Dissection of the mitotic and nuclear functions of Chromator, a nuclear-derived spindle matrix component in Drosophila

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Dissection of the mitotic and nuclear functions of Chromator, a nuclear-derived spindle matrix component in *Drosophila*

by

Yun Ding

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

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To my family
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A spindle matrix has long been proposed to serve as a stationary or elastic molecular matrix substrate for the organization and activities of microtubules and motors, based on the consideration of a mechanical and functional support for the stabilization of microtubule spindle during force generation from mitotic motors. Recently, the identification of four Drosophila proteins, Skeletor, Megator, EAST and Chromator has provided molecular evidence for the existence of this macromolecular matrix structure during mitosis. All of these four proteins have been shown to interact with each other within a protein complex and redistribute from the nucleus at interphase to form a fusiform spindle-like structure that does not rely on polymerized microtubules from prophase until telophase. Especially, the discovery of the large coiled-coil domain in Megator suggests that Megator may provide the structural element of this matrix. Characterizations of these molecules all indicate their potential to constitute a bona fide spindle matrix. However, functional analysis is missing due to the limitation of existing mutant alleles and the multiple essential roles played by at least some of these proteins at different stages of the cell cycle.

Taking advantage of newly generated hypomorphic alleles of the Chro gene, different functional roles of Chromator were dissected and the results are presented in this dissertation. In transheterozygous Chro^{71}/Chro^{612} mutants, interphase polytene chromosome structures are disrupted with misalignment of the band/interband pattern and numerous ectopic contacts between non-homologous regions. During mitosis a dramatically disorganized spindles and chromosome segregation defects are observed in dividing neuroblasts from mutant third instar larval brains. In addition, an interphase- specific interaction between Chromator and
the H3 Serine 10 kinase JIL-1 as well as mitotic interactions of Chromator with the molecular motor Ncd and microtubules are described.

This dissertation provides the first reported detailed functional analysis of one spindle matrix candidate, Chromator, in animal system. Impaired Chromator function in the mutant allele causes improper spindle assembly and chromosome segregation during mitosis as well as defective interphase polytene chromosome structures. These findings, with the characteristic cell-cycle dependent distribution pattern of Chromator reveal that Chromator is a nuclear-derived multifunctional protein that performs its essential functions from interphase to mitosis.
CHAPTER 1: GENERAL INTRODUCTION

DISSERTATION ORGANIZATION

This dissertation is comprised of four chapters. The first chapter is a general introduction reviewing the current knowledge about the mechanisms of mitotic spindle assembly. The properties and functions of microtubules and microtubule-based motor proteins, two major components of a mitotic spindle are discussed. Recent studies of the effects of non-motor microtubule-associated proteins on microtubule spindle organization are described including structural participants and regulatory mitotic kinases. The spindle matrix hypothesis is then introduced with discussion about current evidence and research progress to support existence of such a macromolecular complex. Finally, four putative molecular candidates of the spindle matrix in Drosophila, Skeletor, Megator, EAST and Chromator, are reviewed with the focus on a primary characterization of Chromator protein functions.

The second and third chapters are organized in paper format. The second chapter is a paper published in Journal of Cell Science in June, 2006 on the analysis of Chromator functions in the regulation of Drosophila polytene chromosomes structures. Dr. Uttama Rath and Yun Ding are the co-first authors of this paper. Yun Ding analyzed the disrupted interphase polytene chromosome structures in Chromator transheterozygous mutant, Chro^{71}/Chro^{612} and initiated the study of genetic interaction between Chromator and JIL-1. Dr. Uttama Rath reported a direct interaction between Chromator and JIL-1 kinase. Besides, Huai Deng contributed to the analysis of ultrastructure of chromosomes in Chromator mutant and Dr. Hongying Qi showed that chromosomal localization of JIL-1 is independent of
Chromator level by RNAi assay in S2 cells. These results indicated that Chromator plays an important role at interphase, together with JIL-1 and/or other proteins to maintain the polytene chromosome structure in *Drosophila*.

The third chapter is a manuscript being prepared for submission. In this paper, Changfu Yao made the NP-Chro$^{N41D}$ rescue construct. Yun Ding built up most of the rest required *Drosophila* transgenes or strains, and did all the analyses to describe Chromator functions during mitosis. Using Drosophila neuroblast cells as an experimental system, severely disorganized spindles and chromosome segregation defects were observed in the Chromator transheterozygous mutant allele, $Chro^{71}/Chro^{612}$. Furthermore, an interaction between Chromator and Ncd, a microtubule-based motor protein as well as direct microtubule binding ability of Chromator was demonstrated through biochemical assays. The experimental data from this paper supports mitotic roles for Chromator in helping to assemble the microtubule spindle and facilitate chromosome segregation during cell division, roles that are consistent with the predictions of Chromator acting as a spindle matrix component.

The fourth chapter is the general conclusions for the work presented in this dissertation and proposed potential experiments for further dissection of Chromator functions. Finally, an amino acids sequence alignment of Chromator and its homologues in other Drosophila species is included in the appendix. In addition, I also compare the sequences from NH$_2$-terminal end and COOH-terminal end of Chromator with the most similar annotated protein sequences among different insect species.
LITERATURE REVIEW

Mitosis and the Mitotic Spindle

Mitosis, the division of the mother cell into two identical daughter cells, is the fundamental cellular process accounting for the proliferation capability of living organisms. Chromosome segregation, the “ultimate goal” of mitosis, is accomplished through five mitotic stages, each characterized by several specific events. The replicated DNA molecules condense into mitotic chromosomes at prophase. These condensed chromosomes congress at prometaphase and align at the metaphase plate, marking the completion of metaphase. Then the chromatids separate and chromosomes move towards the opposite directions from anaphase through telophase. This faithful segregation of the genetic material during mitosis relies on a complicated macromolecular machine: the mitotic spindle.

The characterization of a mitotic spindle can go back as far as in the early 1880s, when the German anatomist Walther Flemming first described the morphology of the mitotic spindle and “thread”-like mitotic chromosomes (reviewed in Mitchison and Salmon, 2001). Since then, the basic analysis of the mitotic spindle assembly and the mechanism of its regulation were studied with the development of new microscope techniques, such as the sensitive polarized light microscope, the electron microscope and the fluorescence microscope (Kline-Smith and Walczak, 2004). In the last decade, a wealth of molecular approaches has emerged including the complete genome sequence project, high through-put RNA interference techniques and investigation of small molecular inhibitors of mitotic proteins, along with high resolution imaging combined with advanced microtubule labeling methods. All these powerful techniques have made mitotic spindle research an exciting area
and provided increasingly detailed pictures about how this macromolecular complex is organized (Gadde and Heald, 2004).

**Microtubule Spindle Structure and Dynamics**

The key structural component of a mitotic spindle is the bipolar, antiparallel array of microtubules. Together with various associated mitotic motor proteins and other important factors, this microtubule-based spindle structure is present in all eukaryotic cells (Wittmann et al., 2001). The biochemical and structural properties of microtubules make them an ideal cytoskeletal structural component to build a spindle and exert the essential roles required for a mitotic apparatus.

Microtubules are hollow cylindrical tubes of 13 parallel protofilaments, assembled from αβ-tubulin heterodimers arranged head to tail longitudinally (Desai and Mitchison, 1997). Based on this form of polymerization, microtubules show structural polarity, with the α-tubulin subunit exposed at the minus end and the β-tubulin subunit exposed at the plus end (Hirose et al., 1995; Nogales et al., 1999). The microtubule polymer is highly dynamic mainly due to the status of GTP binding and hydrolysis at the β-tubulin subunits. A GTP nucleotide can bind to the β-tubulin subunit of a heterodimer. After the addition of this heterodimer to the growing polymer, this “GTP-cap” (Carlier and Pantaloni, 1981) tends to stabilize the microtubule and provide a new polymerization site for next heterodimer. However, GTP is hydrolyzed to GDP rapidly after the tubulin heterodimer polymerizes to the microtubule and this nucleotide cannot be exchanged. Therefore, the “cap” of microtubule main body is GDP-bound-β-tubulin, which tends to be unstable and favors depolymerization (“catastrophe”, Davis et al., 1994). The “catastrophe” situation can be reversed by addition
of GTP-capped tubulin subunits, named the “rescue” process (Hyman et al., 1992; Mitchison, 1993). The switch between growth (polymerization) and shrinkage (depolymerization) states occurs rapidly to the microtubule polymers *in vivo* and *in vitro*. This intrinsic property of microtubule is known as “dynamic instability” and is required for the reorganization of the microtubule network through the cell cycle (Mitchison and Kirschner, 1984; Cassimeris et al., 1987).

In terms of the microtubule cytoskeletal network remodeling during mitosis, the most characteristic change is the formation of a fuisform-shaped spindle structure at metaphase. The mitotic spindle extends from two opposing spindle poles, with the less dynamic minus end of microtubules located at the spindle poles while the more dynamic plus end points away from the poles. The microtubules within the mitotic spindle are usually categorized into three classes based on their morphological and functional differences. (*i*) Kinetochore MTs originate from spindle poles and terminate in the outer layer of the kinetochore, a specific protein complex structure assembled at the centromere of the mitotic chromosomes. This class of MTs is often bundled into *K-fibers* that connect the chromosomes to the poles. *K-fibers* are the direct participants involved in the chromosome congression, alignment and segregation. (*ii*) Interpolar MTs extend from pole towards the spindle mid region without attaching to the chromosomes. They often overlap with the MTs from the opposite pole in an antiparallel pattern, which can stabilize the bipolar spindle from prometaphase to metaphase and help spindle pole separation during late anaphase (Compton, 2000; Scholey et al., 2003). (*iii*) Astral MTs radiate from the centrosome into the cell cortex and help determine spindle orientation and positioning.
Judging from an immunofluorescence picture of a metaphase spindle, the microtubule length is relatively constant and the shape and size of the spindle is static, though this view is somewhat deceptive. Taking advantage of the recent development of the “fluorescent speckle microscopy” technique, the “microtubule flux” phenomenon can be observed (Mitchison, 1989). In contrast to the traditional level of immunofluorescently-labeled tubulin subunits in immunocytochemistry, photobleaching and photoactivation experiments (which largely saturates the endogenous MTs), low levels of fluorescently labeled tubulin cannot incorporate uniformly into the MTs. This leads to a small fraction of the labeled microtubule “bar” as a mark to be recognized on the microtubule over time (Waterman-Storer et al., 1998; Waterman-Storer and Danuser, 2002). These experiments show that the small fluorescently tubulin labeled “speckles” are continuously translocating to the spindle poles along the microtubule, despite that the whole length of the microtubule is not changed. This constant microtubule flux occurs on the kinetochore-MTs and interpolar MTs, which requires three activities: continuous depolymerization of minus ends located in the pole region, continuous polymerization of plus ends near the spindle mid region and poleward translocation of spindle tubulin subunits.

Taken together, the microtubule spindle is a highly dynamic structure. Although the microtubules are the primary structural components of the spindle, the inherent “dynamic instability” and “microtubule flux” suggests that a certain number of the microtubules are undergoing polymerization and depolymerization rapidly while being constantly translocated to the spindle poles. The dynamics of MTs are required for the normal functions of a mitotic spindle in at least two ways: the rapid polymerization and depolymerization at the microtubule ends are necessary for microtubules to encounter the kinetochore surface and
catch the chromosome; the function of microtubule flux is involved in the chromosome segregation at anaphase (Maddox et al., 2002).

**Microtubule-based Motor Proteins**

Mitotic spindle assembly is a complicated process involving multiple organizational forces in the eukaryotic cells. From most animal somatic cells, there are two different subcellular structure formed by large protein complexes to generate and maintain the bipolarity of a normal spindle morphology. One is the centrosome, which duplicates before nuclear envelope breakdown (NEB) to nucleate the microtubules from opposite directions. Together with other “PCM” (pericentriolar matrix) proteins, it forms the organization centers of the spindle poles. The other is the kinetochores on the sister chromatids. Microtubules can capture and attach the chromosomes through these sites (Heald, 2000). However, microtubules with these structures themselves are necessary but not sufficient to assemble the spindle. One of the important “missing pieces” is the source to generate force within the spindle: the motor proteins. The search for mitotic motors was inspired by the hypothesis that the movement in the spindle could be explained by a “sliding filament” model (McIntosh et al., 1969).

Motor proteins are a group of well-characterized mechanochemical enzymes that hydrolyze a nucleotide triphosphate to provide the kinetic energy for movement along the cytoskeletal polymer. Two families of the molecular motors use microtubules as a track and exert functions in mitosis: the dyneins and kinesins. Cytoplasmic dynein is the only subfamily of dyneins that functions in spindle organization. In association with the dynactin complex, it works as a homodimer and moves specifically towards the minus end of
microtubules (Holzbaur and Vallee, 1994). Mitotic kinesin family members are categorized based on the location of the motor domain within the peptide sequence and their function (Vale and Fletterick, 1997). This classification results in three main groups: KIN N, KIN C and KIN I. KIN N has an N-terminal motor domain and includes the bipolar kinesins and the chromokinesins. All of the KIN N members move towards the plus end of microtubules. In contrast, KIN C members are microtubule minus-end directional kinesins and their motor domains are in the C-terminus. The final group is the KIN I kinesins whose motor domain is in the middle of the protein. Although they have a motor domain, KIN I members are actually not motile or localized to the kinetochores.

At least three mechanisms are suggested for mitotic motor functions: (1) cross-linking and sliding MTs relative to the neighboring MTs or other cytoskeletal networks; (2) transporting necessary mitotic components along the spindle MTs; (3) coupling the movement to MT assembly dynamics (Sharp et al., 2000). The motors utilize these mechanisms to generate force and help stabilize the spindle structure during different stages of mitosis. For instances, during the spindle pole separation, bipolar KIN N kinesins form tetramers. The motor domains on each end walk towards the plus end and slide the microtubules from opposite poles to push the spindle poles apart. This pole-pole outward force is helped by the function of cytoplasmic dynein to slide the astral MTs to the cell cortex, while this force can be antagonized by the minus-end directed KIN C kinesins in pole separation. At metaphase, the integrity and bipolarity of the spindle is maintained by various motor activities. The bipolar kinesins cross-bridge and slide the interpolar antiparallel MTs to exert outward forces in the central spindle. To balance this outward force, the inward force is generated by the KIN C (Chandra et al., 1993), CHO1/MKLP1 kinesins (Kuriyama et al.,
1994) and the dynein/dynactin complex (Waterman-Storer et al., 1995). In addition, the same mechanisms used for force generation used by motors also applies to spindle pole focusing. In different animal systems, dyneins and/or KIN C kinesins (both minus-end directed motors) bundle and slide the MTs released from centrosomes to generate the focusing force (Compton, 1998) as well as to oppose the outward force generated by KIN N motors (Merdes et al., 2000). The movement of chromosomes within the spindle also requires the participation of motors. Motor-drive transportation of chromosomes is believed to occur at the kinetochores. During chromosome congression, dynein/dynactin is involved in the initial capture of microtubules by kinetochores. Then the plus end directed motor CENP-E (KIN N family) helps k-fibers connect the chromosomes to position them at the metaphase plate. This anti poleward movement is assisted by another group motors, the chromokinesins. They are specific KIN N, plus-end directed motors that localize on the chromosome arms instead of at the kinetochores. Their function is to move chromosome arms along the microtubules toward the metaphase plate, which leads to the generation of the “polar ejection force” or “polar wind” for chromosome congression. KIN I family members are found at the kinetochores. This kind of motor does not show any motility but can directly induce the depolymerization of K-fibers. When it comes to anaphase, the microtubule-depolymerizing activity of KIN I coordinates with the plus-end-achoring activity of KIN N, contributing to the K-fiber shortening and poleward forces that segregate chromosomes.

The above model is the simplified version of activities of different microtubule-based motor families correlating their functions to spindle morphogenesis, force generation and chromosome movement. However, the actual working mechanisms underlying the mitotic motors in various animal systems are far more complicated. It should be noted that individual
motors always work in the context with other motors. This functional multiplicity is represented by cooperativity and redundancy. A delicate balance of complimentary and antagonistic forces generated by bipolar KIN N and KIN C kinesins have been identified throughout eukaryotic organisms (Pidoux et al., 1996; Sharp et al., 1999; Mountain et al., 1999). Also, functional redundancy by similar motors could be important to provide a backup way to maintain the high fidelity of chromosome segregation in case of disruption of the other motor’s activity.

A good example of the functional cooperation of mitotic motors was revealed by Sharp et al. (2000). In this study living, fluorescent tubulin-labeled *Drosophila* embryos were used as a model and the activity of one or more motors were specifically inhibited in order to observe the consequence through time-lapse confocal microscopy. Three mitotic motors were chosen: KLP61F, Ncd and cytoplasmic dynein, which represent the canonical kinesin and cytoplasmic dynein superfamilies. KLP61F belongs to the bipolar kinesins subfamily and moves unidirectionally towards the plus-end of microtubules. Evidence indicated that KLP61F motor could cross-link the interpolar MTs bundles resulting in the maintenance spindle bipolarity. Cytoplasmic dynein, together with its “activator” dynactin, forms a large complex that moves in the opposite direction of KLP61F to the minus-end of microtubules. Their results showed that dynein, which is anchored by dynactin to the cell cortex provides the major force to separate the spindle poles in the initial interphase-prophase transition. After nuclear envelope breakdown, KLP61F was released from the nucleus and joined the force production with cytoplasmic dynein to push the poles further apart. The pushing force of KLP61F was generated through cross-linking and sliding the antiparallel interpolar MTs.
The similar additive pushing force from cytoplasmic dynein and KLP61F might also explain the spindle elongation during anaphase B.

There is a delicate force balance in these steps through the activity of the C-terminal kinesin, Ncd. The ability of Ncd to cross-link interpolar MTs antagonizes the initial spindle poles pushing force from cytoplasmic dynein. From prophase to prometaphase, the pulling force of Ncd is counterbalanced by KLP61F. Finally, the pushing force generated by KLP61F and cytoplasmic dynein and pulling force generated by Ncd reaches a balance, which forms a uniform, bipolar metaphase spindle with a constant microtubule length and static shape and size.

Ncd (nonclaret disjunctional) was among the first of several *Drosophila* mitotic kinesins discovered in the early 1990’s. It was classified as a member of the minus end-directed kinesin-14 subfamily and originally identified as a meiotic kinesin. It plays important roles in the assembly of bipolar meiotic spindles and chromosome segregation in oocytes (Hatsumi and Endow, 1992a, b). Later, by both observation of live Ncd-GFP fusion proteins and specific Ncd antibody stainings in early Drosophila embryos, this kinesin was localized to the spindle poles, including centrosomes and forms a fusiform, spindle-like structure. It colocalizes extensively with the microtubule spindle in a cell-cycle dependent pattern. This Ncd-formed spindle is maintained from early metaphase to mid anaphase. Then it redistributes into the midbody through telophase. In contrast to the microtubule spindle, Ncd-GFP forms filaments across the metaphase chromosomes where the metaphase plate was excluded by tubulin staining (Endow and Komma, 1996). A mutant form of Ncd-GFP fusion protein (a missense mutation in its ATP-binding region that might affect its motor activity) could still localize to the centrosomes and mitotic spindle fibers. However, multiple
dominant spindle abnormalities were observed. In those mutant embryos, centrosomes frequently detached from interphase nuclei. At metaphase, spindles were frayed and spurred and often connected to adjacent spindles by branched microtubule fibers. As a result of the abnormal spindle, chromosome segregation defects such as lagging chromosomes were seen at anaphase. This phenotype is consistent with the observation in Ncd RNAi depletion S2 cells. The K-fibers are unfocused when Ncd expression level is knocked down (Goshima and Vale, 2003). Furthermore, the turnover rate of Ncd on the microtubules is rapid as indicated by FRAP experiments, which means in vivo, Ncd can bind and release from K-fibers and cannot serve as a static antiparallel MTs cross-linker (Goshima et al., 2005). The frayed spindles and splayed K-fibers all suggest a function for Ncd in spindle poles focusing. Ncd can cross-link inter-K fibers and transport them along the astral MTs (centrosomal MTs) by its minus end-directed motor activity.

In conclusion, the interplay between microtubules and microtubule motors is indispensable for mitosis and meiosis. The mitotic motor activities and resultant forces production directly contributes to various aspects of spindle assembly and function including separation of initial duplicated centrosomes, regulation of microtubule dynamics, maintenance of spindle bipolarity, organization of spindle poles, attachment and movement of chromosomes and cytokinesis.

Nonmotor Microtubule-associated Proteins

While the dynamics of microtubules and the force production activities of mitotic motors attract much attention of cytologists in their search for the mechanisms underlying how the mitotic spindle assembles, recent studies have indicated that a large number of
nonmotor microtubule-associated proteins also play essential roles in the organization of the spindle. Because the assembly of the eukaryotic spindle is a highly coordinated process required for the accuracy of genetic material segregation, it is reasonable to expect other factors besides microtubules and motor proteins would be involved in the fine-tuning of spindle functions. It was noted that the physiological functions of those nonmotor microtubule-associated proteins are diverse including structural roles to nucleate and stabilize microtubules organization and regulatory roles to influence motor functions and cell cycle control (Manning and Compton, 2007).

NuMA (nuclear mitotic apparatus protein) is a large coiled–coil protein identified in mammalian cells (Compton and Cleveland, 1994). So far, orthologs of NuMA have been identified in Xenopus, Drosophila, mouse, human and other species. It is sequestered in the interphase nucleus. After NEB, NuMA interacts with cytoplasmic dynein and is transported to the spindle poles where it can help to focus the microtubule minus ends and maintain the integrity of the pericentriolar matrix to connect centrosomes to the spindle body (Merdes et al., 1996; Merdes et al., 2000). Interruption of NuMA function causes splayed microtubule minus ends and dissociation of the spindle poles, suggesting that it can form multivalent microtubule-binding matrices that cross-link and stabilize microtubule minus ends at the spindle poles (Haren and Merdes, 2002).

TPX2 (targeting protein for Xklp2) was originally identified as an interaction partner of a Xenopus KIN N motor, Xklp2. This plus end-directed kinesin can accumulate at the minus end of microtubules during mitosis through the activity of the cytoplasmic dynein-dynactin complex and TPX2 (Wittmann et al., 1998). Functional orthologs of TPX2 are also found in C. elegans, Drosophila, mouse and human. TPX2 starts to localize to the center of
the mitotic asters at the NEB. From prometaphase until anaphase, TPX2 continues to associate with spindle poles as well as spindle microtubules. At late anaphase and telophase, TPX2 redistributes to the midbody of the spindle. Immunodepletion of TPX2 in frog egg extracts leads to disintegration of spindle poles, fewer spindle microtubules between poles and the chromosomes, and sometimes multipolar spindles. This loss-of-function phenotype and the ability of TPX2 to direct bind microtubules in vitro suggests that TPX2 can directly bundle microtubules in order to maintain the spindle pole architecture and stabilize the spindle microtubules (Wittmann et al., 2000).

HURP (hepatoma up-regulated protein) was discovered through a functional genomic screen for mitotic regulators in HeLa cells. Its orthologs exist in all model system from S. cerevisiae, C. elegans, Drosophila, Xenopus to mouse. During mitosis HURP colocalizes with the mitotic spindle, especially the K-fibers, in a concentration gradient that increases towards the chromosomes. When HURP was depleted in HeLa cells by RNAi, the spindle check point proteins such as Mad2 and BubR1 were activated due to persistence of unaligned chromosomes and a decrease in tension across the sister kinetochores on aligned chromosomes. In vitro data showed that HURP is a direct microtubule binding protein and enhances the polymerization of microtubules. This enhancement could stabilize spindle microtubules to promote their polymerization into a bipolar spindle. By potentially regulating spindle microtubule dynamics, HURP facilitates efficient capture of kinetochores from K-fibers and maintains the proper interkinetochore tension that is crucial for the initiation of anaphase (Wong and Fang, 2005).

NuSAP (nucleolar spindle-associated protein) is a novel vertebrate protein that exhibits high mRNA and protein levels in proliferating mitotic cells. NuSAP shows an
intriguing cell-cycle dependent distribution pattern. At interphase NuSAP is concentrated in
the nucleoli. During mitosis, it localizes to the central spindle surrounding the chromosomes
and also colocalizes with the microtubule spindle. In NuSAP-depleted cells, spindle
microtubule arrays are disorganized and less dense and there are chromosome condensation
and alignment defects. These dramatic spindle defects often resulted in aberrant segregation
of less condensed chromosomes and abnormal cytokinesis. NuSAP can interact with
microtubules through its C-terminus and its overexpression results in unusually long, curved
and thick microtubule bundles in the interphase cytoplasm (Raemaekers et al., 2003). Similar
properties of NuSAP were also observed in *Xenopus*. The *Xenopus* NuSAP induces extensive
bundling of spindle microtubules in egg extracts. The mechanism underlying the microtubule
bundling effect of excess NuSAP is revealed by *in vitro* reconstitution experiments. NuSAP
first can stabilize the microtubules against depolymerization (catastrophe phase). Secondly, it
can cross-link microtubules into an aster-like fibrous network. Interestingly, these two effects
can be blocked by specific Importins (Imp). Imp β can suppress the cross-linking activity of
NuSAP, while Imp α and Imp7 decrease its microtubule-stabilizing activity. Besides its
interaction with microtubules, NuSAP shows high concentration in the vicinity or on the
chromosomes. In this way NuSAP can target microtubule nucleation to the chromosomes and
assist in maintaining bipolar spindle integrity through its microtubule stabilizing and cross-
linking activities (Ribbeck et al., 2006, 2007).

Astrin (aster-associated protein) is another protein that is purified with the
microtubules from mammalian mitotic extracts. It contains a large predicted coiled-coil
domain in its C-terminus. At the beginning of mitosis Astrin concentrates on the spindle
poles. It then associates with spindle microtubules as well as the poles at metaphase and
anaphase and goes to the spindle midzone from anaphase to telophase. Astrin also shows a spotted localization at the kinetochores of bioriented chromatids suggesting that it might stabilize the kinetochore connected K-fibers. Domain analysis indicates that the spindle targeting region of Astrin is mainly in its C-terminus (Mack and Compton, 2001).

TOGp (tumor overexpressed gene) belongs to the XMAP215/ Dis1 MAP (microtubule-associated protein) family that is believed to promote microtubule plus-end assembly by antagonizing the depolymerization activity of KIN I kinesins. It is highly expressed in tumors and brains. In mitotic cells TOGp localizes to the centrosomes and spindle microtubules. In vitro TOGp can co-sedimente with taxol stabilized microtubules and is able to promote the nucleation and polymerization of microtubules from a nucleation center (Charrasse et al., 1998). Recent siRNA experiments show that TOGp- depleted cells form multipolar spindles with decreased microtubule length and density. The microtubule shortening phenotype is opposite to the observation from MCAK (kinesin-13 motors, KIN I)-depleted cells, which is consistent with the hypothesis that the antagonization of these two family proteins contributes to the bipolarity of the spindle. Absence of TOGp also causes centrosome fragmentation and unfocused microtubule minus ends at the spindle poles. Thus, TOGp plays essential roles during mitosis in maintaining centrosome integrity, focusing of microtubule minus ends at the spindle poles, and bipolar morphology of spindle (Cassimeris and Morabito, 2004).

RHAMM (receptor for hyaluronic-acid-mediated motility), and its Xenopus homolog XRHAMM, are centrosome-associated proteins. They localize to the spindle microtubules with highest concentration at the spindle poles. This polar localization depends on the activity of the dynein-dynactin complex because inactivation of this complex blocks the
spindle pole localization of XRHAMM. Furthermore, interaction between human RHAMM and dynein is detected by coimmunoprecipitation experiments. XRHAMM associates with TPX2 and \( \gamma \)-TuRC (\( \gamma \)-tubulin ring complex) at the spindle poles. Inhibition of human RHAMM expression results in abnormal spindle morphology including formation of multipolar spindles and a block in mitotic progression. Functional experiments show that XRHAMM and its associated protein such as TPX2 and \( \gamma \)-tubulin can promote anastral spindle assembly in \textit{Xenopus} egg extracts and even directly induce the nucleation of microtubules in a pure tubulin solution (Maxwell et al., 2003; Groen et al., 2004).

The Toucan protein was originally described for its function during \textit{Drosophila} oogenesis. Later its localization to the mitotic spindle was observed in syncytial embryos. By employing the inducible \textit{in vivo} RNAi technique in early embryos, loss of Toucan function was found to lead to abnormal pseudometaphase spindles with unfocused spindle poles and overcondensed chromosomes. Anaphase was also affected with loss of interpolar MTs and absence of the central spindle, which accounted for the obvious chromosome segregation defects. \textit{In vitro} MT overlay assays showed a direct binding of Toucan to the microtubules. This interaction could help to stabilize the interpolar MTs, which is supported by the stabilization of microtubule network in Toucan over-expressed mammalian cells (Debec et al., 2001; Mirouse et al., 2005).

Nuclear envelope breakdown marks the beginning of prometaphase and the whole nucleus undergoes reorganization from disassembly to reassembly during mitosis. During this process several key structural components are found in the mitotic spindle such as nuclear pore complex members. The Nup107-160 complex is the major component after nuclear pore disassembles at mitosis. In human cells immunostaining shows that four
components of this complex localize to prometaphase spindle poles, kinetochores and proximal spindle fibers. In *Xenopus* egg extracts the Nup107-160 complex also goes to reconstituted spindles. In the absence of this complex nucleated microtubules are disassembled very quickly leaving only a small number of bipolar spindles with fewer microtubules and largely unattached mitotic chromosomes. This data suggests that the Nup107-160 complex not only functions at the nuclear pore complex at interphase, but also plays important roles in mitosis to promote spindle assembly (Orjalo et al., 2006).

Because centrosomes are the major microtubule nucleation sites during mitosis, recent high throughput screening of centrosome-associated proteins has identified several centrosomal proteins that are involved in spindle assembly. One of them is the human Cep192 (centrosomal protein of 192 kDa). It is required for the mature centrosome machine and Cep192 siRNA depleted cells completely lose functional centrosomes during mitosis. In addition, spindle bipolarity is disrupted and the pericentriolar matrix is disorganized with mislocalization of γ-tubulin, pericentrin and other PCM components. In contrast, overexpression of Cep192 causes multiple, extracentriolar foci. Taken together, these findings indicate that Cep192 stimulates the formation of the scaffold for a functional PCM to nucleate microtubules and assemble the spindle (Gomez-Ferreria et al., 2007). Another example is Pontin, a component of a chromatin remodeling complex that also interacts with γ-TuRC (γ-tubulin ring complex). The loss of function analysis in different systems including *Drosophila* S2 cells, mammalian cells and *Xenopus* egg extract reveals that Pontin contains a mitotic specific function to regulate the centrosomes integrity, spindle pole organization and spindle microtubule assembly (Ducat et al., 2008).
With the development of genome-wide screening techniques for mitotic regulators some unexpected factors have been identified, which change the traditional view of the organization of a functional mitotic spindle. Rae1, an mRNA export factor, was purified as a spindle assembly factor from a screen based on an aster formation activity assay in *Xenopus* egg extracts. Rae1 localizes to spindle microtubules and is enriched at the spindle poles. Direct interaction of Rae1 with microtubules is indicated by *in vitro* MT pellet assays, although the binding affinity is low. Depletion of Rae1 in either in *Xenopus* egg extracts or in human cells results in significant spindle assembly defects including unorganized spindle poles, long bundled microtubules with fewer fibers, multipolar spindle, and chromosome alignment and segregation defects. In association with other mRNA binding proteins, Rae1 forms a large RNP complex and this entire complex functions to nucleate and stabilize microtubules. Surprisingly, RNA is required for both the integrity of this Rae1-RNP complex and the assembly of mitotic spindle. RNase treatment can severely inhibit spindle and asters assembly in egg extract and the presence of RNA or RNA-containing RNP particles on the spindle has also been observed, although translation does not seem to play roles in the functions of RNA involved in spindle assembly (Blower et al., 2005). Rae1 might be involved in several ways to regulate normal spindle formation since a mitotic-specific interaction is characterized between Rae1 and NuMA and this interaction may enhance the microtubule cross-linking ability of NuMA (Wong et al., 2006).

Recent studies discovered some novel mitotic functions of previously characterized multifunctional proteins. β-catenin, whose functions are involved in cell-cell adhesion and the Wnt-stimulated transcriptional activation pathway, has been found to redistribute to spindle poles and the midbody during mitosis. It co-fractionates with centrosomes.
Centrosomes fail to completely separate when β-catenin levels are reduced in HeLa cells by RNAi, resulting in a dramatic increased frequency of monoastral mitotic spindles. These results demonstrate that β-catenin is a functional component of the mammalian mitotic spindle and it might regulate centrosome separation and establishment of spindle bipolarity (Kaplan et al., 2004). The Drosophila tRNA methyltransferase Dnmt2 has also recently been shown to localize to mitotic spindles. It is present in both cytoplasmic and nuclear fractions and is bound to a hypothesized nuclear matrix structure. In Drosophila syncitial embryos, Dnmt2 can form a spindle-like structure during mitotic divisions and this localization is microtubule dependent (Schaefer et al., 2008). It will be interesting to see the effect on the mitotic spindle as well as the nuclear matrix when the normal function of Dnmt2 is disrupted and what activities are dependent on its tRNA methyltransferase enzymatic activity.

Many motor proteins associate with nonmotor proteins to find their correct targeting sites on the microtubules or maintain the physiological ability to exert force within the spindle. For instance, cytoplasmic dynein is able to focus the minus end of microtubules at spindle poles but requires the presence of NuMA. With the help of TPX2, kinesin Xklp2 is targeted to spindle poles to perform its motor activity (Wittmann et al., 1998). Furthermore, a recent discovery from budding yeast demonstrated that a nonmotor protein Vik1 can directly form a heterodimer with a motor protein Kar3 (kinesin-14 family, KIN C). The microtubule binding capability of Vik1 is higher than that of Kar3, in which facilitates the cooperative microtubule interaction of the Kar3/Vik1 heterodimer without losing motility (Allingham et al., 2007). On the other hand, the mitotic localization of many nonmotor proteins also depends on their interaction with motor proteins. As mentioned above, the spindle pole targeting of RHAMM relies on transportation by cytoplasmic dynein. Another example is the
spindle pole localization of the TOGp homolog, Msps in *Drosophila*, which is driven by the minus end-directed Ncd kinesin (Cullen and Ohkura 2001).

During the cell cycle the dynamics of microtubules and the functions of mitotic regulators must be strictly coordinated spatially and temporally in order to assemble the normal spindle. To reach this goal mitotic kinases play very important roles to control this process and regulate the proper cell cycle progression. Eighty kinases were shown in an RNAi screen in Drosophila S2 cells to have potential functions in mitosis (Bettencourt-Dias et al., 2004), including two kinase families that had been well characterized to be the main mitotic regulation kinases.

The Aurora kinase family belongs to the serine/threonine kinase family. It is an evolutionarily conserved family identified in various eukaryotic systems. Three homologs, Aurora A, B, and C are present in mammals, while only Aurora A and B exist in other species including *Drosophila, Xenopus* and *C elegans*. Aurora A localizes to the spindle poles throughout mitosis and provides both regulatory and structural roles for pole organization. Aurora A can recruit γ-tubulin, the TACC/MAP215 family and CNN (centrosomin) proteins to the centrosome for its maturation into form a functional “microtubule organization center” (MTOC) that can nucleate microtubules (Ducat and Zheng, 2004). Evidence indicated that TPX2 is one of the intrinsic regulators of Aurora A activity. It binds Aurora A and blocks the access of protein phosphatase 1 to keep Aurora A in its active phosphorylated form (Eyers et al., 2003). Aurora B is part of the chromosome passenger complex and localizes to the centromeres before anaphase and relocates to the spindle midzone at the site of the future cleavage furrow from anaphase until the cytokinesis.
Aurora B is in association with INCENP and Survivin to form a complex and is believed to regulate chromosome alignment and segregation (Andrews et al., 2003).

Polo-like kinase (Plk) is a large serine/threonine kinase family that participates in many mitotic events. The first Plk, Polo, was identified in a *Drosophila* screen for mutants affecting spindle pole behavior (Sunkel and Glover, 1988). Afterwards, four Plk members were found in mammalian cells (Plk1, Plk2/Snk, Plk3/Fnk/Prk, and Plk4/Sak), three in *Xenopus* (Plx1-3), while only one was found in *Drosophila* and yeast. As the example of polo-like kinase family, mammalian Plk1 expression is mostly found in highly proliferating tissues and is most active in late S phase to mitosis. Plk1 localization is dynamic and cell-cycle dependent. It is primarily targeted to the centrosomes and kinetochores from early mitosis although a smaller fraction of Plk1 also goes to the spindle midbody in late anaphase (Golsteyn et al., 1995). By phosphorylating different substrates such as Cyclin/Cdk complex subunits, γ-tubulin, centrosomal protein Nlp, kinetochore protein Hec1/Ndc80, anaphase-promoting complex/cyclosome (APC/C) members, and CHO1/MKLP-1, Plk1 provides essential functions required for mitotic entry, maturation of centrosomes, assembly of bipolar spindles, condensation and alignment of chromosomes, transition from metaphase to anaphase and promotion of cytokinesis. The regulatory mechanism and upstream regulators of Plk1 is still elusive, although evidence suggests that Cdk1, MAPK and PKA might be the possible kinases to phosphorylate Plk1 for its activation (Weerdt and Medema et al., 2006).

*Model of Mitotic Spindle Assembly*

Modeling of mitotic spindle assembly has been a big challenge to cell biologists since this macromolecular machinery is comprised of a huge number of molecules including the
basic structural component comprised of microtubules along with mitotic motors and regulators. In addition, all of these functional elements must be regulated coordinately to ensure the correct morphology and functions of a mitotic spindle. With the expansion of knowledge about different mitotic molecular participants and the development of advanced techniques and mathematical and computational modeling methods, two mechanisms are proposed to describe the pathway of bipolar spindle formation.

The first “search and capture” model was hypothesized based on the discovery of the intriguing “dynamic instability” property of microtubules (Mitchison and Kirschner, 1984). The core MT nucleation sites in this model are the duplicated centrosomes. Centrosomes are made up of a pair of cylindrical centrioles surrounded by a pericentriolar matrix that contains the MT-nucleating γ-tubulin ring complex (Scholey et al., 2003). A radius of highly dynamic astral microtubules is nucleated from two opposite centrosomes. The plus ends of these microtubules randomly undergo cycles of growth and shrinkage, exploring the cytoplasm away from centrosomes until they reach the kinetochores of chromosomes. The “capture” of the microtubules by kinetochores results in stabilization of microtubule ends and establishes a relatively stable connection between chromosomes and spindle poles. Over time, increasing numbers of “free” astral MTs are repetitively captured by kinetochores leading to the bundling of K-fibers. Finally, when sister kinetochores are in contact with microtubules emanating from both poles, a typical fusiform bipolar spindle is formed (Kirshner and Mitchison, 1986).

This hypothesis accounted well for the intrinsic properties of microtubules and the MT nucleation capability of centrosomes for the assembly of spindles and explains several key features of mitotic spindle formation which were supported by direct visualization of
capture of microtubules by kinetochores in live newt lung cells (Hayden et al., 1990). However, several questions also arose from this model. First, the inefficiency of random capture cannot explain the typical relatively short duration of prometaphase (15-30 minutes), especially in the cells with large numbers of kinetochores (Wollman et al., 2005). Second, the pole movement of chromosomes termed as “mono-orientation” (Skibbens et al., 1993) before their congression, a common observation in many cells, is another conceptual difficulty with this model. Lastly, the generality of this model does not apply to those acentrosomal animal systems such as large meiotic eggs and higher plant cells. Thus, an alternative “chromosome-directed” pathway was proposed to address these inconsistencies.

Chromosome driven mitotic spindle formation was first identified in Xenopus eggs in which injection of nuclei or DNA could induce the assembly of spindle-like structures (Karsenti et al., 1984). In this model, mitotic chromosomes are the core MT nucleation sites. Microtubules are nucleated and growth in the vicinity of chromatin is promoted by the signals from chromosomes. Then the randomly oriented microtubules are self organized into a bipolar structure with the help of mitotic motors and various microtubule associated proteins. This hypothesis was supported by independent discoveries from several animal systems that successfully formed a spindle without centrosomes, such as Xenopus (Heald et al., 1996) and Drosophila (Basto et al., 2006). The strong evidence for this “chromosome-directed” pathway was provided by the recent characterization of the activities of small GTPase Ran involved in mitosis.

Ran belongs to the Ras GTPase superfamily and was identified as an important cofactor for nucleocytoplasmic transportation (Moore and Blobel, 1993). Similar to other GTPases, Ran functions as a molecular switch which means the GTP or GDP bound status
determines its interactions with effectors. RanGTP is concentrated in the nucleus by the chromatin-associated guanine nucleotide-exchange factor (GEF) RCC1 which catalyzes the exchange of GDP for GTP on Ran (Bischoff and Ponstingl, 1991). In contrast, RanGTP is hydrolyzed into RanGDP in the cytoplasm by RanGAP1, a cytoplasmic GTPase-activating protein (GAP), assisted by cytoplasmic Ran-binding proteins RanBP1 and RanBP2 (Clarke and Zhang, 2008). When nuclear envelope breaks down in mitosis, a concentration gradient of RanGTP is established around the mitotic chromosomes through the activity of RCC1 on the chromosomes (Carazo-Salas et al., 1999). This RanGTP gradient promotes microtubule nucleation and facilitates spindle formation by two mechanisms (Carazo-Salas et al., 2001). Microtubule nucleation is directly stimulated by this gradient around chromosomes. The other mechanism is that significant numbers of spindle assembly factors (SAFs) are released by RanGTP from inhibitory complexes with importin-α-importin-β dimer. RanGTP can interact with importin β resulting in dissociation of these factors and creation of a local concentration of microtubules stabilizing regulators around the chromosomes that promote the capture of astral microtubules (Bastiaens et al., 2006). In fact, a number of spindle assembly factors have been identified whose activities are regulated by RanGTP. TPX2 is one of the important targets released by RanGTP and is involved in targeting the motor protein XKLP2 to microtubule minus ends and activate Aurora A kinase for the assembly of the normal centrosomes (Trieselmann et al., 2003). The K-fiber stabilization factor HURP is another direct target of importin β, and can be released and activated in a similar pathway as TPX2 through the interaction of RanGTP with importin β (Sillje et al., 2006).

Although a Ran-GTP gradient is observed in various cells, it is not an essential pathway in those cells containing centrosomes. In such cells, injection of dominant-negative
regulators of Ran-GTP gradient only has minimal effect which delay the transition from mono- to bipolar/spindle organization (Kalab et al., 2006). While the Ran-GTP induced spindle assembly could be more important in large cells without centrosomes such as eggs and early embryos, in normal cells this pathway has important implications for the “search and capture” hypothesis. The nucleated microtubules around chromosomes increase the efficiency of astral MTs “capture” by kinetochores. In addition, it might provide a backup pathway in case the normal nucleation activity of centrosomes is disrupted in cells. Therefore, the “centrosome-directed” model and “chromosome-directed” model are not mutually excluded. They work cooperatively and kinetochores, centrosomes and microtubules all contribute to spindle assembly (O’Connell and Khodjakov, 2007).

Whatever spindle assembly pathway, centrosome-directed or chromosome-directed, is chosen by the cell, microtubule-based motors are always required for the organization of mitotic spindle. Through different mechanism, they can crosslink and slide the adjacent antiparallel MTs, transport chromosomes and other mitotic cargos along the MTs and regulate dynamics of MTs (Goshima and Vale, 2003; Goshima et al., 2005; Tao et al., 2006). In addition, bipolar (plus-end-directed) and C-terminal (minu-end-directed) kinesins continue to generate the antagonistic outward and inward forces on ipMTs to position the spindle poles and stabilize the metaphase spindle. These force generation motors together with microtubule dynamics cause constant tension to be extended on the mitotic apparatus. Physical calculations indicate that the large forces produced in a spindle are in the range of nanonewtons. Although from theoretically calculation microtubule bundles and motor proteins are able to generate corresponding forces, it is still far below the measured force developed on the spindle and the force difference would result in the buckling of MTs and
collapse of spindle. Thus, a stabilizing structural element to anchor the motor proteins or MT-MT crosslinkers might exist to provide a scaffold or strut (Nicklas, 1983; Scholey and Mogilner, 2003; Johansen and Johansen, 2002). Some other theoretically considerations also imply the existence of such a scaffold or “spindle matrix” structure. Spindles are not expected to be curved if there is no other tensile structure to act against. And from proteomic analysis, out of 795 proteins purified from a human spindle, only 151 were previously known to be associated with mitotic spindles (Sauer et al., 2005). Taken together, our knowledge about the mechanism of the assembly of a mitotic spindle and how the force is generated with the spindle to segregate the chromosomes is incomplete. The studies of such a “spindle matrix” might give us a better understanding to explain many “missing pieces” of the mitotic spindle apparatus, one of the most complicated cellular macromolecular complexes.

**Spindle Matrix**

The spindle matrix is a structure hypothesized to provide a more or less stationary substrate that functions as a backbone or strut for mitotic motor molecules to interact with during force generation and microtubule sliding (Pickett-Heaps et al., 1997). In the past 40 years, a variety of experimental results in different eukaryotic systems have suggested the existence of a spindle matrix through manipulation of spindle apparatus and characterization of a number of potential spindle matrix components.

_Evidence for a spindle matrix_
Back in the 1960s in some spindle isolation procedures it was found that the volume of the nonmicrotubule part was greater than that of the microtubules. The presence of nonmicrotubule components was further demonstrated by the discovery of linear arrays of particles in the absence of microtubules (Goldman and Rebhun, 1969; Forer, 1969). Later, the concept of a “spindle remnant” was raised from the observations by different groups. Antibody-labeled kinesin remained associated with the spindle remnant in an amorphous, spindle shaped structure after MT disassembly in sea urchins (Leslie et al., 1987). Another group showed that tektin related proteins were localized to the spindle remnant as well as to spindles after tubulin depletion (Steffen and Link, 1992). All these data indicated that microtubules are not the only members of the mitotic apparatus and this nonmicrotubule portion or “spindle remnant” left after disassembly of MTs might be correspond to a “spindle matrix”.

Strong evidence for a spindle matrix comes from the observation of UV microbeam irradiation experiments. A UV microbeam can induce disassembly of a small region on the K-fibers that connect chromosomes to the spindle poles. During anaphase the poleward movement of chromosomes were thought to be based on a “Pac-man” model, in which disassembly of kMTs at the kinetochores powered this activity. If this was true, cutting of the K-fibers should stop the segregation of chromosomes. However, the observation from the experiments was in the opposite. The chromosomes continued to move towards the spindle poles even after the UV microbeam mediated kMT disassembly (Pickett-Heaps et al., 1997; Sillers and Forer, 1983; Spurck et al., 1997). Not only that, some chromosomes showed a transient acceleration until the kMT-stub reconnected to the centrosomes. This data suggests that there is another force generation source element acting on chromosome poleward
movement and that kMTs is essential for this process. This microtubule-independent element is probably associated with a spindle matrix and generates the force required for chromosome segregation, while the function of spindle MTs is to resist this force indicating a kind of tensegrity present in spindle structure (Pickett-Heaps et al., 1997).

Mitoic kinesin Eg5 is a conserved microtubule plus-end directed motors with an essential role in establishing bipolarity of the spindle. It is known to be localized to the entire spindle with enrichment at the poles in both *Xenopus* extracts and vertebrate somatic cells (Sawin and Mitchison, 1995; Kapoor et al., 2000). To explore the mechanism by which Eg5 targets to the spindles and its exact functions involved in the promotion of spindle bipolarity, the fluorescent speckle microscopy method was used to track Eg5 dynamics in *Xenopus* extract spindles. Surprisingly, a significant population of Eg5 was static over a short period despite that microtubule flux continued towards the spindle poles. This observation suggested that a static, nonmicrotubule scaffold structure might exist within a spindle to affect the Eg5 dynamics and be consistent with the concept of a spindle matrix (Kapoor and Mitchison, 2001). Although a lack of direct molecular information on the biochemical composition of the spindle matrix has been the major concern and argument about the existence of this structure, several proteins have been identified recently from different organism displaying the properties as a potential spindle matrix component.

*Molecular Components of Spindle Matrix*

NuMA is a large protein containing a unique head and tail domain and a long internal coiled-coil domain that is predicted to oligomerize (Compton et al., 1992; Yang et al., 1992). Its capability to form a filament structures was revealed by different experiments.
Immunogold EM showed that NuMA localized to core filaments of the hypothesized nuclear matrix (Zeng et al., 1994). Artificially expressing this nuclear protein in the cytoplasm by removing its NLS (nuclear localization sequence) led to the formation of networks of interconnected 5-nM filaments of NuMA protein (Saredi et al., 1996). Though it was originally thought to be a nuclear matrix protein, NuMA also plays indispensable structural roles for mitotic spindle organization, as suggested by functional analysis. Immunodepletion of NuMA blocks the mitotic progression and promotes collapse of fully assembled spindles (Yang and Snyder, 1992). In addition, expression of a dominant-negative C-terminal truncation of NuMA causes a similar mitotic failure and micronucleation phenotype (Compton and Cleveland, 1993). Based on its spindle pole localization, its capacity to form a matrix-like structure, and its ability to bind and bundle microtubules (Haren and Merdes, 2002), NuMA is proposed to from a matrix structure to stabilize the mitotic spindle pole organization (Merdes et al., 1996). This hypothesis was supported by the observation of irregular and unfocused MT arrays due to the immunodepletion of NuMA in mitotic assembly extracts. The polar localization of NuMA depends on the dynein-dynactin complex and NuMA-based matrix functions to promote and stabilize the fusiform spindle. The existence of a NuMA-based matrix surrounding the centrosomes was strongly supported by immunogold EM techniques that showed NuMA was localized to an electron-dense matrix at the spindle pole. NuMA remained predominantly in the insoluble fraction even after microtubules were disassembled by nocodazole treatment in cell fractionation experiments (Dionne et al., 1999). In conclusion, NuMA was proposed to be part of the mitotic spindle matrix and its pericentrosomal matrix helped to focus MT minus end and counterbalance the forces exerted by mitotic motors at the spindle poles. However, due to its constrained
localization to the spindle poles at metaphase, the NuMA-based matrix is not likely to provide support for the entire spindle structure. There must be some other components to perform this function.

Another novel coiled-coil domain protein was identified in *S. cerevisiae* named Fin1p (filaments in between nuclei). It contains two putative coiled-coil regions in its C-terminus and was identified as a 14-3-3-interacting protein. Expression of GFP-tagged Fin1p in yeast cells indicated that Fin1p can form a filamentous structure extending between the spindle pole bodies and its subcellular localization is cell-cycle dependent. Fin1p reorganizes from a nonfilamentous structure in nondividing nuclei to a filament localization which is largely colocalized with spindle microtubule fibers between mother and daughter nuclei during mitosis. Detailed analysis by EM and AFM showed that purified His-tagged Fin1p is capable of self-assembly into 10-nm filaments *in vitro* (van Hemert et al., 2002) Recent studies have been focused on the dissection of Fin1p functional domains contributing to spindle stability. Activity of Fin1p is carefully regulated through its phosphorylation status during cell cycle. Dephosphorylation at N-terminus of Fin1p in anaphase promotes its localization to the spindle where it forms extended filaments to stabilize the spindle microtubules. Premature localization of Fin1p’s phosphor-mutnat (Fin1p\(^{3A}\)) to the spindle affects the metaphase spindle structure and causes cell lethality due to the impairment of chromosome segregation, which is similar to the dominant-negative effect when His-tagged Fin1p was overexpressed in cells. The C-terminal coiled-coil domain is involved in self-assembly and Fin1p localization to spindle microtubules while the N-terminal part is sufficient for Fin1p targeting to the spindle poles (Woodbury and Morgan, 2007a, b). Although the microtubule-independent Fin1p-filamentous structure potentially comprise a spindle matrix scaffold in yeast and is suggested
to play a role in stabilizing spindle microtubules, the Fin1p null mutant is viable without any significant spindle defects. This indicated that Fin1p might not be the only spindle matrix candidate in *S. cerevisia* and a redundant pathway might supplement the loss of Fin1p.

The identification of another coiled-coil protein, Ase1p (anaphase spindle elongation), supports this idea. Ase1p localizes to the spindle midzone and works as a homodimer to bind and bundle spindle microtubules. Its function is necessary for anaphase spindle elongation. Moreover, FRAP assay indicated Ase1p is relatively static within the midzone and during spindle elongation in the anaphase, and was thus proposed to form a matrix-like structure to cross-link the midzone spindle and maintain anaphase spindle integrity (Schuyler et al., 2003). Taken together, a potential spindle matrix scaffold in *S. cerevisia* may be comprised of two redundant molecular components, Fin1p and Ase1p and it is very likely that more candidates would be identified since there are more nonmotor elements existing in the spindle midzone of *S. cerevisia* (Woodbury and Morgan, 2007 a, b).

The intermediate filament protein Lamin is the major component of the interphase nuclear lamina. Based on biochemical properties, lamins can be categorized into A and B types. Lamin B is the abundant form ubiquitously expressed in metazoans and essential for cell viability (Goldman et al., 2002). Lamins not only provide mechanical support for the structural integrity of the nucleus but also are involved in important nuclear functions including nuclear envelope assembly, DNA replication, and gene expression. Because of the observation that lamin B associates with mitotic spindles in mammalian cells (Beaudouin et al., 2002; Maison et al., 1997), potential mitotic functions of lamin B were studied. In both *Xenopus* egg extracts and HeLa cells, lamin B was associated with mitotic spindles and the region enclosing the spindle. Reduced expression of lamin B either by siRNA or
immunodepletion led to various mitotic defects such as splayed spindle poles, abnormal spindle organization with chromosome congression defects, and delayed or prolonged prometaphase and metaphase. Furthermore, lamin B was found to assemble a matrix structure on mitotic spindle that was dependent on the activity of Ran-GTP, not the polymerized microtubules. This nonmicrotubule lamin B-defined matrix tethered several key spindle assembly factors (SAFs) including NuMA, Eg5, and XMAP215 even after microtubules were disassembled by nocodazole treatment. Consequently, disruption of the lamin B matrix caused mislocalization of SAFs on spindles during mitosis. Therefore, besides its nuclear functions in interphase, lamin B also is involved in the formation of a hypothesized spindle matrix during mitosis. The lamin B-defined matrix does not depend on microtubules but requires Ran-GTP. This matrix can promote spindle assembly by anchoring several essential spindle assembly factors (Tsai et al., 2006).

In addition to proteins and DNA (chromosomes) molecules, other macromolecules also associate with mitotic spindle. Among these molecules, poly (ADP-ribose) (PAR) received recent attention because poly (ADP-ribose) polymerase (PARP) was found to localize to the spindle in vertebrate cells (Smith, 2001). PAR is a large, branched and negatively charged polymer which belongs to the post-translational modification. PARP catalyzes its polymerization onto the substrate while poly (ADP-ribose) glycohydrolase (PARG) regulates its glycohydrolysis from the acceptor. Examination of PAR in metaphase spindle showed a filamentous and punctate appearance colocalized with spindle microtubules. The population of PAR is enriched in the spindle and a direct association between PAR and spindles was suggested. Perturbation of PAR by either addition of extra PARG or immunodepletion causes rapid disorganization of pre-assembled spindle structures
resulting in unfocused spindle poles, disconnected spindle halves, and decreased interpolar microtubules. When PAR is disrupted before spindle assembly, it completely blocks formation of bipolar spindles whereas monopolar microtubule aster formation is unaffected. Interestingly, PARP and PARG, the PAR regulatory enzymes, show similar localization with PAR on the spindle microtubules. These observations indicate that PAR may be involved in the organization and structure of bipolar spindles. Two possibilities might explain the essential involvement of PAR in spindle assembly. Through the activity of PARP or PARG, the functions of spindle proteins might be regulated by PARsylation. Alternatively, PAR itself might play a structural role to form a potential spindle matrix. The size, branching and high negative charge of PAR suggests the possibility it may mediate the assembly of a matrix scaffold, which is consistent with the low turnover rate of PAR in mitotic spindle (Chang et al., 2004).

**Putative Spindle Matrix Components in Drosophila**

*Skeletor, Megator and East*

In the *Drosophila* model system recent studies have revealed several promising molecular candidates contributing to the formation of a bona fide spindle matrix complex (Johansen and Johansen, 2007). Skeletor encodes an 81-kDa protein that was identified by screening a nuclear antigen with an intriguing dynamic redistribution pattern during mitosis in *Drosophila* embryos (Walker et al., 2000). Skeletor-specific antibody demonstrates that Skeletor is a chromosome-associated protein in interphase nuclei. At prometaphase, it is released from nuclei and forms a spindle-like structure prior to the microtubule spindle
assembly. During metaphase, the Skeletor-defined spindle and microtubule spindle are largely co-aligned with each other. The Skeletor spindle maintains its fusiform structure extensively across the metaphase plate from end to end through anaphase when chromosome segregation occurs. At telophase, Skeletor still appears as a spindle in the midbody even when chromosomes begin to decondense and reassociate with Skeletor at the poles. Strong evidence indicating that Skeletor is a spindle matrix candidate comes from nocodazole treatment experiments in *Drosophila* embryos. The Skeletor-defined spindle persists when the microtubules are depolymerized by nocodazole. Therefore, all the properties displayed by the Skeletor-defined spindle make Skeletor an excellent molecular candidate for a spindle matrix component. However, Skeletor is a low-complexity protein without any predicted conserved structural domains. Thus, it is possible that Skeletor is just one member of a spindle matrix comprised of a complex of multiple proteins and is not itself the structural component.

In order to search for structural elements of spindle matrix, the Bx34 antigen, a nuclear rim and nuclear extra-chromosomal-localized protein (Zimowska et al., 1997) was revisited. The Bx34 antigen, now named Megator, is a 260-kDa protein with a large N-terminal coiled-coil domain and a short C-terminal acidic region. Newly generated Megator antibodies reveal a specific mitotic redistribution of Megator. Megator reorganizes into a fusiform structure during prophase and maintains its spindle structure from prometaphase to anaphase in a pattern that is identical to the Skeletor staining pattern. At telophase the majority of Megator goes to the spindle midbody while Skeletor reassociates with decondensed chromosomes. To understand the relationship between the Megator-defined spindle and the microtubule spindle, cold treatment was used to disassemble the microtubules
at metaphase. In the absence of microtubules, the Megator spindle remains intact and shows extensive co-alignment with the Skeletor spindle. This indicated that formation of the spindle-like structure comprised of Megator and Skeletor does not necessarily require microtubules. The overall protein sequence and structure comparison suggests Megator is the ortholog of the mammalian nuclear pore complex Tpr protein and the large N-terminal coiled-coil domain makes it possible that Megator might contribute to the structural scaffold of a spindle matrix. Intriguingly, this hypothesis is supported by the observation that the N-terminal coiled-coil domain of Megator is able to independently self-assemble into spherical structures in the cytoplasm of transfected S2 cells, whereas the C-terminal acidic region is the targeting domain involved in the nuclear and spindle localization of Megator. Megator is an essential gene during development as indicated by the early lethality observed in homozygous mutants. In S2 cells, RNAi depletion of Megator leads to a dramatic decrease of the mitotic index suggesting that loss of Megator function may prevent cells from entering metaphase, thus strongly inferring a requirement for Megator during mitosis (Qi et al., 2004).

The third protein is EAST, another large 265-kDa protein identified in *Drosophila* that shows an inter-chromosomal localization in the interphase nucleus. Analysis of EAST mutants showed a high frequency of mitotic errors and chromosome congression defects at prometaphase (Wasser and Chia, 2000; 2003). These observations raised the possibility that EAST may be a potential spindle matrix component. Indeed, EAST shows a similar redistribution pattern as Megator during mitosis. Both proteins form a fusiform spindle structure from prometaphase through anaphase with extensive colocalization. However, at telophase these two proteins diverge with EAST redistributing back to the forming daughter nuclei whereas Megator remains at the spindle midbody. The colocalization of EAST and
Megator suggested that they may interact within the same complex, the spindle matrix structure. This possibility was demonstrated by the ability to co-immunoprecipitate each other from S2 cells (Qi et al., 2005).

**Characterization of Chromator**

In addition to explore putative spindle matrix candidates from known *Drosophila* genes like Megator and EAST, effort was also directed towards identifying new components of the spindle matrix structure. For this purpose, a yeast two-hybrid assay was performed to screen *Drosophila* embryonic libraries for potential interaction with a partial sequence of Skeletor (215aa-474aa) as a bait. Several independent clones were found that represented an unknown gene region, CG10712. From the sequencing results of the ESTs, three alternative transcripts were identified due to variant use of 5’ exons. However, these transcripts all use the same start codon as well as the original reading frame (ORF) suggesting an identical protein was encoded. This novel protein contains 926 amino acids with a predicted size of 101kDa. It was named Chromator because of the presence of a predicted chromodomain in its N-terminal (216aa-260aa) (Rath et al., 2004).

Chromator specific antibody was generated and the immunocytochemical labeling indicated that Chromator shares a very similar cell-cycle dependent distribution pattern to Skeletor. Both of them localize to the chromosomes in interphase nuclei. After the commencement of mitosis, Chromator co-localizes with Skeletor during prophase and reorganizes to form a fusiform spindle structure at metaphase that still co-localizes with Skeletor-defined spindle. It should be noted that Chromotor also localizes to the centrosomes at this stage. At telophase the centrosome localization of Chromator becomes more obvious.
together with a significant level on the spindle midbody whereas Skeletor redistributes to the decondensed chromosomes. This redistribution pattern is consistent in both *Drosophila* syncytial embryos and S2 cells. Furthermore, the Chromator C-terminus is sufficient to target to the microtubule spindle, as revealed by the expression of different Chromator deletion constructs in S2 cells (Rath et al., 2004).

The overall co-aligned spindle structures observed with Skeletor, Megator, EAST and Chromator during mitosis along with their abilities to co-immunoprecipitate from cell lysates indicate that these four proteins are part of the same protein complex. Direct physical interactions have been demonstrated between Chromator and Skeletor by GST-pull down assays and between Chromator and Megator by co-immunoprecipitation assays (Rath et al., 2004).

It is evident that Chromator plays important functional roles in mitosis from the RNAi experiment in S2 cells. Depletion of Chromator leads to abnormal spindle morphology and chromosome segregation defects. Interestingly the scattered chromosome phenotype at anaphase resembles that observed after knockdown of some kinesin motor proteins in S2 cells (Goshima and Vale, 2003; Rogers et al., 2004). Therefore, these data suggested that Chromator is a nuclear-derived spindle matrix component and its functions are required for proper spindle assembly leading to chromosome separation during mitosis (Rath et al., 2004). Further studies of Chromator functions, especially in *in vivo* animal systems, had been limited because the P-element insertion null Chromator mutant is homozygous lethal at embryonic or first instar larvae stage. Thus, generation of hypomorphic allele of Chromator that could survive to late stage would be helpful to dissect its functions in details.
Another implication regarding Chromator functions is from its specific interband region localization on interphase polytene chromosomes. In *Drosophila* accumulating evidence indicates that the formation of the interband/band pattern reflects a common organization mechanism underlying higher-order chromatin packing in general. Chromator was independently identified by another group as an interaction partner of Z4, the interband-specific zinc-finger protein (Eggert et al., 2004; Gortchakov et al., 2005). The function of Z4 is thought to be involved in the organization of polytene chromosomal structure due to the observation of chromatin decompaction and loss of an interband/band pattern in the Z4 mutant. The ability to interact with Z4, together with the specific localization to the polytene chromosomes suggests that Chromator may also play a functional role in maintaining chromatin structure during interphase in addition to its mitotic spindle organizational function during mitosis. A hypomorphic allele of Chromator would be useful for the study of interphase as well as mitotic functions of Chromator.
CHAPTER 2. THE CHROMODOMAIN PROTEIN, CHROMATOR, INTERACTS WITH THE JIL-1 KINASE AND PARTICIPATES IN REGULATING THE STRUCTURE OF DROSOPHILA POLYTENE CHROMOSOMES

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ABSTRACT

In this study we have generated two new hypomorphic Chromator alleles and analyzed the consequences of reduced Chromator protein function on polytene chromosome structure. We show that in Chro\textsuperscript{71}/Chro\textsuperscript{612} mutants the polytene chromosome arms were coiled and compacted with a disruption and misalignment of band and interband regions and with numerous ectopic contacts connecting non-homologous regions. Furthermore, we demonstrate that Chromator co-localizes with the JIL-1 kinase at polytene interband regions and that the two proteins interact within the same protein complex. That both proteins are necessary and may function together is supported by the finding that a concomitant reduction in JIL-1 and Chromator function synergistically reduces viability during development. Overlay assays and deletion construct analysis suggested that the interaction between JIL-1 and Chromator is direct and that it is mediated by sequences in the COOH-terminal domain of Chromator and by the acidic region within the COOH-terminal domain of JIL-1. Taken
together these findings indicate that Chromator and JIL-1 interact in an interband-specific complex that functions to establish or maintain polytene chromosome structure in *Drosophila*.

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**INTRODUCTION**

A striking feature of *Drosophila* polytene chromosomes is the stable and reproducible organization of chromatin into band and interband regions (Zhimulev, 1996). Interband regions are made up of parallel oriented 10 nm chromosome fibrils loosely aligned whereas in banded regions the chromatin is further compacted into 30 nm fibrils forming higher order loops or toroidal structures, the exact nature of which is still ill-defined (Mortin and Sedat, 1982; Ananiev and Barsky, 1985; Schwartz et al., 2001; Zhimulev et al., 2004). It is generally thought that this difference in chromatin organization correlates with important aspects of how gene expression is regulated (Zhimulev and Belyaeva, 2003). However, little is known about the molecules and molecular mechanisms that are responsible for controlling the establishment and maintenance of polytene chromatin morphology. With the goal of identifying such molecules we have recently characterized a novel tandem kinase in *Drosophila*, JIL-1, that localizes specifically to euchromatic interband regions of polytene chromosomes (Jin et al., 1999) and which is the predominant kinase regulating histone H3S10 phosphorylation at interphase (Wang et al., 2001). Analysis of JIL-1 null and hypomorphic alleles showed that JIL-1 is essential for viability and that reduced levels of
JIL-1 protein lead to a misalignment of the interband polytene chromatin fibrils that is further associated with coiling of the chromosomes and an increase of ectopic contacts between non-homologous regions. (Jin et al., 2000; Wang et al., 2001; Zhang et al., 2003, Deng et al., 2005). This results in a shortening and folding of the chromosomes with a non-orderly intermixing of euchromatin and the compacted chromatin characteristic of banded regions (Deng et al., 2005). The intermingling of non-homologous regions can be so extensive that these regions become fused and confluent further shortening the chromosome arms. Based on these findings a model was proposed where JIL-1 functions to establish or maintain the parallel alignment of interband chromosome fibrils as well as to repress the formation of contacts and intermingling of non-homologous chromatid regions (Deng et al., 2005).

Another protein that localizes specifically to interband regions of polytene chromosomes is the chromodomain protein, Chromator (Rath et al., 2004). Chromator was originally identified in a yeast two-hybrid screen as an interaction partner of the putative spindle matrix component, Skeletor, and localizes to the spindle and the centrosomes during mitosis (Rath et al., 2004). Furthermore, functional assays using RNAi mediated depletion in S2 cells suggest that Chromator directly affects spindle function and chromosome segregation (Rath et al., 2004). However, Chromator's localization to polytene interbands suggests it also may play a functional role in maintaining chromatin structure during interphase. Such a role is supported by the finding of Eggert et al. (2004) that Chromator (which these workers refer to as Chriz) is found in a protein complex together with the interband-specific zinc-finger protein Z4 (Eggert et al., 2004; Gortchakov et al., 2005). That Z4 participates in regulating polytene chromosomal structure is likely since Z4 null mutant chromosomes show a decompaction of chromatin and a loss of a clear band/interband pattern
(Eggert et al., 2004). However, the effect of Chromator on polytene chromosome morphology has been difficult to study since null alleles of Chromator die as embryos or first instar larvae before salivary gland polytene chromosomes can be analyzed (Rath et al., 2004; Gortchakov et al., 2005). For this reason we performed an EMS mutagenesis screen that generated two new Chromator hypomorphic alleles. The analysis of these alleles shows that impaired Chromator function leads to disorganization and misalignment of band/interband regions resulting in coiling and folding of the polytene chromosomes. In addition, we demonstrate that Chromator directly interacts with the JIL-1 kinase and that the two proteins extensively co-localize at polytene interband regions. Taken together these findings indicate that Chromator and JIL-1 interact in an interband-specific complex that functions to establish or maintain polytene chromosome structure in Drosophila.

**MATERIALS AND METHODS**

**Drosophila stocks and generation of new Chromator alleles**

Fly stocks were maintained according to standard protocols (Roberts, 1998). Canton-S was used for wild-type preparations. The lethal Chromator P-element insertion allele KG03258 is described in Rath et al. (2004) and the JIL-1<sup>z2</sup> null allele is described in Wang et al. (2001) and in Zhang et al. (2003). New Chromator mutant alleles were generated by ethyl methyl sulfonate (EMS) mutagenesis using standard procedures (Grigliatti, 1986). In this screen <i>w<sup>1118</sup></i> males that had wild-type alleles of Chromator were treated with 25 mM EMS in a 1% sucrose solution for 18-20 hours. EMS treated males were then mass mated with <i>w<sup>1118</sup> TM2 Ubx e/TM6 Sb Tb e</i> virgin females. Male <i>w<sup>1118</sup> Y; */TM6 Sb Tb e</i> progeny from these mass matings were selected and individually mated with <i>y w<sup>1118</sup> KG03258/TM6 Sb</i>
Tb e virgin females. The progeny of these single male matings were screened for potential new Chromator alleles that reduced adult survival rates by more than 50% when heterozygous with the KG03258 allele. Twelve such new Chromator alleles were identified in this screen and outcrossed for 6 generations to w^{118} TM2 Ubx e/TM6 Sb Tb e flies to eliminate non-specific second site mutations on the other chromosomes. Complementation tests between the newly generated alleles revealed that individuals heteroallelic for two of these alleles, Chro^{71} and Chro^{612}, survive to third instar larval stages. The molecular lesions of Chro^{71} and Chro^{612} were determined by PCR mapping and sequencing as described in Zhang et al. (2003). Recombinant JIL-1^{i2} Chro^{71} and JIL-1^{i2} Chro^{612} chromosomes were generated by standard crosses as described in Ji et al. (2005) and the genotypes confirmed by PCR analysis as in Zhang et al. (2003). Balancer chromosomes and markers are described in Lindsley and Zimm (1992).

**Antibodies**

The Chromator-specific mAbs 6H11 and 12H9 have been previously characterized (Rath et al., 2004) and the anti-MSL-1 rabbit antiserum was the generous gift of Drs. M. Kuroda and R. Kelley. The affinity purified Hope rabbit anti-JIL-1 polyclonal antibody was described in Jin et al. (1999) and the anti-GST mAb 8C7 in Rath et al. (2004). The anti-α-tubulin and anti-V5 antibodies were obtained from commercial sources (Sigma-Aldrich and Invitrogen, respectively).

**Immunohistochemistry and ultrastructural analysis**
Polytene chromosome squash preparations were performed as in Kelley et al. (1999) using the 5 minute fixation protocol and labeled with antibody as described in Jin et al. (1999). S2 cells were affixed onto poly-L-lysine coated coverslips and fixed with Bouin’s fluid for 10 min at 24°C and methanol for 5 min at -20 °C. The cells on the coverslips were permeabilized with PBS containing 0.5% Triton X-100 and incubated with diluted primary antibody in PBS containing 0.1% Triton X-100, 0.1% sodium azide, and 1% normal goat serum for 1.5 h. Double and triple labelings employing epifluorescence were performed using various combinations of antibodies and Hoechst to visualize the DNA. The appropriate species- and isotype- specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution) to visualize primary antibody labeling. 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 high resolution Spot CCD camera. Confocal microscopy was performed with a Leica confocal TCS NT microscope system equipped with separate Argon-UV, Argon, and Krypton lasers and the appropriate filter sets for Hoechst, FITC, Texas Red, and TRITC imaging. A separate series of confocal images for each fluorophor of double labeled preparations were obtained simultaneously with z-intervals of typically 0.5 µm using a PL APO 100X/1.40-0.70 oil objective. Images were imported into Photoshop where they were pseudocoloured, image processed, and merged. In some images non-linear adjustments were made for optimal visualization of Hoechst labeling of chromosomes. For ultrastructural studies we prepared polytene chromosome squash preparations of wild-type and Chro71/Chro612 third instar larvae according to the procedure of Semeshin et al. (2004) as described in Deng et al. (2005).
**Biochemical Analysis**

*SDS-PAGE and immunoblotting.* SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) with transfer buffer containing 20% methanol and in most cases including 0.04% SDS. For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 µm nitrocellulose, and using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody diluted 1:1000 in Blotto. The signal was visualized using chemiluminescent detection methods (SuperSignal kit, Pierce). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680). Immunoblot analysis of *Chro*\(^{71}\)/*Chro*\(^{612}\) mutants was performed as described in Wang et al. (2001) and Zhang et al. (2003) using extracts from third-instar larvae with wild-type larvae as controls.

**Overlay experiments.** The four truncated GST-JIL-1 fusion proteins, JIL-1-NTD (residues 1-211), JIL-1-KDI (residues 251-554), JIL-1-KDII (residues 615-917), and JIL-1-CTD (residues 927-1207) have been previously described in Jin et al. (2000) and the constructs JIL-1-CTD-A (residues 887-1033) and JIL-1-CTD-B (residues 1034-1207) were described in Bao et al. (2005). Two Chromator GST-fusion proteins, Chro-NTD (residues 1-346) and Chro-CTD (residues 329-926) were cloned into the pGEX4T vector using standard techniques (Sambrook and Russell, 2001). The respective GST-fusion proteins were expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich) according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech). For the overlay interaction assays approximately 2 µg of GST or of the appropriate JIL-1 GST-fusion proteins were fractionated by SDS-PAGE and electroblotted to
nitrocellulose. The blots were subsequently incubated with approximately 2 µg of either the Chro-NTD or the Chro-CTD GST-fusion protein overnight at 4 °C in PBS with 0.5% Tween-20 on a rotating wheel. The blots were washed 4 times for 10 minutes each in PBS with 0.5% Tween-20 and binding detected by antibody labeling with either Chromator mAb 6H11 or 12H9. Input proteins were analyzed by SDS-PAGE and immunoblotting with GST-antibody.

**Immunoprecipitation assays.** For co-immunoprecipitation experiments, anti-JIL-1 or anti-Chromator antibodies were coupled to protein A beads (Sigma) as follows: 10 µl of affinity purified Hope anti-JIL-1 serum or 10 µl of mAb 6H11 was coupled to 30 µl protein-A Sepharose beads (Sigma) for 2.5 h at 4 °C on a rotating wheel in 50 µl ip buffer (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 1 mM Phenylmethylsulfonyl fluoride, and 1.5 µg Aprotinin). The appropriate antibody-coupled beads or beads only were incubated overnight at 4 °C with 200 µl of S2 cell lysate on a rotating wheel. Beads were washed 3 times for 10 min each with 1 ml of ip buffer with low speed pelleting of beads between washes. The resulting bead-bound immunocomplexes were analyzed by SDS-PAGE and Western blotting according to standard techniques (Harlow and Lane, 1988) using mAb 6H11 to detect Chromator and Hope antiserum to detect JIL-1. For V5-antibody immunoprecipitation experiments in S2 cells we used a full length Chromator (926 aa) construct with an in-frame V5 tag at the COOH-terminal end previously described by Rath et al. (2004). The S2 cells were transfected with this construct using a calcium phosphate transfection kit (Invitrogen) and expression was induced by 0.5 mM CuSO₄. Cells expressing the Chromator construct or mock transfected control cells were harvested 18-24 h after induction. Nuclear extracts were prepared as in
(Smith et al., 2000), immunoprecipitated with 10 µl of anti-V5 antibody coupled to 30 µl of protein-A Sepharose beads as described above, fractionated by SDS-PAGE, and immunoblotted using Hope JIL-1 antiserum for detection.

**RNAi interference**

dsRNAi in S2 cells was performed according to Clemens et al. (2000) and as described in Rath et al. (2004). A 780 bp fragment encoding the 5′ end of Chromator cDNA was PCR amplified and used as templates for *in vitro* transcription using the Megascript™ RNAi kit (Ambion). 40 µg of synthesized dsRNA was added to 1 X 10^6 cells in six-well cell culture plates. Control dsRNAi experiments were performed identically except pBluescript vector sequence (800 bp) was used as template. The dsRNA treated S2 cells were incubated for 6-7 d and then processed for immunostaining and immunoblotting. For immunoblotting 10^5 cells were harvested, resuspended in 50 µl of S2 cell lysis buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, and 1% Nonidet P-40), boiled and analyzed by SDS-PAGE and western blotting with anti-Chromator antibody (mAb 6H11) and anti-α tubulin antibody.

**RESULTS**

**Chromator and the JIL-1 kinase directly interact.**

The interphase localization of Chromator to polytene chromosome interband regions (Rath et al., 2004; Eggert et al., 2004; Gortchakov et al., 2005) is very similar to that reported for the JIL-1 histone H3S10 kinase (Jin et al., 1999; 2000; Wang et al., 2001). For this reason we explored whether the two proteins were present in the same macromolecular
complex by performing co-immunolocalization and biochemical studies. Figure 1 shows confocal images of a double labeling with JIL-1 and Chromator specific antibodies of a third instar larval polytene chromosome squash preparation. JIL-1 localization is shown in green, Chromator protein localization in red, and co-localization is indicated by yellow regions in the composite image. The predominantly yellow labeling in the composite image indicates that JIL-1 co-localizes with Chromator extensively along the entire length of the chromosome arms (Fig. 1A). At some band locations in the composite image the color is more green or orange than pure yellow due to slight differences in the relative staining intensity of the two antibodies which can vary from preparation to preparation. However, in all cases examined the banding patterns themselves appeared identical (Fig. 1B, C) providing evidence that Chromator and JIL-1 are co-localized at most if not all interband regions.

In order to further probe for a potential interaction we performed immunoprecipitation (ip) experiments using S2 cell lysates. For these immunoprecipitation experiments, proteins were extracted from S2 cells, immunoprecipitated using either JIL-1 or Chromator specific antibodies, fractionated on SDS-PAGE after the ip, immunoblotted, and probed with antibodies to Chromator and JIL-1, respectively. Figure 2A shows an ip experiment using Chromator antibody where the immunoprecipitate is detected by JIL-1 antibody as a 160 kDa band that is also present in the S2 cell lysate. This band was not present in the lane where immunobeads-only were used for the ip (Fig. 2A). Figure 2B shows the converse experiment: JIL-1 antiserum immunoprecipitated a 130 kDa band detected by Chromator antibody that was also present in S2 cell lysate but not in control ips with immunobeads only. Furthermore, we stably/transiently transfected S2 cells with a V5-tagged full-length Chromator construct and prepared nuclear extracts from lysed cells. Figure
2C shows an ip experiment using V5 antibody where a 160 kD a band detected by JIL-1 antibody that was also present in the S2 cell nuclear extract was co-immunoprecipitated. This band was not present in the lane where the V5 antibody ip was performed in nuclear extracts from untransfected control S2 cells (Fig. 2C). These results strongly indicate that Chromator and the JIL-1 kinase are present in the same protein complex.

To further characterize the interaction between Chromator and JIL-1 and to identify the domains mediating the interaction we performed overlay assays with GST-fusions of various regions of the two proteins. For the initial screening of JIL-1 we used four GST-fusion proteins covering the NH$_2$-terminal domain (JIL-1-NTD), the first kinase domain (JIL-1-KDI), the second kinase domain (JIL-1-KDII), and the COOH-terminal domain (JIL-1-CTD) as diagrammed in Fig. 3A. For Chromator we generated a NH$_2$-terminal construct including the chromodomain (Chro-NTD) as well as a COOH-terminal construct (Chro-CTD) (Fig. 3A). Figure 3B, C show JIL-1 GST-fusion proteins that were coupled with glutathione agarose beads, washed, fractionated by SDS-PAGE, transferred to nitrocellulose paper, and incubated with glutathione agarose bead purified Chro-CTD and Chro-NTD GST-fusion constructs, respectively. Protein interactions were detected with Chromator COOH-terminal mAb 6H11 in Fig. 3B and with the NH$_2$-terminal Chromator mAb 12H9 in Fig. 3C. As illustrated in Fig. 3B (arrows) only the JIL-1-CTD and Chro-CTD fusion constructs were found to interact in these assays. Western blot analysis of the GST proteins purified in these experiments and detected with GST-antibody showed that similar levels of JIL-1-NTD, JIL-1-KDI, JIL-1-KDII, and JIL-1-CTD fusion proteins were present in these assays (Fig. 3D). Immunoblots of the purified Chro-CTD and Chro-NTD fusion constructs used for the overlays are shown in Fig. 3B (lane 6) and Fig. 3C (lane 6), respectively. The region of JIL-
1 that was found to interact with Chromator, the JIL-1 CTD-domain, can be further divided into two distinct regions (Bao et al., 2005): an acidic region from residue 887-1033 that has a predicted pI < 4 and a basic region from residue 1034-1207 that has a pI > 11 (Fig. 3A). Thus, in order to better define the sequences of JIL-1 responsible for the molecular interaction between JIL-1 and Chromator, we generated GST fusion proteins comprising these two regions, JIL-1-CTD-A and JIL-1-CTD-B (Fig. 3A), and performed overlay experiments with the Chro-CTD construct as described above. As shown in Fig. 3E the JIL-1-CTD and JIL-1-CTD-A fusion proteins both interacted with Chro-CTD as detected by Chromator mAb 6H11 (arrows) whereas JIL-1-CTD-B or GST alone did not. Western blot analysis of the GST proteins purified in these experiments and detected with GST-antibody showed that approximately equivalent levels of JIL-1-CTD, JIL-1-CTD-A, and JIL-1-CTD-B fusion proteins were present in these assays (Fig. 3F). An immunoblot of the purified Chro-CTD fusion construct used for the overlay is shown in Fig. 3E (lane 5). Taken together these results suggest that the interaction between JIL-1 and Chromator is direct and that it is mediated by sequences in the COOH-terminal domain of Chromator and by the acidic region within the COOH-terminal domain of JIL-1.

**Generation of hypomorphic Chromator alleles.**

We have previously demonstrated that the P-element insertion KG03258 is a lethal loss-of-function mutation in the Chromator gene (Rath et al., 2004). Unfortunately, most homozygous KG03258 animals die as embryos and none survive past the first instar larval stages, thus precluding the analysis of polytene chromosome structure in third instar larval salivary gland cells. Attempts to generate new hypomorphic loss-of-function Chromator
alleles that survive to third instar larval stages by imprecise P-element excisions have so far been unsuccessful (Rath et al., 2004; Gortchakov et al., 2005). In addition, such studies are likely to be complicated by the close proximity of the essential neighboring ssl1 gene to the Chromator locus (Rath et al., 2004; Gortchakov et al., 2005). For these reasons we generated EMS-induced point mutations in the Chromator gene using standard protocols (Grigliatti, 1986). We identified a total of 12 new alleles that reduced adult survival rates by more than 50% when heterozygous with the KG03258 allele as compared to a wild-type allele. Complementation tests between the newly generated alleles revealed that individuals heteroallelic for two of these alleles survive to third instar larval stages. These two alleles were subsequently sequenced and further characterized in this study. The Chro71 allele is comprised of a G to A nucleotide change at nucleotide position 402 of the Chromator coding sequence that introduces a premature stop codon resulting in a truncated 71 amino acid protein (Fig. 4A). The truncated NH2-terminal fragment does not contain the chromodomain and Chro71 is likely to act as a strong hypomorphic or null allele. Chro71 is homozygous embryonic lethal with no first instar larval escapers. The Chro612 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 (Fig. 4A). Chro612 is homozygous embryonic lethal with a few first instar larval escapers. However, Chro71/Chro612 transheterozygotes survived to third instar larval stages although no larvae have been observed to pupate. This suggests that Chro612 is a severe hypomorphic loss-of-function allele that nonetheless in combination with Chro71 can provide partial function sufficient for development to third instar stages. Although genetic crosses were performed to replace the other chromosomes it should be noted that the presence of second site mutations
on the third chromosome cannot be ruled out and may account for the early lethality of homozygous $\text{Chro}^{612}$ mutants. However, the effect of such potential mutations are likely masked in the $\text{Chro}^{71}/\text{Chro}^{612}$ transheterozygotes. The immunoblot of protein extracts from wild-type and $\text{Chro}^{71}/\text{Chro}^{612}$ third instar larvae in Fig. 4B demonstrates that no detectable full-length Chromator protein was present in the mutant larvae. The immunoblot was labeled with Chromator specific mAb 6H11 (Rath et al., 2004) which was generated to COOH-terminal sequence deleted in both the $\text{Chro}^{71}$ and $\text{Chro}^{612}$ alleles.

**Polytene chromosome structure is disrupted in hypomorphic Chromator mutants.**

The generation of severely hypomorphic Chromator alleles that as transheterozygotes survived to third instar larval stages allowed for an analysis of their effect on polytene chromosome structure. Figure 4C and D show a comparison of polytene squashes from wild-type and $\text{Chro}^{71}/\text{Chro}^{612}$ larvae labeled with Hoechst. Whereas wild-type polytene chromosomes show extended arms with a regular pattern of Hoechst stained bands (Fig. 4C), this pattern is severely perturbed in $\text{Chro}^{71}/\text{Chro}^{612}$ mutant larvae (Fig. 4D). In the latter preparations band/interband regions were disrupted and the chromosome arms were coiled and condensed (Fig. 4D). In order to better understand the underlying causes of these defects we performed an ultrastructural analysis by preparing squashes of polytene chromosomes from $\text{Chro}^{71}/\text{Chro}^{612}$ third instar larvae for transmission electron microscopy (TEM) and comparing them with squashes from wild-type larvae (Fig. 5). Figure 5A shows the orderly segregation into interband and the more electron dense banded regions in TEM of a wild-type autosome. However, in $\text{Chro}^{71}/\text{Chro}^{612}$ mutants the alignment of the chromatids in the interbands was disrupted and the orderly arrangement of compacted chromatin in the
banded regions was severely affected as well (Fig. 5B and C). Another feature of the phenotype was the folding and coiling of the chromosomes with numerous ectopic contacts connecting non-homologous regions (Fig. 5C). However, in spite of these disruptions distinct band and interband regions were still clearly discernable in the mutant chromosomes. These findings suggest that normal Chromator function is required for maintaining the orderly segregation of bands and interbands in polytene chromosome structure.

**Localization of Chromator and JIL-1 in mutant polytene chromosomes.**

The finding that Chromator and JIL-1 directly interact within the same protein complex raised the question whether one of the proteins is required for the polytene chromosome localization of the other. To address this issue we performed double labelings of polytene squashes from wild-type, *Chro*\(^{71}/Chro^{612}\), and *JIL-1^{z2}/JIL-1^{z2}* third instar larvae with the JIL-1 pAb Hope and the Chromator mAb 6H11. The *JIL-1^{z2}* allele is a true null that when homozygous produces no JIL-1 protein (Wang et al., 2001; Zhang et al., 2003). Banded regions of the polytene chromosomes were labeled with Hoechst. Figure 6A (upper panel) shows that in wild-type polytene squashes both Chromator and JIL-1 colocalizes to interband regions in a pattern complementary to the Hoechst labeling. In *Chro*\(^{71}/Chro^{612}\) mutants the polytene chromosome morphology is perturbed; however, JIL-1 continued to be localized to interband regions not labeled by Hoechst (Fig. 6A, middle panel). No full-length Chromator protein was detected by mAb 6H11 which recognizes a COOH-terminal epitope (Rath et al., 2004) in these preparations. In the *JIL-1* null mutants with no detectable JIL-1 protein the chromosome morphology was also severely perturbed as previously described (Wang et al., 2001; Deng et al., 2005); nonetheless, Chromator still localized to chromosome
regions in a complementary pattern to the Hoechst labeling of compacted chromatin (Fig. 6A, bottom panel). These data strongly suggest that Chromator localization to the polytene chromosomes does not depend on the JIL-1 protein. However, a JIL-1 dependence on the Chromator protein could not be ruled out by these experiments because the Chro612 allele can give rise to a truncated Chromator protein that contains the chromodomain and that has enough COOH-terminal sequence to potentially recruit JIL-1. The Chro71 allele is unlikely to play such a role because it lacks the entire COOH-terminal domain and because the NH$_2$-terminal region of Chromator is without a functional nuclear localization signal (Rath et al., 2004). Labelings of Chro71/Chro612 mutant polytene squashes with the NH$_2$-terminal Chromator mAb 12H9 showed that Chro612 protein indeed localizes to the interband regions of the polytene chromosomes (Fig. 6B). We therefore employed RNAi methods in S2 cells to completely deplete Chromator levels and assayed for the consequences on JIL-1 localization by labeling with JIL-1 antibody. In control cells as shown in Fig. 7 JIL-1 antibody specifically labels the chromatin and is upregulated on the X-chromosome (the S2 cell line is a "male" cell line) as previously described (Jin et al., 1999; 2000; Wang et al., 2001). To verify that it indeed was the X chromosome that had upregulated JIL-1 levels the preparations were double labeled with anti-MSL-1 antibody. MSL-1 is a member of the MSL (male specific lethal) dosage compensation complex and is found only on the male X chromosome (Kuroda et al., 1991; Kelley and Kuroda, 1995). However, the distribution of JIL-1 did not change in Chromator RNAi treated cell cultures (Fig. 7A, bottom panel) where Chromator protein levels were reduced below detectable limits by immunoblot analysis (Fig. 7B). These results suggest that Chromator is not required for JIL-1 localization to chromatin nor for upregulation of JIL-1 on the male X chromosome. Similar results were also obtained
for male Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant polytene chromosomes labeled with JIL-1 antibody (Fig. 6C). Furthermore, while the phenotype of JIL-1 and Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant polytene chromosomes generally resemble each other a notable difference is that the morphology of the male X chromosome in Chro\textsuperscript{71}/Chro\textsuperscript{612} larvae was similar to that of the autosomes (Fig. 6C) and not "puffed" as in JIL-1 null mutants (Wang et al., 2001; Deng et al., 2005). Although these experiments indicate that Chromator does not directly recruit JIL-1 to polytene chromosomes the presence of truncated Chromator proteins in the Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant does not allow us to completely exclude the possibility of an indirect requirement.

**Genetic interactions between Chromator and JIL-1 alleles**

To determine whether Chromator and JIL-1 genetically interact in vivo we explored interactions between mutant alleles of Chromator and JIL-1 by generating double mutant individuals. Since Chromator and JIL-1 both are located on the third chromosome we first recombined each of the Chro\textsuperscript{71} and Chro\textsuperscript{612} alleles onto the JIL-1\textsuperscript{z2} chromosome. Subsequently, JIL-1\textsuperscript{z2} Chro\textsuperscript{71}/TM6 Sb Tb males were crossed with JIL-1\textsuperscript{z2} Chro\textsuperscript{612}/TM6 Sb Tb virgin females generating JIL-1\textsuperscript{z2} Chro\textsuperscript{71}/JIL-1\textsuperscript{z2} Chro\textsuperscript{612} progeny. In control experiments we crossed JIL-1\textsuperscript{z2}/TM6 Sb Tb males with JIL-1\textsuperscript{z2}/TM6 Sb Tb virgin females generating JIL-1\textsuperscript{z2}/JIL-1\textsuperscript{z2} progeny as well as Chro\textsuperscript{71}/TM6 Sb Tb males with Chro\textsuperscript{612}/TM6 Sb Tb virgin females generating Chro\textsuperscript{71}/Chro\textsuperscript{612} progeny. In these crosses the TM6 chromosome was identified by the Tb marker. Consequently, the experimental genotypes could be distinguished from balanced heterozygotic larvae by being non-Tb and the expected Mendelian ratio of non-Tb to Tb larvae would be 1:2 since TM6/TM6 is embryonic lethal. Table 1 shows that individuals of both the Chro\textsuperscript{71}/Chro\textsuperscript{612} and JIL-1\textsuperscript{z2}/JIL-1\textsuperscript{z2} genotype
develop into third instar larvae in numbers that were not statistically different ( \( P>0.9, \chi^2 \)-tests) from the expected Mendelian ratio with that of TM6 balanced heterozygotes. However, in the \( JIL-1^{2} \ Chro^{71}/JIL-1^{2} \ Chro^{612} \) double mutant combination there was a clear statistically significant difference (\( P<0.001, \chi^2 \)-test) as no non-\( Tb \) third instar larvae were observed. This suggests that a simultaneous reduction in both JIL-1 and Chromator function synergistically reduces viability during development and is consistent with the hypothesis that the two proteins interact in vivo.

**DISCUSSION**

In this study we have generated two new hypomorphic \( Chromator \) alleles and analyzed the consequences of reduced Chromator protein function on polytene chromosome structure. We show that in \( Chro^{71}/Chro^{612} \) mutants the polytene chromosome arms were coiled and compacted with a misalignment of band and interband regions and with numerous ectopic contacts connecting non-homologous regions. Furthermore, we demonstrate that Chromator co-localizes with JIL-1 at polytene interband regions and that the two proteins interact within the same protein complex. Overlay assays and deletion construct analysis suggested that the interaction between JIL-1 and Chromator is direct and that it is mediated by sequences in the COOH-terminal domain of Chromator and by the acidic region within the COOH-terminal domain of JIL-1. However, studies in S2 cells with RNAi mediated Chromator depletion and in \( JIL-1^{2} \) homozygous null mutant backgrounds demonstrated that neither protein is likely to be dependent on the other for its chromatin localization. This suggests that other member(s) of the complex serving as chromatin targeting factors remain to be discovered or that both proteins may have the ability to directly bind to DNA.
Although the *Drosophila* polytene chromosome has served as a widely used model for studying chromatin structure, remarkably little is known about its spatial organization or about the molecular basis for the conjugation of homologous chromatids in the process of polytenization (Ananiev and Barsky, 1985; Schwartz et al., 2001). Recently it has been demonstrated that the JIL-1 kinase which phosphorylates histone H3 Ser10 in interband regions plays a crucial role in maintaining polytene chromosome structure (Wang et al., 2001; Deng et al., 2005; Zhang et al., 2006). In the absence of JIL-1 there is a shortening and folding of the chromosomes with a non-orderly intermixing of euchromatin and the compacted chromatin characteristic of banded regions (Deng et al., 2005) and there is a striking redistribution of the heterochromatin markers dimethyl H3K9 and HP1 to ectopic chromosome sites (Zhang et al., 2006). This suggested a model where JIL-1 kinase activity functions to maintain chromosome structure and euchromatic regions by counteracting heterochromatization mediated by histone H3 dimethylation and HP1 recruitment (Zhang et al., 2006). However, the *Chromator* mutant analysis presented here suggest that JIL-1 activity is necessary but not sufficient for maintaining some of these aspects of polytene chromosome morphology and that Chromator function is also required. Nonetheless, it should be noted that although the polytene chromosome phenotypes of *JIL-1* and *Chromator* mutants resemble each other with coiled and compacted chromosome arms they are not identical. In contrast to *JIL-1* mutant polytene chromosomes, in *Chromator* mutants there is still a clear demarcation between band and interband regions on the ultrastructural level although these bands are severely misaligned. Furthermore, in *JIL-1* null mutants the male X chromosome is differentially affected with a "puffed" appearance whereas in *Chromator* mutants the morphology of the male X chromosome is similar to that of the autosomes.
Thus, it is likely that JIL-1 and Chromator control different but related aspects of chromosome morphology within the complex. That both proteins are necessary and may function synergistically is supported by the finding that a concomitant reduction in JIL-1 and Chromator function dramatically reduces viability during development.

An important feature of the Chromator protein is the presence of a chromodomain. The function of most chromodomain proteins identified thus far has been related to the establishment or maintenance of a variety of chromatin conformations (Cavalli and Paro, 1998; Brehm et al., 2004). For example, HP1 binds to methylated histone H3 and is essential for the assembly of heterochromatin (Nielsen et al., 2001; Jacobs et al., 2002; Peters et al., 2001). Thus, it is possible that Chromator through interactions mediated by its chromodomain participates in a complex with JIL-1 that is required for maintaining properly separated and aligned interband regions as well as a more open chromatin configuration. However, loss of JIL-1 or Chromator function also influences the coherence and organization of bands although neither protein is present in these regions. This suggests that JIL-1 and Chromator function may affect the distribution and/or activity of other molecules important for influencing chromatin structure such as boundary elements and/or the molecular machinery regulating heterochromatin formation and spreading. In support of this notion it has recently been demonstrated that the lethality as well as some of the chromosome morphology defects observed in JIL-1 null or hypomorphic mutant backgrounds may be the result of ectopic histone methyltransferase activity (Zhang et al., 2006).

In addition to the present studies demonstrating an interaction with JIL-1, Chromator has been shown to interact with the spindle matrix protein Skeletor (Walker et al., 2000; Rath et al., 2004) and with the zinc-finger protein Z4 (Eggert et al., 2004; Gortchakov et al.,
The interaction with 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 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 being localized to centrosomes that are devoid of Skeletor-antibody labeling. The extensive co-localization of the two proteins is compatible with a direct physical interaction between Skeletor and Chromator. 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. 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). However, Chromator and Z4 do not appear to associate directly and their chromosomal binding is independent of each other (Gorthakov et al., 2005). Interestingly, the phenotype of loss of Z4 function is somewhat different from that of loss of JIL-1 or Chromator function. *Z4* mutant chromosomes while losing their band/interband organization decompact and attain a cloudy appearance (Eggert et al., 2004) instead of coiling and shortening as in *JIL-1* and *Chromator* loss-of-function mutations. This differential effect on polytene chromosome banding patterns and morphology may reflect that these constituents contribute different activities within one complex or may indicate the presence of more than one molecular assembly, each with
different functions. Thus, future studies will be necessary to further clarify Chromator's interactions with interband specific proteins and its functional role in establishing or maintaining polytene chromosome structure.

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REFERENCES


FIGURE LEGENDS

**Figure 1. Co-localization of JIL-1 with Chromator at polytene chromosome bands.** Double labeling of a female polytene chromosome squash preparation with antibodies against JIL-1 (B) and Chromator (C). The composite image (A) shows the extensive overlap between JIL-1 (green) and Chromator (red) labeling at a large number of chromosome bands as indicated by the predominantly yellow color. The images are from confocal sections.

**Figure 2. Chromator and JIL-1 immunoprecipitation assays.** (A) Immunoprecipitation (ip) of lysates from S2 cells were performed using Chromator antibody (mAb 6H11, lane 4) and JIL-1 antibody (Hope antiserum, lane 3) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using JIL-1 pAb for detection. JIL-1 antibody staining of S2 cell lysate is shown in lane 1. JIL-1 is detected in the JIL-1 and Chromator immunoprecipitation samples as a 160 kD band (lane 3 and 4, respectively) but not in the control sample (lane 2). (B) Immunoprecipitation (ip) of lysates from S2 cells were performed using Chromator antibody (mAb 6H11, lane 3) and JIL-1 antibody (Hope antiserum, lane 4) coupled to immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using Chromator mAb 6H11 for detection. Chromator antibody staining of S2 cell lysate is shown in lane 1. Chromator is detected in the JIL-1 and Chromator immunoprecipitation samples as a 130 kD band (lane 4 and 3, respectively) but not in the control sample (lane 2). The relative migration of molecular weight markers are indicated in kD. (C)
Immunoprecipitation (ip) of nuclear extracts from S2 cells were performed using V5 antibody from cells transfected with a V5-tagged full-length Chromator (lane 2) or from untransfected cells as a control (lane 3). The immunoprecipitations were analyzed by SDS-PAGE and Western blotting using JIL-1 antiserum for detection. JIL-1 antibody staining of S2 cell nuclear extract is shown in lane 1. JIL-1 is detected as a 160 kD band in V5-antibody ips from V5-tagged Chromator transfected S2 cells (lane 2) but not in the untransfected control samples (lane 3).

**Figure 3. Mapping of the JIL-1 interaction domain with Chromator.**

Diagrams of the JIL-1 and Chromator proteins indicating the domains to which GST-fusion proteins were made for mapping. In the overlay experiments various truncated JIL-1 GST-fusion protein constructs to the domains diagrammed in (A) or a GST-only control were fractionated by SDS-PAGE, Western blotted, incubated with Chro-CTD (B) or Chro-NTD (C) GST-fusion protein, and interactions detected with the COOH-terminal Chromator mAb 6H11 in (B) or the NH$_2$-terminal Chromator mAb 12H9 in (C). The only interaction detected was between the JIL-1-CTD and Chro-CTD fusion proteins (arrows in B). Immunoblots of the overlay GST-fusion proteins Chro-CTD and Chro-NTD are shown in (B, lane 6) and in (C, lane 6), respectively. (D) Immunoblot of the input GST-fusion proteins used for the overlay experiments in (B) and (C) detected with the anti-GST mAb 8C7. (E) Overlay experiments with truncated COOH-terminal JIL-1 GST-fusion protein constructs to the subdomains diagrammed in (A) or a GST-only control were fractionated by SDS-PAGE, Western blotted, incubated with Chro-CTD, and interactions detected with the COOH-terminal Chromator mAb 6H11. In these experiments interactions between Chro-CTD and
JIL-1-CTD as well as JIL-1-CTD-A were detected (arrows) but not between Chro-CTD and JIL-1-CTD-B. Immunoblot of the overlay GST-fusion protein Chro-CTD is shown in lane 5. (F) Immunoblot of the input GST-fusion proteins used for the overlay experiments in (E) detected with the anti-GST mAb 8C7. This defined the JIL-1 COOH-terminal acidic domain as sufficient for mediating interactions with the COOH-terminal domain of Chromator. The relative migration of molecular weight markers is indicated to the right of the immunoblots in kD.

**Figure 4. EMS induced Chromator alleles.** (A) Diagram of the wild-type Chromator protein and the potential truncated protein products of the EMS induced Chromator alleles, Chro\(^{71}\) and Chro\(^{612}\). The Chro\(^{71}\) allele is comprised of a G to A nucleotide change at nucleotide position 402 of the Chromator coding sequence that introduces a premature stop codon resulting in a truncated 71 amino acid protein. The Chro\(^{612}\) 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. (B) 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 and with anti-tubulin antibody as a loading control. Full-length Chromator is detected as a 130 kD protein by mAb 6H11 in wild-type larvae; however, no full-length Chromator is detectable in the mutant larvae. The relative migration of molecular weight markers is indicated to the left of the immunoblots in kD. (C, D) Polytene chromosome preparations from third instar larvae were labeled with Hoechst to visualize the chromatin. Preparations are shown from a wild-type female larvae (C) and
from a female \textit{Chro}^{71}/\textit{Chro}^{612} mutant larvae (D). Reduced levels of wild-type Chromator protein have a severe effect on the structure and organization of larval polytene chromosomes. Note the disruption and misalignment of interband and banded regions and the extensive coiling and folding of the chromosome arms in \textit{Chro}^{71}/\textit{Chro}^{612} mutant chromosomes (D).

**Figure 5. Ultrastructure of \textit{Chro}^{71}/\textit{Chro}^{612} mutant polytene chromosomes.** (A) TEM micrograph of a wild-type polytene chromosome. Note the clear segregation into bands and interbands and the orderly alignment of euchromatic chromatid fibrils. (B, C) Chromosomes from \textit{Chro}^{71}/\textit{Chro}^{612} polytene salivary gland nuclei. The micrograph in (B) shows the disorganization and misalignment of band/interband polytene chromosome regions (arrows). The micrograph in (C) shows the folding and coiling of the chromosomes with numerous ectopic contacts connecting non-homologous regions (arrows).

**Figure 6. Localization of JIL-1 and Chromator in mutant polytene chromosomes.** (A) Triple labelings with the JIL-1 pAb Hope (green), the COOH-terminal Chromator mAb 6H11 (red), and Hoechst (blue) of polytene squashes from wild-type (upper panel), \textit{Chro}^{71}/\textit{Chro}^{612} (middle panel), and \textit{JIL-1}^{22}/\textit{JIL-1}^{22} (lower panel) female 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 (red) and Hoechst (blue) of polytene squashes from a \textit{Chro}^{71}/\textit{Chro}^{612} female third instar larvae. The composite image (comp) is shown to the left. (C) Labeling with the JIL-1 pAb Hope of polytene squashes from wild-type (left micrograph)
and Chro$^{71}$/Chro$^{612}$ (right micrograph) male third instar larvae. Note the upregulation of JIL-1 on the male X chromosome (X) of both wild-type and Chro$^{71}$/Chro$^{612}$ mutant larvae.

**Figure 7. RNAi depletion of Chromator in S2 cells does not affect JIL-1 chromosome localization.** (A) Triple labelings with the JIL-1 pAb Hope (green), anti-MSL-1 antibody (red), and Hoechst (blue) of Chromator dsRNA treated S2 cells (lower panel) and mock treated control cells (upper panel). The composite image (comp) is shown to the left and the location of the X chromosome is indicated with an X. (B) Western blot with Chromator mAb 6H11 of control treated and Chromator RNAi treated S2 cells from the cultures shown in (A). In the RNAi sample Chromator protein levels (Chro) was substantially reduced compared to the level observed in the control cells. Tubulin levels (tub) are shown as a loading control.
Table 1.

Genetic interaction between JIL-1 and Chro alleles

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<sup>a</sup> In these crosses the TM6 chromosome was identified by the Tubby marker. Consequently, the experimental genotypes could be distinguished from balanced heterozygotic larvae by absence of the Tubby marker. The expected Mendelian ratio of non-Tubby to Tubby larvae was 1:2 since TM6/TM6 is embryonic lethal. The percentage of expected genotypic ratios were calculated as: observed non-Tubby larvae X 300/total observed larvae.
Figure 1

Figure 2
Figure 3
CHAPTER 3. THE SPINDLE MATRIX COMPLEX PROTEIN, CHROMATOR, IS REQUIRED FOR PROPER MICROTUBULE SPINDLE FORMATION AND MITOSIS IN DROSOPHILA NEUROBLASTS
ABSTRACT

To study the mitotic function of the spindle matrix complex protein, Chromator, we used two new loss-of-function alleles in the Chromator gene, Chro\textsuperscript{71} and Chro\textsuperscript{612}. We show that neuroblasts from transheterozygous Chro\textsuperscript{71}/Chro\textsuperscript{612} brains display severely disrupted spindle morphology and chromosome segregation defects. The microtubule spindles at metaphase are rudimentary, unfocused and/or without recognizable spindle poles. At anaphase chromosomes are lagging and scattered along the microtubule fibers. To confirm that these defects were due to impaired Chromator functions, a full length Chromator transgene expressed under its own promoter was introduced into the mutant, resulting in a dramatic decrease in spindle defects to near wild type levels. The spindle defects could also be partially rescued by expression of a Chromator C-terminus transgene indicating that the C-terminal domain of Chromator might be important for its functions in spindle organization. Furthermore, we demonstrate using biochemical and immunoaffinity assays that Chromator can directly interact with microtubules as well as microtubule-based mitotic motors, Ncd. These findings strongly support the spindle matrix hypothesis and indicate that Chromator plays a functional role during mitosis.

INTRODUCTION
During cell division an identical set of replicated genetic material must be faithfully distributed to two daughter cells. This purpose is achieved by a mitotic spindle, which is present in all known eukaryotic cells (Mitchison and Salmon, 2001). The spindle apparatus is a complex macromolecular “nanomachinery” arising from the reorganization of an interphase cytoskeletal network. The mitotic spindle is mainly comprised of a bipolar array of oriented microtubules and various associated motors (Karsenti and Vernos, 2001; Scholey, 2003). Although several models have been established to explain spindle structure and function based on knowledge of microtubule dynamics and motor activities, it is still unclear what stabilizes the assembly of the spindle and how mechanical forces are applied to pull the chromosomes to the spindle poles (Pickett-Heaps et al., 1997; Scholey et al., 2001). Thus, the existence of a hypothesized spindle matrix has long been proposed to provide a static substrate to stabilize the spindle during force generation and microtubule sliding (Pickett-Heaps et al., 1997). However, direct molecular evidence for this structure has been elusive. Recently, several proteins have been characterized to carry the properties of a potential spindle matrix component in different systems, such as Fin1p/Ase1p in *S. cerevisiae*, Lamin B in *Xenopus*, and NuMA in mammalian cells (Hemert et al., 2002; Schuyler et al., 2003; Tsai et al., 2006; Merdes et al., 1996). In *Drosophila*, four proteins that derived from different interphase nuclear compartments were identified as promising molecular candidates to form an extensive spindle matrix structure. Two of them, Skeletor and Chromator are associated with chromosomes at interphase (Walker et al., 2000; Rath et al., 2004), while the other two large proteins, Megator and EAST are localized to the interchromosomal region with Megator also present on the nuclear rim (Qi et al., 2004, 2005). All four proteins interact with each other and redistribute to form a spindle-like structure during late prophase prior to
nuclear envelope breakdown. This spindle matrix is largely coaligned with microtubule spindle at metaphase and remains intact through anaphase (Johansen and Johansen, 2002, 2007). Especially, the discovery that Skeletor- and Megator-defined fusiform structures persist despite the depolymerization of microtubules by nocodazole or cold treatment makes them ideal candidates to form a scaffold that structurally supports the mitotic spindle.

Although these four proteins exhibit several characteristic properties consistent with spindle matrix components, their direct functions in mitosis are difficult to discern mainly due to the early lethality from the existing loss-of-function mutants combined with presence of maternal mRNA in the embryos. P-element insertion homozygous mutants of Megator and of Chromator show lethality at the embryonic or first instar larval stage, indicating these proteins might play essential and multiple roles during animal development. However, evidence for functional roles of Chromator in proper spindle organization and chromosome segregation was indicated by RNAi depletion of Chromator in S2 cells, in which a clear disruption of metaphase spindle morphology and scattered chromosomes phenotype during segregation at anaphase were observed. To further study the potential function of Chromator in spindle matrix organization and microtubule spindle assembly, Chro^{71} and Chro^{612}, two hypomorphic alleles of the Chromator gene were used. In brains from third instar larvae of Chro^{71}/Chro^{612} transheterozygotes, the formation of neuroblast spindles was severely disrupted leading to obvious chromosome segregation defects. Moreover, the expression of a full length or a C-terminal truncation of Chromator in transgenic animals rescued the disorganized spindle defects, thus indicating the phenotype was specifically caused by impaired Chromator functions. We further demonstrate that Chromator may directly interact with microtubules and is in the same protein complex with Ncd (nonclaret disjunctional), the
microtubule-based mitotic motors. The Ncd (nonclaret disjunctional) motor is a KIN C subtype kinesin and belongs to the kinesin-14 subfamily. It moves unidirectionally towards the minus end of microtubules. Ncd attracts our special interest because it is a well characterized mitotic motor in Drosophila system. Actually, it is among the first several Drosophila mitotic kinesins discovered back to early 1990s. Interestingly, both specific Ncd antibody labeling or Ncd-GFP live image in early syncytial embryos revealed a cell-cycle dependent redistribution pattern (Endow and Komma, 1996), which is very similar to Chromator and other Drosophila spindle matrix candidates. Ncd is localized to the spindle poles and forms an extensive fusiform spindle-like structure from pole to pole, which is co-aligned with whole microtubule spindles during mitosis. Functional studies firstly indicated that Ncd was required for the assembly of bipolar meiotic or mitotic spindles in oocytes or early embryos to segregate chromosomes. Taken together, these findings suggest that Chromator performs a significant function in helping microtubule spindle organization to facilitate chromosome segregation during mitosis and are strongly consistent with a predicted role of a spindle matrix component.

MATERIALS AND METHODS

Drosophila stocks and generation of new Chromator alleles

Fly stocks were maintained according to standard protocols (Roberts, 1998). Canton S. was used for wild-type preparations. The lethal Chromator P-element insertion allele KG03258 is described in Rath et al. (2004). New Chromator mutant alleles were generated by ethyl methyl sulfonate (EMS) mutagenesis using standard procedures (Grigliatti, 1986). Detailed procedure was described in Rath et al. (2004). Twelve such new Chromator alleles
were identified in this screen and outcrossed for 6 generations to \( w^{118} TM2 Ubx e/TM6 Sb Tb \) flies to eliminate non-specific second site mutations on the other chromosomes. Complementation tests between the newly generated alleles revealed that individuals heteroallelic for two of these alleles, \( Chro^{71} \) and \( Chro^{612} \), survive to third instar larval stages. The molecular lesions of \( Chro^{71} \) and \( Chro^{612} \) were determined by PCR mapping and sequencing as described in Zhang et al. (2003). \( ncd\text{-gfp} \) transgenes were described in Endow and Komma (1996). Balancer chromosomes and markers are described in Lindsley and Zimm (1992).

**Generation of Chromator transgenes**

For NP-Chro\(^{N41D}\) transgene, part of Chromator genomic region including 353 bps promoter sequence and first three introns was PCR amplified from Canton S. genomic sequence and replaced the overlapping region in Chromator cDNA sequence. The resulting full length Chromator plus its potential 5’-expression regulatory elements was fused with GFP tag on its C-terminus and inserted into pUAST vector (Brand and Perrimon, 1993). For Chro-NTD transgene, cDNA sequence corresponding to Chromator residues 1-346 was fused with three tandem NLS sequence cut from pECFP-Nuc vector (BD Biosciences Clontech) and GFP tag on C-terminus and inserted into pUASP vector (Rorth, 1998). For Chro-CTD transgene, cDNA sequence corresponding to Chromator residues 329-926 was fused with GFP tag on its N-terminus and inserted into pUASP vector. The fidelity of the constructs was verified by sequencing at the Iowa State University DNA Facility. Transgenic animals were generated by standard P-element transformation (Roberts 1986). The expression of the transgenes was induced using nervous system specific GAL4 driver: \( P\{w[+mW.hs]=GawB\}elav[C155] \) (Bloomington Stock Center).
Antibodies

The Chromator specific mAb 6H11 and 12H9 have been previously characterized (Rath et al., 2004). Ncd pAb was the generous gift of Dr. Sharyn A. Endow (Duke University, Durham, NC). Anti-GST mAb 8C7 was described in Rath et al. (2004). Phospho-Histone 3 Serine 10 pAb was used as a mitotic chromosome marker from Upstate Inc. The anti-α-tubulin mAb and anti-GFP pAb were obtained from commercial sources (Sigma-Aldrich and Invitrogen, respectively).

SDS-PAGE and immunoblotting

SDS-PAGE was performed according to standard procedures (Laemmli, 1970). Electroblot transfer was performed as in Towbin et al. (1979) with transfer buffer containing 20% methanol and in most cases including 0.04% SDS. For these experiments we used the Bio-Rad Mini PROTEAN II system, electroblotting to 0.2 μm nitrocellulose, and using anti-mouse HRP-conjugated secondary antibody (Bio-Rad) (1:3000) for visualization of primary antibody diluted 1:1000 in Blotto. The signal was visualized using chemiluminescent detection methods (SuperSignal kit, Pierce). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680). For specified experiments, immunoblot analysis was performed using whole extracts or brain extracts from third instar larvae with wild type as controls.

Immunohistochemistry

Larval brain preparations generally followed the protocol of Bonaccorsi et al., (2000) with some modifications. Third instar larval brains were dissected in 0.7% physiological insect saline solution then rinsed in PBS. For antibody labelings the brains were fixed with
4% PFA for 30 min, postfixed for 3 min in 45% acetic acid, and subsequently gently squashed in 60% acetic acid. Squashed samples on the slides were washed in PBT (PBS containing 0.4% Triton X-100) three times (10 minutes each), then blocked for 1 hour in 1% NGS in PBT. Immunostaining was performed by incubation with diluted primary antibody in PBS containing 0.4% Triton X-100, 0.1% sodium azide, and 1% normal goat serum for 1.5 h to overnight. Working dilutions are 1:200 for tubulin antibody, 1:100 for GFP and phospho-Histone 3 Serine 10 antibody; 1:30 for Ncd antibody. Double and triple labelings employing epifluorescence were performed using various combinations of antibodies and Hoechst (0.2µg/µL) to visualize the DNA. The appropriate species- and isotype- specific Texas Red-, TRITC-, and FITC-conjugated secondary antibodies (Cappel/ICN, Southern Biotech) were used (1:200 dilution) to visualize primary antibody labeling. 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 high resolution Spot CCD camera. Images were imported into Photoshop where they were pseudocoloured, image processed, and merged. In some images non-linear adjustments were made for optimal visualization of Hoechst labeling of chromosomes.

**Immunoprecipitation Assays**

For co-immunoprecipitation experiments, anti-Chromator or anti-GFP antibodies were coupled to protein G beads (Sigma) as follows: 100 µl of mAb 12H9 or 10 µl of pAb GFP was coupled to 30 µl protein-G Sepharose beads (Sigma) for 2.5 h at 4°C on a rotating wheel in 50 µl ip buffer (20 mM Tris-HCl pH 8.0, 10 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100, 0.1% Nonidet P-40, 1 mM Phenylmethylsulfonyl fluoride, and 1.5 µg Aprotinin). The appropriate antibody-coupled beads or beads only were incubated
overnight at 4˚C with 200 μl of 0-5 h embryonic lysates from wild type or ncdgfp line on a rotating wheel. Beads were washed 3 times for 10 min each with 1 ml of ip buffer with low speed pelleting of beads between washes. The resulting bead-bound immunocomplexes were analyzed by SDS-PAGE and Western blotting according to standard techniques (Harlow and Lane, 1988) using pAb Ncd (1:300) to detect Ncd and mAb 6H11 (1:2000) to detect Chromator.

**GST pull-down assay**

In pull-down assays Chromator GST-fusion proteins were used to pull down endogenous Ncd protein from 0-5h embryonic lysates. Initially the Chromator GST-fusion proteins Chro-NTD (residues 1-346) and Chro-CTD (residues 329-926) which have been previously described (Rath et al., 2006) and a full length Chromator (residues 1-926) that was PCR amplified from cDNA sequence were cloned into the pGEX4T vector using standard techniques (Sambrook and Russell, 2001). The respective GST-fusion proteins were expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich) according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech). For the in vitro protein-protein interaction assays, approximately 2 μg of GST or the appropriate GST-fusion protein were coupled with glutathione agarose beads and incubated with 500 μl of embryonic lysate at 4˚C overnight on a rotating wheel. The beads were washed 4 times for 10 minutes each in 1 ml PBS with 0.5% Tween-20, and proteins retained on the glutathione agarose beads were analyzed by SDS-PAGE and Western blotting with signals detected Ncd pAb (1:300). Input proteins were analyzed by SDS-PAGE and immunoblotting with GST antibody 8C7 (1:200).

**Microtubules overlay assays**
The two truncated GST-Chromator fusion proteins, Chro-NTD (residues 1-346) and Chro-CTD (residues 329-926) have been previously described (Rath et al., 2006) and construct of Chro-421 (residues 601-926) was described in Rath et al. (2004). Full length Chromator (residues 1-926) and Chro-M (residues 601-926) that were PCR amplified from cDNA sequence were cloned into the pGEX4T vector using standard techniques (Sambrook and Russell, 2001). The respective GST-fusion proteins were expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich) according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech). For the overlay interaction assays approximately 2.5 μg of GST or of the appropriate Chromator GST-fusion proteins were fractionated by SDS-PAGE and electroblotted to nitrocellulose membrane. 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 (80mM Pipes; 2mM MgCl2; 0.5mM EGTA; 25mM NaF) plus 1mM GTP. The blot was then incubated with 8 μg/ml purified bovine tubulin (Cytoskeleton Inc.) in PEMF supplemented with 1mM GTP and 20mM taxol overnight at room temperature. The bound microtubules were detected by standard Western blot analysis using anti-tubulin antibody (1:2000). Input proteins were analyzed by SDS-PAGE and immunoblotting with GST-antibody 8C7 (1:200).
RESULTS

Generation of Chromator hypomorphic alleles

In a previous study we have reported that a P-element insertion KG03258 results in a lethal loss-of-function mutation in the Chro gene with severely reduced Chromator protein levels (2.2± 2.1%, n=6) (Rath et al., 2004). Unfortunately, the KG03258 null allele is homozygous lethal and most animals die in the embryonic stage with very few escaping to the first instar larval stage. This strong lethality makes it difficult to study the potential mitotic functions in homozygous KG03258 embryos due to the maternal Chromator protein which marks any possible phenotypes. Also, the escaped animals die as early as in the first instar larval stage which cannot provide a well developed brain to be used for reliable analysis of dividing neuroblasts by squashes. Therefore, an EMS mutagenesis method was introduced to generate new point mutation alleles in the Chro gene locus following the standard protocols (Grigliatti, 1986). Detailed results of the mutagenesis were reported in a related study about the functions of Chromator pertaining to maintenance of polytene chromosome structure (Rath et al., 2006). Here, we are interested in two new hypomorphic alleles encoding potential truncated proteins. Chro71 contains a G to A nucleotide change which changes tryptophan to a premature stop codon. It leaves only 71 amino acids in the N-terminus of Chromator and removes the chromodomain, suggesting Chro71 is a strong hypomorphic or null allele (Fig. 1 A). Another allele Chro612 is comprised of a C to T nucleotide change that changes glutamine to a premature stop codon, leading to a truncated 612 amino acid polypeptide that consists of the chromodomain and two thirds of the protein (Fig.1 A). Both Chro71 and Chro612 alleles are homozygous lethal at the embryonic stage with very few exceptions of Chro612/Chro612 developing into the first instar larval stage.
However, $Chro^{71}/Chro^{612}$ transheterozygotes can survive to the third instar larvae which indicates the combination of these two strong hypomorphic alleles can exert partial function of Chromator sufficient for the animals to develop into a later stage. Genetic outcrosses to $w^{1118}\ TM2\ Ubx\ e/TM6\ Sb\ Tb\ e$ flies of the new $Chro$ alleles were performed for six generations to remove non-specific second site mutations on the other chromosomes, but second site mutations on the same third chromosome as $Chro$ locus might not be ruled out in $Chro^{71}$ or $Chro^{612}$ alleles. However, the use of $Chro^{71}/Chro^{612}$ animals would probably mask the effect of such mutations and provide a way to analyze the effect of loss of Chromator function.

To check the expression of Chromator in the transheterozygous mutant, an immunoblot of protein extracts from wild-type and $Chro^{71}/Chro^{612}$ third instar larvae was probed with by Chromator-specific mAb 6H11. Fig.1 B demonstrates that no full length Chromator was present in the mutant larvae. Interestingly, compared to wild type, the size of the third instar laval brain from $Chro^{71}/Chro^{612}$ transheterozygotes was deceased by around 50% (Fig. 1C). This characteristic small brain and small imaginal discs (data not shown) phenotype led us to further investigate the mitotic defects in the Chromator mutant.

*Neuroblast spindle organization is disrupted in hypomorphic Chromator mutants*

Reduction in the size of brains and imaginal discs in *Drosophila* is often correlated with perturbation of cell division. Thus it is reasonable to propose that the small brain and imaginal discs of $Chro^{71}/Chro^{612}$ larvae is likely to arise from mitotic abnormalities, and their extended survival to the third instar larval stage allowed us to analyze the mutant effect on mitosis in neuroblasts from brains. Neuroblasts are one of the best model systems for the
study of cell division because they undergo repeated mitoses to generate the majority of the cells of the central nervous system. To test our hypothesis, brain squashes were performed and the preparations were labeled with tubulin mAb, phospho-Histone 3 Serine 10 pAb (mitotic chromosome marker) and Hoechst to indicate chromosomes. Fig. 2 A shows a comparison of dividing neuroblasts from wild type and Chro71/Chro612 larval brains. Most strikingly, various severe spindle defects were observed throughout mitosis in the mutant. Whereas two obvious spindle poles were formed and separated towards the opposite direction to nucleate the microtubules in prometaphase in wild type, no clear pole structure formation was detected in Chro71/Chro612. The bipolar organization of the metaphase spindle was disrupted in mutant neuroblasts with increasing number of cells showing unfocused spindle poles, splayed microtubule fibers, and rudimentary spindles. As a consequence of spindle assembly abnormalities, chromosome segregation defects were found in most anaphase neuroblasts from Chro71/Chro612 larval brains. The separated mitotic chromosomes were always scattered or lagging along the spindle axis. Interestingly, this phenotype resembles that seen in Chromator-depleted S2 cells by RNAi (Rath et al., 2004). Similar mitotic chromosome segregation defects were also observed after RNAi knock-down of specific mitotic motor proteins in S2 cells such as KLP59C (Rogers et al., 2004) and KLP67A (Goshima and Vale, 2003), suggesting a functional connection between Chromator and mitotic motor proteins. To quantify the spindle defects, 595 mitotic neuroblasts (from 76 brains) were scored and 90.2% of the cells showed disorganized spindle assembly or chromosome segregation defects, which is a statistically significant different from the 8.2% defects (364 neuroblasts from 22 brains) observed in wild type ($P<0.001$, $\chi^2$-test, Table 1, Fig. 2 E).
To address whether these defects were due to impaired Chromator function, a transgenic fly expressing a full-length Chromator rescue construct was generated. This rescue construct NP-Chro consists of the complete coding sequence and 353 base pairs of upstream promoter sequence at the 5’ end as well as the first three introns of the gene locus and a GFP tag (Fig. 3 A). The inclusion of the promoter and intronic sequences was to mimic the native regulation of Chromator in the transgene expression. It should be noted that there is one amino acid change (asparagine to aspartate) in this construct and therefore it was renamed NP-Chro\textsuperscript{N41D}. Nevertheless, correct localization of expressed NP-Chro\textsuperscript{N41D} to the mitotic spindles in syncytial embryos and to the interband region of polytene chromosomes in wild type (data not shown) suggest this full length rescue transgene was expressed sufficiently and could localize properly to the endogenous protein sites. After NP-Chro\textsuperscript{N41D} was introduced into Chro\textsuperscript{71}/Chro\textsuperscript{612} transheterozygotes, immunoblots of protein extract from third instar larval brains showed clear expression of Chromator full-length rescue transgene as detected by both GFP pAb and the C-terminal directed Chromator mAb 6H11 in the mutant allele, whereas labeling was absent in Chro\textsuperscript{71}/Chro\textsuperscript{612} larvae (Fig. 3 B). In rescue animals, the small brain was restored to the normal size, as compared to wild type (Fig. 1C). Furthermore, brain squash preparations labeled by GFP pAb, tubulin mAb and Hoechst confirmed the expression of this rescue construct and localization of the protein to the metaphase spindle where it was co-aligned with the microtubule spindle (Fig 3C). Most importantly, expression of this full length Chromator transgene dramatically rescued the spindle defects to near wild type level in neuroblasts. In Fig. 2A, brain squash preparations of rescued animals show the restored spindle organization at different stages during mitosis. Just as in wild type, two spindle poles were formed normally in prometaphase while a fusiform bipolar microtubule
spindle was established in metaphase that acted to segregate chromosomes correctly in anaphase. The spindle defects ratio was decreased to only 17.0% (583 neuroblasts from 16 brains) indicating a statistically significant difference from that observed in Chro$^{71}$/Chro$^{612}$ mutants ($P<0.001$, $\chi^2$-test, Table 1, Fig. 3 D). Above all, these findings suggest loss of Chromator function could specifically disrupt microtubule spindle organization followed by abnormal chromosome segregation behavior, which implies an important role of Chromator during mitosis.

**The COOH-terminus of Chromator is involved in its spindle function**

In the analysis of previous deletion constructs in S2 cells, the COOH-terminal fragment of Chromator without the chromodomain was found to be sufficient for spindle localization whereas the chromodomain containing NH$_2$-terminus was not (Rath et al., 2004). An interesting question raised from these results is whether the COOH-terminus is required for Chromator’s mitotic functions because of its capacity for spindle targeting. The Chro$^{71}$/Chro$^{612}$ mutant allele provides a good system to test this possibility. Two truncated transgenes were made using the same fragments expressed in S2 cells as deletion constructs. The NH$_2$-terminus named Chro-NTD consists of amino acids 1-346 fused with a nuclear localization sequence (NLS), and the COOH-terminus named Chro-CTD consists of amino acids 329-926. Both truncations contain a GFP tag that is used for immunocytochemical analysis (Fig. 4 A). Chro-NTD and Chro-CTD were introduced into Chro$^{71}$/Chro$^{612}$ transheterozygous mutants and expression of the transgenes was induced in brains by the nerve specific driver, Elav-GAL4. To confirm efficient expression, immunoblots of third instar larval brain protein extracts from wild type, Chro$^{71}$/Chro$^{612}$ and the transheterozygotes
expressing either Chro-NTD or Chro-CTD transgene were labeled with Chromator mAb 12H9 and 6H11. 12H9 specifically recognizes the NH$_2$-terminus of Chromator while 6H11 specifically recognizes COOH-terminus of Chromator (Rath et al., 2004). By using these antibodies, both Chro-NTD and Chro-CTD were shown to be expressed in mutant backgrounds (Fig. 4 B) with the expected size. However, the expression level was lower than the endogenous full length Chromator, as shown in the wild type lane. In the next step, we followed the same brain squash preparations as we did in NP-Chro$^{N41D}$ rescue experiments and asked whether the presence of exogenous Chromator truncations would have any effect on mitotic neuroblast defects in the brain of Chro$^{71}$/Chro$^{612}$. No obvious difference was observed in the mutant neuroblasts in the presence or absence of Chro-NTD: various disorganized microtubule spindle structures were observed including disrupted spindle poles, decreased microtubule intensity and occasional unfocused or half spindles. As a result, scattered or lagging chromosomes were seen in most anaphase neuroblasts as an indication of mitotic chromosome segregation defect (Fig. 4 C). In contrast, expression of Chro-CTD in Chro$^{71}$/Chro$^{612}$ brains partially rescued the mitotic defects. As shown in Fig. 3 C, some prometaphase neuroblast cells formed clearly mature spindle pole structures which were capable of nucleating microtubules, while at metaphase many focused bipolar mitotic spindles were established and the majority of the anaphase neuroblasts segregate mitotic chromatids normally towards the newly forming daughter cells. Furthermore, brain squashes showed that Chro-CTD protein labeled by GFP pAb could express and redistribute to mitotic spindle and largely co-aligned with the microtubule spindle that was labeled by tubulin mAb (Fig. 4 D). This finding is similar to the observation in S2 cells that COOH-terminal Chromator sequence is sufficient for spindle targeting. Quantification data from scoring more
than 400 neuroblasts (from 17 brains) indicated that 89.8% of the dividing cells had abnormal spindles in \( \text{Chro}^{71}/\text{Chro}^{612} \) larval expressing Chro-NTD, which is not statistically different from the 90.2% spindle defects observed in transheterozygotes \( (P>0.05, \chi^2\text{-test}, \text{Fig. } 4 \text{ E, Table } 1) \). In contrast, expression of Chro-CTD in \( \text{Chro}^{71}/\text{Chro}^{612} \) animals significantly decreased the spindle defects (410 neuroblasts from 27 brains; \( P<0.001, \chi^2\text{-test} \)) to only 49.8% (Fig. 3 E, Table 1). Taken together, the ability of Chro-CTD to rescue the mitotic defects in neuroblasts from \( \text{Chro}^{71}/\text{Chro}^{612} \) third instar larval brains provides strong evidence that the COOH-terminus of Chromator is the functional domain for Chromator’s roles during mitosis. However, we cannot exclude the possibility that other sequences of Chromator such as the chromodomain or other sequences in the NH2-terminus may also help Chromator to perform its mitotic functions since the COOH-terminal fragment could not fully rescue the spindle defects.

**Molecular interaction between Chromator and the microtubule associated motor, Ncd**

Based on the hypothesis that the spindle matrix provides a static strut or backbone for mitotic motor proteins to interact with during force production and microtubule sliding, a prediction corresponding to the mitotic function of this structure would be one or some spindle matrix components interact with certain mitotic motors. To extend our studies, we further investigated the potential interaction between Chromator and Ncd, a microtubule-based molecular motor. Ncd motor was chosen because Ncd mutant alleles could cause frequent centrosome and spindle disruption leading to abnormal chromosomes configuration and segregation and sometimes chromosomes loss (Hatsumi and Endow, 1992). Later, RNAi experiments in Drosophila S2 cells suggested the microtubule minus end directional
movement properties of Ncd is involved in pole focusing activity during mitosis (Goshima et al., 2005). Therefore, the typical phenotype of unfocused spindle poles and chromosome segregation defects observed in the neuroblasts of \textit{Chro}^{71}/\textit{Chro}^{612} alleles prompted the possible functional relations between Ncd and Chromator.

To address whether there is a molecular interaction between Chromator and Ncd, we performed coimmunoprecipitation experiments using \textit{Drosophila} embryo lysates. For these experiments, proteins were extracted from either wild type embryos or Ncd-GFP embryos which constitutively express transgenic Ncd-GFP proteins regulated by the wild type \textit{ncd} promoter. Protein extracts were immunoprecipitated with Chromator- or GFP- antibody, fractionated on SDS-PAGE after the immunoprecipitation, immunoblotted, and probed with antibodies to Ncd and Chromator, respectively. Fig. 5A shows an immunoprecipitation experiment using an anti-Chromator antibody where the immunoprecipitate is detected by Ncd antibody as a 79 kDa band that is also present in the embryos lysate. This band was not present in the lane where only immunobeads were used for the immunoprecipitation. The efficiency of the Chromator antibody immunoprecipitation was confirmed by detection of Chromator itself in the immunoprecipitated sample. Fig 5B shows the converse experiment: GFP antibody immunoprecipitated a 130 kDa band detected by Chromator antibody that was also present in the Ncd-GFP embryonic lysate but not in negative control immunoprecipitations with immunobeads only or using a wild type embryonic lysate that did not contain expressed Ncd-GFP protein. The presence of Ncd-GFP itself in the immunoprecipitates was confirmed by labeling the immunoblot with GFP antibody.

To further characterize the interaction between Chromator and Ncd and identify the domains mediating their interaction, we performed GST pull-down assays with various GST-
tagged Chromator truncations. We generated a full length construct, an N-terminal construct including the chromodomain (Chro-NTD) and a C-terminal construct (Chro-CTD) depicted in Fig 5C. Fig 5D shows the results of an immunoblot in which glutathione agarose beads coupled with different Chromator GST-fusion proteins were incubated with wild type embryonic lysate, then washed, fractionated by SDS-PAGE and the protein interactions were detected with Ncd specific antibody. As illustrated in Fig. 5D, only full length Chromator (Chro-FL) and the N-terminal fragment (Chro-NTD) could pull down Ncd from the lysate. Western blot analysis of the GST proteins purified in this experiment and detected with anti-GST antibody showed that similar levels of Chro-FL, Chro-NTD, Chro-CTD fusion proteins and GST only were present in this assay. Taken together, these results suggest that Chromator interacts with Ncd in the same protein complex and sequences in the N-terminal domain of Chromator mediate this molecular interaction.

**Chromator is required for Ncd localization on the microtubule spindle at metaphase**

The identification of a molecular interaction between Chromator and Ncd raises the question whether one of the proteins is required for the other to be targeted to the correct location. To address this issue, we took the advantage of newly generated Chromator loss-of-function transheterozygous mutants to make double labelings of brain squashes from wild type or Chro<sup>71</sup>/Chro<sup>612</sup> third instar larvae with the Ncd pAb and tubulin mAb. Fig 6A (upper panel) shows that Ncd clearly relocalizes to the mitotic spindle and forms a fusiform structure largely colocalized with the microtubule spindle in metaphase neuroblasts. In contrast, in Chro<sup>71</sup>/Chro<sup>612</sup> third instar larval brains, Ncd pAb could detect hardly any Ncd protein in the spindle region that is labeled by tubulin mAb (Fig 6A, lower panel). Consistent
with the immunocytochemistry findings, immunoblots of brain protein extracts from wild type and \( \text{Chro}^{71}/\text{Chro}^{612} \) third instar larvae labeled with Ncd pAb demonstrated that Ncd protein levels were dramatically reduced in the Chromator mutant allele (Fig 6B). This decreased protein level might be due to the degradation of Ncd resulting from its mislocalization in \( \text{Chro}^{71}/\text{Chro}^{612} \). To confirm that the mislocalization of Ncd in \( \text{Chro}^{71}/\text{Chro}^{612} \) is not caused by differential penetration of Ncd pAb in various genotypes, we also tested levels of the GFP-fused Ncd transgene (Endow and Komma, 1996) in the Chro mutant background. Two copies of the NcdGFP transgene were crossed into a \( \text{Chro}^{71}/\text{Chro}^{612} \) mutant background or into a double balancer TM2/TM6 background as a control. Brain squashes were performed from \( \text{NcdGFP}; \text{TM2}/\text{TM6} \) or \( \text{NcdGFP}; \text{Chro}^{71}/\text{Chro}^{612} \) third instar larvae and double labelings were performed with tubulin mAb and GFP pAb to recognize exogenous expression of the GFP tagged Ncd protein. As shown in Fig 6C (upper panel), GFP pAb detected an obvious spindle localization of NcdGFP at metaphase that co-aligned with microtubules in \( \text{NcdGFP}; \text{TM2}/\text{TM6} \) animals. However, GFP pAb staining signal was difficult to detect on metaphase microtubule spindles in the neuroblasts from \( \text{NcdGFP}; \text{Chro}^{71}/\text{Chro}^{612} \) larval brains. Similarly, immunoblots of protein extracts of third instar larval brains from these two genotypes probed with GFP pAb showed significantly reduced level of NcdGFP expression in \( \text{Chro}^{71}/\text{Chro}^{612} \) mutant background compared to the control, TM2/TM6 double balancer background (Fig 6D). Therefore, these findings suggest that function of Chromator during mitosis is essential for normal protein levels of Ncd motor on the metaphase spindle, as supported by the observations from both endogenous Ncd and exogenous expressed NcdGFP transgene.
Direct association of Chromator with microtubules

As implied by the spindle matrix hypothesis, one predicted function of this structure would be the stabilization of the microtubule spindle during mitosis. Thus, it is interesting to test whether there is direct interaction between microtubules and spindle matrix components. In order to explore this possibility, a microtubule overlay assay was performed with various GST-fused Chromator truncated proteins. Fig. 7A described the protein region used to generate the Chromator truncations including full length (Chro-FL), N-terminal fragment containing chromodomain (Chro-NTD) and C-terminal fragment (Chro-CTD). Chro-CTD was further divided into two parts: the middle region (Chro-M, 329-600aa) and the remaining part named Chro-421, the Skeletor interacting domain on Chromator. For this experiment, five purified Chromator GST-fusion proteins and a GST protein control were fractionated on SDS-PAGE, then transferred to a nitrocellulose membrane, and incubated with taxol stabilized microtubules polymerized from tubulins purified from bovine brains. The direct binding of microtubules was revealed by tubulin mAb detection on the immunoblot because microtubule would attach to its potential interacting partner on the membrane. As illustrated in Fig. 7B, Chro-FL, Chro-CTD and Chro-M fusion constructs were found to be able to directly interact with polymerized microtubules while Chro-NTD, Chro-421 and GST itself could not bind to microtubules. Western blot analysis of the different GST fusion Chromator proteins purified in this experiments were probed with anti-GST antibody to show the relatively equal loading input of Chro-FL, Chro-NTD, Chro-CTD, Chro-M, Chro-421 and GST present in the overlay assay (Fig. 7B, right panel). These results strongly indicate that the *Drosophila* spindle matrix component Chromator can directly associate with microtubules and its microtubule-interacting region may be located into the middle domain
of Chromator (Chro-M, 329-600aa), a region within the C-terminal fragment that is suggested to be the mitotic spindle targeting domain of Chromator and required for its mitotic functions from previous experiment (Fig. 4C and Fig. 4D).

**DISCUSSION**

In this study, two new hypomorphic *Chro* alleles were used to analyze the consequences of reduced Chromator protein function on the organization of microtubule spindles during mitosis. Using *Drosophila* brain neuroblast cells, we show that in *Chro*<sup>71</sup>/Chro<sup>612</sup> mutants various defective microtubule spindle structures and chromosome segregation defects were observed throughout cell division. The metaphase spindles from third instar larval brain neuroblasts of *Chro*<sup>71</sup>/Chro<sup>612</sup> transheterozygotes are always rudimentary with unfocused or no spindle poles, lower density microtubule fibers and disrupted organization of bipolar spindle resulting in scattered or lagging chromosomes at anaphase. These phenotypes are consistent with the findings discovered by Rath et al., (2004) using RNAi in S2 cells to deplete Chromator levels. The abnormal spindle morphology and perturbed chromosome segregation caused by loss of Chromator function would be consistent if Chromator functions as a spindle matrix-associated protein that mediates interactions between motor proteins and a static scaffold and if these interactions were essential for chromosome movement. Chromator is one of the four recently identified promising molecular candidates for constituting a bona fide spindle matrix complex, a structure hypothesized to provide a more or less stationary substrate that functions as a backbone to help stabilize the microtubule spindle and for mitotic motors to interact with during force production and microtubules sliding (Pickett-Heaps et al., 1997). Chromator,
together with the other three proteins, Skeletor, Megator and EAST redistributes during prophase to form a fusiform spindle structure that persists in the absence of polymerized microtubules and are indicated to interact with each other (Walker et al., 2000; Rath et al., 2004; Qi et al., 2004, 2005). Our functional analysis of Chromator in vivo strongly supports the mitotic roles proposed for a spindle matrix component during cell division.

Rescue of spindle defects in Chro\textsuperscript{71}/Chro\textsuperscript{612} neuroblasts by a full length Chromator transgene whose regulation is controlled by its own promoter indicates that impaired Chromator function is the cause of the spindle abnormalities observed in mutants. Furthermore, we tested whether the Chromator N-terminus (1-346aa) or C-terminus (329-926aa) could rescue the defects and found that expression of only the C-terminal fragment was able to largely recover the normal microtubule spindle assembly. Although there is no predicted motif present in the Chromator C-terminus, this region was previously shown to be sufficient for spindle targeting by deletion analysis in S2 cells (Rath et al., 2004). Data shown here in \textit{Drosophila} brains confirms its spindle targeting roles and more importantly indicates that it may be the functional domain of Chromator for mitotic roles in spindle organization. However, the C-terminus is not the exclusive domain required for spindle function since it cannot rescue spindle defects to wild type levels. On the other hand, the N-terminus of Chromator that cannot rescue spindle defects in Chro\textsuperscript{71}/Chro\textsuperscript{612} neuroblasts contains a predicted conserved chromodomain (chromatin organization modifier) motif. Chromodomain-involved proteins are always associated with chromosomes or related to the establishment or maintenance of chromatin structures (Brehm et al., 2004) and Chromator function was previously shown to affect the polytene chromosome structure (Rath et al., 2006). Chromator localizes to the interband region in interphase polytene chromosomes. This
clear band/interband pattern of polytene chromosomes was disrupted in \( \text{Chro}^{71/\text{Chro}^{612}} \) transheterozygotes with coiled and compacted chromatin structures accompanied by numerous ectopic contacts connecting non-homologous regions and a misalignment of band and interbands. Moreover, Chromator was demonstrated to directly interact with JIL-1 kinase in the same protein complex (Rath et al., 2006). JIL-1 is an interphase histone H3 Ser10 kinase and its function is essential to maintain polytene chromosome structure (Wang et al., 2001). In a JIL-1 null mutant, polytene chromosomes are shortened and folded with an intermixing of euchromatin region and compacted band region (Deng et al., 2005), which is similar to the disorganized polytene chromosome structure in Chromator null alleles. Besides its molecular interaction, Chromator also genetically interacts with JIL-1 as revealed by the synergistic lethality of the double mutant. In addition to interacting with JIL-1, Chromator (named as Chriz in this study) was shown to also be an interaction partner of Z4, a zinc-finger protein that extensively colocalizes with Chromator at interband regions of polytene chromosomes (Eggert et al., 2004; Gortchakov et al., 2005). Thus, these findings indicate that Chromator is a nuclear-derived protein that plays multifunctional roles during mitosis as well as interphase. These distinct but related functions between different cell stages are likely to be regulated through the participation from either the N-terminal domain or C-terminal domain of Chromator.

As suggested by a spindle matrix hypothesis, a spindle matrix protein is involved in the formation of a scaffold structure for mitotic motor molecules to interact with during force generation and help stabilize microtubule spindle in mitosis. For these reasons, it is very possible that one spindle matrix protein may interact with microtubule-based motors or the microtubules themselves. From our studies, we show that Chromator interacts with Ncd in a
stable protein complex and this interaction is potentially mediated by the N-terminal domain of Chromator as suggested by the GST pull-down results. Ncd contains a motor domain in its C-terminus and moves uni-directionally towards the microtubule minus end. It was first identified in *Drosophila* as the only member of the KIN C type kinesin and a mutant allele of Ncd exhibited severe pole-focusing chromosome distribution defects during meiosis and early embryonic mitosis (Endow et al., 1994; Matthies et al., 1996). Later, the spindle pole focusing function of Ncd was confirmed by RNAi assays in S2 cells where depletion of Ncd led to a large number of spindles with unfocused poles and multiple γ-tubulin foci (Goshima and Vale, 2003; Goshima et al., 2005). Therefore, the typical unfocused spindle pole phenotype in neuroblasts from *Chro*⁷¹/*Chro*⁶¹² transheterozygous mutants might be the consequence of perturbed Ncd function during mitosis. In support of this speculation, we observed that Ncd or an expressed NcdGFP trangene was reduced in levels and not detectable on the metaphase spindles due to the absence of Chromator.

After demonstrating the connection between Chromator and the motor protein Ncd, we extended our studies into the relationship between Chromator and microtubules, the basic structural component of the mitotic spindle. Through microtubule overlay assays, we demonstrate that Chromator can directly interact with polymerized microtubules *in vitro* and the middle region (329-600aa) of the C-terminal part of Chromator is involved in mediating this association. This is the first evidence to show a direct microtubule binding ability of one of the *Drosophila* spindle matrix candidates, although it may be the a common property for potential spindle matrix components identified in other system, such as NuMA (Haren and Merdes, 2002) and Ase1p (Schuyler et al., 2003). NuMA was shown to bind to taxol-stabilized microtubules from metaphase-arrested *Xenopus* egg extracts. This direct
interaction is mediated by a 100-amino acid domain within the C-terminal tail of NuMA that is able to induce bundling and stabilization of microtubules in transfected culture cells. In a similar way in yeast, both in vitro-translated Ase1p and purified endogenous Ase1p can bind and bundle microtubules, as revealed by a MT-copelleting assay and a MT-bundling assay. Interestingly, two different regions of Chromator are involved in the interactions with microtubules or microtubule-associated motors. Such functional domain organization provides the possibility that Chromator could coordinate the microtubule dynamics and motor activities simultaneously to stabilize the spindle assembly and maintain the integrity of a bipolar organization during mitosis.

In conclusion, the mutant analysis of Chromator transheterozygous Chro\textsuperscript{71}/Chro\textsuperscript{612} in Drosophila brain neuroblasts presented here provides strong evidence to support the spindle matrix hypothesis. The mitotic defects we observed in the Chromator loss-of-function mutant are consistent with the prediction of such a hypothesized structure that mutations or perturbed functions in components of the spindle matrix compromising this scaffold or inducing its disassembly would result in abnormal microtubule spindles and chromosome segregation defects (Johansen and Johansen, 2007). The discovery of interactions between Chromator and Ncd or microtubules indicates its potential functional roles played during mitosis. Together with Skeletor, Chromator may associate with Megator, the possible structural element of Drosophila spindle matrix (Qi et al., 2004), to provide a tethering site for anchoring the mitotic motor proteins during their sliding on the dynamic spindle microtubules. In this way, it is similar to the functions postulated for the lamin B-constituted matrix structure that is able to tether essential spindle assembly factors including NuMA, Eg5, PAR and XMAP215 (Tsai et al., 2006). Future analysis would be interesting to clarify
whether interaction between Ncd and Chromator is direct or requires interaction with other proteins and whether this association exists exclusively for Ncd or is a more general mechanism for mitotic roles of Chromator. We can explore the molecular or functional connections of Chromator to other microtubule-based molecular motors such as KLP 67A (Goshima and Vale, 2003) and KLP59C (Rogers et al., 2004). As for the microtubule binding capability of Chromator, other assays would be helpful to confirm this data including MT-copelleting or MT-assembly assays. The affinity of Chromator to the microtubules might also be measured through these methods. In addition, more detailed dissection of Chromator’s functional domains will be necessary to differentiate the distinct cellular events involving Chromator during interphase or mitosis.

ACKNOWLEDGMENTS

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REFERENCES:


**FIGURE LEGENDS**

Figure 1. EMS induced Chro alleles. (A) Diagram of the wild type Chromator protein and the potential truncated protein products of the EMS induced Chro alleles, Chro$^{71}$ and Chro$^{612}$. The Chro$^{71}$ allele consists of a premature stop codon resulting in a truncated 71 amino acids protein. The Chro$^{612}$ allele is comprised of a premature stop codon resulting in a
truncated 612 amino acid protein that contains the chromodomain. (B) Chromator protein expression in Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant third instar larvae compared with wild type larvae. The immunoblots were detected with the C-terminal Chromator mAb 6H11 and with anti-tubulin antibody as a loading control. Full-length Chromator is recognized as a 130 kDa protein by mAb 6H11 in wild type, however, no full-length Chromator is detectable in the mutant. The relative migration of molecular weight markers is indicated to the left of the immunoblots in kDa. (C) Comparison of the brain size of third instar larvae from wild type (Canton S), Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant and Chro\textsuperscript{71}/Chro\textsuperscript{612} transheterozygotes expressing Chromator full-length transgene NP-Chro\textsuperscript{N41D}. Note the significantly reduced size of the brain in the Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant compared to wild type. However, expression of full length Chromator in the mutant restores the brain to normal size.

Figure 2. Mitotic spindle structures in third instar larval brain neuroblasts from wild type, Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant and Chro\textsuperscript{71}/Chro\textsuperscript{612} transheterozygous line expressing Chromator full-length transgene NP-Chro\textsuperscript{N41D}. All preparations are triple labeled with tubulin mAb (red), the phospho-histone 3 Ser 10 pAb (green, mitotic marker) and Hoechst 33258 (blue) and presented in different mitotic stages. (A) Microtubule spindle morphology from wild type larvae showing clear focused spindle pole structures, establishment of bipolar organization and normal segregation of mitotic chromosomes at anaphase. (B) Defective spindle and impaired chromosome segregation in the Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant. Microtubule spindles are rudimentary with disrupted spindle poles and splayed microtubules at the minus end. Lagging and scattered chromosomes were seen during anaphase. (C) Expression of Chromator full-length transgene NP-Chro\textsuperscript{N41D} in Chro\textsuperscript{71}/Chro\textsuperscript{612} background rescued spindle defects. Bipolar spindles form resulting in proper chromosome segregation at anaphase.
Figure 3. Expression of Chromator full-length transgene NP-Chro$^{N41D}$ in $Chro^{71}/Chro^{612}$. (A) Diagram of NP-Chro$^{N41D}$ transgene. The construct includes 353 bps containing the promoter region of Chromator and the first three introns besides the coding sequence. Furthermore, there is a C-terminal GFP tag that is used for immunocytochemistry detection. (B) NP-Chro$^{N41D}$ transgene expression in the brains of $Chro^{71}/Chro^{612}$ mutant third instar larvae. The immunoblots were labeled with GFP pAb and C-terminal Chromator mAb 6H11 as well as anti-tubulin antibody as a loading control. The Full-length Chromator transgene is detected as a 160 kDa protein by both GFP pAb and mAb 6H11 in the mutant line expressing the transgene. This protein is not detectable in wild type and mutant larvae by GFP pAb whereas mAb 6H11 recognizes the endogenous Chromator in wild type as a 130 kDa protein. The relative migration of molecular weight markers is indicated to the left of the immunoblots in kDa. (C) Immuno-labeling of a neuroblast expressing NP-Chro$^{N41D}$ in a $Chro^{71}/Chro^{612}$ transheterozygous background with GFP pAb (green), tubulin mAb (red) and Hoechst 33258 (blue). NP-Chro$^{N41D}$ expression is obviously recognizable by GFP pAb labeling and the expressed protein is localized on the metaphase spindle and is largely co-localized with the microtubule spindle (see merge picture). (D) Bar graph shows the comparison of overall neuroblast spindle defects from wild type (Canton S), Chromator $Chro^{71}/Chro^{612}$ mutant, and transgenic NP-Chro$^{N41D}$ expressed in the $Chro^{71}/Chro^{612}$ mutant. The overall spindle defects present in Chromator mutants allele is 90.2% (595 neuroblasts from 76 brains), which is significantly different from the 8.2% (364 neuroblasts from 22 brains) observed in wild type ($P<0.001$, $\chi^2$-test). Expression of NP-Chro$^{N41D}$ largely rescues the spindle abnormalities in $Chro^{71}/Chro^{612}$ to 17.0% (583 neuroblasts from 16 brains) that is statistically significant from the transheterozygous mutant ($P<0.001$, $\chi^2$-test).
Figure 4. Expression of Chromator’s C-terminus or N-terminus in a Chromator mutant. (A) Schematic description of Chromator N-terminus (Chro-NTD) and C-terminus (Chro-CTD) transgenes. Chro-NTD consists of amino acids 1-346 fused with a nuclear localization sequence (NLS) and includes the chromodomain, and Chro-CTD consists of amino acids of 329-926 without the chromodomain. Both of the Chromator truncations were expressed in brains driven by nerve specific driver elav-gal4 and have a GFP tag used for potential immunocytochemistry detection. (B) Immunoblots of brain protein extracts of third instar larvae from wild type, Chro\(^{71}/\text{Chro}\^{612}\) transheterozygotes and the transheterozygotes line carrying the Chro-NTD transgene was labeled with the N-terminal Chromator mAb 12H9 and anti-tubulin antibody as a loading control. 12H9 detected endogenous full length Chromator in wild type and Chro-NTD expression in the mutant lane carrying this transgene but no signal was observed in the Chro\(^{71}/\text{Chro}\^{612}\) line (left panel). Similar immunoblots were probed with the C-terminal Chromator mAb 6H11 by which Chro-CTD expression is absent in the Chro\(^{71}/\text{Chro}\^{612}\) lane but is detected in the mutant carrying this transgene and endogenous full length Chromator is detected in wild type as well (right panel). The relative migration of molecular weight markers is indicated to the left of the immunoblots in kDa. (C) Triple labeling with tubulin mAb (red), the phosphor-histone 3 Ser 10 pAb (green, mitotic marker) and Hoechst 33258 (blue) of third instar neuroblast preparations from Chro\(^{71}/\text{Chro}\^{612}\) and Chro\(^{71}/\text{Chro}\^{612}\) expressing Chro-NTD or Chro-CTD. In Chro\(^{71}/\text{Chro}\^{612}\) transheterozygotes that express Chro-NTD, similar abnormal spindle morphology and chromosome segregation defects are observed (middle panel) as seen in the mutant (top panel). However, expression of Chro-CTD in Chro\(^{71}/\text{Chro}\^{612}\) partially rescued spindle morphology and chromosomes segregation defects (lower panel). Focused bipolar
microtubule spindles and proper chromosome segregation are often observed. (D) Expressed Chro-CTD in Chro\textsuperscript{71}/Chro\textsuperscript{612} is localized to the metaphase spindle, as revealed by GFP pAb labeling, and co-aligned with the microtubule spindle labeled with tubulin mAb. Hoechst 33258 marks the mitotic chromosomes. (E) Quantification data of neuroblasts spindle defects from different genotype shows no significant difference in Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant lines with or without Chro-NTD expression (89.8% in 402 neuroblasts from 17 brains and 90.2% in 595 neuroblasts from 76 brains respectively, $P>0.05$, $\chi^2$-test). In contrast, expression of Chro-CTD in Chro\textsuperscript{71}/Chro\textsuperscript{612} allele significantly reduces the spindle defects ratio to 49.8% (410 neuroblasts from 27 brains; $P<0.001$, $\chi^2$-test).

**Figure 5. Chromator and Ncd immunoprecipitation assays and interaction domain mapping.** (A) Immunoprecipitations (IP) of lysates from Canton S. embryos were performed using Chromator mAb 12H9 (lane3) coupled with immunobeads or with immunobeads only as a control (lane 2). The immunoprecipitations were probed with Ncd pAb and Chromator mAb 6H11. Ncd is detected in the input lysate (lane 1) and Chromator IP lanes (lane 3) as a 79 kDa band but not in the control sample (lane 2). Efficiency of immunoprecipitation from mAb 12H9 was revealed by detection of Chromator in Chromator IP lane (lane 4) and input lysate (lane 5) as 130 kDa band. (B) Immunoprecipitations (IP) of lysates from embryos that constitutively express GFP- tagged Ncd were performed using GFP pAb (lane 4) coupled with immunobeads and immunobeads coupled with Canton S. embryonic lysate that does not contain expressed GFP tagged Ncd or immunobeads only as controls (lane 3 and 2). The immunoprecipitations were probed with Chromator mAb 6H11 and GFP pAb. Chromator was detected in the input lysate (lane 1) and GFP IP lanes (lane 4) as a 130 kDa band but not in the control samples (lane 2 and 3). Efficiency of
immunoprecipitation from GFP pAb was revealed by detection of GFP tagged Ncd in GFP IP lane (lane 5) and input lysate (lane 6) as 110 kDa band. (C) Diagram of Chromator indicating the domains fused to GST tag that were made for mapping. (D) GST-pull down of lysates from Canton S. embryos were performed using different Chromator GST fusion proteins coupled to glutathione beads or GST protein only coupled to glutathione beads as a control. After probing with Ncd pAb, Ncd was as 79 kDa band detected in the lanes of pull down samples from Chro-FL, Chro-NTD and the input lysate (left panel). Right panel shows the input GST-fusion proteins used for GST-pull down assays detected with anti-GST mAb 8C7. The relative migration of molecular weight markers is indicated to the left of the immunoblots in kDa.

**Figure 6. Localization of Ncd in a Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant.** (A) Brain squashes were prepared from wild type (Canton S) and Chro\textsuperscript{71}/Chro\textsuperscript{612} third instar larvae and triple labeled with Ncd pAb (green), tubulin mAb (red) and Hoechst 33258 (blue). Noted the decreased or mislocalized Ncd on the microtubule spindle in a Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant (lower panel) compared to wild type (upper panel). (B) Western analysis of brain extracts from wild type and Chro\textsuperscript{71}/Chro\textsuperscript{612} labeled with Ncd pAb and tubulin mAb as a loading control demonstrates dramatically decreased expression levels of Ncd protein in the Chromator mutants. (C) Brain squashes were prepared from GFP tagged-Ncd-expressing lines either in TM2/TM6 or Chro\textsuperscript{71}/Chro\textsuperscript{612} mutant third instar larvae and triple labeled with GFP pAb (green), tubulin mAb (red) and Hoechst 33258 (blue). In Chro\textsuperscript{71}/Chro\textsuperscript{612} mutants, GFP tagged Ncd level is largely undetectable by GFP pAb on the metaphase spindle (lower panel) while a clear GFP tagged Ncd protein is localized to the spindle in the TM2/TM6 background (upper panel). (D) Immunoblots of brain extracts from GFP tagged Ncd expressing lines either in
TM2/TM6 or $Chro^{71}/Chro^{612}$ mutant third instar larvae probed with GFP pAb and tubulin mAb as a loading control shows significantly decreased levels of GFP-tagged Ncd in the $Chro^{71}/Chro^{612}$ background. The relative migration of molecular weight markers is indicated to the left of the immunoblots in kDa.

**Figure 7. Microtubule overlay assay with different Chromator domains.** (A) Diagram of GST fused Chromator truncated domains used for microtubules overlay assay. (B) Different truncated Chromator GST fusion proteins purified with glutathione beads were fractionated by SDS-PAGE, transferred to nitrocellulose membrane, and incubated with taxol stabilized microtubules. Interactions of polymerized microtubules were observed in the Chro-FL, Chro-CTD and Chro-M lanes as detected by tubulin mAb (left panel). Immunoblot of the input GST-fusion proteins used for the overlay experiment was detected with anti-GST mAb 8C7 (right panel).
Figure 1
Figure 2
Figure 2

A

Promotor

ATG

cDNA

GFP

Intron1

Intron2

Intron3

B

Canton S.
Chro71/Chro612

NP-ChroN41D, Chro71/Chro612

Canton S.
Chro71/Chro612

NP-ChroN41D, Chro71/Chro612

Rabbit α-GFP

6H11

tubulin

C

NP ChroN41D Rescue

GFP

Tubulin

DNA

Merge

D

Overall Spindle Defects

Percentage of Cell (%)

Canton S.
Chro71/Chro612
NP-ChroN41D Rescue

N=364 (22 brains)
N=595 (76 brains)
N=583 (16 brains)

8.2%
90.2%
17.0%

Figure 3
Figure 4
Figure 6
Figure 7
Table 1. Counting of spindle defects in different genotypes

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<th>Genotype</th>
<th>Normal Spindle</th>
<th>Abnormal Spindle</th>
<th>Total</th>
<th>Spindle Defects (%)</th>
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<td>Canton S.</td>
<td>334</td>
<td>30</td>
<td>364</td>
<td>8.2%</td>
</tr>
<tr>
<td>$Chro^{71}/Chro^{612}$</td>
<td>58</td>
<td>527</td>
<td>595</td>
<td>90.2%</td>
</tr>
<tr>
<td>NP-Chro$^{N41D; Chro^{71}/Chro^{612}}$</td>
<td>494</td>
<td>99</td>
<td>583</td>
<td>17.0%</td>
</tr>
<tr>
<td>Chro-NTD$^i$</td>
<td>41</td>
<td>361</td>
<td>402</td>
<td>89.8%</td>
</tr>
<tr>
<td>Chro-CTD$^i$</td>
<td>206</td>
<td>204</td>
<td>410</td>
<td>49.8%</td>
</tr>
</tbody>
</table>

#NP-ChroN41D transgene expression is regulated under the wild type Chromator promoter
*Chro-NTD and Chro-CTD tranregen expression is driven by the neuron-specific driver, elav-GAL4

Only mitotic neuroblast cells are scored from third instar larval brains of different genotype. The spindle defects ratio is calculated as: observed abnormal spindles/total scored spindles x 100%
CHAPTER 4: GENERAL CONCLUSIONS

Primary characterization of Chromator

Chromator is a novel chromodomain protein, originally identified as a direct interaction partner of Skeletor identified by yeast two-hybrid assay. Skeletor is the first characterized candidate of a multi-protein spindle matrix complex in *Drosophila*. It is an 81 kDa protein with an interesting cell cycle dependent distribution pattern during mitosis in *Drosophila* syncytial embryos (Walker et al., 2000). Skeletor is associated with chromosomes at interphase and redistributes to form a spindle-like scaffold structure from late prophase through anaphase. Chromator is comprised of 926 amino acids and migrates on a SDS-PAGE gel as a 130 kDa protein. Immunocytochemistry indicated that Chromator and Skeletor show extensive co-localization during the cell cycle. Both Chromator and Skeletor localize on interphase polytene chromosomes to the interband regions. After commencement of mitosis, Chromator dissociates from the chromosomes and forms a fusiform structure that is largely co-aligned with the Skeletor-defined spindle and the microtubule spindle at metaphase. Biochemical and immunoaffinity assays show that Chromator is part of a large protein complex, interacting with other *Drosophila* spindle matrix candidates such as Skeletor (Rath et al., 2004) and Megator (Qi et al., 2004). A molecular interaction between Chromator and EAST shown by yeast two-hybrid results and its effect on gene regulation during *Drosophila* muscle development were also suggested (Wasser et al., 2007). In order to understand the protein functions of Chromator, an RNAi experiment was performed in S2 cells and clear spindle assembly defects were observed after Chromator depletion. Curved or irregular-shaped metaphase microtubule spindles were often seen with misaligned
chromosomes. During anaphase a significant number of cells showed chromosome segregation defects with scattered or lagging chromosomes along the spindle axis. To dissect the functional domains of Chromator, it was then divided into two truncations: N-terminal fragment (residues 1-346) containing the predicted chromodomain and C-terminal fragment (residues 329-926) containing the potential Skeletor and EAST interaction domains. Using transfected deletion construct analysis in S2 cells, the C-terminus of Chromator was shown to be sufficient for spindle targeting (Rath et al., 2004).

**Functional roles of Chromator during mitosis**

In light of the results from Chromator depletion RNAi assays in S2 cells, it is important to test whether similar phenotypes could be observed in vivo in *Drosophila* animals. If such is the case, that would provide strong evidence supporting that Chromator plays an essential role during mitosis. However, due to the embryonic lethality of existing Chromator mutant alleles induced by P-element insertions and the masking of early embryonic phenotypes by maternal mRNA, it was not possible to analyze the mitotic phenotype. To address this question and gain a further understanding of Chromator function related to its spindle matrix functions, new Chromator alleles were generated through EMS mutagenesis. Two hypomorphic loss-of-function alleles were identified, *Chro*<sup>71</sup> and *Chro*<sup>612</sup>, both of which consist of a premature stop codon resulting in potential 71 kDa and 612 kDa truncated proteins, respectively. Transheterozygous *Chro*<sup>71</sup>/*Chro*<sup>612</sup> mutant animals survive to the third instar larval stage, thus providing neuroblast cells from mutant brain tissues as a good experimental system to study Chromator functions in mitosis. In neuroblasts from *Chro*<sup>71</sup>/*Chro*<sup>612</sup> mutant brains, severe spindle assembly and chromosome segregation defects
were found in the absence of a fully functional Chromator protein. The bipolar organization of microtubule spindle was largely disrupted with rudimentary microtubules appearing frayed at their minus ends. The spindle pole structures were not well developed and in the worst cases, the spindle poles were totally missing. As a consequence of impaired spindle assembly, improper chromosome segregation occurred at anaphase as revealed by lagging and scattered chromosomes. Intriguingly, this phenotype was consistent with the observation from S2 cells depleted of Chromator by RNAi and was similar to the behavior of defective chromosome segregation defects after knock-down of several kinesin proteins in S2 cells including KLP67A (Goshima and Vale, 2003) and KLP59C (Rogers et al., 2004). That these mitotic defects were due to perturbed Chromator function was verified by rescue experiments showing significant recovery after introduction of a full length Chromator rescue construct in the mutant background. We further demonstrated that the C-terminus of Chromator, not the N-terminus, was the major domain required for spindle function because exogenous expression of Chro-CTD could partially rescued the spindle and chromosome segregation defects. This rescue activity corresponded to the specific mitotic spindle localization of this Chromator C-terminal construct in both S2 cells and transgenic animals.

In addition to its interaction with other *Drosophila* spindle matrix components, Chromator was also shown to interact with the microtubule-based motor protein, Ncd. Ncd is a microtubule minus-end motor and it functions in formation of bipolar meiotic spindles, segregation of meiotic chromosomes (Hatsumi and Endow, 1992) and organization of spindle poles during mitosis (Goshima and Vale, 2003; Goshima et al., 2005; Morales-Mulia and Scholey, 2005). Chromator and Ncd were found to be present within the same immunoprecipitation complex and this interaction was potentially mediate through the N-
terminal fragment of Chromator. Besides microtubule-associated proteins, Chromator was also shown to directly bind microtubules. Using microtubule overlay assays, Chromator was capable of interacting with polymerized microtubules and this microtubule association property was mediated by the middle region in the C-terminus of Chromator.

Significance of mitotic roles of Chromator

From the analysis of newly generated Chromator loss-of-function mutant alleles, Chro⁷¹/Chro⁶¹², we investigated the abnormal spindle morphology and chromosome segregation defects caused by disrupted Chromator functions in detail. These findings fulfill the prediction of a spindle matrix model that mutations or functional perturbation in one or more components of the spindle matrix would compromise the assembly of this scaffold and the abnormalities of this matrix structure would result in disorganized microtubule spindles and chromosome segregation defects (Johansen and Johansen, 2007). From this point of view, it strongly supports the spindle matrix hypothesis and suggests that Chromator plays an important role during mitosis. Moreover, Chromator’s ability to interact with the microtubule associated motor protein Ncd as well as microtubules themselves raises the possibility that Chromator may function as a “bridge” protein or “connector”. As a spindle matrix component, Chromator might interact with mitotic motors to organize the formation of a co-polymer (Nedelec, 2002; Scholey et al., 2003). In this way, it could then help to connect the motor to the microtubules during force generation and counterbalancing activities. Two different functional domains within the Chromator sequence may participate to associate with different proteins or scaffold structures to coordinate microtubule spindle assembly and motor protein activities simultaneously during mitosis. This hypothesized working model for
Chromator will still require more experiments in order to define the precise molecular mechanisms underlying Chromator function.

**Functional roles of Chromator during interphase**

Spindle and chromosome segregation defects of dividing neuroblast cells was not the only observed phenotype in $Chro^{71}/Chro^{612}$ mutant animals. In salivary gland interphase cells, the polytene chromosome arms showed an abnormal coiled and condensed appearance with a disruption and misalignment of interband and band patterns and numerous ectopic contacts connecting non-homologous regions. Chromator localizes to the interband region and is largely co-localized with JIL-1 kinase. JIL-1 kinase is the major histone H3 Ser 10 kinase at interphase (Wang et al., 2001). Reduced levels of JIL-1 caused similar compacted chromosomes as seen in $Chro^{71}/Chro^{612}$ with disordered intermixing of euchromatin and heterochromatin structures (Deng et al., 2005). In a JIL-1 mutant allele there is also a striking redistribution of the heterochromatin markers to ectopic chromosome sites (Zhang et al., 2006), while such phenotype was not observed in $Chro^{71}/Chro^{612}$ mutant. Chromator was shown to physically interact with JIL-1 within the same protein complex and genetically interact with JIL-1, as represented by a synergetic lethality of double mutant alleles. Furthermore, through biochemical methods interaction between Chromator and JIL-1 was shown to be direct and mediated by sequences in Chromator’s C-terminal domain and JIL-1’s C-terminal acidic region. From these results, it appears that together with JIL-1, Chromator is present in an interband-specific complex that functions to establish or maintain interphase polytene chromosome structure in *Drosophila*. The presence of Chromator in the interband-specific complex and its effect on the polytene chromosome structure are
supported by the identification of Chromator interaction with another interband region specific Zinc-finger protein Z4 (Eggett et al., 2004; Gortchakove et al., 2005).

**Summary and future research directions**

The identification of Chromator with Skeletor, Megator and EAST in *Drosophila* provides direct molecular evidence to support the existence of a spindle matrix structure. After characterization of the basic properties of Chromator, we have explored its detailed functions during mitosis as well as interphase. By analysis of transheterozygous *Chro* \(^{71}/Chro\)^ \(^{612}\) mutant animals, we show that Chromator is a nuclear-derived member of a spindle matrix macromolecular complex with multiple functions. It plays essential roles for microtubule spindle assembly and proper chromosome segregation during mitosis that is consistent with the concept of spindle matrix. On the other hand, 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, purification of the Chromator-involved protein complex may be an efficient way to find more interaction partners that could indicate its functions in more details. Taking advantage of the high specificity of Chromator mAb’s 6H11 or 12H9, immunoaffinity purification from embryonic protein extracts would be a good choice. It should be noted that Chromator is present in different protein complexes from interphase to mitosis. Therefore, during the purification process, it would be important to separate the nuclear and cytoplasmic fractions.

Another promising direction would be the domain analysis of Chromator. Although we showed preliminary results that the C-terminus of Chromator is involved in spindle
function and directly binding polymerized microtubules, it would be interesting to determine
the function of the chromodomain-containing N-terminus of Chromator, especially in the
interphase nucleus. Is it responsible for the chromosome targeting of Chromator? The
analysis of various truncation transgenic animals would provide a useful system to answer
these questions.

Finally, the interaction between Chromator and microtubules or microtubule-
associated proteins is worthy of extensive investigation. For instance, is the interaction
between Chromator and Ncd direct or does it require assistance of other proteins? *In vitro*
protein-protein interaction assays might answer this question. To confirm the binding
capability of Chromator to microtubules, microtubule co-pelleting experiments and
fluorescent-labeled microtubule assembly assays could be performed and the affinity of
Chromator to microtubules could also be calculated from these methods. Taken together,
answers to all these questions would help us to get a full understanding of Chromator as a
spindle matrix component and provide exciting information about spindle organization as
well as nuclear organization in *Drosophila*. 
APPENDIX

Alignment of Chromator and its homologue Sequences in different Drosophila species

In order to search the homologues of Chromator in different *Drosophila* species and check whether Chromator protein sequence is conserved during evolution, I used amino acids sequence of Chromator to blast the annotated protein database of different *Drosophila* species and aligned the resulted amino acid sequences by “ClustalW” (EMBL-EBI) program.

In details, full length Chromator (*Drosophila melanogaster*) amino acid sequence (1-926) were used and “blastp” program (FlyBase) was chosen to search the annotated protein database of *Drosophila simulans, Drosophila sechellia, Drosophila yakuba, Drosophila erecta, Drosophila ananassae, Drosophila pseudoobscura, Drosophila persimilis, Drosophila willistoni, Drosophila mojavensis, Drosophila virilis, Drosophila grimshawi*. Sequences alignment results (Fig. 1) indicate that Chromator is highly conserved in *Drosophila* species. The percentage of amino acids identity is various from 67% to 96%.

Sequence comparison of Chromator N-terminus and C-terminus among different insect species

In addition to search the annotated database of Drosophila species, I also used the amino acids sequence of Chromator to search the annotated protein database of two different insect species: mosquito (*Aedes aegypti*) and beetle (*Tribolium castaneum*). Chromator sequence is aligned with the most related sequences from these two insects by “ClustalW” (EMBL-EBI) program. Although the whole protein sequences are not similar based on the score of amino acids identity (data not shown), there are two relative consensus sequences. One is in the N-terminus of Chromator: amino acids 1-128 (Fig. 2); the other is in the C-
terminal end of Chromator: amino acids 862-926 (Fig 3.) However, no predicted conserved domains are in these regions. The conservation of the amino acid among different insect species may still suggest that the N-terminus and C-terminus of Chromator are important functional domains involved in its mitotic and interphase activities.
Chromator

<table>
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<th>Sequence</th>
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Figure 1
Figure 2

Figure 3
REFRENCE CITED


ACKNOWLEDGMENTS

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