Characterization of three spindle matrix proteins, Megator, EAST and Asator, in Drosophila

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Characterization of three spindle matrix proteins, Megator, EAST and Asator, in
*Drosophila*

by

Hongying Qi

A dissertation submitted to the graduate faculty
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To my family
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ABSTRACT

Two previously identified proteins Skeletor and Chromator provide the molecular evidence for the existence of a spindle matrix complex in *Drosophila*. The spindle matrix has been proposed to be a macromolecular complex, which plays a role in organization and stabilization of the mitotic spindle as well as in providing structural support for counterbalancing force production.

In this dissertation, I present the identification and characterization of three more putative spindle matrix candidate proteins, Megator, EAST and Asator. Megator, an ortholog of the mammalian TPR protein, contains a large coiled-coil domain at its NH$_2$-terminus and an acidic, non-structural COOH-terminus. Immunohistochemistry studies show that Megator localizes to the nuclear rim and interchromatin region during interphase, while it reorganizes and coaligns with Skeletor and Chromator to form a fusiform spindle structure during metaphase. The Megator defined spindle structure persists when microtubules are depolymerized by cold or nocodazole treatment, indicating the Megator spindle is independent of the microtubule spindle. Expression of the NH$_2$-terminal truncated protein in S2 cells shows the coiled-coil domain can form a large spherical structure in cytoplasm, suggesting it is capable of self-assembly, thus suggesting that Megator may serve a structural role in spindle matrix.

EAST is a novel protein that does not contain any predicted functional motifs. It was previously shown to form an expandable nuclear endoskeleton at interphase. Our immunocytochemical studies demonstrate that EAST colocalizes with Megator at the intranuclear space surrounding the chromosomes at interphase and redistributes during mitosis to colocalize with spindle matrix proteins, Skeletor and Megator.
Immunoprecipitation experiments indicate that EAST and Megator are in the same protein complex. We propose that Megator and EAST interact to form a nuclear endoskeleton and as well are important components of the putative spindle matrix complex during mitosis.

The third protein, Asator, a conserved tau-tubulin kinase family member, was identified in yeast two-hybrid screen using a Megator NH2-terminal fragment and the physical interaction between Megator and Asator was confirmed by \textit{in vitro} pull-down assays. Immunocytochemistry demonstrates that V5/GFP tagged Asator protein colocalizes with tubulin throughout the cell cycle. Asator is distributed in the cytoplasm at interphase and relocated to the spindle structure during mitosis. Analysis of an Asator mutant line indicates Asator is an essential gene. These findings suggest that Asator plays a role in proper microtubule dynamics during the cell cycle.
CHAPTER 1: GENERAL INTRODUCTION

DISSERTATION ORGANIZATION

This dissertation is composed of five chapters. The first chapter is the general introduction of the current knowledge of the mitotic spindle. The mechanism of spindle assembly, motor proteins, mitotic kinases and other molecular components involved are discussed. The concept of spindle matrix is introduced and current progress in this field is reviewed. The spindle matrix components in Drosophila, Skeletor and Chromator are further discussed. Finally the advantage of the Drosophila model system is briefly introduced.

The second, third and fourth chapters are organized in the paper format. The second chapter is a paper published in Molecular Biology of the Cell in Nov. 2004 on the analysis of Megator, a coiled coil protein that localizes to the putative spindle matrix during mitosis in Drosophila. In this paper, I made several Megator truncated protein constructs expressed in Drosophila S2 cells. This study shows that the NH2-terminal coiled-coil domain of the Megator protein can self-assemble and the COOH-terminus is responsible for its spindle localization during metaphase, suggesting that Megator may serve a structural role in spindle matrix.

The third chapter is a paper published in the Journal of Cellular Biochemistry in Aug. 2005. Biochemical and molecular evidence suggests that EAST interacts with Megator and localizes to the putative spindle matrix during mitosis in Drosophila. The fourth chapter is a manuscript in preparation. This paper describes the identification and characterization of Asator, a novel conserved tau-tubulin kinase family member in Drosophila. Interaction with
the spindle matrix protein Megator and co-localization with tubulin during metaphase suggest that Asator might play a role in microtubule dynamics.

The fifth chapter is the general conclusions for the work presented in the dissertation. I then include an appendix on the analysis of Megator mutants generated by EMS mutagenesis, showing mitotic defects observed in third instar larval brain neuroblasts. Finally, references cited in chapter 1 and 5 are listed in the reference section.

BACKGROUND

Mitotic spindle

In all eukaryotic cells the formation of a metaphase spindle, a bipolar, microtubule-based structure with centrally aligned chromosomes, is a prerequisite for the faithful segregation of a cell's genetic material into the daughter cells during cell division (Compton, 2000; Wittmann et al., 2001). As far back as 1882 since the emergence of light microscopy, the German anatomist Walther Flemming already described the basic steps of spindle assembly and anaphase chromosome segregation through mitosis (Gadde and Heald, 2004). Though the importance of accurate chromosome segregation was evident to early cytologists, detailed analysis of the mitotic spindle was made possible only after the development of new techniques, such as polarization microscopy, electron microscopy, advances in tubulin biochemistry and microtubule-labeling techniques, (Mitchison and Salmon, 2001; Kline-Smith and Walczak, 2004). Over the last decades, molecular approaches empowered by complete genome sequences and genome-wide surveys continue to identify new proteins
involved in microtubule regulation. Advances in fluorescence imaging allow visualization of mitotic processes that previously have never been observed.

**Spindle structure and dynamics**

The mitotic spindle consists of microtubules and a variety of microtubule-associated proteins. The primary structural element of the spindle is an antiparallel array of microtubules (MTs). MTs are hollow, cylindrical polymers formed by the parallel association of 13 protofilaments, linear polymers of αβ-tubulin heterodimers that are bound head to tail. MTs are highly dynamic and can switch stochastically between growing and shrinking phases, both *in vitro* and *in vivo*. This phenomenon is known as dynamic instability, and can be described by four parameters: the rates of polymerization and depolymerization, the frequency of rescues (transition from shrinkage to growth) and catastrophes (transitions from growth to shrinkage) (Heald and Walczak, 1999). The GTP cap model was proposed to explain dynamic instability (Desai and Mitchison, 1997). Each tubulin monomer can bind to one molecule of GTP, but only the binding to β-tubulin is exchangeable. Once this GTP bound dimer is polymerized, this nucleotide is hydrolyzed and becomes nonexchangeable. The body of the MT made of GDP-tubulin subunits is unstable, only a layer of tubulin subunits at the ends still retain their GTP, which stabilizes the whole MT structure. When this cap is stochastically lost, the MT rapidly depolymerizes.

MTs *in vivo* differ from pure tubulin primarily in their rapid polymerization rates and their high transition frequencies (Desai and Mitchison, 1997), suggesting that MTs assembly and stability are modified in the cell by different stabilizing and destabilizing factors. These cellular factors are also regulated at different stages during the cell cycle since mitotic MTs
turn over much faster than interphase MTs (Kline-Smith and Walczak, 2004). MT dynamics are reduced by a variety of structural microtubule associated proteins (MAPs) such as tau and MAP2 that regulated by phosphorylation (Paudel, 1997). Microtubule severing enzymes, Katanin, Spastin and Fidgetin can stimulate MT depolymerization (Zhang et al., 2007).

While the metaphase spindle maintains a constant shape and size, spindle microtubules are continuously being polymerized, depolymerized and transported towards the two spindle poles, a phenomenon known as “microtubule flux”. Imaging studies, early photobleaching and photoactivation of fluorescently labeled tubulin subunits, especially recent fluorescent speckle microscopy (FSM) have allowed a detailed analysis (Mitchison and Salmon, 1992; Mitchison, 1989; Waterman-Storer and Danuser, 2002). In FSM, a small amount of fluorescently labeled tubulin is incorporated into the spindle to generate speckles along the length of MTs. The rates of MT flux can be measured by following the speckle movements. During metaphase, flux requires plus end polymerization, minus end depolymerization and poleward translocation of MT polymers. The detailed mechanism of flux is still elusive so far, but recent studies have indicated that Kinesin-5’s, which can drive the relative sliding of microtubules, and kinesin-13’s, which regulate microtubule polymerization, are directly involved in microtubule poleward flux (Benjamin and Kapoor, 2007). Another mechanism might be the force generated by MTs polymerization at the plus ends. RNAi of a MT plus-end tracking protein CLASP in S2 cells inhibits poleward translocation and blocks the recovery of laser microsurgery-cut MT bundles, suggesting that kinetochore microtubules may require CLASP to modulate MT polymerization at kinetochores (Maiato et al., 2005). Plus-end-directed motor proteins, such as CENP-E and
chromokinesins, may also contribute to the poleward translocation of MTs (Sharp et al., 2000).

In each half mitotic spindle MTs have uniform polarity. The less dynamic minus ends are located and focused at the spindle poles, and the more dynamic plus ends extend towards the cell cortex or chromosomes. Each spindle pole contains a centrosome, a specialized organelle responsible for MT nucleation. Chromosomes, another principal structural element of spindles, also play an active role in spindle structure and dynamics. The kinetochore, a specialized proteinaceous structure assembled on the outer layer of the centromere, attaches spindle MTs to the sister chromatids. Kinetochore MTs (kMTs) maintain attachment of chromosomes and allow them to align and segregate. Interpolar MTs (ipMTs) link two poles and stabilize the bipolar spindle during prometaphase and metaphase and push the poles apart during anaphase (Compton, 2000). Another set of morphologically distinct MTs is aster MTs. Plus ends of the aster MTs interact with cell cortex, which is important in positioning of spindle.

Mechanisms of spindle assembly

Spindle formation begins during prophase and pro-metaphase when MTs, motors, chromosomes and centrosomes interact and organize into a bipolar structure. Bipolar spindles form mainly via two pathways, the centrosome-directed or the chromosome-directed assembly pathway (Karsenti and Vernos, 2001; Scholey et al., 2003).

Centrosomes are the principal centers for organization and nucleation of MTs in animal cells. Based on dynamic instability, highly dynamic MTs nucleated from a centrosome undergo cycles of growth and shrinkage, randomly exploring the cytoplasm
space until they encounter the target kinetochores. Over time, MTs from duplicated centrosomes make connection with the bivalent kinetochore, and a bipolar spindle forms. This classic ‘search-and-capture’ hypothesis can explain several key features of mitotic spindle formation, including why mitotic MTs are more dynamic than during interphase and why multipolar spindles form in presence of supernumerary centrosomes (Wadsworth and Khodjakov, 2004). But recent mathematical models of kinetocore capture indicate that search-and-capture is not efficient enough to explain the typical observed duration of prometaphase without any bias toward the chromosomes (Wollman et al., 2005). Meanwhile, this modeling cannot explain mitosis in cells that lack centrosomes, for example plant cells and oocytes.

Growing evidence indicates that chromatin plays a key role in spindle assembly in acentrosomal cells (Karsenti and Vernos, 2001). In metaphase arrested Xenopus egg extracts, the addition of DNA-coated beads can induce the formation of bipolar spindles (Heald et al., 1996). Spindles can still be assembled after centrosomes are destroyed by laser microsurgery in animal cells (Khodjakov et al., 2000). Centrosomes either are lost or their functions are disrupted in Drosophila DSas-4 or centrosomin (cnn) mutants. However, most mitotic spindles assemble normally and mutant flies develop into morphologically normal adults with near normal timing (Megraw et al., 2001; Basto et al., 2006). In this pathway, MTs are randomly nucleated near chromosomes, then “sorted” into a bipolar array by MT sliding motors and crosslinked at their minus ends to form focused poles (Scholey et al., 2003; Gadde et al., 2004). Much work has revealed that the small GTPase Ran can promote MT nucleation and stabilization in the vicinity of chromatin (Kalab et al., 1999; Wilde and Zheng, 1999; Carazo-Salas et al., 2001). Consistently, the Ran guanine nucleotide exchange
factor RCC1 is required for MT polymerization in mitotic extracts. RCC1 activates Ran by facilitating the exchange of bound GDP to GTP and thus generates a spatial gradient of active Ran-GTP around mitotic chromosomes (Carazo-Salas et al., 1999; Caudron et al., 2005). Ran may stimulate MT nucleation directly or indirectly by creating a local concentration of MT stabilizing factors to promote the capture of astral MTs (Heald et al., 1996; Bastiaens et al., 2006; O’Connell and Khodjakov, 2007). Ran-GTP promotes the release of spindle assembly factors such as TPX2 (target protein for Xenopus kinesin-like protein 2), NuMA (nuclear-mitotic apparatus protein) and XCTK2 (Xenopus COOH-terminal kinesin 2) from the inhibitory binding of importin-α and -β (Gruss et al., 2001; Nachury et al., 2001).

Though the conditions and factors in these two pathways are different, they are not mutually exclusive. Recent studies have shown that cooperative interactions also function during spindle formation. Chromosome-driven MT formation is present in centrosome-containing cells. An anastral spindle forms near the chromosomes in treated Drosophila spermatocytes where the centrosomes are retained at the peripheral cell membrane and astral MTs cannot extend to the chromosomal region (Rebollo et al., 2004). Live cell imaging studies in Drosophila S2 cells revealed that both the search-and-capture and self-organization pathways act together to form a bipolar spindle (Maiato et al., 2004).

Recently, after depletion of gamma-tubulin or centrosomin via RNAi in S2 cells, Mahoney et al. (2006) revealed that bipolar spindles form through self-organization of MTs nucleated from chromosomes, acentrosomal poles and from within spindles, as well as through the incorporation of MTs from the preceding interphase network. So in addition to the above two well-described pathways of MT nucleation, the metaphase spindle possesses a
mechanism (or mechanisms) for propagating its own architecture by promoting microtubule assembly.

**Motor proteins**

One important component of the mitotic spindle is the MT-based motor protein. MT-based motor proteins are ATP-dependent force-generating enzymes, including two major families, kinesins and dyneins. These mechanochemical enzymes utilize the energy of ATP hydrolysis to translocate along or destabilize MTs. Mitotic motor proteins have been proposed to function in spindle assembly, chromosome movement and control of spindle length (Walczak and Mitchison, 1996; Kwon and Scholey, 2004). At least three mechanisms are applied to exert their functions during mitosis: cross-bridging and sliding MTs relative to adjacent MTs or other structures, mitotic cargo transportation along the MTs, and regulation of MT dynamics (Sharp et al., 2000). Studies in multiple systems, such as the yeast, *Drosophila* embryo and HeLa cell extracts have established that concept that a delicate balance of complementary and antagonistic forces generated by multiple mitotic motors in parallel are essential for spindle assembly and function (Sharp et al., 2000; Gadde and Heald, 2004).

Functional studies have shown a remarkable level of conservation among related motors. During the transition from interphase to prophase, several subfamilies of mitotic motors including the BimC/Eg5 family, the minus-end directed motor Ncd, and cytoplasmic dynein are involved in spindle pole separation and spindle bipolarity establishment (Goshima and Vale, 2003; Gadde and Heald, 2004; Kwon and Scholey, 2004). Centrosomes migrate around the nucleus to the opposite poles under the influence of balanced forces. Using the
sliding filament mechanism, cortical dynein/dynactin complex motor proteins slide astral MTs along cortical actin and generate outward-pulling forces that are antagonized by inward forces generated by the C-terminal minus-end-directed motor Ncd acting on ipMT bundles. The bipolar homotetramer of plus-end directed kinesin BimC cross-links and slides antiparallel microtubules apart to establish spindle bipolarity. Ncd appears to counteract the BimC kinesin and functions to focus microtubule minus-ends at the spindle poles (Sharp et al., 1999; Kwon and Scholey, 2004; Tao et al., 2006). In addition, Chromokinesin such as Xklp1 also organizes bundles of ipMTs and contributes to the formation of a bipolar array (Kwon et al., 2004).

Plus-end-directed and minus-end-directed motors bind chromosome arms as cargo and transport them along the adjacent MTs and position chromosomes on the spindle. Chromokinesin such as KLP38B in Drosophila, kid from Homo sapiens, or Xkid in Xenopus contain a chromatin-binding motif and may bind to the non-kinetochore chromosome regions and generate plateward or polar ejection forces directed toward the metaphase plate (Ruden et al., 1997; Funabiki and Murray, 2000; Antonio et al., 2000). The kinetochore is another major site to move chromosomes as it seem to be the source of strong poleward forces. Cytoplasmic dynein, the plus-end-directed kinesin CENP-E and MCAK, a member of the MT-destabilizing KinI subfamily localize on the kinetochore and function in chromosome segregation (Schaar et al., 1997; Sharp et al., 2000).

Recent study in Drosophila gives a more detailed description of Kin I kinesin function in chromosome segregation (Rogers et al., 2004). Two Kin I mitotic motors, KLP10A and KLP59C were identified in Drosophila embryos. These two proteins localize to the spindle poles and kinetochores respectively, allowing specific inhibition studies.
Anaphase A was found to utilize a Kin-I dependent pacman-flux mechanism in which both KLP10A and KLP59C depolymerize MTs at both ends of kMTs simultaneously. A “feeder and chipper” model proposed that dynein may also contribute to this mechanism by feeding MTs into the kinetochore for KLP59C dependent depolymerization (Sharp et al., 2000; Gadde and Heald, 2004). Thus, the combination of both plus- and minus-end depolymerization of MTs allows chromatid segregation to opposite poles in Anaphase A.

**Mitotic regulators: Mitotic kinases**

Spindle assembly is a very complicated process. In order to account for the dynamics of MTs in cells, much effort has been directed toward identifying proteins that regulate MT dynamics and understanding how these proteins are regulated. Many factors are regulated temporally and spatially by associated proteins, such as kinases and phosphatases. Genome-wide surveys of protein kinase or phosphatase by RNAi in *Drosophila* S2 cells identified 80 kinases and 22 phosphatases that are involved in cell cycle progression and/or mitosis (Bettencourt-Dias et al., 2004; Chen et al., 2007). Among all these proteins, the Aurora kinase, Polo kinase and NIMA-related kinases (Nrk) have emerged as important regulators of many mitotic events (O'Connell et al., 2003) and are being studied widely.

Aurora kinase, a conserved serine/threonine kinase family crucial in cell cycle control, derived its name originally from a *Drosophila* mutant displaying a monopolar spindle phenotype (Glover, et al., 1995). Since then, homologs were identified in a variety of species. Three genes, Aurora A, B, C exist in mammals while only a single gene was identified in yeast. Aurora A and B are essential for mitosis and may play a role in tumorigenesis (Fu et al., 2007). Aurora B is part of the chromosome passenger complex. I
will discuss its function in the next chapter. Aurora A is concentrated onto centrosomes and spindle microtubules during mitosis. It is mainly involved in centrosome function and spindle assembly. Centrosome Aurora A may recruit CNN, TACC/MAP215 and SPD-2 to promote centrosome maturation and MT nucleation (Ducat and Zheng, 2004). MT Aurora A is activated by TPX2 and targeted to the spindle, whereas Aurora A exerts its spindle assembly function by phosphorylating unknown substrates (Kufer et al., 2002; Tsai et al., 2003).

Polo kinase is another serine/threonine kinase family required for spindle assembly (Sumara et al., 2004; Glover, 2005). It is not only enriched at the centromere but is also found at kinetochores, the midspindle and throughout the nucleocytoplasmic space of mitotic cells (Barr et al., 2004). Mutation of Polo in Drosophila causes monopolar spindles, spindles with broad poles, or multipolar spindles, indicating its function in centrosome maturation and spindle assembly (Sunkel and Glover, 1988). It has been shown that Polo kinase may phosphorylate and activate the abnormal spindle protein (Asp) to recruit the γ-tubulin ring complex to the centrosome (de Carmo Avides et al., 2001). Polo is also involved in activating the anaphase-promoting complex or cyclosome (APC/C) (Eckerdt and Strebhardt, 2006) although the detailed mechanism is still unknown. In polo mutant spermatocytes, the central spindle fails to form in many cells and Pavarotti kinesin is not recruited to a ring-like structure at the equator, suggesting the functional role for Polo in cytokinesis is partially mediated by the Pavarotti kinesin-like motor protein (Carmena et al., 1998; Herrmann et al., 1998).

Compared to Aurora and Polo kinase, the NIMA-related kinases is less well characterized. Though only a single member was identified in yeast, the mammalian Nrk family constitutes of at least 11 structurally conserved proteins (O’Connell et al., 2003; Li
The founder kinase, never in mitosis, gene A (NIMA), was originally identified as a central mitotic regulator in the filamentous fungus *Aspergillus nidulans*. It plays an important role in chromatin condensation, nuclear entrance of Cdc2/cyclin B complex into the nucleus, and reorganization of the microtubule network during mitosis (O’Connell *et al.*, 2003; Yissachar *et al.*, 2007). A recent study of Nek7, a mammalian Nrk, shows that Nek7 is enriched at the centrosomes. Depletion of Nek7 by siRNA results in multipolar spindles, suggesting Nek7 may regulate proper spindle assembly and mitotic progression. Several members of the mitotic kinase families Aurora, Polo and Nek have been connected to tumorigenesis (Giet *et al.*, 2005; Takai *et al.*, 2005; Hayward and Fry, 2006), suggesting that further studies towards functional mechanisms may suggest therapies for cancer treatment.

*Other components*

In addition to MTs and motor proteins, mitotic spindles consist of a variety of accessory factors that may regulate MT dynamics and spindle function. As previously mentioned MAPs like tau or MAP2 bind to the surface of the MT, bridging several tubulin subunits and possibly neutralizing the negative charges on the MT surface to stabilize MTs. Other MAPs such as the XMAP215/Stu20/Msp/TOGp family contain a TOG domain, which can bind to the tubulin dimer and promote MT polymerization at plus end (Spittle *et al.*, 2000; Al-Bassam *et al.*, 2007). XMAP215/TOGp is localized to the spindle poles by interacting with Maskin/TACC (transforming acidic coiled-coil) protein (Lee *et al.*, 2001). The depletion of TOGp by siRNA results in centrosome fragmentation and multipolar spindle, suggesting TOGp functions in focusing MT minus ends at spindle poles, maintaining
centrosome integrity, and contributing to spindle bipolarity (Gergely et al., 2003; Cassimeris and Morabito, 2004). Plus-end-tracking proteins, such as CLIP-170, EB1 and CLASP (Orbit/MAST in Drosophila) localize to the growing plus ends of MTs. In Drosophila S2 cells, Orbit was shown to be required for microtubule subunit incorporation into fluxing k-fibers (Maiato et al., 2005). Further RNAi experiments found that Klp10A works to antagonize Orbit in the regulation of bipolar spindle formation and maintenance (Laycock et al., 2006).

Over the last decades, many more proteins were identified to be localized on to the spindle and to regulate the spindle apparatus. Among them, chromosome passenger proteins show mitotic specific distribution patterns and are important for chromosome alignment, histone modification, the spindle assembly checkpoint and cytokinesis (Vagnarelli and Earnshaw, 2004; Vader et al., 2006). These nuclear proteins associate with the condensing chromosomes in prophase, accumulate at the kinetochores in prometaphase and metaphase. During anaphase, they transfer to the central spindle and finally concentrate in the midbody at cytokinesis. To date, six chromosome passenger proteins have been described: INCENP, Aurora B, Survivin, Borealin/Dasra B, CSC-1 (chromosome segregation and cytokinesis defective-1) and TD-60 (telophase disc-60).

INCENP is essential in mouse, Drosophila and human (Cutts et al., 1999; Adams et al., 2001; Honda et al., 2003). Depletion of INCENP causes chromosome congestion defects and failure of cytokinesis (Ainsztein et al., 1998). Aurora B kinase is a conserved Serine- Threonine kinase phosphorylating a wide variety of substrates, including histone H3, CENP-A, INCENP, Myosin II regulatory light chain, MCAK (mitotic centromere associated kinesin) and Survivin (Murnion et al., 2001; Bishop and Schumacher, 2002; Wheatley et al.,
Aurora B phosphorylates INCENP or Borealin, and in turn, this phosphorylation enhances Aurora B kinase activity (Bishop and Schumacher, 2002; Honda et al., 2003). The activity of Aurora B is required to localize the CENP-E, dynein, MCAK and Dam-1 complex to the centromere and kinetochore regions (Adams et al., 2001; Gassman et al., 2004; Maiato et al., 2004). The interaction of Aurora B with mitotic centromere-associated kinesin (MCAK) could provide a mechanism linking Aurora B to proper chromosome biorientation and alignment. Aurora B may also be involved in regulating the spindle checkpoint. In budding yeast, Ipl1 defective cells often proceed through anaphase despite the presence of misaligned chromosomes (Biggins and Murray 2001). The loss of the spindle checkpoint may be due to a lower concentration of Mad2 and BubR1 at kinetochores in Aurora B-deficient cells (Hauf et al., 2003). Chromosome passenger proteins also play an important role in cytokinesis. Depletion or interference with the function of any member of the passenger complex produces a defect in cytokinesis with consequent multinucleation (Honda et al. 2003; Gassmann et al. 2004).

In addition, interphase nuclear components may be used to regulate mitosis. Nuclear pore complexes (NPCs) have long been considered to be responsible for regulating the molecular traffic between the nucleoplasm and the cytoplasm (Lei and Silver, 2002; Fahrenkrog and Aebi, 2003). NPCs may also control the spatial orientation and transcriptional activity of chromatin (Pai and Corces, 2002). In yeast, nuclear pore complex extensions formed by the conserved TPR homologues MLP1 and MLP2 are responsible for the structural and functional organization of perinuclear silent chromatin. Double deletion of MLP1 and MLP2 disrupts the clustering of perinuclear telomeres and releases telomeric gene repression (Galy et al., 2000). Recently, several NPC subunits nucleoporins (Nups) were
found to be recruited to the kinetochores during mitosis in vertebrates (Salina et al., 2003; Loiodice et al., 2004; Joseph et al., 2004). In the absence of Nup358, the kinetochore structure is aberrant and chromosome congression and segregation are severely perturbed (Salina et al., 2003). In yeast, two NPC proteins Mad1 and Mad2 function in the spindle assembly checkpoint (Iouk et al., 2002).

The spindle matrix

Although different models including microtubule dynamics and the sliding of MTs by MT-based motor proteins have been proposed for force generation and transmittal in driving chromosome congression and separation during mitosis (Scholey et al., 2001; Bloom, 2002), none of them is satisfying. Since mitotic microtubules are highly dynamic and alternate quickly between phases of polymerization and depolymerization, constant microtubule flux at both ends of spindle microtubules and treadmilling of tubulin dimers toward the poles occurs continuously. Based on such an unstable structure, it is difficult to conceive how forces to move chromosomes would be generated. To explain the phenomenon that spindle length is constant while microtubule keep translocating towards the spindle pole, a spindle matrix has been proposed to help in organization and stabilization of spindle microtubules and provide a stationary substrate for motors during force generation (Pickett-Heaps et al., 1997).

Numerous experimental results indicate the existence of the spindle matrix. Eg5 is a plus-end-directed motor protein and helps to establish the spindle bipolar organization (Kapoor et al., 2000). Kapoor and Mitchison (2001) studied the distribution and dynamic behavior of kinesin Eg5 in Xenopus spindles using the “fluorescence speckle microscopy”
technique. Surprisingly, they found that the majority of Eg5 in the spindle is static even though the microtubules are in constant poleward flux. One possibility for “static” Eg5 is that Eg5 is itself “walking” to the opposite direction of microtubule flux with the same speed. But after adding monastrol, an Eg5 motor activity inhibitor, most Eg5 still stays in place instead of moving poleward with the microtubule flux. Thus it may due to an interaction with a static spindle matrix.

Foreret et al., (1997) observed that chromosomes still move to the spindle poles during mitosis, despite that kinetochore microtubules have been severed in UV-microbeam experiments. Kinesin is observed to be associated with a nonmicrotubule component of the spindle (Leslie et al., 1987). Nuclear components including chromosome-associated elements may also have a functional role in spindle apparatus assembly and function. During prophase, rapid spindle assembly happens if nuclear envelope ruptures and chromosomes and other nuclear contents are prematurely exposed to the centrosomes and microtubules (Zhang and Nicklas, 1995a); while removal of nucleus at late prophase inhibits the microtubule assembly (Zhang and Nicklas, 1995b).

Another nuclear protein NuMA (Nuclear Mitotic Apparatus Protein) is a well-characterized spindle matrix component. NuMA is a 236kD protein restricted in the nucleus during interphase and relocated to the spindle pole region during mitosis. Secondary structure prediction indicates that NuMA has a very long α-helical domain (169kD) flanked by globular head and tail domains (Yang et al., 1992). NuMA is capable of self-assembly and can form lattice-like structures when overexpressed in HeLa cells and form a multiarm oligomer by interaction of the C-terminal globular domains when assembled in vitro (Saredi et al., 1996; Gueth-Hallonet et al., 1998; Harborth et al., 1999). NuMA has been shown to
be essential for the integrity of the spindle poles and proper completion of mitosis. It can interact with dynein/dynactin and keeps the minus ends of microtubules focused around the centrosomes (Compton, 1998).

The MT-associated protein, Asp (abnormal spindle) has been proposed to be the functional homolog for NuMA in Drosophila (Wakefield et al., 2001). Asp is highly basic, and mainly composed of α-helix. The Asp protein is enriched at the spindle poles and centrosomes where it cross-links MTs (Wakefield et al., 2001). Asp is also localized at the central spindle and is required for cytokinesis (Riparbelli et al., 2002). A protein complex of two MAPs, Msps and D-TACC may also serve a similar role as NuMA. This complex localizes to the spindle poles and is proposed to regulate MT organization (Cullen and Ohkura, 2001; Lee et al., 2001). However, these proteins only localize to the pericentrosomal region and do not form a complete spindle. So additional components may contribute to this spindle matrix in addition to NuMA.

Astrin is another mitotic MT-associated protein, which also can oligomerize to form an aster-like structure in vitro (Gruber et al., 2002). Astrin shows a cell cycle specific localization. It is concentrated at spindle poles in prophase, then localizes throughout the spindle during metaphase and anaphase. At telophase it transfers to the midzone region. Depletion of astrin by RNAi in HeLa cells resulted in multipolar and highly disordered spindles. Astrin contains an N-terminal globular domain and a large coiled-coil domain in its C-terminus. Both domains can target astrin to spindle poles, indicating astrin may bind to multiple spindle components through different domains (Gruber et al., 2001; Mack and Compton, 2001). The astrin oligomers may have the potential to provide a scaffold for cross-linking regulatory and structural components at the spindle.
Fin1p (filament in between nuclei), an interacting partner of 14-3-3 protein was identified in *S. cerevisiae*. Fin1p contains two putative coiled-coil domains in its C-terminus. GFP-tagged Fin1p was observed to form a filamentous structure extending between the two nuclei of dividing cells during mitosis. Yeast-two-hybrid or *in vivo* FRET studies show that the coiled-coil domain is sufficient for Fin1p-Fin1p interaction (van Hemert *et al*., 2003). Purified 6xHis-tagged Fin1p could self-assemble *in vitro* into a 10nm filamentous structure independent of MT and other proteins, suggesting Fin1p may be part of the “spindle matrix” in yeast (van Hemert *et al*., 2002; Johansen and Johansen, 2002). More recently, mutation of Fin1p into a non-phosphorylatable form showed limited chromosome segregation defects and no obvious spindle defects was detected (Woodbury and Morgan, 2007). It is possible that there are redundant pathways to build a spindle matrix in yeast.

Another coiled-coil protein in yeast, Ase1p (anaphase spindle elongation) is a member of the conserved Ase1p/PRC1/Map65 family, that functions in organizing the spindle midzone during mitosis (Schuyler *et al*., 2003; Loïodice *et al*., 2005). Ase1p behaves as a homodimer and binds and bundles MTs *in vitro*. Interestingly, FRAP experiments revealed that Ase1p is much more static within the spindle midzone compared to other MAPs. Based on these results, it was proposed that Ase1p may function as a spindle midzone matrix cross-bridging MTs (Schuyler *et al*., 2003).

In the nucleus chromosomes have been observed to be arranged in well defined, separate chromosome territories using different techniques, such as fluorescence *in situ* hybridization (FISH) or labeling/segregation (L/S) approach (Zink *et al*., 1998; Manders *et al*., 1999; Cremer and Cremer, 2001). Among the nuclear proteins, nuclear lamins might play a role in maintaining the interphase nuclear structure. In the past, lamins have been assumed
to only form a structural scaffold at the nuclear periphery, but recent studies reveal that lamins are also distributed in the nuclear interior. Nuclear lamins are involved directly or indirectly in many nuclear activities, including DNA replication and transcription, cell cycle regulation, nuclear and chromatin organization (Goldman et al., 2002; Spann et al., 2002; Herrmann and Foisner, 2003).

More interestingly, recent studies suggest that a fraction of Lamin B may associate with the mitotic spindle (Maison et al., 1997; Beaudouin et al., 2002). Reduction of Lamin in C. elegans resulted in mitotic defects and embryonic lethality (Liu et al., 2000). These results suggest that Lamin B may be involved in spindle assembly and regulation of mitosis. Tsai et al. (2006) found that a fraction of Lamin B associates with the mitotic spindle both in mammalian Hela cells and Xenopus egg extracts using fluorescence immunostaining. Depletion of lamin B3 (the main isoform of Lamin B) in Xenopus egg extracts caused a reduction of bipolar spindles and an increase in asters or half spindles, which could be partially rescued by purified lamin B3. Furthermore, Tsai et al. (2006) found Lamin B3 remained in a spindle-like structure in the absence of spindle MTs, and purified Lamin B3 did not associate with MTs directly or promote the MT assembly. In addition, two hypothetical spindle matrix proteins, XMAP215 and Eg5 also associated with the lamin B structure. However, this spindle structure was not affected by the depletion of XMAP215 and Eg5. So it was proposed that lamin B might serve as a structural component of the spindle matrix to support spindle assembly (Tsai et al., 2006).

Long, branched, polymeric macromolecule Poly(ADP-ribose) or PAR was discovered more than 40 years ago as a type of post-translational modification. It has long been considered to function in DNA repair, telomere replication, tissue-specific expression, and
cellular transport (Smith, 2001; Schreiber et al., 2006). PAR is synthesized by enzymes known as poly(ADP-ribose) polymerases or PARPs, whose activity is antagonized by the enzyme poly(ADP-ribose) glycohydrolase (PARG). Several PARPs localize to the spindle suggesting PAR may play a role in spindle function (Smith, 2001). Recently, Chang et al. (2004) discovered that both PAR and PARG localize throughout the spindle in frog egg extracts and mammalian tissue culture cells. Reduction of PAR by adding excess PARG in *Xenopus* egg extracts results in monopolar asters that are associated with chromosomes, suggesting PAR is not required for MT nucleation but is critical for establishing and maintaining spindle bipolarity which is consistent with the character proposed for the spindle matrix molecule. It was also found that PAR appears to be more stable since has a longer half-life than that of other MAPs (Chang et al., 2004). So far, it is still unknown how PAR may function in the spindle. One possibility is that PAR may form a matrix structure by mediating charge-charge interaction with non-modified proteins. Another possibility is that activities of MAPs or motor proteins in the spindle may be regulated by PARsylation. NuMA has been found to be a major acceptor of PAR by tankyrase 1 in mitosis (Chang et al., 2005). While NuMA is mainly localized to the pericentrosomal region, other targets need to be identified.

A number of recent studies have revived interest in the spindle matrix. Two novel proteins have been identified in *Drosophila* to be the putative spindle matrix components.

**Skeletor and Chromator, two spindle matrix proteins in *Drosophila***

Skeletor protein is an 81 kD protein that was identified from the study of a nuclear antigen with a dynamic localization pattern during mitosis in *Drosophila* embryos (Walker et
Antibody staining indicates that Skeletor associates with chromosomes at interphase. During early prophase, Skeletor disassociates from the chromosomes and redistributes into a spindle like structure apparently before nuclear envelope breakdown and microtubule spindle formation. The Skeletor spindle maintains its fusiform spindle structure from end to end across the metaphase plate until anaphase when chromosomes segregate. During telophase, Skeletor reassociates with chromosomes and forms a meshwork like structure again. The Skeletor spindle remains stable after nocodazole treatment indicating that the Skeletor spindle is independent of microtubule spindle structures. Furthermore, antibody perturbation experiments show Skeletor plays an essential role in cell cycle progression. Thus the all the properties the Skeletor-defined spindle exhibits suggest that Skeletor is an excellent candidate for a spindle matrix component.

However, Skeletor encodes a low-complexity protein with no obvious motifs, making it unlikely that Skeletor itself is a structural component. Thus it is likely that Skeletor comprises only a member of the spindle matrix complex.

Chromator is a Skeletor interacting partner identified by yeast two hybrid screening (Rath et al., 2004). The same protein was independently identified by Eggert et al. (2004), who named it Chriz (chromo domain protein interacting with Z4). Chromator is a single copy gene mapping to the 79F region on the 3rd chromosome close to the centromeric heterochromatin. The Chromator locus gives rise to at least three different alternative transcripts, encoding the same protein of 926 amino acids. The calculated molecular weight of Chromator is 101 kD, although it is recognized as a doublet of approximately 130 kD on western blots, which may represent posttranslational modifications (Rath et al., 2004; Gortchakov et al., 2005).
Chromator is ubiquitously expressed and essential for development. Chromator has a predicted chromodomain at its NH$_2$-terminus. Chromator shows a very dynamic cell cycle dependent localization. Chromator is associated with chromatin in interphase nuclei. On *Drosophila* third instar larval polytene chromosomes, Chromator localizes to the interband region, colocalizing with zinc fingers protein Z4 and JIL-1 kinase (Eggert *et al.*, 2004; Gortchakov *et al.*, 2005; Rath *et al.*, 2006). Transheterozygotes mutant polytene chromosome arms show a number of defects including coiling and misalignment of band and interband regions (Rath *et al.*, 2006). This suggests that Chromator has a functional role in maintaining chromatin structure.

Chromator shows a very similar dynamic staining pattern during mitosis as Skeletor. It colocalizes with Skeletor on the chromosomes at interphase forming a meshwork like structure. From prophase to metaphase, Chromator also colocalizes with the Skeletor spindle-like structure. The main difference is at anaphase and telophase when Skeletor is localized to the spindle remnants and chromosomes, Chromator seems to be present in the midbody and centrosomes. The co-localization of Chromator with the Skeletor defined spindle matrix suggests that Chromator may be involved in spindle matrix function, though it might also play some other roles from Skeletor in the cell cycle. Depletion of Chromator by RNAi in S2 cells results in abnormal spindles and mis-segregated chromosomes scattered throughout the spindle (Rath *et al.*, 2004). These types of defects would be expected if Chromator functions as a spindle matrix protein interacting with motor proteins to help the movement of chromosomes. Interestingly, depletion of some kinesin motor proteins such as KLP67A and KLP59C by RNAi in S2 cells resulted in similar mitotic chromosome segregation defects (Goshima and Vale, 2003; Rogers *et al.*, 2004). Thus, these data indicate that Chromator
plays a role in proper spindle dynamics and chromosome separation during mitosis and Chromator may constitute a functional component of a spindle matrix molecular complex.

Studies on Skeletor and Chromator support that both proteins are members of a macromolecular spindle matrix complex. However, neither Skeletor nor Chromator contains molecular motifs capable to self assemble into polymer and form a structural scaffold independently. In this study, I will report on the identification of three additional putative spindle matrix component in Drosophila, one of which potentially plays a structural role in the spindle matrix.

**Advantages of using Drosophila melanogaster as a model system**

The fruit fly known as *Drosophila melanogaster* is a valuable research tool for studying mitosis and spindle matrix components. Early embryos of *Drosophila melanogaster* provide a powerful system for studying spindle and chromosome dynamics in the mitotic cycle. This stage is characterized by a rapid succession of 13 synchronous divisions occurring in a syncytium. At late syncytial blastoderm stage from nuclear cycles 10-13, nuclei form a monolayer on the embryo surface, providing as many as 5,000 geometrically related examples of nuclear structures in a single embryo well suited for time-lapse imaging. Spindles from *Drosophila* larval neuroblasts, cultured S2 cells and oocytes, are different in their spindle machinery composition. They are ideal for different types of experiments to eventually identify common core mechanisms. The salivary glands in the larval stage provide a excellent tool to study the distribution of chromosomal proteins. Furthermore, its entire genome with 165 million bases and an estimated 14,000 genes has been completely sequenced and molecularly defined P-element insertions and deletions that span the entire
genome are available for study. Other powerful tools, including inhibitor microinjection, RNA interference (RNAi), and genome-wide surveys greatly facilitate the identification and characterization of unknown genes involved in mitosis (Miklos and Rubin, 1996; Celniker, 2000; Celniker and Rubin, 2003; Kwon and Scholey, 2004)
CHAPTER 2: MEGATOR, AN ESSENTIAL COILED-COIL PROTEIN THAT LOCALIZES TO THE PUTATIVE SPINDLE MATRIX DURING MITOSIS IN DROSOPHILA\textsuperscript{1}

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ABSTRACT

We have used immunocytochemistry and cross-immunoprecipitation analysis to demonstrate that Megator (Bx34 antigen), a Tpr ortholog in Drosophila with an extended coiled-coil domain, co-localizes with the putative spindle matrix proteins Skeletor and Chromator during mitosis. Analysis of P element mutations in the Megator locus showed that Megator is an essential protein. 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 in the absence of microtubule spindles, strongly implying that the existence of the Megator-defined spindle does not require

\textsuperscript{1}Reprinted with permission of Molecular biology of the Cell, 2004, 15: 4854-4865,

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polymerized microtubules. Deletion construct analysis in S2 cells indicates that the COOH-terminal part of Megator without the coiled-coil region was sufficient for both nuclear as well as spindle localization. In contrast, the NH$_2$-terminal coiled-coil region remains in the cytoplasm; however, we show that it is capable of assembling into spherical structures. Based on these findings we propose that the COOH-terminal domain of Megator functions as a targeting and localization domain whereas the NH$_2$-terminal domain is responsible for forming polymers that may serve as a structural basis for the putative spindle matrix complex.

**INTRODUCTION**

Although much work has been directed towards understanding mitotic spindle apparatus structure and function, it is still unclear how mechanical forces are applied to pull the chromosomes to the spindle poles (Pickett-Heaps *et al.*, 1982; 1997; Scholey *et al.*, 2001). The involvement of a spindle matrix that can act as a stationary substrate to stabilize the spindle during force production and microtubule sliding has long been proposed (Pickett-Heaps *et al.*, 1982; 1997); however, direct evidence for its existence has remained elusive (Scholey *et al.*, 2001; Wells, 2001; Bloom 2002; Kapoor and Compton, 2002; Johansen and Johansen, 2002). Recently, a putative spindle matrix protein, Skeletor, was identified in *Drosophila* (Walker *et al.*, 2000). Skeletor is associated with chromosomes at interphase, but preceding microtubule spindle formation and nuclear lamina breakdown, it redistributes into a true fusiform spindle at prophase. During metaphase the Skeletor defined spindle and the microtubule spindles are coaligned and when embryos are treated with nocodazole to disassemble microtubules, the Skeletor spindle persists (Walker *et al.*, 2000). Thus, many of the features of the Skeletor
defined spindle are consistent with the spindle matrix hypothesis. Using a yeast two-hybrid screen with Skeletor sequence as bait Rath et al. (2004) identified another potential component of a spindle matrix, Chromator, that interacts directly with Skeletor. Chromator contains a chromodomain and co-localizes with Skeletor on the chromosomes at interphase as well as to the Skeletor-defined spindle during metaphase. Furthermore, functional assays using P-element insertion mutants and RNAi in S2 cells suggest that Chromator is an essential protein that affects spindle function and chromosome segregation (Rath et al., 2004).

The above findings supports the hypothesis that Skeletor and Chromator are members of a macromolecular spindle matrix complex constituted by several nuclear components (Walker et al., 2000; Rath et al., 2004). However, for a spindle matrix to form independently or to form a structural scaffold aligned with the microtubule spindle one or more of its molecular components would be predicted to have the ability to form polymers. Neither Skeletor nor Chromator appear to contain molecular motifs with such properties. In this study we report the identification of another molecular component that localizes to the putative spindle matrix and is a candidate to play such a structural role. The monoclonal antibody Bx34 was previously shown to recognize a 260 kDa protein with a large NH₂-terminal coiled-coil domain and a shorter COOH-terminal acidic region that shows overall structural and sequence similarity to the mammalian nuclear pore complex Tpr protein (Zimowska et al., 1997). Zimowska et al. (1997) showed that the Bx34 antigen during interphase was localized to the nuclear rim as well as occupying the intranuclear space surrounding the chromosomes. Here we show using immunocytochemistry and analysis of P-element mutations that the Bx34 antigen is an essential protein that colocalizes with Skeletor and Chromator to the putative spindle matrix as it is defined by these proteins during mitosis. Furthermore, based on the
presence of the large coiled-coil domain we propose the Bx34 antigen may serve as a structural component of the spindle matrix and have named the protein Megator.

MATERIALS AND METHODS

Drosophila Stocks

Fly stocks were maintained according to standard protocols (Roberts, 1986). Oregon-R or Canton-S was used for wild-type preparations. The y¹ w⁶⁷c²³; P{w³mC= lacW}/l(2)k03905⁵k03905/CyO line was obtained from the Bloomington Stock Center and was originally part of the István Kiss collection (Trk et al., 1993). To facilitate identification of homozygous mutant Megator embryos, P{w³mC= lacW}/l(2)k03905⁵k03905 was balanced over one of two different GFP-tagged CyO balancers obtained from the Bloomington Stock Center line: w*, In(2LR)nocScorv9R, b1/CyO, P{w³mC=Act-GFP}JMR1 or CyO, P{w³mC = GAL.4-Kr.C}DC3, P{w³mC= UAS-GFP.S65T}DC7. Control antibody labelings were performed on embryos from these lines.

Antibodies

Residues 1433-1703 of the predicted Megator protein were subeloned using standard techniques (Sambrook et al., 1989) 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. After the third boost, mouse spleen cells were fused with Sp2 myeloma cells and monospecific hybridoma lines were established using standard procedures (Harlow and Lane, 1988). The mAb 12F10 is of the IgG1 subtype. All procedures for mAb production were performed by the Iowa State University Hybridoma Facility. The anti-Skeletor mAb 1A1 (Walker et al., 2000), anti-Chromator mAbs 6H11 and 12H9 (Rath et al., 2004), anti-Bx34 antigen mAb Bx34 and polyclonal antiserum (Zimowska et al., 1997), and anti-lamin mAb ADL195 (Klapper et al., 1997) have been previously described. mAb ADL195 was obtained from the Developmental Studies Hybridoma Bank at University of Iowa. Anti-a-tubulin (mouse mAbs of the IgG1 (Sigma-Aldrich) and IgM (Abcam) subtypes and a rat mAb (Abcam)) as well as anti-V5 antibody (Invitrogen) were obtained from commercial sources.

**Biochemical Analysis**

*SDS-PAGE and immunoblotting.* 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) (1:3000) for visualization of primary antibody diluted 1:1000 in Blotto. The signal was visualized using chemiluminescent detection methods (ECL kit, Amersham). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680). For quantification of immunolabeling, digital images of exposures of immunoblots on Biomax ML film (Kodak) were analyzed using the ImageJ software as previously described (Wang et al., 2001).
these images the grayscale was adjusted such that only a few pixels in the wild type lanes were saturated. The area of each band was traced using the outline tool and the average pixel value determined. Homozygous mutant Megator embryos selected from $P\{w^{+mC}=lacW\}l(2)k03905^{k03905}/CyO, P\{w^{+mC}=Act-GFP\}JMR1$ parents and identified by virtue of lack of GFP signal were obtained from 15-20 hour embryo collections. Heterozygous $l(2)k03905/CyO$ and $CyO/CyO$ embryos from the same embryo collection served as a reference for the reduction in Megator protein levels in homozygous embryos. Similar experiments were performed using $P\{w^{+mC}=lacW\}l(2)k03905^{k03905}/CyO, P_t^\{w^{+mC}=\text{GAL.4-Kr.C}\}/DC3, P\{w^{+mC}=UAS-GFP.S65T\}/DC7$ parents to minimize maternal GFP levels. Quantification of labeling on Western blots of $l(2)k03905$ mutant embryos were determined as a percentage relative to the level determined for control embryos using tubulin levels as a loading control. In RNAi experiments Megator levels in experimental and control S2 cell cultures were normalized using tubulin loading controls for each sample.

**Immunoprecipitation assays.** For co-immunoprecipitation experiments, anti-Megator or anti-Chromator antibodies were bound to protein G beads (Sigma) as follows: 10 µl of mAb 12F10 ascites or 100 ml of mAb 12H9 supernatant was bound to 30 µl protein-G Sepharose beads (Sigma) for 2.5 h at 4°C on a rotating wheel in 50 µl ip buffer. The appropriate antibody-coupled beads or beads only were incubated overnight at 4°C with 200 µl of 0-3 h embryonic lysate on a rotating wheel. Beads were washed 3 times for 10 min 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 according to standard techniques (Harlow and Lane, 1988) using mAb 6H11 to detect Chromator and mAb 12F10 to detect Megator.
**Immunohistochemistry**

Antibody labelings of 0-3 h embryos were performed as previously described (Johansen et al., 1996, Johansen and 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 and Sedat, 1983) at room temperature for 30 sec. S2 cells were affixed onto poly-L-lysine 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 Megator (mAb 12F10, IgG1), Chromator (mAb 6H11, IgG1), Skeletor (mAb 1A1, IgM), anti-a-tubulin mouse IgG1 or IgM antibody, anti-a-tubulin rat IgG2a, anti-lamin antibody (IgM), V5-antibody (IgG2a), and Hoechst to visualize the DNA. The appropriate species and isotype specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) 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, Texas Red, 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 μm 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. Polytene chromosome squash preparations from late third instar larvae were immunostained by the Skeletor antibody mAb 1A1 and Megator antibody mAb 12F10 essentially as previously described by Zink and Paro (1989), Jin et al. (1999), and by Wang et al. (2001).

Microtubule depolymerization experiments

Dechorionated embryos from 0-2.5 h collections were added to heptane containing 10 mM nocodazole (Sigma-Aldrich) and shaken for 1.5 min, before adding fixative and incubating for a further 20 min. Cold-treated embryos were dechorionated on ice for 2 min and incubated for 1 min with pre-chilled heptane. Pre-chilled Bouin's fluid was then added to the heptane layer, shaken for 30 s, and rotated at 4°C for 20 min. Immunolabeling was performed as described above.

Expression of Megator constructs in transfected S2 cells

A full length Megator (2346 aa) construct, a NH₂-terminal domain construct of Megator from residue 1-1431 containing 87% of the coiled-coil region, and a COOH-terminal domain construct of Megator from residue 1758-2346 were cloned into the
pMT/V5-HisB vector (Invitrogen) with in-frame V5 tags at the COOH-termini using standard methods (Sambrook et al., 1989). Similarly, a middle construct from residue 1432-1709 were subcloned into the pMT/V5-HisA vector with an in-frame V5-tag at the COOH-terminus. The fidelity of all constructs was verified by sequencing at the Iowa State University Sequencing facility.

*Drosophila* Schneider 2 (S2) cells were cultured in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal or newborn bovine serum, antibiotic/antimycotic solution and L-Glutamine (Gibco/BRL/Life Technologies) at 25°C. The S2 cells were transfected with different Megator subclones using a calcium phosphate transfection kit (Invitrogen) and expression was induced by 0.5 mM CuSO₄. Cells expressing Megator constructs were harvested 12-24 h after induction and affixed onto poly-L-lysine coated coverslips for immunostaining and Hoechst labeling.

**RNAi interference**

dsRNAi in S2 cells was performed according to Clemens et al. (2000). A 784 bp fragment encoding sequence from the coiled-coil region of Megator cDNA was PCR amplified and used as template for *in vitro* transcription using the Megascript™ RNAi kit (Ambion). 40 μg of synthesized dsRNA was added to 1 X 10⁶ cells in six-well cell culture plates. Control dsRNAi experiments were performed identically except pBluescript vector sequence (800 bp) was used as template. The dsRNA treated S2 cells were incubated for 120 h and then processed for immunostaining and immunoblotting. For immunoblotting 10⁵ 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-Megator antibody (mAb 12F10) and anti-α tubulin antibody. The mitotic index defined as the number of cells in metaphase and anaphase as a percentage of total cell number were compared between experimental and control S2 cell cultures. At least 500 cells were examined in each individual experiment (range: 500-2,500 cells).

**Analysis of P-element mutants**

*PCR mapping.* The insertion site flanking sequence provided by the Berkeley *Drosophila* Genome Project for the P{w+mC= lacW}l(2)k03905k03905 element (Accession # AQ025733) placed the P-element insertion near the transcription start site for the Megator gene. By designing several sets of nested forward and reverse primers from genomic sequence encompassing this region we performed PCR from mutant flies as previously described (Preston and Engels, 1996). PCR fragments were subcloned and sequenced according to standard protocols (Sambrook *et al.*, 1989).

*Viability assays.* In order to determine the viability of Megator mutants we analyzed the offspring from crosses of l(2)k03905/CyO, P{w+mC= Act-GFP}JMR1 parents in which the balancer chromosome is labeled with GFP allowing for the identification of homozygous l(2)k03905/l(2)k03905 embryos and larvae. For these assays eggs were collected on standard yeasted agar plates and incubated at 21°C. No homozygous l(2)k03905/l(2)k03905 larvae were found among 200 third instar larvae examined from such crosses and among 300 embryos only one homozygous l(2)k03905/l(2)k03905 first instar larvae emerged.

**P-element excision.** The P element of y1w67c23; P{w+mC= lacW}l(2)k03905k03905/CyO was mobilized by a Δ2-3 transposase source (y1 w+; CyO, H{w+mC= PΔ2-3}HoP2.1/BcEgfrE1) (Robertson *et al.*, 1988). Several fly lines in which the P
element had been excised were identified by their white eye color. Three precise excisions were confirmed by PCR analysis using primers corresponding to the P element and/or the genomic sequences flanking it. DNA isolation from single flies and PCR reactions were performed as described in Preston and Engels (1996). The precise excision lines were further analyzed for viability as described above and for restoration of Megator protein levels by immunoblotting. Protein extracts were prepared by homogenizing adult flies in IP buffer. Homozygous excised l(2)k03905 flies were identified by the absence of the Curly marker. Proteins were separated on SDS-PAGE and analyzed by western blotting with anti-Megator antibody (mAb 12F10) and anti-α tubulin antibody.

RESULTS

The putative spindle matrix protein Skeletor colocalizes with the Bx34 antigen (Megator) during mitosis

In a search for candidate proteins that potentially could interact with the putative spindle matrix we conducted labeling studies with the Bx34 mAb (Zimowska et al., 1997). The Bx34 antigen (Megator) previously was found to be localized to the nuclear rim and to the nuclear extra-chromosomal space during interphase; however, considerable Bx34 immunoreactivity was also reported to be present around the metaphase plate during mitosis, although the nature of this labeling was not resolved (Zimowska et al., 1997). For this reason we revisited the issue of mAb Bx34's labeling during the cell cycle in syncytial Drosophila embryos fixed with Bouin's fluid, a precipitative fixative characterized by its rapid penetration and efficient fixation of nuclear proteins (Johansen and Johansen, 2003). As illustrated in Fig. 1 the Bx34 mAb in addition to its characteristic interphase staining pattern also labeled what
appeared to be fusiform spindle structures at meta- and anaphase. We observed this
distribution of Megator both in Bouin's fluid and PFA fixed preparations as well as with a
polyclonal antiserum made toward a synthetic peptide based on Megator's amino acid sequence
(Zimowska et al., 1997). While the spindle-like labeling of the Bx34 mAb was intriguing and
suggested a potential colocalization with the putative spindle matrix proteins the antibody was
insufficiently robust for double labeling studies. We therefore generated new Megator mAbs,
12F10 and 11E10, against a GST fusion protein containing residues 1433-1703 of the Megator
protein. Both mAbs label a single protein band of approximately 260 kDa on immunoblots of
S2 cell protein extracts consistent with the predicted molecular mass of Megator of 262 kDa
(Fig. 2A) and recapitulate the reported interphase distribution of Megator at interphase. This is
shown for polystene nuclei in Fig. 2B where the Megator labeling surrounds the chromosomes
labeled with Hoechst and in confocal sections of embryonic syncytial nuclei in Fig. 2C where
the nuclear rim labeling coincides with that of lamin antibody. We subsequently used mAb
12F10 (IgG1) to perform double labeling studies with the Skeletor antibody 1A1 (IgM) on
fixed syncytial blastoderm embryos at different stages of mitosis (Fig. 3). Fig. 3A shows that
whereas Megator and Skeletor labeling are intermingled in the nuclear interior only Megator
staining is prominent at the nuclear rim. While embryonic interphase nuclei do not afford
sufficient resolution to determine whether Skeletor and Megator labeling are separate in the
nuclear interior this can be clearly demonstrated in light squashes of polystene salivary gland
nuclei where Skeletor is localized on the chromosomes which are surrounded by Megator
labeling (Fig. 3B). However, as mitosis commences Megator reorganizes during prophase into
a fusiform spindle structure the pattern of which at prometaphase and anaphase appears
identical to that of the putative spindle matrix protein Skeletor (Fig. 3A). At telophase Skeletor
begins to redistribute back to the decondensing chromosomes whereas at this stage the majority of Megator is localized to the spindle midbody (Fig. 3A).

In order to address the relationship between the Megator and microtubule spindles, we conducted triple labeling studies in embryos where microtubules were disassembled by either nocodazole- or cold-treatment as previously described (Walker et al., 2000). Figure 4A shows an image of a Megator spindle from a cold treated embryo arrested at metaphase (lower panel) compared to a control labeling (upper panel). In the control labeling the Megator-defined spindle and the microtubule spindle are co-aligned (Fig. 4A, upper panel). In contrast, after cold treatment there was no detectable tubulin antibody labeling indicating complete disassembly of the microtubules (Fig. 4A, lower panel). However, even in the absence of microtubule spindles, the Megator spindle remains intact implying that the existence of the Megator spindle does not require polymerized microtubules. Furthermore, under such depolymerized tubulin conditions both Megator and Skeletor spindle labeling are present and showing extensive co-localization (Fig. 4B). This suggests that both Megator and Skeletor may be contributing to the formation of a spindle-like structure the integrity of which is largely independent of microtubules.

The spindle localization of Megator is not restricted to the early embryonic cycles of nuclear division that lack the normal cell cycle checkpoints. We analyzed Megator distribution in the S2 cell line which is a cell line that was originally derived from later stage embryonic cells (~16 hour). In these cells, Megator shows a similar distribution pattern to that of syncytial blastoderm embryos (Fig. 5). At interphase Megator is present in the nuclear interior and co-localizes with lamin at the nuclear rim (Fig. 5A) whereas at metaphase Megator and Skeletor
are co-localized at a spindle-like structure distinct from the chromosomes congregated at the metaphase plate (Fig. 5B).

**Megator molecularly interacts with the putative spindle matrix complex**

To address whether Megator may interact with the putative Skeletor/Chromator spindle matrix complex we performed coimmunoprecipitation experiments designed to test for molecular interactions. For these experiments proteins were extracted from *Drosophila* embryos, immunoprecipitated with Megator or Chromator antibody, fractionated on SDS-PAGE after the immunoprecipitation, immunoblotted, and probed with antibody to Chromator and Megator, respectively. Figure 6A shows such an immunoprecipitation experiment where Chromator antibody co-immunoprecipitated a 260 kDa protein that is detected by Megator antibody on Western blots. Western blot analysis also confirms that this band co-migrates with Megator protein from total embryo lysate or from Megator antibody immunoprecipitation samples. In the converse experiment the immunoprecipitate of Megator antibody contained a 130 kDa band detected by Chromator antibody that was also present in the lysate and in the Chromator immunoprecipitate sample (Fig. 6B). This band was not present in lanes where immunobeads only were used for the immunoprecipitation (Fig. 6A and B). These results provide evidence that Chromator and Megator are present in the same protein complex.

**Megator is an essential gene**

Megator has been previously cloned and sequenced and encodes a large 2,346 amino acid protein of 262 kD in which the NH$_2$-terminal 70% is predicted to form an extended
coiled-coil region while the COOH-terminal 30% is unstructured and acidic (Zimowska et al., 1997) (Fig. 7A). It contains a putative nuclear localization signal (NLS) in the COOH-terminal part (Fig. 7A). By PCR mapping and sequencing we determined that the P-element present in the l(2)k03905 line (Spradling et al., 1999) is inserted at the start of the published cDNA of Megator (Zimowska et al., 1997) at position +1 (Fig. 7A). This insertion event also resulted in a 9 bp duplication including 8 bp of upstream genomic sequence and a duplicated +1 residue. The site and nature of the insertion suggests that a functional Megator transcript is not likely to be made from the mutant gene and thus this insertion may represent a null mutation. In order to determine the viability of Megator mutants we analyzed the offspring from crosses of l(2)k03905/CyO, P/w+mc=Act-GFP/JMR1 parents in which the balancer chromosome is labeled with GFP allowing for the identification of homozygous l(2)k03905/l(2)k03905 embryos and larvae. No homozygous l(2)k03905/l(2)k03905 larvae were found among 200 third instar larvae examined from such crosses and among 300 embryos only one homozygous l(2)k03905/l(2)k03905 first instar larvae emerged. This suggests that the Megator protein is essential and that the lethality caused by the P-element mutation largely occurs during embryonic development as maternal stores are exhausted. Consistent with this we find that Western blots (Fig. 7B) of homozygous 15-20 hour l(2)k03905 mutant Megator embryos show decreased Megator protein levels of only 28.5 ± 7.6% (n = 4) that of Megator levels in l(2)k03905/CyO and CyO/CyO embryos from the same embryo collection. We quantified this difference by determining the average pixel density of mAb Bx34 immunoblot staining of equal numbers of homozygous l(2)k03905 mutant Megator embryos and control embryos. The remaining low level of Megator protein observed in the homozygous mutant is likely due to residual maternal stores.
In a recent study, it was found that in a significant percentage of lethal mutant lines carrying characterized P insertions, the lethal mutation was not directly associated with the P insertion event itself (Bellotto et al., 2002). For this reason it was essential to confirm that the P insertion is the source of lethality for the l(2)k03905 allele. In order to address this concern, we screened for precise excision events by introducing the Δ2-3 transposase to mobilize the transposon and then selecting for loss of the mini-white marker that is carried by the P-element. Stocks established from such flies were then analyzed by PCR to characterize the nature of the excision event to identify those lines with precise excisions of the P element. Test crosses of such lines demonstrated that the precise excision of the P element restored Megator expression and viability to flies that were homozygous for the second chromosome that had previously carried the l(2)k03905 insertion (data not shown). That precise excision of the l(2)k03905 P element in three independent lines restores Megator expression and viability supports that the lethality observed in the l(2)k03905 mutant line was directly due to the insertion of the P element in the Megator region.

**Functional consequences of reduced Megator protein levels**

The cross immunoprecipitation experiments and the immunolabeling results are consistent with that Megator and Chromator are present in the same macromolecular complex during mitosis. This suggest that Megator has the potential to play a functional role in proper cell division. Unfortunately, this hypothesis cannot be tested in homozygous l(2)k03905 early embryos due to the presence of maternally derived Megator protein which masks any potential phenotypes. Furthermore, these animals die before hatching precluding larval neuroblast analysis. For these reasons, we employed RNAi methods in S2 cells to
deplete Megator protein levels (Fig. 8). While we did not observe any obvious perturbation phenotypes of tubulin spindle morphology or chromosome segregation defects by anti-tubulin and Hoechst labeling of the cells (data not shown) the number of cells undergoing mitosis was greatly reduced in Megator RNAi treated cultures (Fig. 8A). In five separate experiments we determined the mitotic index defined as the number of cells in meta- and anaphase as a percentage of total cell number. Experimental cultures had a mitotic index of 1.8±0.3% (n=5) versus an index of 4.3±0.5% (n=5) in mock treated control cultures representing a reduction of nearly 60% of cells undergoing cell division (Fig. 8A). This difference is statistically significant on the p<0.0025 level (Student's t-test). The degree of Megator knock down in the cultures was determined by immunoblot analysis (Fig. 8B) and averaged 86±7% (n=3) that of mock treated controls. These results suggest that depletion of Megator may prevent cells from entering metaphase.

**The COOH-terminal fragment of Megator is sufficient for nuclear and spindle localization**

Sequence analysis of Megator identified only one previously known domain, the extended NH2-terminal coiled-coil domain, in addition to a putative nuclear localization signal (NLS) in the COOH-terminal part. Coiled-coil domains are known to be protein-protein interaction domains that often are involved in self assembly of filamentous structures (Fuchs and Weber, 1994). We therefore tested whether the coiled-coil domain plays a role in the localization of Megator to the putative spindle matrix structure. We made four constructs containing Megator sequences for expression in S2 cells carrying a COOH-terminal V5-tag. The four constructs were a full length Megator construct (Meg-FL), an NH2-terminal
construct (Meg-NT) containing sequence from the starting methionine to residue 1431 that includes 87% of the coiled-coil domain, a COOH-terminal construct (Meg-CT) from residue 1758 to the terminal proline residue containing the putative NLS motif, and a smaller middle construct (Meg-M) from residue 1432 to 1709. Figure 9 shows examples of expression of these constructs in transiently or stably transfected S2 cells detected with V5-antibody and double-or triple-labeled with lamin or tubulin antibody and Hoechst. The Meg-FL construct localizes to the nucleus (Fig. 9A) although its overexpression often leads to aggregation. It is present at the nuclear rim in lamin double labelings at interphase (Fig. 9A, upper panel) and it is localized to the spindle at metaphase although the distribution is abnormal with aggregation around the spindle poles (Fig. 9A, lower panel, white arrows). The Meg-NT construct containing the coiled-coil domain is not targeted to the nucleus and remains in the cytoplasm typically forming small aggregates (Fig. 9B-1). However, in about 30% of transfected S2 cells (n=320) the Meg-NT construct forms several large spheres outside the nucleus. Three examples of this is shown in Fig. 9B. Figure 9B-4 shows a maximum projection image from a transfected S2 cell double labeled with Hoechst whereas Fig. 9B-5 shows a single confocal section from a different cell demonstrating that the spheres are hollow. Fig. 9B-6 is a stereo image illustrating the spatial relationship between the spheres. These data suggest that the coiled-coil domain while not targeted to the nucleus nevertheless has the ability to self assemble into hollow spherical structures. In contrast, the Meg-CT construct is localized to the nucleus including the nuclear rim at interphase and co-localizes with the tubulin spindle at metaphase (Fig. 9C). Thus the localization of the COOH-terminal Megator construct during the cell cycle phenocopies that of endogenous Megator observed with Megator antibody labeling. This indicates that the coiled-coil domain is not necessary
for targeting of Megator to the nucleus but rather that COOH-terminal sequences are sufficient for both nuclear and spindle localization. The Meg-M construct localizes to the cytoplasm, is not present in the nucleus, and does not appear to form aggregates (Fig. 9D) suggesting that the behavior of the three other constructs are independent of the V5-tag.

**DISCUSSION**

In this study we show that the Bx34 antigen in addition to the previously reported localization to the extrachromosomal space and nuclear rim at interphase (Zimowska et al., 1997) also interacts with the putative spindle matrix proteins, Skeletor and Chromator, during mitosis. The organization of the Bx34 antigen with a large NH$_2$-terminal coiled-coil domain and a shorter acidic COOH-terminal domain is similar to the structure of the mammalian Tpr (*translocated promoter region*) protein (Mitchell and Cooper, 1992) and like Tpr the Bx34 antigen is found at the nuclear rim, likely in association with nuclear pore complexes (Zimowska et al., 1997). However, comparison of Tpr and the Bx34 antigen sequences show a very low level of identity on the amino acid level (Zimowska et al., 1997) and whereas the Bx34 antigen is abundant in the nuclear interior, mammalian Tpr is restricted to the nuclear periphery (Frosst et al., 2002). Furthermore, mammalian Tpr has not been observed to localize to the spindle at metaphase. Thus, while structurally similar there is likely to be significant functional differences between the Bx34 antigen and mammalian Tpr wherefore we have named the Bx34 antigen in *Drosophila*, Megator.

The presence of a large coiled-coil domain in Megator raises the intriguing possibility that it could comprise the structural element of a potential spindle matrix. Since both Chromator and Skeletor localize to chromosomes as well as to the spindle-like structure,
it was not clear whether the physical interactions observed in co-ip and pull-down experiments between these molecules reflected interactions in chromosomal complexes or interactions on the spindle-like structure or both (Rath et al., 2004). However, since Megator is not localized to the chromosomes during interphase nor on centrosomes during metaphase through telophase, the molecular interaction of the complex observed likely occurs on the spindle-like structure. Interestingly, the Megator deletion construct analysis in S2 cells indicate that the NH$_2$-terminal coiled-coil containing domain has the ability to self assemble into spherical structures in the cytoplasm. This is in contrast to the acidic COOH-terminal domain which is targeted to the nucleus implying the presence of a functional nuclear localization signal. Furthermore, the COOH-terminal domain is sufficient for localization to the nuclear rim as well as for spindle localization. Thus, an attractive hypothesis is that the COOH-terminal domain functions as a targeting and localization domain whereas the NH$_2$-terminal domain may be responsible for forming polymers that may serve as a structural basis for the putative spindle matrix complex. Supporting this notion is the finding that Megator spindles persist in the absence of microtubules depolymerized by cold or nocodazole treatment. The localization of Megator to at least three cellular compartments (nuclear rim, extrachromosomal nuclear space, spindle matrix complex) and reorganization during the cell cycle suggest it is highly dynamic and that it may exist in several structural forms (Zimowska and Paddy, 2002). This is underscored by the finding that one hour after heat shock treatment the amount of Megator protein in the extrachromosomal space diminishes while accumulation occurs at a single chromosomal heat shock puff, 93D; however, as this occurs Megator localization to the nuclear rim remains unchanged (Zimowska and Paddy, 2002).
The co-localization of Megator with the Skeletor and Chromator-defined spindle matrix during mitosis suggests that Megator may be involved in spindle matrix function. A spindle matrix has been hypothesized to provide a stationary substrate that anchors molecules during force production and microtubule sliding (Pickett-Heaps et al., 1997). Such a matrix could also be envisioned to have the added properties of helping to organize and stabilize the microtubule spindle (Johansen and Johansen, 2002). Previously, we demonstrated using RNAi assays in S2 cells that depletion of Chromator protein leads to abnormal spindle morphology and that chromosomes are scattered in the spindle indicating defective spindle function in the absence of Chromator (Rath et al., 2004). However, we are not able to infer a clear functional role for Megator based on the results obtained in the present study. When Megator levels are knocked down by RNAi in S2 cell cultures the number of cells undergoing mitosis was greatly reduced. However, we did not observe any cells with obvious defects in tubulin spindle morphology or chromosome segregation defects suggesting that depletion of Megator prevents cells from entering metaphase. This could be due to an essential function of Megator in maintaining nuclear structure and/or in maintaining the integrity of the nuclear rim and pore complexes during interphase or a necessary function for nuclear reorganization during prophase. Thus, if Megator plays multiple functional roles as its dynamic localization pattern suggests (Zimowska and Paddy, 2002) it would prevent us from analyzing a mitotic function using RNAi approaches. That Megator is an essential protein necessary for viability is supported by the embryonic lethality observed as a consequence of P-insertions in the Megator gene.

Studies using preparations spanning the evolutionary spectrum from lower eukaryotes to vertebrates have provided new and intriguing evidence that a spindle matrix may be a
general feature of mitosis (Bloom, 2002; Johansen and Johansen, 2002). Here we show that at least three proteins, Megator, Chromator, and Skeletor, from two different cellular compartments reorganize to form a putative spindle matrix during mitosis in *Drosophila*. Furthermore, the Megator and Skeletor defined fusiform spindle structure remains intact even in the absence of polymerized microtubules. The identification of several potential spindle matrix molecules in *Drosophila* together with P-element mutations in their genes should provide an avenue for further genetic and biochemical experiments. Especially, the future isolation and characterization of point mutations in *Megator* promises to provide the means to separate Megator's role in spindle matrix function from its role at other stages of the cell cycle.

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**REFERENCES**


FIGURE LEGENDS

Figure. 1. Syncytial Drosophila embryo nuclei labeled by mAb Bx34 and Hoechst from various stages of the cell cycle (inter-, meta-, and anaphase). The labeling by mAb Bx34 is shown in green and the labeling of DNA by Hoechst in blue. The composite (comp) images of the stainings are to the left. At interphase the Bx34 antibody labels the nuclear rim together with interior nuclear labeling. At meta- and anaphase the Bx34 antibody labels a spindle-like structure. All images in these panels are from confocal sections.

Figure. 2. Immunoblot and interphase nuclear labeling of the Megator mAb 12F10. (A) Western blot analysis of Drosophila embryonic protein extract shows that mAb 12F10 recognizes Megator protein as a 260 kD band. The migration of molecular weight markers are indicated to the right in black numerals. (B) Larval polytene nucleus labeled with mAb 12F10 (green) and Hoechst (blue). The composite image (comp) clearly indicates that the Megator labeling by mAb 12F10 surrounds the chromosomal DNA labeled by Hoechst. (C) Triple labelings using mAb 12F10 to visualize Megator (green), anti-lamin antibody to visualize the nuclear lamina (red), and Hoechst to visualize the DNA (blue) of interphase syncytial embryonic nuclei. The composite image (comp) shows that Megator and lamin labeling overlaps at the nuclear rim (yellow color) whereas interior nuclear Megator is interspersed with the DNA labeling of Hoechst. The images are from confocal sections.

Figure. 3. The dynamic redistribution of Megator relative to the putative spindle matrix protein Skeletor during the cell cycle. The images are from double labelings of
Megator with mAb 12F10 (green) and Skeletor with mAb 1A1 (red). The composite images (comp) are shown to the left. (A) At interphase Skeletor and Megator labeling are intermingled in the nuclear interior whereas Megator labeling is prominent at the nuclear rim. During prometa- and anaphase the composite images (comp) show extensive overlap between Megator and Skeletor labeling as indicated by the predominantly yellow color. At telophase where Skeletor begins to redistribute back to the chromosomes Megator appears to be preferentially localized to the spindle midbody. The images are from confocal sections of syncyntial embryonic nuclei. (B) Light squash of a larval polytene nucleus where Skeletor localized on the chromosomes are surrounded by Megator labeling.

**Figure. 4. Nuclei from cold- or nocodazole-treated embryos at metaphase.** (A) Control (upper panel) and cold-treated (lower panel) embryos triple labeled with mAb 12F10 (green), rat α-tubulin antibody (red), and Hoechst (DNA in blue). In the cold-treated embryo microtubule spindles have completely depolymerized as indicated by the absence of microtubule labeling. The mAb 12F10 labeled spindle (green) is still intact demonstrating that this structure persists independently of the microtubule spindle. (B) Triple-labeling with mAb 12F10 (Megator in green), mAb 1A1 (Skeletor in red), and Hoechst (DNA in blue) from an embryo where microtubules were depolymerized with nocodazole. Both Megator and Skeletor labeling are still present and show extensive co-localization (yellow color in the composite [comp] image). All images are from confocal sections.

**Figure. 5. Nuclear localization of Megator in S2 cells.** (A) Interphase nucleus labeled with mAb 12F10 (Megator in green), lamin antibody (red), and Hoechst (DNA in
blue. The composite image (Megator/lamin) shows considerable overlap (yellow color) between Megator and lamin at the nuclear rim whereas only Megator is present in the nuclear interior. (B) Metaphase cell labeled with mAb 12F10 (Megator in green), mAb 1A1 (Skeletor in red), and Hoechst (DNA in blue). Megator and Skeletor labeling show extensive overlap (yellow color in the composite image [comp]) at the Skeletor defined spindle. All images are from confocal sections.

**Figure. 6. Megator and Chromator immunoprecipitation assays.** (A) Immunoprecipitation (ip) of lysates from *Drosophila* embryos were performed using Chromator antibody (mAb 12H9, lane 4) and Megator antibody (mAb 12F10, lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Megator mAb 12F10 for detection. Megator antibody staining of embryo lysate is shown in lane 1. Megator is detected in the Megator and Chromator immunoprecipitation samples as a 260 kD band (lane 3 and 4, respectively) but not in the control sample (lane 2). (B) Immunoprecipitation (ip) of lysates from *Drosophila* embryos were performed using Chromator antibody (mAb 12H9, lane 3) and Megator antibody (mAb 12F10, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator mAb 6H11 for detection. Chromator antibody staining of embryo lysate is shown in lane 1. Chromator is detected in the Megator and Chromator immunoprecipitation samples as a 260 kD band (lane 4 and 3, respectively) but not in the control sample (lane 2).
Figure. 7. P-element insertion in the Megator gene. (A) Diagram of the Megator genomic locus. The locus has five exons separated by four introns. The P-element insertion site of line l(2)k03905 at the +1 position of the Megator cDNA is indicated by the triangle. The ORF coding for the Megator protein including the position of the coiled-coil region and predicted nuclear localization signal (NLS) is depicted underneath. (B) Megator protein expression in homozygous l(2)k03905 mutant embryos from l(2)k03905/CyO parents. The level of Megator expression in l(2)k03905/CyO and CyO/CyO embryos from the same cross served as a control. The immunoblots were labeled with the anti-Megator Bx34 antibody and with anti-tubulin antibody. Protein extracts from thirty-five 15-20 hour embryos per lane were separated by SDS-PAGE. The relative level of Megator protein expression in mutant embryos as a percentage of Megator expression in control embryos is shown to the right.

Figure. 8. RNAi depletion of Megator in S2 cells leads to a reduction of cell undergoing mitosis. (A) Comparison of the mitotic index of Megator RNAi treated (n=5) and control (n=5) S2 cell cultures. The mitotic index was defined as the number of cells in metaphase and anaphase as a percentage of total cell number. Megator RNAi treated S2 cell cultures had nearly 60% fewer cells undergoing cell division than mock treated control cultures. This difference is statistically significant on the p<0.0025 level (Student's t-test). (B) Western blot with Megator antibody of control-treated and Megator RNAi-treated S2 cells. In the RNAi sample Megator protein level is reduced to about 8% of the level observed in the control cells. Tubulin levels are shown as a loading control.

Figure. 9. Expression of V5-tagged Megator deletion constructs in S2 cells. The
expressed constructs are diagrammed beneath the micrographs. (A) Full-length V5-tagged Megator (Meg-FL) localizes to the nuclear interior and nuclear rim (arrows) of S2 cells at interphase (upper panel). The cells were triple-labeled with V5-antibody to visualize the Meg-FL construct (green), lamin antibody (red), and Hoechst to visualize the DNA (blue). The lower panel shows S2 cells at metaphase labeled with V5-antibody (green), tubulin antibody (red), and Hoechst (DNA in blue). As shown in the composite image (comp) Meg-FL labeling overlaps that of tubulin (yellow color). However, the overexpressed Meg-FL construct also show some aggregation (white arrows). (B) V5-tagged NH₂-terminal Megator deletion construct (Meg-NT) truncated just before the end of the coiled-coil region localizes to the cytoplasm and is mainly absent from the nucleus (upper panel). The Meg-NT construct was visualized with V5-antibody (green) and the DNA with Hoechst (blue). In 30% of S2 cells the Meg-NT construct formed several large spheres outside the nucleus (lower panel). (B4) Maximum projection image from a Meg-NT (green) transfected S2 cell double labeled with Hoechst (blue). (B5) Single confocal section through a transfected S2 cell demonstrating that the spheres are hollow. (B6) Stereo image of a Meg-NT transfected cell illustrating the spatial relationship between the spheres. (C) S2 cells at inter- and metaphase expressing a V5-tagged COOH-terminal deletion construct (Meg-CT) lacking the coiled-coil domain. At interphase Meg-CT localizes to the nuclear interior and to the nuclear rim (white arrows). The nucleus was labeled with V5-antibody (green), lamin antibody (red), and the DNA with Hoechst (blue). At metaphase (lower panel) Meg-CT co-localizes with the microtubule spindle as indicated by the yellow color in the composite image (comp). The cell was labeled with V5-antibody (green), tubulin antibody (red), and the DNA with Hoechst (blue). (D) Interphase labeling in the cytoplasm of an S2 cell expressing the Meg-
M construct. The cell was labeled with V5-antibody (green), tubulin antibody (red), and the DNA with Hoechst (blue). All images are from confocal sections. On the diagrams the coiled-coil region is in black, the NLS is indicated by a black bar, and the V5-tag by a gray circle.
Figure 1

Figure 2
Figure 3
Figure 4
Figure 5

Figure 6
Figure 7

Figure 8
**Figure 9**

**A**  
Interphase:  
- comp  
- Meg-FL  
- lamin  
- DNA  

Metaphase:  
- comp  
- Meg-FL  
- tubulin  
- DNA  

**B**  
Interphase:  
- comp  
- Meg-NT  
- DNA  

Metaphase:  
- Meg-NT/DNA  
- Meg-NT/DNA  
- Meg-NT  

**C**  
Interphase:  
- comp  
- Meg-CT  
- lamin  
- DNA  

Metaphase:  
- comp  
- Meg-CT  
- tubulin  
- DNA  

**D**  
Interphase:  
- comp  
- Meg-M  
- tubulin  
- DNA
CHAPTER 3: EAST INTERACTS WITH MEGATOR AND LOCALIZES TO THE PUTATIVE SPINDLE MATRIX DURING MITOSIS IN DROSOPHILA

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ABSTRACT

We have used immunocytochemistry to demonstrate that the EAST protein in Drosophila, which forms an expandable nuclear endoskeleton at interphase, redistributes during mitosis to colocalize with the spindle matrix proteins, Megator and Skeletor. EAST and Megator also 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. However, we show by immunoprecipitation experiments that EAST is likely to molecularly interact with Megator which has a large NH₂-terminal coiled-coil domain with the capacity for self assembly. On the basis of these findings we propose that Megator and EAST interact to form a nuclear endoskeleton and as well are important components of the putative spindle matrix complex during mitosis.

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INTRODUCTION

During cell division the entire nucleus undergoes a dynamic and tightly orchestrated reorganization. Major structural components of the nucleus such as the nucleolus and the nuclear lamina are completely dismantled and are reassembled in the forming daughter nuclei only after chromosome segregation. Many of the proteins making up these structures are either degraded or are recycled through incorporation into vesicles (Moir et al., 2000; Olson et al., 2000). Other proteins known as "the chromosomal passengers" become associated with the condensing chromosomes during prophase, accumulate at the inner centromeres in prometaphase, then at the onset of anaphase leave the chromosomes and transfer to the central spindle before concentrating at the midbody at cytokinesis (Vagnarelli and Ernshaw, 2004). A third class of nuclear proteins reorganizes during pro- and/or prometaphase to form or associate with various parts of the mitotic spindle apparatus (Merdes et al., 1996; Dionne et al., 1999; Wittman et al., 2001; Raemaekers et al., 2003).

In Drosophila we have recently identified three nuclear proteins, Skeletor, Chromator, and Megator (Walker et al., 2000; Rath et al., 2004; Qi et al., 2004) that redistribute during prophase forming a fusiform spindle structure that persists in the absence of polymerized tubulin (Walker et al., 2000; Qi et al., 2004). The features of this structure are compatible with those of a spindle matrix which on theoretical grounds has been proposed to provide a stationary substrate that anchors molecules during force production and microtubule sliding (Pickett-Heaps et al., 1982; 1997; Johansen and Johansen, 2002). Two of these proteins, Skeletor and Chromator, are localized to chromosomes during interphase (Walker et al., 2000; Rath et al., 2004) whereas the third, Megator (Bx34 antigen), occupies the intranuclear space surrounding the chromosomes in addition to being localized
to the nuclear rim (Zimowska et al., 1997; Qi et al., 2004). Megator is a 260 kD protein with a large NH₂-terminal coiled-coil domain and a shorter COOH-terminal acidic region that shows overall structural and sequence similarity to the mammalian nuclear pore complex Tpr protein (Zimowska et al., 1997). Another large protein in Drosophila that associates with an interior nonchromosomal compartment of the interphase nucleus is EAST (Wasser and Chia, 2000; 2003). Loss-of-function east mutations lead to an increased frequency of mitotic errors as well as to abnormal congression of chromosomes in prometaphase (Wasser and Chia, 2003). Here we show using cross-immunoprecipitations and immunocytochemistry that EAST interacts with Megator and that EAST colocalizes with Skeletor and Megator to the putative spindle matrix as it is defined by these proteins during mitosis.

MATERIALS AND METHODS

Drosophila Stocks

Fly stocks were maintained according to standard protocols (Roberts, 1986). Oregon-R or Canton-S was used for wild-type preparations.

Antibodies

Residues 242-480 and 704-820 of the predicted EAST protein (Wasser and Chia, 2000) were subcloned using standard techniques (Sambrook et al., 1989) into the pGEX-4T-1 vector (Amersham Pharmacia Biotech) to generate the constructs GST-239 and GST-117. The correct orientation and reading frame of the inserts were verified by sequencing. The GST-239 and GST-117 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). The mAb 5B1 was generated by injection of 50 µg of GST-239 into BALB/c mice at 21 d intervals. After the third boost, mouse spleen cells were fused with Sp2 myeloma cells and monospecific hybridoma lines were established using standard procedures (Harlow and Lane, 1988). The mAb 5B1 is of the IgM subtype. All procedures for mAb production were performed by the Iowa State University Hybridoma Facility. Anti EAST IgY antibodies were purified from eggs of chickens that had been injected with the purified GST-117 fusion protein by Aves Labs. The anti-Skeletor mAb 1A1 (Walker et al., 2000), anti-Megator mAb 12F10 (Qi et al., 2004), and the anti-EAST polyclonal mouse antiserum ED3 (Wasser and Chia, 2000) have been previously described. An anti-a-tubulin rat mAb was obtained from a commercial source (Abcam).

Biochemical Analysis

**SDS-PAGE and immunoblotting.** 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) (1:3000) for visualization of primary antibody diluted 1:1000 in Blotto. The signal was visualized using chemiluminescent detection methods (ECL kit, Amersham).

**Immunoprecipitation assays.** For co-immunoprecipitation experiments, anti-Megator (mAb 12F10) or anti-EAST antibodies (mAb 5B1 or pAb ED3) were bound to 30 µl protein-G Sepharose beads (Sigma) for 2.5 h at 4°C on a rotating wheel in 50 µl ip buffer (20 mM
Tris-HCl pH 8.0, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 1 mM Phenylmethylsulfonyl fluoride, and 1.5 µg Aprotinin). The appropriate antibody-coupled beads or beads only were incubated overnight at 4°C with 200 µl of S2 cell lysate on a rotating wheel. Beads were washed 3 times for 10 min 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 according to standard techniques (Harlow and Lane, 1988) using mAb 12F10 to detect Megator and pAb ED3 to detect EAST.

**Immunohistochemistry**

Antibody labelings of 0-3 h embryos were performed as previously described (Johansen et al., 1996, Johansen and 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 and Sedat, 1983) at room temperature for 30 s. Double and triple labelings employing epifluorescence were performed using various combinations of antibodies against Megator (mAb 12F10, IgG1), EAST (mAb 5B1, IgM; pAb ED3; pAb IgY), Skeletor (mAb 1A1, IgM), anti-a-tubulin rat IgG2a, and Hoechst to visualize the DNA. The appropriate species and isotype specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) 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, Texas Red, 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 µm 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 (http://rsb.info.nih.gov/ij/). 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. Polytene chromosome squash preparations from late third instar larvae were immunostained by EAST antibodies and Megator antibody essentially as previously described by Zink and Paro (1989), Jin et al. (1999), and by Wang et al. (2001).

RESULTS

The interphase localization of EAST to the extrachromosomal nuclear domain (Wasser and Chia, 2000) is very similar to that of the putative spindle matrix protein Megator (Qi et al., 2004). Furthermore, considerable EAST immunoreactivity has been reported to be present around the metaphase plate during mitosis although the exact nature of this labeling was not resolved (Wasser and Chia, 2000; 2003). For these reasons we revisited the issue of EAST antibody labeling during the cell cycle in syncytial Drosophila embryos fixed with Bouin's fluid, a precipitative fixative characterized by its rapid penetration and efficient fixation of nuclear proteins (Johansen and Johansen, 2003). Figure 1 shows double labelings
with EAST antibody and the mAb 1A1 to the spindle matrix protein, Skeletor (Walker et al., 2000). The EAST antibody is the mouse antiserum (pAb ED3) used in the original EAST localization study by Wasser and Chia (2000). The double labelings were made possible by the fact that mAb 1A1 is of the IgM subtype that can be selectively recognized with TRITC-conjugated IgM specific secondary antibody and that EAST pAb ED3 can be detected with FITC-conjugated IgG (IgG1, IgG2A, IgG2B) specific secondary antibody that does not cross react with IgM antibody. Furthermore, control experiments in which pAb ED3 single labelings were incubated with the TRITC-conjugated IgM secondary antibody showed that the EAST antiserum contained no detectable levels of EAST antibody of the IgM subtype at the antibody dilutions used for staining. Therefore, there was no cross reactivity in the detection of the two antigens in the employed labeling protocol. Figure 1 shows that as mitosis commences EAST reorganizes during prophase into a fusiform spindle structure the pattern of which during metaphase appears identical to that of the putative spindle matrix protein Skeletor. At telophase both EAST and Skeletor redistribute back to the forming daughter nuclei. This localization of EAST during mitosis was also obtained in single labeling studies with pAb ED3 (data not shown).

In order to further confirm these findings and to generate probes for double labeling experiments with other potential spindle matrix proteins we made new EAST antibodies against GST fusion proteins containing residues 242-480 and 704-820 of the EAST protein. One antibody was a chicken antiserum (pAb IgY) that labels a single protein band of ~265 kD on immunoblots of S2 cell extracts consistent with the predicted molecular mass of EAST of 253 kD (Fig. 2A). This band is also labeled by the original EAST antibody pAb ED3 (Fig. 2A). A second antibody we generated was a mouse monoclonal antibody (mAb) 5B1. mAb
5B1 is of the IgM subtype and did not label immunoblots. However, in immunoprecipitation assays of S2 cell lysates mAb 5B1 immunoprecipitated a 265 kD protein detected by the EAST antibody pAb ED3 (Fig. 2B). A similarly sized band was labeled by pAb ED3 in the S2 cell lysate but not in the beads only immunoprecipitation control (Fig. 2B). These findings demonstrate that mAb 5B1 immunoprecipitates the EAST protein. Immunocytologically, the staining patterns of pAb IgY and mAb 5B1 were indistinguishable from that of pAb ED3 in the poltene salivary gland and early syncytial embryo preparations examined.

To address the relationship between EAST and Megator localization in interphase nuclei as well as during mitosis we used the newly generated antibodies for double labeling studies. Figure 3 shows such labelings with EAST pAb IgY and Megator mAb 12F10 of light squashes of poltene salivary gland nuclei where both EAST and Megator labeling surround the chromosomes. Furthermore, EAST and Megator appear to colocalize as indicated by the predominantly yellow color in the composite image of Fig. 3D. Figure 4A shows the relative distribution of EAST as labeled by mAb 5B1 and Megator as labeled by mAb 12F10 during mitosis in syncytial Drosophila embryos. Both proteins reorganize as mitosis commences into fusiform spindle structures at metaphase where the two proteins are colocalized (Fig. 4A) and where the EAST-defined spindle is co-aligned with the microtubule spindle apparatus (Fig. 4B). At telophase EAST redistributes back to the forming daughter nuclei whereas Megator can be observed only in the spindle midbody. Thus, while EAST and Megator mostly are colocalized during the cell cycle their distributions are not identical. Another difference is that Megator shows strong localization to the nuclear rim during inter- and prophase whereas EAST does not (Fig. 4A) suggesting
that EAST and Megator may have overlapping as well as distinct functions in the interphase nucleus.

The extensive overlap in the distribution of EAST and Megator suggested that the two proteins may interact within the same molecular complex in vivo. To test this possibility we performed co-immunoprecipitation experiments. In these assays proteins were extracted from S2 cell lysates, immunoprecipitated with EAST or Megator antibody, fractionated on SDS-PAGE after the immunoprecipitation, immunoblotted, and probed with EAST or Megator antibody, respectively. Figure 5A shows that the Megator mAb 12F10 pulled down EAST protein as a 265 kD band that is also detected in the S2 cell lysate but is not present in the control immunoprecipitated with immunobeads only. Figure 5B shows the converse experiment where EAST pAb ED3 pulled down a 260 kD band detected by Megator mAb 12F10 that was also present in the lysate but not in the immunobeads only control. A similar result was obtained with EAST mAb 5B1 (Fig. 5C). These results indicate that EAST and Megator are present in the same protein complex.

**DISCUSSION**

In this study we show that in addition to the previously reported localization of EAST to the extrachromosomal space at interphase (Wasser and Chia, 2000), during mitosis EAST redistributes to colocalize with the putative spindle matrix proteins, Skeletor and Megator (Walker et al., 2000; Qi et al., 2004). Based on overexpression studies of EAST in polyploid cells it has been proposed that EAST may be part of an internal nucleoskeleton that at interphase regulates nuclear volume and the distribution of some nuclear proteins such as CP60 and actin (Wasser and Chia, 2000). However, recent studies of chromosome behavior
in *east* loss-of-function mutations during mitosis and meiosis suggested that EAST may also play an important role in the congression and alignment of chromosomes during pro- and prometaphase as well as in regulating chromosome movement at metaphase (Wasser and Chia, 2003). Interestingly, abnormal chromosome localization in *east* mutants can be observed in prophase of male meiosis before nuclear envelope breakdown and before the chromosomes can establish interactions with microtubules (Wasser and Chia, 2003). These findings provide evidence that EAST may function to guide or constrain chromosome congression (Wasser and Chia, 2003). Moreover, the continued colocalization of EAST with Megator during prophase suggests that Megator may interact with EAST to play a role in this process as well. When Megator levels were knocked down by RNAi in S2 cell cultures no defects in tubulin spindle morphology or chromosome segregation were apparent; however a significant reduction in the number of cells undergoing mitosis was observed suggesting that depletion of Megator prevents cells from entering metaphase (Qi *et al.*, 2004). These results suggest that both EAST and Megator have important functions in nuclear reorganization during prophase.

The colocalization of EAST with the Skeletor- and Megator-defined spindle matrix during metaphase suggests that EAST may be involved in spindle matrix function. In its simplest formulation a spindle matrix is hypothesized to provide a more or less stationary substrate that provides a backbone or strut for motor molecules to interact with during force generation and microtubule sliding (Pickett-Heaps *et al.*, 1997; Johansen and Johansen, 2002). Thus, a prediction of the spindle matrix hypothesis is that if such a scaffold were interfered with in a way that it could not properly anchor motor proteins, the dynamic behavior of spindle components such as motors would be affected leading to abnormal
chromosome alignment and segregation (Rath et al., 2004). Such a phenotype of chromosomes scattered within the metaphase spindle was observed in S2 cells after RNAi depletion of the putative spindle matrix protein Chromator (Rath et al., 2004). Similar abnormal arrangements of chromosomes where the chromosomes strayed away from the metaphase plate and adopted a scattered configuration were also observed by Wasser and Chia (2003) in approximately 50% of east mutant spermatocytes in metaphase. Thus, these findings taken together indicate that EAST is likely to be the fourth member of a group of proteins derived from two different nuclear compartments that reorganize during mitosis to form a spindle matrix.

EAST is a novel protein of 2362 amino acids which apart from seven potential nuclear localization sequences and twelve potential PEST sites does not have any other previously characterized motifs or functional domains (Wasser and Chia, 2000). However, we show by immunoprecipitation experiments that EAST is likely to be present in a molecular complex together with Megator. Megator has a large NH$_2$-terminal coiled coil domain and we have demonstrated by expression construct analysis in S2 cells that this domain has the capacity for self assembly (Qi et al., 2004). This raises the intriguing possibility that Megator can form polymers which could serve as the structural basis for both a possible nuclear extrachromosomal endoskeleton and for the spindle matrix. Because both EAST and Megator localize to the extrachromosomal nuclear compartment at interphase and the spindle matrix at mitosis, it is not clear whether the interaction observed in the co-immunoprecipitation experiments reflected interactions in an interphase complex or interactions on the spindle-like structure or both. The future challenge will be to determine
how the reorganization of these proteins is coordinated and how the resulting molecular complex affects chromosome dynamics and cell division.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. The dynamic redistribution of EAST relative to the putative spindle matrix protein Skeletor during the cell cycle. The images are from triple labelings of EAST with pAb ED3 (green), Skeletor with mAb 1A1 (red) and with Hoechst (blue). The composite images (comp) are shown to the left. During pro-, meta-, and telophase the composite images (comp) show extensive overlap between Megator and Skeletor labeling as indicated by the yellow color. The images are from confocal sections of syncytial embryonic nuclei.

Figure 2. EAST antibody labeling of immunoblots of S2 cell lysates. (A) Western blot analysis of protein extracts from S2 cells shows that both pAb ED3 and pAb IgY recognize EAST protein as a 265 kD band. The relative migration of molecular weight markers in kD are indicated to the right in grey numerals. (B) Immunoprecipitation (ip) of S2 cell lysate was performed using the mAb 5B1 (lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using EAST pAb ED3 for detection. EAST pAb ED3 staining of S2 cell lysate is shown in lane 1. EAST is detected in the mAb 5B1 immunoprecipitation sample as a 265 kD band (lane 3) but not in the control sample (lane 2).

Figure 3. Light squash of a larval polytene nucleus where the chromosomes are surrounded by EAST and Megator labeling. The images are from a triple labeling of EAST with pAb IgY (green), Megator with mAb 12F10 (red), and the DNA with Hoechst (blue). The composite image in (A) shows that EAST surrounds the chromosomes and the
composite image in (D) shows that Megator and EAST largely co-localize as indicated by the predominantly yellow color.

**Figure 4.** The localization of EAST relative to the putative spindle matrix protein Megator and to tubulin during the cell cycle. (A) The images are from triple labelings of EAST with mAb 5B1 (green), Megator with mAb 12F10 (red), and DNA with Hoechst (blue). The composite images (comp) are shown to the left. At prophase EAST and Megator labeling overlap in the nuclear interior whereas Megator labeling is prominent at the nuclear rim. During metaphase the composite image (comp) shows extensive overlap between EAST and Megator labeling as indicated by the predominantly yellow color. At telophase where EAST begins to redistribute back around the chromosomes Megator appears to be preferentially localized to the spindle midbody. (B) The images are from triple labelings of EAST with mAb 5B1 (green), tubulin with a rat anti-a-tubulin mAb (red), and DNA with Hoechst (blue). The EAST antibody clearly labels a true fusiform spindle structure that is co-aligned with the microtubule spindle except for the centrosomes. The images in (A) and (B) are from confocal sections of syncytial embryonic nuclei.

**Figure 5.** EAST and Megator immunoprecipitation assays. (A) Immunoprecipitation (ip) of S2 cell lysate was performed using the Megator mAb 12F10 (lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using EAST pAb ED3 for detection. EAST pAb ED3 staining of S2 cell lysate is shown in lane 1. EAST is detected in the Megator immunoprecipitation sample as a 265 kD band (lane 3) but not in the
control sample (lane 2). (B) Immunoprecipitation (ip) of S2 cell lysate was performed using EAST pAb ED3 (lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Megator mAb 12F10 for detection. Megator mAb 12F10 staining of S2 cell lysate is shown in lane 1. Megator is detected in the EAST immunoprecipitation sample as a 260 kD band (lane 3) but not in the control sample (lane 2). (C) Immunoprecipitation (ip) of S2 cell lysate was performed using EAST mAb 5B1 (lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Megator mAb 12F10 for detection. Megator mAb 12F10 staining of S2 cell lysate is shown in lane 1. Megator is detected in the EAST immunoprecipitation sample as a 260 kD band (lane 3) but not in the control sample (lane 2).
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Figure 5
CHAPTER 4: ASATOR, A NOVEL TAU-TUBULIN KINASE, INTERACTS WITH THE SPINDLE MATRIX PROTEIN MEGATOR DURING MITOSIS IN DROSOPHILA

A paper to be submitted to Journal of Cellular Biochemistry

Hongying Qi¹, Yun Ding, Jack Girton, Kristen M. Johansen and Jørgen Johansen

ABSTRACT

In this study, we identified Asator, a conserved tau-tubulin kinase family member that interacts directly with the spindle matrix protein Megator by a yeast two-hybrid interaction assay. Immunocytochemistry demonstrated that V5/GFP tagged Aastor protein colocalizes with tubulin throughout the cell cycle. During interphase, Asator is distributed in the cytoplasm, while during mitosis Asator is relocated to the spindle structure. Analysis of an Asator mutant line indicates Asator is an essential gene. These findings suggest that Asator plays a role in proper microtubule dynamics during cell cycle.

INTRODUCTION

In all eukaryotic cells, the formation of a metaphase spindle is a prerequisite for the faithful segregation of chromosomes into the daughter cells during cell division (Compton,

¹Primary researcher and author.
When the cell progresses from interphase to metaphase, microtubule dynamics change dramatically. A variety of protein kinases and phosphatases are involved in the microtubule dynamic properties by phosphorylation or dephosphorylation of different associated proteins or the αβ-tubulin dimer itself, such as Cdk1, calmodulin kinase, CDK5/P25 complex, and PP2A (Fourest-Lieuvin et al., 2006; Wang et al., 2007; Hayashi et al., 2006).

During this process, a spindle matrix has been proposed to help to organize and stabilize spindle microtubules and provide a stationary substrate for motor to generate forces (Pickett-heaps et al., 1997; Johansen and Johansen, 2002). Four spindle matrix proteins, Skeletor, Chromator, Megator and EAST have recently been identified to redistribute and form a fusiform spindle structure during mitosis in Drosophila (Walker et al., 2000; Qi et al., 2004; Rath et al., 2004; Qi et al., 2005). Among them, Megator is a 260 kDa protein with a large coiled-coil domain at its N-terminus and a shorter non-structural acidic C-terminal region that shows overall structural and sequence similarity to the mammalian nuclear pore complex TPR protein (Zimowska et al., 1997). Megator is an essential protein and may serve as a structural component of spindle matrix (Qi et al., 2004).

How the spindle matrix is involved in microtubule dynamics and regulates spindle assembly is largely unknown. We used a yeast two-hybrid screen to identify a protein interacting with Megator that we have named Asator. Sequence analysis shows that Asator is a tau-tubulin kinase (TTBK), which encodes a Ser/Thr kinase motif. Previous studies showed that TTBK can directly phosphorylate tau and tubulin at multiple sites and thus may be involved in the paired helical filaments (PHFs) that accumulate in the brain of Alzheimer’s patients (Sato et al., 2006; Katalashiki et al., 1995; Tomizawa et al., 2001). Though TTBK1 is
neuron-specific in human, TTBK2 is ubiquitously distributed in rat tissues (Seta et al., 2006; Tomizawa et al., 2001).

In this paper, we report the identification of the Drosophila Asator gene, which is highly conserved from nematode to human. We show that Asator interacts with the spindle matrix protein Megator by in vitro pull-down assays. Using immunocytochemistry and analysis of P-element mutations, Asator is an essential protein that colocalizes with tubulin throughout the cell cycle. We suggest that interaction with the spindle matrix protein Megator, targets Asator to its substrates, tubulin or the microtubule associated protein tau, which in turn, regulates spindle assembly via phosphorylation during mitosis.

MATERIALS AND METHODS

Drosophila Stocks

Fly stocks were maintained according to standard protocols (Roberts, 1986). Oregon-R or Canton-S was used for wild-type preparations. The $P^{SUPor-P}$Asator$^{KG0370}$ and $P^{SUPor-P}$Asator$^{KG0501}$ lines were obtained from the Bloomington Stock Center. Asator-PB with a GFP tag was inserted into pUAST vector in frame and then the Asator transgenes were generated by BestGene Inc.

Sequence alignment and phylogenetic tree construction

Homologous protein sequences were aligned by the Clustalw version 1.7 program with default settings. A maximum parsimony tree was constructed based on the multiple alignments after removal of all columns in the alignment containing one or more gaps. Trees were constructed using the PAUP computer program version 4.0b (Swofford, 1993).
Confidence of the tree topology was assessed by a bootstrap test with 1000 replicates and indicated on the consensus trees.

**Identification and molecular characterization of Asator**

Three different Megator cDNA sequences, MtorN, MtorM, and MtorC, containing residues 173-360, 1435-1712, and 2188-2346 respectively were subcloned in-frame into the yeast two hybrid bait vector pGBKT7 (Clontech) using standard methods (Sambrook et al., 1989) and verified by sequencing (Iowa State University (ISU) Sequencing Facility). The Megator baits were used to screen the Clontech Matchmaker 0-21 h embryonic Canton-S yeast two-hybrid cDNA library according to the manufacturer’s instructions. Positive cDNA clones were isolated, retransformed into yeast cells containing the Megator bait to verify the interaction, and sequenced. Homology searches identified the interacting clone as comprised of partial coding sequences from the *CG11533* locus. The Asator sequence was compared with known and predicted sequences using the National Center for Biotechnology Information BLAST server. The sequence was further analyzed using SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/) to predict the domain organization of the protein.

**Biochemical analysis**

*SDS-PAGE and immunoblotting.* 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 rabbit HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody GFP (Invitrogen) diluted 1:1000 in Blotto. The signal was visualized using chemiluminescent detection methods (ECL kit, Amersham).

**Pull-down experiments.** For *in vitro* pull down assays, an Asator fragment of the last 468aa was subcloned in-frame into the Pinpoint Xa-2 vector (Promega) and Mtor N was subcloned into pGEX4T-1 vector. Then biotinylated Asator protein and GST-Mtor N fusion protein were expressed in XL-1 Blue cells (Stratagene). For GST pull down assays, approximately 3 µg of GST-MtorN and GST protein alone were coupled to glutathione agarose beads (Sigma) and incubated with 0.5 ml cell extract expressing biotinylated Asator in immunoprecipitation (ip) buffer (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 1 mM Phenylmethylsulfonyl fluoride, and 1.5 µg Aprotinin) overnight at 4°C. The protein complex coupled beads were washed with 1 ml of IP buffer for three times and analyzed by SDS-PAGE and western blotting using Streptavidin tagged Alkaline Phosphatase according to the manufacturer's instructions (Promega). Similarly for avidin pull down assays, Bio-Asator or the biotinylation tag alone was bound to immobilized Streptavidin beads (Pierce) and incubated with 3 µg of GST-MtorN in 500 µl of immunoprecipitation buffer. The resulting complexes were then analyzed by SDS PAGE and western blotting using anti-GST antibody.

**Immunohistochemistry**

S2 cells were affixed onto Con A (Sigma) 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 labelings employing epifluorescence were performed using anti-α-tubulin mouse IgG1 antibody (Sigma), V5-antibody (IgG2A, Invitrogen), and Hoechst to visualize the DNA. The Texas Red conjugated anti-mouse IgG2A (Southern Biotech), and FITC-conjugated anti-mouse IgG1 secondary antibodies (Cappel,) 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 Texas Red imaging. 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 neuroblast cell squash preparations from third instar larvae were immunostained by tubulin (Sigma) and GFP (Invitrogen) antibody as previously described by Bonaccorsi et al. (2000).

*Expression of Asator constructs in transfected S2 cells*

Full length Asator isoform B (811 aa) was cloned into the pMT/V5-HisC vector (Invitrogen) with an in-frame V5 tags at the COOH-terminus using standard methods (Sambrook et al., 1989). The fidelity of the construct was then verified by sequencing at the Iowa State University Sequencing facility.

*Drosophila* Schneider 2 (S2) cells were cultured in Shields and Sang M3 insect medium (Sigma) supplemented with 10% fetal or newborn bovine serum, antibiotic/antimycotic solution and L-Glutamine (Gibco/BRL/Life Technologies) at 25°C. The S2 cells were transfected with the Asator clone using a calcium phosphate transfection
kit (Invitrogen) and expression was induced by 0.5 mM CuSO₄. Then cells were harvested 12-24 h after induction and affixed onto poly-L-lysine coated coverslips for immunostaining and Hoechst labeling.

**Analysis of P-element mutants**

*PCR mapping.* The insertion site flanking sequences provided by the Berkeley Drosophila Genome Project for the P\{SUPor-P\}Asator\(^{KG03370}\) and P\{SUPor-P\}Asator\(^{KG05051}\) element placed the P-element insertions close to the 5’ end of the Asator gene locus. By designing several sets of nested forward and reverse primers from genomic sequence, we performed PCR from mutant flies as previously described (Preston and Engels, 1996). PCR fragments were then sequenced according to standard protocols (Sambrook *et al.*, 1989).

*P-element excision.* The P element P\{SUPor-P\}Asator\(^{KG05051}\) was mobilized by a Δ2-3 transposase source (y\(^{1}\) w\(^{2}\); CyO, H\{w\(^{+mC}=P\Delta2-3\}HoP2.1/Bc\(^{E1}\)Egfp\(^{E1}\)) (Robertson *et al.*, 1988). Several fly lines in which the P element had been excised were identified by their white eye color. One precise excision line was confirmed by PCR analysis using primers corresponding to the P element and/or the genomic sequences flanking it. DNA isolation from single embryo and PCR reactions were performed as described in Preston and Engels (1996).

**RESULTS**

*Identification of Asator and phylogenetic analysis of the Asator protein family*

In order to identify candidates for the spindle matrix macromolecular complex, we performed yeast two-hybrid interaction assays using three different Megator bait constructs.
Residues 173-360, 1433-1712, and 2172-2346 of the Megator protein were cloned in-frame into the pGBKT7 vector respectively to generate the construct MtorN, MtorM and MtorC (Fig. 1A). The Drosophila matchmaker 0-21 hour embryonic library (Clontech) was screened. One interacting clone comprised of a partial COOH-terminus (1407bp) of CG11533 coding sequence, which we named Asator, was identified only from the MtorN screening.

To confirm the physical interaction with Megator, we conducted in vitro pull down experiments using a PinPoint vector construct that produces biotinylated Asator fusion protein and GST-Megator fusion protein produced in E. coli. Biotinylated Asator protein pulled down a band corresponding to the size of GST-Megator, while the biotin alone was not able to pull down Megator when purified using avidin beads, (Fig. 1B). In the converse experiment, GST-Megator fusion protein was able to pull down biotinylated Asator using glutathione beads whereas GST protein alone cannot (Fig. 1B). These results support the existence of a direct physical interaction between Megator and Asator.

Analysis of the Asator gene locus shows three alternative transcripts as depicted in Fig. 2A. Each transcript uses the same stop codon but different starting methionine sites to generate three different open reading frames. RT-PCR showed that the isoform B has the highest expression level in Drosophila S2 cells (data not shown). All three isoforms contain a serine/threonine kinase domain at their NH2-termini (black box in Fig. 2A). Proteins containing this kinase domain have been shown to be able to phosphorylate tau and tubulin specifically (Sato et al., 2006; Katahashi et al., 1995; Tomizawa et al., 2001). Other than the kinase domain, the long COOH-terminus of Asator does not contain any previously described conserved motifs. Homologs were also identified in different organisms such as
nematode, zebrafish, chicken, mouse and human (Fig 2B). Among all these tau-tubulin kinase (TTBK) family members, all the homologs contain a conserved kinase domain at their NH2-termini, in which the sequence identity between Asator and the mammalian homolog is as high as 78%, while their C-termini are highly variable. The sequence identity between the tau-tubulin kinase domain and casein kinase domain is only 36%, suggesting they are two different protein kinase families (Fig. 2B).

To further analyze the evolutionary relationship of this protein family, we constructed phylogenetic trees using the maximum parsimony method. Figure 2C shows a consensus tree based on conserved kinase domain sequence from the protein family members. The phylogenetic analysis indicates that Asator evolve earlier than other vertebrate homologs.

**Localization of Asator during the cell cycle**

To determine localization of Asator during the cell cycle, we made a construct containing full length Asator isoform B sequences for expression in S2 cells with a COOH-terminal V5-tag. Figure 3 shows examples of expression of this construct in transiently transfected S2 cells detected with V5-antibody and triple-labeled with tubulin antibody and Hoechst. Asator shows a very dynamic cell cycle dependent localization pattern. Asator is distributed in the cytoplasm during interphase and reorganizes to form a spindle structure at metaphase, co-localizing with tubulin throughout the cell cycle.

To confirm its localization pattern in the cell, we further expressed the Asator protein in live animals. Full-length Asator isoform B with a GFP tag at its COOH-terminus was cloned into the pUAST vector to generate transgenic fly lines. A protein band migrating at approximately 116 kDa was detected by GFP antibody, which is consistent with the predicted
molecular mass (Fig. 4A). We also drove the protein expression in Drosophila larval brains and performed neuroblast squash labeling with GFP- and tubulin-antibody and Hoechst to illustrate DNA. Neuroblast staining shows an identical staining pattern as that in S2 cells. It co-localizes with tubulin, distributing in the cytoplasm during interphase and reorganizing into spindle structure during mitosis (Fig. 4B).

**Asator is an essential gene**

Two SUPor-P (Roseman et al., 1995) elements have been found to be inserted into the CG11533 region (Fig. 2A). We verified the P element insertion sites by polymerase chain reaction (PCR) analysis using primers corresponding to genomic sequences flanking the region and sequencing the PCR product. In P{SUPor-P}Asator\(^{KG05051}\) flies the P element is inserted within the third intron of transcript A 998 bp before the fourth exon of Transcript A (Fig. 2A). In P{SUPor-P}Asator\(^{KG03370}\) flies the P element is inserted within the first exon of transcript A and 27bp upstream of the starting Methionine site for transcript A. Both insertion lines are homozygous viable. The viability may be due to that the insertions do not interrupt the open reading frames of all three splicing isoforms.

In order to generate an allelic series of hypomorphic Asator alleles including a complete Asator null allele, we mobilized the P element in P{SUPor-P}Asator\(^{KG05051}\) flies using the Δ2–3 transposase (Robertson et al., 1988) and screened for imprecise excision events. No homozygous flies can survive to adult stage in one line. By PCR mapping, it was determined that the excision event removed part of the P element and most of the fourth exon of the isoform A, suggesting the excision may interrupt the protein expression from all three
isoforms. Lethality caused by loss of Asator protein indicates that Asator is an essential gene for fly development.

**DISCUSSION**

In this paper, we report the identification and characterization of Asator, a *Drosophila* serine/threonine dual kinase. It belongs to a novel tau-tubulin kinase family, which is highly conserved from nematode, fly, zebrafish, *Xenopus*, chick, mouse and human. At least two TTBK isoforms were identified in human and mouse. TTBK1 in human was found to be expressed specifically in brain tissues. It is involved in tau phosphorylation at multiple serine sites, which are phosphorylated in paired helical filaments (PHFs) of Alzheimer’s disease (Sato *et al.*, 2006). While TTBK2 was shown to be ubiquitously distributed in the rat tissues (Tomizawa *et al.*, 2001). Since only one member of TTBK family was identified in Drosophila, Asator might be temporally and spatially regulated to be expressed in different tissues.

Immunohistochemistry studies show that V5 or GFP tagged Asator colocalizes with tubulin throughout the cell cycle in either S2 cells or third instar larval neuroblasts. Asator interacts with the putative spindle matrix protein Megator, which was first detected in a yeast two-hybrid screen and subsequently confirmed by pull-down assays. The Megator defined spindle matrix was previously shown to organize into a fusiform spindle like structure, co-localizing with the microtubule spindle structure (Qi *et al.*, 2004), suggesting that Asator might be another member of the spindle matrix complex. Asator may localize to the proper position by interacting with the spindle matrix protein Megator during metaphase and phosphorylate its substrates, microtubule associated protein tau or tubulin to regulate
microtubule dynamics and microtubule spindle assembly. Phosphorylation of a tubulin subunit at multiple sites can interfere with its incorporation into microtubules (Fourest-Lieuvin et al., 2006). Hyperphosphorylation of tau interferes with its binding to microtubule.

An Asator mutant that disrupts all three transcripts shows that Asator is an essential gene for fly viability. While no obvious mitotic phenotype was observed in CG11533 RNAi experiments in Drosophila S2 cells (our data, not shown; Bettencourt-Dias et al., 2004). One reason might be that if the protein knockdown is not efficient, a little amount of protein can carry out the sufficient function in cells. Another reason might be that redundant pathways exist in cells to perform the same function. For example, Cdk1 can directly phosphorylate β-tubulin in vitro and in mitotic cells and CKiδ phosphorylates tau and disrupts its binding to microtubules (Fourest-Lieuvin et al., 2006; Li et al., 2004).

**ACKNOWLEDGEMENTS**

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**REFERENCES**


**FIGURE LEGENDS**

**Figure 1. Protein interaction between Asator and Megator.** (A). Three Megator fragments were cloned in-frame into the pGBK vectors and used as baits to screen the *Drosophila* Matchmaker 0-21 hours’ library (Clontech). (B). *In vitro* pull-down assays of Asator and Megator. In left panel, an Asator-biotin construct pulls down Megator-GST as detected by GST antibody (lane 1). A biotin only pulldown control was negative (lane 2). Lane 3 shows the position of the Megator-GST fusion protein. In the right panel, A Megator-GST construct pulls down biotinylated Asator as detected by Streptavidin alkaline phosphatase (Avidin-AP) (lane 1). A GST only pull down control was negative (lane 2). Lane 3 shows the position of the Asator-biotin fusion protein.

**Figure 2. Analysis of Asator gene locus and sequence alignment of TTBK family.** (A) Diagram of Asator alternative transcripts. The Asator locus gives rise to three different transcripts (A, B, and C). Two SUPor-P elements are inserted in the 5’ of the Asator gene, all three transcripts share the same stop codon. The location of the Serine/Threonine kinase domain is indicated by a black box. (B). Diagram and alignment of Asator-PB and its homologs in human, chick, *X. tropical.is*, zebrafish, and *C. elegans*. Numbers in the diagrams are the percentage of sequence identity on the amino acid level compared to the Asator kinase domain. (C) Phylogenetic tree of the TTBK family and Casein kinases based on the alignment of the kinase domain. The unrooted tree is depicted with associated bootstrap values from 1000 iterations.

**Figure 3. Expression of Asator constructs in S2 cells.** The expressed construct is diagrammed beneath the micrographs. S2 cells expressing a V5-tagged full-length Asator-PB
were triple-labeled with V5-antibody to visualize the Asator-PB-V5 construct (green),
tubulin-antibody to visualize the microtubules (red) and Hoechst to visualize the DNA (blue).
At interphase Asator-PB-V5 localizes to the cytoplasm whereas at metaphase it reorganizes
into a spindle structure. The composite images show the colocalization of Asator and tubulin
throughout the cell cycle (in yellow).

**Figure 4. Expression of Asator constructs in Drosophila live animals.** The
expressed construct is diagrammed beneath the micrographs. (A) Western blot analysis
shows the expressed full length Asator-PB-GFP protein in Drosophila embryo extracts as a
single band about 116 kDa, consistent with its predicted molecular size. (B) Third instar
larval neuroblasts expressing GFP-tagged full-length Asator-PB. The cells were triple-
labeled with GFP-antibody to visualize the Asator-PB-GFP construct (green), tubulin-
antibody to visualize the microtubules (red) and Hoechst to visualize the DNA (blue). At
interphase Asator-PB-GFP localizes to the cytoplasm whereas at metaphase it reorganizes
into spindle structure. The composite images show the colocalization of Asator and tubulin
throughout the cell cycle (in yellow).
Figure 1

A

Megator

Coiled-coil

NLS

Acidic

Baits: Mtor N Mtor M Mtor C

Library: Drosophila Matchmaker 0-21hours' library

B

Asator-biotin pulldown Biotin pulldown MtorN-GST pulldown

detected with GST antibody

detected with Avidin-AP
Figure 2
Figure 3

Figure 4
CHAPTER 5: GENERAL CONCLUSION

Megator and EAST interact to form a nuclear endoskeleton

Megator (Bx34 antigen) is a 260 kD protein with a large NH\textsubscript{2}-terminal coiled-coil domain and a shorter COOH-terminal acidic region that shows overall structural and sequence similarity to the mammalian nuclear pore complex Tpr (translocated promoter region) protein (Zimowska et al., 1997). Yet the protein sequences of Tpr and Megator have only a 28% amino acid identity. Moreover, immunostaining data indicates their functional differences. Different from Tpr protein, Megator is not only localized to the nuclear rim, associated with the nuclear pore complex, but also occupies the intranuclear space surrounding the chromosomes during interphase (Zimowska et al., 1997; Qi et al., 2004).

EAST (Enhanced adult sensory threshold) is another large protein in Drosophila that distributes in the extrachromosomal region in the interphase nucleus (Wasser and Chia, 2000; Wasser and Chia, 2003). EAST does not have any previously characterized motifs or functional domains. Increase in nuclear EAST results in the expansion of the extrachromosomal domain. Studies in east loss-of function mutations suggested that EAST play an important role in the congression and alignment of chromosomes during pro/prometaphase as well as in regulating chromosome movement during metaphase. More recently, it has been shown that EAST interacts with Chromotor, has the ability to bind polytene chromosomes and may be involved in gene regulation (Wasser and Chia, 2007; Wasser et al., 2007). Cross-immunoprecipitation experiments show that EAST molecularly interacts with Megator. Immunocytochemistry studies show the co-localization of EAST and Megator, which associates with an interior extrachromosomal compartment of the interphase.
nucleus, suggesting that Megator interacts with EAST as part of a nuclear endoskeleton that is involved in the spatial organization of the nucleus.

**Megator, a structural component of the spindle matrix complex**

Immunohistochemistry studies show that Megator has a very dynamic cell cycle dependent localization during mitosis. Megator is localized to the nuclear envelope and the nuclear interior during early prophase and reorganizes to form a fusiform spindle-like structure during late pro- to prometaphase. This Megator spindle structure colocalizes extensively with the fusiform Skeletor defined spindle structure from prometaphase to anaphase. Both Megator and the Skeletor spindle also coalign with the mitotic microtubule spindle during meta- and anaphase. At telophase, Megator relocates to the midbody region and Skeletor is redistributed into the daughter nuclei. Co-immunoprecipitation analysis indicates that Megator also molecularly interacts with Chromator, a putative functional component of the spindle matrix. Furthermore, nocodazole or cold treatment in *Drosophila* syncytial embryos does not interfere the Megator defined spindle structure during metaphase when microtubules are depolymerized. These results taken together indicate that Megator is a part of the macromolecular spindle matrix complex of proteins.

Both of the previously described putative spindle matrix proteins, Skeletor and Chromator, do not have any predicted structural motifs that are known to form polymers in their protein sequences. The NH$_2$-terminus large coiled-coil domain in Megator raises the possibility that it may serve as a structural candidate of the spindle matrix complex. A Megator NH$_2$- terminal construct containing the coiled-coil domain when transfected into the *Drosophila* S2 cells was able to self-assemble into spherical structures in the cytoplasm. The
COOH- terminal construct containing the acidic domain of Megator upon transfection shows similar localization as the endogenous protein. These findings suggest that the COOH-terminus of Megator is required for the targeting and localization of the protein to the spindle and the NH₂- terminus is responsible for forming polymers and serving as the structural basis of the spindle matrix. Functional analysis using dsRNAi to deplete Megator protein in Drosophila S2 cells shows a lower mitotic index but no obvious defects in the mitotic spindle morphology or chromosome congression/segregation. Recently, we generated 46 Megator mutant alleles via EMS mutagenesis, which can be classified into three complementation groups. Among them, alleles 22-6 and 53-3 when heteroallelic over the P-element null allele P537 show obvious mitotic defects, including incomplete spindles, misaligned chromosomes, failure of chromosome segregation (shown in the appendix). All these studies suggest that Megator is structural component of the spindle matrix complex and regulates the spindle function during mitosis. Further analysis of EMS generated Megator point mutation alleles and future Megator self-assembly assays will provide more evidences for the multiple functions of Megator in nuclear organization and spindle matrix.

**EAST, another spindle matrix protein, interacts with Megator**

EAST is a novel nuclear protein of 2,362 amino acids, which apart from seven potential nuclear localization sequences and twelve potential PEST sites does not have any other previously characterized motifs or functional domains. Immunoprecipitation experiments suggest that EAST is likely to be present in a molecular complex together with Megator. Immunostaining of EAST shows a cell cycle dependent localization pattern very similar to the spindle matrix protein Megator. During metaphase, EAST reorganizes into a
spindle structure and extensively co-localizes with the Skeleter- and Megator- defined spindle matrix, suggesting that EAST may be involved in spindle matrix function. In about 50% *east* loss of function mutant spermatocytes, abnormal arrangements of chromosomes where the misaligned chromosomes strayed away from the metaphase plate were observed (Wasser and Chia, 2003), which is consistent with the spindle matrix hypothesis to provide a more or less stationary substrate that provides a backbone or strut for motor molecules to interact with during force generation and microtubule sliding. Disruption of the spindle matrix structure will affect the motors and lead to abnormal chromosome alignment and segregation. Thus, these findings taken together indicate that EAST is likely to be the fourth member of a group of proteins derived from two different nuclear compartments that reorganize during mitosis to form a spindle matrix. Further studies on how motor proteins are involved will be much helpful for characterizing its spindle matrix functions.

**Asator, a novel tau-tubulin kinase, interacts with Megator and localizes onto the spindle matrix**

We identified Asator, a serine/threonine dual kinase through a yeast two-hybrid interaction assay using a fragment from the Megator N-terminal coiled-coil domain. In vitro pull-down experiments confirm that Asator can physically interact with Megator directly. The kinase domain in Asator is highly conserved from nematode, fly, zebrafish, *Xenopus*, chick to human. Asator belongs to a novel tau-tubulin kinase family, which can specifically phosphorylate its substrates including tubulin and the microtubule associated protein tau in its –SR motif (Sato *et al.*, 2006; Tomizawa *et al.*, 2001). The substrate specificity raises the
intriguing possibility that Asator may be involved in regulation of microtubule dynamics and thus spindle dynamics. Expression of V5 or GFP tagged Asator-PB protein in Drosophila S2 cells or third instar larval brain neuroblasts show the dynamic cell cycle dependent localization pattern. Asator distributes in the cytoplasm during interphase, while it reorganizes into a spindle structure co-localizing with microtubule spindle during metaphase. Previously, it was shown that Megator defined spindle matrix also co-localizes with microtubule spindle at metaphase. All these findings suggest that Asator may be another component of spindle matrix complex. During mitosis, Asator interacts with spindle matrix protein Megator, which may direct Asator to its substrates, thus regulates the microtubule dynamics and further the mitotic spindle function by phosphorylating tubulin or tau. The future challenge will be to determine how the reorganization of these proteins is coordinated and how the resulting molecular complex affects spindle dynamics and cell division.

Asator is an essential gene, as the homozygous Asator mutant allele generated from P-element imprecise excision cannot survive to adult stage. However, no obvious mitotic phenotype in microtubule spindle morphology or chromosome alignment/segregation was detected in dsRNAi experiments when depleting Asator protein in Drosophila S2 cells. There might exist a redundant pathway since a variety of protein kinases present in cells and it have been shown that many enzymes share the same substrates, for example Cdk1 can also phosphorylate β-tubulin in vitro and in vivo in metaphase cells (Fourest-Lieuvin et al., 2006). Inhibiting the kinase activity of Asator may result in the enhancement of the activity of other kinases. Overexpression of Asator protein or a kinase dead mutant might overcome some of
these effects and provide more hints on how Asator is involved in spindle matrix function and cell division.

**The Spindle Matrix Complex**

The “spindle matrix” is hypothesized as a stationary non-microtubule structure that provides a backbone or a strut for interaction of motor proteins during force generation and microtubule sliding in the metastable spindle apparatus during mitosis. In addition, it may help organize and stabilize the microtubule spindle (Pickett-Heaps *et al.*, 1997; Johansen and Johansen, 2002). Various hints as to the existence of such a “spindle matrix” structure have been provided but the molecular components of this structure have remained elusive. In *Drosophila*, Skeletor and Chromator have been identified as candidate proteins since they possess many characteristics predicted for a hypothesized spindle matrix protein (Walker *et al.*, 2000, Rath *et al.*, 2004). However, both proteins do not contain any conserved motifs known to have the capability of self-assembly to form a microtubule independent spindle matrix structure. Using yeast two hybrid and co-immunoprecipitation analysis we have identified three more putative spindle matrix candidates Megator, EAST and Asator. At interphase, Megator and EAST proteins are present in the nucleus, while Asator is distributed in cytoplasm, co-localizing with microtubules. Different from Skeletor and Chromator on the chromosomes, Megator and EAST co-localize at the interchromosomal region. Megator also is present at the nuclear rim associated with the nuclear pore complex. During mitosis, these proteins redistribute to form a spindle structure independent of the microtubule spindle. Especially, the self-assembly capability of Megator coiled-coil domain suggests that Megator serves as a structural role in the spindle matrix complex. An incomplete spindle structure
with chromosome alignment/segregation defects observed in Megator, EAST mutants as well after depletion of Chromator are consistent with the predictions of the spindle matrix hypothesis.

In summary, we have identified three more proteins Megator, EAST and Asator as putative components of the stationary spindle matrix complex in Drosophila. Analysis of these proteins has provided insights into the organization and function of the proposed spindle matrix structure. Further studies involving point mutations of these genes, motor protein interaction, overexpression or kinase dead Asator mutant in animals or cell line will shed more light into the mechanistic details of the spindle matrix complex of proteins during mitosis.
APPENDIX

Characterization of Megator mutants

Using EMS mutagenesis, 46 Megator mutant alleles were generated using standard protocols (Grigliatti, 1986). Complementation analysis shows that 43 of the 46 alleles fall into three distinct complementation groups (Jack Girton, unpublished data), suggesting that Megator may serve three different roles in the cell. Among them, 10 alleles which can survive to second or third instar larval stages when heteroallelic with P537 (or l(2)k03905) were further analyzed for mitotic defects in neuroblasts from third instar larval brains. The result is shown in table A1 that only two alleles, 22-6 from complementation group I and 53-3 from complementation group III show clear mitotic defects.

In 22-6/P537 third instar larvae, full-length Megator protein can be detected as a single band migrating at approximately 260kD, same as observed in the wildtype control (Fig. A1A). In 22-6/P537 neuroblasts, a much higher percentage (70%) of the mitotic neuroblast cells were observed at pro/prometaphase, as compared to only 37% of cells are at this phase in wildtype. Chromosomes were stretched and not well condensed, the two spindle poles were not deposited at the opposing positions, and fewer microtubule fibers were present during prophase. At metaphase, the tubulin spindles were misaligned, incompletely formed, and with abnormal congression of chromosomes. Very few cells could proceed to anaphase and chromosome segregation was abnormal (Fig. A1B). We quantified this difference by counting the number of such phenotypes in 22-6/P537 Megator mutant brains.
and compared it to wild type. In mutant brains (n=9) 317 out of 346 (91.6%) neuroblasts examined had such phenotypes versus only 13 out of 265 (4.9%) neuroblasts in control brains (n=12) (Fig. A1C). This difference is statistically significant on the $P<0.001$ level ($\chi^2$-test).

Full length Megator protein was detected by 12F10 antibody in 53-3/P537 third instar larvae extract (Fig. A2A). In 53-3/P537 mitotic neuroblasts, aberrant monopolar spindles were observed with chromosomes congressed to the pole position during metaphase. In contrast, normal polar spindles with well-aligned chromosomes at the metaphase plate were observed in control metaphase cells (Fig A2B).

**Materials and methods**

**Drosophila stocks.**

Fly stocks were maintained according to standard protocols (Roberts, 1986). Canton-S was used for wild-type preparations. The $y^{1} w^{67c23}; P_{f^{w}+mC}=lacW/l(2)k03905k03905/CyO$ line was obtained from the Bloomington Stock Center.

**Biochemical analysis**

**SDS-PAGE and immunoblotting.** 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 rabbit HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody diluted 1:1000 in Blotto. The signal was visualized using chemiluminescent detection methods (ECL kit, Amersham).
**Immunohistochemistry**

Brains from third instar larvae were dissected and fixed according to Bonaccorsi et al. with minor modification (Bonaccorsi et al., 2000). Brains from late-third-instar larvae were dissected 0.7% NaCl solution and fixed in 3.7% formaldehyde in PBS for 30 min, and then in 45% acetic acid for 3 min. They were gently squashed in 60% acetic acid under a 20x20 mm coverslip and frozen in liquid nitrogen. After removal of the coverslip, the slides were washed in PBST (PBS containing 0.4% Triton X-100) two times (10 min each), then blocked for 30 min in 1% NGS (Cappel) in PBST at room temperature. The slides were then incubated with diluted primary antibody in PBS containing 0.4% Triton X-100, and 1% normal goat serum at 4 °C overnight. Double labelings employing epifluorescence were performed using anti-tubulin mouse IgG1 antibody (Sigma 1:200) and rabbit polyclonal histone 3 phospho-Serine 10 (Upstate, 1:100) antibody, and Hoechst to visualize the DNA. The Texas Red-conjugated anti-mouse IgG1 (1:400, Southern Biotech), and FITC-conjugated anti-rabbit IgG (1:200, Cappel) secondary antibodies were used 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, Texas Red, and TRITC imaging.
Table A1. Summary of examined Megator mutants by neuroblast squash staining.

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**Figure A1. Western blot and immunostaining analysis of the Megator 22-6 mutant.** (A) Western blot analysis of *Drosophila* third instar larval protein extract shows that mAb 12F10 recognizes Megator protein as a 260 kD band in both wild type control (right lane) and 22-6/P537 mutant (left lane). (B) Neuroblasts from third instar larval brain squashes of control brains (upper panel) and 22-6/P537 *Megator* mutant brains (lower panel) double labeled with tubulin antibody (red) and histone H3S10ph antibody (green). (C) Quantification of mitotic defects in neuroblasts from third instar larval brain squashes. The grey bar indicates the number of normal mitotic cells and the black bar indicates the abnormal mitotic neuroblasts. The difference is statistically significant on the \( P<0.001 \) level (\( \chi^2 \)-test).

**Figure A2. Western blot and immunostaining analysis of the Megator 53-3 mutant.** (A) Western blot analysis of *Drosophila* third instar larval protein extract shows that mAb 12F10 recognizes Megator protein as a 260 kD band in both wild type control (right lane) and 53-3/P537 mutant (left lane). (B) Neuroblasts from third instar larval brain squashes of control brains (upper panel) and of 53-3/P537 *Megator* mutant brains (lower panel) double labeled with tubulin antibody (red) and histone H3S10ph antibody (green). Individual panels show multiple examples of control wild-type or mutant monopolar spindles with mislocalized chromosomes in the 53-3/P537 *Megator* mutant neuroblasts at metaphase.
Figure A1
Figure A2
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