The *Drosophila* Centromeric Protein MEI-S332: Its Role and Regulation in Sister-Chromatid Cohesion

by

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ABSTRACT

The physical association between sister chromatids is a necessary prerequisite for proper chromosome segregation in both mitosis and meiosis. Defects in sister-chromatid cohesion can result in aneuploidy, which is associated with tumorigenesis, spontaneous abortions, and congenital disorders such as Down syndrome. The MEI-S332 protein has been shown to be essential for centromeric sister-chromatid cohesion during both female and male meioses of *Drosophila melanogaster*. Loss-of-function mutations in *mei-S332* cause precocious sister-chromatid separation in late anaphase I, resulting in chromosome loss and missegregation in meiosis II. In this thesis, the analysis of the MEI-S332 protein began with the determination of its role in sister-chromatid cohesion. Immunofluorescence microscopy showed that MEI-S332 functions to maintain sister-chromatid cohesion at the centromere, rather than to establish cohesion, as the protein localizes to chromosomes during prometaphase and its localization is independent of intact microtubules. Results from both yeast two-hybrid assay and immunoprecipitation experiments demonstrated that MEI-S332 is capable of homotypic interactions, suggesting that self interaction between MEI-S332 molecules could be the mechanism by which the protein holds sister chromatids together. A structure-function analysis of the domains of MEI-S332 revealed that MEI-S332 has at least two functional domains and that the carboxy-terminal basic region is essential for localization to centromeres. In addition to its role in meiotic sister-chromatid cohesion, MEI-S332 seems to also play a role in strengthening cohesion between sister centromeres during mitosis. Finally, during both mitosis and meiosis, MEI-S332 has a very interesting pattern of localization: it assembles onto the centromeres during prometaphase, and at the time of sister-chromatid separation it dissociates from the chromosomes. This observation led to the examination of the regulation of the MEI-S332 protein during the cell cycle. MEI-S332 was found to be post-translationally modified by phosphorylation, which seems to be cell cycle-regulated. MEI-S332 appears to be phosphorylated during interphase and anaphase, when it is dissociated from the chromosomes, and dephosphorylated during metaphase, when it is localized to the centromere.

Thesis Supervisor: Terry L. Orr-Weaver
Title: Professor of Biology

Dedicated to

My Mother and Father
Wen-Ying and Yu-Tseng Tang

and

My Graduate Advisor
Dr. Terry L. Orr-Weaver

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Chapter One

Introduction

When a cell divides, the segregation of genetic material must occur with high fidelity to prevent the formation of aneuploid daughter cells, which are cells with abnormal number of chromosomes. Aneuploidy, arising from errors in chromosome segregation in somatic mitotic cells, appears to contribute to the formation of some colorectal cancers and is associated with tumorigenesis (Lengauer et al. 1997). Chromosomes can also missegregate in meiosis, an essential process in the production of eggs or sperm, which must have half the normal number of chromosomes. Errors in chromosome segregation actually occur in as many as 10% of human female meioses (Hassold et al. 1993; Orr-Weaver 1996). The consequences of fertilization between sperm or eggs with incorrect number of chromosomes are spontaneous abortions, birth defects, and/or congenital disorders such as Down syndrome (Hassold et al. 1993; Orr-Weaver 1996). Approximately 25% of naturally aborted fetuses are trisomic (Hassold et al. 1993); about 20% of trisomy 21 cases result from maternal meiosis II chromosome missegregation (Lamb et al. 1996).

During its lifetime, a dividing cell typically progresses through a cycle of four phases (for a review of the cell cycle, see Murray and Hunt 1993). During S phase, chromosomes are replicated, giving rise to what are called sister chromatids. Then in M phase, the replicated chromosomes are segregated. The S and M phases are separated by two gap phases, G1 and G2, during which the cell grows and prepares for dynamic processes such as chromosome replication and chromosome segregation (Figure 1-1). Within M phase, or mitosis, there are several stages, which are defined by chromosome morphology and chromosome movement (Figure 1-2). During the first phase, prophase, the chromosomes are condensing, and by late prophase, the centrosomes have duplicated. Then in prometaphase, the nuclear envelope breaks down, allowing the microtubules nucleating from the centrosomes to

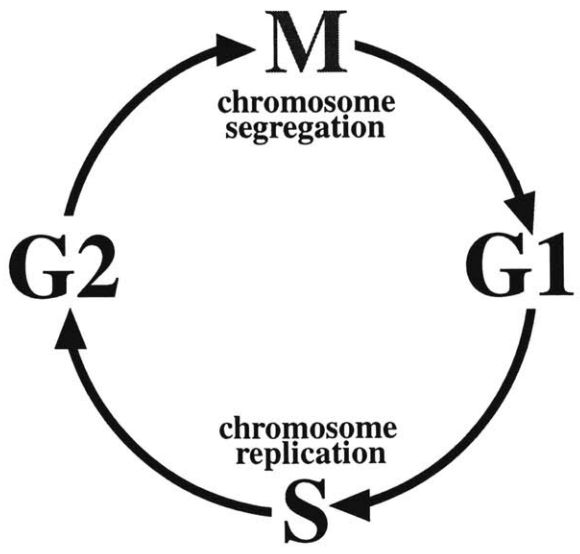

Figure 1-1. The canonical cell cycle

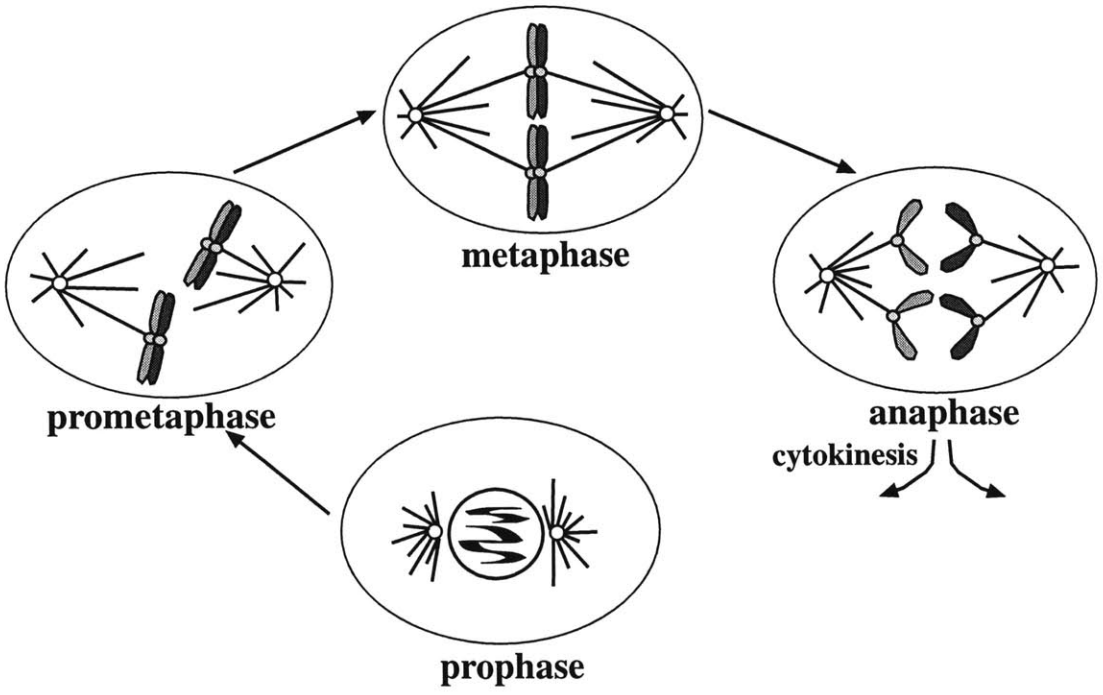

Figure 1-2. Mitosis

enter into the nucleus. Within the nucleus, microtubules engage in interactions with kinetochores on the chromosomes. Kinetochores are protein structures that can capture microtubules and associate with motor proteins. The regions of chromosomes where kinetochores are located are called the centromeres. By metaphase, the kinetochores have captured microtubules emanating from opposite spindle poles, the bipolar spindle is established, and chromosomes are aligned on the metaphase plate. Since the time of DNA replication, sister chromatids have been held together along their entire lengths. This physical association between the replicated chromosomes is a necessary prerequisite for proper chromosome segregation in both mitosis and meiosis (see below). In anaphase, the linkage between sister chromatids is released, allowing sister chromatids to separate from each other and segregate towards opposite spindle poles. In telophase, daughter nuclei are reformed, and cytokinesis occurs to produce two daughter cells that then enter into interphase and start a new cell cycle.

Meiosis is a variant of the mitotic cell cycle. Unlike mitosis, meiosis consists of two rounds of chromosome segregation following a single round of DNA replication (Figure 1-3). During meiosis I, the homologous chromosomes pair and disjoin from each other while the sister chromatids remain attached at their centromere regions. This centromeric association persists until meiosis II, during which the sister chromatids, as in mitosis, separate from each other and migrate to opposite spindle poles. These two meiotic divisions occur successively without intervening DNA synthesis. An important distinction between mitosis and meiosis is that meiotic sister-chromatid cohesion is released in two steps--arm cohesion is released first in meiosis I and centromeric cohesion is released later in meiosis II--while in mitosis both arm

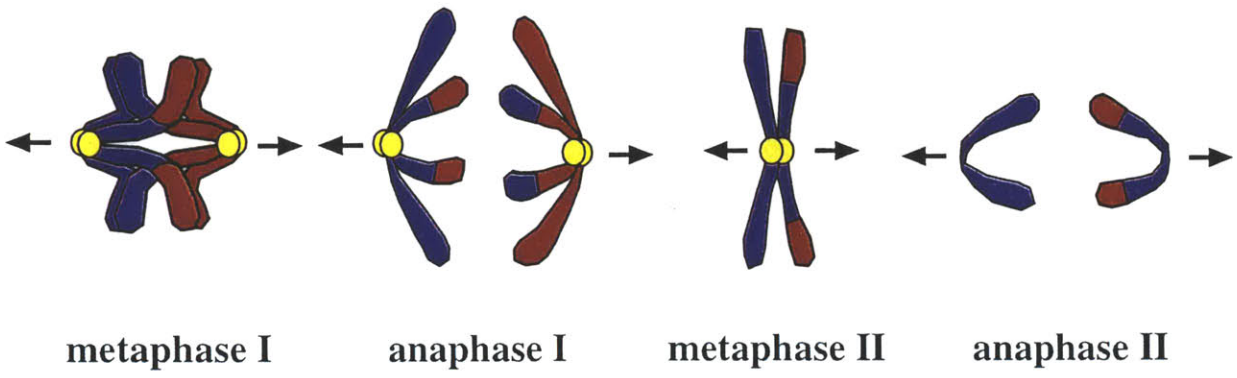

Figure 1-3. Meiosis

and centromere cohesion are released at the same time at the metaphase/ anaphase transition (Miyazaki and Orr-Weaver 1994).

In this chapter, the roles and regulation of sister-chromatid cohesion as well as components involved in the mechanism of cohesion are discussed. In the past several years, research in understanding the mechanism of sister-chromatid cohesion at the molecular level has been tremendously productive. Several players involved in this fundamental and essential cellular process have been isolated and characterized. Work in several organisms, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and *Xenopus*, has elucidated a picture of how replicated chromosomes are held together during mitosis and how this association is released at the onset of anaphase. The role of the *Drosophila* centromeric protein MEI-S332 in sister-chromatid cohesion and the structure-function analysis of this protein are presented in Chapter Two. The localization of MEI-S332 during mitosis and female meiosis is shown in Chapter Three. Chapter Four reveals that MEI-S332 not only is essential for meiotic sister-chromatid cohesion but also plays a role in mitosis in strengthening sister-chromatid cohesion at the centromere. Finally, the regulation of the MEI-S332 protein during the cell cycle is discussed in Chapter Five.

A. Roles of sister-chromatid cohesion

In meiosis, sister-chromatid cohesion is a necessary prerequisite for proper chromosome segregation. Sister chromatids must be held together to move concertedly to the same spindle poles during meiosis I (Miyazaki and Orr-Weaver 1994; Figure 1-3). Then in meiosis II, the persistent centromeric chromatid cohesion provides the force to counteract the poleward pulling forces, creating tension necessary to keep the sister chromatids on the metaphase II plate until their separation in anaphase II (Nicklas 1974; Figure 1-3). For example, no metaphase II plates are observed in *Drosophila ord* or *mei-S332* mutant spermatocytes (Goldstein 1980; Kerrebrock et al. 1992; Miyazaki and Orr-Weaver 1992). Mutations in the *ord* and *mei-S332* genes cause precocious separation of sister chromatids in meiosis I.

Sister-chromatid cohesion may also influence the behavior of the homologous chromosomes during meiosis I. The cohesion along the sister-chromatid arms has been proposed to be involved in the maintenance of stable chiasmata, preventing their resolution until anaphase I (Bickel and Orr-Weaver 1996; Moore and Orr-Weaver 1998; Maguire 1982; Maguire 1993). In organisms that undergo meiotic recombination (e.g., female *Drosophila melanogaster*), chiasmata, the cytologically defined structures representing the points of crossover, are thought to provide the physical association between the homologs. The tension resulting from the counteraction of the forces pulling the homologs toward opposite poles and the forces from chiasmata holding the homologs together orients the bivalents and establishes their bipolar attachment on the meiotic spindle (Hawley 1988). The resistance to the pulling of the spindle microtubules must persist until the homologs are ready to segregate, and hence, the maintenance of stable chiasmata is required for the fidelity of homolog segregation.

In addition, sister-chromatid cohesion appears to play an important role in controlling the orientation of the sister kinetochores, which is essential for the correct movement of the sister chromatids (Nicklas 1974). In meiosis I, the sister kinetochores lie on the same face of the chromosomes to capture microtubules emanating from the same spindle pole. This monopolar attachment of sister kinetochores ensures that sister chromatids move into the same daughter cells at the end of meiosis I (Bickel and Orr-Weaver 1996; Nicklas 1977). Prior to meiosis II, the sister kinetochores must establish an opposite orientation such that they attach to microtubules nucleating from opposite poles. This bipolar attachment then allows the segregation of sister chromatids away from each other (Nicklas 1977). Failure to reorient sister kinetochores to opposite poles will lead to sister-chromatid nondisjunction and possibly chromosome loss in meiosis II. It has been proposed that the centromeric chromatid cohesion inherently provides for the polarity of the sister kinetochore orientation during meiosis II. By restricting the movement of the sister chromatids via physical association, sister-chromatid cohesion potentially forces the sister kinetochores to face opposite poles and establishes their bipolar attachment to the spindle microtubules in meiosis II (Bickel and Orr-Weaver 1996).

In mitosis, like in meiosis II, sister-chromatid cohesion also acts to create the tension that is necessary to keep the chromosomes aligned on the metaphase plate (McNeill and Berns 1981; Skibbens et al. 1995). After severing the linkage between sister kinetochores on bi-oriented chromosomes in newt lung cells by use of laser ablation, Skibbens et al. (1995) observed that without the physical association with one another, sister kinetochores failed to congress to the spindle equator and their movement became uncoordinated. Furthermore, recent observation in *S. pombe* suggests that sister-chromatid

cohesion functions to orient the sister kinetochores towards opposite directions in mitosis and hence ensures that they capture only microtubules emanating from opposite spindle poles. *S. pombe* cells defective in the *mis6* gene exhibit disrupted centromere heterochromatin structure, precocious separation of sister chromatids in metaphase, and disordered positioning of centromeres in metaphase, leading to random segregation of chromosomes into daughter nuclei (Saitoh et al. 1997). Saitoh et al. (1997) proposed that the Mis6 protein functions as a "glue" that keeps the sister centromeres in a bi-oriented fashion until the onset of anaphase and that the biorientaton of the sister centromeres is abolished in the *mis6* mutant.

Finally, the phenotypes of *S. cerevisiae* mutants that have defects in sister-chromatid cohesion also support the proposal that sister-chromatid cohesion plays an important role in controlling the orientation of sister kinetochores during mitosis. Specifically, the *mcd1*, *smc1*, and *ctf7/eco1* mutants have been reported to exhibit not only precocious separation of sister chromatids but also aberrant spindle phenotypes and delay during mitosis (Strunnikov et al. 1993; Guacci et al. 1997; Michaelis et al. 1997; Skibbens et al. 1999). In addition, the mitotic delay of *ctf7* mutant cells requires the spindle assembly checkpoint, a control mechanism that ensures all kinetochores are attached to microtubules from opposite spindle poles before anaphase is triggered (Skibbens et al. 1999; see Spindle Assembly Checkpoint in this chapter). These observations suggest that in the absence of cohesion between sister chromatids, sister kinetochores are not restricted to face opposite orientations and consequently, fail to form stable interactions with microtubules emanating from opposite spindle poles. As a result, the spindle assembly checkpoint is activated, and the progression through mitosis is delayed.

B. Establishment of sister-chromatid cohesion during S phase

To ensure normal chromosome segregation, sister-chromatid cohesion must be established properly. The simplest way to assure that the connections are indeed formed between two sister chromatids, instead of randomly between two non-sister chromatids, would be to establish these linkages as sister chromatids are being generated during or immediately after DNA replication. Results from fluorescence in situ hybridization (FISH) experiments in *S. cerevisiae* provide evidence that sister-chromatid cohesion is established during S phase (Guacci et al. 1994). Using probes specific to multiple regions of the chromosomes, Guacci et al. (1994) found that they could never detect two dots of FISH signals until anaphase when sister chromatids have separated. The presence of only one dot of FISH signal prior to anaphase indicates that sister chromatids are already held together during or immediately after DNA replication.

1. DNA catenation

Several mechanisms have been proposed to account for the physical connections between sister chromatids. It has been suggested that catenation of sister-chromatid strands resulting from DNA replication could contribute to the intertwining of the sister chromatids (Murray and Szostak 1985). Sundin and Varshavsky (1981) have shown that during the final stage of the SV40 DNA replication, as the two replication forks approach each other from opposite directions, the replicated DNA duplexes become two interlocking circles called catenanes. To separate the two circular DNA duplexes, the activity of topoisomerase II is needed. The enzyme has the ability to cut a double-stranded DNA molecule, pass a DNA duplex through the cut, and

religate the cut (for review, see Wang 1985). Murray and Szostak (1985) proposed that, until anaphase, sister chromatids remain associated after DNA replication by catenation. Only in anaphase would topoisomerase II be activated to destroy the catenation between sister chromatids, allowing them to separate from each other.

Studies in yeast, frog extracts, and mammalian cell lines have demonstrated that topoisomerase II is essential for the accurate separation of sister chromatids in anaphase (Holm 1994). Mutations in *S. cerevisiae* topoisomerase II have been shown to cause lethality at the nonpermissive temperature if mutant cells were allowed to pass through the cell cycle (Holm et al. 1985). Lethality was prevented if cells were treated with nocodazole, a microtubule-depolymerizing drug that arrests cells in prometaphase, indicating that topoisomerase II activity is required during mitosis. This is consistent with the hypothesis that topoisomerase II acts to separate the intertwining sister chromatids during mitosis. Furthermore, in *S. pombe*, mutants for topoisomerase II have been shown to cause defects in chromosome condensation and sister-chromatid separation (Uemura et al. 1987). Using inhibitors of topoisomerase II in *Xenopus* egg extracts, Shamu and Murray (1992) were also able to demonstrate that in the absence of topoisomerase II activity anaphase was delayed and chromosomes bridges were observed, suggesting that the topoisomerase II-mediated decatenation of sister chromatids was required for sister-chromatid separation.

Although these studies on topoisomerase II seem to support that the interlocking between sister chromatids as a intrinsic result of DNA replication is what holds sister chromatids together, the idea that catenation alone is responsible for sister-chromatid cohesion during mitosis remains questionable. The catenation model for sister-chromatid cohesion predicts that

topoisomerase II is only activated at the metaphase/anaphase transition to resolve catenation and allow separation of sister chromatids. However, Shamu and Murray (1992) found that topoisomerase II activity was highest in metaphase frog egg extracts, at the time when sister chromatids remain associated, and there was no increase in topoisomerase II activity at the metaphase/anaphase transition. Furthermore, although topoisomerase mutants appear to have defects in chromosome condensation and chromosome segregation, studies in topoisomerase II mutants do not provide direct evidence for the involvement of catenation in sister-chromatid cohesion. In fact, minichromosome segregation in *S. cerevisiae* occurs faithfully without detectable catenation of sister-chromatid strands (Koshland and Hartwell 1987).

2. Cohesion proteins

If catenation is not the sole factor that tethers sister chromatids together, what are other factors involved in establishing the physical connections between the replicated DNA duplexes? Sister chromatids can be held together by the action of non-histone chromosomal proteins. Three independent genetic screens in *S. cerevisiae* have identified a gene known as *MCD1/SCC1* that is necessary for sister-chromatid cohesion (Guacci et al. 1997; Michaelis et al. 1997). Budding yeast cells mutant for the *MCD1/SCC1* gene exhibit precocious separation of sister chromatids and fail to condense chromosomes properly during mitosis (Guacci et al. 1997; Michaelis et al. 1997). Michaelis et al. (1997) found that Mcd1p/Scc1p associates with the chromatin during late G1/early S phase and dissociates from the chromosomes at the onset of anaphase, when sister chromatids separate. Furthermore, Guacci et al. (1997) found that the levels of Mcd1p/Scc1p are cell cycle-

regulated, being the most abundant during S phase, declining in late S phase, and remaining constant through telophase. These results provided strong evidence for a role of Mcd1p/Scc1p in the establishment of sister-chromatid cohesion. Further studies with Mcd1p/Scc1p showed that the protein must be associated with the chromosomes as they are being replicated to assure proper sister-chromatid cohesion (Uhlmann and Nasmyth 1998). Mcd1p/Scc1p can associate with chromosomes in the absence of DNA replication as well as in G2 phase, but sister chromatids separate precociously if cells are allowed to progress through S phase in the absence of Mcd1p/Scc1p.

In addition to *MCD1/SCC1*, Michaelis et al. (1997) also isolated *SMC1* and *SMC3*, which are members of the SMC (Structural Maintenance of Chromosomes) family, a family of conserved chromosomal ATPases, as important for sister-chromatid cohesion in their screens. Like *mcd1/scc1*, *smc1* and *smc3* mutants separated their sister chromatids in the absence of the anaphase-promoting complex (see below). Interestingly, Guacci et al. (1997) showed that Mcd1p/Scc1p physically interacts with SMC1 by immunoprecipitation experiments. Thus, this complex of proteins, consisting of Mcd1p/Scc1p, SMC1p, and SMC3p, has now been termed the cohesin complex. A homologous complex has also been isolated from *Xenopus* and shown to be required for the establishment of sister-chromatid cohesion (Losada et al. 1998). In addition, ORFs encoding proteins related to Mcd1p/Scc1p are found in human, mouse, *C. elegans*, and *Drosophila* (Guacci et al. 1997; Michaelis et al. 1997). Therefore, the mechanism of establishing sister-chromatid cohesion appears to be evolutionarily conserved.

Another protein that was identified in *S. cerevisiae* and shown to be important for the establishment of sister-chromatid cohesion is Ctf7p/Eco1p, an essential chromatin-associated protein that is not a component of the

cohesin complex (Skibbens et al. 1999; Toth et al. 1999). Skibbens et al. (1999) and Toth et al. (1999) found that *ctf7/eco1* mutants exhibited a delay in metaphase with precociously separated sister chromatids. Furthermore, like the cohesin complex, Ctf7p/Eco1p is required during S phase to establish cohesion. However, unlike the cohesin complex, it is not needed for the maintenance of sister-chromatid cohesion. Interestingly, Ctf7p/Eco1p was found to interact genetically with components of the DNA replication machinery, PCNA and a RF-C-like protein, providing a compelling, direct link between sister-chromatid cohesion and DNA replication (Skibbens et al. 1999). An attractive model is that Ctf7p/Eco1p physically interacts with PCNA, and, hence, links sister chromatids together as they emerge from the replication forks. Ctf7p/Eco1p is not required for the association of the cohesin complex with the chromosomes during S phase. Therefore, it remains an intriguing question of how Ctf7p/Eco1p and the cohesin complex work together to establish sister-chromatid cohesion during S phase.

In *S. pombe*, the *MCD1/SCC1* homolog is the *Rad21* gene, which has been shown to be involved in DNA repair (Birkenbihl and Subramani 1992; Tatebayashi et al. 1998). Although it is not clear whether *Rad21* is required for sister-chromatid cohesion, the *Rec8* gene, another *MCD1/SCC1* homolog in *S. pombe*, has been demonstrated to be necessary for sister-chromatid cohesion specifically in meiosis (Molnar et al. 1995; Parisi et al. 1999). Thus, it appears that similar complexes of proteins are involved in the establishment of sister-chromatid cohesion in both mitosis and meiosis and that the activities of these proteins are specific to either type of cell division.

Finally, another protein that could function to establish sister-chromatid cohesion during meiosis is the *Drosophila* ORD protein. Mutations in the *ord* gene have been shown to cause high frequencies of chromosome

missegregation during meiosis I and meiosis II (Miyazaki and Orr-Weaver 1992; Bickel et al. 1996). Cytological analysis demonstrated that *ord* mutants exhibit precocious separation of sister chromatids during prometaphase I (Goldstein 1990; Miyazaki and Orr-Weaver 1992).

C. Chromosome condensation during mitosis

To facilitate chromosomal movement during mitosis, interphase chromosomes compact 5- to 10-fold in mammalian cells and 2-fold in yeast cells (for review, see Koshland and Strunnikov 1996). Like sister-chromatid cohesion, chromosome condensation must occur properly to ensure the accurate segregation of chromosomes. Recently, a family of conserved chromosomal ATPases, the SMC family, has been identified from bacteria to humans (Saka et al. 1994; Strunnikov et al. 1995; Hirano et al. 1997; Schmiesing et al. 1998; Britton et al. 1998; for review, see Hirano 1999); two of the members, SMC2 and SMC4, are integral components of the condensation machinery.

Mutations in the *cut3* (SMC4-type) and *cut14* (SMC2-type) genes in *S. pombe* lead to a reduction in chromosome compaction during mitosis and phenotypes indicating defects in chromosome segregation (Saka et al. 1994). In *S. cerevisiae*, the *smc2* mutant displays a similar phenotype (Strunnikov et al. 1995). Components of the condensation machinery, a 13S protein complex termed condensin, have also been identified in *Xenopus* (Hirano et al. 1997) and demonstrated to be required for both establishing and maintaining the condensation state of mitotic chromosomes (Hirano and Mitchison 1994; Hirano et al. 1997). Two of these proteins, XCAP-C (SMC4-type) and XCAP-E (SMC2-type), are members of the SMC family, and one, XCAP-H, is a homolog

of the *Drosophila* BARREN protein which when mutated leads to chromosome segregation defects (Bhat et al. 1996).

The BARREN protein has been shown to interact with topoisomerase II and modulate its activity in vitro (Bhat et al. 1996). Studies with mutants (Uemura et al. 1987) and inhibitors of topoisomerase II revealed that topoisomerase II is required for the establishment but not the maintenance of mitotic chromosome condensation (reviewed by Koshland and Strunnikov 1996). It is likely that while the condensin complex compacts the chromatin, the activity of topoisomerase II is needed to keep the chromosomes untangled.

D. Maintenance of sister-chromatid cohesion

The physical associations between sister chromatids established during or immediately after DNA replication must be maintained until anaphase. During metaphase, sister kinetochores are attached to microtubules emanating from opposite spindle poles, and they experience forces exerted by the microtubules pulling them towards opposite spindle poles. If the connections between sister kinetochores and chromatid arms are not strong enough to withstand the poleward pulling forces, sister chromatids will be pulled apart before the onset of anaphase.

In addition to establishing sister-chromatid cohesion during S phase, the *S. cerevisiae* cohesin complex, containing Mcd1p/Scc1p [it is XRAD21 in *Xenopus* (Losada et al. 1998)], SMC1p, and SMC3p, is also required for maintaining cohesion during mitosis. Temperature-sensitive *mcd1/scc1* mutants, when arrested in metaphase by nocodazole at the permissive temperature and then shifted to the nonpermissive temperature, exhibited precocious separation of sister chromatids as detected by FISH (Guacci et al.

1997; Michaelis et al. 1997). This observation indicates that after establishing cohesion during S phase, the cohesin must remain active during mitosis to keep sister chromatids associated. Unlike in *S. cerevisiae*, the cohesin complex in *Xenopus* dissociates from the chromosomes at the onset of mitosis, before the time of sister-chromatid separation (Losada et al. 1998). It is thought that because metazoans have a much higher degree of chromosome condensation than *S. cerevisiae* (Koshland and Strunnikov 1996), the cohesin complex in metazoans dissociates early in order to reduce steric hindrance and allow the condensin complex to localize onto the chromosomes and carry out condensation (Losada et al. 1998). However, the condensin complex does not appear to have any cohesion activity. Therefore, in metazoans some other factor(s) must localize to chromosomes as the cohesin dissociates from the chromosomes at the onset of mitosis to maintain cohesion until the onset of anaphase.

Another protein that is required for the maintenance of sister-chromatid cohesion is the *Drosophila* MEI-S332 protein (see below). Mutants in *mei-S332* fail to maintain sister-chromatid cohesion in late anaphase I and meiosis II (Kerrebrock et al. 1992). It has been demonstrated that in meiosis MEI-S332 first assembles onto the centromeres during prometaphase I and remains there until anaphase II when sister chromatids separate (Kerrebrock et al. 1995; Moore et al. 1998; Tang et al. 1998). Similarly, in mitosis, MEI-S332 does not localize onto the chromosomes until prometaphase and disappears from the chromosomes in anaphase (Moore et al. 1998; Tang et al. 1998). Consistent with it playing a role in maintaining cohesion, MEI-S332 localization to chromosomes is independent of intact spindle (Tang et al. 1998). Because of the premature dissociation of the cohesin complex from the chromosomes in *Xenopus* and the role and localization pattern of the MEI-S332

protein in *Drosophila*, an attractive model is that a homolog of MEI-S332 exists in metazoans. It functions to maintain cohesion specifically at the centromeres at the time when microtubules are exerting poleward-pulling forces on the kinetochores. However, as yet no homolog of the *Drosophila* MEI-S332 protein has been identified in *Xenopus* or other organisms.

E. Regulation of sister-chromatid cohesion

While microtubule attachment to kinetochores during prometaphase is a stochastic event and chromosomes congress to the metaphase plate at different times, sister chromatids separate synchronously at the onset of anaphase. Thus, it appears that a control mechanism exists in the cell to ensure that anaphase is initiated only when all kinetochores have achieved stable bipolar attachment to microtubules, and once anaphase is triggered, the physical connections between every pair of sister chromatids are somehow dissolved.

1. Spindle assembly checkpoint

As mentioned briefly above, the spindle assembly checkpoint functions during mitosis and meiosis as a surveillance system to ensure that before anaphase is initiated kinetochores are bound to microtubules nucleating from opposite spindle poles. This control mechanism detects the presence of unattached kinetochores (Rieder et al. 1994), impaired kinetochore proteins and centromeric DNA (Wang and Burke 1995; Pangilinan and Spencer 1996), and spindle depolymerization (Li and Murray 1991; Hoyt et al. 1991; for review, see Rudner and Murray 1996). All of these defects seem to affect the binding of kinetochores to microtubules. Studies in vertebrate somatic cells

and insect spermatocytes provided compelling evidence that the spindle assembly checkpoint monitors the integrity of the kinetochore-microtubule interaction by sensing a signal produced by unattached kinetochores, that inhibits the onset of anaphase, or in other words, activates the spindle assembly checkpoint (Rieder et al. 1994 ; Rieder et al. 1995; Zhang and Nicklas, 1996). Using the 3F3/2 antibodies, Gorbsky and Ricketts (1993) demonstrated that this inhibitory signal from the unattached kinetochores involves phosphorylation of unidentified proteins localized specifically on the kinetochores.

A major breakthrough in the field of spindle assembly checkpoint was the discovery of the protein components of the checkpoint machinery, the BUB, MAD, and Msp1 proteins (for review, see Rudner and Murray 1996). The BUB and MAD genes were originally identified in *S. cerevisiae* because mutants in these genes exhibit hypersensitivity to microtubule depolymerizing drugs (Li and Murray 1991; Hoyt et al. 1990) and fail to induce metaphase arrest in response to the loss of microtubules. In yeast, the checkpoint genes are not essential for normal mitosis but become necessary only when kinetochore-spindle interactions are perturbed. Subsequently, BUB1, BUB3, MAD1, and MAD2 proteins were also identified in worms, flies, frogs, mice, and humans (Basu et al. 1998; Chen et al. 1996; Li and Benezra 1996; Taylor and McKeon 1997; Basu et al. 1999, submitted). The large chromosome size of some of these systems allows the determination of the cellular localization of the checkpoint components. The *Xenopus* MAD1 and MAD2 (XMAD1 and XMAD2), human MAD2 (hsMAD2), and mouse BUB1 (mBUB1) proteins are all found on the kinetochores in prophase, prometaphase, and nocodazole-treated cells but not in metaphase or anaphase (Chen et al. 1996; Li and Benezra 1996; Taylor and McKeon 1997; Chen et al. 1998), suggesting that

they localize to kinetochores that have not attached to microtubules. The localization pattern of MAD1, MAD2 and BUB1 is analogous to the 3F3/2 antibody staining. However, it remains to be determined whether any of these proteins is a 3F3/2 antigen, although no differentially modified forms have been detected for MAD2 on Western blots (Chen et al. 1996).

The function of MAD1, MAD2 and BUB1 protein have been investigated in metazoans by mutant analysis, antibody microinjection experiments, and use of dominant-negative protein fragments (Basu et al. 1999, submitted; Chen et al. 1996; Gorbsky et al. 1998; Li and Benezra 1996; Taylor and McKeon 1997; Chen et al. 1998). The results from these experiments suggest that unlike in yeast, the spindle assembly checkpoint in metazoans not only acts to arrest cell cycle in response to the lack of microtubules but also plays an essential role in a timing mechanism for normal mitosis. Microinjection of MAD2 antibodies into mammalian cells during prophase or prometaphase induce premature onset of anaphase, which occurs even before all the chromosomes have arrived at the metaphase plate (Gorbsky et al. 1998). Similarly, Taylor and McKeon (1997) demonstrated that a dominant-negative domain of mBUB1 accelerates the progression through mitosis in the absence of microtubule inhibitors.

Using taxol to substantially reduce the tension on the sister kinetochores, Waters et al. (1998) were able to show that spindle assembly checkpoint senses kinetochore attachments to microtubules rather than tension on the kinetochores in mitotic cells. XMAD2 was detected only on kinetochores that have dissociated from microtubules even though tension was nearly absent on all the kinetochores. Furthermore, in *S. cerevisiae cdc6* and *cdc7* mutants, DNA replication does not occur and thus, no cohesion between sister chromatids and consequently no tension can be established. However,

these mutants still progress through mitosis (Toyn et al. 1995; Piatti et al. 1995). Assuming that in these mutants kinetochore-microtubule interactions are normal, the lack of metaphase arrest further indicates that spindle assembly checkpoint in mitosis monitors the integrity of kinetochore-microtubule interactions, rather than tension on the kinetochores.

Unlike in mitosis, spindle assembly checkpoint in meiosis I appears to respond to tension on the bivalents. Praying mantid spermatocytes containing a mono-oriented X chromosome are delayed in metaphase I, but this delay is lifted if tension is placed on the mono-oriented kinetochore by pulling the chromosome with a microneedle (Li and Nicklas 1995). Furthermore, in the absence of tension, kinetochores of grasshopper spermatocytes are labeled brightly with the 3F3/2 antibodies, indicating that certain kinetochore proteins are phosphorylated (Nicklas et al. 1995). When tension is generated either by natural microtubule forces or by microneedle manipulation, 3F3/2 signal diminishes from the kinetochores, indicating that tension leads to dephosphorylation of kinetochore proteins recognized by the 3F3/2 antibodies. When chromosomes are manually detached from the spindle with a microneedle, tension on the kinetochores is abolished, kinetochore proteins recognized by the 3F3/2 antibodies become rephosphorylated, and anaphase I is delayed. Nicklas et al. (1995) proposed that tension mediated by kinetochore dephosphorylation is the "all clear" signal to the spindle assembly checkpoint in meiotic cells.

It remains to be addressed whether during meiosis II, spindle assembly checkpoint also monitors tension. As mentioned above, the persistent physical association between sister centromeres during meiosis II provides the force that is necessary to create tension on the sister kinetochores. In *Drosophila mei-S332* mutant spermatocytes, which lose centromeric cohesion

precociously in late anaphase I, no tension would be generated between the sister kinetochores during meiosis II (Goldstein 1980; Kerrebrock et al. 1992). If the spindle assembly checkpoint monitors tension in meiosis II, *mei-S332* mutant spermatocytes would be expected to arrest in metaphase II due to a lack of tension. Components of the spindle assembly checkpoint have been isolated in *Drosophila* and shown to localize to the kinetochores during meiosis in spermatocytes (Basu et al. 1998; Basu et al. 1999, submitted). However, *mei-S332* mutants are able to complete meiosis, as gametes are produced, albeit with abnormal number of chromosomes (Kerrebrock et al. 1992). Perhaps, like in mitosis, spindle assembly checkpoint in meiosis II monitors kinetochore attachment to microtubules rather than tension on the kinetochores. Alternatively, MEI-S332 could play a role in spindle assembly checkpoint (See Afterword in this thesis).

The recent discovery in *S. cerevisiae* that Mad1p, Mad2p, and Mad3p interact with Cdc20p, a protein required for mitotic exit, finally linked the spindle assembly checkpoint to the master regulator of the mitotic cell cycle (Hwang et al. 1998; see below). Similar interactions were observed in *S. pombe* and humans (Kim et al. 1998; Fang et al. 1998; Kallio et al. 1998; Wassmann and Benezra 1998). Furthermore, hsMAD2 was found to be in a complex not only with p55CDC [a human homolog of CDC20 (Weinstein et al. 1994); it is Fizzy or Fizzy-related in *Drosophila* (Dawson et al. 1993; Sigrist and Lehner 1997)] but also with some components of the anaphase-promoting complex (APC)/cyclosome (Li et al. 1997; Fang et al. 1998; Kallio et al. 1998; Wassmann and Benezra 1998). Interestingly, the addition of hsMAD2 in *Xenopus* extracts leads to the inhibition of ubiquitin conjugation to mitotic cyclins and cyclin proteolysis in vitro (Li et al. 1997; Fang et al. 1998). Thus, Li et al. (1997) and Fang et al. (1998) proposed that hsMAD2 is involved in the

inactivation of APC by forming a hsMAD2-p55CDC-APC complex. Consistent with this, Kallio et al. (1998) found that p55CDC is required for hsMAD2 binding to CDC27 and CDC26, which are components of the APC/cyclosome. Therefore, it is possible that CDC20 mediates the association of MAD2 with the APC/cyclosome and that this association inhibits the APC activity. Subsequently, this blocks the ubiquitin-dependent proteolysis of anaphase inhibitors and mitotic cyclins and halts the cell cycle at metaphase with associated sister chromatids and high levels of cyclin-dependent kinase activity.

2. Separation at the metaphase/anaphase transition

Once the spindle assembly checkpoint senses that all sister kinetochores have formed bipolar attachment to the spindle, anaphase is triggered with the separation of sister chromatids. Evidence from *S. cerevisiae* and *Xenopus* has indicated that ubiquitin-dependent proteolysis of proteins other than mitotic cyclins is necessary for mitotic sister-chromatid separation (Holloway et al. 1993; Surana et al. 1993). Using a nondegradable mitotic cyclin, Holloway et al. (1993) were able to prevent the cell from exiting mitosis. However, sister-chromatid separation still occurred when cyclin was not degraded. Only when methylated ubiquitin or peptide containing the destruction box was used, was separation of sister chromatids blocked. Surana et al. (1993) also found that sister-chromatid separation and mitotic cyclin degradation occur independently of each other.

The ubiquitin-dependent proteolysis involves three enzymatic activities, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase; for review, see Ciechanover 1994). During the cell cycle, E1 and E2 are constitutively active, but E3 has been shown to be active

specifically during late mitosis and G1 phase (King et al. 1995; Irniger et al. 1995). This mitosis-specific ubiquitin ligase has been termed the anaphase-promoting complex (APC) or the cyclosome (King et al. 1995; Sudakin et al. 1995). It consists of several protein subunits (e.g., CDC16, CDC23, and CDC27) and is necessary for the ubiquitination and hence subsequent proteolysis of both mitotic cyclins and inhibitors of anaphase (King et al. 1995; Irniger et al. 1995; Zachariae et al. 1996; Funabiki et al. 1996; Cohen-Fix et al. 1996).

It has been demonstrated that besides cyclins, APC also targets the degradation of Pds1p in *S. cerevisiae* and Cut2p in *S. pombe* (Cohen-Fix et al. 1996; Funabiki et al. 1996); Pds1p can be directly ubiquitinated by immuno-purified *Xenopus* APC in an in vitro reconstituted system (Cohen-Fix et al. 1996). Studies in yeast have shown that Pds1p and Cut2p are anaphase inhibitors (Yamamoto et al. 1996; Cohen-Fix et al. 1996; Funabiki et al. 1996). The proteins are rapidly degraded before the initiation of anaphase in an APC-dependent manner. Nondegradable derivatives of Pds1p and Cut2p block the separation of sister chromatids. Consistent with this, *pds1* mutants exhibit precocious separation of sister chromatids in the presence of microtubule inhibitors (Yamamoto et al. 1996). It is, however, interesting that *cut2-deleted* mutants have similar phenotype as cells containing nondegradable Cut2p.

How does the degradation of Pds1p and Cut2p lead to the separation of sister chromatids? Ciosk et al. (1998) recently showed that the destruction of Pds1p by the APC-dependent pathway triggers the dissociation of Mcd1p/Scc1p, the cohesin, from the chromatin via the action of the Esp1 protein. They proposed that before the onset of anaphase, Esp1p is physically associated with and inhibited by Pds1p. When APC-dependent proteolysis is activated, Pds1p is degraded, and Esp1p is free to induce the dissociation of the

cohesin complex from the chromatin, leading to the separation of sister chromatids.

In addition to ubiquitin-dependent proteolysis, a growing evidence suggests that phosphorylation/ dephosphorylation is involved with the separation of sister chromatids. *S. cerevisiae* cells defective in the *cdc55* gene, which encodes a protein homologous to the regulatory subunit (PR55) of the rabbit skeletal muscle protein phosphatase 2A (PP2A), display precocious separation of sister chromatids in the presence of nocodazole (Healy et al. 1991; Minshull et al. 1996). Similarly, in *Drosophila aar* (also known as *twins*) mutants, which are defective in the *Drosophila* PR55 homolog, abnormal anaphase figures are observed (Uemura et al. 1993; Gomes et al. 1993; Mayer-Jaekel et al. 1993). Quantitative analysis of metaphase and anaphase figures showed that *aar* homozygous mutants exhibited a reduction in the ratio of metaphase to anaphase cells, indicating acceleration through the metaphase/ anaphase transition (Gomes et al. 1993). However, the separation of sister chromatids, as shown by FISH using probes specific to the centromere and telomeres, appears to be normal in the *aar* homozygotes (Mayer-Jaekel et al. 1993). Therefore, it is possible that in *aar* homozygotes, the apparent increase in the frequency of anaphase figures relative to the frequency of metaphase figures is a consequence of precocious sister-chromatid separation. Biochemical studies in vitro showed that addition of the PR55 subunit reduces the activity of PP2A, and the extent of reduction is dependent on the substrate used (for review, see Mayer-Jaekel and Hemmings 1994). Thus, in *cdc55* and *aar/twins* mutants PP2A is expected to be more active, and this would implicate PP2A activity in the separation of sister chromatids. Consistent with this hypothesis, overcondensed chromatin and a block in mitosis between prophase and the initiation of anaphase are observed in embryos homozygous

for the P-element that is inserted 251 bp upstream of the PP2A gene and substantially reduces the levels of PP2A mRNA and activities (Snaith et al. 1996).

In addition to PP2A, protein phosphatase 1 (PP1) has also been proposed to play a role in the regulation of sister-chromatid separation (Ghosh and Paweletz 1992). Treating mitotic HeLa cells with different concentrations of okadaic acid to inhibit PP2A activity alone or both PP2A and PP1 activities, Ghosh and Paweletz (1992) found that at a concentration that is assumed to block PP2A, okadaic acid has no visible effect on mitotic progression. Only when higher concentrations of okadaic acid are used, presumably inhibiting both PP2A and PP1, are metaphase arrest and formation of diplochromosomes observed, indicating a block in sister-chromatid separation. In *S. pombe*, there are two PP1 catalytic subunits and they are encoded by the *dis2*$^+$ and *sds21*$^+$ genes (Ohkura et al. 1988; Ohkura et al. 1989). Deletion of both of these genes results in metaphase arrest as indicated by a short metaphase spindle and condensed, unseparated chromosomes (Ishii et al. 1996). Similarly, mutations in one of the four genes encoding PP1 isoenzymes in *Drosophila* cause overcondensed chromosomes and failure in anaphase spindle elongation (Axton et al. 1990). A block in mitosis with short metaphase spindles and condensed chromosomes is also observed in *Aspergillus nidulans BimG* (PP1) mutants (Doonan and Morris 1989). Furthermore, microinjection of anti-PP1 antibodies into late G2 mammalian cells induces metaphase arrest (Fernandez et al. 1992). Thus, the phenotypes associated with disrupting PP1 activities are consistent with PP1 playing a role in sister-chromatid separation in multiple organisms.

Recently, studies in *S. cerevisiae* showed that conditional alleles of the PP1 catalytic subunit Glc7p also arrest in metaphase with short metaphase

spindles, and interestingly, the arrest requires the spindle assembly checkpoint (Bloecher and Tatchell 1999; Sassoon et al. 1999). In vitro microtubule-binding assays demonstrated that *glc7* mutant cells have reduced kinetochore-microtubule binding activity. Furthermore, a component of the kinetochore protein complex is hyperphosphorylated in *glc7* mutant extracts (Sassoon et al. 1999). Although these results suggest that PP1 is involved in regulating the attachment of kinetochores to microtubules, they do not eliminate the possibility that PP1 also plays a role in regulating sister-chromatid separation.

The studies with *Drosophila pimples* and *three rows* genes suggest that additional pathways are involved in the separation of sister chromatids. In *pimples* and *three rows* mutants, sister centromeres fail to dissociate during mitosis, resulting in the production of polyploid nuclei (Stratmann and Lehner 1996). This suggests that the protein products of *pimples* and *three rows* genes are required for the separation of sister chromatids specifically at the centromere. Interestingly, the PIMPLES protein is rapidly degraded at the onset of anaphase.

F. MEI-S332 and *Drosophila melanogaster* as a model system

As mentioned above, the *Drosophila mei-S332* gene was identified as being essential for sister-chromatid cohesion during meiosis (Davis 1971; Goldstein 1980; Kerrebrock et al. 1992). Cytological analysis of *mei-S332* mutant spermatocytes indicated precocious separation of sister chromatids in late anaphase I (Goldstein 1980; Kerrebrock et al. 1992) and the lack of metaphase plates in meiosis II. In anaphase II, lagging chromatids were observed. Genetic analysis showed that mutations in *mei-S332* cause high frequencies of chromosome loss and missegregation in meiosis II in both

females and males. (Kerrebrock et al. 1992). In addition to being essential for meiotic sister-chromatid cohesion, the *mei-S332* gene has also been found to play a role in mitotic sister-chromatid cohesion. *mei-S332* mutants display a weakening of the centromeric cohesion in mitosis (Chapter Four of this thesis).

The *mei-S332* gene has been cloned, and it encodes a novel protein consisting of a predicted coiled-coil domain at the amino terminus, an acidic region in the middle, and a basic region at the carboxyl terminus of the protein (Kerrebrock et al. 1995). Interestingly, there are two putative PEST sequences and thirty putative phosphorylation sites in the protein, raising the possibility that proteolysis and/or phosphorylation are involved in the regulation of this protein (Kerrebrock et al. 1995). PEST sequences are common in proteins that have high turnover rates (Rogers et al. 1986; Rechsteiner 1988). It is not surprising that because MEI-S332 is an essential player in sister-chromatid cohesion, its protein levels and/or activity would be tightly regulated during the cell cycle. Consistent with this, MEI-S332 protein has a very interesting pattern of localization. During meiosis, the protein localizes to centromeres during prometaphase I, remains on the centromeres from metaphase I through metaphase II, and dissociates from the centromeres at anaphase II (Kerrebrock et al. 1995; Moore et al. 1998; Tang et al. 1998). Similarly, in mitosis, the protein does not localize to the chromosomes until prometaphase and dissociates from them in anaphase (Moore et al. 1998; Tang et al. 1998).

Finally, because meiosis is different between the two sexes in *Drosophila*, it is of great interest that there exist sex-predominant alleles of *mei-S332* (Kerrebrock et al. 1992). Mutations in the carboxy-terminal basic region cause a higher frequency of chromosome loss and missegregation in female meiosis than in male meiosis, whereas mutations in the amino-terminal

coiled-coil domain lead to a more severe segregation phenotype in males than in females. Understanding how these mutations result in sex-predominant phenotypes will help elucidating the function of MEI-S332 in both sexes and the differential mechanisms of chromosome segregation in *Drosophila* male and female meiosis.

Drosophila melanogaster offers a great system to investigate the mechanism and regulation of sister-chromatid cohesion. Both mitosis and meiosis can be studied easily because while embryos and larval brains and imaginal discs provide enriched pools of mitotic cells, ovaries and testes contain large, cytologically well-characterized meiotic cells. Furthermore, mutants that cause defects in mitotic and/or meiotic chromosome segregation have been isolated and characterized. Sister-chromatid cohesion is apparently regulated differently in mitosis and meiosis, as cohesion is released in one step in mitosis but two steps in meiosis. Thus, understanding the differences between mitotic and meiotic sister-chromatid cohesion will shed light on the mechanism of sister-chromatid cohesion as a whole. Finally, another advantage of using *Drosophila* to study chromosome segregation cannot be ignored, and that is the large size of its chromosomes. Easily visualized under the microscope, *Drosophila* chromosomes greatly facilitate the determination of cellular localization of proteins involved in chromosome segregation and sister-chromatid cohesion.

G. Summary

Sister-chromatid cohesion plays a key role in ensuring the faithful segregation of chromosomes; defects in sister-chromatid cohesion can lead to aneuploidy, which is a contributive factor to tumorigenesis, miscarriages, and

congenital disorders such Down syndrome. If cohesion is not established or maintained properly, sister chromatids can randomly capture microtubule nucleating from either spindle pole (Figure 1-4A). If both sister chromatids happen to capture microtubules emanating from the same spindle pole, they will be pulled towards the same pole and end up in the same daughter cell. This results in the formation of two aneuploid daughter cells, one having one extra chromosome and the other lacking one chromosome. It is not only important to establish and maintain cohesion properly, it is also crucial to regulate cohesion precisely such that it is released at the right time. If cohesion is not released properly, sister chromatids will be sheared by the pulling forces of microtubules or will be dragged towards the same spindle poles. The consequence is again aneuploidy (Figure 1-4B). Because of its importance, the cells have evolved multiple mechanisms to ensure that the cohesion between sister chromatids is established, maintained, and regulated properly. Many components involved in these mechanisms have been identified, and it is certain that more will be isolated. We are only beginning to understand how these components work together to ensure proper cohesion and accurate separation of sister chromatids.

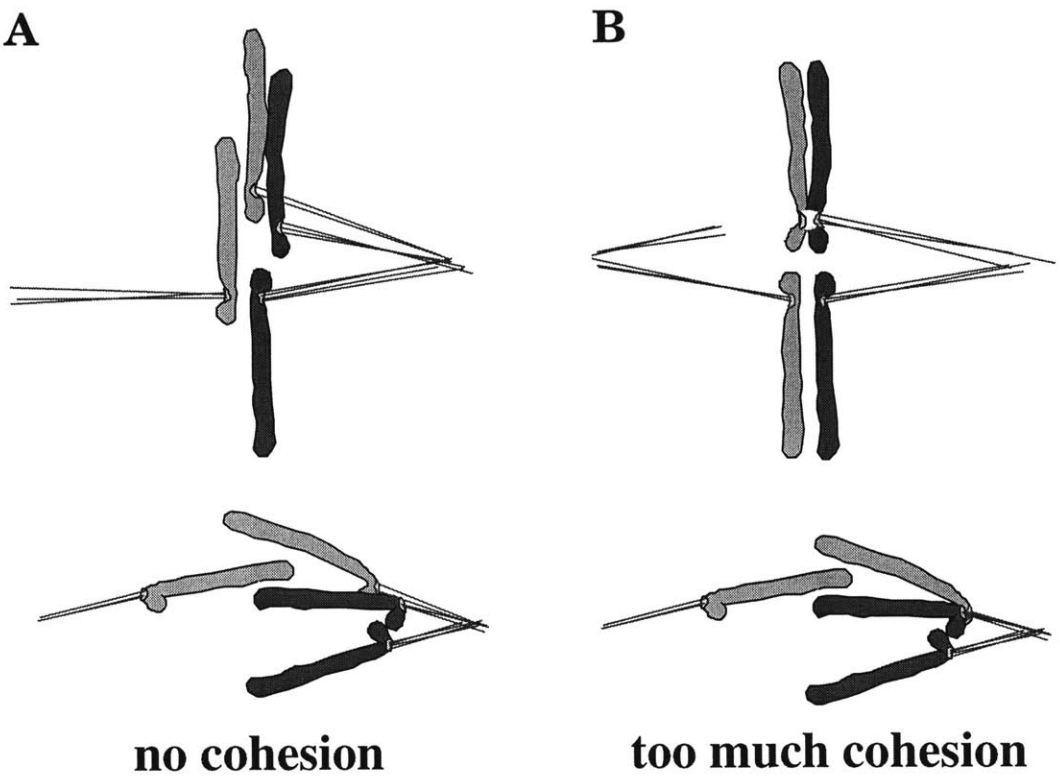

Figure 1-4. Improper cohesion leads to aneuploidy.

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Chapter Two

Maintenance of sister-chromatid cohesion at the centromere by the *Drosophila* MEI-S332 protein

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*T.T.-L.Tang expressed and purified the GST-MEI-S332 fusion protein, generated and characterized the guinea pig anti-MEI-S332 antiserum, performed the colchicine experiments and all of the immunofluorescence experiments in embryos, oocytes, and spermatocytes, determined the amino acid changes of the two new *mei-S332* alleles by PCR and sequencing, prepared protein extracts, ran the Western blots, characterized the two new alleles by nondisjunction tests, carried out the intragenic complementation experiments, and determined that MEI-S332 is in a multimeric complex by immunoprecipitation and glycerol gradients.

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Abstract

Sister-chromatid cohesion is essential for the faithful segregation of chromosomes during cell division. Recently biochemical analysis with *Xenopus* extracts suggests that cohesion is established during S phase by a cohesion complex but that other proteins must maintain it in mitosis. The *Drosophila melanogaster* MEI-S332 protein is present on centromeres in mitosis and meiosis and is essential for cohesion at the centromeres in meiosis II. Here, we analyze the timing of MEI-S332 assembly onto centromeres and the functional domains of the MEI-S332 protein. We find that MEI-S332 is first detectable on chromosomes during prometaphase, and this localization is independent of microtubules. MEI-S332 contains two separable functional domains, as mutations within these domains show intragenic complementation. The carboxy-terminal basic region is required for chromosomal localization. The amino-terminal coiled-coil domain may facilitate protein-protein interactions between MEI-S332 and male meiotic proteins. MEI-S332 interacts with itself in the yeast two-hybrid assay and in immunoprecipitates from *Drosophila* oocyte and embryo extracts. Thus it appears that MEI-S332 assembles into a multimeric protein complex that localizes to centromeric regions during prometaphase and is required for the maintenance of sister-chromatid cohesion until anaphase, rather than its establishment in S phase.

Introduction

Accurate segregation of the genetic material is one of the most fundamental and essential cellular processes. Errors in chromosome segregation during mitosis or meiosis result in aneuploidy, which is associated with tumorigenesis, miscarriages, and congenital disorders such as Down syndrome. Several events must be linked and coordinated to occur in a timely manner to ensure accurate segregation of chromosomes. First, cohesion between duplicated sister chromatids must be established during or immediately after DNA replication. Second, the dispersed interphase chromosomes must condense to facilitate chromosomal movement during segregation. Third, during mitotic and meiotic spindle formation and chromosome congression, sister-chromatid cohesion must be maintained stably to resist the poleward pulling forces as kinetochores engage in microtubule interaction.

To ensure proper chromosome segregation, cohesion must be established between sister chromatids, and the simplest way to ensure the attachments are indeed between sisters would be to make these connections during or immediately after DNA replication. Results from FISH experiments in *Saccharomyces cerevisiae* indicate that sister-chromatid cohesion is established during S phase (Guacci et al. 1994). Recent identification of *S. cerevisiae* proteins necessary for sister-chromatid cohesion (Guacci et al. 1997; Michaelis et al. 1997) provides additional evidence that sister-chromatid cohesion is established during S phase. Guacci et al. (1997) demonstrated that one of these proteins, Mcd1p/Scc1p, physically associates with a member of the SMC family, SMC1, and that its levels are cell cycle regulated, peaking in S phase, declining by late S phase, and remaining constant through telophase. Michaelis et al. (1997) found that Mcd1p/Scc1p associates with chromatin in

late G1/early S phase and dissociates from it at the onset of anaphase. Recently it was shown that Scc1p must associate with sister chromatids in S phase to ensure cohesion. Although Scc1p can assemble onto chromosomes in G2 phase, chromosome nondisjunction nevertheless occurs if cells undergo S phase in the absence of Scc1p (Uhlmann and Nasmyth 1998).

Upon entry into mitosis, in prophase, chromosomes compact 5- to 10-fold in mammalian cells or 2-fold in yeast cells (for review, see Koshland and Strunnikov, 1996). The isolation, in multiple species, of mutants defective in mitotic chromosome condensation demonstrated that condensation is a necessary prerequisite for proper chromosome segregation. Mutations in the *S. cerevisiae smc* genes and in some of the *Schizosaccharomyces pombe cut* genes lead to a reduction in chromosome compaction during mitosis and phenotypes indicating defects in chromosome segregation (Saka et al. 1994; Strunnikov et al. 1995). Components of the condensation machinery, a 13S protein complex termed condensin, have also been identified in *Xenopus* (Hirano et al. 1997) and demonstrated to be required for both establishing and maintaining the condensation state of mitotic chromosomes (Hirano and Mitchison 1994; Hirano et al. 1997).

During and after condensation, the sister-chromatid cohesion previously established must be maintained until the metaphase/anaphase transition. The physical association between sister chromatids appears to counteract the poleward forces, creating tension that keeps the sister chromatids on the metaphase plate until their separation at the onset of anaphase (for review, see Miyazaki and Orr-Weaver 1994). Sister-chromatid cohesion also likely plays a role in sister kinetochore orientation. By restricting the movement of sister chromatids via physical association, sister-chromatid cohesion forces the sister kinetochores to face opposite spindle poles and establish the bipolar

attachment to the spindle microtubules during mitosis and meiosis II (for review, see Bickel and Orr-Weaver, 1996).

Recent work in *Xenopus* identified a cohesin complex consisting of homologs of the yeast SMC1 and SMC3 proteins as well as a homolog of *S. cerevisiae* Mcd1p/Scc1p (Rad21p in *Schizosaccharomyces pombe*; Losada et al. 1998). In contrast to yeast, in *Xenopus* extracts, the cohesin complex does not persist through mitosis, rather it dissociates at the onset of mitosis and is replaced by the condensin complex (Losada et al. 1998). Although the condensin complex could, in theory, maintain cohesion until anaphase, mutations in the *S. pombe* or *S. cerevisiae* components of the condensin complex do not exhibit defects in sister-chromatid cohesion, making it possible that condensins are not sufficient for cohesion (Saka et al. 1994; Strunnikov et al. 1995). Therefore, a function to maintain cohesion during mitosis after the cohesin complex has dissociated from the chromosomes would be particularly important at the sister centromeres which are subjected to microtubule pulling forces.

The *Drosophila* MEI-S332 protein is required for cohesion between the centromeres of the sister chromatids. Mutations in the *mei-S332* gene lead to precocious separation of sister chromatids in late anaphase I, resulting in chromosome loss and missegregation in meiosis II (Kerrebrock et al. 1992). They also seem to cause a weakening of the centromeric cohesion in mitotic cells, indicating a role for MEI-S332 during mitosis (LeBlanc, H., Tang, T. T.-L., Wu, J., and Orr-Weaver, T. L., in prep.). Although *mei-S332* mutants are defective in sister-chromatid cohesion, they are not affected in chromosome condensation (Kerrebrock et al. 1992). Furthermore, the MEI-S332 protein localizes to the centromeric regions during meiosis and mitosis, while it dissociates from the chromosomes at the onset of anaphase when sister

chromatids separate from one another (Kerrebrock et al. 1995; Moore et al. 1998).

The mutant phenotypes and the cellular localization of the MEI-S332 protein make it a strong candidate to maintain sister-chromatid cohesion at the centromere. Although previous mutant analysis in meiosis showed that MEI-S332 is essential at the centromeres, it did not address whether MEI-S332 is involved in establishing or maintaining cohesion. In addition, because MEI-S332 acts at the centromeres, an important step in understanding its mechanism of action is to determine the relationship between MEI-S332 localization, microtubule attachment, and spindle assembly. Thus, in this study we used cytological, genetic, and biochemical experiments to demonstrate that MEI-S332 functions to maintain rather than establish sister-chromatid cohesion at the centromeres. MEI-S332 assembles onto condensed chromosomes during prometaphase independent of intact microtubules, and its chromosomal localization requires the carboxy-terminal basic region.

Results

MEI-S332 assembles onto condensed chromosomes during prometaphase

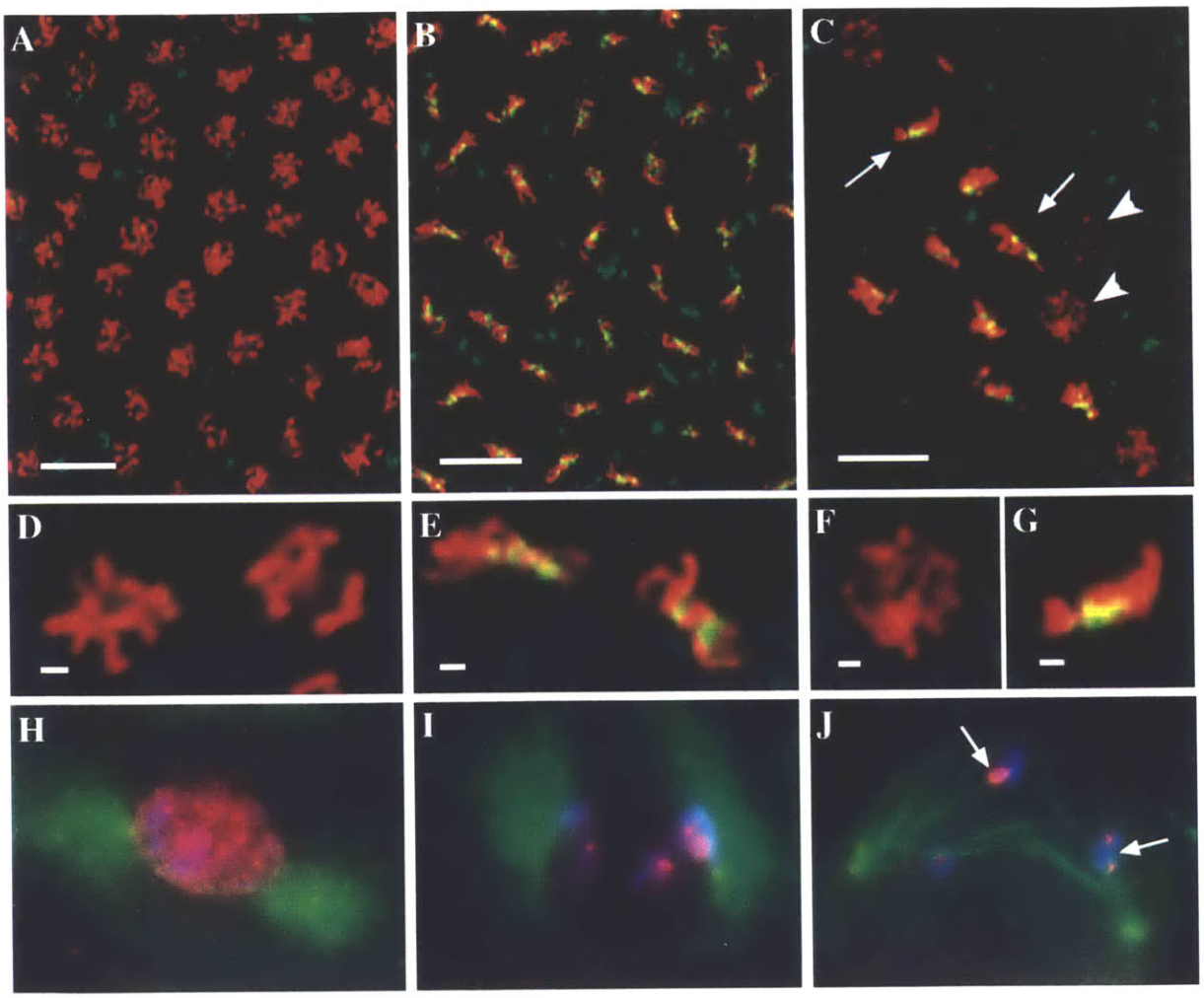
To determine whether MEI-S332 is involved in the establishment or maintenance of sister-chromatid cohesion, we investigated the onset of MEI-S332 assembly onto chromosomes by double-labeling syncytial blastoderm and postblastoderm embryos with anti-phosphorylated histone H3 (anti-phospho H3) and anti-full-length MEI-S332 antibodies (For a review of *Drosophila* embryogenesis, see Foe et al. 1993). With anti-phospho H3 antibodies, Hendzel et al. (1997) have shown that mitotic phosphorylation of histone H3 initiates in pericentromeric heterochromatin in late G2 interphase cells and spreads throughout the condensing chromosomes, completing just prior to the formation of prophase chromosomes. By comparing the timing of MEI-S332 chromosomal localization relative to histone H3 phosphorylation and hence, to chromosome condensation, we found that MEI-S332 assembled onto the chromosomes during prometaphase (Figure 2-1A-G).

MEI-S332 became visible on the chromosomes only when they appeared to be congressing, and its signal on the chromosomes was the most obvious at metaphase. Similar results were obtained with different MEI-S332 antibodies that recognize only a carboxy-terminal 15-mer epitope (data not shown and Moore et al. 1998). While we can not exclude the possibility that MEI-S332 is present on chromosomes earlier than prometaphase but is not detectable by either the anti-full-length or the anti-peptide antibodies, it is unlikely. On the basis of DNA morphology and the phosphorylated histone H3 staining, the degree of chromosome compaction appears to be the same in prophase and prometaphase, and hence, if MEI-S332 were on the chromosomes during earlier cell-cycle stages, we should have detected it.

Figure 2-1. MEI-S332 assembles onto mitotic and meiotic chromosomes during prometaphase.

(*A-C*) Embryos doubled-labeled with anti-MEI-S332 (green) and anti-phospho H3 (red) antibodies. (Yellow) Areas of overlap. (*H-J*) Spermatocytes triple-labeled with DAPI (blue), anti-MEI-S332 (red) and anti-tubulin (green) antibodies. (*A, B*) Images from the same S-M syncytial blastoderm embryo. In a portion of the nuclei, MEI-S332 is not detected on the chromosomes stained with anti-phospho H3; these chromosomes do not appear to be congressing (*A*). In nuclei where MEI-S332 is observed on the chromosomes, the chromosomes seem to be congressing (*B*). (*C*) A portion of a mitotic domain in a S-G2-M postblastoderm embryo demonstrates that MEI-S332 is not observed on the chromosomes in some of the nuclei that have already initiated histone H3 phosphorylation (arrowheads). MEI-S332 is seen on chromosomes that appear to be congressing (arrows). (*D, E*) Enlargements of two nuclei from *A* and *B*, respectively. (*F, G*) Enlargements of two nuclei shown in C. (*H*) In late meiotic prophase I, tubulin staining reveals two centrosomes that have not yet migrated completely to opposite sides of the nucleus, and the chromosomes appear to be condensing. MEI-S332 seems to localize in a punctate fashion inside the nucleus (stage M1a; Cenci, et al. 1994). (*I*) In this prometaphase I spermatocyte (stage M1b; Cenci, et al. 1994), the chromosomes have condensed further, and the centrosomes have completed their migration to opposite sides of the nucleus. MEI-S332 is now detected on the chromosomes in distinct foci. (*J*) The nuclear-cytoplasmic demarcation has disappeared in this M2 (Cenci, et al. 1994) prometaphase I spermatocyte, allowing microtubule interactions with the kinetochores. MEI-S332 is observed in two foci on each bivalent, corresponding to two pairs of sister centromeres in each bivalent. Microtubule fibers emanating from one

centrosome can be clearly seen co-localizing with both MEI-S332 foci on a bivalent (arrows), indicating that bipolar attachment has not yet been established. (*A-C*) Bars, ~10μm; (*D-G*) Bars, ~1 μm.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It was also important to determine when MEI-S332 assembles onto the chromosomes in meiosis. Previous analysis of oocytes showed that MEI-S332 does not localize on the chromosomes in the prophase I karyosome (Moore et al. 1998). Instead, it appeared to assemble at a time when the nuclear envelope breaks down and the spindle begins to form. However, cytological analysis with oocytes is limited in that individual chromosomes are not distinguishable in the chromosome mass until egg activation and the beginning of anaphase I movement. Therefore, we addressed this issue using spermatocytes where individual bivalents, pairs of homologous chromosomes, can be visualized. In addition, in this current study of MEI-S332 localization, we used antibodies that specifically recognize MEI-S332 (see Materials and Methods), which greatly enhanced the sensitivity of detection relative to the green fluorescence protein (GFP) used in the previous studies (Kerrebrock et al. 1995; Moore et al. 1998).

Triple-labeling of spermatocytes with anti-MEI-S332 and anti-tubulin antibodies and a DNA dye, DAPI, demonstrated that MEI-S332 exhibited nuclear localization at late meiotic prophase I (Figure 2-1H). At the onset of prometaphase I, it became detectable in a few foci on the condensing chromosomes (Figure 2-1I). By the time the nuclear/ cytoplasmic separation was no longer visible in prometaphase I, MEI-S332 was clearly seen to localize in two dots on each bivalent (Figure 2-1J).

These immunofluorescence results from embryos and spermatocytes showed that MEI-S332 is not detected on the chromosomes immediately after DNA replication when cohesion has been established and indicate that MEI-S332 is involved in the maintenance of sister-chromatid cohesion.

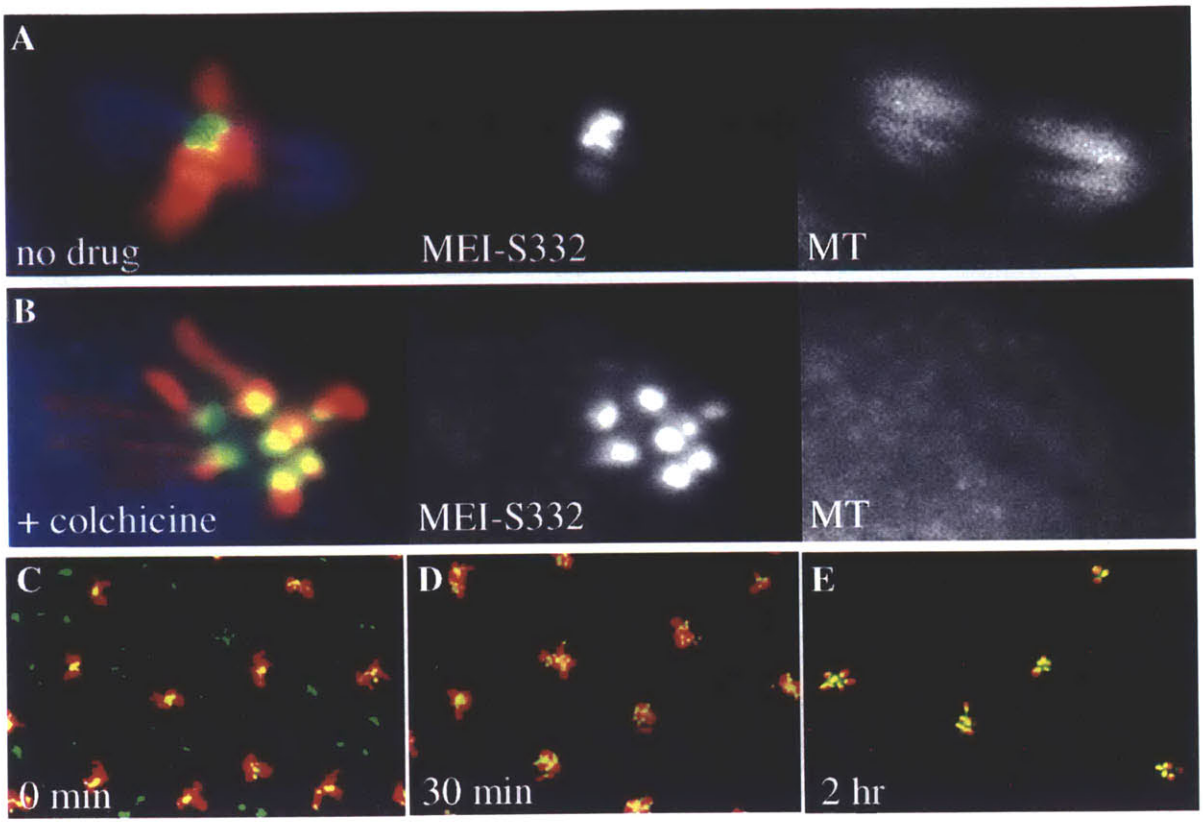
Spindle assembly is not required for MEI-S332 localization

In meiosis and mitosis, chromosomal localization of MEI-S332 correlated with the onset of spindle assembly and chromosome movement or congression, suggesting that MEI-S332 may not assemble onto the chromosomes until microtubule binding at the kinetochores. Additional cohesion proteins might be necessary when poleward forces that could separate the sister chromatids are exerted on the kinetochores. To examine the relationship between MEI-S332 and spindle forces, we tested whether the spindle was required for MEI-S332 localization.

First, we looked at whether the MEI-S332 molecules that had already assembled onto the centromeres would remain in the absence of intact microtubules. We treated embryos with a short incubation (30 minutes) of colchicine, a microtubule depolymerizing drug, followed by fixation and staining with anti-MEI-S332 and anti-phospho H3 antibodies. In both syncytial blastoderm S-M and postblastoderm S-G2-M embryos, MEI-S332 still localized on the chromosomes when the microtubules had been depolymerized (Figures 2-2A, B; and data not shown). Anti-tubulin staining confirmed that colchicine had destabilized the spindle in these embryos (Figure 2-2B). This result shows that microtubules are not required for maintaining MEI-S332 localization on the centromeres.

To test whether MEI-S332 needs microtubules to assemble onto the centromeres, we incubated early embryos longer with colchicine (2 hours). In the early embryos, the cell cycles are very rapid, between 8 and 18 minutes, so all nuclei would enter mitosis during a 2-hr colchicine treatment. Thus, we could determine whether microtubules were necessary for MEI-S332 assembly by seeing whether all nuclei in colchicine treated embryos had MEI-S332 localized on the chromosomes. Indeed, we still observed MEI-S332 on the

Figure 2-2. MEI-S332 localizes to chromosomes independent of microtubules. A collection of 2-hour wild-type embryos was incubated for 30 minutes without (*A*) or with (*B*) colchicine (100µg/ml), fixed, and stained with DAPI, anti-MEI-S332 and anti-tubulin antibodies. Representative nuclei from S-M syncytial blastoderm embryos are shown. Artificial colors are used: (red) DNA; (green) MEI-S332; and (blue) spindle. Under colchicine treatment, chromosomes are more spread out, MEI-S332 is seen as eight dots, corresponding to the 4 pairs of homologous chromosomes in *Drosophila*, and a metaphase plate is not visible. (*C-E*) Embryos untreated or treated with colchicine (100µg/ml) for 30 minutes or 2 hours, fixed, and stained with anti-MEI-S332 and anti-phospho H3 antibodies. More MEI-S332 (green) signals are detected on chromosomes (red) in embryos that were treated with colchicine for 2 hours (*E*) than ones for 30 minutes (*D*) or ones that were not treated (*C*). With prolonged colchicine treatment (*E*) the chromosomes became hypercondensed.


A
no drug
MEI-S332
MT
B
+ colchicine
MEI-S332
MT
C
0 min
D
30 min
E
2 hr

mitotic chromosomes in all the nuclei after the longer treatment with colchicine (Figure 2-2E). Again, anti-tubulin staining confirmed that microtubules were depolymerized by colchicine (data not shown). MEI-S332 signal and the apparent levels on the chromosomes consistently was higher in embryos incubated longer in colchicine (Figure 2-2, cf. E to C and D).

In addition to showing that intact microtubules are dispensable for MEI-S332 assembly and maintenance on centromeres, these results suggest that there is a period of time during which MEI-S332 can assemble onto the chromosomes. This period is immediately after prophase but prior to microtubule binding to the kinetochores. Colchicine treatment arrests cells in this period of time, and consequently, more MEI-S332 is able to assemble.

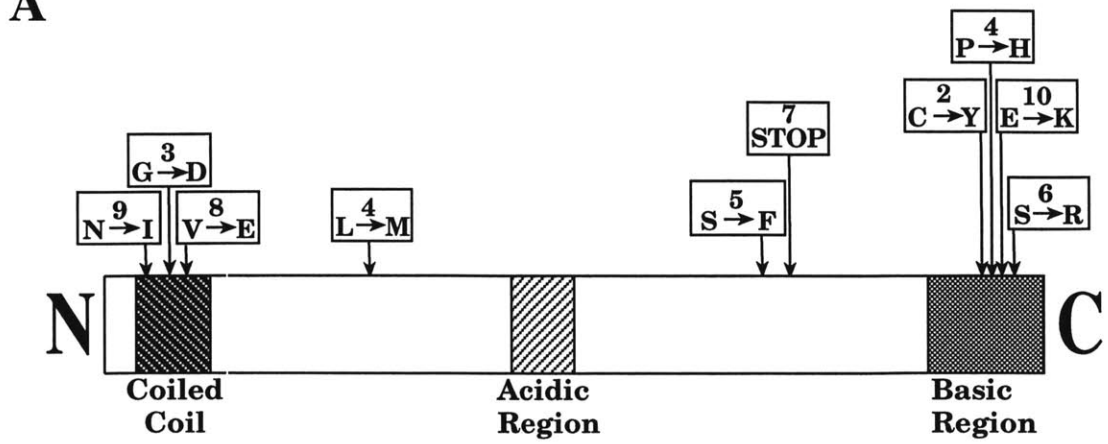
Mutations in MEI-S332 highlight two domains

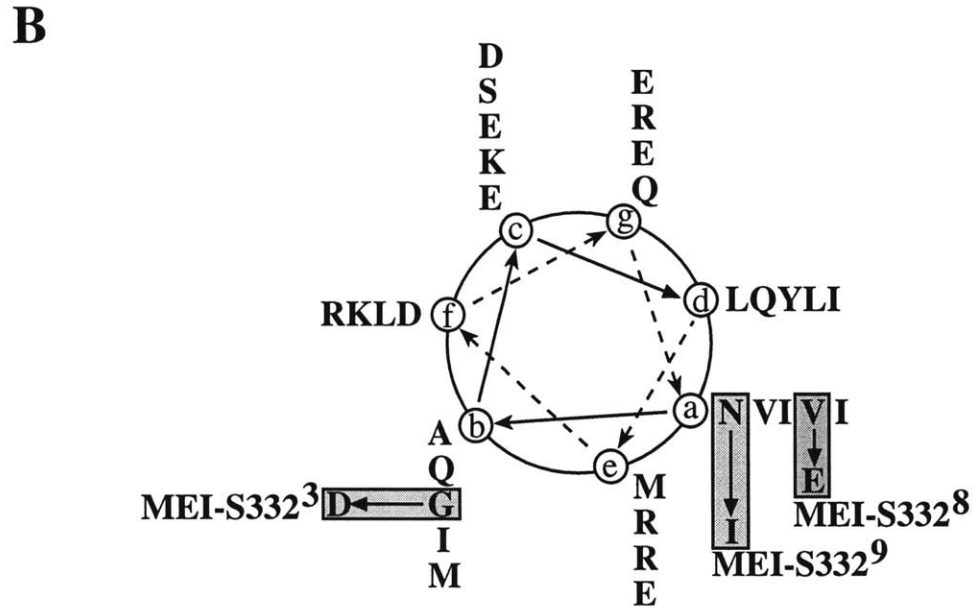
Knowing the timing of MEI-S332 assembly onto chromosomes, we next defined the domain(s) of the MEI-S332 protein necessary for its chromosomal localization. Mutations in *mei-S332* highlight two distinct domains of MEI-S332, a predicted coiled-coil domain near the amino terminus and a basic region at the carboxyl terminus of the protein (Kerrebrock et al. 1995; Figure 2-3A). Furthermore, mutations in the predicted coiled-coil domains are male-predominant alleles, because they cause high frequencies of chromosome loss and missegregation in male meiosis but low frequencies in female meiosis (Kerrebrock et al. 1992). In contrast, mutations in the basic region are female-predominant alleles and result in stronger missegregation phenotype in females than in males.

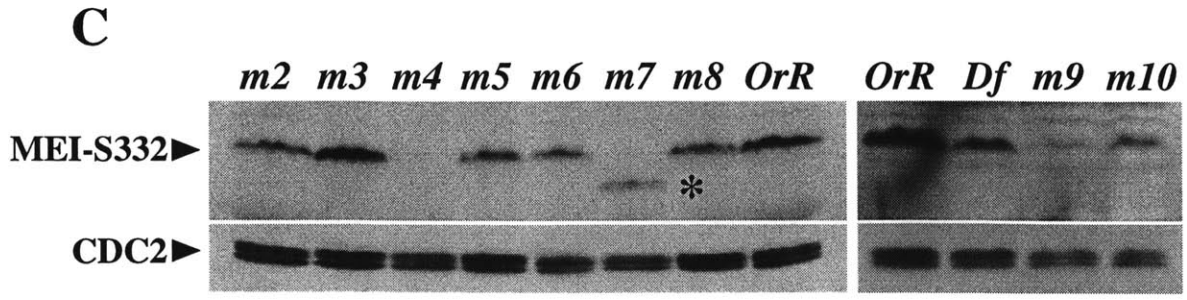
We recovered two new alleles of *mei-S332* in a noncomplementation screen with *mei-S332*^{1} (Bickel et al. 1997). One allele, *mei-S332*^{9}, is a mutation of asparagine-13 to isoleucine at the start of the predicted coiled coil,

Figure 2-3. Amino acid alterations in MEI-S332.

(*A*) The MEI-S332 protein has several distinct structural features. There are 10 alleles of *mei-S332*, most of which map to two domains, the amino-terminal predicted coiled coil and the carboxy-terminal basic region. (Downward arrows) Relative positions of the mutations. Numbers in the boxes designate the allele numbers; below are the amino acid changes. (*B*) The predicted coiled coil of MEI-S332 is represented in this helical wheel diagram showing the amino acids corresponding to positions a-e in the coil. Both *mei-S332*^{8} and *mei-S332*^{9} mutations are located at position a in the hydrophobic face of the predicted coiled coil, but *mei-S332*^{3} is more proximal to the hydrophilic face of the coil at position b. (*C*) Immunoblots of ovary extracts from *mei-S332* mutant females bound to anti-MEI-S332 antibodies reveal the stability of the mutant forms of the MEI-S332 protein. CDC2 levels are shown as the loading control. Except for the truncated, faster-migrating MEI-S332^{7}(*), mutant forms of the MEI-S332 protein do not exhibit any altered mobility rate in gels. They are designated by *m* followed by a corresponding allele number. The relative protein levels of MEI-S332^{9} and MEI-S332^{10} should be compared to that in ovaries from females carrying one copy of the *mei-S332* gene (*Df*; see Materials and Methods).


A
4 P→H
2 C→Y
10 E→K
7 STOP
3 G→D
9 N→I
8 V→E
4 L→M
5 S→F
6 S→R
N
C
Coiled Coil
Acidic Region
Basic Region


B
D S E K E
E R E Q
c
g
d
LQYLI
RKLD
f
a
N VI V I
E
b
A Q
e
MEI-S332³ D G
I M
M R R E
MEI-S332⁸
MEI-S332⁹


C
m2 m3 m4 m5 m6 m7 m8 OrR
OrR Df m9 m10
MEI-S332►
CDC2►
*

and the other, *mei-S332*^{10}, is a change from glutamate to lysine at residue 382 in the basic region (Figures 2-3A, B). We also sequenced the weakest allele, *mei-S332*^{5}, and found that it changes serine-277 to phenylalanine.

Because *mei-S332*^{9} and *mei-S332*^{10} map to the coiled-coil domain and the basic region, respectively, we predicted that they would exhibit sex-predominant chromosome segregation phenotypes. By standard genetic tests (see Materials and Methods) we found that *mei-S332*^{9} and *mei-S332*^{10} mutations resulted in high frequencies of chromosome loss and missegregation in both male and female meiosis II (Tables 2-1 and 2-2). Three previously described alleles, *mei-S332*^{6}, *mei-S332*^{7}, and *mei-S332*^{8}, were included in the tests as controls (Kerrebrock et al. 1992). Furthermore, in males, *mei-S332*^{9} caused a stronger missegregation phenotype than *mei-S332*^{10} (Table 1-2). On the other hand, in females, *mei-S332*^{10} resulted in higher missegregation frequency than *mei-S332*^{9} (Table 2-2). Thus, the genetic results were consistent with our previous observation and strongly suggest that the amino-terminal coil domain plays a male-specific role while the carboxy-terminal basic region is more important in females.

Prior to testing the effects of these mutations on MEI-S332 localization in mitosis as well as in male and female meioses, we wanted to confirm that the mutant MEI-S332 proteins were stable. We were particularly interested in this for the two strong alleles, *mei-S332*^{4} and *mei-S332*^{7}. Ovary extracts were prepared from mutant *mei-S332* females, and immunoblots were bound to guinea pig anti-MEI-S332 antibodies (Figure 2-3C). For alleles 2 through 8, ovaries were dissected from homozygous mutant females, whereas for alleles 9 and 10, ovaries were from mutant over deficiency females.

The results from these experiments revealed that we have a null allele of *mei-S332*, there is a stable truncated form of the protein, and that the mutant

Table 2-1

Sex chromosome missegregation in males with the indicated allele over *Df(2R)X58-6*

	% Regular Sperm		% Exceptional Sperm					
allele	*X*	*Y(Y)*	*0*	*XY(Y)*	*XX*	*XXY(Y)*	Total Progeny	Total % Missegregation
mei-S332 9	34.2 (480)[a]	26.5 (373)	26.7 (375)	1.6 (22)	11.0 (155)	0.0 (0)	1405	39.3
mei-S332 7	41.1 (363)	26.3 (232)	23.9 (211)	0.7 (6)	8.0 (71)	0.0 (0)	883	32.6
mei-S332 10	39.4 (551)	28.5 (398)	21.0 (293)	0.8 (11)	10.3 (144)	0.0 (0)	1397	32.1
mei-S332 8	38.3 (415)	31.8 (345)	19.3 (209)	0.6 (6)	10.1 (109)	0.0 (0)	1084	29.9
mei-S332 6	52.0 (654)	41.1 (517)	5.2 (66)	0.3 (4)	1.4 (17)	0.0 (0)	1258	6.9

[a]Numbers in parentheses indicate the numbers of progeny counted.

Table 2-2

Sex chromosome missegregation in females with the indicated allele over *Df(2R)X58-6*

	% Regular Ova	% Exceptional Ova				
allele	*X*	*0*	*XX*	Total Progeny	Adjusted Total[a]	Total % Missegregation
mei-S332 7	42.3 (423)	24.2 (121)	33.4 (167)	711	999	57.7
mei-S332 10	51.9 (710)	33.6 (230)	14.5 (99)	1039	1368	48.1
mei-S332 6	52.6 (572)	21.3 (116)	26.1 (142)	830	1088	47.4
mei-S332 9	55.2 (1034)[b]	25.5 (239)	19.2 (180)	1453	1872	44.8
mei-S332 8	74.7 (1072)	8.8 (63)	16.6 (119)	1254	1436	25.3

[a] The total progeny is adjusted to correct for the recovery of only half of the total number of exceptional progeny.

[b] Numbers in parentheses indicate the numbers of progeny counted.

missense forms are stable. Specifically we found the following: (1) MEI-S332^4 was completely absent from ovaries (Figure 2-3C, lane 3) and could be considered as a null in females, consistent with previous genetic results (Kerrebrock et al. 1992); (2) The MEI-S332^7 protein was seen as a stable and truncated protein by the use of the guinea pig antibodies generated against the full-length MEI-S332 protein (Figure 2-3C, lane 6); (3) Mutant MEI-S332 proteins were present in ovaries from *mei-S332^2*, *mei-S332^5*, *mei-S332^6*, *mei-S332^8*, *mei-S332^9*, and *mei-S332^{10}* females, although with decreased levels (Figure 2-3C, lanes 1, 4, 5, 7, 11, and 12); (4) MEI-S332^3 appeared to be present in wild-type levels (Figure 2-3C, lane 2); and (5) Western blots of testis extracts from mutant *mei-S332* males showed similar results, except that MEI-S332^4 was present at very low levels (data not shown). Again, this observation is consistent with previous genetic analysis (Kerrebrock et al. 1992); unlike in females, *mei-S332^4* did not behave genetically as a null in males.

The carboxy-terminal basic domain of MEI-S332 is required for chromosomal localization

We looked at the effect of mutations in the MEI-S332 basic region on the ability of the protein to localize onto meiotic and mitotic chromosomes. We were able to test the effects of amino acid substitutions using the *mei-S332^2*, *mei-S332^6*, and *mei-S332^{10}* alleles and to also examine the consequence of complete loss of the carboxy-terminal basic region with the truncation allele, *mei-S332^7* (Figure 2-3A).

In both spermatocytes and oocytes, either amino acid substitution within or truncation prior to the basic domain ablated detectable MEI-S332 chromosomal localization. The wild-type pattern of localization was not

observed with MEI-S332^{6} or MEI-S332^{7} mutant proteins in either spermatocytes or oocytes (Figures 2-4, cf. A and B to C-F). Anti-tubulin staining on spermatocytes confirmed the stages of meiosis (data not shown). Failure of centromere localization was observed also with MEI-S332^{2} and MEI-S332^{10} mutant proteins (data not shown).

It was surprising that missense mutations in the basic region of MEI-S332 disrupted MEI-S332 chromosomal localization in spermatocytes, given that two of these alleles exhibit only weak defects in male meiosis. The cloud of MEI-S332^{6} signal concentrated around the chromosomes (Figure 2-4C) leaves open the possibility that a small amount of the mutant protein localized onto the chromosomes and was capable of ensuring cohesion in males.

We examined the requirements for the carboxy-terminal basic region for mitotic chromosomal localization by staining mutant embryos. The truncated MEI-S332^{7} protein failed to localize during the S-M cycles and the postblastoderm divisions, demonstrating that the carboxyl terminus of the protein is essential for centromere localization in mitosis as well as meiosis (Figures 2-5A, B).

The analysis of MEI-S332^{6} protein in embryos gave unexpected results. No localization of MEI-S332^{6} was observed during the S-M cycles (Figure 2-5C). However, in postblastoderm divisions we observed MEI-S332^{6} localized to the mitotic chromosomes (Figure 2-5D). The MEI-S332^{6} signals appeared dimmer and more diffuse than wild type. The ability of MEI-S332^{6} to localize correlated with the length of the cell cycle. The rapid S-M cycles at syncytial blastoderm stage lack a G2 phase and at most have an interphase of 13 minutes and mitosis of 4.5 minutes (Foe et al. 1993). The S-G2-M postblastoderm cycles, when MEI-S332^{6} did localize, have a G2 of 30 to > 150 minutes (Edgar and O'Farrell 1989) and a mitosis of 10-60 minutes (Foe et al.

Figure 2-4. Mutations in the carboxy-terminal basic region alter MEI-S332 localization in spermatocytes and oocytes.

(Green) MEI-S332; (red) chromosomes. (*A*) Wild-type MEI-S332 protein localizes to two distinct foci on each bivalent in a prometaphase I spermatocyte. Each of these foci represents one pair of sister-chromatid centromeres in each homolog. All four chromosome bivalents can been seen here. The tiny fourth chromosome bivalent, which is often difficult to visualize, is seen clustered with a large bivalent (arrow). (*B*) In a metaphase I-arrested oocyte, wild-type MEI-S332 protein is seen on two opposite ends of the condensed karyosome (arrowheads). Unlike the foci in spermatocytes, these represent two clusters of sister-chromatid centromeres of all four chromosomes. (*C*) MEI-S332^{6} mutant protein fails to localize to the typical two foci on each bivalent in a prometaphase I spermatocyte. Three large bivalents can be seen clearly here. Instead, this mutant form of the protein seems to be concentrating around the chromosomes in the nucleus. Among all the spermatocytes (~100 cells) examined in eight separate trials using either the rabbit carboxy-terminal peptide MEI-S332 antibodies (Moore et al., 1998) or the guinea pig full-length MEI-S332 antibodies (this study), MEI-S332^{6} protein has never been clearly observed on the chromosomes in the two-foci-per-bivalent fashion. (*D*) MEI-S332^{6} protein also has never been observed on the karyosome in metaphase I-arrested oocytes. (*E, F*) Deletion of the carboxy-terminal basic region ablates the ability of the MEI-S332 protein to localize properly. MEI-S332^{7} mutant protein is absent from the chromosomes in both prometaphase I spermatocyte (*E*) and metaphase I oocyte (*F*). Bars, ~1μm.

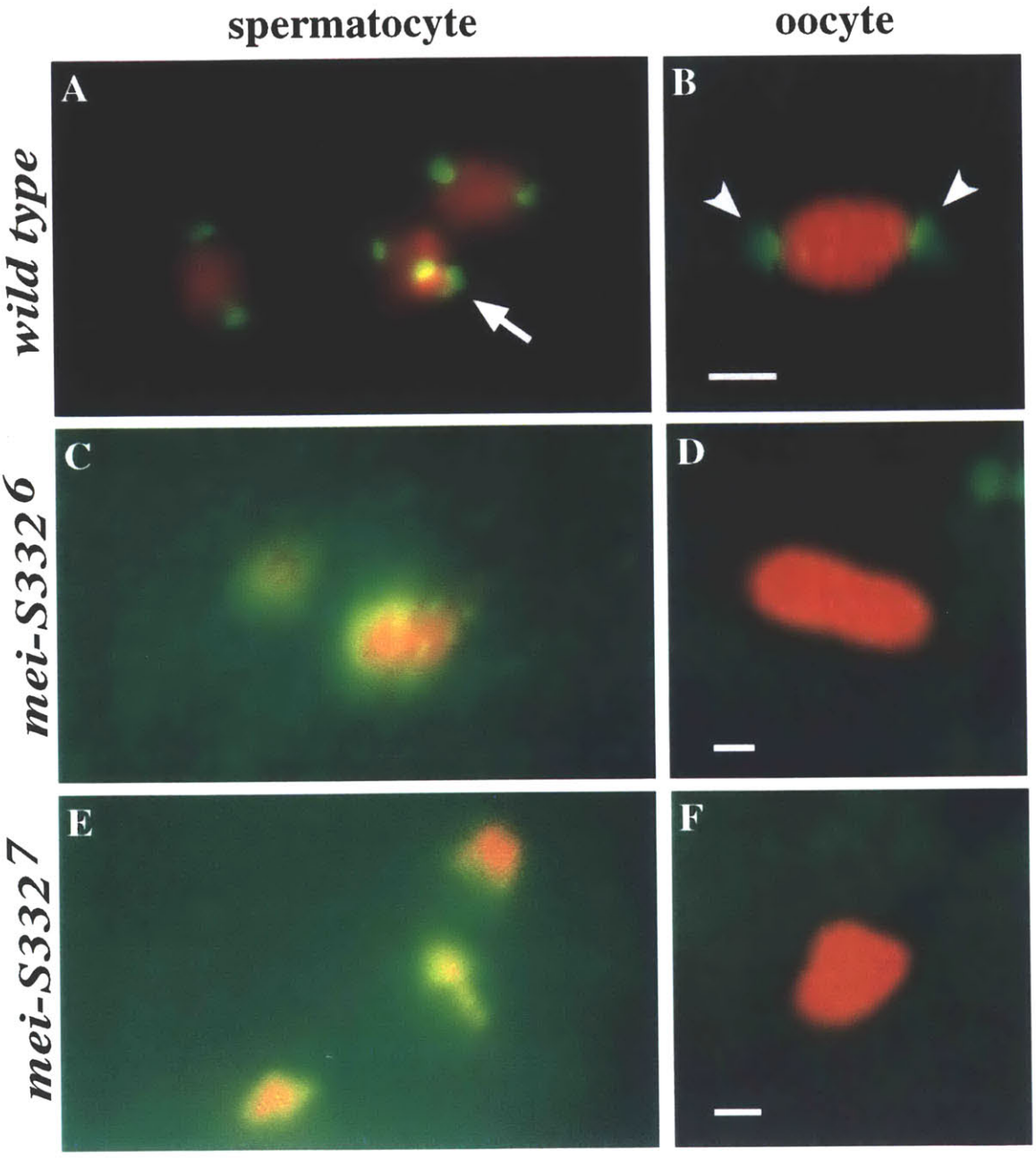
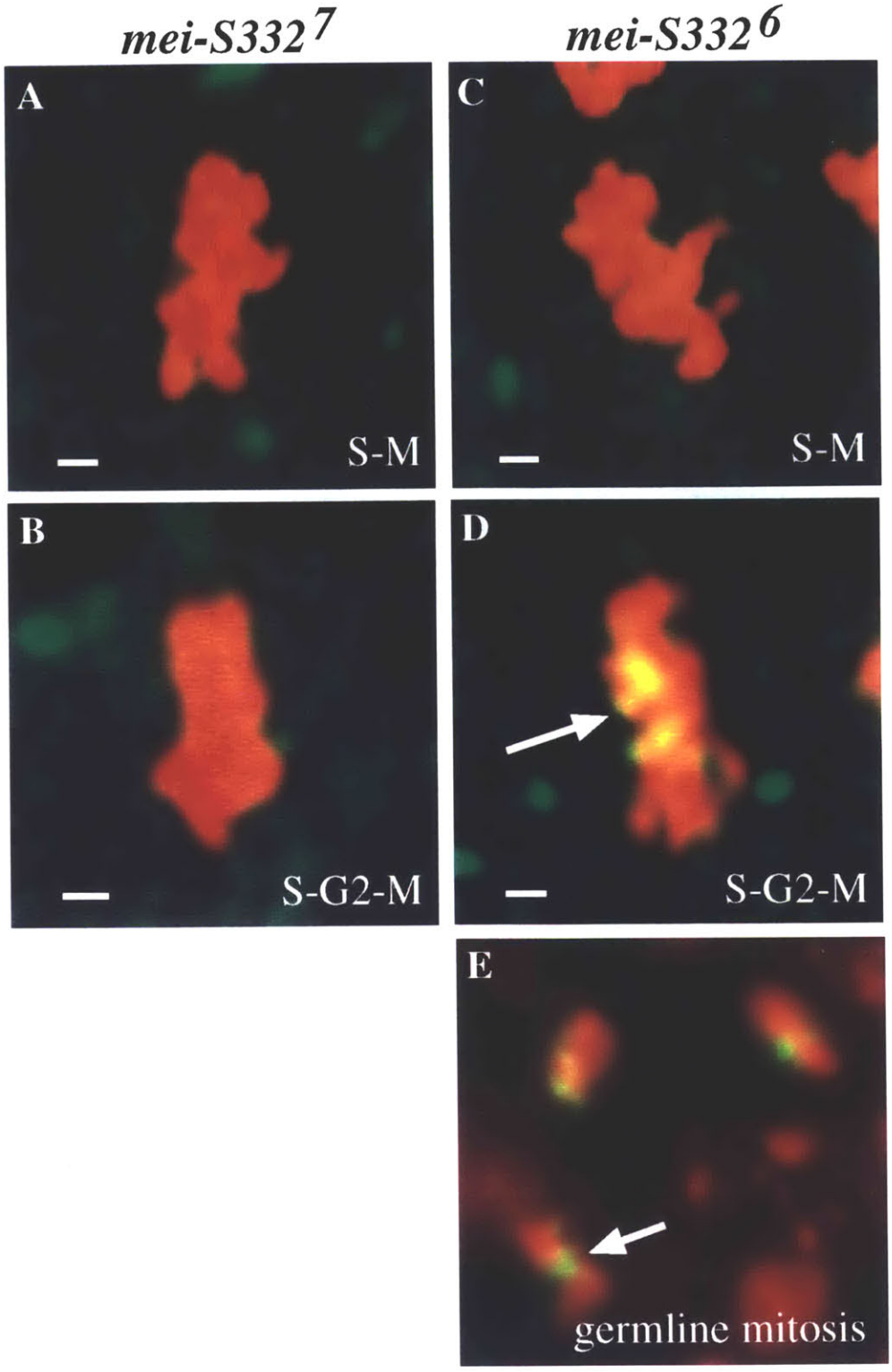

spermatocyte
oocyte
A
B
C
D
E
F
wild type
mei-S332 6
mei-S332 7

Figure 2-5. Missense mutations in the carboxy-terminal basic region impede but do not preclude chromosomal localization.

(Green) MEI-S332; (red) chromosomes; (yellow) areas of overlap. (*A, B*) Representative metaphase nuclei from a *mei-S332*^{7} S-M syncytial blastoderm embryo (*A*) and a S-G2-M postblastoderm embryo (*B*) are shown. MEI-S332^{7} is not detectable on the condensed chromosomes. (*C*) MEI-S332^{6} protein is not observed on metaphase chromosomes in S-M syncytial blastoderm embryos. (*D*) As the cell cycles lengthen with an addition of the G2 phase and a longer M phase in postblastoderm embryos, MEI-S332^{6} localizes to the condensed metaphase chromosomes (arrow). (*E*) In spermatogonial mitotic divisions with the canonical cell cycle, MEI-S332^{6} localizes to the condensed metaphase chromosomes. Chromosomes in embryos were visualized by use of anti-phospho H3 antibodies, and chromosomes in spermatogonial mitotic nuclei were stained with DAPI. Bars, ~1μm.


mei-S332 7
mei-S332 6
A
S-M
C
S-M
B
S-G2-M
D
S-G2-M
E
germline mitosis

1993). We hypothesize that while deletion of the basic region ablates the ability of MEI-S332 to localize, missense mutations in the basic region only weaken it. Thus missense mutant proteins can localize given enough time. Consistent with this idea, we detected MEI-S332^{6} in spermatogonial mitotic divisions with the canonical cell cycle (Figure 2-5E).

To test this hypothesis further, we provided the mutant proteins with an unlimited amount of time to assemble onto the centromeres by arresting the S-M cycles in prometaphase with colchicine. Whereas MEI-S332^{6} was not detected on the chromosomes in the syncytial blastoderm S-M cycles under normal conditions, it was seen on the chromosomes in these early cycles after the embryos had been treated with colchicine (Figure 2-6A). On the other hand, MEI-S332^{7} still failed to localize to the chromosomes when prometaphase was arrested by colchicine (Figure 2-6B). Therefore, the localization studies on MEI-S332^{6} and MEI-S332^{7} show that the carboxyl terminus is essential for centromere binding and that amino acid substitutions in the basic region impede but do not preclude chromosomal localization.

MEI-S332 proteins with alterations in the predicted coiled-coil domain localize to meiotic and mitotic chromosomes

Next, we tested the role of the predicted coiled-coil domain of MEI-S332 for chromosomal localization in meiosis and mitosis. In spermatocytes, oocytes, and the S-M cycles, both MEI-S332^{3} and MEI-S332^{8} mutant proteins localized normally to the centromeres (Figures 2-7A-F). This was also true for postblastoderm and spermatogonial divisions (data not shown). The signals on the chromosomes were more intense for MEI-S332^{3} than MEI-S332^{8}, most likely reflecting the endogenous mutant protein levels as revealed by Western blotting (Figure 2-3C). We could not detect MEI-S332^{9} on the

Figure 2-6. MEI-S332 proteins with missense mutations in the carboxy-terminal basic region require more time to achieve chromosomal localization. Syncytial blastoderm (S-M) embryos from *mei-S332*^{6} (*A*) and *mei-S332*^{7} (*B*) females were treated with 100µg/ml colchicine for 2 hours, fixed, and stained with anti-MEI-S332 (green) and anti-phospho H3 (red) antibodies.

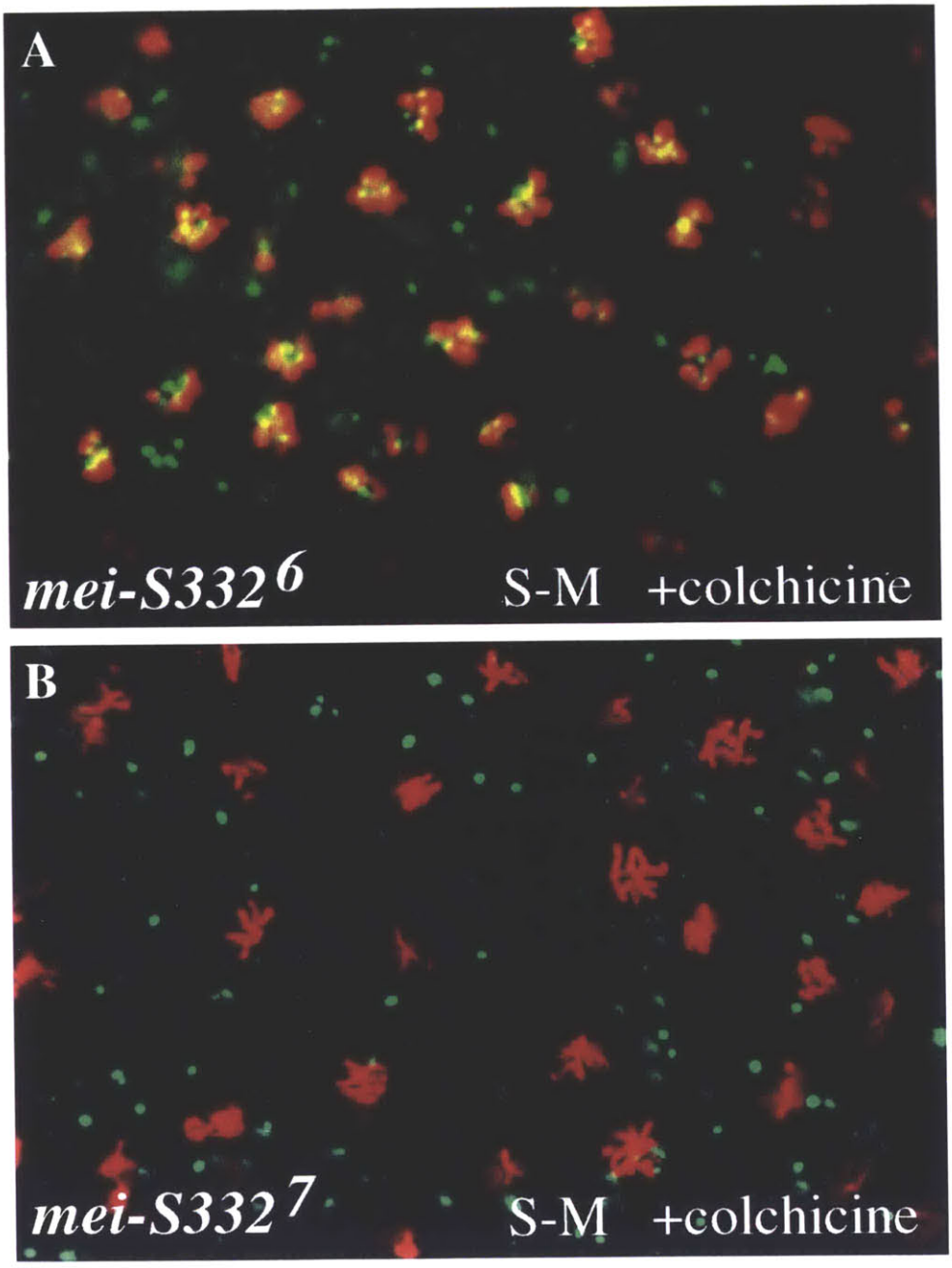
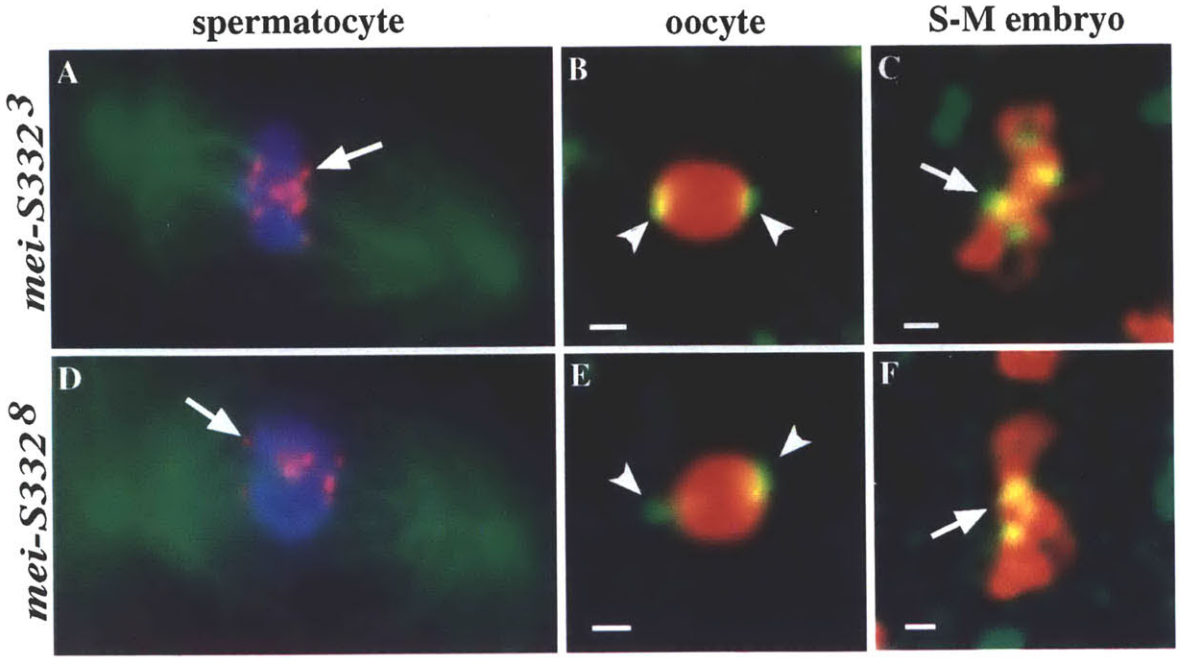

A
mei-S332 6
S-M +colchicine
B
mei-S332 7
S-M +colchicine

Figure 2-7. MEI-S332 proteins with mutations in the predicted coiled-coil domain still localize to mitotic and meiotic chromosomes. (*A, D*) MEI-S332 is red, chromosomes stained by DAPI are blue, and the spindle is in green. (*B, C, E, F*) MEI-S332 is green, and anti-phospho H3-stained chromosomes are red. (*A*) In metaphase I spermatocytes, MEI-S332^{3} mutant protein is observed on the chromosomes in eight foci corresponding to the eight pairs of sister-chromatid centromeres. Microtubules can be seen colocalizing with every MEI-S332^{3} dot (arrow). (*B*) MEI-S332^{3} mutant protein is also capable of localizing to the meiotic centromeric regions in oocytes. The pattern of localization resembles that of wild type (arrowheads; see Figure 2-4B). (*C*) In S-M syncytial blastoderm embryos, MEI-S332^{3} localizes properly to the metaphase chromosomes. (*D*) A nonconservative amino acid substitution (valine to glutamate) in the hydrophobic face of the predicted coiled coil does not disrupt the ability of MEI-S332 to localize onto meiotic chromosomes in spermatocytes. Eight dots of MEI-S332^{8} are detected on the metaphase I chromosomes, each colocalizing with microtubules (arrow). (*E*) This amino acid change also fails to perturb chromosomal localization of MEI-S332 in metaphase I-arrested oocytes. Like the wild-type protein, MEI-S332^{8} localizes to opposite sides of the karyosome (arrowheads). (*F*) MEI-S332^{8} continues to localize properly onto mitotic chromosomes during embryogenesis (arrow). Bars, ~1μm.


spermatocyte
oocyte
S-M embryo
mei-S332 3
mei-S332 8
A
B
C
D
E
F

chromosomes in *mei-S332^{9}/Df(2R)X58-6* spermatocytes (data not shown), even in conditions under which we could see MEI-S332 chromosomal localization in *Df(2R)X58-6/+* spermatocytes. One possibility is that the reduced levels of MEI-S332^{9} protein render it difficult to detect by immunofluorescence microscopy. Nevertheless, given the nature of *mei-S332^{8}* mutation, these results suggest that the predicted coiled-coil structure is not required for MEI-S332 chromosomal localization.

MEI-S332 has homotypic interactions and is in a multimeric complex

In vivo studies in *S. cerevisiae* with dimerized LacI-GFP fusion proteins suggested that protein-protein interactions may be sufficient to mediate sister-chromatid cohesion (Straight et al. 1996). Thus we determined whether MEI-S332 was capable of interacting with itself, a potential mechanism by which MEI-S332 could provide cohesion activity. We used two approaches to address this possibility: (1) The yeast two-hybrid system to test whether MEI-S332 was capable of binding to itself; and (2) Immunoprecipitation to determine whether a complex containing more than one MEI-S332 protein subunit exists in vivo.

To determine whether MEI-S332 would bind itself in the yeast cell, we employed the LexA-based interaction system (Gyuris et al. 1993). Full-length MEI-S332 fused to an activation domain interacted strongly with full-length MEI-S332 fused to the LexA DNA-binding domain, resulting in high levels of expression from two reporter genes (Figure 2-8A). A fusion containing only the amino-terminal third of MEI-S332 and the DNA-binding domain exhibited comparable levels of interaction with the full-length MEI-S332 fused to activation domain, showing that the carboxy-terminal two-thirds of MEI-S332 was not required for homotypic interaction in the yeast cell. A fusion

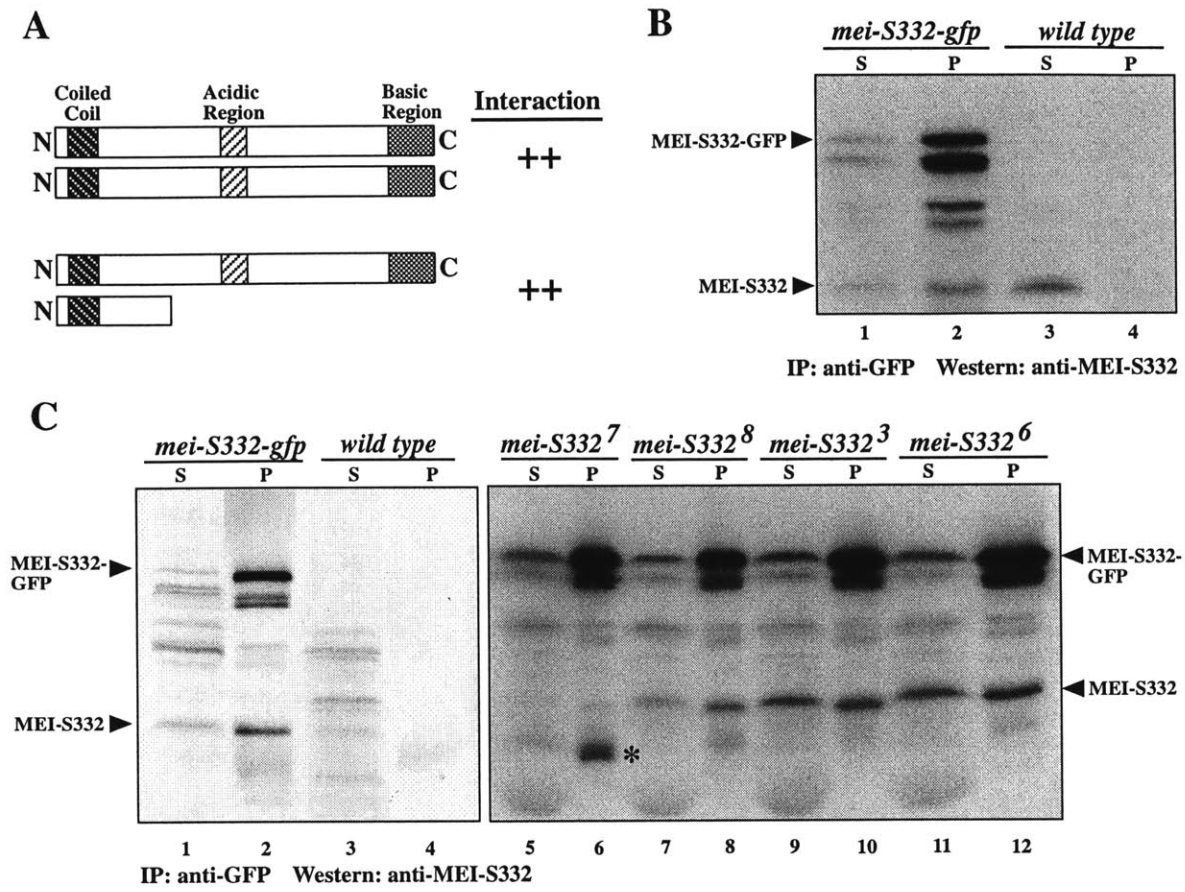


Figure 2-8. MEI-S332 is capable of homotypic interactions and exists in multimeric complex in vivo.

(*A*) In a yeast two-hybrid assay, MEI-S332 is found to interact with itself. The amino-terminal third of MEI-S332, consisting of the predicted coiled-coil domain, was sufficient to mediate this interaction. (*B*) Endogenous MEI-S332 coimmunoprecipitates with MEI-S332-GFP fusion protein from 2- to 6-hour embryos. By use of anti-GFP antibodies, the MEI-S332-GFP fusion protein was precipitated from embryos of flies carrying the *mei-S332-gfp* transgene, and the immunocomplex was analyzed by immunoblotting with guinea pig anti-MEI-S332 antibodies. In addition to MEI-S332-GFP, a band corresponding to the endogenous MEI-S332 is also present in the immunoprecipitate from *mei-S332-gfp* extracts. This band is absent from wild-type immunoprecipitate lacking the MEI-S332-GFP fusion.

(S) Supernatant fractions from the immunoprecipitation; (P) pellets from the immunoprecipitation. The amounts of proteins seen in S represent only ~1/10 of the total immunoprecipitation supernatant, whereas all of the pellet was loaded. Other bands in S and P from *mei-S332-gfp* extracts are likely to be degradation products of the MEI-S332-GFP fusion protein. (*C*) Endogenous MEI-S332 also coimmunoprecipitates with MEI-S332-GFP fusion protein from mature metaphase I-arrested oocytes. Probed with anti-MEI-S332 antibodies, the immunoblot of the immunoprecipitates demonstrates that the band corresponding to MEI-S332 is the endogenous MEI-S332 protein rather than a degradation product of MEI-S332-GFP (The band is absent from the *mei-S332*^{7} immunoprecipitate). This complex is not disrupted by mutations in the coiled-coil domain or in the basic region, and consistent with the yeast two-hybrid result, the carboxy-terminal portion of MEI-S332 is not necessary for the formation of this multimeric MEI-S332 complex. Four mutant forms of MEI-S332 coimmunoprecipitate with MEI-S332-GFP. MEI-S332^{3}, MEI-S332^{6}, and MEI-S332^{8} have the same mobility as the wild-type protein (arrow), while the truncated MEI-S332^{7} protein migrates faster (*). Again, the endogenous MEI-S332 protein is absent in extracts from flies lacking the *mei-S332-gfp* transgene.

containing only the carboxy-terminal two thirds of MEI-S332 and the DNA-binding domain activated on its own and hence could not be tested.

To examine the association between MEI-S332 subunits in vivo, we immunoprecipitated a MEI-S332-GFP fusion protein and tested whether the endogenous MEI-S332 was present in the immunocomplex by immunoblotting the immunoprecipitates. Rabbit polyclonal anti-GFP antibodies pulled down not only MEI-S332-GFP but also the endogenous MEI-S332 present in *mei-S332-gfp* transgenic embryos (Figure 2-8B). As a negative control, no MEI-S332-GFP was present in the parallel immunoprecipitate from wild-type non-transgenic embryo extracts; MEI-S332 was also absent from this immunoprecipitate. Similar results were seen with oocytes (Figure 2-8C, cf. lanes 3 and 4 to lanes 1 and 2). We confirmed that the indicated band (Figure 2-8B) was the endogenous MEI-S332 by performing a parallel immunoprecipitation using oocyte extracts from homozygous *mei-S332*^{7} mutant females that expressed MEI-S332-GFP. Although the fusion protein was detected in the immunocomplex, the band corresponding to the endogenous wild-type MEI-S332 protein was absent from the complex (Figure 2-8C, lane 6). Instead, a faster migrating band corresponding to the endogenous truncated MEI-S332^{7} protein was seen on the blot. Therefore, in embryos and oocytes, MEI-S332 is in a multimeric complex with more than one subunit of MEI-S332.

Combining the immunoprecipitation results with the results from yeast two-hybrid, we postulated that MEI-S332 interaction with itself was mediated by the predicted coiled-coil domain at the amino terminus. One prediction of this model is that the *mei-S332*^{8} mutation would disrupt MEI-S332 self-interaction. However, immunoprecipitation of *mei-S332*^{8} mutant, *mei-S332-gfp* transgenic oocyte extracts with anti-GFP antibodies showed that MEI-

S332^8 was still in the complex with MEI-S332-GFP (Figure 2-8C, lane 8). Nevertheless, this result does not exclude the idea that MEI-S332 self-interaction is mediated through the coiled-coil domain, and we present several possibilities for this in the Discussion.

Preliminary results from gel-filtration and glycerol-gradient experiments indicated that MEI-S332 with a predicted molecular mass of 44.4 kD is present in two populations in embryos (data not shown). Most of the MEI-S332 protein is in a large complex of 200-1000 kD, indicating that MEI-S332 is in a multimeric complex. The less abundant form is 45-200 kD, suggesting a dimer of MEI-S332. The Multicoil program (Wolf et al. 1997) predicts that the coiled coil of MEI-S332 has a higher probability of forming a dimer than a trimer.

Intragenic complementation between the two MEI-S332 domains

Given that MEI-S332 can multimerize, it was possible that mutations disrupting the coiled-coil domain might complement mutations in the basic region, and vice versa. We tested this hypothesis by measuring the frequencies of meiotic chromosome nondisjunction and loss in female and male meioses in *mei-S332^6/mei-S332^8*. Strikingly, we observed complementation between the two alleles, *mei-S332^6* and *mei-S332^8* (Table 2-3). In males, *mei-S332^8* chromosome segregation was improved by the presence of *mei-S332^6* mutation. Similarly, *mei-S332^6* was improved by *mei-S332^8* in females. These results are due to intragenic complementation rather than the activity of the *mei-S332^6* gene in males or the *mei-S332^8* gene in females. *mei-S332^8/Df* females had 22.3% nondisjunction, and *mei-S332^6/Df* males had 12.0% nondisjunction (Kerrebrock et al. 1992; data not shown). Thus it appears that the MEI-S332^8 mutant protein was able to tether MEI-S332^6

Table 2-3

Genetic complementation between two *mei-S332* alleles

Male Tests	% Regular Sperm		% Exceptional Sperm					
genotype	*X*	*Y(Y)*	*0*	*XY(Y)*	*XX*	*XXY(Y)*	Total Progeny	Total % Missegregation
pr cn mei-S332^{6} bw sp / cn mei-S332^{8} px sp	55.0 (991)[a]	43.9 (792)	0.4 (8)	0.2 (4)	0.4 (8)	0.0 (0)	1803	1.1
pr cn mei-S332^{8} bw sp / cn mei-S332^{6} px sp	50.2 (915)	46.8 (854)	1.8 (33)	0.1 (2)	1.1 (20)	0.0 (0)	1824	3.0
pr cn mei-S332^{6} bw sp / cn mei-S332^{6} px sp	49.3 (240)	47.4 (231)	1.8 (9)	0.2 (1)	1.2 (6)	0.0 (0)	487	3.3
pr cn mei-S332^{8} bw sp / cn mei-S332^{8} px sp	47.0 (239)	33.9 (172)	12.4 (63)	1.2 (6)	5.5 (28)	0.0 (0)	508	19.1

Female Tests	% Regular Ova	% Exceptional Ova				
genotype	*X*	*0*	*XX*	Total Progeny	Adjusted Total[b]	Total % Missegregation
pr cn mei-S332^{6} bw sp / cn mei-S332^{8} px sp	90.8 (3686)[a]	4.1 (84)	5.1 (103)	3873	4060	9.2
pr cn mei-S332^{8} bw sp / cn mei-S332^{6} px sp	92.4 (3333)	3.5 (64)	4.0 (73)	3470	3607	7.6
pr cn mei-S332^{6} bw sp / cn mei-S332^{6} px sp	62.3 (878)	21.6 (152)	16.2 (114)	1144	1410	37.7
pr cn mei-S332^{8} bw sp / cn mei-S332^{8} px sp	92.6 (1336)	3.7 (27)	3.6 (26)	1389	1442	7.4

[a] Numbers in parentheses indicate the numbers of progeny counted.

[b] The total progeny is adjusted to correct for the recovery of only half of the total number of exceptional progeny.

mutant protein, which is itself unable to localize to chromosomes, to the meiotic chromosomes in both males and females. Once on the chromosomes, the wild-type coiled-coil domain of MEI-S332^{6} may have compensated for the mutant coil in MEI-S332^{8}. This genetic complementation strongly suggests that MEI-S332 is in a complex with itself in vivo, not only in females as seen with immunoprecipitations in oocyte extracts, but also in males.

Discussion

We found that the MEI-S332 centromere cohesion protein first assembles onto the chromosomes during prometaphase, and its chromosomal localization does not require intact microtubules. There are two functional domains of MEI-S332 that can act in *trans* to complement each other. The carboxy-terminal basic region of MEI-S332 is essential for chromosomal localization. Although the amino-terminal coiled-coil domain may not be necessary for localization, it may be involved in mediating protein-protein interactions with a yet-unidentified male-specific factor. Furthermore, MEI-S332 is capable of self-interaction, and this may facilitate its function in sister-chromatid cohesion.

MEI-S332 maintains sister-chromatid cohesion

To ensure proper chromosome segregation, the physical association between the duplicated sister chromatids appears to be established during or immediately after DNA replication, and it must be maintained until sister chromatids separate at the onset of anaphase. The timing of association and dissociation of the *Xenopus* cohesin complex raised the possibility of additional cohesion proteins to maintain cohesion in mitosis (Losada et al. 1998). It is intriguing that MEI-S332, shown to be required for sister-chromatid cohesion, does not assemble onto the chromosomes until prometaphase. Our interpretation is that MEI-S332 acts to maintain cohesion specifically at the centromere.

Why would a cell require multiple, different complexes for sister-chromatid cohesion? Losada et al. (1998) speculated that upon entry into mitosis, the cohesin complex must be cleared from the chromosomes to relieve steric hindrance that could otherwise prevent the condensin-mediated

chromosome condensation. Consequently, the cohesin complex is dissociated from the chromosomes early, prior to the onset of sister-chromatid separation. If the condensins do not contribute to sister-chromatid cohesion activity, a possibility raised by the yeast mutants (Saka et al. 1994; Strunnikov et al. 1995), we postulate that additional cohesion proteins are required in mitosis. This would be particularly true at the centromeres. When kinetochores engage in microtubule attachment, additional centromere cohesion is likely to be needed to counteract the poleward pulling forces exerted on the kinetochores. On the basis of its spatial and temporal pattern of chromosomal localization, MEI-S332 may be a component of the maintenance complex, counteracting the poleward spindle forces by maintaining sister-chromatid cohesion at the centromeres. Consistent with this model, we found that MEI-S332 assembly onto the chromosomes is not dependent on microtubule attachment to the kinetochores. To maintain sister-chromatid cohesion against the poleward pulling forces, MEI-S332 would have to localize to the centromeres before the kinetochores capture microtubules.

A defined period of time when MEI-S332 has accessibility to chromosomes

The observation that more MEI-S332 localizes onto the chromosomes when nuclei are arrested in prometaphase suggests that there is a defined period of time, with both an onset and an end, when MEI-S332 can localize onto the chromosomes. The fact that lengthening prometaphase allowed a mutant MEI-S332 protein with a crippled basic region to localize onto chromosomes supports the model that there is a defined period of time when MEI-S332 has accessibility to the chromosomes. Whereas these results demonstrate an onset for when MEI-S332 is capable of assembling on the

centromere, our failure to observe localization of MEI-S332^{6} mutant protein onto the chromosomes of mature oocytes suggests an endpoint beyond which MEI-S332 can not localize. This endpoint would be marked by microtubule binding and spindle assembly. Mature oocytes are arrested indefinitely in metaphase I until the egg is activated (Theurkauf and Hawley 1992). Although the metaphase-I arrest in mature oocytes should provide sufficient time for MEI-S332 mutant proteins with a crippled basic region to localize, we never observed MEI-S332 proteins with mutations in the basic regions on the karyosome. The distinction between our failure to observe MEI-S332^{6} localized in oocytes and its localization in colchicine-treated embryos is that the kinetochores are already attached to microtubules during metaphase I. We propose that microtubule attachment blocks the ability of MEI-S332 to localize. Thus, if MEI-S332 fails to localize in the preceding short prometaphase stage, it will not localize in mature oocytes regardless of how long the metaphase-I arrest lasts.

Functional domains within MEI-S332

Mutations in *mei-S332* highlight two domains of the protein, a predicted coiled-coil domain at the amino terminus and a basic region at the carboxyl terminus. We found that these two domains have distinct functions. The basic region is essential for MEI-S332 chromosomal localization, whereas mutations in the coiled-coil domain do not have any effect on localization. The results from the intragenic complementation tests between mutations in the two domains provide compelling evidence that these two domains play essential but different functions. The basic region of MEI-S332 may bind to DNA directly, perhaps by recognizing specific DNA sequences in the centromeric

heterochromatin. Alternatively, the basic region could be critical for protein-protein interactions between MEI-S332 and other DNA-binding factors.

The yeast two-hybrid and immunoprecipitation results demonstrate that MEI-S332 is capable of interacting with itself. The self-interaction of MEI-S332 could be mediated through the coiled-coil domain, even though *mei-S332*^{8}, a mutation in the hydrophobic side of the coil, does not disrupt this self-interaction in the coimmunoprecipitation experiments. If more than two subunits of MEI-S332 are associated in a single complex and/or if there is another protein in the MEI-S332 complex, we would not expect to detect the effect of the coiled-coil mutation on self-interaction by coimmunoprecipitation. Because cohesion at the centromere may need to spread over a chromosomal domain and not be restricted to the kinetochore, it is reasonable that there may be more than two subunits of MEI-S332 in a complex and/or that they are associated with bridging proteins. This hypothesis is supported by the observation that on glycerol gradients and gel-filtration columns MEI-S332 migrates with a high molecular mass complex. Previous work with a LacI-GFP fusion protein in *S. cerevisiae* showed that sister-chromatid cohesion can be mediated via protein-protein interaction (Straight et al. 1996). It is attractive to think that the mechanism by which MEI-S332 functions to hold sister chromatids together is protein-protein interactions between MEI-S332 subunits on separate sister chromatids.

Mutations in the coiled-coil domain affect chromosome segregation more severely in males than in females. However, they do not have a detectable effect on MEI-S332 chromosomal localization. Therefore, we postulate that the coiled-coil domain interacts with some protein(s) necessary for male but not female meiosis. Because the basic region of MEI-S332 is required for chromosomal localization in both sexes, it is surprising that mutations in the

basic region cause only limited chromosome missegregation in males. Perhaps the basic-region mutant proteins, MEI-S332^{2} and MEI-S332^{6}, do localize onto the chromosomes, although at a level lower than wild type and not detectable by the antibodies. Low levels of MEI-S332 on the chromosomes may be sufficient for proper sister-chromatid cohesion in males, but higher levels might be needed for females. Alternatively, MEI-S332 could be required on the male meiotic chromosomes only transiently but must remain associated with the female meiotic chromosomes from prometaphase until sister-chromatid separation at the onset of anaphase II. The reason could be that in addition to MEI-S332 there are other factors participating in sister-chromatid cohesion which are present in males but not in females.

All of the proteins necessary for sister-chromatid cohesion identified so far either do not localize to chromosomes (eg., Pds1p; Ciosk et al. 1998) or localize all along the length of the chromosomes (e.g., the cohesin complex; Michaelis et al. 1997). MEI-S332 is the only protein that localizes specifically to the centromeres. It functions to maintain sister-chromatid cohesion at the centromeres and appears to counteract the poleward pulling forces. It is attractive to think that in addition to being a structural component necessary for maintaining sister-chromatid cohesion, MEI-S332 also plays a regulatory role in sister-chromatid cohesion. Because the spindle assembly checkpoint components are observed at the centromeres along with the anaphase-promoting complex, MEI-S332 could interact with these proteins. Finally, MEI-S332 can be used as a bait for finding sex-specific factors necessary for proper chromosome segregation. Meiosis is different between males and females in *D. melanogaster*, and the identification and characterization of these sex-specific factors will help us understand the differences in the two sexes.

Materials and methods

Fly strains

To generate homozygous *mei-S332* mutant flies, two different stocks of each allele were crossed to each other (Kerrebrock et al. 1992), and progeny of the genotype *pr cn mei-S332 bw sp/cn mei-S332 px sp* were selected. Alleles 9 and 10 are exceptions in that they were analyzed in *trans* to the *Df(2R)X58-6* deficiency (Kerrebrock et al. 1992). The *Df(2R)X58-6 pr cn/SM1* stock and *Oregon-R* were used as the wild-type control for Western blotting and MEI-S332 localization in embryos, oocytes, and spermatocytes. In addition, *Oregon-R* was also used as the negative control for immunoprecipitation experiments.

A $y/y^{+}Y$; +/*SM1* stock was used to isogenize the sex chromosomes in the *mei-S332* mutant stocks. The isogenized *mei-S332* and deficiency stocks were crossed to each other to generate *mei-S332/Df(2R)X58-6* flies for the nondisjunction tests. *C(1)RM,* y^2 *su(*w^a*)*w^a $Y^S X \bullet Y^L$, *In(1)EN* y^+ *v f B*, carrying attached *XX* and *XY* chromosomes, was used to measure sex chromosome nondisjunction and loss in meiosis.

In the studies of the MEI-S332 multimeric complex, females of the genotype *y w P[*w^{+mc} *5.6KK mei-S332*$^+$*::GFP = GrM]-7;* +/*SM1* and *y w P[GrM]-7;* +/+ were used to provide the MEI-S332-GFP-expressing oocytes and embryos for immunoprecipitation extracts. *P[GrM]-7*, an insertion of the fusion transgene *mei-S332*$^+$*::GFP* on the *X* chromosome (Moore 1997), was crossed into both stocks of *mei-S332* alleles 3, 6, 7, and 8 to generate *mei-S332* mutants that express MEI-S332-GFP. This *mei-S332-gfp* transgene is functional in meiosis (Moore 1997).

Immunofluorescence in embryos, oocytes, and spermatocytes

Antibodies against a full-length MEI-S332 recombinant protein fused to GST were generated in guinea pigs. The cloning of the GST-MEI-S332 expression construct was described previously (Moore et al. 1998). The GST-MEI-S332 fusion protein was expressed in BL21(λDE3)pLysS cells by IPTG induction. The small fraction of soluble GST-MEI-S332 was purified using glutathione agarose beads (Sigma Chemicals), combined with the purified inclusion bodies containing majority of the GST-MEI-S332 protein, and separated on standard 10% SDS polyacrylamide gels. The band corresponding to GST-MEI-S332 was excised, eluted, and injected into guinea pigs for antibody production (Covance, Denver, PA).

For immunofluorescence studies in *mei-S332* mutant embryos, embryos were collected for 6 hours from females of the genotype *pr cn mei-S332 bw sp / cn mei-S332 px sp* as well as from *Oregon-R* control females, fixed essentially as described (Whitfield et al. 1990), and stained first with anti-MEI-S332 antibodies (1:5000 dilution), followed by Cy3-conjugated anti-guinea pig (1:150 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), and then with anti-phospho H3 (1:500 dilution; D. Allis), followed by Cy2-conjugated anti-rabbit antibodies (1:100 dilution; Jackson ImmunoResearch Laboratories, Inc.).

Mature unactivated oocytes were prepared by use of protocols described by Theurkauf (1994) and Page and Orr-Weaver (1997) from fattened females of the genotype *pr cn mei-S332 bw sp / cn mei-S332 px sp* for alleles 2 through 8 and *cn bw sp If mei-S332 / Df(2R)X58-6 pr cn* for alleles 9 and 10 as well as from *Oregon-R* and *Df(2R)X58-6 pr cn / SM1* females. They were bound to MEI-S332 antibodies as described above for embryos, and stained with YOYO-1

iodide (Molecular Probes) at 1:1000 in PBS before dehydration in methanol and mounting in clearing solution.

To stain spermatocytes for MEI-S332 and tubulin, testes were dissected from newly eclosed males of the genotype described above and processed for immunostaining as described in Hime et al. (1996) with slight modifications. Slides were incubated with anti-MEI-S332 (1:10,000 dilution), followed by Cy3-conjugated anti-guinea pig, and then with rat monoclonal anti-tubulin (YL1/2 and YOL1/34 at 1:5 dilution; Sera-Lab Ltd., Sussex, UK), followed by Cy2-conjugated anti-rat antibodies (1:100 dilution; Jackson ImmunoResearch Laboratories, Inc.). DNA was stained with DAPI at 1μg/ml in PBS before the slides were mounted in glycerol containing 50mg/ml n-propyl gallate.

Colchicine treatment of embryos

To arrest the mitotic cell cycles at prometaphase, *Oregon-R* embryos from a 2-hour or a 4-four collection were dechorionated in 50% Clorox bleach, rinsed with Grace's medium (GIBCO/BRL), permeabilized by Grace's medium-saturated octane (Aldrich; Ashburner 1989), and incubated for 30 minutes or 2 hours, respectively, in Grace's medium with or without colchicine at 100μg/ml. After colchicine treatment, embryos were immediately fixed and processed either for MEI-S332 and phosphorylated histone H3 immunostaining or for MEI-S332 and tubulin immunostaining followed by DAPI staining.

Microscopy

Two types of epifluorescence microscopy were used in our studies of MEI-S332 localization. In cases where tissues were triple-labeled, a Nikon Eclipse E800 microscope equipped with a 100x oil Plan Apo objective was

employed to visualize the chromatin, spindles, and MEI-S332. Images were captured by a Photometrics CE200A cooled CCD video camera and subsequently processed with the CELLscan 2.1 system (Scanalytics) to create volume views from focal planes separated by 0.25μm. When tissues were double-labeled, specimens were viewed with a confocal laser scanning head (MRC 600; BioRad) that was equipped with a kryopton/argon laser and mounted on a Zeiss Axioskop microscope (Oberkochen, Germany). A 40x oil Plan Neofluar objective was used. Adobe Photoshop 3.0 was used to process and merge the images; artificial colors were used.

PCR and Sequencing of *mei-S332* alleles

Genomic DNA from female flies of genotypes *cn bw sp If mei-S332^{9}/Df(2R)X58-6 pr cn*, *cn bw sp If mei-S332^{10}/Df (2R)X58-6 pr cn*, *pr cn mei-S332^{5} bw sp/Df(2R)X58-6 pr cn*, or *cn mei-S332^{5} px sp/Df(2R)X58-6 pr cn* was prepared by use of the single-fly DNA preparation protocol described by Gloor and Engels (1992), amplified by PCR with primers flanking the *mei-S332* ORF, and subjected to automated DNA sequencing (Research Genetics). Two independently amplified PCR products were sequenced for each mutation. In addition, both strands of DNA were sequenced.

Western Blot Analysis

Protein extracts were prepared and Western blotting was performed as described previously (Moore et al. 1998) with the exception that ovaries were dissected in IB buffer [55mM NaOAc, 40mM KOAc, 100mM sucrose, 10mM glucose, 1.2mM $MgCl_2$, 1mM $CaCl_2$, and 100mM HEPES (pH7.4)], and anti-full-length MEI-S332 antibodies were used. Blots were first incubated with both guinea pig anti-MEI-S332 whole serum at 1:20,000 and rabbit anti-CDC2

antibodies (Edgar et al. 1994) at 1:5000 and then with horseradish peroxidase (HRP)-conjugated anti-rabbit antibodies (Promega) diluted 1:2500 and alkaline phosphatase-conjugated anti-guinea pig antibodies (Jackson ImmunoResearch Laboratories, Inc.) at 1:5000. Visualization of bound HRP-conjugated antibodies was done first with the ECL chemiluminescent detection (Amersham), followed by that of bound alkaline phosphatase-conjugated antibodies with CDP-Star as the chemiluminescent substrate (Tropix, Bedford, MA).

For *mei-S332* alleles 2-8, ovary extracts were prepared from homozygous mutant females, and hence their relative MEI-S332 levels could be compared to that in the wild-type *Oregon-R* ovary extract. For *mei-S332* alleles 9 and 10, the extracts were made from *mei-S332/Df* females, and thus, their relative MEI-S332 levels should be compared to that in *Df/+* ovary extract. CDC2 protein levels were used as the loading controls.

Nondisjunction Tests

Genetic assays used to measure the frequencies of sex chromosome nondisjunction and loss in both female and male meiosis were performed as described previously (Kerrebrock et al. 1992). Isogenized *mei-S332/Df(2R)X58-6* virgin females or males were crossed to attached *XY* males or attached *XX* virgin females, respectively (see Fly Strains). The parents of the crosses were removed at day 7, and progeny were scored on days 13 and 18. Crosses with *mei-S332/SM1* and *Df(2R)X58-6/SM1* were included in the tests as controls. All crosses were kept at 25°C during the testing period.

Yeast two-hybrid and immunoprecipitation experiments

The full length MEI-S332 coding sequence was cloned into the prey and bait vectors of Gyuris et al. (1993) by inserting a *Bam*HI/*Dra*I fragment of the *mei-S332* cDNA. In addition, a *Bgl*II fragment that contains the amino-terminal third of the protein was cloned into the bait vector. Both the *lacZ* and *leu2* reporter genes were used to test interaction as described (Golemis et al. 1997).

For immunoprecipitation experiments, embryos and mature unactivated oocytes were homogenized in IP buffer [150mM or 500mM NaCl, 50mM Tris (pH 8), 2.5mM EDTA, 2.5mM EGTA, 0.2%NaN_3, 0.3mM Na_3VO_4, 0.1mM PMSF, 10µg/ml pepstatin A, 10µg/ml aprotinin, 100µg/ml chymostatin, 10µg/ml leupeptin, and 10µg/ml soybean tripsin inhibitor]. Then, NP-40 was added to the extracts to a final concentration of 1% before extracts were cleared by centrifugation at 4°C for 5 minutes. Extracts were frozen in liquid nitrogen, and stored at -80°C. Rabbit anti-GFP antibodies (Clontech) were added to the IP extracts and allowed to incubate overnight at 4°C before binding to protein A-sepharose 6MB beads for 1 hour. Beads were washed 10 times at 4°C with NP-40 buffer [150mM or 500mM NaCl, 50mM Tris (pH 8), 2.5mM EDTA, 2.5mM EGTA, 0.2% NaN_3, 0.3mM Na_3VO_4, and 1% NP-40]. Finally, 2X SDS sample buffer was added to the beads, and the samples were heated at 95°C for 5 minutes and centrifuged for 5 minutes. The immunocomplexes were separated on SDS-polyacrylamide gels and analyzed by immunoblotting with guinea pig anti-MEI-S332 antibodies.

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Chapter Three

The Cohesion Protein MEI-S332 Localizes to Condensed Meiotic and Mitotic Centromeres until Sister Chromatids Separate

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*T.T.-L.Tang constructed the pGEX.MEI plasmid, expressed and purified the GST-MEI-S332 fusion protein, generated the fusion protein-bound immobilon-P strips, affinity purified the rabbit anti-MEI-S332 peptide antibodies, prepared protein extracts from ovaries, embryos, and unactivated oocytes, and ran all of the Western blots.

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Abstract

The *Drosophila* MEI-S332 protein has been shown to be required for the maintenance of sister-chromatid cohesion in male and female meiosis. The protein localizes to the centromeres during male meiosis when the sister chromatids are attached, and it is no longer detectable after they separate. *Drosophila melanogaster* male meiosis is atypical in several respects, making it important to define MEI-S332 behavior during female meiosis, which better typifies meiosis in eukaryotes. We find that MEI-S332 localizes to the centromeres of prometaphase I chromosomes in oocytes, remaining there until it is delocalized at anaphase II. By using oocytes we were able to obtain sufficient material to investigate the fate of MEI-S332 after the metaphase II/ anaphase II transition. The levels of MEI-S332 protein are unchanged after the completion of meiosis, even when translation is blocked, suggesting that the protein dissociates from the centromeres but is not degraded at the onset of anaphase II. Unexpectedly, MEI-S332 is present during embryogenesis, localizes onto the centromeres of mitotic chromosomes, and is delocalized from anaphase chromosomes. Thus, MEI-S332 associates with the centromeres of both meiotic and mitotic chromosomes and dissociates from them at anaphase.

Introduction

Cohesion between sister chromatids is essential for proper segregation of chromosomes during mitosis and meiosis. By counteracting spindle forces pulling chromosomes towards the poles, cohesive forces between sister chromatids enable stable bipolar attachments to be established; these in turn allow the sister chromatids to be partitioned appropriately during anaphase. The consequences of inappropriate partitioning can be severe: aneuploidy is observed in many tumors and also in individuals with congenital disorders such as Down syndrome. Defects in sister-chromatid cohesion have been suggested as an important factor that might be involved in oncogenesis or meiotic errors (Orr-Weaver, 1996; Lamb et al., 1996; Lengauer et al., 1997).

In both meiosis and mitosis, cohesion exists between the arms and the centromere regions of the sister chromatids after their replication, but release of sister-chromatid cohesion occurs differently in these two types of cell division (Moore and Orr-Weaver, 1998) In mitosis, the sister chromatids segregate from one another in a single cell division, and thus cohesion is released from both the chromosome arms and centromere regions at the same time, the onset of anaphase. Meiosis consists of two cell divisions that follow a single round of replication: the homologs segregate from one another in the first division, the sister chromatids in the second division. The homologs are typically connected at sites on their arms called chiasmata, and sister-chromatid cohesion along the chromosome arms is believed to be important for the maintenance of chiasmata (Maguire, 1974, 1993). With the onset of anaphase I, this arm cohesion is lost, but cohesion between the centromeric regions of the sister chromatids is maintained. This cohesion in the centromeric region is required to align the sister chromatids for metaphase II

and is released at the beginning of anaphase II. Thus, meiosis is a specialized cell division that requires a two-step release of sister-chromatid cohesion.

The *Drosophila* protein MEI-S332 has been demonstrated both to be essential for cohesion between sister chromatids and to be localized to chromosomes (Goldstein, 1980; Kerrebrock et al., 1992, 1995). These cytological studies were performed in spermatocytes. In male meiosis, MEI-S332 localizes to the centromeric regions of meiotic chromosomes and is maintained there through the metaphase I/ anaphase I transition (Kerrebrock et al., 1995). MEI-S332 is observed on chromosomes in metaphase II but is no longer detectable with the commencement of anaphase II, the time when cohesion between sister chromatids is released. The protein is required primarily for proper segregation during the second meiotic division, because by genetic assays, *mei-S332* mutant males and females have nearly normal segregation during the first meiotic division and high levels of missegregation during the second meiotic division (Davis, 1971; Goldstein, 1980; Kerrebrock et al., 1992). Precociously separated sister chromatids are observed in *mei-S332* spermatocytes in late anaphase I, suggesting that MEI-S332 is vital for centromeric cohesion after the metaphase I/ anaphase I transition (Goldstein, 1980; Kerrebrock et al., 1992). Previous studies have not described the localization of MEI-S332 during female meiosis.

The structure of the meiotic chromatin and the meiotic spindle differs between the sexes in *Drosophila melanogaster* (for review see Orr-Weaver, 1995), so it cannot be assumed that localization of MEI-S332 is the same in both spermatocytes and oocytes. In females, but not in males, synaptonemal complex forms during prophase and reciprocal exchange occurs, resulting in the chiasmata that are assumed to hold homologs together. In males, pairing sites hold the homologs together without synaptonemal complex or reciprocal

exchange between the homologs (for review see McKee, 1996). Another significant difference is that the oocyte metaphase I spindle is thought to be organized by the chromatin rather than by centrosomes (Theurkauf and Hawley, 1992), and this function could require that the meiotic chromosomes have a different structure in females. Finally, oocytes arrest during metaphase I, while spermatocytes normally do not, thus requiring cohesion to be maintained longer. Differences between meiosis in male and female *Drosophila* could impact MEI-S332 localization. Moreover, the existence of alleles that affect male and female meiosis with different severity suggests that there must be some differences in MEI-S332 mechanism between the sexes (Kerrebrock et al., 1992). Whereas *Drosophila* male meiosis has several unusual features, *Drosophila* female meiosis is more typical of meiosis in most eukaryotes; thus, localization of MEI-S332 in oocytes is of particular interest.

Sister chromatids are believed to be held together by proteins until anaphase (for review see Bickel and Orr-Weaver, 1996). The cohesive proteins that hold sister chromatids together could dissociate or could be degraded at the time when the chromatids separate. Studies in both yeast and *Xenopus* extracts have shown that release of cohesion is dependent on proteolysis of some substrates by the cyclin degradation machinery, the anaphase-promoting complex (Holloway et al., 1993; Irniger et al., 1995; Funabiki et al., 1996). This complex could directly proteolyze the cohesive proteins at the chromosomes, or indirectly promote sister-chromatid separation by degrading inhibitors of anaphase. Recent work in budding yeast demonstrates that the Pds1p protein, which acts as an inhibitor of separation, is degraded by the anaphase-promoting complex at the initiation of anaphase (Cohen-Fix et al., 1996; Yamamoto et al., 1996). A second protein more integrally involved in cohesion, the Mcd1p/Scc1p protein, has also been identified (Guacci et al.,

1997; Michaelis et al., 1997). Mcd1p localizes to mitotic chromosomes and dissociates at the metaphase/anaphase transition, but its degradation is slow, and the protein persists after anaphase. Thus, both dissociation and degradation may play important roles in the release of sister-chromatid cohesion. Although the cohesion protein MEI-S332 is not observed on the chromatids after the sister chromatids separate during meiosis II, it is not known whether the protein simply dissociates or is degraded.

In this paper, we look at the localization of MEI-S332 during meiosis in females, and we find that, as in males, the protein disappears from centromeres at anaphase II. The fate of MEI-S332 at the metaphase II/ anaphase II transition is examined using Western blots, and we find that MEI-S332 is not degraded detectably at that time. Because the protein is not degraded, we examine its localization during embryonic mitoses. Although centromeric cohesion also occurs in mitosis, *mei-S332* is not essential for mitotic divisions (Kerrebrock et al., 1992, 1995). Strikingly, we find that the MEI-S332 protein is localized to the centromeric regions of mitotic chromosomes in the embryo.

Results

MEI-S332 localizes to centromeric regions in oocytes

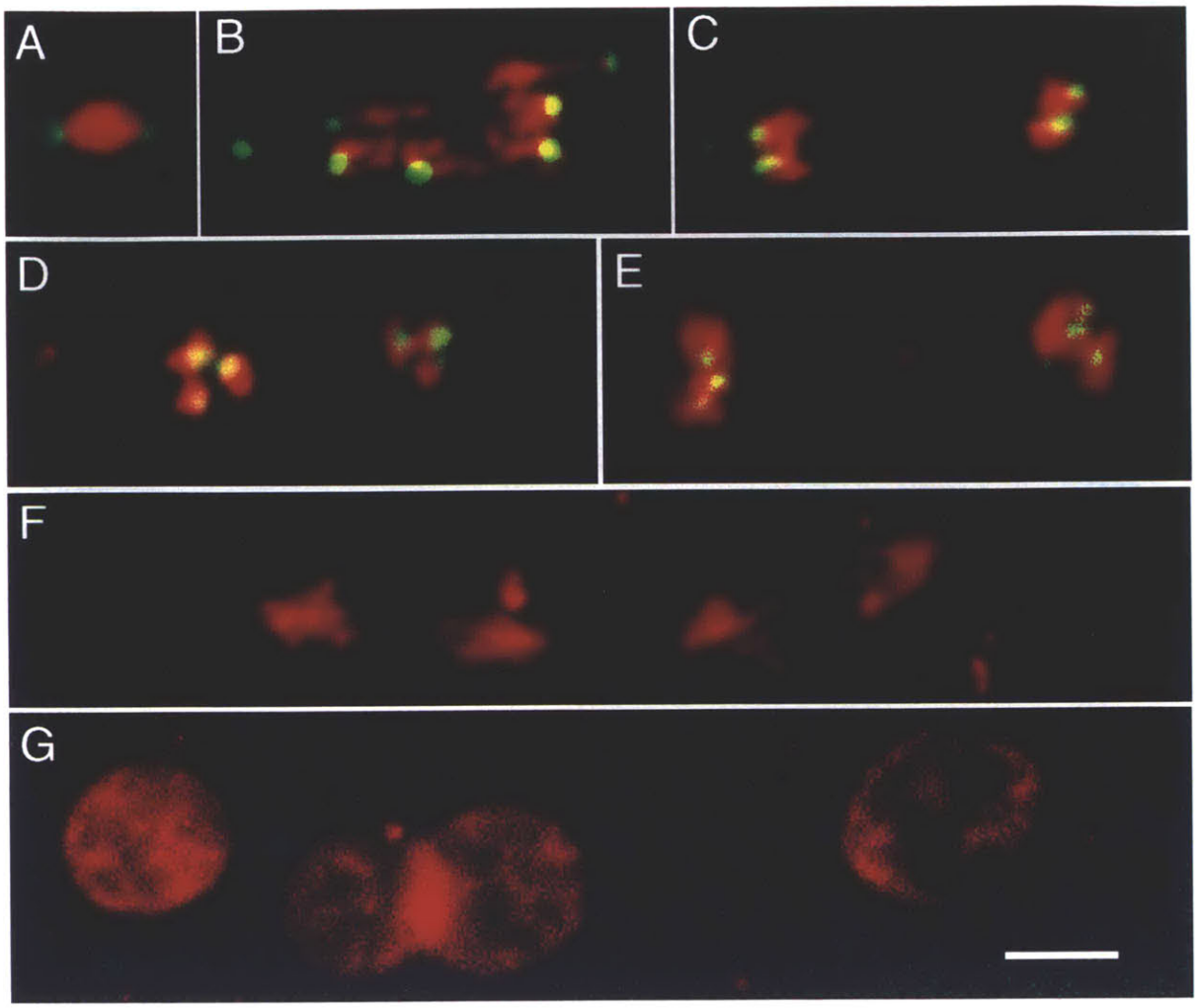
Although the localization of MEI-S332 has been determined in spermatocyte meiosis (Kerrebrock et al., 1995), the differences between male and female meiosis in *Drosophila melanogaster* and the existence of *mei-S332* alleles that affect the two sexes with different severity led us to ask where MEI-S332 is localized in oocyte meiotic divisions. Specifically, we asked whether it localizes to meiotic centromeres, and if so, what is the fate of the protein when the sisters separate at anaphase II.

To visualize the MEI-S332 protein in oocytes, we used a fusion of GFP to the NH_2-terminal end of *mei-S332* (*mei-S332*$^+$::*GFP*) that has been shown to complement fully the mutant phenotype in both males and females (Kerrebrock et al., 1995). In *Drosophila*, mature oocytes arrest at metaphase I with a tapered spindle and an elongated nucleus. We examined fixed oocytes stained for DNA and observed that MEI-S332-GFP was present in two caps at opposite ends of the oocyte nucleus (Figure 3-1A). The orientation of the caps with respect to the morphology of the oocyte nucleus suggested that these caps were facing the poles of the metaphase I spindle, and tubulin staining later confirmed this interpretation (see below). Because it has been shown that the centromeric regions of chromosomes are positioned on opposite sides of the chromatin mass during the metaphase I arrest in *Drosophila* oocytes (Dernburg et al., 1996), it was likely that caps of MEI-S332-GFP represented centromeric localization.

We wanted to determine what happens to these caps of MEI-S332 when the meiotic cell cycle resumes after the oocyte arrest. In particular, we sought to observe the localization of the protein during anaphase I, when centromeric localization would be most apparent, and observe what happens to the protein

Figure 3-1. MEI-S332-GFP localizes to centromeric regions of female meiotic chromosomes until anaphase II.

MEI-S332-GFP is shown in green and chromatin in red. (*A*) Unactivated stage-14 oocytes are arrested in metaphase I, with MEI-S332-GFP localized to two discrete sites on the opposite ends of the condensed chromosomes. (*B*) At the onset of anaphase I, eight dots of MEI-S332-GFP are visible at the leading edges of the separating anaphase chromosomes, one per pair of sister chromatids, with the fourth chromosomes closest to the poles. Chromosome *4* in *Drosophila* is very small and sometimes difficult to visualize. (*C*) In late anaphase I, MEI-S332-GFP is still detected at the leading edges the chromosomes, which become shorter and rounder as they approach the poles. (*D*) Between the first and second meiotic divisions, nuclear decondensation does not occur. Rather, two clusters of 3-4 chromatin balls are observed. Each ball most likely represents a pair of sister chromatids and is associated with a dot of MEI-S332-GFP. (*E*) In metaphase II, the chromatin balls move together to form metaphase plates, and MEI-S322-GFP localizes to the middle of the chromatin. (*F*) When sister-chromatid cohesion is released at anaphase II, the sister chromatids separate, and MEI-S332-GFP is no longer detectable on the meiotic chromosomes. (*G*) During the postmeiotic interphase, MEI-S332-GFP is not visible on the decondensed chromosomes. Oocytes were isolated from females carrying four copies of the *mei-S332*$^{+}$::*GFP* transgene, activated in vitro, fixed, and stained with propidium iodide. Images were collected using confocal microscopy. Bar, ~5 μm.


A
B
C
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at the metaphase II/ anaphase II transition when the sister chromatids separate. Historically, it has been difficult to observe any of the stages of female meiosis that follow the metaphase I arrest in *Drosophila* oocytes, but recent advances in egg activation in vitro now allow all the stages of meiosis to be examined (Page and Orr-Weaver, 1997). Accordingly, oocytes from mothers carrying the *mei-S332*$^{+}$::*GFP* transgene were activated in vitro to complete meiosis, then fixed and stained for DNA. Oocytes in anaphase I had 8 pairs of sister chromatids, four on each side, as is expected since the haploid chromosome number in *Drosophila* is four. Such oocytes also had 8 dots of MEI-S332-GFP visible at the leading edges of the separating chromosomes, one per pair of sister chromatids (Figure 3-1B). The observation that each pair of sister chromatids had MEI-S332 at their leading edge argued strongly that MEI-S332 is localized at the centromeric regions of chromosomes in female meiosis. MEI-S332-GFP was continually visible on the chromosomes between anaphase I and metaphase II (Figure 3-1B-E and see below). When sister-chromatid cohesion was released at anaphase II, the sister chromatids separated, and for the first time during the meiotic divisions, MEI-S332 was not observed on the chromosomes (Figure 3-1F). After the meiotic divisions, the chromatin decondensed into four nuclei (three polar bodies and one pronucleus) in the post-meiotic interphase. MEI-S332-GFP was not detectably localized during the post-meiotic interphase (Fig. 3-1G).

The cytology of nuclei between the meiotic divisions has been difficult to observe in oocytes. Indeed, even with the in vitro activation system, the lack of familiar cytological landmarks between anaphase I and metaphase II has meant that it was still unknown what happened to chromosome morphology between the divisions. Although it is known that in *Drosophila* male meiosis the telophase I nuclei decondense and then recondense for meiosis II (Cenci et

al., 1994), it was unclear whether such decondensation occurred in *Drosophila* females. In experiments activating hundreds of *mei-S332*$^{+}$::*GFP* transgenic oocytes, we never observed oocytes with only two decondensed nuclei, in agreement with our unpublished observations with oocytes from non-transgenic flies. Thus it appears that *Drosophila* oocyte nuclei remain condensed throughout meiosis until telophase II.

Since it was clear from the early anaphase I figures that MEI-S332-GFP labels the centromeric regions of oocyte meiotic chromosomes (Figure 3-1B), we were able to use it as a tool in deducing the order of events in chromatin remodeling between anaphase I and metaphase II. We observed that late anaphase I chromosomes appear to become shorter and rounder as they approach the poles, but despite these morphological changes they could always be identified by the leading edge of MEI-S332-GFP at the centromere (Figure 3-1C). Between the divisions, the chromosomes rounded up and formed two clusters of three or four individual balls of chromatin (Figure 3-1D). Each ball was associated with a dot of MEI-S332-GFP, but the dots were no longer oriented at the leading (outside) edge of the chromosomes. We think it likely that each ball represents the sister chromatids of each of the three large chromosomes, with the small fourth chromosome only sometimes visible. Metaphase II was evident when the clusters of chromatin balls compacted to form metaphase plates, usually parallel to each other, with MEI-S332-GFP in the middle of the compacted chromatin (Figure 3-1E). Often, as in Figure 3-1E, the two nuclei were slightly out of synchrony. Even though there is no decondensation between the meiotic divisions, a series of interesting changes occurs in chromosome morphology between anaphase I and metaphase II.

When does MEI-S332 localize to centromeres?

In spermatocytes, MEI-S332 protein is observed in the cytoplasm during prophase I, and it is localized to the chromosomes as they compact for prometaphase I (Kerrebrock et al., 1995). We examined when and how MEI-S332 is localized prior to metaphase I in oocytes, since there are marked differences between spermatocytes and oocytes during prophase I. The origin of the cytoplasm in oocytes differs from that in spermatocytes, because much of it is created in the nurse cells, and the volume of cytoplasm is much greater in oocytes than in spermatocytes. Another important difference is that synaptonemal complex is seen on oocyte chromosomes but not on spermatocyte chromosomes. Sex-specific differences in the origin and amount of cytoplasm or in the structure of the meiotic chromosomes suggested that the timing of MEI-S332 localization should be examined in oocytes to see if it differed from spermatocytes.

To examine MEI-S332 localization in oocytes during early developmental stages, ovaries were dissected from females carrying the *mei-S332*$^{+}$*::GFP* transgene, fixed and stained for DNA (data not shown). MEI-S332-GFP was not observed in egg chambers during prophase I, corresponding to oocyte development through stage 12, either in the cytoplasm or on the condensed meiotic chromosomes in the karyosome. Multiple foci of MEI-S332-GFP were first observed on the meiotic chromatin after the chromatin compacted into the small round mass characteristic of prometaphase I. Using egg chamber morphology to judge developmental stage, we determined that these foci first appeared in stage 13. By stage 14, MEI-S332-GFP was observed in two caps on either side of the nucleus (Figure 3-1A and see below).

Because the meiotic spindle is organized shortly after the chromatin compacts, we further characterized the localization of MEI-S332-GFP with

respect to formation of the spindle by isolating stage 13 and 14 oocytes, and labeling both the DNA and tubulin. After compaction of the chromatin in stage 12, the nuclear envelope breaks down and short microtubule fibers captured by the chromatin subsequently coalesce into a bipolar spindle during stage 13 (Theurkauf and Hawley, 1992). The earliest stage at which MEI-S332-GFP was observable was coincident with the beginning of spindle formation. A small number of dots of MEI-S332-GFP were distributed throughout the chromosomal mass (Figure 3-2A). When spindles appeared more bipolar and elongated, typical of late stage 13 and stage 14 oocytes, the MEI-S332-GFP foci were more clearly combined into caps on the ends of the chromatin mass that face the spindle poles (Figure 3-2B,C).

The metaphase II/anaphase II transition

In both female and male meioses MEI-S332 was not visible on the sister chromatids after they separated at anaphase II; consequently we investigated what happened to the protein when sister-chromatid cohesion was released. In yeast and *Xenopus* mitosis, an inhibitor of sister-chromatid separation is degraded by the cyclin destruction machinery at the metaphase/anaphase transition (Holloway et al., 1993; Irniger et al., 1995; Cohen-Fix et al., 1996). Because MEI-S332 is essential for sister-chromatid cohesion, it seemed plausible that it might be degraded at the metaphase II/ anaphase II transition.

To study protein levels directly, we generated polyclonal rabbit antibodies against a peptide corresponding to the COOH-terminal fragment of the MEI-S332 protein (Figure 3-3A). Affinity-purified antibodies recognized a band of ~55 kD on a Western blot of ovary and oocyte extracts (Figure 3-3B). This band was absent in extracts made from *mei-S332*^{7} oocytes and ovaries

Figure 3-2. MEI-S332-GFP assembly onto female meiotic chromosomes correlates with spindle formation. MEI-S332-GFP is shown in green, tubulin in blue, and chromatin in red. The images are also separated to show the individual channels. (*A*) MEI-S332-GFP is first observed on the meiotic chromosomes at multiple discrete sites before the formation of a bipolar spindle. (*B*) As the spindle becomes increasingly elongated and bipolar, the discrete dots of MEI-S332-GFP begin to cluster at opposite ends of the chromatin mass. (*C*) When the spindle is fully elongated, MEI-S332-GFP is observed in two caps at the opposite ends of the chromatin mass, aligned with the bipolar spindle. Oocytes were isolated from females carrying four copies of the *mei-S332*$^{+}$::*GFP* transgene, fixed, and stained with anti-tubulin antibodies and DAPI. Images were collected using a CCD camera. Bar, ~5 μm.

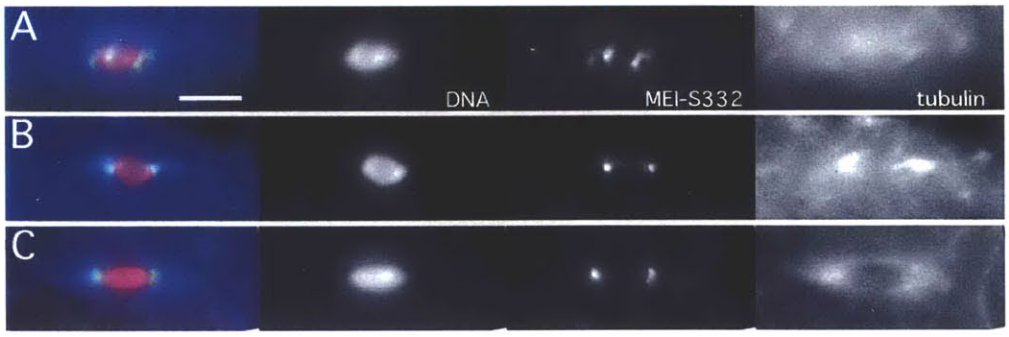
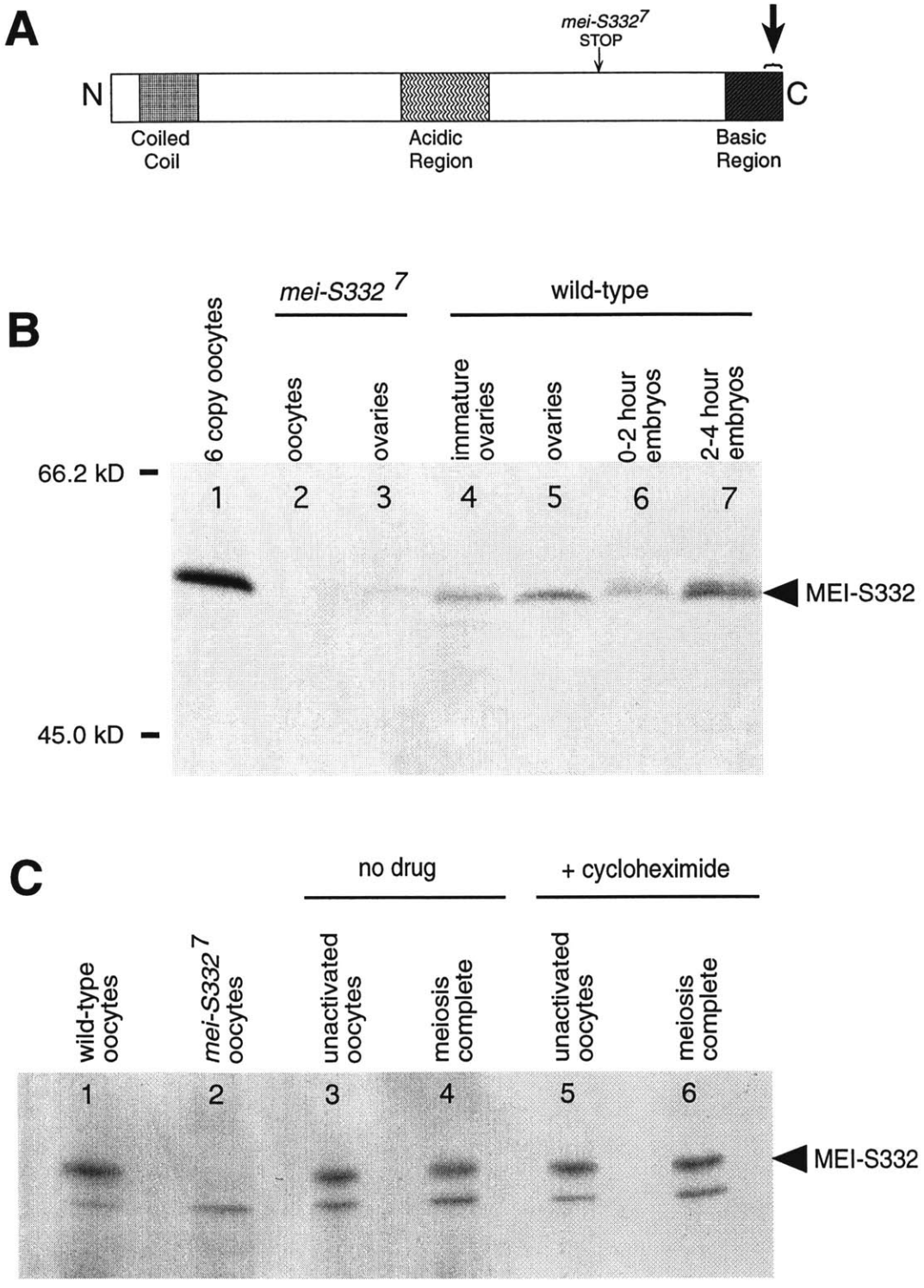

A
DNA
MEI-S332
tubulin
B
C

Figure 3-3. The MEI-S332 protein is present in embryos and is not globally degraded at the metaphase II/ anaphase II transition.

(*A*) A schematic of the MEI-S332 protein. Anti-MEI-S332 antibodies were generated against a COOH-terminal 15-amino acid peptide of MEI-S332 (large arrow). Tissues from *mei-S332*^{7} flies were used as negative controls for the antibodies because the *mei-S332*^{7} mutation generates a truncated form of the protein that lacks the epitope for the COOH-terminal peptide antibodies (small arrow). (*B*) The MEI-S332 protein, predicted to be 44kD, is recognized as a 55-kD band on Western blots by affinity-purified anti-MEI-S332 peptide antibodies. Higher levels of MEI-S332 (lane 1) are seen in oocytes isolated from females carrying six copies of the *mei-S332*$^{+}$ gene (two endogenous copies and 4 copies of a genomic fragment). MEI-S332 is present in previtellogenic ovaries (lane 4), mature ovaries (lane 5), 0-2 hour embryos (lane 6), and 2-4 hour embryos (lane 7). There appear to be different mobility forms of MEI-S332 in embryos. As expected, the 55-kD band is not detected in *mei-S332*^{7} oocytes and ovaries (lanes 2 and 3). (*C*) MEI-S332 protein levels remain essentially unchanged in activated eggs that have completed meiosis (compare lanes 3 and 4). Although MEI-S332 is no longer detectable on the chromosomes when sister-chromatid cohesion is lost, it is not degraded globally. Protein levels remain unchanged when meiosis is completed in the presence of the translational inhibitor cycloheximide (compare lanes 5 and 6). *Oregon-R* and *mei-S332*^{7} unactivated oocytes were used as positive and negative controls, respectively, for the antibodies (lanes 1 and 2). The lower non-specific band, probably an artifact of this sample preparation, is not MEI-S332 as it is still present in extracts from *mei-S332*^{7} oocytes (lane 2).


A
mei-S332 7
STOP
N
C
Coiled Coil
Acidic Region
Basic Region
B
mei-S332 7
wild-type
6 copy oocytes
oocytes
ovaries
immature ovaries
ovaries
0-2 hour embryos
2-4 hour embryos
66.2 kD
45.0 kD
1 2 3 4 5 6 7
MEI-S332
C
no drug
+ cycloheximide
wild-type oocytes
mei-S332 7 oocytes
unactivated oocytes
meiosis complete
unactivated oocytes
meiosis complete
1 2 3 4 5 6
MEI-S332

(Figure 3-3B, lanes 2 and 3). Extracts from *mei-S332*^{7} homozygotes and hemizygotes provided a critical negative control, as this mutation creates a nonsense codon that prematurely truncates the protein so that it lacks the epitope for the COOH-terminal peptide antibodies (Figure 3-3A). As additional evidence that the identified band is MEI-S332, we probed extracts from transgenic ovaries that had 4 extra copies of a genomic *mei-S332*$^{+}$ fragment, in addition to the two endogenous copies, and we found that the band was significantly more intense (Figure 3-3B, lane 1). These data lead us to conclude that the peptide antibodies recognize the MEI-S332 protein as a 55-kD band on Western blots. This protein migrates during electrophoresis as a 55-kD band even though its predicted size is 44 kD.

To determine whether MEI-S332 is degraded at the metaphase II/ anaphase II transition we analyzed in vitro activated oocytes. Sixty minutes after activation, eggs can be selected so that 95-99% have completed meiosis (Page and Orr-Weaver, 1997). We compared MEI-S332 protein levels between extracts of unactivated oocytes, which have MEI-S332 localized to the chromosomes (Figure 3-1A), and extracts of eggs that have passed through the metaphase II/ anaphase II transition after activation for 60 minutes. On Western blots, these protein levels remained essentially unchanged (Figure 3-3C, lanes 3 and 4), a result that was repeated several times. This suggests that although the protein dissociated from the chromosomes at anaphase II, it was not degraded.

Although the total levels of MEI-S332 remained constant before and after meiosis was completed, we were concerned that continuing translation of new MEI-S332 protein might mask protein degradation. To address this concern, we activated oocytes in the presence of the translational inhibitor cycloheximide. Metabolic labeling experiments have demonstrated that

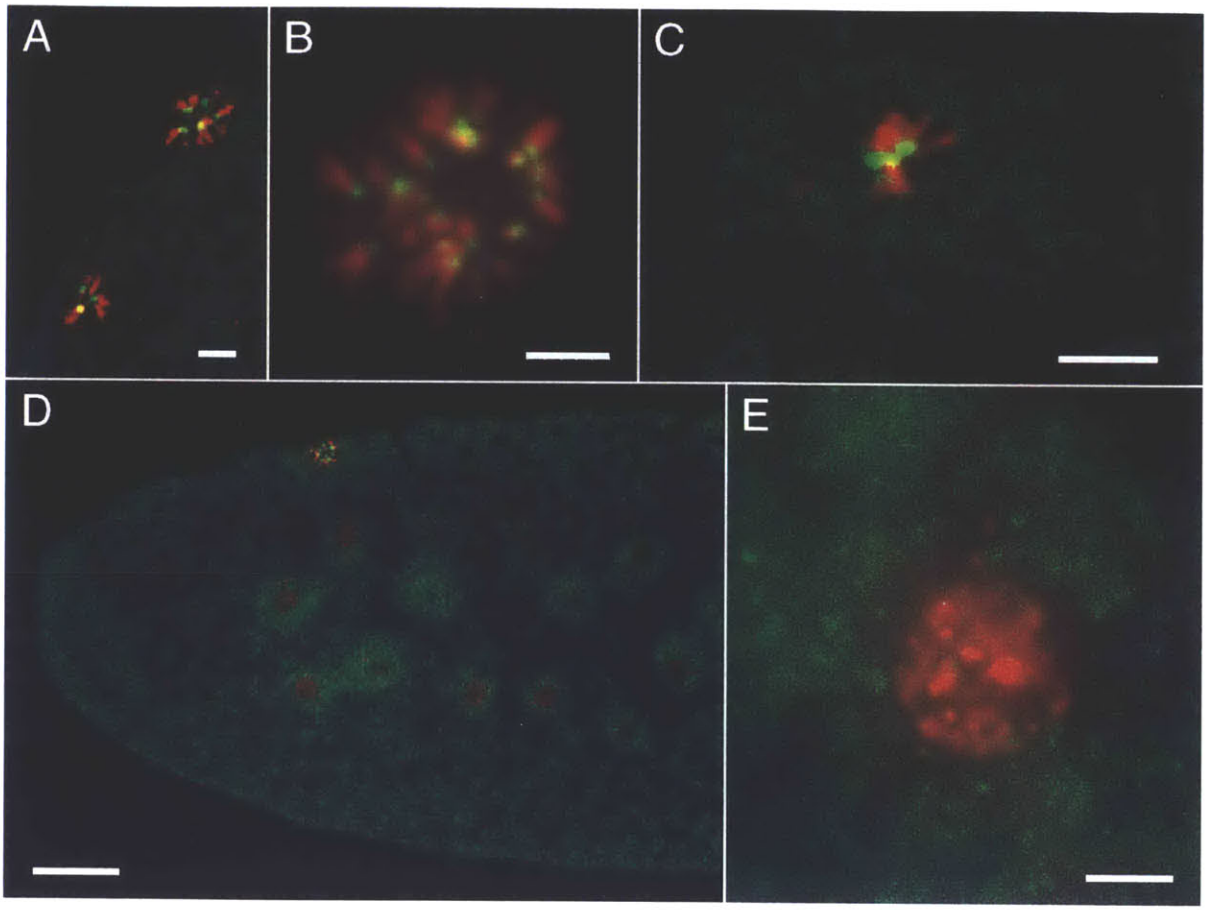
oocytes activated in the presence of cycloheximide have protein synthesis inhibited to about 5% of wild-type levels, but that about 95% of oocytes still complete meiosis under these conditions, arresting at the post-meiotic interphase (Page and Orr-Weaver, 1997). Western blotting of extracts from arrested, unactivated oocytes incubated in cycloheximide, compared to extracts from oocytes activated in the presence of cycloheximide, further demonstrated that there was no detectable degradation of MEI-S332 during meiosis, suggesting that it instead delocalized (Figure 3-3C, lanes 3-6).

MEI-S332 during mitosis

The phenotype of *mei-S332* mutants was previously shown to be exclusively meiotic and not mitotic: no cytological defect has been detected in proliferating tissues, mutants are completely viable, and no increase in somatic clones from mitotic errors is observed (Kerrebrock et al., 1992, 1995). However, our finding that the protein was not degraded at anaphase II led us to ask whether the protein persisted in the developing embryo. We examined extracts from wild-type oocytes and embryos by Western blotting, and we found significant amounts of MEI-S332 in a collection of embryos of ages 0-2h (Figure 3-3B, lane 6). The protein level appeared to increase in populations of embryos of ages 2-4h (Figure 3-3B, lane 7), suggesting that MEI-S332 did not merely persist into embryogenesis, but could be playing a role there. Additionally, we noted that there appeared to be different mobility forms of MEI-S332, an observation that is currently under investigation.

We used the *mei-S332*$^{+}$::*GFP* transgene to determine whether MEI-S332 could localize onto chromosomes in the embryo, and we observed persistent localization of the protein on polar body rosettes (Figure 3-4A, B). Chromosomes from the unused meiotic products are pulled into a radial

Figure 3-4. MEI-S332-GFP localizes to condensed chromosomes in embryos. MEI-S332-GFP is shown in green and DNA in red. (*A*) MEI-S332-GFP is present on the polar body rosettes. (*B*) A close-up image of a polar body rosette shows punctate MEI-S332-GFP localization on the inside ring of the rosette where centromeres are believed to be pulled to the center. 22 dots of MEI-S332-GFP can be counted in the single rosette found in this embryo. (*C*) MEI-S332-GFP localizes to discrete dots on a mitotic metaphase plate, resembling those on meiotic metaphase II chromosomes. In addition, a cloud of diffuse MEI-S332-GFP is observed around each mitotic nucleus. (*D*) MEI-S332-GFP is detected in clouds surrounding the interphase nuclei. The nuclei are not centered within the clouds. (*E*) A close-up image of the interphase nucleus demonstrates the absence of MEI-S332-GFP localization on the decondensed interphase chromatin. Embryos were collected from females carrying four copies of the *mei-S332*$^{+}$::*GFP* transgene, fixed, and stained with either propidium iodide or DAPI. Images in (*A*), (*C*), and (*D*) were collected using confocal microscopy, and images in (*B*) and (*E*) were collected using a CCD camera. Bars: (*A-C*, *E*) ~ 5 μm; (*D*) ~ 30 μm.

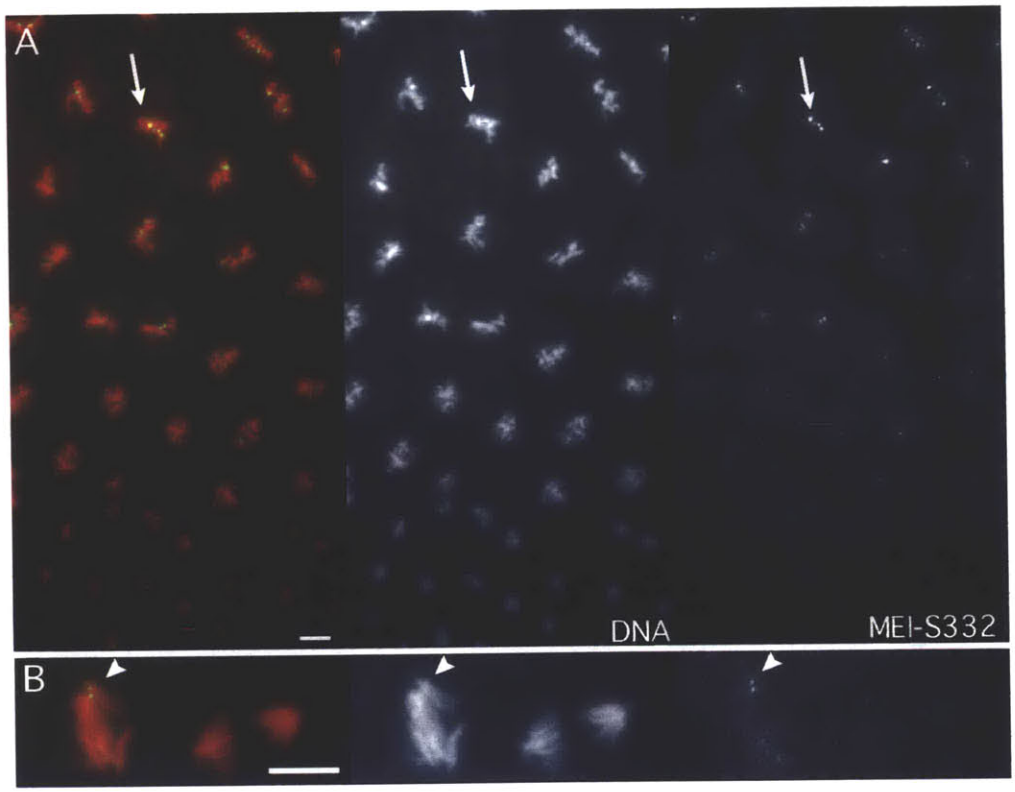

A
B
C
D
E

formation by a sphere of tubulin, after replicating and condensing into a metaphase-like state. These are found in the anterior dorsal quadrant of early embryos, typically fused so that there exist only one or two rosettes (Foe et al., 1993). MEI-S332-GFP localized to the condensed chromosomes facing the inside of the rosette, where centromeres are expected to be located (Foe et al., 1993). Moreover, when all the unused meiotic chromosomes have fused into a single rosette formation, the number of chromosomes should be 12, or after replication 24, and we count ~24 foci of MEI-S332-GFP in a typical single rosette formation (Fig. 3-4B). As in meiosis, MEI-S332 localized to the apparent centromeric regions of replicated sister chromatids.

MEI-S332-GFP also localized to condensed chromosomes in the early mitotic divisions. *Drosophila* embryos have 13 syncytial nuclear division cycles before gastrulation. On condensed prometaphase and metaphase chromosomes of these early cycles we observed MEI-S332-GFP in punctate dots resembling those on meiotic chromosomes, consistent with centromeric localization (Figure 3-4C). These punctate dots were not observed in interphase nuclei (Figure 3-4D, E). In addition to chromosome localization, diffuse clouds of fluorescence were observed in the vicinity of each mitotic nucleus (Figure 3-4C). Similar diffuse clouds of MEI-S332-GFP fluorescence were evident near interphase nuclei (Figure 3-4D,E) and produced a signal brighter than the background autofluorescence in embryos lacking the transgene (data not shown). These clouds of fluorescence may correspond to energids, regions of yolk-free cytoplasm that have been observed in the early cycles of *Drosophila* embryos (Foe et al., 1993). Immunofluorescence with anti-peptide antibodies confirmed the localization to polar body chromosomes and condensed mitotic chromosomes (data not shown).

In later syncytial divisions, the nuclei migrate to the surface of the embryo, and mitosis proceeds in a wave across the embryo. We examined mitotic chromosomes in these easily visualized nuclei to analyze localization of MEI-S332 during the metaphase/anaphase transition in mitosis. To simulate the same *mei-S332* gene dosage as that of wild-type oocytes, embryos from mothers hemizygous for *mei-S332*^{7} and carrying two copies of the *mei-S332*$^{+}$::*GFP* transgene were examined after fixation in formaldehyde and DNA staining. MEI-S332-GFP was observed in bright dots aligned on the metaphase plates with the chromatin (Figure 3-5A; see arrow for one example). Sometimes much dimmer dots of MEI-S332-GFP were observed on chromosomes in early anaphase (Figure 3-5B, arrowhead). The residual MEI-S332-GFP was found on the leading edge of chromosomes. By late anaphase and telophase, no MEI-S332-GFP was observed on any of the chromatin. Thus, the metaphase/anaphase transition begins a process of delocalization of MEI-S332. The alignment of the dots on the metaphase plate and the association of residual MEI-S332-GFP with the leading edges of chromosomes strongly suggests that MEI-S332 is localized to the centromeric regions of mitotic chromosomes. Thus, in mitosis as in meiosis, MEI-S332 is localized to the centromeric regions of chromosomes condensed for metaphase, and MEI-S332 begins to dissociate from the chromatin when cohesion is lost and the sister chromatids segregate.

Figure 3-5. MEI-S332-GFP disappears from centromeres at the metaphase/ anaphase transition in embryos. MEI-S332-GFP is shown in green, DNA in red. Images are also separated to show individual channels as labeled. (*A*) A field of syncytial nuclei in a cycle 12 embryo is in the process of mitosis. In each panel, metaphase figures are on the top, anaphase figures in the middle, and late anaphase figures on the bottom. MEI-S332-GFP localizes to discrete dots on the mitotic metaphase plates (arrow). MEI-S332-GFP is no longer detectable on mitotic chromosomes in late anaphase. (*B*) MEI-S332-GFP can be seen at the leading edge of the chromosomes in early anaphase (arrowhead), but it is no longer detectable on mid-anaphase chromosomes. Embryos were collected from *mei-S332*^{7} females carrying two copies of the *mei-S332*$^{+}$*::GFP* transgene. Images were collected using a CCD camera. Bars, ~ 5 μm.


A
DNA
MEI-S332
B

Discussion

In this study we examined the expression and localization of MEI-S332 in *Drosophila* oocytes and embryos. We found that in oocytes, MEI-S332 localizes to the centromeric region of condensed meiotic chromosomes from prometaphase I until the metaphase II/ anaphase II transition, when sister chromatids separate. This is essentially the same localization pattern as has been observed in spermatocyte meiosis (Kerrebrock et al., 1992). It is striking that although no mitotic phenotype has been observed in *mei-S332* mutants (Kerrebrock et al., 1995), MEI-S332 protein has a similar localization pattern in the early mitotic divisions in the embryo, where it appears bound to condensed chromosomes until the sister chromatids separate at anaphase. On the chromosomes of polar bodies, which are constitutively condensed in a configuration analogous to metaphase, MEI-S332 is consistently observed at the expected centromeric regions. Thus MEI-S332 appears localized to centromeres of metaphase chromosomes in each of these three different cell cycles, and it is dispersed each time sister chromatids separate.

MEI-S332 and the metaphase/anaphase transition

Precisely what happens to MEI-S332 when sister chromatids separate at anaphase is a question of great interest. One possibility is that the protein is degraded at the metaphase/anaphase transition. To test this idea, we examined the levels of MEI-S332 in oocytes before and after the completion of meiosis. We found that even in the presence of cycloheximide to prevent new protein synthesis, the levels of MEI-S332 appeared unchanged before and after the metaphase II/ anaphase II transition. This result demonstrates that on a global level MEI-S332 is not degraded at anaphase II. Although we have not directly examined the question of degradation in mitosis, the observation

that MEI-S332 protein is visible in clouds around interphase nuclei strongly supports the idea that it is not degraded on a global level in the developing embryo during the syncytial divisions. Still, we cannot exclude the possibility that centromere-localized protein is locally degraded at either the metaphase II/ anaphase II transition in oocytes or at the mitotic metaphase/anaphase transition. If a subpopulation of MEI-S332 was degraded at anaphase II, however, the amount degraded would have to be insignificant compared to the persisting fraction, since we do not observe any decrease in protein levels by Western blotting.

A second possibility is that dissociation of MEI-S332 from the centromeric regions triggers sister-chromatid separation. An analogous mechanism may occur in the yeast *S. cerevisiae*, because the Mcd1p/Scc1p cohesion protein localized on the chromosomes is not degraded until after anaphase. Instead it is removed from the chromosomes beginning at anaphase (Guacci et al., 1997; Michaelis et al., 1997). Noting that MEI-S332 appears to run as a doublet on Western blots, we speculate that dissociation of MEI-S332 may be regulated at some level by phosphorylation. Consistent with this speculation, the MEI-S332 protein has 30 possible phosphorylation sites recognized by protein kinase C, casein kinase II, cAMP-dependent protein kinase, and tyrosine protein kinase.

There is a third possibility, however, that MEI-S332 may first be inactivated to permit anaphase movement and subsequently dissociate from the chromosomes. This model is supported by our detection of MEI-S332 on the centromeres of chromosomes in early anaphase, although the levels are reduced compared to metaphase. Similarly, some Mcd1p/Scc1p remains localized to the chromosomes in anaphase (Michaelis et al., 1997; Guacci et al., 1997). We cannot distinguish between these two latter models at this point,

because it is possible that sufficient amounts of MEI-S332 or Mcd1p/Scc1p dissociate at the metaphase/anaphase transition to permit sister-chromatid separation. Residual levels may then be removed subsequently.

Establishment versus maintenance of sister-chromatid cohesion

In spermatocytes, oocytes, and early embryos, MEI-S332 is not detectable on the chromosomes until prometaphase. It is possible that sister-chromatid cohesion is not fully established until this point and that the localization of MEI-S332 marks the establishment of cohesion. It may be the case, however, that cohesion is established immediately after DNA replication. In FISH studies done in yeast, separate signals from the two sister chromatids were not observed until anaphase, indicating that sister chromatids are tightly associated from the time of their replication (Guacci et al., 1993, 1994). This suggests that cohesion is established during S phase. If this is true, then MEI-S332 may be required to maintain or augment cohesion when spindle forces come into play, rather than to establish cohesion. For example, it may serve to protect and preserve proteins directly attaching the sister chromatids until anaphase.

A mitotic role for MEI-S332?

We were surprised to find that MEI-S332 localizes to mitotic chromosomes in much the same way it localizes to meiotic chromosomes in spermatocytes and oocytes because no function has been ascribed to MEI-S332 in mitosis. The presence of MEI-S332 on mitotic chromosomes is not unique to the early embryonic cycles. MEI-S332 protein is present in dividing larval tissues and can localize to the chromosomes during mitosis (LeBlanc, H., T.T. Tang, and T.L. Orr-Weaver, unpublished results). We and our colleagues

have undertaken careful phenotypic analyses of *mei-S332* mutants in order to determine whether the protein is required for mitosis. Viability studies have demonstrated that *mei-S332* homozygotes and their heterozygous siblings survive equally (Kerrebrock et al., 1992), even when the maternal *mei-S332* contribution is eliminated (LeBlanc, H., and T.L. Orr-Weaver, unpublished data). Examinations of large numbers of larval brains, a mitotically active tissue that when squashed flat gives excellent mitotic cytology, demonstrated no significant difference in mitotic index or premature sister-chromatid separation between *mei-S332* hemizygous (*mei-S332/Df*) and wild-type larval brains (Kerrebrock et al., 1995). Furthermore, experiments testing the frequency of chromosome missegregation in the developing wing demonstrated no significant difference between *mei-S332* hemizygotes and their heterozygous siblings (Kerrebrock et al., 1995).

If MEI-S332 is localized to mitotic centromeres, why do we not see a phenotype in *mei-S332* mutants? One possibility is that in mitosis there is redundancy in the mechanisms that hold sister chromatids together. The simplest model for redundancy is that both MEI-S332 and another protein act independently to bind sister chromatids together at the centromeric regions in mitosis, and therefore no phenotype is observed when *mei-S332* is mutated. Currently there are no candidates for such a protein. Although mutations have been characterized in three genes that encode *Drosophila* centromere-binding proteins, none appear to promote sister-chromatid cohesion. The HP1 and PROD proteins affect centromere condensation and presumably kinetochore function (Kellum and Alberts, 1995; Torok et al., 1997), whereas ZW10 may monitor spindle attachment to the kinetochore (Williams et al., 1996). Another version of this redundancy model is that whereas MEI-S332 acts at mitotic centromeres to attach sister chromatids, other proteins act

along the lengths of the chromatid arms to ensure cohesion and proper orientation with respect to the mitotic spindle. The loss of MEI-S332 would result in the loss of centromeric cohesion, but this would not have phenotypic consequences in mitosis because arm cohesion would be sufficient to hold the chromatids together. This redundancy is not provided solely by ORD, a *Drosophila* protein required for arm cohesion in meiosis, because flies lacking both *mei-S332* and *ord* have demonstrated no abnormalities in somatic mitoses (Bickel, S.E., D.P. Moore, C. Lai, and T.L. Orr-Weaver, manuscript submitted for publication).

Alternatively, it is possible that MEI-S332 does play a nonredundant role in mitosis, but it is required only in response to perturbations of the cell cycle. For example, if it were necessary for a cell to delay the onset of anaphase, persistence of MEI-S332 at the centromeric regions could, in principle, restrain the sister chromatids from separating. The discovery of a mitotic phenotype, under any conditions, would greatly enhance our understanding of the mitotic function of MEI-S332.

Meiotic cytology

Because MEI-S332 localizes to centromeres throughout meiosis until anaphase II, we were able to use it as a tool to examine meiotic chromosome morphology. In metaphase I arrested oocytes, the two caps of MEI-S332-GFP demonstrate that the centromeric regions of homologs are closest to the spindle poles during metaphase I, as would be anticipated if homologs are connected by chiasmata on the chromosome arms. Again using MEI-S332 to identify centromeres and chromosome orientation, we were able to infer an order of events after anaphase I. We found that in *Drosophila* as in *Xenopus* and other organisms, oocyte chromosomes do not decondense between the two

meiotic divisions (Murray and Hunt, 1993), in contrast to spermatocyte chromosomes that do decondense between the divisions (Cenci et al. 1994).

Conclusions

Our finding that MEI-S332 is present on both meiotic and mitotic chromosomes reinforces the idea that meiosis and mitosis are highly conserved processes, even at the molecular level. In both types of divisions, it is localized to the centromeric regions of sister chromatids aligned on a bipolar spindle, and it is no longer present on the sister chromatids when they segregate from one another in anaphase. The function of MEI-S332 is essential during meiosis and not mitosis probably because of the meiosis-specific requirement that sister chromatids remain attached in the centromeric region during the first meiotic cell division. It is ironic that MEI-S332 is now implicated in mitosis, because if it had a strong mitotic phenotype, lethality would have hindered the genetic and cytological analyses that defined its role in sister-chromatid cohesion. Our findings indicate that the analysis of meiosis will lead to a deeper understanding of chromosome segregation mechanisms in general.

Materials and methods

Fly Strains

In the studies of MEI-S332-green fluorescent protein (GFP) localization in oocyte meiosis, females of genotype *y w P{+mc* 5.6KK *meiS332+::GFP= GrM}-13; P{w+mc* 5.6KK *mei-S332+::GFP=GrM}-1*, containing four copies of the fusion transgene *mei-S332+::GFP* (Kerrebrock et al., 1995) and two endogenous copies of *mei-S332+*, were used. (The insertion of the transgene on the *X* chromosome is named *P{GrM}-13*; the insertion on chromosome *2* is named *P{GrM}-1.*) For localization of MEI-S332-GFP in embryos, mothers of the genotype described above or mothers carrying only two copies of the fusion transgene *mei-S332+::GFP* in the *y; mei-S332^{7} / Df(2R)X58-6* background were used. The latter flies were generated by crossing *y w P{GrM}-13; cn mei-S332^{7} px sp / SM1* females to *y w P{GrM}-13 / y+Y; Df(2R)X58-6 pr cn / SM1* males.

In studying the MEI-S332 levels in oocytes before and after activation (Figure 3-3C), *y w* females were used. Embryos and oocytes from *y; pr cn mei-S332^{7} bw sp / Df(2R)X58-6, pr cn* and *y; pr cn mei-S332^{7} bw sp / cn mei-S332^{7} px sp* females were used as negative controls for the anti-MEI-S332 peptide antibodies (Kerrebrock, et al., 1992 and see below). *Oregon-R* (wild type) was used as the negative control for GFP fluorescence microscopy and positive control for Western blot analysis (Figure 3-3B). For protein extracts from overexpressing oocytes, oocytes were obtained from females carrying 6 copies of the *mei-S332+* gene (two endogenous copies and 4 copies from homozygous insertions of *P{w+mc* 5.6 KK *mei-S332+}* on the second and third chromosomes; Kerrebrock et al., 1995). In all the *mei-S332* transposons the gene was expressed from the normal genomic regulatory regions.

Meiosis in Activated Eggs

The cytology of activated eggs was performed essentially as described in Page and Orr-Weaver (1997) with changes in the fixation conditions to preserve the GFP fluorescence. 300 females of genotype *y w P{GrM}-13; P{GrM}-1* were fattened on wet yeast for several days. Flies were disrupted in IB (55 mM NaOAc, 40 mM KOAc, 110 mM sucrose, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM HEPES, final pH 7.4) in a blender, and oocytes were isolated by filtration and gravity settling. This isolation step took 10-11 minutes. Oocytes were activated by the addition of AB (3.3 mM NaH_2PO_4, 16.6 mM KH_2PO_4, 10 mM NaCl, 50 mM KCl, 5% PEG 8000, 2 mM $CaCl_2$, final pH 6.4) for a five-minute incubation, and then the buffer was changed to ZAB (9 mM $MgCl_2$, 10 mM $MgSO_4$, 2.9 mM NaH_2PO_4, 0.22 mM NaOAc, 5 mM glucose, 27 mM glutamic acid, 33 mM glycine, 2 mM malic acid, 7 mM $CaCl_2$, final pH 6.8) for an additional incubation of 10 minutes (for anaphase I and metaphase II) or 25 minutes (for anaphase II and the post-meiotic interphase). Eggs with cross-linked vitelline membranes, a hallmark of activation, were selected by a 3-minute incubation in 50% Clorox bleach, and fixed in 8% EM-grade, MeOH-free formaldehyde (Ted Pella Inc., Irvine, CA) in cacodylate buffer (100 mM cacodylic acid, 100 mM sucrose, 40 mM KOAc, 10 mM NaOAc, 10 mM EGTA, pH to 7.2 with KOH; Theurkauf, 1994) for 10-15 minutes, and washed in PBST (PBS with 0.3% Triton X-100) containing ~1% BSA to prevent sticking to glassware. Vitelline membranes were removed by rolling the fixed eggs between two microscope slides (Theurkauf, 1994), again using PBST/BSA as a lubricant. Eggs were incubated in 1% RNase A (boiled to destroy DNase activity) for 20 minutes, and then incubated with 1 µg/ml propidium iodide (Sigma Chemical Co., St. Louis, MO) for 30 minutes. Samples

were mounted in Vectashield containing propidium iodide (Vector Labs Inc., Burlingame, CA).

Tubulin Immunofluorescence

Oocytes were prepared using the protocol described by Theurkauf (1994) for isolation and fixation of egg chambers. Tubulin was labeled using two anti-tubulin rat monoclonal antibodies, YL1/2 and YOL1/34 (Sera-Lab Ltd., Sussex, UK), overnight at room temperature at a dilution of 1:5 in 0.1% BSA in PBST, followed by a 3-hour incubation with a Texas red-conjugated donkey anti-rat antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA) at room temperature at a dilution of 1:200. The oocytes were further stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) at 1 µg/ml in PBS for 10 minutes, followed by two 15-minute rinses in PBS before mounting in 50% glycerol.

MEI-S332-GFP Localization in Embryos

Embryos were collected for 2.5 hours from females of the genotype *y w P{GrM}-13; P{GrM}-1*. The embryo in Figure 3-5 was from a 4-hour collection from females of the genotype *y w P{GrM}-13; cn mei-S332*^{7} *px sp / Df(2R)X58-6 pr cn. Oregon-R* embryos were used as a control for background autofluorescence.

Embryos were dechorionated in 50% bleach, and fixed for 30 minutes in 8% MeOH-free formaldehyde in cacodylate buffer (see above). After washing in PBS, embryos were rolled out of their vitelline membranes between 2 glass slides (Theurkauf, 1994). To stain for DNA, two methods were used. Embryos in Figures 3-4A, C, and D were treated with 1 mg/ml RNase A for 30 minutes, stained with 1 µg/ml propidium iodide for 30 minutes, and mounted in

Vectashield with propidium iodide (Vector Laboratories). The embryos shown in Figures 3-4B, 4-4E, and 4-5 were stained with DAPI at 1 μg/ml in PBS for 10 minutes, followed by two 15-minute rinses in PBS before mounting in 50% glycerol.

Microscopy

Two kinds of epifluorescence microscopy were used in our investigations. Conventional epifluorescence microscopy was performed using either a Nikon Optiphot-2 microscope or a Nikon Eclipse E800 equipped with a Nikon 60x oil objective (Garden City, NY). A Photometrics CE200A cooled CCD video camera was used to photograph images. The images were further processed with the CELLscan 2.0 system (Scanalytics) to create volume views from focal planes separated by 0.25 μm. 32 focal planes are shown for the oocyte images in Figure 3-2, 45 focal planes for the rosette in Figure 3-4B, 20 focal planes for the mitotic interphase nucleus in Figure 3-4E, and 7 focal planes for the images in Figure 3-5. Chromatin and MEI-S332-GFP in Figures 3-1A-G, and 3-4A, C, and D were visualized on a BioRad MRC 600 confocal laser scanning head (MRC 600; BioRad, Hercules, CA) equipped with a krypton/argon laser, mounted on a Zeiss Axioskop microscope (Oberkochen, Germany), with 20 and 40x oil Plan Neofluar objectives. In some cases, optical sections were taken and projected into a single plane. All images were further processed, colorized, and merged using Adobe Photoshop 3.0 on a Macintosh Power PC.

Western Blot Analysis

The rabbit anti-MEI-S332 antibodies (Covance Research Products Inc., Denver, PA) were generated against a COOH-terminal MEI-S332 peptide

conjugated to keyhole limpet hemacyanin. This 15-mer peptide (residues 386-400), (C)KNKLRNGSKGKAKAK, was chosen as the antigen because of the availability of the *mei-S332*^{7} allele, which lacks the COOH-terminal region of the protein because of a nonsense mutation at residue arg^{293} (Kerrebrock et al., 1995) and, hence, provides a negative control for the antibodies. The anti-peptide antibodies were affinity purified from rabbit serum using GST-MEI-S332 fusion protein bound to immobilon-P strips. The antibodies were eluted from the strips by acid elution buffer (5mM glycine-HCl pH2.5, 150mM NaCl) and immediately neutralized by 1M $NaPO_4$ buffer, pH8. The GST-MEI-S332 fusion protein was generated by cloning a 1.35 kb *Bam*HI-*Eco*RI *mei-S332* cDNA fragment in frame with GST in the pGEX-4T-3 expression vector (Pharmacia Biotechnology, Inc., Piscataway, NJ). The resulting pGEX.MEI plasmid allowed for expression of the full-length MEI-S332 protein, fused to GST at the NH_2-terminus, in BL21(λDE3)pLysS cells.

Embryonic extracts were made by dechorionating *Oregon-R* embryos in 50% Clorox bleach and homogenizing in urea sample buffer (USB: 8 M urea, 2% SDS, 5% β-mercaptoethanol, 100 mM Tris pH7.6, and 5% Ficoll) at 5:1 USB/embryo (vol/vol). Oocyte extracts were made from mature oocytes isolated as described in Page and Orr-Weaver (1997). Females were fattened for 3-5 days with yeast before blender isolation. Oocytes were homogenized in urea sample buffer at 3:1 USB/oocyte (vol/vol). Ovary extracts were made by dissecting previtellogenic, immature ovaries or mature ovaries in PBS from newly eclosed females or females that were fattened on yeast for 3 days, respectively, and homogenizing pooled ovaries in USB (~1 µl buffer/ovary). All protein extracts were cleared by centrifugation, frozen in liquid nitrogen, and stored at -80°C.

For the analysis of MEI-S332 levels in oocytes before and after activation, oocytes were isolated in IB, in either the presence or absence of 100 µg/ml cycloheximide (Fluka), from 300 *y w* females fattened on wet yeast for 3 days, as described above. After isolation, half of the oocytes were fixed by immersion in MeOH (unactivated) and the other half were activated in AB and ZAB in either the presence or absence of 100 µg/ml cycloheximide, as described above. The total incubation time in AB+ZAB was 60 minutes. These activated eggs were then fixed by incubation in MeOH. After several hours fixation in MeOH at room temperature, oocytes and eggs were rehydrated in PBS. Rehydrated samples were mixed with 1:1 EB/4 x Laemmli Sample Buffer (EB: 10 mM Tris 7.5, 80 mM Na β-glycerophosphate, pH 7.5, 20 mM EGTA, 15 mM $MgCl_2$, 2 mM Na_3VO_4, 1 mM benzamidine, 1 mM sodium metabisulfite, 0.2 mM PMSF) by crushing with the melted tip of a glass pipette. The ratio of sample to buffer added was 1:4 (vol/vol). Samples were boiled for 15 minutes, cleared by centrifugation, and frozen in a dry ice/MeOH bath. Control extracts for this experiment were made by isolating and fixing unactivated oocytes from *pr cn mei-S332^7 bw sp / cn mei-S332^7 px sp* and *Oregon-R* females as above. A cross-reacting band on the Western blot, just below the MEI-S332 signal, is also present in the *mei-S332^7* negative control, and is perhaps an artifact of this sample preparation.

Protein extracts were separated on 12% 150:1 (acrylamide/bis-acrylamide) gels and blotted onto immobilon-P membranes (Millipore Corp., Waters Chromatography, Milford, MA). About 200 µg of total protein was loaded per lane, and Ponceau S staining was used to verify equivalent protein loading before immunoblotting. Blots were blocked in 5% nonfat dry milk and 2% BSA in TBST (0.01 M Tris, pH7.5, 0.9% NaCl, and 0.1% Tween 20) for one hour at room temperature, and then incubated overnight at room temperature

with affinity-purified anti-MEI-S332 peptide antibodies diluted at 1:40 in the block solution. Alkaline phosphatase-conjugated anti-rabbit secondary antibodies (Promega Corp., Madison, WI), diluted 1:7500 in the block solution, were used to detect bound anti-peptide antibodies. The MEI-S332 protein was visualized using the BCIP/NBT color development substrate (Promega). Although it is predicted to be 44 kD, the MEI-S332 protein migrates as a 55-kD band.

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Chapter Four

MEI-S332: A Mitotic Centromere Cohesion Protein

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Abstract

Faithful segregation of sister chromatids during cell division requires properly regulated cohesion between the sister centromeres. The sister chromatids are attached along their lengths, but particularly tightly in the centromere regions. Therefore specific cohesion proteins may be needed at the centromere. Here we show that *Drosophila* MEI-S332 protein localizes to mitotic metaphase centromeres. In *mei-S332* mutants the ratio of metaphase to anaphase figure is lower than wild type, but it is higher if MEI-S332 is overexpressed. Centromere attachments appear weaker in *mei-S332* mutants than wild type and stronger when MEI-S332 is overexpressed. Both overexpression and mutation of MEI-S332 increase the number of apoptotic cells. Thus MEI-S332 contributes to centromeric sister-chromatid cohesion in a dose-dependent manner. MEI-S332 is the first member identified of a predicted class of centromeric proteins that maintain centromeric cohesion.

Introduction

During cell division it is crucial that the correct complement of chromosomes is accurately distributed to each daughter cell. This requires that the sister chromatids are physically attached until anaphase. This is particularly true at the centromere regions, which must be oriented towards opposite poles of the spindle, facilitating binding of the kinetochores to microtubules emanating from different centrosomes. Cohesion at the centromeres is also likely to contribute to tension that stabilizes kinetochore-microtubule interaction once bipolar attachments are made (Nicklas and Staehly 1967; Nicklas and Koch 1969; Skibbens et al. 1995). This bipolar attachment is required for the proper segregation of chromosomes at the metaphase/anaphase transition. Aneuploidy resulting from failure in chromosome segregation is likely to be a major contributor to cancer. For example, in 90% of colorectal tumors the cells are aneuploid (Lengauer et al. 1997). Mutations in components of the spindle assembly checkpoint, which monitors kinetochore attachment to the spindle, have recently been found in human colorectal cancers (Cahill et al. 1998). Because it is needed for proper segregation, defects in sister-chromatid cohesion may also contribute to the onset of cancer.

In mitosis the sister chromatids are physically associated along their lengths, indicating that there is cohesion both on the chromosome arms and at the centromeres. It appears that the tightest attachments are in the centromere regions because the individual sister chromatids are not distinguishable in the centric heterochromatin (Sumner 1991). A complex of proteins, the cohesins, has been shown to localize to mitotic chromosomes and to be necessary to promote sister-chromatid cohesion (Michaelis et al. 1997;

Losada et al. 1998; Guacci et al. 1997). The cohesins are likely to act along the length of the chromatids.

The cytological distinction between arm and centromere cohesion suggests that centromere-specific cohesion proteins might exist, but proteins that localize to centromeres and maintain sister-chromatid attachments in mitotic cells have yet to be identified. Such a protein has been characterized in *Drosophila* meiosis: MEI-S332 has been shown by genetic and cytological criteria to be required specifically for centromere cohesion (Kerrebrock et al. 1992). In meiosis arm cohesion is released at the metaphase I/anaphase I transition, while centromere cohesion persists until anaphase II. The MEI-S332 protein localizes to the centromeric regions of meiotic chromosomes at prometaphase I and delocalizes at the second meiotic metaphase/anaphase transition when centromeric cohesion is released (Moore et al. 1998; Kerrebrock et al. 1995; Tang et al. 1998). Mutations in *mei-S332* cause premature separation of the sister chromatids after arm cohesion has been released, late in anaphase I (Kerrebrock, et al. 1992). Here we evaluate the role of MEI-S332 in sister-chromatid cohesion in mitosis by first showing the protein is present in mitotic cells and localized on centromeres, and next analyzing its activity during mitosis.

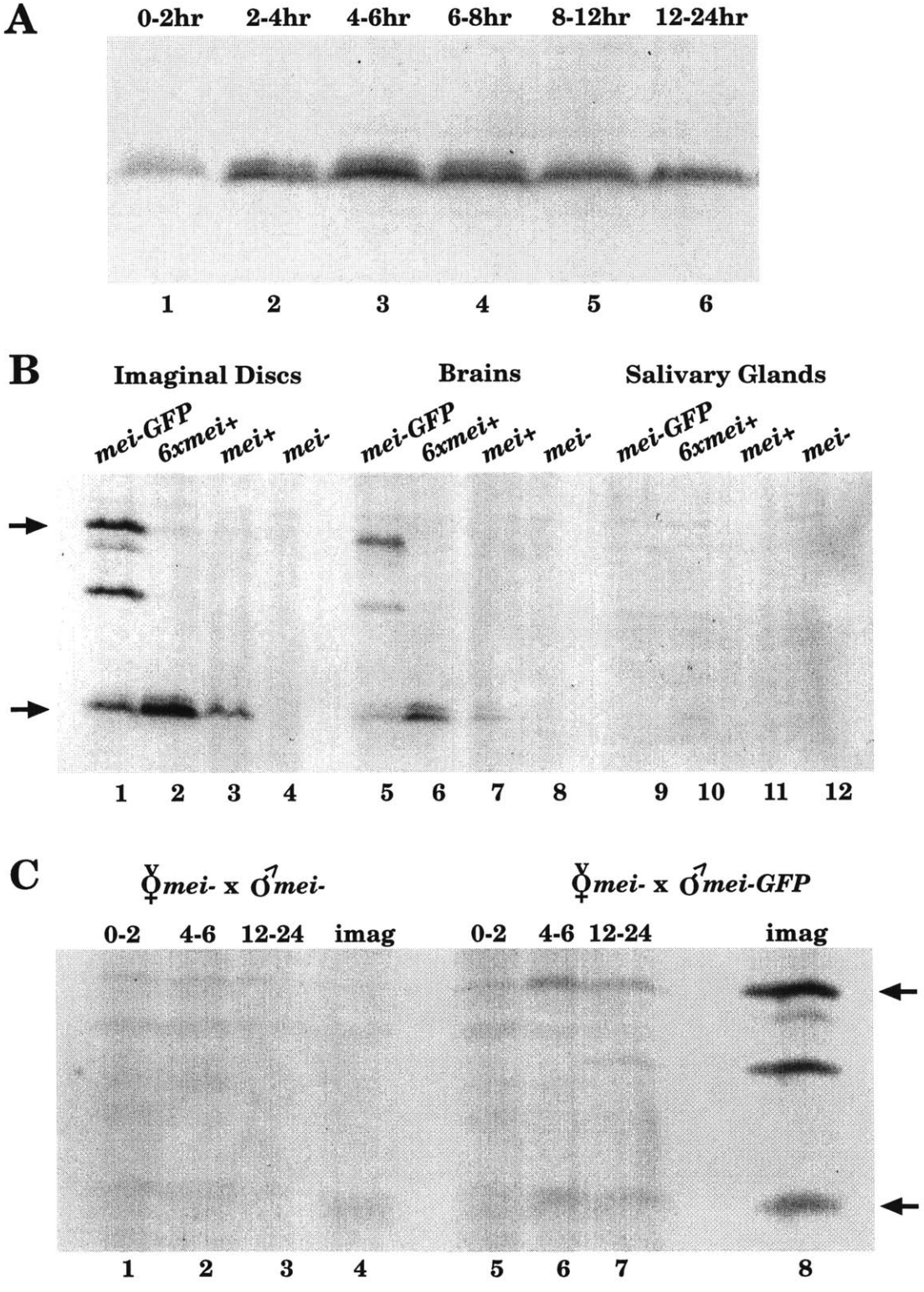
Results

MEI-S332 is expressed in mitotic tissues throughout development

We determined that MEI-S332 is present in mitotic cells throughout development. We have previously shown that maternal MEI-S332 survives meiosis and persists in early embryos, up to 4 hours after egg-laying (Moore, et al. 1998). This period is characterized by rapid (10 minute) S/M cycles driven by maternal products in a nuclear syncytium. By Western blotting we examined MEI-S332 protein levels in later stages of embryogenesis, following cellularization and the onset of zygotic gene expression. In these cycles mitosis is preceded by a G2 phase, as in the archetypic cell cycle (Foe et al. 1993). We found that MEI-S332 was present throughout embryogenesis (Figure 4-1A). We then analyzed the protein levels on Western blots of extracts from third instar larvae, a developmental stage five days after embryogenesis. We examined larval brains and imaginal discs, which undergo mitosis in a cycle with G1 and G2 phases (Figure 4-1B). A band corresponding to MEI-S332 was present in neuroblasts and imaginal discs from wild-type larvae (Figure 4-1B, lanes 3 and 7). The antibody used was specific for MEI-S332 as this band is not present in tissues from *mei-S332*^{7}, an allele in which the protein is truncated and thus lacks the carboxy-terminal epitope used to generate the antibodies (Figure 4-1B, lanes 4 and 8). In addition, the intensity of the band increases in neuroblasts and imaginal discs from a wild-type strain containing four additional copies of a genomic *mei-S332*$^{+}$ transgene (Figure 4-1B, lanes 2 and 6), demonstrating that this is the bona fide MEI-S332 protein. A second transgenic strain containing a similar *mei-S332* fragment with an in-frame fusion to GFP shows, in addition to MEI-S332^{+}, a larger band (Figure 4-1B, lanes 1 and 5) that is not present in any of the other strains, as further confirmation of the presence of MEI-S332 in mitotic tissues.

Figure 4-1. Expression of *mei-S332* in mitotic tissues.
Immunoblots of MEI-S332 were probed with antibodies directed against a carboxy-terminal peptide. (*A*) MEI-S332 is present throughout embryogenesis. Protein extracts from wild-type embryos of different ages (indicated above the blot) all contain MEI-S332. (*B*) *mei-S332* is expressed in larval tissues that have a canonical cell cycle. A Western blot was prepared with protein extracts from imaginal discs (lanes 1-4), brains (lanes 5-8), and polytene salivary glands (lanes 9-12). A wild-type strain (*mei+*) and two transgenic lines expressing MEI-S332 (*6x mei+*) or a MEI-S332-GFP fusion (*mei-GFP*) from the native promoter all showed bands of the appropriate sizes (arrows) in the mitotic tissues but not in the salivary gland samples. The bands were absent from *mei-S332*^{7} samples (*mei-*), demonstrating the specificity of the antibodies raised against a carboxy-terminal peptide. The middle bands in lanes 1 and 5 probably represent degradation products of the MEI-S332-GFP fusion protein, as they are only seen in lines expressing this construct. (*C*) Embryonic MEI-S332 is maternally derived; larval MEI-S332 is zygotically expressed. Females homozygous for the *mei-S332*^{7} allele were crossed to wild-type males containing additional *mei-S332-GFP* transgenes expressed from the native promoter (lanes 5-8) or, as a negative control, to homozygous mutant males (lanes 1-4), and their progeny were tested for MEI-S332 by immunoblotting. No MEI-S332 is seen in the 0-2 hour embryos (lanes 1 and 5) before zygotic transcription begins, or in the 4-6 hour (lanes 2 and 6) collections when zygotic transcription has begun. A small amount of protein may be expressed in the 12-24 hour collection (lanes 7 compared to lane 3). The imaginal discs of third instar larvae from the offspring of the *mei-S332-GFP* males express both the transgenic fusion protein and the endogenous MEI-S332 (lane 8, compared to lane 4). Larval tissues were normalized by

dissecting equal numbers of larvae, and so were comparable within a tissue type. Less protein was loaded in the imaginal disc lanes in (*C*), and there were considerably higher levels of total proteins in salivary gland samples than brains or imaginal discs, as determined by Ponceau S staining.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Imaginal Discs
Brains
Salivary Glands
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6xmei+
mei+
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We asked whether the MEI-S332 present in mitotically active embryos and larval tissues was persisting maternally-contributed product or if it was being expressed from the zygotic genome. Females homozygous for the truncated form of *mei-S332* were crossed to wild-type males carrying additional copies of the *mei-S332-GFP* transgene, or to homozygous mutant males as a negative control, and the progeny were tested for the presence of MEI-S332 by immunoblotting (Figure 4-1C). Any MEI-S332 seen in the progeny must have been expressed from the zygotic genome because the mothers could not contribute MEI-S332 that can be recognized by the antibodies. The high levels of MEI-S332 found in embryos from wild-type mothers were not detected in the progeny of either cross (compare Figure 4-1C, lanes 1-3 and 5-7 with Figure 4-1A, lanes 1, 3, and 6). The MEI-S332 present in embryos thus must derive from persisting maternal protein and/or translation of maternally-supplied mRNA. *mei-S332* mRNA is not detectable 12 hours after egg laying (Kerrebrock, et al. 1995), thus the protein appears to be very stable at this time in development. By contrast, imaginal disc samples from the late larval progeny of the *mei-S332-GFP* fathers (Figure 4-1C, lane 8) showed strong expression of both the endogenous *mei-S332* gene and the transgenic *mei-S332-GFP* fusion. However, neither the endogenous MEI-S332 nor the MEI-S332-GFP fusion protein was present in imaginal disc samples from late larval progeny of the mutant fathers (Figure 4-1C, lane 4). Therefore, MEI-S332 is expressed zygotically in mitotic tissues at later developmental times.

In polytene chromosomes the endoreplicated sister chromatids are tightly aligned, and the centromere regions of all the chromosomes are clustered together in a chromocenter. Given the essential role MEI-S332 plays in maintaining sister-chromatid cohesion in meiosis, we wanted to test whether

it might function in attaching sister chromatids in polytene chromosomes, or in chromocenter formation. We found, however, that MEI-S332 was not expressed in the polytene salivary glands of third instar larvae (Figure 4-1B, lanes 9-12). Consistent with this are our observations that ectopically expressed MEI-S332 did not localize to the salivary gland chromosomes (data not shown) and the polytene chromosomes from *mei-S332* mutants had normal morphology (A. Kerrebrock and T.L. Orr-Weaver, unpublished results).

MEI-S332 localizes to metaphase centromeres

Since MEI-S332 was zygotically expressed, it was of interest to determine the localization pattern of MEI-S332 in tissues with a canonical cell cycle and constitutive heterochromatin. We have shown that a MEI-S332-GFP fusion protein localizes to the centromeric region of metaphase chromosomes in early embryos (Moore, et al. 1998), but these cell cycles are unusual, as noted above, for their rapid S/M division cycles and for having under-condensed heterochromatin. In addition, large amounts of maternally-provided proteins that control these rapid divisions are present. It was possible that the localization of MEI-S332 in early embryos was a fortuitous consequence of the presence of high concentrations of the protein that survived from the female meiotic divisions. Furthermore, previous localization of MEI-S332 on mitotic chromosomes relied on transgenic expression of MEI-S332-GFP so the formal possibility remained that the pattern seen was a result of expression of higher than normal levels of the protein.

We confirmed that endogenous MEI-S332 localized to the centromeres of metaphase chromosomes in tissues with a canonical cell cycle and normally condensed heterochromatin. We prepared whole-mounts of wing imaginal discs stained with antibodies raised against full-length MEI-S332 and counter-

stained with antibodies raised against the phosphorylated form of histone H3, which specifically bind to condensed chromosomes (Hendzel et al. 1997; Figure 4-2A). MEI-S332 clearly localized to distinct foci on the middle of metaphase chromosomes, consistent with the centromeric region. In anaphase cells, however, MEI-S332 was absent from the leading edge of the chromosomes.

We confirmed that MEI-S332 was bound to the centromere of metaphase chromosomes by squashing wing imaginal discs from third instar larvae and immunolocalizing MEI-S332 as above (Figure 4-2B). MEI-S332 was clearly localized to discrete dots at the centers of the chromosomes (Figure 4-2B, arrow). Thus zygotically-expressed MEI-S332 does localize to the centromeric regions of mitotic chromosomes in a regulated manner.

Levels of MEI-S332 influence cell viability

Mutations in genes with an important role in mitosis should result in reduced viability. Although we did not previously observe decreased viability in *mei-S332* mutants (Kerrebrock, et al. 1992), substantial pools of MEI-S332 contributed by heterozygous mothers were present (see Figure 4-1A). Thus, we compared the viability of homozygous and heterozygous mutant progeny of mothers homozygous for a strong allele of *mei-S332*. When homozygous mutant females were crossed to heterozygous mutant males, the ratio of the homozygous and heterozygous progeny was the same (51.0% homozygotes, n=776) as in the reciprocal control cross (51.6% homozygotes, n=2040). MEI-S332 is therefore not required for viability, even in the absence of maternally-contributed protein during embryogenesis. We tested the effects of overexpressing MEI-S332 by creating a transgenic line where MEI-S332 expression was dependent on GAL4 (Brand and Perrimon 1993). When MEI-S332 was ubiquitously overexpressed under the control of a heat-shock

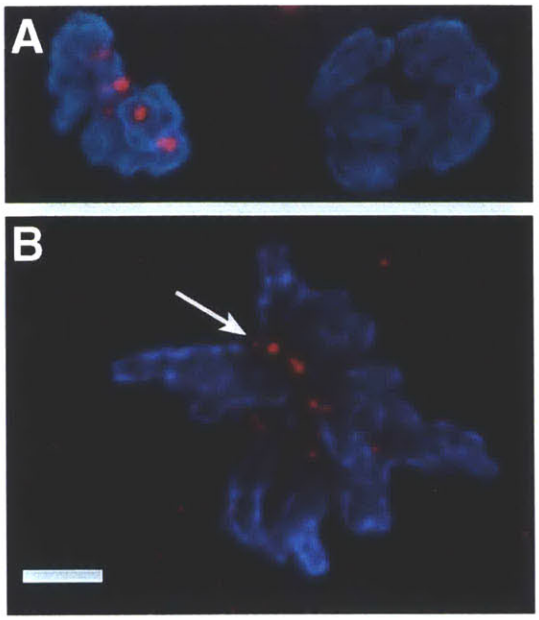

Figure 4-2. MEI-S332 localizes to the centromeres of mitotic metaphase chromosomes.

Wing imaginal discs from *y w* larvae were immunostained for MEI-S332 (red) and the phosphorylated form of histone H3, present only on condensed chromosomes (Hendzel et al. 1997; shown in blue). (*A*) In whole mount preparations, MEI-S332 is present at the center of the congressed metaphase chromosomes (left nucleus) but is not detected at anaphase (right nucleus). (*B*) Immunolocalization of MEI-S332 on squashed preparations confirmed that MEI-S332 localizes specifically to the centromeric region of metaphase chromosomes. Paired dots of MEI-S332 signal can be seen at the center of one of the metacentric major autosomes (arrow). There was no residual MEI-S332 associated with the leading edge of the chromosomes in anaphase figures, even after prolonged exposure (data not shown). Bar, ~2 μM.

inducible GAL4 transgene (Brand et al. 1994), virtually no progeny containing both transgenes were recovered (7 overexpressing progeny vs. 400 non-expressing siblings, 1.7% compared to 50.9% in non-heat-shocked controls). Thus, although MEI-S332 is not required for viability, its overexpression results in lethality, raising the possibility that excess MEI-S332 perturbs mitosis.

The lethality of MEI-S332 overexpression coupled with the zygotic expression and regulated localization of MEI-S332 in mitotic tissues strongly suggested that MEI-S332 plays a role in mitosis, despite the viability of mutant animals. It is well documented that *Drosophila* can tolerate extensive mitotic failures without an effect on organismal viability (Baker et al. 1982). Plasticity during development allows proliferation of neighboring cells (Milan et al. 1996) or even cell growth (Neufeld et al. 1998) to compensate for defects resulting in cell lethality. We therefore examined the pattern of acridine orange staining in wing imaginal discs to see if perturbing levels of MEI-S332 resulted in increased apoptosis.

As previously reported (Milan et al. 1996), we saw small clusters of acridine orange-staining cells in wild-type wing discs (Figure 4-3A). Consistent with observed lethality, overexpression of MEI-S332 driven by GAL4 in imaginal discs resulted in a dramatic increase in apoptosis (Figure 4-3C). Most interestingly, wing discs from larvae mutant for *mei-S332* also showed a significant increase in cell death. We counted an average of 10.6 clusters of acridine orange-staining cells per mutant wing disc, compared to 5.4 clusters in wild type discs ($p<0.0001$). Furthermore, there were generally more cells per cluster in mutant discs compared to wild type (Figure 4-3B). Loss of *mei-S332* therefore results in decreased cell viability.

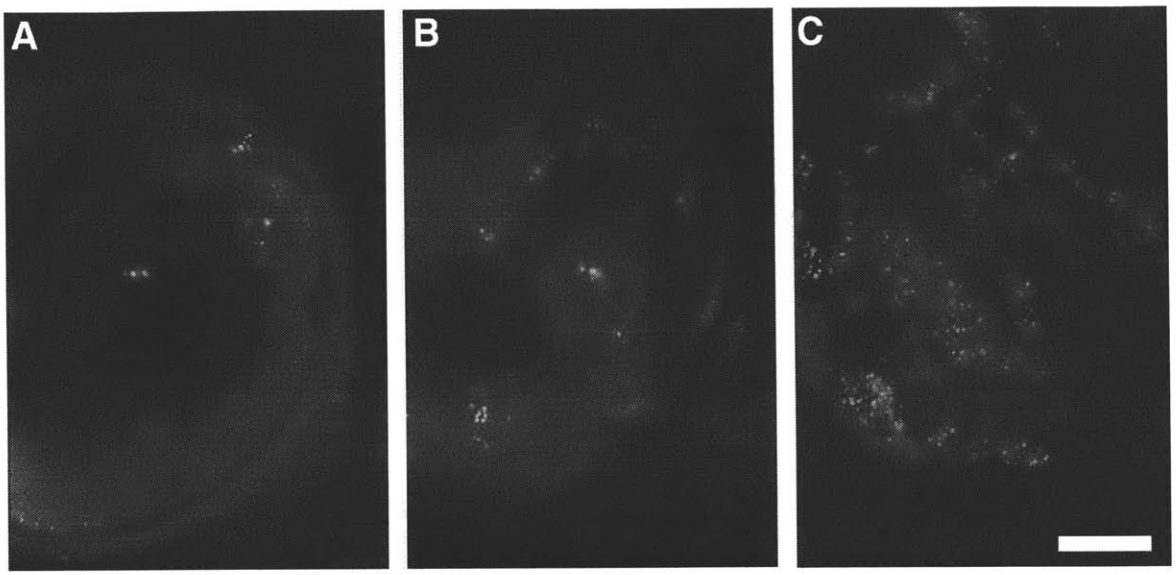

Figure 4-3. Acridine orange staining of wing imaginal discs.

(*A*) Wild-type discs have a few small clusters of acridine orange-stained cells. (*B*) *mei-S332* mutant discs show an increase in the number of acridine orange cell clusters and tend to have more cells per cluster. (*C*) Discs overexpressing MEI-S332 under the control of GAL4 have a dramatic increase in cell death.

Bar, ~25 μM.

MEI-S332 contributes to centromeric cohesion in mitosis

We looked directly for evidence of MEI-S332 function in mitosis. We first quantified the mitotic frequency, comparing wild-type strains to *mei-S332* mutants and larvae overexpressing MEI-S332 in imaginal discs and neuroblasts under the control of GAL4. The mitotic index was scored by quantifying the relative frequency of mitotic cells in larval neuroblasts (Figure 4-4A) or imaginal discs (Figure 4-4B), both mitotically dividing tissues. The mitotic index of *mei-S332* mutants was not different from that of wild-type controls (0.99 vs. 1.02 mitotic cells per field, p=0.78; Table 4-1). In contrast, when MEI-S332 was overexpressed the frequency of mitotic cells increased significantly (1.56 vs. 1.00 mitotic cells/field, $p<0.0001$), suggesting that high levels of MEI-S332 prolong passage through mitosis.

While we identified no effect of mutating *mei-S332* on the mitotic index, the metaphase-to-anaphase ratio was altered by changing *mei-S332* dosage. Because the defining event of the metaphase/anaphase transition is the loss of sister-chromatid cohesion, we predicted that we would see an increase in the frequency of anaphases in *mei-S332* mutants relative to wild type, and a decrease in tissues overexpressing MEI-S332. When we measured the metaphase to anaphase ratios, that was what we observed (Table 4-1). The metaphase/anaphase ratio was similar in both wild-type tissues tested (3.7:1). The ratio decreased in *mei-S332* mutant neuroblasts to 1.96:1 while it increased to 5.70:1 in tissues overexpressing MEI-S332. Thus we saw a decrease in anaphase frequency as levels of MEI-S332 expression increased.

By analogy to meiosis, we expect MEI-S332 to have a role in centromeric cohesion in mitosis, which is consistent with the effects we see on mitotic index and metaphase/anaphase ratios. We did not see precocious separation of sister chromatids (PSSC) in neuroblasts from *mei-S332* mutants

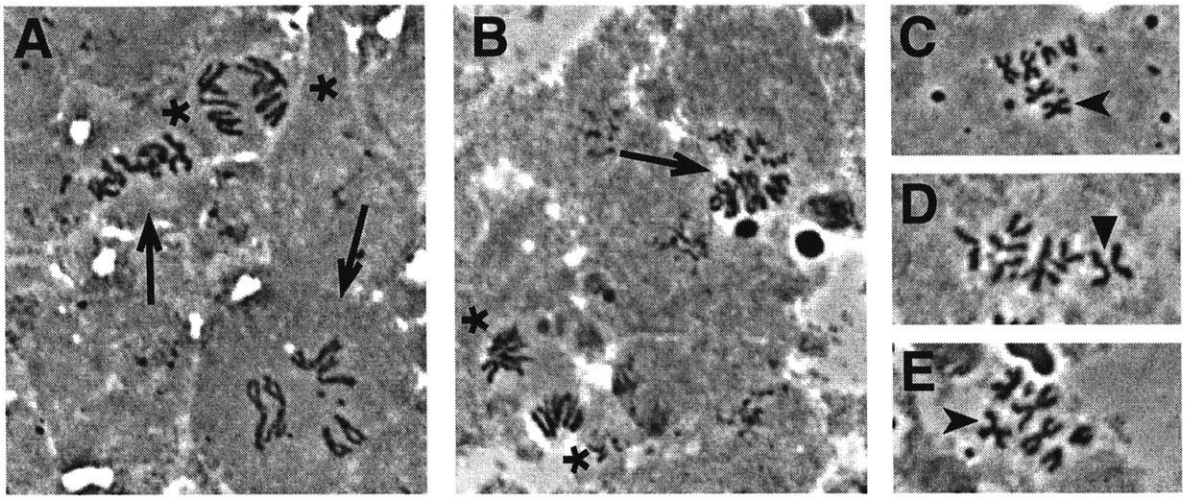

Figure 4-4. Orcein-stained squashes of mitotic tissues.

The mitotic cytology of neuroblasts (*A*) and wing imaginal discs (*B*) are indistinguishable. Chromosome morphology was identical in *mei-S332* mutants (*A*), tissues overexpressing MEI-S332 (*B*), and wild type (not shown). Metaphase cells are indicated with arrows, the poles of anaphase figures are shown by *. Hypotonically treated neuroblasts from *mei-S332* mutants (*D*) showed increased precocious separation of sister chromatids (big arrowhead) under conditions where wild-type sister chromatids are held together only at their centromeric regions (arrowhead in *C*). Overexpression of MEI-S332 seems to protect centromeric cohesion (*E*). Bar, ~2 μM.

Table 4-1. Effects of MEI-S332 dosage on mitosis.

	mei-S332 mutant	Wild Type *Canton S*	Wild Type *y w*	MEI-S332 overexpression
mitotic index[a]	0.99[b] (491, 6)[c]	1.02[b] (499, 6)	1.00 (480, 24)	1.56 (338, 17)
meta:ana[a]	1.96	3.75	3.70	5.70
PSSC[d]	17.2% (255, 9)		9.9% (350, 14)	5.3% (375, 15)

a Mitotic index and metaphase:anaphase ratios were measured in neuroblasts from *mei-S332*^{7} / *Df(2R)X-58-6* mutants and *Canton S* larvae and in wing imaginal discs from *y w* and *P{UGM}29e* / *P{GAL4}69B* MEI-S332 overexpressing larvae.

b These data were collected using a 63x objective. For ease of comparison, the original values of 2.49 and 2.59 have been divided by 2.52 to correct for the relative difference in field size between 63x and 100x magnification.

c Numbers in parentheses show the number of fields scored, followed by the number of individual brains or wing discs scored.

d Precocious separation of sister chromatids was measured in neuroblasts for all three genotypes and refers to the percentage of all prometaphase cells that showed PSSC.

(Kerrebrock, et al. 1995 ; Figure 4-4A), however in mitosis arm cohesion could suffice to keep the sisters together even if centromeric cohesion is defective. We assayed centromeric cohesion in the absence of arm cohesion by exposing cells to hypotonic treatment, which releases arm cohesion (Figure 4-4C; Gatti and Goldberg 1991). To increase the population of prometaphase cells we preincubated the discs with the microtubule-depolymerizing drug colchicine.

In hypotonically-treated neuroblasts we found that *mei-S332* mutants indeed show PSSC (Figure 4-4D and Table 4-1). The conditions used led to a background rate of PSSC in wild type (Table 4-1), but the frequency of precocious separation was doubled in the *mei-S332* mutant ($p<0.02$) (Table 4-1). Conversely, increased levels of MEI-S332 decreased the rate of PSSC (Figure 4-4E and Table 4-1), although the data are only marginally significant ($p<0.09$). Thus the absence of MEI-S332 results in an increase in PSSC under conditions where arm cohesion is compromised; conversely, increased MEI-S332 appears to protect the attachments between the sister centromeres. These observations complement the effects observed on metaphase/anaphase ratios in untreated tissues containing increased or decreased MEI-S332 levels. Centromeric cohesion in mitosis thus correlates with the levels of MEI-S332 present, indicating a functional role for MEI-S332 in mitosis.

Discussion

We have shown that MEI-S332 is expressed and localized in mitotic cells, and that it contributes to centromeric cohesion in mitosis as it does in meiosis. *mei-S332* is not required for the viability of the organism: for most chromosomes, in most cells, arm cohesion may be sufficient for proper segregation during mitosis. However, the increase in apoptosis, premature separation of sister chromatids, and the relative frequency of anaphase cells in *mei-S332* mutant tissues suggests that many cells in the developing organism fail to complete mitosis properly when centromeric cohesion is reduced. Over-expression of MEI-S332 had more severe consequences, probably because centromeric cohesion is in excess and is not properly dissolved at the metaphase/anaphase transition.

The properties of *Drosophila* development permit a plasticity that is likely to compensate for mitotic defects in *mei-S332* mutants and overexpressing cells. In the embryo, aneuploid nuclei are shuttled into the yolk, eliminating the products of defective mitoses (Sullivan et al. 1990). In imaginal discs, even when the majority of cells die, increases in cell size and division occur to compensate for the loss of cells, producing a viable adult with normal patterning (Neufeld, et al. 1998; Baker and Rubin 1992). We did observe an increase in apoptosis in *mei-S332* mutants, suggesting that chromosome segregation may be defective in many cells. It is perhaps counterintuitive that cells were undergoing apoptosis, yet we did not find aneuploid cells. It is possible that improper chromosome segregation during mitosis in the imaginal discs is followed by immediate death of the daughter cells.

A picture is emerging of the hierarchy that regulates proper segregation of sister chromatids at the metaphase/anaphase transition. The spindle assembly checkpoint monitors the attachment of sister kinetochores to the

spindle microtubules and prevents premature onset of the metaphase/anaphase transition. Once all the chromosomes have formed stable bipolar attachments and congressed to the metaphase plate, the regulatory mechanism governing the onset of anaphase activates kinetochore motor proteins and releases sister-chromatid cohesion in the defining event of the metaphase/anaphase transition (Elledge 1998). Proteins associated with most of these functions localize to mitotic centromeres: 1) MAD2, BUB1, and BUB3, components of the spindle assembly checkpoint, are present at the kinetochore region (Li and Benezra 1996; Chen et al. 1996; Taylor and McKeon 1997; Taylor et al. 1998); 2) Cdc20, which activates the anaphase-promoting complex to degrade inhibitors of anaphase, binds MAD2 (Hwang et al. 1998; Kim et al. 1998); and 3) CENP-E has long been known as a kinetochore protein (Yen et al. 1992) and has recently been characterized as a plus-end directed kinetochore motor (Wood et al. 1997). Thus three of the four classes of proteins that must participate in the faithful segregation of sister chromatids are functionally and cytologically associated with the centromere. The *Saccharomyces cerevisiae MCD1/SSC1* gene and the Xenopus homologue are required for cohesion along the lengths of sister chromatids (Guacci, et al. 1997; Michaelis, et al. 1997; Losada, et al. 1998), but in Xenopus the cohesins establish but do not appear to maintain cohesion. No proteins specific for centromeric cohesion have previously been identified. MEI-S332 thus represents the first member of the fourth class of centromere proteins that must regulate chromosome segregation, maintaining sister-chromatid cohesion at the centromere.

Finally, given the resiliency of developing *Drosophila* tissues, many important mitotic genes have no doubt escaped detection in lethality-based screens. The phenotypes resulting from overexpression of MEI-S332 provide a

powerful means to identify other components of mitotic sister-chromatid cohesion and its regulation by genetic interactions.

Materials and methods

Fly strains

Wild-type strains were either *Oregon-R, Canton-S,* or *y w,* as indicated. Transformant strains containing extra copies of the *mei-S332* gene or a functional *mei-S332-GFP* gene have been described previously: *6x mei+*, a strain carrying 4 copies of homozygous insertions of a genomic fragment containing *mei-S332*$^{+}$ (*P{w*$^{+mc}$ *5.6 KK mei-S332*$^{+}$*}*) on the second and third chromosomes in addition to the 2 endogenous copies of *mei-S332* (Kerrebrock, et al. 1995); and *mei-GFP*, *y w* containing 4 copies of the same genomic fragment as above with *GFP*$^{+}$ inserted in-frame at the amino-terminus of *mei-S332* [*P{GrM}13* on the *X* chromosome and *P{GrM}1* on the second chromosome (Kerrebrock, et al. 1995)]. The *mei-S332* mutant strains *y; pr cn mei-S332*^{7} *bw sp/cn mei-S332*^{7} *px sp* (*mei-S332*^{7}*A/mei-S332*^{7}*B*), *y; pr cn mei-S332*^{4} *bw sp / cn mei-S332*^{4} *px sp*, and *y; Df(2R)X58-6 / SM1* have been described (Kerrebrock, et al. 1992). *mei-S332*^{7} contains a mutation causing termination of translation prior to the epitope recognized by the antibodies used for immunoblotting (Kerrebrock, et al. 1995). *mei-S332*^{4} mutant females do not have detectable protein in their ovaries (Tang, et al. 1998) and genetically behave as nulls (Kerrebrock, et al. 1992). To test for maternal versus zygotic origins of *mei-S332* expression, virgin *mei-S332*^{7}*A/mei-S332*^{7}*B* females were crossed to *mei-S332*^{7}*A/mei-S332*^{7}*B* males or to *mei-GFP* males. To test for organismal lethality *y; pr cn mei-S332*^{4} *bw sp / cn mei-S332*^{4} *px sp* females were mated to *y; Df(2R)X58-6 / SM1* males, and the number of *mei-S332*^{4} */ Df(2R)X58-6* mutant progeny was scored. In control experiments the genotypes of the parents were reversed.

Immunoblots and Immunolocalization of MEI-S332

Immunoblot analysis was carried out as described in Moore et al. (1998) using a rabbit polyclonal antibody generated against a C-terminal peptide from MEI-S332 (Moore, et al. 1998). Protein extracts were prepared from brains, salivary glands, and eye and wing imaginal discs isolated from 15 third instar larvae as described for embryos (Moore, et al. 1998).

Whole-mount and squashed preparations of imaginal discs from third instar larvae were prepared as described (Gonzalez and Glover 1993) MEI-S332 was localized using guinea pig antibodies raised against full-length MEI-S332, as described (Tang, et al. 1998). To visualize condensed chromosomes the tissues were immunolabeled with rabbit antibodies specific for the phosphorylated form of histone H3 (Hendzel, et al. 1997), a kind gift of D. Allis, and detected with FITC conjugated donkey anti-rabbit antibodies (Jackson Immunoresearch Laboratories). Similar localization patterns were seen in imaginal discs and neuroblasts when MEI-S332 was detected by fusion to GFP or with the antibody raised against the carboxy-terminal peptide (data not shown).

Serial sections of whole mounts were collected on a Nikon Eclipse E800 epifluorescent microscope equipped with a Nikon 100x oil objective and a Hamamatsu Orca C4742-95 cooled CCD digital camera. The images were processed with the CELLscan 2.0 system (Scanalytics) to reassign out-of-focus light and to create volume views. Serial sections of squashed preparations were collected on an Olympus IX70 epifluorescent inverted microscope equipped with an Olympus 100x oil objective and a Photometrics CH350L cooled CCD digital camera. The images were collected using the DeltaVision system (Applied Precision), and volume views were created after the images were processed with the Constrained Iterative Deconvolution

Method to reassign out-of-focus light. Individual images were rescaled to use the complete 256 gray-value spectrum and then merged in Adobe Photoshop 5.0.

Overexpression of MEI-S332

Transgenic flies were created in which the *mei-S332-GFP* gene fusion was under the control of the GAL4 UAS (Brand, et al. 1994). The GFP-coding region of the previously described *mei-S332-GFP* fusion (Kerrebrock, et al. 1995) was replaced with a mutated version of *GFP* (S65T, M. Dobles and P. Sorger, personal communication) with enhanced fluorescence (Heim et al. 1995). The fusion gene was cloned into pUAST (Brand and Perrimon 1993), using an introduced *Bam*HI site and a sequence for improved translation (Cavener 1987). The resulting transposon, *P{w*$^{+mC}$ *UAS-GFP:mei-S332=UGM}*, was used to transform *y w* flies by injection (Spradling 1986), and several independent transformant lines were recovered.

mei-S332 was induced by crossing in *P{GAL4}69B* (*P{w*$^{+mW.hs}$ *GawB}69B*) that expresses GAL4 in imaginal discs (Brand and Perrimon 1993) and larval brains (data not shown). Overexpression of MEI-S332 under the control of GAL4 was confirmed by immunoblotting (data not shown). Of the two lines used in this report, *P{UGM}3d* gives fairly high levels of *mei-S332-GFP* expression in response to GAL4 induction, while levels of expression from *P{UGM}29e* are so high that no viable adults were recovered from any of the GAL4 lines tested (data not shown).

Heat shock overexpression of MEI-S332

In duplicate vials virgin *y w*; *P{UGM}3d* females were crossed to *hsGAL4/CyO* males [*P{w*$^{+mC}$ *GAL4-Hsp70.PB}2/CyO*; (Brand, et al. 1994)]. The vials were

incubated at 25°C and the parents were transferred to fresh vials every 2 days. On the ninth day, when some progeny had begun to pupate in the original vial, one set of vials was heat shocked twice for 1 hour at 37°C. The vials were allowed to develop at 25°C. Control vials were left at 25°C. Progeny were scored for *Cy* on the *CyO* chromosome. Expression of the *hsGAL4* construct had no effect in the absence of *P{UGM}3d* (data not shown).

Acridine orange staining and scoring

Wing imaginal discs were dissected from third instar larvae in phosphate buffered saline (PBS). Discs were stained for 5 minutes in 10μM acridine orange in PBS, destained for 5 minutes in PBS, and mounted in PBS. The discs were photographed immediately onto Kodak Ectachrome 64T 35 mm slide film on a Zeiss Axiophot microscope using a Zeiss PlanNeofluar 40X objective and fluorescence optics. The approximate number of clusters of acridine orange-stained cells was estimated visually.

Cytological analysis of mitosis

To calculate the mitotic index and metaphase to anaphase ratios for *mei-S332* mutants, neuroblasts from wandering third instar larvae were prepared by standard orcein-acetic acid squashing techniques (Ashburner 1989, Protocol III). We found that the same technique provided excellent cytology for mitotic figures from wing imaginal discs. The samples were examined (and photographed onto Kodak Ectachrome 64T 35 mm slide film) on a Zeiss Axiophot microscope using a Zeiss PlanNeofluar 100X objective or a PlanApo 63X objective, as noted in Table 1, and phase optics. A field is defined as the photo frame viewed through the 100X or 63X objective and 10X oculars. At least 20 fields were scored for each sample.

To calculate rates of precocious separation of sister chromatids (PSSC), brains were prepared as above, and cultured for one hour in 10μM colchicine/0.7% NaCl then transferred individually to 0.5% NaCitrate hypotonic solution for exactly 12 minutes before squashing. This time was chosen to increase the background rate of PSSC in wild-type neuroblasts (Gatti and Goldberg 1991) so any decrease in PSSC could be detected. For each brain at least 25 fields containing prometaphase figures were scored.

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Chapter Five

Regulation of the *Drosophila* Centromeric Protein MEI-S332 during the Cell Cycle

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*T.T.-L. Tang did all the experiments presented in this chapter as well as constructing the *myc-mei-S332*$^{+}$ transgenic flies by P-element-mediated transformation and performing nondisjunction tests to determine whether the *myc-mei-S332*$^{+}$ insert is capable of rescuing the *mei-S332* mutant phenotype.

Abstract

The physical associations between sister chromatids are crucial for the faithful segregation of chromosomes during cell division. The *Drosophila* MEI-S332 protein is essential for the maintenance of meiotic sister-chromatid cohesion and seems to play a role in strengthening cohesion at the centromeres during mitosis. The protein localizes to centromeres in both meiosis and mitosis until sister chromatids separate. Previous study showed that the MEI-S332 protein is not degraded globally at the metaphase II/anaphase II transition. In this study, we present data indicating that the MEI-S332 protein is post-translationally modified by phosphorylation. Interestingly, this modification correlates with the cell cycle. It appears that MEI-S332 is dephosphorylated in metaphase, when the protein is localized on the chromosomes, and phosphorylated in interphase and anaphase, when it is dissociated from the chromosomes. Therefore, the MEI-S332 protein is cell-cycle regulated. Dephosphorylation may be necessary for MEI-S332 chromosomal localization and/or cohesion activity, and phosphorylation may be required to delocalize MEI-S332 from the chromosomes.

Introduction

To prevent the formation of aneuploid cells, which are associated with tumorigenesis (Lengauer et al., 1997), chromosomes must be faithfully segregated during cell division. For proper segregation of chromosomes, it is not only important to establish and maintain the physical associations between sister chromatids, it is also important to regulate sister-chromatid cohesion precisely such that it is released at the right time. In order to allow controlled separation of the sister chromatids at the metaphase/ anaphase transition, it makes sense to have a regulated mechanism that removes the cohesion protein from the chromosomes. Proteolysis and phosphorylation are two potential mechanisms that can regulate the release of sister-chromatid cohesion.

Evidence from *S. cerevisiae* and *Xenopus* indicated that ubiquitin-dependent proteolysis of proteins other than the mitotic cyclins is necessary for the separation of sister chromatids at the metaphase/ anaphase transition (Holloway et al., 1993; Surana et al., 1993). Structural proteins that physically hold the sister chromatids together and regulatory proteins that inhibit the onset of anaphase are potential candidates for proteins that must be degraded to allow sister-chromatid separation.

The targeted degradation of the *S. cerevisiae* Pds1p protein and the *S. pombe* Cut2p protein by the anaphase-promoting complex (APC) has been shown to be required for sister-chromatid separation (Cohen-Fix et al., 1996; Funabiki et al., 1996; King et al., 1995; Irniger et al., 1995). Although it localizes to the nucleus, Pds1p is not associated with the chromatin (Yamamoto et al., 1996; Ciosk et al., 1998). Similarly, Cut2p is not detected on the chromosomes, instead it is concentrated along the metaphase spindle (Funabiki et al., 1996). Because they do not localize to chromosomes, these

two proteins cannot be the structural proteins that physically hold the sister chromatids together. Rather, they are inhibitors of anaphase; their destruction initiates anaphase. Recent work in *S. cerevisiae* has shown that Pds1p forms a stable complex with the 180-kD Esp1p protein and that the APC-dependent degradation of Pds1p triggers the dissociation of Mcd1p/Scc1p, a component of the cohesin complex, from the chromatin (Ciosk et al., 1998; Guacci et al., 1997; Michaelis et al., 1997). It appears that the APC-dependent proteolysis of Pds1p releases Esp1p, that then promotes the dissociation of the cohesin complex from the chromatin, leading to the release of sister-chromatid cohesion. Thus, it remains to be determined what--if any--structural proteins involved in sister-chromatid cohesion are targeted for proteolysis at the onset of anaphase.

Mutations in the regulatory subunit (PR55) of protein phosphatase 2A (PP2A) have been shown to cause precocious separation of sister chromatids or aberrant anaphase figures (Minshull et al., 1996; Gomes et al., 1993; Mayer-Jaekel et al., 1993). In addition, Ghosh and Paweletz (1992) have shown that okadaic acid, a potent inhibitor of protein phosphatase 1 (PP1) and PP2A, inhibits sister-chromatid separation. Furthermore, disrupting the catalytic activity of PP1 has been shown to cause metaphase arrest, as characterized by short metaphase spindles and condensed and unseparated chromosomes in many organisms (Ohkura et al., 1988; Ohkura et al., 1989; Ishii et al., 1996; Axton et al., 1990; Doonan and Morris, 1989; Fernandez et al., 1992). These results strongly indicate that phosphorylation/ dephosphorylation also plays a role in controlling sister-chromatid cohesion.

In *Drosophila melanogaster*, *mei-S332* has been shown to be essential for sister-chromatid cohesion during meiosis (Goldstein, 1980; Kerrebrock et al., 1992). It also appears to play a role in strengthening cohesion at the

centromeres in mitosis (H. LeBlanc, T.T.-L. Tang, J. Wu, and T.L. Orr-Weaver, submitted). The protein localizes onto the meiotic centromeres during prometaphase I and does not dissociate from chromosomes until anaphase II when sister chromatids separate (Kerrebrock et al., 1995; Moore et al., 1998). Similarly, in mitosis MEI-S332 first assembles onto the centromeres during prometaphase and remains there until anaphase (Moore et al., 1998; Tang et al., 1998). Its striking disappearance from the chromosomes at the time of sister-chromatid separation suggests that MEI-S332 could be regulated by proteolysis and that the degradation of MEI-S332 at the metaphase/ anaphase transition is necessary for the proper release of sister-chromatid cohesion. The presence of two PEST sequences in the MEI-S332 protein makes this model attractive (Kerrebrock et al., 1995). PEST sequences are common in proteins that have high turn-over rates (Rogers et al., 1986; Rechsteiner, 1988). However, Western blots of oocyte extracts show that MEI-S332 protein is still present after the completion of meiosis, suggesting that it is not degraded globally at the metaphaseII/ anaphase II transition (Moore et al., 1998).

Alternatively, MEI-S332 may be delocalized from the chromosomes at the metaphase II/anaphase II transition by a post-translational modification that presumably reduces its affinity for DNA and/or protein complexes on the DNA. It is possible that phosphorylation or dephosphorylation could control the activity of MEI-S332 and/or its ability to localize onto the chromosomes. MEI-S332 has many possible phosphorylation sites (Moore et al., 1998).

In this study, we showed that MEI-S332 is post-translationally modified, and this modification depends on phosphorylation. In addition, MEI-S332-GFP and MYC-MEI-S332 fusion proteins can be phosphorylated in vitro. Interestingly, the modification on MEI-S332 correlates with the cell cycle. The

protein appears to be phosphorylated in interphase and anaphase, and dephosphorylated in metaphase. These results indicate that MEI-S332 is regulated by phosphorylation during the cell cycle. Phosphorylation of MEI-S332 may induce the delocalization of the cohesion protein, permitting the separation of sister chromatids.

Results

MEI-S332 is post-translationally modified

Because MEI-S332 is not degraded globally at the metaphase II/ anaphase II transition, we tested whether the protein is regulated by an alternative mechanism at this transition. Developmental Western blots of *Drosophila* embryonic extracts show not only that MEI-S332 is present throughout embryogenesis but also that MEI-S332 migrates as a doublet on SDS polyacrylamide gels (H. LeBlanc, T.T.-L. Tang, J. Wu, and T.L. Orr-Weaver, submitted; Figure 5-1A). This strongly indicates that the MEI-S332 protein is post-translationally modified. In whole embryonic crude extracts that consisted of a population of cells in different stages of the cell cycle, including interphase, metaphase, and anaphase, the bottom band of the doublet was consistently observed as the more predominant form (Figure 5-1A). A similar result was seen with brain and imaginal disc extracts, which also contained cells in different stages of the cell cycle (Figure 5-1B; data not shown). In *Drosophila* tissue culture S2 cell extracts, only one MEI-S332 band was detected on Western blots (Figure 5-1C). This band co-migrated with the bottom MEI-S332 band of embryo, brain, and imaginal disc extracts (data not shown). This is consistent with the fact that in the tissue culture cell population, most cells are in interphase.

Modification of MEI-S332 involves phosphorylation

Since MEI-S332 has thirty potential phosphorylation sites (Moore et al., 1998), we wanted to determine if the post-translational modification of MEI-S332 involves phosphorylation. To test this possibility, we added lambda protein phosphatase to wild-type 2- to 6-hour embryo crude extracts in the presence or absence of phosphatase inhibitors and separated the proteins on

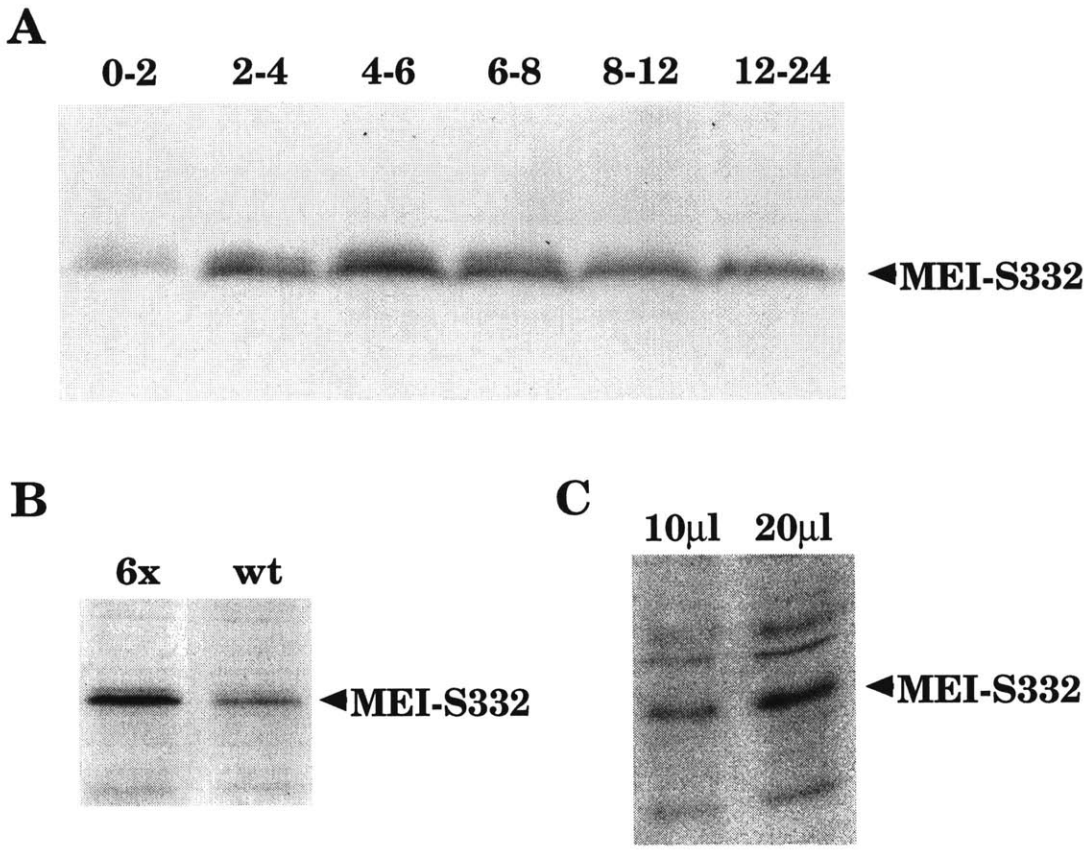

Figure 5-1. MEI-S332 runs as a doublet in gels, indicating post-translational modification.

(*A*) Developmental Western blot of staged wild-type embryos probed with MEI-S332 antibodies. Numbers on top of blot indicate developmental stages of the embryos in hours. (*B*) Western blot of protein extracts from imaginal discs dissected from wild-type flies (wt) and flies carrying 6 copies of the *mei-S332* gene (6x) probed with MEI-S332 antibodies. (*C*) Western blot of *Drosophila* S2 cell extracts (10µl and 20µl) probed with MEI-S332 antibodies.

SDS polyacrylamide gels. Lambda protein phosphatase is a Mn^{+2}-dependent protein phosphatase that is capable of removing phosphates from serine, threonine, and tyrosine amino acid residues (Cohen and Cohen 1989; Zhuo et al. 1993; also see Materials and Methods). Western blots probed with guinea pig anti-full length MEI-S332 antibodies demonstrated that lambda protein phosphatase completely shifted the doublet of the MEI-S332 protein to the top band (Figure 5-2, lanes 3 and 4). In the presence of sodium vanidate and sodium phosphate, potent inhibitors of lambda protein phosphatase, this shift was not observed (Figure 5-2, lanes 5 and 7). Sodium fluoride, a weak inhibitor of lambda protein phosphatase, failed to block the shift efficiently (Figure 5-2, lane 6). Therefore, the post-translational modification of the MEI-S332 protein involves phosphorylation. Furthermore, the top band of the doublet appears to be the dephosphorylated form and the bottom band the phosphorylated form.

MEI-S332 can be phosphorylated in vitro

Treatment of embryonic crude extracts with lambda protein phosphatase indicated that post-translational modification on the MEI-S332 protein involves phosphorylation. To determine whether MEI-S332 is directly phosphorylated, MEI-S332-GFP fusion protein was immunoprecipitated, by use of polyclonal rabbit anti-GFP antibodies, from extracts of embryos laid by females carrying and expressing the *mei-S332-gfp* transgene, and the immunocomplex was subjected to a kinase assay (see Materials and Methods). Figure 5-3 shows that the MEI-S332-GFP fusion protein was indeed labeled by ^{32}P in the kinase assay, indicating that MEI-S332 can be phosphorylated. We could not determine if the endogenous MEI-S332 protein, which coimmuno-precipitates with the MEI-S332-GFP fusion protein, is phosphorylated, because a nonspecific band with the same mobility as MEI-S332 was also

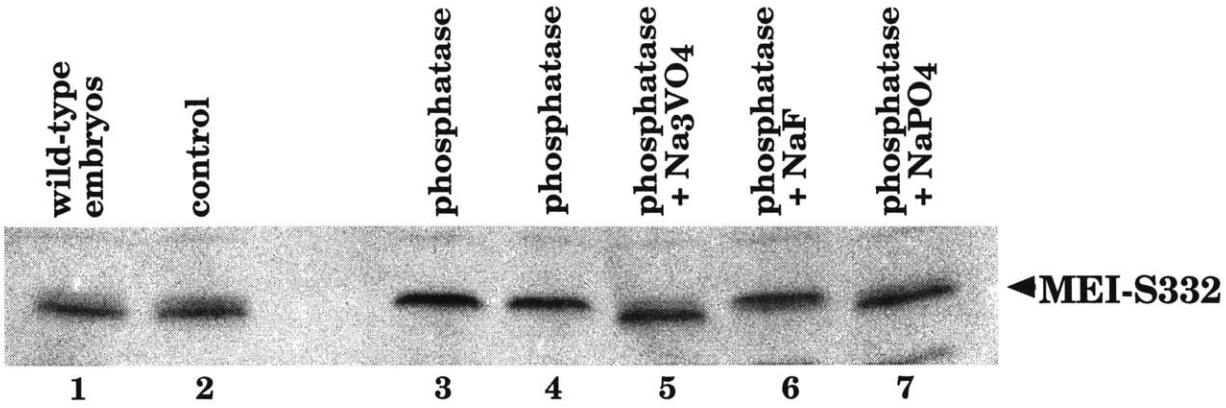

Figure 5-2. Post-translational modification of MEI-S332 involved phosphorylation. Anti-MEI-S332 bound Western blot of embryo crude extracts treated lamba protein phosphatase (500 units/25µl reaction) in the presence or absence of protein phosphatase inhibitors. Embryos were homogenized in the lambda protein phosphatase reaction buffer (see Materials and methods) and aliquoted into 7 reaction samples. (Lane 1) Sample was immediatedly mixed with equal volume of urea sample buffer. MEI-S332 can be seen as a doublet with the bottom band being the more predominant form. (Lane 2) Sample was incubated at 30°C for 30 minutes without phosphatase or phosphatase inhibitor. Again, MEI-S332 is seen as a doublet. (Lanes 3 and 4) Samples were incubated in the presence of lambda protein phosphatase. MEI-S332 is now shifted to the top band only. (Lane 5) Sodium vanidate (10mM) was added into the sample before the phosphatase was added. It inhibited the effect of lambda protein phosphatase on the doublet. (Lane 6) Sodium fluoride (50mM) does not block lambda protein phosphate efficiently; the formation of the top band can be seen. (Lane 7) In the presence of sodium phosphate (100mM), lambda protein phosphatase fails to shift MEI-S332 to the slower migrating form.

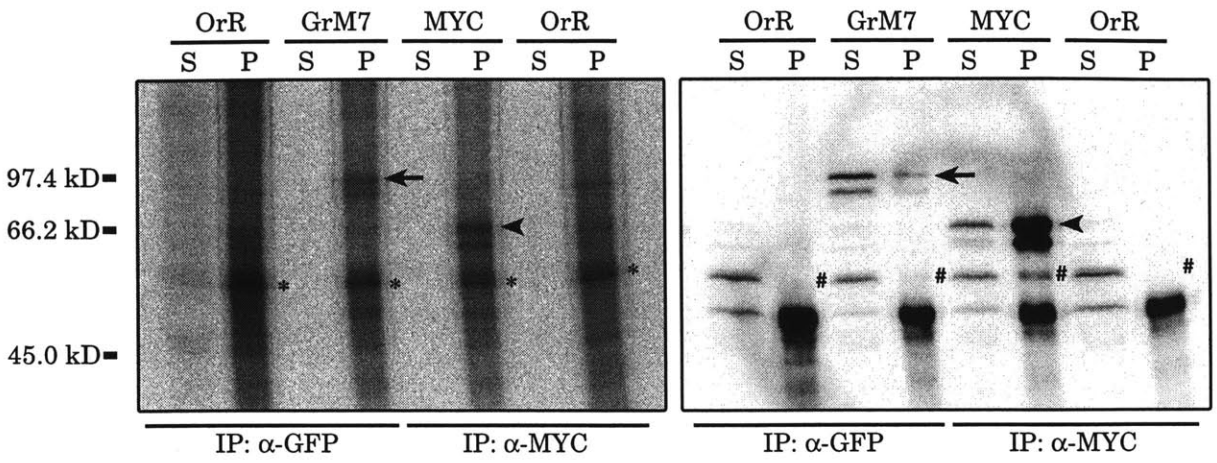

Figure 5-3. MEI-S332 can be phosphorylated in vitro.

The phosphoimage of in vitro ^{32}P-labeled MEI-S332 immunoprecipitates is shown in the left panel. The same blot was probed with guinea pig anti-MEI-S332 antibodies and is shown in the right panel. (Left panel) Both the MEI-S332-GFP band (arrow) and the MYC-MEI-S332 band (arrowhead) are labeled with ^{32}P. A band (*) running with the same mobility as MEI-S332 is also labeled. However, this ^{32}P-labeled band is present in all the immunoprecipitate pellets, including the *Oregon-R* negative controls. (Right panel) The Western blot confirms that this band is not MEI-S332, and it is masking the radioactive signal, if any, from MEI-S332. The Western also confirms that the two radioactively labeled bands (arrow and arrowhead) are MEI-S332-GFP and MYC-MEI-S332, respectively. The MEI-S332 protein (#) coimmunoprecipitated with the fusion proteins and is not present in the OrR immunoprecipitate pellets. The levels of MEI-S332 in the GrM7 IP pellet are low but can be seen more clearly with a longer exposure (data not shown). S designates immunoprecipitate supernatant, and P denotes immunoprecipitate pellet. OrR is wild-type extract. GrM7 is extract containing MEI-S332-GFP fusion protein. MYC is extract with MYC-MEI-S332 fusion protein.

labeled in this assay, hence, masking the radioactive signal, if any, from MEI-S332 (* in Figure 5-3).

Similar results were obtained with MYC epitope-tagged MEI-S332 (Figure 5-3). Transgenic flies carrying and expressing two copies of a *myc-mei-S332*$^+$ insert were generated by P-element-mediated transformation (see Materials and Methods); the insert is capable of rescuing the *mei-S332* chromosome missegregation phenotype in both male and female meiosis (data not shown). The MYC-MEI-S332 protein was immunoprecipitated by monoclonal anti-MYC antibodies from embryos laid by the *myc-mei-S332*$^+$ transgenic flies and labeled by ^{32}P in the kinase assay (Figure 5-3). Therefore, MEI-S332 can be phosphorylated in vitro, and a kinase capable of phosphorylating MEI-S332 seems to be associated with the MEI-S332 immunocomplex.

Post-translational modification of MEI-S332 correlates with the cell cycle

The presence of a doublet in extracts from mixed populations of cells at various cell cycle stages implicated that MEI-S332 protein could be modified differently in different stages of the cell cycle. In order to determine whether MEI-S332 exists in differentially modified forms in different stages of the cell cycle, we isolated single staged embryos that contain interphase, metaphase, or anaphase nuclei (Figure 5-4) and separated them on SDS polyacrylamide gels. Indeed, post-translational modification of MEI-S332 correlates with the cell cycle (Figure 5-5).

Wild-type embryos were fixed with methanol and stained with DAPI to reveal the chromosomes, which indicated the stages of cell cycle (Figure 5-4; Edgar et al., 1994). Under the microscope, embryos containing interphase,

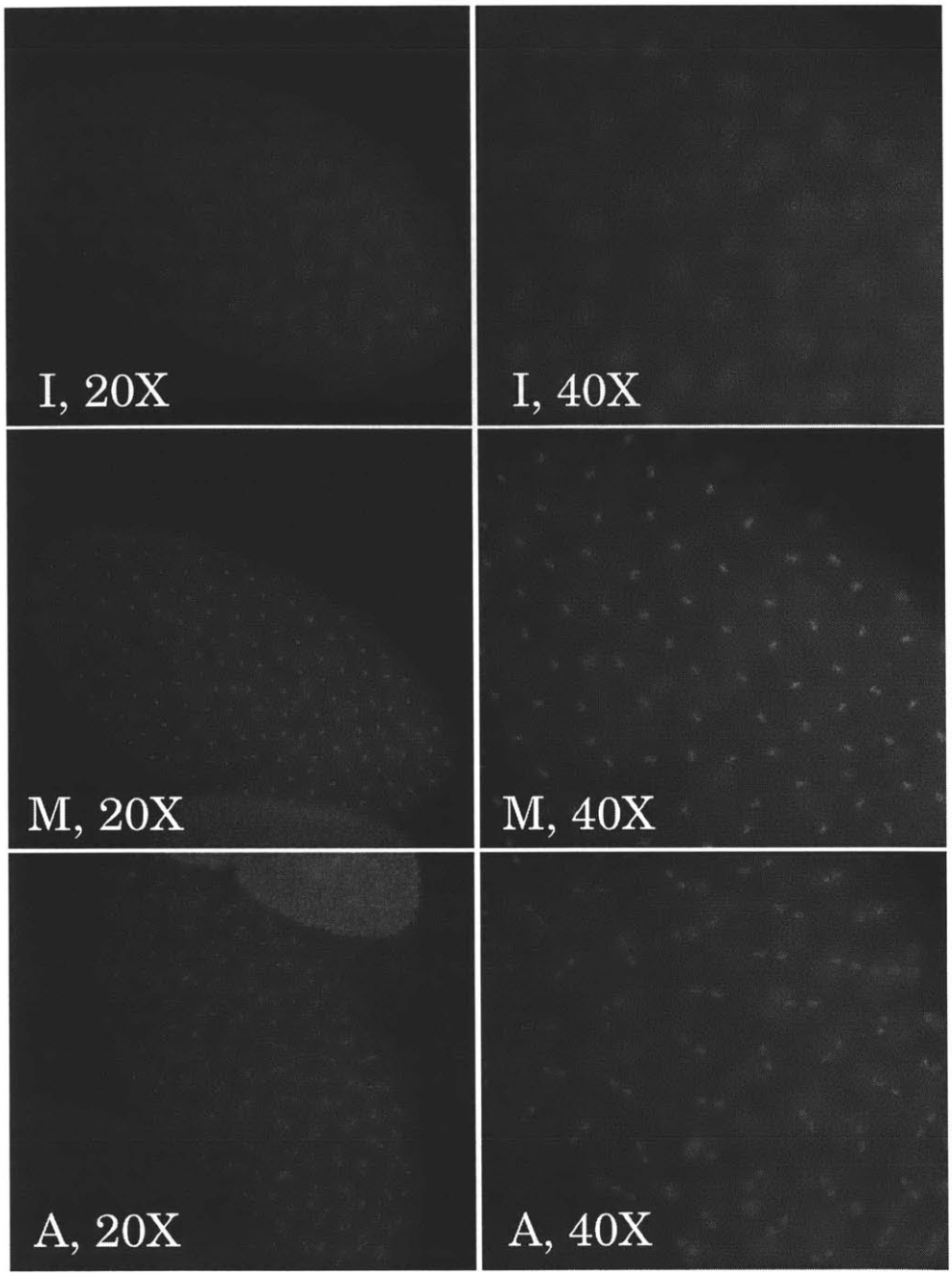

Figure 5-4. Wild-type embryos were fixed and stained with DAPI. Embryos containing interphase (I), metaphase (M), or anaphase (A) nuclei that were manually selected under the microscope using a 20X objective (20X) resemble the ones shown in the images on the left. The images on the right were taken with a 40X objective (40X) to show that these embryos did contain nuclei that were in the expected cell cycle stages. Thus, it was possible to distinguish the cell cycle with a 20X objective.

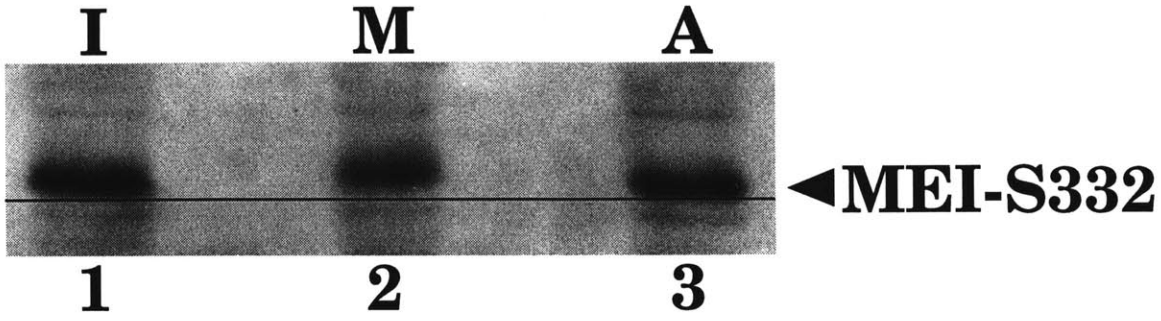

Figure 5-5. Post-translational modification of the MEI-S332 protein correlates with the cell cycle.

Manually isolated embryos containing interphase (I; lane 1), metaphase (M; lane 2), and anaphase (A; lane 3) nuclei were resolved on a Western blot and probed with anti-full length MEI-S332 antibodies. Thirty embryos were used per lane. MEI-S332 in embryos consisting of interphase or anaphase nuclei is seen as the faster-migrating form (bottom band of the doublet). In embryos containing only metaphase nuclei, MEI-S332 has a slower mobility in the gels (top band of the doublet). This modification correlates with MEI-S332 chromosomal localization. That is, MEI-S332 is not on the chromosomes in interphase and anaphase nuclei but localizes to centromeres in metaphase. The bands are fuzzy probably because the protein extracts were prepared from fixed embryos.

metaphase, or anaphase nuclei were isolated manually. Thirty embryos were pooled for each sample in order to obtain enough MEI-S332 protein to be detected on Western blots. MEI-S332 migrated as the bottom band in embryos consisting of only interphase or anaphase nuclei (Figure 5-5, lanes 1 and 3). In contrast, in embryos containing only metaphase nuclei, MEI-S332 migrated as the top band (Figure 5-5, lane 2). These results, in combination with the data from the lambda protein phosphatase experiment, suggest that MEI-S332 is phosphorylated during interphase and anaphase but dephosphorylated during metaphase. Interestingly, MEI-S332 localizes to chromosomes during metaphase, but it dissociates from the chromosomes in interphase and anaphase. The phosphorylation/ dephosphorylation of MEI-S332 correlates with whether MEI-S332 is localized onto the chromosomes.

Dephosphorylation of MEI-S332 is independent of chromosomal association

Since MEI-S332 appears as the top, dephosphorylated form only when it is on the chromosomes during metaphase, we wondered whether chromosomal localization of MEI-S332 is required for this dephosphorylation to occur. That is, does MEI-S332 have to be on the chromosomes in order to get dephosphorylated? To address this question, we isolated staged single *mei-S332*^{6} mutant embryos and separated the extracts on Western blots to determine if MEI-S332^{6} mutant protein was similarly modified during the cell cycle. MEI-S332^{6} mutant protein fails to localize properly to chromosomes in early embryos (Tang et al., 1998).

Figure 5-6 shows that the same pattern of post-translational modification occurs on the mutant MEI-S332 protein even though it does not localize to the mitotic chromosomes in embryos. This result indicates that

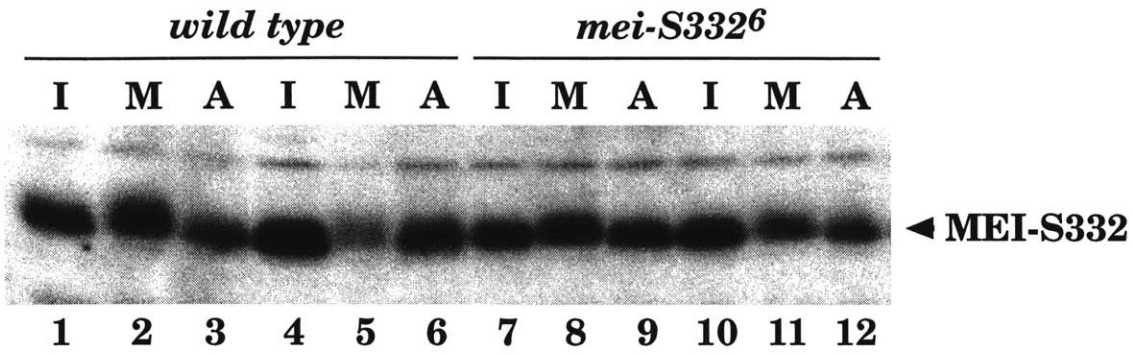

Figure 5-6. Chromosomal association is not necessary for MEI-S332 modification to occur.

Manually isolated wild-type or *mei-S332*^{6} mutant embryos consisting of interphase (I), metaphase (M), or anaphase (A) nuclei were separated on a SDS polyacrylamide gel and transferred to an immobilon membrane. The Western blot was probed with anti-MEI-S332 antibodies. (Lanes 1-6) Wild-type embryos. (Lanes 7-12) *mei-S332*^{6} mutant embryos. Extracts from fifteen to thirty embryos were loaded in each lane; protein levels are different among the lanes. Although MEI-S332^{6} mutant protein fails to localize to mitotic chromosomes (Tang et al. 1998), the same pattern of post-translational modification is observed in the mutant embryos as in wild type. MEI-S332 protein from interphase and anaphase nuclei migrates faster than MEI-S332 from metaphase nuclei. The bands are fuzzy probably because the extracts were prepared from fixed embryos.

MEI-S332 does not have to be on the chromosomes in order to become dephosphorylated.

Mutation of the PP2A regulatory subunit (PR55) does not affect MEI-S332 doublet

Because protein phosphatase 2A (PP2A) has been implicated in the regulation of sister-chromatid cohesion (Minshull et al., 1996; Gomes et al., 1993; Mayer-Jaekel et al., 1993), we wondered if PP2A could be involved in the post-translational modification of MEI-S332. Specifically, we looked at whether or not the MEI-S332 doublet is affected in the existing PP2A regulatory subunit (PR55) mutant, *twins*^{01436} (Berkeley Drosophila Genome Project, 1994; Meister and Braun, pers. comm., 1995). Brain and imaginal disc protein extracts were prepared from the *twins* mutant, separated on SDS polyacrylamide gels, and blotted with anti-MEI-S332 antibodies. MEI-S332 still migrates as a doublet in *twins* extracts, with the bottom band being the more predominant form (Figure 5-7). Therefore, this particular mutation in the PP2A regulatory subunit PR55 does not affect the post-translational modification of MEI-S332.

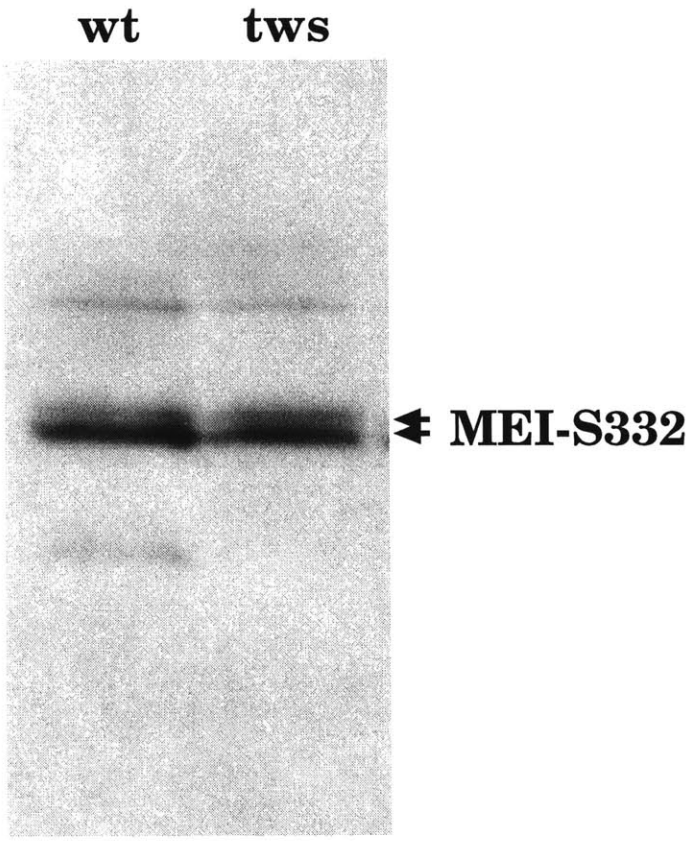

Figure 5-7. MEI-S332 doublet in a *twins* mutant.

Brains and imaginal discs were dissected from wild-type (wt) and *twins* (tws) mutant larvae (15 larvae per genotype) and homogenized in urea sample buffer. Extracts were separated on a SDS-polyacrylamide gel and blotted with anti-MEI-S332 antibodies. MEI-S332 migrates as a doublet in both wild-type and *twins* mutant brain and imaginal disc extracts. Thus, reducing the amount of the PP2A regulatory subunit PR55 does not affect the post-translational modification of MEI-S332.

Discussion

In this study, we examined the regulation of the *Drosophila* centromeric protein MEI-S332 during the cell cycle. We found that the MEI-S332 protein is post-translationally modified by phosphorylation. We also showed that MEI-S332 does not have to be on the centromere to be modified. Because the modification occurs on a global level, we believe that MEI-S332 is cell-cycle regulated. MEI-S332 is phosphorylated during interphase and anaphase, when it is dissociated from the chromosomes, but dephosphorylated during metaphase, when it is localized to the centromere.

MEI-S332 is post-translationally modified during the cell cycle

During meiosis, MEI-S332 localizes onto the centromeres in prometaphase I and does not dissociate from the chromosomes until anaphase II when sister chromatids separate (Kerrebrock et al., 1995; Moore et al., 1998). Similarly, in mitosis, MEI-S332 assembles onto the chromosomes in prometaphase and delocalizes from the chromosomes in anaphase (Moore et al., 1998; Tang et al., 1998). Its localization pattern and many putative phosphorylation sites suggest that the MEI-S332 protein may be regulated by phosphorylation during the cell cycle.

The result from the lambda protein phosphatase experiment in *Drosophila* embryo crude extracts provides compelling evidence that the MEI-S332 protein is phosphorylated. The fact that lambda protein phosphatase converted the faster-migrating form of MEI-S332 into the slower-migrating form suggests that the latter form is dephosphorylated. In embryos containing only metaphase nuclei, MEI-S332 exists as the slower-migrating form. Thus, it appears that MEI-S332 is dephosphorylated during metaphase. The faster-

migrating form is likely to be the phosphorylated form of MEI-S332, and it is present in embryos containing only interphase or anaphase nuclei.

An attractive model is that MEI-S332, when phosphorylated, is incapable of interacting with the centromere and/or centromere-assoicated proteins and that dephosphorylation is required to promote chromosomal localization of MEI-S332, which starts during prometaphase (Figure 5-8). During interphase, MEI-S332 is phosphorylated and, thus, remains in the cytoplasm. Then during prometaphase, MEI-S332 gets dephosphorylated; now capable of binding to the centromere and/or centromere-associated proteins, MEI-S332 localizes to the chromosomes. In its dephosphorylated form, MEI-S332 remains bound to the centromeres in metaphase. Then at the onset of anaphase, MEI-S332 becomes phosphorylated, and this decreases the affinity of MEI-S332 for the centromere and/or centromere-associated proteins, leading to its dissociation from the chromosomes and consequent separation of sister chromatids. Results from the *mei-S332*^{6} mutant embryos showed that MEI-S332 does not have to be on the chromosomes to become modified.

MEI-S332 can be phosphorylated in vitro

In vitro kinase assay of MEI-S332 immunoprecipitates showed that MEI-S332 could be phosphorylated directly in vitro. However, an important caveat of this experiment remains to be addressed. That is, the portions of the fusion and tagged proteins that were radioactively labeled in the in vitro kinase assay could be the GFP moiety of the fusion protein and the MYC epitope of the tagged protein, and MEI-S332 may not have been labeled. To test this possibility, we are in the process of repeating the in vitro kinase assay with GFP immunoprecipitates. If GFP is not radioactively labeled in the assay,

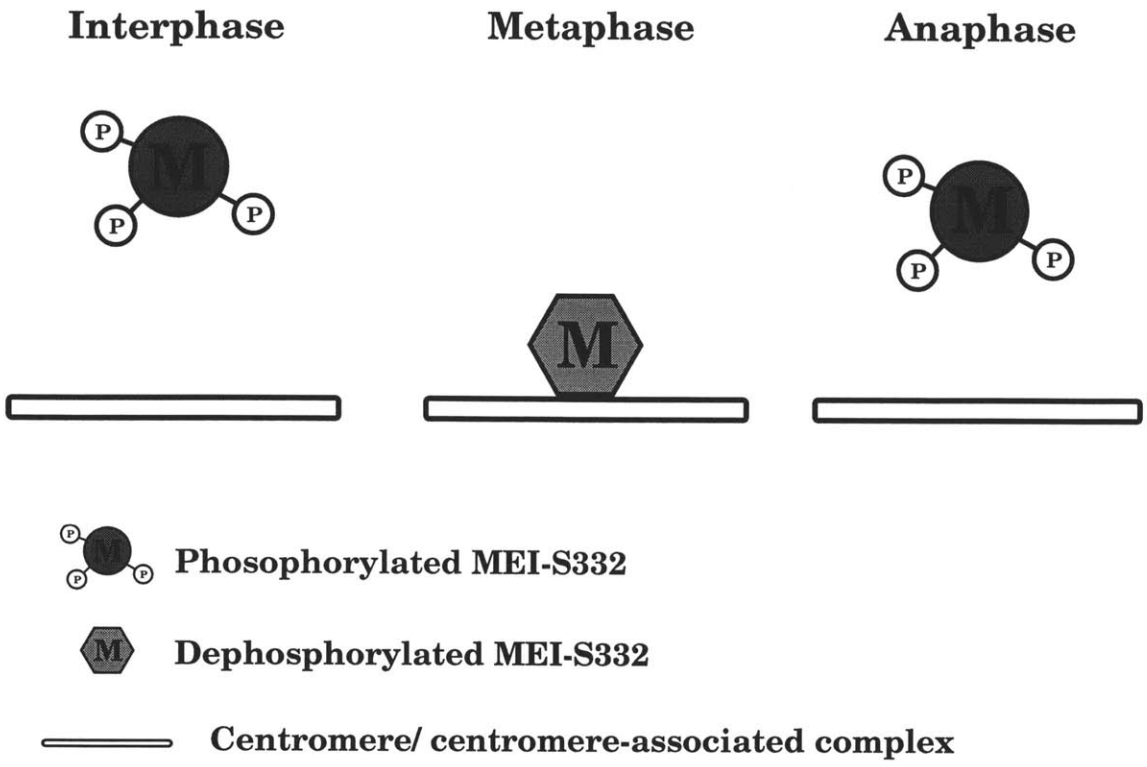

Figure 5-8. The MEI-S332 protein is post-translationally modified by phosphorylation, and this modification is cell-cycle regulated.

then we can safely conclude that the radioactive label on the MEI-S332-GFP fusion protein is due to the direct phosphorylation of MEI-S332. However, if GFP is also radioactively labeled in the assay, then we cannot rule out the possibility that what we observed in the kinase assay was the phosphorylation of the GFP protein.

The fact that the MEI-S332 immunoprecipitates can be radioactively labeled in vitro in the kinase assay without the addition of any exogenous kinase strongly indicates that at least one kinase is coimmunoprecipitated. It will be interesting to identify this kinase.

Is PP2A involved in the dephosphorylation of MEI-S332?

Although the *twins*^{01436} allele (Berkeley Drosophila Genome Project, 1994; Meister and Braun, pers. comm., 1995) does not alter the post-translational modification on MEI-S332, we cannot yet rule out the involvement of PP2A in the regulation of MEI-S332. The core structure of PP2A consists of a catalytic subunit and a 65-kD regulatory subunit (PR65); this core dimer is associated with a third variable regulatory subunit, ranging from 54 to 74 kD (for review, see Mayer-Jaekel and Hemmings, 1994). It is this third regulatory subunit that is thought to confer distinct properties, such as substrate recognition and binding, on the holoenzyme. Thus, it is possible that PR55 encoded by *twins* does not target MEI-S332 to PP2A for dephosphorylation, but another regulatory subunit is present to mediate specifically the interaction between PP2A and MEI-S332. Consistent with this hypothesis, one of the interactors recently isolated in a yeast two-hybrid screen using MEI-S332 as a bait was homologous to a PP2A regulatory subunit different than PR55 (L. Dang, C. Raymond, and T.L. Orr-Weaver, unpublished result). Additional experiments are necessary to confirm the

physical interaction between this newly isolated PP2A regulatory subunit and MEI-S332. It will be interesting to see whether this regulatory subunit is involved in specifically targetting MEI-S332 to PP2A.

In addition to testing the newly isolated PP2A regulatory subunit, we also need to determine directly the role of PP2A on MEI-S332 regulation. As a first step in the investigation of whether PP2A is involved in the dephosphorylation of MEI-S332, the *Drosophila* strain *l(2)k09822* can be used. This strain has been shown to carry a *P[lacW]* element inserted 251 bp upstream of the initiating ATG of the gene encoding the catalytic subunit of PP2A (Snaith et al., 1996). Flies heterozygous for this insertion have reduced levels of *PP2A* mRNA and PP2A activity; homozygotes for this insertion die during embryogenesis (Snaith et al., 1996). Overcondensed chromatin and a block in mitosis between prophase and the initiation of anaphase are observed in embryos homozygous for the P insertion (Snaith et al., 1996). If PP2A plays a role in dephosphorylating MEI-S332, we would expect to see only the faster-migrating form of MEI-S332 (i.e., the phosphorylated form) in protein extracts from mutant embryos deficient in PP2A activity.

If we are able to determine that PP2A plays a role in the dephosphorylation of MEI-S332 at the onset of mitosis, we are forced to face a conundrum. Our model predicts that in the absence of PP2A activity, MEI-S332 remains phosphorylated and incapable of associating with the centromere and/or centromere-associated proteins, and consequently, sister-chromatid cohesion at the centromere would not be maintained properly. Then, why does the reduction of PP2A activity caused by the insertion of the *P[lacW]* element in the strain *l(2)k09822* lead to a mitotic block between prophase and the initiation of anaphase? We speculate that during mitosis, arm cohesion can compensate for the loss of centromeric cohesion and that

PP2A is also required for other aspects of mitosis, as PP2A activity seems to be important for several substrates that are phosphorylated by the $p34^{cdc2}$/cyclin B kinase (Mayer-Jaekel et al., 1994).

MEI-S332 modification appears normal in a *twins* mutant

Using a P-element allele, $twins^{01436}$, of the *Drosophila* PP2A regulatory subunit *PR55* gene (Berkeley Drosophila Genome Project, 1994; Meister and Braun, pers. comm., 1995), we found that mutation in the PP2A regulatory subunit PR55 does not affect the post-translational modification of MEI-S332. It is possible that no alteration of the MEI-S332 doublet was observed in this particular allele because it is a weak mutation, as $twins^{01436}$ homozygotes survive to third instar larval stage. Thus, examining the MEI-S332 doublet in stronger *twins* alleles, ones that result in embryonic lethality, may be necessary to determine with certainty whether PR55 is involved in regulating the dephosphorylation of MEI-S332. However, we do not think that stronger *twins* alleles will affect the MEI-S332 doublet, because separation of the centromeres is apparently normal as judged by FISH experiments in another allele of PR55, aar^{1}, even though abnormal anaphase figures were observed in this allele (Mayer-Jaekel et al., 1993).

Although the MEI-S332 doublet appears normal in this $twins^{01436}$ allele, the presence of this mutation has been observed to suppress the wing phenotype caused by the overexpression of MEI-S332 in imaginal discs (H. LeBlanc and T.L. Orr-Weaver, unpublished result). We think that the *twins* suppression of MEI-S332 overexpression phenotype is an indirect effect of the *twins* mutation and that MEI-S332 does not actually interact with PR55. Quantitative analysis of metaphase and anaphase figures showed that *aar* (*twins* and *aar* are the same gene; see Mayer-Jaekel and Hemmings, 1994 for

review) homozygotes exhibited a reduction in the ratio of metaphase to anaphase cells, indicating acceleration through the metaphase/anaphase transition (Gomes et al., 1993). This acceleration could cancel out the effect of the delay at the metaphase/anaphase transition caused by overexpressing MEI-S332 (see Chapter Four in this thesis). Mutations in the *PR55* gene lead to aberrant anaphase figures and acceleration through the metaphase/ anaphase transition perhaps because PP2A is also involved in the regulation of some other substrates that are involved in the progression of the cell cycle.

Another protein phosphatase candidate for MEI-S332 is the type 1 protein phosphatase

In addition to PP2A, type 1 protein phosphatase (PP1) may also be involved in the regulation of MEI-S332 during the cell cycle. Recent work in *S. cerevisiae* has shown that PP1 is important for the dephosphorylation of a component of the kinetochore complex and this dephosphorylation is necessary for the proper binding of kinetochore to microtubules (Sassoon et al., 1999). Conditional alleles of the *S. cerevisiae* PP1 catalytic subunit Glc7p arrest in metaphase with short metaphase spindles, most likely because the spindle assembly checkpoint is activated in these mutants as kinetochores fail to form stable interactions with microtubules. Consistent with this, the arrest is abolished when the cells are also mutated for components of the spindle assembly checkpoint (Bloecher and Tatchell, 1999; Sassoon et al., 1999). These results suggest that the component of the kinetochore complex, whose phosphorylation state seems to be regulated by PP1, must be dephosphorylated during prometaphase and metaphase to allow stable microtubule attachments to the kinetochores. Thus, it is intriguing that MEI-S332, localizing to the centromere during prometaphase, also appears to be

dephosphorylated during metaphase. It will be interesting to see if PP1 is directly involved in the regulation of MEI-S332, and this question can be addressed by the use of *Drosophila* PP1 mutants (Axton et al., 1990).

What are some potential kinases that could phosphorylate MEI-S332?

During interphase and anaphase, MEI-S332 appears to be phosphorylated. It is possible that phosphorylation is required to delocalize MEI-S332 from the chromosomes, allowing sister chromatids to separate. At least three kinases are known to function during mitosis: the $p34^{cdc2}$/cyclin B kinase, the AURORA kinase, and the POLO kinase. In addition, the *Drosophila* LK6 kinase (Kidd and Raff, 1997) has been found to interact with MEI-S332 in yeast two-hybrid assay and in GST-pulldown experiments (Page 1998).

The $p34^{cdc2}$/cyclin B kinase, the universal inducer of mitosis, is involved in regulating many aspects of mitosis (for review, see Nigg 1995; Nigg et al., 1996). Because the CDC2 kinase activity is high during mitosis, it is possible that it is involved in the rephosphorylation of MEI-S332 at the onset of anaphase. We set out to test this possibility first by determining whether MEI-S332 interacts with CDC2. By probing MEI-S332 immunoprecipitate with antibodies specific to the *Drosophila* CDC2 protein (Edgar et al., 1994), we found that MEI-S332 is not associated with the CDC2 protein (see Appendix I; Figure I-1C). Because our in vitro kinase assay indicated that the kinase that is capable of phosphorylating MEI-S332 in vitro is coimmuno-precipitated with MEI-S332, it is unlikely that CDC2 plays a role in phosphorylating MEI-S332.

The *Drosophila* AURORA kinase also functions during mitosis. Mutations in the *Drosophila aurora* gene block the separation of centrosomes

and result in the formation of monopolar spindles (Glover et al., 1995). Although AURORA kinase functions during mitosis, it is unlikely to be involved in the phosphorylation of MEI-S332 because chromosomes can be see to enter anaphase in embryos derived from *aurora* females, indicating that sister-chromatid separation is unaffected by the *aurora* mutations (Glover et al., 1995). Moreover, the *S. cerevisiae* IPL1 kinase is homologous to the *Drosophila* AURORA kinase, and sister chromatids apparently separate normally in *ipl1* mutants (Biggins et al., 1999).

LK6 is a potential kinase that phosphorylates MEI-S332 at the onset of anaphase, because it was isolated in a yeast two-hybrid screen using MEI-S332 as a bait and shown to interact physically with MEI-S332 in GST-pulldown experiments (Page 1998). Overexpression of LK6, identified as a microtubule-associated kinase, causes defects in microtubule organization in both *Drosophila* eggs and early embryos (Kidd and Raff, 1997). Thus, it is possible in these embryos, the excess activity of the LK6 kinase causes inappropriate phosphorylation of the MEI-S332 protein, leading to an aberrant progression through the cell cycle. However, MEI-S332 chromosomal localization is not affected in embryos from two LK6-overexpressing lines (T.T.-L. Tang and T.L. Orr-Weaver, unpublished results). Western blots of protein extracts from these LK6-overexpressing embryos also showed no alteration of the MEI-S332 doublet, indicating that excess LK6 activity does not perturb the post-translational modification of MEI-S332 (T.T.-L. Tang and T.L. Orr-Weaver, unpublished results). Furthermore, LK6 does not co-immunoprecipitate with MEI-S332 (see Appendix I in this thesis). Thus, LK6 does not appear to act as a kinase that controls MEI-S332 delocalization or cohesion activity.

Finally, the *Drosophila* POLO kinase remains a potential candidate for MEI-S332 phosphorylation. A family of polo-like kinases has been isolated from many organisms, and they appear to play multiple functions during mitosis (for review, see (Glover et al., 1996; Glover et al., 1998; Nigg 1998). Originally, the *polo* gene was isolated in *Drosophila* because *polo* mutants exhibit highly disorganized spindle microtubules (Sunkel and Glover, 1988) In these mutants, the CP190 centrosomal antigen fails to assemble into centrosomes (Sunkel and Glover, 1988), and centrosomes fail to separate (Llamazares et al., 1991). In addition, defects in chromosome segregation in meiosis have been reported for the $polo^1$ allele, and most of the nondisjunction occurs during the second meiotic division, suggesting a defect in the separation of sister chromatids (Sunkel and Glover, 1988). These observations suggest that POLO is a kinase for MEI-S332 phosphorylation at the onset of anaphase. Because the lambda protein phosphatase experiments were performed in embryos, the results from the experiments only indicate MEI-S332 rephosphorylation at the onset of mitotic anaphase. However, because of the similarity between mitosis and meiosis II, it is likely that MEI-S332 is phosphorylated at the onset of anaphase II when sister chromatids separate. Then, the anaphase II nondisjunction phenotype (i.e., failure of sister-chromatid separation) in the $polo^1$ allele is consistent with the proposal that POLO normally phosphorylates MEI-S332. Thus, in the absence of POLO activity, MEI-S332 remains dephosphorylated and bound to the centromere and/or centromere-associated proteins, continuing to maintain cohesion at the sister centromeres even when anaphase II is triggered and meiosis is completed to produce gametes. Therefore, POLO is the best candidate for the kinase of MEI-S332 at the onset of mitotic anaphase and meiotic anaphase II.

Protein extracts from *polo* mutant embryos should be separated on SDS polyacrylamide gels to see if the MEI-S332 doublet is affected.

Conclusion

We have shown in this study that MEI-S332 is regulated by phosphorylation during the cell cycle. MEI-S332 appears to be phosphorylated during interphase and anaphase, and dephosphorylated in metaphase. Thus, dephosphorylation may be required for chromosomal localization of MEI-S332. Removing the negatively charged phosphates from MEI-S332 may be a necessary prerequisite for promoting the physical interactions between MEI-S332 and centromere and/or centromere-associated proteins. Then at the onset of anaphase, MEI-S332 has to be phosphorylated to induce its dissociation from the centromere and/or centromere-associated proteins, allowing sister chromatids to separate. PP1 and PP2A are two potential protein phosphatases that may play a role in the dephosphorylation of MEI-S332 at the onset of mitosis, while the *Drosophila* POLO kinase may function to phosphorylate MEI-S332 at the onset of mitotic anaphase and meiotic anaphase II. Further investigation on the involvement of PP1, PP2A, and POLO in the phosphorylation/ dephosphorylation of the MEI-S332 protein will be important for the understanding of the regulation of sister-chromatid cohesion.

Materials and methods

Fly strains

Oregon-R was used as the source of wild-type embryo, brain, and imaginal disc protein extracts. Embryos from the *mei-S332+::GFP* transgenic flies, *y w P[GrM]-7; +/+*, described previously (Tang et al., 1998), were used to prepare extracts for immunoprecipitation and subsequent kinase assays. Embryos from *myc-mei-S332*$^{+}$ transgenic flies were also used for making extracts for immunoprecipitation and kinase assays. The *myc-mei-S332*$^{+}$ insert was constructed by inserting at the *Bgl*II site of *mei-S332* in the transposon, P[w^{+} 5.6KK *mei-S332*] (Kerrebrock et al., 1995), a tandem repeat of three *myc* gene fragments encoding the epitope EQKLISEEDLN. The resulting transposon, P[w^{+} 5.6KK mid-*myc-mei-S332*], was used to transform flies by injection (Spradling, 1986). Multiple transformant lines were recovered and tested for complementation (data not shown). This *myc-mei-S332*$^{+}$ insert was shown to rescue *mei-S332* chromosome missegregation phenotype in both females and males by nondisjunction tests (data not shown; Kerrebrock et al., 1992). The *twins* mutant used in this study is a P-element allele, *P1532*, from the Bloomington Stock Center; it is also known as *twins*^{01436} (Berkeley Drosophila Genome Project, 1994; Meister and Braun, per. comm.1995). It was balanced over the *TM6, Tb* balancer; brains and imaginal discs were dissected from non-tubby larvae, which are homozygous for the *twins* mutation, to make protein extracts for Western blots.

Western blot analysis

Protein extracts were separated on 12% 150:1 (acrylamide/bis-acrylamide) gels and blotted onto immobilon-P membranes. To ensure that a good resolution of the MEI-S332 doublet was achieved, the gels were run at

20mA until the red marker (Lysozyme, ~18kD) of Kaleidoscope Prestained Standards (BioRad) reached the bottom of the gels, taking approximately 4-5 hours. The vertical slab gel unit (The Sturdier; model SE 400) from Hoefer Scientific Instruments (San Francisco, CA) was used for the electrophoresis.

The blots were probed with guinea pig anti-full length MEI-S332 antiserum at 1:20,000 as described in Tang et al. (1998).

Isolation of single embryos

Collections of 0- to 3-hour embryos were dechorionated, fixed in methanol, and stained with DAPI exactly as described by Edgar et al. (1994). Embryos containing interphase, metaphase, or anaphase nuclei were visually selected under an Axiophot microscope with a 20X objective; a fine brush was used to manipulate the embryos. For each sample, selected embryos were kept in PBS until approximately thirty embryos were found. Then, PBS was removed completely with a drawn-out Pasteur pipet and replaced with 20µl 2X SDS sample buffer [2% SDS, 10% glycerol, 80mM Tris-HCl (pH6.8), 2mM EDTA, 0.1M DTT]. Embryos were homogenized in the sample buffer, and extracts were cleared by centrifugation, quick frozen in dry ice, and stored at –80°C. The selected embryos were in cycles 8/9 to 13 as the nuclei in these embryos had migrated to the surface of the embryos. The MEI-S332 band was detected on Western blots with good intensity only when at least 28-30 embryos were pooled in each sample; 10-15 embryos gave a very reduced signal on Western blots that could be seen only with a longer exposure of the films. It was not possible to detect MEI-S332 on Western blots if only one or five embryos were used.

Phosphatase experiments

2- to 6-hour embryos were dechorionated in 50% Clorox, rinsed with water, and homogenized in 1X lambda protein phosphatase reaction buffer supplemented with 2mM $MnCl_2$ (provided by New England Biolabs with the phosphatase) in a glass 2-ml dounce homogenizer using the tight pestle. Extracts were incubated at 30°C for 30 minutes in the presence or absence of lambda protein phosphatase (NEB; 20units/μl reaction). According to the manufacture's protocol, in 0.1 nanomole of protein in 30 minutes in a 50μl reaction, 20-200 units of the enzyme will typically release >95% of phosphates from serine/threonine residues, and 100-1000 units will typically release >95% of phosphates from tyrosine residues (New England Biolabs, Inc.). In our experiment, 500 units of the enzyme were used in 25μl reactions. In cases where phosphatase inhibitors were included in the reactions [10mM Na_3VO_4, 50mM NaF, or 100mM $NaPO_4$ (pH8)], the inhibitors were added into the extracts before the addition of the phosphatase. At the end of the 30-minute incubation, equal volume of urea sample buffer [USB: 8M urea, 2% SDS, 5% β-mercaptoethanol, 100mM Tris (pH 7.6), and 5% Ficoll] was added to each reaction to quench the enzyme; samples were quick frozen in dry ice and stored at –80°C. It was important that samples in USB were not heated because heating in the presence of urea leads to carbamylation on proteins, which can alter the protein mobility in gels. The effect of adding lambda protein phosphatase in embryonic crude extracts was visualized by separating the extracts on SDS polyacrylamide gels and blotting with anti-MEI-S332 antibodies as described above.

Immunoprecipitation and kinase assay

Embryo extracts for immunoprecipitation were prepared as previously described (Tang et al., 1998) with the exception that the final concentration of NaN_3 was 0.02%. MEI-S332-GFP fusion protein was immunoprecipitated using rabbit polyclonal anti-GFP antibodies (Clontech; Tang et al., 1998). MYC-MEI-S332 was immunoprecipitated with cMyc (9E10) mouse monoclonal IgG (Santa Cruz Biotechnology) by incubating the extracts with the antibodies overnight at 4°C and subsequently with protein A-sepharose CL4B beads for one hour at 4°C. Immunocomplex-bound beads were washed once with IP buffer (see Tang et al., 1998) containing 500mM NaCl, five times with IP buffer, and twice with kinase buffer [20mM HEPES, (pH7.5), 150mM KCl, 10mM $MgCl_2$, 2mM DTT, 1mM EDTA, 5μM ATP, and 174ng/ml PMSF] before the kinase reaction.

For the kinase assay, washed immunocomplex-bound beads were incubated in kinase buffer in the presence of 20μCi γ^{32}P-ATP for one hour at room temperature. Then, the beads were washed 3-5 times with RIPA buffer [150mM NaCl, 50mM Tris (pH 8), 1% NP-40, 0.5% sodium deoxycolate, 0.1% SDS, 2.5mM EDTA, 0.02% NaN_3, 20mM NaF, 0.3mM Na_3VO_4, 174ng/ml PMSF] to remove excess nonspecific background and twice with kinase buffer, and heated in 50μl kinase buffer and 10μl 6X SDS sample buffer [for a 10-ml stock: 7ml 4X Tris-Cl/SDS (pH6.8), 3ml 100% glycerol, 1g SDS, 0.93g DTT, 1.2mg bromphenol blue] at 95°C for 5 minutes. Finally, samples were quick frozen in dry ice and stored at –80°C overnight. The day after the kinase reaction, the immunocomplexes were separated on SDS polyacrylamide gels as described above and transferred to immobilon-P membranes. ^{32}P-labeled bands on the membranes were detected using a Fuji phosphoimager; usually an overnight exposure was taken. Then the same membranes were probed

with anti-MEI-S332 antibodies to determine the location of the MEI-S332-GFP, MYC-MEI-S332, and the endogenous MEI-S332 proteins.

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Afterword

I. Post-translational modification on MEI-S332

I have found that MEI-S332 is post-translationally modified. Treating embryo crude extracts with lambda protein phosphatase, I showed that this post-translational modification involved phosphorylation. Because MEI-S332 possesses as many as thirty putative phosphorylation sites, I set out to determine whether MEI-S332 is directly phosphorylated. In vitro ^{32}P-labeling experiments demonstrated that MEI-S332-GFP fusion protein and MYC-tagged MEI-S332 were phosphorylated, suggesting that MEI-S332 can be phosphorylated. However, a caveat in these experiments remains to be addressed. That is, the portions of the fusion and tagged proteins that were radioactively labeled in the in vitro labeling experiments could be GFP and the MYC epitope alone, and MEI-S332 was actually not labeled. To test this possibility, I am in the process of repeating the in vitro labeling experiments with the inclusion of GFP immunoprecipitates as a control. Namely, GFP will be precipitated by anti-GFP antibodies from protein extracts prepared from embryos laid by flies carrying and expressing GFP under the actin promoter. If GFP is not radioactively labeled in the kinase assay, then I can conclude that MEI-S332 can be directly phosphorylated.

Precocious sister-chromatid separation and aberrant anaphase figures are observed in *S. cerevisiae cdc55* mutant and *Drosophila twins/aar* mutant, respectively (Minshull et al. 1996; Uemura et al. 1993; Gomes et al. 1993; Mayer-Jaekel et al. 1993). Because both of these genes encode the PR55 regulatory subunit of protein phosphatase 2A (PP2A), I wondered if PP2A plays a role in dephosphorylating MEI-S332. Although a P-element allele of *twins* does not alter the post-translational modification on MEI-S332, I cannot yet rule out the involvement of PP2A in MEI-S332 regulation. The core structure of PP2A, consisting of a catalytic subunit and a 65-kD regulatory

subunit (PR65), is associated with a third variable regulatory subunit that usually shows tissue- and development-specific expression pattern and is likely to be involved in substrate recognition and binding (for review, see Mayer-Jaekel and Hemmings 1994). In other words, in different tissues and different developmental stages, the PP2A core enzyme can be associated with different variable regulatory subunits. It is likely that each of these regulatory subunits targets the enzyme to specific substrates. PR55 is one of these variable regulatory subunits. Perhaps, it is not involved in targetting PP2A to MEI-S332. However, it is possible that there exists another regulatory subunit that functions to target PP2A specifically to MEI-S332.

Consistent with this hypothesis, one of the interactors isolated in a yeast two-hybrid screen using MEI-S332 as a bait was homologous to a PP2A regulatory subunit different than PR55 (L. Dang, C. Raymond, and T.L. Orr-Weaver, unpublished results). I plan to confirm the physical interaction between MEI-S332 and the newly isolated PP2A regulatory subunit by the GST-pull-down approach. In this experiment, MEI-S332, in vitro translated and labeled with ^{35}S in rabbit reticulocyte lysate, will be incubated with the PP2A regulatory subunit fused to GST. The amounts of MEI-S332 bound to glutathione agarose beads via the fusion protein will be quantitated. By comparing to GST alone controls, I will be able to determine if significant amounts of MEI-S332 physically interact with the PP2A regulatory subunit.

To determine whether this newly isolated PP2A regulatory subunit is involved in modulating the post-translational modification of MEI-S332 in vivo, I plan to first map the chromosomal location of the gene on polytene chromosomes. Then I will search for existing deficiencies that uncover the region of the chromosome where this gene is located. By crossing the *twins* P-element allele mentioned above into the various deficiency background, I will be

able to determine whether reducing the levels of two regulatory subunits of PP2A alters the mobility of MEI-S332 doublet in the gels. A change in protein mobility will indicate that PP2A is involved in the regulation of MEI-S332.

Another approach to test whether PP2A is involved in the dephosphorylation of MEI-S332 is to use the *Drosophila* strain *l(2)k09822*. This strain has been shown to carry a *P[lacW]* element inserted 251 bp upstream of the initiating ATG of the gene encoding for the catalytic subunit of PP2A (Snaith et al. 1996). Flies heterozygous for this insertion have reduced levels of PP2A mRNA and PP2A activities; homozygotes for this insertion die during embryogenesis (Snaith, et al. 1996). I can first distinguish the embryos homozygous for this P insertion from the heterozygous embryos by staining for β-galactosidase and then separate the protein extracts from the homozygous embryos on SDS polyacrylamide gels. If PP2A plays a role in dephosphorylating MEI-S332, I would expect to see only the faster-migrating form of MEI-S332 (i.e., the phosphorylated form) in these extracts.

Other potential phosphatases and kinases can also be tested by looking at whether mutations in these enzymes affect the mobility of the MEI-S332 doublet on SDS polyacrylamide gels. Some of these candidates include the *Drosophila* type 1 protein phosphatase (PP1), CDC2 protein kinase, AURORA protein kinase, and POLO kinase (see Chapter Five in this thesis). Mutants for these genes have all been isolated and characterized in *Drosophila* (Axton et al. 1990; Stern et al. 1993; Sigrist et al. 1995; Glover et al. 1995; Sunkel and Glover 1988).

II. Isolation of proteins that interact with MEI-S332

Preliminary results from gel-filtration and glycerol-gradient experiments indicated that MEI-S332 is in a large, multimeric complex. Furthermore, staining *mei-S332*^{3} and *mei-S332*^{8} mutant spermatocytes with anti-MEI-S332 antibodies, I found that mutations in the predicted coiled-coil domain of MEI-S332 do not affect its localization to chromosomes. However, these mutations do cause high frequencies of chromosome loss and chromosome missegregation predominantly in males. Thus, the predicted coiled coil may interact physically with factors that are present only in males, and these protein-protein interactions may be disrupted by the *mei-S332*^{3} and *mei-S332*^{8} mutations. Therefore, I predict that the MEI-S332 protein complex consists of proteins that are common in the two sexes as well as proteins that are sex-specific.

One approach to purify MEI-S332 and its interactors from mature oocyte extracts is immuno-affinity chromatography with low-affinity polyclonal guinea pig anti-full length MEI-S332 antibodies. The major advantage of using low-affinity antibodies is that the protein complex can be eluted under relatively mild conditions and hence its native function(s) is likely to be preserved (Kellogg and Alberts 1992). Thus, if in the near future one wishes to study the DNA-binding ability of the MEI-S332 protein, this purification strategy can be used to isolate the native MEI-S332 protein and protein complexes for DNA-protein interaction assays.

Extracts from mature oocytes of *Oregon-R* (wild type), *mei-S332*^{7} (a truncation mutation that lacks the carboxyl-terminal basic region), and *mei-S332*^{8} (a missense mutation in the hydrophobic phase of the predicted coiled-coil domain) will be made and passed through a series of chromatography columns. First, a Sepharose column will remove any protein aggregates that may have formed in the extracts before entry into the column. Then a control

IgG column will filter out some of the nonspecific interactors. Finally, a low-affinity anti-MEI-S332 antibody column will retain specifically the MEI-S332 protein and its interactors after several washes. The success of the purification procedure will be monitored by detecting the presence of MEI-S332 with rabbit anti-MEI-S332 peptide antibodies on Western blots of the crude extracts and the different fractions from the columns.

The MEI-S332-containing fractions eluted from the anti-MEI-S332 antibody column will be pooled and run on SDS polyacrylamide gels. The presence of MEI-S332 and its interactors in the gels will be detected by silver-staining. If multiple protein bands are present, a few bands for the follow-up experiments will be selected based on the following criteria. Bands that are present in the purified fractions from *Oregon-R* oocyte extracts but missing in those from the *mei-S332*^{7} oocyte extracts will likely to be interesting, since they may be interacting with the MEI-S332 protein through its basic region. Similarly, bands missing in fractions from *mei-S332*^{8} extracts will potentially represent proteins that interact with the coiled-coil domain of the MEI-S332 protein. Finally, any major bands will also be chosen.

The identities of the interactors will be determined by mass spectrometric protein sequencing (Shevchenko et al. 1996) and running the sequences through the databases for homology searches. Some of the interactors could have homology to components of the kinetochores, and others to non-histone chromosomal proteins that are required for the formation of the higher order chromatin structure at the centromere. Novel proteins that are specifically required for centromeric sister-chromatid cohesion may also be identified. For example, proteins that appear to interact with the basic region of MEI-S332 may be involved in the recruitment of MEI-S332 to the centromeres. The sequences of these interactors will be compared with those isolated in the two yeast two-hybrid screens that have been performed using

MEI-S332 as the bait (A. Frank, A.W. Page, L. Dang, C. Raymond, and T.L. Orr-Weaver, unpublished results).

To isolate sex-specific interactors, yeast two-hybrid screen will be done with testis cDNA library. Factors that are isolated in this screen but not in the previous yeast two-hybrid screens using ovarian cDNA library are potential candidates for male-specific proteins that interact with MEI-S332. These factors will be tested for interaction with MEI-S332^{8} mutant protein, and the ones that fail to interact with the mutant protein could be interactors that associate with MEI-S332 via the predicted coiled-coil domain.

As a follow-up experiment, antibodies against portions of the interactors isolated by immuno-affinity chromatography and/or yeast two-hybrid screens will be raised and used to determine whether these interactors co-localize with MEI-S332 during meiosis. Although some of these proteins (e.g., kinetochore components and chromatin proteins), may not co-localize with MEI-S332 at every point in the meiotic cell cycle, their localization patterns will still be interesting. The antibodies can also be used to determine the expressions of these proteins during development by Western blotting embryos, and larval and adult tissues. It will be interesting also to see whether expressions of some of these proteins are sex-specific.

III. Relationship between MEI-S332 and the spindle assembly checkpoint

The observation that mutations in *mei-S332* fail to arrest spermatocytes in metaphase II raises that possibility that MEI-S332 plays a role in spindle assembly checkpoint (see Introduction). In addition to being a structural component that holds sister chromatids together, it is possible that MEI-S332 acts upstream of the spindle assembly checkpoint. That is, it functions to sense the tension between kinetochores or kinetochore attachments to microtubules in meiosis II and to then relay the signal to the spindle assembly checkpoint machinery. Before tension or microtubule attachment is established, MEI-S332 keeps the spindle assembly checkpoint activated, and/or when tension or microtubule attachments are formed, it transfers an inhibitory signal to the checkpoint. Alternatively, MEI-S332 may be the downstream effector of spindle assembly checkpoint. In the absence of tension or microtubule attachments, the spindle assembly checkpoint keeps MEI-S332 activated. Once tension is achieved and microtubule attachments are made, the checkpoint sends out a signal to inhibit the MEI-S332 protein.

Because of the possibility that MEI-S332 plays a role in spindle assembly checkpoint by acting either upstream or downstream of the checkpoint, I wondered how mutations in *mei-S332* would affect the localization of *Drosophila* BUB1, a component of the spindle checkpoint, to the chromosomes. Using chicken anti-BUB1 antibodies (Basu et al. 1999, submitted), I have found that in *mei-S332* mutant spermatocytes BUB1 is still capable of localizing to chromosomes during prometaphase I. It remains unclear whether BUB1 localization to or delocalization from chromosomes is affected in meiosis II, during which *mei-S332* mutant phenotype is manifested.

More immunofluorescence microscopy is necessary to address this question; this awaits the production of higher-affinity anti-BUB1 antibodies.

Staining *Drosophila* spermatocytes mutant for *bub1* with anti-MEI-S332 antibodies will also be informative in elucidating a potential pathway between the spindle assembly checkpoint and MEI-S332. Two alleles of *bub1* have been isolated (Basu et al. 1999, submitted). Unfortunately, male *bub1* mutant larvae have very small testes; this makes cytological analysis in *bub1* spermatocytes very difficult. Alternatively, the effect of *bub1* mutations on MEI-S332 localization can be investigated in larval brains and imaginal discs, and these experiments are currently in progress (H. LeBlanc and T.L. Orr-Weaver, pers. comm.).

The fact that BUB1 is a protein kinase and MEI-S332 is a potential phospho-protein raises the possibility that BUB1 is involved in a pathway that leads to the phosphorylation of MEI-S332, which would place MEI-S332 and hence, sister-chromatid cohesion downstream of the spindle assembly checkpoint. Brain and imaginal disc extracts from *bub1* mutant larvae were separated on SDS polyacrylamide gels and blotted with anti-MEI-S332 antibodies. MEI-S332 still migrates as a doublet in *bub1* mutant extracts, indicating that BUB1 is not involved in the post-translational modification of MEI-S332. Alternatively, cohesion proteins, such as MEI-S332, could act upstream of the spindle assembly checkpoint (see Introduction). Basu et al. (1999, submitted) reported that Drosophila BUB1 protein migrates as a doublet on Western blots. Thus, it will be interesting to see if BUB1 protein mobility in SDS polyacrylamide gels is affected by mutations in the *mei-S332* gene.

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Appendix I

Immunoprecipitation of the MEI-S332 protein

One approach to understand further how the MEI-S332 protein acts to hold sister chromatids together is identifying proteins that interact with MEI-S332. Preliminary results from gel-filtration and glycerol-gradient experiments indicate that MEI-S332 is in a large, multimeric complex (Tang et al. 1998). Thus, it is of great interest to determine what proteins are in this complex. I addressed this question by immunoprecipitating MEI-S332 from 2- to 6-hour embryo extracts and probing the Western blots of immunocomplexes with antibodies against potential interactors.

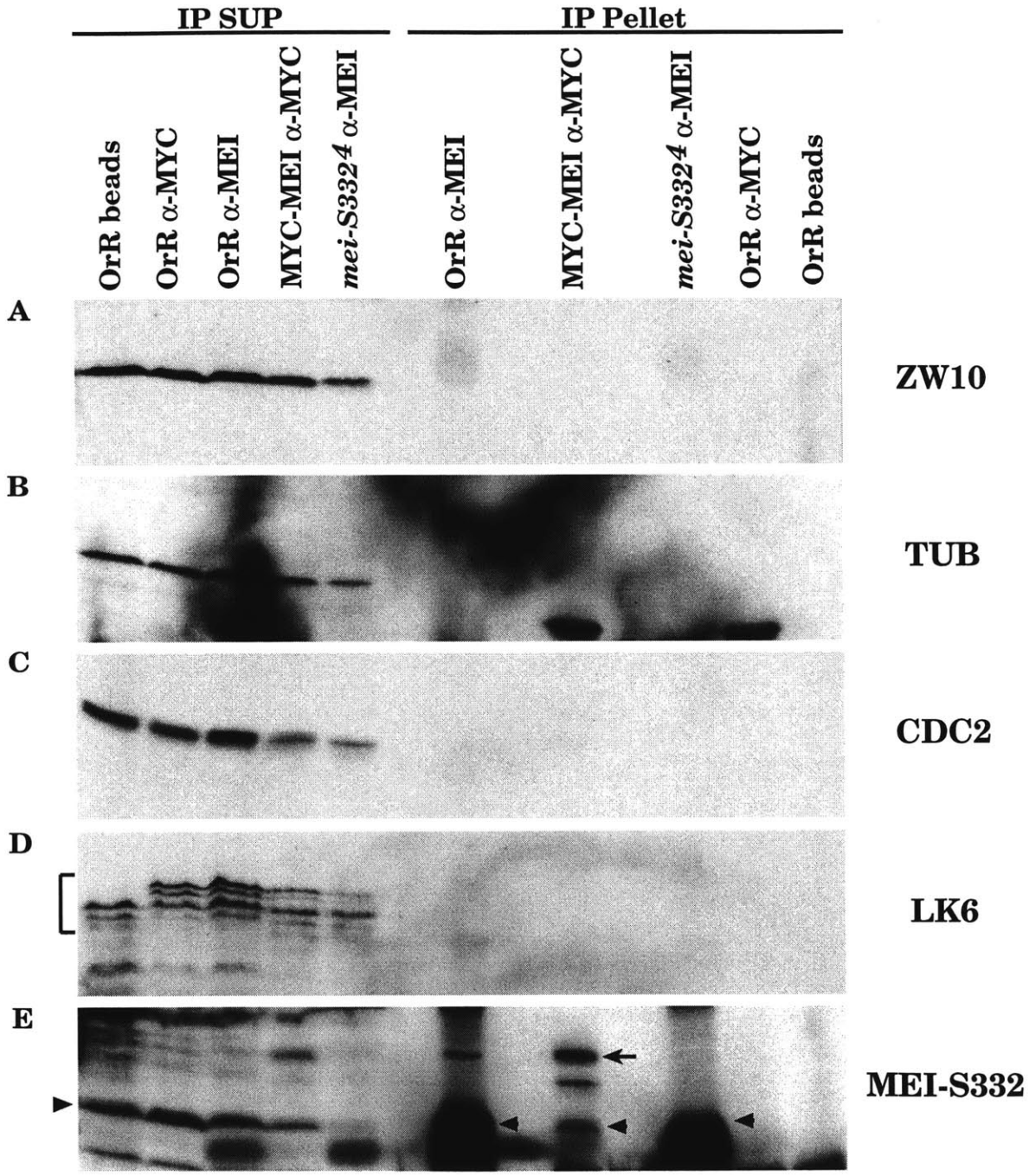
The ZW10 protein

Because MEI-S332 is detected on the centromeres of condensed mitotic and meiotic chromosomes (Kerrebrock et al. 1995; Moore et al. 1998), I wondered if MEI-S332 is associated with the kinetochores. To test this possibility, I probed the MEI-S332 immunocomplex for the *Drosophila* ZW10 protein, which is believed to be a component of the *Drosophila* kinetochores (Williams and Goldberg 1994). It appears that MEI-S332 and ZW10 are not associated in the same complex (Figure I-1A). ZW10 is present in all of the supernatants from MEI-S332 immunoprecipitation but not in any of the immunoprecipitation pellets.

Therefore, although MEI-S332 localizes to the centromere regions, it is not on the kinetochores. This result is consistent with the finding that MEI-S332 and ZW10 do not colocalize in spermatocytes, even though they are adjacent to each other (J. Lopez and T.L. Orr-Weaver, unpublished result). Furthermore, studies with minichromosome derivatives have led Murphy and Karpen (1995) to propose that sister-chromatid cohesion is mediated by a 200-kb centromeric heterochromatin region flanking the centromere core, the region of kinetochore activity (Murphy and Karpen 1995).

Figure I-1. MEI-S332 immunoprecipitation.

The Western blot of various immunoprecipitates was probed with rabbit anti-ZW10 (*A*), rat anti-α-tubulin (*B*), rabbit anti-CDC2 (*C*), rabbit anti-LK6 (*D*), and guinea pig anti-MEI-S332 (*E*) antibodies. ZW10, tubulin, CDC2, and LK6 are not in a complex with MEI-S332. MEI-S332 (arrowhead in *E*) coimmunoprecipitates with MYC-MEI-S332 (arrow in *E*). Because MEI-S332 was detected on the blot using the same antibodies that were in the OrR *α*-MEI and *mei-S332*^{4} α-MEI IP reactions, the anti-guinea pig secondary antibodies used on the blot also reacted with the IgG in the IP pellets, making it difficult to see the MEI-S332 bands. (OrR beads) Wild-type extracts immunoprecipitated with protein A beads alone. (OrR α-MYC) Wild-type extracts immunoprecipitated with anti-MYC antibodies. (OrR α-MEI) Wild-type extracts immunoprecipitated with guinea pig anti-MEI-S332 antibodies. (MYC-MEI α-MYC) Extracts containing MYC-MEI-S332 protein immunoprecipitated with anti-MYC antibodies. (*mei-S332*^{4} α-MEI) *mei-S332*^{4} mutant extracts immunoprecipitated with guinea pig anti-MEI-S332 antibodies; *mei-S332*^{4} mutants have reduced levels of MEI-S332. Bracket in *D* indicates LK6 bands.


IP SUP
IP Pellet
OrR beads
OrR α-MYC
OrR α-MEI
MYC-MEI α-MYC
mei-S332⁴ α-MEI
OrR α-MEI
MYC-MEI α-MYC
mei-S332⁴ α-MEI
OrR α-MYC
OrR beads
A
ZW10
B
TUB
C
CDC2
D
LK6
E
MEI-S332

α-tubulin subunit

Consistent with the result that MEI-S332 is not associated with the kinetochores, it does not interact with the α-tubulin subunit (Figure I-1B). This result suggests that MEI-S332 does not associate with the microtubules.

The CDC2 protein

The regulation of MEI-S332 seems to involve phosphorylation (Chapter Five in this thesis). Whether this phosphorylation depends on the activity of the mitotic cyclin-dependent kinase is of great interest. Thus, I probed the MEI-S332 immunoprecipitate with antibodies specific to the *Drosophila* CDC2 protein (Edgar et al. 1994). Figure I-1C shows that CDC2 is not associated with MEI-S332.

The LK6 protein

The LK6 protein kinase was isolated in a yeast two-hybrid screen using MEI-S332 as a bait and shown to interact physically with MEI-S332 in GST-pulldown experiment (Page 1998). It is important to test if LK6 is associated with MEI-S332 in vivo. Thus, I probed the MEI-S332 immunoprecipitates for LK6 using antibodies that were raised against the LK6 protein (Kidd and Raff 1997). Surprisingly, LK6 was not found in the immunoprecipitation pellets; it remained in the supernatants (Figure I-1D).

Among the four proteins investigated, none was detected in the MEI-S332 immunoprecipitate. Therefore, it remains to be determined what proteins are present in the MEI-S332 multimeric complex. Preliminary results from silver-stained, two-dimensional polyacrylamide gels of MEI-S332 immunoprecipitate showed additional protein dots that were not present in the negative controls (data not shown). Thus, it is unlikely that the absence of

ZW10, tubulin, CDC2, and LK6 in MEI-S332 immunoprecipitates was due to a failure in immunoprecipitating other proteins with MEI-S332. Also, under the same immunoprecipitation condition, MEI-S332 was found to coimmuno-precipitate with MEI-S332-GFP fusion protein and MYC-tagged MEI-S332 protein (Figure I-1E; also see Figure 2-8B, C). It is also unlikely that CDC2 and LK6 failed to co-immunoprecipitate with MEI-S332 because the enzyme-substrate interactions are weak. In vitro kinase assay showed that at least one kinase is apparently co-immunoprecipitated with MEI-S332, allowing the fusion and tagged proteins as well as other nonspecific proteins to get radioactively labeled (see Chapter Five in this thesis).

Protocols for MEI-S332 immunoprecipitation

I. Prepare IP extracts

(1) Isolate starting material:

- embryos:
  - (a) collect embryos from population cages or collection bottles.
  - (b) dechorionate with 50% Clorox bleach and rinse with H_2O.
  - (c) transfer embryos to an eppendorf tube in embryo wash buffer and estimate embryo volume visually.
  - (d) transfer embryos in embryo wash buffer to a glass 2-ml dounce homogenizer and continue on with step (2).

- mature oocytes:
  - (a) isolate oocytes from fattened females by blender in IB buffer as described (Theurkauf 1994; Page and Orr-Weaver 1997).
  - (b) transfer oocytes to an eppendorf tube in IB buffer and estimate oocyte volume visually.
  - (c) transfer oocytes in IB buffer to a glass 2-ml dounce homogenizer and continue on with step (2).

(2) Remove as much embryo wash buffer or IB buffer as possible and immediately add 2X embryo (or oocytes) volume of IP buffer and PMSF (10mg/ml) at 1/100 volume of IP buffer.

Example: 100µl embryo (or oocyte)
200µl IP buffer
2µl 17.4mg/ml PMSF

(3) Homogenize embryos (or oocytes) using Pestle B (tight) with 30-45 strokes.

(4) Transfer extract to an eppendorf tube and add 100% NP-40 to final 1%. From now on keep extract on ice.

(5) Spin 5 minutes at 4°C (Brinkmann 14K in cold room).

(6) Transfer supernatant to a fresh eppendorf tube and make 50-60µl aliquots.

(7) Quick freeze in liquid N_2 and store at -80°C.

II. IP reactions

(1) Thaw extracts on ice. One aliquot equals one IP reaction.

(2) Add antibodies to extracts and incubate on ice (see Notes).

(3) Add 30µl 1:1 vol/vol protein A sepharose/NP-40 buffer to each IP reaction.

(4) Ice 1 hour (flick tubes every 10-15 min).

(5) Quick spin (Brinkmann 10K or Nanofuge) and save supernatant (when running on SDS-PAGE 6-7µl supernatant works well).

(6) Wash beads 8 to 10 times with NP-40 buffer.

(7) Remove all NP-40 buffer and add 50µl 2X SDS sample buffer. I have been loading all 50µl of the IP pellet sample per lane on SDS-PAGE. However, 20-30µl works as well.

(8) 95°C 5 min and spin 5 min.

(9) Quick freeze in liquid N_2 and store at -80°C.

Notes:

Rabbit anti-GFP (Clontech):
-use 6µl per 50-60µl IP extract
-ice in cold room overnight

Guinea pig anti-MEI-S332:
-use 1µl whole serum per 50-60µl IP extract
-works with following conditions: RT 1hr, 4°C 1hr, or 4°C overnight.

Mouse monoclonal anti-MYC (9E10):
(200µg/ml; Santa Cruz Biotech cat#SC-40)
-use 10µl per 50-60µl IP extract
-4°C 1hr.

Buffers:

Embryo wash buffer: 0.7% NaCl and 0.03% Triton X-100

IB buffer: 55mM NaOAc, 40mM KOAc, 100mM sucrose, 10mM glucose, 1.2mM $MgCl_2$, 1mM $CaCl_2$, and 100mM HEPES (pH7.4)

IP buffer: 150mM NaCl, 50mM Tris (pH8), 2.5mM EDTA, 2.5mM EGTA, 0.02% NaN_3, 0.3mM Na_3VO_4, 10µg/ml pepstatin A, 10µg/ml aprotinin, 100µg/ml chymostatin, 10µg/ml leupeptin, and 10µg/ml soybean trypsin inhibitor.

NP-40 buffer: 150mM NaCl, 50mM Tris (pH8), 2.5mM EDTA, 2.5mM EGTA, 0.02% NaN_3, 0.3mM Na_3VO_4, and 1% NP-40.

2X SDS sample buffer: 2% SDS, 10% glycerol, 80mM Tris-HCl (pH6.8), 2mM EDTA, 0.1M DTT.

III. Western Blotting

(A) 12% (150:1) SDS-PAGE:

Separating gel: 25ml total

H_2O	7.75ml
4X Tris-Cl/SDS pH8.8	6.25ml
30% acrylamide	10ml
2% bis-acrylamide	1ml

mix and degas
add 106µl 10%AP and 12.5µl TEMED

Stacking gel: 10ml total

H_2O	6ml
4X Tris-Cl/SDS pH6.8	2.5ml
30% acrylamide	1ml
2% bis-acrylamide	400µl

mix and add 100µl 10%AP and 15µl TEMED

Use Hoefer "The Sturdier" SE 400 gel apparatus and 1X SDS electrophoresis buffer.

Run at 20mA (takes approximately 5 hours)

Transfer to Immobilon P using Hoefer Semi-Phor at 150mA for 2 hrs.

Ponceau S stain, dry blot on Wattman paper, and store in Saran wrap at 4°C.

(B) Immunoblot:

(1) Wet blot with methanol and rinse well with H_2O.

(2) Block 1hr at RT in TBST +5% nonfat dry milk + 2% BSA.

(3) 1° Ab: dilute antibodies in Block solution (see above) + 0.01% thimerosal
guinea pig anti-MEI-S332 at 1:20,000
rabbit anti-CDC2 (Sprenger) at 1:5,000
rabbit anti-ZW10 (Goldberg) at 1:500-1:1,000
rabbit anti-LK6 (Raff) at 1:500-1:1,000
rat anti-tubulin (YL1/2 and YOL1/34) at 1:200
incubate overnight at RT in seal-a-meal bag

Note: (i) 6ml works well for a big blot; I have also used as little as 3 ml.
(ii) Antibodies from two different species can be incubated together (for example, anti-MEI-S332 and anti-CDC2).

(4)Wash with TBST + 5% nonfat dry milk + 1% BSA: 3X quick and 3X5min.

(5) 2°Ab: dilute in "wash" buffer [see (4)]
alkaline phosphatase anti-guinea pig (Jackson)at 1:5,000
alkaline phosphatase anti-rabbit (Promega) at 1:7,500
alkaline phosphatase anti-rat (Jackson) at 1:3,000
HRP anti-rabbit (Promega) at 1:2,500
incubate 40-60 minutes at RT in seal-a-meal bag

Note: If two 1° antibodies were used, two 2° antibodies can be incubated together as long as one is conjugated to alkaline phosphatase and the other to HRP (for example, AP anti-GP and HRP anti-rabbit).

(6) Wash as before.

(7) Detection:

(a) To detect bound HRP-conjugated antibodies, wash off milk with Superblock in TBS (Pierce) and transfer blot to a clean tupeware containing 6ml each of ECL solutions 1 and 2. Incubate for 2 min. Go to (c).

(b) To detect bound AP-conjugated antibodies, wash blot with Developer buffer (a few quick washes and 1X5min wash) and transfer blot to a clean tupeware containing 6ml Tropix RTU. Incubate for 5 min. Go to (c).

(c) Drip off excess liquid and place blot between two plastic sheets (seal-a-meal bag works well). Place in a cassette and bring to dark room along with a timer and BioMax MR-1 film (Kodak). In the dark room, place a film on top of blot and count up (exposure time). Develop film with X-Omat.

Note: (i) If two antibodies were used, detect HRP with ECL first (a), and then detect AP with RTU (b).
(ii) Usually exposure time of ~12min works well for guinea pig anti-MEI-S332, and ~17sec for rabbit anti-CDC2 (Sprenger).

Buffers:

10X SDS electrophoresis buffer (1000ml): 30.2g Trizma base, 144g glycine, 10g SDS, dH_2O to 1000ml. Store at 4°C.

10X Transfer buffer (1000ml): 30.2g Trizma base, 144g glycine, dH20 to 1000ml. Store at RT.

TBST: 100mM Tris pH7.5, 154mM NaCl, and 0.1% Tween 20

Developer buffer: 100mM Tris pH9.5, 100mM NaCl, and 5mM $MgCl_2$

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Appendix II

Protocols for Purifying the GST-MEI-S332 Fusion Protein from *Escherichia coli*

Protocol I: Quick way to get insoluble GST-MEI-S332 protein

Day 1: (1) Inoculate a few colonies of pGEX.MEI containing BL21(λDE3)pLysS cells into 50ml LB + 50µl 100mg/ml ampicillin (amp).

(2) Grow at 37°C overnight.

Day 2: (1) Inoculate 6X(500ml LB+ amp) with 5ml overnight each.

(2) Grow at 37°C until OD_{600}=0.6

(3) Save 1ml uninduced cells* and add IPTG to final 0.1mM.

(4) Grow at 37°C for 2 hrs.

(5) Save 1ml induced cells* and spin rest for 10 minutes at 5K at 4°C (repeat this twice to pool 1 liter cultures into each 500ml bottles).

(6) Discard supernatant; place pellets on ice.

(7) Resuspend cell pellets in total of 20ml ice cold 1XPBS/EDTA buffer by vortexing.

(8) Transfer to 50ml Falcon.

(9) Add 200µl 17.4mg/ml PMSF and 1ml 10mg/ml lysozyme. Ice 30 min.

(10) Freeze in liquid nitrogen for 5 min.

(11) Thaw rapidly in 50°C water bath. Do not let contents of tube exceed 4°C.

(12) Repeat steps 10 and 11 if necessary to improve lysis. Good lysis is indicated by viscosity.

(13) Add 10 ml of PBS/EDTA.

(14) Sonicate 4-6X 30 sec at 90% cycle setting 4 on ice. Viscosity should disappear.

(15) Add 2ml 15% Triton X-100 (final 1%) and mix.

(16) Spin 12K 10 min at 4°C.

(17) Save 100µl supernatant for analysis and transfer supernatant to 5ml 50% slurry of glutathione-agarose beads in 50ml Falcon if purification of soluble GST-MEI-S332 is desired (see Protocol II).

(18) Resuspend pellet with 8M urea buffer (use as little buffer as possible).

(19) Spin 10K 10min at 4°C.

(20) Save urea supernatant and pellet. Add 2X SDS sample buffer to pellet.

(21) Run the urea supernatant on 10% standard polyacrylamide gel.
-mix and load: 2.6ml urea supernatant, 800µl 10% SDS, 400µl 1M Tris (pH 7.5), 100µl β-mercaptoethanol, 40µl 4% bromphenol blue, and any white precipitate from the GST purification (see Protocol II).
-Run stacking gel at 100V and then turn down to 50V.
-Run gel overnight at 50V.

(22) Cut the edges from the gel, Coomassie stain them for 30 minutes, and destain. Meanwhile cover the rest of the gel with Saran wrap and store at 4°C.

(23) Determine the GST-MEI-S332 band based on the staining pattern. Cut the GST-MEI-S332 band out and fragment it through a 10ml syringe (no needle) into a 15ml Falcon tube.

(24) Add 1X SDS electrophoresis buffer, just enough to allow good shaking of the gel pieces.

(25) Incubate overnight on notator in the cold room.

(26) Filter out the acrylamide gel pieces. GST-MEI-S332 should now be in the 1X SDS electrophoresis buffer.

(26) Determine protein concentration by running small samples (1µl and 10µl) on 10% standard polyacrylamide mini-gel along with IgG standards and by Bradford assay.

(27) Aliquot the protein sample, quick freeze in liquid nitrogen, and store at –80°C.

*Spin cells down and remove supernatant. Resuspend pellet in 50µl lysis buffer and add 50µl 2X SDS sample buffer. Heat at 95°C for 5 minutes. Spin and store at –20°C.

Buffers:

10XPBS (1 liter): 80g NaCl, 2g KCl, 6.1g Na_2HPO_4, and 2g KH_2PO_4 in water.

PBS/EDTA: 1XPBS + 10mM EDTA.

8M urea buffer (20ml): 9.6g urea, 10ml 1M Tris (pH 8), 80µl 250mM EDTA, fill up to 20ml with sterile water, and add 41.6µl β-mercaptoethanol.

2X SDS sample buffer: 2% SDS, 10% glycerol, 80mM Tris-HCl (pH6.8), 2mM EDTA, 0.1M DTT.

Lysis buffer: 50mM Tris (pH 8), 1mM EDTA, 25% sucrose.

10X SDS electrophoresis buffer (1liter): 30.2g Tris base, 144g glycine, 10g SDS, in water.

Protocol II: A way to get more soluble GST-MEI-S332 protein

Day 1: (1) Streak from frozen stock of pGEX.MEI#5 in BL21(λDE3)pLysS -on ampicillin (amp), kanamycin (kan), and chloramphenicol (cm) LB plate.

(2) Grow to single colonies at 37°C overnight.

Day 2: (1) Inoculate 2X 5ml cultures (amp + kan + cm with single colonies. Grow at 37°C overnight.

Day 3: (1) Inoculate 4X 500ml LB + amp (2 liters total) with 1ml overnight each. Grow at 18°C for 24 hours.

Day 4: (1) Induce with 12mg IPTG powder per 500ml culture when culture is very dense.

(2) Grow at 18°C for 4 hours.

(3) Spin cells down at 5000 rpm at 4°C for 5 minutes. Pour off supernatant.

(4) Resuspend all cell pellets in a total of 20ml ice cold PBS/EDTA by vortexing.

(5) Transfer to a 50ml Falcon tube.

(6) Add 200µl 17.4mg/ml PMSF and 1ml 10mg/ml lysozyme. Mix.

(7) Ice 30 minutes.

(8) Free in liquid nitrogen for 5 minutes.

(9) Thaw rapidly in 50°C water bath.

(10) Add 10ml PBS/EDTA.

(11) Sonicate 6X 30seconds on ice.

(12) Add 2ml 15% Triton X-100 (Final 1%) and mix.

(13) Spin 12K for 10 minutes at 4°C.

(14) Transfer supernatant to 5ml 50% slurry of glutathione-agarose beads in a 50ml Falcon tube.

(15) Incubate with gentle rocking for 2 hours at room temperature.

(16) Spin 500g for 5 minutes.

(17) Save supernatant as "Flow Through."

(18) Wash 3X with 1XPBS + 1% Triton X-100 and 4X with 1XPBS.

(19) Transfer beads to a 15ml Falcon tube with the last PBS wash.

(20) Remove all PBS.

(21) Add 2ml Elution Buffer. Incubate on notator for 15 minutes at room temperature. Spin at 500g for 5 minutes. Save supernatant.

(22) Repeat step (21) 5-6 times.

(23) Quick freeze eluates in liquid nitrogen and store at –80°C.

Note: GST-MEI-S332 will precipitate out of solution (white precipitate) during elution if no salt is included in the Elution Buffer. I have eluted GST-MEI-S332 in the presence of 600mM NaCl, and the protein remains in solution. Lower concentrations of salt have not been tested.

Buffers:

10XPBS (1 liter): 80g NaCl, 2g KCl, 6.1g Na_2HPO_4, and 2g KH_2PO_4 in water.

PBS/EDTA: 1XPBS + 10mM EDTA.

Elution Buffer (20ml): 1ml 1M Tris (pH8) and 0.092g reduced glutathione (15mM). Bring to 20ml with sterile water.

Appendix III

Affinity Purification of the Rabbit MEI-S332 Peptide Antibodies (α-TOW5)

A. Prepare GST-MEI-S332-bound immobilon strips:

(1) Purify GST-MEI-S332 and run it on 10% standard polyacrylamide gel as described in Appendix I Protocol I.

(2) Transfer proteins from the gel to Immobilon P at 150mM for 2 hours.

(3) Ponceau S stain the blot. Store blot in saran wrap at 4°C.

(4) Immunostain strips from the blot with Rabbit anti-MEI-S332 peptide anti-serum at 1:20,000 to confirm the location of the GST-MEI-S332 band.

(5) Cut the GST-MEI-S332 band out into 10 lanes. Cut each lane into 2-4 strips. Put strips from each lane into an eppendorf tube (dry) and store at –20°C. Make sure that the strips are not sticking to one another.

B. Blot affinity purification:

(1) Use strips from a lane (in one eppendorf tube; see above) for each prep.

(2) Soak strips in methanol and rinse in water.

(3) Block strips in a small petri dish with 3ml block solution on shaker.

(4) Transfer strips into diluted serum in an eppendorf tube.

(5) Incubate on notator overnight at room temperature.

(6) Put 100µl 1M NaPO4 (pH8) in a fresh eppendorf tube.

(7) Remove diluted serum; save it as "Flow Through."

(8) Wash strips in the eppendorf tube 5-6 times with 1XPBS/ 0.5% Tween 20.

(9) Remove all PBS/Tween buffer.

(10) Acid elution: Add 300µl Acid Elution Buffer to strips. Run the buffer over the strips for 30 seconds by pipetting up and down, and then quickly transfer the buffer into the $NaPO_4$-containing eppendorf tube. Mix well to neutralize the eluate.

(11) Repeat step (10) two more times.

(12) Wash strips with PBS/Tween 20.

(13) Store strips and Acid Eluates at 4°C.

(14) The Acid Eluate can now be used on Western blots at 1:40 to 1:100 dilution.

Buffers:

Block Solution: 3% BSA, 0.5% Tween 20, 1XPBS.

Diluted Serum: 200µl rabbit anti-peptide serum, 0.5% Tween 20, 0.01% thimerosal, 1XPBS.

Acid Elution Buffer (4ml): 5mM glycine (pH 2.5), 150mM NaCl.

10X PBS (1 liter): 80g NaCl, 2g KCl, 14.4g Na_2HPO_4, 2.4 KH_2PO_4, adjust pH to 7.4 and adjust volume to 1 liter with water.

NOTE: Rabbit anti-peptide serum is from Rabbit HM196.

The End