Analysis of potential interaction of spindle matrix proteins and the motor protein Ncd in Drosophila

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Analysis of potential interaction of spindle matrix proteins and the motor protein Ncd in Drosophila

by

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ABSTRACT

The microtubule-based mitotic spindle is dynamic rather than static, as polymerization and depolymerization of microtubules occur in sequence. Motor proteins including a minus end directed Ncd are associated with the microtubule-based spindle, and have long been proposed to generate force in microtubules by crossbridging and sliding microtubules relative to adjacent microtubules.

However, the force exerted by motor proteins is only part of the force exerted in the spindle. A stationary “spindle matrix” structure has been proposed to provide a strut on which motor proteins and microtubules interact during force generation. So far at least four molecular components of the spindle matrix, Skeletor, Chromator, Megator and EAST, were characterized to be in the same complex and all localize to the fusiform spindle matrix.

Based on the distribution of spindle matrix proteins and the motor protein Ncd in the spindle, a potential interaction between Ncd and spindle matrix proteins was proposed. In this thesis, RNAi was performed to generate Ncd depleted Drosophila S2 cells. After depletion of Ncd, S2 cells displayed a range of spindle abnormalities including multipolar spindles or loss of pole focus. In addition, spindle matrix protein structure was altered. Results obtained from larval neuroblast squashes were consistent with those observed in S2 cells. In the loss-of-function mutant ncd^D, the Megator-defined spindle was found to be widely extended. Moreover, immunoprecipitation experiments showed that Ncd and the spindle matrix proteins Chromator and
Megator are in the same complex. These findings suggest that the motor protein Ncd interacts with the spindle matrix. My observations also support the hypothesis that the spindle matrix provides a strut for interaction of motor proteins. However, this strut might also depend on a stabilizer such as Ncd that is required for spindle matrix assembly and maintenance.
CHAPTER 1 GENERAL INTRODUCTION

The process of mitosis has fascinated biologists since its discovery in the late 1870s. The term “mitosis” originates from the Greek word for describing the shape of mitotic chromosomes (Flemming, 1882), but now is defined as a process of distributing identical copies of replicated chromosomes to the daughter cells at each cell division. Mitosis begins at the stage when condensing chromosomes become visible in the nucleus. In the transition of prophase and prometaphase, nuclear envelope breakdown initiates and chromosomes start to attach to kinetochore microtubules (k-fibers) and position to the mitotic spindle. During metaphase, all the chromosomes align to the spindle equator. In the anaphase cells, sister chromatids separate and move toward the two opposite poles. Finally, segregated chromosomes form two new nuclei in the telophase followed by cytokinesis. During mitosis, replicated chromosomes segregate equally and precisely to two opposite poles depend upon the functional mitotic spindle machinery. The action of the mitotic spindle is essential for maintaining the normal cell cycle and cell division.

Thesis organization

This thesis is organized into five chapters. The first chapter begins with the outline of the thesis. This is followed by background knowledge about
mitosis. Current knowledge in the second chapter about the mechanisms of mitotic spindle assembly, microtubule flux, chromosome congression and segregation, involvement of motor proteins, and other molecular components in the process of mitosis are reviewed. Later on, the inconsistencies in the process of mitosis are discussed and the concept of “spindle matrix” is introduced.

The third chapter is organized in the paper format. To test the potential interaction between motor proteins and the spindle matrix proteins, RNAi treatment to S2 cells and brain squash in loss-of-function mutant ncdD were performed. Moreover, immunoprecipitation analysis showed that the motor protein Ncd and the spindle matrix proteins Chromator and Megator are in the same complex.

A general conclusion for the work presented in the dissertation is drawn in the fourth chapter. Chapter 5 is a list of references cited. In the end, I have acknowledged the people who have helped and supported me throughout my thesis work.
Mitotic spindle

The mitotic spindle that functions to segregate duplicated chromosomes into two separate nuclei is a complex macromolecule machine. This machinery is a transient apparatus, which changes its morphology during the stages of mitosis. Early cytologists (Flemming, 1882; Wilson, 1928; Schrader, 1953) described a fusiform-shaped mitotic spindle made of filaments parallel to the direction of chromosome movement using fixed images (Dustin, 1984; Bajer & Mole-Bajer, 1972; Inoue & Sato, 1967). Formation of a fusiform spindle begins during prophase and prometaphase when microtubules, motors, chromosomes and centrosomes self-organize into the biopolar metaphase spindle, where chromosomes are aligned at the spindle equator facing opposite poles. Then, during anaphase, duplicated chromosomes are moved to opposite poles (anaphase A) while the poles themselves separate (anaphase B), and spindle length become longer.

Mitotic spindle assembly in cultured animal cells is strongly influenced by the centrosome, which is a nucleating structure for the organization and nucleation of microtubules. When centrosomes are present they provide a dominant nucleation site that overrides the centrosome-independent pathway (Heald et al., 1997). However Drosophila oocytes and plant cells are noncentrosomal, and experiments where centrosomes are destroyed
specifically by laser microsurgery have confirmed that animal cells that normally contain centrosomes can also utilize a centrosome-independent pathway to form bipolar spindles (Khodjakov et al., 2000). In addition, when only one of the two centrosomes in a tissue-culture cell is destroyed by laser microsurgery, the cell still assembles a bipolar spindle with a centrosome at only one pole (Khodjakov et al., 2000). This result indicates that there is no absolute difference in potency of the two assembly pathways in cells. A significant conceptual contribution of these studies has been to clearly distinguish between the centrosome and the spindle pole. The spindle pole does not require centrosomes for either its establishment or its maintenance but is rather a distinct structure that self-organizes during spindle assembly. In most animal cells, centrosomes are the principal microtubule organizing and nucleating centers during interphase. Mitotic chromosomes are hypothesized to generate a microtubule nucleating and/or stabilizing activity to make up a spindle when centrosomes are absent in cells.

The centrosome consists of a pair of centrioles and pericentriolar material that contains the γ-tubulin ring structures that are highly conserved complexes composed of γ-tubulin and associated proteins, and in fission yeast αβ-tubulin complex components are essential for cytoplasmic MT organization (Weise and Zheng, 2006). Other proteins, such as pericentrin, NEDD1 and ninein contribute to centrosome function by recruiting and tethering γ-TuRCs to the centrosome and anchoring newly formed MTs (Luders et al., 2006;
Mogensen et al., 2000). The centrosome duplicates exactly once per cell cycle to generate two daughter centrosomes (Winey, 1999). Mitotic cells have two centrosomal arrays while interphase cells have a single centrosomal array. Those arrays generated by MT nucleation and anchoring by the centrosome (Andersen, 1999; Hyman & Karsenti, 1998) are radial and the fast growing plus end of the MT is distal to the centrosome and the slow-growing minus end is proximal to the centrosome. In mitosis of *Drosophila* cultured S2 cells, the two radial arrays contribute to the generation of bipolar spindle. MT arrays in differentiated animal and plant cells are usually linear and stabilized as compared to that most MTs in proliferating and migrating cells are radial and very dynamic (Bulinski & Gundersen, 1991). The features of overall radial MT arrays such as self-assembly of MTs arrays from tubulin subunits, dynamic instability of MTs (Cassimeris & Spittle, 2001), and capture of MTs by chromosomes and cortical sites (Gadde & Heald, 2004; Gundersen et al., 2004; Schuyler & Pellman, 2001) need the contribution of +TIP family (such as CLIP-170 and EB1). Those key players at the growing plus end of MTs (Akhmanova & Hoogenraad, 2005) can also mediate interactions with cortical or intracellular structures and regulate MT organization and stability (Gundersen, 2002). Microtubules, fascinating filaments, are prominent elements of the cytoskeleton that contribute to cell division, migration and polarity and required for a variety of cellular processes such as spindle assembly and vesicular transport. The microtubule lattice in a mitotic spindle
has two properties. Firstly, microtubule ends undergo dynamic instability from a polymerizing to a depolymerizing state (Desai & Mitchison, 1997) dependent on the hydrolysis of β-tubulin associated GTP. Secondly, the polar microtubule lattice serves as a track for the mechanochemical motor proteins of the dynein and kinesin superfamilies; these proteins convert energy from ATP hydrolysis into spatial displacement along microtubules (Goldstein & Philp, 1999; Hirokawa et al., 1998; Kim & Endow, 2000). Most studies focus on dynamics of two components of the mitotic machinery: microtubules and various motor proteins that are dominant in spindle assembly (Karsenti & Vernos, 2001; Scholey et al., 2003). I will discuss microtubule dynamics and motors in the following paragraphs.

Microtubules have a feature that is crucial for microtubule organization in the spindle called “dynamic instability” which proposed that the microtubule-based spindle is dynamic rather than static, polymerization and depolymerization of microtubule fibers alternatively occur, and addition and loss of tubulin dimers happens sequentially (Desai and Mitchison, 1997). Microtubules are 25 nm in diameter constituted of 12-15 protofilament polymers from noncovalent polar αβ-tubulin heterodimers and have very complex polymerization characteristics with a fast-growing plus and a slow-growing minus end. The plus and minus ends of microtubules have similar but not the same dynamic properties. It has been proposed that the minus end undergoes less depolymerization than the plus end and also seems
to be more intrinsically stable (Tran et al., 1997). Microtubule growth can be interrupted by a stochastic transition to depolymerization termed “catastrophe” (Chretien et al., 1995). When GTP hydrolysis rates exceed the plus-end growth rates, the association between microtubule filaments is lost, catastrophe occurs, and microtubules shrink. A molecular mechanism is generally accepted that the ends of growing microtubules have a GTP cap structure, and that the disappearance of this cap results in catastrophes. Addition of tubulin heterodimers to microtubules requires that the β-tubulin subunit has bound GTP, the so-called “GTP cap” (Chretien et al., 1999; Nogales et al., 1999) whereas the α-tubulin subunit bound GTP is not hydrolyzed. Growing microtubule ends are not blunt but contain two-dimensional sheets of straight protofilaments. In shrinking microtubules, protofilament sheets are bent and curl to prevent tube closure. The “dynamic instability” model proposed a good mechanistic description of microtubule polymerization, explaining the regulation of rapid changes in microtubule polymer mass during the cell cycle (Kirschner & Mitchison, 1986). During mitosis, microtubules become short and dynamic because of a great increase in the catastrophe frequency compared with that in interphase (Saxton et al., 1984). In the dynamics of MTs, MAPs are abundant factors that stably bind to and stabilize microtubules. The small GTPase Ran and its GDP–GTP exchange factor RCC1 play important roles in inducing microtubule polymerization around chromosomes (Heald & Weis, 2000; Sazer & Dasso,
MTs in the spindle are classified according to their position relevant to their functions, respectively. Kinetochoore MTs (k-fibers) connect to the kinetochore, a multiple protein structure dock to centromere in the chromosome. Interpolar MTs (ip-MTs) extend fibers from one pole to the spindle equator and may interact with MTs from the opposite pole to form an overlapping antiparallel array. Astral MTs grow from poles toward the cell cortex. Accurate chromosome segregation depends upon proper assembly and function of the MT arrays (Mitchison & Salmon, 2001). MT flux is generally proposed as necessary for chromosome alignment and precise separation. Major contribution to our understanding of spindle dynamics came from imaging analysis. As early as the 1960s, Forer (1965) observed that an area irradiated by ultraviolet microbeam would move to the nearest pole. He then pointed out that birefringence revealed poleward continuous movement of spindle fibers. Based on the discovery of MT treadmilling (Margolis & Wilson, 1978), Margolis proposed a theoretical model of spindle function that the parallel MTs in each half-spindle treadmill while their plus-ends (at kinetochores and in the spindle equator) assemble by tubulin subunit addition and their minus ends (at spindle poles) simultaneously disassemble by tubulin loss. Sliding apart of the anti-parallel ip-MTs would result in poleward translocation in the region of two half-spindle overlap at the spindle equator where MTs interact with each other. If k-fibers were linked to the translocating
ip-MTs by crossbridges, chromosomes attached to the k-fibers would experience a poleward force. During metaphase and anaphase, the coordinated poleward sliding of crosslinked, treadmilling MTs would create an isometric tension in the spindle. Then the cessation of k-fiber plus-end assembly would lead to the segregation of disjoined sister chromatids to their respective poles. Treadmilling describes specific steady-state polymer dynamics at both ends where the addition of subunits from one end is balanced by subunit loss at the opposite end at constant rate (Margolis & Wilson, 1981). However, MTs assembled from pure tubulin do not treadmill at steady-state as initially thought by Margolis and Wilson (Margolis and Wilson, 1978); instead their plus- and minus-ends exhibit dynamic instability (Grego et al., 2001). While analyzing the MT lattice motion of metaphase spindles, Mitchison gave a descriptive name “polewards MT flux” distinct from the treadmilling process (Mitchison, 1989). At first, “flux” was defined as the poleward movement of k-fibers and balanced rates of plus-end assembly and minus-end disassembly. Later they found at anaphase, MTs depolymerization not only occurred at minus ends but also at both ends when k-fibers continue to move polewards (Mitchison & Salmon, 1992). This led to a model in which rapid microtubule turnover during mitosis is driven by a phosphorylation-dependent change in the activity of an XMAP215-type MAP that reduces its ability to protect microtubule ends from the action of constitutively active catastrophe factors (Vasquez et al., 1994). In addition to
rapid turnover, the entire ensemble of spindle microtubules undergoes concerted poleward movement. Therefore, Maddox et al., (2003) proposed a definition which can be applied through the mitotic stage: flux is the poleward movement of MTs that is coupled to minus-end disassembly at the spindle pole. Photoactivation of caged fluorescent tubulins and photobleaching studies have observed poleward microtubule flux (Mitchison, 1989; Waterman-Storer et al., 1998) in the kinetochore microtubules of spindles from several different vertebrate cell types and *Xenopus*-extract spindle assembly (Waters et al., 1996). Therefore, k-fiber plus-ends attached to moving chromosomes must disassemble faster than the k-fiber flux, utilizing a “Pacman”-based mechanism that couples kinetochore motility to plus-end disassembly (McIntosh et al., 2002). It has been found that robust flux occurs in both k-fibers and ip-MTs assembled in vitro in *Xenopus* egg extracts (Sawin & Mitchison, 1991). The technique of fluorescent speckle microscopy (FSM) dramatically enhanced the resolution and ease of imaging flux (Waterman-Storer et al., 1998) by allowing observation of spindle dynamics in living cells by creating fluorescent “speckles” (Brust-Mascher & Scholey, 2002; Maddox et al., 2002). These experiments have also revealed that flux does not occur in astral MTs but is restricted to k-fibers and ip-MTs (Waterman-Storer et al., 1998).

This flux does not occur in the interphase MT arrays and is unique to spindles (Zhai et al., 1995). During metaphase, flux displays an additional
treadmilling-like activity, continuous depolymerization of minus ends near the spindle poles, and polymerization of plus ends in the central spindle, achieving a balance that maintains a constant spindle length. Importantly, ATP dependence of this process comes from the evidence that the nonhydrolyzable ATP analog AMP-PNP completely inhibits flux when added to preformed extract spindles. Studies found Eg5 in the *Xenopus* extract can prompt this flux and the non-hydrolyzable ATP analogue AMP-PNP, a broad-range inhibitor of kinesin-family motor proteins (Sankararaman et al., 2004), could inhibit flux. Large numbers of microtubules rapidly polymerize and depolymerize while being continually translocated towards the poles. Highly dynamic spindle-microtubule ends are thought to be necessary to ensure that the small surface of the kinetochore encounters a microtubule.

In addition, spindle MT flux rates change between mitotic stages in cultured mammalian tissue cells (Mitchison & Salmon, 1992; Zhai et al., 1995). During anaphase, MT flux continues but slows to a rate approximately half the metaphase rate. Comparisons of the rates of flux and chromosome movement during a particular mitotic stage indicate that the flux rate is only approximately a third of the rate of chromosome movement, irrespective of the mitotic stage (Skibbens et al., 1993). Furthermore, it has been proposed that k-fibers are made to slide poleward by a plus-end-directed force produced by kinesin-like motors anchored to a potential non-MT “spindle matrix” (Scholey et al., 2001).
**Motor proteins**

The motor proteins have been categorized into three classes: myosin, kinesin and dynein. Those proteins share several common features according to their name of motor (Endow, 1999; Schliwa et al., 2004). First, their motor domain uses the energy from ATP hydrolysis to undergo conformational changes to generate a “step” along the track. The crystal structures of myosin and kinesin have uncovered an unexpected structural similarity, indicating that they have a common evolutionary origin (Wesche et al., 2003). Furthermore, nonmotor domains are also important for motor function, including coiled-coil domains for dimerization and regulatory or cargo-binding domains. Also, motors can generate a force sufficient to move even large cargos through a networked cytoplasm.

Motor proteins are essential for spindle organization and mitotic movements by a “sliding filament” mechanism (Scholey et al., 2001; McIntosh & McDonald, 1989) that could explain the spindle MTs search for mitotic motors and could use ATP hydrolysis to drive the sliding to adjacent MTs. Dynein and kinesin are two families of MT based motors, that are ATP-dependent force-generating proteins (Vale & Fletterick, 1997; Holzbaur & Vallee, 1994). These mechanochemical ATPases can move microtubules unidirectionally toward their plus or minus ends. Over the half century since finding of the first kinesin, the kinesin superfamily has expanded to 14 classes (Lawrence et al., 2004), many of which contribute to mitosis. They localize to
different positions of the mitotic spindle, including midzone, kinetochore, cortex, and they belong to different families.

The motor "head" is the only structural element shared among all members of each motor superfamily. These head domains are linked to a wide variety of "tails," which bind to different types of cargo and enable the various family members to perform different functions in the cell. Force generation is proposed to involve a rotation of the entire head (Burgess et al., 2003). Specifically, dynein is an ATPase associated with various cellular activities (AAA+) protein. These AAA+ domains have an intact P-loop motif that signifies a nucleotide-binding site (Desai & Mitchison, 1997), and is the primary site of ATP hydrolysis. The microtubule-binding site is located at the end of the stalk that extends from the side opposite to the first AAA+ unit.

It has long been proposed that motor-driven MT sliding contributes to force generation in the spindle (Bonaccorsi et al., 1998). Great efforts were aimed at understanding the basic motor mechanisms in mitosis. It appears that mitotic motors can move on the surface of the MT lattice and transport specific mitotic cargoes. More importantly, motors generate force in MTs by crossbridging and sliding MTs relative to adjacent MTs or other filaments and regulating MT depolymerization in the plus end. In the hypothesis of “sliding filament” mechanism (McIntosh & McDonald, 1989), spindle movements are driven by motors that cross-link and slide adjacent MTs in relation to one another. Motors could crosslink and slide overlapping antiparallel MTs within
interpolar MT bundles and sliding astral MTs in relation to a stationary cell cortex to position spindle poles (Sharp et al., 2000). In addition, analyses of the functionally relevant mitotic motors have revealed those specific mitotic movements such as chromosome separation and MT sliding are driven by dynamic forces from multiple motors that function cooperatively or antagonistically. The strongest evidence that mitotic motors function by MT–MT sliding is from analyses of the fast-growing (plus)-end-directed bipolar (bimC) kinesins. Members of this kinesin subfamily form bipolar homotetramers, with two MT-motor domains positioned at opposite ends of a central rod (Hinchcliffe et al., 2001; Sawin & Mitchison, 1991; Heald et al., 1996), indicating that they simultaneously generate force along adjacent MTs. Moreover, in *Drosophila* functional inhibition of bipolar kinesins results in the formation of spindles with abnormally close poles (Khodjakov et al., 2000; Karki & Holzbaur, 1999; Heald et al., 1997). EM showed that the four Klp61F motors within this complex are tetramerized with two MT-motor domains positioned at both ends of a central rod (Heck et al., 1993). These data strongly suggest that these mitotic motors such as Cin8 and Kip1 slide antiparallel spindle MTs apart to generate “outward” forces between spindle poles and therefore maintain the bipolar spindle in metaphase and anaphase.

Another major family of kinesin motors with members that are known to play a role in mitosis is the C-terminal kinesins. These motors are so named because their ATP-dependent motor domain is positioned at the carboxy-terminal end
of the motor polypeptide, opposite to most other kinesins (McDonald et al., 1979). Moreover, C-terminal kinesins have also been shown to display the opposite transport properties to most other kinesins, moving toward the MT minus end (Karabay & Walker, 1999). As with the bipolar kinesins, evidence shows that at least some C-terminal kinesins function to pull the poles together during mitosis by driving antiparallel MT-MT sliding. These motors include members of the carboxy-terminal as well as dynein/dynactin. They contain nucleotide-insensitive MT-binding sites distal to their motor domains (Chandra et al., 1993; Kuriyama et al., 1995; Pidooux et al., 1996), allowing them to bind the MT as cargo and slide it in relation to adjacent MTs. This hypothesis is supported by the observation that members of these motors can crosslink MTs into bundles, \textit{in vitro} or in extracts (Brust-Mascher et al., 2002; Nogales, 2000). Moreover, there is evidence that the C-terminal and CHO1/MKLP1 kinesins position spindle poles and organize interpolar MT bundles, consistent with an antiparallel-MT bundling and sliding mechanism (Sharp, 2000\textsuperscript{c}; Adams et al., 1998; Raich et al., 1998). MT-sliding forces within the spindle appear not to be confined to MT–MT sliding. It is probable that mitotic motors exert force by crosslinking and sliding astral MTs in relation to a dense cortex of actin filaments and associated proteins that form just beneath the cell surface. Dynein/dynactin, for example, which positions mitotic spindle poles (Sharp et al., 2000\textsuperscript{b}), clearly localizes to the cell cortex during mitosis in many systems (Yeh et al., 1995; Busson, 1998; O’Connell & Wang, 2000) and thus is
positioned appropriately to capture and slide MTs extending away from the central spindle. In addition to the role of MT-sliding motors in positioning spindle poles, motor-driven MT bundling and sliding may also focus MTs at spindle poles (Merdes & Cleveland, 1997). For example, the *Drosophila* C-terminal kinesin, Ncd, can “zip” together MTs at their minus ends in the anastral (centrosome-free) spindles that form during female meiosis (Matthies et al., 1996).

Multiple mitotic motors have been identified as potentially being involved in chromosome movements by transporting specific chromosomal regions as cargo along spindle MTs via the kinetochore. Consistent with the hypothesis that kinetochores move by motor-driven transport along the surface lattice of MTs, both the minus-end-directed cytoplasmic dynein and the plus-end-directed kinesin can slide in the microtubules to generate force. Moreover, functional analyses indicate that both of these motors are important for properly positioning chromosomes on the spindle in a manner consistent with their transport properties. Inhibition of any single motor cannot completely abolish kinetochore movement suggesting the redundancy in the mechanisms driving its movement.

Besides transporting kinetochores along spindle MTs, mitotic motors probably couple MT assembly dynamics to kinetochore motility. Studies indicate that CENP-E could use its plus-end-directed transport properties in two ways; to transport kinetochores toward the MT plus-ends through
metaphase and subsequently to anchor kinetochores to the shortening plus-ends of MTs during anaphase (Wood et al., 1997). More recent data support the notion that motors can also regulate MT disassembly directly. Two related \textit{Xenopus laevis} motors containing motor domains in the interior of their polypeptides, XKCM1 and XKIF2, destabilize MTs at their ends \textit{in vitro} (Desai et al., 1999). Functional analyses indicate roles in both spindle assembly and chromosome movements. Kif18A is a dual-functional kinesin-family MT depolymerase and a key component of chromosome congression in mammalian cells (Mayr et al., 2007). By combining RNAi-depletion experiments with \textit{in vitro} biochemical assays, Mayr et al. (2007) demonstrated that the human kinesin Kif18A is a motile microtubule depolymerase essential for chromosome congression in mammalian tissue culture cells and it possesses microtubule depolymerizing activity that depolymerizes longer microtubules more quickly than shorter ones. The depletion of Kif18A induces aberrantly long mitotic spindles and loss of tension across sister kinetochores, and a complete failure of chromosomal alignment at the spindle equator.

Many motors contained in the mitotic spindle have similar properties. For example, Cin8p and Kip1p are two members of the bipolar kinesin family found in yeast. Although double knockouts of these motors result in defects in positioning of spindle poles, cells with single knockouts have no observable phenotype leading to the proposal that similar mitotic motors have functional redundancy (Hoyt et al., 1992; Roof et al., 1992). Thus, these similar motors
perform complementary but not entirely overlapping functions. Besides the cooperation, there is another relationship between different motors, that is, antagonism. For example, bipolar and C-terminal kinesins function antagonistically (Sharp et al., 1999; Pidoux et al., 1996; Saunders, 1992). In Drosophila syncytial embryos, the positioning of centrosomes within bipolar spindles is determined by a balance of opposite forces generated by a bipolar kinesin motor, KLP61F, directed to microtubule plus ends, and a carboxy-terminal kinesin motor, Ncd, directed towards microtubule minus ends. This activity maintains the spacing between separated centrosomes and spindle bipolarity during prometaphase and metaphase. KLP61F and Ncd may function in related to each other by crosslinking and sliding antiparallel spindle microtubules, allowing KLP61F to push centrosomes apart and Ncd to pull them together (Sharp et al., 1999; Sharp et al., 2000b). These data indicate that cells use multiple mitotic motors in parallel to generate a delicate balance of complementary and antagonistic forces.

**Spindle matrix**

As mentioned above, there are likely to be multiple mechanisms of chromosome movement in mitosis might multiple. When UV-microbeam disrupts the microtubules, chromosomes still move toward spindle poles (Sillers & Forer, 1983; Forer et al., 1997). Eg5 is static over the entire spindle assembled in frog egg extracts despite microtubules flux (Kapoor & Mitchison,
Yeast protein Fin1p was found to form filaments extending throughout the spindle (van Hemert et al., 2002). Furthermore, in anaphase microtubules depolymerize at both plus and minus end (Mitchison & Salmon, 1992; Rogers et al., 2005). This suggests that constant depolymerization might be expected to decrease the length of spindle leading to its eventual collapse. However, the mitotic spindle does not become short, hinting at the existence of a structure that keeps the bipolar spindle intact. Based on these observations and force production concerns (Elbaum et al., 1996; Fygenson et al., 1997; Freitas et al., 1999; Nicklas, 1983), the concept of a static non-microtubule structure has been proposed.

The “spindle matrix” structure is hypothesized to be a stationary structure that provides a backbone or strut for interaction of motor proteins during force generation and microtubule sliding in the mitotic spindle apparatus in contrast to the dynamic microtubules (Pickett-Heaps et al., 1997; Johansen & Johansen, 2002). Much evidence to supports the existence of such a “spindle matrix”. In the early 1980’s the “microtrabecular lattice” model (Pickett-Heaps et al., 1982) was proposed invoking an elastic spindle matrix structure based on studies in sea urchin and diatom spindles that showed non-microtubule spindle remnant-kinesin and kinesin-like proteins (Scholey et al., 1985, 2001; Pickett-Heaps, 1986; Leslie et al., 1987). Chromosome movement persists even after UV microbeam disrupts the kinetochore microtubules (Skibbens et al., 1995). These observations lead to the
hypothesis that a “microtrabecular lattice” acts as a recoiling spring like structure that extends from each of the poles to the kinetochores on the chromosomes at the equator in each half spindle. At metaphase, this spring like lattice was stretched and under tension due to concerted plateward movement of the MT motors attached to the lattice. Induction of anaphase leads to poleward recoiling of this elastic matrix that pulls the kinetochores linked to the chromatids along with it towards their respective poles (Pickett-Heaps et al., 1997; Forer et al., 1997; Scholey et al., 2001). More recently, indirect evidence for existence of a spindle matrix came from spindle force measurements (Freitas Jr., 1999). During anaphase, physical estimates suggest that the spindle apparatus develops forces in the nanoNewton range, which would cause microtubule buckling unless MTs are supported by a matrix like structure (Scholey et al., 2003; Johansen & Johansen, 2007).

Molecular components of spindle matrix in Drosophila so far include at least Skeletor, Chromator, Megator and EAST. Skeletor was cloned and characterized that proposed to be a part of a macromolecular complex forming such a spindle matrix. Skeletor is associated with the chromosomes at interphase, but redistributes into a true fusiform spindle structure that precedes microtubule spindle formation at prophase. During metaphase the spindle matrix defined by Skeletor and the microtubule spindle are coaligned. Walker et al. (2000) found that the Skeletor-defined spindle maintains its fusiform spindle structure from end to end across the metaphase plate during anaphase.
when the chromosomes segregate.

Yeast two-hybrid interaction assays using Skeletor as bait identified Chromator (Rath et al., 2004), a protein with a chromodomain. Immunocytochemistry demonstrated that Chromator shows extensive co-localization with Skeletor throughout the cell cycle. During interphase Chromator is localized on chromosomes to interband chromatin regions in a pattern that overlaps that of Skeletor. However, during mitosis both Chromator and Skeletor detach from the chromosomes and align together in a spindle like structure. Deletion construct analysis in S2 cells showed that the COOH-terminal half of Chromator without the chromodomain was sufficient for both nuclear as well as spindle localization. Analysis of P-element mutations in the Chromator locus shows that Chromator is an essential protein. Furthermore, RNAi depletion of Chromator in S2 cells leads to abnormal microtubule spindle morphology and to chromosome segregation defects.

Immunocytochemistry and cross-immunoprecipitation analysis were used to demonstrate that Megator (Qi et al., 2004), a Tpr ortholog in Drosophila with an extended coiled-coil domain, colocalizes with the putative spindle matrix proteins Skeletor and Chromator during mitosis. During interphase Megator is localized to the nuclear rim and occupies the intranuclear space surrounding the chromosomes. However, during mitosis Megator reorganizes and aligns together with Skeletor and Chromator into a fusiform spindle structure. The Megator metaphase spindle persists even if the
microtubule spindle is depolymerized, strongly implying that the existence of the Megator-defined spindle does not require microtubules. Based on the localization studies using truncated Megator constructs (Qi et al., 2004), it has been proposed that the COOH-terminal domain of Megator functions as a targeting and localization domain, whereas the NH2-terminal domain is responsible for forming polymers that may serve as a structural basis for the putative spindle matrix complex.

Immunocytochemical stainings have identified to demonstrate another spindle matrix component in *Drosophila*, EAST that forms an expandable nuclear endoskeleton at interphase (Wasser & Chia, 2000), redistributes during mitosis to colocalize with the spindle matrix proteins, Megator and Skeletor (Qi et al., 2005). Megator and EAST interact to form a nuclear endoskeleton and as well are important components of the putative spindle matrix complex during mitosis. They colocalize to the intranuclear space surrounding the chromosomes at interphase. EAST is a novel protein that does not have any previously characterized motifs or functional domains (Wasser & Chia, 2003). But immunoprecipitation experiments show that EAST is likely to molecularly interact with Megator which has a large NH2-terminal coiled-coil domain for self assembly (Qi et al., 2005).

We have proposed a potential interaction between motor proteins and spindle matrix proteins based on that some motor proteins such as Ncd coalign with the mitotic spindle in mitosis, and show a similar localization with
the spindle matrix. In this thesis, I performed RNAi to generate Ncd depleted S2 cell and analyze ncd mutant flies. Results observed in larval neuroblast cells are consistent with that in S2 cells. Moreover, immunoprecipitations were performed to test whether Ncd and spindle matrix proteins are in the same complex.
CHAPTER 3 ANALYSIS OF POTENTIAL INTERACTION BETWEEN SPINDLE MATRIX PROTEINS AND THE MOTOR PROTEIN NCD

ABSTRACT

The microtubule-based mitotic spindle is dynamic rather than static, as polymerization and depolymerization of microtubules occur in sequence. Motor proteins including a minus end directed Ncd are associated with the microtubule-based spindle, and have long been proposed to generate force in microtubules by crossbridging and sliding microtubules relative to adjacent microtubules.

However, the force exerted by motor proteins is only part of the force exerted in the spindle. A stationary “spindle matrix” structure has been proposed to provide a strut on which motor proteins and microtubules interact during force generation. So far at least four molecular components of the spindle matrix, Skeletor, Chromator, Megator and EAST, were characterized to be in the same complex and all localize to the fusiform spindle matrix.

Based on the distribution of spindle matrix proteins and the motor protein Ncd in the spindle, a potential interaction between Ncd and spindle matrix proteins was proposed. In this thesis, RNAi was performed to generate Ncd depleted Drosophila S2 cells. After depletion of Ncd, S2 cells displayed a range of spindle abnormalities including multipolar spindles or loss of pole
focus. In addition, spindle matrix protein structure was altered. Results obtained from larval neuroblast squashes were consistent with those observed in S2 cells. In the loss-of-function mutant ncd\(^\chi\), the Megator-defined spindle was found to be widely extended. Moreover, immunoprecipitation experiments showed that Ncd and the spindle matrix proteins Chromator and Megator are in the same complex. These findings suggest that the motor protein Ncd interacts with the spindle matrix. My observations also support the hypothesis that the spindle matrix provides a strut for interaction of motor proteins. However, this strut might also depend on a stabilizer such as Ncd that is required for spindle matrix assembly and maintenance.

INTRODUCTION

Ncd has been shown to concentrate on interpolar MT bundles during mitosis (Endow, 2003), positioning it appropriately to crosslink antiparallel MTs and generate forces that pull the poles together. Ncd null early embryos contain mitotic spindles that are abnormally spurred or branched and often become multipolar (Hatsumi & Endow, 1992).

The “spindle matrix” is hypothesized to be a stationary structure that provides a backbone or strut for interaction of motor proteins during force generation and microtubule sliding in the mitotic spindle apparatus (Pickett-Heaps et al., 1997; Johansen & Johansen, 2002). Molecular components of the spindle matrix identified so far include Skeletor, Chromator,
Megator and EAST. These four proteins colocalize with the mitotic spindle (Walker et al., 2000; Rath et al., 2004; Qi et al., 2004; Qi et al., 2005).

Motor proteins are proposed to interact with spindle matrix proteins based on force calculations that suggest stabilizing elements must exist in the spindle as well as that some motors such as Ncd have been found to coalign with the mitotic spindle. Moreover, published results (Goshima et al., 2005; Cytrynbaum et al., 2005; Morales-Mulia et al., 2005) showed that depletion of Ncd led to abnormal spindle phenotypes including multipolar spindles, loss of pole focusing and elongated spindles. To examine whether depletion of Ncd would affect the structure of spindle matrix, I performed RNAi to generate Ncd depleted S2 cells. Results showed that when Ncd was depleted in S2 cells or reduced by mutation in neuroblast cells, the spindle matrix defined by Skeletor, Chromator, Megator and EAST changed its localization as did the microtubule based spindle. Moreover, immunoprecipitation was performed to test whether Ncd was in the same complex as the spindle matrix proteins.

**METHODS**

**Drosophila Stocks**

Fly stocks were maintained according to standard protocols (Roberts, 1986). Canton-S was used for wild-type preparations. *Drosophila* carrying 2 or 4 copies of the *ncd-gfp* transgene (Endow & Komma, 1996) was used. The *gfp* refers to the gene encoding the S65T mutant GFP that shows ~6-fold
increased fluorescence compared to wild-type GFP. Loss-of-function mutant $ncd^D$ is from *Drosophila* stock center, Bloomington.

**Antibodies**

The anti-Skeletor mAb 1A1 has been previously described (Walker et al., 2000). Residues 552–668 of the predicted Skeletor protein were subcloned into pGEX-3 (Amersham Pharmacia Biotech) to generate the construct 3gexF. The mAb1A1 was generated by injection of 50 μg of 3gexF into BALB/c mice at 21 d intervals. Hybridomas were generated by the Iowa State University Cell and Hybridoma Facility. The mAb1A1 is of the IgM subtype.

The anti-Chromator mAb 6H11 and 12H9 have been previously described (Rath et al., 2004). Residues 601–926 and 1–260 of the predicted Chromator protein were subcloned into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) to generate the constructs GST-421 and GST-260. The GST-421 and GST-260 fusion proteins were expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich), according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech). Chromator mAb 12H9 by injection of 50 μg of GST-260 into BALB/c mice at 21 d intervals. The mAbs 6H11 is generated by injection of 50 μg of GST-421. Hybridomas were generated by the Iowa State University Cell and Hybridoma Facility. The mAb 6H11 is of the IgG1 subtype.

The anti-Megator mAb 12F10 has been previously described (Qi et al.,
Residues 1433–1703 of the predicted Megator protein were subcloned into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) to generate the construct GST-270. The correct orientation and reading frame of the insert was verified by sequencing. GST-270 fusion protein was expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich), according to the pGEX manufacturer’s instructions (Amersham Pharmacia Biotech). The mAbs 12F10 and 11E10 were generated by injection of 50 μg of GST-270 into BALB/c mice at 21-d intervals. Hybridomas were generated by the Iowa State University Cell and Hybridoma Facility. After the third boost, mouse spleen cells were fused with Sp2 myeloma cells, and monospecific hybridoma lines were established. The mAb 12F10 is of the IgG1 subtype.

The anti-Ncd polyclonal Ab was the generous gift of Dr. Sharyn Endow and has been previously described (Hatsumi & Endow, 1992). Antisera directed against the Ncd N terminus were raised by injecting rabbits.

Anti-α-tubulin (mouse mAbs of the IgG1 [Sigma-Aldrich] and IgM [Abcam] subtypes and a rat mAb [Abcam]) as well as anti-GFP (Rabbit polyclonal, Invitrogen) were obtained from commercial sources. The appropriate Texas Red-, and FITC-conjugated secondary antibodies (Cappel/ICN) were used (1:200 dilution) to visualize primary antibody labeling.

Biochemical Analysis
SDS–PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) with transfer buffer containing 20% methanol and in most cases including 0.04% SDS. For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3,000) for visualization of primary antibody diluted 1:1,000 in Blotto. The signal was visualized using chemiluminescent detection methods (ECL kit, Amersham Pharmacia Biotech). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680). In RNAi experiments Ncd levels were normalized using tubulin loading controls for each sample.

Immunoprecipitation assays

For co-immunoprecipitation experiments, anti-Chromator (mAb 12H9) or anti-Megator antibody (mAb 12F10) was bound to 10μl protein-G Sepharose beads (Sigma) for 2.5 hours at 4°C on a rotating wheel in 50μl ip buffer (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM Na3VO4, 0.2% Triton X-100, 0.2% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and 1.5 μg/ml Aprotinin). Protein extracts prepared from Canton-S embryos or brains from GFP-Ncd transgenic larvae were homogenized in immunoprecipitation (ip) buffer, sonicated three times, and the supernatant cleared by centrifugation at 16,000 g for 10 minutes at
4°C. The appropriate antibody-coupled beads or beads only were washed and incubated overnight at 4°C with 250μl of S2 cell lysate on a rotating wheel. Beads were washed four times for 10 minutes each with 1 ml of ip buffer with low speed pelleting of beads between washes. The resulting bead-bound immunocomplexes were analyzed by SDS-PAGE and western blotting using rabbit polyclonal Ab anti-Ncd (generous gift from Dr. Endow’s lab) to detect ncd and pAb GFP (Invitrogen) to detect GFP-Ncd. In addition, control ips were performed without adding protein lysate (Wang et al., 2001).

**Immunohistochemistry**

Antibody labelings of 0–3 h embryos were performed as previously described (Johansen et al., 1996; Johansen & Johansen, 2003). The embryos were dechorionated in a 50% Chlorox solution, washed with 0.7 M NaCl/0.2% Triton X-100 and fixed in a 1:1 heptane:fixative mixture for 20 min with vigorous shaking at room temperature. The fixative was either 4% paraformaldehyde in phosphate buffered saline (PBS) or Bouin’s fluid (0.66% picric acid, 9.5% formalin, 4.7% acetic acid). Vitelline membranes were then removed by shaking embryos in heptane-methanol (Mitchison & Sedat, 1983) at room temperature for 30 s. S2 cells were affixed onto conA coated coverslips and fixed with Bouin’s fluid for 10 min at 24°C and methanol for 5 min at -20°C. The cells on the coverslips were permeabilized with PBS containing 0.5% Triton X-100 and incubated with diluted primary antibody in
PBS containing 0.1% Triton X-100, 0.1% sodium azide, and 1% normal goat serum for 1.5 h. Double and triple labelings employing epifluorescence were performed using various combinations of antibodies against Chromator (mAb 6H11, IgG1), Skeletor (mAb 1A1, IgM), EAST (mAb 5B1, IgM) anti-\(\alpha\)-tubulin mouse IgG1 antibody (Sigma-Aldrich), anti-\(\alpha\)-tubulin mouse IgM (Sigma-Aldrich), GFP-antibody (rabbit polyclonal, Invitrogen), and Hoechst to visualize the DNA. The appropriate TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN) were used (1:200 dilution) to visualize primary antibody labeling. Confocal microscopy was performed with a Leica confocal TCS NT microscope system equipped with separate Argon-UV, Argon, and Krypton lasers and the appropriate filter sets for Hoechst, FITC, and TRITC imaging. A separate series of confocal images for each fluorophor of double labeled preparations were obtained simultaneously with z-intervals of typically 0.5 mm using a PL APO 100X/1.40–0.70 oil objective. A maximum projection image for each of the image stacks was obtained using the ImageJ software. In some cases individual slices or projection images from only two to three slices were obtained. Images were imported into Photoshop where they were pseudocolored, image processed, and merged. In some images non-linear adjustments were made for optimal visualization especially of Hoechst labelings of nuclei and chromosomes.
Brain Squash

Brain tissues were dissected from third instar larvae, fixed in Bouin’s fluid for 30 minutes and 45% acetic acid for 3 minutes, and then squashed in 60% acetic acid. Squashed tissue was washed in PBS+ 1% Triton X-100 3 times for 10 minutes each followed by incubation with Megator Ab (mAb 12F10) and phospho-histone H3S10 rabbit antiserum (Upstate Biotechnology). DNA was visualized by Hoescht. Fixed images were taken by a Zeiss fluorescent microscope.

RNA Interference

dsRNAi in S2 cells was performed according to Clemens et al. (2000). A 726 bp fragment (primer sequence showed in Table 1) in the middle region of the ncd gene was PCR amplified (Figure 1) and used as the template for in vitro transcription using the Megascript RNAi kit (Ambion). 40 μg synthesized dsRNA was added to 1X10^6 cells in 6-well cell culture plates. Control dsRNAi experiments were performed identically except pBluescript vector sequence (800 bp, described in [Rath et al., 2004]) was used as template. The dsRNA treated S2 cells were incubated for 6 days (cell was passaged once and 40 μg dsRNA addition on the first and third day) and then processed for immunostaining and immunoblotting. For immunoblotting 10^5 cells were harvested, resuspended in 50 μl of S2 cell lysis buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, and 1% Nonidet P-40), boiled and analyzed by
SDS–PAGE and Western blotting with anti-Ncd antibody and anti-α-tubulin antibody.

RESULTS

A 726 bp fragment (primer sequence showed in Table 1) in the middle region of the ncd gene was amplified by PCR (Fig. 1A) and further used to generate a fragment with the T7 promoter sequence (Fig. 1B) as the template for in vitro transcription (described in the instruction of the Megascript™ RNAi kit, Ambion). Forty microgram dsRNA (Fig. 1C) was added to cultured S2 cell. Control dsRNAi experiments were performed identically except pBluescript vector sequence (800 bp, described in [Rath et al., 2004]) was used as template. Immunoblots showed that Ncd was knocked down in RNAi cell lysate, and tubulin as loading control (Fig. 1D). Significant reduction of Ncd levels after a 6-day RNAi treatment was confirmed. To better understand the pole focusing process in Drosophila S2 cells, we performed indirect immunostaining to visualize fixed mitotic cells. The control cells shown in Figure 2 clearly indicate the k-fiber focusing at the spindle pole toward the centrosome. Generally, the bundling of k-fibers were focused at the minus end and attached to the centrosome in the mitotic spindle.

I next performed RNAi of Ncd, and then examined pole focusing of mitotic spindles by fixed cell immunofluorescence. Two phenomena involved in pole focusing were examined: the lateral spread of the kinetochore minus ends
(herein termed k-fiber distance; and the distance between the centrosome and the base of the k-fibers (centrosome to k-fiber distance).

Abnormal microtubule spindles including multipolar spindles and k-fiber-unfocused spindles were observed. Interestingly, Ncd depletion by RNAi did not alter the centrosome to k-fiber distance. In the cells with multi-polar spindles, it was striking that three to five poles in a mitotic spindle could be seen, yet chromosomes could still congress and align well in the metaphase plate. And during anaphase, chromosomes separated along the k-fibers and moved to the minus ends of the microtubules. The spindle matrix components Megator, Chromator, and Skeletor (as shown in Fig.3, Fig.5B and Fig.6C, respectively) appeared to align along with the microtubule bundles, but the spindle matrix lost its fusiform shape as did the microtubule based spindle. Although the overall shape of the spindle matrix was similar to that of the microtubule spindle, the spindle matrix component, Megator displayed strong immunochemical reactivity in the region of low density of microtubules (Fig. 3C). In the cells with unfocused k-fibers, one or two centrosomes were observed to be detached from the spindle pole(s), k-fiber distances became wide. Chromosomes were still observed to congress and align at the spindle equator. If only one centrosome was disassociated (Fig. 4A, B), the half-spindle still attached to the other centrosome, and chromosomes were found to be aligned at the metaphase plate. In the cells with two detached centrosomes (Fig.4C, D, Fig. 5C), the k-fibers were completely unfocused. The
mitotic spindle was found to extend extensively and the width of k-fiber distances was similar to the width at the spindle equator. Chromosomes still could congress and position at the metaphase plate, but presented to be split at spindle equator rather than align properly as a consecutive line. In the cells in which the metaphase spindle lost one or both focused poles, the spindle matrix defined by Skeletor, Megator, Chromator and EAST was localized along the microtubule based abnormal mitotic spindle (Fig. 3, 4, 5, 6, 7).

Furthermore, I performed squashes followed by immuno-histochemical experiments in neuroblast cells from larval brains of the ncd loss-of-function allele ncdD as compared to control Canton-S. The spindle matrix protein Megator localized to a fusiform structure in the control neuroblast metaphase cells (Fig. 8A). Chromosomes were observed to congress and align at the metaphase plate. However, for the mutant ncdD (Fig. 8B), which has a point mutation in the motor domain and lacks motor function, the staining pattern of Megator became widely expanded and lost its fusiform appearance. Chromosomes in the mutant cells displaying an expanded spindle matrix structure showed partial congression but failed to position to the spindle equator properly. These findings were consistent with the results obtained in the RNAi treated S2 cells.

Since depletion of minus-end directed Ncd leads to loss of the fusiform shape of the spindle matrix, I performed immunoprecipitation (ip) experiments in order to probe further for a potential interaction between Ncd and spindle
matrix proteins. For these immunoprecipitation experiments, proteins were extracted from Drosophila syncytial embryos, immunoprecipitated using either Chromator or Megator antibodies (mAb 12H9, mAb 12F10), fractionated on SDS-PAGE after the ip, immunoblotted, and probed with antibodies to Ncd (Rabbit pAb), respectively. A 79 kD band which is also present in the embryo lysate was detected by Ncd antibody in the immunoprecipitates (Fig. 9A, Fig.10A).

In order to confirm this potential interaction of the spindle matrix with Ncd, I did similar ip experiments using extracted protein lysate from brains of Ncd-GFP transgenic larvae, then immunoprecipitated by antibodies against Chromator or Megator. A band of 109 kD of the GFP-Ncd fusion protein can be detected by GFP Ab in the immunoprecipitates, as well as in the lysate input (Fig. 9B, Fig.10B). Also, uncoupled beads incubated with protein lysate were used as negative control (beads). The immunoprecipitation experiments suggest that motor protein Ncd physically interacts with the spindle matrix complex of Chromator and Megator.

DISCUSSION

It is perhaps not surprising that cells use multiple mechanisms for chromosome motility, including motor sliding along MTs. Members of kinesin families play integral roles in maintaining and elongating bipolar spindles. Some of these motors are thought to drive the initial separation of spindle
poles such as the minus-end directed motor protein Ncd (Sharp et al., 2000a). In normal cells, k-fibers can interact with and transport along centrosome MTs, resulting in a close connection to centrosomes and a tightly focusing at the minus ends of k-fibers (Goshima et al., 2005). In cells depleted of Ncd, mitotic spindles often involute in multipolar or unfocused k-fibers at the poles. This suggests that Ncd acts to prevent the centrosome premature in the early centrosome duplication or overseparation of spindle poles and drives the initial separation of spindle poles (Sharp et al., 2000a). While Ncd generates an inward force on the poles, it is possible that KLP61F crosslinks and slides antiparallel interpolar spindle microtubules to generate an outward force on the poles. This antagonism of KLP61F pushing centrosomes apart and Ncd pulling them together maintains the space between separated centrosomes and the length of spindle during prometaphase and metaphase (Sharp et al., 1999). In these experiments, spindle matrix proteins lost their fusiform shape in the S2 cells depleted Ncd as well as in Ncd mutant neuroblast cells, suggesting that when Ncd is depleted, not only do microtubule k-fibers lose their pole focus, but also the spindle matrix changes its morphology along with the microtubules. Immunoprecipitation results further support a functional relationship, as Ncd and spindle matrix proteins were found in the same complex. These observations reveal that at least one motor protein is not only binding to the MT in the mitotic spindle (Surrey et al., 2001), but also interacts with other elements within the mitosis apparatus, such as the spindle matrix. It is
interesting that the spindle matrix also lost its fusiform shape when Ncd is depleted. The possible explanation is that the spindle matrix functions as a scaffold with elastic properties involved in motor sliding and generating force. The spindle matrix might cross link to plus end directed motor proteins such as KLP61F that slide microtubules to generate outward forces to balance the inward forces generated by Ncd. I do not rule out the possibility of other functions of the spindle matrix besides as a scaffold structure for microtubule spindle in mitosis. In a review paper, a spring-like or elastic element based on the motor proteins has been proposed to generate force or amplify force (Endow, 2003). This element might potentially interact with a spindle matrix components.

The second possibility is that Ncd might be a factor that stabilizes the assembly of the spindle matrix. At least four components including Megator, Chromator, Skeletor and EAST have been already characterized as comprising a spindle matrix. However, the molecular mechanism of spindle matrix assembly and manner of MT interaction is unknown. Ncd may be an important stabilizer of the spindle matrix based on the distribution of Ncd in the mitotic spindle and the mislocalization of the spindle matrix component when Ncd is depleted.
Figure 1 dsRNA treatment in S2 cell

(A) the fragment of ncd with a length of 726 bp (primer sequence shown in table 1) is amplified from genomic DNA. (B) DNA fragment of ncd (described in A) associated with T7 promoter. (C) dsRNA of ncd and control (template from pBluescript as described in Rath et al., 2004) from in vitro transcription. (D) immunoblot shows Ncd knock down in RNAi treated S2 cell lysates, tubulin (anti-α-tubulin, IgG1, Sigma-Aldrich) was used as a loading control.

Table 1 Primer sequence to generate template for dsRNA of ncd

3' GGTTCATCTTTGGCCGTGT

5' GCACAGAAGTGGTGCATCTG
**Figure 2 Immunostaining of control RNA treated S2 cells**

S2 cells were fixed with Bouin’s fluid and incubated with anti-α-tubulin Ab (green, IgM, Sigma-Aldrich), anti-Megator (red, mAb 12H10) and Hoechst for visualizing DNA (blue). k-fibers clearly focus at the spindle pole toward the centrosomes. The Megator-defined spindle matrix appears fusiform.
Multiple poles in a microtubule (green, anti-α-tubulin, IgM) spindle could be seen (A, B, C), yet chromosomes (blue) could still congress and align well on the metaphase plate (B). During anaphase (A, C), chromosomes separate along the k-fibers and move toward the minus end of the microtubules. The spindle matrix component Megator (red) appeared to change its fusiform shape as did the microtubule spindle (A, B, C). In the region of low density of microtubules, Megator displays strong immunochemistry reactivity (C).
Figure 4 k-fibers’ loss of pole focus after depletion of Ncd

One or two centrosomes detach from the spindle pole, and the width of k-fibers (green, anti-α-tubulin, IgM) becomes large. Chromosomes (blue) still were observed to congress and align at the spindle equator. If only one centrosome is disassociated (A, B), the half-spindle still attaches to the centrosome, and chromosomes align at the metaphase plate. In cells where both centrosomes
were detached (C, D), k-fibers totally lost their pole focus. The mitotic spindle extended widely, the width of k-fiber distance at the poles is similar to that at the spindle equator. Chromosomes could congress and position at the metaphase plate, but were not positioned at spindle equator properly. The spindle matrix defined by Megator (red) colocalized with the abnormal microtubule spindle.

Figure 5 Chromator changes structure after depletion of Ncd

The Chromator (green)-defined spindle matrix appeared fusiform in a control.
cell (A). When Ncd is depleted, the MT (red, rat anti-α-tubulin) spindle becomes multipolar (B) or loses pole focus (C). The spindle matrix defined by Chromator localizes along the abnormal microtubule spindle.

Figure 6 Skeletor changes structure after depletion of Ncd

Skeletor (green)-defined spindle matrix appeared fusiform in control cell (A). When Ncd is depleted, the MT (red, rat anti-α-tubulin, IgG1)-based spindle becomes multipolar (C) or loses pole focus (B). The spindle matrix defined by
Skeletor localizes along the abnormal microtubule-based metaphase spindle (A, C). In an anaphase cell, Skeletor was observed to localize around the chromosomes (B).

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\text{Figure 7 EAST defined spindle matrix changes structure after depletion of Ncd}
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The EAST (green)-defined spindle matrix appeared fusiform in control cell (A). When Ncd is depleted, the MT (red, rat anti-\(\alpha\)-tubulin)-based spindle loses its pole focus (B). The spindle matrix defined by EAST (green) loses its fusiform structure and is localized along the abnormal microtubule spindle.
Figure 8 Neuroblast squash in the *ncd* loss-of-function mutant *ncd^D*

Squash followed by immuno-histochemistry experiments in neuroblast cells from larval brains from the animals with *ncd* loss-of-function allele *ncd^D* (B) immunostainings of control Canton-S (A) neuroblasts. The spindle matrix protein Megator (red) localizes to a fusiform structure in the neuroblast metaphase cells in control (A). Chromosomes were observed to congress and align at the metaphase plate. However, for the mutant *ncd^D* (B), which has a point mutation in its motor domain that eliminates the motor function, the staining pattern of Megator becomes widely expanded and the fusiform shape is lost. Chromosomes in mutant cells with expanded spindle matrix structure showed partial congression but fail to align at the spindle equator properly.
Figure 9 Immunoprecipitation to test the potential interaction of Chromator with Ncd

(A) Immunoprecipitates by Chromator antibody (mAb 12H9) were fractionated by SDS-PAGE, immunoblotted, and probed with antibodies to Ncd (Rabbit pAb). A 79 kD band was detected in the immunoprecipitates by Ncd antibody, and is also present in the embryo lysate. (B) Protein lysate was extracted from larval brains of GFP-Ncd transgenic larvae, incubated with Sepharose beads coupled to antibody against Chromator (mAb 12H9), fractionated on SDS-PAGE after the ip, immunoblotted, and the western blot probed with antibody against GFP. A band of 109 kD of GFP-Ncd fusion protein can be detected by the GFP Ab in the immunoprecipitates, and also is present in the lysate input.
**Figure 10 Immunoprecipitation to test the potential interaction of Megator with Ncd**

(A) Proteins were extracted from *Drosophila* syncytial embryos, immunoprecipitated using Sepharose beads coupled Megator antibody (mAb 12F10), fractionated by SDS-PAGE after the ip, immunoblotted, and the western blots probed with antibody against Ncd (Rabbit pAb). A 79 kD band was detected in the immunoprecipitates by Ncd antibody, and is also present in the embryo lysate input. (B) shows protein lysate that was extracted from brains of GFP-Ncd transgenic larvae, incubated with Sepharose beads coupled antibody to Megator (mAb 12F10), fractionated by SDS-PAGE after the ip, immunoblotted, and western blot probed with the GFP antibody. A band of 109 kD of GFP-Ncd fusion protein can be detected by GFP Ab in the immunoprecipitates, and also appeared in the lysate input.
CHAPTER 4 CONCLUSIONS AND PERSPECTIVE

In mitosis, the mitotic spindle is the important protein machinery that consists of complex macromolecules that primarily serve to ensure the segregation of duplicated chromosomes into two separate nuclei. The microtubule based mitotic spindle has a crucial feature called “dynamic instability”, that reflects that the spindle is dynamic rather than static, and polymerization and depolymerization of MT fibers occurs sequentially. Poleward microtubule flux (Mitchison, 1989; Waterman-Storer et al., 1998) has been shown to be essential for chromosome segregation.

Motor proteins are associated with the MT-based spindle. They can not only move on the surface of the MT lattice and transport specific mitotic cargoes, but they also generate force on the MTs by crossbridging and sliding adjacent MT and regulating MT depolymerization. In the hypothesis of the “sliding filament mechanism” (McIntosh, 1969), spindle movements are driven by motors that cross-link and slide adjacent MTs in relation to one another.

However, the force exerted by motor proteins is only part of the force that is exerted on spindle. Therefore a “spindle matrix” structure has been hypothesized to be a stationary structure that provides a backbone or strut for interaction of motor proteins during force generation and microtubule sliding in the mitotic spindle apparatus comparing to dynamic microtubules (Pickett-Heaps et al., 1997; Johansen & Johansen, 2002). So far at least four
molecular components of a spindle matrix, Skeletor, Chromator, Megator and EAST have been characterized. Walker et al. (2000) found that the Skeletor-defined spindle maintains its fusiform spindle structure from end to end across the metaphase plate during anaphase when the chromosomes segregate. Chromator is a novel protein with chromodomain that interacts directly with the putative spindle matrix protein Skeletor. Cross-immunoprecipitation analysis show that Megator (Qi et al., 2004) and EAST (Qi et al., 2005) together with Skeletor and Chromator exist in the same complex. They all localize to the spindle matrix as defined by Skeletor.

Ncd is a minus end directed motor that functions to focus the spindle poles. Based on the distribution of spindle matrix proteins and the motor protein Ncd in the spindle, a potential interaction was proposed between motor proteins and spindle matrix proteins. RNAi results observed in S2 cells as well as with ncd mutant neuroblast cells consistently showed that the spindle matrix altered its fusiform structure when cells lost functional Ncd. Spindle defects varied including multipolar spindles or loss of pole focus. Furthermore, spindle matrix proteins were mislocalized in the cell, and misaligned along the abnormal microtubule bundles. In the ncd loss-of-function mutant ncd\textsuperscript{D}, similar phenotypes of spindle matrix protein was observed. Megator-defined spindle was found to extend widely. Moreover, immunoprecipitation experiments demonstrated that Ncd and spindle matrix protein are in the same complex. Therefore, a spindle matrix, proposed to interact with motor proteins to
generate force or to amplify forces in the spindle, might be elastic and adjust its morphology when the motor protein Ncd is depleted. After Ncd is lost, MT bundles are splayed. The spindle matrix appeared to change its distribution concomitant with microtubules. However, a second possibility can’t be ruled out that Ncd acts as a stabilizer for spindle matrix assembly and maintenance. Without Ncd stabilizer, the spindle matrix hardly maintains its fusiform shape.
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