Universiteit Utrecht

THE ROLE OF THE CENTROSOME IN MITOTIC SPINDLE FORMATION

Roderick P. Tas
Supervision: B. Etemad; G.J.P.L. Kops

Abstract

During cell division, correct chromosome segregation between the two daughter cells is important to maintain the genetic balance in the organism. A bipolar mitotic spindle consisting of microtubules is formed to generate force to separate the chromosomes. For a long time the centrosome has been described to be the main microtubule organizing center in the cell during mitotic spindle formation. Meiotic cell divisions however, do not require the centrosomes for chromosome segregation. Currently, several papers show that mitotic spindle formation can also occur in the absence of the centrosome. This review discusses the role of the centrosomes during the formation of the microtubule spindle. We propose that although the centrosomes are important to increase the fidelity of bipolar spindle formation and microtubule organization, they are not essential to form the bipolar spindle. Furthermore, we will highlight the alternative mechanisms that can order non-centrosomal microtubules into a functional spindle. We conclude that the cell possesses sufficient cellular machinery to form the bipolar spindle in the absence of the centrosomes.

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Introduction

During cell division the chromosomes need to be equally distributed between mother and daughter cell. To separate the chromosomes a mitotic spindle is formed. Defects in chromosome separation lead to aneuploidy and cause several severe disease. Additionally, cell aneuploidy due to mitotic spindle defects is often observed in cancer[1]. Early studies showed that the centrosome, consisting of centrioles and the pericentral matrix (PCM) is located at the heart of this mitotic spindle and has been found to be involved in microtubule organization and nucleation. The centrosome is therefore considered to be the main Microtubule Organizing Center (MTOC) during cell division. Centrosomes, containing centrioles, were already present in the early eukaryotes and have been implicated to function in sensation, cell migration and cell division [1, 2]. More recently, several studies have implicated that the mitotic spindle can be formed in the absence of the centrosome. They propose that the microtubules can be nucleated and organized into a mitotic spindle in the absence of the centrosome [3-5]. For many years non-centrosomal microtubule microtubules have been known to organize complex microtubule networks [6, 7]. These findings raise questions about the role of the centrosome during spindle formation. What is the role of the centrosome during (mitotic) spindle formation? Can microtubules self-organize into a bipolar spindle in the absence of the centrosome?

This review will focus on the current status of the field regarding these questions. We will first revisit the described data for mitosis without a centrosome. Subsequently, current data focused on the organization of the microtubules during non-centrosomal spindle formation will be discussed. From this data it can be concluded that the mitotic spindle, although less efficient, can be formed in the absence of the centrosome.

Chapter 1 Cell division without the active centrosome

1.1 The centrosome as spindle organizing center

During the cell cycle, after chromosome duplication, the sister chromosomes need to be separated to produce two daughter cells. To separate the chromosomes, a bipolar spindle of microtubules is formed that attaches to the kinetochores and can exert force to separate the sister chromosomes during anaphase (Fig. 1A). The formation of such a bipolar spindle is important for correct chromosome separation. Microtubules nucleate from the centrosomes at both spindle poles. Astral microtubules attach to the cell cortex by cortical dynein and other anchoring factors while kinetochore and polar microtubules localize to the center of chromosome division[8] (Fig. 1A). This microtubule organization allows for force generation between the two spindle poles to separate the sister chromosomes. Cortical dynein which anchors astral microtubules to the cortex of both poles pulls on the spindle by minus end directed force. Additionally, plus-end directed kinesins walk between microtubules from both poles and generates a pushing force to separate the spindles (Fig. 1B). Furthermore, MT depolymerization dynamics at the pull on the chromosomes and the centrosomes[8, 9] (Fig. 1C,D [8, 10]).

The main role of the centrosome in spindle formation appears to be the nucleation and central organization of the microtubules. These processes result in further organization and force generation by the above described motor and microtubule dynamics.

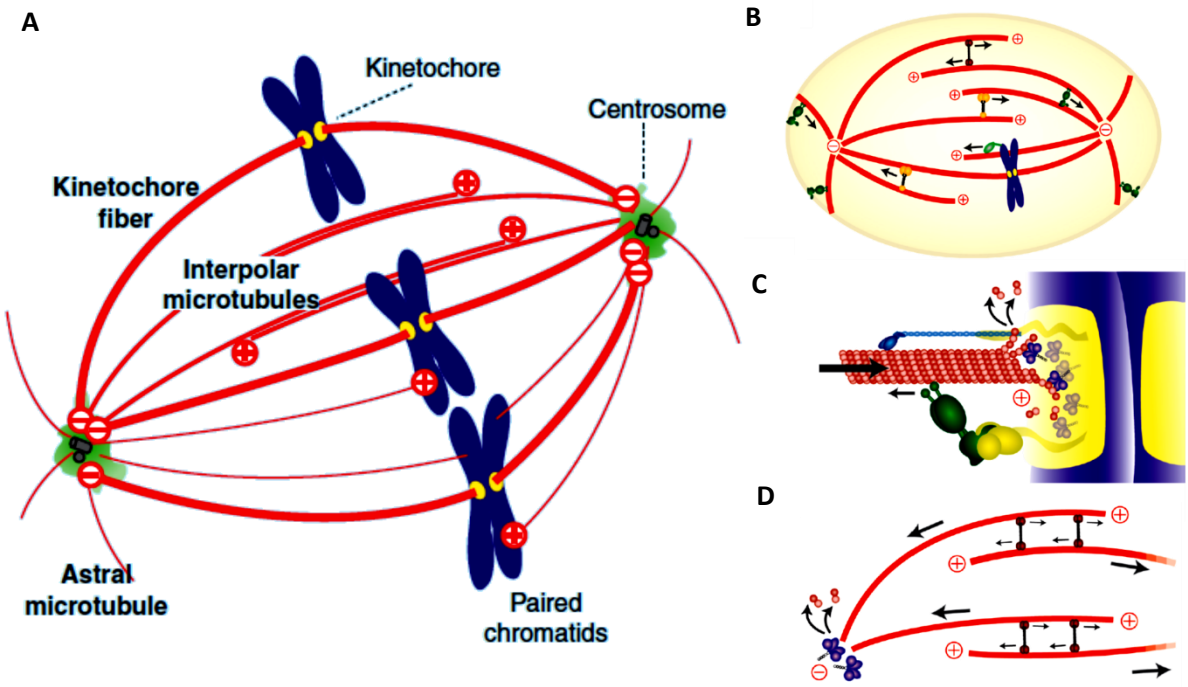

Fig. 1 (Adapted from: Gadde & Heald., 2004). A) Schematic representation of the mitotic spindle during anaphase. B) Representation of the motor forces during chromosome separation. C) Microtubule depolimerization pulls on the kinetochore. (Blue = kinetochore, green structure= dynein). D) Representation of the microtubule dynamics at the centrosome during chromosome separation

Centrosome composition is optimized for microtubule nucleation and anchoring

The composition of the centrosome is highly dedicated to the nucleation and attachment of microtubules. At the core of each centrosome are two centrioles. One of these centrioles is the mother centriole which is inherited from the previous cell division. The other centriole is the immature daughter centriole, created during centrosome duplication. The centrioles are surrounded by a cloud of proteins that forms the Pericentriolar Matrix (PCM). Together, the centrioles and the PCM build the centrosome (Fig. 2). Microtubule nucleation from the centrosome is performed by the γ-Tubulin Ring Complex (γ-TuRC) in the PCM. This complex consist of γ-tubulin and Dgrip subunits that together form a ring-like structure that serves as template for α- and β-tubulin recruitment resulting in microtubule nucleation [11, 12]. To form a centrally organized aster, these newly formed microtubules need to be anchored to the centrosome. Microtubule anchoring is performed by multiple mechanisms in the centrosome. Microtubules can be attached to the distal appendages that are located on the mature mother centriole. These appendages contain the ninein protein which is essential for microtubule anchorage [13, 14]. Furthermore it has been suggested that ninein promotes microtubule anchoring by binding the γTuRC to the PCM, promoting microtubule nucleation through γTuRC recruitment at the same time[13]. Another microtubule anchoring mechanism is through pericentrosomal satellites. Since the minus-ends of the nucleated microtubules are located at the center of the centrosome, dynein associated dynactin can recruit these satellites that supply proteins which anchor the microtubules to the matrix of the centrosome [12, 15]. Furthermore dynactin, EB1 and other microtubule binding proteins anchored to the PCM have been described to facilitate in microtubule anchorage at the centrosome (For reviews see: [12, 16]).

The centrosome thus functions as an ideal hub for microtubule organization into the mitotic spindle by microtubule nucleation and anchoring. Many more identified proteins, like pericentrin and others, have been described to be important for the function of the centrosome as primary MTOC in many cell types. However, we will not go into further details about the additional centrosomal proteins since they are less relevant for the rest of this review.

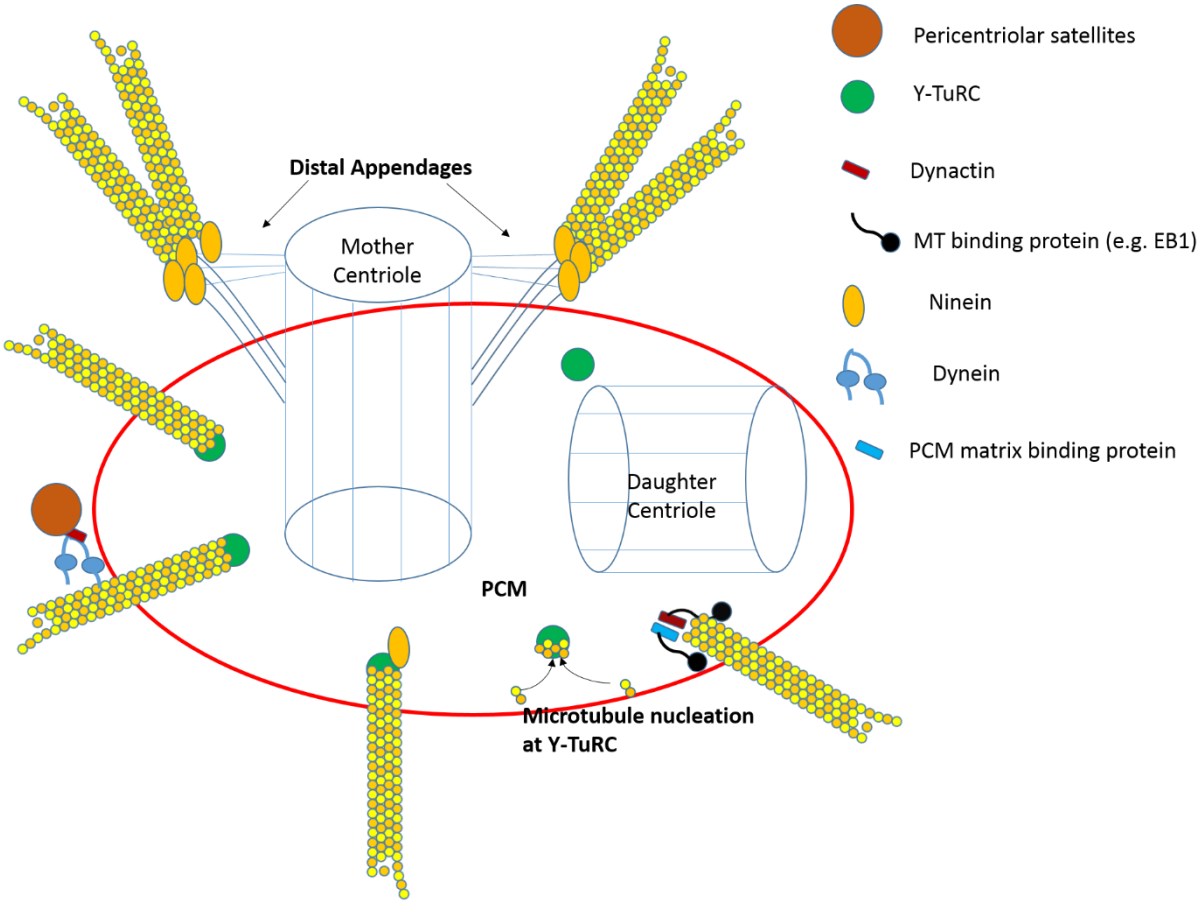

Fig. 2. Schematic representation of several mechanisms of nucleation and anchoring at the centrosome

1.2 Spindle formation in the absence of a centrosome

Meiosis in the oocytes does not require the centrioles.

Although the centrioles were generally considered to be the primary MTOC in most cells, an early electron microscopy study showed that the centrioles were absent from the meiotic spindle during meiosis I and II of mouse oocytes [17]. Absence of the centrioles during has also been observed in other mammalian species like cows, rabbits, *Xenopus,* humans and other species [18, 19]. However, despite centrosome loss, meiotic spindle formation and chromosome segregation take place during oocyte development. The microtubules that compose the meiotic spindle are centrally organized by several acentriolar MTOCs which nucleate and attach microtubules [17, 20]. These MTOCs consist of proteins that can also be found in the centrosome such as γ-tubulin, NuMa and pericentrin [21-24]. Although there are no centrioles present at the center of the meiotic spindle poles, an alternative nucleation center is organized which consist of proteins that can be found in the centrosome. These MTOCs are able to self-organize at the spindle pole and cluster the microtubules in a meiotic spindle. This self-organization is mediated by microtubule attachment to the MTOCs and motor protein dynamics in the presence of chromatin [20, 25, 26]. We will focus on these mechanisms of self-assembly in chapter 2.

Mitosis without the centrosome

Although meiosis occurs in the absence of the centrosome, mitotic cells of many organisms contain an active centrosome which is involved in centering the microtubule spindle during development. This section focusses on the current data that investigated the role of the centrosome during mitotic spindle formation.

Early work in *Sciara* embryos investigated the contribution of the centrosomes to cell division during early development [27]. Unfertilized oocytes do not contain the centrosomes since these are contributed by the male sperm cell. Nonetheless, unfertilized oocytes displayed chromosome segregation by a spindle. However, in unfertilized oocyte the distance between the separated chromosomes was reduced and incorrect distribution of nuclei in the embryonic syncytium was observed, which indicates that the centrosomes mediate in spindle length control and organization. In addition to *Sciara* oocytes, it has been shown that spindle formation and chromosome segregation can occur independent of the centrosome in mammalian cell lines [3-5]. Photo-ablation of one or both centrosomes during prophase resulted in spindle formation and chromosome segregation comparable to wild type cells[3]. Furthermore, it was shown that the microtubules of the spindle were assembled at one central point in the absence of the centrosome[3-5]. Multiple studies which further investigated the efficiency of cell division in the absence of the centrosome confirmed that, although there are some changes in dynamics and efficiency, cultured cells can divide without the centrosome [5, 28, 29]. Although these cells were able to form a spindle, the failure rate of cytokinesis and cleavage furrow positioning was increased resulting in cells that contained multiple nuclei[5, 29]. Cells with ablated centrosomes also displayed aberrant chromosome numbers [28] and an extra centrosome resulted in chromosomal instability in cultured cells [30]. Also, time spent in mitosis after chromosome ablation was observed to be longer than in control cells[4, 31]. The major impact of centrosome ablation however was that daughter cells without the centrosome failed to enter S-phase for the next replication round [4, 5].

These previous studies thus show that the centrosome is not necessarily required for the formation of a mitotic spindle that can perform chromosome segregation. However, other mechanisms such as cell cycle progression and cytokinesis are affected. These processes could be affected by processes involved in centrosomal microtubule nucleation or other centrosomal processes. Multiple studies showed that the effect of centrosome depletion on cell cycle progression is not likely to be microtubule nucleation dependent [4, 31, 32]. It was shown that after microsurgery of the centrosome a new MTOC is generated that contained γ -tubulin and pericentrin but centrioles were not regenerated [4]. However, these cells were still unable to enter S-phase for the next round of cell division. In contrast, RNA interference in *Drosophila* S2 cells of centrosomin and γ-tubulin which depleted microtubule nucleation at the centrosome did not arrest the cell cycle [31]. Cells were able to nucleate microtubules from non-centrosomal sites and the mitotic spindle was formed. A *Drosophila* centrosomin null mutant which failed to form microtubules from the centrosome could even develop into an adult fly [33]. These studies show that microtubule nucleation localized at the centrosome is not specifically required to progress through the cell cycle, where other centrosome-associated factors are. Recent studies have now focussed on the elucidation of these centrosome associated proteins that are necessary for the progression of cell division.

These studies provide insights in the role and necessity of the centrosome during cell division. One conclusion could be that although the centrosomes increase the efficiency of correct chromosome segregation and cytokinesis, they are not required for microtubule nucleation and the formation of a mitotic spindle that can separate the chromosomes during anaphase. It seems that an important aspect of the centrosomes during cell division is positioning of the mitotic spindle since nuclei distribution is disturbed in *Sciara* syncytia [27]. The positioning role of the centrosome rather than spindle formation is also nicely illustrated by its effect on asymmetric cell divisions. Incorrect organization of

the spindle in an Abnormal Spindle-like Microcephaly-associated protein (ASPM) mutant mouse cell line leads to cell divisions at an incorrect plane resulting in reduced neuronal stem cell maintenance. This decrease of neuronal stem cells was associated with microcephaly [34]. ASPM was associated with incorrect distribution of the centrosomes, further suggesting a spindle orientation role for the centrosome. A better described model for spindle positioning is the *Drosophila* oocyte. A mutant line defective in the DSas-4 gene which is essential for centriole duplication shows a 30% decrease in asymmetric cell divisions [35]. These mutants however progress through development quite normally but die because they lack cilia in their sensory neurons. This development in the absence of centrioles confirms alternative centrosome independent mechanisms for spindle formation and mitosis.

Since the centrosomes are not required for the formation of a mitotic spindle, other mechanisms should be able to organize the microtubules during mitosis. The next sections of this review will focus on the alternative mechanisms of microtubule nucleation and non-centrosomal organization of the microtubules which can be used during cell division.

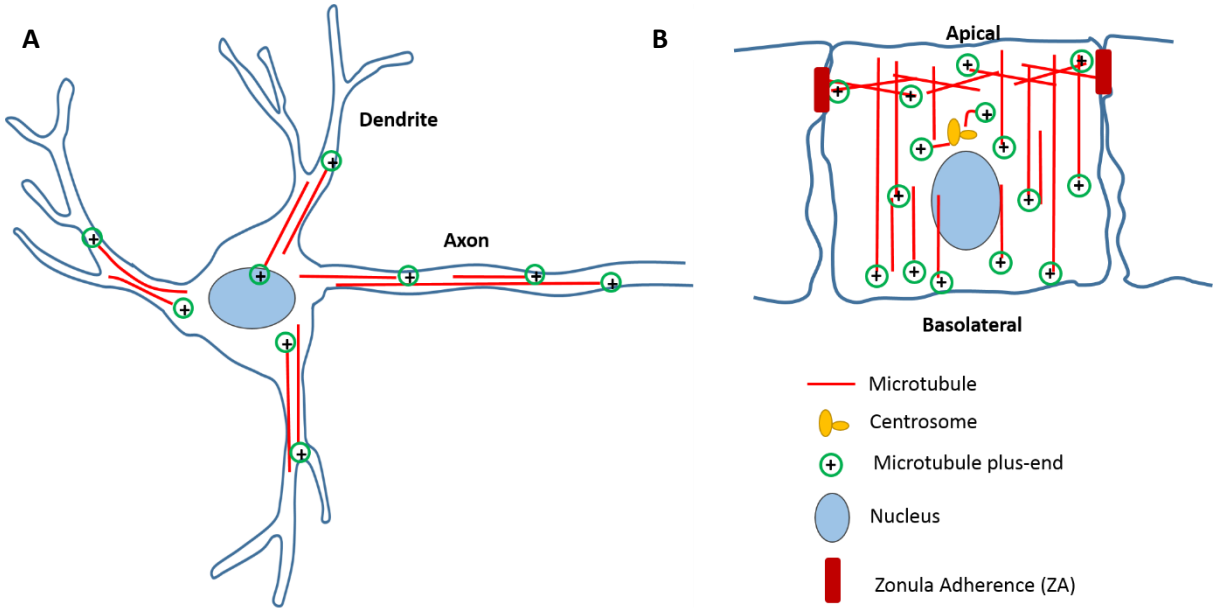

Fig. 3. A) microtubule orientation in neural cells. Note: axonal and dendritic are differentially organized. B) Microtubule orientation in epithelial cells.

1.3 Non-centrosomal microtubule organization in polarized cells

In the previous section it has discussed that the centrosome is not required for mitotic spindle assembly per se. Although the centrosomes are an optimized hub for microtubule nucleation it has also been reported, in several differentiated and dedicated cell types, that specialized microtubule arrays can be organized without a centrosome. This further confirms alternative pathways for microtubule nucleation and organization. These cell types are: neuronal cells, epithelial cells, *S.Pombe,* plants and myotubes. Here, we discuss the first two to illustrate how non-centrosomal microtubules are organized for specific functions in the cell. In addition, organization of the microtubules unattached from the centrosome can provide additional advantages for cellular organization. The non-centrosomal microtubule network of the latter three is reviewed in [7].

Neuronal cells

The microtubule network in neurons is highly specialized. The axon consists of microtubules oriented with their plus-ends pointing to the distal tip of the axon, while the dendrites have a mixed orientation of microtubules (Fig. 3A)[6, 36, 37]. This differential organization of the non-centrosomal microtubules between the axon and the dendrites has been described to be important in selective transport of cargo. Dynein can selectively enter the dendrites through their minus-end pointing out microtubules[37, 38]. Specific non-centrosomal microtubule organization can thus provide advantages for selective transport. The mixed orientation with the minus-end out cannot be accomplished when the microtubules originate from one central centrosome. At the onset of neurite development non-centrosomal microtubules need to be organized. It has been described that during neurogenesis the centrosomes are important to maintain neuronal progenitors through asymmetric cell division [34, 39]. However, during differentiation and the organization of the mixed microtubules in the dendrites the pool of centrosomal microtubules is depleted and non-centrosomal microtubules account for the complex non-radial organization [40-42]. Consistent with loss of centrosomal microtubules, ablation of the centrosome during neuritogenesis results in axon outgrowth with microtubule organization and nucleation similar to wild type [41].

Epithelia

Epithelia are highly polarized cells and distinguish between the apical and the basolateral site. Consistently with this polarity non-centrosomal microtubules are organized in linear arrays. Microtubules span the cell from the apical to the basal site where the plus-ends are basally located [43]. Furthermore a non-centrosomal apical microtubule meshwork between the zonula adherens is observed in different types of epithilia (Fig. 3B) [44, 45]. In contrast to nucleation of the neuronal non-centrosomal microtubules, the centrosomes still contribute to the nucleation of epithelial non-centrosomal microtubules which detach from the centrosome in polarized cochlear epithelial cells [46].Recent evidence further suggests that localization of the centrosome in post-mitotic *C.elegans* epithelia is important to localize γ-tubulin to the apical membrane [47]. Further research will provide more detailed information in the role of the centrosome in establishing polarity. In the differentiated epithelial cells the non-centrosomal microtubules contribute to the functional polarity of the epithelia. Several studies describe that the linear microtubule array originating apically is required mainly for apical polarized transport [48]; reviewed in [49, 50]. The exact molecular mechanisms for polarized transport and the role of the microtubule network in basolateral transport needs to be further investigated. Furthermore the apical attachment of the microtubules to the adherence junctions appear to enhance the structural integrity of these junctions [44, 45]

The complex non-centrosomal microtubule organization in these cell types show that cells have the ability to organize their microtubule network in the absence of the centrosome. Although the centrosome is not necessary for microtubule nucleation in neuronal cells, several epithelial cells rely on centrosomal microtubule nucleation. This shows that the centrosome can be involved in different steps of microtubule organization and that during differentiation cells can rely on alternative pathways for a complex organization. In addition, microtubule dynamics regulation independent of the centrosomes can provide advantages utilized in specific cellular functions. These advantages are illustrated by the neuronal cells and the epithelial cells. In neuronal cells the non-radial organization of the microtubules provides an additional mechanism for selective transport through a minus end directed motor [37, 38]. Furthermore the non-centrosomal microtubules can provide an additional non-radial meshwork for structural stabilization of cell-cell junctions in epithelial cells.

The microtubule organization in these cells together with the data discussed in section 1.2 show that the biological machinery in cells can organize their microtubules with high complexity

independent of the centrosome. This can also lead to the formation of a mitotic spindle. The next chapter will focus on the cellular machinery that accounts for non-centrosomal microtubule organization. Recent work will be discussed focused on how this machinery can organize a mitotic spindle in the absence of the centrosome and what the advantages of these processes are.

Chapter 2: Organization of non-centrosomal proteins into an active mitotic spindle

The formation and maintenance of non-centrosomal microtubules requires alternative processes that are normally performed by the centrosomes. New non-centrosomal microtubules need to be nucleated from alternative sites. Maintenance of these microtubules further requires stabilization through minus-end capping which is usually performed by centrosomal γ-TuRC and other centrosomal proteins as described in section 1.1. These processes thus require alternative use of the nucleation machinery and other microtubule stabilizing mechanisms. Subsequently, the newly formed non-centrosomal microtubules need to be organized. The next sections will focus on the formation, stabilization and organization of non-centrosomal microtubules. Recent data will be discussed to illustrate the processes involved in non-centrosomal microtubule formation and spindle organization.

2.1 non-centrosomal microtubule generation, maintenance and properties

Generation of non-centrosomal microtubules

Non-centrosomal microtubules have been shown to be formed dependent and independent of the centrosome. The three described mechanisms for nucleation are: release from the centrosome, nucleation at alternative sites and generation from existing microtubules (Fig. 4). The basics of these processes are very well reviewed by Bartolini & Gundersen[7]. We will therefore only briefly discuss these mechanisms and include more recent studies in which these mechanisms contribute to non-centrosomal microtubule arrays.

Release from the centrosome

It has been shown that the centrosome contributes to the pool of non-centrosomal microtubules through microtubule nucleation and release in epithelial and migrating cells [7, 46, 51]. The microtubules that are released from the centrosomes need to be transported to other sites in the cells to form non-radial microtubule arrays. Release from the centrosome is therefore paired with translocation of the microtubules. It has been shown that upon release of the centrosomes in epithelial cells, microtubules are actively transported or treadmilled until the microtubules are anchored by end binding proteins[51-53]. Whether microtubule release from the centrosomes occurs bound to the γ-TuRC on the minus-end or unbound remains unclear. Recent data however describes a new family of proteins that can stabilize the minus-ends independent of γ-tubulin. These proteins will be discussed in the next section.

Nucleation at alternative sites

The first data for non-centrosomal microtubule nucleation originates from plant cells which do not possess centrosomes. In these plants, nucleation of microtubules occurs in the nuclear periphery and at the cortex during different stages of the cell cycle. Similar alternative nucleation sites have been observed in *S.pombe*[7]. These non-centrosomal nucleation processes in plant and yeast have been shown to be dependent on the γ-TuRC that is recruited to the nucleating compartments in these organisms[54, 55]. More recent studies in mammalian cells provide strong evidence that the golgi is involved in non-centrosomal γ-tubulin dependent nucleation[56]. Furthermore, dendritic nucleation of non-centrosomal microtubules in neurons has been shown to occur at golgi outposts serving as alternative MTOCs in the dendrites[57]. However, whether the golgi outposts are required for microtubule nucleation in neurons in currently under debate[58]. Another important mechanism of non-centrosomal microtubule nucleation is microtubule-dependent microtubule nucleation. Augmin has been shown to be important for γ-TuRC dependent nucleation of microtubules on the lattice of existing microtubules[59, 60]. We will focus on augmin-dependent nucleation in section 2.2 because it is important

in microtubule nucleation/organization during spindle assembly in the presence and absence of the centrosome.

Generation from existing microtubules

To generate microtubules from existing ones, they need to be severed. The resulting fragments can then continue growing as two individual microtubules. Breaking the microtubule is performed by two mechanisms, protein mediated breaking and breaking by mechanical stress. Protein mediated breakage of microtubules is performed by either katanin, spastin and fidgetin[61]. Katanin was the first identified enzyme to break microtubules *in vitro*[62]. However, a distinct cellular function was not yet known. Subsequently, *C. elegans* studies suggested that microtubule severing by katanin increases the number of meiotic spindle microtubules which is required for proper bipolar spindle formation[63, 64]. Furthermore, katanin and spastin have recently been shown to be important in neurite outgrowth and branching during neuronal development[65, 66]. These combined results therefore suggest that microtubule severing *in vivo* is responsible for spatiotemporal microtubule amplification which can lead to regulation of microtubule dependent processes like neurite branching and spindle formation.

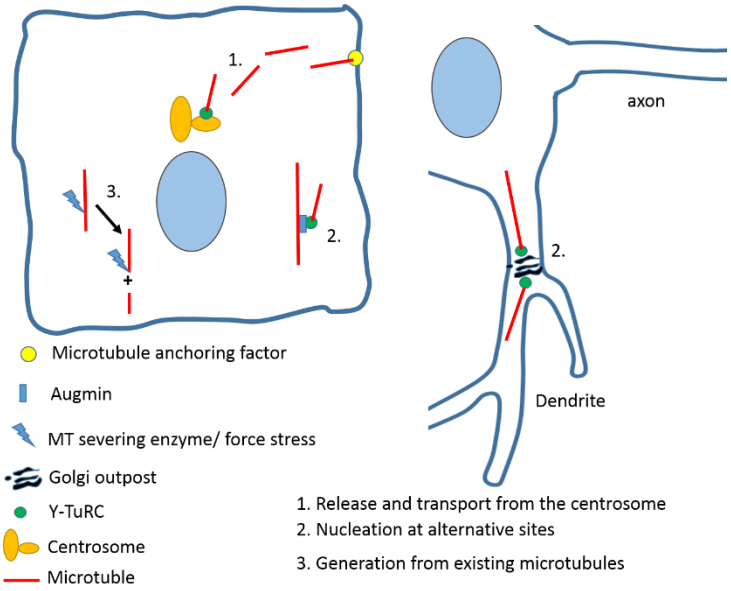

Fig. 4. Schematic representation of the mechansisms involved in non-centrosomal microtubule generation.

Minus-end capping and microtubule stabilization maintains the non-centrosomal microtubule pool

Minus-end capping

To maintain the non-centrosomal microtubules, the microtubules need to be stabilized. Microtubule conservation is highly dependent on the dynamic tips. Therefore, microtubules can be stabilized by capping the tips to prevent microtubule depolymerization. The plus-ends are very dynamic with polymerization, depolymerization and polymerization rescue events. These dynamics are sufficient for microtubule growth *in vitro.* In contrast, the minus-ends have been shown to be more static. Initial depolymerization of the minus-ends almost always leads to full depolymerization of the microtubule without rescue events[52, 53]. Stabilization of the minus-ends through capping is thus a good mechanism to reduce microtubule depolymerization significantly.

It has been shown that purified non-centrosomal microtubules display less polymerization from the minus-end than centrosomal microtubules[67]. This suggests that minus-end properties are tightly regulated and differ between centrosomal and non-centrosomal microtubules. In the presence of the centrosome, the γ-TuRCs that are anchored by the centrioles and PCM have been described to be the main mechanism for microtubule capping. Various proteins like augmin, ninein, pericentrin and others, have been described are involved in γ-TuRC recruitment to the centrosome resulting in minus-end stabilized, centrally organized microtubules[68]. In cells without the centrosomes, the γ-TuRC has also been shown to be important for microtubule stabilization. A study in neurons which do not contain active centrosomes showed that depletion of γ-tubulin drastically reduced the pool of dendritic

microtubules[40]. However, this study also showed that depletion of another microtubule minus-end protein, CAMSAP2, resulted in loss of these microtubules. They therefore propose that in addition to γ-tubulin nucleation and capping, non-centrosomal microtubules are capped by other minus-end binding proteins to stabilize the non-centrosomal microtubule pool. More studies now showed that proteins of the CAMSAP/Nezha/Patronin family are involved in minus-end stabilization and that these proteins are required to maintain the pool of non-centrosomal microtubules[68-70]. It has been suggested that CAMSAP3 (Nezha) anchors and stabilizes minus-ends at the zonula adherence in epithelia and therefore regulates non-centrosomal microtubule organization[71]. Furthermore, it was shown that depletion of CAMSAP2 and 3 results in a loss of non-centrosomal microtubules in epithelial CaCo cells[69]. After nocodazole treatment in these CAMSAP depleted cells microtubules were nucleated and maintained at the centrosome. The authors showed that depletion of non-centrosomal microtubules results in misorganization of several organelles. The Nezha stabilized non-centrosomal microtubules have also been implicated to possess different properties which result in capturing Rho-GEFs to regulate stress fiber formation. Disruption of the balance by Nezha knockdown in HeLa cells resulted in more centrosomal microtubules and faster stress fiber formation through up-regulation of RhoA[72]. These studies therefore show that maintenance of the non-centrosomal microtubule pool is important for cellular functions like microtubule organization (in neurons), organelle positioning and stress fiber formation. Since these minus-end binding proteins are now discovered linked with non-centrosomal microtubules maintenance, future studies should focus on the exact contribution and importance of the non-centrosomal microtubule pool in cellular processes.

Stabilization of non-centrosomal microtubules

The second mechanism to stabilize microtubules is through stabilization of the microtubule lattice. Early nocodazole washout experiments suggested that the non-centrosomal microtubules in cultured cells were more stable than the centrosomal microtubule pool[73]. The non-centrosomal microtubules of MDCK cells were highly decorated by detyrosinated tubulin which is a marker for stable microtubules. These detyrosinated microtubules were found to be more resistant to nocodazole washouts and therefore more stable. In addition, neuronal microtubules microtubules are highly stable when subjected to cold treatment which usually depolymerizes the microtubules[74, 75]. This stability is suggested to be microtubule transglutamylation dependent resulting in a more stable microtubule lattice[75]. Stabilization of microtubule lattices in neurons by a small molecule taxol has also been shown to be sufficient for axon initiation[74]. Furthermore, different Microtubule Associated Proteins (MAPs) can enhance microtubule lattice stability[76, 77]. Although these studies show that microtubule stability might be an important property of non-centrosomal microtubules, the exact mechanisms, importance and functions of this stability need to be elucidated.

The above discussed literature shows that the non-centrosomal microtubules can have different properties and functions than centrosomal microtubules. Recently it has been suggested that Herpes Simplex Virus (HSV) infection results in non-centrosomal reorganization of the microtubule network for efficient transport of viral proteins to form a capsid[78]. This study thus suggest that non-centrosomal microtubules are a mechanism for directed transport, which is consistent with dendritic selectivity by dynein in neurons as described above. Although the exact properties of non-centrosomal microtubules are still under investigation, it is clear that non-centrosomal microtubules have an important role in cellular functions and dynamics in different cell types. These microtubules might be important for directed transport in a non-radial manner and several mechanistic processes. The identification of the minus-end binding proteins which can be used manipulate the non-centrosomal microtubule balance and current microscopy techniques can provide us with more information about the role of these microtubules in the future. Structural and cell biological studies should further focus on the different properties of these microtubules.

2.2 Non-centrosomal microtubule organization into a bipolar spindle

Self-organization of the mitotic spindle in the absence of the centrosomes

Section 1.2 showed that a mitotic spindle can form in the absence of the centrosome. To organize the microtubules in a mitotic spindle in the absence of the centrosome, alternative mechanisms of microtubule organization are necessary. Here we will discuss the mechanisms that are involved in the formation of an acentriolar spindle. These mechanisms should perform the functions usually executed by the proteins of centrosome and bipolar spindle formation. These results will also consider the mechanisms of meiotic spindle formation since this process naturally occurs without the centrosome. We consider the functions of the centrosome as MTOC during mitotic spindle formation to be: aster formation, bipolar spindle orientation and microtubule nucleation.

Aster formation

Recent studies started to elucidate the mechanisms that can explain microtubule self-organization into a mitotic spindle. It has been shown that minus-end directed motors contribute to the localization of microtubules to the spindle. During plant mitosis without centrosomes, kinesin-14 is required to transports microtubules to the spindle poles[79], while dynein is responsible during meiosis in *Xenopus* oocytes[26]. Upon inhibition of dynein in *Xenopus* egg extracts, the microtubules attached to chromatin failed to assemble into a bipolar spindle[26]. Additionally inhibition of dynein by multiple dynein inhibitors resulted in loss of centrally focused microtubules at the spindle pole in mammalian cells[80]. Similar to dynein, the minus-end directed motor HSET was found to contribute to microtubule focusing at the spindle poles[81]. Although HSET blocking by antibodies did not affect mitotic spindle formation in the presence of the centrosome, meiotic spindles were affected and lacked focused spindle poles. Furthermore, in cancer cells, depletion of HSET leads to extranumerary spindles and incorrect segregation of the chromosomes[82].

These studies show that different minus-end directed motors are important in the central organization of the microtubules even in the presence of the centrosomes. These motors should bind one microtubule and transport it to the minus-end of another. In this way all microtubule minus-ends will be focused at one central point (Fig. 5A). For kinesin-14, it has been shown that the tail domain is able to bind another microtubule[83]. In this manner microtubules can be transported on one another. In contrast, for cytoplasmic dynein no specific microtubule binding partners have been identified. It has been proposed that microtubules are transported by dynein through association with NuMa[84]. Future research should provide further insights in the dynein, microtubule interaction. Complementary to dyneins function in aster formation in oocyte extracts, addition of kinesin-14 to microtubules *in vitro* was sufficient to form microtubule asters with a minus end-in microtubule orientation[85]. Minus-end directed transport of microtubules is thus sufficient for microtubule aster formation, independent of the centrosome.

Bipolar spindle orientation

Aster formation alone is not sufficient to form the mitotic spindle. Correct chromosome segregation requires the formation of two focused spindles that both attach to one set of sister chromatids. Bipolar spindle formation is highly dependent on two different processes that contribute to the formation of a bipolar spindle with two opposing asters: 1) microtubule plus-end attachment to the chromatin and 2) the antiparallel microtubule array between the spindle poles. An early study described that DNA itself provides guidance cues favoring bipolar spindle formation. Introduction of small DNA beads (similar to DNA) that interacted with microtubules led to the formation of a mitotic

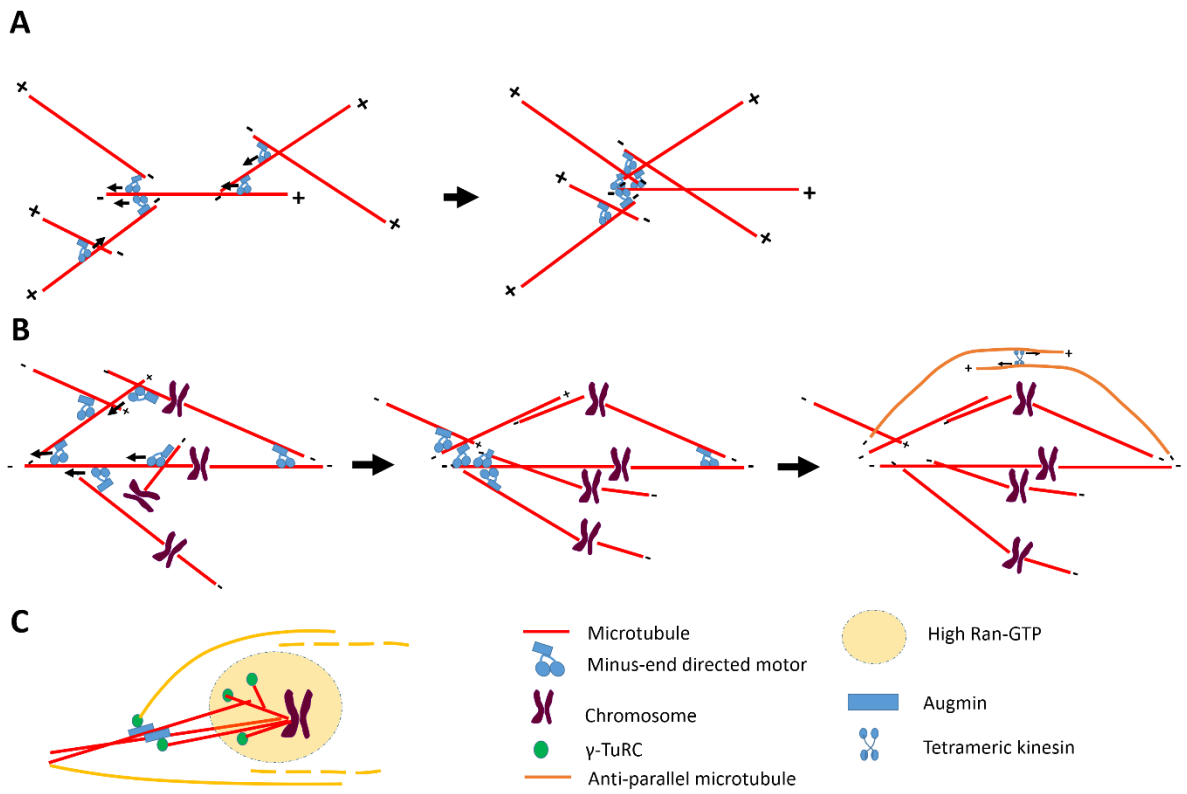

Fig. 5 Microtubule self-organization into a spindle in the absence of the centrosomes. A) minus-end directed motors are required for aster formation. B) Chromosome alignment and bipolar spindle formation by motor proteins. C) Non-centrosomal nucleation mechanisms strengthen chromosome attachment to the spindle poles

spindle in *Xenopus* cell extracts [26]. These newly formed spindles were bipolar in approximately 85% of the observations suggesting that spindle bipolarity cues are implemented in the area around the DNA. The efficiency of bipolar spindle formation is kinesin and dynein dependent. Addition or depletion of these motors results in aberrant spindle pole number[26, 86]. The motor activity of dynein is hereby necessary to focus the spindle poles. Kinesins can then increase the robustness of the system by centering the microtubules in the antiparallel microtubule array between the spindle poles. One important factor that plays a role in the configuration and number of spindle poles is the surface on which the microtubules attach. Microtubules attached to a chromatin coated artificial elongated rod or slightly asymmetric sphere formed opposing spindles on the longest axis of the shape[87, 88].

From these controlled experiments on chromatin coated surfaces it can be hypothesized that *in vivo* self-organization of microtubules in the presence of chromatin will lead to bipolar spindle formation. Intuitively the microtubule attachment to the back-to-back configuration of the kinetochores and the minus-end directed focusing by dynein can increase the favorability of a bipolar spindle (Fig. 5B). The importance of microtubule attachment to the kinetochores has been illustrated by several studies *in vivo* and in culture. These studies observed defects in bipolar spindle formation and chromosome segregation and alignment upon deletion of the microtubule kinetochore interaction[89-91].

Another factor important for the assembly of a bipolar spindle is antiparallel microtubule bundling by kinesins. Kinesin-5 has been shown to slide antiparallel motors apart by moving on both microtubules[92]. Likewise, dimeric kinesin-12 has been implicated to generate pushing force between

the anti-parallel microtubules of the mitotic spindle. Kinesin-12 interacts with the microtubules through association with the microtubule binding TPX2 protein [93]. Although kinesin-5 is the main force generating motor between the antiparallel microtubules of the spindle these motors work in synergy to separate the antiparallel microtubules[93]. These sliding interactions between the antiparallel microtubules through kinesins contribute to the formation of a bipolar spindle and chromosome separation. Depletion of kinesin-5 activity by monastrol leads to the monopolar spindles[94]. It is now generally accepted that these antiparallel microtubules generated by kinesin-5 and kinesin-12 is essential to form the bipolar spindle[9, 93]. Upon spindle assembly these motors can thus align the microtubules with a 180 degrees rotation leading to a favored bipolar spindle (Fig. 5B). In addition to the kinesins which can form antiparallel microtubules in the mitotic spindle, Ase1/MAP65 has been described to be involved in the stabilization of this antiparallel microtubule conformation in plants and fission yeast[95, 96]. However, a role for these MAPs in animal spindle formation needs investigation.

Microtubule nucleation

To organize a spindle in the absence of the centrosome, not only the microtubules need to be clustered and organized in a bipolar spindle but they also need to be nucleated to add to the spindle. Since the centrosome cannot anchor the γ-TuRCs, other sites are required to nucleate focused microtubules. During meiosis in mouse oocytes, MTOCs cluster at the spindle poles similar to the centrosomes. Live cell imaging of microtubule plus-ends revealed that these MTOCs have similar microtubule nucleation and microtubule growth dynamics compared to the centrosomes[20]. Furthermore, after ablation of the mitotic centrosomes γ-tubulin has been shown to focus at the newly formed spindle poles[4].

Recently, in *Drosophila* a complex of 5 genes has been identified to be involved in γ-tubulin localization to the spindle poles resulting in microtubule nucleation [59]. These genes were the *Dgt*2-5 genes (dim gamma tubulin) and were later identified to be the human augmin complex[60]. This complex has been described to be important in the localization of γ-tubulin at the spindle poles in *Drosophila* oocyte meiosis resulting in microtubule nucleation biased at the spindle poles[97]. Augmin generates new microtubules from existing ones by recruiting γ-tubulin alongside the microtubule lattice, resulting in enhanced microtubule nucleation along the spindle body[31, 98]. Depletion of centrosomin in *Drosophila* oocytes resulted in bipolar spindle formation where co-depletion of the *Dgt* genes completely abolished spindle formation[60]. Furthermore, depletion of augmin decreases the number of nucleation events and resulted in detachment of microtubules from the spindle pole[60, 98]. In addition, augmin is important for the creation of a bipolar focus in cancer cells. Depletion of augmin in these cells resulted in supernumerary spindle poles and aneuploidy[82]. The augmin-dependent processes in spindle enhancement are timed by Aurora-A and Polo-like kinase 1 which are activated during cell division[97]. These results therefore show that augmin-dependent microtubule nucleation is important in the amplification of spindle microtubules resulting in strengthening of the spindle and spindle poles (Fig. 5C).

Another mechanism which has been well described to be involved in microtubule nucleation independent of the centrosome is chromatin-dependent. A high Ran-GTP gradient near the chromatin results in the recruitment and assembly of γ-tubulin-dependent microtubule nucleation in both meiotic and mitotic cells[99] (Fig. 5C). Ran-GDP is converted to its GTP form by the RCC1 protein (Regulator of Chromatin Condensation) and this gradient recruits the microtubule nucleation factors (nicely reviewed in: Meunier & Vernos, 2012[100]). This mechanism has been well studied and contributes significantly to the initial microtubule pool in mitotic cells.

The three above described processes of microtubule nucleation, aster formation and bipolar spindle formation can all occur in the absence of the centrosome upon exposure to the chromatin that becomes available after nuclear envelope breakdown. Although these processes are likely to be affected by a pool of additional factors in the cells, the demands of mitotic spindle assembly can be fulfilled in the absence of the centrosomes. However, cooperation of the necessary processes for bipolar spindle formation requires an exact timing at the right stage of cell division. The complexity of this timing might increase the chance of aneuploidy and extranumerary spindle formation. This increase has been illustrated by the discussed papers, which show that although bipolar spindle assembly is favored, also aberrant numbers of spindle poles occur in the absence of the centrosome[26, 86]. These defects are also described by the cellular assays that show increased chromosomal instability upon ablation of the centrosomes[28, 30]. Although these studies explain the formation of a bipolar spindle in the absence of the centrosomes, it appears to be that the centrosomes are important in the regulation and timing of these above described processes. Microtubule nucleation and focusing are clustered in the two replicated centrosomes. These combined features of the centrosomes can therefore favor high fidelity bipolar spindle assembly and correct chromosome segregation because it will intrinsically form two microtubule nucleating asters. Further research to confirm this hypothesis is required but the discussed loss of mitotic fidelity is one of the first clues for this hypothesis.

Because the microtubule dynamics within these self-generated spindle poles are very complex and hard to study all at the same time, recently modeling was applied to shed light on the interplay between al the involved processes[101]. The meiotic spindle formation in the absence of the centrosomes was modelled. The authors implemented the minus-end directed transport of microtubules, microtubule nucleation at branches (Augmin pathway), microtubule nucleation near the chromatin (Ran-GTP pathway) and antiparallel bundling/ sliding of microtubules. They found that all these forces resulted in the formation of a bipolar spindle. The minus-end directed force and microtubule amplification on other microtubules were required to form the bipolar spindle because microtubules would align with the same direction in both antiparallel bundles. Additionally the minus-end directed force was necessary for microtubule focusing at the poles. Furthermore, they confirmed *in silico* that depolymerizing microtubule dynamics at the anti-parallel bundles and the spindle poles were crucial to determine the spindle length. These depolymerizing factors have been assigned to the depolymerizing kinesins which indeed have been shown to be located at these positions[8]. This study further revealed that microtubule length distribution was not important for the spindle morphology *in silico*.

Computational modeling can thus be a powerful tool to investigate the microtubule dynamics required for spindle pole formation. Not only can it be applied to confirm previous cellular assays, complex microtubule dynamic parameters that influence spindle formation and shape can be studies. Although powerful new super resolution microscopy techniques (e.g. dSTORM, STED, PALM) and live cell imaging techniques are now widely available to image individual processes, modeling studies can help to implement and understand the complex data that is generated. Furthermore it can be a powerful tool to elucidate important key features of microtubule organization which can be studied in a controlled modeling environment based on experimental data. Future modeling studies can focus on the mechanisms that result in decreased chromosome segregation fidelity upon centrosome loss.

Chapter 3: Discussion and future perspectives

Mitotic spindles formation in the presence of the centrosomes is a highly conserved process. However, mitotic spindle formation can occur in the absence of the centrosomes. Although the centrosomes are necessary to pass the mitotic checkpoints, a functional mitotic spindle can form without the centrosomes as MTOCs through non-centrosomal microtubule organization. Microtubule organization independent of the centrosome has been described to be the main mechanism of microtubule organization in several organisms and cell types ranging from plants and *S.Pombe* to mammalian cell types like neurons, epithelia and myotubes. These cell systems all consist of non-radial linear microtubule arrays[7] that need to be tightly organized. In this review we discussed how non-centrosomal microtubules organize into a functional mitotic spindle. This occurs through minus-end directed motor transport of non-centrosomal microtubule, antiparallel bundling by kinesins and nucleation at alternative sites. Although the exact minimal system necessary for non-centrosomal microtubule organization into a bipolar spindle has yet to be determined, recent cellular and modeling studies have begun to elucidate the factors that are essential. Microtubule organization through motor transport has not only been described to be important during non-centrosomal spindle formation. In *Drosophila* neurons, kinesins attached to the plus-end of microtubules in one neurite have been shown to transport the attached microtubule into a second neurite to organize the cytoskeleton[102]. In addition to non-centrosomal microtubule organization, the non-centrosomal microtubules are maintained by distinct stabilization mechanisms. Recent studies now discovered proteins that are important in the stabilization of these non-centrosomal microtubules. Furthermore, the non-centrosomal microtubules have been suggested to possess distinct properties and functions. Although the importance of non-centrosomal microtubules has been discovered in the recent years, more research is necessary to identify all the molecular mechanisms for stabilization, organization and the distinct properties of these microtubules.

It has to be noted that these non-radial non-centrosomal microtubule arrays in animal cell types have been described in highly differentiated and specialized cells. Non-centrosomal microtubules can be arranged and are highly functional in a distinct non-radial specialized network. In contrast, in dividing somatic cells, the centrosomes are maintained and depletion of the centrosomes results in a decrease in the fidelity of chromosome separation. This suggests that the centrosomes have a distinct conserved function among these cell types. We propose that the function of the centrosome in mitotic spindle formation is best illustrated by the implications that are paired with centrosome loss during mitosis. The main implications as described in section 1.2 are not in spindle formation but in chromosome segregation; time spent in mitosis; and asymmetric cell division. It can therefore be suggested that the centrosome's main function during spindle formation is to ensure smooth temporal progression through mitosis, bipolar spindle formation and spindle orientation. As discussed in section 1.2 and 2.2, self-organization of the microtubules favors bipolar spindle formation but with decreased fidelity compared to control situations. Although the mitotic checkpoint ensures the capture of all chromatids[103] it does not control the amount of spindles to separate these chromatids. We therefore propose that the fidelity of bipolar spindle formation is assured by the centrosomes. Duplication of the centrosomes during S-phase inherently produces two MTOCs resulting in two asters for correct chromosome segregation into the daughter cells. The discussed studies also show that the non-centrosomal nucleation and organization processes by for example augmin and dynein further strengthen these two spindles poles. The process of spindle pole strengthening by the non-centrosomal processes rather than their importance in spindle formation has already been proposed by Mahoney et al., 2006[31]. They proposed that these non-centrosomal processes are required to strengthen bipolar spindle pole formation and increase the fidelity of

chromosome segregation resulting in faster mitosis. A recent review focused on the kinetochore-microtubule interaction suggested that fast progression through mitosis is important because during mitosis, transport; DNA repair; and other core functions for survival are inhibited. A delay in mitosis could then lead to apoptosis since these processes are necessary for cell survival[103]. The reduced time spent in mitosis, in the presence of the centrosomes, could therefore also be important for the cell survival signals. So although a bipolar spindle is favored through self-organization of microtubules we propose that the centrosomes are essential to safeguard mitotic divisions. This is necessary because incorrect chromosome segregation results in aneuploidy which can then be amplified in all daughter cells which has been implicated to be causal in diseases like cancer[104]. In addition, regulation of spindle orientation during asymmetric cell division by the centrosomes is essential during development to maintain the progenitor stem cell pool as discussed in section 1.2.

Although we focused on the importance of the centrosome in spindle formation, the centrosome has also been shown to be important for cytokinesis, cilia formation and cell migration[1, 105]. All these processes are essential for organism survival, further illustrating the importance of the centrosome.

The role of the centrosome in mitotic fidelity then leads to the question: how can meiosis progress with high efficiency in the absence of the centrosome? Deficiencies in meiosis equally lead to development with aberrant chromosome number causing developmental diseases of which the most well-known is down-sydrome[106]. Although oocyte meiosis occurs in the absence of the centrosomes, bipolar spindle formation during meiosis of male spermatogenesis has been described to occur in the presence of the centrioles after which they degenerate in some species[18, 107]. Although in male meiosis it can be suggested that meiotic fidelity is partially provided by the centrosomes, female meiosis occurs in the absence of centrosomes. Meiosis thus requires additional checkpoints to control chromosome number in both male and female gametes. Recent studies are now starting to discover and elucidate these additional control mechanisms that ensure correct chromosome number during meiosis and fertilization[108-110]. These additional mechanisms however do not explain why female gametogenesis meiosis occurs in the absence of the centrosomes. Centrosome degradation has been suggested to be important to ensure that one copy is present in the fertilized egg and prevent premature parthenogenesis[107]. Maintenance of one centrosome in the fertilized embryo can occur through multiple mechanisms. Female gamete meiosis in the absence of centrosome might be an emergent property arising from centrosome reduction. Future research will provide more information about the difference in the need for centrosomes between mitotic and meiotic divisions.

In conclusion the discussed papers show that the mitotic spindle can be formed in the absence of the centrosomes through self-organization of the microtubules. These self-organizational processes are similar to the ones applied to form the meiotic spindle. Both mitosis and meiosis are two distinct processes that require non-centrosomal microtubules to either strengthen or form the spindle. From the discussed literature we propose that although these processes are similar they both utilize different mechanisms for high fidelity chromosome segregation. During mitosis the centrosomes ensure bipolar spindle formation without additional poles and therefore correct chromosome segregation. Furthermore, the centrosomes are key players in spindle orientation which has shown to be important in many processes. So although the centrosomes are not necessary for spindle formation they are essential to regulate orientation, time spend in mitosis and high fidelity chromosome segregation. These mitotic spindle processes are all required to maintain correct chromosome distribution in the organism

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Layman's summary

To develop into an organisms, cells need to divide to replicate. During this division, the genetic material consisting of the chromosomes, need to be equally distributed between the resulting daughter cells. To separate the chromosomes between the cells, two opposing spindles of microtubules are formed. Microtubules are hollow tubes that can serve as tracks for transport in the cell. The microtubules in these spindles attach to one set of chromosomes each. After attachment force is generated to pull one set of chromosomes to one spindle pole. Usually, the center of each spindle pole consist of a centrosome. This centrosome forms and organizes the microtubules of the spindle. Recently however, several studies have described cell division and microtubule organization in the absence of such a centrosome at the spindle pole center. This review discusses the formation of such a spindle in the absence of the centrosomes. Furthermore, we focus on the alternative processes that can organize the microtubules in such a spindle for chromosome segregation. In the absence of the centrosomes, the bipolar spindle still forms and the chromosomes are separated between the daughter cells. These spindles are formed from non-centrosomal microtubules that are not attached at the centrosomes. These microtubules have been described to be important in neuronal cells and other cell types.

To organize a spindle from these non-centrosomal microtubulesit is necessary to center them at the spindle pole, organize two spindle poles to form a bipolar spindle and form new microtubules. We discuss that the main forces driving these processes are motor protein related. Motor proteins can walk along one microtubule and transport cargo on them. When motors transport microtubules all to the same end of another microtubule, the microtubules will be organized into a spindle resembling one pole of the spindle during cell division. In addition to the centrosomes, the cell contains other mechanisms that can form new microtubules. At last, the attachment of the microtubules from two spindle poles will result in the bipolar spindle. These occurring processes make it possible to form a spindle in the absence of centrosomes. However, cell division will be slower and sometimes the chromosomes will be unequally separated between the daughter cells. We therefore propose that although the centrosomes are not necessary for spindle formation per se, they are necessary for safeguarding the process and increasing the efficiency. Increasing efficiency is a very important function. Low efficiency chromosome separation can lead to diseases like cancer and Down syndrome. Therefore it is likely that that is the reason for centrosome conservation in many organisms including humans. Furthermore an important function of the centrosome is the orientation of the division. Deletion of centrosomes results in loss of control of the division plane for chromosome segregation. A correct division plant is important for maintaining cells that are able to form new cells like brain cells. Loss of this ability to form new cells can lead to diseases like microcephaly in which insufficient brain cells are made. In conclusion in this review the importance of the centrosome during cell division is highlighted.