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A nuclear-derived proteinaceous matrix embeds the microtubule spindle apparatus during mitosis

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A nuclear-derived proteinaceous matrix embeds the microtubule spindle apparatus during mitosis

by

Changfu Yao

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

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Iowa State University
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2013
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To my family
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ABSTRACT

The nucleus undergoes a dramatic reorganization as the cell prepares to segregate its duplicated chromosomes during cell division. For many years, the prevailing view was that in open mitosis the nucleus completely disassembled during early mitotic stages, thus enabling cytoplasmic microtubules emanating from the separated centrosomes to form a mitotic spindle. This cytocentric view largely discounted any nuclear contributions to regulation of mitotic progression (Johansen and Johansen, 2009; De Souza and Osmani, 2009; Simon and Wilson, 2011; Sandquist et al., 2011).

A spindle matrix has long been proposed to serve as a relatively stable or elastic molecular matrix that interacts with the microtubule spindle apparatus, based on the consideration of a mechanical and functional support for the stabilization of the microtubule spindle during force generation; however, whether such a structure exists and its molecular and structural composition has remained controversial.

In Drosophila we have identified four nuclear proteins, Skeletor, Chromator, Megator, and EAST from two different nuclear compartments that interact with each other (reviewed in Johansen et al., 2012) and that redistribute during prophase to form a dynamic, gel-like spindle matrix that embeds the microtubule spindle apparatus, stretching from pole-to-pole (Yao et al., 2012a). In this dissertation, I present the dynamic distribution of the spindle matrix components using a live imaging approach by expressing GFP tagged spindle matrix complex components in Drosophila syncytial embryos. As shown in the dissertation, this matrix forms prior to nuclear
envelope breakdown and specific interactions between spindle matrix molecules are necessary for complex formation and cohesion (Yao et al., 2012a). When microtubules are depolymerized with colchicine just prior to metaphase, the spindle matrix contracts and coalesces around the chromosomes suggesting that microtubules act as "struts" stretching the spindle matrix. Furthermore, in colchicine treated embryos free tubulin accumulates co-extensively with the spindle matrix proteins suggesting that this enrichment is dependent on one or more proteins within the spindle matrix with tubulin binding activity. Biochemical interaction assays show a potential direct interaction between Chromator and polymerized microtubules or free tubulin. This tubulin binding activity of Chromator provides support for the hypothesis that reorganization of nuclear proteins into a spindle matrix may play a wider functional role in spatially regulating cell cycle progression factors in conjunction with contributing to microtubule spindle assembly and dynamics. Moreover, we have demonstrated that the coiled-coil domain of Megator is responsible and required for Megator’s spindle matrix localization and function.

During interphase Chromator localizes to the interband region of Drosophila polytene chromosomes and is required for the maintenance of chromosome morphology. Here I show that the N-terminus domain of Chromator is required for proper localization to chromatin during interphase and that chromosome morphology defects observed in Chromator hypomorphic mutant backgrounds can be largely rescued by expression of this domain. Furthermore, the Chromodomain can interact
with histone H1 and this interaction is necessary for correct chromatin targeting (Yao et al., 2012b).
CHAPTER 1: GENERAL INTRODUCTION

DISSERTATION ORGANIZATION

This dissertation is comprised of five chapters. The first chapter is a general introduction reviewing the current knowledge about the mitotic spindle. The importance of mitosis, microtubule structure and dynamic properties, and the mechanism of spindle assembly are discussed. Recent studies of the effects of microtubule associated proteins (motor proteins and non-motor proteins including structural participants and regulatory mitotic kinases) on mitotic spindle organization are described. The spindle matrix hypothesis is introduced and current evidence supporting existence of such a macromolecular complex and research progress in the field are reviewed. Further, the four putative molecular candidates of the spindle matrix in *Drosophila*, Skeletor, Chromator, Megator and EAST are discussed. Finally, the advantages of the *Drosophila* model system are 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 Sep. 2012 on the analysis of nuclear derived proteins forming a gel-like matrix that embeds the microtubule spindle apparatus during mitosis. Dr. Uttama Rath contributed the spinning disc live imaging of Chromator-GFP. All other figures were generated by Changfu Yao. In this paper, using a live imaging approach in *Drosophila* syncytial embryos, we found that nuclear proteins Chromator and Megator reorganize during mitosis and form a highly dynamic and viscous gel-like spindle matrix that embeds the mitotic spindle from pole to pole. This spindle matrix structure is a unique structure that is distinct from the
microtubule spindle and from a lamin B-containing spindle envelope. The coiled-coil domain of Megator is shown to be responsible for Megator’s spindle matrix localization and function. The work about spindle matrix helps to understand microtubule dynamics and the viscous-elastic properties of the spindle during cell division.

The third chapter is a manuscript being prepared for submission. In this paper, all the constructs are contributed by Dr. Yun Ding and Dr. Uttama Rath. Dr. Yun Ding contributed the figure of microtubule overlay assay and Dr. Uttama Rath contributed the figure of microtubule spin-down assay in *Drosophila* embryo extract. All other figures are contributed by Changfu Yao. The paper uses a variety of biochemistry methods to explore the possibility of a direct interaction between Chromator and microtubules. Further determination of the interaction status of tubulin (free tubulin or polymerized microtubules) and the functional domain of Chromator that is responsible for the interaction were performed. The potential for a direct interaction between Chromator and free tubulin, Chromator and polymerized microtubules suggests a connection between the spindle matrix complex and the microtubule spindle, which is important for further understanding the function of the spindle matrix during mitosis.

The fourth chapter is a paper published in *Chromosoma* in Apr. 2012 on the analysis of Chromator functions and domain requirements in regulation of *Drosophila* polytene chromosomes structures. Dr. Yun Ding and Dr. Uttama Rath contributed the N-terminal and C-terminal transgenic constructs, the Chro-FL, Chro-NTD and ChroCTD GST fusion constructs as well as the Chromator mutants *Chro*\(^71\) and *Chro*\(^612\). Changfu Yao made all the remaining transgenic and GST fusion constructs. Changfu Yao performed all the tests and made all the figures in the paper. In Chromator
transheterozygous mutant allele, $Chro^{71}/Chro^{612}$, polytene chromosomes show a compacted and coiled morphology. By introducing different Chromator truncated domain transgenic constructs into the mutant background, we found that the N-terminus of Chromator is responsible for Chromator's chromosome localization and function in maintaining chromosome structure at interphase in *Drosophila*. The chromodomain, which could interact with linker histone H1, is required for proper localization and function of Chromator at interphase.

The fifth chapter contains the general conclusions for the work presented in the dissertation, and proposes potential experiments for further dissection of the function of the spindle matrix complex.

**LITERATURE REVIEW**

**Mitosis and microtubule spindle**

Mitosis, the term originally coined from the Greek word for thread by the German anatomist Walther Flemming in the 1880s to reflect the shape of mitotic chromosome (reviewed in Mitchison and Salmon, 2001), is the process in which a mother cell reproduces by duplicating its own contents and dividing into two genetic identical daughter cells. It is one of the most fundamental cellular processes for living organisms to keep proliferation capability. Faithful DNA replication and accurate chromosome segregation, the ultimate goal of mitosis, are accomplished through several well-characterized phases. During cell cycle, after the DNA has replicated in interphase, the
nucleus starts to reorganize. The reorganization leads to chromosome condensation at prophase. During prometaphase, the phosphorylation of lamin triggers nuclear envelope breakdown and the condensed chromosomes start to congress. A highly dynamic microtubule spindle is formed at metaphase; with the chromosomes aligning at its equator. Then the chromatids separate and chromosomes move towards to the opposite spindle poles from anaphase through telophase (reviewed in Martins et al., 2000). The mitotic spindle is a macromolecular complex known to be comprised of polymerized microtubule and various associated motor or non-motor proteins (reviewed in Johansen and Johansen 2007). The proper organization, stabilization and eventual disintegration of the fusiform mitotic microtubule spindle apparatus are essential for the progression of mitosis.

**Microtubule structure and dynamics**

Microtubules are hollow cylindrical tubes of 13 parallel protofilaments, assembled from αβ-tubulin heterodimers arranged head to toe fashion that curve to form a tube. Microtubules show structural polarity on basis of this form of polymerization, with the α-tubulin subunit exposed at the minus end and the β-tubulin subunit exposed at the plus end (Fan et al., 1996; Hirose et al., 1995; Nogales et al., 1999). Lateral interactions between homologous subunits, α–α and β–β, forming a “B lattice” are applied to stabilize the tubular organization of protofilaments (Song and Mandelkow, 1993). The microtubule polymers are highly dynamic due to the status of the guanine nucleotide bound to the β-tubulin subunit of the heterodimers. Both tubulin subunits can bind GTP nucleotides, but only the GTP on the β-tubulin subunit would hydrolyze to
GDP (David-Pfeytay et al., 1977; Spiegelman et al., 1977). When the GTP binds to β-tubulin subunits at the end of a growing polymer (a “GTP-cap”; Carlier and Pantaloni, 1981), this “GTP-cap” tends to stabilize the microtubule and provide a new polymerization site for next heterodimer. However, the GTP eventually hydrolyzes to GDP in a sequential manner after the tubulin heterodimer polymerizes to the microtubule and this nucleotide cannot be exchanged. Therefore, the growing microtubule always has a GTP cap at the end but the main body is GDP-bound-β-tubulin subunits. GDP-bound-β-tubulin heterodimers have less affinity to each other, which tends to be unstable and favors depolymerization (“catastrophe”, Davis et al., 1994). The “catastrophe” situation can be reversed by a “rescue” event with addition of GTP-capped tubulin subunits (Hyman et al., 1992; Mitchison, 1993). The rapid switch between growth (polymerization) and shrinkage (depolymerization) states of the microtubule polymers is the hallmark of “dynamic instability” for which microtubules are known and is a key feature for the reorganization of microtubule networks through the cell cycle (Cassimeris et al., 1987; Mitchison and Kirschner, 1984).

The fusiform-shaped mitotic spindle is the most characteristic change for the microtubule cytoskeletal network during mitosis. The mitotic spindle is anchored by two poles with the more dynamic plus end pointing away from the pole and less dynamic minus end microtubules located at the spindle poles. Within the mitotic spindle, the microtubules are usually categorized into three different classes based on their morphological and functional properties: (i) Astral Microtubules, which radiate from the spindle poles toward the cell cortex and are believed to help determine spindle orientation, positioning and cleavage plane specification. (ii) Kinetochore Microtubules (kMTs),
which typically are bundled into *k-fibers*, originate from spindle pole and attach to the outer layer of the kinetochores. Kinetochores are specific protein complex structures that assemble at the centromere region of the mitotic chromosomes. The k-fibers are believed to be involved in the chromosome congression, alignment and segregation. (iii) Interpolar Microtubules (*ipMTs*), which extend from one spindle pole across the spindle midzone without attaching to the chromosomes and overlap with the microtubules originating from the opposite half spindle in an antiparallel pattern to stabilize the bipolar spindle at prometaphase and metaphase, help spindle pole separation during anaphase (Compton, 2000).

Based on immunofluorescence staining pictures of a metaphase microtubule spindles, the length, size and shape are relatively constant and static in given cell types. However, this observation is somewhat deceptive. A constant pole-ward “tubulin flux” could be observed by photobleaching and photoactivation of fluorescently labeled tubulin subunits, especially using the newly developed technique, “fluorescent speckle microscopy” (FSM) (Mitchison, 1989). By conventional fluorescence imaging technique, which would largely saturate the endogenous microtubules (even only 10% of the content is fluorescently labeled), bundles of k-fiber microtubules are evident whereas the astral microtubules are not because of the high background fluorescence caused by the unfocused microtubules and unincorporated fluorescently labeled tubulin (Waterman-Storer *et al.*, 1998). In contrast with conventional methods, low levels of fluorescently labeled tubulin (less than 0.5%) are applied in the FSM experiment. The background fluorescence is greatly reduced, and the signal cannot incorporate uniformly into the microtubule spindle. This leads to a small fraction of the labeled microtubule forming a
“bar” shape structure and serving as a mark to be recognized on the microtubule over time (Waterman-Storer et al., 1998; Waterman-Storer and Danuser, 2002). The rates of tubulin flux can be measured by following the speckle movements. Judging from the FSM imaging series, the small fluorescently labeled tubulin “speckles” are constantly translocating toward the spindle poles along the microtubule without changing the length of the microtubule. This constant tubulin flux occurs on the kinetochore-microtubules and the interpolar microtubules, but not the astral microtubules. Three activities are required for this process: continuous polymerization at the plus ends near the spindle mid region, depolymerization at the minus ends located in the pole region and poleward translocation of spindle tubulin subunits.

Thus, the microtubule spindle is a highly dynamic structure. And the "dynamic instability" and “tubulin flux” are required for the normal functions of the microtubule spindle: the rapid growth and shrinkage at the microtubule ends are necessary for the kinetochore surface attachment and chromosome capture; the constant poleward tubulin flux is believed to be required for the segregation of chromosomes at anaphase (Maddox et al., 2002).

**Mechanism of spindle assembly**

The mitotic spindle is a transient organizational state of microtubules that provides tracks for precise chromosome movement during segregation (Kapoor & Mitchison, 2001). But how the mitotic spindle is assembled has been a huge challenge for cell biologists, since it is a macromolecular machine comprised of a large group of molecules including the basic structural element (polymerized microtubule), various
associated mitotic motor proteins and regulators (Johansen and Johansen 2007). In addition, all of these functional elements must be regulated coordinately in a precise temporospatial manner to ensure the correct morphology and functions of the mitotic spindle. Two major models are proposed to explain the formation of bipolar spindles on the basis of expansion of knowledge about the molecular participants of the mitotic spindle, development of advanced imaging techniques and mathematical and computational modeling methods. They are the “search and capture” model and the “self-organization” model (Johansen and Johansen 2007).

The “search and capture” model was proposed based on the finding of microtubule “dynamic instability” (Mitchison and Kirschner, 1984). Highly dynamic astral microtubules nucleating from the centrosomes, undergoing multiple rounds of growth and shrinkage, randomly scan the cytoplasm until they become stabilized by “capture” of a chromosome kinetochore (Kirschner and Mitchison, 1986). Centrosomes are comprised of a pair of cylindrical centrioles surrounded by pericentriolar matrix, which contains microtubule nucleating template γ-tubulin ring complex (Scholey et al., 2003). Once the bivalent kinetochore on the sister chromatid is “captured” by the astral microtubules emanating from the opposite pole, the mitotic chromosomes will start to congress and align at the metaphase plate, eventually forming a typical fusiform bipolar spindle (Nicklas and Kubai, 1985). This model could well explain several key features of the mitotic spindle assembly, since it is accounts well for the intrinsic dynamic properties of the microtubules and capability of microtubule nucleation from the centrosomes. However, although centrosomes are required for spindle assembly in some systems (Sluder and Rieder, 1985; Rieder and Alexander, 1990; Zhang and Nicklas, 1995a,b), in
other systems like acentrosomal animal oocytes and higher plant cells, it appears to be dispensable (Steffen et al., 1986; Heald et al., 1996). A different mechanism of spindle assembly must occur in these cells. Thus, an alternative chromosome-directed, "self-organization" pathway was proposed to exist.

In the "self-organization" model, instead of the MTOC, mitotic chromosomes are believed to be the major microtubule nucleation sites. Microtubules are nucleated from the vicinity of chromatin under the control of signals from chromosomes. Then the randomly oriented microtubules are progressively focused by the actions of the motor proteins and other scaffolding proteins to form a bipolar spindle. This hypothesis was supported by evidence from different animal systems that successfully formed a spindle without centrosomes. For example, addition of DNA-coated beads into metaphase arrested Xenopus egg extracts could induce the formation of bipolar spindles (Heald et al., 1996); most mitotic spindles assemble normally in Drosophila DSas-4 or centrosomin (cnn) mutants, in which centrosomes either are lost or functionally disrupted, and mutant flies develop into morphologically normal adults with near normal timing (Megraw et al., 2001; Basto et al., 2006). The small GTPase Ran has been shown to play an important role in promoting microtubule nucleation and stabilization in the vicinity of chromatin by generating a spatial gradient of active Ran-GTP around mitotic chromosomes (Kalab et al., 1999; Wilde and Zheng, 1999; Carazo-Salas et al., 2001). Ran is a Ras GTPase superfamily member, which was identified as an important cofactor of nucleocytoplasmic transportation (Moore and Blobel, 1993). The activation of Ran is dependent on GTP or GDP bound status as is the case for other GTPases. Ran-GTP is restrained inside the nucleus by the guanine nucleotide-exchange factor RCC1, which is associated with
chromatin. RCC1 activates Ran by facilitating the exchange of GDP to GTP on Ran (Bischoff and Ponstingi, 1991; Caudron et al., 2005). On the other hand, in the cytoplasm, RanGTP is hydrolyzed to RanGDP by RanGAP1, a cytoplasmic GTPase-activating protein (GAP) (Clarke and Zhang, 2008). Thus, when the cell enters mitosis, nuclear envelope breaks down; a spatial RanGTP concentration gradient is established surrounding the mitotic chromosomes through the activities of RCC1 and RanGAP1. RanGTP stimulates microtubule nucleation and facilitates spindle formation directly or indirectly by creating a local concentration of microtubule stabilizing regulators that are released from the inhibitory binding of importin-α and -β to promote capture of astral microtubules (Gruss et al., 2001; Nachury et al., 2001). Similarly, the K-fiber stabilization factor HURP and spindle assembly factors such as TPX2 (target protein for Xenopus kinesin-like protein), NuMA (nuclear-mitotic apparatus protein) and XCTK2 (Xenopus COOH-terminal kinesin) are also shown to be released and activated by Ran-GTP through its interaction with importin-β (Trieselmann et al., 2003; Sillje et al., 2006).

The Ran-GTP gradient is not an essential pathway for mitotic spindles assembly in cells containing centrosomes, even though it has been observed in these cells. By injection of dominant-negative regulators of Ran-GTP gradient into centrosome-containing cells, only minimal effect delaying the transition from mono- to bipolar-spindle organization could be observed (Kalab et al., 2006). The “self-organization” model could be more important for the cells without centrosomes such as higher plant cells and eggs. However, it does have importance in cells directed by the “search and capture” model as it might serve as a backup plan for normal spindle assembly or help with the process of “search and capture”. The two models are not mutually exclusive. In
Drosophila S2 cells, although the centrosome-mediated pathway is dominant, by high-resolution imaging revealed that both pathways are operational (Maiato et al., 2004). More recently, a third model for microtubule nucleation in mitotic spindle has been proposed, “a microtubule based microtubule amplification” model, in which existing microtubules in the spindle serve as a template to promote the nucleation and polymerization of microtubules, thereby efficiently amplifying microtubules in the spindle (Zhu et al., 2009; Zheng and Iglesias, 2013). Thus, a “combined model”, in which multiple redundant mechanisms such as centrosome-directed microtubule nucleation, chromosome-mediated microtubule assembly, and recruitment, clustering and sliding, and bundling of microtubules from other sites interact together to give rise to spindle assembly is becoming more favored (Gadde and Heald, 2004; Janson et al., 2007; Mahoney et al., 2006; Wadsworth and Khodjakov, 2004).

Motor proteins

The assembly of the mitotic spindle in eukaryotic cells is a complicated process involving multiple precise organizational forces. For most animal somatic cells, microtubules are mainly nucleated from the centrosomes and chromosomes to form and maintain a normal bipolar spindle at metaphase. However, the protein complexes at the two locations are necessary but not sufficient to assemble the spindle. One of the important missing pieces is the microtubule based motor proteins, which is the source to generate force within the spindle. During mitosis, motor proteins play at least three functions: cross-bridging and sliding microtubules relative to adjacent microtubules or other structures, transporting mitotic cargo along the microtubule fibers, and regulating
microtubule dynamics and spindle length (Sharp et al., 2000a,b; Goshima and Scholey, 2010). Furthermore, multiple motor proteins are known to function co-operatively to create a dynamic balance of complementary and antagonistic forces in parallel, which are essential for spindle assembly and function in multiple systems such as yeast, *Drosophila* embryo and HeLa cell extracts (Sharp et al., 2000a,b; Gadde and Heald, 2004).

Microtubule based motor proteins are a group of well-characterized ATP-dependent force-generating enzymes. Functional studies have shown a remarkable level of conservation among related motors across different species. Two families of molecular motors use microtubules as a track and exert functions in mitosis: the kinesins and the dyneins. Based on the location of the motor domain within the peptide sequence and its function, the mitotic kinesin family members are categorized into three main groups: KIN N, KIN C and KIN I. Members of KIN N all have an N-terminal motor domain and move towards the plus end of microtubules. In contrast, members of KIN C are all minus end directed kinesins with a C-terminal motor domain. Finally, the motor domains of the KIN I kinesins are in the middle of the proteins. Although they have a motor domain, KIN I members are actually not motile. The only subfamily of dyneins that function in spindle assembly and function is cytoplasmic dynein. Together with dynactin as a complex, it works as a homodimer and moves specifically towards the minus end of microtubules (Holzbaur and Vallee, 1994).

Mitotic motors play an important role during different stages of mitosis by functional cooperation. During the transition from interphase to prophase that is characterized by spindle pole separation, bipolar plus-end-directed KIN N kinesins including the BimC/Eg5, the minus-end directed motor KIN C kinesins including Ncd,
and cytoplasmic dynein are all involved in the process as well as further bipolar spindle establishment and maintenance (Goshima and Vale, 2003; Gadde and Heald, 2004; Kwon and Scholey, 2004). The KIN N kinesins form homo-tetramers and motor domains on each end move towards the plus end and slide the overlapping antiparallel microtubules from the two opposite poles (centrosomes) to push them apart. This pole to pole outward force is helped by cortical dynein/dynactin complex motor proteins that slide astral microtubules along cortical actin. Meanwhile the outward forces are antagonized by inward forces generated by minus-end-directed motors including Ncd during the pole separation. At metaphase, the bipolarity and integrity of the spindle is maintained by the cooperation and balance of various motor proteins. The plus end-directed bipolar homo-tetramer N-terminal kinesins like BimC kinesin cross-link and slide antiparallel interpolar microtubule apart to establish spindle bipolarity. Thereby, the length of the antiparallel overlap is reduced and the distance between the two spindle poles is increased. Conversely, the outward force is balanced by the inward force generated by C-terminal kinesins like Ncd, which functions to focus microtubule minus-ends at the spindle pole, CHO1/MKLP1 kinesins and the dynein/dynactin complex (Kuriyama et al., 1994; Waterman-Storer et al., 1995; Sharp et al., 1999; Kwon and Scholey, 2004; Tao et al., 2006, Goshima and Schoely, 2009). The minus-end directed motor proteins cross-link and slide the interpolar microtubule bundles, thus expanding the antiparallel overlap and decreasing the distance between the two poles. Thus, a balance of force together with the balance spindle length is reached by the help of various motor proteins. In addition, Chromokinesin such as Xklp1 also organizes bundles of interpolar microtubules and contributes to the formation of a bipolar array (Kwon et al., 2004).
Chromosome movement within the spindle also requires the participation of motors. During chromosome congression, the initial capture of the microtubules by kinetochores requires the activity of the dynein/dynactin complex. After capture, with the help of plus end directed motor protein CENP-E, k-fibers connect the chromosomes to the metaphase plate. Chromokinesins, a set of chromosome-associated plus-end-directed kinesin motor proteins, also generate a “plateward” or a “polar ejection force”. They localize on the chromosome arms instead of at the kinetochores. They can bind chromosomes as cargo and transport them towards the metaphase plate along the microtubule fibers, thus generating a “plateward” force (Tokai et al., 1996; Molina et al., 1997; Ruden et al., 1997; Sharp et al., 2000b). Destruction of these chromokinesins is required for proper anaphase A chromosome movement.

KIN I (internal catalytic domain) family members are found at the kinetochores and spindle pole. This kind of motor does not show any motility but can directly induce the depolymerization of K-fibers. Work in Drosophila shows more details about Kin I kinesin function in chromosome segregation at anaphase A (Rogers et al., 2004). Two KIN I members, KLP10A and KLP59C, which localize to the spindle poles and kinetochores respectively, were identified in Drosophila embryos. During anaphase A, KLP10A and KLP59C depolymerize microtubules at both ends of k-fibers simultaneously.

Anaphase A was found to utilize a KIN I dependent pacman-flux mechanism in which KLP10A and KLP59C depolymerize microtubules at both ends of kMTs simultaneously. Dynein is proposed to contribute to this mechanism by feeding microtubules into the kinetochore for KLP59C dependent depolymerization in a “feeder
and chipper” model (Sharp et al., 2000 a,b; Gadde and Heald, 2004). Coordinately, the plus-end-anchoring activity of KIN N also contributes to shortening the K-fiber and generating poleward forces. Thus, the combination of both plus- and minus-end depolymerization of microtubules allows chromatid segregation to opposite poles in anaphase A.

**Non-motor microtubule associated proteins**

The mitotic spindle is formed by the metastable microtubule polymers and their associated factors, which include both motor proteins and non-motor proteins. Spindle assembly is a complicated process. The dynamics of the microtubules and force production draws much attention of cytologists. While much effort has been directed to identify proteins that regulate microtubule dynamics and mechanisms understand how they are regulated, recent studies have indicated that a large number of non-motor microtubule-associated proteins (MAPs) also play essential roles in the formation, organization and regulation of the mitotic spindle. It is not surprising that other factors besides microtubules and motor proteins would be involved in the fine-tuning of spindle function, since the assembly and segregation of the mitotic spindle in eukaryotic organisms is a highly coordinated process that is required to faithfully separate the genetic material. The non-motor microtubule associated proteins have been shown to play various functions including structural roles to nucleate and stabilize microtubule organization, and regulatory roles to influence motor functions and cell cycle control (Manning and Compton, 2007). Here I will introduce some of these proteins.

Classical non-motor microtubule associated proteins like Tau and MAP2 bind to
the surface of microtubules, bridge several tubulin subunits and possibly stabilize microtubule by neutralizing the negative charge on the microtubule surface. Another distinct class of the non-motor MAPs are the “plus-end-tracking” proteins or the +TIPs, which include EB1 and the CLASPs. EB1 localizes to the growing plus ends of the microtubules. It autonomously tracks the growing plus ends of the microtubules, recruits additional plus-end-tracking proteins and promotes microtubule growth (Akhmanova and Steinmetz, 2008; Bieling et al. 2007, Rogers et al. 2002). This makes EB1 a master regulator of microtubules plus-end dynamics. In functional analysis in Drosophila S2 cells, EB1 depletion leads to shorter spindle formation (Goshima et al., 2005; Rogers et al., 2002), which indicates it is essential for proper spindle assembly. It is shown to be required for proper spindle positioning during mitosis and localization of other motor proteins at the microtubule plus-end during interphase (Rogers et al., 2002; Mennella et al., 2005). MAPs such as CLIP170, kinesin-14, kinesin-13, Klp10A and CLASP are recruited by EB1 to the microtubule plus ends (Akhmanova &Steinmetz 2008). However, how it promotes microtubules growth still remains an open question (Komarova et al. 2009). CLASP (Orbit/MAST in Drosophila) localize to the growing plus ends of microtubules, stabilizes dynamically unstable interphase microtubules by inducing pauses or rescue events (Galjart 2005; Mimori-Kiyosue et al., 2005; Sousa et al., 2007). During mitosis, CLASP (Orbit) depends on CENP-E to localize to outer kinetochores, where it promotes incorporation microtubule subunits into fluxing kfibers (Maffini et al., 2009; Maiato et al., 2003, 2005). RNAi depletion of CLASP in S2 cells causes spindles to continuously shorten and eventually collapse into monopolar structures, which is antagonized by co-depletion of Klp10A. This suggests that microtubule depolymerization
at the pole is counterbalanced with CLASP-dependent kMT polymerization to control metaphase spindle length (Inoue et al., 2000; Lemos et al., 2000; Goshima et al., 2005b; Laycock et al. 2006).

XMAP215/Stu20/Msps/TOGp family proteins, which contain a TOG domain, are believed to bind tubulin dimers and promote microtubule 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 siRNA depletion of TOGp results in centrosome fragmentation and multipolar spindles with decreased microtubule length and density, suggesting TOGp functions in focusing microtubule minus ends at spindle poles, maintaining centrosome integrity, contributing to spindle bipolarity and counterbalancing the microtubule depolymerization effect by MCAK (kinesin-13 motors, KIN I) (Gergely et al., 2003; Cassimeris and Morabito, 2004).

TPX2 (targeting protein for Xklp2) was originally identified as a MAP which links Xklp2 (Xenopus KIN N motor) to the microtubules. It is a coiled-coil domain protein with protein size 82.4 kDa. Functional orthologs of TPX2 have also been discovered in C. elegans, Drosophila, mouse and human. TPX2 is released by Ran-GTP from the inhibition of importin-α/β, which frees it to promote spindle assembly. TPX2 has been shown to be required for proper spindle formation in vitro using chromatin coated beads in Xenopus egg extract. Depletion of TPX2 from the egg extract leads to disintegration of spindle poles, less microtubule intensity, and sometimes multipolar spindles. It also interacts with Aurora A kinase and Eg5, and is important for proper function of these two proteins. The loss of function phenotype of TPX2 suggests that
TPX2 can directly bundle microtubules in order to maintain the architecture of the spindle pole as well as regulate the microtubule based microtubule amplification pathway (Wittmann et al., 2000; Zheng and Iglesias, 2013).

HURP (Hepatoma Up-Regulated Protein) was identified as a cancer-related marker for detecting transitional cell carcinoma through a functional genomic screen for mitotic regulators in HeLa cells. Its functional orthologs have been found in all model system from S.cerevisiae, C. elegans, Drosophila, Xenopus to mouse. HURP was found to be part of a Ran-GTP–regulated spindle assembly factor complex that includes TPX2, Aurora A, XMAP215 and Eg5. During mitosis, it is released by Ran-GTP from the inhibition by importin-β, and co-localizes with the mitotic spindle, especially kMTs, in a concentration gradient manner that increases towards the chromosome (Koffa et al., 2006). In vitro spindle assembly experiments show that HURP is a direct microtubule binding partner and enhances microtubule polymerization. Spindle check point proteins such as Mad2 and BubR1 are recruited to the kinetochores and activated when HURP is depleted by RNAi in HeLa cells due to the persistence of unaligned chromosomes and tension decrease across the sister kinetochores on aligned chromosomes. This indicates that HURP is important for efficient capture of kinetochores from K-fibers and maintaining the proper inter-kinetochore tension that is crucial for the initiation of anaphase (Wong and Fang, 2005).

NuSAP (Nucleolar Spindle-Associated Protein) is a 55 kDa, basic protein that is upregulated in proliferating mitotic cells. It contains an N-terminal SAP domain, a helix-extension-helix motif that is proposed to be involved in organizing nuclear architecture by binding MARs (AT-rich nuclear Matrix Attachment Regions of the DNA) and/or
RNA (Aravind and Koonin, 2000), and a C-terminal–charged helical domain. Immunostaining shows that NuSAP localizes in the nucleoli during interphase; it redistributes to the central spindle surrounding the chromosomes and also colocalizes with the microtubule spindle during metaphase. NuSAP is shown to be able to directly bind to microtubules via its C-terminal domain in an in vitro microtubule spin down assay. Overexpression of NuSAP results in unusually long, curved and highly bundled microtubules in the interphase cytoplasm (Raemaekers et al., 2003). Under this condition, the microtubules are extremely stable, even in the presence of nocodazole. When NuSAP expression level is reduced, the cell shows delayed mitotic entry and defects in chromosome condensation, chromosome alignment, spindle organization and cytokinesis. NuSAP is also regulated by Ran-GTP in a complex manner. Its cross-linking activity needs to be released from inhibition by importin-β, while its microtubule stabilization activity needs to be released from the blockage by importin-α and -7. Its high concentration in the vicinity or on the chromosomes indicate a function for of NuSAP in targeting microtubule nucleation to the chromosomes and assisting in maintaining bipolar spindle integrity through its microtubule stabilizing and crosslinking activities (Ribbeck et al., 2006, 2007).

In addition, interphase nuclear components may also be involved in regulation of mitosis. Nuclear envelope breakdown is the mark of the beginning of prophase and the whole nucleus starts to reorganize, from disassembly to reassembly during mitosis. During the process of this reorganization, several key structural components are found in the mitotic spindle such as nuclear pore complex members. Nuclear pore complexes (NPCs) have long been believed to be responsible for the molecular traffic between the
nucleoplasm and the cytoplasm (Lei and Silver, 2002; Fahrenkrog and Aebi, 2003). NPCs may also be involved in controls the spatial orientation and transcriptional activity of chromatin (Pai and Corces, 2002). The Nup107-160 complex in human cells localizes to prometaphase spindle poles, kinetochores and proximal spindle fibers. Depletion of the complex results in quick disassembly of nucleated microtubules, leaving only a small number of bipolar spindles with less intensity of microtubules and largely unattached mitotic chromosomes (Orjalo et al., 2006). FG-Nups (nucleoporins with multiple FG-repeats) including Nup153 (Katsani et al., 2008), Nup214 (Hashizume et al., 2010) and Nup358 (RanBP2) (Joseph et al., 2002) are also been localized to the spindle. In the absence of Nup358, chromosome congression and segregation are severely perturbed (Salina et al., 2003). Disruption of Nup214 localization causes formation of multipolar spindles and chromosome separation defects (Hashizume et al., 2010). This suggests that these nucleoporins not only function at the nuclear pore complex at interphase, but also play important roles in mitosis to promote spindle assembly.

The dynamics of microtubules and the function and activity of mitotic regulators must be strictly regulated temporally and spatially during the cell cycle. In order to account for this purpose, efforts have been directed to identify proteins that are responsible for the regulation, such as the mitotic kinases and phosphatases. By genome wide screening in Drosophila S2 cells by RNAi, at least 80 kinases and 22 phosphatases have been found to be involved in cell cycle progression and/or mitosis (Bettencourt-Dias et al., 2004; Chen et al., 2007), including two well studied kinase families: Aurora kinases and Polo kinase.
The Aurora kinase family belongs to the serine/threonine kinase family, which is conserved through eukaryotic systems. Three homologs, Aurora A, B and C, are present in mammals, two homologs, Aurora A and B, in other species including Drosophila, Xenopus and C elegans, while only one is found in yeast. Aurora A and B are essential for mitosis and may play a role in tumorigenesis (Fu et al., 2007). Aurora A localizes to the spindle poles during the whole process of mitosis and provides structural and regulatory roles for centrosome maturation and spindle pole organization. Centrosome Aurora A may recruit CNN, TACC/MAP215 and γ-tubulin to the centrosome for its maturation and promote the formation of a functional "microtubule organization center" (Ducat and Zheng, 2004). TPX2 binds Aurora A and shields it from protein phosphatase I to maintain Aurora A in its active form so it can exert a spindle assembly function by phosphorylating unknown substrates (Kufer et al., 2002; Tsai et al., 2003). Aurora B is part of the chromosome passenger complex. It localizes to the centromeres before anaphase and relocalizes to the spindle midzone, which is the future cleavage furrow from anaphase until the cytokinesis. Aurora B has a variety of substrates including histone H3, CENP-A, INCENP, MCAK (mitotic centromere associated kinesin), Myosin II regulatory light chain and Survivin (Murnion et al., 2001; Bishop and Schumacher, 2002; Wheatley et al., 2004; Andrews et al., 2004). Aurora B phosphorylates and associates with INCENP and Survivin into a complex, which in turn enhances Aurora B kinase activity (Bishop and Schumacher, 2002; Honda et al., 2003; Andrews et al., 2003). Localization of the CENP-E, dynein, MCAK and Dam-1 complex to the centromere and kinetochore regions depends on the activity of Aurora B (Adams et al., 2001; Gassman et al., 2004; Maiato et al., 2004). The interaction and feedback loop provides a mechanism
linking Aurora B to proper chromosome bi-orientation, alignment and segregation. Furthermore, Aurora B depletion results in deactivation of spindle checkpoint activity with lower concentration of Mad2 and BubR1 at kinetochores (Hauf et al., 2003).

The Polo like kinases (Plk) are another large serine/threonine kinase family required for spindle assembly (Sumara et al., 2004; Glover, 2005). Polo, the first Plk, was identified in Drosophila through mutant screening to identify genes that affect spindle pole behavior. Currently, four Plk members have been identified in mammalian cells (Plk1, Plk2/Snk, Plk3/Fnk/Prk, and Plk4/Sak), three in Xenopus (Plx1-3), and one in Drosophila and yeast. In Drosophila, Polo localization is dynamic and cell cycle dependent. During mitosis, Polo is enriched at the centrosome as well as at kinetochores, the midspindle and throughout the nucleocytoplasmic space of mitotic cells (Barr et al., 2004). Monopolar spindles, spindles with broad poles, or multipolar spindles were observed in Drosophila Polo mutants, suggesting it functions in centrosome maturation and spindle assembly (Sunkel and Glover, 1988). Polo kinase may also be responsible for recruitment of the γ-tubulin ring complex to the centrosome by phosphorylating and activating the abnormal spindle protein (Asp) (do Carmo Avides et al., 2001). Polo is also involved in anaphase-promoting complex or cyclosome (APC/C) activation (Eckerdt and Strebhardt, 2006) although the detailed mechanism is still unknown. Polo kinase provides essential temporal and spatial functions and regulation required for centrosome maturation, mitotic entry, chromosome condensation and alignment, bipolar spindle assembly, metaphase to anaphase transition and promotion of cytokinesis. Although evidence indicates that Cdk1, MAPK and PKA might potentially phosphorylate Polo
kinase (Plk1 in mammalian cells) for its activation, the detailed regulatory mechanism and upstream regulators of Polo kinase are still elusive (Weerdt and Medema et al., 2006).

**Spindle Matrix**

Much work has been directed to understand the spindle assembly and function, and numerous models have been proposed for force generation (Mitchison and Salmon, 2001; Scholey et al., 2001; Wittmann et al, 2001; Kapoor and Compton, 2002; Bloom, 2002; Gadde and Heald, 2004). However, none of those models can really fully account for force generation (Scholey et al., 2001; Sharp et al, 2000). For example, the different microtubule-associated motor proteins are believed to be the main force generators. These motor proteins, such as bipolar kinesins, move along a microtubule track carrying another adjacent microtubule fiber as cargo, thus causing a microtubule-microtubule sliding. But the question is how these motor proteins generate forces against the microtubule tracks which themselves are also in a highly dynamic state. Based on theoretical considerations of the requirement for force production at the spindle, the concept of a spindle matrix has been put forward (reviewed in Pickett-Heaps et al., 1982, 1997; Wells, 2001; Johansen and Johansen, 2002, 2007, 2009; Johansen et al 2011). In its simplest formulation a spindle matrix complex would provide a relatively stable or elastic molecular matrix that interacts with and stabilizes the microtubule based spindle apparatus (including both microtubule and motor proteins) during force production (Pickett-Heaps et al., 1997; Johansen and Johansen 2007).

Evidence for the existence of a putative spindle matrix has accrued by numerous experiments in different organisms. A non-microtubule “spindle remnant” was yielded in
the absence of microtubules by different extraction protocols (Goldman and Rebhun, 1969). Furthermore, rather than the early accepted “PAC-MAN” model, which proposed chromosome segregation is powered by the disassembly of kMTs at the kinetochore, in living crane-fly spermatocytes and newt lung epithelia cells, the chromosomes still continually moved to the spindle poles even after k-fibers were severed in the middle of the half-spindle by UV-microbeam irradiation and even after k-fiber disassembly (Sillers and Forer, 1983; Spurck et al., 1997; Pickett-Heaps et al., 1997). These results suggest that the force for chromosome poleward motion is not produced only by kMTs but rather acts on them; the full integrity of kMTs is not required for movement. In metaphase PtK2 cells, mitotic spindle was cut across by laser microbeam, resulting in movement of spindle poles toward the spindle equator. The cut side pole moved first, the other pole moved later, ultimately forming a shorter symmetric spindle. The cut side pole movement towards the equator caused intervening microtubules to bend and buckle. Since there were no detectable microtubules within the ablation zone, the movement of the spindle pole might be due to restraining forces generated by the spindle matrix which embeds the spindle (Sheykhani et al., 2013). In *Xenopus* by using the “fluorescent speckle microscopy” technique, the mitotic kinesin Eg5 was found to be static within the spindle while microtubules exhibit constant polewards flux (Kapoor and Mitchison, 2001). This static behavior was still observed after adding monastrol to inhibit Eg5 motility, which would rule out the possibility that Eg5 moved to the opposite direction of tubulin flux at the same speed. This result suggests that either Eg5 could be static itself or it could interact with a certain static matrix structure.

However, among the “suspicious clouds” around the spindle matrix model, the
major concern for many years was the inability to identify the direct molecular components of a putative spindle matrix. Molecules which could be candidates for a spindle matrix complex would be expected to exhibit several characteristics: 1) they should associate together and form a fusiform structure co-aligned with the microtubule spindle, 2) the fusiform structure should be independent with microtubule spindle, 3) perturbation of one or more components of the spindle matrix complex should affect spindle assembly and/or function, 4) one or more components of the complex should interact with microtubule or microtubule associated molecules (building up the connection between spindle matrix and spindle) (Johansen and Johansen, 2007).

The coiled-coil protein NuMA, which has been shown to be necessary for proper spindle formation and function (Merdes et al., 1996; Dionne et al., 1999), is capable of self-assembly and can form multiarm oligomers by interaction of its C-terminal globular domains in vitro. It also forms an extensive filamentous network with dynamic microtubules in cytoplasm when over-expressed in HeLa cells (Saredi et al., 1996; Gueth-hallonet et al., 1998; Harborth et al., 1999). NuMA is essential for bipolar spindle formation and forms a pericentriolar matrix which possibly provides a static site for microtubules minus ends to anchor. A more extensive member of the spindle matrix complex is the human nuclear pore complex protein TPR, which forms a relatively stable fusiform spindle-like structure during mitosis and can spatially regulate the recruitment of the spindle assembly checkpoint protein Mad2 and Msp1 to unattached kinetochores (Lince-Faria et al., 2009). In Aspergillus nidulans, the TPR homologue Mlp1 similarly serves as a mitotic scaffold and spatially regulates Mad2 localization and function (De Souza et al., 2009). In yeast, the TPR homologue Mlp2 binds directly to core components
of the spindle pole body (SPB) and is required for proper SPB function and normal cell division (Niepel et al., 2005). Lamin B3 (the major lamin B isoform in Xenopus) was found localized to the spindle and peripheral region surrounding the spindle and is required for spindle assembly. In Nocodazole-treated normally assembled spindles, the MTs depolymerized completely but Lamin B3 still remained spindle-associated (Tsai et al., 2006). Mitchison et al. (2005) suggested that there should be an unidentified tensile element that acts in parallel with conventional microtubule lattice factors to generate spindle shortening forces on the base of microtubule destabilization experiments in Xenopus egg extracts. Poly (ADP-ribose) was proposed to be the molecular candidate for such an internal matrix in this system. Approximately 10-fold higher levels of PAR were found at the mitotic spindle than in the surrounding cytoplasm during mitosis. When PAR polymerase levels were enzymatically decreased or functionally blocked with purified anti-PAR antibodies, the microtubule spindle rapidly broke down, microtubules splayed outwardly and the two half-spindles became disconnected (Chang et al., 2004). Thus the existence of a putative spindle matrix has been consistently proved in a wide range of experimental observations in different organisms. However, a definitive correlation of structure with function is still lacking.

In Drosophila, our laboratory has recently identified four nuclear proteins, Skeletor, Chromator, Megator and EAST (Walker et al., 2000; Rath et al., 2004; Qi et al., 2004; 2005; 2009, Ding et al., 2009) as a complex of putative spindle matrix candidates. They interact with each other and redistribute during prophase, forming a fusiform spindle structure that persists in the absence of polymerized tubulin(Walker et al., 2000; Qi et al., 2004). Skeletor encodes an 81kD protein that was identified by screening an
expression library to isolate a nuclear antigen with an intriguing dynamic redistribution pattern during mitosis in Drosophila syncytial embryos (Walker et al., 2000). At interphase, Skeletor is localized on the chromosomes (interband region of polytene chromosomes). During metaphase, Skeletor forms a fusiform spindle-like structure and colocalizes with the microtubule spindle during metaphase. During anaphase, the fusiform spindle-like structure extends from end to end when chromosomes segregation occurs. At telophase, Skeletor still can be observed as a spindle remnant in the midregion even when chromosomes start to decondense. Striking evidence for Skeletor to be a putative spindle matrix candidate is from nocodazole treatment experiments in Drosophila embryos. When microtubule spindles were depolymerized by nocodazole, the Skeletor defined spindle like structure persisted but in a compressed state surround the mitotic chromosome (Walker et al., 2000). This phenotype indicates that the Skeletor-defined spindle-like structure is a relatively independent structure from the microtubule spindle, but its spindle-like shape requires the mitotic spindle as a scaffold. Line scan across the half spindle, measuring fluorescence intensities of tubulin and Skeletor stained by antibodies, reveals that the peaks of tubulin labeling are notably distinct from the peaks of Skeletor labeling (Johansen et al., 2011). Thus, all the properties displayed by Skeletor make it an excellent molecular candidate for spindle matrix complex component. However, based on secondary structure prediction, Skeletor is a low-complexity protein without any conserved or known functional domains. Thus, it is more likely Skeletor is a nonstructural member of the spindle matrix complex and some other protein may provide the structural framework.
The second candidate is Chromator, which was identified by yeast two-hybrid experiments using part of the Skeletor ORF as bait. Chromator encodes a predicted 101 kDa novel Drosophila protein with a predicted chromodomain at its N-terminus. Chromator largely colocalizes with Skeletor during the cell cycle. The difference between Chromator and Skeletor localization is during anaphase and telophase, where rather than localizing to the spindle remnants, Chromator strongly localizes to the midbody and centrosomes. Either by RNAi experiments in S2 cells or mutational analysis, decreasing the expression level of Chromator resulted in severe defects of spindle assembly and chromosome segregation (Rath et al., 2004, 2006; Ding et al., 2009). Additionally, RNAi depletion of Chromator in S2 cells causes failure of recruitment of spindle checkpoint protein Mad2 to the unattached kinetochore. A live imaging approach using fluorescently tagged Chromator-GFP and α-tubulin-mCherry was initiated to assess the dynamics of both microtubules and matrix proteins during formation of the mitotic spindle. The result showed that Chromator dissociates from the chromosomes as they begin to condense to fill the entire nuclear space, and there is no obvious spindle-like morphology until the microtubules begin invading the nuclear space. The observation suggests that the Chromator-defined spindle matrix exists as a malleable gel-like structure, and its spindle-like appearance is a reorganization in response to the incoming microtubules (Johansen et al., 2011).

Megator, the Bx34 antigen, is a nuclear rim and nuclear extra chromosomal-localized protein that was identified in the search for potential structural elements of the spindle matrix. Megator is the Drosophila homolog of the mammalian nuclear pore complex protein-TPR and encodes a 260 kDa protein with a large NH2-terminal coiled-
coil domain and a shorter COOH-terminal acidic region (Zimowska et al., 1997). By antibody labeling in Drosophila embryos, Megator shows a specific mitotic redistribution pattern. Megator reorganizes into a spindle-like structure from late prophase, maintains its spindle-like structure from prometaphase to anaphase, and goes to the spindle midbody at telophase. The Megator-defined spindle persists and co-aligns with the Skeletor spindle in the absence of a microtubule spindle, as shown when Qi et al. (2004) used cold treatment to disassemble microtubules in metaphase Drosophila embryos. This result indicates that the Megator- and Skeletor- defined spindle is independent from the microtubule spindle. Live imaging of colchicine treated S2 cells expressing Tubulin-GFP and Megator-mcherry showed that when microtubule is depolymerized, the Megator-defined spindle-like structure compresses and shrinks to surround the mitotic chromosomes without any signal loss. FRAP experiments showed little or no exchange of Megator signal between the spindle and cytoplasm, nor exchange between two spindles in the unusual case when there are two spindles in the same cell (Lince-Faria et al., 2009). Taken together the information, these results suggest that the Megator-defined spindle matrix structure is a relatively static, elastic gel-like structure. It needs the microtubule spindle to serve as a scaffold to stretch out from pole to pole to form its spindle-like structure. When the outward force from the scaffold is lost, based on its intrinsic elasticity, the Megator defined gel-like structure shrinks back to the middle, surrounding the mitotic chromosomes. The large N-terminal coiled-coil domain may form the structural scaffold for the spindle matrix since the expression of N-terminal coiled-coil domain itself without a nuclear localization signal in S2 cells; it can self-assemble into spherical structures in the cytoplasm. Homozygous mutants of Megator are
embryonic lethal indicating that Megator is an essential gene during development. However, RNAi of Megator did not cause major spindle defects in S2 cells, but resulted in a poorly defined metaphase plate and $\sim 15\%$ faster progression into mitosis due to the failure to recruit SAC protein Mad2 and Mps1 to unattached kinetochores. Therefore, considering all the information, Megator is also an important gene during mitosis (Qi et al., 2004; Lince-Faria et al., 2009).

EAST is another large protein that has been identified in the putative spindle matrix complex in *Drosophila*. EAST encodes a 265 kD protein which shows an interchromosomal localization in the interphase nucleus. Loss of function mutations showed a high frequency of mitotic errors and abnormal chromosome congression in prometaphase (Wasser and Chia, 2000; 2003). Co-immunoprecipitation assay in S2 cells shows that EAST and Megator are in the same complex. Furthermore, EAST also shows a specific redistribution pattern during mitosis and co-localizes with Megator forming a fusiform spindle like structure from prometaphase to anaphase. The only difference between EAST localization and Megator localization happens in telophase. During telophase, unlike Megator which localizes to the spindle midbody, EAST redistributes back to the forming daughter nuclei (Qi et al., 2005). The colocalization and interaction between EAST and Megator strongly suggests that EAST could be a potential candidate of spindle matrix. The evidence from different species supports that a spindle matrix may be a general feature of mitosis.

However, many questions remain to be addressed, including how the spindle matrix affects the spindle assembly, what other mitotic functions the spindle matrix may have and so forth. The function of such a spindle matrix complex still remains elusive. As
we hypothesized: 1) the spindle matrix complex could function to stabilize the microtubule spindle apparatus and assist in force production as originally envisioned for a spindle matrix; 2) the spindle matrix may serve a general role in physically confining and organizing cell cycle factors in the spindle region in organisms with open or semi-open mitosis; 3) the viscoelastic properties of the matrix may constrain spindle length; 4) it may facilitate microtubule dynamics and chromosome segregation; 5) it may help exclude organelles and vesicles from the spindle region; 6) it may function as a "spindle matrix passenger protein complex" ensuring equal distribution to the daughter nuclei of essential proteins that for structural reasons are difficult to degrade or resynthesize and reassemble on a rapid time scale; 7) it may interact directly with the microtubule-based spindle apparatus (eg. Chromator); 8) it may have different combinations all of the above functions.

Advantages of using *Drosophila melanogaster* as a model system

Other than a tractable genetic model system, *Drosophila melanogaster* is also a powerful research tool for studying mitosis and spindle matrix components. The first thirteen nuclear cycles of *Drosophila* embryonic development are synchronous divisions occurring in a syncytium without cell membranes. From nuclear cycles 10-13, nuclei form a monolayer on the surface of the embryo, providing as many as 5,000 geometrically related examples of nuclear structures in a single embryo well suited for time-lapse imaging. During this stage, due to less of stringent quality control checkpoints than found in later stages of the *Drosophila* life cycle that would cause a delay in mitotic progression due to defects in chromosome alignment during metaphase, a more direct
effect of different cell cycle related components can be observed for inhibition studies. *Drosophila* larval neuroblasts, cultured S2 cells and oocytes provide different spindle machinery composition for different types of experiments to eventually identify common core mechanisms. The larval salivary glands provide an excellent tool to study the distribution of chromosomal proteins. Furthermore, the entire genome with 165 million base pairs and an estimated 14,000 genes has been completely sequenced and annotated. Molecularly defined P-element transposon insertions and deletions that span the entire genome are available for study. Other powerful tools, including inhibitor microinjection, genome-wide RNA interference (RNAi), genome-wide *in situ* analysis and genome-wide yeast two-hybrid assays greatly facilitate the identification and characterization of unknown genes involved in mitosis (Spradling *et al.*, 1995; Miklos and Rubin, 1996; Celniker, 2000; Celniker and Rubin, 2003; Kwon and Scholey, 2004).
CHAPTER 2. A NUCLEAR-DERIVED PROTEINACEOUS MATRIX EMBEDS THE MICROTUBULE SPINDLE APPARATUS DURING MITOSIS

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ABSTRACT

The concept of a spindle matrix has long been proposed; however, whether such a structure exists and its molecular and structural composition has remained controversial. In this study using a live imaging approach in Drosophila syncytial embryos we demonstrate that nuclear proteins reorganize during mitosis to form a highly dynamic, viscous spindle matrix that embeds the microtubule spindle apparatus, stretching from pole-to-pole. We show that this "internal" matrix is a distinct structure from the microtubule spindle and from a Lamin B containing spindle envelope, and by injection of 2000 kDa dextrans, that the disassembling nuclear envelope does not present a diffusion barrier. Furthermore, when microtubules are depolymerized with colchicine just prior to metaphase the spindle matrix contracts and coalesces around the chromosomes suggesting that microtubules act as "struts" stretching the spindle matrix. Additionally, we demonstrate that the spindle matrix protein Megator requires its coiled-coil amino-terminal domain for spindle matrix localization suggesting that specific interactions

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between spindle matrix molecules are necessary for them to form a complex confined to the spindle region. The demonstration of an embedding spindle matrix lays the groundwork for a more complete understanding of microtubule dynamics and of the visco-elastic properties of the spindle during cell division.

**INTRODUCTION**

During cell division the entire nucleus undergoes a dramatic reorganization as the cell prepares to segregate its duplicated chromosomes. For many years the prevailing view in organisms possessing an open mitosis has held that the nucleus completely disassembled during early mitotic stages, thus enabling cytoplasmic microtubules emanating from the separated centrosomes to form a mitotic spindle. This cytocentric view largely discounted any nuclear contributions to the formation and/or function of the mitotic spindle (Johansen and Johansen, 2009; Simon and Wilson, 2011; Sandquist et al., 2011). However, in *Drosophila* we have recently identified two nuclear proteins, Chromator (Rath et al., 2004; Ding et al., 2009; Yao et al., 2012) and Megator (Qi et al., 2004; Lince-Faria et al., 2009), from two different nuclear compartments that interact with each other and that redistribute during prophase to form a molecular complex that persists in the absence of polymerized tubulin (Johansen et al., 2011). Chromator is localized to polytene chromosome interbands during interphase (Rath et al., 2004; 2006; Yao et al., 2012) whereas Megator occupies the nuclear rim and the intranuclear space surrounding the chromosomes (Zimowska et al., 2007; Qi et al., 2004). Chromator has no known orthologs in other species; however, Megator is the homolog of mammalian Tpr
(Zimowska et al., 1997). The Megator/Tpr family of proteins is highly conserved through evolution and structural homologs are present from yeast to humans (De Souza and Osmani, 2009). Moreover, in addition to Megator the Aspergillus Mlp1 and human Tpr spindle matrix proteins have been demonstrated to have a shared function as spatial regulators of spindle assembly checkpoint proteins during metaphase (Lee et al., 2008; De Souza et al., 2009; Lince-Faria et al., 2009). Both Chromator and Megator are essential proteins required for normal mitosis to occur in Drosophila (Qi et al., 2004; Lince-Faria et al., 2009; Ding et al., 2009). These findings suggest that these proteins are molecular components of the hitherto elusive spindle matrix that based on theoretical considerations of the requirements for force production has been proposed to help constrain and stabilize the microtubule-based spindle apparatus (Pickett-Heaps et al., 1982; Pickett-Heaps and Forer, 2009). Here we demonstrate that this nuclear-derived "internal" spindle matrix is a highly dynamic, self-contained structure that embeds the microtubule spindle apparatus from pole to pole. The findings further suggest that the spindle matrix may directly contribute to the visco-elastic micromechanical properties (Shimamoto et al., 2011) of the spindle.

RESULTS

The spindle matrix embeds the microtubule spindle apparatus

Figure 1 shows time lapse imaging of Chromator-GFP and tubulin-mCherry during mitosis in syncytial Drosophila embryos. The results show that Chromator has reorganized away from the chromosomes as they begin to condense and fills the entire
nuclear space prior to microtubule invasion (Fig. 1A). As spindle microtubules form, Chromator distribution attains a spindle-like morphology while also translocating to the centrosomes (Fig. 1A). At anaphase and telophase Chromator dynamics closely mirror that of the microtubules before relocating back to the chromosomes in the forming daughter nuclei. This dynamic behavior of Chromator during mitosis is very different from MAPs such as Jupiter (Karpova et al., 2006). While Chromator is present throughout the spindle its poleward boundary does not extend all the way to the centrosome (Fig. 1B) as has also been observed for the putative spindle pole matrix protein NuMA (Radulescu and Cleveland, 2010). Interestingly, in linescans of pixel-intensity across the spindle we found that peak-intensities of the MAP Jupiter coincide with that of microtubules, indicating co-localization (Figs. 1C, E), whereas peak-intensities of Chromator are notably distinct from those of microtubules and in many cases show an alternating pattern (Figs. 1D, G). Moreover, pixel intensities in linescans across the spindle for Jupiter-GFP and tubulin-mCherry were strongly correlated (Pearson's r = 0.73±0.10, n=17) (Fig. 1F) whereas pixel intensities in linescans of Chromator-GFP and tubulin-mCherry showed little correlation (Pearson's r = 0.32±0.07, n=17) (Fig. 1H). Taken together these observations are consistent with the hypothesis that the Chromator-defined spindle matrix is part of a viscous, gel-like structure that embeds the microtubule-based spindle apparatus. Furthermore, the findings suggest that while this matrix forms independently of microtubules, its morphology and dynamic behavior during mitosis is governed by microtubule spindle dynamics.

To further test this hypothesis we depolymerized tubulin by injecting colchicine into embryos expressing GFP-Chromator and tubulin-mCherry or histone H2Av-RFP
prior to prophase (Fig. 2). Under these conditions Chromator still relocates from the chromosomes to the matrix (Figs. 2 A, B); however, in the absence of microtubule spindle formation the Chromator-defined matrix did not undergo any dynamic changes but instead statically embedded the condensed chromosomes for extended periods (>20 min). The movement observed within the matrix is caused by Brownian motion of the chromosomes. Interestingly, Chromator under these conditions still relocated to the centrosomes suggesting that this is a microtubule-independent process. Control embryos injected with vehicle only underwent normal mitosis indistinguishable from wild-type preparations. Moreover, as illustrated in Fig. 2C unpolymerized tubulin accumulates within the nuclear space as measured by relative average pixel intensity to 1.6±0.2, n=12 (from 5 different preparations) times the levels outside the nuclear space in the colchicine injected embryos (see also Figs. 2A, C ). This finding suggests the presence of one or more tubulin binding proteins within the spindle matrix.

The nuclear envelope and lamin B do not contribute to the internal spindle matrix

*Drosophila* embryos have semi-open mitosis where the nuclear envelope (NE) initially breaks down only in the region of the centrosomes and NE breakdown and dispersal of nuclear lamins such as Lamin B (Lamin Dm0 in *Drosophila*) is not completed until just before the end of metaphase (Stafstrom and Staehelin, 1984; Paddy *et al.*, 1996; Civelekoglu-Scholey *et al.*, 2010). This raises the question whether the NE or the nuclear lamina presents a diffusion barrier during the early stages of mitosis and thus may contribute to the confinement of spindle matrix proteins. To test whether this is the case we injected fluorescein labeled 70, 500, or 2000 kDa molecular mass dextrans,
that are up to 10 times the molecular mass of the spindle matrix proteins Chromator and Megator, into tubulin-mCherry expressing embryos treated with colchicine. The results showed that all three molecular mass dextrans entered the nuclear space after NE breakdown on approximately the same time scale as tubulin-mCherry (Figs. 3, 4) indicating the absence of any significant diffusion barriers to spindle matrix proteins. Furthermore, in colchicine injected embryos Lamin B disperses within 2 min on a similar time scale to that of uninjected embryos (Fig. 5) and does not accumulate in the nuclear space. In contrast, the Chromator-defined matrix persists around the chromosomes for at least 10 times longer. Taken together these findings suggest that the Chromator-defined "internal" spindle matrix is a distinct and independent structure from both the microtubule-based spindle apparatus and from the Lamin B containing spindle envelope previously described in Xenopus egg extracts (Zheng 2010) and that the spindle matrix is held together by cohesive molecular interactions within the matrix.

70 and 500 kDa dextrans incorporate into the spindle matrix

Interestingly, we noted that 70 and 500 kDa dextrans accumulated within the nuclear space in a way similar to tubulin in colchicine injected embryos as illustrated in Fig. 3 for 500 kDa dextran. This suggested that branched macromolecular polysaccharides can be incorporated into the spindle matrix. To further explore this possibility we injected fluorescein-conjugated 70, 500, and 2000 kDa dextrans into tubulin-mCherry expressing embryos without colchicine treatment. As exemplified in Fig. 4A for 70 kDa dextran, both 70 and 500 kDa dextrans accumulate in the nuclear space prior to microtubule spindle formation, and its dynamics during mitosis until the
end of telophase where it gets excluded from the forming daughter nuclei closely resembles that of the spindle matrix proteins Chromator and Megator. In contrast, although the 2000 kDa dextran did enter and equilibrate within the nuclear space at the time of NE breakdown, it did not show any enrichment within the spindle region (Fig. 4B). We speculate that this difference between 70 and 2000 kDa dextrans is due to potential size exclusionary properties of the spindle matrix. These data provide additional support for the concept of a viscous matrix made up of macromolecules enriched in the spindle region by cohesive interactions.

The amino-terminal region of Megator is required for its spindle matrix localization

Megator is a large 260 kDa protein (Mtor-FL) with an extended amino-terminal coiled-coil domain (Mtor-NTD) and an unstructured carboxy-terminal domain (Mtor-CTD). Coiled-coil domains are known protein interaction domains as has been previously demonstrated for the spindle pole matrix protein NuMA (Radulescu and Cleveland, 2010). Therefore, to explore whether Megator's coiled-coil domain is required for Megator's spindle matrix localization we conducted time-lapse imaging of full-length YFP-tagged Megator (Mtor-FL), GFP-tagged Mtor-CTD, and GFP-tagged Mtor-NTD together with histone H2Av-RFP in syncytial embryos (Fig. 6). As illustrated in Fig. 6A, Mtor-FL localizes to the nuclear interior as well as the nuclear rim at interphase and to the spindle matrix at metaphase. In contrast, Mtor-CTD, which contains the native nuclear localization signal (NLS), is diffusively present in the nucleoplasm without detectable nuclear rim localization at interphase and is absent from the spindle region at metaphase (Fig. 6B). Mtor-NTD is present at the nuclear rim with no or very
little interior nuclear localization but relocalizes to the spindle matrix at metaphase (Fig. 6C). The localization patterns of Mtor-FL, Mtor-NTD, and Mtor-CTD at interphase are illustrated at higher magnification in Fig. 6D. These data suggest that the amino-terminal coiled-coil domain of Megator is required for localization to both nuclear pore complexes as well as to the spindle matrix, whereas Megator's carboxy-terminal domain facilitates Megator's interchromosomal localization during interphase. Furthermore, if microtubules are prevented from forming by colchicine injection prior to prophase both Mtor-FL and Mtor-NTD still relocate to the spindle matrix and, as with the Chromator-defined matrix, do not undergo any dynamic changes but statically embeds the condensed chromosomes (Fig. 6E). In contrast, under these conditions Mtor-CTD disperses on a rapid time-scale in less than 2 min after NE breakdown (Fig. 6E). These findings provide further evidence that the cohesiveness of the spindle matrix depends on specific molecular interactions among the spindle matrix proteins.

**Depolymerization of microtubules at metaphase collapses but does not disassemble the spindle matrix**

In order to test the dependence of the spindle matrix on microtubule dynamics we injected colchicine into Chromator-GFP and tubulin-mCherry expressing embryos during metaphase. As shown in the image sequence of Fig. 7, as the microtubules undergo depolymerization the Chromator-defined matrix contracts and coalesces around the chromosomes. The reduction in the length of the spindle matrix was almost 60% from when the first image was obtained after colchicine injection to when microtubules were depolymerized (Fig. 7B). This suggests that the spindle matrix is stretched by the
microtubules. A similar result has been obtained in S2 cells expressing the spindle matrix protein Megator (Lince-Faria et al., 2009) suggesting the properties of the spindle matrix described here are a general feature of mitosis and not confined to only syncytial nuclei. Furthermore, the expectation would be that if microtubules were stabilized at metaphase instead of depolymerized that the shape and form of the spindle matrix would not change. To test this prediction we injected the microtubule stabilizing agent taxol into Mtor-FL and tubulin-mCherry expressing embryos during metaphase, under these conditions both the spindle matrix and microtubules do not undergo any dynamic changes but maintain their metaphase fusiform spindle morphology for extended time periods of greater than 14 min.

Discussion

In this study we have shown that at least two proteins from different nuclear compartments reorganize during mitosis to form a spindle matrix that embeds the microtubule spindle apparatus and that is likely to be part of a molecular complex stretching from pole to pole. As also indicated by previous experiments in S2 cells (Lince-Faria et al., 2009) our present observations are not compatible with a rigid matrix structure but rather with a highly dynamic viscous matrix made up of protein polymers forming a gel-like meshwork. For such a matrix to be stretched infers that components of the matrix physically be linked to microtubules and that changes to the shape and form of the matrix in turn are governed by microtubule dynamics. One possible mechanism to
accomplish this is exemplified by NuMA which together with dynein functions as a spindle pole matrix that tethers and focuses the majority of spindle microtubules to the poles largely independently of centrosomes (Dumont and Mitchison, 2009; Radulescu and Cleveland, 2010). Thus, we propose that a spindle pole matrix may be a constituent of a larger pole-to-pole matrix that couples this matrix to microtubule dynamics.

In *Xenopus* egg extracts it has been suggested that a membranous Lamin B-containing envelope derived from the nuclear membrane could be part of the spindle matrix (Tsai *et al.*, 2006; Zheng 2010). However, our findings clearly demonstrate that the "internal" matrix as defined by the Chromator and Megator proteins is physically distinct from such a structure and that the internal matrix persists after dispersal of Lamin B in nuclei arrested at metaphase. Nonetheless, the interplay between microtubules, the spindle matrix, and NE dynamics during mitosis is likely to be finely tuned and mutually dependent (Zheng 2010). For example, evidence has been provided that the NE and Lamin B in systems with semi-open mitosis may contribute to the robustness of spindle function and assembly during prometaphase and that the gradual disassembly of the Lamin B envelope is coupled to proper spindle maturation during metaphase (Civelekoglu-Scholey *et al.*, 2010).

In this study we present evidence by injection of high molecular weight dextrans that the disassembling NE and nuclear lamina after their initial breakdown, are not likely to present a diffusion barrier to most known proteins. Interestingly, even in the absence of such a diffusion barrier we show that free tubulin (possibly as a/b-tubulin dimers) accumulates co-extensively with the spindle matrix protein Chromator in colchicine-treated embryos independently of tubulin polymerization. We propose that this
enrichment is dependent on one or more proteins within the spindle matrix with tubulin binding activity. A similar enrichment within the nuclear region of free tubulin after NE breakdown has recently been reported in *C. elegans* embryos (Hayashi *et al.*, 2012). The enhanced accumulation of free tubulin within the nascent spindle region may serve as a general mechanism to promote the efficient assembly of the microtubule-based spindle apparatus (Hayashi *et al.*, 2012) and be mediated by spindle matrix constituents. The accumulation of tubulin in the nucleus under microtubule depolymerization conditions is not a general property of cytoplasmic proteins as exemplified by the dynactin complex component DNC-1 in the nematode (Hayashi *et al.*, 2012).

A surprising finding of the present study is that non-proteinaceous polysaccharide macromolecules such as dextrans have the ability to be incorporated into the spindle matrix. However, the results of previous studies have shown that the spindle pole protein NuMA is highly poly(ADP-ribosyl)ated (Radulescu and Cleveland, 2010) and that poly(ADP-ribose) is required for spindle assembly and function in *Xenopus* (Chang *et al.*, 2004). Thus, it is possible that the size, branching, and charge distribution of such polymeric carbohydrate modifications of spindle matrix proteins may play a role in regulating its assembly and function. Furthermore, these modifications may contribute directly to the visco-elastic properties of the spindle and contribute to the modulation of microtubule dynamics and spindle stabilization.

An issue for the spindle matrix hypothesis has been to account for its molecular composition and structure especially as the number and diversity of its possible constituents has grown (reviewed in Johansen *et al.*, 2011). In *Drosophila*, in addition to Megator and Chromator, the nuclear proteins Skeletor, EAST and Mad2 have been
demonstrated to be associated with the spindle matrix (Walker et al., 2000; Qi et al., 2005; Katsani et al., 2008; Lince-Faria et al., 2009; Ding et al., 2009). Another candidate nuclear spindle matrix protein that relocates to the spindle region during mitosis in a microtubule-independent manner is the nucleoporin, Nup107 (Katsani et al., 2008). Thus, it is becoming clear that during mitosis many disassembled components of interphase nuclear structure do not simply disperse but rather reorganize, making important contributions to mitotic progression (De Souza and Osmani, 2009; Johansen and Johansen, 2007; 2009; Simon and Wilson, 2011). For example, many nuclear pore complex constituents in addition to Megator/Tpr and Nup107 have been demonstrated to relocate to the spindle region in both invertebrates and vertebrates (reviewed in refs. De Souza and Osmani, 2009; Johansen et al., 2011). Interestingly, certain nuclear pore proteins have been shown to form a three-dimensional polymer meshwork with hydrogel-like properties within the nuclear pore (Frey et al., 2006). If as suggested here the spindle matrix is a similar gel-like assembly of weakly associated protein polymers its exact stoichiometry and composition may not be critical and it likely would be able to accommodate the inclusion of a wide array of proteins. However, it is important to note that not all nuclear proteins relocate to the spindle matrix during mitosis. For example, both Lamin B and C (Paddy et al., 1996; Katsani et al., 2008) disperse as does the nucleoporin Nup58 (Katsani et al., 2008). Furthermore, in this study we demonstrate that the amino-terminal coiled-coil region of Megator is required for its spindle matrix localization during mitosis whereas the carboxy-terminal region disperses. In future experiments it will be of interest to determine the nature of the specific molecular interactions that governs which proteins are incorporated into the matrix.
Regardless of the exact composition and structure of the spindle matrix, the demonstration here of a self-contained macromolecular structure embedding the spindle apparatus during mitosis will have important implications for our understanding of microtubule dynamics (Dumont and Mitchison, 2010). Furthermore, in a recent study of the micromechanical properties of the metaphase spindle, the effective viscosity of the spindle region was measured to be about 100 times higher than in the surrounding cytoplasm (Shimamoto et al., 2011). This difference was attributed largely to the actions of motor and non-motor proteins crosslinking microtubules with the assumption of negligible contributions from the spindle medium. However, the results of this study suggest that a gel-like spindle matrix is likely to directly contribute to the visco-elastic mechanical properties of the spindle.

**MATERIALS AND METHODS**

*Drosophila melanogaster stocks and transgenic flies.* Fly stocks were maintained according to standard protocols (Roberts 1998) and Canton S was used for wild-type preparations. Full-length GFP-tagged Chromator constructs under native or *GAL-4* promoter control have been previously characterized (Ding et al., 2009). Tubulin-mCherry, Jupiter-GFP, and Lamin-GFP fly stocks (stock 25774, 6836, 7378) and a *tubulin-GAL-4* driver line (stock 7062) were obtained from the Bloomington Stock Center. The Megator YFP-trap fly line (*w[1118]; PBac[602.P.SVS-1]Mtor[CPT1001044]*) was obtained from the Kyoto Stock center (stock 115129). The *H2AvDmRFP1*
transgenic line was the gift of Dr. S. Heidmann and has been previously described (Deng et al., 2005). For the Megator-CTD construct under native promoter control a genomic region of 949 nucleotides upstream and 9 nucleotides downstream of the ATG start codon was PCR amplified and fused with an in frame GFP-tag as well as with Megator carboxy-terminal coding sequence corresponding to residues 1758-2347 and inserted into the pUAST vector using standard techniques (Sambrook and Russell, 2001). For the Megator-NTD construct under native promoter control the same upstream region as for the Mtor-CTD construct was fused with an in frame GFP-tag, with Megator amino-terminal coding sequence corresponding to residues 1-1757, and with the NLS from Clontech's NLS-pECFP vector and inserted into the pPFHW vector (Murphy, 2003) using standard techniques (Sambrook and Russell, 2001). Transgenic Mtor-CTD and Mtor-NTD fly lines were generated by P-element transformation by BestGene Inc. Fly lines expressing combinations of transgenes were generated by standard genetic crosses.

Timelapse confocal microscopy and injections. Timelapse imaging of the fluorescently-tagged constructs in live syncytial embryos were performed using a Leica TCS SP5 tandem scanning microscope or an Ultraview spinning-disk confocal system (Perkin Elmer) as previously described (Ding et al., 2009). In short, 0-1.5 h embryos were collected from apple juice plates, and aged 1 h. The embryos were manually dechorinated, transferred onto a cover slip coated with a thin layer of heptane glue, and covered with a drop of Halocarbon oil 700. Timelapse image sequences of a single z-plane or of z-stacks covering the depth of the mitotic apparatus were obtained using a Plan-apochromat 63X 1.4 NA objective. For colchicine injections, colchicine (Sigma
Aldrich) was dissolved in DMSO to a concentration of 100 mg/ml as a stock solution. The final concentration of colchicine for injection was 1 mg/ml by diluting the stock solution with PEM buffer (80mM Na-PIPES pH 6.9, 1 mM MgCl₂, 1mM EGTA, 5% Glycerol). Injections of approximately 100-200 pl of 1 mg/ml of colchicine into each embryo were performed with a Narishige Programmable Microinjector IM 300 system connected to the Leica confocal TCS SP5 microscope system as previously described (Brust-Mascher and Scholey, 2009). For taxol injections, approximately 100-200 pl of 20 mg/ml of taxol (Sigma Aldrich) in DMSO was injected into each embryo. Control injections were performed with DMSO alone or with PEM buffer with 1% DMSO. Fluorescein labeled 70, 500, or 2000 kDa molecular mass dextrans (Invitrogen) were injected into syncytial embryos using standard methods (Brust-Mascher and Scholey, 2009).

**Image quantification and analysis.** Image processing and quantification were carried out with the ImageJ 1.45 software (NIH) or with Photoshop (Adobe). Quicktime movies were generated with Apple Quicktime Pro 7.6.6. Scatter plots, average pixel intensities of regions of interest, and determination of Pearson's Correlation Coefficient of the measured fluorescence intensity of line scans generated in ImageJ were performed and calculated using Microsoft Excel (Microsoft Corporation).
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FIGURE LEGENDS

Figure 1. Confocal timelapse analysis of Chromator-GFP during mitosis in syncytial Drosophila embryos. (A) Relative dynamics of Chromator-GFP (in green) and tubulin-mCherry (in red) during a complete mitotic cycle. Scale bar, 10 mm. (B) Chromator-GFP at metaphase. Arrowheads indicate the gap between Chromator-GFP's spindle matrix and centrosomal localization. Scale bar, 10 mm. (C) Relative localization of Jupiter-GFP (in green) and tubulin-mCherry (in red) at metaphase. Scale bar, 5 mm. (D) Relative localization of Chromator-GFP (in green) and tubulin-mCherry (in red) at metaphase. Scale bar, 5 mm. (E and G) Linescan plots of pixel intensity across the spindle along the white lines in (C) and (D) for Jupiter-GFP/tubulin-mCherry and Chromator-GFP/tubulin-mCherry, respectively. The images in (C) and (D) are both from a single confocal optical plane. The asterisks indicate the likely position of microtubule K-fibers. (F and H) Plots of the correlation between pixel intensity between Jupiter-GFP/tubulin-mCherry and Chromator-GFP/tubulin-mCherry across the spindle along the white lines in (C) and (D), respectively. The regression line and the value of Pearson's coefficient are indicated for each plot.

Figure 2. Spindle matrix dynamics after colchicine injection prior to nuclear envelope breakdown. (A) Two image panels from the beginning and end of a timelapse sequence of Chromator-GFP (in green) and tubulin-mCherry (in red) after colchicine injection. (B) Two image panels from the beginning and end of a timelapse sequence of Chromator-GFP (in green) and histone H2Av-RFP (in red). (C) Plot of the average pixel
intensity in regions of interest (ROIs) outside the nucleus (in red) and inside the nucleus (in blue) as a function of time in a colchicine injected embryo. The two image inserts correspond to the area outlined by a white boxes in (A) prior to and after NE breakdown, respectively. The ROIs are indicated by white squares. The difference in expression levels of Chromator-GFP in (A) and (B) is due to use of high and low expression driver lines, respectively.

**Figure 3.** 500 kDa dextran enters and accumulates in the nuclear space on the same time-scale as tubulin in colchicine-injected embryos. (A) Image panels from a timelapse sequence from a tubulin-mCherry (in red) expressing embryo co-injected with fluorescein labeled 500 kDa molecular mass dextrans (in green) and colchicine. Time is in s. Scale bar, 10 mm. (B) Plot of the normalized average pixel intensity in regions of interest (ROIs) outside the nucleus and inside the nucleus of tubulin (in red) and 500 kDa dextran (in green) as a function of time in a colchicine injected embryo. The solid and stippled lines correspond to areas inside and outside a nucleus, respectively, as outlined by the white boxes in (A). The approximate time of NE breakdown is indicated by an arrow.

**Figure 4.** 70 kDa but not 2000 kDa dextrans incorporate into the spindle matrix during the cell cycle. (A) Image panels from a timelapse sequence from a tubulin-mCherry (in red) expressing embryo injected with fluorescein labeled 70 kDa molecular mass dextrans (in green). (B) Image panels from a timelapse sequence from a tubulin-mCherry (in red) expressing embryo injected with fluorescein labeled 2000 kDa
molecular mass dextrans (in green). Time is in min and s. Scale bars, 10 mm.

**Figure 5.** Lamin B in colchicine injected embryos disperses on a similar time scale to uninjected embryos during mitosis. (A) Image panels from a timelapse sequence from a histone H2Av-RFP (in red) and Lamin B-GFP (in green) expressing embryo. (B) Image panels from a timelapse sequence from a histone H2Av-RFP (in red) and Lamin B-GFP (in green) expressing embryo injected with colchicine prior to nuclear envelope breakdown. Time is in min and s. Scale bars, 10 mm.

**Figure 6.** Timelapse analysis of the spindle matrix protein Megator in syncytial embryos. (A) Relative dynamics of full-length Megator-YFP (Mtor-FL) and histone H2Av-RFP (H2Av) during a complete mitotic cycle. The images show their distribution at interphase 1, metaphase, and interphase 2, respectively. The diagram beneath the images shows the domain structure of Megator with the coiled-coil region in black, the CTD in white, and the endogenous nuclear localization signal (NLS) in red. Scale bar, 20 mm. (B) Relative dynamics of a truncated GFP-tagged carboxy-terminal construct of Megator (Mtor-CTD) and histone H2Av-RFP (H2Av) during a complete mitotic cycle. The images show their distribution at interphase 1, metaphase, and interphase 2, respectively. Mtor-CTD is diagrammed below the images. Scale bar, 20 mm. (C) Relative dynamics of a truncated GFP-tagged amino-terminal construct of Megator (Mtor-NTD) and histone H2Av-RFP (H2Av) during interphase and metaphase. Mtor-NTD is diagrammed below the images. Scale bar, 10 mm. (D) The Localization patterns of Mtor-FL, Mtor-NTD, and Mtor-CTD at interphase. Mtor-FL localizes to the nuclear
interior as well as the nuclear rim, Mtor-NTD is present at the nuclear rim with no or very little interior nuclear localization, and Mtor-CTD is diffusively present in the nucleoplasm without detectable nuclear rim localization. (E) Upper panel: three images from a timelapse sequence of Mtor-FL-YFP (in green) and histone H2Av-RFP (in red) after colchicine injection at interphase. Middle panel: three images from a timelapse sequence of Mtor-CTD-GFP (in green) and histone H2Av-RFP (in red) after colchicine injection at interphase. Lower panel: three images from a timelapse sequence of Mtor-NTD-GFP (in green) and histone H2Av-RFP (in red) after colchicine injection at interphase. Time is in min and s. Scale bars, 10 mm.

**Figure 7.** Depolymerization of microtubules at metaphase leads to contraction of the spindle matrix. (A) Two image panels from the beginning and end of a timelapse sequence of Chromator-GFP (in green) and tubulin-mCherry (in red) after colchicine injection. The image sequence begins approximately 30 s after colchicine injection. Scale bar, 10 mm. (B) Image sequence of Chromator-GFP after colchicine injection in the spindle outlined by white rectangles in (A). Time is in min and s. Scale bar, 5 mm.
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ABSTRACT

As shown in Yao et. al (2012a), in colchicine treated interphase embryos free tubulin accumulates co-extensively with the spindle matrix proteins suggesting that this enrichment is dependent on one or more proteins within the spindle matrix with tubulin binding activity. The chromodomain containing protein Chromator is likely to be a candidate. In this study using a variety of biochemistry assays we demonstrate the possibility of a potential direct interaction between spindle matrix protein Chromator and polymerized microtubules or free tubulin. Taxol treated Chro-GFP and Tub-mcherry co-expression Drosophila syncytial embryos shows a co-extensively co-localization of Chromator and polymerized microtubule. Co-immunoprecipitation in S2 cell lysate and cosentimnetation assay in embryo extract showed the possibility of that Chromator may interact with polymerized microtubules in the same complex. The overlay assay and microtubule in vitro assembly and spin down assay further showed the possibility of a direct interaction. Pull-down assays with the presence of colchicines either in S2 cell lysate and direct protein-protein pull-down indicate that not only polymerized microtubules but also the free tubulin could direct interact with Chromator through its ----

A paper to be submitted
middle domain. This tubulin binding activity of Chromator provides support for the hypothesis that reorganization of nuclear proteins into a spindle matrix may play a wider functional role in spatially regulating cell cycle progression factors in conjunction with contributing to microtubule spindle assembly and dynamics.

**INTRODUCTION**

During cell division the entire nucleus undergoes a dramatic reorganization as the cell prepares to segregate its duplicated chromosomes. In *Drosophila* we have identified four nuclear proteins, Skeletor, Chromator, Megator, and EAST from two different nuclear compartments that interact with each other (Walker *et al*., 2000; Rath *et al*., 2004; Qi *et al*., 2004; 2005) and that redistribute during prophase to form a dynamic, gel-like spindle matrix that embeds the microtubule spindle apparatus, stretching from pole-to-pole (Yao *et al*., 2012a). This matrix forms prior to nuclear envelope breakdown and specific interactions between spindle matrix molecules are necessary for complex formation and cohesion (Yao *et al*., 2012a). When microtubules are depolymerized with colchicine just prior to metaphase the spindle matrix contracts and coalesces around the chromosomes suggesting that microtubules act as "struts" stretching the spindle matrix. For such a matrix to be stretched infers that components of the matrix physically be linked to microtubules and that changes to the shape and form of the matrix in turn are governed by microtubule dynamics (Yao *et al*., 2012a). Furthermore, in colchicine treated embryos free tubulin accumulates co-extensively with the spindle matrix proteins (Yao *et al*., 2012a) suggesting that this enrichment is dependent on one or more proteins within
the spindle matrix with tubulin binding activity.

A candidate spindle matrix protein for having tubulin binding activity is the chromodomain containing protein, Chromator, which during interphase is localized to interband regions of chromosomes (Rath et al., 2004). Chromator can be divided into two main domains, an NH₂-terminal domain (NTD) containing the chromodomain (ChD) and a COOH-terminal domain (CTD) containing a nuclear localization signal (Rath et al., 2004). Recently, Yao et al. (2012b) provided evidence that the NTD of Chromator is responsible for correct targeting to chromatin, that it interacts with histone H1, and that the chromodomain is required for these interactions. Interestingly, the studies of Ding et al. (2009) showed that the CTD of Chromator was sufficient for localization to the spindle matrix and that expression of this domain alone could partially rescue Chrom mutant spindle defects. Furthermore, the presence of frayed and unstable microtubule spindles during anaphase after Chromator RNAi depletion in S2 cells indicated that Chromator may directly interact with microtubules (Ding et al., 2009). Therefore, in this study we have explored this hypothesis by performing a variety of biochemical tubulin binding and interaction assays. The results show that a novel amino acid sequence in the CTD of Chromator has the capacity to bind both free and polymerized tubulin.

MATERIALS AND METHODS

Drosophila melanogaster stocks and transgenic flies

Fly stocks were maintained according to standard protocols (Roberts, 1998). Transgenic flies expressing full-length, GFP-tagged Chromator under GAL-4 promoter
control have been previously characterized (Ding et al., 2009; Yao et al., 2012a). Tubulin-mCherry (stock 25774) and a tubulin-GAL-4 driver line (stock 7062) were obtained from the Bloomington Drosophila Stock Center, Indiana University (Bloomington, IN).

**Time-lapse confocal microscopy and injections**

Time-lapse imaging of the fluorescently tagged constructs in live syncytial embryos was performed using a TCS SP5 tandem scanning microscope (Leica, Wetzlar, Germany) as previously described (Yao et al., 2012a). In brief, 0 to 1.5 h embryos were collected from apple juice plates and aged 1 h. The embryos were manually dechorinated, transferred onto a coverslip coated with a thin layer of heptane glue, and covered with a drop of halocarbon oil 700. Time-lapse image sequences of a single z-plane or of z-stacks covering the depth of the mitotic apparatus were obtained using a Plan-Apochromat 63×/1.4 numerical aperture objective. For taxol injections, ∼100-200 pl of 20 mg/ml taxol (Sigma-Aldrich) in DMSO was injected into each embryo using an IM-300 programmable microinjector system (Narishige, Tokyo, Japan) connected to the Leica confocal TCS SP5 microscope system as previously described (Brust-Mascher and Scholey, 2009; Yao et al., 2012a).

**Immunoblot analysis**

Protein lysates were separated by SDS-PAGE and immunoblotted according to standard procedures (Sambrook and Russell, 2001). For these experiments we used the Bio-Rad Mini PROTEAN III system, electroblotting to 0.2 µm nitrocellulose, and using anti-mouse, anti-goat or anti-rabbit HRP-conjugated secondary antibody (Bio-Rad)
Primary antibodies used in this study included Chromator mAbs 6H11 and 12H9 (Rath et al., 2004), anti-GST mAb 8C7 (Rath et al., 2004), and mouse anti-tubulin (Sigma). Antibody labeling was visualized using chemiluminescent detection methods (SuperSignal West Pico Chemiluminescent Substrate or the SuperSignal kit from Pierce). The immunoblots were either digitized using a ChemiDoc-It®TS2 Imager equipped with an epifluorescence attachment (UVP) or with a flatbed scanner (Epson Expression 1680).

Overlay experiments

For the overlay experiments GST-tagged versions of the full-length or truncated Chromator constructs, Chro-FL (1–926), Chro-NTD (1-346), Chro-CTD (329-926), Chro-M (329-600), and Chro-421 (601-926) were generated using standard methods (Sambrook and Russell 2001) and as previously described (Rath et al., 2006). The respective GST fusion proteins and GST only were expressed in BL21 cells and purified over a glutathione agarose column (Sigma-Aldrich) according to the pGEX manufacturer's instructions (Amersham Biosciences). For the overlay interaction assays approximate relative molar ratios of Chro-FL (10 mg), Chro-NTD (6 mg), Chro-CTD (8 mg), Chro-M (6 mg), Chro-421 (6 mg), and GST (2 mg) were fractionated by SDS-PAGE and electroblotted to nitrocellulose. The membrane was subsequently blocked in 5% non-fat dry milk in TBST (TBS with 0.1% Tween-20) for 1 h, washed once in 1% non-fat dry milk in TBST for 15 min, and washed once in PEMF buffer (80 mM Pipes; 2 mM MgCl₂; 0.5 mM EGTA; 25 mM NaF) supplemented with 1 mM GTP. The blot was then incubated with 8 mg/ml purified bovine tubulin (Cytoskeleton) in PEMF buffer
supplemented with 1 mM GTP and 20 mM taxol overnight at room temperature. After being washed twice in PEMF buffer the bound microtubules were detected by standard immunoblot analysis using anti-tubulin antibody. Input proteins were analyzed by SDS-PAGE and immunoblotting with GST antibody. The cDNA sequence for all fusion proteins was verified by sequencing at the Iowa State University DNA Facility.

**Spindown assays**

For in vitro spin down assays microtubules were assembled from 16 mg of commercial bovine brain tubulin monomers (Cytoskeleton) in PEM buffer and stabilized with 20 μM taxol and 2 mM GTP at 37°C for 20 min. The assembled microtubules were then incubated with approximate relative molar ratios of Chro-FL (10 mg), Chro-NTD (6 mg), Chro-CTD (8 mg), Chro-M (6 mg), Chro-421 (6 mg), and GST (2 mg) at room temperature for 30 min. Assembled microtubules and associated proteins were then pelleted by centrifugation at 75,000 rpm for 20 min. For immunoblot analysis the pellet and supernatant were carefully separated, fractionated by SDS-PAGE, immunoblotted and probed with anti-GST and anti-tubulin antibody.

For in vivo spin down assays 0-3 hour embryo protein lysates were prepared as described in Qi et al. (2004) and treated with either 20 μM taxol and 2 mM GTP or with 1 mg/ml nocodazole (Sigma Aldrich). Subsequently the respective lysates were subjected to centrifugation at 75,000 rpm for 20 min. The resulting pellet and supernatant fractions were carefully separated and fractionated by SDS-PAGE, immunoblotted and probed with Chromator mAb 6H11 and anti-tubulin antibody.
Pull-down experiments

For in vitro pull-down assays 2 mg of GST–Chromator fusion proteins or GST protein alone were coupled to glutathione agarose beads (Sigma) and incubated with 2 mg of TRITC labeled commercial bovine brain tubulin monomers (Cytoskeleton) in 500 µl of 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 PMSF, and 1.5 mg aprotinin) overnight at 4°C. Following this incubation colchicine was added to a final concentration of 1 mg/ml to prevent tubulin polymerization. The protein complex coupled beads were washed five times for 10 min each with 1 ml of 2X PBS-T. After separation by SDS-PAGE and immunoblotting TRITC-tubulin was detected by epifluorescence using the UVP imaging system.

For in vivo pull-down assays of native tubulin, Drosophila S2 cell lysate was prepared as described in Yao et al. (2012b). 2 mg of GST–Chromator fusion proteins or GST protein alone were coupled to glutathione agarose beads (Sigma) and incubated with 500 ml of S2 cell lysate at 4°C overnight. The beads were washed five times for 10 min each in 1 ml of ip buffer, and proteins retained on the glutathione agarose beads were analyzed by SDS-PAGE and immunoblotting using anti-tubulin antibody.

Immunoprecipitation assays

For co-immunoprecipitation experiments 5 ml of mouse anti-α-tubulin antibody, 100 ml of mAb 12H9 supernatant, or 5 ml of mAb anti-GST antibody 8C7 was bound to 30 ml protein G-Sepharose beads (Sigma) for 2.5 h at 4°C on a rotating wheel in 300 ml ip buffer. Subsequently antibody-coupled beads or beads only were incubated overnight
at 4°C with 500 ml of S2 cell lysate on a rotating wheel. The beads were washed three times for 10 min each with 1 ml of ip buffer with low-speed pelleting of beads between washes. The resulting bead-bound immuno-complexes were analyzed by SDS-PAGE and immublotting using mAb 6H11 to detect Chromator and anti-tubulin antibody to detect tubulin.

RESULTS

Chromator localization is coextensive with taxol stabilized microtubules

Recently, using timelapse imaging Yao et al. (2012a) followed the dynamics of Chromator localization during the cell cycle in Drosophila syncytial embryos. The results showed that Chromator reorganizes away from the chromosomes as they begin to condense and fills the entire nuclear space prior to microtubule invasion. As spindle microtubules form, Chromator distribution attains a spindle-like morphology while also translocating to the centrosomes. At anaphase and telophase Chromator dynamics closely mirror that of the microtubules before relocating back to the chromosomes in the forming daughter nuclei. In order to further determine the localization and relationship between Chromator and microtubules, we stabilized microtubules by injecting taxol into embryos expressing GFP-Chromator (Chro-GFP) and tubulin-mCherry at metaphase (Fig. 1A; Movie 1) arresting the cell cycle at the metaphase-anaphase transition. At the concentration of taxol injected, microtubule depolymerization was inhibited but not polymerization, leading to an increase of spindle and especially of astral microtubules (Fig. 1A; Movie 1). Under these conditions timelapse imaging showed that the
localization of Chro-GFP became coextensive with that of the microtubules including the growing astral microtubules and that this association was maintained for extended time periods (>30 min). These results indicate that Chromator can be recruited by polymerizing microtubules.

**Chromator interacts with tubulin in in vivo interaction assays**

In order to further probe for a potential in vivo interaction between Chromator and tubulin we performed immunoprecipitation (IP) experiments and a pull-down experiment using S2 cell lysate. For the IP experiments proteins were extracted from S2 cells, immunoprecipitated with tubulin or Chromator antibody, fractionated on SDS-PAGE after the immunoprecipitation, immunoblotted, and probed with antibody to Chromator and tubulin, respectively. Figure 2A shows an example of a tubulin antibody IP experiment labeled by Chromator antibody. Chromator was detected by the antibody both in the lysate as well as in the immunoprecipitate lanes but not in the GST IP control lane. Figure 2B shows an example of a Chromator antibody IP experiment labeled by tubulin antibody. Tubulin was detected by the antibody both in the lysate as well as in the immunoprecipitate lanes but not in the beads only control lane. Furthermore, we performed a pull-down experiment using a full-length Chromator GST-tagged construct (Chro-FL-GST). In the pull-down experiment, Chro-FL-GST was coupled to glutathione-agarose beads, incubated with S2 cell lysate, washed, fractionated by SDS-PAGE, and analyzed by immunoblot analysis using a tubulin specific antibody. A GST protein only pull-down served as control. Whereas the GST only control showed no pull-down activity, Chro-FL-GST was able to pull-down tubulin as detected by tubulin
antibody (Fig. 2C). Taken together these experiments present further evidence for an in vivo interaction between Chromator and tubulin.

To determine whether Chromator interacted with polymerized microtubules we performed spindown assays using lysate from 0-3 h Drosophila embryos under conditions where polymerized tubulin and associated proteins were separated into the pellet fraction and free tubulin into the supernatant fraction. In the experiments embryo lysates were treated with taxol to generate polymerized microtubules or with nocodazole to destabilize microtubules into free tubulin. Subsequently, after ultracentrifugation of the lysates the pellet and supernatant were carefully separated, fractionated by SDS-PAGE, immunoblotted, and probed with Chromator and tubulin antibody. As illustrated in Fig. 3 after taxol treatment and tubulin polymerization into microtubules the majority of both tubulin and Chromator was found in the pellet fraction (lane 2) whereas no detectable tubulin and very little Chromator was present in the supernatant (lane 3). In contrast, after nocodazole treatment and microtubule depolymerization into free tubulin the majority of both tubulin and Chromator was found in the supernatant fraction (lane 5) whereas no detectable tubulin and very little Chromator was present in the pellet (lane 4). The co-precipitation of Chromator and microtubules in these spindown assays strongly suggest that Chromator can interact with microtubules in vivo.

**A region in the carboxy-terminal domain of Chromator binds directly to microtubules**

To further characterize the interaction between Chromator and microtubules and to identify the domain mediating the interaction we performed in vitro overlay assays
with polymerized tubulin of GST-fusion proteins of various regions of Chromator (Fig. 4). We used five GST-fusion proteins covering full-length (Chro-FL), the NH$_2$-terminal domain (Chro-NTD), the COOH-terminal domain (Chro-CTD), and two truncated COOH-terminal domains (Chro-421 and Chro-M) as diagrammed in Fig. 4A. Figure 4C shows Chromator GST-fusion proteins that were fractionated by SDS-PAGE, transferred to nitrocellulose paper, and incubated with 5\,$\mu$g/ml of tubulin polymerized with 20\,$\mu$M taxol and 1 mM GTP. Protein interactions were detected with tubulin antibody. As illustrated in Fig. 4C Chro-FL, Chro-CTD as well as Chro-M were found to interact with tubulin in these assays but not Chro-NTD, Chro-421, or the GST control. Immunoblot analysis of the GST proteins purified in these experiments and detected with GST-antibody showed that similar levels of the GST-fusion proteins were present in the overlay assay (Fig. 4B). Thus, these results indicate that Chromator sequences in the Chro-M domain can directly bind to microtubules.

In order to confirm the above results we also performed in vitro spindown assays. In these experiments microtubules were assembled from bovine tubulin monomers with 20\,$\mu$M taxol and 1 mM GTP and incubated with the different Chromator GST-fusion proteins (Fig. 5). Subsequently, after ultracentrifugation of the samples the pellet and supernatant were carefully separated, fractionated by SDS-PAGE, immunoblotted, and probed with Chromator and tubulin antibody. As illustrated in Fig. 5B all three Chromator GST-fusion proteins containing the M-domain, Chro-FL, Chro-CTD, and Chro-M were found in the pellet fraction but not in the supernatant. In contrast, Chro-421 and Chro-NTD were largely present in the supernatant. Furthermore, almost all the tubulin for all five experimental conditions were present in the pellet. Immunoblot
analysis of each of the input GST fusion proteins probed with anti-GST antibody showed comparable levels of GST fusion proteins in each of the spindown assays (Fig. 5C). Thus, the findings from the spindown assays were identical to those of the overlay assays further confirming a direct interaction of Chromator's M-domain with microtubules.

**Chromator directly interacts with unpolymerized free tubulin**

Yao *et al.* (2012a) recently presented evidence by injection of high molecular weight dextrans into syncytial embryos that the disassembling nuclear envelope and nuclear lamina after their initial breakdown, are not likely to present a diffusion barrier to most known proteins during mitosis. Interestingly, even in the absence of such a diffusion barrier free tubulin (possibly as a/b-tubulin dimers) accumulates co-extensively with Chromator in colchicine-treated embryos independently of tubulin polymerization (Yao *et al.*, 2012a). The level of unpolymerized tubulin enrichment within the Chromator defined matrix in the nuclear space was about 1.6 fold the levels outside the nuclear space (Yao *et al.*, 2012a). Thus, in order to determine whether Chromator has the capacity to interact with free tubulin in addition to microtubules we performed pulldown assays from S2 cell lysate with Chromator-GST fusion proteins under conditions where microtubules were depolymerized by colchicine. In the experiments the different Chromator GST-fusion constructs (Fig. 6A) were coupled to glutathione beads and incubated with 1 mg/ml colchicine treated S2 cell lysate. Bound proteins were washed, fractionated by SDS-PAGE, immunoblotted, and analyzed using a tubulin specific antibody. A GST protein only pull-down served as a control. Whereas Chro-NTD and the GST only control showed no pull-down activity, Chro-FL, Chro-CTD and Chro-M were all able to pull-
down tubulin as detected by tubulin antibody (Fig. 6B). In order to confirm these results we applied the same experimental paradigm except for substituting purified bovine TRITC-labeled tubulin for the S2 cell lysate. Bound proteins were washed, fractioned by SDS-PAGE, blotted, and the blots analyzed for TRITC immunofluorescence. As illustrated in Fig. 6C an identical result to that for tubulin pulldown from S2 cell lysate was obtained. Chro-NTD and the GST only control showed no pull-down activity, whereas Chro-FL, Chro-CTD and Chro-M were all able to pull-down tubulin (Fig. 6C). Gel analysis of each of the input GST fusion proteins labeled with coomassie blue showed comparable levels of GST fusion proteins in each of the pulldown assays (Fig. 6D). Taken together these experiments indicate that the M-domain of Chromator has the capacity to bind to unpolymerized free tubulin as well as to microtubules.

DISCUSSION

The concept of a spindle matrix has long been proposed (Pickett-Heaps et al., 1982; Pickett-Heaps and Forer, 2009); however, whether such a structure exists and its molecular composition and how it may interact with the microtubule-based spindle apparatus has remained controversial (reviewed in Johansen and Johansen, 2002; 2007; 2009; Zheng, 2010; Johansen et al., 2011). In this study using a variety of biochemical assays we show that the spindle matrix protein, Chromator, can directly interact with microtubules as well as with free tubulin. Furthermore, we have mapped this interaction with tubulin to a relatively small stretch of 271 aa in the carboxy-terminal region of
Chromator. This sequence is likely to contain a novel tubulin binding interface since database searches did not find any sequence matches with known tubulin binding motifs. These findings are consistent with the hypothesis that Chromator may serve as a constituent of a viscous gel-like spindle matrix that through its microtubule binding capacity couples this matrix to microtubule dynamics governing the changes to the shape and form of the matrix during mitosis (Yao et al., 2012a).

The microtubule-based spindle apparatus provides a conserved mechanism to segregate chromosomes during mitosis. However, how this process is coordinated with disassembly and reassembly of nuclear structures during mitotic progression is poorly understood (De Souza and Osmani, 2009). It is also not clear how cell cycle regulators and other diffusible molecules are localized and confined to the spindle region in the absence of diffusion barriers following nuclear envelope breakdown (Johansen and Johansen, 2009; Wozniak et al., 2010; Johansen et al., 2011). To begin to address these issues Yao et al. (2012a) depolymerized tubulin by injecting colchicine into syncytial embryos prior to prophase. Under these conditions Chromator still relocated from the chromosomes to the matrix; however, in the absence of microtubule spindle formation the Chromator-defined matrix did not undergo any dynamic changes but instead statically embedded the condensed chromosomes for extended periods. Moreover, unpolymerized tubulin accumulated within the nuclear space relative to the levels outside the nuclear space in the colchicine injected embryos. A similar enrichment within the nuclear region of free tubulin after nuclear envelope breakdown has been reported in C. elegans embryos (Hayashi et al., 2012). Thus, the enhanced accumulation of free tubulin within the nascent spindle region may serve as a general mechanism to promote the
efficient assembly of the microtubule-based spindle apparatus (Hayashi et al., 2012) and
be mediated by spindle matrix constituents. Based on Chromator's ability to bind free
tubulin we propose that Chromator may fulfill such a role in Drosophila.

Moreover, it has recently been demonstrated that Megator and its human homolog
Tpr act as spindle matrix proteins that have an evolutionarily conserved function as
spatial regulators of the spindle assembly checkpoint that ensure the efficient recruitment
of Mad2 and Mps1 to unattached kinetochores in eukaryotes from fungi to humans
during mitosis (Lee et al., 2008; De Souza and Osmani, 2009; De Souza et al., 2009;
Lince-Faria et al., 2009). Taken together with the present demonstration of Chromator's
tubulin binding activity these findings provide support for the hypothesis that
reorganization of nuclear proteins into a spindle matrix may play a wider functional role
in spatially regulating cell cycle progression factors in conjunction with contributing to
microtubule spindle assembly and dynamics. Thus, future studies of Chromator and other
spindle matrix proteins are likely to provide new insights into how cell cycle factors are
physically confined and organized in the spindle region in organisms with open or semi-
open mitosis allowing for spatial and temporal control of mitotic progression and
chromosome segregation.

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FIGURE LEGENDS

**Figure. 1.** Confocal timelapse analysis of transgenically expressed full-length Chromator-GFP (in green) and tubulin-mCherry (in red) in a syncytial Drosophila embryo after injection of taxol at metaphase. The image sequence shows that under these conditions the localization of Chro-GFP became coextensive with that of the microtubules including the growing astral microtubules.

**Figure 2.** Chromator and tubulin immunoprecipitation and pulldown assays. (A) Immunoprecipitation of lysate from S2 cells using tubulin antibody and detected with Chromator antibody. Chromator is detected in the tubulin ip (lane 3) and in the S2 cell
lysate (lane 1) but not in the GST antibody control ip (lane 2). (B) Immunoprecipitation of lysate from S2 cells using Chromator antibody and detected with tubulin antibody. Tubulin is detected in the Chromator ip (lane 3) and in the S2 cell lysate (lane 1) but not in the beads only control (lane 2). (C) A full-length Chromator GST-fusion construct (Chro-FL-GST) pulls down tubulin from S2 cell lysate as detected by tubulin antibody (lane 3). A GST only pull down control was negative (lane 2). Lane 1 shows the position of tubulin in the S2 cell lysate.

Figure 3. Tubulin spindle assays from 0-3 h embryonic lysates. Microtubules were either stabilized with addition of 20 µM taxol and 2 mM GTP (lane 2 and 3) or disassembled by addition of 1 mg/ml nocodazole (lane 4 and 5). Subsequently the respective lysates were subjected to centrifugation at 75,000 rpm for 20 min. The resulting pellet and supernatant fractions were carefully separated and fractionated by SDS-PAGE, immunoblotted and probed with Chromator mAb 6H11 and anti-tubulin antibody. Lane 1 shows migration of Chromator and tubulin from untreated embryonic lysate.

Figure 4. Overlay assay mapping of the Chromator interaction domain with tubulin. (A) Diagram of Chromator indicating the domains to which GST-fusion proteins were made for mapping. (B) Immunoblot of the respective GST fusion proteins and GST only labeled with a GST mAb. (C) In the overlay experiments the Chromator GST-fusion protein constructs and GST only shown in (B) were incubated with taxol stabilized microtubules and interactions detected with tubulin antibody. In these
experiments interactions with Chro-FL, Chro-CTD, and Chro-M were detected (lane 1, 3, and 4) but not with Chro-NTD, Chro-421, and GST (lane 2, 5, and 6). This defined the Chro-M domain as sufficient for mediating interactions with tubulin. The relative migration of molecular weight markers is indicated to the left of the immunoblots in kDa.

**Figure 5.** Spindown assay mapping of the Chromator interaction domain with tubulin. (A) Diagram of Chromator indicating the domains to which GST-fusion proteins were made for mapping. (B-C) In the spindown experiments the Chromator GST-fusion protein constructs were incubated with taxol stabilized microtubules. Assembled microtubules and associated proteins were then pelleted by centrifugation at 75,000 rpm for 20 min. For immunoblot analysis the pellet (B) and supernatant (C) were separated, fractionated by SDS-PAGE, immunoblotted and probed with anti-GST and anti-tubulin antibody. In these experiments Chro-FL, Chro-CTD, and Chro-M were detected in the pellet fraction (B) whereas Chro-NTD and Chro-421 were detected in the supernatant (C). This confirmed the Chro-M domain as sufficient for mediating interactions with tubulin. Tubulin for all five experimental conditions were only detectable in the pellet (B). (D) Immunoblot of the respective GST fusion proteins used in the spindown assays labeled with a GST mAb. The relative migration of molecular weight markers is indicated to the right of the immunoblots in kDa.

**Figure 6.** Chromator directly interacts with unpolymerized free tubulin. (A) Diagram of Chromator indicating the domains to which GST-fusion proteins were made for mapping. (B) Pulldown assays from S2 cell lysate incubated with Chromator-GST
fusion proteins or GST only under conditions where microtubules were depolymerized by colchicine. Bound proteins were washed, fractionated by SDS-PAGE, immunoblotted, and analyzed using a tubulin specific antibody. Whereas Chro-NTD and the GST only control showed no pull-down activity (lane 2 and 4), Chro-FL, Chro-CTD and Chro-M were all able to pull-down tubulin (lane 3, 5, and 6) as detected by tubulin antibody. Lane 1 shows tubulin from untreated S2 cell lysate. (C) Pulldown assays with bovine TRITC-labeled tubulin incubated with Chromator-GST fusion proteins or GST only under conditions where microtubules were prevented from forming by colchicine. Bound proteins were washed, fractionated by SDS-PAGE, immunoblotted, and analyzed for TRITC fluorescence. Whereas Chro-NTD and the GST only control showed no pull-down activity (lane 2 and 4), Chro-FL, Chro-CTD and Chro-M were all able to pull-down tubulin (lane 3, 5, and 6) as detected by tubulin antibody. Lane 1 shows TRITC-tubulin from untreated S2 cell lysate. These experiments defined the Chro-M domain as sufficient for mediating interactions with unpolymerized free tubulin. (D) Immunoblot of the respective GST fusion proteins and GST used in the pulldown assays labeled with a GST mAb. The relative migration of molecular weight markers is indicated to the right of the immunoblots in kDa.
Fig. 4

Fig. 5

Fig. 6
CHAPTER 4. THE CHROMODOMAIN-CONTAINING NH$_2$-TERMINUS OF CHROMATOR INTERACTS WITH HISTONE H1 AND IS REQUIRED FOR CORRECT TARGETING TO CHROMATIN

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ABSTRACT

The chromodomain protein, Chromator, can be divided into two main domains, a NH$_2$-terminal domain (NTD) containing the chromodomain (ChD) and a COOH-terminal domain (CTD) containing a nuclear localization signal. During interphase Chromator is localized to chromosomes; however, during cell division Chromator redistributes to form a macro molecular spindle matrix complex together with other nuclear proteins that contribute to microtubule spindle dynamics and proper chromosome segregation during mitosis. It has previously been demonstrated that the CTD is sufficient for targeting Chromator to the spindle matrix. In this study we show that the NTD domain of Chromator is required for proper localization to chromatin during interphase and that chromosome morphology defects observed in Chromator hypomorphic mutant backgrounds can be largely rescued by expression of this domain. Furthermore, we show that the ChD domain can interact with histone H1 and that this interaction is necessary for correct chromatin targeting. Nonetheless, that localization to chromatin still occurs in

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the absence of the ChD indicates that Chromator possesses a second mechanism for chromatin association and we provide evidence that this association is mediated by other sequences residing in the NTD. Taken together these findings suggest that Chromator's chromatin functions are largely governed by the NH$_2$-terminal domain whereas functions related to mitosis are mediated mainly by COOH-terminal sequences.

INTRODUCTION

The chromodomain protein, Chromator, has multiple functions depending on the developmental context (Rath et al., 2006, Mendjan et al., 2006; Wasser et al., 2007; Ding et al., 2009). During interphase Chromator is localized to interband regions of Drosophila polytene chromosomes (Rath et al., 2004; Gortchakov et al., 2005) and has been demonstrated to interact with other chromosomal proteins such as the zinc-finger protein Z4 (Eggert et al., 2004; Gan et al., 2011) and the histone H3S10 kinase JIL-1 (Rath et al., 2006) and to contribute to the maintenance of polytene chromosome morphology (Rath et al., 2006). However, during cell division Chromator redistributes to form a macro molecular spindle matrix complex together with at least three other nuclear derived proteins Skeletor, Megator, and EAST (Walker et al., 2000; Rath et al., 2004; Qi et al., 2004; 2005). It has recently been proposed that this structure may take the form of a hydrogel-like matrix with viscoelastic properties that contribute to microtubule spindle dynamics and proper chromosome segregation during mitosis (Johansen et al., 2011). Evidence that Chromator may participate in spindle matrix function has been provided by
mutational analysis with two loss-of-function alleles, \textit{Chro}^{71} \text{ and } \textit{Chro}^{612} \text{ (Ding et al., 2009). The analysis showed that neuroblasts from } \textit{Chro}^{71}/\textit{Chro}^{612} \text{ brain squash preparations have severe microtubule spindle and chromosome segregation defects that were associated with a developmental small brain phenotype. Furthermore, time-lapse analysis of mitosis in S2 cells depleted of Chromator by RNAi treatment suggested that the chromosome segregation defects were the results of incomplete alignment of chromosomes at the metaphase plate, possibly due to a defective spindle-assembly checkpoint, as well as of frayed and unstable microtubule spindles during anaphase (Ding et al., 2009).}

Chromator can be divided into two main domains, an NH$_2$-terminal domain (NTD) containing the chromodomain (ChD) and a COOH-terminal domain (CTD) containing a nuclear localization signal (Rath et al., 2004). The studies of Ding et al. (2009) showed that the CTD of Chromator was sufficient for localization to spindles and that expression of this domain alone could partially rescue mutant spindle defects. However, the function of the NTD and whether it plays a role in targeting Chromator to chromatin was not determined. Here we provide evidence that the NTD of Chromator is responsible for correct targeting to chromatin, that it interacts with histone H1, and that the chromodomain is required for these interactions.

**MATERIALS AND METHODS**

**Chromator transgenic constructs**

A Chromator full-length (1-926) construct (FL) was inserted into the pUASP
vector (Brand and Perrimon, 1993) with a N-terminal TAP-tag (3xHA, 3xFlag) and a C-terminal GFP-tag using standard methods (Sambrook and Russell, 2001). The Chromator NTD construct (1–346) and the CTD construct (329-926) in the pUASP vector have been previously described in Ding et al. (2009). The CTD of Chromator contains the endogenous NLS (Rath et al., 2004). The ChD construct (219-277) was cloned into the pUAST vector and included three in-frame NLS sequences cut from the pECFP-Nuc vector (Clontech) followed by in-frame V5- and GFP-tags. The NTD-DChD construct (1-219) was cloned into the pUAST vector and contained three in-frame NLS sequences in addition to an in-frame V5-tag. The fidelity of all constructs was verified by sequencing at the Iowa State University DNA Facility.

**Drosophila melanogaster stocks**

Fly stocks were maintained according to standard protocols (Roberts, 1998). Canton S was used for wild type preparations. The Chromator mutant alleles Chro\textsuperscript{71} and Chro\textsuperscript{612} as well as the transheterozygous Chro\textsuperscript{71}/Chro\textsuperscript{612} allelic combination have been previously described in Rath et al. (2006) and in Ding et al. (2009). Chromator construct pUAST or pUASP transgenic lines were generated by standard P-element transformation (Best-Gene, Inc.), and expression of the transgenes was driven using the hsp\textsuperscript{70-GAL4} (P\{w[+mC]=GAL4-hsp\textsuperscript{70.PB}\}) driver or the Sgs3-GAL4(P\{w[+mC]=Sgs3-GAL4.PD\}TP1) driver (obtained from the Bloomington Stock Center; stocks 5704, and 6870, respectively) introduced by standard genetic crosses. For heat shock experiments, larvae were subjected to 30 minutes of heat shock treatment at 37°C as described previously (Nowak et al., 2003). Balancer chromosomes and markers are described in

**Immunohistochemistry**

Standard polytene chromosome squash preparations were performed as in Cai et al. (2010) using either 1 or 5 minute fixation protocols and labeled with antibody as described in Jin et al. (1999) and in Wang et al. (2001). Primary antibodies used included chicken anti-GFP (Aves Labs), anti-V5 antibody (Invitrogen), anti-H1 antibody (Active Motif), as well as anti-Chromator mAbs 6H11 and 12H9 (Rath et al., 2004). DNA was visualized by staining with Hoechst 33258 (Molecular Probes) in PBS. The appropriate species- and isotype-specific Texas Red-, TRITC-, and fluorescein isothiocyanate-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution) to visualize primary antibody labeling. The final preparations were mounted in 90% glycerol containing 0.5% n-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope, and images were captured and digitized using a Spot CCD camera. Images were imported into Photoshop where they were pseudocolored, image processed, and merged. In some images non-linear adjustments were made to the channel with Hoechst labeling for optimal visualization of chromosomes.

For live imaging of polytene chromosomes third instar larvae salivary glands were dissected and mounted in physiological saline (110 mM NaCl, 4 mM KCl, 2 mM CaCl$_2$, 10 mM glucose, 10 mM HEPES, pH 7.4) as in Deng et al. (2005). In some cases 25-50% glycerol was added to the physiological saline in order to prevent drift of the preparations. The larvae were from transgenic animals carrying the GFP-tagged FL,
NTD, ChD, or CTD expressed in a $Chro^{71}/Chro^{612}$ mutant background. Confocal images were obtained using a Leica confocal TCS SP5 microscope system.

**Immunoblot analysis**

Protein extracts were prepared from whole third instar larvae or in some experiments from dissected salivary glands homogenized in a buffer containing: 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 0.2% NP-40, 2 mM Na$_3$VO$_4$, 1 mM PMSF, 1.5 µg/ml aprotinin. Proteins were separated by SDS-PAGE according to standard procedures (Sambrook and Russell, 2001). 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 III system, electroblotting to 0.2 µm nitrocellulose membrane, and using anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody. In some experiments labeling with anti-tubulin antibody (Sigma-Aldrich) was used as a loading control. Antibody labeling was visualized using chemiluminescent detection methods (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680).

**Overlay Experiments**

For overlay experiments GST-tagged versions of the full-length or truncated Chromator constructs, GST-FL (1-926), GST-NTD (1-346), GST-ChD (219-277), GST-NTD-DChD (1-218), and GST-CTD (329-926) were generated using standard methods.
The respective GST fusion proteins were expressed in BL21 cells and purified over a glutathione agarose column (Sigma-Aldrich) according to the pGEX manufacturer’s instructions (Amersham Biosciences). In addition, a full-length *Drosophila* histone H1 fusion protein with a maltose binding protein-tag (MBP) was generated in the pMAL-c2x vector (NEB), expressed in BL21 cells, and purified over an amylose resin column (NEB) according to the pMAL manufacturer’s instructions (NEB). For the overlay interaction assays, either individually purified bovine histones (Roche Applied Science), *Drosophila* histone H1 (MBP-H1) or MBP-only were fractionated by SDS-PAGE and electroblotted to nitrocellulose. The blots were subsequently incubated with 2 µg of either GST-FL, GST-NTD, GST-ChD, GST-NTD-DChD, or GST-CTD fusion protein overnight at 4°C in PBS with 0.5% Tween 20 and 5% nonfat milk on a rotating wheel. The blots were washed 4 times for 10 min each in 2X PBS with 0.1% Tween 20 (PBS-T), and binding was detected by anti-GST mAb 8C7 (Rath et al., 2004).

In addition, the overlay proteins were separated by SDS-PAGE, electroblotted to nitrocellulose, and visualized by Ponceu S or Coomassie Blue staining (Sambrook and Russell, 2001). The cDNA sequence for all fusion proteins was verified by sequencing.

**Pull-down experiments.**

For *in vitro* pull down assays with the GST-tagged Chromator fusion proteins, a His-tagged (6x) *Drosophila* histone H1 fusion protein (His-H1) as well as a His-tagged control fusion protein (His-JIL-1) containing the NH2-terminal of JIL-1 (1-260) (Jin et al., 1999) were generated in the pET-28a vector (Novagen). The His-H1, His-JIL-1, and Chromator GST fusion proteins were expressed in BL-21 cells. For GST pull down
assays, approximately 3 µg of GST-Chromator fusion proteins or GST protein alone were
coupled to glutathione agarose beads (Sigma) and incubated with 3 µg His-H1 protein 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 five times for 10 minutes each with 1 ml of 2X PBS-T and analyzed by SDS-
PAGE and immunoblotting using anti-H1 antibody (Active Motif). For pull down assays
with His-tagged proteins, 3 µg of His-H1 or His-JIL-1 was bound to Ni-NTA beads
(QIAGEN) and incubated with 3 µg of Chromator GST fusion protein in 500 µl of
immunoprecipitation buffer. The protein complex coupled beads were washed five times
for 10 minutes each with 1 ml 2X PBS-T and analyzed by SDS-PAGE and
immunoblotting using the GST mAb 8C7 (Rath et al., 2004).

For native protein pull down assays, Drosophila S2 cell nuclear extract was
prepared as described in Kusch et al. (2003). For GST pull down assays GST-ChD (2 µg),
GST protein alone (2 µg), or GST-CTD (6 µg) at molar ratios was coupled with
 glutathione-agarose beads and incubated with 500 µl of S2 cell nuclear extract at 4°C
overnight. The beads were washed 5 times for 10 min each in 1 ml of ip buffer, and
proteins retained on the glutathione-agarose beads were analyzed by SDS-PAGE and
immunoblotting using anti-H1 antibody. For pull down assays with His-tagged proteins,
3 µg of His-H1 or 3.5 µg of His-JIL-1 was bound to Ni-NTA beads (QIAGEN) and
incubated with 500 µl of S2 cell nuclear extract at 4°C overnight. The protein complex-
coupled beads were washed five times for 10 minutes each with 1 ml ip buffer and
proteins retained on the beads analyzed by SDS-PAGE and immunoblotting using the
Chromator mAb 6H11 (Rath et al., 2004).

**Co-immunoprecipitation experiments**

For co-immunoprecipitation experiments the GFP-tagged FL, NTD, ChD, and CTD transgenes were each expressed in adult flies using the *hsp70-GAL4* driver. Protein lysate was prepared for each genotype as well as wild-type controls by homogenizing 50 adult flies in 2 ml of ip buffer and collecting the supernatant after centrifugation at 4000 rpm for 10 min. Transgene protein expression in the lysates was verified by SDS-PAGE and immunoblot analysis using a rabbit anti-GFP mAb (Cell Signaling). Immunobeads were prepared by coupling 5 µl of chicken anti-GFP antibody (Aves Lab) to 30 µl of anti-IgY immobilized Agarose beads (Pierce) for 2.5 hours at 4°C on a rotating wheel in 300 µl ip buffer. The antibody-coupled beads were subsequently incubated in 500 µl of fly lysate overnight at 4°C. The protein complex coupled beads were washed five times for 10 minutes each with 1 ml ip buffer, and proteins retained on the beads analyzed by SDS-PAGE and immunoblotting using anti-H1 antibody.

**Modeling**

The structure of the chromodomain of Chromator was modeled with the I-TASSER protein prediction server (Zhang, 2007; Roy et al., 2010) and compared to the crystal structure of *Drosophila* HP1a's chromodomain (PDB ID: 1Q3L). I-TASSER generates models of proteins by excising continuous fragments from Local Meta-Threading-Server multiple-threading alignments and then reassembling them using
replica-exchange Monte Carlo simulations (Zhang, 2008). The comparison and visualization between the model of Chromator's chromodomain and HP1a's chromodomain was processed and rendered by PyMOL.

RESULTS

The NTD is required for correct targeting of Chromator to chromatin.

In order to undertake a structure/function analysis of the Chromator protein we expressed deletion constructs transgenically in flies heterozygous for the two hypomorphic loss-of-function Chromator alleles, Chro\textsuperscript{71} and Chro\textsuperscript{612} as in Rath et al. (2006) and in Ding et al. (2009). The Chro\textsuperscript{71} allele is comprised of a G to A nucleotide change at nucleotide position 402 of the Chromator transcribed sequence that introduces a premature stop codon resulting in a truncated 71 amino acid protein (Rath et al., 2006). The truncated NH\textsubscript{2}-terminal fragment does not contain the chromodomain and Chro\textsuperscript{71} is likely to act as a null allele. Chro\textsuperscript{71} is homozygous embryonic lethal with no first instar larval escapers. The Chro\textsuperscript{612} allele consists of a C to T nucleotide change at nucleotide position 2024 that introduces a premature stop codon resulting in a truncated 612 amino acid protein that retains the chromodomain (Rath et al., 2006) but is missing parts of the COOH-terminal domain important for spindle localization (Rath et al., 2004) and for interactions with Skeletor (Rath et al., 2004) and EAST (Wasser et al., 2007). Chro\textsuperscript{71}/Chro\textsuperscript{612} transheterozygotes survive to third instar larval stages although no larvae have been observed to pupate. Figure 1A and 1B show a comparison of polytene squashes from wild-type and Chro\textsuperscript{71}/Chro\textsuperscript{612} larvae labeled with Hoechst and the
Chromator mAbs 6H11 and 12H9, respectively. mAb 6H11 recognizes an epitope specific to the CTD whereas mAb 12H9 recognizes an epitope specific to the NTD (Rath et al., 2004; 2006; Ding et al., 2009). Whereas wild-type polytene chromosomes show extended arms with a regular pattern of Hoechst stained bands (Fig. 1A and 1B), this pattern is severely perturbed in *Chro*⁷¹/*Chro*⁶¹² mutant larvae (Fig. 1A and B). In the latter preparations band/interband regions were disrupted and the chromosome arms were coiled and condensed (Fig. 1A and 1B) (Rath et al., 2006). The immunoblot of protein extracts from wild-type and *Chro*⁷¹/*Chro*⁶¹² third instar larvae in Fig. 1C demonstrates that no detectable full-length Chromator protein was present in the mutant larvae. However, weak labeling of *Chro*⁷¹/*Chro*⁶¹² mutant polytene chromosomes could occasionally be detected with mAb 12H9 (Fig. 1B).

In order to explore the role of the different Chromator domains in chromosome targeting we expressed five GFP- and/or V5-tagged Chromator UAS P-element insertion constructs transgenically in *Chro*⁷¹/*Chro*⁶¹² mutant animals: a full-length construct (FL), a construct without the COOH-terminal domain (NTD), a construct containing only the COOH-terminal region (CTD), a construct containing the NTD but without the chromodomain (NTD-DChD), and a construct with the ChD only (Fig. 2). In these studies a *hsp70-GAL4* or a *SgG3-GAL4* driver line was used. As previously reported (Ding et al., 2009) expression of a full-length Chromator construct in the *Chro*⁷¹/*Chro*⁶¹² mutant background rescued all aspects of the mutant phenotype studied including lethality, microtubule spindle morphology, brain and salivary gland size, and polytene chromosome morphology.

As a first approach to determine which domain of Chromator is responsible for
localization to chromatin we expressed GFP-tagged FL, NTD, ChD, and CTD constructs in a Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant background and obtained confocal images from live polytene nuclei. As illustrated in Fig. 3 the FL and NTD localized to the polytene chromosomes in a banded pattern, whereas the localization of the ChD, while clearly present on the chromosomes, was more diffuse. In contrast, the CTD was found exclusively in the intra-nuclear space surrounding the chromosomes. These findings suggested that Chromator's affinity for chromatin is mediated by sequences in the NTD. To further explore this possibility at higher resolution we expressed the ChD and the NTD-DChD in addition to the FL, NTD, and CTD in the Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant background and prepared polytene chromosome squash preparations labeled with Chromator, GFP, or V5 antibody to identify the constructs as well as with Hoechst. As illustrated in Fig. 4 expression of the FL rescued all aspects of the mutant polytene chromosome morphology and the localization of the FL to interband regions was indistinguishable from that of native Chromator in wild-type preparations (Fig. 1) as also previously reported for a full-length Chromator construct under native promoter control (Ding et al., 2009). Interestingly, the NTD construct also substantially rescued polytene chromosome morphology although rescue was not complete with some remaining coiled regions of the chromosome arms (Fig. 4). It should be noted that the NTD unlike the FL did not rescue any aspects of the reduced size of brains, imaginal disks, or salivary glands. However, localization of the NTD to a majority of interband polytene chromosome regions was clearly discernable. In contrast, while both the Chd and the NTD-DChD localized to chromatin no distinct banding pattern was apparent and there was no or very little improvement in the mutant polytene chromosome morphology (Fig. 4). For comparison,
the CTD showed little or no chromatin binding affinity and there was no rescue of the mutant polytene chromosome morphology (Fig. 4). These findings confirm the results from the imaging of live salivary gland nuclei that the NTD is largely responsible for correct targeting of Chromator to chromatin and further indicate that sequences from both the ChD and the NTD-DChD contribute to this localization.

**The chromodomain of Chromator interacts with histone H1.**

The above polytene chromosome localization studies of the various Chromator domains indicated that both the ChD and the NTD-DChD may have the ability to bind to chromatin. Major constituents of chromatin include the linker histone H1 and the histones H2A, H2B, H3, and H4 that together with DNA form nucleosomes (reviewed in Khorasanizadeh 2004). We therefore used overlay assays to test for interactions between these chromatin components and the various Chromator domains. For the screening we used GST-tagged versions of the Chromator constructs diagrammed in Fig. 2. In the overlay assays purified bovine histones were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with glutathione agarose bead-purified GST-FL, GST-NTD, GST-ChD, GST- NTD-DChD, and GST-CTD, respectively. Protein interactions were detected with a mAb to GST. As illustrated in Fig. 5A to 5F we found that the FL, NTD, and ChD, but not the NTD-DChD or the CTD, could specifically interact with bovine histone H1 in these assays. Taken together these results indicate that the chromodomain of Chromator can interact with bovine histone H1. However, histone H1 is the least phylogenetically conserved histone and to verify the interaction we made a MBP-tagged *Drosophila* histone H1 construct. As illustrated in Fig. 5G and 5H, overlay
assays of the MBP-H1 construct performed as described above demonstrated that the Chromator chromodomain can interact with *Drosophila* histone H1. It should be noted that the very weak interaction of NTD-DChD with bovine H1 in Fig. 5E was not present with native *Drosophila* H1 (Fig. 5G) and was therefore likely to be non-specific.

To further confirm the physical interaction of Chromator's chromodomain with histone H1, we performed *in vitro* pull down experiments using a His-tagged histone H1 (His-H1) construct together with ChD-GST. Whereas CTD-GST or GST alone was not able to pull down His-H1, ChD-GST pulled down a band corresponding to the size of His-H1 (Fig. 6A). In the converse experiment, His-H1 was able to pull down ChD-GST using Ni-NTA-beads whereas a His-tagged construct of the NH$_2$-terminal domain of JIL-1 (Jin *et al*., 1999) or beads alone were not (Fig. 6B). These results support the existence of a direct physical interaction between Chromator's chromodomain and histone H1.

In order to explore whether the physical interaction of Chromator with histone H1 was physiological, we performed pull down experiments with the His-H1 and ChD-GST constructs using S2 cell nuclear lysate (Fig. 6C and 6D). Whereas CTD-GST or GST alone was not able to pull down histone H1, ChD-GST pulled down a band corresponding to the size of histone H1 in the nuclear lysate as detected by anti-H1 antibody (Fig. 6C). In the converse experiment using Ni-NTA-beads, His-H1 was able to pull down a band corresponding to the size of Chromator in the nuclear lysate as detected with Chromator mAb 6H11 (Fig. 6D). In control lanes with a His-tagged construct of the NH$_2$-terminal domain of JIL-1 (Jin *et al*., 1999) or beads alone no bands were detected (Fig. 6D). These results support the existence of a physical interaction between Chromator and histone H1.
To further confirm the physiological interaction we performed ip experiments from flies expressing each of the four GFP-tagged transgenes FL, NTD, CTD, and ChD using GFP antibody. Protein lysate from 50 adult flies was prepared for each genotype as well as wild-type controls. Transgene protein expression in the lysates was verified by SDS-PAGE and immunoblot analysis using a rabbit anti-GFP mAb (Fig. 7A). The ChD and CTD were robustly expressed at comparable levels whereas the relative levels of FL and NTD were lower. In the ip experiments with anti-GFP-antibody from these lysates we found that a 38 kd protein band detected by H1 antibody also present in lysate of wild-type flies (Fig. 7B, lane 1) was immunoprecipitated from lysate of flies expressing the FL, NTD, and ChD, but not from lysate of flies expressing the CTD (Fig. 7B, lane 3-6). Furthermore, this 38 kD band was not present in anti-GFP antibody ips from lysates of wild-type flies without transgene expression (Fig. 7B, lane 2).

**Polytene chromosome immunolocalization of Chromator and H1.**

To determine the relative distribution of H1 and Chromator we double labeled polytene squash preparations with Chromator mAb 6H11 and H1 antibody. As illustrated in Fig. 8A histone H1 is predominantly present at band regions whereas Chromator is localized to interbands. However, as a linker histone H1 is also present at a lower density in the euchromatic interband regions (Hill et al., 1989). The interaction between H1 and Chromator is therefore likely to occur with the fraction of H1 present in interband chromatin or at the interface between interband and band regions. As illustrated in Fig. 8B and 8C neither the ability to localize to chromatin nor the amount of H1 was affected in the Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant background as compared to wild-type.
Modeling of the Chromator chromodomain.

The chromodomain as well as the chromo-related domains constitute an evolutionary conserved module of about 50 amino acids that are widespread among eukaryotes (Paro and Hogness, 1991; Gortchakov et al., 2005) and that perform a wide range of diverse functions (Brehm et al., 2004). The chromodomain in Drosophila most closely related to that of Chromator in structural database searches was that of HP1a. HP1a is essential for the assembly of heterochromatin and its chromodomain is responsible for its binding to methylated histone H3 (Jacobs and Khorasanizadeh, 2002). In order to compare the structure of Chromator's chromodomain with that of HP1a we modeled it using the I-TASSER structure prediction program (Roy et al., 2010). As illustrated in Fig. 9 the spatial structure of the two chromodomains are very similar; however, two out of the three aromatic amino acids essential for binding to methylated histone H3 (Nielsen et al., 2002; Fischle et al., 2003) in the chromodomain of HP1a are not conserved and have been substituted with an Arginine and Aspartate residue, respectively (Gortchakow et al., 2005). In addition, the chromodomain of Chromator has an a-helical stretch before the main a-helix instead of a b-strand (Fig. 9). Thus, these changes may contribute to the chromodomain of Chromator's affinity for histone H1 instead of for methylated histone H3.

DISCUSSION

In this study we show that the NTD domain of Chromator is required for proper
localization to chromatin and that chromosome morphology defects observed in Chromator mutant backgrounds can be largely rescued by expression of this domain. We furthermore provide evidence that the ChD domain can interact with histone H1 suggesting that this interaction is necessary for the correct chromatin targeting. Nonetheless, that localization to chromatin still occurs in the absence of the ChD indicates that Chromator possesses a second mechanism for chromatin association and we provide evidence that this association is mediated by other sequences residing in the NTD. Such an association could in principle be mediated by other molecular interaction partners of Chromator that also localize to chromatin such as JIL-1 or Z4. However, studies in S2 cells with RNAi mediated Chromator depletion and in JIL-1²² homozygous null mutant backgrounds demonstrated that neither protein was dependent on the other for its chromatin localization (Rath et al., 2006). The interaction of Chromator with Z4 was identified in co-immunoprecipitation experiments and the two proteins colocalize extensively at interband polytene regions (Eggert et al., 2004) Recently, Gan et al. (2011) provided evidence that Chromator and Z4 may directly interact and that localization of Z4 to chromatin depends on Chromator, but not vice versa. Another candidate for mediating chromatin localization is Skeletor (Walker et al., 2000). The interaction between Chromator and Skeletor was first detected in a yeast two-hybrid screen and subsequently confirmed by pull-down assays (Rath et al., 2004). Immunocytochemical labeling of Drosophila embryos, S2 cells, and polytene chromosomes demonstrated that the two proteins show extensive co-localization during the cell cycle although their distributions are not identical (Rath et al., 2004). During interphase Chromator is localized on polytene chromosomes to interband chromatin regions in a pattern that
overlaps that of Skeletor. During mitosis both Chromator and Skeletor detach from the chromosomes and align together in a spindle-like structure with Chromator additionally localizing to centrosomes that are devoid of Skeletor-antibody labeling. Thus, the extensive co-localization of the two proteins is compatible with a direct physical interaction; however, at present it is not known whether such an interaction occurs throughout the cell cycle or is present only at certain stages, with additional proteins mediating complex assembly at other stages (Rath et al., 2006). Regardless, it is likely that Chromator together with Skeletor functions in at least two different molecular complexes, one associated with the spindle matrix during mitosis and one associated with nuclear and chromatin structure during interphase (Rath et al., 2004). Furthermore, taken together the findings of the present study and those of Ding et al. (2009) suggest that Chromator's chromatin functions are largely governed by the NH$_2$-terminal domain whereas functions related to mitosis are mediated by COOH-terminal sequences. The molecular mechanisms of how the two distinct chromatin binding affinities residing within the NH$_2$-terminal domain of Chromator interact to confer proper localization to interbands remains to be elucidated.

An important feature of the Chromator protein is the presence of a chromodomain, the only conserved motif found in database searches (Rath et al., 2004; Gortchakov et al., 2005). Structure determination of the prototype chromodomain has revealed a small, three-stranded antiparallel b-sheet supported by an a-helix that runs across the sheet (Ball et al., 1997; Brehm et al., 2004). Classic chromodomains contain three conserved aromatic amino acids that confer binding affinity for methylated histone H3 (Nielsen et al., 2002; Fischle et al., 2003). However, several chromodomains have been identified
that vary at some of these structurally important positions but that still conform well to the overall folding of the prototype chromodomain (Brehm et al., 2004). One example of this is the chromo-shadow domain also found in HP1a that is a protein-protein interaction domain that allows HP1a to homodimerize via its a-helices (Brasher et al., 2000; Cowieson et al., 2000). In addition, various chromodomains have been demonstrated to bind to a wide variety of proteins including transcription corepressors, remodeling ATPases, lamin B receptor, and chromatin assembly factors (reviewed in Jones et al., 2000). Thus, relatively small sequence variations in the otherwise conserved structural scaffold of chromodomains can confer considerable variation in molecular interactions (Brehm et al., 2004). We provide evidence by modeling that the chromodomain of Chromator is likely to adopt the canonical chromodomain tertiary configuration very similar to the chromodomain of HP1a. However, due to amino acid substitutions at two of the three conserved aromatic amino acid positions it is not likely to bind to methylated histone H3. Rather we provide evidence by overlay and pull down assays that it binds to the linker histone H1. A candidate region for providing such a binding fold or surface is the additional a-helical stretch found in the chromodomain of Chromator just prior to the main a-helix of the chromodomain structure. In future experiments it will be of interest to further determine the structural basis for the interaction of Chromator with histone H1 and specifically how the chromodomain contributes to Chromator's role in nucleosome and chromatin organization.
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FIGURE LEGENDS

**Fig. 1** Chromator expression in the hypomorphic *Chro*\(^{71}/Chro^{612}\) mutant background. (A) Double labelings with the COOH-terminal Chromator mAb 6H11 (green) and Hoechst (blue/gray) of polytene squashes from wild-type (upper panel) and *Chro*\(^{71}/Chro^{612}\) (lower panel) third instar larvae. The composite image (comp) is shown to the left. The mAb 6H11 epitope is not present in either of the truncated *Chro*\(^{71}\) or *Chro*\(^{612}\) proteins. (B) Double labeling with the NH\(_2\)-terminal Chromator mAb 12H9 (green) and Hoechst (blue/gray) of polytene squashes from a *Chro*\(^{71}/Chro^{612}\) third instar larvae. The composite image (comp) is shown to the left. (C) Immunoblot analysis of Chromator protein expression in *Chro*\(^{71}/Chro^{612}\) mutant third instar larvae as compared to wild type larvae. The immunoblots were labeled with the COOH-terminal Chromator mAb 6H11 (left panel), with the NH\(_2\)-terminal Chromator mAb 12H9 (right panel), and with anti-tubulin antibody as a loading control. Full-length Chromator is detected by both mAb 6H11 and 12H9 in wild-type larvae; however, no full-length Chromator is detectable in the mutant larvae.

**Fig. 2** Diagrams of the transgenic Chromator constructs analyzed.

**Fig. 3** Localization of transgenic Chromator GFP-tagged constructs in live salivary gland nuclei in a *Chro*\(^{71}/Chro^{612}\) mutant background. The FL (A) and NTD (B) localizes to banded regions of the polytene chromosomes whereas the ChD (C), while present on the chromosomes, had a more diffuse localization. In contrast, the CTD was
exclusively found in the intranuclear space surrounding the chromosomes (D). The images are from confocal sections.

**Fig. 4** Expression of Chromator deletion constructs transgenically in a *Chro*¹/²⁻/Chro²⁻ mutant background. Polytenic chromosome squash preparations from *Chro*¹/²⁻/Chro²⁻ third instar larval salivary glands expressing the FL, NTD, ChD, NTD-DNTD, and CTD, respectively. Transgene localization (green) was identified using either mAb 6H11 or anti-V5 or anti-GFP antibodies. DNA (blue/gray) was labeled by Hoechst.

**Fig. 5** The chromodomain of Chromator interacts with histone H1. (A-F) In overlay experiments purified bovine histones were fractionated by SDS-PAGE, immunoblotted, incubated with Chromator GST-FL, GST-NTD, GST-ChD, GST-NTD-DChD, or GST-CTD fusion protein, and interactions detected with an anti-GST mAb (B-F). A representative Ponceau S labeling of the fractionated histone proteins is shown in (A). (G) In overlay experiments a *Drosophila* histone H1 MBP-tagged fusion construct or MBP only was fractionated by SDS-PAGE, immunoblotted, incubated with Chromator GST-FL, GST-NTD, GST-ChD, GST-NTD-DChD, or GST-CTD fusion protein, and interactions detected with an anti-GST mAb. (H) Ponceau S labeling of the fractionated MBP-H1 and MBP proteins used for the overlay assay in (G). (I) A representative immunoblot of the GST-fusion proteins used for the overlay experiments in (B-G) detected with Coomassie Blue.

**Fig. 6** Pull-down assays with Chromator's chromodomain and histone H1. (A)
ChD-GST construct pulled down His-tagged *Drosophila* histone H1 (His-H1) as detected by anti-H1 antibody (lane 4). A CTD-GST as well as a GST only control pull down were negative (lane 2 and 3, respectively). Lane 1 shows the His-H1 fusion protein. (B) A *Drosophila* His-H1 construct pulls down ChD-GST as detected by anti-GST antibody (lane 4). A control pull down with a His-tagged construct of the NH$_2$-terminal domain of the JIL-1 kinase (His-JIL-1) or with beads only was negative (lane 2 and 3, respectively). Lane 1 shows the ChD-GST fusion protein. (C) A ChD-GST construct pulled down a band corresponding to the size of histone H1 from S2 cell nuclear lysate as detected by anti-H1 antibody (lane 1). A CTD-GST as well as a GST only control pull down was negative (lane 2 and 3, respectively). Lane 4 shows the band in the nuclear lysate detected by H1 antibody. (D) A *Drosophila* His-H1 construct pulls down Chromator from S2 cell nuclear lysate as detected by the Chromator mAb 6H11 (lane 1). A control pull down with a His-tagged construct with His-JIL-1 or with beads only was negative (lane 2 and 3, respectively). Lane 4 shows the band in the nuclear lysate detected by mAb 6H11 antibody.

**Fig. 7** Expression and co-immunoprecipitation analysis of protein lysates of flies expressing each of the four GFP-tagged transgenes FL, NTD, CTD, and ChD using anti-GFP antibody. (A) Protein lysate from wild-type (wt) flies and flies expressing the FL, NTD, CTD, or ChD transgenes analyzed by SDS-PAGE and immunoblotted using a rabbit anti-GFP mAb. The arrow indicates a background band also detected by the anti-GFP-antibody in wild-type fly lysate (lane 1). This band served as a loading control. The relative migration of molecular size markers is indicated to the right in kD. (B) Anti-
GFP-antibody ips from lysates of wild-type (wt) flies and of flies expressing the FL, NTD, CTD, or ChD transgenes analyzed by SDS-PAGE (4-15% gradient gel) and immunoblotted using H1 antibody (lane 2-6). A 38 kd protein band detected by anti-H1 antibody also present in lysate of wild-type flies (lane 1) was co-immunoprecipitated from lysate of flies expressing the FL, NTD, and ChD, but not from lysate of flies expressing the CTD (lane 3-6). This 38 kD band was not present in anti-GFP antibody ips from lysates of wild-type flies without transgene expression (lane 2).

**Fig. 8** H1 and Chromator chromosome localization. (A) Triple labeling with anti-H1 antibody (red), Chromator mAb 6H11 (green), and Hoechst (blue/gray) of a polytene squash from a wild-type third instar larvae. The composite image of H1 and Chromator labeling (comp) is shown to the left. (B) Double labeling with anti-H1 antibody (red) and Hoechst (blue/gray) of a polytene squash from a Chro^{71}/Chro^{612} third instar larvae. The composite image (comp) is shown to the left. (C) Immunoblot analysis of H1 protein expression in Chro^{71}/Chro^{612} mutant third instar larvae as compared to wild type larvae. The immunoblots were labeled with anti-H1 antibody and with anti-tubulin antibody as a loading control.

**Fig. 9** Comparison of the structure of Chromator's chromodomain with that of HP1a. (A) Model of Chromator's chromodomain using the I-TASSER structure prediction platform (Roy *et al.*, 2010). (B) The crystal structure of *Drosophila* HP1a's chromodomain (PDB ID: 1Q3L). (C) Overlay of the predicted structure of Chromator's chromodomain with that of HP1a. The three aromatic amino acids essential for binding to
methylated histone H3 in the chromodomain of HP1a and the corresponding amino acids in Chromator's chromodomain are highlighted. The arrow in (A) indicates an a-helical stretch before the main a-helix instead of a b-strand in the chromodomain of Chromator. The structures were rendered in PyMOL.

Fig. 1
Fig. 2

Fig. 3
Fig. 4
Fig. 5

Fig. 6
Fig. 7

A

GFP antibody

transgene expression

B

H1 antibody

lysate

GFP antibody ip

Fig. 8
CHAPTER 5: GENERAL CONCLUSIONS

Dynamic distribution of Chromator during the cell cycle

Chromator was originally identified as a direct interaction partner of Skeletor by a yeast two hybrid assay. It is comprised of 926 amino acids and migrates around 130 kDa on a SDS-PAGE gel. Since the only conserved functional domain identified in Chromator is a chromodomain, it was named Chromator. As Walker et al., (2000) described by immunocytochemistry labeling, Skeletor is associated with chromosomes during interphase and redistributes to form a spindle-like structure from late prophase in Drosophila syncytial embryo. Similarly, antibody labeling of Chromator shows that Chromator extensively co-aligns with Skeletor during the cell cycle and also attains a fusiform spindle-like structure at metaphase. However, the more detailed dynamic properties of Chromator and the putative spindle matrix complex proteins need to be visioned by live imaging approach instead of fix preparation.

Live imaging in a Chromator–GFP- and tubulin-mCherry- expressing Drosophila syncytial embryo during mitosis shows that Chromator reorganizes away from its interphase localization, the chromosomes, as the chromosomes begin to condense. Chromator fills the entire nuclear space before microtubule invasion. Chromator distribution attains a spindle-like structure after microtubule spindle formation, while also translocating to the centrosomes. At anaphase and telophase Chromator dynamics closely mirror that of the microtubules before relocating back to the chromosomes in the forming daughter nuclei. This dynamic behavior of Chromator during mitosis is very different from microtubule-associated proteins (MAPs) such as Jupiter.
By analyzing line scans of pixel intensity across the spindle, we found that peak intensities of the MAP Jupiter coincide with that of microtubules, indicating co-localization, whereas peak intensities of Chromator are notably distinct from those of microtubules and in many cases show an alternating pattern. The dynamic distribution of Chromator during the cell cycle indicates that the Chromator-defined spindle matrix structure is a viscous-elastic gel-like structure which forms before nuclear envelope breakdown and is relatively independent of the microtubule spindle. This hypothesis is further supported by colchicine microinjection experiments. If colchicine is injected at interphase before nuclear envelope breakdown, all microtubules are depolymerized before nuclear envelope breakdown, preventing microtubule spindle formation. Under these conditions Chromator still relocates from the chromosomes to the matrix; however, in the absence of microtubule spindle formation the Chromator-defined matrix does not undergo any dynamic changes but instead statically embeds the condensed chromosomes for extended periods (>20 min). If colchicine is injected at metaphase, as the microtubules undergo depolymerization the Chromator-defined matrix contracts and coalesces around the chromosomes. The reduction in the length of the spindle matrix was almost 60% from when the first image was obtained after colchicine injection to when microtubules were depolymerized. Taken together, the data suggests Chromator belongs to a protein complex which forms a viscous-elastic gel-like structure during the cell cycle. This gel-like structure is relatively independent of the microtubule spindle, but uses microtubules in the spindle to serve as a "struts" in order to attain a spindle-like structure at metaphase (Yao et al., 2012a).
The spindle matrix protein, Chromator, is a tubulin binding protein

In colchicine treated *Drosophila* syncytial embryos free tubulin accumulates co-extensively with the spindle matrix proteins (Yao *et al.*, 2012a) suggesting that this enrichment depends on one or more proteins within the spindle matrix with tubulin binding activity. A candidate spindle matrix protein for having tubulin binding activity is the chromodomain containing protein, Chromator, which during interphase is localizes to interband regions of *Drosophila* polytene chromosomes (Rath *et al.*, 2006). If embryos expressing GFP-Chromator (Chro-GFP) and tubulin-mCherry at metaphase are injected with taxol, the cell cycle arrestes at the metaphase-anaphase transition. Microtubule depolymerization but not polymerization is inhibited, leading to an increase of spindle and especially of astral microtubules. Chro-GFP extensively co-localizes with the microtubules, including the growing astral microtubules, and this association is maintained for extended time periods. Co-immunoprecipitation and GST pull-down experiments in *Drosophila* S2 cell lysate show an *in vivo* interaction between Chromator and tubulin. Furthermore, microtubule spin-down assays using *Drosophila* embryonic lysate or *in vitro* assembled microtubules as well as protein overlay assay indicate a direct interaction between Chromator and polymerized microtubules through its 271 aa middle domain. Additionaly, *in vivo and in vitro* pull-down assays in the presence of colchicine indicate that a direct interaction could happen between Chromator and free tubulin too. Thus, the enhanced accumulation of free tubulin within the nascent spindle region may serve as a general mechanism to promote efficient assembly of the microtubule-based spindle apparatus (Hayashi *et al.*, 2012) and may be mediated by spindle matrix constituents. Based on Chromator's ability to bind free tubulin we propose
that Chromator may fulfill such a role in *Drosophila*.

**The chromdomain-containing NH₂-terminus of Chromator interacts with histone H1 and is required for correct targeting to chromatin**

Chromator is localized to interband regions of *Drosophila* polytene chromosomes during interphase (Rath *et al*., 2004; Gortchakov *et al*., 2005) and has been shown to interact with other chromosomal proteins such as the zinc-finger protein Z4 (Eggert *et al*., 2004; Gan *et al*., 2011) and the histone H3S10 kinase JIL-1 (Rath *et al*., 2006) and to contribute to the maintenance of polytene chromosome morphology (Rath *et al*., 2006). Domain analysis has demonstrated that the COOH-terminus domain is sufficient for targeting Chromator to the spindle matrix (Ding *et al*., 2009), whereas the NH₂-terminus domain of Chromator is required for proper localization to chromatin during interphase and that chromosome morphology defects observed in Chromator hypomorphic mutant backgrounds can be largely rescued by expression of this domain. Furthermore, the chromo-domain of Chromator has been shown to interact with histone H1 and that this interaction is necessary for correct chromatin targeting. Nonetheless, that localization to chromatin still occurs in the absence of the Chromodomain indicates that Chromator possesses a second mechanism for chromatin association. Collectively, these findings suggest that Chromator's chromatin functions are largely governed by the NH₂-terminal domain whereas functions related to mitosis are mediated mainly by COOH-terminal sequences (Yao *et al*., 2012b).

**The coiled-coil domain is required for Megator's spindle matrix localization.**

Megator (Bx34 antigen) encodes a 260kDa protein (Mtor-FL) with a large NH₂-terminal coiled-coil domain (Mtor-NTD) and a shorter COOH-terminal acidic region
(Mtor-CTD). It shows overall structural and sequence similarity to the mammalian nuclear pore complex Tpr (translocated promoter region) protein (Zimowska et al., 1997). We conducted time-lapse imaging of Mtor-FL-YFP, Mtor-NTD-GFP and Mtor-CTD-GFP together with histone H2Av-RFP in syncytial embryos. Mtor-FL localizes to the nuclear interior as well as to the nuclear rim at interphase and to the spindle matrix at metaphase. In contrast, Mtor-CTD, which contains the native nuclear localization signal (NLS), is diffusively present in the nucleoplasm without detectable nuclear rim localization at interphase and is absent from the spindle region at metaphase. Mtor-NTD is present at the nuclear rim with no or very little interior nuclear localization but relocates to the spindle matrix at metaphase. If the embryos were injected with colchicine at interphase, Mtor-FL and Mtor-NTD will relocate to the spindle matrix and, as with the Chromator-defined matrix, do not undergo any dynamic changes but statically embed the condensed chromosomes. In contrast, Mtor-CTD disperses rapidly after NE breakdown (Yao et al., 2012a). Thus, these findings suggest that the coiled-coil domain of Megator is required for Megator’s spindle matrix localization and function.

**Summary and future research direction**

The identification of Chromator, Skeletor, Megator and EAST in *Drosophila* provides direct molecular evidence to support the existence of a spindle matrix structure. By characterization of the dynamic properties of Chromator and Megator, we show that the Chromator-and Megator-defined spindle matrix structure is a nuclear derived gel-like matrix structure which forms before nuclear envelope breakdown. This viscous-elastic gel-like structure could interact with the microtubule spindle and facilitate the assembly of the microtubule spindle. The spindle matrix complex uses the microtubule spindle as a
strut to stretch out from pole to pole and forms a fusiform spindle-like structure. And this structure is relatively independent of the microtubule spindle, since after depolymerization of the microtubules by colchicine at metaphase, the spindle matrix structure still persists although contracts and coalesces around the chromosomes. This result further supports the idea of a viscous-elastic gel-like spindle matrix structure where the loss of the microtubule scaffold results in loss of outward forces generated by the microtubule spindle. The shrinkage of the spindle matrix structure is due to the intrinsic elasticity of the gel itself. The viscous-elastic properties of the matrix may thus constrain spindle length, stabilize the microtubule spindle apparatus, and assist in force production as originally envisioned for a spindle matrix (Johansen and Johansen, 2007). Distinct from the Lamin B matrix, the spindle matrix structure could form a relatively static gel-like structure that imbeds the mitotic chromosomes if the microtubules are depolymerized at interphase. Our data of dextran injection showed that 70 kDa and 500 kDa but not 2000 kDa dextrans could incorporate into the spindle apparatus and enrich in the region defined by the Chromator-defined spindle matrix region after nuclear envelope breakdown when the embryo is treated by colchicine at interphase, but not 2000kDa. This suggests a potential role for the spindle matrix to help exclude organelles and vesicles from the spindle region. Failure of recruitment of Mad2 to the unattached kinetochores in Chromator and Megator RNAi S2 cells suggest that the spindle matrix may serve a general role in physically confining and organizing cell cycle factors in the spindle region in organisms with open or semi-open mitosis. Thus, the significance of such a spindle matrix structure is well supported.

Apart from the important spindle matrix role of Chromator in mitosis, its
functions are also necessary for nuclear organization, especially the maintenance of normal chromosome structure at interphase. For further dissection of Chromator’s physiological roles, identification of additional interaction partners could indicate its functions in more details. A tandem affinity purification from separated nuclear and cytoplasmic fractions would be a good choice to decipher its function during different cell stages. Taking advantage of the high specificity of Chromator mAb’s 6H11, an antibody neutralization experiment would be helpful to study its role during the cell cycle. In initial studies, 6H11 injection into Drosophila early syncytial embryos does not affect the first cycle but blocks the dissociation of Chromator from the condensing mitotic chromosomes and further blocks the formation of the spindle matrix in the next cycle. Without the formation of the spindle matrix, the cell cycle is blocked before nuclear envelope breakdown. But this block is uncoupled from the duplication and division of centrosomes (unpublished data). Triple knockdown of mitotic cyclins A, B and B3 by RNAi also showed a similar phenotype (McCleland et al., 2009). This indicates a strong possibility for the spindle matrix to play a role in regulating cell cycle factors. Further tests to explore these possibilities would help to elucidate Chromator’s role as a spindle matrix component and provide exciting information about spindle organization as well as nuclear organization in Drosophila.
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