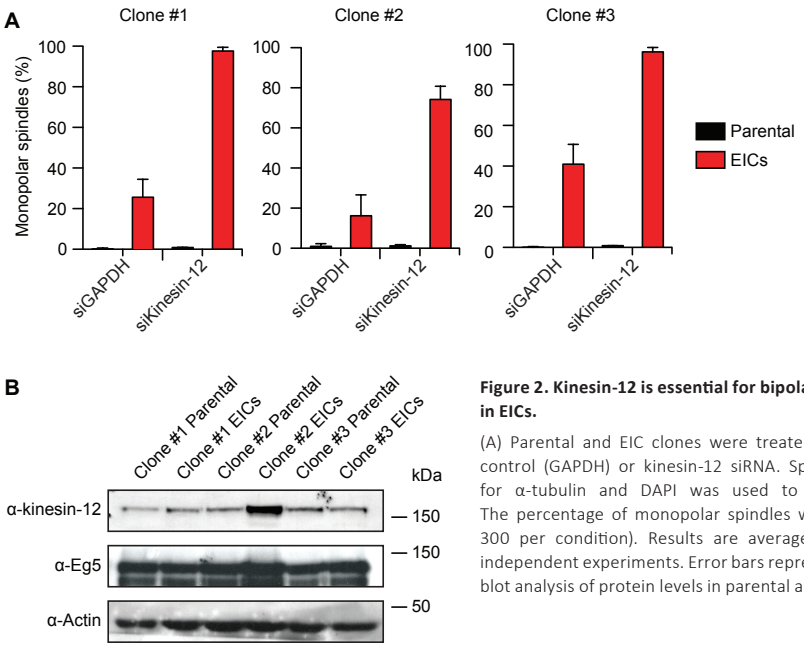

Figure 2. Kinesin-12 is essential for bipolar spindle assembly in EICs.

(A) Parental and EIC clones were treated for 48 hours with control (GAPDH) or kinesin-12 siRNA. Spindles were stained for α-tubulin and DAPI was used to visualize the DNA. The percentage of monopolar spindles was determined (n = 300 per condition). Results are averages of at least three independent experiments. Error bars represent SD. (B) Western blot analysis of protein levels in parental and EIC clones.

The dynein complex drives prophase centrosome separation in EICs

Eg5 was shown to be essential for prophase centrosome separation in human cells[30,31,224-226] and Fig.3A,B). Surprisingly, all three clones of EICs separated their centrosomes in prophase in the absence of Eg5 activity to almost the same extent as parental cells (Fig.3A,B and Fig.S1A-C), indicating that an Eg5-independent pathway takes over prophase centrosome separation in these cells. Interestingly, washout of STLC in EICs resulted in excessive prophase centrosome separation (Fig.3A,B), suggesting that EICs have hyperactivated the Eg5-independent pathway for prophase centrosome separation.

In contrast to bipolar spindle assembly after NEB, we found that prophase centrosome separation is not dependent on kinesin-12 (Fig.3C), consistent with the fact that kinesin-12 does not act before NEB[54,57,58]. Previous studies implicated the minus-end directed motor dynein in prophase centrosome separation in certain cell types[38,39,232]. Furthermore, NE-associated dynein can transport nuclei along microtubules, indicating it is capable of producing significant forces on microtubules[233]. Therefore, we tested whether dynein was involved in centrosome separation in the EICs. Indeed, depletion of dynein completely blocked prophase centrosome separation in all three clones of EICs (Fig.3C and Fig.S1A-C). In contrast, robust centrosome separation was observed after dynein depletion in all three parental HeLa clones (Fig.3C and Fig.S1A,C), although a small decrease in centrosome separation was observed after dynein RNAi in one of the three clones ($p=<0.001$, Fig.S1B). Similarly, depletion of Lis1 or dynein intermediate chain 2 (DIC), two other proteins essential for dynein function[37], completely blocked prophase centrosome separation in EICs, while they did not inhibit centrosome separation in parental cells (Fig.S1A). Together, these results show that dynein is required for prophase centrosome separation in the absence of Eg5 activity.

The NE-associated pool of dynein drives prophase centrosome separation

How can dynein promote prophase centrosome separation? Dynein localizes to several distinct intracellular compartments[25,37], including the cortex, intracellular vesicles, microtubules plus-ends and the NE[37,234] and could therefore exert force from distinct locations. Recent studies identified BICD2 and CENPF as independent specific recruiters of dynein and the dynein-activating proteins Nde1/L1 at the NE, respectively[43,235], allowing us to address if the NE-associated pool of dynein is involved in centrosome separation. Indeed, we were able to confirm that depletion of BICD2 and CENPF resulted in loss of dynein and Nde1/L1 from the NE, respectively (Fig. S2A,B). Depletion of BICD2 and CENPF does not affect localization of dynein to the centrosomes (Fig. S2C). Furthermore, Nde1/NdeL1 are not found at the centrosomes during prophase, indicating that its dynein activating function is restricted to the NE during prophase (Fig. S2C). Nde1/NdeL1 localization is not affected by BICD2 depletion (Fig.S2A,B.), consistent with previous findings[43]. Surprisingly, we did not observe a detectable decrease in DIC or p150glued levels at the NE upon CENPF depletion, while we were able to effectively deplete CENPF, as judged by western blot and by the strongly decreased Nde1/L1 levels at the NE after CENPF depletion (Fig. S2A,B and S3A). Furthermore, we found that CENPF depletion resulted in an increased distance between centrosomes and the NE, confirming that CENPF is likely required for dynein activity at the NE (Fig. S3B,[43]). It should be noted that, in contrast to Bolhy et al., 2011, our experiments where done in the presence of nocodazole to better visualize NE-dynein, and this may explain the difference between the two studies. In any case, these results validate siRNAs targeting BICD2 and CENPF as good tools to specifically inactivate dynein at the NE, either by preventing dynein recruitment to the NE, preventing NE-dynein activation or both.

Strikingly, depletion of either BICD2 or CENPF resulted in an almost complete block of prophase centrosome separation in EICs, similar to depletion of dynein itself (Fig.3D,E and Fig.S1B,C). These results indicate that specifically the NE pool of dynein drives prophase centrosome separation.

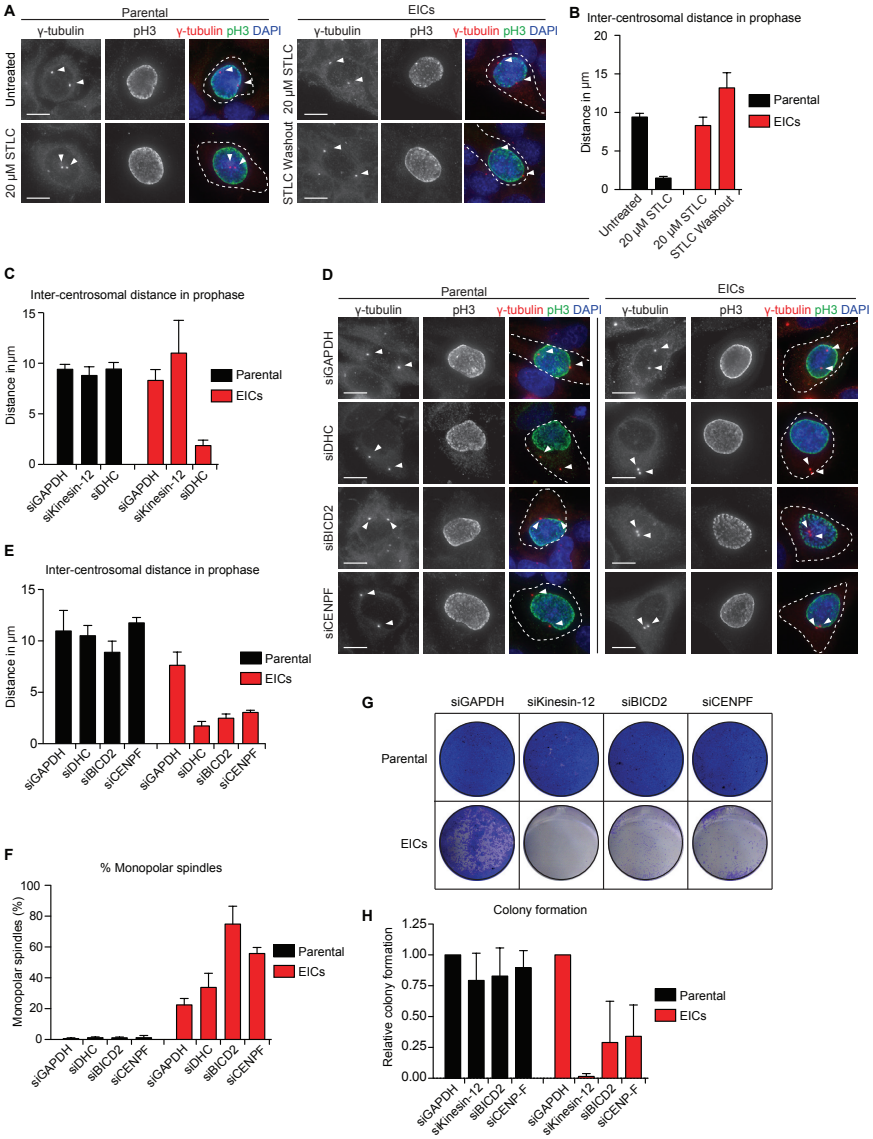

Figure 3. NE-Dynein is required for prophase centrosome separation in EICs.

(A) Representative images of parental and EICs (clone #1) treated as indicated. Cells were stained for γ-tubulin to visualize the centrosomes, phospho-H3 (pH3) to mark prophase cells and DAPI to visualize the DNA. Arrowheads mark the centrosomes. (B) Quantification of inter-centrosomal distance in prophase from (A) (n = 45 per condition). (C) Parental and EICs (clone #1) were treated for 48 hours with either control (GAPDH), kinesin-12 or dynein heavy chain (DHC) siRNA. Inter-centrosomal distance in prophase was calculated as in (B) (n = 45 per condition). (D) Parental and EICs (clone #1) were treated for 72 hours with either control (GAPDH), DHC, BICD2, or CENPF siRNA. Cells were stained for centrosomes (γ-tubulin), pH3 (prophase cells) and DNA (DAPI). Arrowheads mark the centrosomes. (E) Quantification of inter-centrosomal distance in prophase from (D) (n = 45 per condition). (F) Parental and EICs were treated as in (D), stained for α-tubulin and DAPI to visualize the DNA and the percentage of monopolar spindles was determined (n = 300 per condition). (G) Colony formation of parental and EICs (clone #1). Cells were treated for 7 days with either control (GAPDH), kinesin-12, BICD2, CENPF siRNA and stained as in Fig. 1A. Re-transfection was performed every 3 days. (H) Quantification of the colony formation in (G). Results in (B), (C), (E), (F) and (H) are averages of at least three independent experiments. Error bars represent SD. Scale bars represent 10 μm. See also Figure S1, S2, S3, S4.

Interestingly, inhibition of NE-dynein by depletion of either BICD2 or CENPF not only blocked prophase centrosome separation, but also robustly inhibited bipolar spindle assembly in EICs (Fig.3F), demonstrating the importance of prophase centrosome separation for Eg5-independent bipolar spindle assembly. Note that dynein depletion itself did not increase the fraction of cells that formed a monopolar spindle, likely because dynein has a second, independent function in prometaphase in pulling centrosomes together[31,63,64]. This result also confirms that depletion of BICD2 or CENPF does not perturb dynein function in general, but specifically inhibits NE-associated dynein activity.
Since depletion of kinesin-12 or removal of dynein from the NE in EICs results in a dramatic increase in monopolar spindles, these pathways may be key to survival of EICs. Indeed, depletion of kinesin-12 or removal of NE-associated dynein in EICs potently blocked their proliferation, while having no substantial effect on parental cells (Fig.3G,H). These results show that NE-associated dynein-dependent centrosome separation during prophase and subsequent kinesin-12 activity during prometaphase drive bipolar spindle assembly and long-term cellular proliferation in cells lacking Eg5 activity.

The balance of motor activities at the NE is altered in EICs

Previous studies found that during late G2 and prophase a balance of NE-dynein and kinesin-1 activity controls proper localization of centrosomes relative to the nucleus[235]. Depletion of dynein results in detachment of centrosomes from the nucleus in normal cells, which is dependent on kinesin-1 activity (Fig.3D and Fig.S4A and[235]. Surprisingly, centrosome detachment from the nucleus was strongly reduced in EICs depleted of dynein compared to parental cells (Fig.S4A) Importantly, there was no apparent difference in the depletion of dynein when comparing both cell lines (Fig,S4B) and the difference effect was also not due to STLC treatment of EICs (Fig.S4A and S4C,D). These results indicate that the balance of forces that link the centrosomes to the NE is altered in EICs (i.e. either kinesin-1 activity is reduced in EICs or NE-dynein activity is increased) and could explain why dynein is able to drive centrosome separation in the absence of Eg5 in EICs, while it is unable to do so in parental HeLa cells.
To obtain further insights into the altered motor balance in EICs, we first tested the involvement of kinesin-1 in prophase centrosome separation. Depletion of kinesin-1 did not significantly affect prophase centrosome separation in EICs, nor did it affect prophase centrosome separation in U2OS cells treated with a low dose of STLC (Fig.S5A,B). However, depletion of kinesin-1 in cells treated with a high dose of STLC (20μM) promoted centrosome separation in U2OS cells ($p=0.0024$, Fig.S5C), consistent with the model that dynein promotes centrosome separation and is counteracted by kinesin-1.
We also examined the levels of several components of the dynein complex at the NE, including DIC, p150glued, CENPF and BICD2, to test if the EICs have altered the expression of these dynein components at the NE. However, we found that NE levels of these proteins were unchanged (Fig.S6A,B), suggesting that the altered motor balance is due to a change in NE-dynein activity rather than an increase in protein levels.

Dynein cooperates with Eg5 to drive prophase centrosome separation

Since NE-associated dynein drives prophase centrosome separation in EICs, we wondered if a similar pathway is active in parental cells. Depletion of dynein does not result in a significant block in centrosome separation in most of the parental cell lines (Fig.3C and[31], suggesting that high Eg5 activity may compensate for a lack of dynein activity during prophase centrosome separation. To test this, Eg5 activity was partially inhibited using increasing concentrations of STLC in U2OS and HeLa cells (Fig.4A and Fig.S7A). Strikingly, concentrations as low as 250nM of STLC, which barely

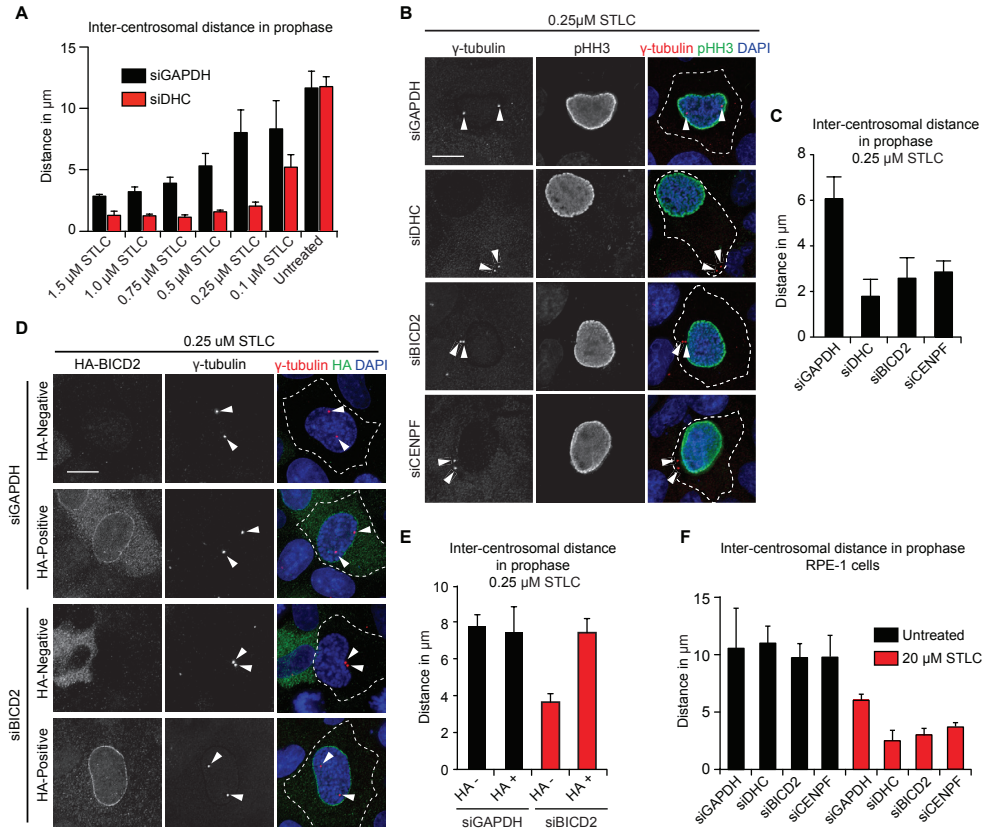

Figure 4. NE-Dynein cooperates with Eg5 to drive prophase centrosome separation.

(A) STLC titration curve. U2OS cells were transfected with siRNAs targeting either control (GAPDH) or DHC. The indicated concentrations of STLC were added to the cells 16 hours before fixation (n = 30 per condition). (B) U2OS cells were treated for 72 hours with either control (GAPDH), DHC, BICD2, or CENPF siRNA. 16 hours before fixation, cells were treated with 0.25 µM STLC. Centrosomes (γ-tubulin), prophase cells (pH3) and DNA (DAPI) were stained. (C) Quantification of inter-centrosomal distance in prophase from (B) (n = 30 per condition). (D) U2OS cells were treated for 72 hours with either control (GAPDH) or BICD2 siRNA. 24 hours after siRNA transfection, cells were transfected with HA-BICD2. 16 Hours before fixation, cells were treated with 0.25 µM STLC. Cells were stained for centrosomes (γ-tubulin), DNA (DAPI) and HA. Arrowheads mark the centrosomes. (E) Quantification of inter-centrosomal distance in prophase from (D) (n = 30 per condition). (F) RPE-1 cells were transfected with indicated siRNA's. Cells were left untreated or treated with 20 µM STLC 6 hours before fixation. Inter-centrosomal distance in prophase was determined (n = 45 per condition). Results in (A), (C), (E) and (F) are averages of at least three independent experiments. Error bars represent SD. Scale bars represent 10 µm.

affected centrosome separation in control cells, almost completely eliminated prophase centrosome separation in cells lacking dynein. Similar results were observed for depletion of Lis1 or DIC (Fig.S7B and Fig.S8), confirming the importance of the dynein complex for prophase centrosome separation. These results indicate that, while dynein is not *essential* for prophase centrosome separation in cells with full Eg5 activity, it becomes essential when Eg5 activity is slightly compromised.

Similar to dynein depletion, BICD2 and CENPF depletion also potently blocked prophase centrosome separation in parental cells when Eg5 activity is reduced (Fig.4B,C and Fig.S7C), confirming the

involvement of the NE pool of dynein. The block in centrosome separation was observed with 4 different siRNA's targeting CENPF and in case of BICD2 RNAi, the block could be reverted by expression of an RNAi-insensitive HA-tagged BICD2 (Fig 4D,E).

To obtain more insights into the kinetics of centrosome separation and bipolar spindle assembly, time-lapse imaging was performed to follow prophase centrosome separation and spindle assembly in living cells. Consistent with fixed cell experiments, live-cell imaging revealed that depletion of dynein, BICD2 or CENPF inhibited prophase centrosome separation when Eg5 was slightly inhibited (Fig.5A). Furthermore, depletion of BICD2 or CENPF also increased the fraction of cells that form a monopolar spindle, consistent with the notion that robust prophase centrosome separation is important for subsequent spindle bipolarity (Fig.5B,C and Fig.3F). Again, dynein depletion did not increase the fraction of cells that formed a monopolar spindle (Fig.5C), likely due to loss of the inward force produced by dynein in the spindle in prometaphase[31,63,64].

To test the contribution of Eg5- and dynein-dependent pathways in non-transformed cells, we inhibited Eg5 and/or dynein activity in RPE-1 cells. Surprisingly, in contrast to HeLa and U2OS cells, inhibition of Eg5 in RPE-1 cells did not fully block prophase centrosome separation (Fig.4F) whereas it did block bipolar spindle formation after NEB (data not shown). This Eg5-independent centrosome separation depends on NE-associated dynein, as depletion of DHC, BICD2 and CENPF resulted in a substantial decrease in inter-centrosomal distance in cells lacking Eg5 activity (Fig.4F). Taken together, these results show that NE-associated dynein cooperates with Eg5 to drive prophase centrosome separation in human cells, but the relative contribution of each pathway appears to differ per cell type. Consistent with this, depletion of dynein resulted in a decrease in prophase centrosome separation in one of the parental HeLa clones (Fig. S1B).

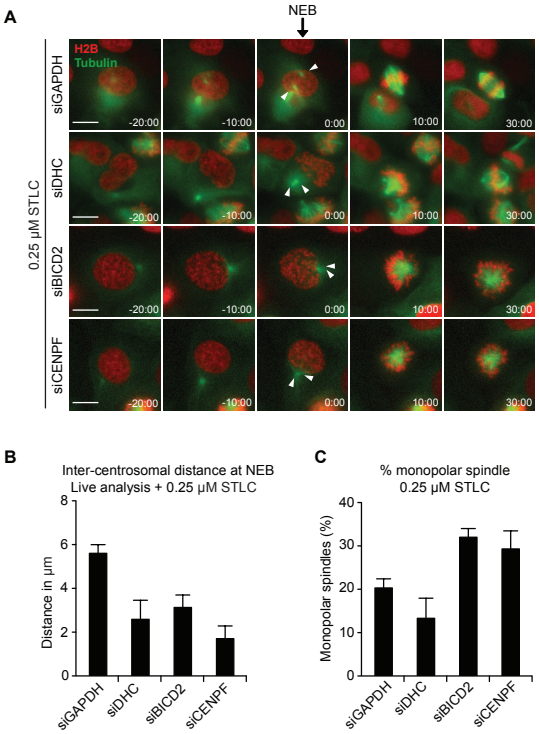

Figure 5. Live-cell analysis of prophase centrosome separation in U2OS cells.

(A) U2OS cells stably expressing mCherry-α-tubulin and GFP-H2B were transfected with the indicated siRNA's and imaged using time-lapse microscopy. Images were acquired every 2.5 minutes. Arrow indicates NEB, arrowheads mark the centrosomes. (B) Quantification of the inter-centrosomal distance from the movies in (A). Inter-centrosomal distance was measured one frame (2.5 min) before NEB (n = 30 per condition). (C) Quantification of the number of monopolar spindles from the movie in (A).(n = 150 per condition).

Dynein pulls on individual centrosomes, while Eg5 acts on centrosome pairs

Eg5 is known to slide two anti-parallel microtubules apart, and this activity is thought to allow Eg5 to exert an outward pushing force on both centrosomes simultaneously. In contrast, we hypothesize that NE-associated molecules of dynein, by continuously walking towards the minus ends of microtubules emanating from either the one or the other centrosome, generate a pulling force on individual centrosomes. To test this, we generated cells possessing only one centrosome by depletion of Plk4 to inhibit centriole duplication[236]. 48 hours post-transfection more than 92% of Plk4-depleted prophase cells contained only one centrosome (Fig.S9A,B). Time-lapse imaging of prophase centrosome movement in these cells showed that a large fraction of the individual centrosomes moved substantial distances along the nuclear envelope (Fig.6A,B and Movie S1). Importantly, treatment with a high dose of STLC did not significantly affect these movements (p=0.251, Student's *t*-test), demonstrating that Eg5 does not act on individual centrosomes (Fig.6A,B). In contrast, depleting DHC or BICD2 in cells with a single centrosome resulted in a substantial reduction of the total observed movement of the centrosome (p<0.0001, Students *t*-test) (Fig.6A,B and Movie S2,S3). Together, these results show that, while NE-associated dynein and Eg5 cooperate in prophase centrosome separation, they act mechanistically different; NE-associated dynein pulls on individual centrosomes, while Eg5 pushes centrosome pairs apart.

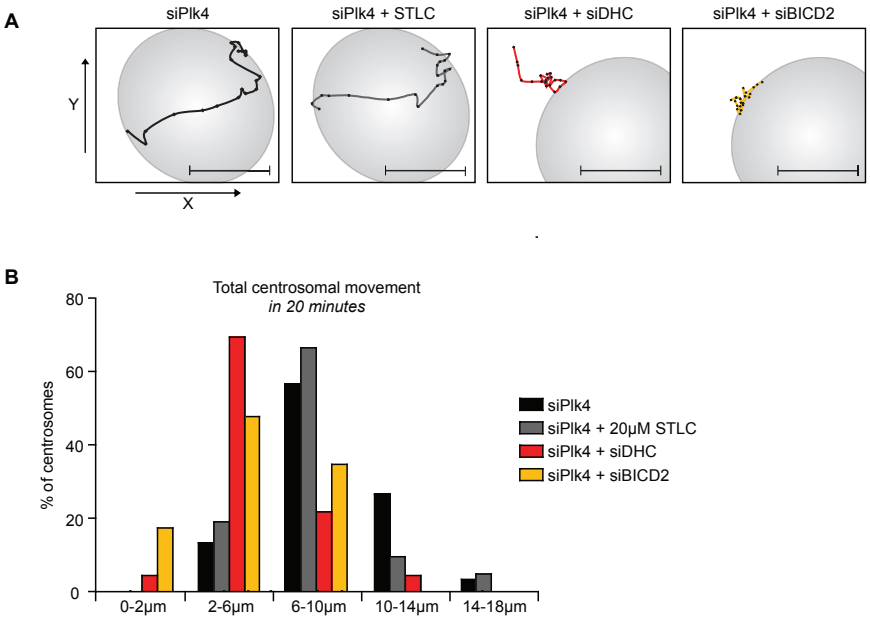

Figure 6. NE-Dynein moves individual centrosomes.

(A-B) Cells stably expressing mCherry-α-tubulin were depleted of Plk4 and centrosome movements were analyzed by time-lapse microscopy. Time interval for each tracking point is 60 seconds for the duration of 20 minutes prior to NEB. Movements were corrected for nuclear movement for the duration of the movie. (A) Representative tracks from individual centrosome movements are shown (B) Histogram of the total movements of individual centrosomes from (A) (n >20 cells per conditions). See also Fig.S9 and Movie S1, S2, S3.

Discussion

Eg5 is thought to be the key regulator of centrosome separation and bipolar spindle assembly, making it an attractive drug target for cancer therapy. However, relatively little is known about Eg5-independent pathways that contribute to spindle bipolarity. Here, we have generated human cells that divide and proliferate in the absence of Eg5 activity. Using this approach, we show that kinesin-12 is essential for bipolar spindle assembly in the absence of Eg5. Furthermore, we identify a pathway involving NE-associated dynein that can substitute for Eg5 in prophase centrosome separation and find that this pathway is essential for bipolar spindle assembly in the absence of Eg5. Importantly, the action of this NE-dynein pathway is not restricted to EICs, but is also seen in Eg5-dependent cells, where it acts together with Eg5 to coordinate prophase centrosome separation.

Mechanisms of prophase centrosome separation

Initial centrosome separation occurs in late G2/prophase with the migration of the two centrosomes to opposite sides of the nucleus, allowing spindle assembly in prometaphase to initiate with separated centrosomes. However, the mechanism of prophase centrosome separation has remained unclear, in part due to conflicting data from various organisms concerning the involvement of different motors[31,34,38-40,232]. In this study, we show that in human cells dynein and Eg5 act together to drive prophase centrosome separation. Interestingly, while both Eg5 and dynein are involved in prophase centrosome separation in all cell types we tested, the relative importance of each pathway differs between cell types. Perhaps a similar effect might also explain why studies have found contradictory results regarding the involvement of these motor proteins in prophase centrosome separation in different organisms. Future experiments in other systems involving double inhibition of dynein and Eg5 will hopefully address this intriguing notion and lead to a unifying model of prophase centrosome separation.

While Eg5 and dynein have redundant functions in prophase centrosome separation, their activities are mechanistically distinct. Our results show that Eg5 specifically generates an outward force on centrosome pairs, likely by cross-linking and sliding antiparallel inter-centrosomal microtubules apart (Fig.7). In contrast, dynein pulls on single microtubules emanating from centrosomes, enabling dynein to generate forces specific to each individual centrosome (Fig.7).

An interesting question is how NE-dynein can generate asymmetric pulling forces on centrosomes, required for juxtaposed movement of the two centrosomes along the NE. One possibility, albeit speculative, is that productive microtubule interactions with NE-dynein occur stochastically, resulting in random walk of individual centrosomes. In this respect it is of interest to note that we observed discrete periods of prolonged unidirectional movement of single centrosomes (Fig.6A). In cells with two centrosomes, it is possible that microtubules emanating from one centrosome physically collide with MTs from the other aster, resulting in catastrophes specifically between the two centrosomes[237]. This would result in biased MT growth away from the opposing aster and could result in an asymmetric distribution of pulling forces and movement of centrosomes away from each other. Addition of the Eg5-dependent outward sliding force between centrosomes will further skew movement of centrosomes in opposite directions.

Mechanisms for prophase and prometaphase centrosome separation are redundant

While most cells separate centrosomes in prophase (Figs. 3,4 and [139], in certain HeLa clones a fraction of cells does not undergo prophase centrosome separation and these cells can still form a bipolar spindle[238]. However, without prophase centrosome separation, subsequent chromosome segregation

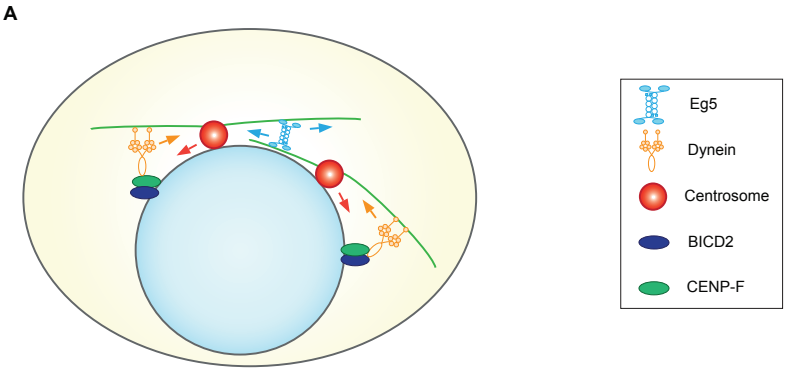

Figure 7. Model of NE-dynein and Eg5 function in prophase centrosome separation.

Both Eg5-dependent antiparallel microtubule sliding, as well as minus-end-directed microtubule pulling forces generated by NE-dynein mediate centrosome separation. During late G2/prophase, CENPF and BICD2 are recruited to the NE and in turn recruit/activate dynein at the NE. NE-dynein can bind centrosomal microtubules and pull centrosomes apart through its minus-end-directed motility. Blue and orange arrows indicate the direction of Eg5 and dynein motility, respectively. Red arrows indicate the movement of the centrosomes.

becomes more error-prone[239]. Furthermore, the prophase centrosome separation becomes essential for spindle bipolarity, when spindle assembly in prometaphase is put under stress[238]. Consistent with this, we found that inhibition of prophase centrosome separation in cells with reduced Eg5 activity also decreased spindle bipolarity (Fig.5). Thus, it is likely that prophase and prometaphase mechanisms for centrosome separation act redundantly to allow robust bipolar spindle assembly. When such redundancy is removed, as is the case in EICs, grown in the presence of Eg5 inhibitor, bipolar spindle assembly becomes less robust and all remaining pathways become essential.

Mechanism of resistance to Eg5 inhibitors

How can EICs build a bipolar spindle in the absence of Eg5 activity? Previously, we reported that 5- to10-fold overexpression of kinesin-12 is sufficient to establish spindle bipolarity in the absence of Eg5[57]. However, EICs can form a bipolar spindle without kinesin-12 overexpression, so other mechanisms must exist. Dynein-dependent prophase centrosome separation in EICs allows spindle assembly in prometaphase to initiate with highly separated centrosomes, a situation which likely results in a strong bias towards spindle bipolarity[57,64] and thus, relatively low kinesin-12 activity might be sufficient to tip the balance towards spindle bipolarity. Consistent with this, endogenous kinesin-12 activity is not sufficient for spindle bipolarity in parental HeLa cells treated with Eg5 inhibitors, as these cells enter prometaphase with unseparated centrosomes.

However, other changes have likely occurred in EICs as well that could enhance spindle bipolarity in prometaphase, and future work will be directed towards identification of these additional changes. Nonetheless, eliminating prophase centrosome separation in EICs by depletion of BICD2 or CENPF, results in a dramatic decrease in spindle bipolarity, clearly pointing at an important role for prophase centrosome separation as a modulator of bipolar spindle assembly in the absence of Eg5 activity. Although the exact mutations/changes that allow dynein to drive prophase centrosome separation in EICs are currently unknown, these might involve increased activation of NE-dynein or weakening of the linkage that holds centrosomes together[240], and could be different for each clone.

Taken together, the development of EICs has allowed us to delineate multiple levels of redundancy in centrosome separation and bipolar spindle assembly. This approach will be useful to identify additional pathways involved in spindle assembly that have previously been overlooked due to redundancy. A similar approach can easily be adopted for other motor and non-motor proteins, allowing widespread identification of redundancy, as well as potential resistance to clinically relevant drugs.

Materials and Methods

Cell culture, transfection and drug treatment

HeLa and U2OS cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) with 6% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. siRNA was transfected with HiPerFect (QIAGEN) according to the manufacturer's guidelines. DNA was transfected using X-tremeGENE (Roche) according to the manufacturer's guidelines. The following siRNAs were used in this study: GAPDH OTP SMARTpool (Dharmacon), Eg5 OTP SMARTpool (Dharmacon), Hec1 OTP SMARTpool (Dharmacon), dynein HC AAGGAUCAAACAUGACGGAAU[241] (Dharmacon), kinesin-12 OTP SMARTpool (Dharmacon), Lis1 GAGTTGTGCTGATGACAAG[242] (Dharmacon), dynein IC OTP GUAAAGCUUUGGACAACUA (Dharmacon), BICD2 SMARTpool and OTP AGACGGAGCGCGAACAGAA (Dharmacon), CENPF SMARTpool and OTP GAAGCUAUGCUAAGAAAUA (Dharmacon), Kinesin-1 OTP CGAGGAACGUCUAAGAGUA (Dharmacon) and Plk4 OTP GGACUUGGUCUUACAACUA (Dharmacon). The following expression construct was used: Mouse HA-BICD2[243].

Generation of EICs

Different HeLa clones were treated with increasing concentrations of STLC over the course of 5 weeks. Cells were washed three times a week and passaged into increased STLC concentrations twice a week. After reaching the final concentration of 20 µM STLC, Eg5 RNAi was used to test if the cells were truly dividing independently of Eg5.

Immunofluorescence

Cells were grown on 10 mm glass coverslips and fixed with either 4% formaldehyde with 1% triton X-100 in PBS at room temperature or ice cold methanol for 15 min. α-Tubulin antibody (Sigma) was used at 1:10.000, γ-tubulin (Abcam) was used at 1:500, phospho-H3 (Ser10) (Millipore) was used at 1:1.500, BICD2 antibody [235] was used 1:300, P150glued antibody (BD) was used 1:500, CENPF (Novus) was used 1:500, Dynein Intermediate Chain (70.1, Sigma) was used 1:1.000, Nde1/NdeL1 [244] was used 1:100, HA (HA11, Covance) was used 1:1.000, Lamin B (Santa Cruz) and Lamin A/C (Santa Cruz) antibodies were used 1:200. Primary antibodies were incubated overnight at 4°C and secondary antibodies (Alexa 488 and 561, Molecular Probes) were incubated for 1h at room temperature. DAPI was added before mounting using Vectashield (Vectorlabs). Prophase cells were selected based on phospho-H3 (Ser10) signal, DNA condensation status and the lack of cytoplasmic proteins in the nucleus and inter-centrosomal distances were measured in 3D images using Zeiss LSM 510 confocal software. Images were acquired on a Zeiss LSM510 META confocal microscope (Carl Zeiss) with a Plan Apochromat 63x NA 1.4 objective. Brightness and contrast were adjusted with Adobe Photoshop.

Mitotic indexes were determined using automated image acquisition and analysis based on the pHH3 signal using a Cellomics ArrayScan VTI (Thermo Scientific) as described previously[118].

Time-lapse microscopy

U2OS cells stably expressing mCherry-α-tubulin and GFP-H2B were plated on 8-well glass-bottom dishes (Labtek). Cells were imaged in a Zeiss Axiovert 200M microscope equipped with a Plan-Neofluar 40x/1.3 NA oil objective in a permanently heated chamber in Leibovitz L15 CO_2-independent medium. Images were acquired every 60 seconds using a Photometrics Coolsnap HQ charge-coupled device (CCD) camera (Scientific) and GFP/mCherry filter cube (Chroma Technology Corp.). Z-stacks were acquired with 3.33 µm intervals between Z-slices. Images were processed using Metamorph software (Universal Imaging). Centrosome movement was tracked in 3D using ImageJ. Statistical analysis was carried out using Prism 5 (Graphpad Software Inc.)

Colony formation

Parental and STLC-resistant HeLa cells were seeded at a density of 5.000 cells per well in a 96-well plate. Cells were treated with the indicated concentrations of STLC and siRNAs and grown for 7 days. siRNA transfections were repeated every 72 hours. Cells were fixed with methanol and stained with crystal violet. Colony density was quantified using ImageJ.

Western blotting

Cells were lysed with Laemmli buffer (120mM Tris (ph 6.8), 4% SDS, 20% glycerol). Protein levels were analysed by western blot. Kinesin-12 antibody[57] was used 1:500, Actin antibody (Santa Cruz) was used 1:1.500, BICD2 antibody[38235] was used 1:1.000, Eg5 antibody (Abcam) was used 1:500, Hec1 antibody (Genetex) was used 1:1.000, CENPF antibody (Novus) was used 1:500 and Dynein Intermediate Chain (70.1, Sigma) was used 1:1.000. Actin levels served as a loading control.

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Author contributions

JAR, RGHPH, RHM and MET designed the experiments. JAR, RGHPH and JLM carried out and analyzed the experiments. BFG and EFG provided technical assistance. JAR, RGHPH, RHM and MET wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Chapter 3

Aurora A, MCAK and Kif18b promote Eg5-independent spindle formation

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Abstract

Inhibition of the microtubule (MT) motor protein Eg5 results in a mitotic arrest due to the formation of monopolar spindles, making Eg5 an attractive target for anti-cancer therapies. However, Eg5-independent pathways for bipolar spindle formation exist, which might promote resistance to treatment with Eg5-inhibitors. To identify essential components for Eg5-independent bipolar spindle formation we performed a genome-wide siRNA screen in Eg5-independent cells (EICs). We find that the kinase Aurora A and two kinesins, MCAK and Kif18b are essential for bipolar spindle assembly in EICs and in cells with reduced Eg5 activity. Aurora A promotes bipolar spindle assembly by phosphorylating Kif15, hereby promoting Kif15 localization to the spindle. In turn, MCAK and Kif18b promote bipolar spindle assembly by destabilizing the astral MTs. In the absence of MCAK and Kif18b, excessive astral MTs generate inward pushing forces on centrosomes at the cortex that inhibit centrosome separation. Together, these data reveal a novel function for astral MTs in force generation on spindle poles and how proteins involved in regulating microtubule growth control, indirectly control bipolar spindle assembly.

Introduction

The bipolar spindle is a microtubule (MT)-based structure required for successful chromosome segregation during mitosis. Assembly of the bipolar spindle requires tight regulation of a wide variety of microtubule-associated proteins (MAPs), including MT motors from the kinesin family of proteins[245]. An essential and highly conserved protein for bipolar spindle assembly is kinesin-5 (Eg5 in humans). Eg5 forms a unique tetrameric configuration, hereby enabling it to crosslink and slide antiparallel MTs apart and thereby driving centrosome separation and bipolar spindle assembly[27,28]. Inhibition or depletion of Eg5 results in a mitotic arrest and subsequent cell death due to the formation of monopolar spindles in nearly all organisms tested[26]. Therefore, Eg5 is an attractive anti-mitotic target for cancer therapy[246].

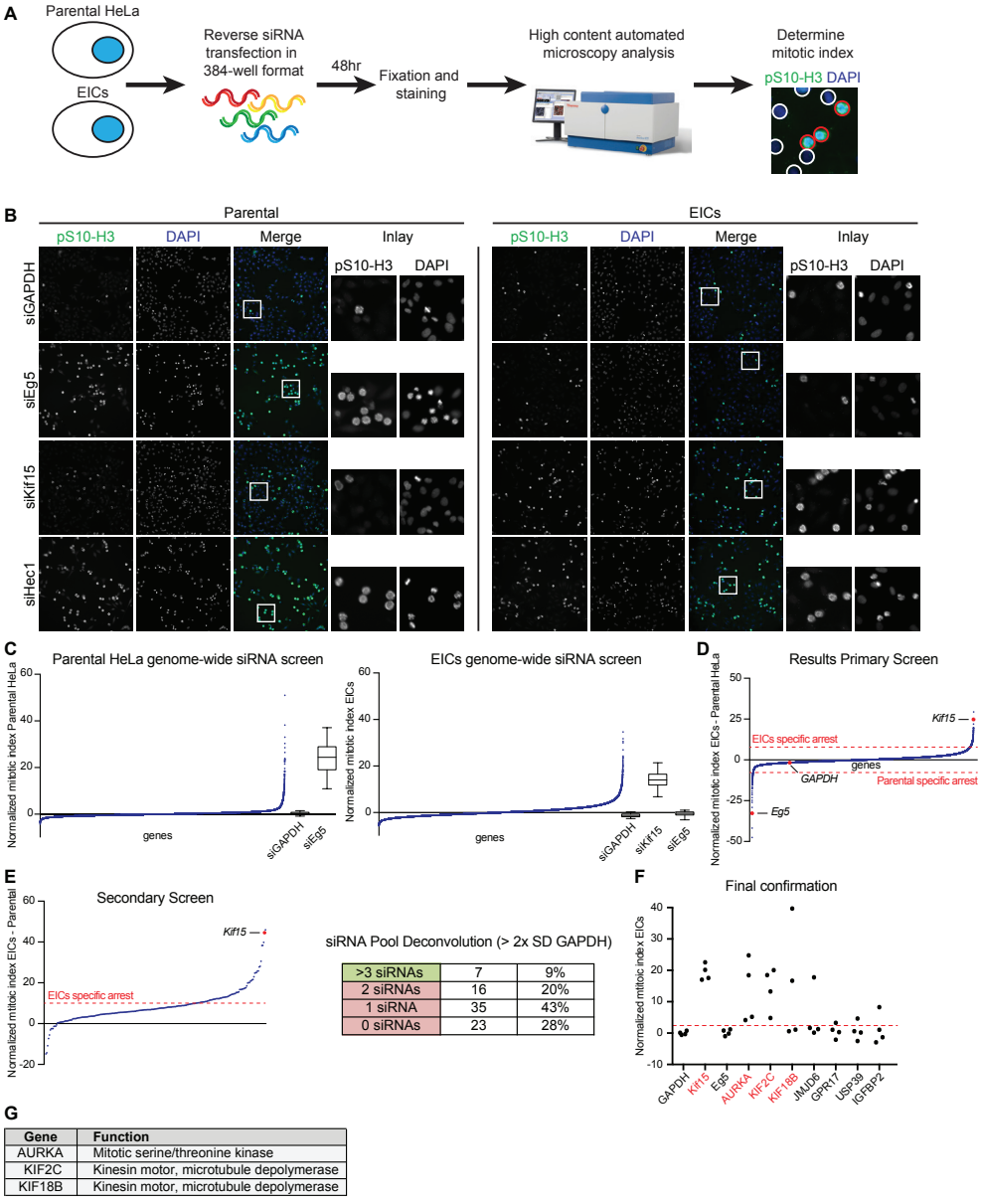
Recent studies reported the existence of redundant pathways, cooperating with Eg5 to drive centrosome separation and bipolar spindle assembly. In human cells, kinesin-12 (Kif15/Hklp2 in humans) was identified to cooperate with Eg5 in bipolar spindle assembly[57,58]. Ectopic over-expression of Kif15 bypasses the requirement for Eg5 in bipolar spindle assembly[57]. In addition, we and others have shown that human cells, treated with Eg5 inhibitors can easily acquire the ability to build a bipolar spindle in the absence of Eg5 activity, but become dependent on Kif15 for bipolar spindle formation[32,45,60,247].

To identify genes that are required for Eg5-independent bipolar spindle assembly, we performed a genome-wide siRNA screen in HeLa and HeLa-derived Eg5-independent cells (EICs, [32]. We searched for genes that specifically arrested EICs in mitosis, using a high content, fixed cell assay. We identified the mitotic kinase Aurora A and two kinesins that regulate MT dynamics, MCAK (Kif2C/kinesin-13) and Kif18b (kinesin-8), to be essential for bipolar spindle assembly in EICs. Our data reveals two novel mechanisms that are required for Eg5-independent bipolar spindle assembly and uncover three potential targets for combination therapy with Eg5 inhibitors.

Results

A genome-wide siRNA screen identifies three genes required for bipolar spindle assembly in Eg5-independent cells.

In order to identify genes contributing to centrosome separation and bipolar spindle assembly in EICs, we performed a high content, image-based genome-wide siRNA screen in these cells, cultured in the presence of a high dose (20 μM) of the Eg5-inhibitor S-trityl-L-cysteine (STLC;[32,230], see Figure 1A for experimental setup). In short, the cells were transfected with pools of ON-TARGET plus siRNAs containing four duplexes per gene, targeting 18,104 human genes in total in a 384-well format (approximately 80% of the human genome, see experimental procedures for detailed information about the siRNA library). To visualize the effect of gene knockdown on mitotic progression, we fixed the cells 48 hours after siRNA transfection and determined the mitotic index by staining the cells using the mitotic marker phospho-Histone H3 (Figure 1A and B). The screen was performed in both parental HeLa cells and HeLa-derived EICs to identify genes that specifically arrest EICs in mitosis. As positive controls in our screen setup, we used siRNA targeting Eg5 to specifically arrest the parental cells in mitosis and siRNAs targeting Kif15 to specifically arrest the EICs in mitosis (Figure 1B). We used siRNAs targeting the Hec1 gene, encoding an essential outer-kinetochore (KT) component, as a second positive control, since its depletion leads to a mitotic arrest in both cell lines (Figure 1B). GAPDH siRNA was used as a negative control for both cell lines (Figure 1B). The primary screen was performed



>3 siRNAs	7	9%
2 siRNAs	16	20%
1 siRNA	35	43%
0 siRNAs	23	28%

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Gene	Function
AURKA	Mitotic serine/threonine kinase
KIF2C	Kinesin motor, microtubule depolymerase
KIF18B	Kinesin motor, microtubule depolymerase

◄ Figure 1: Genome-wide siRNA screen identifies three genes specifically affecting mitosis in Eg5-independent cells.

(A) Experimental setup of the screen. Parental and EICs HeLa cells were reverse transfected in a 384-well format with a genome wide ON-Target plus siRNA library from Dharmacon. 48 hr after transfection, the cells were fixed and mitotic cells were stained using the marker phospho-histone H3. The mitotic index was determined using an automated fluorescence microscope. (B) Representative pictures of parental cells and EICs, treated with the indicated siRNAs. GAPDH and Hec1 siRNA served as non-specific negative and positive controls respectively. Eg5 siRNA served as a parental-specific control and Kif15 as a EICs-specific control. (C) Results from the primary screen. The left graph and middle graph show the normalized mitotic indexes, ordered from lowest to highest for the parental cells and the EICs respectively. Box plots on the right site of the graphs show the controls for the indicated cell line. Note that depletion of Eg5 shows a high mitotic index in the parental cells, while it has no significant effect in the EICs. Kif15 served as a EICs specific positive control. (D) The Graphs shows the normalized mitotic index after subtraction of parental screen scores from the EICs screen scores. Note that as expected, Eg5 and Kif15 were found as clear outliers. Genes above the upper red-dotted line indicates an EICs-specific mitotic arrest, genes below the lower red-dotted line show a parental specific mitotic arrest. (E) Results from the secondary screen, after subtraction of the normalized mitotic of the parental cells from the scores of the EICs. The 85 genes above the red-dotted line were selected for siRNA deconvolution. The right table shows the results from the siRNA deconvolution. 7 genes from the original 85 were confirmed on-target and selected for final confirmation. (F,G) The final confirmation experiment identified three hits to be specific for the EICs.

in duplicate in both cell lines and the results from both screens were analyzed using CellHTS2 and normalized using sample-based normalization (Figure 1C,[248], see experimental procedures for more detailed information about the analysis method).

In order to identify genes specifically arresting one of the two cell lines, we subtracted the normalized mitotic index of the parental cells from the normalized mitotic index of the EICs (Figure 1D). The scores after subtraction of the normalized mitotic index were plotted and showed Eg5 and Kif15 as clear outliers, specific for the parental cells and the EICs, respectively (Figure 1D). We selected a total of 250 EIC-specific genes for follow-up screening. After subtraction of the mitotic indexes, the selected genes had a normalized difference of at least 8 for the EIC-specific genes. The hits from the primary screen were rescreened in triplicate using a similar setup as the primary screen (Figure 1E). Consistently, we identified Kif15 to be one of the strongest hits after analysis. We selected 85 genes from the secondary screen with a normalized difference of at least 10. We further validated the EIC-specific hits by performing siRNA pool deconvolution (Figure 1E, and experimental procedures). From these 85 candidates, seven genes were confirmed on-target using the criteria that at least three siRNAs of the pool scored with a minimum of two times standard deviation (SD) of the siGAPDH (see experimental procedure). We tested the seven genes in a final confirmation experiment (Figure 1F). From these seven initial hits, we were able to confirm three genes, which upon depletion led to a dramatic increase in the mitotic index in the EICs and showed loss of the corresponding protein on western blot (Figure 1F and Supplementary Figure 1A). The genes identified in the screen, Aurora A, Kif2C (MCAK) and Kif18b (Figure 1G) where all previously implicated in mitosis, but their exact contribution to bipolar spindle assembly is unclear.

MCAK, Kif18b and Aurora A are essential for bipolar spindle assembly in EICs and in cells with reduced Eg5-activity.

In order to validate the role of the hits in Eg5-independent pathways of bipolar spindle assembly, we depleted MCAK, Kif18b and Aurora A from parental and EICs and scored the percentage of bipolar spindles (Figure 2A and B). Similar to Kif15 depletion, the EIC-specific hits from the screen efficiently blocked bipolar spindle assembly while their depletion did not affect spindle bipolarity in the parental cells (Figure 2A and B). Next, we determined if the contribution of MCAK, Kif18b and Aurora A to bipolar spindle assembly was restricted to EICs or if they also contribute to bipolar spindle assembly in parental cells. To test this, we partially inhibited Eg5 activity in parental HeLa cells using a low dose

(0.75 μM,[32] of STLC. Similar to our results in EICs, siRNA depletion of MCAK, Kif18b and Aurora A in parental HeLa cells, treated with a low dose of STLC, fully blocked bipolar spindle assembly (Figure 2C). This indicates that the function of these proteins in bipolar spindle assembly is not restricted to EICs, but that their function is masked by the major centrosome-separating force produced by Eg5 in normal cells.

Both Eg5 and Aurora A inhibitors are promising anti-cancer drugs that are currently investigated in a number of clinical trials[221,249]. The dramatic increase in monopolar spindles in cells treated with Aurora A siRNA and Eg5-inhibitors made us wonder if combined treatment of Eg5 inhibitors with Aurora A inhibitors might lead to a synergistic effect in blocking bipolar spindle formation and as a long-term consequence, in decreased cell proliferation. To test this, we treated both parental and EICs with the selective Aurora A inhibitor MLN8054[250]. Similar to siRNA treatment, concentrations up to 300 nM MLN8054 did not affect bipolar spindle formation in parental cells, but efficiently blocked bipolar spindle formation in EICs (Figure 2D) and in parental cells with reduced Eg5 activity (Figure 2E). In addition, long-term treatment with low doses of MLN8054 (50 – 100nM) blocked proliferation in EICs, while similar doses did not affect EICs after removal of STLC (Figure 2F). These data suggest that combining Eg5 and Aurora A inhibitors might have increased efficacy versus monotherapy. In addition it might prevent the development of resistance to Eg5 inhibitors.

MCAK, Kif18b and Aurora A are required for bipolar spindle maintenance in the absence of Eg5 activity.

We have previously shown that EICs critically depend on nuclear-envelope (NE) dynein-mediated centrosome separation in prophase and Kif15-activity during prometaphase in order to build a bipolar spindle[32,45]. In order to determine which pathway for bipolar spindle assembly the genes identified in our screen act, we first tested if their depletion affected centrosome separation during prophase. While dynein depletion efficiently blocked prophase centrosome separation in EICs, depletion of MCAK, Kif18b and Aurora A did not have a significant effect on prophase centrosome separation that could explain the number of monopolar spindles in prometaphase (Figure 3A and B). This indicates that the function of these proteins in bipolar spindle formation is most likely restricted to prometaphase.

To test if the action of MCAK, Kif18b and Aurora A is restricted to the assembly of the bipolar spindle during prometaphase, or if their function is also required for the maintenance of the metaphase bipolar spindle, we arrested parental HeLa cells in metaphase using the proteasome inhibitor MG132 and subsequently inhibited all Eg5 activity using a high dose of STLC[57,59]. As we have shown previously, control cells maintain a bipolar spindle after treatment with STLC, due to the action of Kif15[57]. Similar to Kif15 depletion, MCAK, Aurora A, and to a lesser extent Kif18b depletion, results in collapse of the bipolar spindle upon Eg5 inhibition (Figure 3C). This indicates that the action of these proteins is not restricted to the assembly of the bipolar spindle, but is also required for maintenance of the metaphase bipolar spindle.

Excessive astral microtubule nucleation blocks centrosome separation and bipolar spindle assembly

Regulation of MT dynamics during mitosis is a tightly regulated process[251]. The MT motors from the kinesin-13 and kinesin-8 family have both been shown to control MT depolymerization[252]. MCAK (kinesin-13) is a non-processive motor that diffuses along the MT lattice to reach the ends of MTs[253], and contains an internal motor domain required for its MT depolymerizing activity[254]. Besides that, MCAK can also track the growing plus-ends of MTs through a direct interaction with EB1[255,256]. In contrast to kinesin-13, kinesin-8 motors contain a N-terminal motor domain and have shown to

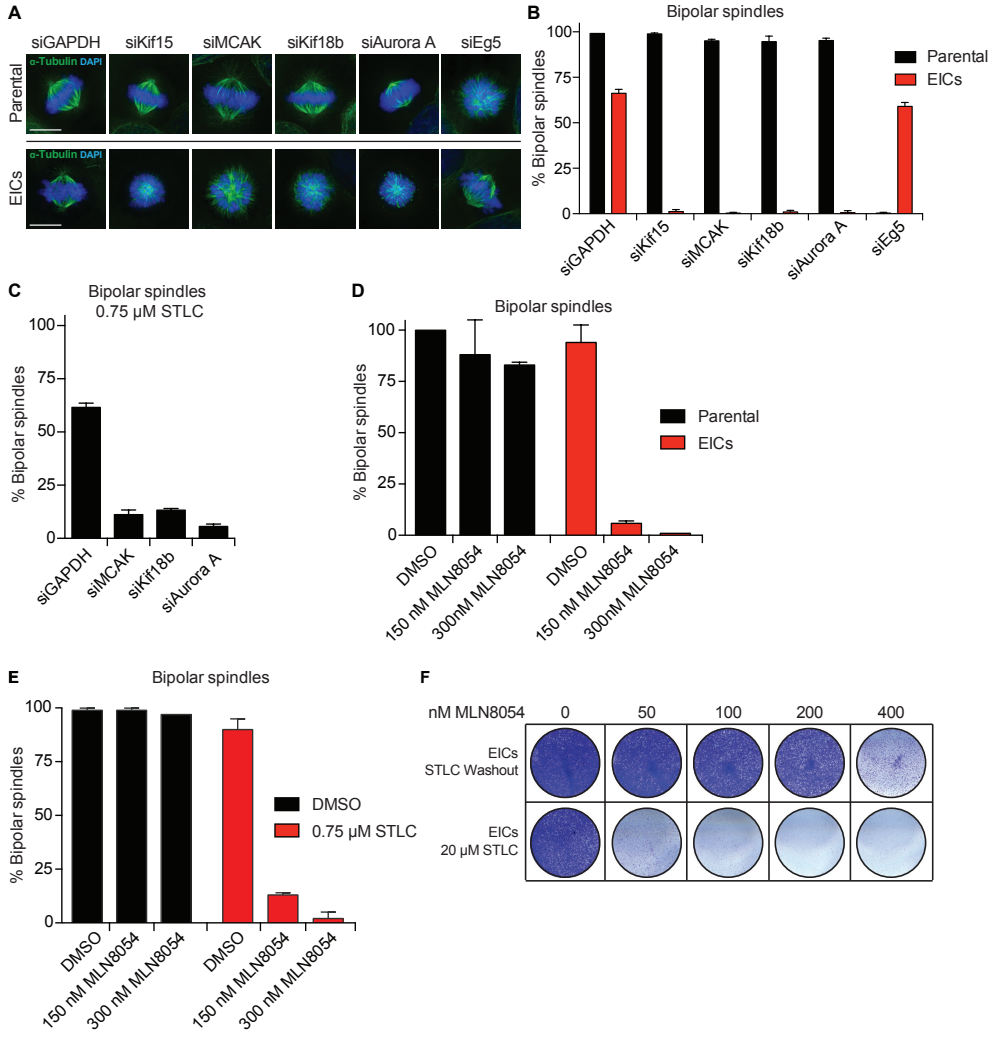
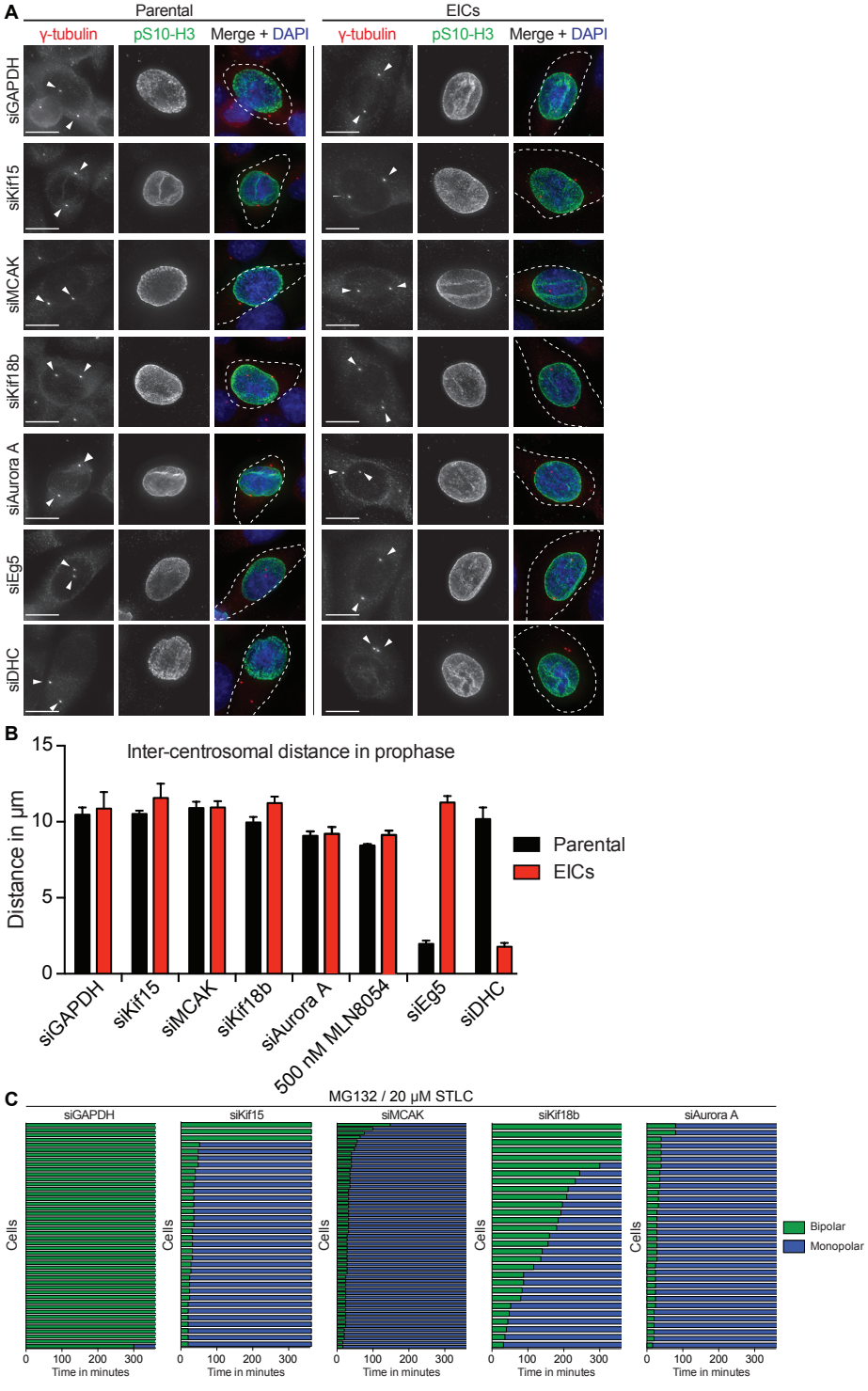


Figure 2: MCAK, Kif18b and Aurora A are essential for bipolar spindle assembly in EICs and cells with reduced Eg5-activity.

(A) Parental and EICs HeLa cells were transfected with the indicated siRNAs, fixed and stained for α-tubulin. DAPI was used to stain the DNA. (B) Percentage of bipolar spindles from the cells treated in (A). (C) Parental cells were transfected with the indicated siRNAs and treated for five hours with 0.75 µM STLC. The percentage of bipolar spindles was scored after fixation and staining of the cells. (D,E) Parental and EICs HeLa cells were treated as indicated. The percentage of bipolar spindles was scored after fixation and staining of the cells. (F) Colony formation assay of EICs. The cells were treated for 5 days with the indicated drug combination, fixed with methanol and stained with crystal violet. STLC was washed out at the start of the experiment. Results in (B,C,D,E) are averages of at least three independent experiments. Error bars represent s.d. Scale bars represent 10 µm.



◄ Figure 3: MCAK, Kif18b and Aurora Aare required for bipolar spindle maintenance in the absence of Eg5 activity.

(A) Representative images of parental and EICs HeLa cells treated with the indicated siRNAs. Cells were stained for Y-tubulin to visualize the centrosomes, phospho-H3 (pH3) to mark prophase cells and DAPI to visualize the DNA. Arrowheads in the pictures mark the centrosomes. (B) Quantification of the inter-centrosomal distance in prophase from the cells in (A). (C) Quantification of the mitotic timing of HeLa cells expressing H2B-mCherry and GFP-α-tubulin. Cells were treated for 48 hr with the indicated siRNAs. Before starting the time-lapse acquisition, cells were arrested in metaphase for 1 hr using the proteasome inhibitor MG132. Cells were then treated with 20 μM STLC and images were acquired every 4 minutes. Results from (B) are averages of at least three different experiments. Error bars represent s.d. Scale bars represent 10 μm.

be processive, plus-end directed motors[257-259], that depolymerize MTs at the plus-ends in a length dependent manner[257,260]. Kif18b has also been shown to accumulate at MT plus-ends through a direct interaction with EB1[95,261]. In addition, Kif18b interacts with MCAK hereby promoting the plus-end accumulation of each other[95]. While MCAK regulates MT depolymerization at different locations in the cell, including kinetochores, centrosomes and astral MTs[145,262,263], the localization of Kif18b is negatively regulated by Aurora kinases and has only been found at the plus-tips of astral MTs[95,261]. Taking into account that Kif18b localization is restricted to astral MTs and MCAK is a non-processive motor, it is unlikely that they act in sliding anti-parallel MTs. However, the fact that MCAK and Kif18b both regulate astral MT depolymerization by forming a mitosis-specific complex[95], we wondered if astral MT length control could influence bipolar spindle assembly.

Depletion of either MCAK or Kif18b results in the formation of excessive and long astral MTs (Figure 4A and[95,145,261,264]. Furthermore, a single astral MT can grow towards the cell cortex where it can push against the cell membrane for a short amount of time before it undergoes catastrophe and subsequent shrinkage[265]. During this short contact time, a single MT can exert a substantial amount of force, that is comparable to the force exerted by a single kinesin molecule[237,266]. Indeed, upon depletion of MCAK and Kif18b, we observed high numbers of astral MTs reaching the cortex and buckling of astral MTs indicating continuous polymerization and force generation by these astral MTs (Figure 4A). To test if the excessive astral MTs that form in the absence of MCAK and Kif18b might generate forces by continuous growth against the cortex, we lowered cortical membrane tension by disrupting the actomyosin cytoskeleton using Cytochalasin D. This prevents polymerizing astral MTs from generating forces via the cortical membrane on spindle poles and polymerizing astral MTs would rather deform the cortex upon loss of the actomyosin network. Upon disruption of the actomyosin network in MCAK or Kif18b depleted cells, we indeed observed cortical membrane protrusions in which astral MTs continued to polymerize (Figure 4A, middle panel). Disruption of the actomyosin network hardly affected the number of bipolar spindles formed in control-treated cells in which Eg5-activity was partially inhibited (Figure 4B and C). However, disruption of the actomyosin network in MCAK- or Kif18b-depleted cells produced a marked increase in the amount of bipolar spindles (Figure 4B and C), indicating that the force that perturbs bipolar spindle formation in MCAK- or Kif18b-depleted cells depends on cortical tension. Second, we suppressed excessive astral MT polymerization by partial depletion of the MT polymerase TOGp[267]. Upon partial depletion of TOGp, we noticed a marked decrease in the amount and length of astral MTs in control, MCAK or Kif18b-depleted cells and we did not observe astral MTs that reached all the way to the cortex (Figure 4A, lower panel). This treatment lowered the number of bipolar spindles formed in control-treated cells in which Eg5-activity was partially inhibited, probably due to essential function of TOGp in general MT nucleation. However, cells in which MCAK or Kif18b was depleted showed an increase in the number of bipolar spindles after partial depletion of TOGp. These results suggest that upon disruption of the cortical actomyosin network, or upon suppression of the MT polymerization rate, astral MTs cannot generate sufficient force on the centrosomes to counteract the forces that drive the separation of centrosomes.

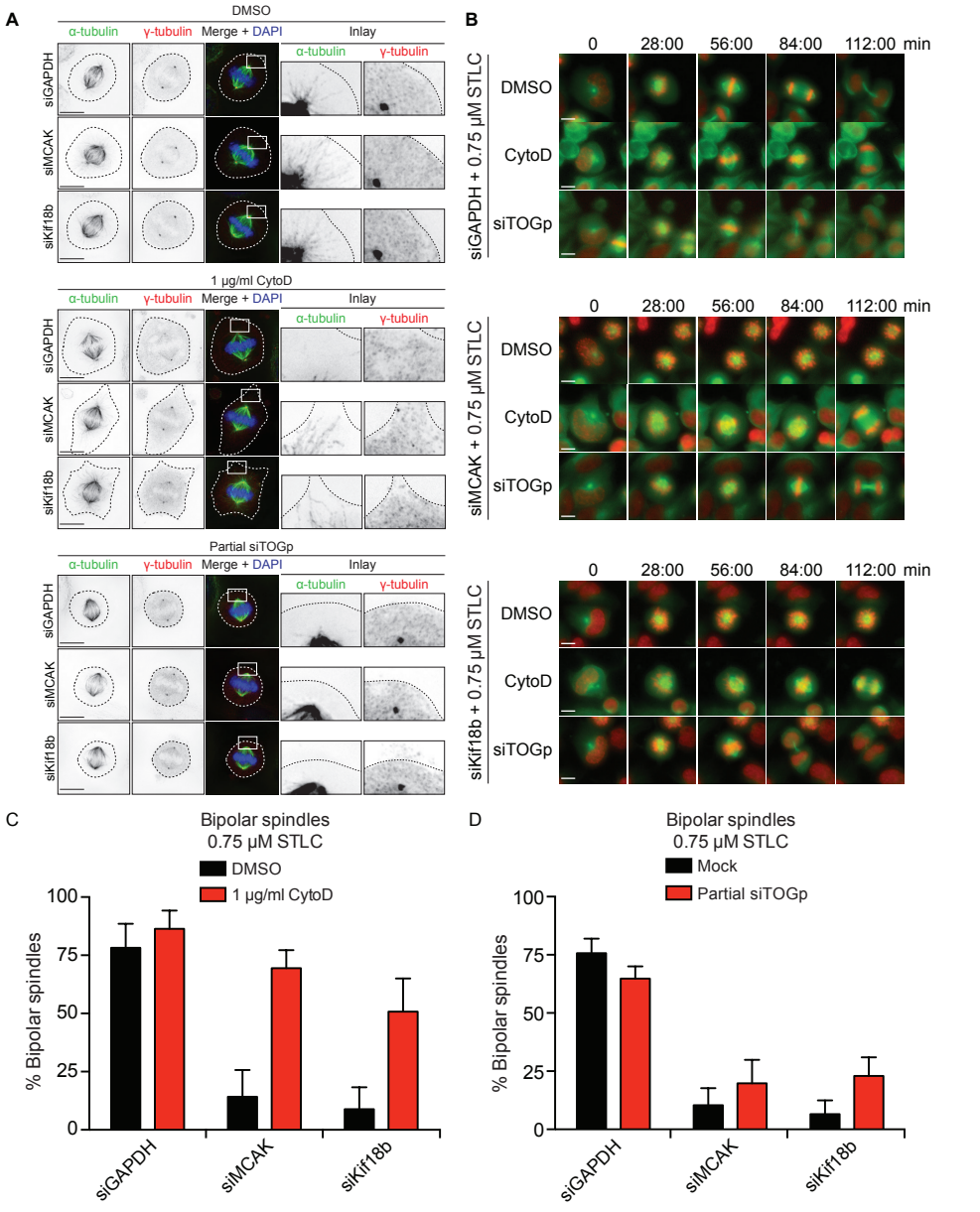


Figure 4: Excessive astral MT nucleation block centrosome separation and bipolar spindle formation.

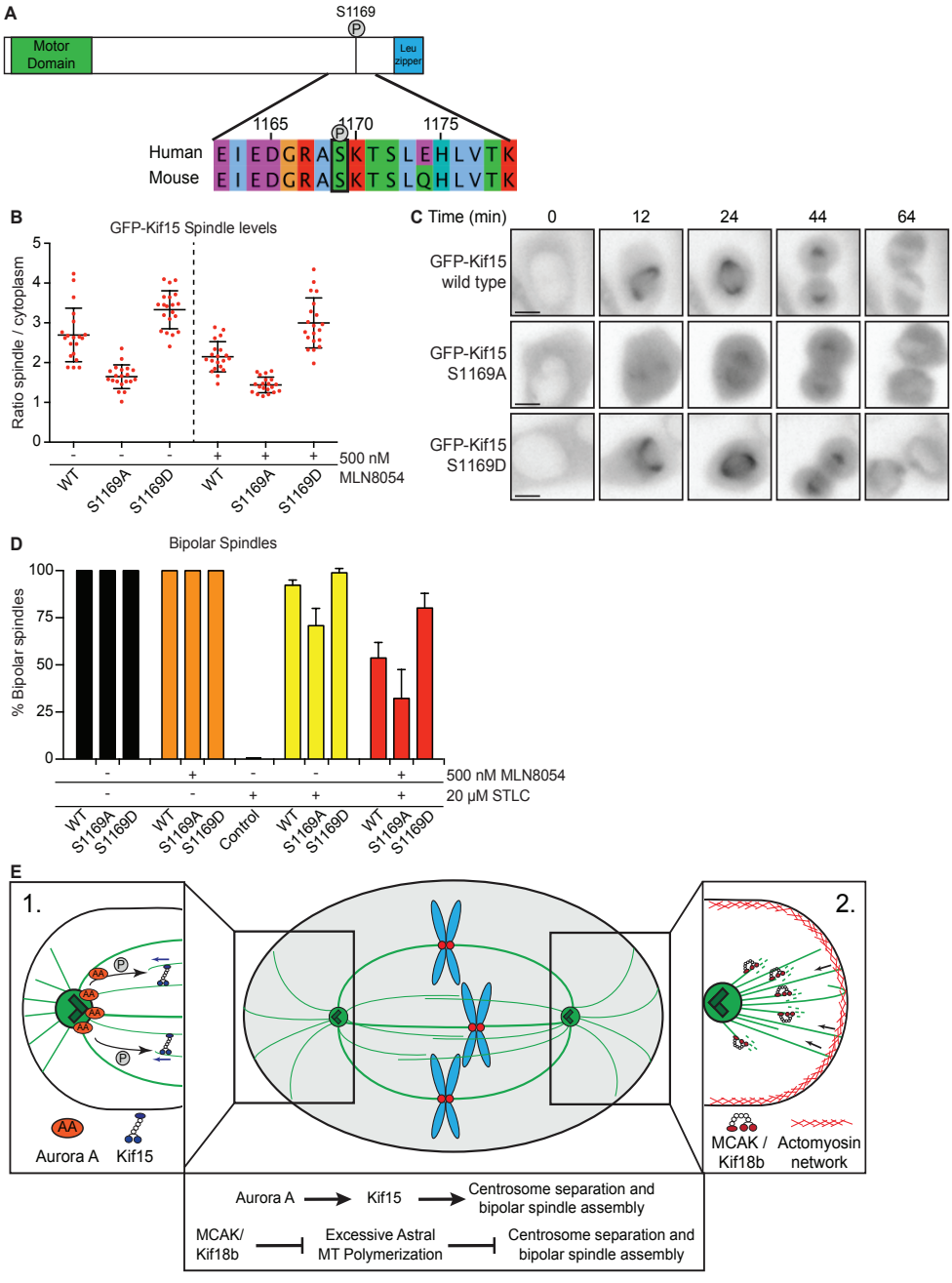
(A) Representative images of parental HeLa cells treated with the indicated siRNAs and drug combinations. 48 hr after siRNA transfection, the cells were fixed and stained for α-tubulin and Y-tubulin. DAPI was used to stain the DNA. The dotted line indicates the cell cortex. Boxed area is enlarged in the inlay. Note the deformation of the cortex by astral MTs in the cytochalasin D-treated cells. (B) Representative stills of parental HeLa cells, expressing H2B-mCherry and GFP-α-tubulin, treated with the indicated siRNAs and drugs combinations. Note the rescue in spindle bipolarity in MCAK and Kif18b-depleted cells after treatment with cytochalasin D and partial TOGp siRNA. (C,D) Quantification of the percentage of bipolar spindles from the cells in (B). Results in C and D are averages of at least three different experiments. Error bars represent s.d. Scale bars represent 10 μm.

Phosphorylation of S1169 by Aurora A is required to target Kif15 to the spindle

The role of Aurora A in centrosome separation and bipolar spindle formation is controversial. Early studies in Drosophila showed that mutations in Aurora A led to centrosome separation defects and monopolar spindle formation[268]. Consistently, studies in mouse embryonic fibroblasts showed that Aurora A deletion led to the formation of monopolar spindles[269]. In contrast, Aurora A deletion in chicken DT40 cells led to the formation of small bipolar spindles[270] and studies in human cells observed a wide variety of phenotypes, including chromosome misalignments, multipolar spindles and monopolar spindles[271,272]. The discrepancies observed between model systems could be explained by the methods used to inactivate or deplete Aurora A from cells, or by different contributions of parallel pathways involved in centrosome separation and bipolar spindle assembly[225]. Despite the fact that a wide variety of Aurora A substrates have been identified[249,273], clear downstream targets involved in centrosome separation and bipolar spindle assembly are poorly understood. Eg5 was described to be phosphorylated by Aurora A in Xenopus[274], but since we identified Aurora A in an Eg5-independent background, this cannot be its only target for its function in bipolar spindle assembly. Furthermore, centrosome maturation and MT nucleation might also indirectly affect centrosome separation, although these function are likely not affected in our system, since we did not observe major defects in prophase centrosome separation in both normal cells and EICs (Figure 3A and B). At last, the fact that we observed a rapid bipolar spindle collapse in cells in which we blocked both Eg5 and Aurora A activity simultaneously (Figure 3C) points towards a target that is also involved in the maintenance of the bipolar spindle.

Since Kif15 and Aurora A depletion have overlapping phenotypes in EICs, we wondered if Aurora A might directly regulate Kif15. To identify potential Aurora A phosphorylation sites in Kif15, we performed an in vitro kinase assay using recombinant Kif15 and Aurora A. Mass-spectrometry of Kif15 identified multiple residues being phosphorylated by Aurora A (Supplementary Figure S2). One particular conserved residue we identified, serine 1169, matched the Aurora A phosphorylation consensus sequence R-X-[S/T] (Figure 5A and Supplementary Figure S2;[275], and was also previously identified in vivo to be specifically phosphorylated during mitosis[276]. To examine the role of S1169 phosphorylation on Kif15 by Aurora A, we expressed GFP-tagged versions of mouse Kif15[57], as well as a non-phosphorylatable Kif15-S1169A and phosphomimetic Kif15-S1169D mutant in U2OS cells under the control of a tetracycline-inducible promoter. While the wild type and Kif15-S1169D localized normally to the spindle (Figure 5B and movie S1), recruitment of the S1169A mutant to the spindle was markedly reduced (Figure 5B and movie S1). This suggests that S1169 phosphorylation on Kif15 by Aurora A promotes its spindle localization during mitosis.

Next we tested if overexpression of the Kif15 mutants could bypass the requirement of Eg5[57]. As expected, overexpression of the different constructs did not affect bipolar spindle assembly in the absence or presence of MLN8054 (Figure 5C, black and orange bars). However, while full Eg5 inhibition efficiently blocked bipolar spindle formation in control-transfected cells (Figure 5C, middle bar), overexpression of wild type and S1169D mutated Kif15 fully restored bipolar spindle formation in Eg5-inhibited cells (Figure 5C, yellow bars). In contrast, expression of S1169A mutated Kif15 required higher levels of expression in order to restore bipolar spindle formation and about 30% of the cells were not able to form a bipolar spindle (Figure 5C, yellow bars). Finally, when we combined Eg5 inhibition with partial Aurora A inhibition (500 nM MLN8054), we observed a pronounced decrease in the amount of bipolar spindles when we overexpressed either wild type or S1169A mutated Kif15 (Figure 5C, red bars). Strikingly, the S1169D mutant was only mildly affected and over 80% of the cells formed bipolar spindles upon combined inhibition of Eg5 and Aurora A (Figure 5C, red bars). These results indicate that Aurora A directly regulates Kif15 by targeting it to the spindle during mitosis through phosphorylation on S1169.



◄ **Figure 5: Phosphorylation of S1169 by Aurora A is required for targeting Kif15 to the spindle.**

(A) Schematic representation of Kif15. The conserved domain spanning serine 1169 is shown in the inlay. (B) Spindle levels of GFP-Kif15 phospho-mutants. U2OS cells were transfected with the indicated construct and treated as indicated. The spindle level of the different GFP-Kif15 mutants was determined by dividing the spindle levels over the levels of the cytoplasm (n=20 cells). (C) Representative stills of U2OS cells expressing the annotated GFP-Kif15 construct. Note the reduced spindle levels after expression of the GFP-Kif15 S1169A mutant. (D) Quantification of the percentage of bipolar spindles in U2OS cells. The cells were transfected with the annotated GFP-Kif15 constructs and treated as indicated. Results in (D) are averages of at least three different experiments. Error bars represent s.d. Scale bar represent 10 µm. (E) Model about the contribution of MCAK, Kif18b and Aurora A in bipolar spindle assembly. (1) Aurora A phosphorylates Kif15 to target it to the spindle, hereby promoting its function in bipolar spindle assembly. (2) MCAK and Kif18b control the number and length of astral MTs, hereby preventing astral MTs from generating forces at the cortex that counteract centrosome separation and bipolar spindle assembly.

Discussion

Here, we performed a genome-wide siRNA screen in parental and EICs cells to identify novel factors involved in Eg5-independent bipolar spindle formation. Using our setup, we identified three genes required for bipolar spindle assembly in EICs. We show that the microtubule motors MCAK and Kif18b are required for bipolar spindle assembly in EICs and normal cells with reduced Eg5-activity. While Eg5 and likely Kif15 directly drive bipolar spindle assembly by sliding antiparallel MTs apart[27,28,57,59], we show that the contribution of MCAK and Kif18b to bipolar spindle assembly is mediated by their function in regulating the length and number of astral MTs during mitosis. In the absence of either MCAK or Kif18b, excessive astral MTs generate inward pushing forces on centrosomes when these MTs collide with the cortex (Figure 5E). Under normal conditions, these forces are not sufficient to counteract outward forces, but when Eg5 activity is compromised, growing astral MTs can generate sufficient amounts of force on the cortex to counteract the remaining centrosome separation forces. Our results indicate that MCAK and Kif18b have an equal and non-redundant contribution in regulating astral MT dynamics. However, we did observe a more rapid collapse of the preassembled bipolar spindle after MCAK depletion compared to Kif18b. This might be due to additional functions of MCAK in the regulation of KT-MT turnover as has been shown previously[145,262]. In addition to MCAK and Kif18b, we identified Aurora A in our screen to be required for bipolar spindle assembly in EICs. Aurora A phosphorylates many substrates in mitosis; however, its downstream targets for controlling centrosome separation are poorly understood. We now show that Aurora A functions in bipolar spindle formation by controlling the localization and activity of Kif15. Spindle localization of Kif15 is decreased upon inhibition of Aurora A. While Kif15 function is under normal conditions redundant for bipolar spindle assembly, its function is essential for EICs and cells with reduced Eg5-activity[32,57,59], explaining the high sensitivity for Aurora A inhibition under this condition. Interestingly, both Aurora A and Kif15 require TPX2 for their function and depletion of TPX2 prevents spindle targeting of Kif15. How phosphorylation of S1169 contributes to spindle targeting of Kif15 is still unclear, but it might affect the interaction of TPX2 with the C-terminal leucine zipper[57], or affect the previously proposed non-motor MT-binding domain of Kif15[277].

Although we find direct phosphorylation of Kif15 by Aurora A, it has likely more targets required for bipolar spindle assembly. We observed that cells expressing high levels of the phospho-null S1169A Kif15 were still able to form bipolar spindles. This could indicate that additional Aurora A phosphorylation sites on Kif15 are present. Aurora A also contributes to MT nucleation and KT-MT stability[278,279], which has been shown to contribute to bipolar spindle assembly and maintenance[59,60]. Most likely, a combination of regulating MT dynamics and kinesins like Kif15, explains the synergistic effect we see after combined inhibition of Eg5 and Aurora A.

Both Eg5 and Aurora A inhibitors are currently being tested as potential anti-cancer drugs in clinical trails[246,280]. In order to enhance efficacy, we propose that combination therapy of Eg5 and Aurora A inhibitors might be beneficial because of three main reasons. First, the combined treatment shows a very strong synergistic effect in the formation of monopolar spindles, even when both proteins are only partially inhibited. Second, the development of resistance mechanisms for Eg5 inhibitors[32,57] will likely be prevented by combining the Eg5 and Aurora A inhibitors. And last, there are currently no Kif15 inhibitors available, which makes Aurora A inhibitors currently the most attractive candidate to increase the efficacy for Eg5 inhibitors. Taken together, we unrevealed new mechanisms for bipolar spindle assembly that might have promising translational applications.

Experimental Procedures

Screen setup, analysis and normalization

The human ON-TARGETplus siRNA SMARTpool library (Dharmacon) was used for the primary screen. The siRNAs for the secondary screen were manually picked and re-tested. For the deconvolution screen, the four single siRNAs of the SMARTpool were tested separately. The primary screen was performed in duplicate, the secondary and deconvolution screen were screened in triplicate.

For the primary and secondary screen, siRNA libraries were aliquoted in a 384-well format using a Sciclone liquid handling robot (Caliper). Deconvolution screen was performed in a 96-well format. A final concentration of 20nM siRNA per well was used. Per transfection, 0.075 µl RNAiMAX (Invitrogen) and 10 µl Opti-MEM (GIBCO) was added to the siRNA and incubated for about 20 minutes. 1500 cells diluted in 40 µl media were added to the wells after incubation of the transfection reagents, using a MultiDrop Combi bulk dispenser (Thermo).

After 48 hours of culturing, the cells were fixed for 10 minutes using a final concentration of 4% formaldehyde (3X formaldehyde in PBS was added to the wells). Fixation reagent was added using a Multidrop Combi bulk dispenser (Thermo), primary and secondary antibodies were added using the Sciclone (Caliper) and all washing step were performed in an AquaMax 2000 plate washer (MDC).

After staining of the wells, the mitotic index of the wells were analyzed using a Cellomics Arrayscan VTI (Thermo Scientific) using a 10x (0.50 NA) objective. Four images were acquired per well. Image analysis was performed using Cellomics Target Activation Bioapplication (Thermo Scientific). Cells were identified based on the DAPI staining and were scored to be mitotic if the phospho-Histone H3 signal reached a set threshold.

The raw mitotic index data was normalized using the CellHTS2 package (Boutros et al., 2006). For the primary screen, sample-based normalization was used. For the secondary and deconvolution screen, control-based normalization was used. After subtraction of the normalized mitotic indexes, the top genes (EICs specific) from the primary screen, that had a normalized difference in the mitotic index of 8, were selected for the secondary screen. Similar criteria were used for the secondary screen. For the deconvolution screen, a siRNA duplex was confirmed on-target when the increase in the normalized mitotic index was > 2 times standard deviation of the negative control (siGAPDH) in all replicates.

Cell culture, transfection and drug treatment

Cells were cultured in DMEM (GIBCO), supplemented with 6% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. siRNAs were transfected using RNAiMax (Invitrogen) according to the manufactures guidelines. DNA was transfected using FuGENE 6 (Promega) according to the manufactures guidelines. The following siRNAs were used in this study: GAPDH OTP SMARTpool (Dharmacon), MCAK/Kif2C OTP SMARTpool (Dharmacon), Kif18b OTP SMARTpool (Dharmacon),

Aurora A OTP SMARTpool (Dharmacon), Eg5/Kif11 OTP SMARTpool (Dharmacon), Kif15/HKlp2 OTP SMARTpool (Dharmacon), Dynein heavy chain [281] and TOGp OTP SMARTpool (Dharmacon). The following expression constructs were used in this study: mouse pTON-bEGFP-Kif15 [95]. Phosphomutants of Kif15 were generated using site-directed mutagenesis. STLC (Sigma) was used at a concentration of 20 µM and 0.75 µM for EICs and parental cells respectively. MLN8054 (Millenium Pharmaceuticals), MG132 (Sigma), nocodazole (Sigma), cytochalasin D (Sigma), were all used at the indicated concentrations.

Immunofluorescence

Cells were grown on 10mm glass coverslips and pre-extracted for 60 seconds in PEM buffer (100 mM PIPES, 10 mM EGTA, 1mM MgCl and 0.1% Triton X-100) followed by fixation in 4% formaldehyde in PEM buffer with 0.3% Triton X-100 for 10 minutes at room temperature. The following primary antibodies were used: α-tubulin antibody (Sigma) was used 1:10,000, phospho-H3 (Serine 10, Millipore) was used at 1:1,500, γ-tubulin antibody (Abcam) was used 1:500. All antibodies were incubated overnight at 4°C. Secondary antibodies (Alexa 488, 568, 647, Molecular Probes) were incubated for 1hr at room temperature. DAPI was added before mounting using ProLong Gold (Invitrogen). Images were acquired using a Deltavision deconvolution microscope (Applied Precision) with a 60x (NA 1.42) or a 100x (NA 1.40) oil objective, Softworx (Applied Precision), Fiji image software and Adobe Photoshop and Illustrator CS6.

Time-lapse microscopy

Cells were plated on 8-well glass-bottom dished (LabTek). Cells were imaged using a Deltavision deconvolution microscope (Applied Precision) equipped with a heated chamber and cultured in L-15 CO_2-independent medium (GIBCO). Images were acquired every 4 minutes using a 20x (NA 0.25) objective. Z-stacks were acquired with 2.5 µm intervals. Images were processed using Softworx (Applied Precision), Fiji image software and Adobe Photoshop and Illustrator CS6.

Colony formation

Cells were plated at a density of 10,000 cells per well in a 48-well plate, treated as indicated and grown for about 7 days. Cells were fixed and stained using methanol and crystal violet.

Western blot

Cells were counted and lysed using Laemmli buffer (120mM Tris pH 6.8, 4% SDS, 20% glycerol). Protein levels were analyzed by western blot. The following antibodies were used: MCAK [281] was used 1:1,000, Kif18b [95] was used 1:500, Aurora A (Cell Signalling) was used 1:1,000, α-tubulin (Sigma) was used 1:10,000, Hsp90 (Santa Cruz) was used 1:2,000.

Identification of phosphorylation sites

5µg of recombinant mouse His-GFP-Kif15, purified from SF9 cells, was incubated with 0.75 µg recombinant human His-Aurora A (Enzo Lifesciences) for 30 minutes in kinase buffer (50mM Tris pH 7.5, 15mM MgCl, 2mM EGTA, 0.5mM Vanadate, 1mM DTT) in the presence of 60 µM ATP. Kinase assay using recombinant His-Aurora A and recombinant human histone-3 (NEB) served as a control. Phosphorylation sites on Kif15 were identified by mass-spectrometry with a nano-LC-LTQ-Orbitrap (Thermo Scientific).

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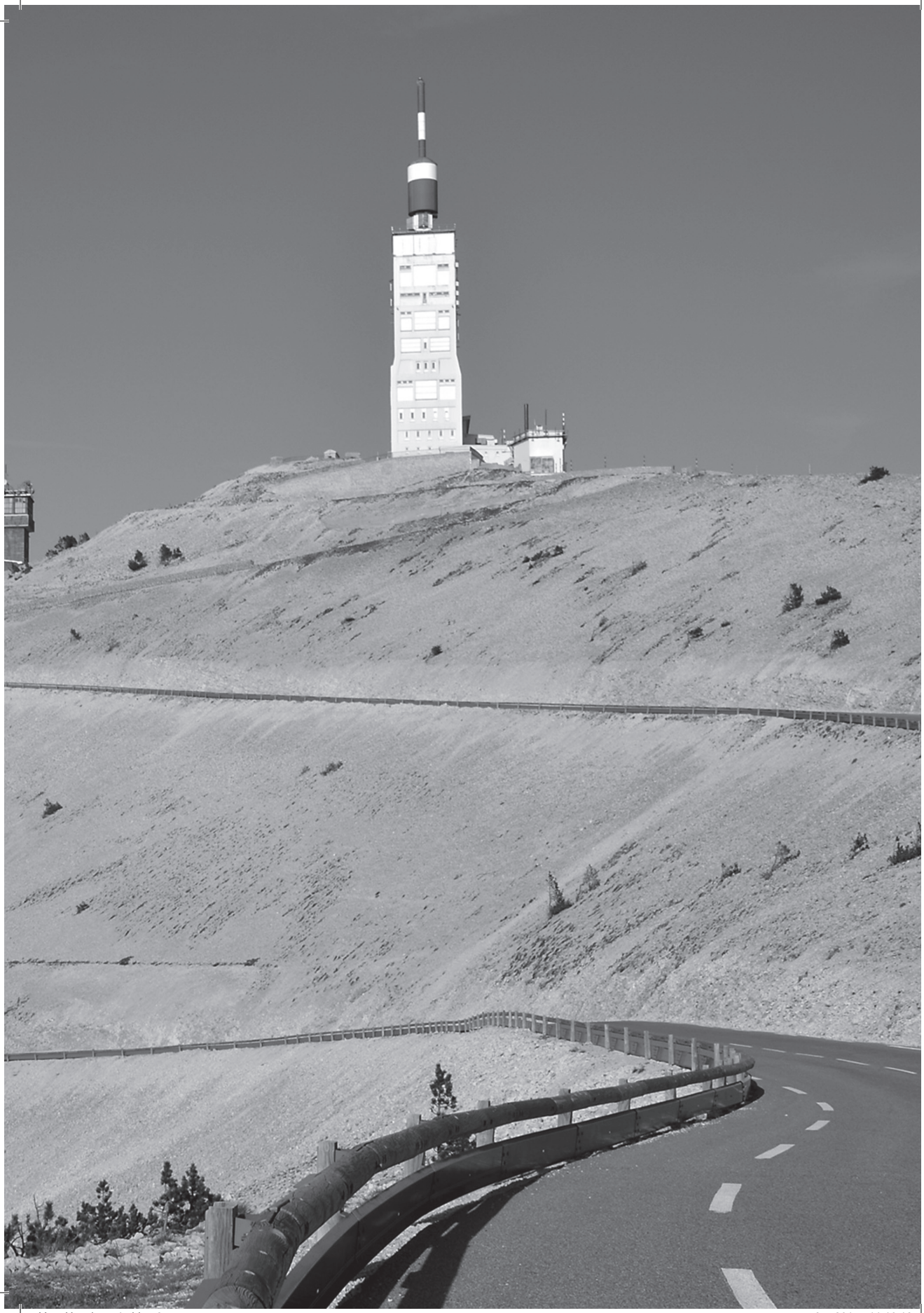
Author contribution
RGHPH, MET, DAE and RHM designed the experiments. RGHPH carried out the experiments. RGHPH analyzed experiments. RGHPH and DAE analyzed screen data. JAR, CL, and DL provided technical assistance. VAH and AJRH carried out and analyzed mass-spectrometry analysis. RGHPH and RHM wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.



Chapter 4

Balanced activity of three mitotic motors is required for bipolar spindle assembly and chromosome segregation

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Abstract

Bipolar spindle assembly requires force to organize the microtubule network. Here we show that three motor proteins, namely Eg5, Kif15 and dynein, act together to produce the right force balance in the spindle. Excessive inward force results in monopolar spindle formation, while excessive outward force generation results in unstable spindles with splayed spindle poles. Blocking activity of all three motors prevents bipolar spindle formation, but established bipolar spindles are refractory to loss of all motor activity. Further analysis shows that although these preformed spindles remain bipolar, outward force generation is required to establish sufficient tension on kinetochores and to accomplish successful chromosome segregation. Together, these results show how Eg5, Kif15 and dynein work together to build a bipolar spindle and reveal an important role for antagonistic motors in chromosome segregation.

Introduction

The microtubule motor protein Eg5 (kinesin-5) is one of the main drivers of bipolar spindle formation[25,57]. Eg5 can crosslink microtubules into an antiparallel configuration and slide them apart, thereby generating an outward-directed pushing force on centrosomes[27,28,57,58,228,282]. Depletion or inhibition of Eg5 activity prevents bipolar spindle formation and arrests cells in mitosis with unseparated centrosomes.

Eg5's outward-directed pushing force on spindle poles can be antagonized by minus-end directed motors. In human cells, as well as in Xenopus egg extracts inhibition of the minus-end directed motor dynein efficiently rescues bipolar spindle assembly in the absence of Eg5 activity, indicating that dynein antagonizes Eg5 activity[31,57,58,63-65,283]. Besides dynein, the minus-end-directed kinesin-14 can also antagonize the activity of the kinesin-5 homolog Klp61F in Drosophila[33,57,58]. However, it is currently unclear what the relative contribution of dynein and HSET is in antagonizing Eg5 in human cells.

Recently, we and others identified an additional motor, kinesin-12 (Kif15/hklp2), that is important for bipolar spindle formation[57,58,75,284]. While Kif15 is not essential for bipolar spindle assembly under normal conditions, loss of Kif15 sensitizes cells to partial loss of Eg5 activity, suggesting that it acts in concert with Eg5[57,58,70]. Indeed, when expressed at high levels, Kif15 can compensate for full loss of Eg5 activity[57,70,285,286]. This suggests that Kif15 can also produce an outward-directed force on spindle poles.

While it is clear that the abovementioned motors can cooperate or antagonize each other, it is debated whether the existing data support a simple "push-pull" model, in which the outward-directed force generated by Eg5 and Kif15 is directly antagonized by an inward-directed force generated by kinesin-14 and/or dynein[75,287]. Such a push-pull model makes two predictions. 1) In the absence of all outward and inward forces, centrosome separation should not occur and monopolar spindles should be formed. 2) If all outward and inward forces are removed after bipolar spindle formation, the spindle should remain bipolar. However, previous studies showed that complete inhibition of both dynein and Eg5 still enables bipolar spindle formation[31,62,75,283,288]. If dynein and Eg5 are the main force generators in the spindle, this result contradicts the first prediction stated above, and argues that the push-pull model is not correct[283]. However, additional force-generating motors like Kif15 and HSET exist, but it remains unclear how they contribute to spindle bipolarity in the absence of dynein and Eg5.

In Xenopus egg extracts and mammalian cells, inhibition of either dynein or Eg5 results in severe defects in spindle assembly[29,50,51,70,124,137,289,290]. However, simultaneous inhibition of both motors results in bipolar spindles that are functional for chromosome segregation[31,63]. A similar effect has been observed for the kinesin-14 NCD and kinesin-5 in Drosophila[33]. Thus, it has remained unclear why antagonistic motors exist if functional bipolar spindles can assemble in their absence.

Results

Eg5, Kif15 and dynein produce balanced in- and outward forces essential for bipolar spindle formation

To study the antagonism between Eg5 and dynein, we used time-lapse microscopy to film U2OS cells expressing mCherry-α-tubulin and GFP-H2B treated with the Eg5 inhibitor S-trityl-L-cysteine (STLC,[230] in combination with either control (GAPDH) or dynein heavy chain (DHC) siRNA (Figure 1A). Increasing concentrations of STLC in control cells reduced the percentage of bipolar spindles (Figure 1B, 1D,

S1B and movie S1). As shown previously, depletion of dynein led to a variety of mitotic defects; centrosomes are not incorporated in the spindle, spindle poles are poorly focused and chromosome alignment is perturbed (movie S1), leading to a mitotic delay. However, we observed that the majority of cells eventually entered anaphase with misaligned chromosomes (movie S1). This indicates that the spindle assembly checkpoint (SAC) in U2OS cells is not very effective in sensing a low number of unattached kinetochores (KTs). In contrast to control-depleted cells, increasing concentrations of STLC in dynein-depleted cells minimally affected the capacity to form bipolar spindles (Figure 1B, S1A, S1C and movie S1). Consistent with previous results, the rescue of bipolar spindle assembly seen in dynein-depleted cells is very efficient, even when all Eg5 activity is blocked (Figure 1B, S1C, 40μM STLC,[31,283]). Importantly, ~40% of the bipolar spindles that formed in the dynein-depleted cells treated with STLC had not separated their centrosomes, but formed monoastral bipolar spindles; a focused spindle pole on one side with one or more centrosomes incorporated, and an unfocussed array of MTs on the other side, devoid of a centrosome (Figure 1B, 1D and S1C). Thus, dynein not only counteracts Eg5's function in centrosome separation, it also antagonizes Eg5's role in centrosome-independent bipolar spindle assembly, consistent with results in Xenopus egg extracts[63]. The rescue of bipolar spindle formation in cells lacking both dynein and Eg5 is particularly noteworthy, as cells invariably enter mitosis with unseparated centrosomes in the absence of both motors[32,45]. Our data show that despite this failure to separate centrosomes in prophase, the majority of cells (90%) lacking dynein and Eg5 form a bipolar spindle after NEB, of which approximately half separate their centrosomes.

How can centrosome separation and bipolar spindle formation occur in the absence of Eg5? Kif15 was identified as a second motor that promotes bipolar spindle assembly[57,58], and therefore we tested if Kif15 is responsible for the rescue of spindle bipolarity in cells lacking both Eg5 and dynein activity. Depletion of Kif15 increased the sensitivity of cells to (partial) Eg5 inhibition[57] Figure 1C and movie S2). Treatment with 2 μM STLC fully blocked bipolar spindle assembly in cells lacking Kif15, while in control cells ~30% formed bipolar spindles at this concentration of STLC (Figure 1B, 1C and S1D). Strikingly, while dynein depletion efficiently rescued bipolar spindle assembly in Eg5-inhibited cells, co-depletion of Kif15 fully abolished bipolar spindle assembly (Figure 1B, 1C, S1E and movies S2). Thus, the force generated by Kif15 is absolutely required for bipolar spindle assembly in the absence of dynein and Eg5. Conversely, bipolar spindle formation in cells lacking dynein and Kif15 is fully dependent on Eg5, since Eg5 inhibition (>2 μM STLC) fully blocked bipolar spindle assembly (Figure 1C and S1E). Interestingly, HSET depletion did not rescue spindle bipolarity in cells that were treated with STLC (Figure S2A, S2B), while our siRNA reduced HSET expression by an estimated 90% (Figure S1A) and this was sufficient to produce a clear spindle phenotype (see below). Co-depletion of other kinesin-14 members failed to produce a clear phenotype (Figure S2C and S2D), indicating that the contribution of kinesin-14 motors to inward force generation is very minimal. Taken together, these data support a model in which Eg5 and Kif15 generate an outward-directed force on spindle poles, which is counteracted by an inward-directed force generated by dynein.

In- and outward forces are dispensable for the maintenance of a bipolar spindle

Acute inhibition of Eg5 does not result in collapse of a metaphase spindle, while simultaneous inhibition of Kif15/Eg5 does. A three-motor push-pull model would predict that removing the inward force in cells lacking Kif15 and Eg5, should prevent spindle collapse. To test this, we blocked cells in metaphase using the proteasome inhibitor MG132 and subsequently added a high dose (40 μM) of STLC to inhibit Eg5 activity (see Figure 2A for experimental setup). Control cells treated with MG132 and STLC maintained a bipolar spindle for several hours, consistent with previously published work. Nonetheless, spindle collapse could be observed after a prolonged mitotic arrest (Figure 2B, 2C

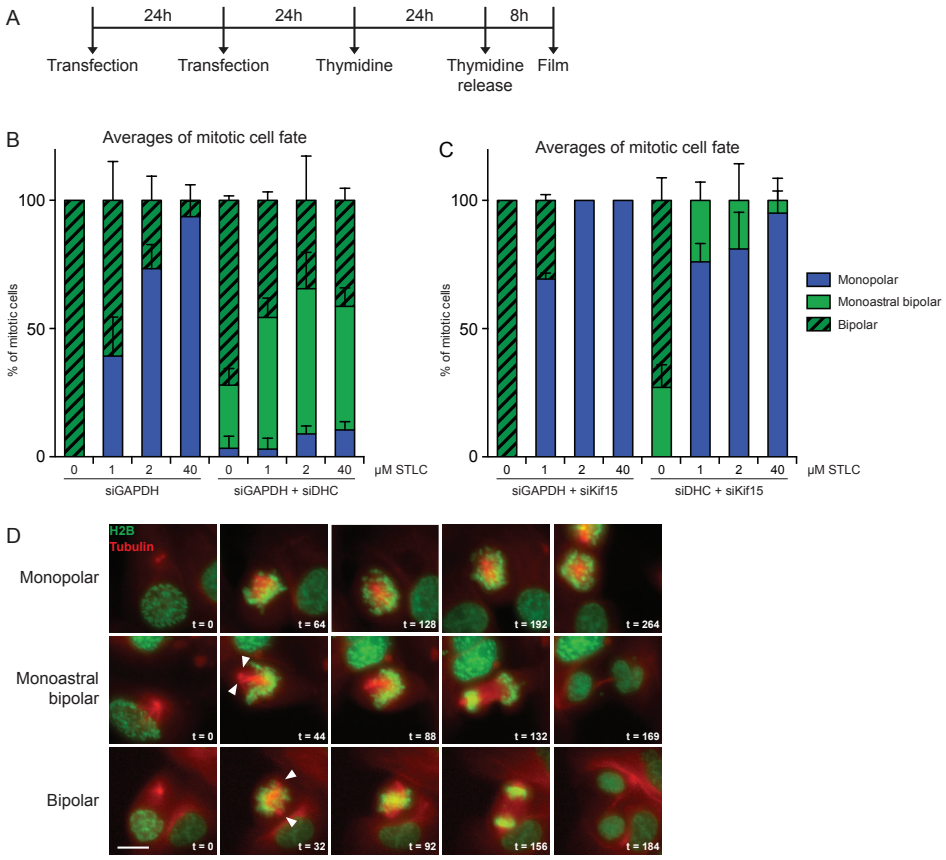


Figure 1. Kif15 is required for the rescue of spindle bipolarity in the absence of Eg5 and dynein.

(A) Experimental setup for the experiments performed in Figure 1. (B, C) Averages of mitotic fate of U2OS cells, stably expressing mCherry-α-tubulin and GFP-H2B. Cells were treated for 72 hours with control (GAPDH, B), DHC siRNA (B), Kif15 (C) or a combination of DHC and Kif15 siRNA (C). Cells were blocked in thymidine 48 post-transfection. After 24 hours, cells were released from the thymidine block. Live cell imaging with the indicated STLC concentration started 6 hours after thymidine release. Images were acquired every 4 minutes. (D) Representative images of cells with a monopolar, monoastral bipolar, and bipolar spindle. Results in (B and C) are averages of at least 3 different experiments. Error bars represent standard deviation. Scale bar in (D) represents 10 μm.

and movie S3), indicating that Eg5 has a minor contribution to spindle stability in metaphase. STLC treatment of dynein-depleted metaphase cells failed to induce spindle collapse (Figure 2B, 2C and movie S3). No monoastral bipolar spindles formed under these conditions, since these cells have previously entered mitosis with separated centrosomes. Spindles in cells depleted of Kif15 rapidly collapsed after treatment with STLC (Figure 2B, 2C and movie S3,[57,58]. This clearly indicates that Kif15 generates the major outward force required for maintenance of spindle bipolarity in the absence of Eg5 activity. Importantly, we found that dynein depletion efficiently blocked spindle collapse in metaphase cells lacking both Kif15 and Eg5 activity (Figure 2B, 2C and movie S3). This is in striking contrast to the situation where cells enter mitosis in the absence of all three motors, exclusively resulting in monopolar spindle formation (Figure 1C). These results suggest that simultaneous inhibition of Eg5,

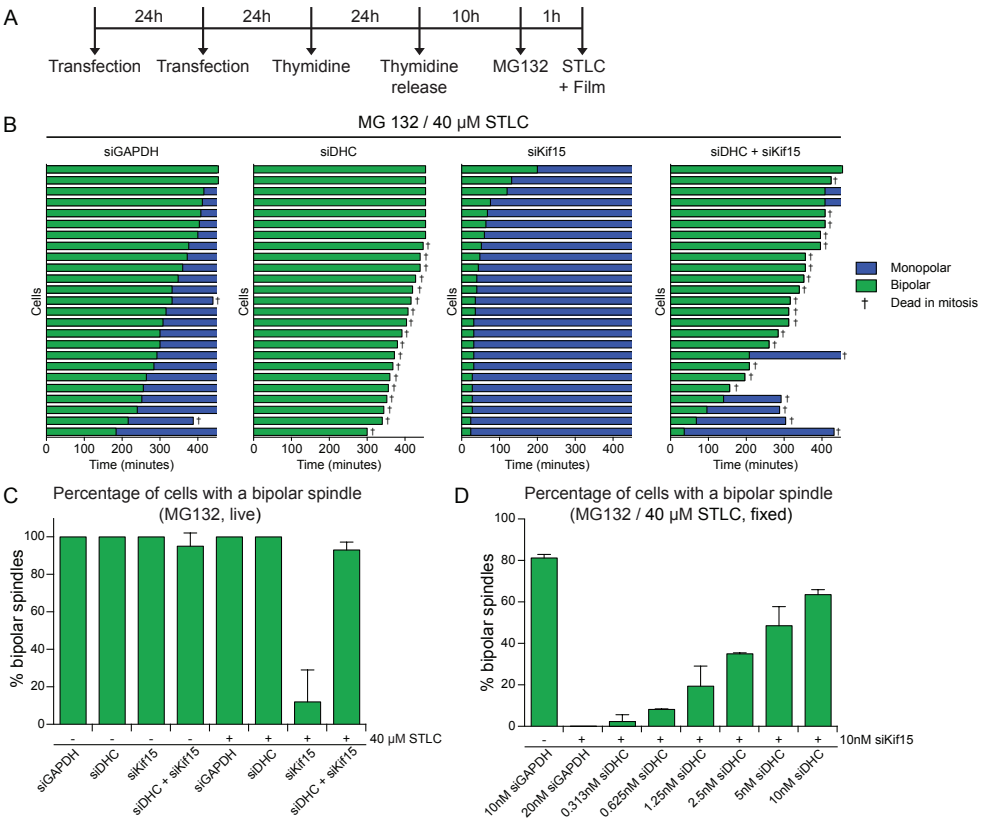


Figure 2. In- and outward forces are dispensable for maintenance of a bipolar spindle.

(A) Experimental setup for the experiments performed in Figure 2, 3 and 4. (B) Quantification of mitotic timing and fate of mitotic U2OS cells stably expressing mCherry-α-tubulin and GFP-H2B. Cells were treated for 72 hours with control (GAPDH), DHC, Kif15 or a combination of DHC and Kif15 siRNA. The cells were blocked in thymidine 48 hours post transfection. After 24 hours, cells were released from the thymidine block and 8 hours later, cells were arrested in metaphase for 1 hour using the proteasome inhibitor MG132. Cells were then treated with 40 µM STLC and images were acquired every 4 minutes. (C) Averages of the quantification in (B) after 1 hour of imaging. (D) Titration of DHC siRNA in cells treated with Kif15 siRNA and 40 µM STLC. Cells were treated as in (B), fixed after 1 hour of STLC treatment and stained for α- and γ-tubulin. DNA was stained using DAPI. Results from (C) and (D) are averages of at least 3 different experiments. Error bars represent standard deviation.

Kif15 and dynein neutralizes the force balance in the spindle causing the spindle configuration that is established at the moment of inhibition (monopolar or bipolar) to remain constant. Interestingly, the effect produced by dynein depletion on a metaphase spindle was nicely titratable (Figure 2D), consistent with a push-pull model in which the outward force generated by Eg5 and Kif15 is directly antagonized by the inward force generated by dynein. Together, these results shows that spindle bipolarity is directly correlated to the sum of outward and inward forces present.

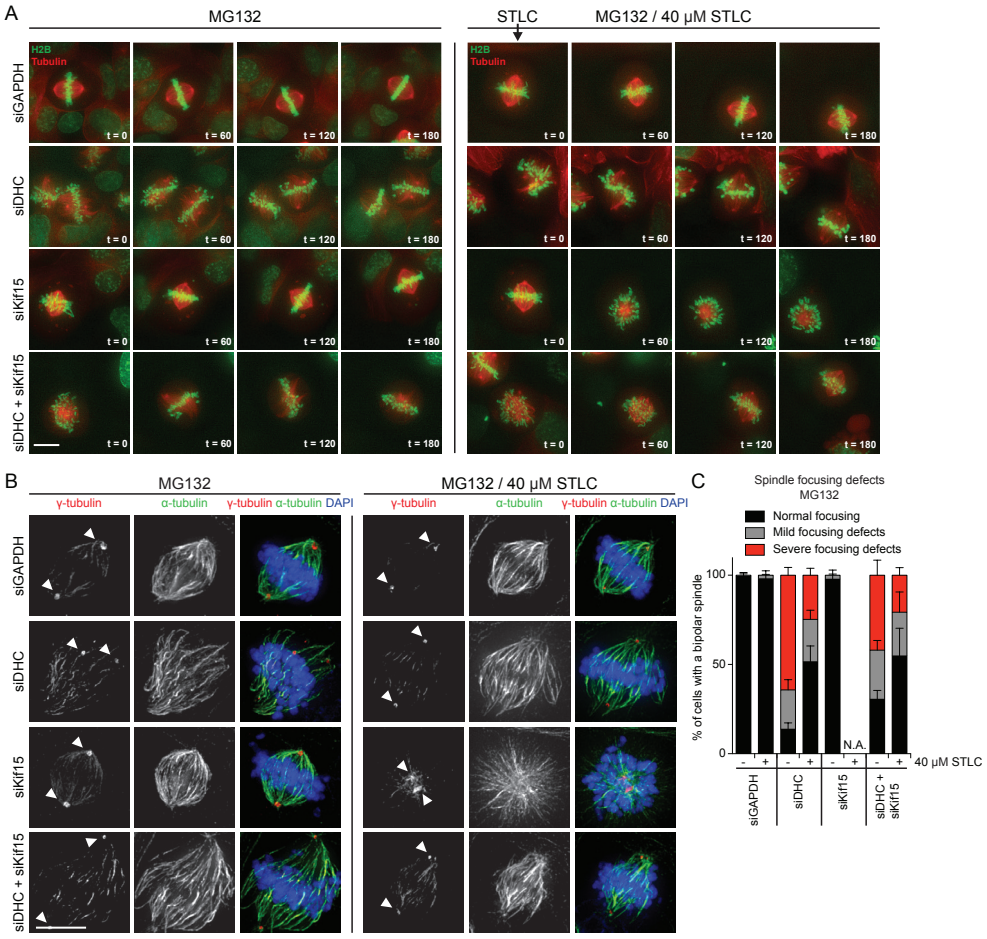


Figure 3. Spindle pole focusing in the absence of dynein can be alleviated by inhibition of Eg5 or Kif15.

(A) Representative stills of mitotic spindles in U2OS cells stably expressing mCherry-α-tubulin and GFP-H2B. Cells were treated for 72 hours with control (GAPDH), DHC, Kif15 or a combination of DHC and Kif15 siRNA. The cells were blocked in thymidine 48 hours post transfection. After 24 hours, cells were released from the thymidine block and 8 hours later, cells were arrested in metaphase for 1 hour using MG132. Cells were then treated with DMSO or 40 µM STLC and imaged were acquired every 4 minutes. Note the loss of centrosome incorporation in dynein-depleted cells and the reincorporation of centrosomes after addition of STLC in dynein-depleted cells. (B) Representative images of U2OS cells transfected with the indicated siRNAs and treated with MG132 to arrest the cells in metaphase for 1 hour. Cells were then treated for 1 hours with either MG132 or MG132/40 µM STLC. Cells were fixed and stained for α- and γ-tubulin. DNA was stained using DAPI. Arrowheads mark the centrosomes. (C) Quantification of the spindle focusing defects from the cells in (B). Note that spindle pole focusing defects were not scored in cells lacking Kif15 and Eg5 activity due to the formation of monopolar spindles. Error bars represent standard deviation. Results in (C) are averages of at least 3 different experiments. Scale bar in (A) and (B) represents 10 µm.

Spindle pole focusing defects in cells lacking dynein can be corrected by inhibition of Eg5 or Kif15

Dynein transports K-fibers to the spindle poles in Drosophila S2 cells[75,284], and this process contributes to spindle pole focusing. Similarly, transport of NuMA by dynein is thought to contribute to spindle

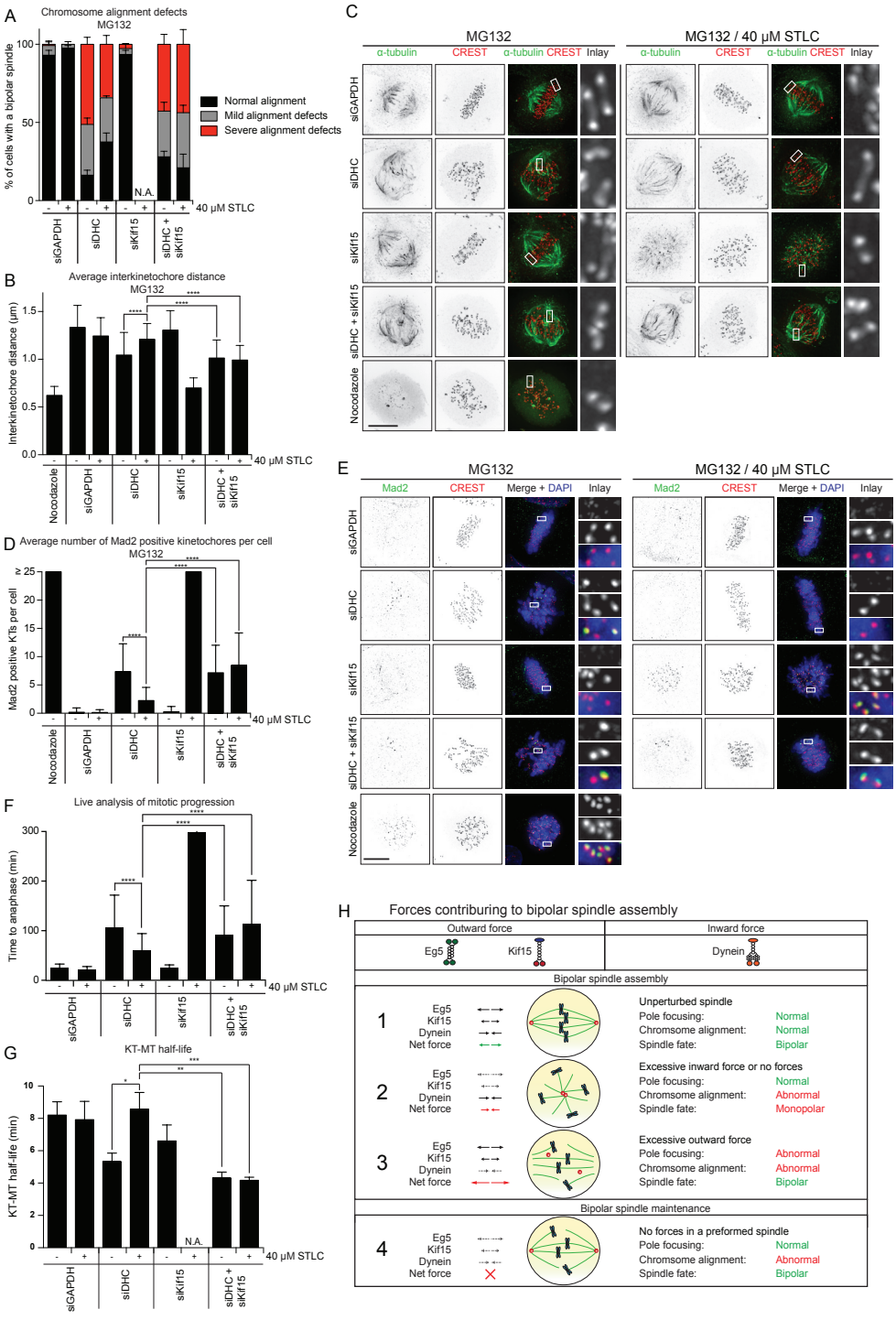
pole focusing in Xenopus egg extracts[70]. Surprisingly, when we inhibited Eg5 or Kif15 activity in mitotic cells lacking dynein, spindle pole focusing was improved (Figure 3A–C and movie S3. Note that spindle pole focusing defects were not scored in cells lacking Kif15 and Eg5 activity due to the formation of monopolar spindles). This suggests that the spindle defects observed after dynein depletion are due to excessive outward force. In addition, this suggests that other motor proteins might act redundantly with dynein in spindle pole focusing. Indeed, in line with previous results, we find slight changes in the spindle distribution of NuMa and TPX2 in the absence of dynein, although Kif2a localization did not seem to be affected (Figure S3A-S3C, Merdes et al., 1996;[70,285,286]. However, Eg5 inhibition can rescue spindle pole focusing in dynein-depleted cells, without rescuing the distribution of NuMa and TPX2 (Figure S3A and S3B), suggesting that spindle pole recruitment of these factors is not required for pole focusing under these circumstances. The minus-end-directed motor kinesin-14, HSET in humans, is also involved in spindle pole focusing[75]. As expected, HSET depletion alone resulted in a small defect in spindle pole focusing (Figure S3D-S3F), consistent with previous reports[62,75,288]. However, this defect could not be rescued by inhibition of Eg5, suggesting that the focusing defects observed in HSET-depleted cells are not due to an imbalance in forces (Figure S3D-S3F). Double depletion of HSET and dynein resulted in slightly more severe spindle pole focusing defects than depletion of dynein alone, indicating that dynein and HSET cooperate in spindle pole focusing. Furthermore, pole focusing in the absence of both dynein and HSET could not be rescued by Eg5 inhibition (Figure S3F). These results show that efficient pole focusing is dependent on a correct balance of inward and outward forces, but that at least some inward force is required, even if outward forces are low.

A correct force balance in the spindle is required for chromosome alignment and segregation

Surprisingly, chromosome alignment defects observed after dynein depletion were also partially rescued by inhibition of Eg5 (Figure 4A). This suggests that chromosome alignment defects after dynein depletion are, at least in part, secondary to defects in spindle morphology. However, inhibition of Eg5 in cells lacking both dynein and Kif15 failed to rescue chromosome alignment (Figure 4A), despite the fact that spindle pole focusing was partially restored (Figure 3C). Thus, chromosome alignment is perturbed in the absence of Eg5, Kif15 and dynein, even though the forces appear to be in balance.

► Figure 4. A correct force balance in the spindle is required for chromosome alignment and segregation.

(A) Quantification of the chromosome alignment defects in U2OS cells. Cells were transfected with the indicated siRNAs and incubated for 72 hours. The cells were blocked in thymidine 48 hours post transfection. After 24 hours, cells were released from the thymidine block and treated 8 hours later with MG132 to arrest the cells in metaphase for 1 hour. Cells were then treated for 1 hours with either MG132 or MG132/40 μM STLC. Cells were fixed and stained for α- and γ-tubulin. DNA was stained using DAPI. Note that chromosome alignment defects were not scored in cells lacking Kif15 and Eg5 activity due to the formation of monopolar spindles. (B) Analysis of inter-KT tension. U2OS cells were treated as in (A). Nocodazole-treated cells were used as a positive control. Cells were fixed and stained for CREST and α-tubulin. DNA was stained using DAPI. The distance between CREST signals was measured of at least 400 KTs. ****, $P < 0.0001$, Mann-Whitney *t* test. (C) Representative images from the cells in (B). Inlays are enlargements of individual KT pairs. (D) Quantification of Mad2 positive KTs. U2OS were transfected with the indicated siRNA and treated as in (B). Cells were fixed and stained for Mad2 and CREST. DNA was stained using DAPI. The average number of Mad2 positive KTs was quantified from at least 400 KTs. ****, $P < 0.0001$, Mann-Whitney *t* test. (E) Representative images from the cells in (D). Inlays are enlargements of individual KT pairs. (F) Live analysis of mitotic progression. U2OS cells were treated as in (A) without MG132 treatment. DMSO or STLC was added at the start of the movie. Only (pro)metaphase cells were included in the quantification. Images where acquired every four minutes. ****, $P < 0.0001$, Mann-Whitney *t* test. Error bars in represent standard deviation. (G) Calculated KT-MT half-life of U2OS cells treated as indicated. Error bars represent standard error. See Figure S4 for experimental details. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, t-test. Results from (A), (B), (D), (F), (G) are averages of at least 3 different experiments. Scale bar in (C) and (E) represent 10 μm. (H) Summary of the results obtained in this study. 1,2 and 3 indicate spindle fates during bipolar spindle assembly. 4 indicates spindle fate for preassembled bipolar spindles.



To better understand why chromosome alignment was perturbed in the absence of Eg5, Kif15 and dynein, we performed detailed analysis of kinetochore-MT (KT-MT) attachments. We confirmed a clear reduction in inter-KT distance in dynein-depleted cells compared to control cells (Figure 4B and 4C), consistent with a role for dynein in the formation of stable KT-MT attachments[33,124,137]. In addition, several unaligned chromosomes in dynein-depleted cells stained positive for Mad2 (Figure 4D and 4E), known to localize to unattached KTs. Strikingly, Eg5 inhibition in dynein-depleted cells increased the average inter-KT distance and reduced the number of Mad2 positive KTs (Figure 4D and 4E). This indicates that the rescue of chromosome alignment defects when depletion of dynein is combined with inhibition of Eg5, is a result of restoring normal tension on KTs and subsequent stabilization of KT-MT attachments. While inhibition of Eg5 alone was able to restore KT-MT attachments in dynein-depleted cells, simultaneous inhibition of both Kif15 and Eg5 was unable to restore KT-MT attachments (Figure 5B-E). It should be noted that the strong decrease in inter-KT distance and high number of Mad2 positive KTs in cells depleted for Kif15 and Eg5 activity are due to the formation of monopolar spindles. To determine if a normal force balance directly influences the dynamic properties of both spindle and KT-MTs, we used photoactivatable GFP-α-tubulin and determined the loss of fluorescence over time (Figure 4G and S4A-S4C). The normalized fluorescence intensity plots were fit by a double exponential curve where the fast decaying fluorescence corresponds to the highly dynamic non-KT-MTs and the slow decaying fluorescence corresponds to the more stable KT-MT[57,58,291]. While the nonKT-MT turnover rates were only slightly changed between the tested conditions (Figure S4C), we observed a significant lower stability of KT-MTs in cells lacking dynein (Figure 4G). We could rescue the lower KT-MT stability in dynein-depleted cells by inhibiting Eg5 activity in these cells, hereby reducing outward force generation (Figure 4G). Strikingly, the rescue of KT-MT stability was dependent on Kif15, since we observed in the morphologically normal bipolar spindles, lacking dynein, Kif15 and Eg5 activity no increase in KT-MT stability (Figure 4G). Taken together, our results show that bipolar spindles are maintained in the absence of inward and outward forces, but these spindles are not functional to form stable KT-MT attachments.

To test if Eg5 inhibition could also alleviate the mitotic delay induced by dynein depletion, we analyzed the ability of control cells and dynein-depleted cells to progress to anaphase in the presence or absence of STLC. To by-pass the requirement for Eg5 in bipolar spindle formation and to prevent the formation of monoastral bipolar spindles, we added STLC after cells had formed a bipolar spindle. Control cells, Kif15-depleted cells and control cells treated with STLC all rapidly entered anaphase (Figure 4F). In contrast, Kif15-depleted cells treated with STLC collapsed into a monopolar spindle and arrested in mitosis for a prolonged amount of time (Figure 4F). Dynein-depleted cells showed a strong mitotic delay, consistent with an important role for dynein in mitosis. However, addition of STLC to dynein-depleted cells reduced the time to enter anaphase with almost 50% compared to dynein-depletion alone, (Figure 4F), further demonstrating the importance of a correct force balance in the spindle for KT-MT attachment and chromosome alignment. Interestingly, inhibiting both Eg5 and Kif15 in dynein-depleted cells could not alleviate the mitotic delay (Figure 4F), consistent with the lack of stable KT-MT attachments in the absence of all three motors. Together, these results show that while an apparently normal bipolar spindle can be maintained in the absence of Eg5, Kif15 and dynein, these spindles are in fact highly defective and unable to sustain KT-MT attachments or satisfy the spindle checkpoint.

Discussion

To investigate the functional interaction between several force-producing motors in mitosis, we inhibited inward and outward force-generating motors and investigated the effect on spindle bipolarity and chromosome segregation. Consistent with previous reports[29,57,227] we find that inhibition of Eg5 from the start of mitosis results in monopolar spindles. Simultaneous depletion of dynein allows bipolar spindle formation, and we now show that this is driven by Kif15. In addition, we show that Eg5 and dynein directly oppose each other and that the percentage of bipolar spindles in cells lacking Kif15 activity is dependent on the ratio of Eg5 and dynein. In the absence of both Eg5 and Kif15 centrosome separation is completely blocked, independent of the presence or absence of inward force generators. Interestingly, when Eg5, Kif15 and dynein are simultaneously inhibited in preassembled bipolar spindles, spindles remain bipolar. Thus, when the major inward force producer dynein is absent, spindles remain bipolar, even when there is no outward force present. Interestingly, loss of dynein results in severe defects in spindle pole focusing, but pole focusing is significantly improved upon inhibition of either Kif15 or Eg5. This shows that excessive outward force is also damaging to the spindle and results in spindle pole splaying, indicating that the activity of antagonistic motors must be carefully balanced.

Besides Eg5, Kif15 and dynein, several other factors contribute to force generation in the spindle. Many different motors and MAPs contribute to proper bipolar spindle assembly. For example, kinesins associated with chromosome arms can generate an outward pushing force[287,292,293], dynein at the cortex can provide a pulling force on astral microtubules[25,31,283,294] and a role for kinetochores was also found in pushing centrosomes apart[238,283]. Nonetheless, we find that in the absence of Eg5 and Kif15 none of these forces are sufficient to allow bipolar spindle assembly, even when the major minus-end directed force generator dynein was removed. Similarly, we were unable to find any condition that leads to collapse of a metaphase spindle in the absence of dynein. Taken together, this suggests that in mammalian cells Eg5 and Kif15 are the main drivers of spindle bipolarity, while dynein is their major antagonizer.

When all three motors are inhibited in preformed bipolar spindles, spindles do not collapse. Nonetheless, cells delay in mitosis with kinetochores that lack tension and stain positive for Mad2. But why are KT-MT attachments defective in the absence of the major inward and outward forces? In order to maintain stable KT-MT attachments, tension has to be generated on sister KT pairs by MTs of the mitotic spindle[29,50,51,70,124,137,151,289,290]. Although attachments are formed in the absence of Eg5, Kif15 and dynein, there is a clear reduction in KT-MT stability, which may lead to less stable attachments, reduced inter-KT distance and defects in chromosome alignment. Based on these findings, we propose that a correct force balance is required to establish sufficient tension on kinetochores to stabilize KT-MT attachments.

While our data clearly show how the balance between inward and outward forces acting on centrosomes contribute to bipolar spindle assembly, they do not resolve the direct consequences of this force balance on KT positioning and inter-KT tension. We assume that the reduced inter-KT tension that we observe upon perturbation of the force balance is an indirect consequence of destabilization of KT-MT attachments. The exact molecular mechanism of how in- and outward forces on spindle poles are translated to kinetochore positioning and tension will need further investigation.

In contrast to HSET, dynein and Eg5, antiparallel MT sliding has so far not been demonstrated for Kif15, so it remains to be determined how Kif15 generates force in the spindle. A recent study implied that Kif15 acts exclusively through K-fibers[60,230]. However, cells depleted of Hec1, and thus lacking K-fibers, do not readily collapse upon inhibition of Eg5 (Figure S1A, S4D, S4E and[63,295], but co-depletion

of both Hec1 and Kif15 does result in robust spindle collapse upon Eg5 inhibition (Figure S4D and S4E), strongly arguing that Kif15 can function independently of KT-MT fibers. Furthermore, it was suggested that Kif15 can only promote spindle bipolarity when overexpressed[60], but we show here that in the absence of Eg5 and dynein, endogenous Kif15 fully supports bipolar spindle assembly, further confirming its role as a key player in spindle assembly.

Taken together, our results show that tight coordination of three motors, Eg5, Kif15 and dynein controls proper spindle assembly, demonstrating the importance of a proper force balance; 1) when excessive inward forces are present, monopolar spindles are formed, 2) when excessive outward forces are present, spindle poles splay. Our results address an important outstanding question; why are antagonistic motors present if their removal results in morphologically normal bipolar spindles? We show that in the absence of the major inward and outward force producing motors, spindles may superficially appear normal, but they are incapable of forming correct KT-MT attachments and chromosome segregation. Together, these results provide new insights into to the intricate force balance that controls robust bipolar spindle assembly and ensures faithful chromosome segregation.

Experimental Procedures

Transfections, drug treatment, immunofluorescence and time-lapse microscopy

U2OS cells were transfected twice with the indicated siRNAs to a final concentration of 20nM. In experiments using double siRNA treatment, the indicated siRNAs were diluted 1:1 to a final concentration of 20 nM. Cells were blocked in thymidine 48 post-transfection. After 24 hours, cells were released from the thymidine block and treated as indicated, followed by live-cell imaging, fixation or lysis. STLC was diluted in DMSO and used with the indicated concentrations. For MG132/STLC treatment, a final concentration of 40 μM STLC was used. MG132 and nocodazole were dissolved in DMSO and used with a final concentration of 5 μM and 250ng/ml respectively. (Live-cell) images were acquired using a Deltavision deconvolution microscope (Applied Precision). See Extended Experimental Procedures for a detailed description.

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Author contribution

RGHPH, MET and RHM designed the experiments. RGHPH carried out and analyzed the experiments. RGHPH, MET and RHM wrote the manuscript.

Chapter 5

Kif15; a useful target for anti-cancer therapy?

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Abstract

Kif15 (kinesin-12) was discovered a decade ago as a kinesin motor involved in bipolar spindle assembly. Although its exact molecular function is still under debate, data from recent studies indicate that Kif15 cooperates with Eg5 (kinesin-5) to promote centrosome separation and bipolar spindle assembly. Due to the essential function of Eg5 in bipolar spindle assembly, inhibitors of this protein recently entered clinical trails as anti-cancer therapy. However, several studies from past years indicate that Eg5 is not essential for bipolar spindle assembly under certain conditions and Kif15 seems to the central player in mediating Eg5-independent bipolar spindle assembly. In this chapter, we describe the function of Kif15 during mitosis and discuss if Kif15 could be a target for drug development in anticancer therapy.

Introduction

Centrosome separation and bipolar spindle formation is essential for chromosome segregation during mitosis. The bipolar spindle is a highly dynamic structure composed of microtubules (MTs) and many microtubule associated proteins (MAPs). In mammalian cells, the centrosomes play an important role in regulating spindle bipolarity, by acting as the major microtubule-organizing center (MTOC) in cells. Before nuclear envelope breakdown (NEB), the centrosomes separate and move to opposite sites of the nucleus. After NEB, MTs with their minus ends embedded in the centrosomes, interact with the chromosomes with their plus ends to form the bipolar spindle[25].

One of the main drivers of bipolar spindle formation is the highly conserved homotetrameric kinesin Eg5 (also known as kinesin-5 / Kif11). Due to its unique tetrameric configuration, it is thought that Eg5 can crosslink antiparallel MTs and subsequently slide them apart using its plus-end-directed motor activity. Indeed, *in vitro* studies showed that Eg5 preferentially crosslinks MTs in an antiparallel configuration and moves on both MTs that it crosslinks[27,28,53] Depletion or inhibition of Eg5 blocks centrosome separation both before as after NEB and these cells arrest in mitosis with a characteristic monopolar spindle[27,29-31,52].

While previous data clearly indicate an essential role for Eg5 in bipolar spindle assembly, recent studies indicate that additional, Eg5-independent pathways exist that cooperate with Eg5 to drive bipolar spindle formation. It was shown that bipolar spindle formation could be rescued by simultaneous inhibition of Eg5 and a minus-end-directed motor (dynein or kinesin-14[31-33,62,64]). In addition, Eg5 activity is not essential for maintenance of the metaphase spindle, since established bipolar spindles do not collapse into a monopolar state upon Eg5 inhibition[57,58,228,295]. Moreover, it was shown that both *C. elegans* and *Dictostelium* do not require Eg5 for bipolar spindle formation[48,49].

The fact that Eg5 is dispensable for bipolar spindle formation under certain conditions led to the suggestion that additional motors might exist that cooperate with Eg5 to drive bipolar spindle formation. One of these main candidates to cooperate with Eg5 is the kinesin-12 family member Kif15.

Identification of Kif15

Kif15 belongs to the kinesin-12 family and was originally identified as a novel kinesin-like protein (KLP) in Xenopus oocytes[296]. Early studies showed that Xklp2 (Xenopus Kif15) is a relatively slow plus-end-directed motor[54] and is localized to the centrosomes and to the spindle MTs. The first evidence for a role in centrosome separation and bipolar spindle assembly came from *in vitro* spindle assembly experiments using injection of dominant negative Xklp2 protein and inhibitory antibodies to the extracts[54]. Addition of a truncated Xklp2 construct, lacking the N-terminal motor domain, blocked centrosome separation and led to the formation of abnormal spindles, including monopolar spindles[54]. Similar to addition of truncated Xklp2 constructs, injection of Xklp2 inhibitory antibodies also efficiently blocked centrosome separation and bipolar spindle formation and resulted in the formation of monopolar spindles[54]. Interestingly, the study of Boleti *et al.,* showed that the C-terminal domain of Xklp2 was sufficient for its localization to spindle poles[54]. This led to the suggestion that the C-terminus of Xklp2, containing a conserved leucine-zipper (Figure 1A), acts a non-motor MT targeting domain. Follow-up studies by the Vernos lab identified a novel MAP, named TPX2 (Targeting Protein for Xklp2) required for MT targeting of Xklp2 C-terminal leucine zipper[297].

Kif15 domain structure

Similar to most other kinesins, Kif15 contains an N-terminal motor domain of about 350 amino acids that has about 40% identity to other kinesins (Figure 1A). The C-terminal part of the protein is predicted

to form a coiled coil structure, hereby making it a unique member of the kinesin family. In addition to that, Kif15 contains two conserved leucine zipper domains (Figure 1A). The second, C-terminal leucine zipper was previously identified to be required for MT targeting of Xenopus Xklp2[54,297]. Studies in human cells confirmed that Kif15 is recruited to spindle MTs due to interaction between the C-terminal Leucine zipper and TPX2 and that the motor domain is not required for MT targeting[57].
While Human Kif15, mouse Kif15 and Xenopus Xklp2 are highly conserved (86% and 61% identical respectively), the C. elegans kinesin-12 KLP-18 is only about 41% identical and lacks a part of the extended C-terminus present in other kinesin-12 members (Figure 1B). Similar to other kinesin-12 family members, its entire C-terminus is predicted to form a coiled coil structure. Although the C-terminus of KLP-18 contains a leucine zipper motif, it is currently unknown if KLP-18, similar to human and Xenopus Kif15, requires an interaction with a targeting protein for its spindle localization. An obvious ortholog of TPX2 has not been identified in C. elegans, indicating why the C-terminus of KLP-18 might diverge so much from the Kif15 orthologs in organisms that do have a clear TPX2 ortholog. A recent study proposed the existence of a second, non-motor MT-binding domain in the first coiled-coil of Kif15[277]. The exact function of the second MT-binding domain will is discussed below in more detail.

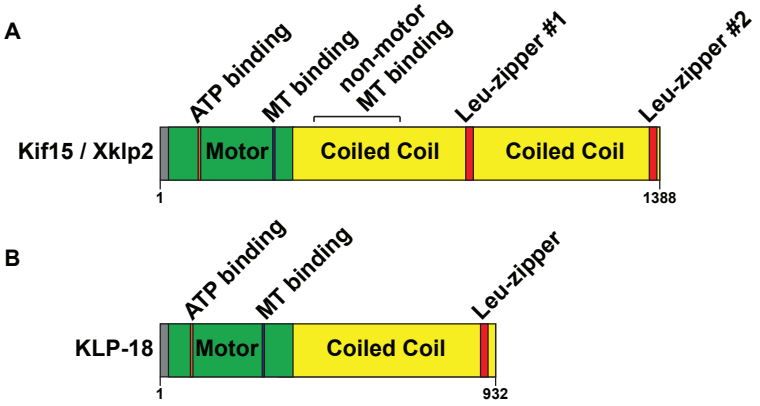

Figure 1. Schematic representation of protein domains present in Kif15.

(A) Human Kif15 and Xenopus Xklp2 are about 61% identical and contain a N-terminal kinesin motor domain, a long C-terminal coiled coil structure that is characteristic for kinesin-12 members. Furthermore, the protein contains two conserved leucine zippers, of which Leucine zipper #2 is thought to be required for MT targeting of the protein by its interaction with the MT binding protein TPX2. The recently proposed second, non-motor MT-binding site is also depicted. (B) The *C. elegans* ortholog KLP-18 is less well conserved (about 41% identical). Similar to other family members, its C-terminus is predicted to form a coiled coil structure and it contains also a leucine zipper in the C-terminus. Currently it is unknown if KLP-18 requires a TPX2-like protein for spindle localization.

Kif15 function in centrosome separation and bipolar spindle assembly

While early studies proposed important roles for Kif15 in centrosome separation and bipolar spindle assembly, subsequent studies in different experimental spindle assembly systems suggested that Kif15 is not essential for bipolar spindle assembly[298,299]. For example, studies using plasmid DNA-coated bead incubated with Xenopus extracts showed that Xklp2 does not have an important role during spindle assembly[298]. Only conditions in which Xklp2 was inhibited in combination with either dynein or the

minus-end-directed kinesin-14 XCTK2 led to increased spindle defects compared to either inhibiting dynein or XCTK2 alone[298]. These results where somewhat surprising as they contradicted the data from the original study that led to the identification of Kif15[54]. However, in the study by Walczak et al., the experimental setup lacked centrosomes, which might suggest that Xklp2 is specifically required to push centrosomes apart in Xenopus and is therefore not absolutely required for acentrosomal spindle formation.

In addition to spindle assembly in Xenopus egg extracts, Kif15 was also found not to be essential for spindle formation in human cells. This was demonstrated by Zhu et al., in a systematic RNAi screen to test mitotic functions of all human kinesins and the dynein motor complex[299]. This also, suggested that Kif15 function is either redundant or not required for centrosome separation and bipolar spindle assembly.

Subsequent evidence for a potential role of Kif15 in bipolar spindle assembly came from studies conducted in sea urchins and *C. elegans*[55,300]. The sea urchin kinesin-12 KRP_{180} localizes to interpolar MTs and was shown to be important to maintain a bipolar spindle in prometaphase embryos[300]. Injection of inhibitory antibodies or expression of a dominant negative mutant in prometaphase resulted in bipolar spindle collapse in which the poles moved towards each other in a linear fashion[300]. This data suggested that KRP_{180}, similar to Eg5 in other experimental systems, might slide MTs outward, hereby promoting centrosome separation and spindle elongation. In the absence of KRP_{180}, antagonistic minus-end-directed motors, such as dynein and/or kinesin-14 could possibly drive this spindle collapse and promote monopolar spindle formation.

While Eg5/kinesin-5 was reported to be essential for bipolar spindle assembly in nearly all organisms tested, the sole *C. elegans* kinesin-5, BMK-1 was shown not to be essential[48]. *Bmk-1* homozygous deletion mutants do display reduced fertility, but these animals are otherwise fully viable and do not display any defects in bipolar spindle formation or cytokinesis, suggesting that in *C. elegans*, other motors must be present that drive centrosome separation and bipolar spindle assembly[48]. Interestingly, while Eg5/kinesin-5 is not essential in *C. elegans*, the *C. elegans* kinesin-12 KLP-18 is essential[55]. Similar to kinesin-12 homologs in other organisms, KLP-18 localizes to the spindle poles during prometaphase, metaphase and early anaphase and subsequently transfers to the spindle midzone during late anaphase and telophase[55]. RNAi depletion of KLP-18 showed that its function is specifically required for acentrosomal (female meiotic) spindle formation, causing the mutant to produce embryos with abnormal maternal DNA content[55]. This is in striking contrast to its proposed function in Xenopus, where Xklp2 seems to be required for spindle assembly in the presence of centrosomes[54]. The exact molecular mechanism how KLP-18 contributes to meiotic spindle assembly in *C. elegans* is not fully understood, but its depletion prevents parallel bundling of MTs that are nucleated near meiotic chromosomes and as a result, the formation of disorganized spindles[55]. This might also indicate why KLP-18 is not absolutely required for mitotic divisions that occur in the presence of centrosomes, as centrosomes can play a dominant role in the ordering of the MTs, by sequestering the minus ends towards the centrosomes. MTs in acentrosomal spindles are nucleated in random directions at or near the chromatin and under these circumstances KLP-18 is likely essential to organize and bundles these MTs into (anti)-parallel arrays. A more recent study indicated that KLP-18 is required for the establishment and/or maintenance of spindle bipolarity in oocytes; high-resolution analysis of KLP-18 RNAi-treated worms indicated the spindles in oocytes are monopolar[56]. Similar to previous studies, this study shows that KLP-18 is only required for bipolar spindle formation in meiosis, suggesting that in mitotic cell divisions of C. elegans, the possible contribution of KLP-18, but also of Eg5/kinesin-5 in bipolar spindle formation, might be masked by other motors.

In addition to C. elegans, recent studies in human cells also provided clear evidence for a role of Kif15 in bipolar spindle formation[57-59]. Depletion of Kif15 in human cells strongly sensitized cells to partial Eg5 inhibition[57-59], indicating that Kif15 cooperates with Eg5 during bipolar spindle assembly. In addition to that, the maintenance of a bipolar spindle in Eg5-inhibited cells[57,58], and bipolar spindles formed in the complete absence of Eg5 and the minus-end-directed motor dynein[31,59], were shown to be fully dependent on Kif15. This indicates that Kif15 acts redundant with Eg5 during bipolar spindle formation; cells form normal bipolar spindles in the absence of Kif15 and do not show major spindle defects[57,58] while metaphase spindle length in Kif15-depleted cells was on average only 11% shorter compared to control cells[57,58]. Conversely, inhibition of Eg5 in these human cells prevents bipolar spindle formation, indicating that the activity of Kif15 is insufficient to promote centrosome separation and bipolar spindle formation in the absence of Eg5 activity. However, over-expression of Kif15 in Eg5-inhibited cells fully rescued bipolar spindle formation [57]. These results strongly suggest that Kif15 is, similar to Eg5, able to generate an outward force during bipolar spindle formation. In line with data from Xenopus, human Kif15 requires TPX2 for its function[57,58]. Depletion of TPX2 or expression of Kif15 truncation mutants lacking the C-terminal leucine zipper, prevents Kif15 from binding to the spindle MTs[57,58] and these mutants fail to promote bipolar spindle formation in cells lacking Eg5 activity. It is however, not completely clear why the endogenous Kif15 is incapable of promoting bipolar spindle formation under circumstances where Eg5 is inhibited. Possibly, this is due to low levels of endogenous expression, but it should also be noted that TPX2 is strictly nuclear during interphase and prophase. Due to the physical separation of Kif15 and TPX2 during prophase, Kif15 is unable to replace Eg5's function during prophase centrosome separation[57]. In the absence of Eg5 activity cells will therefore enter mitosis with the centrosomes in close proximity, regardless of the expression level of Kif15. Indeed, the capacity of Kif15 to drive bipolar spindle formation strictly depends on the initial separation of the centrosomes; cells with partial loss of Eg5 activity only assembled bipolar spindles when their centrosomes were at least partially separated during prophase[57]. In addition to this, Eg5-independent human cells (EICs) described in a recent study, also critically depend on prophase centrosome separation in order to assemble a bipolar spindle[32]. These cells, that can proliferate in the complete absence of Eg5 activity, require both nuclear envelope (NE)-associated dynein and Kif15 for prophase centrosome separation and bipolar spindle formation respectively[32,45]. Thus, the data available at present are most compatible with a model in which Kif15, in complex with TPX2 can crosslink and slide antiparallel MTs (Figure 2).

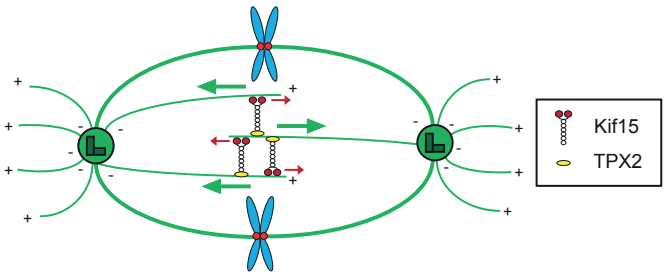

Figure 2. Model of Kif15 function during bipolar spindle formation.

TPX2 targets Kif15 to the spindle through the interaction with the C-terminal leucine zipper of Kif15. The complex of Kif15 and TPX2 can crosslink anti-parallel MTs and the plus-end-directed motility of Kif15 can slide anti-parallel MTs apart, hereby generating a force similar to Eg5. The arrows indicate the movement of motor (red) and the MTs (green).

Kif15 function in the regulation of kinetochore-microtubule dynamics

A recent study suggested that Kif15 and Eg5 act on distinct sets of MTs[60]. While Eg5 does not seem to discriminate between kinetochore-microtubules (KT-MTs) and interpolar MTs, Kif15 recruitment to the spindle seems to depend primarily on KT-MTs and the spindle-associated pool of Kif15 increases in metaphase cells compared to prometaphase cells[60]. Indeed, bipolar spindles collapsed upon Eg5 inhibition in cases where the KT-MT where removed, while unperturbed bipolar spindles did not collapse upon Eg5 inhibition. These data indicate that Kif15's contribution to bipolar spindle maintenance is likely mediated via KT-MTs, instead of interpolar MTs[60]. Strikingly, Kif15-depleted cells initially build a longer metaphase spindle, followed by a contraction phase until the spindle reaches the characteristically shorter spindle[60]. This contraction phase is KT-MT-dependent since cells co-depleted of Kif15 and Nuf2 produced longer spindles that do not show the subsequent contraction phase[60]. These data suggest that at physiological levels, Kif15 depends on KT-MTs for force generation. Related to Kif15's function in regulating KT-MT length, a recent paper identified Eg5 and Kif15 as key regulators of chromosome movements, by being part of a possible KT-MT crosslinking structure[301]. This potential crosslinking structure mediates coupled movement of neighboring chromosomes during mitosis. Although the molecular mechanism underlying this coupled movement of neighboring chromosomes is still unknown, Kif15 is suggested to act as a mechanical coupling factor of KT-MTs in metaphase and is limited in its action by Eg5[301]. Inhibition of Eg5 leads to a loss of periodic chromosome oscillations and increased coupling of neighboring chromosomes that can be partially rescued by Kif15 depletion[301]. Similar to the study of Sturgill and Ohi, Eg5 and Kif15 seem to antagonize each other in this process.

Kif15-dependent bipolar spindle assembly in Eg5-independent cells

While the study of Sturgill et al. suggests that Kif15 can antagonize the activity of Eg5, there is a considerable amount of evidence that indicates that Kif15 can produce a force within the spindle that can separate the centrosomes. The strongest support in favor of a role for Kif15 during centrosome separation comes from the fact that Kif15 overexpression can fully rescue bipolar spindle assembly when the activity of Eg5 is blocked[57]. Additionally, several labs have recently generated human cell lines that can proliferate in the absence of Eg5, but in contrast to the parental cells the Eg5-independent derivatives have become absolutely dependent on Kif15 activity for bipolar spindle assembly[32,60]. The OL-EIC1 cells, generated by the Ohi lab depend on the over-expression of Kif15 for survival. The data generated with these cells indicate that while Kif15, localized to KT-MTs opposes Eg5 activity during bipolar spindle assembly in normal cells, Kif15 over-expression in OL-EIC1 cells results in mislocalization of Kif15 to interpolar MTs, hereby generating a force that can separate centrosomes[60]. EICs generated independently in our own lab were also shown to depend on Kif15 activity, but in addition, we found that NE-associated dynein is also essential in these cells in order to promote prophase centrosome separation when Eg5 activity is absent[32]. Due to the activity of this dynein-dependent prophase pathway, these cells enter prometaphase with separated centrosomes, hereby creating a situation that likely results in a strong bias towards bipolar spindle assembly as has been shown previously[57,64]. Importantly, two of the three EICs clones that were generated in our lab did not show any signs of Kif15 over-expression, which shows that physiological levels of Kif15 can drive centrosome separation. If this outward force produced by endogenous Kif15 depends on its binding to KT-MTs, it seems most logical that this KT-MT-associated Kif15 can crosslink with an anti-parallel interpolar MT and slide the MTs apart.

In vitro properties of Kif15

Recently, two studies were published in which the in vitro properties of Kif15 were tested to gain more insights into its in vivo function[277,302]. The first study by Drechsler et al., showed that Kif15 is a processive, plus-end-directed motor[302]. In addition to plus-end-directed movement, also short durations of minus-end-directed movement and diffusive movement along the MT lattice were also observed[302]. However, the most striking result from the study of Drechsler et al., was the observation that Kif15 forms a homotetrameric complex, similar to Eg5[27,302]. The authors show that the homotetrameric nature of Kif15 enables it to efficiently switch MT tracks, hereby allowing Kif15 to navigate through MT networks[302]. In addition to track switching, Kif15 complexes are also able to dynamically crosslink MTs bundles[302]. Its ability to crosslink MTs and its tetrameric configuration hints towards an additional function in which Kif15 slides MTs apart as has been shown previously for Eg5[28]. This would also fit with the current models for Kif15 function in vivo[57-59]. However, the authors found only short, bidirectional MT sliding events and no evidence for continuous MT sliding as has been shown for Eg5. This result still does not fully rule out a function of Kif15 in anti-parallel sliding of MTs and additional factors might be involved in this function in vivo. One of the candidate factors supporting Kif15 function in vivo might be TPX2[57]. Drechsler et al., tested the contribution of TPX2 to Kif15 function in vitro. They did not observe strong evidence for a stable complex between Kif15 and TPX2 at low concentrations of both proteins. However, in agreement with in vivo results, TPX2 did enhance the MT binding affinity of Kif15[57,303], suggesting that TPX2 promotes Kif15 function. However, rather than enhancing Kif15 activity, TPX2 blocked processive runs of Kif15 and increased the force-holding capability of MT-bound Kif15[302]. This suggests that TPX2 rather acts as an inhibitor of Kif15 processivity and might promote its MT crosslinking activity.

The second study investigating the in vitro properties of Kif15 by the Ohi lab showed some contradictory results[277]. In contrast to the first study, Sturgill et al., found that Kif15 forms homodimers rather than appearing as a homotetramer and is self-repressed during interphase by its C-terminal tail[277]. Indeed, while recombinant expressed full-length Kif15 did not show any signs of motility on glass-anchored MTs, the same construct showed processive behavior when it was in complex with an antibody targeted to the C-terminal tail of Kif15[277]. The exact nature of self-repression is still unclear, but based on size-exclusion chromatography, the self-repressed state is salt-dependent suggesting that intermolecular electrostatic interactions maintain the self-repressed form[277]. Similar to the study by the McAinsh lab, Sturgill et al., found that Kif15 is able to crosslink MTs. However, while Drechsler et al., postulate that MT crosslinking by Kif15 is mediate by its tetrameric configuration, Strurgill et al., identified a second, non-motor MT-binding site in the first long coiled-coil domain of Kif15, required for its MT crosslinking activity. In addition to crosslinking, the authors also show that Kif15 can slide crosslinked MTs, which was not observed in high frequencies in the study by Drechler et al. While many self-repressed kinesins become activated upon binding of activating proteins or by phosphorylation[304], Kif15 was not able to bind MTs upon addition of its proposed co-factor TPX2[277]. In contrast, Kif15 was able to bind MTs when it encountered MT bundles, suggesting that a second MT relieved its self-repressed state[277]. Since TPX2 is an important factor for MT nucleation and bundling in the spindle[305], this observation could also explain how TPX2 might indirectly mediates spindle localization of Kif15.

Although some contradictory results, the studies by Drechler et al., and Sturgill et al., shed new light on the function of Kif15 in the spindle. Further in vitro analysis of Kif15 will be required to determine the exact function of Kif15 in the spindle.

Kif15 as a potential target for drug development

Currently, it is unclear if Kif15 plays an important role in cancer. Data from cancer genome expression show no clear up- or downregulation of Kif15. Also, Kif15 mutations are found in not more than 1% of cancer genomes present in the COSMIC database, from which 75% are missense substitutions, 13% are synonymous substitutions and 4% are nonsense substitution. None of the identified missense mutations have thus far been shown to interfere with Kif15 function.

In contrast to cancer genome data, recent studies indicate that alteration in Kif15 expression levels might have major implications for anticancer therapies that employ inhibitors of Eg5 to inhibit cell proliferation[32,57,60]. Tanenbaum et al., showed that 5- to 10-fold ectopic overexpression of Kif15 is sufficient to drive bipolar spindle assembly in the absence of Eg5 activity[57]. Follow-up studies in which EICs were generated using an *in vitro* 'directed evolution' approach indicated that enhanced expression of the endogenous Kif15 can be induced by prolonged exposure to Eg5 inhibitors and this might contribute to resistance against these inhibitors[32,60]. While it is clear that other mechanisms besides Kif15 over-expression can result in the growth of EICs[32], all EICs lines that have currently been tested invariably depend on Kif15 activity, also in cases where the protein is not overexpressed[32,60].

In addition to the studies describing EICs, a recent study found that Eg5 activity becomes dispensable for bipolar spindle assembly in cells with altered MT dynamics[306]. Treatment of cancer cells with low doses of nocodazole (suppresses MT dynamics) or treatment with siRNAs against TOGp (a MAP required for MT nucleation) rescued bipolar spindle assembly in cells treated with an Eg5 inhibitor[306]. Live cell analysis of spindle formation in the cells with reduced MT dynamics revealed that these cells enter mitosis with non-separated centrosomes, followed by the formation of multiple MTOCs that eventually organize into a bipolar spindle with separated centrosomes[306]. Similar to EICs, these cells now fully depend on the activity of Kif15 for bipolar spindle assembly[306].

The identification of these Eg5-independent mechanisms for centrosome separation and bipolar spindle assembly have important implications anticancer therapies that employ Eg5 inhibitors to perturb cell division. The data from these studies indicate that Kif15 is the central player driving Eg5-independent bipolar spindle assembly. First, Kif15 is required for maintenance of bipolar spindle in metaphase when Eg5 is inhibited[57,58]. Second, Kif15 is required for bipolar spindle assembly in Eg5-inhibited cells with reduced MT dynamics[306]. Third, Kif15 over-expression can bypass the requirement of Eg5 in human cells[57]. In fact these cells can form a normal bipolar spindle when Eg5 activity is completely blocked, and their proliferative capacity is not compromised by the inhibition of Eg5[57]. As such, mere overexpression of Kif15 appears to be sufficient to promote resistance to Eg5 inhibitors. Finally, bipolar spindle assembly in cells co-depleted of both Eg5 and the minus-end-directed motor dynein depends on Kif15 activity[59]. Thus, while Eg5 inhibitors show potent anti-tumor activity both in vitro and in vivo (reviewed in[246], these recent studies indicate that changes in MT dynamics or upregulation of Kif15 expression can easily bypass Eg5 inhibition and promote drug resistance. Currently, it is unclear if upregulation of Kif15 occurs in tumors that are treated with Eg5 inhibitors and show signs of resistance. Nonetheless, since multiple Kif15-dependent mechanisms can easily promote resistance to Eg5 inhibitors, the eventual success of an anti-tumor strategy involving inhibition of Eg5 will likely require co-administration of Kif15 inhibitors to prevent rapid acquisition of therapy resistance and the combination might also prove to have a more potent anti-tumor activity. Obviously, such a combinatorial regimen might trigger more pleiotropic side effects, especially if Kif15 also has functions that are not limited to driving bipolar spindle assembly in mitosis. In this respect it is important to note that the essential co-factor for Kif15's role in spindle assembly, TPX2, is localized in the nucleus in interphase. Thus, if one were to be able to specifically target the interaction between Kif15 and TPX2, this could possible limit interference to the mitotic function of Kif15 only.

Summary and future perspectives

Members of the kinesin-12 family play an evolutionary conserved role during bipolar spindle assembly. Although the exact contribution of kinesin-12 members to bipolar spindle assembly in different organisms is still not fully understood, it is clear that in human cells, Kif15 is a central player in Eg5-independent mechanisms of bipolar spindle assembly. Inhibitors of Kif15 might therefore increase the efficacy of Eg5 inhibitors and are likely to reduce the occurrence of resistance to inhibitors of Eg5. Development of specific Kif15 inhibitors could therefore be of great value in therapy and will certainly be useful to study the functions of Kif15 in more detail.

Chapter 6

A haploid genetic screen in human cells identifies novel factors that control chromosome segregation

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Abstract

The spindle assembly checkpoint (SAC) is a highly conserved signaling cascade that ensures faithful segregation of chromosomes during mitosis. Deletion of essential SAC components, including Mad1 and Mad2 leads to severe aneuploidy and is thought to be detrimental for all human cells. Here, we show that haploid human HAP1 cells can survive without a functional SAC. By performing an insertional mutagenesis screen in SAC-deficient HAP1 cells we were able to identify genes that are essential for viability of SAC-deficient cells. Besides known regulators of chromosome congression, such as CENP-E, HURP and EB1, we identified several unexpected synthetic lethal interactors of Mad1 and 2. Amongst those were all of members of the condensin II complex, and Rod1 and Zwint from the RZZ complex. We show that in the absence of condensin II, decatenation of sister chromatids is delayed, leading to massive segregation errors in SAC-deficient cells. We show that cells lacking the RZZ complex are delayed in mitosis due to the presence of transient chromosome alignment defects. Taken together, we have successfully used haploid genetics in human cells to uncover novel factors that promote correct chromosome segregation and that are essential for the viability of cells that lack SAC function.

Introduction

Equal segregation of sister chromatids during mitosis is essential to prevent aneuploidy and maintain cellular fitness. This process is mediated by a microtubule-based structure, called the mitotic spindle, that captures and aligns chromosomes in the center of the mitotic cell[25]. The sister chromatids connect to the mitotic spindle via a large protein complex called the kinetochore (KT), that is assembled on centromeric DNA of every single chromatid (reviewed in[307]). Entry into anaphase, the state in which the sister chromatids are physically separated over the two newly formed daughter cells, will only occur when all chromatids are correctly attached to the spindle in a bipolar fashion (Reviewed in[161]). To prevent premature segregation of the chromosomes, the spindle assembly checkpoint (SAC, also known as the mitotic checkpoint) has evolved (reviewed in[308]). Right after mitotic entry, the molecular components of the SAC localize to the unattached KTs, where they generate a diffusible signal, known as the mitotic checkpoint complex (MCC), that prevents anaphase onset by inhibiting the anaphase promoting complex / cyclosome (APC/C,[307]). Upon attachment of spindle MTs to the KTs, the SAC components are displaced from the KT and the downstream signaling pathway that prevents anaphase onset is turned off[309]. Elegant genetic screens in yeast identified the core components of the SAC[162,163]. These components, Mad1, Mad2, Bub1, BubR1 and Bub3 are essential SAC components that all localize to KTs. Mad2, BubR1 and Bub3 together form the MCC, that sequesters Cdc20 from the APC/C, hereby preventing its activation[167]. In addition, Mad1 and Bub1 mediate the assembly of the MCC by acting as a "hub" at unattached KTs to catalyze the formation of this complex[161,310]. Unless mitosis is perturbed, yeast and *Drosophila* are able to live without a functional SAC[163,311]. In contrast, the SAC is essential in higher organisms where deletion of SAC components leads to massive chromosome missegregation and aneuploidy[312-316]. Curiously, a recent study in mice showed that conditional deletion of Mad2 in the skin is tolerated by epidermal cells but not by hair follicle stem cells[317], indicating that loss of SAC function can be tolerated in certain cell types.

Here we have applied the power of haploid genetics to identify factors that mediate chromosome segregation in human cells. The HAP1 cell line is derived from the chronic myeloid leukemia cell line KBM7[318], and the unique near-haploid nature of these cells allows it to use insertional mutagenesis screening methods[319-323]. Here, we tested the essentiality of the SAC in multiple human cell lines using RNA-guided Cas9 nuclease-based genome editing technology (CRISPR,[324,325]). Using this technique, we found that the HAP1 cell line was able to survive without a functional SAC. In this study, we used SAC-deficient HAP1 cells for an insertional mutagenesis screen to identify genes that become synthetic lethal with loss of the SAC. Using this setup we identified several factors involved in timely chromosome alignment, but are not essential for the formation of stable end-on KT-MT attachments.

Results

HAP1 cells do not require a functional spindle assembly checkpoint

To test the essentiality of the SAC, we knocked out Mad2 in multiple human cell lines using CRISPR/Cas9 (Figure 1A[324]). We selected the cells by co-transfection of a donor plasmid, containing a blasticidin resistance cassette (see materials and method). The blasticidin-resistant colonies were screened by both PCR as by western blot. We did not obtain colonies using HeLa, RPE1 or U2OS cells. However, we obtained many colonies for HAP1 cells and about 90% of the resistant colonies showed integration of the blasticidin cassette in the *Mad2* locus and had no detectable Mad2 protein left on western blot (Data not shown and Figure 1B). We used the same strategy to generate HAP1 *Mad1* knockouts and

similar to *Mad2*, we obtained multiple colonies showing integration of the blasticidin cassette into the first exon of *Mad1* (data not shown).

To confirm the loss of Mad2 function in *ΔMad2* cells, we treated cells with microtubule (MT) depolymerizing drug nocodazole and the proteasome inhibitor MG132 to arrest cells in mitosis with unattached kinetochores. While Mad2 localized to the unattached KTs in wild-type cells, we did not observe any detectable Mad2 on KTs in the *ΔMad2* cells (Figure 1C). Interestingly, we found that anaphases in untreated *ΔMad2* cells were very similar to wild-type cells (Figure 1D) with less than 25% of anaphase cells presenting chromosome segregation defects (Figure 1E). Next we checked if our *ΔMad2* cells showed functional loss of the mitotic checkpoint. We filmed both wild-type and *ΔMad2* cells stably expressing H2B-RFP with or without nocodazole (Figure 1F). Wild-type cells spent on average about 25 to 30 minutes from NEB to anaphase (Figure 1F, left graph). *ΔMad2* cells underwent mitosis even faster and entered anaphase within 20 to 25 minutes after NEB (Figure 1F, left graph). When we treated wild-type cells with nocodazole, they arrested in mitosis for approximately 8 to 10 hours and subsequently underwent apoptosis (Figure 1F, right graph). As expected, *ΔMad2* cells did not arrest in mitosis upon treatment with nocodazole and exited mitosis after about 25 minutes, similar to untreated *ΔMad2* cells (Figure 1F, left graph), indicating that SAC function is completely lost in these cells. Next we performed a growth assay in which we treated wild-type and *ΔMad2* cells with increasing doses of the MT poisons taxol and nocodazole. Lower doses (nanomolar range) of both drugs result in aberrant MT dynamics and induce chromosome congression and alignment defects[220]. Wild-type cells managed to proliferate in concentrations up to 64 nM and 8 nM nocodazole and taxol respectively. However, *ΔMad2* cells only proliferated in concentrations up to 32 nM nocodazole and 1 nM taxol, indicating that the SAC promotes cell survival in cells in which MT dynamics are perturbed. We obtained similar data for *ΔMad1* cells (data not shown) underlining that HAP1 cells do not require a functional SAC during an unperturbed mitosis but the SAC becomes essential when the process of chromosome alignment is delayed.

Haploid genetic screen identifies factors involved in chromosome congression

The haploid nature of the HAP1 cell line allowed us to use our SAC-deficient cells for a genetic screen to identify genes that are synthetic lethal with loss of the SAC. In short, we mutagenized wild-type, *ΔMad1*, and *ΔMad2* cells with a retroviral gene trap vector that randomly inserts into the genome[320]. The gene trap inserts preferentially in intronic regions near the start of actively transcribed genes and can either integrate in sense (thereby inactivating the gene) or in antisense orientation (not inactivating the gene when inserted into intronic regions (Figure 2A,[319,320]). After infection with the gene trap, we cultured the cells for 10 days. After 10 days, we extracted the genomic DNA from the cells and used deep-sequencing to retrieve all the gene trap insertion locations[320]. Next, we determined for each individual gene the ratio of the number of sense integrations, divided by the number of total (sense + antisense) integrations. If a particular gene is not essential for cell survival, the expected ratio will be near 0.5 since the direction of the integrated gene trap is random. However, in case of an essential gene, cells with integrations sense direction will be depleted from the population and the expected ratio will be below 0.5.

After mapping all integrations of the gene trap in the wild-type, *ΔMad1*, and *ΔMad2* cells, we plotted the number of integrations versus the ratio of the number of sense integrations divided by the number of total integrations for every targeted gene (Figure 2B-C). The majority of the genes in all cell lines have a ratio near 0.5, indicating that disruption of these genes does not have a significant effect on cell viability. In addition, a number of genes show a bias towards integration in the antisense direction (ratio < 0.5), indicating that these genes are important for cell viability (sense integrations are depleted

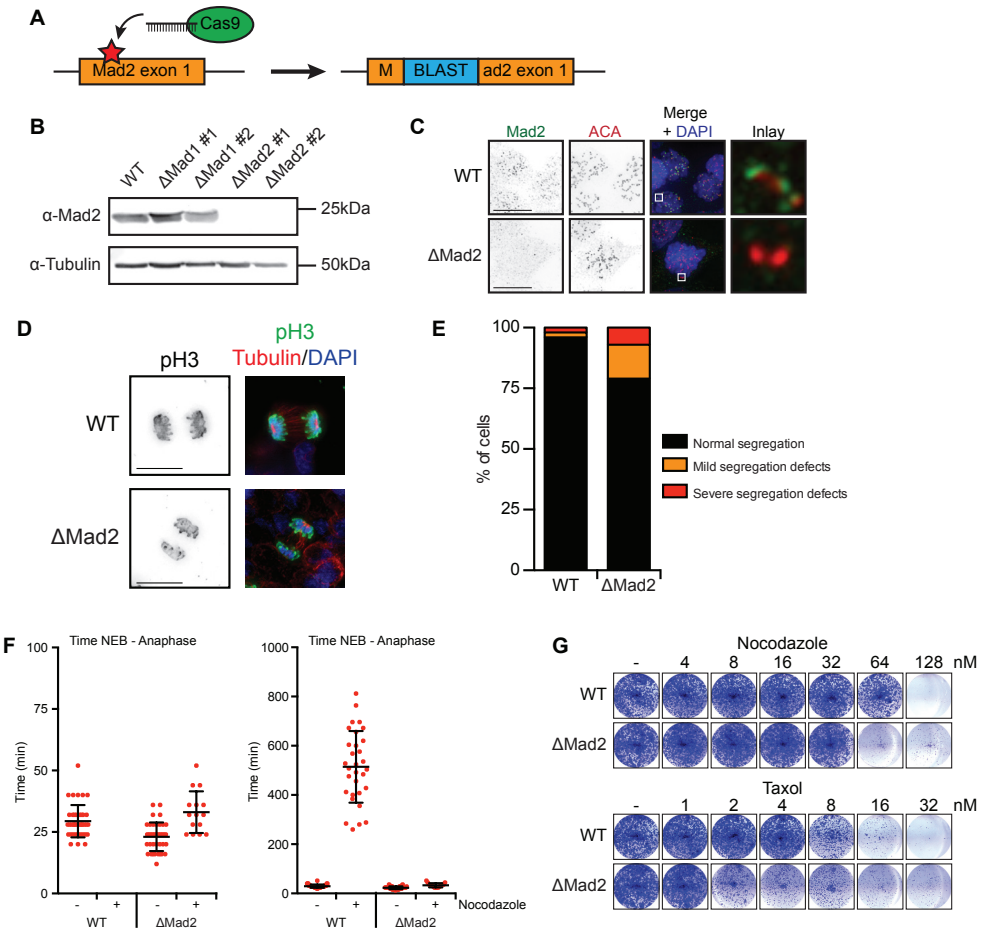
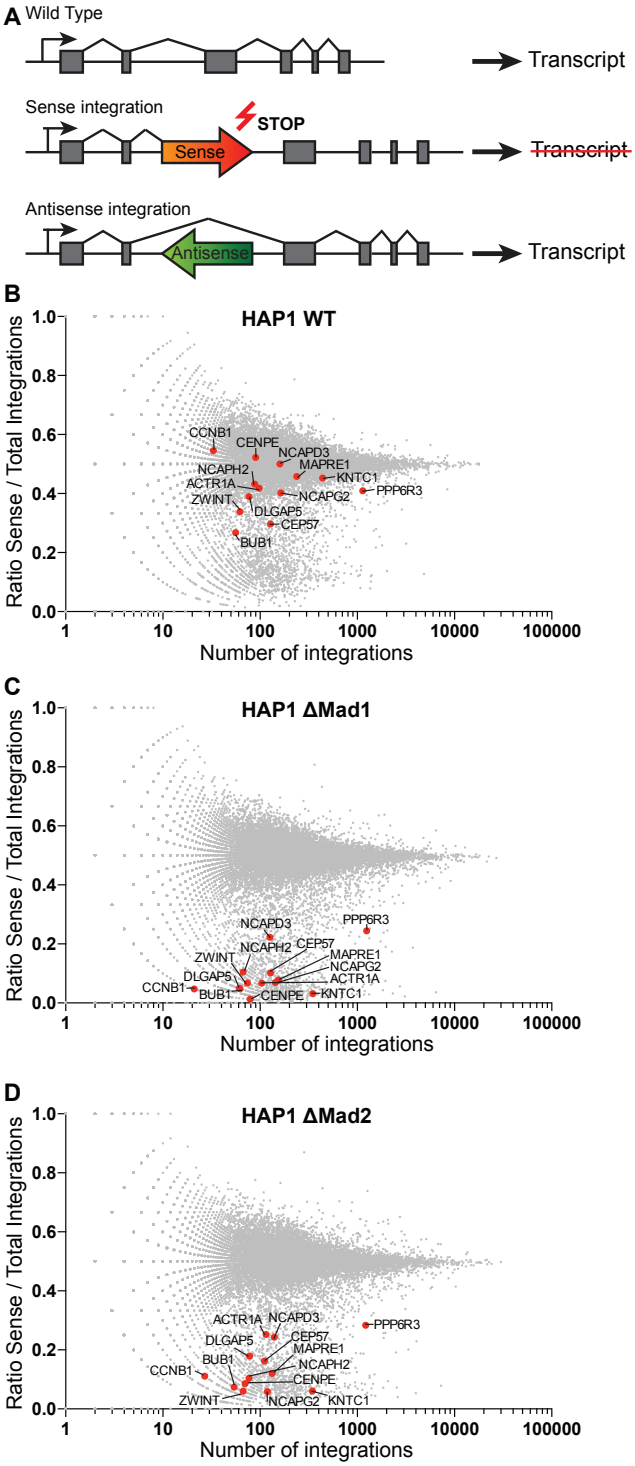


Figure 1: HAP1 cells do not require a functional SAC.

(A) Schematic representation of CRISPR/Cas9-mediated genome editing for the generation of SAC-deficient HAP1 cells. Integration of a blasticidin resistance cassette was used to disrupt the first exon of *Mad2*. (B) Wild-type, *ΔMad1* and *ΔMad2* cells were harvested and Mad2 protein levels were analyzed by western blot. Tubulin was used as loading control (C) Representative images of Wild-type and *ΔMad2* cells, arrested in mitosis with nocodazole. Mad2 is shown in green, centromeres (ACA) in red and DNA (DAPI) in blue. Insets are enlargements of individual KT pairs. (D) Representative images of Wild-type and *ΔMad2* cells in anaphase. Phosphorylated Histone H3 is shown in green, tubulin in red and DNA (DAPI) in blue. (E) Quantification of the amount of chromosome missegregation in wild-type and *ΔMad2* cells. (F) Quantification of wild-type and *ΔMad2* cells, expressing H2B-RFP and treated as indicated. Time from NEB to anaphase was scored and each dot represents an individual cell. Right graph shows the same data as the left graph but with a different time scale on the y-axis. (G) Colony formation of wild-type and *ΔMad2* cells, treated for seven days with increasing doses of taxol or nocodazole. Cells were fixed with methanol and stained with crystal violet. Error bars in (F) represent standard deviation. Scale bars represent 10 µm.

from the population). The majority of these genes have functions in essential cellular processes including transcription, splicing and mitosis (data not shown). We calculated the average ratio sense/total integrations of every single gene from three wild-type screens and from four screens in SAC-deficient cell lines (two *ΔMad1* and two *ΔMad2* screens) and subtracted the average ratio from the



◄ **Figure 2: Haploid genetic screen identifies novel factors involved in chromosome congression.**

(A) Schematic representation of insertional mutagenesis using a gene trap virus. About 50 million haploid HAP1 cells were infected with gene trap virus and expended for seven days. After expansion, genomic DNA from the cells was harvested and DNA flanking the integrated gene trap was amplified and sequenced. Insertion sites in genes were mapped genome-wide. Gene trap integration in the same transcriptional orientation (sense) of a gene will disrupt the transcript and result in a knock out of the particular gene. Integration of the gene trap in the antisense direction will not affect the transcript of the particular gene. (B, C, D) Plots of all gene trap integrations mapped in wild-type (B), *ΔMad1* (C) and *ΔMad2* (D) cells. Dots represent individual genes. Number of integrations is plotted on the X-axis. Ratio sense / total number of integrations is plotted on the Y-axis. Genes that are synthetic lethal with loss of the SAC are shown in red. Note the drop in ratio for the depicted genes in the *ΔMad1* and *ΔMad2* screen.

ΔMad1/2 from the wild-type. We highlighted the genes that had an absolute difference between the wild-type and the *ΔMad1/2* of at least 0.2 and only selected the genes that had a false discovery rate (FDR) lower than 0.01 (Figure 2B-D and Table 1). We found several known mitotic genes to be synthetic lethal with loss of the SAC (table 1). One of the top hits, CENPE, has been shown to be required for efficient chromosome congression[123,125], indicating that our screen setup can indeed identify genes involved in this process. In addition, we identified *MAPRE1* (EB1) and *DLGAP5* (HURP), which both have been shown to contribute to chromosome alignment by regulating MT dynamics and stability during mitosis[84,326]. Besides known regulators of chromosome congression, we also identified other known mitotic genes. These included the members of the condensin II complex (NCAPH2, NCAPG2 and NCAPD3[327]) and the Rod-Zwilch-Zw10 (RZZ) complex member *Rod1* and its upstream regulator *Zwint*[328].

Gene	# Screens scored as HIT	Av. Ratio WT	Av. Ratio Mad1/2	Absolute difference WT – Mad1/2	Cellular Function
CCNB1	3	0.55	0.09	0.45	G2/mitotic-specific cyclin-B1
NCAPH2	4	0.47	0.08	0.40	Condensin-2 complex subunit H2
KNTC1	4	0.47	0.08	0.39	Kinetochore-associated protein 1 / ROD1
MAPRE1	4	0.48	0.10	0.38	Microtubule-associated protein RP/EB family member 1
CENPE	4	0.45	0.08	0.37	Centromere-associated protein E
NCAPG2	4	0.38	0.08	0.30	Condensin-2 complex subunit G2
ZWINT	4	0.43	0.13	0.30	ZW10 interactor protein 1
DLGAP5	3	0.40	0.13	0.27	Disks large-associated protein 5 / HURP
BUB1	4	0.33	0.07	0.26	Mitotic checkpoint serine/threonine-protein kinase BUB1
NCAPD3	4	0.49	0.25	0.24	Condensin-2 complex subunit D3
ACTR1A	2	0.40	0.18	0.22	Dynactin complex subunit Alpha-centractin
CEP57	2	0.34	0.14	0.20	Centrosomal protein of 57 kDa

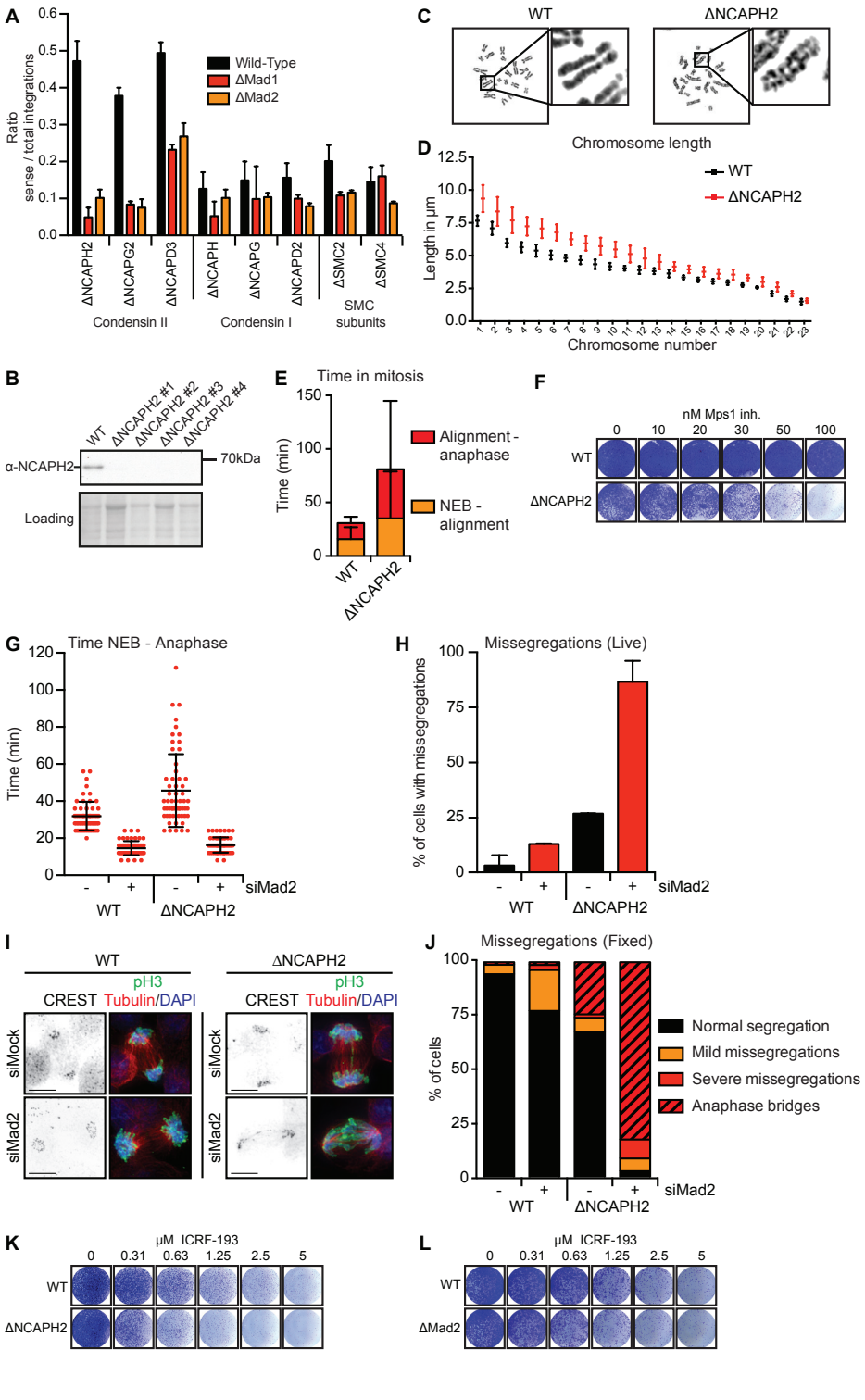
The condensin II complex is required for efficient chromosome alignment and decatenation of sister chromatids

Three members of the condensin II complex score as a hit in the screen (figure 3A[327]). Condensin is a highly conserved protein complex, which plays an essential role in the structural and functional organization of chromosomes during mitosis[2]. Condensin is structurally related to cohesion and two different condensin complexes exist in higher eukaryotes[327]. Both complexes share the two SMC ATPase subunits (SMC2 and SMC4) but differ in their accessory subunits[2]. Condensin I and II have differential contributions to chromosome compaction. While condensin I is cytoplasmic during interphase and

starts to condense chromosomes only after NEB, condensin II localizes to the nucleus and initiates condensation already in prophase[329]. While both complexes are thought to be essential during mitosis[330], our screen data suggests that only condensin I is essential for viability in HAP1 cells, while condensin II is not (Figure 3A). Interestingly, upon loss of SAC function, condensin II becomes essential for cell proliferation (Figure 3A). In order to determine the molecular cause of this synthetic lethal interaction, we used CRISPR/Cas9 to knock out *NCAPH2* in HAP1 cells. We managed to get multiple knockout clones of *NCAPH2* that all show complete loss of the protein on western blot (Figure 3B). To address the contribution of condensin II to chromosome condensation, we made chromosome spreads of mitotic cells. *ΔNCAPH2* cells showed relatively mild defects in chromosome condensation (Figure 3C). The chromosomes displayed a twisted morphology, consistent with previous studies (Figure C and[329,330]), and we only found a minor increase in the average length of the chromosomes (Figure 3D). When we filmed *ΔNCAPH2* cells, we observed an almost threefold increase in the time in mitosis (Figure 3E). Both time in prometaphase as well as time in metaphase increased in these cells, although due to lack of resolution in our experiments, we cannot rule out that the increase time spent in metaphase might be caused by mild defects in chromosome alignment. To confirm the synthetic lethal interaction observed in the screen, we performed a colony assay and treated wild-type and *ΔNCAPH2* cells with increasing doses of an Mps1 inhibitor, known to inactivate the SAC (Figure 3F). While the compound did not affect proliferation of wild-type cells in doses up to 100nM, the Mps1 inhibitor severely affected proliferation of *ΔNCAPH2* cells and we did not observe colony outgrowth at a dose of 50nM Mps1 inhibitor. In addition, we also depleted Mad2 from wild-type and *ΔNCAPH2* cells using siRNAs and filmed mitotic progression (Figure 3G). Depletion of Mad2 from wild-type cells decreased the time spent in mitosis similar to *ΔMad2* cells (Figure 3H and supplementary Figure S1) and we did observed only a small increase in chromosome segregation errors, comparable to *ΔMad2* cells (Figure 3H and Figure 1E). Untreated *ΔNCAPH2* cells already showed an increase in chromosome segregation defects (Figure 3H). However, in contrast to wild-type cells, depletion of Mad2 in *ΔNCAPH2* cells resulted in a massive increase in chromosome segregation defects (Figure 3H). To characterize the type of segregation defects in more detail, we fixed and stained wild-type and *ΔNCAPH2* cells (Figure 3I). Depletion of Mad2 from *ΔNCAPH2* resulted in a massive increase in anaphase bridges (Figure 3J) and sister chromatids were frequently still entangled during anaphase (Figure 3I). Previous studies have shown that condensin might regulate the localization and activity of type II topoisomerase during mitosis and that the activity of topoisomerase II is essential for decatenation of sister chromatids[331-334]. Our data suggest that *ΔNCAPH2* cells indeed have problems with decatenation since we already observed some anaphase bridges in *ΔNCAPH2*. Upon inactivation of the SAC, these

► Figure 3: The condensin II complex is required for efficient chromosome alignment and decatenation of sister chromatids.

(A) Graph of average ratio sense / total number of integrations in wild-type, *ΔMad1* and *ΔMad2* cells for all subunits of the condensin I and condensin II complex. Note the drop in the ratio of condensin II-specific complex members in *ΔMad1* and *ΔMad2* cells. (B) Wild-type and *ΔNCAPH2* cells were harvested and NCAPH2 protein levels were analyzed by western blot. (C) Representative images of chromosome spreads from wild-type and *ΔNCAPH2* cells. (D) The average length of individual chromosomes from the spreads in (C). (E) Quantification of wild-type and *ΔNCAPH2* cells, expressing H2B-RFP. Time from NEB to alignment and alignment to anaphase was scored. (F) Colony formation of wild-type and *ΔNCAPH2* cells, treated for seven days with increasing doses of Mps1 inhibitor. Cells were fixed with methanol and stained with crystal violet. (G) Quantification of wild-type and *ΔNCAPH2* cells, expressing H2B-RFP and treated as indicated. Time from NEB to anaphase was scored and each dot represents an individual cell. (H) Quantification of the amount of missegregations from the cells in (G). (I) Representative images of wild-type and *ΔNCAPH2* cells in anaphase. Phosphorylated Histone H3 is shown in green, tubulin in red and DNA (DAPI) in blue. Error bars represent standard deviation. Scale bars represent 10 μm.



defects dramatically increase, indicating that decatenation does occur in *ΔNCAPH2* cells, but likely occurs at a slower pace.

Indeed, we found that *ΔNCAPH2* cells were also more sensitive to the topoisomerase II inhibitor ICRF-193 (Figure 3K,[335]). In addition, we found that also *ΔMad2* cells were also slightly more sensitive to ICRF-193 (Figure 3L), again indicating that *ΔNCAPH2* cells require more time in mitosis to efficiently decatenate sister chromatids. These results suggest that condensin II is required for efficient chromosome congression and in addition, they indicate that condensing II stimulates the activity of topoisomerase II hereby promoting decatenation of sister chromatids.

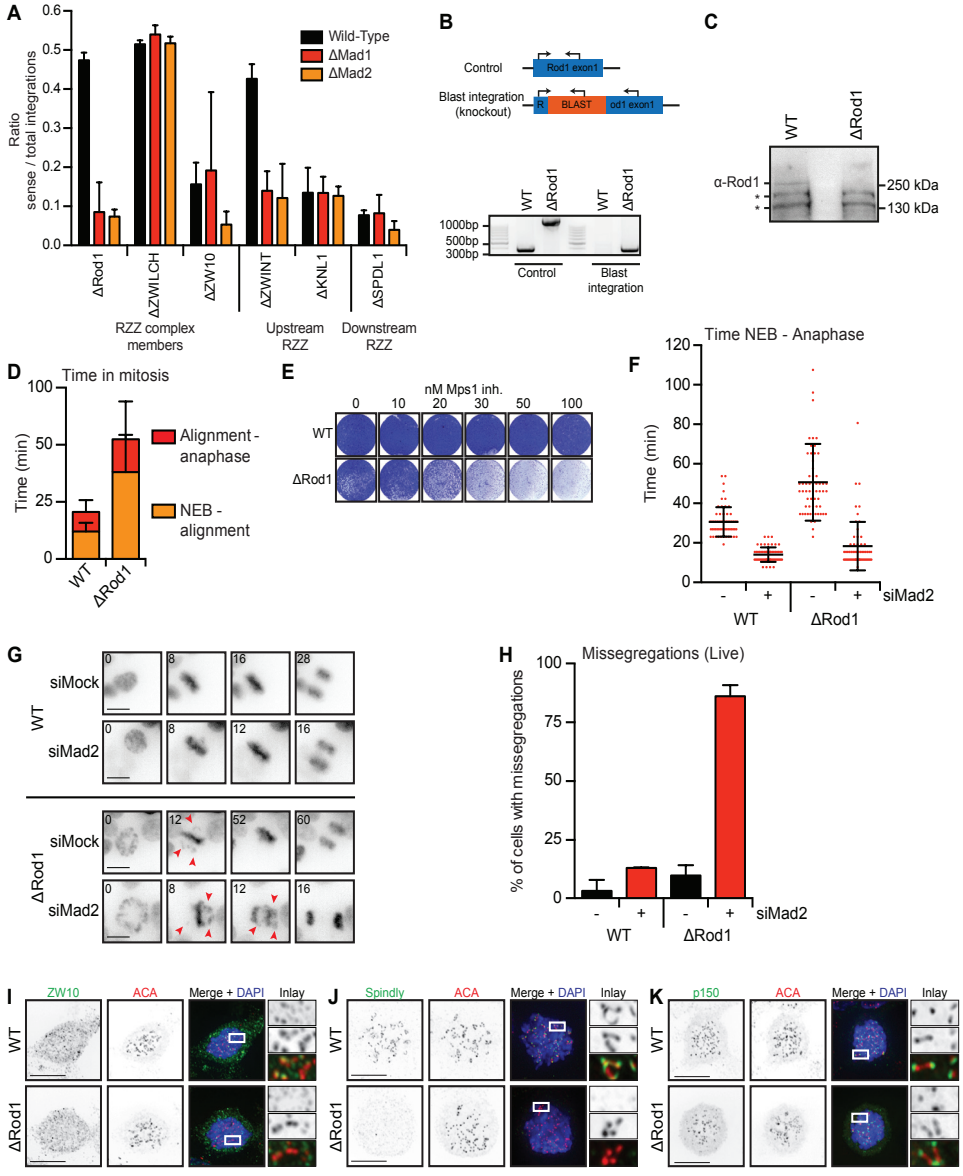
Rod1 knockout cells have delayed chromosome alignment and depend on the SAC for survival

The screen results indicate that loss of specific components of the RZZ complex is synthetic lethal with SAC deficiency. The RZZ complex is only present in higher eukaryotes and is composed of Rod1/KNTC1, Zw10 and Zwilch[328]. The RZZ complex is recruited to the KT by Zwint[328], which interacts with the KT by binding to the C-terminus of KNL-1, a component of the KNL-1, MIS-12 complex and NDC80 (KNM) network, the main MT-binding complex of the KT[96]. Interestingly, based on our screen results, not all components of the RZZ seem to functionally overlap (Figure 4A). Loss of the RZZ component Rod1 and the upstream recruiting factor Zwint does not affect the viability of HAP1 cells by itself, but are synthetic lethal with loss of the SAC (Figure 4A). Interestingly, in contrast to Rod1 and Zwint, loss of Zwilch does not affect viability in the absence of a functional SAC, indicating that Zwilch is not essential for the RZZ function that is required to sustain cell viability in SAC-deficient cells (Figure 4A). Finally, the screen data indicates that Zw10, KNL-1 (upstream of the RZZ complex) and Spindly (*Spdl1*, downstream of the RZZ complex) are essential for HAP1 cells (Figure 4A). Zw10 has been shown to also function outside mitosis, by regulating dynein-mediated vesicle transport[336,337], and KNL-1 plays an essential role during mitosis by acting as a scaffold for several additional outer-KT components[338], explaining their essential role in HAP1 cells. Surprisingly, while HAP1 cells tolerate loss of RZZ function, loss of its downstream component Spindly is detrimental for HAP1 cells (Figure 4A).

Previous studies showed that RZZ is required for SAC signaling but how it contributes to the SAC is still under debate[199,200]. Since we found a synthetic lethal interaction between the RZZ complex and the SAC in our screen, this would imply that RZZ complex has a more complicated function during mitosis than merely contributing to checkpoint signaling. To elucidate the function of the RZZ complex

► Figure 4: Rod1 knockout cells have delayed chromosome alignment and depend on the SAC for survival.

(A) Graph of average ratio sense / total number of integrations in wild-type, *ΔMad1* and *ΔMad2* cells for the RZZ complex and up- and downstream factors. (B) Generation of *Rod1* knockout cells using CRISPR/Cas9. Schematic representation of the genomic PCR used to confirm integration of the blasticidin cassette. Arrows indicate the position of the primers used for the PCR. *ΔRod1* cells have a bandshift in the control PCR and a band in blasticidin PCR, confirming integration of the blasticidin cassette in the *Rod1* locus. (C) Wild-type and *ΔRod1* cells were harvested and Rod1 protein levels were analyzed by western blot. Asterisks mark crossreacting bands. (D) Quantification of wild-type and *ΔRod1* cells, expressing H2B-RFP. Time from NEB to alignment and alignment to anaphase was scored. (E) Colony formation of wild-type and *ΔRod1* cells, treated for seven days with increasing doses of Mps1 inhibitor. Cells were fixed with methanol and stained with crystal violet. (F) Quantification of wild-type and *ΔRod1* cells, expressing H2B-RFP and treated as indicated. Time from NEB to anaphase was scored and each dot represents an individual cell. (G) Representative stills from individual cells from (F), treated as indicated. Not the single misaligned chromosomes in the *ΔRod1* cells, marked with the red arrows. (H) Quantification of the amount of missegregations from the cells in (G) and (F). (I) Representative images of wild-type and *ΔRod1* cells treated with nocodazole. Zw10 is shown in green, KTs (ACA) in red and DNA (DAPI) in blue. (J) Representative images of wild-type and *ΔRod1* cells treated with nocodazole. Spindly is shown in green, KTs (ACA) in red and DNA (DAPI) in blue. (K) Representative images of wild-type and *ΔRod1* cells treated with nocodazole. p150 (dynactin) is shown in green, KTs (ACA) in red and DNA (DAPI) in blue. Insets in (I), (J), and (K) are enlargements of individual KT pairs. Error bars represent standard deviation. Scale bars represent 10 μm.



in more detail, we used CRISPR/Cas9 to knockout *Rod1* from HAP1 cells. We confirmed deletion of *Rod1* by PCR and western blot (Figure 4B and C). A genomic PCR showed integration of the blasticidin cassette in the *Rod1* locus (Figure 4B) and we observed loss of a 250 kDa band (expected molecular weight of Rod1) on western blot after probing with Rod1 antibody (Figure 4B). Live-cell imaging of *ΔRod1* cells showed a dramatic increase in the time spent in prometaphase compared to wild-type cells (Figure 4D) and we observed an increased sensitivity of *ΔRod1* to Mps1 inhibition, confirming our results from the screen (Figure 4E). Next, we filmed wild-type cells and *ΔRod1* combined with Mad2 siRNA (Figure 4F-H). We observed clear chromosome congression defects in *ΔRod1* cells (Figure 4G), explaining the increased time these cells spent in prometaphase. Importantly, the congression defects were only transient, since *ΔRod1* cells were able to form a normal metaphase plate after about 45 minutes (Figure 4G) and subsequently exited mitosis without major defects (Figure 4H). Strikingly, while depletion of Mad2 did not affect chromosome segregation in wild-type cells, Mad2 depletion in *ΔRod1* reduced the time in mitosis and the majority of cells entered anaphase with misaligned chromosomes, leading to chromosome missegregations (Figure 4F-H). This indicates that *ΔRod1* cells have a functional SAC, and depend on the SAC for congression of all chromosomes. To confirm that loss of Rod1 indeed disrupted the RZZ complex as shown previously[339], we assessed the KT localization of Zw10, Spindly and dynactin (p150glued subunit) in nocodazole-treated cells (Figure 4I-K). Zw10, Spindly and dynactin clearly localized to KT in nocodazole-treated wild-type cells (Figure 4I-K) and as expected, Zw10 and Spindly were lost from KTs in *ΔRod1* cells (Figure 4I and J). Surprisingly, we could still detect dynactin on KTs in *ΔRod1* cells (Figure 4K), indicating that a second, RZZ-independent recruitment pathway for dynactin at the KT exists. These data suggest that cells that have lost the RZZ complex from KTs, display chromosome congression defects and require a functional SAC in order to achieve full chromosome alignment.

Discussion

Efficient spindle assembly and chromosome bi-orientation explains tolerance to SAC-deficiency in HAP1 cells

It is thought that organisms with low chromosome numbers, like yeast and *Drosophila* do not require a functional SAC. Cells in these organisms rapidly form spindles with all chromosomes correctly bi-oriented within minutes. Indeed, *Drosophila* cells that can live without a SAC enter anaphase within 15 minutes[311], providing a possible explanation why these cells do not depend on the SAC. Here we characterize for the first time a human cell line, HAP1 that can live without a functional SAC. One feature of this cell line is that the duration of mitosis is relatively short; cells enter anaphase in about 25 minutes and alignment of the chromosome takes on average about 15 minutes. The haploid nature of HAP1 cells, and thus their relative low amount of chromosomes, might explain why the process of spindle assembly and bi-orientation can occur in a short time. This does not seem to be the complete explanation, because we also managed to obtain diploid and tetraploid *ΔMad2* clones (Supplementary Figure S1). Interestingly, while the diploid clones behave relatively similar to haploid clones, we found tetraploid clones to have a dramatic increase in chromosome segregation defects (Supplementary Figure S1). Although this indicates that the number of chromosomes indeed influences the dependency on the SAC, the haploid nature of the HAP1 cells does not fully explain their independency of the SAC. A recent study by the Khodjakov lab proposed that the formation of a compact spindle with a high density of MTs between centrosomes supports efficient, rapid and error free chromosome

alignment[139]. It remains to be determined if this mode of spindle assembly also occurs in HAP1, which would explain why these cells can live without a functional SAC.

A screen in SAC-deficient HAP1 cells uncovers a role for condensin II and the RZZ complex in timely chromosome alignment

The congression of chromosomes to the spindle equator is an important process during mitosis to promote biorientation and correct chromosome segregation. Previous studies have identified multiple factors contributing to this process, including MT motor proteins like dynein, CENPE, and chromokinesins[144]. However, no systematic, genome-wide screens have been performed to specifically identify factors contributing to this complex process. By using a unique screen setup, involving insertional mutagenesis screen in SAC-deficient haploid cells, we successfully identified factors in HAP1 cells that are important for timely chromosome congression. Our screen-setup will only identify factors whose deletion will result in a delay in chromosome alignment, not factors whose deletion results in a persistent block in chromosome alignment, since the latter will also be essential in cells with a functional SAC.

Condensin II is required for efficient decatenation of sister chromatids

We show that cells lacking a functional condensin II complex require the SAC for their survival. Loss of condensin II does not affect proliferation of HAP1 cells, indicating that its function in controlling interphase chromatin is not important for survival in 2D cell culture, whereas its function in mediating mitotic chromosome condensation might be redundant with condensin I. Interestingly, from our results it would appear that condensin I is the major player in mitotic chromosome condensation; all three non-SMC subunits from this complex are essential for HAP1 survival. In agreement with previous literature, we found a small, but significant increase in length of mitotic chromosomes in the absence of condensin II[330,340], indicating that it does contribute to chromosome condensation, albeit to a limited extent.

Our data suggest that cells lacking condensin II depend on the SAC for two main reasons. First, cells lacking condensin II display a delay in chromosome congression and would therefore require the SAC to prevent premature anaphase onset. This defect might be caused by increased chromosome length, hereby altering the dynamics of the individual chromosomes. In addition, forces generated at chromosome arms by chromokinesins have been shown to promote chromosome congression[144], and these forces might be less efficient when the compaction of chromosome arms is altered. The second reason why the SAC is important in cells lacking condensin II, might be due to inefficient decatenation of sister chromatids by topoisomerase II. Cells lacking condensin II show an increase in anaphase bridges and a previous study in yeast showed that mutations in condensin and topoisomerase II are synthetic lethal[341]. In addition, a recent study in yeast by the Uhlmann lab showed that condensin prevents anaphase bridges during chromosome segregation by promoting the decatenation of sister chromatids[334]. Interestingly, while the percentage of mitotic cells with anaphase bridges is relatively low in cells lacking condensin II, this percentage dramatically increases upon inactivation of the SAC and virtually all cells end up with entangled chromosome arms during anaphase. In addition, cells lacking a functional SAC were more sensitive to topoisomerase II inhibitors. This suggests that cells require a certain amount of time in mitosis to decatenate all sister chromatids and that upon loss of condensin II, decatenation is occurs very inefficient. It will be interesting to resolve exactly how condensin promotes sister chromatid decatenation at the molecular level.

The RZZ complex mediates chromosome congression

Our results using *Rod1* knockout cells show that loss of Rod1 results in transient chromosome alignment problems that require an active SAC to prevent missegregation of unaligned chromosomes. The RZZ complex has previously been shown to be involved in chromosome congression by recruiting the Spindly/Dynein/Dynactin complex to KTs[125,135,137,138]. In addition, evidence from studies in *C. elegans* indicated that the RZZ complex by itself can prevent premature formation of KT-MT attachments through inhibition of the NDC80 complex[133,138]. Thus, there are two possible scenarios by which loss of the RZZ complex could lead to perturbed chromosome congression. First, loss of RZZ could perturb dynein-dependent congression. However, in remarkable contrast to previous studies, we find that dynactin still localizes to unattached KTs in the absence of Rod1, while Spindly and Zw10 are clearly absent. Second, loss of the RZZ complex could lead to premature stabilization of end-on attachments, prior to proper biorientation. Although we cannot rule out the possibility that dynein function is perturbed despite the presence of dynactin, our data would suggest that the congression defects in the absence of Rod1 are most likely a consequence of premature stabilization of KT-MT attachments by the NDC80 complex, in line with previous studies in *C. elegans*[133,138]. Our screen also generated new insights in the contribution of the individual members of the RZZ complex. In contrast to Rod1 and Zwilch, Zw10 is essential for viability in the parental HAP1 cells. Since we observed no remaining Zw10 at KTs in cells lacking Rod1, we propose that Zw10 also functions outside the kinetochore, and that these functions are essential for viability of HAP1 cells. This hypothesis is further strengthened by the fact that cells lacking Zwint, the recruitment factor for the RZZ at the kinetochore, is not essential for viability of the parental HAP1 cells. Only upon deletion of Mad1/Mad2 does Zwint become essential, similar to Rod1. Since we did observe a small increase in lethality when Zw10 and Mad2 loss are combined (Figure 4A), we propose that Zw10 and Rod1, as well as their upstream recruitment factor Zwint, are essential for the mitotic functions of the RZZ complex. Surprisingly, we found in our screen that the function of the third member of the RZZ complex, Zwilch, is non-essential for RZZ complex function, in contrast to previous studies in *Drosophila* which showed that *Zwilch* mutants exhibit similar mitotic phenotypes as *Zw10* and *Rod1* mutants[342].

The RZZ complex has previously been shown to be important for SAC signaling due to its role in the recruitment of Mad1 and Mad2 to the KT[199-201]. KT recruitment of Mad1 and Mad2 was shown to be essential and sufficient for SAC signaling[343-345], but our data indicate that in the absence of Rod1, cells are still able to activate the SAC. We therefore propose that in human cells an alternative receptor for Mad1/Mad2 at the KT exists and that therefore the RZZ complex is not absolutely essential for SAC signaling.

Taken together, SAC-deficient haploid cells have allowed us to uncover factors involved in chromosome congression and in sister chromatid decatenation. Our approach revealed novel insights how two previously identified mitotic complexes have additional functions during mitosis. Our screening approach to identify synthetic lethal interactions in human cells can also easily be applied to study other processes.

Experimental Procedures

Cell culture, transfection and drug treatments

HAP1 cells were cultured in IMDM (Gibco) with 8% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. DNA transfections were performed with FuGENE 6 (Promega) according to the manufactures guidelines. siRNA transfections were performed using RNAiMax (Invitrogen) according to the manufactures guidelines. The following siRNAs were used in this study: Mad2 OTP SMARTpool (Dharmacon), Nocodazole and MG321 were used at a final concentration of 250 ng/ml and 5 µM

respectively, or as indicated. Taxol, ICRF-193, and Mps1 inhibitor (compound-5, Koch et al., manuscript in revision) were used at the indicated concentrations.

Generation of knockout cell lines
Guide sequences for the CRISPR/Cas9-mediated genome editing were designed using CRISPR design (Zhang lab). All guides used in this study to generate knockout cell lines were targeted against exon 1 or 2 of the gene of interest. Guides were cloned into the pX330 vector [324]. pX330 and a blasticidin donor vector (gift from Thijn Brummelkamp) were co-transfected in HAP1 cells and selected with 5 µg/ml blasticidin. Individual clones were selected and knockouts were confirmed using either PCR to confirm integration of the blasticidin cassette or by western blot.

Insertional mutagenesis screen
Gene trap virus was produced in 293T cells and harvested as described previously [320]. About 50 million HAP1 cells (wild-type, *Mad1* and *Mad2* knockouts) were mutagenized by transduction with gene trap virus in T175 flasks and selected for 7 days. Screens were performed in triplicate (wild-type) or in duplicate (*Mad1* and *Mad2*) After selection, the haploid population was sorted out based on cell size and genomic DNA was harvested as described previously [320]. Gene trap insertion sites were mapped using PCR and deep sequencing of the genomic DNA flanking the gene trap insertion site as described previously [320]. In all screens, we obtained at least 5 million independent insertion sites. Sense or antisense integration of the gene trap was classified as described previously [320].

Immunofluorescence
Cells were grown on 10mm glass coverslips and pre-extracted for 60 seconds in PEM buffer (100 mM PIPES, 10 mM EGTA, 1mM MgCl and 0.1% Trinton X-100), followed by fixation for 10 minutes at room temperature in 4% formaldehyde in PEM buffer with 0.3% Triton X-100. The following antibodies were used: rabbit phospho-histone H3 (Millipore), mouse α-tubulin (Sigma), human ACA (Cortex Biochem), Rabbit Mad2 (Bethyl), Rabbit Zw10 (Abcam), Rabbit Spindly (Bethyl), mouse p150 (Transduction Laboratories). All antibodies were incubated over night at 4°C. Secondary antibodies (Molecular probes, Invitrogen) were incubated 1 hr at room temperature. DAPI was added before mounting using ProLong Gold (Invitrogen). Images were acquired using a Deltavision deconvolution microscope (Applied Precision) with a 100x 1.40 NA oil objective. Softworx (Applied Precision, ImageJ and Adobe Photoshop and Illustrator CS6 were used to process acquired images.

Time-lapse microscopy
Cells were plated on 8-well glass-bottom dishes (LabTek) and cultured in L-15 CO_2 independent medium (Gibco). Cells were imaged using a Deltavision deconvolution microscope (Applied Precision) equipped with a heat chamber. Images were acquired every four minutes using a 20x (0.25 NA) or 40x (1.40 NA) objective. Z-stacks were acquired with 3 µm intervals. Images were analyzed and processed using Softworx (Applied Precision) and ImageJ.

Colony formation
Cells were plate in 48-well plates at a density of 17,000 cells per well and treated as indicated for 7 days. After 7 days, cells were fixed using methanol and stained using crystal violet.

Western Blot

Cells were counted and lysed using Laemmli buffer (120 mM Tris pH 6.8, 4% SDS, 20% glycerol). Protein levels were analyzed using western blot. The following antibodies were used in this study: mouse α-tubulin (Sigma), mouse Mad2 (MBL), rabbit NCAPH2 (Santa Cruz), mouse Rod1 (Santa Cruz).

Chromosome spreads

Cells were treated with nocodazole for 4 hours and harvested. Spreads were prepared as described previously [346]. Length of individual chromosomes was measured using ImageJ software.

Acknowledgements

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Chapter 7

The RZZ complex and Bub1 cooperate in the kinetochore recruitment of Mad1

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Abstract

The spindle assembly checkpoint (SAC) prevents the onset of anaphase until all of the chromosomes have properly attached to the spindle. Chromosomes that are not attached to the mitotic spindle recruit the checkpoint protein Mad1 to their unattached kinetochores, which can subsequently activate Mad2. Active Mad2 stimulates the formation of the mitotic checkpoint complex (MCC) that acts to prevent anaphase onset. How exactly Mad1 is recruited to unattached kinetochores is still a matter of debate. Here, we show that Bub1 and the Rod1/Zw10/Zwilch (RZZ) complex can both independently recruit Mad1 to unattached kinetochores. We find that the RZZ complex is required for the maintenance of Mad1 at unattached kinetochores throughout mitosis, while Bub1 cooperates with the RZZ complex in the early recruitment of Mad1. Surprisingly, we find that the functions of Bub1 in chromosome alignment and SAC signaling are not dependent on its role in the recruitment of BubR1 to kinetochores. We show that BubR1 is dispensable for stabilization of kinetochore-microtubule attachments and is solely required for SAC signaling. By promoting centromeric accumulation of Aurora B, Bub1 stimulates the positive feedback loop between Aurora B and Mps1, hereby contributing to both chromosome alignment and establishment of the SAC. Taken together, we elucidated a novel interplay between Bub1 and the RZZ complex in the regulation of Mad1 recruitment and the control of chromosome alignment.

Introduction

The spindle assembly checkpoint (SAC) prevents premature separation of sister chromatids during mitosis by inhibiting the anaphase promoting complex or cyclosome (APC/C[161]). The molecular components of the SAC localize to kinetochores (KTs) that are not attached to the mitotic spindle, where they generate a diffusible signal, known as the mitotic checkpoint complex (MCC). This complex, composed of Mad2, BubR1 and Bub3, prevents anaphase onset by binding to the APC/C co-activator Cdc20 to prevent premature APC/C activation[307]. Mad1 is an essential SAC component that localizes to unattached KTs, were it forms a tetrameric complex with Mad2. This complex catalyzes the conversion of cytoplasmic open Mad2 (O-Mad2) into closed Mad2 (C-Mad2), that can in turn interact with Cdc20, hereby stimulating MCC formation[161].

The SAC components Bub1 and BubR1 are highly similar at the sequence level, but exhibit different functions during mitosis[187]. BubR1 is an essential for the SAC by being part of MCC[161]. In addition, both BubR1 and Bub1 heterodimerize with Bub3 in order to localize to unattached KTs were they contribute to the formation of stable KT-microtubule (MT) attachments[156,192,195,347]. BubR1 participates in this process by recruiting PP2A-B56, which dephosphorylates substrates of Aurora B at the KTs, hereby stabilizing KT-MT attachments[155,156]. Bub1 is thought to participate in stabilizing KT-MT attachments in two ways; first by recruiting BubR1/Bub3 heterodimers to KTs[188,348], and second by promoting Aurora B accumulation at centromeres through phosphorylation of histone H2A[192,196]. While the function of BubR1 in the SAC is well established, the function of Bub1 in the SAC is not fully clear. Bub1 is thought to contribute MCC assembly by recruiting individual MCC components to the KT [161,349]. In addition, Bub1 promotes recruitment of Aurora B to the inner centromere, thereby establishing the positive feedback loop between Aurora B and Mps1, essential for establishment of the SAC in early mitosis[192-194,196]. And lastly, Bub1 is essential for the recruitment of Mad1 and Mad2 to unattached KTs in yeast and *C. elegans*[189,191]. Although no direct interaction has been found to date, Bub1 is also thought to be involved in KT recruitment of Mad1 and Mad2 in higher eukaryotes[197,198].

Besides Bub1, the Rod1/KNTC1, Zw10 and Zwilch (RZZ) complex, only present in metazoans, is also thought to be involved in KT recruitment of Mad1 and Mad2 and has been shown to be required for SAC signaling[199,201]. Nevertheless, the exact contributions of the RZZ complex and Bub1 in the recruitment of Mad1 are currently unclear, nor is it known which component is the direct KT receptor for Mad1 and Mad2 tetramers.

The human HAP1 cell line is a near-haploid cell line, which makes it a very useful tool for the execution of insertional mutagenesis screens[319-323]. We previously showed that HAP1 cells do not require a functional SAC and we successfully generated Mad1 and Mad2 knockout cell lines. We used these cell lines to perform insertional mutagenesis screens to identify genes that act synthetic lethal with the loss of Mad1 and Mad2 (chapter 6). Surprisingly, while the parental HAP1 cells tolerate loss of Bub1, BubR1 and the RZZ complex, Bub3 is essential for viability. Interestingly, upon deletion of Mad1 or Mad2, Bub1 and the RZZ complex become essential for cell proliferation, while BubR1 remains non-essential. This suggests that Bub1, BubR1, Bub3 and the RZZ complex play a more complex and potentially redundant function in SAC signaling. In this study, we tried to elucidate this paradox by examining the mitotic functions of Bub1, BubR1 and the RZZ complex in HAP1 cells in more detail.

Results

Bub1 contributes to chromosome alignment

In the synthetic lethality screen we performed in the SAC-deficient HAP1 cells, we obtained some contradictory results compared to previous studies (chapter 6). First, we found that Bub1 and BubR1 are not essential for viability in parental HAP1 cells (Figure 1A). This finding contrast with the general view that deletion of BubR1 should not be compatible with viability, as this would affect SAC function and alignment, leading to massive segregation errors. Second, in contrast to Bub1 and BubR1, Bub3 is essential in HAP1 cells (Figure 1A), which is not in line with the general view that Bub1/BubR1 and Bub3 act in complex with each other. Curiously, while we find that Bub1 becomes essential in SAC-deficient HAP1 cells, BubR1 does not become essential in SAC-deficient HAP1 cells (Figure 1A). This indicates that Bub1 and BubR1 must have separate roles in mitosis that can explain this paradox. To unravel the molecular details of this contradiction, we used CRISPR/Cas9 to knockout *Bub1* from HAP1 cells. We managed to get viable *ΔBub1* clones that show complete loss of Bub1 protein on western blot (Figure 1B). We imaged H2B-RFP expressing *ΔBub1* cells and unexpectedly these cells spent about double the amount of time in mitosis compared to wild-type cells and this delay was mainly due to a prolonged prometaphase (Figure 1C). This indicates that *ΔBub1* cells have a functional checkpoint, at least sufficient to maintain a mitotic delay in response to a few unattached kinetochores. We confirmed the synthetic lethal interaction of Bub1 with loss of the SAC by colony formation assays in which we treated wild-type and *ΔBub1* cells with increasing doses of an Mps1 inhibitor (Figure 1D). In addition, treatment of *ΔBub1* cells with siRNAs targeting Mad2 decreased the time in mitosis (Figure 1E), and we observed a dramatic increase in the amount of missegregations (Figure 1F). Strikingly, when we looked in more detail to *ΔBub1* knockout cells, we noticed that *ΔBub1* cells entering mitosis had problems with alignment, but did manage to form a normal metaphase plate after spending more time in prometaphase (Figure 1G). After depletion of Mad2, *ΔBub1* cells did not align all chromosomes and entered anaphase while missegregating multiple chromosomes (Figure 1G). These results indicate that Bub1 is required for normal chromosome alignment in HAP1 cells and is not absolutely essential for SAC function.

BubR1 is not required for chromosome alignment in HAP1 cells

Loss of BubR1 from KTs has previously been shown to result in severe defects in KT-MT attachments that persist over time[155,156,195,347]. To test if BubR1 is lost from KTs in *ΔBub1* cells, we treated wild-type and *ΔBub1* cells with nocodazole and stained for Bub1 and BubR1 (Figure 2A). While both Bub1 and BubR1 clearly localized to unattached KTs in wild-type cells, we did not observe any residual Bub1 protein in *ΔBub1* cells and nearly all BubR1 protein was lost from KTs, consistent with previous literature (Figure 2A and[188,348]). However, unlike loss of Bub1, loss of *BubR1* is not synthetic lethal with loss of the SAC (Figure 1A), suggesting that chromosome alignment must occur relatively normal in BubR1 knockout cells. If so, this would mean that Bub1 acts independently from BubR1 in chromosome alignment, possibly by controlling recruitment of Aurora B to the centromeres[192,196]. To study this, we tested if centromeric accumulation of Aurora B was affected in *ΔBub1* cells. To this end, we stained phosphorylated histone H2A and Aurora B in nocodazole-treated wild-type and *ΔBub1* cells (Figure 2B and C). Consistent with previous studies, histone H2A phosphorylation was absent and centromeric accumulation of Aurora B was reduced in *ΔBub1* cells (Figure 2B and C), indicating that Bub1 likely controls chromosome alignment by controlling centromeric Aurora B accumulation.

Next, we wanted to test whether BubR1 is indeed not required for chromosome alignment in HAP1 cells. To this end, we knocked out *BubR1* in HAP1 cells using CRISPR/Cas9 and confirmed loss of BubR1

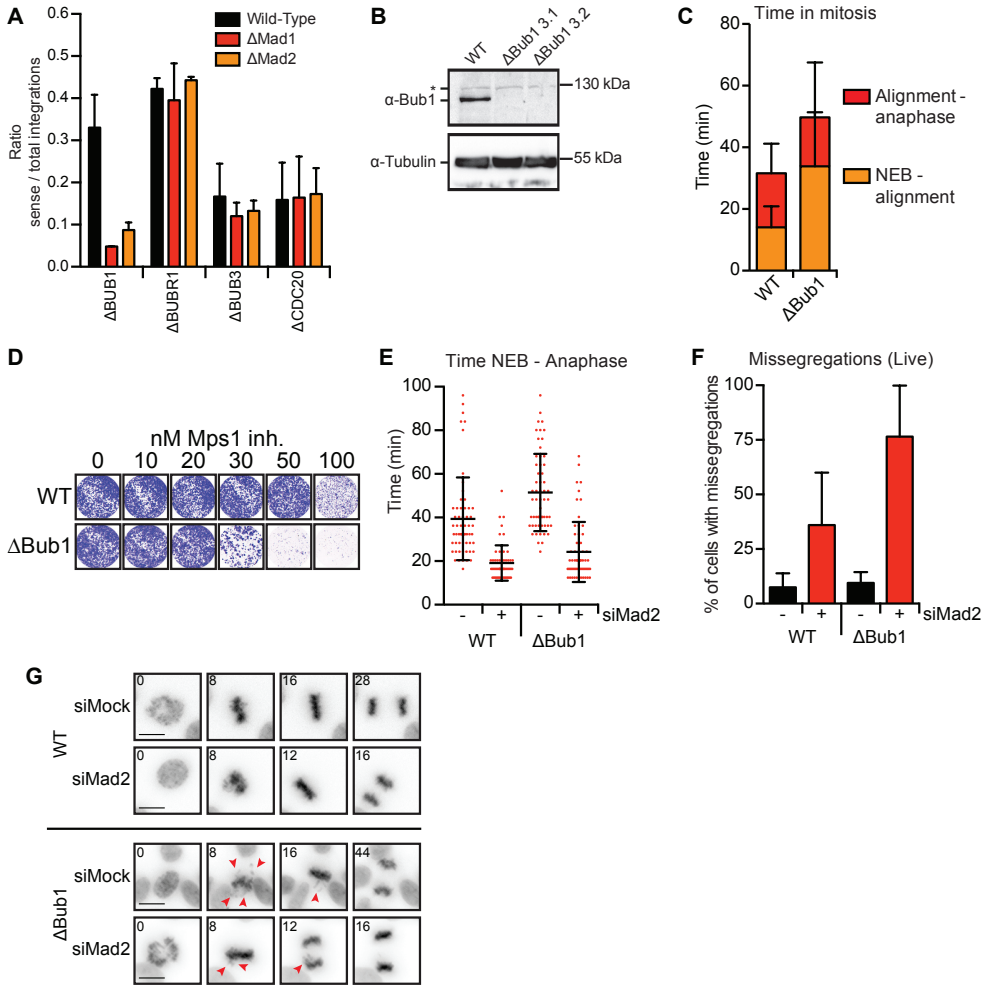


Figure 1: Bub1 contributes to chromosome alignment independently of BubR1.

(A) Graph of average ratio sense / total number of gene trap integrations in wild-type, *ΔMad1* and *ΔMad2* cells for *Bub1*, *BubR1*, *Bub3* and *Cdc20* from the screen performed in chapter 6. A bias towards integration in the antisense direction (ratio < 0.5) indicates that the gene is important for cell viability (sense integrations are depleted from the population). (B) Confirmation of *Bub1* knockout cells. Wild-type and *ΔBub1* cells were harvested and Bub1 protein levels were analyzed by western blot. Asterisks mark crossreacting band. Tubulin served as a loading control. (C) Quantification of wild-type and *ΔBub1* cells, expressing H2B-RFP. Time from NEB to alignment and alignment to anaphase was scored. (D) Colony formation of wild-type and *ΔRod1* cells, treated for seven days with increasing doses of Mps1 inhibitor. Cells were fixed with methanol and stained with crystal violet. (E) Quantification of wild-type and *ΔRod1* cells, expressing H2B-RFP and treated as indicated. Time from NEB to anaphase was scored and each dot represents an individual cell. (F) Quantification of the amount of missegregations from the cells in (E). (G) Representative stills from individual cells from (E), treated as indicated. Not the single misaligned chromosomes in the *ΔBub1* cells, marked with the red arrows. Error bars represent standard deviation. Scale bars represent 10 µm.

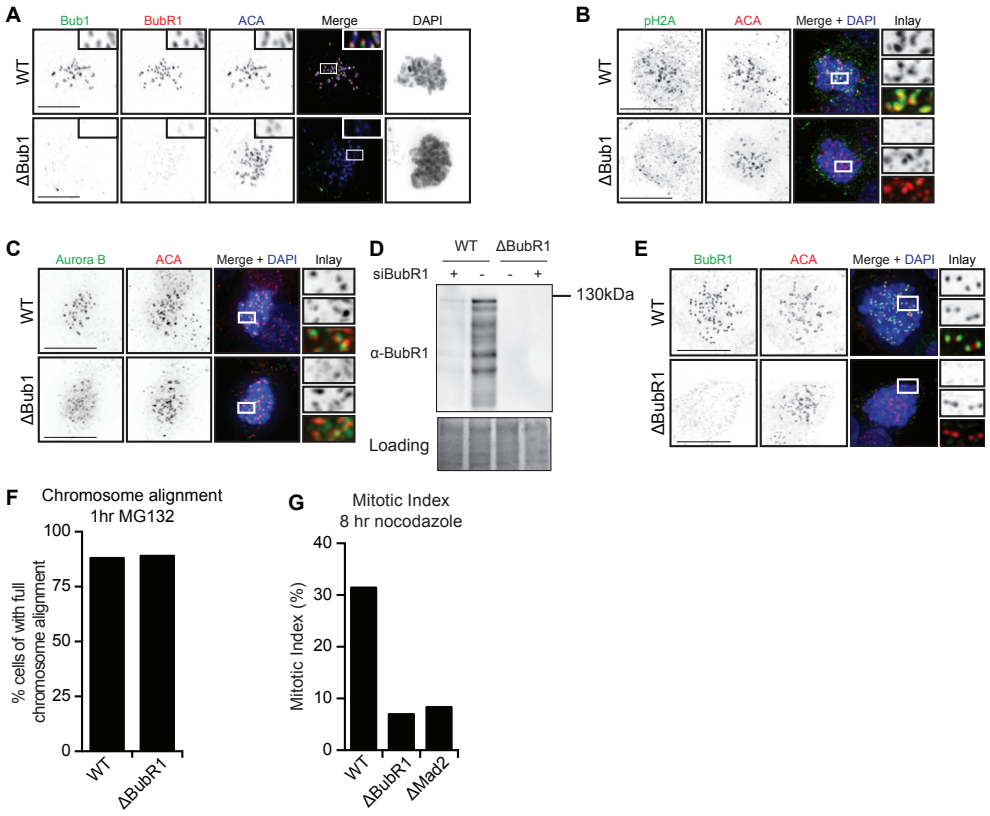


Figure 2: BubR1 is not required for chromosome alignment in HAP1 cells.

(A) Representative images of wild-type and *ΔBub1* cells treated with nocodazole. Bub1 is shown in green, BubR1 is shown in red, KTs (ACA) in blue. (B) Representative images of wild-type and *ΔBub1* cells treated with nocodazole. Phosphorylated H2A is shown in green, KTs (ACA) in red, DNA (DAPI) in blue. (C) Representative images of wild-type and *ΔBub1* cells treated with nocodazole. Aurora B is shown in green, KTs (ACA) in red, DNA (DAPI) in blue. (D) Confirmation of *BubR1* knockout cells. Wild-type and *ΔBubR1* cells were treated with or without BubR1 siRNAs, harvested after 48 hours and BubR1 protein levels were analyzed by western blot. (E) Representative images of wild-type and *ΔBubR1* cells treated with nocodazole. BubR1 is shown in green, KTs (ACA) is shown in red, DNA (DAPI) in blue. (F) Chromosome alignment in wild-type and *ΔBubR1* cells, treated with MG132 for 1 hour. Graph represents the percentage of cells with full chromosome alignment. (G) Mitotic index of wild-type and *ΔBubR1* cells treated for 8 hours with nocodazole and stained for phospho-H3. Insets in (A), (B), (C) and (E) are enlargements of individual KT pairs. Scale bars represent 10 μm.

protein on western blot (Figure 2D) and by immunofluorescence (Figure 2E). Strikingly, we observed no chromosome alignment defects in *ΔBubR1* cells (Figure 2F), which is in contrast to previous observations obtained using RNA interference[155,156,195,347]. To test if loss of BubR1 results in loss of SAC function, we treated wild-type, *ΔBubR1* and *ΔMad2* cells with nocodazole and determined the mitotic index (Figure 2G). While wild-type cells arrested in mitosis and reached a mitotic index of around 30% after eight hours, both *ΔBubR1* and *ΔMad2* cells displayed a low mitotic index of around 5% (Figure 2G), indicating that these latter cells lack a functional SAC. Thus, loss of SAC function is the only obvious mitotic phenotype of cells lacking BubR1, which also explains why BubR1 loss is

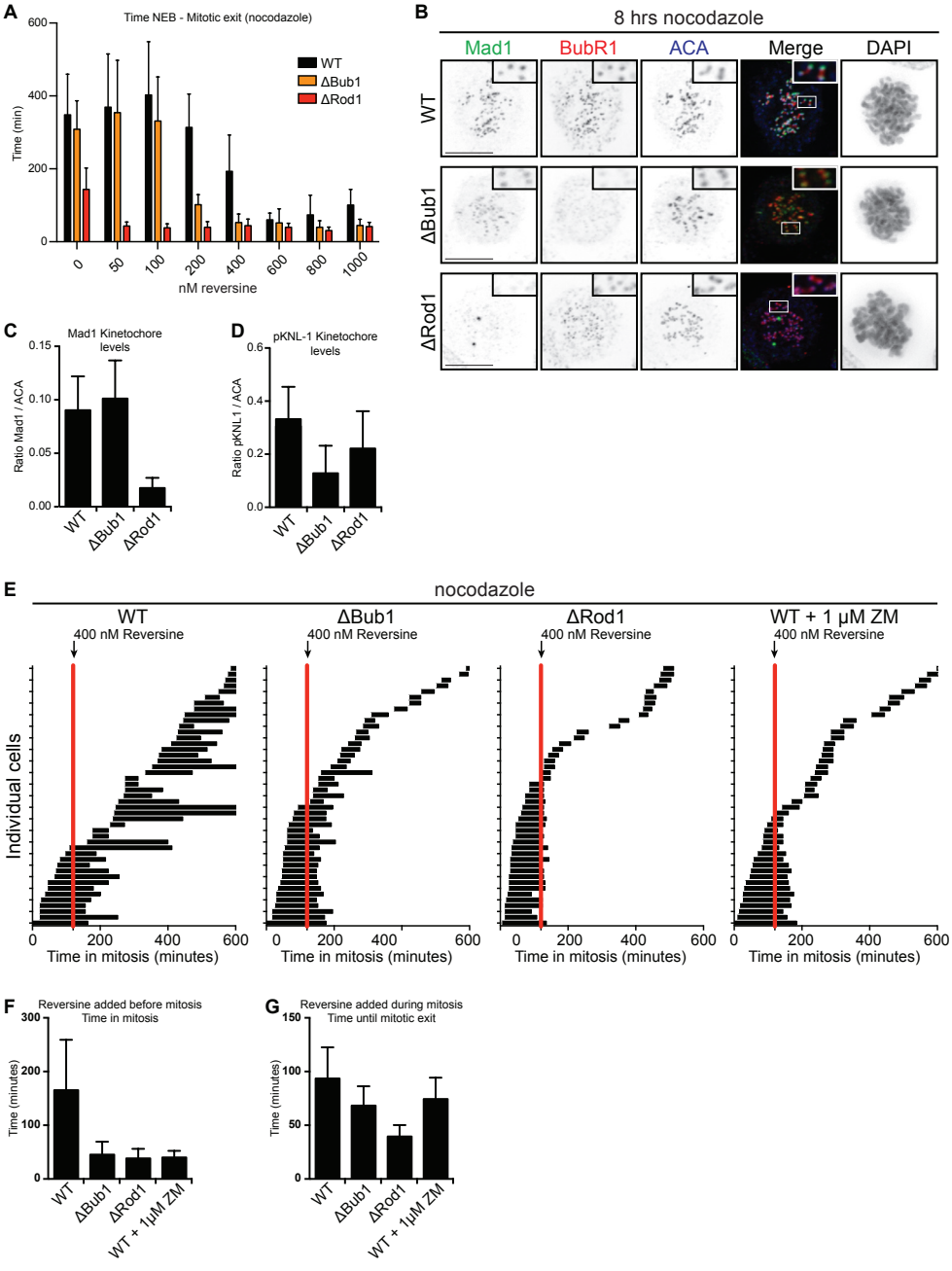
not lethal by itself and why we did not found a synthetic lethal interaction with Mad1 or Mad2 loss. Interestingly, Bub3 appears to perform a dual role in mitosis. First, Bub3 is required for chromosome alignment as a consequence of its role in KT recruitment of Bub1[108,184,348,350]. Second, Bub3 is required for SAC function as it is part of the MCC that prevents premature activation of the APC/C[167]. Deletion of Bub3 will therefore comprise alignment and result in loss of SAC function, two defects that are not compatible with cell viability when combined.

Bub1 promotes robust SAC signaling by establishing high Mps1 activity

The synthetic lethality screen described in chapter 6 indicates that both *ΔRod1* and *ΔBub1* cells have a functional SAC and depend on the SAC for their survival. This is in contrast to previous studies that proposed a role for both the RZZ complex and Bub1 in SAC signaling. To examine the functionality of the SAC in more detail, we treated wild-type, *ΔBub1,* and *ΔRod1* cells with nocodazole and followed the cells by live cell microscopy (Figure 3A). Wild-type cells arrested in mitosis for about 400 minutes before undergoing apoptosis (Figure 3A). Similar to wild type cells, *ΔBub1* cells also maintained a mitotic delay for 400 minutes on average (Figure 3A), indicating that the SAC indeed is functional in these cells. In contrast, *ΔRod1* cells slipped out of mitosis after approximately 150 minutes, suggesting that cells lacking the RZZ-complex can initiate a SAC response, but are unable to maintain an active SAC over time. To test whether the checkpoint was completely unaffected in *ΔBub1* cells, we sensitized the SAC by titrating in increasing concentrations of the Mps1 inhibitor reversine[205]. Wild-type cells started to slip out of mitosis when treated with 400 nM reversine or higher (Figure 3A). However, while *ΔBub1* cells arrested to the same extent as wild-type cells after nocodazole treatment alone, they slipped out when treated with 200 nM reversine or higher (Figure 3A). This suggests that although *ΔBub1* cells have a functional SAC, it lacks robustness and these cells are more sensitive to further impairment of the SAC. Interestingly, SAC function was completely compromised in *ΔRod1* cells at the lowest concentrations of reversine we tested (Figure 3A), indicating that these cells are extremely sensitive to further SAC perturbation.

As mentioned above, both Bub1 and the RZZ complex are implicated in KT recruitment of Mad1[189,191,197,198,351]. In order to test whether Mad1 and its downstream effector Mad2 could still localize to unattached KTs in the absence of either Bub1 or the RZZ complex, we treated wild-type, *ΔBub1* and *ΔRod1* cells with nocodazole and stained for Mad1 and BubR1 (Figure 3B). As expected, BubR1 was almost completely lost from KTs in the *ΔBub1* cells and we did not observe major differences in BubR1 localization in *ΔRod1* cells compared to wild-type cells (Figure 3B). In contrast to BubR1, we did observe normal Mad1 localization at KTs in *ΔBub1* cells, indicating that Bub1 is not required for KT recruitment of Mad1 in HAP1 cells (Figure 3B and C). On the contrary, Mad1 recruitment was severely affected in *ΔRod1* cells (Figure 3B and C), indicating that the RZZ complex is required to load Mad1 at KTs, explaining the extreme sensitivity of these cells to partial SAC inhibition (Figure 3A).

Since we did not observe defects in KT recruitment of Mad1 in *ΔBub1* cells, we wondered what could be the cause of the weakened SAC of *ΔBub1* cells. *ΔBub1* cells rapidly exit mitosis upon treatment with 400 nM reversine and in addition, we showed that centromeric accumulation of Aurora B is reduced in *ΔBub1* cells. Since centromeric accumulation of Aurora B is important for timely SAC establishment by stimulating the Mps1-Aurora B positive feedback loop[193], we hypothesized that the weakened SAC in *ΔBub1* cells might be a direct consequence of reduced centromeric accumulation of Aurora B and Mps1 activation. In order to test this, we arrested cells in mitosis using nocodazole for 1 hour and assessed Mps1 activity using phospho-specific antibodies against the MELT-motif on KNL-1, which is phosphorylated by Mps1[109,111,183]. Indeed, *ΔBub1* cells showed a reduction in pKNL-1 levels compared to wild-type cells (Figure 3D), explaining why these cells are more sensitive to partial Mps1 inhibition.



◄ Figure 3: Bub3 promotes robust SAC signaling by establishing high Mps1 activity.

(A) Quantification of wild-type, *ΔBub1*, and *ΔRod1*, cells, expressing H2B-RFP and treated as indicated. Time from NEB to mitotic exit was scored. (B) Representative images of wild-type, *ΔBub1,* and *ΔRod1* cells treated for 8 hours with nocodazole. Mad1 is shown in green, BubR1 is shown in red, KTs (ACA) in blue. (C) Quantification of the KT levels of Mad1 as a ratio over ACA intensity. (D) Quantification of the KT levels of phosphorylated KNL-1 as a ratio over ACA intensity. (E) Quantification of the mitotic timing of wild-type, *ΔBub1, ΔRod1*, and wild-type cells treated with 1 μM ZM. The start of the black bars represents a single cell entering mitosis. The end of the black bar indicates mitotic exit. Reversine was added at the indicated time point (red line). (F) Quantification of the average remaining time spent in mitosis of wild-type, *ΔBub1, ΔRod1*, and wild-type cells treated with 1 μM ZM, to which reversine was added during mitosis. (G) Quantification of the average time spent in mitosis of wild-type, *ΔBub1, ΔRod1*, and wild-type cells treated with 1 μM ZM to which reversine was added before mitosis. Error bars represent standard deviation. Scale bars represent 10 μm.

Curiously, we also noticed a decrease in pKNL-1 levels in *ΔRod1* cells (Figure 3D). This might be due to the fact that *ΔRod1* cells cannot maintain a robust SAC response and cells might progressively slip out of mitosis, resulting in a reduction of mitotic phosphorylation. Because Aurora B is predominantly required to establish the SAC, we next tested if the SAC defect in *ΔBub1* cells is limited to SAC establishment and not to SAC maintenance. To test this, we treated cells with nocodazole and added reversine during the mitotic arrest (Figure 3E-G). *ΔBub1, ΔRod1,* and wild-type cells treated with a partial dose (1μM) of the Aurora B inhibitor ZM447439[352], were not able to arrest in mitosis when reversine was added before mitotic entry (Figure 3E and F). Wild-type cells were able to sustain a checkpoint-dependent arrest for some time, similar to what we observed before (Figure 3A, E, F). In contrast, *ΔRod1* cells rapidly exited from mitosis when reversine was added to cells that had already entered mitosis (Figure 3E and G), whereas *ΔBub1* cells managed to maintain a partial mitotic arrest when reversine was added during mitosis, similar to wild-type cells treated with or without ZM447439 (Figure 3E and G). The fact that *ΔBub1* behave similar to wild-type cells with reduced Aurora B activity indicates that the effect of Bub1 on SAC signaling is entirely mediated via its contribution to the centromeric recruitment of Aurora B, hereby establishing high Mps1-activity at KTs.

The RZZ complex and Bub1 cooperate to load Mad1 at KTs

Multiple studies indicated that the KT localization of Mad1 and Mad2 is not only strictly required, but also sufficient for SAC activation[343-345,353]. However, *ΔRod1* cells do not display clear KT localization of Mad1 in nocodazole-treated cells, while these cells have a partially active Mad1/Mad2-dependent SAC response (Figure 3A and chapter 6). We further investigated this paradox by testing the recruitment of Mad1 and Mad2 in untreated, asynchronous growing cells (Figure 4A). We could clearly observe KT-localization of Mad1 and Mad2 in wild-type and *ΔBub1* cells that just entered mitosis and on unattached chromosomes in late prometaphase in *ΔBub1* cells (Figure 4A). Surprisingly, we were also able to detect Mad1 and Mad2 at KTs of unattached chromosomes in early and late prometaphase *ΔRod1* cells (Figure 4A, left panel), while again we did not detect Mad1 and Mad2 in cells treated for 5 hours with nocodazole (Figure 4A, right panel). These data indicate that a Rod1-independent Mad1 KT recruitment pathway exists in early mitosis that is sufficient to trigger a temporary SAC response in *ΔRod1* cells.

Next, we tested which factor could be responsible for KT-targeting of Mad1 in early mitosis. Since multiple studies suggested that Bub1 is also involved in KT recruitment of Mad1 in human cells[197,351], we tested if Bub1 is responsible for the transient recruitment of Mad1 to KTs in *ΔRod1 c*ells. We treated wild-type and *ΔRod1* cells for 1 hour with nocodazole, with or without siRNA-mediated depletion of Bub1 (Figure 4B). As expected, we observed a prominent recruitment of Mad1 to KTs following a short nocodazole treatment in wild-type cells. Also, Bub1 depletion did not affect the KT

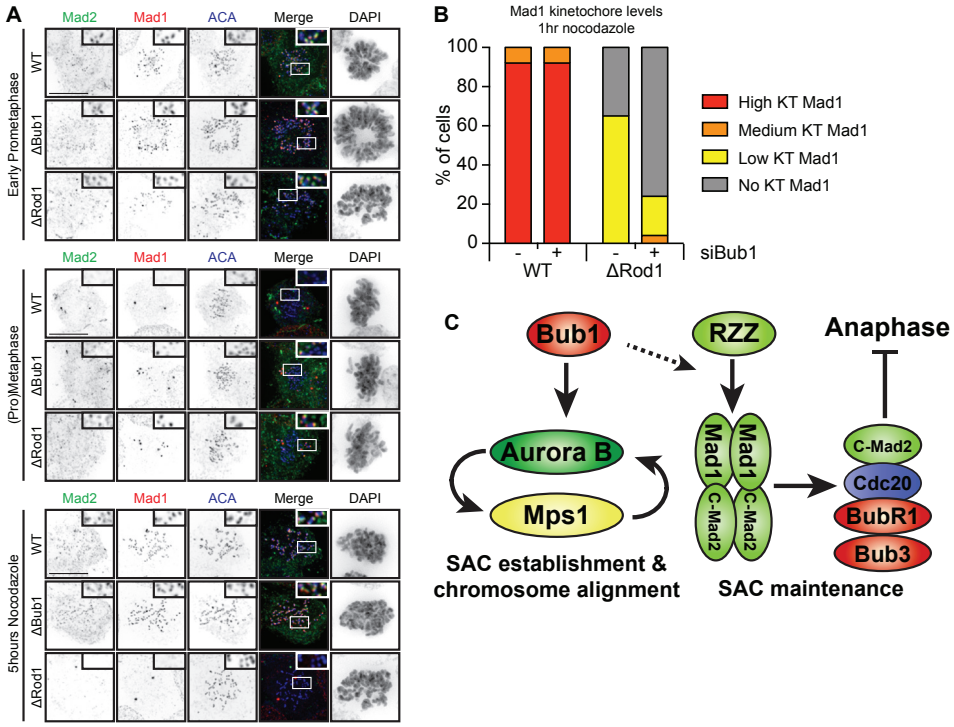


Figure 4: Bub1 and the RZZ complex cooperate with each other to target Mad1 to KTs.

(A) Representative images of wild-type, *ΔBub1,* and *ΔRod1* cells at different stages in mitosis. Mad2 is shown in green, Mad1 in red, KTs (ACA) in blue. Insets are enlargements of individual KT pairs. Note the recruitment of Mad1 and Mad2 to unattached KTs in early and late prometaphase in *ΔBub1* and *ΔRod1* cells and the absence of Mad1 and Mad2 at unattached KTs in *ΔRod1* cells treated for 8 hours with nocodazole. Scale bars represent 10 µm. (B) Quantification of the relative Mad1 KT levels in wild-type and *ΔRod1* cells, treated with or without siRNAs targeting Bub1. (C) Model of Bub1 and the RZZ complex function in the SAC. Bub1 helps to initiate the SAC by promoting the Aurora B – Mps1 positive feedback loop. The RZZ complex is required for the SAC by stably recruiting Mad1 and Mad2 to KTs. Bub1 likely accelerates this process by transiently binding to Mad1.

levels of Mad1 in these nocodazole-treated cultures. Furthermore, using this short-term nocodazole treatment, we could observe low levels of Mad1 on KTs in about 60% of *ΔRod1* cells (Figure 4B). Interestingly, the remaining KT Mad1 was significantly reduced after depletion of Bub1 in *ΔRod1* cells (Figure 4B), indicating that the RZZ complex is required for stable KT-targeting of Mad1. In addition, Bub1 likely transiently interacts with Mad1 in early mitosis, which is sufficient to establish a temporary SAC response in the absence of the RZZ complex. This temporary checkpoint activation likely enables cells lacking Rod1 to survive a short delay chromosome alignment, as we observed in chapter 6.

Discussion

Here we determined the contribution of Bub1, BubR1 and the RZZ complex to SAC signaling and chromosome alignment. Bub1 has previously been implicated in chromosome alignment by functioning in two separate pathways. First, it promotes the recruitment of BubR1-Bub3 dimers to KTs[188,348].

BubR1 in turn recruits PP2A-B56 which promotes stabilization of KT-MT interactions[155,156]. Second, it promotes centromeric accumulation of Aurora B by phosphorylating H2A, hereby stimulating the Aurora B-Mps1 positive feedback loop[192,193,196]. Here, we show that in HAP1 cells, the main function of Bub1 for chromosome alignment is to promote centromeric accumulation of Aurora B, since deletion of BubR1 does not result in any chromosome alignment defects. A recent study showed that transformed glioblastoma cells are more sensitive to BubR1 depletion, in contrast to their non-transformed equivalents, due to presence of altered KT-MT dynamics[354]. Hyperstable KT-MTs are a common feature of many cancer cells[355], and although we do not know if HAP1 cells have abnormal KT-MT dynamics, our results suggest that BubR1's function in chromosome alignment might be more important for transformed cell lines harboring this phenotype and could potentially explain why HAP1 cells do not depend on BubR1 for chromosome alignment.

The RZZ complex and Bub1 have both been shown to be important for SAC function, but the molecular details of their contribution to SAC signaling remain controversial mostly due to conflicting data obtained in different organisms[161]. Despite their previously described roles in SAC function, we found both Bub1 and the RZZ subunit Rod1 to be synthetic lethal with SAC-deficiency, indicating that the SAC must be, at least partially, functional in cells lacking Bub1 or Rod1 to maintain their proliferative capacity. Indeed, our data show that cells lacking Bub1 have a functional SAC that is indistinguishable from wild-type cells in an unperturbed mitosis. However, a minor defect in SAC function does become apparent in cells lacking Bub1 when they are challenged with inhibitors of Mps1. So what is the exact role of Bub1 in the SAC? Since cells lacking Bub1 arrest in mitosis as long as wild-type cells, its function in recruiting SAC proteins like BubR1, Bub3 and Cdc20 is likely not required for the maintenance of a robust SAC. Furthermore, our results also show that, in contrast to yeast and *C. elegans*, Bub1 is not essential for KT-recruitment of Mad1 and Mad2 in HAP1 cells[189,191]. Importantly, our results show that SAC function in cells lacking Bub1 is only perturbed during the initial stages of SAC activation, whereas SAC maintenance is not affected. Given the established role of Aurora B during the establishment of the SAC[193], combined with our observation that centromeric recruitment of Aurora B and Mps1-depedent KNL-1 phosphorylation are reduced in Bub1 knockout cells, we propose that Bub1 contributes to robust SAC signaling in HAP1 cells predominantly by stimulating the Aurora B-Mps1 positive feedback loop rather than via the recruitment of BubR1.

In contrast to previous literature[199,339,356], we found that in the absence of Rod1, cells still are able to activate the SAC, albeit only briefly. Interestingly, we found that Mad1 was recruited to unattached KTs in *ΔRod1* cells that just entered mitosis. Thus, our results indicate that the RZZ complex is not required for the early recruitment of Mad1/Mad2 to unattached KTs, but acts to sustain recruitment of Mad1 and Mad2. Indeed, we found that Mad1 and Mad2 were lost of KTs after prolonged nocodazole treatment in *ΔRod1* cells. This observation explains why *ΔRod1* cells are still able to briefly activate the SAC and can survive a short delay in chromosome congression as we showed in chapter 6.

It is currently unclear how Bub1 would promote recruitment of Mad1 at the onset of mitosis. Studies in yeast, that do not have a RZZ-complex, showed that Bub1 targets Mad1/Mad2 to KTs by a direct interaction between the RKL motif that is present in Mad1[190], and the CD1 motif, present in Bub1[349]. Although no direct interaction in human cells with Bub1 and Mad1 has been shown previously, we do find a further reduction in Mad1 recruitment in early mitotic cells lacking Rod1 that are depleted of Bub1. Interestingly, a recent study indicated that two different populations, a stable and a dynamic KT-bound Mad1 pool exist in *Drosophila*[357]. Although the molecular details of these two populations are still lacking, this might reflect the existence of a Bub1- and RZZ complex-bound Mad1 pool. We hypothesize that Bub1 initially targets Mad1 to the KT and subsequently hands the Mad1 over to the RZZ complex to maintain a prolonged SAC signal. Future studies will be required to test if Mad1 indeed

can be targeted to the KT by direct binding to the RZZ complex and Bub1, and to further understand the molecular details underlying the partial redundancy between Bub1 and the RZZ complex in the KT recruitment of Mad1.

Taken together, by using knockout cell lines for different SAC components, we unraveled the relative contributions to different SAC components to checkpoint establishment, checkpoint maintenance and chromosome alignment for each individual component. Using this approach, we uncovered a novel interplay between Bub1 and the RZZ complex in the early versus sustained recruitment of Mad1 to the kinetochore. Finally, we find that checkpoint components differ in their contribution to chromosome alignment not only between species, but also across human cell lines.

Experimental Procedures

Cell culture, transfection and drug treatments

HAP1 cells were cultured in IMDM (Gibco) with 8% FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin. DNA transfections were performed with FuGENE 6 (Promega) according to the manufactures guidelines. siRNA transfections were performed using RNAiMax (Invitrogen) according to the manufactures guidelines. The following siRNAs were used in this study: Mad2 OTP SMARTpool (Dharmacon), BubR1 custom siRNA [156], Bub1 (Dharmacon, gift from Geert Kops). Nocodazole and MG321 were used at a final concentration of 250 ng/ml and 5 µM respectively, or as indicated. Mps1 inhibitor (compound-5 Koch et al., in revision), reversine and ZM447439,[352] were used at the indicated concentrations.

Generation of knockout cell lines

Guide sequences for the CRISPR/Cas9-mediated genome editing were designed using CRISPR design (Zhang lab). All guides used in this study to generate knockout cell lines were targeted against exon 1, 2 or 3 of the gene of interest. Guides were cloned into the pX330 vector [324]. pX330 and a blasticidin donor vector (gift from Thijn Brummelkamp) were co-transfected in HAP1 cells and selected with 5 µg/ml blasticidin. Individual clones were selected and knockouts were confirmed using either PCR to confirm integration of the blasticidin cassette or by western blot.

Immunofluorescence

Cells were grown on 10mm glass coverslips and pre-extracted for 60 seconds in PEM buffer (100 mM PIPES, 10 mM EGTA, 1mM MgCl and 0.1% Trinton X-100), followed by fixation for 10 minutes at room temperature in 4% formaldehyde in PEM buffer with 0.3% Triton X-100. The following antibodies were used: rabbit phospho- H3 (Millipore), mouse α-tubulin (Sigma), human ACA (Cortex Biochem), Rabbit Mad2 (Bethyl), mouse Bub1 (Abcam), rabbit BubR1 (Bethyl), rabbit phosho-histone 2A (Upstate), rabbit Aurora B (Abcam), mouse Mad1 (Santa Cruz), rabbit phospho-KNL-1 (custom, a gift from Geert Kops). All antibodies were incubated over night at 4°C. Secondary antibodies (Molecular probes, Invitrogen) were incubated 1 hr at room temperature. DAPI was added before mounting using ProLong Gold (Invitrogen). Images were acquired using a Deltavision deconvolution microscope (Applied Precision) with a 100x 1.40 NA oil objective. Softworx (Applied Precision, ImageJ and Adobe Photoshop and Illustrator CS6 were used to process acquired images. Mitotic index was determined as described previously [118].

Time-lapse microscopy

Cells were plated on 8-well glass-bottom dishes (LabTek) and cultured in L-15 CO_2 independent medium (Gibco). Cells were imaged using a Deltavision deconvolution microscope (Applied Precision) equipped

with a heat chamber. Images were acquired every four minutes using a 20x (0.25 NA) or 40x (1.40 NA) objective. Z-stacks were acquired with 3 µm intervals. Images were analyzed and processed using Softworx (Applied Precision) and ImageJ.

Colony formation
Cells were plate in 48-well plates at a density of 17,000 cells per well and treated as indicated for 7 days. After 7 days, cells were fixed using methanol and stained using crystal violet.

Western Blot
Cells were counted and lysed using Laemmli buffer (120 mM Tris pH 6.8, 4% SDS, 20% glycerol). Protein levels were analyzed using western blot. The following antibodies were used in this study: mouse α-tubulin (Sigma), mouse Bub1 (Abcam), rabbit BubR1 (Bethyl).

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Chapter 8

Summary and General Discussion

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Summary

Successful chromosome segregation during mitosis depends on the formation of a highly robust mitotic spindle. The cooperative behavior of many different pathways participating in mitotic spindle assembly ensure the formation of correct and stable attachments of all chromosomes to the mitotic spindle in a timely manner. Aberrations in pathways regulating mitotic spindle assembly can result in the formation of a defective mitotic spindle and as a consequence, errors in chromosome segregation. Drugs that perturb spindle formation are used extensively in anti-cancer treatments, but their efficacy is limited, in large part due to neurotoxicity. This is a consequence of the fact that the commonly used spindle poisons, such as taxanes and vincristines, perturb microtubule dynamics that are not only important for proper spindle assembly, but also for neuronal functions. As a result, investigators have been seeking alternative anti-mitotic drugs that strictly affect dividing cells. Prime examples of this class of drugs are inhibitors of the Eg5 microtubule motor protein. Inhibition of Eg5 blocks centrosome separation in human cells, resulting in perturbation of bipolar spindle formation, and a prolonged arrest in mitosis with monopolar spindles. This has spurred a lot of interest in Eg5 as an attractive target for anti-cancer therapy. However, in **chapter 2** of this thesis, we provide the evidence that cells can easily acquire the ability to divide in the complete absence of Eg5-activity. We showed that bipolar spindle assembly in cells lacking Eg5 critically depends on the function of the dynein motor protein. We identified a novel function for dynein in prophase centrosome separation in these Eg5-indepdendent cells and we show that this pathway also cooperates with Eg5 in normal cells. In addition, we showed that Kif15, whose function is normally redundant with Eg5, becomes essential for bipolar spindle formation in cells lacking Eg5-activity. In **chapter 3**, we identified three additional factors that are specifically required for bipolar spindle formation in Eg5-independent cells. We show that two kinesins with microtubule-depolymerase activity, MCAK and Kif18b, promote mitotic spindle assembly by controlling astral microtubule length. In addition, we show that the mitotic kinase Aurora A phosphorylates Kif15 to promote its association to the spindle. In **chapter 4**, we showed that Eg5, dynein and Kif15 act together to produce the right force balance in the spindle. By manipulating the function of these motors either alone or in combination, we show that alterations in the force balance result in the formation of aberrant mitotic spindles. In **chapter 5**, we reviewed the current literature about the function of Kif15 in mitotic spindle assembly and discuss the possibility to use Kif15 as a target in anti-cancer therapy. In **chapter 6** and **7**, we made use of haploid human cells, lacking a functional spindle assembly checkpoint (SAC) to identify novel genes controlling chromosome segregation. We identified several genes that participate in timely chromosome congression and confirm that the SAC becomes essential for proliferation in cells lacking these particular genes. In addition, we investigated the contribution of Bub1 and the RZZ-complex to SAC signaling in HAP1 cells. In contrast to previous studies, we show that cells either lacking Bub1 or the RZZ-complex have a partial functional SAC and that these cells depend on the SAC for their survival.

General Discussion

The mitotic spindle is the driving force behind chromosome segregation during mitosis. This highly dynamic structure is composed of microtubules that are organized into a bipolar array through the action of many different microtubule-associated proteins (MAPs). One important group of proteins that acts to organize the mitotic spindle is the kinesin superfamily. Kinesins are microtubule-dependent motor proteins that use the energy of ATP hydrolysis to produce a force stroke. The forces these motors produce can organize microtubules in the mitotic spindle by transport and sliding of microtubules. Here, we will discuss the results from this thesis in relation to the current literature on the contribution of several mitotic motor proteins and additional factors in mitotic spindle assembly.

Dynein cooperates with Eg5 during prophase centrosome separation

The first step of mitotic spindle assembly occurs during prophase. During this phase, the duplicate centrosomes move apart to opposite sides of the nucleus. Centrosome separation in prophase occurs in nearly all organisms that undergo an open mitosis, in which the nuclear envelope breaks down at the onset of mitosis. However, the extent of centrosome separation in prophase, as well as the factors that control it seem to differ across organisms. Previous studies indicate that Eg5 is the main driver of centrosome separation in human cells[30-32,224-226]. The tetrameric configuration of Eg5 allows it to crosslink and slide antiparallel microtubules[27,28], and this unique feature makes Eg5 a likely candidate to drive centrosome separation during prophase. However, if Eg5 would be the sole driver of prophase centrosome separation, centrosomes would be expected to move in a coordinated manner since Eg5-dependent forces are mediated via the sliding of anti-parallel microtubules that act on both centrosomes simultaneously. Nonetheless, observations from mammalian cells indicate that centrosomes move relatively independent from each other in prophase[35], indicating that other forces must contribute to centrosome separation. In line with these results, studies in *C. elegans* and *Drosophila* showed that Eg5 is dispensable for prophase centrosome separation[34,358]. In these organisms, the minus-end-directed motor dynein seems to produce the major force, driving prophase centrosome separation[38,39].

By applying selective pressure on human cell lines grown in tissue culture, we were able to induce directed evolution, resulting in the generation of Eg5-independent cells. Interestingly, we find that human cells can rapidly acquire the ability to proliferate in the complete absence of Eg5-activity[32,45]. These Eg5-independent cells (EICs) were able to efficiently separate their centrosomes in prophase, indeed confirming that in human cells additional factors participate in this process as well. We showed that this separation was fully dependent on the function of dynein, demonstrating that the role of dynein in centrosome separation in prophase is not limited to *C. elegans* and *Drosophila*. Importantly, we show that the function of dynein is not restricted to EICs, but also cooperates with Eg5 in normal cells[32], explaining why centrosomes move in an uncoordinated manner in prophase[359].

Whereas in *Drosophila* the cortical pool of dynein is thought to drive prophase centrosome separation[40,41,360], we showed that specifically removing dynein from the nuclear envelope in human cells perturbs its function in prophase centrosome separation in mammalian cells[32]. Thus, it is the nuclear envelope-associated pool of dynein that produces a force on centrosomes in prophase, not the cortical-bound pool, in line with previous observations that in mammalian cells, dynein does not localize to the cortex before nuclear envelope breakdown (NEB,[361]). Importantly, dynein is recruited to the nuclear envelope, in late G2 phase, just prior to the initiation of centrosome separation[42], and this pool has also been implicated in facilitating NEB and in keeping centrosomes in close proximity of the nucleus[42,44,362].

The molecular details how dynein recruitment to the nuclear envelope is regulated in space and time is still under debate. Recruitment can occur via at least four distinct pathways. Studies in *C. elegans* and the mammalian brain cells indicated that dynein-mediated nuclear migration, depends on its nuclear envelope recruitment by SUN-nesprin proteins[363,364]. In contrast, the late G2 nuclear envelope recruitment of dynein seems to depend on three other, separate pathways[42,43,365]. These studies showed that recruitment of dynein in late G2 is mediated by interactions with the nuclear pore-tethered complexes RanBP2-BicD2, Nup133-CENPF and Asunder-Lis1 [42,43,365]. RanBP2 binds to the dynein adaptor protein BicD2, hereby directly recruiting the dynein-dynactin complex to the nuclear pores[42]. The exact contribution of Nup133 in tethering dynein to the nuclear envelope is still under debate; while the Doye lab showed that dynein is lost upon depletion of Nup133 and CENPF[43], data from our lab indicated that not dynein itself, but rather the dynein activators NudE/L were lost from the nuclear envelope, hereby mimicking a dynein-depletion phenotype[32]. In addition, depletion of the nuclear envelope-associated protein Asunder also causes a reduction in dynein recruitment to the nuclear envelope[365]. This latter study showed that Asunder likely acts via the dynein adaptor protein Lis1, since it reported a direct interaction between both proteins[365]. Although the exact contribution of the pathways that recruit dynein or dynein adaptor proteins to the nuclear envelope is not fully resolved, it is interesting to note that all three pathways are required for dynein function at the nuclear envelope. How these pathways are connected and talk to each other needs to be determined in future studies.

In addition to its recruitment, another important question that remains is how the homogeneously distributed nuclear envelope-bound pool of dynein can produce asymmetric pulling forces on centrosomes to drive their separation. One plausible mechanism is that microtubule nucleation from centrosomes is asymmetric. Longer microtubules can bind more dynein molecules, hereby generating asymmetric pulling forces on centrosomes, which has previously been shown to be important for cleavage plane determination in amphibian and fish embryos[366]. Differences in centrosomal microtubule length along the nuclear envelope can simply be a consequence of the oval geometry of the nucleus. In addition, microtubules show increased catastrophe rates when they collide against physical barriers[237]. When centrosomes are still in close proximity of each other, microtubules from one centrosome might collide against the other centrosome, or against microtubules nucleated from the other centrosome, resulting in increased catastrophe rates in the area between two centrosomes and asymmetric aster growth. Dynein could pull on these asymmetric asters, hereby driving centrosome separation. Future studies, using for example *in vitro* reconstitution or high resolution imaging of prophase centrosomal asters, will hopefully help us to understand how nuclear envelope-bound dynein generates asymmetric pulling forces leading to centrosome separation.

Another remaining question is whether prophase centrosome separation is important in human cells. Our results indicate that EICs and normal cells with reduced Eg5 activity are more prone to form monopolar spindles when prophase centrosome separation is inhibited[32]. This suggests that prophase centrosome separation increases robustness of bipolar spindle formation. In addition, the spatial arrangement of all of the chromosomes between the two separated centrosomes at the time of NEB has been shown to support efficient mitotic spindle assembly, lowering the chance of forming incorrect kinetochore-microtubule attachments[139]. Indeed, a recent study showed that cells that fail to undergo centrosome separation in prophase display an increase in chromosome segregation errors[239]. Together, this indicates that prophase centrosome separation improves robust spindle assembly and increases the fidelity of chromosome segregation, providing an explanation for the evolutionary pressure to maintain this first step in mitotic spindle assembly. Our setup using directed *in vitro*

evolution was very helpful to uncover the relevant players of the prophase pathway, and can also be applied to study other essential processes in human cells and to uncover novel, redundant pathways.

Multiple antagonistically acting motors drive robust mitotic spindle assembly

The discovery of Eg5 was an important breakthrough in understanding centrosome separation and mitotic spindle formation. Its antiparallel microtubule sliding activity was shown to organize spindle microtubules into bipolar arrays and by sliding the antiparallel interpolar microtubules in the spindle midzone apart, Eg5 has been shown to play a key role in bipolarization of the mitotic spindle[27,52]. Indeed, the function of Eg5 is conserved throughout evolution and nearly all organisms require the function of Eg5 for mitotic spindle assembly[26]. However, studies from the past decade also indicate that Eg5 does not act alone and additional motors have been shown to participate in bipolarization of the spindle. For example, Kif15 was recently identified as an outward force generator in the mitotic spindle[57,58]. This function of Kif15 in human cells is fully redundant with Eg5's function in centrosome separation, but is obscured by the dominant action of Eg5 in human cells[57,59]. Nonetheless, over-expression of Kif15 can completely bypasses the requirement for Eg5 in bipolar spindle formation[57]. Furthermore, Kif15 is essential for bipolar spindle assembly in EICs[32].

An important remaining question is why cells require a second outward force-generating motor during mitotic spindle bipolarization? The most plausible explanation would be that Eg5 and Kif15 perform non-overlapping functions during mitotic spindle assembly. Thus, while Kif15 can drive bipolar spindle assembly in the absence of Eg5, it might not be able to perform all of Eg5's functions. Indeed, as mentioned above, Eg5 plays a key role in prophase centrosome separation in human cells, but this function cannot be executed by Kif15. This difference is most likely caused by the fact that Kif15 is kept inactive before NEB, because of the physical separation from its nuclear-localized cofactor TPX2[57]. Thus, Eg5 is clearly of crucial importance during the first steps of bipolar spindle assembly. In contrast, Kif15 and Eg5 seem to be equally important for maintenance of mitotic spindle bipolarity[57-59,228]. However, due to the dual function of Eg5 in prophase and prometaphase centrosome separation, it is difficult to study the individual contributions of Kif15 and Eg5 to bipolar spindle formation during prometaphase. In addition, while the mechanistic details of Eg5 function in the spindle are relatively well understood[28,53], the function of Kif15 is still poorly understood at the molecular level and contradictory results have been obtained by different labs[277,302]. First, it is currently not clear if Kif15 acts as a dimer or similar to Eg5, as a tetramer. Second, while we and others showed that TPX2 is absolutely required for Kif15 function in intact cells[57,58], *in vitro* single molecule assays of Kif15 and TPX2 showed that TPX2 blocked Kif15 stepping, suggesting that it acts as an inhibitor of Kif15[302]. However, since TPX2 can directly bind to microtubules, it is difficult to discard the possibility that inhibition of Kif15 stepping is due to TPX2 acting as a roadblock by binding to microtubules, or whether it exerts its effect by binding to Kif15 itself. In addition to TPX2, we identified Aurora A to be a regulator of Kif15 (chapter 3). Aurora A phosphorylates the C-terminus of Kif15, hereby promoting its binding to the spindle. We currently do not know how phosphorylation affects Kif15 at the molecular level. Similar to Kif15, Aurora A requires TPX2 for its function during mitosis and phosphorylation of Kif15 might affect its interaction with TPX2. Alternatively, phosphorylation of Kif15 by Aurora A might induce a conformational change in Kif15. A second, non-motor microtubule-binding site was recently identified in Kif15[277]. This site was shown to be important its microtubule crosslinking and sliding activity and is self-repressed by the C-terminus of Kif15[277]. The self-repressed state of Kif15 was shown to be released upon engagement of microtubules bundles. Possibly, phosphorylation of Aurora A might also affect either the affinity of the non-motor microtubule-binding domain for microtubules or affect self-repression by the C-terminus. Taken together, the contradictory results from previous

studies make it currently impossible to fully understand the mechanistic properties of Kif15. Further studies will hopefully give more insights into the exact function and regulation of Kif15 during mitotic spindle assembly.

The outward forces generated by Eg5 and Kif15 during mitotic spindle assembly are counteracted by minus-end-directed motors, including dynein and kinesin-14[31,33,59,63,64]. While Kinesin-14 seems to constitute the main antagonistic activity for Eg5 in *Drosophila*[33], dynein antagonizes Eg5 in *Xenopus* and human cells[31,59,63,64]. Metaphase spindles collapse in a dynein-dependent manner after simultaneous inhibition of Eg5 and Kif15[59], indicating that antagonistic motor forces continuously operate during mitosis.
So why do cells need these antagonistic motor forces in the mitotic spindle? Depletion of dynein results in a variety of mitotic spindle defects, including defects in spindle pole focusing, a failure to incorporate centrosomes in the spindle and defects in chromosome alignment[137,232]. We showed that the spindle defects observed upon dynein depletion could be reverted upon inhibition of either Eg5 or Kif15[59]. This indicates that a correct balance in the in- and outward forces is essential to maintain a correctly organized mitotic spindle. In the absence of dynein, spindle microtubule minus-ends are pushed out of the spindle pole by the outward force-generating motors, leading to pole focusing defects. Interestingly, we found that metaphase spindles remain bipolar upon when all antagonizing motor activities are inhibited simultaneously, indicating that, once formed, the bipolar spindle can retain its shape without any inward and outward force-generating motors. However, we find that in the absence of force-generating motor activity, the microtubules fail to generate sufficient tension on kinetochore pairs and kinetochore-microtubule interactions are not fully stabilized[59]. This indicates that outward force generation in the spindle is required to establishing proper kinetochore-microtubule interactions. The mechanism by which in- and outward forces are connected to establishment of tension on kinetochore pairs will require further investigation. Taken together, balanced activity of antagonistic motor forces is essential for robust mitotic spindle formation and segregation of chromosomes. *In vitro* reconstitution using multiple motors, including Eg5, Kif15 and dynein will be useful to understand how these factors cooperate in the spindle on the molecular level.

In addition to sliding motors like Eg5, Kif15 and dynein, the microtubule depolymerizing kinesins MCAK and Kif18b also play a role in mitotic spindle bipolarization (chapter 3). We showed that EICs and cells with reduced Eg5 activity depend on these proteins to build a bipolar spindle. MCAK and Kif18b cooperate with each other to regulate astral microtubule dynamics by inducing microtubule catastrophe[95,261,281], and depletion of MCAK and Kif18b results in altered microtubule dynamics and formation of long astral microtubules. *In vitro* studies showed that polymerizing microtubules, colliding into a barrier can generate a substantial amount of force before undergoing catastrophe[237,367]. We showed that in the absence of MCAK and Kif18b, polymerizing astral microtubules push against the cell cortex, hereby generating forces on spindle poles that counteract the activity of Eg5 and Kif15 (chapter 3). Our data indicates that tight regulation of microtubule dynamics by depolymerizing kinesins supports bipolarization of the mitotic spindle and allows microtubule motors like Eg5 and Kif15 to efficiently move spindle poles apart.

Multiple mechanisms drive chromosome alignment

Successful chromosome segregation requires sister chromatids to be stably connected to spindle microtubules from opposite spindle poles. The kinetochore is the main interaction site for chromosomes and spindle microtubules. In order to be captured by microtubules, chromosomes need to be guided

to regions in the cell with a high density of microtubules. The combined movement of chromosomes that eventually lead to the formation of stable kinetochore-microtubule interactions is known as chromosome congression. Multiple mechanisms cooperate with each other during chromosome congression and their combined action is required for timely formation of stable, amphitelic attachments. Defects in pathways involved in chromosome congression have been shown to cause chromosome segregation defects and as a consequence aneuploidy[160,368]. At the onset of NEB, most chromosomes will be positioned in between the two centrosomes and these chromosomes will quickly create interactions with opposing spindle microtubules, leading to fast bi-orientation[139]. However, some peripheral chromosomes might be more distant to spindle microtubules and kinetochores of these particular chromosomes sometimes face away from the spindle, hereby preventing timely bi-orientation. These peripheral chromosomes are usually first transported to spindle poles and subsequently toward the spindle equator[144]. Recent studies have provided more insight into the mechanism of these chromosomal movements[125,127,139,141]. Poleward transport of chromosomes is mediated by lateral attachments of kinetochores to astral microtubules via the minus-end-directed motor dynein[125,132,369]. Once near the pole, chromosomes can be transported to the spindle equator in three different ways that all eventually result in bi-orientation[144]. First, a chromosome can form monotelic attachments that are corrected to an amphitelic attachment that result in the chromosome to subsequently be pulled towards the spindle equator. Second, the monotelic attachments on a chromosome can persist, but the chromosome is transported to the spindle equator through the action of motor proteins. Once at the spindle equator, the monotelic attachment is corrected. Third, the chromosome looses all of the attachments and is first transported away from the pole, to then be bi-oriented near the spindle equator[126,140]. Transport towards the spindle equator is mediated by the plus-end-directed motor CENP-E (kinesin-7) and the chromokinesin Kid (kinesin-10,[123,125,127,129,141,143,370]). The initial attachment of kinetochores to microtubules occurs in a lateral fashion. In order to reach stable biorientation, these lateral attachments need to be converted into end-on attachments. This conversion depends on the microtubule depolymerizing activity of MCAK[145,371] and the microtubule binding factors from the KNL-1, Mis12, Ndc80 (KMN)-network[103]. Combined with chromosome movement, this promotes the formation of a stable metaphase plate of mitotic chromosomes aligned at the spindle equator of the mitotic cell.

In chapter 6 we identify a number of factors that delay chromosome alignment, by screening for synthetic lethal genes in SAC-deficient HAP1 cells. One of the genes we identified was condensin II. We showed that loss of condensin II does not impair chromosome condensation in HAP1 cells, while its loss did result in a delay in chromosome alignment. In contrast, loss of condensin I is not tolerated in HAP1 cells, suggesting condensin I, not II, plays an essential role in chromosome condensation.

So how does the condensin II complex promote efficient chromosome alignment? In contrast to condensin I, condensin II is nuclear during interphase and participates in the early stages of chromosome condensation in prophase[2]. As mentioned above, prepositioning of compacted chromosomes upon NEB facilitates timely formation of amphitelic attachments[139]. One possibility is that a minor delay in chromosome condensation affects the spatial organization of chromosomes during early prometaphase, preventing their efficient capture by microtubules. Chromokinesins might not function efficiently on not fully condensed chromosomes to expel chromosome arms away from the emerging spindles. As a consequence, kinetochore positioning towards the emerging spindle might also be affected. In addition to spatial organization, a slight reduction in chromosome condensation could also affect the molecular “spring” that generates tension between bi-orientated sister kinetochores[372]. Lack of tension can result in destabilization of the kinetochore-microtubule attachment, hereby delaying chromosome alignment. Detailed analysis of chromosome movement

and kinetochore organization in cells lacking condensin II could potentially give more insight into the exact function of chromosome condensation in chromosome alignment.

In addition to condensin II, we found that loss of Rod-Zw10-Zwilch (RZZ) complex is also synthetic lethal with SAC-deficiency (chapter 6). This result was remarkable since previous studies indicated that the RZZ complex itself is required for SAC signaling. In fact, the RZZ complex has a complicated function during mitosis. It has been shown to contribute to SAC signaling by recruiting Mad1-Mad2 to kinetochores[199,201], and is also involved in SAC silencing by removing SAC components from kinetochores upon microtubule attachment[208,209]. In addition, the localization of dynein at the kinetochores depends on the RZZ complex and the RZZ-accessory protein spindly[134-136,138]. The loss of dynein from kinetochores in the absence of the RZZ complex, could explain the alignment defect in cells lacking Rod1. However, we found that although spindly was lost from kinetochores, dynein was still present at kinetochores upon loss of Rod1 (chapter 6).

So why do cells display transient chromosome alignment problems upon loss of Rod1? One simple explanation is that although dynein in our hands still localizes to kinetochores, it is not functional anymore. This explanation seems to be unlikely, since the unaligned chromosomes in cells lacking Rod1 were mostly located near the poles (chapter 6), suggesting that dynein-mediated poleward movement of chromosomes is not affected. A more plausible explanation is based on the recent observation that RZZ-spindly-dynein prevents the premature formation of end-on kinetochore-microtubule attachments by the Ndc80 complex[133,135,138]. The RZZ complex was shown to inhibit Ndc80 in *C. elegans* via control of the CH domain of Ndc80 and spindly-dynein act as a switch to turn off this inhibitory function[133]. Thus, upon lateral attachment of chromosomes by dynein, the RZZ complex inhibits Ndc80. But once the kinetochore comes in contact with the depolymerizing plus-end of the microtubule, dynein will engage in stabilization of the plus-end, releasing its inhibitory action on Ndc80, which in turn can promote the formation of an end-on attachment. Although this model is based on *C. elegans* data, it might function similar in human cells since the RZZ and Ndc80 complex are both conserved throughout metazoans. In addition, the phenotypes observed upon depletion of RZZ and spindly from *C. elegans* and human cells are also similar. It would be interesting to test if the CH domain in the tail of Ndn80 also interacts with RZZ in human cells and how the inhibitory function of RZZ is released during the switch from lateral to end-on attachment. Furthermore, investigating the kinetochores attachment status of misaligned chromosomes in the absence of RZZ in more detail might confirm the formation of premature end-on kinetochore-microtubule attachments, as predicted by the model.

At last, we investigated the role of Bub1 in chromosome alignment and SAC signaling in more detail (chapter 7). While Bub1 was originally identified in yeast as an essential SAC component[162,163], it was also shown to be involved in chromosome alignment in human cells[188,192,373]. Bub1 is thought to participate in this process via two independent mechanisms. First, it regulates the recruitment of BubR1-Bub3 heterodimers to kinetochores[111,184,188,348]. BubR1 in turn recruits the phosphatase PP2A-B56 to the kinetochore to counteract Aurora B activity hereby stabilizing kinetochore-microtubule attachments[155-157,195]. Second, Bub1 promotes centromeric accumulation of Aurora B through phosphorylation of histone 2A, hereby stimulating the error-correction machinery[192,196]. To our surprise, we found that BubR1 is dispensable for survival of HAP1 cells. Although HAP1 cells lacking BubR1 do not have a functional SAC, they do not show any defects in chromosome alignment. Due to this remarkable observation, our results indicate that Bub1 promotes chromosome alignment most likely via its function in regulating centromeric recruitment of Aurora B, independently of its function in recruiting BubR1 to kinetochores. The essential function of BubR1 for chromosome alignment has been confirmed in multiple cell lines[155,156,195,374]. However, our data indicates that the contribution of

BubR1 for stabilization of kinetochore-microtubule attachments might not be essential in certain cell types. Many cancer cell lines display hyper stable kinetochore-microtubule attachments and altered kinetochore-microtubule dynamics in contrast to non-transformed cells[355]. In addition, a recent study indicated that in contrast to glioblastoma cells, non-transformed neuronal stem cells also do not require BubR1 for stabilizing kinetochore-microtubule attachments[354]. This raises the question if the function of BubR1 in chromosome alignment is only required for transformed cells harboring altered kinetochore-microtubule dynamics. Although HAP1 cells are transformed, their kinetochore-microtubule dynamics might not be altered. It would be interesting to test if more non-transformed cell lines do not require BubR1 for chromosome alignment and to exploit this feature for anti-cancer therapies.

Taken together, the process of chromosome alignment is a complex and highly redundant process. Our screen results also show that known mitotic factors have additional direct and/or indirect roles in mediating chromosome alignment. The use of haploid genetics in combination with CRISPR/Cas9-mediated genome editing has proven to be a powerful tool for synthetic lethal screening and might be useful to uncover other redundant pathways involved in different aspects of mitosis.

The RZZ complex and Bub1 promote SAC signaling

We described the first human cell line that can live without a functional SAC (chapter 6). Detailed analysis indicated that mitosis occurs extremely efficient in HAP1 cells; chromosome alignment takes on average only 15 minutes in these cells, which is comparable to the time of alignment in *Drosophila* cells in which is the SAC is also non-essential[311]. By using these cells, we were able to study the contribution of several factors implicated in SAC function in more detail.

The roles of Bub1 and the RZZ complex and in the SAC have been controversial since their initial discovery. Studies in yeast have shown that Bub1 is essential for SAC signaling due to its direct role in Mad1-Mad2 kinetochore recruitment[189,375], and similar observations have been made in human cells[188,351]. Based on our results using CRISPR/Cas9-mediated genome editing, Bub1 is dispensable for kinetochore recruitment of Mad1-Mad2 (chapter 7). Bub1 knockout cells were able to maintain a prolonged SAC response, indicating that it does not play a significant role in SAC signaling. However, upon sensitization of the SAC using partial inhibition of Mps1, we found that Bub1 knockout cells have a less robust SAC response compared to wild-type cells. Our data indicate that the reduced SAC response is likely caused by delayed activation of Mps1 due to a reduction in centromeric Aurora B localization. This is based on the fact that we did not observe increased sensitivity to partial Mps1 inhibition when cells already entered mitosis in the absence of Bub1 (chapter 7,[193]. This is in agreement with previous literature showing an important role for Bub1 kinase signaling and in SAC activation[192,196]. The results from our Bub1 knockout cells also indicate that kinetochore recruitment of BubR1 and Bub3 is not required for MCC formation and SAC signaling, confirming data from previous studies showing that conversion of O-Mad2 into C-Mad2 at kinetochores is the dominant step for MCC formation at kinetochores[376].

In addition to Bub1, the RZZ complex is also thought to be involved in kinetochore recruitment of Mad1-Mad2 in higher organisms[199], explaining its function in SAC signaling. We show that in human cells, the RZZ complex is indeed the main kinetochore receptor for Mad1-Mad2 (chapter 7). In the absence of the RZZ complex subunit Rod1, Mad1-Mad2 was no longer stably associated to the kinetochore and cells lacking Rod1 were unable to maintain a prolonged SAC response. Surprisingly, we found that cells lacking Rod1 were able to transiently recruit Mad1-Mad2 to unattached kinetochores in early mitosis and these cells depend on this temporary Mad1-Mad2 recruitment phase for their survival. This indicates that an additional, likely transient kinetochore receptor for Mad1-Mad2 exists that

functions in parallel with the RZZ complex. We currently do not know which factor is responsible for this temporary recruitment. However, noticed a clear reduction in this temporary recruitment after depletion of Bub1. This could indicate that Bub1 supports the kinetochore recruitment of Mad1-Mad2 by transiently binding to Mad1 and subsequently transferring it to the RZZ complex. The existence of two separate KT-pools of Mad1 that display different turnover rates in *Drosophila* cells, further supports our hypothesis regarding the existence of two parallel Mad1 recruitment pathways[357]. However, the results after Bub1 depletion might also be indirect due to the delayed Mps1 activation in these cells and Mps1 has previously been shown to be essential for Mad1-Mad2 recruitment[168,170,377]. Since the recruitment of Mad1-Mad2 does not require Bub1 kinase activity[189], it would be interesting the test if expression of kinase-dead Bub1 can rescue the transient early recruitment of Mad1-Mad2 to kinetochores in Rod1 knockout cells.

Concluding remarks

Faithfull chromosome segregation during mitosis is essential to maintain genome integrity and is mediated by a dynamic structure known as the mitotic spindle. The results described in this thesis provide novel insights into the complex mitotic processes mediating mitotic spindle assembly and function. By using sensitized backgrounds in our screening approaches, we discovered previously unrecognized parallel pathways involved in mitotic spindle assembly. Our work has contributed to the understanding of how these different pathways function together to mediate robust mitotic spindle assembly. Similar screening approaches can be a valuable tool for the discovery of new genes and pathways that function in other cell biological processes as well.

Addendum

References

Nederlandse Samenvatting

Curriculum Vitae

List of Publications

Dankwoord

References

1. Remeseiro, S. & Losada, A. Cohesin, a chromatin engagement ring. *Current Opinion in Cell Biology* 25, 63–71 (2013).
2. Hirano, T. Condensins: universal organizers of chromosomes with diverse functions. *Genes & Development* 26, 1659–1678 (2012).
3. Gardner, M. K., Zanic, M. & Howard, J. Microtubule catastrophe and rescue. *Current Opinion in Cell Biology* 25, 14–22 (2013).
4. Wittmann, T., Hyman, A. & Desai, A. The spindle: a dynamic assembly of microtubules and motors. *Nat. Cell Biol.* 3, E28–34 (2001).
5. Glotzer, M. The 3Ms of central spindle assembly: microtubules, motors and MAPs. *Nature Reviews Molecular Cell Biology* 10, 9–20 (2009).
6. McNally, F. J. Mechanisms of spindle positioning. *The Journal of Cell Biology* 200, 131–140 (2013).
7. Bornens, M. The centrosome in cells and organisms. *Science* 335, 422–426 (2012).
8. Hinchcliffe, E. H. The centrosome and bipolar spindle assembly: does one have anything to do with the other? *cc* 10, 3841–3848 (2011).
9. Ganem, N. J., Godinho, S. A. & Pellman, D. A mechanism linking extra centrosomes to chromosomal instability. *Nature* 460, 278–282 (2009).
10. Nigg, E. A. & Raff, J. W. Centrioles, centrosomes, and cilia in health and disease. *Cell* 139, 663–678 (2009).
11. Godinho, S. A. *et al.* Oncogene-like induction of cellular invasion from centrosome amplification. *Nature* 1–18 (2014). doi:10.1038/nature13277
12. Lawo, S., Hasegan, M., Gupta, G. D. & Pelletier, L. Subdiffraction imaging of centrosomes reveals higher-order organizational features of pericentriolar material. *Nat. Cell Biol.* 14, 1–13 (2012).
13. Mennella, V. *et al.* Subdiffraction-resolution fluorescence microscopy reveals a domain of the centrosome critical for pericentriolar material organization. *Nat. Cell Biol.* 14, 1159–1168 (2012).
14. Piel, M., Meyer, P., Khodjakov, A., Rieder, C. L. & Bornens, M. The respective contributions of the mother and daughter centrioles to centrosome activity and behavior in vertebrate cells. *The Journal of Cell Biology* 149, 317–330 (2000).
15. Kollman, J. M., Merdes, A., Mourey, L. & Agard, D. A. Microtubule nucleation by γ-tubulin complexes. *Nature Reviews Molecular Cell Biology* 12, 709–721 (2011).
16. Barr, A. R. & Gergely, F. Aurora-A: the maker and breaker of spindle poles. *Journal of Cell Science* 120, 2987–2996 (2007).
17. Maiato, H. & Logarinho, E. Mitotic spindle multipolarity without centrosome amplification. *Nat. Cell Biol.* 16, 386–394 (2014).
18. Mardin, B. R. & Schiebel, E. Breaking the ties that bind: new advances in centrosome biology. *The Journal of Cell Biology* 197, 11–18 (2012).
19. Bahe, S., Stierhof, Y.-D., Wilkinson, C. J., Leiss, F. & Nigg, E. A. Rootletin forms centriole-associated filaments and functions in centrosome cohesion. *The Journal of Cell Biology* 171, 27–33 (2005).

20. Mayor, T., Stierhof, Y. D., Tanaka, K., Fry, A. M. & Nigg, E. A. The centrosomal protein C-Nap1 is required for cell cycle-regulated centrosome cohesion. *The Journal of Cell Biology* 151, 837–846 (2000).
21. Faragher, A. J. & Fry, A. M. Nek2A kinase stimulates centrosome disjunction and is required for formation of bipolar mitotic spindles. *Mol. Biol. Cell* 14, 2876–2889 (2003).
22. Fry, A. M. *et al.* C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *The Journal of Cell Biology* 141, 1563–1574 (1998).
23. Graser, S., Stierhof, Y.-D. & Nigg, E. A. Cep68 and Cep215 (Cdk5rap2) are required for centrosome cohesion. *Journal of Cell Science* 120, 4321–4331 (2007).
24. Bahmanyar, S. *et al.* beta-Catenin is a Nek2 substrate involved in centrosome separation. *Genes & Development* 22, 91–105 (2008).
25. Tanenbaum, M. E. & Medema, R. H. Mechanisms of Centrosome Separation and Bipolar Spindle Assembly. *Developmental Cell* 19, 797–806 (2010).
26. Ferenz, N. P., Gable, A. & Wadsworth, P. Mitotic functions of kinesin-5. *Seminars in Cell and Developmental Biology* 21, 255–259 (2010).
27. Kashina, A. S. *et al.* A bipolar kinesin. *Nature* 379, 270–272 (1996).
28. Kapitein, L. C. *et al.* The bipolar mitotic kinesin Eg5 moves on both microtubules that it crosslinks. *Nature* 435, 114–118 (2005).
29. Blangy, A. *et al.* Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell* 83, 1159–1169 (1995).
30. Whitehead, C. M. & Rattner, J. B. Expanding the role of HsEg5 within the mitotic and post-mitotic phases of the cell cycle. *Journal of Cell Science* 111 (Pt 17), 2551–2561 (1998).
31. Tanenbaum, M. E., Macurek, L., Galjart, N. & Medema, R. H. Dynein, Lis1 and CLIP-170 counteract Eg5-dependent centrosome separation during bipolar spindle assembly. *The EMBO Journal* 27, 3235–3245 (2008).
32. Raaijmakers, J. A. *et al.* Nuclear envelope-associated dynein drives prophase centrosome separation and enables Eg5-independent bipolar spindle formation. *The EMBO Journal* 31, 4179–4190 (2012).
33. Sharp, D. J., Yu, K. R., Sisson, J. C., Sullivan, W. & Scholey, J. M. Antagonistic microtubule-sliding motors position mitotic centrosomes in Drosophila early embryos. *Nat. Cell Biol.* 1, 51–54 (1999).
34. Saunders, A. M., Powers, J., Strome, S. & Saxton, W. M. Kinesin-5 acts as a brake in anaphase spindle elongation. *Current Biology* 17, R453–R454 (2007).
35. Waters, J. C., Cole, R. W. & Rieder, C. L. The force-producing mechanism for centrosome separation during spindle formation in vertebrates is intrinsic to each aster. *J Cell Biol* 122, 361–372 (1993).
36. Raaijmakers, J. A. & Medema, R. H. Function and regulation of dynein in mitotic chromosome segregation. *Chromosoma* (2014). doi:10.1007/s00412-014-0468-7
37. Kardon, J. R. & Vale, R. D. Regulators of the cytoplasmic dynein motor. 1–12 (2009). doi:10.1038/nrm2804
38. Cytoplasmic dynein is required for distinct aspects of MTOC positioning, including centrosome separation, in the one cell stage Caenorhabditis elegans embryo. 147, 135–150 (1999).

39. Robinson, J. T., Wojcik, E. J., Sanders, M. A., McGrail, M. & Hays, T. S. Cytoplasmic dynein is required for the nuclear attachment and migration of centrosomes during mitosis in Drosophila. *The Journal of Cell Biology* 146, 597–608 (1999).

40. Sharp, D. J. *et al.* Functional coordination of three mitotic motors in Drosophila embryos. *Mol. Biol. Cell* 11, 241–253 (2000).

41. Cytrynbaum, E. N., Scholey, J. M. & Mogilner, A. A Force Balance Model of Early Spindle Pole Separation in Drosophila Embryos. *Biophysj* 84, 757–769 (2003).

42. Splinter, D. *et al.* Bicaudal D2, Dynein, and Kinesin-1 Associate with Nuclear Pore Complexes and Regulate Centrosome and Nuclear Positioning during Mitotic Entry. *PLoS Biol* 8, e1000350 (2010).

43. Bolhy, S. *et al.* A Nup133-dependent NPC-anchored network tethers centrosomes to the nuclear envelope in prophase. *The Journal of Cell Biology* 192, 855–871 (2011).

44. Salina, D. *et al.* Cytoplasmic dynein as a facilitator of nuclear envelope breakdown. *Cell* 108, 97–107 (2002).

45. van Heesbeen, R. G. H. P., Raaijmakers, J. A., Tanenbaum, M. E. & Medema, R. H. Nuclear envelope-associated dynein cooperates with Eg5 to drive prophase centrosome separation. *Commun Integr Biol* 6, e23841 (2013).

46. Cao, J., Crest, J., Fasulo, B. & Sullivan, W. Cortical actin dynamics facilitate early-stage centrosome separation. *Curr. Biol.* 20, 770–776 (2010).

47. Vignaud, T., Blanchoin, L. & Théry, M. Directed cytoskeleton self-organization. *Trends in Cell Biology* 22, 671–682 (2012).

48. Bishop, J. D., Han, Z. & Schumacher, J. M. The Caenorhabditis elegans Aurora B kinase AIR-2 phosphorylates and is required for the localization of a BimC kinesin to meiotic and mitotic spindles. *Mol. Biol. Cell* 16, 742–756 (2005).

49. Tikhonenko, I., Nag, D. K., Martin, N. & Koonce, M. P. Kinesin-5 is not essential for mitotic spindle elongation in Dictyostelium. *Cell Motil. Cytoskeleton* 65, 853–862 (2008).

50. Heck, M. M. *et al.* The kinesin-like protein KLP61F is essential for mitosis in Drosophila. *The Journal of Cell Biology* 123, 665–679 (1993).

51. Hoyt, M. A., He, L., Loo, K. K. & Saunders, W. S. Two Saccharomyces cerevisiae kinesin-related gene products required for mitotic spindle assembly. *The Journal of Cell Biology* 118, 109–120 (1992).

52. Sawin, K. E., LeGuellec, K., Philippe, M. & Mitchison, T. J. Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* 359, 540–543 (1992).

53. van den Wildenberg, S. M. J. L. *et al.* The Homotetrameric Kinesin-5 KLP61F Preferentially Crosslinks Microtubules into Antiparallel Orientations. *Current Biology* 18, 1860–1864 (2008).

54. Boleti, H., Karsenti, E. & Vernos, I. Xklp2, a novel Xenopus centrosomal kinesin-like protein required for centrosome separation during mitosis. *Cell* 84, 49–59 (1996).

55. Segbert, C. *et al.* KLP-18, a Klp2 kinesin, is required for assembly of acentrosomal meiotic spindles in Caenorhabditis elegans. *Mol. Biol. Cell* 14, 4458–4469 (2003).

56. Wignall, S. M. & Villeneuve, A. M. Lateral microtubule bundles promote chromosome alignment during acentrosomal oocyte meiosis. *Nat. Cell Biol.* 11, 909–913 (2009).

57. Tanenbaum, M. E. *et al.* Kif15 Cooperates with Eg5 to Promote Bipolar Spindle Assembly. *Current Biology* 19, 1703–1711 (2009).

58. Vanneste, D., Takagi, M., Imamoto, N. & Vernos, I. The Role of Hklp2 in the Stabilization and Maintenance of Spindle Bipolarity. *Current Biology* 19, 1712–1717 (2009).

59. van Heesbeen, R. G. H. P., Tanenbaum, M. E. & Medema, R. H. Balanced activity of three mitotic motors is required for bipolar spindle assembly and chromosome segregation. *CellReports* 8, 948–956 (2014).

60. Sturgill, E. G. & Ohi, R. Kinesin-12 Differentially Affects Spindle Assembly Depending on Its Microtubule Substrate. *Current Biology* 1–11 (2013). doi:10.1016/j.cub.2013.05.043

61. Saunders, W. S. & Hoyt, M. A. Kinesin-related proteins required for structural integrity of the mitotic spindle. *Cell* 70, 451–458 (1992).

62. Mountain, V. *et al.* The kinesin-related protein, HSET, opposes the activity of Eg5 and cross-links microtubules in the mammalian mitotic spindle. *The Journal of Cell Biology* 147, 351–366 (1999).

63. Mitchison, T. J. *et al.* Roles of polymerization dynamics, opposed motors, and a tensile element in governing the length of Xenopus extract meiotic spindles. *Mol. Biol. Cell* 16, 3064–3076 (2005).

64. Ferenz, N. P., Paul, R., Fagerstrom, C., Mogilner, A. & Wadsworth, P. Dynein Antagonizes Eg5 by Crosslinking and Sliding Antiparallel Microtubules. *Current Biology* 19, 1833–1838 (2009).

65. Tanenbaum, M. E., Vale, R. D. & McKenney, R. J. Cytoplasmic dynein crosslinks and slides anti-parallel microtubules using its two motor domains. *eLife* 2, e00943–e00943 (2013).

66. Gaglio, T. *et al.* Opposing motor activities are required for the organization of the mammalian mitotic spindle pole. *The Journal of Cell Biology* 135, 399–414 (1996).

67. Chandra, R., Salmon, E. D., Erickson, H. P., Lockhart, A. & Endow, S. A. Structural and functional domains of the Drosophila ncd microtubule motor protein. *J. Biol. Chem.* 268, 9005–9013 (1993).

68. Fink, G. *et al.* The mitotic kinesin-14 Ncd drives directional microtubule–microtubule sliding. *Nat. Cell Biol.* 11, 717–723 (2009).

69. Hentrich, C. & Surrey, T. Microtubule organization by the antagonistic mitotic motors kinesin-5 and kinesin-14. *The Journal of Cell Biology* 189, 465–480 (2010).

70. Merdes, A., Ramyar, K., Vechio, J. D. & Cleveland, D. W. A complex of NuMA and cytoplasmic dynein is essential for mitotic spindle assembly. *Cell* 87, 447–458 (1996).

71. Galjart, N. CLIPs and CLASPs and cellular dynamics. *Nature Reviews Molecular Cell Biology* 6, 487–498 (2005).

72. Schroer, T. A. Dynactin. *Annu. Rev. Cell Dev. Biol.* 20, 759–779 (2004).

73. Heald, R., Tournebize, R., Habermann, A., Karsenti, E. & Hyman, A. Spindle assembly in Xenopus egg extracts: respective roles of centrosomes and microtubule self-organization. *The Journal of Cell Biology* 138, 615–628 (1997).

74. Loughlin, R., Heald, R. & Nédélec, F. A computational model predicts Xenopus meiotic spindle organization. *The Journal of Cell Biology* 191, 1239–1249 (2010).

75. Goshima, G., Nédélec, F. & Vale, R. D. Mechanisms for focusing mitotic spindle poles by minus end-directed motor proteins. *The Journal of Cell Biology* 171, 229–240 (2005).

76. Moutinho-Pereira, S. *et al.* Genes involved in centrosome-independent mitotic spindle assembly in Drosophila S2 cells. *Proceedings of the National Academy of Sciences* 110, 19808–19813 (2013).

77. Elting, M. W., Hueschen, C. L., Udy, D. B. & Dumont, S. Force on spindle microtubule minus ends moves chromosomes. *The Journal of Cell Biology* 82, 528 (2014).

78. Wakefield, J. G., Bonaccorsi, S. & Gatti, M. The drosophila protein asp is involved in microtubule organization during spindle formation and cytokinesis. *The Journal of Cell Biology* 153, 637–648 (2001).

79. Manning, A. L. & Compton, D. A. Mechanisms of Spindle-Pole Organization Are Influenced by Kinetochore Activity in Mammalian Cells. *Current Biology* 17, 260–265 (2007).

80. Clarke, P. R. & Zhang, C. Spatial and temporal coordination of mitosis by Ran GTPase. *Nature Reviews Molecular Cell Biology* 9, 464–477 (2008).

81. Carazo-Salas, R. E. *et al.* Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. *Nature* 400, 178–181 (1999).

82. Carazo-Salas, R. E., Gruss, O. J., Mattaj, I. W. & Karsenti, E. Ran-GTP coordinates regulation of microtubule nucleation and dynamics during mitotic-spindle assembly. *Nat. Cell Biol.* 3, 228–234 (2001).

83. Gruss, O. J. *et al.* Ran induces spindle assembly by reversing the inhibitory effect of importin alpha on TPX2 activity. *Cell* 104, 83–93 (2001).

84. Koffa, M. D. *et al.* HURP Is Part of a Ran-Dependent Complex Involved in Spindle Formation. *Current Biology* 16, 743–754 (2006).

85. Nachury, M. V. *et al.* Importin beta is a mitotic target of the small GTPase Ran in spindle assembly. *Cell* 104, 95–106 (2001).

86. Groen, A. C. *et al.* XRHAMM Functions in Ran-Dependent Microtubule Nucleation and Pole Formation during Anastral Spindle Assembly. *Current Biology* 14, 1801–1811 (2004).

87. Goshima, G., Mayer, M., Zhang, N., Stuurman, N. & Vale, R. D. Augmin: a protein complex required for centrosome-independent microtubule generation within the spindle. *The Journal of Cell Biology* 181, 421–429 (2008).

88. Zhu, H., Coppinger, J. A., Jang, C.-Y., Yates, J. R. & Fang, G. FAM29A promotes microtubule amplification via recruitment of the NEDD1-gamma-tubulin complex to the mitotic spindle. *The Journal of Cell Biology* 183, 835–848 (2008).

89. Petry, S., Groen, A. C., Ishihara, K., Mitchison, T. J. & Vale, R. D. Branching Microtubule Nucleation in Xenopus Egg Extracts Mediated by Augmin and TPX2. *Cell* 152, 768–777 (2013).

90. Uehara, R. *et al.* The augmin complex plays a critical role in spindle microtubule generation for mitotic progression and cytokinesis in human cells. *Proceedings of the National Academy of Sciences* 106, 6998–7003 (2009).

91. Meunier, S. & Vernos, I. Microtubule assembly during mitosis - from distinct origins to distinct functions? *Journal of Cell Science* 125, 2805–2814 (2012).

92. Carmena, M., Ruchaud, S. & Earnshaw, W. C. Making the Auroras glow: regulation of Aurora A and B kinase function by interacting proteins. *Current Opinion in Cell Biology* 21, 796–805 (2009).

93. Sampath, S. C. *et al.* The chromosomal passenger complex is required for chromatin-induced microtubule stabilization and spindle assembly. *Cell* 118, 187–202 (2004).

94. Kelly, A. E. *et al.* Chromosomal enrichment and activation of the aurora B pathway are coupled to spatially regulate spindle assembly. *Developmental Cell* 12, 31–43 (2007).

95. Tanenbaum, M. E. *et al.* A complex of Kif18b and MCAK promotes microtubule depolymerization and is negatively regulated by Aurora kinases. *Curr. Biol.* 21, 1356–1365 (2011).

96. Cheeseman, I. M. The Kinetochore. *Cold Spring Harbor Perspectives in Biology* 6, a015826–a015826 (2014).

97. Palmer, D. K., O'Day, K., Wener, M. H., Andrews, B. S. & Margolis, R. L. A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. *The Journal of Cell Biology* 104, 805–815 (1987).

98. Palmer, D. K., O'Day, K., Trong, H. L., Charbonneau, H. & Margolis, R. L. Purification of the centromere-specific protein CENP-A and demonstration that it is a distinctive histone. *Proc. Natl. Acad. Sci. U.S.A.* 88, 3734–3738 (1991).

99. Hori, T., Shang, W. H., Takeuchi, K. & Fukagawa, T. The CCAN recruits CENP-A to the centromere and forms the structural core for kinetochore assembly. *The Journal of Cell Biology* (2012). doi:10.1083/jcb.201210106

100. Hori, T. *et al.* CCAN Makes Multiple Contacts with Centromeric DNA to Provide Distinct Pathways to the Outer Kinetochore. *Cell* 135, 1039–1052 (2008).

101. Nishino, T. *et al.* CENP-T-W-S-X Forms a Unique Centromeric Chromatin Structure with a Histone-like Fold. *Cell* 148, 487–501 (2012).

102. Kim, S. & Yu, H. Multiple assembly mechanisms anchor the KMN spindle checkpoint platform at human mitotic kinetochores. *The Journal of Cell Biology* 208, 181–196 (2015).

103. Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M. & Desai, A. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell* 127, 983–997 (2006).

104. Kline, S. L., Cheeseman, I. M., Hori, T., Fukagawa, T. & Desai, A. The human Mis12 complex is required for kinetochore assembly and proper chromosome segregation. *The Journal of Cell Biology* 173, 9–17 (2006).

105. Petrovic, A. *et al.* The MIS12 complex is a protein interaction hub for outer kinetochore assembly. *The Journal of Cell Biology* 190, 835–852 (2010).

106. Screpanti, E. *et al.* Direct binding of Cenp-C to the Mis12 complex joins the inner and outer kinetochore. *Curr. Biol.* 21, 391–398 (2011).

107. Kiyomitsu, T., Obuse, C. & Yanagida, M. Human Blinkin/AF15q14 Is Required for Chromosome Alignment and the Mitotic Checkpoint through Direct Interaction with Bub1 and BubR1. *Developmental Cell* 13, 663–676 (2007).

108. Krenn, V., Wehenkel, A., Li, X., Santaguida, S. & Musacchio, A. Structural analysis reveals features of the spindle checkpoint kinase Bub1-kinetochore subunit Knl1 interaction. *The Journal of Cell Biology* (2012). doi:10.1083/jcb.201110013

109. London, N., Ceto, S., Ranish, J. A. & Biggins, S. Phosphoregulation of Spc105 by Mps1 and PP1 Regulates Bub1 Localization to Kinetochores. *Current Biology* 22, 900–906 (2012).

110. Primorac, I. *et al.* Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling. *eLife* 2, e01030–e01030 (2013).

111. Vleugel, M. *et al.* Arrayed BUB recruitment modules in the kinetochore scaffold KNL1 promote accurate chromosome segregation. *The Journal of Cell Biology* 203, 943–955 (2013).

112. McCleland, M. L. *et al.* The vertebrate Ndc80 complex contains Spc24 and Spc25 homologs, which are required to establish and maintain kinetochore-microtubule attachment. *CURBIO* 14, 131–137 (2004).

113. DeLuca, J. G. *et al.* Kinetochore Microtubule Dynamics and Attachment Stability Are Regulated by Hec1. *Cell* 127, 969–982 (2006).

114. DeLuca, J. G., Moree, B., Hickey, J. M., Kilmartin, J. V. & Salmon, E. D. hNuf2 inhibition blocks stable kinetochore-microtubule attachment and induces mitotic cell death in HeLa cells. *The Journal of Cell Biology* 159, 549–555 (2002).

115. Wei, R. R., Al-Bassam, J. & Harrison, S. C. The Ndc80/HEC1 complex is a contact point for kinetochore-microtubule attachment. *Nat Struct Mol Biol* 14, 54–59 (2007).

116. Guimaraes, G. J., Dong, Y., McEwen, B. F. & DeLuca, J. G. Kinetochore-microtubule attachment relies on the disordered N-terminal tail domain of Hec1. *CURBIO* 18, 1778–1784 (2008).

117. Welburn, J. P. I. *et al.* The Human Kinetochore Ska1 Complex Facilitates Microtubule Depolymerization-Coupled Motility. *Developmental Cell* 16, 374–385 (2009).

118. Raaijmakers, J. A., Tanenbaum, M. E., Maia, A. F. & Medema, R. H. RAMA1 is a novel kinetochore protein involved in kinetochore-microtubule attachment. *Journal of Cell Science* 122, 2436–2445 (2009).

119. Gaitanos, T. N. *et al.* Stable kinetochore–microtubule interactions depend on the Ska complex and its new component Ska3/C13Orf3. *The EMBO Journal* 28, 1442–1452 (2009).

120. Schmidt, J. C. *et al.* The Kinetochore-Bound Ska1 Complex Tracks Depolymerizing Microtubules and Binds to Curved Protofilaments. *Developmental Cell* 23, 968–980 (2012).

121. Cheeseman, I. M. *et al.* Implication of a novel multiprotein Dam1p complex in outer kinetochore function. *The Journal of Cell Biology* 155, 1137–1145 (2001).

122. Lampert, F., Hornung, P. & Westermann, S. The Dam1 complex confers microtubule plus end-tracking activity to the Ndc80 kinetochore complex. *The Journal of Cell Biology* 189, 641–649 (2010).

123. Wood, K. W., Sakowicz, R., Goldstein, L. S. & Cleveland, D. W. CENP-E is a plus end-directed kinetochore motor required for metaphase chromosome alignment. *Cell* 91, 357–366 (1997).

124. Varma, D., Monzo, P., Stehman, S. A. & Vallee, R. B. Direct role of dynein motor in stable kinetochore-microtubule attachment, orientation, and alignment. *The Journal of Cell Biology* 182, 1045–1054 (2008).

125. Barisic, M., Aguiar, P., Geley, S. & Maiato, H. Kinetochore motors drive congression of peripheral polar chromosomes by overcoming random arm-ejection forces. *Nat. Cell Biol.* (2014). doi:10.1038/ncb3060

126. Kapoor, T. M. *et al.* Chromosomes can congress to the metaphase plate before biorientation. *Science* 311, 388–391 (2006).

127. Iemura, K. & Tanaka, K. Chromokinesin Kid and kinetochore kinesin CENP-E differentially support chromosome congression without end-on attachment to microtubules. *Nature Communications* 6, 1–11 (2015).

128. Rieder, C. L. & Salmon, E. D. Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *The Journal of Cell Biology* 124, 223–233 (1994).

129. Funabiki, H. & Murray, A. W. The Xenopus chromokinesin Xkid is essential for metaphase chromosome alignment and must be degraded to allow anaphase chromosome movement. *Cell* 102, 411–424 (2000).

130. Wandke, C. *et al.* Human chromokinesins promote chromosome congression and spindle microtubule dynamics during mitosis. *The Journal of Cell Biology* 198, 847–863 (2012).

131. Sharp, D. J., Rogers, G. C. & Scholey, J. M. Cytoplasmic dynein is required for poleward chromosome movement during mitosis in Drosophila embryos. *Nat. Cell Biol.* 2, 922–930 (2000).

132. Yang, Z., Tulu, U. S., Wadsworth, P. & Rieder, C. L. Kinetochore dynein is required for chromosome motion and congression independent of the spindle checkpoint. *CURBIO* 17, 973–980 (2007).

133. Cheerambathur, D. K., Gassmann, R., Cook, B., Oegema, K. & Desai, A. Crosstalk Between Microtubule Attachment Complexes Ensures Accurate Chromosome Segregation. *Science* 342, 1239–1242 (2013).

134. Griffis, E. R., Stuurman, N. & Vale, R. D. Spindly, a novel protein essential for silencing the spindle assembly checkpoint, recruits dynein to the kinetochore. *The Journal of Cell Biology* 177, 1005–1015 (2007).

135. Barisic, M. *et al.* Spindly/CCDC99 is required for efficient chromosome congression and mitotic checkpoint regulation. *Mol. Biol. Cell* 21, 1968–1981 (2010).

136. Starr, D. A., Williams, B. C., Hays, T. S. & Goldberg, M. L. ZW10 helps recruit dynactin and dynein to the kinetochore. *The Journal of Cell Biology* 142, 763–774 (1998).

137. Raaijmakers, J. A., Tanenbaum, M. E. & Medema, R. H. Systematic dissection of dynein regulators in mitosis. *The Journal of Cell Biology* 201, 201–215 (2013).

138. Gassmann, R. *et al.* A new mechanism controlling kinetochore-microtubule interactions revealed by comparison of two dynein-targeting components: SPDL-1 and the Rod/Zwilch/Zw10 complex. *Genes & Development* 22, 2385–2399 (2008).

139. Magidson, V. *et al.* The Spatial Arrangement of Chromosomes during Prometaphase Facilitates Spindle Assembly. *Cell* 146, 555–567 (2011).

140. Cai, S., O'Connell, C. B., Khodjakov, A. & Walczak, C. E. Chromosome congression in the absence of kinetochore fibres. *Nat. Cell Biol.* 11, 903–908 (2009).

141. Barisic, M. *et al.* Microtubule detyrosination guides chromosomes during mitosis. *Science* (2015). doi:10.1126/science.aaa5175

142. Kim, Y., Holland, A. J., Lan, W. & Cleveland, D. W. Aurora kinases and protein phosphatase 1 mediate chromosome congression through regulation of CENP-E. *Cell* 142, 444–455 (2010).

143. Levesque, A. A. & Compton, D. A. The chromokinesin Kid is necessary for chromosome arm orientation and oscillation, but not congression, on mitotic spindles. *The Journal of Cell Biology* 154, 1135–1146 (2001).

144. Walczak, C. E., Cai, S. & Khodjakov, A. Mechanisms of chromosome behaviour during mitosis. *Nature Reviews Molecular Cell Biology* 11, 91–102 (2010).

145. Kline-Smith, S. L., Khodjakov, A., Hergert, P. & Walczak, C. E. Depletion of centromeric MCAK leads to chromosome congression and segregation defects due to improper kinetochore attachments. *Mol. Biol. Cell* 15, 1146–1159 (2004).

146. Stumpff, J., Wagenbach, M., Franck, A., Asbury, C. L. & Wordeman, L. Kif18A and Chromokinesins Confine Centromere Movements via Microtubule Growth Suppression and Spatial Control of Kinetochore Tension. *Developmental Cell* 22, 1017–1029 (2012).

147. Carmena, M., Wheelock, M., Funabiki, H. & Earnshaw, W. C. The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. *Nature Publishing Group* 13, 789–803 (2012).

148. Hauf, S. *et al.* The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *The Journal of Cell Biology* 161, 281–294 (2003).

149. Suzuki, A. *et al.* Spindle microtubules generate tension-dependent changes in the distribution of inner kinetochore proteins. *The Journal of Cell Biology* 193, 125–140 (2011).

150. Lampson, M. A. & Cheeseman, I. M. Sensing centromere tension: Aurora B and the regulation of kinetochore function. *Trends in Cell Biology* 21, 133–140 (2011).

151. Liu, D., Vader, G., Vromans, M. J. M., Lampson, M. A. & Lens, S. M. A. Sensing chromosome bi-orientation by spatial separation of aurora B kinase from kinetochore substrates. *Science* 323, 1350–1353 (2009).

152. Welburn, J. P. I. *et al.* Aurora B Phosphorylates Spatially Distinct Targets to Differentially Regulatethe Kinetochore-Microtubule Interface. *Molecular Cell* 38, 383–392 (2010).

153. Chan, Y. W., Jeyaprakash, A. A., Nigg, E. A. & Santamaria, A. Aurora B controls kinetochore-microtubule attachments by inhibiting Ska complex-KMN network interaction. *The Journal of Cell Biology* (2012). doi:10.1083/jcb.201109001

154. Liu, D. *et al.* Regulated targeting of protein phosphatase 1 to the outer kinetochore by KNL1 opposes Aurora B kinase. *The Journal of Cell Biology* 188, 809–820 (2010).

155. Foley, E. A., Maldonado, M. & Kapoor, T. M. Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. *Nat. Cell Biol.* 13, 1265–1271 (2011).

156. Suijkerbuijk, S. J. E., Vleugel, M., Teixeira, A. & Kops, G. J. P. L. Integration of Kinase and Phosphatase Activities by BUBR1 Ensures Formation of Stable Kinetochore-Microtubule Attachments. *Developmental Cell* 23, 745–755 (2012).

157. Kruse, T. *et al.* Direct binding between BubR1 and B56-PP2A phosphatase complexes regulate mitotic progression. *Journal of Cell Science* 126, 1086–1092 (2013).

158. Nijenhuis, W., Vallardi, G., Teixeira, A., Kops, G. J. P. L. & Saurin, A. T. Negative feedback at kinetochores underlies a responsive spindle checkpoint signal. *Nat. Cell Biol.* (2014). doi:10.1038/ncb3065

159. Campbell, C. S. & Desai, A. Tension sensing by Aurora B kinase is independent of survivin-based centromere localization. *Nature* 1–5 (2013). doi:10.1038/nature12057

160. Gordon, D. J., Resio, B. & Pellman, D. Causes and consequences of aneuploidy in cancer. *Nat Rev Genet* 13, 189–203 (2012).

161. London, N. & Biggins, S. Signalling dynamics in the spindle checkpoint response. *Nature Publishing Group* 15, 735–747 (2014).

162. Hoyt, M. A., Totis, L. & Roberts, B. T. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell* 66, 507–517 (1991).

163. Li, R. & Murray, A. W. Feedback control of mitosis in budding yeast. *Cell* 66, 519–531 (1991).

164. Rieder, C. L., Schultz, A., Cole, R. & Sluder, G. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *The Journal of Cell Biology* 127, 1301–1310 (1994).

165. King, R. W. *et al.* A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* 81, 279–288 (1995).

166. Zou, H., McGarry, T. J., Bernal, T. & Kirschner, M. W. Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* 285, 418–422 (1999).

167. Sudakin, V., Chan, G. K. & Yen, T. J. Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *The Journal of Cell Biology* 154, 925–936 (2001).

168. Abrieu, A. *et al.* Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. *Cell* 106, 83–93 (2001).

169. Jelluma, N. *et al.* Mps1 Phosphorylates Borealin to Control Aurora B Activity and Chromosome Alignment. *Cell* 132, 233–246 (2008).

170. Tighe, A., Staples, O. & Taylor, S. Mps1 kinase activity restrains anaphase during an unperturbed mitosis and targets Mad2 to kinetochores. *The Journal of Cell Biology* 181, 893–901 (2008).

171. Hardwick, K. G., Weiss, E., Luca, F. C., Winey, M. & Murray, A. W. Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. *Science* 273, 953–956 (1996).

172. Jelluma, N., Dansen, T. B., Sliedrecht, T., Kwiatkowski, N. P. & Kops, G. J. P. L. Release of Mps1 from kinetochores is crucial for timely anaphase onset. *The Journal of Cell Biology* 191, 281–290 (2010).

173. Nijenhuis, W. *et al.* A TPR domain-containing N-terminal module of MPS1 is required for its kinetochore localization by Aurora B. *The Journal of Cell Biology* (2013). doi:10.1083/jcb.201210033

174. Kemmler, S. *et al.* Mimicking Ndc80 phosphorylation triggers spindle assembly checkpoint signalling. *The EMBO Journal* 28, 1099–1110 (2009).

175. Luo, X. *et al.* The Mad2 spindle checkpoint protein has two distinct natively folded states. *Nat Struct Mol Biol* 11, 338–345 (2004).

176. Luo, X., Tang, Z., Rizo, J. & Yu, H. The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. *Molecular Cell* 9, 59–71 (2002).

177. Shah, J. V. *et al.* Dynamics of Centromere and Kinetochore Proteins. *Current Biology* 14, 942–952 (2004).

178. Howell, B. J. *et al.* Spindle Checkpoint Protein Dynamics at Kinetochores in Living Cells. *Current Biology* 14, 953–964 (2004).

179. De Antoni, A. *et al.* The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. *CURBIO* 15, 214–225 (2005).

180. Han, J. S. *et al.* Catalytic Assembly of the Mitotic Checkpoint Inhibitor BubR1-Cdc20 by a Mad2-Induced Functional Switch in Cdc20. *Molecular Cell* 51, 92–104 (2013).

181. Rodriguez-Bravo, V. *et al.* Nuclear Pores Protect Genome Integrity by Assembling a Premitotic and Mad1-Dependent Anaphase Inhibitor. *Cell* 156, 1017–1031 (2014).

182. Schweizer, N. *et al.* Spindle assembly checkpoint robustness requires Tpr-mediated regulation of Mad1/Mad2 proteostasis. *The Journal of Cell Biology* 203, 883–893 (2013).

183. Yamagishi, Y., Yang, C.-H., Tanno, Y. & Watanabe, Y. MPS1/Mph1 phosphorylates the kinetochore protein KNL1/Spc7 to recruit SAC components. *Nat. Cell Biol.* 14, 746–752 (2012).

184. Vleugel, M. *et al.* Sequential Multisite Phospho-Regulation of KNL1- BUB3 Interfaces at Mitotic Kinetochores. *Molecular Cell* 1–13 (2015). doi:10.1016/j.molcel.2014.12.036

185. Zhang, G., Lischetti, T. & Nilsson, J. A minimal number of MELT repeats supports all the functions of KNL1 in chromosome segregation. *Journal of Cell Science* 127, 871–884 (2014).

186. Krenn, V., Overlack, K., Primorac, I., van Gerwen, S. & Musacchio, A. KI Motifs of Human Knl1 EnhanceAssembly of Comprehensive Spindle Checkpoint Complexes around MELT Repeats. *Current Biology* 1–11 (2013). doi:10.1016/j.cub.2013.11.046

187. Vleugel, M., Hoogendoorn, E., Snel, B. & Kops, G. J. P. L. Evolution and function of the mitotic checkpoint. *Developmental Cell* 23, 239–250 (2012).

188. Johnson, V. L., Scott, M. I. F., Holt, S. V., Hussein, D. & Taylor, S. S. Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. *Journal of Cell Science* 117, 1577–1589 (2004).

189. London, N. & Biggins, S. Mad1 kinetochore recruitment by Mps1-mediated phosphorylation of Bub1 signals the spindle checkpoint. *Genes & Development* (2014). doi:10.1101/gad.233700.113

190. Brady, D. M. & Hardwick, K. G. Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. *CURBIO* 10, 675–678 (2000).

191. Moyle, M. W. *et al.* A Bub1-Mad1 interaction targets the Mad1-Mad2 complex to unattached kinetochores to initiate the spindle checkpoint. *The Journal of Cell Biology* 10, 675 (2014).

192. Kawashima, S. A., Yamagishi, Y., Honda, T., Ishiguro, K.-I. & Watanabe, Y. Phosphorylation of H2A by Bub1 prevents chromosomal instability through localizing shugoshin. *Science* 327, 172–177 (2010).

193. Saurin, A. T., van der Waal, M. S., Medema, R. E. H., Lens, S. M. A. & Kops, G. J. P. L. Aurora B potentiates Mps1 activation to ensure rapid checkpoint establishment at the onset of mitosis. *Nature Communications* 2, 316–9 (2011).

194. van der Waal, M. S. *et al.* Mps1 promotes rapid centromere accumulation of Aurora B. *Nature Publishing Group* 13, 847–854 (2012).

195. Lampson, M. A. & Kapoor, T. M. The human mitotic checkpoint protein BubR1 regulates chromosome–spindle attachments. *Nat. Cell Biol.* 7, 93–98 (2004).

196. Ricke, R. M., Jeganathan, K. B., Malureanu, L., Harrison, A. M. & van Deursen, J. M. Bub1 kinase activity drives error correction and mitotic checkpoint control but not tumor suppression. *The Journal of Cell Biology* 199, 931–949 (2012).

197. Kim, S., Sun, H., Tomchick, D. R., Yu, H. & Luo, X. Structure of human Mad1 C-terminal domain reveals its involvement in kinetochore targeting. *Proceedings of the National Academy of Sciences* 109, 6549–6554 (2012).

198. Sharp-Baker, H. & Chen, R. H. Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. *The Journal of Cell Biology* 153, 1239–1250 (2001).

199. Kops, G. J. P. L. ZW10 links mitotic checkpoint signaling to the structural kinetochore. *The Journal of Cell Biology* 169, 49–60 (2005).

200. Chan, G. K., Jablonski, S. A., Starr, D. A., Goldberg, M. L. & Yen, T. J. Human Zw10 and ROD are mitotic checkpoint proteins that bind to kinetochores. *Nat. Cell Biol.* 2, 944–947 (2000).

201. Buffin, E., Lefebvre, C., Huang, J., Gagou, M. E. & Karess, R. E. Recruitment of Mad2 to the Kinetochore Requires the Rod/Zw10 Complex. *Current Biology* 15, 856–861 (2005).

202. Varma, D. *et al.* Spindle assembly checkpoint proteins are positioned close to core microtubule attachment sites at kinetochores. *The Journal of Cell Biology* 21, 1968 (2013).

203. Kasuboski, J. M. *et al.* Zwint-1 is a novel Aurora B substrate required for the assembly of a dynein-binding platform on kinetochores. *Mol. Biol. Cell* 22, 3318–3330 (2011).

204. Sliedrecht, T., Zhang, C., Shokat, K. M. & Kops, G. J. P. L. Chemical Genetic Inhibition of Mps1 in Stable Human Cell Lines Reveals Novel Aspects of Mps1 Function in Mitosis. *PLoS ONE* 5, e10251–12 (2010).

205. Santaguida, S., Tighe, A., D'Alise, A. M., Taylor, S. S. & Musacchio, A. Dissecting the role of MPS1 in chromosome biorientation and the spindle checkpoint through the small molecule inhibitor reversine. *The Journal of Cell Biology* 190, 73–87 (2010).

206. Pinsky, B. A., Nelson, C. R. & Biggins, S. Protein Phosphatase 1 Regulates Exit from the Spindle Checkpoint in Budding Yeast. *Current Biology* 19, 1182–1187 (2009).

207. Vanoosthuyse, V. & Hardwick, K. G. A novel protein phosphatase 1-dependent spindle checkpoint silencing mechanism. *Curr. Biol.* 19, 1176–1181 (2009).

208. Howell, B. J. *et al.* Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. *The Journal of Cell Biology* 155, 1159–1172 (2001).

209. Wojcik, E. *et al.* Kinetochore dynein: its dynamics and role in the transport of the Rough deal checkpoint protein. *Nat. Cell Biol.* 3, 1001–1007 (2001).

210. Mische, S. *et al.* Dynein light intermediate chain: an essential subunit that contributes to spindle checkpoint inactivation. *Mol. Biol. Cell* 19, 4918–4929 (2008).

211. Sivaram, M. V. S., Wadzinski, T. L., Redick, S. D., Manna, T. & Doxsey, S. J. Dynein light intermediate chain 1 is required for progress through the spindle assembly checkpoint. *The EMBO Journal* 28, 902–914 (2009).

212. Gassmann, R. *et al.* Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells. *Genes & Development* 24, 957–971 (2010).

213. Chan, Y. W. Mitotic control of kinetochore-associated dynein and spindle orientation by human Spindly. *The Journal of Cell Biology* 185, 859–874 (2009).

214. Yang, M. *et al.* p31comet Blocks Mad2 Activation through Structural Mimicry. *Cell* 131, 744–755 (2007).

215. Habu, T., Kim, S. H., Weinstein, J. & Matsumoto, T. Identification of a MAD2-binding protein, CMT2, and its role in mitosis. *The EMBO Journal* 21, 6419–6428 (2002).

216. Xia, G. *et al.* Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. *The EMBO Journal* 23, 3133–3143 (2004).

217. Westhorpe, F. G., Tighe, A., Lara-Gonzalez, P. & Taylor, S. S. p31comet-mediated extraction of Mad2 from the MCC promotes efficient mitotic exit. *Journal of Cell Science* 124, 3905–3916 (2011).

218. Nilsson, J., Yekezare, M., Minshull, J. & Pines, J. The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction. *Nat. Cell Biol.* 10, 1411–1420 (2008).

219. Reddy, S. K., Rape, M., Margansky, W. A. & Kirschner, M. W. Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature* 446, 921–925 (2007).

220. Jordan, M. A. & Wilson, L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 4, 253–265 (2004).

221. Janssen, A. & Medema, R. H. Mitosis as an anti-cancer target. *Oncogene* 30, 2799–2809 (2011).

222. Walczak, C. E. & Heald, R. *Mechanisms of Mitotic Spindle Assembly and Function*. 265, 111–158 (Elsevier, 2008).

223. Roostalu, J. *et al.* Directional Switching of the Kinesin Cin8 Through Motor Coupling. *Science* 332, 94–99 (2011).

224. Bertran, M. T. *et al.* Nek9 is a Plk1-activated kinase that controls early centrosome separation through Nek6/7 and Eg5. *The EMBO Journal* 1–14 (2011). doi:10.1038/emboj.2011.179

225. Smith, E. *et al.* Differential control of Eg5-dependent centrosome separation by Plk1 and Cdk1. *The EMBO Journal* 1–13 (2011). doi:10.1038/emboj.2011.120

226. Woodcock, S. A. *et al.* Tiam1-Rac Signaling Counteracts Eg5 during Bipolar Spindle Assembly to Facilitate Chromosome Congression. *Current Biology* 20, 669–675 (2010).

227. Mayer, T. U. Small Molecule Inhibitor of Mitotic Spindle Bipolarity Identified in a Phenotype-Based Screen. *Science* 286, 971–974 (1999).

228. Kapoor, T. M., Mayer, T. U., Coughlin, M. L. & Mitchison, T. J. Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. *The Journal of Cell Biology* 150, 975–988 (2000).

229. Antitumor activity of a kinesin inhibitor. 64, 3276–3280 (2004).

230. DeBonis, S. *et al.* In vitro screening for inhibitors of the human mitotic kinesin Eg5 with antimitotic and antitumor activities. *Mol. Cancer Ther.* 3, 1079–1090 (2004).

231. S-trityl-L-cysteine is a reversible, tight binding inhibitor of the human kinesin Eg5 that specifically blocks mitotic progression. 281, 17559–17569 (2006).

232. Vaisberg, E. A., Koonce, M. P. & McIntosh, J. R. Cytoplasmic dynein plays a role in mammalian mitotic spindle formation. *The Journal of Cell Biology* 123, 849–858 (1993).

233. Reinsch, S. & Karsenti, E. Movement of nuclei along microtubules in Xenopus egg extracts. *Curr. Biol.* 7, 211–214 (1997).

234. Tanenbaum, M. E., Akhmanova, A. & Medema, R. H. Dynein at the nuclear envelope. *Nature Publishing Group* 11, 649–649 (2010).

235. Bicaudal D2, dynein, and kinesin-1 associate with nuclear pore complexes and regulate centrosome and nuclear positioning during mitotic entry. 8, e1000350 (2010).

236. The Polo kinase Plk4 functions in centriole duplication. 7, 1140–1146 (2005).

237. Janson, M. E. Dynamic instability of microtubules is regulated by force. *The Journal of Cell Biology* 161, 1029–1034 (2003).

238. Toso, A. *et al.* Kinetochore-generated pushing forces separate centrosomes during bipolar spindle assembly. *The Journal of Cell Biology* 184, 365–372 (2009).

239. Kaseda, K., McAinsh, A. D. & Cross, R. A. Dual pathway spindle assembly increases both the speed and the fidelity of mitosis. *Biology Open* (2011). doi:10.1242/bio.2011012

240. Mardin, B. R. *et al.* Components of the Hippo pathway cooperate with Nek2 kinase to regulate centrosome disjunction. *Nat. Cell Biol.* 12, 1166–1176 (2010).

241. Draviam, V. M., Shapiro, I., Aldridge, B. & Sorger, P. K. Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. *The EMBO Journal* 25, 2814–2827 (2006).

242. Ndel1 operates in a common pathway with LIS1 and cytoplasmic dynein to regulate cortical neuronal positioning. 44, 263–277 (2004).

243. Bicaudal-D regulates COPI-independent Golgi-ER transport by recruiting the dynein-dynactin motor complex. 4, 986–992 (2002).

244. Stehman, S. A., Chen, Y., McKenney, R. J. & Vallee, R. B. NudE and NudEL are required for mitotic progression and are involved in dynein recruitment to kinetochores. *The Journal of Cell Biology* 178, 583–594 (2007).

245. Walczak, C. & Heald, R. in *Mechanisms of Mitotic Spindle Assembly and Function* 265, 111–158 (Elsevier, 2008).

246. Rath, O. & Kozielski, F. Kinesins and cancer. *Nat Rev Cancer* 12, 527–539 (2012).

247. Ma, H. T., Erdal, S., Huang, S. & Poon, R. Y. C. ScienceDirect. *Molecular Oncology* 1–15 (2014). doi:10.1016/j.molonc.2014.05.007

248. Boutros, M., Brás, L. P. & Huber, W. Analysis of cell-based RNAi screens. *Genome Biol.* 7, R66 (2006).

249. Lens, S. M. A., Voest, E. E. & Medema, R. H. Shared and separate functions of polo-like kinases and aurora kinases in cancer. *Nat Rev Cancer* 10, 825–841 (2010).

250. Manfredi, M. G. *et al.* Antitumor activity of MLN8054, an orally active small-molecule inhibitor of Aurora A kinase. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4106–4111 (2007).

251. Howard, J. & Hyman, A. A. Microtubule polymerases and depolymerases. *Current Opinion in Cell Biology* 19, 31–35 (2007).

252. Walczak, C. E., Gayek, S. & Ohi, R. Microtubule-Depolymerizing Kinesins. *Annu. Rev. Cell Dev. Biol.* 29, 130722103520007 (2012).

253. Helenius, J., Brouhard, G., Kalaidzidis, Y., Diez, S. & Howard, J. The depolymerizing kinesin MCAK uses lattice diffusion to rapidly target microtubule ends. *Nature* 441, 115–119 (2006).

254. Hunter, A. W. *et al.* The kinesin-related protein MCAK is a microtubule depolymerase that forms an ATP-hydrolyzing complex at microtubule ends. *Molecular Cell* 11, 445–457 (2003).

255. Moore, A. T. *et al.* MCAK associates with the tips of polymerizing microtubules. *The Journal of Cell Biology* 169, 391–397 (2005).

256. Lee, T., Langford, K. J., Askham, J. M., Brüning-Richardson, A. & Morrison, E. E. MCAK associates with EB1. *Oncogene* 27, 2494–2500 (2008).

257. Varga, V. *et al.* Yeast kinesin-8 depolymerizes microtubules in a length-dependent manner. *Nat. Cell Biol.* 8, 957–962 (2006).

258. Gupta, M. L., Carvalho, P., Roof, D. M. & Pellman, D. Plus end-specific depolymerase activity of Kip3, a kinesin-8 protein, explains its role in positioning the yeast mitotic spindle. *Nat. Cell Biol.* 8, 913–923 (2006).

259. Mayr, M. I. *et al.* The Human Kinesin Kif18A Is a Motile Microtubule Depolymerase Essential for Chromosome Congression. *Current Biology* 17, 488–498 (2007).

260. Varga, V., Leduc, C., Bormuth, V., Diez, S. & Howard, J. Kinesin-8 Motors Act Cooperatively to Mediate Length-Dependent Microtubule Depolymerization. *Cell* 138, 1174–1183 (2009).

261. Stout, J. R. *et al.* Kif18B interacts with EB1 and controls astral microtubule length during mitosis. *Mol. Biol. Cell* 22, 3070–3080 (2011).

262. Andrews, P. D. *et al.* Aurora B regulates MCAK at the mitotic centromere. *Developmental Cell* 6, 253–268 (2004).

263. Tanenbaum, M. E., Medema, R. & Akhmanova, A. Regulation of localization and activity of the microtubule depolymerase MCAK. *BioArchitecture* 1, 80–87 (2011).

264. Rankin, K. E. & Wordeman, L. Long astral microtubules uncouple mitotic spindles from the cytokinetic furrow. *The Journal of Cell Biology* 190, 35–43 (2010).

265. Tran, P. T., Marsh, L., Doye, V., Inoué, S. & Chang, F. A mechanism for nuclear positioning in fission yeast based on microtubule pushing. *The Journal of Cell Biology* 153, 397–411 (2001).

266. Visscher, K., Schnitzer, M. J. & Block, S. M. Single kinesin molecules studied with a molecular force clamp. *Nature* 400, 184–189 (1999).

267. Brouhard, G. J. *et al.* XMAP215 is a processive microtubule polymerase. *Cell* 132, 79–88 (2008).

268. Glover, D. M., Leibowitz, M. H., McLean, D. A. & Parry, H. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell* 81, 95–105 (1995).

269. Cowley, D. O. *et al.* Aurora-A kinase is essential for bipolar spindle formation and early development. *Molecular and Cellular Biology* 29, 1059–1071 (2009).

270. Hegarat, N. *et al.* Aurora A and Aurora B jointly coordinate chromosome segregation and anaphase microtubule dynamics. *The Journal of Cell Biology* 195, 1103–1113 (2011).

271. Marumoto, T. *et al.* Aurora-A Kinase Maintains the Fidelity of Early and Late Mitotic Events in HeLa Cells. *Journal of Biological Chemistry* 278, 51786–51795 (2003).

272. Hoar, K. *et al.* MLN8054, a Small-Molecule Inhibitor of Aurora A, Causes Spindle Pole and Chromosome Congression Defects Leading to Aneuploidy. *Molecular and Cellular Biology* 27, 4513–4525 (2007).

273. Hochegger, H., Hegarat, N. & Pereira-Leal, J. B. Aurora at the pole and equator: overlapping functions of Aurora kinases in the mitotic spindle. *Open Biology* 3, 120185–120185 (2013).

274. Giet, R., Uzbekov, R., Cubizolles, F., Le Guellec, K. & Prigent, C. The Xenopus laevis aurora-related protein kinase pEg2 associates with and phosphorylates the kinesin-related protein XlEg5. *J. Biol. Chem.* 274, 15005–15013 (1999).

275. Kettenbach, A. N. *et al.* Quantitative Phosphoproteomics Identifies Substrates and Functional Modules of Aurora and Polo-Like Kinase Activities in Mitotic Cells. *Science Signaling* 4, rs5–rs5 (2011).

276. Olsen, J. V. *et al.* Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. *Science Signaling* 3, ra3–ra3 (2010).

277. Sturgill, E. G. *et al.* Kinesin-12 Kif15 targets kinetochore fibers through an intrinsic two-step mechanism. *Curr. Biol.* 24, 2307–2313 (2014).

278. Kinoshita, K. *et al.* Aurora A phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis. *The Journal of Cell Biology* 170, 1047–1055 (2005).

279. Ertych, N. *et al.* Increased microtubule assembly rates influence chromosomal instability in colorectal cancer cells. *Nat. Cell Biol.* (2014). doi:10.1038/ncb2994

280. Malumbres, M. & Pérez de Castro, I. Aurora kinase A inhibitors: promising agents in antitumoral therapy. *Expert Opin. Ther. Targets* 18, 1377–1393 (2014).

281. Walczak, C. E., Mitchison, T. J. & Desai, A. XKCM1: a Xenopus kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. *Cell* 84, 37–47 (1996).

282. Cameron, L. A. Kinesin 5-independent poleward flux of kinetochore microtubules in PtK1 cells. *The Journal of Cell Biology* 173, 173–179 (2006).

283. Florian, S. & Mayer, T. U. The Functional Antagonism between Eg5 and Dynein in Spindle Bipolarization Is Not Compatible with a Simple Push-Pull Model. *CellReports* 1–9 (2012). doi:10.1016/j.celrep.2012.03.006

284. Khodjakov, A., Copenagle, L., Gordon, M. B., Compton, D. A. & Kapoor, T. M. Minus-end capture of preformed kinetochore fibers contributes to spindle morphogenesis. *The Journal of Cell Biology* 160, 671–683 (2003).

285. Gaetz, J. Dynein/dynactin regulate metaphase spindle length by targeting depolymerizing activities to spindle poles. *The Journal of Cell Biology* 166, 465–471 (2004).

286. Ma, N. *et al.* Poleward transport of TPX2 in the mammalian mitotic spindle requires dynein, Eg5, and microtubule flux. *Mol. Biol. Cell* 21, 979–988 (2010).

287. Sharp, D. J., Rogers, G. C. & Scholey, J. M. Microtubule motors in mitosis. *Nature* 407, 41–47 (2000).

288. Kwon, M. *et al.* Mechanisms to suppress multipolar divisions in cancer cells with extra centrosomes. *Genes & Development* 22, 2189–2203 (2008).

289. Enos, A. P. & Morris, N. R. Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in A. nidulans. *Cell* 60, 1019–1027 (1990).

290. Sawin, K. E., LeGuellec, K., Philippe, M. & Mitchison, T. J. Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature* 359, 540–543 (1992).

291. Bakhoum, S. F., Thompson, S. L., Manning, A. L. & Compton, D. A. Genome stability is ensured by temporal control of kinetochore–microtubule dynamics. *Nat. Cell Biol.* 11, 27–35 (2008).

292. Kwon, M. *et al.* The chromokinesin, KLP3A, dives mitotic spindle pole separation during prometaphase and anaphase and facilitates chromatid motility. *Mol. Biol. Cell* 15, 219–233 (2004).

293. Dumont, S. & Mitchison, T. J. Force and length in the mitotic spindle. *Curr. Biol.* 19, R749–61 (2009).

294. Laan, L. *et al.* Cortical Dynein Controls Microtubule Dynamics to Generate Pulling Forces that Position Microtubule Asters. *Cell* 148, 502–514 (2012).

295. Kollu, S., Bakhoum, S. F. & Compton, D. A. Interplay of Microtubule Dynamics and Sliding during Bipolar Spindle Formation in Mammalian Cells. *Current Biology* 19, 2108–2113 (2009).

296. Vernos, I., Heasman, J. & Wylie, C. Multiple kinesin-like transcripts in Xenopus oocytes. *Developmental Biology* 157, 232–239 (1993).

297. Wittmann, T., Boleti, H., Antony, C., Karsenti, E. & Vernos, I. Localization of the kinesin-like protein Xklp2 to spindle poles requires a leucine zipper, a microtubule-associated protein, and dynein. *The Journal of Cell Biology* 143, 673–685 (1998).

298. Walczak, C. E., Vernos, I., Mitchison, T. J., Karsenti, E. & Heald, R. A model for the proposed roles of different microtubule-based motor proteins in establishing spindle bipolarity. *Current Biology* 8, 903–913 (1998).

299. Zhu, C. *et al.* Functional analysis of human microtubule-based motor proteins, the kinesins and dyneins, in mitosis/cytokinesis using RNA interference. *Mol. Biol. Cell* 16, 3187–3199 (2005).

300. Rogers, G. C. *et al.* A kinesin-related protein, KRP(180), positions prometaphase spindle poles during early sea urchin embryonic cell division. *The Journal of Cell Biology* 150, 499–512 (2000).

301. Vladimirou, E. *et al.* Nonautonomous Movement of Chromosomes in Mitosis. *Developmental Cell* 27, 60–71 (2013).

302. Drechsler, H., McHugh, T., Singleton, M. R., Carter, N. J. & McAinsh, A. D. The Kinesin-12 Kif15 is a processive track-switching tetramer. *eLife* 3, e01724 (2014).

303. Wittmann, T., Wilm, M., Karsenti, E. & Vernos, I. TPX2, A novel xenopus MAP involved in spindle pole organization. *The Journal of Cell Biology* 149, 1405–1418 (2000).

304. Verhey, K. J. & Hammond, J. W. Traffic control: regulation of kinesin motors. *Nature Reviews Molecular Cell Biology* 10, 765–777 (2009).

305. Gruss, O. J. *et al.* Chromosome-induced microtubule assembly mediated by TPX2 is required for spindle formation in HeLa cells. *Nat. Cell Biol.* 4, 871–879 (2002).

306. Florian, S. & Mayer, T. U. Modulated microtubule dynamics enable Hklp2/Kif15 to assemble bipolar spindles. *cc* 10, 3533–3544 (2011).

307. Foley, E. A. & Kapoor, T. M. Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. *Nature Publishing Group* 14, 25–37 (2013).

308. Sacristan, C. & Kops, G. J. P. L. Joined at the hip: kinetochores, microtubules, and spindle assembly checkpoint signaling. *Trends in Cell Biology* 25, 21–28 (2015).

309. Kops, G. J. P. L. & Shah, J. V. Connecting up and clearing out: how kinetochore attachment silences the spindle assembly checkpoint. *Chromosoma* (2012). doi:10.1007/s00412-012-0378-5

310. Overlack, K., Krenn, V. & Musacchio, A. When Mad met Bub. *Nature Publishing Group* 15, 326–328 (2014).

311. Buffin, E., Emre, D. & Karess, R. E. Flies without a spindle checkpoint. *Nat. Cell Biol.* 9, 565–572 (2007).

312. Dobles, M., Liberal, V., Scott, M. L., Benezra, R. & Sorger, P. K. Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2. *Cell* 101, 635–645 (2000).

313. Michel, L. S. *et al.* MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells. *Nature* 409, 355–359 (2001).

314. Kalitsis, P., Earle, E., Fowler, K. J. & Choo, K. H. Bub3 gene disruption in mice reveals essential mitotic spindle checkpoint function during early embryogenesis. *Genes & Development* 14, 2277–2282 (2000).

315. Wang, Q. BUBR1 deficiency results in abnormal megakaryopoiesis. *Blood* 103, 1278–1285 (2003).

316. Perera, D. *et al.* Bub1 Maintains Centromeric Cohesion by Activation of the Spindle Checkpoint. *Developmental Cell* 13, 566–579 (2007).

317. Foijer, F. *et al.* Spindle checkpoint deficiency is tolerated by murine epidermal cells but not hair follicle stem cells. *Proceedings of the National Academy of Sciences* 110, 2928–2933 (2013).

318. Kotecki, M., Reddy, P. S. & Cochran, B. H. Isolation and characterization of a near-haploid human cell line. *Experimental Cell Research* 252, 273–280 (1999).

319. Carette, J. E. *et al.* Haploid Genetic Screens in Human Cells Identify Host Factors Used by Pathogens. *Science* 326, 1231–1235 (2009).

320. Carette, J. E. *et al.* Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. *Nat Biotechnol* 29, 542–546 (2011).

321. Jae, L. T. *et al.* Deciphering the Glycosylome of Dystroglycanopathies Using Haploid Screens for Lassa Virus Entry. *Science* (2013). doi:10.1126/science.1233675

322. Jae, L. T. *et al.* Virus entry. Lassa virus entry requires a trigger-induced receptor switch. *Science* 344, 1506–1510 (2014).

323. Bürckstümmer, T. *et al.* A reversible gene trap collection empowers haploid genetics in human cells. *Nat Meth* 10, 965–971 (2013).

324. Cong, L. *et al.* Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* 339, 819–823 (2013).

325. Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* 339, 823–826 (2013).

326. Green, R. A., Wollman, R. & Kaplan, K. B. APC and EB1 function together in mitosis to regulate spindle dynamics and chromosome alignment. *Mol. Biol. Cell* 16, 4609–4622 (2005).

327. Aragón, L., Martinez-Perez, E. & Merkenschlager, M. Condensin, cohesin and the control of chromatin states. *Current Opinion in Genetics & Development* 23, 204–211 (2013).

328. Karess, R. Rod–Zw10–Zwilch: a key player in the spindle checkpoint. *Trends in Cell Biology* 15, 386–392 (2005).

329. Ono, T. *et al.* Differential contributions of condensin I and condensin II to mitotic chromosome architecture in vertebrate cells. *Cell* 115, 109–121 (2003).

330. Green, L. C. *et al.* Contrasting roles of condensin I and condensin II in mitotic chromosome formation. *Journal of Cell Science* 125, 1591–1604 (2012).

331. Bhat, M. A., Philp, A. V., Glover, D. M. & Bellen, H. J. Chromatid segregation at anaphase requires the barren product, a novel chromosome-associated protein that interacts with Topoisomerase II. *Cell* 87, 1103–1114 (1996).

332. Coelho, P. A., Queiroz-Machado, J. & Sunkel, C. E. Condensin-dependent localisation of topoisomerase II to an axial chromosomal structure is required for sister chromatid resolution during mitosis. *Journal of Cell Science* 116, 4763–4776 (2003).

333. Baxter, J. *et al.* Positive Supercoiling of Mitotic DNA Drives Decatenation by Topoisomerase II in Eukaryotes. *Science* 331, 1328–1332 (2011).

334. Charbin, A., Bouchoux, C. & Uhlmann, F. Condensin aids sister chromatid decatenation by topoisomerase II. *Nucleic Acids Research* 42, 340–348 (2013).

335. Roca, J., Ishida, R., Berger, J. M., Andoh, T. & Wang, J. C. Antitumor bisdioxopiperazines inhibit yeast DNA topoisomerase II by trapping the enzyme in the form of a closed protein clamp. *Proc. Natl. Acad. Sci. U.S.A.* 91, 1781–1785 (1994).

336. Varma, D., Dujardin, D. L., Stehman, S. A. & Vallee, R. B. Role of the kinetochore/cell cycle checkpoint protein ZW10 in interphase cytoplasmic dynein function. *The Journal of Cell Biology* 172, 655–662 (2006).

337. Hirose, H. *et al.* Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. *The EMBO Journal* 23, 1267–1278 (2004).

338. Sacristan, C. & Kops, G. J. P. L. Joined at the hip: kinetochores, microtubules, and spindle assembly checkpoint signaling. *Trends in Cell Biology* 1–8 (2014). doi:10.1016/j.tcb.2014.08.006

339. Scaërou, F. *et al.* The ZW10 and Rough Deal checkpoint proteins function together in a large, evolutionarily conserved complex targeted to the kinetochore. *Journal of Cell Science* 114, 3103–3114 (2001).

340. Shintomi, K. & Hirano, T. The relative ratio of condensin I to II determines chromosome shapes. *Genes & Development* 25, 1464–1469 (2011).

341. Saka, Y. *et al.* Fission yeast cut3 and cut14, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis. *The EMBO Journal* 13, 4938–4952 (1994).

342. Williams, B. C. *et al.* Zwilch, a new component of the ZW10/ROD complex required for kinetochore functions. *Mol. Biol. Cell* 14, 1379–1391 (2003).

343. Collin, P., Nashchekina, O., Walker, R. & Pines, J. The spindle assembly checkpoint works like a rheostat rather than a toggle switch. *Nat. Cell Biol.* 15, 1–11 (2013).

344. Dick, A. E. & Gerlich, D. W. Kinetic framework of spindle assembly checkpoint signalling. *Nat. Cell Biol.* 15, 1–13 (2013).

345. Kuijt, T. E. F., Omerzu, M., Saurin, A. T. & Kops, G. J. P. L. Conditional targeting of MAD1 to kinetochores is sufficient to reactivate the spindle assembly checkpoint in metaphase. *Chromosoma* (2014). doi:10.1007/s00412-014-0458-9

346. Haarhuis, J. H. I. *et al.* WAPL-Mediated Removal of Cohesin Protects against Segregation Errors and Aneuploidy. *Current Biology* 1–7 (2013). doi:10.1016/j.cub.2013.09.003

347. Elowe, S., Hummer, S., Uldschmid, A., Li, X. & Nigg, E. A. Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions. *Genes & Development* 21, 2205–2219 (2007).

348. Overlack, K. *et al.* A molecular basis for the differential roles of Bub1 and BubR1 in the spindle assembly checkpoint. *eLife* 4, e05269 (2014).

349. Overlack, K., Krenn, V. & Musacchio, A. When Mad met Bub. *Nature Publishing Group* 15, 326–328 (2014).

350. Taylor, S. S., Ha, E. & McKeon, F. The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase. *The Journal of Cell Biology* 142, 1–11 (1998).

351. Klebig, C., Korinth, D. & Meraldi, P. Bub1 regulates chromosome segregation in a kinetochore-independent manner. *The Journal of Cell Biology* 185, 841–858 (2009).

352. Ditchfield, C. *et al.* Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *The Journal of Cell Biology* 161, 267–280 (2003).

353. Maldonado, M. & Kapoor, T. M. Constitutive Mad1 targeting to kinetochores uncouples checkpoint signalling from chromosome biorientation. *Nat. Cell Biol.* 13, 475–482 (2011).

354. Ding, Y. *et al.* Cancer-Specific requirement for BUB1B/BUBR1 in human brain tumor isolates and genetically transformed cells. *Cancer Discovery* 3, 198–211 (2013).

355. Bakhoum, S. F. & Compton, D. A. Kinetochores and disease: keeping microtubule dynamics in check! *Current Opinion in Cell Biology* 24, 64–70 (2012).

356. Starr, D. A. *et al.* Conservation of the centromere/kinetochore protein ZW10. *The Journal of Cell Biology* 138, 1289–1301 (1997).

357. Défachelles, L. *et al.* RZZ and Mad1 dynamics in Drosophila mitosis. *Chromosome Res* 1–10 (2015). doi:10.1007/s10577-015-9472-x

358. Sharp, D. J. *et al.* The bipolar kinesin, KLP61F, cross-links microtubules within interpolar microtubule bundles of Drosophila embryonic mitotic spindles. *The Journal of Cell Biology* 144, 125–138 (1999).

359. Waters, J. C., Cole, R. W. & Rieder, C. L. The force-producing mechanism for centrosome separation during spindle formation in vertebrates is intrinsic to each aster. *The Journal of Cell Biology* 122, 361–372 (1993).

360. Cytrynbaum, E. N., Sommi, P., Brust-Mascher, I., Scholey, J. M. & Mogilner, A. Early spindle assembly in Drosophila embryos: role of a force balance involving cytoskeletal dynamics and nuclear mechanics. *Mol. Biol. Cell* 16, 4967–4981 (2005).

361. Busson, S., Dujardin, D., Moreau, A., Dompierre, J. & De Mey, J. R. Dynein and dynactin are localized to astral microtubules and at cortical sites in mitotic epithelial cells. *CURBIO* 8, 541–544 (1998).

362. Beaudouin, J., Gerlich, D., Daigle, N., Eils, R. & Ellenberg, J. Nuclear envelope breakdown proceeds by microtubule-induced tearing of the lamina. *Cell* 108, 83–96 (2002).

363. Fridolfsson, H. N. & Starr, D. A. Kinesin-1 and dynein at the nuclear envelope mediate the bidirectional migrations of nuclei. *The Journal of Cell Biology* 191, 115–128 (2010).

364. Zhang, X. *et al.* SUN1/2 and Syne/Nesprin-1/2 complexes connect centrosome to the nucleus during neurogenesis and neuronal migration in mice. *Neuron* 64, 173–187 (2009).

365. Jodoin, J. N. *et al.* Human Asunder promotes dynein recruitment and centrosomal tethering to the nucleus at mitotic entry. *Mol. Biol. Cell* 23, 4713–4724 (2012).

366. Wühr, M., Tan, E. S., Parker, S. K., Detrich, H. W., III & Mitchison, T. J. A Model for Cleavage Plane Determination in Early Amphibian and Fish Embryos. *Current Biology* 20, 2040–2045 (2010).

367. Dogterom, M., Kerssemakers, J. W., Romet-Lemonne, G. & Janson, M. E. Force generation by dynamic microtubules. *Current Opinion in Cell Biology* 17, 67–74 (2005).

368. Holland, A. J. & Cleveland, D. W. Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nature Reviews Molecular Cell Biology* 10, 478–487 (2009).

369. Li, Y., Yu, W., Liang, Y. & Zhu, X. Kinetochore dynein generates a poleward pulling force to facilitate congression and full chromosome alignment. *Cell Res* 17, 701–712 (2007).

370. Antonio, C. *et al.* Xkid, a chromokinesin required for chromosome alignment on the metaphase plate. *Cell* 102, 425–435 (2000).

371. Shrestha, R. L. & Draviam, V. M. Lateral to End-on Conversionof Chromosome-Microtubule Attachment Requires Kinesins CENP-E and MCAK. *Current Biology* 1–13 (2013). doi:10.1016/j.cub.2013.06.040

372. Stephens, A. D. *et al.* Pericentric chromatin loops function as a nonlinear spring in mitotic force balance. *The Journal of Cell Biology* 200, 757–772 (2013).

373. Meraldi, P. & Sorger, P. K. A dual role for Bub1 in the spindle checkpoint and chromosome congression. *The EMBO Journal* 24, 1621–1633 (2005).

374. Suijkerbuijk, S. J. E. *et al.* Molecular Causes for BUBR1 Dysfunction in the Human Cancer Predisposition Syndrome Mosaic Variegated Aneuploidy. *Cancer Research* 70, 4891–4900 (2010).

375. Heinrich, S. *et al.* Mad1 contribution to spindle assembly checkpoint signalling goes beyond presenting Mad2 at kinetochores. *Nature Publishing Group* 15, 291–298 (2014).

376. Kulukian, A., Han, J. S. & Cleveland, D. W. Unattached Kinetochores Catalyze Productionof an Anaphase Inhibitor that Requiresa Mad2 Template to Prime Cdc20 for BubR1 Binding. *Developmental Cell* 16, 105–117 (2009).

377. Hewitt, L. *et al.* Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex. *The Journal of Cell Biology* 190, 25–34 (2010).

Nederlandse samenvatting

Mitose

Elk mens bestaat uit biljoenen cellen die allemaal afstammen van één enkele bevruchte eicel. Deze uitzonderlijke hoeveelheid cellen kan alleen maar worden bereikt door vele rondes van celdeling waarbij een bestaande cel zich deelt en twee identieke dochtercellen vormt (Figuur 1). Het proces van celdeling wordt ook wel mitose genoemd. Deze term is afgeleid van het Griekse woord *mitos*, wat vertaald kan worden als "draad" en refereert naar de draadvormige structuren (de chromosomen) die zichtbaar zijn tijdens het proces van celdeling. Celdeling is niet essentieel voor groei, maar ook voor het functioneren van organismen; cellen worden continue vervangen om alle organen goed te laten functioneren.

Het meest essentiële onderdeel van celdeling is het splitsen van het DNA. Het DNA bevat alle erfelijke informatie en is verdeeld over zesenveertig chromosomen. Voor de celdeling wordt van elk chromosoom een exact kopie gemaakt die vervolgens tijdens het delingsproces verdeeld worden over de twee dochtercellen.

De gelijke verdeling van de chromosomen tijdens mitose is essentieel om de fitheid van de dochtercellen te waarborgen. Het ongelijk verdelen van chromosomen tijdens mitose resulteert in dochtercellen met meer of minder dan zesenveertig chromosomen en als consequentie een disbalans in de hoeveelheid DNA. Een ongelijk aantal chromosomen wordt ook wel aneuploïdie genoemd en resulteert in de meeste gevallen tot een slecht functionerende cel die niet verenigbaar met leven is. Daarnaast kunnen defecten in het splitsen van de chromosomen bijdragen aan het ontstaan van kanker: ongeveer 70% van alle tumoren zijn aneuploïd en verdelen de chromosomen ongelijk tijdens mitose. Daarnaast kan een aanhoudende verandering van de hoeveelheid chromosomen in tumorcellen ook bijdragen aan groeivoordelen en resistentie tegen anti-kanker medicijnen.

Het verdelen van de 2 kopieën van elk chromosoom is een ingewikkeld proces. In het eerste stadium, **prophase,** condenseren de chromosomen zich tot compacte staafachtige structuren in de celkern. Daarnaast hebben cellen tijdens mitose twee centrosomen liggen net buiten het kernmembraan die zich ieder verplaatsen naar één kant van de kern. Deze centrosomen zijn belangrijk voor het bouwen en organiseren van het mitotisch spoelfiguur door een netwerk van dynamische draden (microtubuli) te vormen. **Prometafase** start met het opbollen van de cel en de afbraak van het kernmembraan. De gecondenseerde chromosomen komen hierdoor vrij te liggen in de cel en microtubuli die groeien vanuit de centrosomen kunnen nu binden aan de chromosomen. De chromosomen worden na het binden van microtubuli getransporteerd naar het midden van de cel. Het koppelen van microtubuli aan chromosomen vindt plaats op de kinetochore. Dit complex van tientallen eiwitten speelt, naast het maken van de fysieke contacten met de microtubuli, een belangrijke rol in het controleren van de contacten of deze op de juiste manier zijn gevormd: verkeerde contacten kunnen leiden tot onjuiste chromosoom verdeling en heeft als consequentie aneuploïdie. Het mitotisch spoelfiguur is klaar zodra alle chromosomen op een correcte manier gekoppeld zijn aan de microtubuli van de centrosomen. Dit moment wordt ook wel **metafase** genoemd. Na deze fase volgt **anafase**, waarin alle chromosoom kopieën van elkaar gescheiden worden en door de microtubuli ieder naar een eigen kant getrokken worden. **Telofase** wordt bereikt zodra alle chromosomen zijn aangekomen aan de overzijde van de cel. Tegelijkertijd vormt zich er een nieuw kernmembraan rond de verdeelde chromosomen en worden de cellen fysiek van elkaar gescheiden in een proces dat ook wel cytokinese genoemd wordt.

De formatie van het mitotisch spoelfiguur

Het mitotisch spoelfiguur is een dynamisch netwerk van microtubuli en bevat daarnaast een groot aantal verschillende microtubuli-bindende eiwitten die een essentiële rol spelen voor de integriteit en dynamiek van het spoelfiguur. Een belangrijke groep van deze eiwitten zijn de zogenaamde motoreiwitten zoals kinesine en dyneïne. Deze eiwitten kunnen door te 'lopen' over het oppervlak van microtubuli krachten uitoefenen op de microtubule waarmee chromosomen en centrosomen verplaatst kunnen worden in de cel. Zonder deze groep van eiwitten is het onmogelijk om het mitotisch spoelfiguur te vormen en om chromosomen te scheiden over de twee dochtercellen. Diverse medicijnen die in de kliniek worden gebruikt voor de behandeling van kanker hebben het spoelfiguur als doelwit. Door de formatie van het spoelfiguur te blokkeren zijn cellen niet in staat om de chromosomen op een correcte manier te verdelen, wat resulteert in celdood. Snel delende cellen zoals tumorcellen zullen hier het meeste last van hebben, waardoor de groei van tumoren kan worden afgeremd. De huidige medicijnen die de formatie van het spoelfiguur blokkeren realiseren dit door de dynamiek van de microtubuli te veranderen. De microtubuli spelen echter ook een belangrijke rol in een groot aantal andere cellulaire processen waardoor er tijdens de behandeling met deze medicijnen veel bijwerkingen optreden. Het is daarom een uitdaging voor onderzoekers om meer gerichtere medicijnen te vinden die specifiek werkzaam tijdens de celdeling zodat andere belangrijke cellulaire processen onaangetast blijven.

Inhoud van dit proefschrift

Het onderzoek dat beschreven is in dit proefschrift richt zich met name op functie van motoreiwitten die betrokken zijn bij de formatie van het mitotisch spoelfiguur.

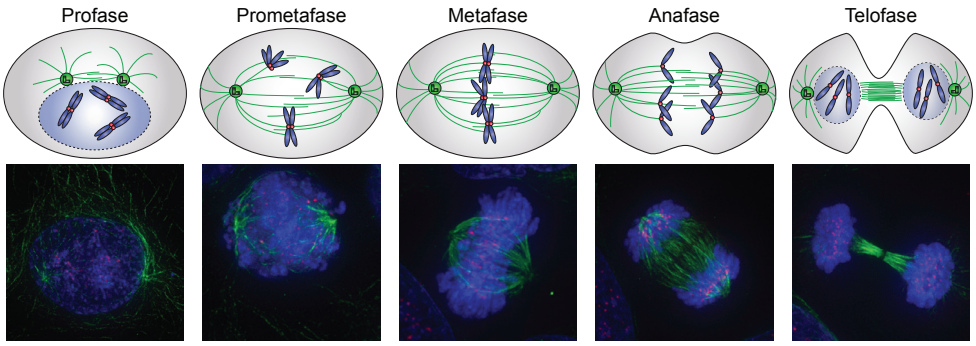
In hoofdstuk 1 wordt een uitgebreid overzicht gegeven van de huidige kennis van de processen die betrokken zijn bij mitose en met name de formatie van het mitotisch spoelfiguur. Het motoreiwit Eg5 is essentieel voor de formatie van het mitotisch spoelfiguur. Eg5 kan microtubuli uit elkaar duwen om hiermee het spoelfiguur te organiseren. Zonder Eg5 zijn cellen niet instaat om de chromosomen te verdelen tijdens mitose en deze essentiële functie maakt Eg5 een interessant doelwit om te inhiberen om op deze manier de groei van tumorcellen tegen te gaan.

Echter, recente studies hebben laten zien dat er mogelijk alternatieve mechanismen zijn om het spoelfiguur te vormen zonder de functie van Eg5.

In hoofdstuk 2 wordt beschreven dat tumorcellen zich door 'gerichte evolutie' gemakkelijk kunnen aanpassen om het mitotische spoelfiguur te formeren zonder het normaal gesproken essentiële motoreiwit Eg5.

Door gebruik te maken van deze Eg5-onafhankelijke cellen hebben wij een nieuw mechanisme blootgelegd dat belangrijk is voor het separeren van de centrosomen tijdens profase en hierdoor Eg5-onafhankelijke formatie van het spoelfiguur toelaat. Het motoreiwit dyneïne dat onder andere te vinden is op het kernmembraan speelt een centrale rol in dit mechanisme. Door dyneïne specifiek te verwijderen van het kernmembraan kunnen we deze vorm van Eg5-onafhankelijke formatie van het spoelfiguur blokkeren. Daarnaast laten we zien dat het motoreiwit Kif15 essentieel is voor de separatie van de centrosomen in prometafase van deze cellen. Dit eiwit werkt samen met Eg5 om microtubuli uit elkaar te duwen. De functie van Kif15 is normaal gesproken niet essentieel voor de formatie van het spoelfiguur, maar wordt wel essentieel in Eg5-onafhankelijke cellen.

In hoofdstuk 3 hebben wij een geautomatiseerd genoomwijd onderzoek uitgevoerd om genen te identificeren die belangrijk zijn voor Eg5-onafhankelijke formatie van het mitotisch spoelfiguur. Door systematisch alle genen één voor één uit te schakelen hebben we twee motoreiwitten geïdentificeerd die hun krachten gebruiken om de dynamiek van microtubuli aan te passen. Zonder deze motoreiwitten



Figuur 1: Fases van mitose.

Een schematische en een werkelijke weergave van de verschillende fases van mitose. Tijdens profase condenseren de chromosomen tot compacte, staafvormige structuren en verplaatsen de twee centrosomen zich ieder naar een kant van het kernmembraan. In prometafase breekt het kernmembraan af en wordt het spoelfiguur gevormd. De chromosomen binden aan die microtubuli van het spoelfiguur en worden vervolgens getransporteerd naar het midden van de cel. Metafase is bereikt zodra alle chromosomen op een correcte manier gebonden zijn. In anafase worden de chromosoom kopieën gescheiden en ieder naar een andere kant getransporteerd. In telofase wordt het kernmembraan gevormd om de gesplitste chromosomen en wordt de cel fysiek in twee dochter cellen gesplitst.

kunnen microtubuli niet optimaal functioneren en blokkeren ze de formatie van het spoelfiguur specifiek in de Eg5-onafhankelijke cellen. Daarnaast hebben we de kinase Aurora A geïdentificeerd, die belangrijk is voor de activatie het motoreiwit Kif15. Zonder de activiteit van Aurora A kan Kif15 zijn functie niet naar behoren uitvoeren waardoor de formatie van het spoelfiguur geblokkeerd wordt.

In hoofdstuk 4 beschrijven we hoe drie motoreiwitten samenwerken om een robuust spoelfiguur te vormen die op een correcte manier de chromosomen kan scheiden. De motoreiwitten Eg5 en Kif15 gebruiken hun krachten om ervoor te zorgen dat microtubuli uit elkaar worden geduwd. Het motoreiwit dyneïne doet daarentegen het tegenovergestelde en gebruikt zijn kracht om de activiteit van Eg5 en Kif15 tegen te gaan om te voorkomen dat de microtubuli niet te ver uit elkaar worden geduwd. Door de gecombineerde activiteit van deze motoreiwitten behoudt het spoelfiguur zijn normale constructie zodat deze instaat blijft om de chromosomen op een correcte manier binden en te verdelen.

In hoofdstuk 5 wordt een uitgebreid overzicht gegeven over de huidige kennis van het motoreiwit Kif15 en de mogelijkheden om dit eiwit, samen met Eg5, als doelwit te gebruiken voor de behandeling van kanker.

In hoofdstuk 6 en 7 onderzoeken we het mitotisch checkpoint. Dit checkpoint zorgt ervoor dat anafase in delende cellen niet start voordat alle chromosomen op een correcte manier gebonden zijn aan het spoelfiguur. Dit checkpoint is in de meeste cellen essentieel; zonder dit checkpoint hebben de cellen te weinig tijd in prometafase om alle chromosomen te koppelen aan het spoelfiguur, waardoor er chromosomen niet in de juiste dochtercel terecht komen. Wij beschrijven in dit hoofdstuk een humane cellijn die kan leven zonder dit checkpoint doordat ze op een zeer efficiënte en snelle manier het spoelfiguur kunnen formeren voordat anafase in deze cellen start. We hebben in deze cellen een genetische onderzoek uitgevoerd om genen te vinden die synthetische letaliteit vertonen met het verlies van het mitotisch checkpoint. Synthetische letaliteit is een type genetische interactie waarbij het verlies van twee genen letaal is voor cellen, maar het afzonderlijk verlies van dezelfde genen

niet letaal is voor de cel. In dit onderzoek karakteriseren we aantal genen die synthetische letaliteit vertonen met het verlies van het mitotisch checkpoint.
In hoofdstuk 8 tenslotte worden alle vindingen van dit proefschrift samengevat en bediscussieerd in relatie tot de huidige literatuur.

Curriculum Vitae

Roy Gerardus Hendrikus Petrus van Heesbeen werd geboren op 24 augustus 1986 te Waalwijk. In 2004 behaalde hij zijn HAVO diploma aan het Willem van Oranje College te Waalwijk. In hetzelfde jaar startte hij met de opleiding Applied Science aan de Fontys Hogeschool te Eindhoven. Tijdens deze opleiding heeft hij een onderzoeksstage doorlopen in het laboratorium van dr. Eric Reits onder begeleiding van dr. Marcel Raspe aan het Academisch Medisch Centrum te Amsterdam. Zijn tweede onderzoeksstage werd doorlopen in het laboratorium van prof. dr. Rik Korswagen onder begeleiding van dr. Martin Harterink aan het Hubrecht Instituut te Utrecht. In 2008 ronde hij zijn bachelor studie *cum laude* af en is hij gestart met de master opleiding Cancer Genomics and Developmental Biology aan de Universiteit Utrecht. Tijdens deze master heeft hij een onderzoeksstage gelopen in het laboratorium van prof. dr. Geert Kops onder begeleiding van dr. Saskia Suijkerbuijk aan het UMC Utrecht. Daarna vertrok Roy voor 10 maanden naar het laboratorium van dr. Jagesh Shah aan Harvard Medical School te Boston, Massachusetts, USA. In 2010 heef hij zijn master opleiding afgerond en is hij in december van datzelfde jaar gestart met zijn promotieonderzoek in het laboratorium van prof. dr. René Medema bij de afdeling Medische Oncologie aan het UMC Utrecht. In januari 2012 is het laboratorium verhuisd naar het Nederlands Kanker Instituut te Amsterdam, waar hij zijn promotieonderzoek heeft voortgezet.

List of publications

van Heesbeen, R.G.H.P., Raaijmakers, J.A., Janssen, L., Blomen, V.A., Brummelkamp, T.R., Medema, R.H. The RZZ complex and Bub1 cooperate to promote kinetochore recruitment of Mad1. *Manuscript in preparation*

van Heesbeen, R.G.H.P.*, Raaijmakers, J.A.*, Blomen, V.A., van Diemen, F.R., Brummelkamp, T.R., Medema, R.H. A haploid genetic screen in human cells identifies novel factors that control proper chromosome segregation. *Manuscript in preparation*

van Heesbeen, R.G.H.P., Tanenbaum, M.E., Halim, V.A., Raaijmakers, J.A., Lelieveld, D., Lieftink, C., Heck, A.J.R., Egan, D.A., Medema, R.H. (2015). Aurora A, MCAK, and Kif18b promote Eg5-independent spindle formation. *Submitted for publication*

van Heesbeen, R.G.H.P., and Medema, R.H. (2015). Kif15: A useful target for anti-cancer therapy? Book chapter, Kinesins and Cancer, 77-86

Maia, A.R.R.*, **van Heesbeen, R.G.H.P.***, and Medema, R.H. (2014). A growing role for Aurora A in chromosome instability. Nat. Cell Biol. *16*, 739–741. *Contributed equally

van Heesbeen, R.G.H.P., Tanenbaum, M.E., and Medema, R.H. (2014). Balanced activity of three mitotic motors is required for bipolar spindle assembly and chromosome segregation. CellReports *8*, 948–956.

van Heesbeen, R.G.H.P.*, Raaijmakers, J.A.*, Tanenbaum, M.E., and Medema, R.H. (2013). Nuclear envelope-associated dynein cooperates with Eg5 to drive prophase centrosome separation. Commun Integr Biol *6*, e23841. *Contributed equally

Manning, D.K., Sergeev, M., **van Heesbeen, R.G.**, Wong, M.D., Oh, J.-H., Liu, Y., Henkelman, R.M., Drummond, I., Shah, J.V., and Beier, D.R. (2013). Loss of the ciliary kinase Nek8 causes left-right asymmetry defects. Journal of the American Society of Nephrology *24*, 100–112.

Raaijmakers, J.A.*, **van Heesbeen, R.G.H.P.***, Meaders, J.L., Geers, E.F., Fernandez-Garcia, B., Medema, R.H., and Tanenbaum, M.E. (2012). Nuclear envelope-associated dynein drives prophase centrosome separation and enables Eg5-independent bipolar spindle formation. The EMBO Journal *31*, 4179–4190. *Contributed equally

Shah, J., **van Heesbeen, R.**, Sergeev, M., and Guan, Y. (2011). Dynamic Quantitative Imaging of Primary Cilium Assembly. Microscopy and Microanalysis, 17 (Suppl. 2), pp 50-51.

Harterink, M., Port, F., Lorenowicz, M.J., McGough, I.J., Silhankova, M., Betist, M.C., van Weering, J.R.T., **van Heesbeen, R.G.H.P.**, Middelkoop, T.C., Basler, K., et al. (2011). A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor Wntless and is required for Wnt secretion. Nat. Cell Biol. *13*, 914–923.

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