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Characterization of two novel spindle matrix proteins in *Drosophila*, Chromator and Megator

by

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Co-Major Professor

For the Major Program

To the STS-107 crew of Space Shuttle Columbia

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GENERAL INTRODUCTION

DISSERTATION ORGANIZATION

This dissertation is organized into four chapters. The first chapter outlines the organization of this dissertation and gives a background introduction. Evidence for nuclear compartmentalization and proteins potentially involved in this process are reviewed. Then the mechanisms of establishing and maintaining chromatin structure are introduced and chromodomain family proteins, an important protein family involved in these processes, are discussed in detail. The review of the mitotic spindle apparatus outlines the current knowledge on microtubule dynamics and motor proteins. Gaps in the knowledge of the mitotic process are also analyzed, which leads to the discussion of the spindle matrix. The knowledge of Skeletor is covered in detail, since it leads directly to this study. Finally, a brief discussion of the advantage of the *Drosophila* system is included.

The second and third chapters are two manuscripts prepared for publication. The first manuscript describes the cloning of the Chromator gene, evidence of the interaction between Skeletor and Chromator, the dynamic distribution pattern of Chromator and the characterization of a recessive lethal allele. The second manuscript describes the evidence indicating Megator to be a spindle matrix component, its interaction with Chromator and the characterization of a Megator recessive lethal allele.

The final chapter is the general conclusions. The current knowledge of Skeletor, Chromator and Megator is summarized, a model for their functions is proposed, two other proposed spindle matrix proteins are discussed and some methodology issues of the spindle matrix research are addressed.

References cited in the general introduction and the general conclusion are listed in the section titled Literature Cited.

BACKGROUND

The Nucleus Is Highly Organized

Even though the nucleus is not divided by membrane into different organelles as in the cytoplasm, ample evidence suggests that the nucleus is in fact highly ordered and compartmentalized (reviewed by Jonathan and Bickmore, 2003). Each chromosome occupies a defined, mutually exclusive space or territory, which has been well demonstrated by fluorescent in situ hybridization (Schardin et al., 1985; Pinkel et al., 1986). Moreover, early- and late-replicating DNA regions occupy distinct domains within a chromosome territory throughout the interphase (Visser and Aten, 1999; Sadoni et al., 1999). Considering the huge amount of biological processes going on in the nucleus, organizing chromosomes into territories might be a way to gain higher efficiency of gene expression and processing by creating specialized local environments. The most obvious example is the nucleolus, where a high concentration of ribosome processing and assembly factors ensures the high efficiency of rRNA processing. Other examples of specialized neighborhoods for the expression of certain genes include The OPT domain (Prombo et al., 1998), Cajal bodies (Frey et al., 1999; Schul et al., 1999) and PML bodies (Shiels et al., 2001). Positioning chromosomes in specific locations might also be involved in gene silencing by the formation of heterochromatin domains (Dernburg et al., 1996; Csink and Henikoff, 1998).

How the nuclear compartmentalization is established and maintained is still unclear. But the most credible clue comes from the study of *Drosophila* proteins EAST and CP60 (Wasser and Chia, 2000). EAST (enhanced adult sensory threshold) was originally identified by studying a mutant defective in response to odor stimuli (VijayRaghavan et al., 1992). Wasser and Chia found that EAST protein occupies the interchromosomal regions (or END for extrachromosomal nuclear domain). Heat shock treatment causes EAST expression to

increase and the END to expand. Over expression of EAST alone similarly causes the interchromosomal region to expand as well as affects DNA replication. In addition, CP60, a protein that shuttles between the centrosomes and the END (Kellog et al., 1995), is abnormally distributed in *east* mutant larvae. These data were interpreted to suggest that EAST may be a component of an expandable extrachromosomal matrix.

Other proteins, including nuclear lamins, TPR and NuMA, might also be involved in maintaining interphase nuclear structure. The lamins are the building blocks of the nuclear lamina, a cage-like structure under the inner nuclear membrane (Zhang et al., 1996). Lamins provide support to the nuclear envelope, anchor other lamina proteins, nuclear pore complexes and chromatin (Lui et al., 2000; Smythe et al., 2000; Gotzmann and Foisner, 1999). Recent studies indicate that lamins are also distributed in the interior of the nucleus and are possibly involved in DNA synthesis and RNA processing mechanisms (Goldberg et al., 1995; Spann et al., 2002). Mammalian Tpr (for Translocated promoter region, Mitchell and Cooper, 1992) is a large coiled-coil protein localized to nuclear pore complexes. Tpr has been observed as being attached to the inner side of the nuclear pore complexes as thin fibers (Cordes et al., 1997). For Tpr in the nuclear interior of cultured mammalian cells, there is debate on whether it forms an intranuclear filament network or exists as numerous discrete foci (Fontoua et al., 2001; Frosst et al., 2002). The function of Tpr is not clear, though roles in mRNA or nuclear protein export have been proposed (Frosst et al., 2002). On the other hand, it is certainly interesting to know whether it is involved in establishing or maintaining the nuclear structure considering its ability to form thin fibers. The Bx34 antigen was identified as a possible *Drosophila* homolog of Tpr (Zimowska et al., 1997), though considering the relatively low homology (28% amino acid identity and 50% similarity), it is not entirely clear whether Bx34 antigen has the same function as Tpr. Zimowska and Paddy (2002) reported both fibrillar and granular distribution patterns of Bx34 antigen and observed dramatic redistribution after heat shock. Thus Bx34 antigen distribution pattern during

interphase is somewhat dynamic. Protein NuMA will be reviewed in the context of its potential as a spindle matrix component. Further study to clarify the function of these proteins in the interior of the nucleus is likely to reveal principles underlying establishment and maintenance of nuclear organization.

Chromatin Structure Remodeling and Chromodomain Proteins

The genome is highly compacted in the form of chromatin. Relatively well understood is the basic unit of the chromatin, the nucleosome. The nucleosome is comprised of a histone core, which includes an H3-H4 tetramer and two H2A-H2B heterodimers, with 160 bp of DNA wrapped around the core in two full turns of left-handed superhelix. The linker DNA connects the nucleosomes forming a beads-on-a-string structure. There is about one core per 200 bp of DNA and also one H1 histone per 200 bp, which links adjacent nucleosomes with its arm (Luger et al, 1997; Kornberg and Lorch, 1999). It is certain that chromatin has higher order packaging in the interphase and during mitosis, though there are healthy debates about the mechanisms of the packaging. The beads-on-a-string structure forms 10 nm fibers which may possibly fold helically into 30 nm chromatin fibers and further into 240 nm chromatid fibers (Cook, 1995). It is also suggested that the higher structural order subunit of metaphase chromosomes is a chromatin loop of roughly 75 kb of DNA anchored by SARs (scaffold associated regions) on a protein scaffold (Saitoh and Laemmli, 1994).

One obvious indication of differential higher order packaging of chromosomes is the distinction between euchromatin and heterochromatin. "Heterochromatin" was originally used to describe a type of chromatin that is densely stained with acetic acid and "euchromatin" was the term for the portion of chromatin that underwent decondensation during anaphase to interphase. It was then discovered that *Drosophila* mutants exhibited

mosaic expression of the *white* gene when the *white* gene was placed close to the heterochromatin (reviewed by Weiler and Wakimoto, 1995), thus linking heterochromatin to gene silencing. A characteristic feature of the constitutive heterochromatin sequence in pericentromeric regions is that it includes long stretches of satellite repeats and is less susceptible to DNaseI and restriction enzymes. It was recently established that the methylation of histone H3 is closely related to the formation of heterochromatin (Rea, 2000). Heterochromatin is required for the normal function of the centromere (Bernard et al., 2001; Perters et al., 2001a) and it has an important role in modulating gene expression (Peters et al., 2001b; Boggs et al., 2001).

It is easy to imagine that the structure of the nucleosomes has to be altered in some way for transcription or replication to take place. This "chromatin remodeling" process involves various enzymes, including histone kinases, histone acetyltransferases, histone deacetyltransferases, histone methyltransferases and ATP-dependent chromatin remodeling factors (Kingston and Narlikar, 1999). ATP-dependent chromatin remodeling factors are multiprotein complexes, ranging from heterodimers to complexes of 12 or more subunits, utilizing the energy of ATP hydrolysis to alter the dynamic property of the nucleosomes. In *Drosophila*, the Brahma protein provides the catalytic subunit of the chromatin remodeling complex Brahma, just as its yeast homolog does in the SWI2/SNF complex (Tamkun et al, 1992). The other three chromatin remodeling complexes in *Drosophila* contain the ISWI ATPase: nucleosome remodeling factor (NURF, Tsukiyama et all, 1995), chromatin accessibility complex (CHRAC, Varga-Weisz et al., 1997) and ATP-utilizing chromatin assembly and remodeling factor (ACF, Ito et al., 1997). ACF and CHRAC assemble and slide nucleosomes to establish regular spacing. NURF, on the other hand, disrupts regular nucleosome arrangement by sliding histone octamer along the DNA.

An important class of proteins involved in establishing and maintaining chromatin structures are chromodomain proteins. The first description of the chromodomain was given

when Paro and Hogness (1991) realized that the Polycomb protein of *Drosophila melanogaster* has a 37 amino acid motif sharing 65% amino acid identity with heterochromatin protein 1 (HP1). Considering that Polycomb is a silencer of homeotic genes and HP1 is associated with heterochromatin, Paro and Hogness named this motif the chromodomain (chromatin organization modifier domain). The chromodomain is usually composed of 50-60 amino acids. The three-dimensional structure of the chromodomain from mouse HP1β has been characterized and shows a three-stranded anti-parallel β-pleated sheet structure that backs onto an α-helix (Ball et al., 1997). The most conserved residues are contained in the hydrophobic core. It is worthwhile to look at some important members of the chromodomain family in detail.

HP1 is a chromodomain protein primarily associated with heterochromatin. In *Drosophila*, HP1 is encoded by Su(var)2-5, which is shown to be a dominant suppressor of position effect variegation (PEV) (Eissenberg et al., 1992). The homologs of HP1 have been found in diverse organisms from yeast to humans. Mice and humans each possesses three HP1 isoforms with similar amino acid sequence, but different distribution patterns (Minc et al., 1999). These proteins are all relatively small, ranging from 15 to 35 kD, with a chromodomain at their N-terminus and a structurally similar motif, termed chromo shadow domain at the C-terminus. These two conserved domains are separated by an unconserved linker sequence. Although it was observed very early on that HP1 is often associated with heterochromatin and its overexpression will increase heterochromatic silencing, the mechanism of this effect has only recently been discovered. In studying murine Suv39h1 protein, another chromodomain protein which is a homolog of the *Drosophila* Su(var)3-9, it was found that the SET domain of Suv39h1 contains methyltransferase activity specific for lysine nine of histone H3 (Bannister et al., 2001; Jacobs et al., 2001). The HP1 chromodomain, but not the chromodomain of some other proteins, binds the methylated lysine 9 of histone H3 tail. It was shown that HP1 and Suv39h1 interact both physically and

genetically in heterochromatic gene silencing (Wustmann et al., 1989, Schotta et al., 2002). This confirms the existence of a "histone code", which allows chromosomal regions to be epigenetically marked as transcriptionally active or repressed. Also, the chromo shadow domain of HP1 was shown to interact with various nonhistone proteins (Zhao et al., 2000; Dellatre et al., 2000). In summary, HP1 seems to connect histones and nonhistone proteins through the interaction of its chromodomain with the methylated histone H3 tail and the interaction of its chromo shadow domain with other proteins. But methylated H3 lysine 9 binding cannot totally explain the distribution pattern of HP1 (Cowell et al., 2002), so there are probably multiple mechanisms for HP1 to associate with the chromosome.

HP1 has been shown to interact with a wide range of proteins, the following are some examples. Besides Su(var)3-9, HP1 was also shown to interact with Su(var)3-7, a zinc finger protein associated with satellite DNA sequences (Cowell et al., 2002). Their colocalization is observed on *Drosophila* polytene chromosomes and in embryos, but the significance of this interaction is still unclear. The proposed interaction between inner centromere protein (INCENP) and HP1 hinge region may imply HP1's role in chromosome dynamics. INCENP is a chromosomal scaffold protein distributed along the chromosome arms at the onset of mitosis. INCENP first becomes concentrated on the centromeres and then detaches from chromosomes to be associated with the central spindle during anaphase. It has been suggested that HP1 is involved in priming INCENP for the anaphase relocation (Ainsztein et al., 1998). CAF1 large subunit, p150, was shown to interact with HP1 chromo shadow domain (Murizna et al., 1999), abolishing this interaction reduces the CAF-1 presence in heterochromatin region. It is possible that CAF-1 helps to stabilize heterochromatin structure during transcription. HP1's association with origin recognition complex (ORC) is reminiscent of the association of ORC with silent information regulator (SIR) in yeast (Hwang et al., 2001). This parallel is consistent with the fact that mutations in ORC or ORC associated protein are suppressors of PEV. HP1's function in chromosome positioning is

suggested by its association with lamin β receptor through the chromo shadow domain (Pyrpasopoulou et al., 1996; Ye et al., 1996; Ye et al., 1997; Kourmouli et al., 2000). HP1 may thus tether the chromosomes to the nuclear envelope and play a role in the nuclear envelope reassembly events at telophase. In summary, the significance of the interactions between HP1 and various proteins is only beginning to be understood, but it is safe to say that HP1 has different interaction partners for different biological functions.

The Polycomb-like chromodomain proteins are larger than HP1, usually more than 300 amino acids in length. They do not have the chromo shadow domain, but share a sequence called the Pc-box in the COOH terminus. Polycomb goup (PcG) proteins are general transcription repressors best known for their function in restricting Hox gene expression along the anterior-posterior animal body axis (reviewed by Simon, 1995). In Drosophila embryos, two different PcG complexes can be purified. The PCR1 complex contains PcG proteins Polycomb (PC), Polyhomeotic (PH) and Posterior sex comb (PSC). Another complex contains ESC and E(Z) (Shao et al., 1999; Ng et al., 2000). Core components of both complexes are conserved in mammals. PRC1 inhibits remodeling by human SWI/SNF, which contains homologs of several trxG proteins. PRC1 also blocks remodeling of nucleosome without histone tails, implying PRC1 interacts with the nucleosome core. It was shown that the C-terminal part of Polycomb binds nucleosomes lacking histone tails (Breiling et al., 1999).

An interesting experiment is the swapping of the HP1 chromodomain and Polycomb chromodomain in Drosophila. When a recombinant HP1 protein with its own chromodomain replaced by that of Polycomb was expressed, endogenous Polycomb protein was mislocalized to the centromeric heterochromatin region (Platero et al., 1995). The simplest explanation is that the Polycomb chromodomain in the chimera recruited endogenous Polycomb through self-association (Cowell and Austin, 1997).

The Suv39 family of chromodomain proteins was mentioned earlier with regarding to its interaction with HP1. For Suv39 proteins of different species, the most conserved region is the 130 amino acid SET domain located near the C-terminus. Another conserved region is located near the N-terminus of the SET domain, it contains several cysteine residues and is proposed to be involved in protein-protein interactions. The chromodomains of this family of proteins are not as well conserved as that of HP1 and Polycomb families (Jenuwein et al., 1998; Aagaard et al., 1999). It has been described above that the SET domain of Suv39h1 has methyltransferase activity specific for histone H3 lysine nine, the methylated H3 tail then recruits HP1 to the heterochromatin.

The CHD-1 family of proteins has two N-terminal chromodomains and a C-terminal helicase. This family contains two classes: CHD1 class and CHD3/4 class. The budding yeast CHD1 protein Chd1p is not essential, but its mutation is lethal in Swi2 mutant cells. This implicates Chd1p in gene activation mechanism since Swi2 is believed to remodel chromatin for transcription (Tran et al., 2000). *Drosophila* CHD1 protein is localized to less condensed region of the polytene chromosomes, which is consistent with a role in gene activation (Stokes et al., 1996). Drosophila CHD3/4 class protein dMi-2 is essential, and was shown to be an enhancer of PcG gene mutaions.

Chromodomains in two proteins were shown to have RNA binding activity, those of MSL3 (male-specific lethal 3) and MOF (males absent on the first), both components of the *Drosophila* dosage compensation complex. Here the function of chromodomain is probably to anchor MSL3 or MOF to the dosage compensation complex through binding to structural RNA roX1 and roX2 (Akhtar et al., 2000).

Above is the review of only a few important chromodomain proteins, a list of known and predicted chromodomain proteins in *Drosophila* can be found in Table 1.

Table 1   Chromodomain proteins in *Drosophila*

Chromodomain protein	Number of chromodomains*	Map position	Chromosomal distribution	References
HP1	2	29A	Heterochromatin, telomeres, few euchromatic sites	James et al., 1989; Fanti et al., 1998
HP1β	2	8C7	Heterochromatin and euchromatin	Smothers and Henikoff, 2001
HP1γ	2	94C	Euchromatin	Smothers and Henikoff, 2001
RHINO	2	54C	Unknown	Volpe and Berg, 1996
CG8120	2	85D	Unknown	GadFly genome annotation
CG7282	2	17E	Unkown	GadFly genome annotation
Chromator	1	80A	Heterochromatin and euchromatin	Wang et al., 2001
Kis	2	21B4	Unknown	Daubresse et al., 1999
CHD-1	2	23C	Euchromatin	Delmas et al., 1993; Stokes et al., 1996
dMi-2	2	76D	Unknown	Kehle et al., 1998
POLYCOMb	1	78C	> 100 euchromatin sites	Zink and Paro, 1989
MOF	1	5C	Male X chromosome	Hilfiker et al., 1997
MSL3	2	65E	Male X chromosome	Gorman et al., 1995
MRG15	1	88E	Unknown	Marin and Baker, 2000; Bertram and Pereria-Smith, 2001

* Including chromo shadow domains.

Microtubule Dynamics, MT Based Motors and Mitosis

The fusiform shape and filamentous organization of mitotic spindles were documented by early cytologists around the beginning of the 20th century. Since the discovery of tubulin in the 1960's, there has been tremendous advancement in the understanding of the mitotic spindles (for a historical review, see Mitchison and Salmon, 2001). The mitotic spindle is made up primarily of microtubules and its associated proteins. Microtubules consist of a core cylinder built from heterodimers of α- and β- tubulin monomers. It has been shown that microtubules are very dynamic and can alternate swiftly between phases of polymerization and depolymerization. This property of "dynamic instability" is described by four parameters: polymerization rate, depolymerization rate, catastrophe frequency (frequency of transition from growth to shrinkage) and rescue frequency (frequency of transition from shrinkage to growth) (Waters et al., 1997; Heald and Walczak, 1999). Once the nucleus enters mitosis, the cytoplasmic microtubules are eliminated and replaced by newly nucleated microtubules assembled around the chromosomes. Mitotic microtubules are more dynamic as a result of increased catastrophe frequency and reduced rescue frequency (Desai and Mitchison, 1998). In most animal cells, centrosomes play a role in spindle formation. At the beginning of mitosis, duplicated centrosomes move to the opposite poles of the cell, nucleating radial arrays of microtubules or asters in the process. The plus ends of the microtubules enter the nucleus once the nuclear envelope is broken down and eventually are captured and stabilized by the kinetochores, leading to the formation of the bipolar spindle. In cells without centrosomes, including higher plant cells and female meiotic cells of *Drosophila*, microtubules are nucleated around the chromosomes and then self-organize into a bipolar spindle (Alberson and Thompson, 1993; Gard, 1992).

Another important component of the mitotic spindle is MT-based motor proteins. MT-based motor proteins are ATP-dependent force-generating enzymes, including the kinesins and dyneins (Holzbaur and Vallee, 1994; Vale and Fletterick, 1997). The functions of the MT-based motor proteins include crosslinking and sliding microtubules relative to adjacent microtubules or other structures, cargo transportation along microtubules and modulating microtubule dynamics (reviewed by Sharp et al., 2000). Since numerous MT-based motor proteins have been identified to date, it is almost certain that there exists cooperation and redundancy. An interesting example is the motor protein pair KLP61F and Ncd. *Drosophila* KLP61F is a bipolar kinesin that *cross-links spindle microtubules within interpolar MT bundles* (Sharp et al., 1999). It does not prevent the initial separation of spindle poles but results in their collapse after nuclear envelope breakdown. The C-terminal kinesin Ncd is a minus-end-directed motor, which is also localized on interpolar microtubule bundles but instead generates outward force. Sharp et al. (1999, 2000) showed that inhibiting the motor activity of either KLP61F or Ncd will significantly affect the spindle enlongation rate, but inhibiting both motors will rescue the spindle activity. The observation that KLP61F and Ncd double mutants are viable and fertile, though not healthy, while a single mutation in KLP61F is lethal and in Ncd causes infertility also suggests that these two motor proteins act in antagonistic fashion. Though considering only KLP61F and Ncd is obviously over simplistic, it surely suggests that the functions of various MT-based motor proteins are elaborately orchestrated.

Even with current knowledge of microtubules and MT-based motor proteins, there is still difficulty explaining mitotic spindle activities. This has led to a proposal for a relatively static structure to anchor motor proteins.

The Spindle Matrix

Both microtubule dynamics and the sliding of microtubules by MT-based motor proteins have been proposed to be the driving force of chromosome congression and separation, but neither explanation is totally satisfactory. During congression, chromosomes dance between poles until their kinetochores are captured by microtubules from both poles, they then move to the equator and oscillate there. It was demonstrated that the polar ejection force is largely generated by the Kid subfamily of kinesin-related proteins (Antoniko et al., 2000; Levesque and Compton, 2001), and supposedly the two polar ejection forces reach equilibrium at the spindle equator, thus giving a cue to the chromosomes about its position. In Kid deficient cells, however, once the chromosome is captured by microtubules from both poles, it can complete congression, though without the oscillation. This implies another mechanism to drive congression (Levesque and Compton, 2001). Then there is poleward microtubule flux, the movement of tubulin subunits from the MT plus ends facing the spindle equator to the MT minus ends facing the poles (Mitchison and Salmon, 1992). To explain the phenomenon that spindle length is constant while microtubules keep translocating towards the spindle poles, the concept of spindle matrix has been proposed (for review see Pickett-Heaps et al., 1997). Skeletor has been identified as the first protein with all characteristics of a spindle matrix protein (Walker et al., 2000) and will be discussed in detail later. Another study strongly supports the existence of spindle matrix. Kapoor and Mitchson (2001) studied the behavior of kinesin Eg5 in *Xenopus* spindles. Eg5 is a plus-end –directed motor of the BimC kinesin family proposed to generate force to push the two poles of the mitotic spindle apart (Sharp et al., 1999). Kapoor and Mitchson used fluorescence speckle microscopy technique by incorporating small amounts of fluorescently-tagged tubulin subunits or labeled Eg5 subunits into the microtubules to create speckles. These speckles can then serve as fiduciary marks to follow the movement of the structure versus time. It was

surprising to find that the majority of Eg5 is static even though the microtubules are in constant flux. One explanation for how Eg5 could remain static on the constantly moving microtubules is that Eg5 is itself "walking" to the opposite direction of microtubule flux with the same speed, thus does not appear to be moving. But after monastrol was added, which inhibits Eg5 motor activity, most Eg5 stayed in place instead of being carried poleward with the microtubule flux as would have been predicted by the "walking" model. It is thus likely that Eg5 interacts with a static spindle matrix.

Besides microtubule flux, earlier evidence for the existence of a spindle matrix was gathered in the studies showing that chromosomes whose kinetochore fibers have been severed by UV-microbeam irradiation still move poleward (Forer et al., 1997) as well as that kinesin appears to associate with a nonmicrotubule component of the spindle (Leslie et al., 1987). As for the component of the spindle matrix, NuMA was the most credible candidate before the identification of Skeletor (Walker et al., 2000). The Nuclear Mitotic Apparatus Protein (NuMA) is a 236 kD protein identified in human autoantibody studies (Lydersen and Pettijohn, 1980), which is restricted in the nucleus during interphase and relocates to the spindle pole region during mitosis. NuMA has a very long α-helical rod (169 kD) flanked by two globular head and tail domains of 24 and 25 kD. Overexpression of NuMA lacking the nuclear localization signal results in 5 nm NuMA fibers in the cytoplasm, while overexpression of intact NuMA protein leads to a hexagonal lattice in the nucleus (Saredi et al., 1996; Gueth-Hallonet et al., 1998). NuMA has also demonstrated an ability to form a multiarm oligomer mediated by the interaction of its C-terminal global domains. So NuMA at least has the potential to form a scaffold structure. During mitosis, it is proposed that many centrosomal microtubules are released from their nucleating sites, but are then 'captured' by complexes of MT-based motor proteins, such as dynein/dynactin anchored on a NuMA matrix. Thus NuMA is needed to keep these microtubules focused around the centrosomes (Compton, 1998). In *Drosophila*, which has no NuMA gene, Asp protein is

probably the functional equivalent (Wakefield et al., 2001). Asp is a 220 kD microtubule-associated protein found at the spindle poles and centrosomes from prophase to early telophase. Asp is also a component of the central spindle and is required for male cytokinesis (Riparbelli et al., 2001). A major problem for NuMA or Asp to constitute a spindle matrix is that they are limited around the spindle pole region (also at spindle midzone for Asp). So if a spindle matrix exists, there has to be another protein that is distributed from pole to pole.

Recently, the concept of a spindle matrix has found renewed interest as a result of the identification of a credible spindle matrix component.

Skeletor, a founding member of a protein complex constituting spindle matrix

Skeletor was identified from the study of a *Drosophila* nuclear antigen with a dynamic distribution pattern (Johansen, 1996; Johansen et al., 1996; Walker et al., 2000). The Skeletor gene maps to the 86C region on the third chromosome, and is contained within a complex locus. Two alternatively spliced transcripts of 6.5 and 1.6 kb were identified. The 1.6 kb transcript corresponds to a 32 kD protein with unknown function. The 6.5 kb transcript has bicistronic potential and the two possible open reading frames were designated ORF1b and ORF2. The product of ORF1b has not been observed, while the ORF2 product corresponds to Skeletor, which is confirmed by immunoblot and immunoprecipitation experiments with various antibodies.

Skeletor antibody labeling reveals that Skeletor appears to be associated with chromosomes during interphase, which is confirmed by the staining pattern of the polytene chromosome squashes. At early prophase, Skeletor dissociates from the chromosomes and reorganizes into a spindle like structure. Interestingly, some Skeletor protein becomes colocalized with the nuclear lamina during this stage. It is worth noting that Skeletor

labeling reveals a spindle like structure even before nuclear envelope breakdown and before microtubules enter the nucleus. During metaphase, Skeletor antibody labels a complete spindle from pole to pole. This Skeletor spindle coaligns with the microtubule spindle. The colocalization of the two spindles continues through anaphase, but microtubules shorten whereas the Skeletor spindle does not. At telophase, Skeletor begins to reassociate with the chromosomes and regain the interphase meshwork like structure. However, the Skeletor staining still can be seen from pole to pole with prominent staining around the midzone region, where cytokinesis will eventually occur. Skeletor still maintains a spindle like structure even after nocodazole treatment abolishes the microtubules, suggesting the structure stained by the Skeletor antibody is independent of the microtubules. On the other hand, the observation that the remaining Skeletor spindles are somewhat deformed after nocodazole treatment may suggest an interaction between the two structures. In the antibody perturbation experiment, embryos injected with anti-Skeletor antibody had fewer nuclei than the control embryos and the DNA appeared fragmented and in various stages of disintegration.

These data suggest that Skeletor is an excellent candidate for being a component of the spindle matrix. Skeletor forms a pole to pole spindle structure during metaphase, which is still intact after nocodazole treatment and takes form before nuclear envelope break down. All these traits were unique to Skeletor and are expected for a nuclear matrix protein. But Skeletor does not have any structural features and it is not clear what function it performs in the spindle matrix. In this study, I will report two interaction partners of Skeletor, one of which is potentially the structural component of the spindle matrix.

The Advantage of Using *Drosophila melanogaster* as a Model System

Drosophila melanogaster is a powerful and convenient system to study mitosis and the components of the spindle matrix. In the first 13 nuclear cycles of *Drosophila* embryo

development, the nuclei divide very quickly within a syncytium without cell membranes. During cycle 10-13, the nuclei migrate to the periphery of the embryo to form a single layer of cells. During this period, the mitotic spindle is prominent and well positioned for photo imaging. The squashes of third instar larval salivary glands are good preparations to study the distribution of proteins on chromosomes. One hundred years' of fly genetics provides various mutants for a wide range of experiments. After the *Drosophila* genomic sequence became available from the Berkeley *Drosophila* Genome Project, fly genetics has incorporated a vast amount of new techniques. In this study, methods from both classical genetics and molecular biology were used.

CHARACTERIZATION OF CHROMATOR, A NOVEL CHROMODOMAIN PROTEIN THAT INTERACTS WITH SKELETOR DURING THE CELL CYCLE

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ABSTRACT

We have previously described the *Drosophila* protein Skeletor and proposed that it is a component of a spindle matrix, a relatively static structure proposed to help organize and stabilize the microtubule spindle during mitosis. In this study, we describe the cloning and characterization of a novel chromodomain protein, Chromator, which is an interaction partner of Skeletor. Chromator interacts with Skeletor in yeast two-hybrid assay as well as in *in vitro* pull down and coimmunoprecipitation experiments. Chromator colocalizes with Skeletor during metaphase on a spindle-like structure but becomes concentrated on the centrosomes and spindle midzone during telophase. A recessive lethal allele of Chromator has been identified. The polytene chromosome squashes prepared from homozygous Chomator mutant larvae demonstrate severe morphological defects. These data suggest that Chromator is an essential gene and is probably required for establishing or maintaining normal chromosome structures. Its association with Skeletor and its dynamic distribution pattern also suggest that Chromator may mediate spindle matrix' involvement in mitotic processes like the pericentrosomal microtubule organization as well as chromosome dynamics.

INTRODUCTION

An essential feature of mitosis is the formation of a mitotic spindle apparatus that helps to distribute chromosomes equally into two daughter nuclei. Though it is well documented that the dynamic instability of the microtubules and microtubule sliding mediated by MT-based motor proteins play important roles in the spindle machinery (Brust-Mascher et al., 2002; Cytrynbaum et al., 2002; Sharp et al., 2000; Wittman et al., 2001), substantial gaps remain in the understanding of the coordination of mitotic apparatus and the driving force of chromosome movement (Kapoor et al., 2002; Mitchison et al., 2001; Levesque et al., 2001). The concept of a spindle matrix has been proposed to explain the fact that chromosome movement is accomplished on constantly fluxing microtubule structure (Pickett-Heaps et al., 1997; Scholey et al., 2001). Recent discovery of Skeletor (in *Drosophila*; Walker et al., 2000), Fin1p (in *Saccharomyces cerevisiae*; Hemert et al., 2002) and Ase1p (in *Saccharomyces cerevisiae*; Schuyler et al., 2003) as potential components of the spindle matrix and the observation that Eg5 is static relative to constantly fluxing spindle microtubules (Kapoor et al., 2001) give new credibility to this concept.

The *Drosophila* protein Skeletor was cloned as a nuclear antigen that exhibited a dynamic distribution pattern during the cell cycle (Johansen, 1996; Johansen et al., 1996; Walker et al., 2000). The Skeletor gene is encoded within a complex locus that gives rise to two transcripts. The product of the smaller transcript is a 32kD protein with unknown function, while the larger transcript has bicistronic potential with one of the open reading frames, ORF2, corresponding to the Skeletor protein. Skeletor is localized on the chromosomes during interphase. But during early prophase, Skeletor dissociates from chromosomes to form an interconnected meshwork like structure and, interestingly, some Skeletor protein also seems to colocalize with nuclear lamina at this stage. By late prophase, Skeletor has already formed a spindle structure even before nuclear envelope break down and

microtubule entry into the nucleus. At metaphase, Skeletor spindle and microtubule spindle colocalize, but the Skeletor spindle appears wider. The colocalization of the two spindles continues through anaphase, until telophase, when Skeletor regains association with chromosomes at the poles. At this stage, Skeletor is still seen from pole to pole with a significant portion of Skeletor distributed around the spindle midzone, where the cytokinesis will occur. Since the spindle structure of Skeletor persists after the microtubules are disassembled by nocodazole treatment, it is conceivable that Skeletor is a component of a spindle matrix that provides a relatively stationary substrate for motor proteins and helps to stabilize the microtubule spindle. But how this spindle matrix interacts with microtubules and motors and what proteins provide the structural element remains unclear.

In this paper, we describe a novel chromodomain protein, Chromator, which was identified through yeast two-hybrid screening with a Skeletor cDNA fragment. Chromator was shown to interact with Skeletor physically. It colocalizes with Skeletor at most cell cycle stages but has a distinctive distribution pattern, including prominent centrosomal and midzone staining at telophase. Analysis of a Chromator mutant suggests that Chromator is an essential gene involved in maintaining proper chromatin structure. The chromodomain, originally identified as a motif shared by *Drosophila* proteins Polycomb and HP1 (Paro and Hogness, 1991, Eissenberg, 1990), has since been found in various other nuclear proteins. Though the function of the chromodomain is not completely understood, it has been proposed to be involved in protein interactions (Cowell et al., 1997; Nielsen et at, 2001; Taverna et al., 2002; Delettre, 2000), DNA binding (Bouazoune et al., 2002) and RNA binding (Akhtar et al., 2000). Most chromodomain proteins identified thus far are involved in establishing and remodeling the chromatin structures (Jones et al., 2000; Pardo et al., 2002; Cheutin et al., 2003; Jenuwein, 1998). The identification of Chromator as an interaction partner of Skeletor may suggests a link between the spindle matrix, chromatin structure, and cytokinesis during the cell cycle.

MATERIALS AND METHODS

Drosophila Stocks

Fly stocks were maintained according to standard protocols (Roberts, 1986). Oregon-R or Canton-S were used for wild-type preparations. The *y1; P[y+mDint2 wBR.E.BR=SUPor-P]CG10712KG03258 ry506/TM3, Sb1Ser1* stock was obtained from Bloomington *Drosophila* Stock Center and the *P[y+mDint2 wBR.E.BR=SUPor-P]KG06256 ry506/TM3, Sb1 Ser1* stocks was obtained from Dr. Hugo J. Bellen at Baylor University. The *w; Δ2-3 Sb/TM2Ubx* stock was the generous gift of Dr. Linda Ambrosio.

Molecular Cloning and Sequence Analysis

Yeast two-hybrid screening was carried out according to the technical manual of Clontech Matchmaker GAL4 Two-Hybrid System 3 & Libraries. A fragment containing residues 215-474 of the predicted Skeletor sequence (accession no. AF321290) was subcloned into pGBKT7 Kan vector (Skeletor-GBK) using standard methods (Sambrook et al., 1989). The construct was sequenced to verify fidelity. Skeletor-GBK was used to screen a *Drosophila* 0-2 h embryonic yeast two-hybrid library (generous gift of Dr. L. Ambrosio, Iowa State University) and a 0-21 h embryonic yeast two-hybrid library from Clontech. Positively interacting clones were identified by growth of yeast colonies on His-, Leu-, Trp-medium as well as by induction of a blue reaction product with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Positive clones were isolated, retransformed with bait vector to confirm interaction and sequenced. ESTs RE33863, RE01873, RE35827, RE37221 LD 39127, LD43522, GM27059 and SD06626 were obtained from the Berkeley *Drosophila* Genome Project and were sequenced with custom made primers at the DNA Sequencing Facility of Iowa State University. DNA sequences were compared with known and predicted

sequences using the BLAST servers of the National Center for Biotechnology Information and of the Berkeley *Drosophila* Genome Project (Adams et al., 2000). Phylogenetic analysis was performed after alignment of the chromodomain sequences by the computer program Clustalw version 1.7. Gaps in the alignment were then removed and trees were constructed by maximum parsimony method using the PAUP program 3.1.1 (Swofford, 1993) on a Power Macintosh G4. All trees were generated by heuristic searches and bootstrap values in percent of 1, 000 replications are indicated on the bootstrap 50% majority rule consensus tree.

Antibody Generation

Residues 601-926 of the predicted Chromator protein sequence were subcloned using standard techniques (Sambrook et al., 1989) into pGEX-4T-1 vector (Amersham Pharmacia Biotech) to generate construct GST-421. The correct orientation and reading frame of the insert was verified by sequencing. GST-421 fusion protein was expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich), according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech). The mAb6H11 was generated by injection of 50μg of GST-421 into BALB/c mice at 21 d intervals. After the third boost, mouse spleen cells were fused with Sp2 myeloma cells and a monospecific hybridoma line was established and used to generate ascites fluid using standard procedures (Harlow and Lane, 1988). The mAb6H11 is of the IgG1 subtype. The Skeletor antibodies mAb1A1, Bashful and Freja were described previously (Walker et al., 2000). All procedures for mAb and ascites production were performed by the Iowa State University Hybridoma Facility.

Western Blot Analysis

Protein extracts were prepared from dechorionated embryos homogenized in lysis buffer (0.137 M NaCl, 20 mM Tris-HCl, pH 8.0, 10% glycerol, 1% NP-40). Protease inhibitors phenylmethanesulfonyl fluoride (PMSF) and aprotinin (Sigma) were routinely added to the homogenization buffers. Proteins were separated on SDS-PAGE gels, transferred to nitrocellulose, incubated with mAb6H11 (1:2000) for 6 hours at room temperature, washed in TBST (0.9% NaCl, 100 mM Tris-HCl, pH 7.5, 0.1% Tween-20), incubated with HRP-conjugated goat anti-mouse antibody (1:3,000) (Bio-Rad Laboratories) for 2 h, washed in TBST, and the antibody complex was visualized using ECL Western Blotting Analysis System (Amersham Phamacia Biotech) according to manufacturer's instructions.

Immunoprecipitation experiments were performed essentially as previously described by Walker et al. (2000). For *in vitro* pull-down experiments, residues 215-474 of the predicted Skeletor sequence was subcloned into Pinpoint Xa-2 vector (Promega) and the open reading frame was confirmed by sequencing. The biotinylated protein Bio-skl was purified according to manufacturer's instructions (Promega). About 15 μl glutathione agarose beads saturated with GST-421 were incubated with 1μg Bio-skl in 100 μl immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate) for 5 h at 4°C, and then washed with 1 ml immunoprecipitation buffer for three times. The resulting complexes were separated by SDS-PAGE, transferred to nitrocellulose and visualized with Streptavidin-Alkaline Phosphatase (Promega) according to manufacturer's instructions. Similarly, Bio-skl coupled with immobilized avidin beads (Pierce) were used to pull down GST-421, the resulting complexes were analyzed by SDS-PAGE and Western blot analysis as described above.

Immunohistochemistry

Antibody labelings of embryos were performed as previously described (Johansen et al., 1996, Walker et al., 2000). Using epifluorescence, double labeling was performed with mAb1A1 anti-Skeletor antibody, anti-α-tubulin antibody (Sigma-Aldrich) or mAb6H11 anti-Chromator antibody and Hoechst to visualize DNA. The appropriate TRITC- and FITC-conjugated secondary antibodies (ICN Biomedicals) were used (1:200 dilutions) to visualize primary antibody labeling. Confocal microscopy was performed as described in Walker et al. (2000).

Polytene chromosome squash preparations from late third instar larvae were performed essentially as previously described (Sullivan et al., 2000). The polytene chromosome spreads were incubated with anti-Skeletor and anti-Chromator antibodies diluted in PBS containing 0.2% Tween-20 and 1% normal goat serum (1:2000 dilutions for mAb1A1, 1:1000 dilutions for mAb6H11) at room temperature for 2 h, washed three times for 10 min, incubated with FITC- and TRITC-conjugated secondary antibodies (ICN Biomedicals), washed in PBS containing 0.2% Tween-20, rinsed in PBS, and stained with 1μg/ml Hoechst 33258 for 10 min. After a final brief rinse with PBS, the preparations were mounted in 90% glycerol containing 0.5% *n*-propyl gallate and imaged using a Zeiss Axioskop microscope with a Diagnostic Instruments high resolution CCD camera. Images were processed using Diagnostic Instruments Spot software.

Rescue Experiments

The full length Chromator coding sequence was cloned into the P element germline transformation vector pCaSpeR-h83, generating a Chromator cDNA sequence in frame with CFP at its N- terminus. This P{h83-CFP-Chromator } was injected into *yw; Δ2-3/TM2Ubx* flies using standard techniques (Roberts, 1986). A total of 11 transgenic lines (7 on the X chromosome and 4 on the second chromosome) were recovered. To increase Chromator-

CFP expression level, the transgenic flies were heat shocked 30 min daily during the experiments. Transgenic lines of the genotypes *P{h83-CFP-Chromator}/Y; +/ P{h83-CFP-Chromator}; KG06256/TM6 Sb Tb* and *P{h83-CFP-Chromator}/+; P{h83-CFP-Chromator}/+; KG06256/TM6 Sb Tb* were established. In their offspring, any non Tb Sb flies were resulted from the rescue of the recessive lethal KG06256 phenotype by P{h83-CFP-Chromator}.

P Element Excision

wBR.E.BR marked SUPor-P element was mobilized by the Δ2-3 transposase source (Robertson et al., 1988). Fly lines in which the P-element sequences had been excised were identified by their white eye color and confirmed by PCR analysis using primers corresponding to genomic sequences flanking the SUPor-P element insertion region. DNA isolation from single flies and PCR reactions were performed as in Preston and Engels (1996).

RESULTS

Molecular Cloning and Characterization of Chromator

Construct Skeletor-GBK was used to screen 0-2 h and 0-21 h *Drosophila* embryonic yeast two-hybrid libraries. Interacting clones comprised of partial Chromator coding sequence were identified from both libraries. This sequence was found to match the CG10712 locus predicted by the Berkeley *Drosophila* Genome Project and various ESTs. Some of these ESTs, including LD 39127, LD43522, GM27059, SD06626, RE33863, RE01873, RE35827, and RE37221 were obtained from the Berkeley *Drosophila* Genome Project and sequenced. The sequences of SD06626, RE37221 and RE35827 all contain an intact open reading frame, but they represent different transcripts due to variant use of the 5'

exons (Figure 1 B). All three transcripts have the same starting AUG codon, and thus encode the same protein product. The predicted Chromator amino acid sequence is shown in Figure 1 A. It has 926 amino acids and a predicted molecular weight of 101 kD. To further study the Chromator protein, a GST fusion protein of Chromator, GST-421, was used to generate monoclonal antibody mAb6H11 specific to Chromator. mAb6H11 recognizes a doublet band of the size 130 kD on immunoblot of embryo protein extracts (Figure 1 C). It is not clear why Chromator protein runs as a doublet on SDS-PAGE or why it runs higher than its predicted molecular weight.

Residues 216-260 of the Chromator protein encode a chromodomain (Paro et al., 1991; Jones et al., 2000). Chromator also has an Asparagine rich region near the C-terminus, and sequences downstream of the chromodomain are overall of relatively low complexity. Figure 2 shows a consensus tree based on the sequences of the chromodomains from Chromator and various other chromodomain proteins using maximum parsimony method. The tree is rooted with the chromodomain sequence from the *Arabidopsis* protein CMT3. Using this analysis, chromodomains of Chromator and its *Anopheles* homolog define their own family distinct from other families like the HP1 family and Polycomb family. Besides its *Anopheles* homolog, Chromator also shares modest homology with *Tetrahymena* protein Pdd1 outside the chromodomain.

Chromator interacts with Skeletor physically

To test for direct physical interactions using a different approach, we performed *in vitro* pull down experiments with biotinylated Skeletor and a GST fusion protein of Chromator. Biotinylated Skeletor was coupled with avidin beads, incubated with GST-Chromator fusion protein, washed, fractionated by SDS-PAGE, and analyzed by immunoblot using GST-specific antibody (Figure 3 A). While the biotinylation target peptide encoded by the Pinpoint vector (Promega) was not able to pull down Chromator, biotinylated Skeletor

pulled down a band corresponding to the size of GST-Chromator. Figure 3 B shows the converse experiment using GST-Chromator to pull down biotinylated Skeletor from the lysate of the *E. coli* cells containing the corresponding construct. GST protein alone showed no pull down activity.

We also performed coimmunoprecipitation (ip) experiments using embryonic extracts. Proteins were extracted from 1-12 h embryos, immunoprecipitated using Skeletor or Chromator specific antibodies, separated on SDS-PAGE after the ip, transferred to nitrocellulose, and probed with antibodies specific to Chromator or Skeletor. Figure 4 A shows an ip experiment using Chromator- or Skeletor- specific antibodies where the immunoprecipitates are detected by Chromator-specific antibody. The 130 kD band appeared in both the Chromator and Skeletor ip lanes but is not present in the lane where immunobeads only was used for the ip. Note only a single Chromator band was detected in this experiment. Figure 4 B shows another ip experiment using Chromator specific antibody and subsequently detected by antibody specific to Skeletor. An 83 kD band corresponding to Skeletor was observed, which is not present in the lane using immunobeads only for the ip experiment. These data together with the result from the yeast two-hybrid assay strongly suggest that Chromator and Skeletor directly physically interact.

Chromator largely colocalizes with Skeletor at interphase, yet has a distinctive distribution pattern during the cell cycle

To compare the distribution patterns of Chromator and Skeletor, we performed double labeling with Chromator and Skeletor antibodies on both polytene chromosome squashes and *Drosophila* syncytial embryos. Figure 5 shows the Chromator distribution pattern (A) compared with that of Skeletor and Hoechst staining (B, C, D, E, F). It shows that Chromator distribution in most cases complements Hoechst staining. Chromator colocalizes with Skeletor on polytene chromosomes, but is absent from some locations where

Skeletor antibody shows staining, most notably the nucleolus. Similar to Skeletor, Chromator also has a dynamic distribution pattern during mitosis, as shown in the double labeled early embryos (Figure 6). During prophase, Chromator antibody stains a meshwork-like structure similar to the Skeletor antibody staining pattern. The major exception is that some Skeletor is seen to be associated with nuclear lamina at this stage while Chromator is not present in that region. At metaphase, Chromator antibody stains a spindle structure with extensive colocalization with the Skeletor spindle from pole to pole, but also with prominent centrosomal staining which is not characteristic of the Skeletor staining pattern. During telophase, Chromator antibody staining is heavily concentrated in the centrosomal region and spindle midzone where cytokinesis will eventually take place. At this stage, Skeletor has begun reassociation with chromosomes. Around the spindle midzone, Skeletor is mostly in regions more poleward relative to Chromator, though colocalization does exist. The extensive colocalization observed for the two proteins is consistent with the physical interaction of Skeletor and Chromator. On the other hand, the differences of the distribution patterns, especially in the centrosomal region and spindle midzone, suggest that Chromator has distinctive functions apart from those that accompanying its interaction with Skeletor.

Characterization of Chromator Mutant Allele, KG06256

Two P element insertion lines carrying SUPor-P (for suppressor-P element, Roseman et al., 1995) inserted into the CG10712 region, corresponding to the Chromator locus, were obtained from the Gene Disruption Project at Baylor University (gift of Dr. H. Bellen) or from the Bloomington *Drosophila* Stock Center. The P element insertion sites were verified by polymerase chain reaction (PCR) analysis using primers corresponding to genomic sequences flanking the SUPor-P element insertion region and sequencing the PCR product. In the KG03258 line, the SUPor-P element is inserted into the first intron of Transcripts B and C and just before the first exon of Transcript A (Figure 7 A). In the KG06256 line, the

SUPor-P element is inserted just before the first exons of transcripts B and C. Both KG03258 and KG06256 are recessive lethal, with KG03258 homozygous animals dying during larval stages and KG06256 homozygous animals dying during larval and pupal stages. KG03258/KG06256 flies are viable, indicating these alleles can complement and raising the possibility that two insertions may affect different genes.

To test whether the Chromator gene can rescue either of these two alleles, we constructed 11 CFP-Chromator transgenic lines. Construct P{h83-CFP-Chromator} were injected into *yw; Δ2-3/TM2 Ubx* flies and 11 transgenic lines (7 on the X chromosome and 4 on the second chromosome) were recovered. Rescue experiment of the KG06256 allele was carried out using the scheme shown in Figure 8. A total of 55 animals homozygous in KG06256 were observed, indicating P{h83-CFP-Chromator} can rescue the recessive lethal phenotype of KG06256. Experiment was also carried out to test whether P{h83-CFP-Chromator} can rescue the KG03258 allele, in which no KG03258 homozygous animals has been observed. To test whether the Chromator protein level is reduced in KG06256 homozygous animals, immunoblot analysis using anti-Chromator antibody was performed on extracts from third instar larvae homozygous in KG06256 or wild type third instar larvae. Figure 7B shows that the Chromator protein level in KG06256 homozygous animals is reduced to about 30% of the protein level in KG06256 heterozygous animals. Moreover, data also suggests that KG03258 does not affect Chromator protein level (data not shown).

To test whether the recessive lethal phenotype of KG06256 is caused by the P element insertion, we mobilized the SUPor-P element in KG06256 flies using the Δ2-3 transposase (Robertson et al., 1988) and screened for precise excision events by selecting white-eyed flies and sequencing the genomic region flanking the original P element insertion site after PCR. A total of 20 precise excision events were characterized, all resulted in homozygous viable and fertile alleles. This shows that the recessive lethality of KG06256 is the result of P element insertion into the Chromator locus.

These evidences suggest that KG06256 is a recessive lethal allele of the Chromator gene, while KG03258 is likely not an allele of Chromator. Possibly the SUPor-P element of KG03258 is spliced away with the first intron of Transcripts B and C, and since KG03258 is also very close to the Ssl1 gene (1270 bp), the recessive lethal phenotype could be caused by its effect on Ssl1 (Figure 7A).

In order to analyze the phenotype of the KG06256 allele, we used Hoechst to stain polytene chromosome squashes from KG06258 homozygous third instar larvae and compared them to similar preparations from KG06256 heterozygous and Canton-S larvae (Figure 9). The polytene chromosomes from KG06256 homozygous larvae were totally fragmented and it was very difficult to find any sizable length of chromosome (Figure 9E, F). Under the same treatment, polytene chromosomes from Canton-S larvae are completely normal in appearance, with minimal fragmentation observed (Figure 9A, B). Appearances of polytene chromosomes from larvae homozygous for the KG06256 allele together with a wild type Chromator allele range from normal to fragmented, with many highly-stretched regions (Figure 9C, D). These results suggest that Chromator is an essential gene in *Drosophila*, loss of Chromator protein affects chromosome structure and this effect appears to be dosage dependent. We did not observe any obvious defects in homozygous mutant Chromator embryos but such effects may be masked by maternal Chromator product.

DISCUSSION

In this study, we reported the cloning and characterization of the *Drosophila* chromodomain protein Chromator. EST evidence shows that the Chromator gene is alternatively transcribed due to variant use of three different 5' exons, but protein coding sequence is not affected. Chromator was identified by virtue of its interaction with Skeletor in yeast two-hybrid assays and their physical interaction was confirmed by *in vitro* pull down

and coimmunoprecipitation experiments. Only a single Chromator band instead of a doublet was detected in the coimmunoprecipitation experiments, which may be due to that only Chromator protein with certain type of post translational modification can bind to Skeletor or one form of Chromator is more susceptible to degradation during the relatively long process of coimmunoprecipitation experiments. Since gel filtration experiments suggests that Chromator chromatographically elutes in a 2 mD protein complex (data not shown), there are probably other proteins that interact with Skeletor and Chromator. Similar to Skeletor, Chromator has a dynamic distribution pattern during mitosis. During interphase, Chromator is localized on the chromosomes, but unlike Skeletor is not found in the nucleolus. During mitosis, Chromator detaches from the chromosomes and forms a pole to pole spindle structure with extensive colocalization with Skeletor. However, a significant difference in the distribution patterns of these two proteins is that Chromator is also seen in the centrosomal regions where Skeletor is absent. During telophase, Chromator is concentrated in the centrosomal region and spindle midzone while Skeletor has begun reassociation with chromosomes and is seen both overlapping with and flanking the Chromator signal in the spindle midzone. The SUPor-P insertion KG06256 was found to be a recessive lethal allele of Chromator, thus indicating that Chromator is an essential gene. Polytene chromosomes from KG06256 homozygous larvae completely fragment upon squashing, whereas KG06256/TM6 Tb polytene chromosomes exhibit much less fragmentation but instead are prone to high degree of stretching. This starkly contrasts with polytene chromosomes from wild type larvae, which have a normal appearance. This suggests that the Chromator protein is required for maintaining normal chromosome integrity. Most of the chromodomain proteins identified so far have functional roles related to chromosome structure. HP1 binds to methylated lysine 9 on histone H3 tail and is essential for the assembly of heterochromatin (Niesen et al., 2001; Jacobs et al., 2002; Peters et al., 2001), Suv39h1 is a heterochromatin-associated histone methyltransferase (Ivanova et al, 1998; Nakayama et al., 2001) and CHD1

family of proteins are helicases, just to name a few. Apart from the *Anopheles* homolog, sequence of Chromator most closely resembles that of Pdd1, the protein involve in programmed DNA elimination in *Tetrahymena* (Taverna et al., 2002). It was suggested that Pdd1 functions through association with histone H3 by a mechanism similar to that used by HP1 in maintaining heterochromatin structure. Thus it will be of interest to determine whether Chromator is associated with chromosomes in a related manner.

In addition to a potential role in chromatin structure or behavior, the dynamic nature of the Chromator distribution pattern points to a possible function related to mitotic spindles and/or chromosome segregation during division. The colocalization of Chromator and Skeletor during metaphase suggests they could function together in the spindle matrix structure. However, the concentration of Chromator in the centrosomal region invites comparison with another dynamically distributed nuclear protein, Asp (abnormal spindle, Saunders et al., 1997; Wakefield et al., 2001; Riparbelli et al., 2002). Asp is suggested to be the *Drosophila* equivalent of NuMA, a protein long proposed to be part of the spindle matrix (Wakefield et al., 2001). Wakefield et al. proposed that "Asp functions by cross-linking microtubule minus ends during cell division, helping to organize both the spindle poles and the central spindle". Like Asp, Chromator is also observed in the centrosomal region. But during metaphase, Chromator is seen on the whole spindle while the Asp distribution is limited to centrosome adjacent areas. During telophase, Asp is located at the minus end of the central spindle (Wakefield et al., 2001; Riparbelli et al., 2002) while Chromator seems to be on the central spindle itself. It will be interesting to see whether Skeletor or Chromator interact with Asp during mitosis. Another protein complex with a dynamic distribution pattern is that of INCENP, Aurora B and survivin (Adams et al., 2001; Terada, 2001; Parra et al., 2002). INCENP is located at the centromere during metaphase, relocates to the spindle midzone during anaphase and is required for proper chromosome congression, separation and completion of cytokinesis. The function of INCENP includes targeting Aurora B kinase to

centromeres and the spindle midzone, which may infer a link between chromosome structure and the function of the central spindles. It is known that central spindle can self-assemble without microtubules emanating from spindle poles (Bonaccorsi et al., 1998), which may suggest that central spindle assembly and organization are influenced by a spindle matrix. Recently Schuyler et al. (2003) demonstrated that the yeast protein Ase1p is a component of semistatic central spindle matrix. It is thus tempting to speculate that the central spindle matrix represented by Ase1p is part of a larger spindle matrix defined by Skeletor and Chromator. Chromator might be the link between Ase1p and the bigger spindle matrix, while Asp and INCENP complexes are also functionally involved. In the centrosomal region, Chromator might have similar interaction with Asp as in the midzone, consistent with the similarity between the central spindle and pericentrosomal spindle proposed by Bonaccorsi et al. (1998).

Skeletor and Chromator are the only proteins identified to date to reveal a pole to pole spindle-like structure independent of the mitotic microtubules. But neither Skeletor nor Chromator have features consistent with their being the structural component of this spindle matrix, judging from their protein sequences. Likely another protein provides the structural component of the matrix while Skeletor and Chromator may have specific role in its function. One of the possibilities is that Skeletor and Chromator mediate the spindle matrix' involvement in various biological processes like chromosome dynamics during cell division.

ACKNOWLEDGEMENTS

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FIGURE LENGENDS

Figure 1 **The organiztion of the Chromator locus.** (A) The predicted protein sequence of Chromator. Chromator is 926 amino acids in length, with a predicted molecular weight of 101 kD. Residues 216-260 form a chromodomain, which is underlined. (B) Diagram of the three transcripts of the Chromator locus. Note the variant use of the 5' exon. All three share the same starting AUG, indicated in the diagram by arrows. Chromodomains are indicated as the shaded regions. (C) Western blot analysis of embryonic protein extract shows that mAb6H11 recognizes an 130 kD band.

Figure 2 **Consensus maximum parsimony tree derived from an alignment with all gaps removed of the chromodomains of Chromator and representative chromodomain proteins.** The tree is rooted using *Arabidopsis* protein CMT3 as an outgroup. The bootstrap 50% majority rule consensus of 1000 maximum parsimony tree is depicted with associated bootstrap values. The proteins used in constructing the tree are (from top to bottom): Chromator, Drosophila melanogaster; Chromotoar homolog, *Anopheles gambiae*; SUV39, mouse; SUV39, human; Polycomb like protein 2, zebra fish; Polycomb like protein 2, mouse; Polycomb like protein3, mouse; Polycomb like protein 3, human; Polycomb like protein 3, zebra fish; Polycomb, *Drosophila melanogaster*; MYST1, human; MYST1, mouse; Pwa33, *Pleurodeles waltl*; Mof, *Drosophila melanogaster*; Msl3, *Drosophila melanogaster*; Msl

homolog, *Anopheles gambiae*; Msl like protein 1, human; Msl like protein 1, rat; MRG15, human; MRG15, rat; MRG15, *Drosophila melanogaster*; Rbp1, human; Rbp1, mouse; CG7282, *Drosophila*; HP1, *Drosophila melanogaster*; HP1, *Anopheles*; HP1α, human; HP1α, mouse; CMT3, *Arabidopsis*; CHD2, mouse; CHD2, rat; CHD2, human; CHD1, human; CHD1, mouse; CHD1 *Drosophila melanogaster*; CHD1, *Anopheles*; Su(var)3-9, *Drosophila melanogaster*.

Figure 3 *In vitro* **pull down assays shows that Chromator and Skeletor interact physically.** (A) Biotinylated Skeletor protein coupled to immobilized avidin beads was used to pull down a GST-Chromator fusion protein. The first panel shows a band recognized by GST antibody. The second panel shows that biotinylated target peptide from the Xa vector can not pull down GST-Chromator fusion protein. The third panel is a Western blot of GST-Chromator. (B) GST-Chromator coupled with glutathione agarose beads was used to pull down biotinylated Skeletor. The first panel shows GST-Chromator pulled down a protein band visualized by strepavidin-phosphatase NBT/BCIP reaction. The second panel shows that GST protein has no pull down activity. The third panel shows the biotinylated Skeletor protein detected by strepavidin-phosphatase NBT/BCIP reaction.

Figure 4 **Co-immunoprecipitation experiments confirms that Chromator interacts with Skeletor.** (A) Anti-Chromator and anti-Skeletor antibodies were coupled with protein A beads, incubated with 0-12 h embryonic extract, washed and detected on immunoblot using anti-Chromator antibody. Note that anti-Skeletor antibody can co-ip a protein corresponding to Chromator while immunobeads cannot. (B) Similar experiment as (A), except using anti-Chromator antibody coupled with protein A beads and detected with anti-Skeletor antibody. Anti-Chromator antibody can co-ip Skeletor while immunobeads cannot.

Figure 5 **Chromator and Skeletor colocalize on polytene chromosomes.** Polytene chromosome squashes were labeled with mAb 6H11, mAb1A1 and Hoechst to visualize Chromator, Skeletor and DNA. (A) mAb 6H11 labeling of Chromator (green). (B) mAb 1A1 labeling of Skeletor (red). (C) Hoechst labeling of DNA (blue). (D) Composite image of Chromator (green), Skeletor (red) and DNA visualized by Hoechst staining (blue). (E) Composite image of Chromator (green) and Skeletor (red). (F) Composite image of Chromator (green) and DNA visualized by Hoechst staining (blue). Note Chromator largely colocalizes with Skeletor but does not localize to the nucleolus while Skeletor does.

Figure 6 **Chromator and Skeletor colocalize on the spindle during mitosis but also have differences in distribution pattern.** Skeletor was visualized by mAb1A1 labeling (red) and Chromator was visualized by mAb6H11 labeling (green). The composite images (comp) are shown in the left column. At prophase, both Skeletor and Chromator demonstrate meshwork-like structure with Skeletor appearing also along the nuclear inner rim. At metaphase, both proteins demonstrate an end-to-end spindle-like structure with Chromator having additional centrosomal localization. At telophase, Skeletor maintains a pole to pole structure and begins reassociation with chromosomes while Chromator is concentrated at centrosomal and midzone regions.

Figure 7 **The P element insertion allele KG06256 affects Chromator protein level.** (A) Two P element insertion lines carry SUPor-P inserted into the Chromator locus. The KG06256 insertion site is at the 5' terminus of Transcripts B and C while the KG03258 insertion site is near the 5' terminus of Transcript A and inside the first introns of Transcripts B and C. The 5' terminus of the Ssl1 gene is also depicted. (B) Western blot analysis of embryonic extract from wild type, KG03258 homozygous and KG06256 homozygous embryos using anti-Chromator and anti-tubulin antibody. After adjusting different tubulin

protein level, KG03258 homozygous embryos have similar Chromator protein levels comparing to the wild type while Chromator protein level is greatly reduced in the KG06256 homozygous embryos.

Figure 8 **Scheme of KG06256 rescue experiment.** P{h83-CFP-Chromator} contains Chromator full length coding sequence in frame with CFP at its N-terminus in the germline transformation vector pCaSpeR-hs83. Flies carrying 4 copies of P{h83-CFP-Chromator} were generated and used to rescue the recessive lethal phenotype of KG06256. Note in G2, any surviving non-Tb Sb flies resulted from the rescue.

Figure 9 **Loss of Chromator protein causes chromosome morphological defects.** Polytene chromosomes from Canton-S, KG06256/TM6 Tb and KG06256/KG06256 third instar larvae were squashed under the same conditions and labeled with Hoechst to visualize DNA. The polytene chromosomes from Cantons-S third instar larvae have normal appearance (A, B). Polytene chromosomes from larvae with one KG06256 allele and one wild-type allele range from normal in appearance to fragmented with a lot of highly stretched regions (C, D). Polytene chromosomes from KG06256 homozygous larvae are totally fragmented (E, F).
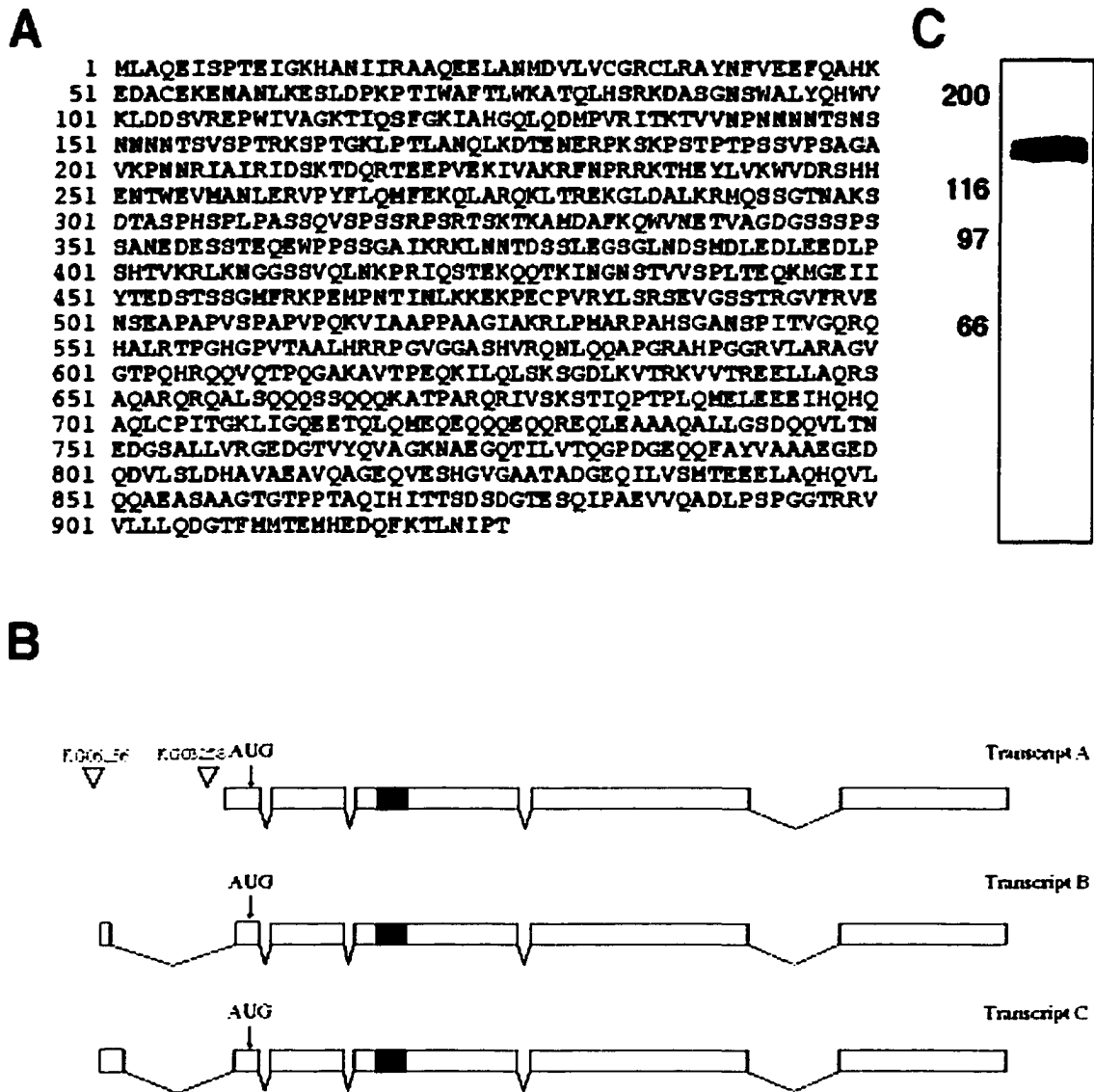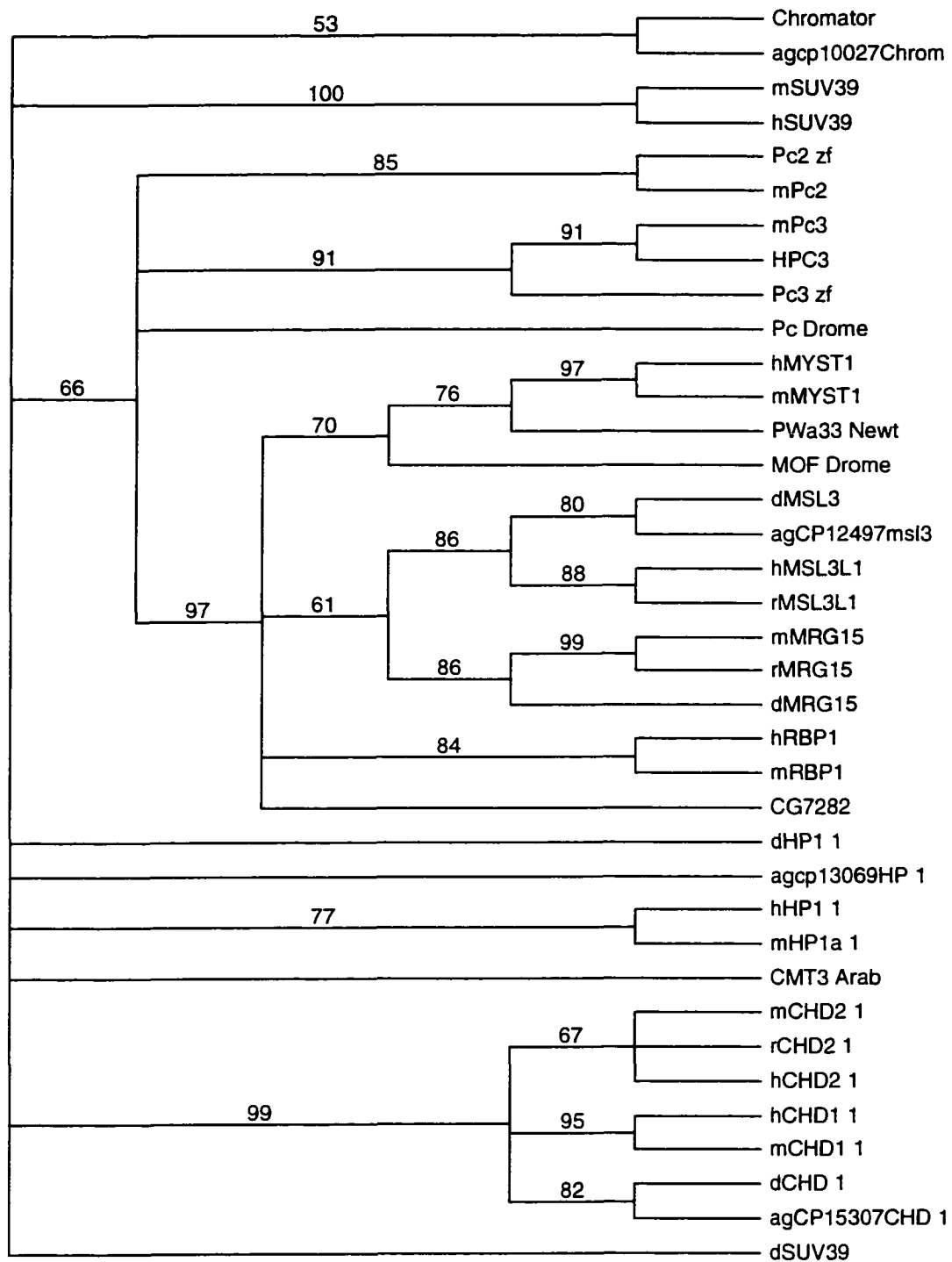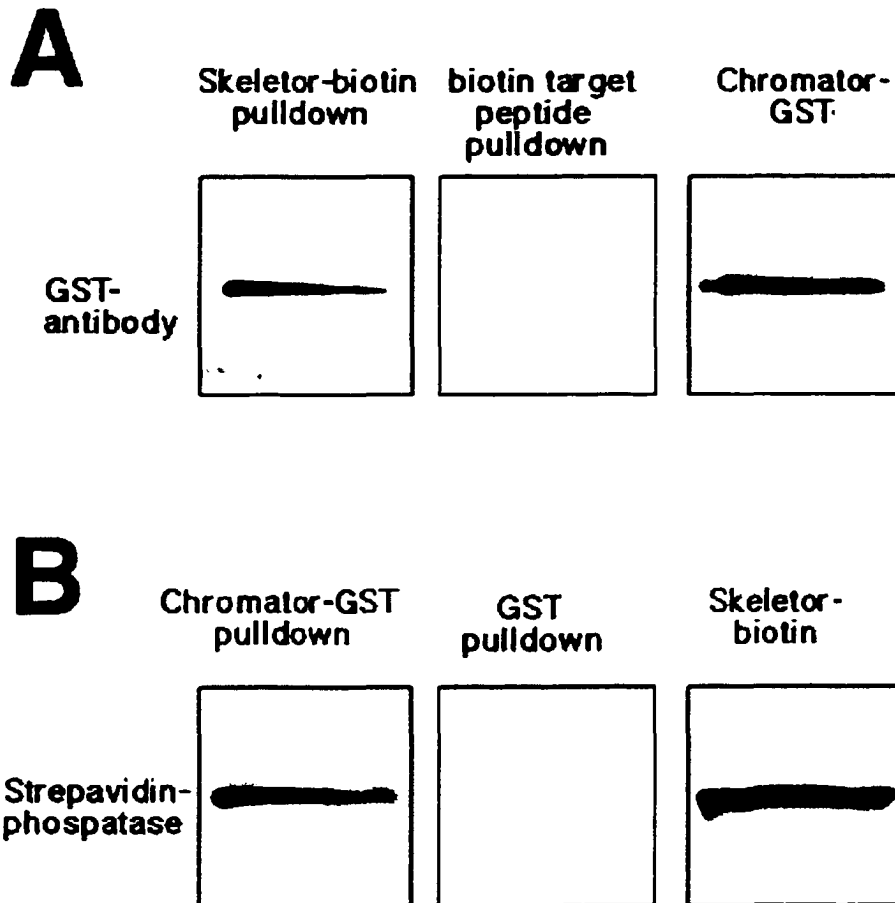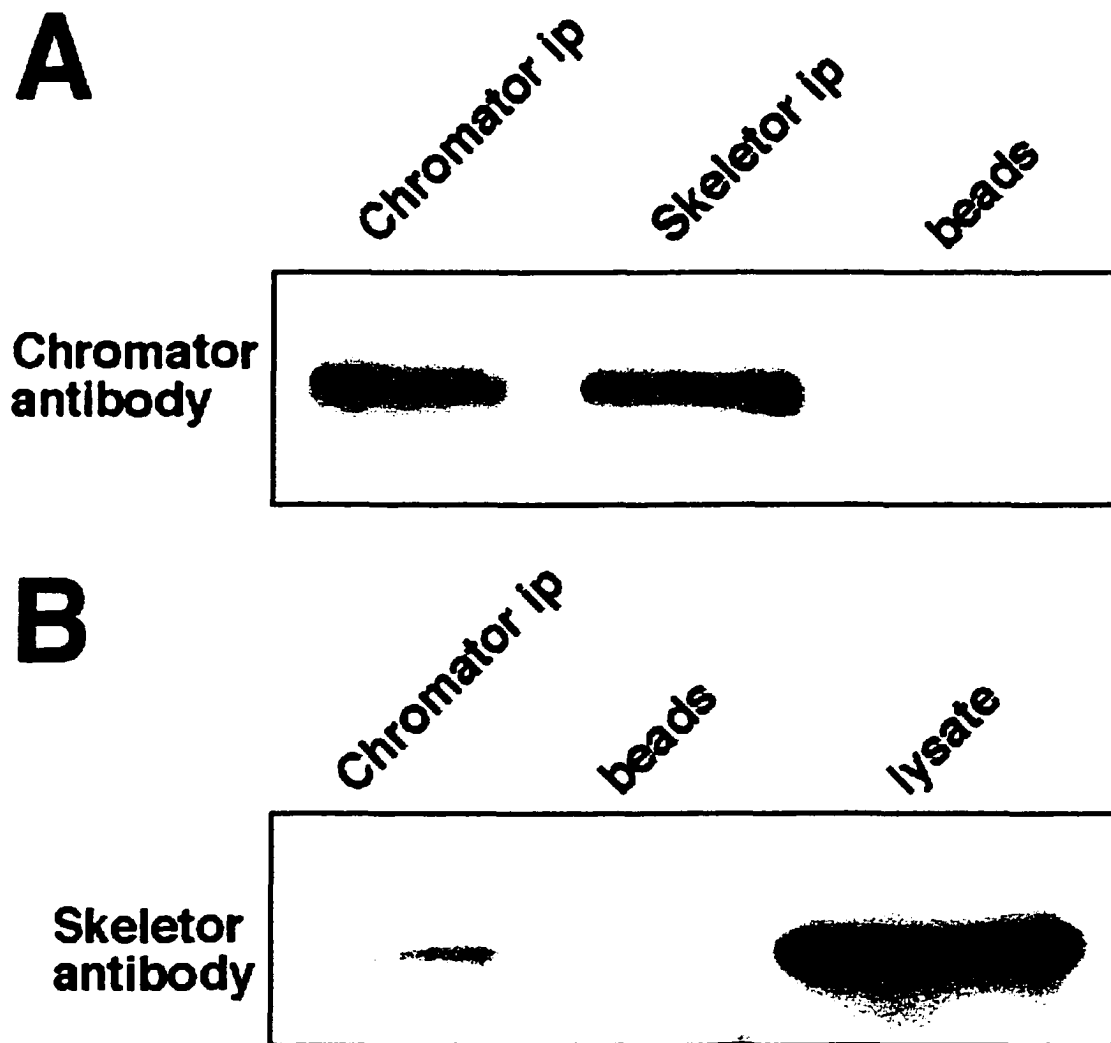
A

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  1 MLAQEISPTEIGKHANIIRAAQEELAHMDVLVCGRCLRAYNFVEEFQAHK
 51 EDACEKENANLKESLDPKPTIWAFTLWKATQLHSRKDASGNSWALYQHWV
101 KLDDSVREPWIVAGKTIQSFGKIAHGQLQDMPVRITKTVVNPNNNNTSNS
151 NNNNTSVSPTRKSPTGKLPTLANQLKDTENERPKSKPSTPTPSSVPSAGA
201 VKPNNRIAIRIDSKTDQRTEEPVEKIVAKRFNPRRKTHEYLVKWVDRSHH
251 ENTWEVMANLERVPYFLQMFEKQLARQKLTREKGLDALKRMQSSGTNAKS
301 DTASPHSPLPASSQVSPSSRPSRTSKTKAMDAFKQWVNETVAGDGSSSPS
351 SANEDESSTEQEWPPSSGAIKRKLNNTDSSLEGSGLNDSMDLEDLEEDLP
401 SHTVKRLKNGGSSVQLNKPRIQSTEKQQTKINGNSTVVSPLTEQKMGEII
451 YTEDSTSSGMFRKPEMPNTINLKKEKPECPVRYLSRSEVGSSTRGVFRVE
501 NSEAPAPVSPAPVPQKVIAAPPAAGIAKRLPHARPAHSGANSPITVGQRQ
551 HALRTPGHGPVTAALHRRPGVGGASHVRQNLQQAPGRAHPGGRVLARAGV
601 GTPQHRQQVQTPQGAKAVTPEQKILQLSKSGDLKVTRKVVTREELLAQRS
651 AQARQRQALSQQQSSQQQKATPARQRIVSKSTIQPTPLQMELEEEIHQHQ
701 AQLCPITGKLIGQEETQLQMEQEQQQEQQREQLEAAAQALLGSDQQVLTN
751 EDGSALLVRGEDGTVYQVAGKNAEGQTILVTQGPDGEQQFAYVAAAEGED
801 QDVLSLDHAVAEAVQAGEQVESHGVGAATADGEQILVSMTEEELAQHQVL
851 QQAEASAAGTGTPPTAQIHITTSDSDGTESQIPAEVVQADLPSPGGTRRV
901 VLLLQDGTFMMTEMHEDQFKTLNIPT
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C

B

Fig. 1

Fig. 2

A

Skeletor-biotin pulldown	biotin target peptide pulldown	Chromator-GST

GST-antibody

B

Chromator-GST pulldown	GST pulldown	Skeletor-biotin

Strepavidin-phospatase

Fig. 3

A

Chromator ip   Skeletor ip   beads

Chromator
antibody

B

Chromator ip   beads   lysate

Skeletor
antibody

Fig. 4

Fig. 5

Fig. 6

47

A.

B.

Fig. 7

G0: ♂♂ $\dfrac{+}{Y}$; $\dfrac{+}{+}$; $\dfrac{KG06256}{TM3\ Sb\ Ser}$ X ♀♀ $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}}{P\{h83\text{-}CFP\text{-}Chromator\}}$; $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}}{P\{h83\text{-}CFP\text{-}Chromator\}}$; $\dfrac{+}{TM6\ Sb\ Tb\ e}$

G1: ♂♂ $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}}{Y}$; $\dfrac{+}{P\{h83\text{-}CFP\text{-}Chromator\}}$; $\dfrac{KG06256}{TM6\ Sb\ Tb\ e}$ X ♀♀ $\dfrac{+}{P\{h83\text{-}CFP\text{-}Chromator\}}$; $\dfrac{+}{P\{h83\text{-}CFP\text{-}Chromator\}}$; $\dfrac{KG06256}{TM6\ Sb\ Tb\ e}$

G2:

♂ or ♀ $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +\ or\ Y}$; $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}$; $\dfrac{KG06256}{KG06256}$ Rescued or Dead

♂ or ♀ $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +\ or\ Y}$; $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}$; $\dfrac{KG06256}{TM6\ Sb\ Tb\ e}$ Heterozygotes

♂ or ♀ $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +\ or\ Y}$; $\dfrac{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}{P\{h83\text{-}CFP\text{-}Chromator\}\ or\ +}$; $\dfrac{TM6\ Sb\ Tb\ e}{TM6\ Sb\ Tb\ e}$ Dead
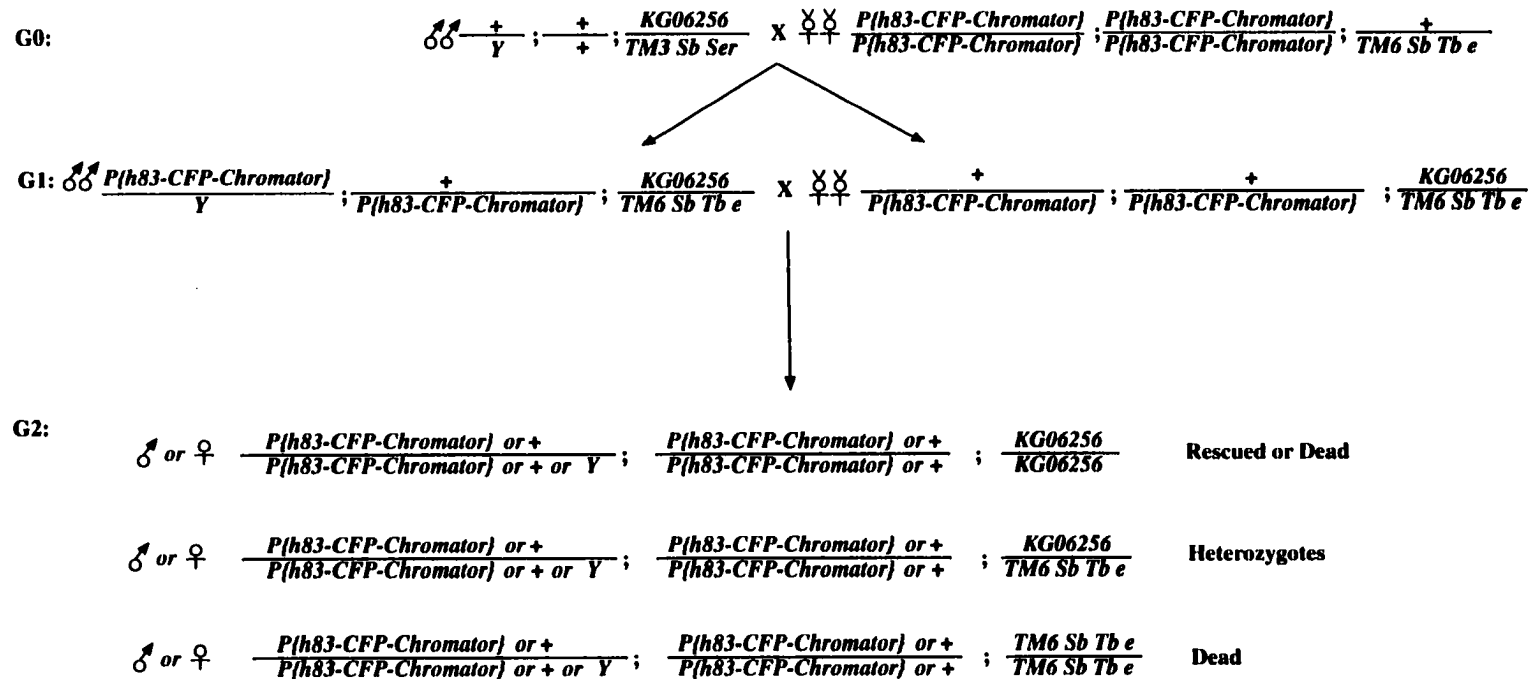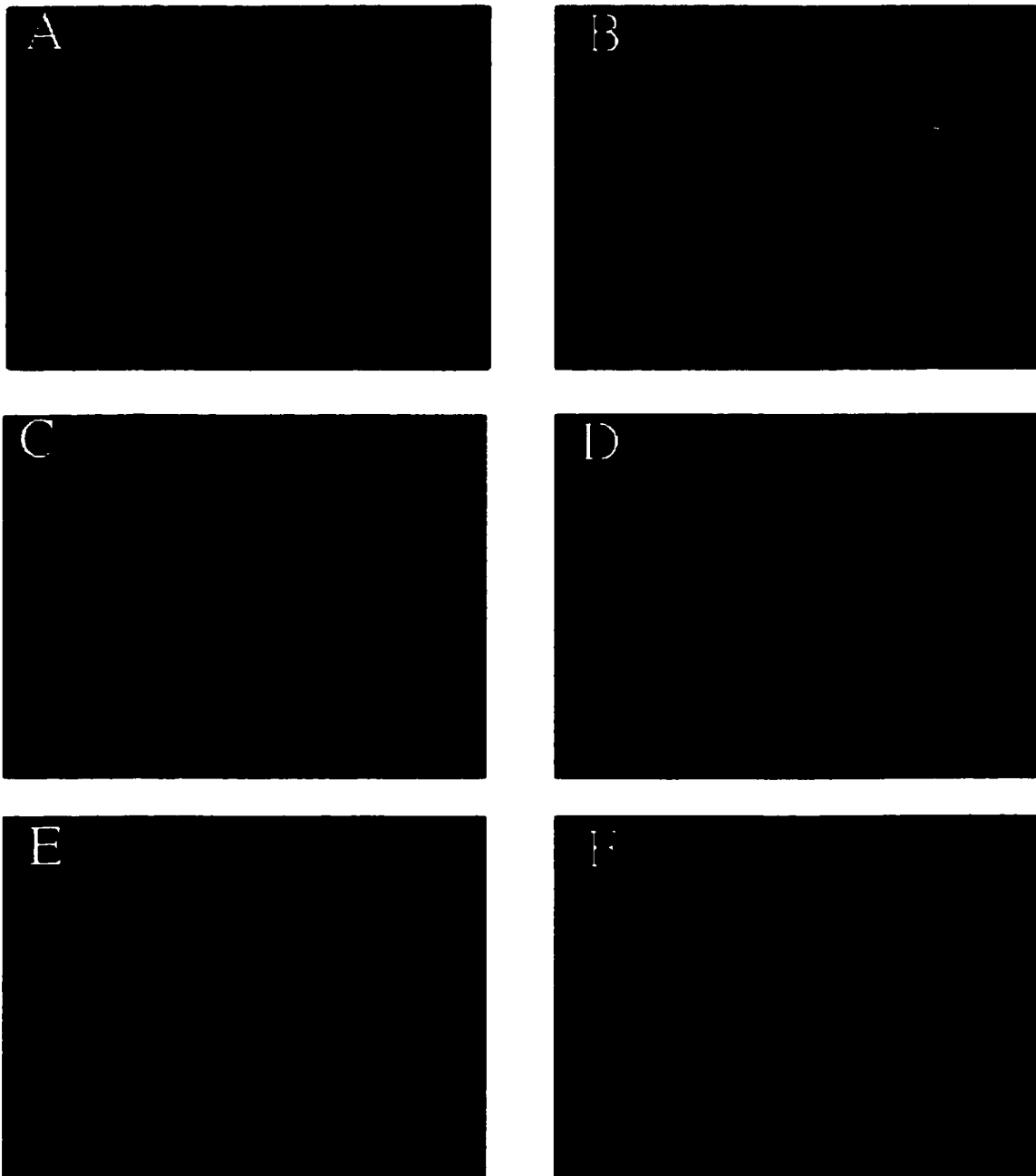
Figure 8

Fig. 9

MEGATOR, A COILED-COIL PROTEIN LOCALIZES TO THE SPINDLE MATRIX AND IS ESSENTIAL FOR MITOSIS IN *DROSOPHILA*

Dong Wang, Yingzhi Xu, Uttama Rath, Michael R. Paddy,

Jørgen Johansen, and Kristen M. Johansen

SUMMARY

Although much work has been directed towards understanding mitotic spindle apparatus structure and function, it is still unclear how mechanical forces are applied to pull the chromosomes apart [1]. The involvement of a stationary spindle matrix has been proposed [2, 3] but its molecular composition has remained elusive. Recently, a spindle matrix protein, Skeletor, and an interaction partner Chromator were identified in *Drosophila*; however, neither of these proteins have molecular features characteristic of structural proteins [4, 5]. We now report the identification of another component that localizes to the spindle matrix and is a candidate to play such a structural role. The monoclonal antibody Bx34 was previously shown to recognize a coiled-coil protein that shows a low level of homology to mammalian nuclear pore complex TPR protein yet is also found at significant levels within the nucleus [6]. We show that the Bx34 antigen is an essential protein that colocalizes with Skeletor during mitosis and physically interacts with Chromator. We propose the Bx34 antigen serves as a structural component of the spindle matrix and have named the protein Megator.

RESULTS AND DISCUSSION

Figure 1 shows the intranuclear distribution of Megator as labeled by the mAb Bx34 (red) compared to the DNA as visualized by Hoechst staining (green) in interphase nuclei. As previously reported [6] Bx34 antibody shows staining at the periphery of the nucleus consistent with Megator's association with the nuclear pore complex. However, there are significant intranuclear levels of Megator as well. This intranuclear localized Megator does not co-localize with the DNA, but instead appears to surround the chromosomes [6] as is evident in squashes of polytene larval chromosomes (Fig. 1, lower panel). However, as mitosis commences (Fig. 2) Megator (green) reorganizes during prophase into a spindle structure the pattern of which at metaphase appears identical to that of the spindle matrix protein Skeletor (red). Skeletor is localized to chromosomes at interphase but redistributes into a true fusiform spindle at prophase which during metaphase is coaligned with the microtubule spindles [4]. Megator and Skeletor continue to be colocalized throughout mitosis including telophase until the interphase distribution pattern is reestablished. We observed this distribution of Megator both in Bouin's and PFA fixed preparations as well as with a polyclonal antiserum made toward a synthetic peptide based on Megator's amino acid sequence [6]. While Zimowska et al. [6] reported considerable interior Bx34 labeling of metaphase nuclei the spindle-like structure was not resolved in their study due to different fixation conditions that were optimized for chromosome preservation.

To explore the possibility of physical interaction between Megator and Chromator, we preformed blot overlay experiments. In the experiment shown in Figure 3, Chromator GST fusion protein GST-421 was separated on SDS-PAGE and transferred to nitrocellulose. The membrane was then incubated with GST-BxM fusion protein containing residues 1436-1697 of the predicted Megator sequence (accession no. U91980), washed and probed with Bx34 antibody. A band corresponding to the size of GST-421 was detected in the lane

loaded with GST-421 protein, while no band was detected in the lane loaded with GST protein. This suggests that Megator physically interacts with Chromaor.

Megator has been previously cloned and sequenced and encodes a large 2,346 amino acid protein of 262 kD in which the N-terminal 70% is predicted to form an extended coiled-coil region while the C-terminal 30% is unstructured and acidic [6] (Fig. 4a). By PCR mapping and sequencing [7] we determined that the P-element present in the *l(2)k03905* line [8] is inserted at the start of the published cDNA [6] of Megator at position +1 (Fig. 4a). This insertion event also resulted in a 9 bp duplication including 8 bp of upstream genomic sequence and a duplicated +1 residue (Fig. 2a). The site and nature of the insertion suggests that a functional Megator transcript is not likely to be made from the mutant gene and thus may represent a null mutation. In order to determine the viability of Megator mutants we analyzed the offspring from crosses of *l(2)k03905/CyO, P{w+mC=Act-GFP}JMR1* parents in which the balancer chromosome is labeled with GFP allowing for the identification of homozygous *l(2)k03905/l(2)k03905* embryos and larvae. No homozygous *l(2)k03905/l(2)k03905* larvae were found among 200 third instar larvae examined from such crosses and among 300 first instar larvae only one homozygous *l(2)k03905/l(2)k03905* larvae emerged. This suggests that the Megator protein is essential and that the lethality caused by the P-element mutation largely occurs during embryonic development as maternal stores are exhausted. Consistent with this we find that Western blots (Fig. 4b) of homozygous 15-20 hour *l(2)k03905* mutant Megator embryos show decreased Megator protein levels of only 28.5 ± 7.6% (n = 4) that of Megator levels in *l(2)k03905/CyO* and *CyO/CyO* embryos from the same embryo collection. We quantified this difference by determining the average pixel density of Bx34 immunoblot staining of equal numbers of homozygous *l(2)k03905* mutant Megator embryos and control embryos. The remaining low levels of Megator protein observed in the homozygous mutant is likely due to residual maternal stores.

A spindle matrix has been hypothesized to provide a stationary substrate that anchors motor molecules during force production and microtubule sliding [3]. Direct evidence that motor proteins are static in bipolar spindles relative to tubulin has been provided by flux experiments with the mitotic kinesin Eg5 in *Xenopus* [9]. The identification and characterization of the Skeletor protein in *Drosophila* was the first molecular evidence for the existence of a complete spindle matrix that forms within the nucleus [4]. Recently, yeast proteins Fin1p [10] and Ase1p [11] have also been proposed to be components of spindle matrix. The identification of the chromodomain protein Chromator as an interaction partner suggests a possible role of spindle matrix in chromosome dynamics and cytokinesis besides stabilizing and modulating microtubules as proposed previously. But neither Skeletor nor Chromator have characteristics consistent with their serving as a structural component, which makes the identification of Megator as a spindle matrix protein more intriguing. The co-localization of Skeletor and Megator persists during most of the mitosis, while Chromator co-localizes with Skeletor at metaphase but becomes concentrated at the centrosomal region and spindle midzone during telophase. It is thus likely that both Chromator and Skeletor interact with the structure formed by Megator during metaphase, with Skeletor remaining associated and Chromator relocating to centrosomes and the midzone region during telophase. These features as well as Megator's prominent coiled-coil domain are consistent with Megator being the structural protein. Further study of the phenotype of Megator mutants are likely to shed light on the function of the spindle matrix in various cell cycle events.

EXPERIMENTAL PROCEDURES

Fly Strains

Stocks were maintained according to standard protocols [12]. Oregon-R was used for wild type preparations. The y^1 w^{67c23}; $P\{w^{+mC}=lacW\}l(2)k03905^{k03905}/CyO$ line was obtained

from the Bloomington Stock Center and was originally part of the István Kiss collection [13].

To facilitate identification of homozygous mutant Megator embryos,

$[w^{+mC}=lacW]l(2)k03905^{k03905}$ was balanced over the GFP-tagged CyO balancer obtained

from the Bloomington Stock Center line y^1 w^1 ; dmr^6 cn^1 bw^1 sp^1 / CyO, $P[w^{+mC}$ =GAL4-

Kr.C]DC3, $P[w^{+mC}$ =UAS-GFP.S65T]DC7.

Constructs

A DNA fragment containing residues 1436-1697 of the predicted Megator sequence was subcloned into pGEX-4T-1 vector (Amersham Pharmacia Biotech) to generate construct GST-BxM using standard techniques [19]. Construct GST-421 was generated by subcloning a fragment containing residues 601-926 of the Chromator sequence into pGEX-4T-1 vector. The correct orientation and reading frame of the inserts was verified by sequencing. GST fusion proteins was expressed in XL1-Blue cells (Stratagene) and purified over a glutathione agarose column (Sigma-Aldrich), according to the pGEX manufacturer's instructions (Amersham Pharmacia Biotech).

Immunohistochemistry

Antibody labelings of *Drosophila* embryos (0-3 hours) and salivary gland polytene nuclei were as previously described [4,14] after fixation with either Bouin's Fluid fixative (0.66% picric acid, 9.5% formalin, 4.7% acetic acid) or paraformaldehyde (4% in PBS pH 7.0). Double and triple labelings employing epifluorescence were performed using the mAb 1A1 IgM antibody against Skeletor [4], mAb Bx34 IgG_{2a} antibody against Megator [16] or anti-α-tubulin mouse IgG_1 antibody (Sigma), and Hoechst 33258 (Molecular Probes) (0.2 μg/ml in PBS) to visualize the DNA. The appropriate TRITC- and FITC-conjugated secondary antibodies (ICN) were used to visualize primary antibody labeling. Confocal microscopy was performed with a Leica confocal TCS NT microscope system equipped with separate

Argon-UV, Argon, and Krypton lasers and the appropriate filter sets for Hoechst, FITC, and TRITC imaging as previously described [14].

PCR mapping

The insertion site flanking sequence provided by the Berkeley Drosophila Genome Project for the $P\{w^{+mC}=lacW\}l(2)k03905^{k03905}$ element (Accession # AQ025733) placed the P-element insertion near the transcription start site for the Megator gene. By designing several sets of nested forward and reverse primers from genomic sequence encompassing this region we performed PCR from mutant flies as previously described [17]. PCR fragments were subcloned and sequenced according to standard protocols [18].

Western Blot analysis

Western blots were performed as previously described [4]. Homozygous mutant Megator embryos selected from $P\{w^{+mC}=lacW\}l(2)k03905^{k03905}/CyO$, $P\{w^{+mC}=GAL4-Kr.C\}DC3$, $P\{w^{+mC}=UAS-GFP.S65T\}DC7$ parents and identified by virtue of lack of GFP signal were obtained from 15-20 hour embryo collections. Heterozygous $l(2)k03905/CyO$ and CyO/CyO embryos from the same embryo collection served as a reference for the reduction in Megator protein levels in homozygous embryos. Quantification of labeling on Western blots was performed as previously described [14].

Protein Blot Overlay Assays

Protein blot overlay assays were performed essentially as described by Bellin et al. [15]. Protein was transferred to nitrocellulose after SDS-PAGE, blocked with 5% milk and 0.1% Tween-20 in PBS, incubated with 10µg overlay protein for 2 h at room temperature, washed and probed by antibody specific to the overlay protein.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1 Nuclear localization of Megator during the cell cycle. The mAb Bx34 [6] was used to label Megator, the mAb 1A1 to label Skeletor [5], and Hoechst to label DNA and chromosomes. The upper panel shows confocal sections of syncytial blastoderm interphase nuclei show the localization of Megator (red) to the nuclear periphery as well as to the nuclear interior. The composite image (comp) shows the relative distribution of DNA (green). The lower panel shows a light squash of a third instar larval salivary gland polytene nucleus. In the composite image (comp) it is evident that Megator (red) surrounds the chromosomes (green).

Figure 2 Megator (green) is colocalized with the spindle matrix protein Skeletor (red) during the mitotic stages. The composite images (comp) show extensive overlap between Megator and Skeletor labeling at pro-, meta-, and telophase as indicated by the predominantly yellow color. All images in these panels are confocal sections of syncytial embryonic nuclei.

Figure 3 Blot overlay experiment suggests a physical interaction between Chromator and Megator. GST-421 was separated on SDS-PAGE, transferred and incubated with GST-BxM. The membrane was probed by Bx34 antibody. Note the band in the Chromator loaded lane.

Figure 4 P-element insertion in the Megator gene. **a,** diagram of the Megator genomic locus. The locus has five exons separated by four introns. The P-element insertion site with flanking nucleotide sequence of line *l(2)k03905* at the +1 position of the Megator cDNA is indicated above. The ORF coding for the Megator protein including the position of the coiled-coil region is depicted underneath. **b,** Megator protein expression in homozygous *l(2)k03905* mutant embryos from *l(2)k03905/CyO* parents. The level of Megator expression in *l(2)k03905/CyO* and *CyO/CyO* embryos from the same cross served as a control. The immunoblots were labeled with the anti-Megator Bx34 antibody and with anti-tubulin antibody. The proteins from thirty-five 15-20 hour embryos were homogenized and separated by SDS-PAGE in each lane. The relative level of Megator protein expression in mutant embryos as a percentage of Megator expression in control embryos is shown to the right.
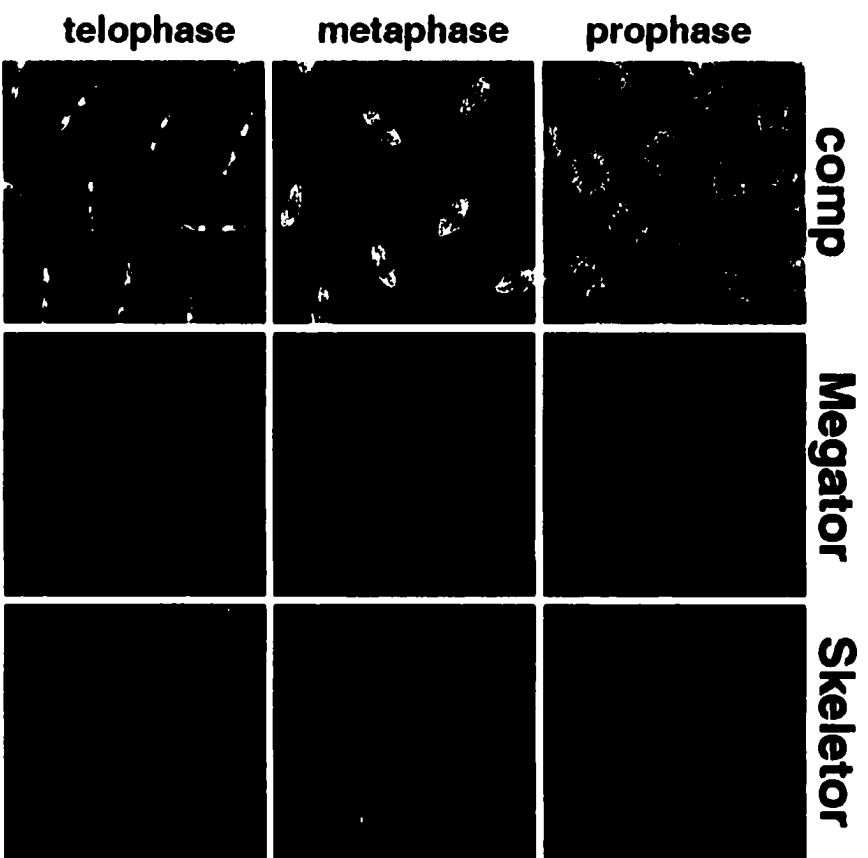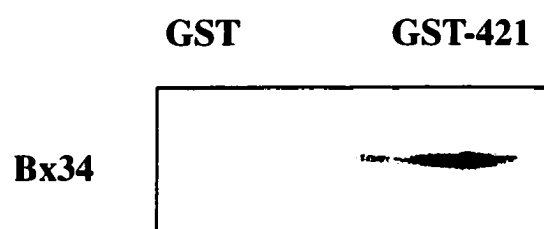
Figure 1

telophase   metaphase   prophase

comp   Megator   Skeletor

Figure 2

62

GST   **GST-421**

Bx34

Figure 3

a

ccctaaagc P ccctaaagc
+1   +1

1 kb

coiled-coil region

b

control   mutant
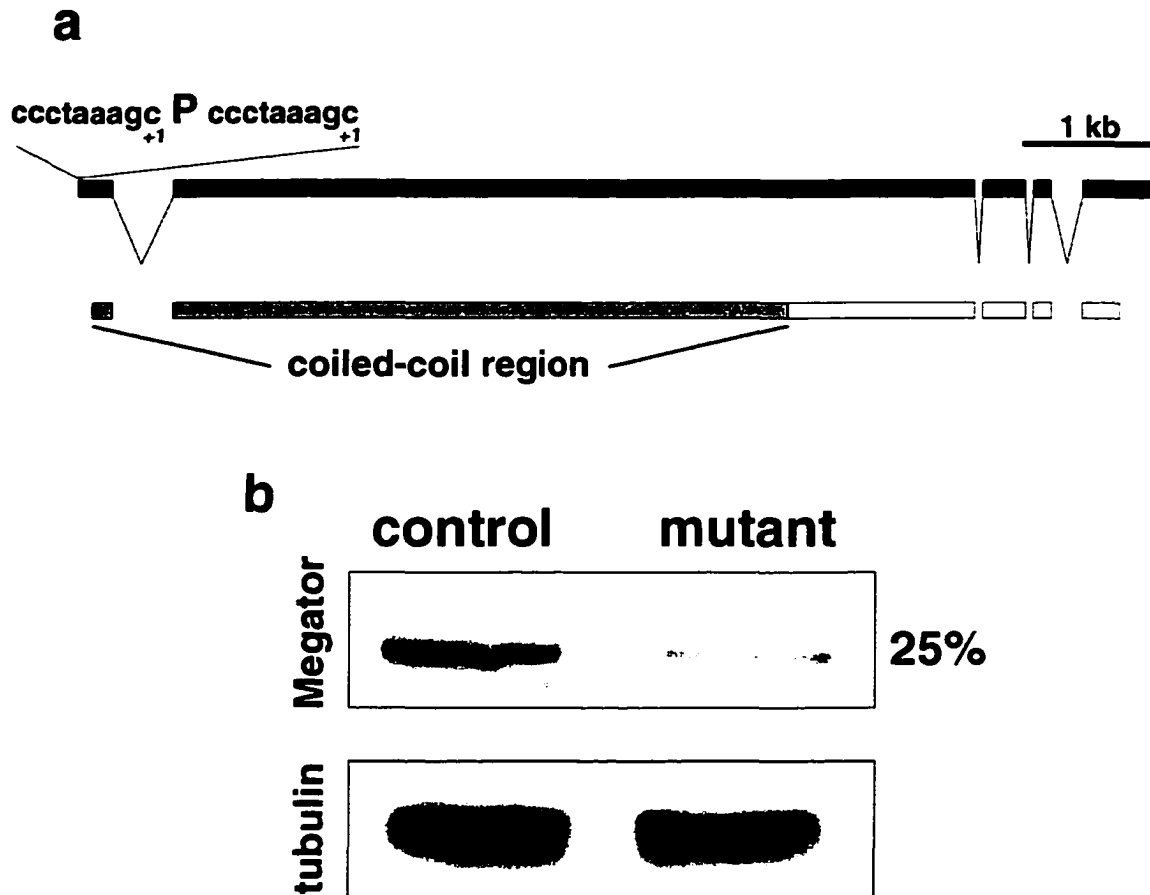
Megator

25%

tubulin
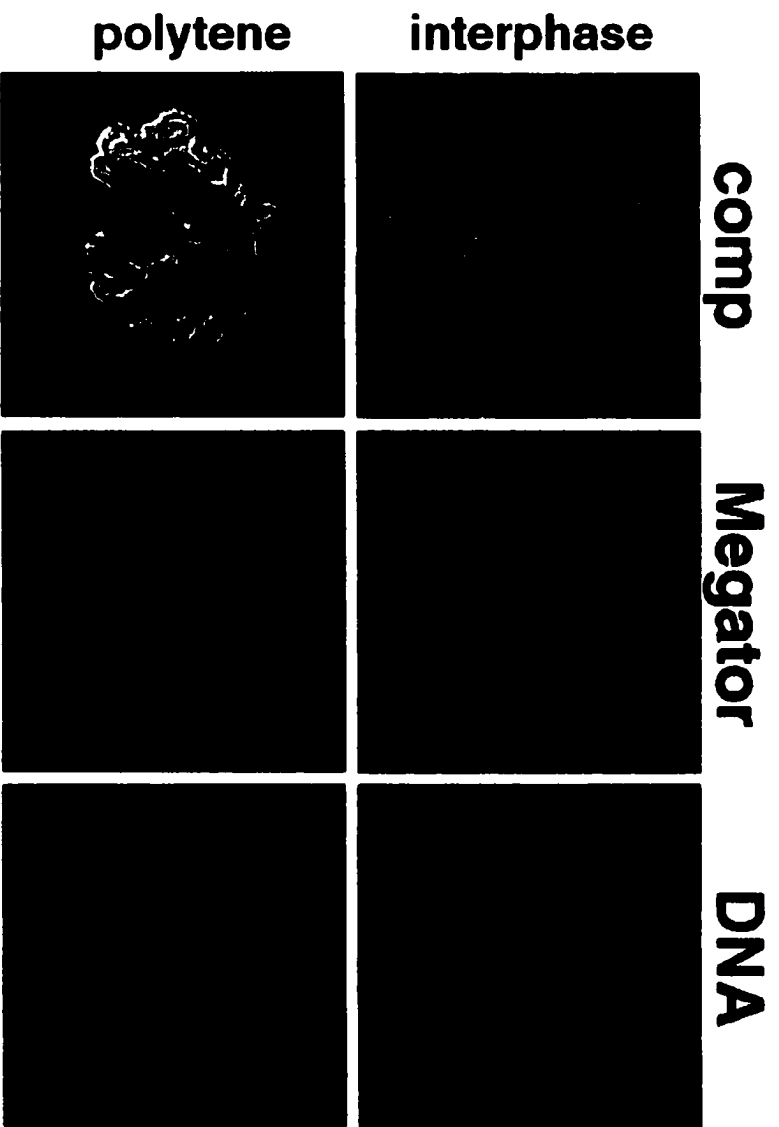
Figure 4

GENERAL CONCLUSIONS

Chromator Is a Novel Chromodomain Protein

The C-terminal portion of Chromator was identified in the screening of two independent *Drosophila* embryonic yeast two-hybrid libraries. The sequences of these fragments were matched to the CG10712 locus predicted by the Berkeley *Drosophila* Genome Project. Through sequencing ESTs of this region, we identified three transcript of Chromator, which encode the same protein sequence. The predicted Chromator sequence has 926 amino acids with a chromodomain near its N-terminus. The protein with most homology to Chromator is the predicted product from two *Anopheles gambiae* genome shotgun sequences, which is expected considering the close relationship between *Drosophila* and *Anopheles*. Clone agCP10027 matches the N-terminal portion of Chromator including the chromodomain region, while agCP9618 matches the C-terminal portion of Chromator. The whole genome of *Anopheles* has not been assembled, but it is a good guess that the sequences in agCP9618 and agCP10027 derive from the same gene. Besides these two *Anopheles* sequences, Chromator has only modest homology with another protein, the *Tetrahymena* protein Pdd1 (for programmed DNA degradation). Comparing the chromodomain sequence of Chromator with that of other chromodomain proteins, the nearest sequence still comes from agCP10027 as expected and these two chromodomains could not be placed in any other known families, like HP1, Polycomb or CHD1, as demonstrated from the phylogenetic tree. This suggests that Chromator and its *Anopheles* homolog represent a new class of chromodomain proteins. There are no other defined domains in the Chromator sequence, though there is an Asn rich region near the C-terminus.

Other features of Chromator sequence include two classic nuclear localization signals, PRRK and PRRKTHE at residue 233, several PEST-like sequences implicated in

protein degradation (Rodgers, et al., 1986) are located at residues 300-400 and 850-900, and several potential SUMO modification sites (Dr. M.J. Blacketer, personal communication).

The structure of Megator has been described by Zimowska and Paddy (1997), the major feature is the coiled-coil structure from the N-terminus to residue 1630. Megator also contains potential SUMO modification sites and many PEST motifs.

Skeletor, Chromator and Megator Are Components of a Multiprotein Complex

The physical interaction between Skeletor and Chromator has been rigorously demonstrated in *in vitro* pull down and coimmunoprecipitation experiments. The biotinylated Skeletor could pull down GST-Chromator and vice versa. In the same fashion, antibody specific to Chromator could immunoprecipitate Skeletor from embryonic extract and vice versa. Also considering the result of yeast two-hybrid assay, there are multiple lines of evidence that Skeletor and Chromator interact physically.

The physical interaction between Chromator and Megator was indicated in the blot overlay experiment, in which Bx34 antibody could detect a band at the position corresponding to GST-421 after the immunoblot was incubated with a Megator GST fusion protein. There is also preliminary evidence that Megator also interacts with Skeletor physically using this type of assay (data not shown).

A gel filtration experiment composed of Superose 12 fractionation of Schneider 2 cell extract has shown that Chromator migrates with a two mega-Dalton protein complex (data not shown). So there are likely other proteins interacting with Skeletor, Chromator and Megator in the same protein complex. Note that the Chromator fragment identified by its interaction with Skeletor in the yeast two-hybrid assay is composed of the C-terminal portion, not including the chromodomain. Yeast two-hybrid screening performed with the

Chromator chromodomain region has recovered various protein including filamin and some transcription factors, which may be interesting for later study.

Skeletor, Chromator and Megator are Dynamically Distributed

Skeletor, as the founding member of this protein complex was identified for its dynamic distribution pattern, Chromator and Megator have this feature as well. All three protein go through a reorganization process to form a pole to pole spindle structure at metaphase, and regain the interphase distribution patterns at the end of telophase. The difference in the distribution patterns during interphase is that Chromator and Skeletor demonstrate a meshwork like structure and seem to be associated with chromosomes, while Megator decorates the nuclear envelope inner rim and is also seen in the interchromosomal space. Skeleor also has a feature of decorating the nucleolus. At metaphase, all three proteins form a full spindle structure coaligned with the microtubule spindle, with Chromator having in addition a centrosome localization. The colocalization of Skeletor and Megator continues through telophase, but Chromator becomes concentrated at centrosomes and the spindle midzone at telophase. At this stage, Chromator still has colocalization with Skeletor and Megator at spindle midzone. This dynamic pattern points to both coordination and independent functions of the three proteins. Among them, the distribution of Chromator seems to be most like microtubules, especially during telophase, possibly indicating Chromator is involved in microtubule dynamics, while Skeletor and Megator decorate an end-to-end structure during most of the mitosis.

Skeletor, Chromator and Megator Are Essential for Nuclear Functions

To date, no mutant of the Skeletor gene has been identified, but antibody perturbation experiments do give some clue of the phenotype of a Skeletor mutant (Figure 1). Embryos injected with anti-Skeletor ascites have fewer nuclei than control embryos and the DNA appears fragmented and in various stages of disintegration. These results suggest that Skeletor specific antibody can perturb nuclear morphology and division. But whether the antibody acts through depletion of Skeletor or by hindering Skeletor interacting proteins like Megator is not resolved.

Chromator and Megator both have recessive lethal alleles, suggesting that they are essential genes. The KG06256 allele is recessive lethal at larval to pupal stages, so polytene chromosome squashes can be prepared. The polytene chromosomes from KG06256 homozygous larvae become totally fragmented while polytene chromosomes from wild type larvae appeared normal under the same squash conditions. The morphology of the polytene chromosomes from KG06256 heterozygotes is intermediate. This suggests Chromator is required for normal chromatin structure, which is consistent with the property of a chromodomain protein. Phenotypes affecting mitotic spindle functions have not been identified so far for Chromator and Megator. An intrinsic difficulty is that both proteins have sizable amount of maternal product.

The Functions of Skeletor, Chromator and Megator

Almost certainly, the functions of these proteins change as the cell cycle progresses. Since Megator is a component of the nuclear pore complex, it is likely to be involved in nuclear import/export. Megator is not the first protein involved both in nuclear transport and mitotic spindle function, importin-β is a component of the nuclear transport machinery and
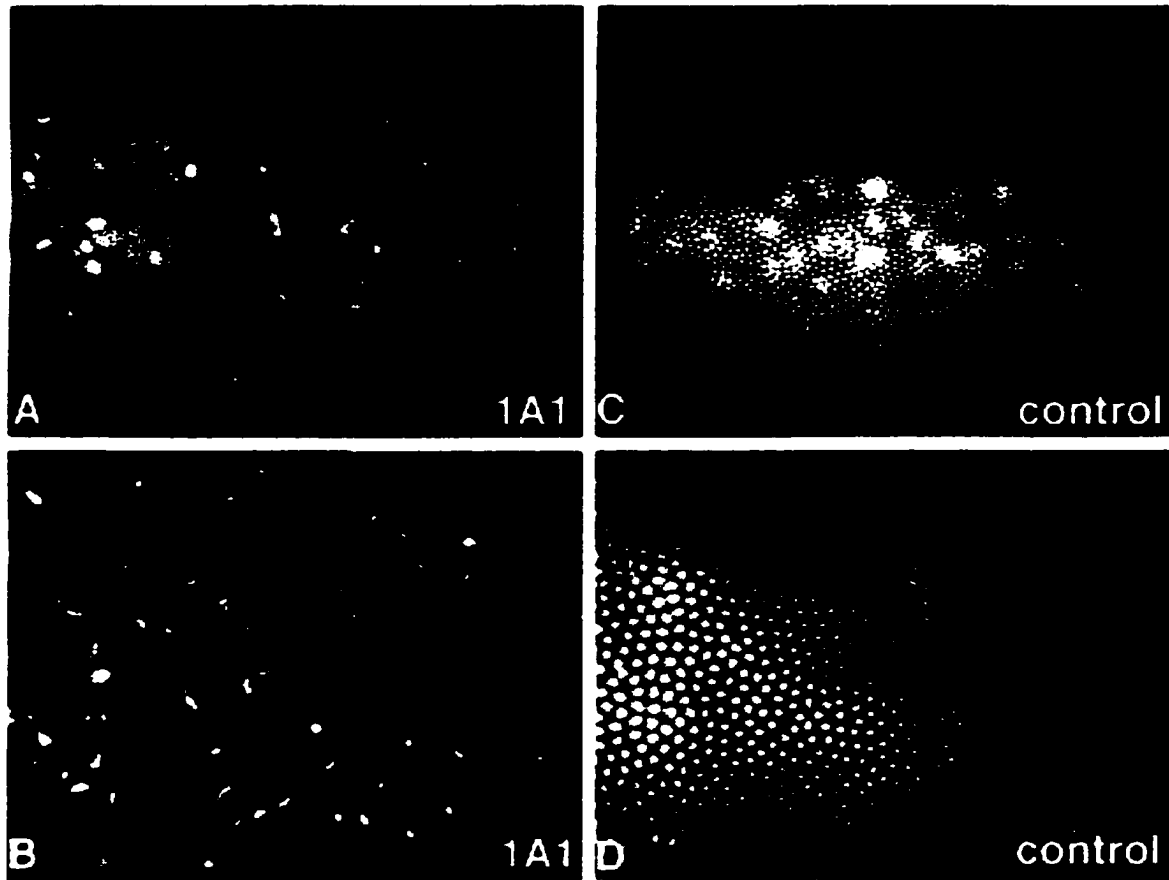
Figure 1. mAb1A1 perturbation analysis of nuclear development in syncytial embryos (figure adapted from Walker et al., 2000). The mAb1A1 (A and B) or control IgM antibody (C and D) was injected into early, syncytial stage embryos that were allowed to develop for 2.5 h before fixation, devitellinization, and Hoechst staining. (A) Experimental embryo injected with mAb1A1 shows fewer nuclei that are disorganized and mislocalized. (B) At higher magnification, nuclei in mAb1A1-injected embryos show lack of nuclear structure and are in various stages of fragmentation (insert). (C) Embryo injected with control antibody develops normally and appears wild-type in its Hoechst-staining pattern. (D) At higher magnification, nuclei from control-injected embryos are synchronized and show normal nuclear morphology.

also modulates the function of Ran in stimulating the formation of mitotic spindles (Nachury et al. 2001; Gruss et al., 2001). The interchromosomal postioning of Megator in the interior of the nucleus may indicate that Megator plays a role in maintaining interphase nuclear structure in the fashion of EAST protein, considering its ability to form filamentous structure. The redistribution of Megator upon heat shock reported by Zimowska and Paddy (2002) may suggest that Megator could respond to different environmental and developmental cues.

The function of Chromator and Skeletor during interphase is not clear, though both localize to the chromosomes. If the modest homology between Chromator and Pdd1 also extends to their function, Chromator might be marking the chromosomes for some other factors to form complexes on specific sites. One of the mechanisms that HP1 uses to establish heterochromatin territories is tethering the chromatin to the nuclear envelope by interacting with lamin β receptors. It is not clear if the interaction of Chromator and Megator could serve a similar purpose. The Skeletor association with chromatin does not depend on Chromator, as there are some sites with only Skeletor but not Chromator localization. The nucleolar localization of Skeletor is very interesting in light of the discovery that proteins regulating mitotic exit are sequestered in the nucleolus (Shou et al., 1999). Zimowska and Paddy (2002) also favor a role for Megator in transportation out of nucleolus. So the functions of all three proteins could be linked during interphase as well as in the mitosis.

It is tempting to speculate that Skeletor localization to the nuclear lamina at early prophase is due to Megator. In any case, a new form of interaction has to be established. The spindle matrix takes form before the microtubule entry into the nucleus and probably provides a cue for the microtubule spindle formation. Megator is likely the structural component of this spindle matrix considering its sequence features. Chromator's localization on centrosomes may suggest that Chromator functions as a bridge between the centrosome and the spindle matrix. It is proposed that many microtubules are released from the centrosomes after nucleation, and then being captured by motor proteins and NuMA or Asp

in *Drosophila* (Wakefield et al., 2001). Asp is localized to the centrosome and the surrounding spindles. Though Asp is proposed to form a matrix to anchor the microtubule minus end motors, it is likely that Asp itself is tethered on the spindle matrix structure. Conceptually, an end-to-end structure is better suited as a substrate for MT-based motors.

Another region where Chromator and Asp might coordinate is the central spindle. The central spindle is a body of microtubules formed between the two late anaphase-telophase nuclei. It has been shown that central spindle plays a vital role in cytokinesis (Gatti et al., 2000). Riparbelli et al. (2001) showed that central spindle can still be formed in male meiotic cells of the *asterless* mutant, which do not have functional centrosomes. This observation could be easily explained invoking the model that the spindle matrix helps to establish and organize microtubule spindles. Just as in the centrosomal region, Asp is required for normal central spindle formation. Riparbelli et al. (2001) proposed that Asp has essentially the same function of focusing the microtubule minus end in the central spindle as in the centrosomal region, which raises the possibility that Chromator has the same coordination with Asp in these two regions too. Another protein related to the central spindle is the inner centromere protein (INCENP). INCENP dissociates from the chromosomes at the metaphase-anaphase transition and is deposited at the central spindle and the cell cortex. INCENP is believed to recruit Aurora B kinase, which is involved in chromosome structure and alignment, as well as in coordinating anaphase and cytokinesis (Adams et al, 2001). This might potentially be related to the regulation of Chromator. It is not clear why Chromator runs on SDS-PAGE as a doublet or why it runs higher than its predicted molecular weight (130 kD vs. 101 kD). Since Chromator has many potential phosphorylation sites, it will be interesting to see if it can be phosphorylated by Aurora B kinase.

The specific function of Skeletor is harder to predict, due to the absence of any identifiable domain. But considering Skeletor colocalizes with Megator during most of the mitosis, their function is likely to be closely related.

Comparison with Other Proposed Spindle Matrix Proteins

After the publication reporting the spindle matrix protein Skeletor, more proteins have been proposed to be components of spindle matrix. In this dissertation I have described two such proteins, Chromator and Megator. Recently other candidate spindle matrix proteins have been identified in other systems.

The *Saccharomyces cerevisiae* protein Fin1p was identified in yeast two-hybrid assay as an interaction partner of the 14-3-3 protein Bmh2p (Hemert et al., 2002). Fin1 is 291 amino acids in length and contains two coiled-coil motifs which are able to mediate self-association of Fin1p (Hemert et al. 2003). In addition, purified Fin1p protein can form 10 nm diameter filaments *in vitro*, similar to known intermediate filament-forming proteins. Fin1p has a dynamic distribution pattern. During interphase, Fin1p is nuclear localized, but does not form filamentous structures. In dividing cells, Fin1p forms a filament between the mother and daughter nuclei, and this filament is coaligned with microtubules. The dynamic distribution of Fin1p and its feature of coiled-coil motifs draw obvious comparison with Megator. But one should note that the budding yeast nucleus is very different to that of *Drosophila*, it has a "closed" mitosis totally in the nucleus, each kinetochore is attached to one microtubule from each pole. Fin1p is not essential for yeast viability, which may suggests some redundant mechanism.

Ase1p is another budding yeast protein with a coiled-coil motif. Ase1p has an extended rod shape and has been shown to form homodimers. It has microtubule binding activity and stimulates microtubule bundling. The absence of Ase1p leads to the collapse of the mitotic spindle during anaphase while its overexpression alone is enough to induce premature spindle enlongation without accordingly progress the cell cycle. Most strikingly, Ase1p seems to be immobile inside the central spindle. In the photo-bleaching experiment, Ase1p-GFP takes more than 15 min to recover while Cin8p-GFP, in which Cin8p is a

microtubule based motor protein, takes only 45 seconds. If the Ase1p homolog in *Drosophila* has the same property, it will be a good candidate as a component of spindle matrix. It will then be interesting to see whether it has interaction with Chromator, Skeletor or Megator.

As more and more spindle matrix proteins are being characterized, the functions of spindle matrix are likely to become much clearer.

Studying Spindle Matrix

The objection that some researchers have against a spindle matrix is that "aster spindles" can be assembled *in vitro*, presumably without the spindle matrix component. But nobody can seriously argue that this kind of "aster spindle" possesses all the functions and properties of a mitotic spindle in living cells. As mentioned in the general introduction, Kapoor and Mitchison showed that most Eg5 is static on spindle microtubules. But Wilde et al. (2001) showed that antibody labeled Eg5 moved towards the plus end of the microtubules assembled from cytostatic factor-arrested *Xenopus* egg extracts with Ran-GTP after adding centrosomes. Kapoor and Mitchison argue that Wilde's results are based on "aster spindles", not bipolar spindles and antibody might block Eg5 interaction with spindle matrix. Moreover, Johansen and Johansen (2002) pointed out that there may not be a spindle matrix in the Ran-GTP induced aster spindles at all. This example shows the danger of extrapolating from *in vitro* assembly experiment even though it is certainly a very powerful tool in studying mitotic process.

The same holds true for mathematical modeling studies. Efforts to establish mathematical models incorporating microtubule dynamics and force generating motors can certainly be informative. But arguing that certain models can simulate the behavior of the mitotic spindle without invoking a spindle matrix component leads to the conclusion that a

spindle matrix does not exist is not valid. A fundamental principle of mathematical modeling is that you can always add a parameter in your model and fit the data at least equally well (since you can always set your parameter to zero). While it might be true that simple is always good in physics, redundancy and coordination is often the norm in biology. Today's mathematical models are likely to be oversimplistic in some or many ways.

In summary, the identification of Chromator and Megator as components of the spindle matrix first defined by Skeletor has shed light on the organization and function of spindle matrix. Further study using the mutants of these proteins, especially germline mosaic flies, will be highly promising.

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ACKNOWLEDGEMENTS