The Role of the Chromosomal Passenger Complex and Condensin Complex in Meiotic Chromosome Cohesion and Segregation

by

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Sc.B. Biology
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Providence, RI, 2000

Submitted to the Department of Biology
In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

at the

Massachusetts Institute of Technology
Cambridge, MA
[June 2007]
May 2007

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ABSTRACT

The canonical mitotic cell cycle is modified in metazoans to achieve a variety of developmental goals. Among these, meiotic divisions reduce the DNA content of the cell, haploid gametes fuse and reenter mitotic cycling, and mitoses of early embryogenesis, in many animals, employ a rapid cell cycle that lacks gap phases. Much less is understood about regulation of these developmentally regulated cell cycles, than about canonical mitosis, because they cannot be studied in tissue culture systems and because mutations that specifically affect these processes are rare. This thesis describes a screen to characterize mutants that display defects in these programs in *Drosophila melanogaster*. This model system allowed use of powerful tools for genetic and cell biological analyses of these mutants. Nineteen mutants with defects in this developmental window were screened and placed them into five phenotypic classes, including mutants that displayed defects in fertilization, pronuclear fusion, and mitosis and DNA synthesis in the early divisions of embryogenesis. Among these mutations, one was mapped and molecularly characterized as an allele of the passenger complex component *incenp*. This allele was used to investigate roles of the passenger complex in meiosis in both male and female Drosophila. *incenp* mutant males displayed missegregation of sex chromosomes in both meiosis I and meiosis II, and this is due, at least in part, to premature loss of cohesion between sister chromatids. *incenp* is required for proper localization of MEI-S332, an essential protector of meiotic centromere cohesion, in spermatocytes. MEI-S332 is phosphorylated by Aurora B/INCENP *in vitro* at a specific Aurora B target site. Mutation of this site disrupts MEI-S332 localization to the centromere, suggesting a model in which phosphorylation of MEI-S332 by the passenger complex is required for proper localization of MEI-S332, and thereby required for maintenance of sister-chromatid cohesion. In female meiosis, *incenp* is required for maintenance of the synaptonemal complex and for proper formation of the metaphase I chromosome configuration. Characterization of female-sterile alleles of the condensin component *dcap-g* revealed a role for the condensin complex in disassembly of the synaptonemal complex and also for metaphase I chromosome behavior, but in a manner distinct from the role played by *incenp*. The differences between the meiotic roles of *dcap-g* and *incenp* are striking, given that both have characterized roles in chromosome condensation in mitosis and that the passenger complex is required for localization of the condensin complex in several systems. In addition, *ord*, another player in meiotic chromosome condensation that also has essential roles in meiotic cohesion, interacts genetically with *incenp*.

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

Dedicated with love to my parents,

Nancy and David Resnick,

who gave me the great gift of believing I could accomplish
anything I put my mind to and unwavering support to
pursue whatever made me happy.

Acknowledgments

Thank you to everyone in the Orr-Weaver lab. I feel very lucky to have worked with such a wonderful group of people. I would especially like to thank Kim Dej who was a very important mentor to me in my early days in the lab and is almost entirely responsible for turning me into a Drosophilist, and Astrid Clarke whose scientific insights, undaunted attitude, and tremendous heart have made her an invaluable baymate and friend. Thank you to Janice Lee who patiently endured an endless stream of questions about chromosome segregation, lab techniques, thesis writing, and postdoc applications.

Thank you to Terry, who is truly a terrific scientist and from whom I have received tremendous graduate training. In addition, I have found Terry to be a principled and deliberate member of the scientific community and to create an expectation within the lab that people will be helpful and fair to each other. I have greatly valued these aspects of my training, and I thank Terry for this as well.

I'd like to thank Jillian Pesin, Jessica Alföldi, Teresa Holm, and Rachel Woodruff, with whom I shared the journey through graduate school. These years would have been so much lonelier, less rewarding and fun without them.

My family has been wonderfully supportive and loving. Both my sisters, Amira and Yael, lived in Boston for parts of my time in graduate school, and seeing them often has made these years very special. My brother, Adam, has provided much-appreciated perspectives on life as a graduate student. Most of all, my parents have given me strong roots and strong wings, and I cannot thank them enough. And I would especially like to thank my mother who, through her curious and observant approach to the world, was truly my first science teacher.

And finally, thank you to Daniel, who unfailingly supports me and challenges me, and helps me to be the best version of myself.

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Chapter One

Introduction

I. COORDINATION OF CHROMOSOME CONDENSATION, COHESION, AND SEGREGATION DURING CELL DIVISION

A. Chromosome dynamics in mitosis

Every time a cell divides, the chromosomes must undergo an exquisite series of dynamic behaviors in order to ensure that each daughter cell receives a precise genetic complement. First the genome is replicated exactly once, with no part over- or under-represented. In prophase, the chromosomes then condense tightly into highly compacted rod-like structures so that they can easily move in relation to each other within the physical space of the nucleus (Mitchison and Salmon 2001). The cohesin complex, which was loaded onto the DNA during S phase, holds sister chromatids together in prophase and metaphase (Uhlmann and Nasmyth 1998). In metazoans, cohesin is released from chromosome arms in prophase without being cleaved (Losada et al. 2000; Waizenegger et al. 2000; Warren et al. 2000). A patch of cohesin retained at the centromere maintains sister-chromatid attachments. Indeed, the physical connection between sister chromatids is essential for stable biorientation on the mitotic spindle and coordination of chromatid segregation in anaphase (Fig. 1-1). At the onset of anaphase the cohesin subunit Rad21 is cleaved by the protease separase, and sister chromatids synchronously move apart (Uhlmann et al. 1999; Uhlmann et al. 2000). Prevention of cohesin cleavage until the onset of anaphase is ensured by securin, an inhibitor of separase, whose degradation is facilitated by the Anaphase Promoting Complex/Cyclosome (APC/C) once the chromosomes have achieved stable, bipolar spindle attachment (Cohen-Fix et al. 1996; Funabiki et al. 1996; Ciosk et al. 1998; Waizenegger et al. 2002).

The chromosomes progress through these characteristic movements in a carefully synchronized manner, so that if even a single chromosome experiences defects in these

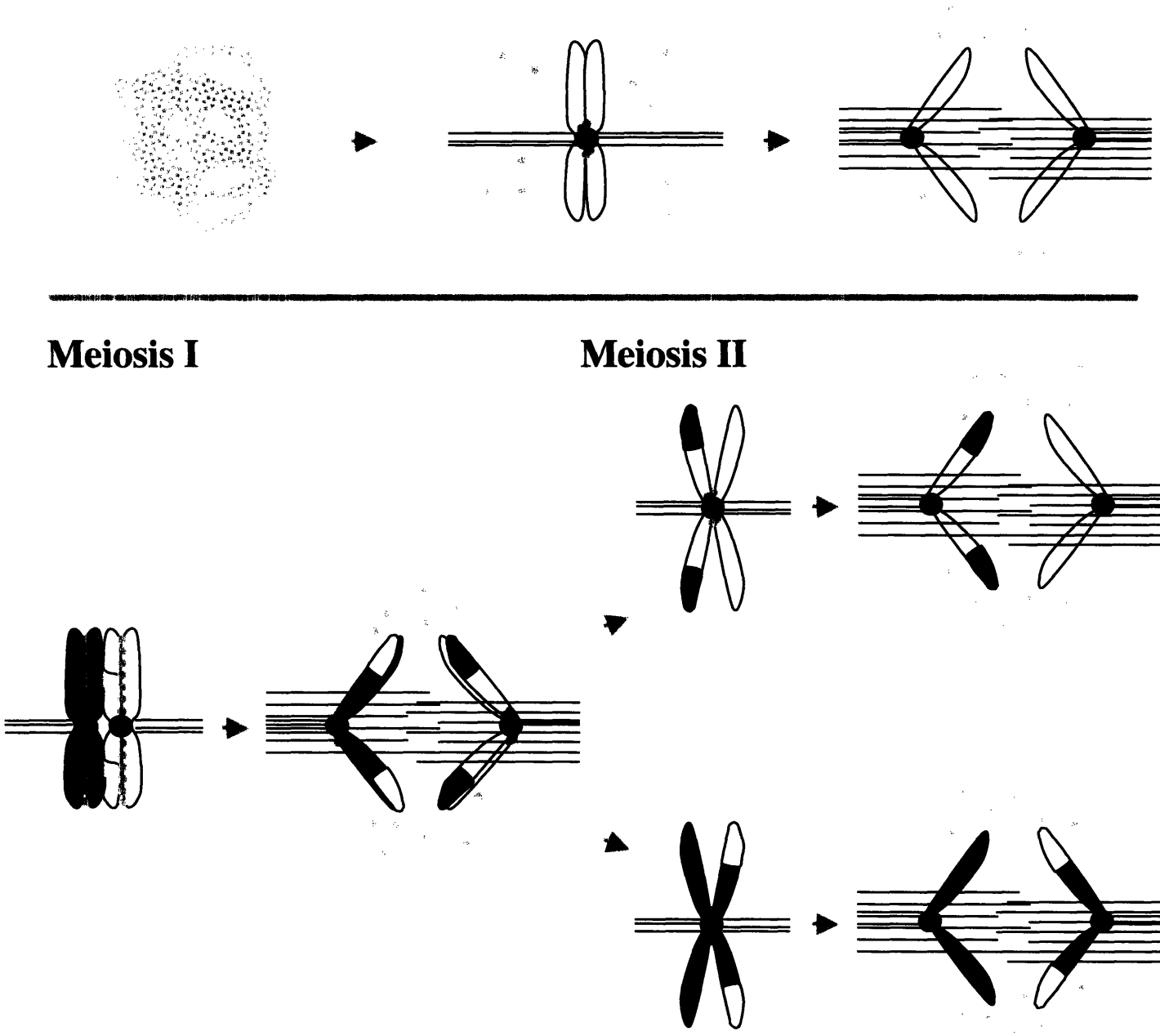

Figure 1-1. Chromosome segregation in mitosis and meiosis
The cohesin complex (orange) is loaded onto chromosomes in S phase. In metazoan mitosis, cohesin is removed from chromosome arms in prophase but is not cleaved (orange circles). At the onset of anaphase, centromere-associated cohesin is cleaved (orange crescents) and sister chromatids separate. In meiosis I homologs are held together by cohesion distal to the chiasmata. Cleavage of cohesin on the chromsome arms allows homologs to separate in anaphase I. Cohesin at the contromere maintains attachment between sister chromatids. Centromere-associated cohesin is cleaved at the onset of anaphase II and sister chromatids move apart.

processes, the progress of the rest of the chromosomes is delayed (Musacchio and Hardwick 2002). In sum, this complex series of events results in an extremely faithful method for generating daughter cells each with exactly one copy of the genome. Precisely regulating the genetic content of each cell is critical because too much or too little DNA can lead to catastrophic consequences including cell death or deregulation of cell regulatory processes and ultimately tumorigenesis (Pellman 2007).

B. Chromosome dynamics in meiosis

Generation of sperm and eggs requires modifications to these stereotypic chromosome dynamics because gametes contain exactly half the genome of somatic cells (for a review see Petronczki et al. 2003). This reduced genetic content is critical so that when sperm and egg come together to form a zygote, the complete genome is reestablished. Once again, precisely regulating the DNA content is of utmost importance because severe developmental abnormalities result from an incorrect genetic complement in the zygote. Most often too much or too little DNA leads to developmental arrest and, in mammals, spontaneous miscarriage. Aberrant DNA content is the most common cause of miscarriage in humans. Genomic abnormalities are estimated to occur in at least 5% of recognized pregnancies and up to a third of pregnancies for women in their forties. In addition, as many as 1 in 300 liveborn infants are approximated to have genomic defects, the most common of these in humans include abnormalities in the number of sex chromosomes and trisomy 21, an extra copy of chromosome 21, which results in Down syndrome. (for a review see Hassold and Hunt 2001)

In order to achieve the precise reduction of genetic complement in meiosis, cells replicate all their DNA and then divide twice without another intervening round of DNA

synthesis. To facilitiate these sequential divisions, meiosis-specific modifications are made to chromosome segregation (for reviews see Lee and Orr-Weaver 2001; Petronczki et al. 2003; McKee 2004; Page and Hawley 2004). In prophase I of meiosis, homologs find each other and pair. In many systems, additional chromosomal behaviors are common in meiotic prophase I. Among these, a proteinaceous structure called the synaptonemal complex assembles between homologs. Double-strand breaks are initiated and repaired off a homolog template, resulting in regions of gene conversion as well as crossovers, which generate covalent linkages between homologs. Chiasmata, the physical structures that result from crossing over, in combination with cohesion on the distal part of the chromosome arm, maintain a physical attachment between homologs in meiosis I.

In metaphase I, homologs biorient on the spindle, while sister chromatids co-orient toward the same pole (Toth et al. 2000). At the onset of anaphase I, cohesin localized to the chromosome arms is removed by separase-mediated cleavage and homologs separate, while sister chromatids move together toward a single spindle pole (Fig. 1-1) (Buonomo et al. 2000; Bickel et al. 2002). A pool of cohesin at the centromere is retained, allowing sister chromatids to remain attached and thereby coordinate their bipolar attachment on the meiosis II spindle (Watanabe and Nurse 1999). Cleavage of cohesin at the centromere allows sister chromatids to separate in anaphse II and they move to opposite poles in a manner more similar to mitosis.

C. Regulation of chromosome dynamics by protein complexes

Execution of these intricate chromosomal maneuvers is carefully regulated by interacting proteins that together ensure proper progression through the cell cycle, and a

number of proteins that play important roles in chromosome dynamics will be discussed in more detail throughout this chapter (Table 1-1). Some proteins are specific to meiosis, including the synaptonemal complex, mentioned above. Also specific to meiosis, ORD plays important roles in chromosome cohesion, as well as condensation, recombination, and segregation. The protein MEI-S332 is essential in meiosis for proper segregation of sister chromatids in the equational division, and it is also involved in mitotic segregation though it is not essential in mitosis.

Many proteins involved in meiotic regulation are also required for mitotic divisions, as chromosomes go through many of the same behaviors in both types of cell cycle. Among these, the cohesin complex, introduced above, forms a physical attachment between sister chromatids that is released in metaphase, in both mitosis and meiosis. The condensin complex is essential for sister-chromatid resolution and also plays a role in chromosome condensation. The chromosomal passenger complex contributes to important events throughout the cell cycle including chromosome condensation and biorientation on the spindle.

Dissecting the important meiotic functions of proteins that are also essential in mitosis has been experimentally difficult. In metazoans, using genetic approaches to address these questions has been hampered by an absence of appropriate alleles that retain sufficient function to allow development of an adult animal, but disrupt function enough to reveal identifiable phenotypes in meiotic progression. In addition, many of the proteins involved in meiotic regulation have been analyzed singly regarding their role in

Table 1-1. Interacting proteins regulate chromosome dynamics in mitosis and meiosis

	Roles in chromosome dynamics	Components of the complex in Drosophila
MEI-S332	Protects sister-chromatid cohesion at the centromere	----
ORD	Required for meiotic cohesion, condensation, recombination	----
Synaptonemal Complex	Consists of a pair of lateral elements interconnected by transverse filaments Forms between homologs at synapsis Facilitates crossing over	C(3)G, C(2)M, cohesin proteins*
Cohesin Complex	Holds sister chromatids together from S phase through metaphase Released in a stepwise manner in meiosis	SMC1, SMC3, Rad21/Scc1, SA/Scc3
Chromosomal Passenger Complex	Required for chromosome condensation, spindle checkpoint, proper biorientation of chromatids	Aurora B, INCENP, Survivin‡, Borealin/Dasra
Condensin Complex	Essential for sister-chromatid resolution, contributes to chromosome condensation	SMC2, SMC4, CAP-D2/D3, CAP-G, CAP-H (BARREN)/H2

* Cohesin proteins have not been characterized in the synaptonemal complex in Drosophila.
Cohesin mutants have not been characterized in Drosophila, except for a male-meiosis specific SA homolog. (Thomas et al., 2005)
‡ Survivin has not been characterized in Drosophila.

chromosome dynamics, and sufficient tools have only recently been developed to examine the interrelated roles of these proteins. In this thesis we describe work identifying alleles in passenger complex and condensin complex members that give rise to meiotic phenotypes, and examine interactions between these protein complexes and others with important roles in meiotic chromosome behavior.

II. MEIOSIS-SPECIFIC PROTEINS FACILITATE SEQUENTIAL DIVISIONS

A. MEI-S332 protects cohesion at the centromere

The founding member of a conserved family of proteins, MEI-S332 plays essential roles in meiotic centromere cohesion (Kerrebrock et al. 1992). Flies that are mutant for *mei-S332* display dramatic defects in chromosome segregation in the second meiotic division, but very few defects in meiosis I segregation. These defects in sister-chromatid segregation arise due to a precocious separation of sister chromatids: in anaphase I, centromere cohesion is released along with arm cohesion, resulting in an inability of sister chromatids to coordinate their movements in meiosis II (Kerrebrock et al. 1992). MEI-S332 localizes to centromeres in prophase I and remains there until the onset of anphase II, the time at which centromere cohesion is also released (Kerrebrock et al. 1995). MEI-S332 is localized to the centromere from prophase until anaphase onset in mitotic cells as well. It contributes to centromere cohesion in these divisions, though its role is not essential (LeBlanc et al. 1999).

MEI-S332 is a phosphoprotein and its phosphorylation state is regulated in coordination with cell-cycle progression (Clarke et al. 2005). POLO kinase can phosphorylate MEI-S332 directly *in vitro* and is required for delocalization of MEI-S332

at anaphase in the second meiotic division and in mitosis. In its absence MEI-S332 is inappropriately maintained at the centromeres, resulting in chromosome segregation defects ((Clarke et al. 2005), A. Clarke, personal communcation). Many other aspects of regulation of MEI-S332 phosphorylation state, localization, and function remain poorly understood.

The family of proteins to which MEI-S332 belongs, now referred to as Shugoshins, shares related functions in protecting a pool of cohesin at the meiotic centromere (Katis et al. 2004; Kitajima et al. 2004; Marston et al. 2004; Rabitsch et al. 2004). Sequence similarity among family members is limited to small regions at the N- and C-termini of the protein (Rabitsch et al. 2004). Although the overall similarity is not robust, additional support for the suggestion that these conserved regions are particularly important comes from the fact that several of the best conserved residues are modified in *mei-S332* alleles that have been characterized genetically and shown to display chromosome segregation defects (Kerrebrock et al. 1992).

In addition to the shared roles in cohesion, the Shugoshin family of proteins also displays functions that seem to have diverged among species. Many organisms, including budding and fission yeasts and mammals, contain two shugoshins; other species contain a single shugoshin (Rabitsch et al. 2004). Drosophila has only one characterized family member, MEI-S332, which is more closely related to Sgo1 in other species (Astrid Clarke, personal communication). In addition, across systems Shugoshins interact in important ways with protein phosphatases, but the specific nature of this interaction varies. Localization of phosphatase PP2A to the centromere requires Sgo2 in human cells and Sgo1 in yeast meiosis, however human Sgo1 requires PP2A for its centromere

localization (Kitajima et al. 2006; Riedel et al. 2006; Tang et al. 2006). Furthermore, in yeast meiosis, ectopic localization of PP2A to the chromosome arm is sufficient for maintenance of Rec8 and sister-chromatid cohesion even without Sgo1 present, but in human cells, upon Sgo1 depletion, PP2A remains at the centromere and is not sufficient for cohesion.

B. ORD is required for meiotic chromosome cohesion and condensation

The meiotic protein ORD plays important roles in several aspects of chromosome dynamics. The initial *orientation disruptor* (*ord*) allele was recovered from a screen in *Drosophila melanogaster* for mutations that resulted in meiotic chromosome missegregation in both meiosis I and meiosis II (Mason 1976). In both male and female *ord* mutants, these segregation defects arise, at least in part, from precocious loss of sister-chromatid cohesion and a resulting inability to coordinate chromatid orientation on the spindle (Miyazaki and Orr-Weaver 1992; Bickel et al. 1997; Balicky et al. 2002; Bickel et al. 2002). *ord*'s role in arm cohesion is supported by the observation that it is the only characterized mutant in which chromosomes that have formed crossovers undergo nondisjunction (Bickel et al. 2002). Segregation and cohesion defects arise in both the first and second meiotic divisions. In male flies, ORD also plays important roles in meiotic chromosome condensation, with defects in chromosome packing visible in *ord* mutants as early as prophase I (Miyazaki and Orr-Weaver 1992).

In female flies, ORD has been shown to colocalize partially with the synaptonemal complex and to be critical for its maintenance (Webber et al. 2004). ORD also functions, perhaps through its roles in sister-chromatid cohesion, to suppress

recombination between sister chromatids and to promote crossing over between homologs (Bickel et al. 1997; Webber et al. 2004). Interhomolog exchange is essential to facilitate segregation in the first meiotic division.

Finally, understanding ORD's role in meiosis is especially intriguing in light of the observation that *ord* mutant female flies show chromosome segregation defects that worsen with maternal age (Jeffreys et al. 2003). In humans, rates of chromosomal defects in female meiosis increase exponentially for women in their 30s (Hassold and Hunt 2001), which creates very real human health and fertility obstacles, especially as average maternal age is increasing. Finding an animal model for understanding these defects has been difficult. The central role of cohesion in long-term maintenance of proper meiotic chromosome organization is highlighted both by the role for ORD in Drosophila female meiosis and by experiments in mice demonstrating age-related segregation defects when the meiosis-specific cohesin SMC1β is disrupted (Hodges et al. 2005).

C. The Synaptonemal Complex forms an axis between homologs

In meiotic prophase I, homologs pair and, in many systems, a proteinaceous structure called the synaptonemal complex forms between homologs (for reviews see (Page and Hawley 2004; Colaiacovo 2006)). The synaptonemal complex assembles transiently and plays a role in holding chromosomes tightly together during pachytene and has been implicated in generation and spacing of crossover events (Sym et al. 1993; Sym and Roeder 1994; Tung and Roeder 1998; Page and Hawley 2001; MacQueen et al. 2002). At the structural level, viewed by electron microscopy, the synaptonemal complex is very well conserved evolutionarily. Axial elements form first along the length of each

homolog, and then these elements, now called lateral elements, are joined together by transverse filaments (Meuwissen et al. 1992; Sym et al. 1993; Page and Hawley 2001; MacQueen et al. 2002; Colaiacovo et al. 2003). These transverse filaments are composed of elongated proteins that orient perpendicularly to the lateral elements and interdigitate in a manner similar to a zipper. Intriguingly, proteins of the transverse filaments have been identified in many species, including budding yeast, flies, worms, and mice, and do not display sequence similarity but do reveal robust structural conservation. These proteins display globular domains at the termini and long coiled-coil domains in between, through which they dimerize. Lateral elements include cohesin subunits and other meiosis-specific proteins (Smith and Roeder 1997; Offenberg et al. 1998; Klein et al. 1999; Zetka et al. 1999; Yuan et al. 2000; Eijpe et al. 2003).

The requirements for synaptonemal complex assembly vary among systems. Double-strand break formation is essential for synaptonemal complex formation in many systems including budding yeast and mammalian systems, but it is dispensible in *C. elegans* and Drosophila (Giroux et al. 1989; Keeney et al. 1997; Dernburg et al. 1998; McKim et al. 1998; Lichten 2001; Burgess 2002). Importantly, the requirements for pairing and synapsis appear to be distinguishable, as suggested by *S. cerevisiae* expressing a catalytically inactive form of Spo11, the enzyme responsible for introducing double-strand breaks, which have been reported to be unable to form the synaptonemal complex, though significant pairing still occurs (Cha et al. 2000).

Recent experiments have raised intriguing questions about synaptonemal complex disassembly. In Drosophila oogenesis, the synaptonemal complex disassembles during the same time window in which the condensin subunit SMC4 is seen to localize

specifically to the chromosomes. Mutation of *nucleosomal histone kinase 1* (*nhk-1*) disrupts both processes, suggesting a possible functional interaction between condensin loading and synaptonemal complex unloading from the chromosomes (Ivanovska et al. 2005). In addition, a study in *C. elegans* suggests a link between synaptonemal complex unloading and Aurora B kinase (AIR-2) localization (Nabeshima et al. 2005). Experiments in this system showed that AIR-2 required the synaptonemal complex protein SYP-1 for its localization in prophase I and seemed to follow the asymmetric localization of SYP-1 to the distal part of the chromosome at the end of meiotic prophase, suggesting a possible role for disassembly of SYP-1 in directing AIR-2 localization. Proper localization of AIR-2 to this region of the chromosome is critical for sequential cohesin release in the meiotic divisions in *C. elegans* (described below, (Kaitna et al. 2002; Rogers et al. 2002)).

III. MITOTIC PROTEINS AND THEIR SPECIALIZED ROLES IN MEIOSIS

A. The chromosomal passenger complex and its multifaceted regulation of mitosis

The chromosomal passenger complex was characterized and named for its characteristic localization pattern (Earnshaw and Bernat 1991). In mitotic prophase the complex is seen across the chromatin, and it restricts to the centromere by metaphase. It rides the chromosomes to the metaphase plate, and then transfers abruptly to the spindle midzone at the onset of anaphase (Figure 1-2. (Schumacher et al. 1998; Terada et al. 1998; Adams et al. 2001b)). As suggested by its dynamic localization pattern, the passenger complex has been implicated in functions throughout mitosis. Depletion or mutation of passenger complex subunits has suggested roles in chromosome

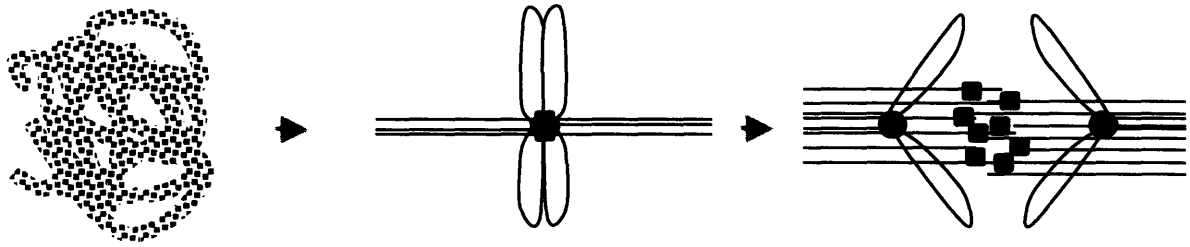

Figure 1-2. Chromosomal Passenger Complex Localization in Mitosis
The chromosomal passenger complex (red) localizes across the chromosomes in prophase, restricts to the centromeres by metaphase, and transfers to the spindle midzone at the onset of anaphase

condensation and proper biorientation on the mitotic spindle, as well as spindle stability and cytokinesis (reviewed (Carmena and Earnshaw 2003; Vagnarelli and Earnshaw 2004)). A discussion of the passenger complex in mitosis follows directly, and we return to important meiotic roles for the complex below.

The passenger complex includes Aurora B, which is a serine-threonine protein kinase and is the enzymatic component of the complex, as well as INCENP, Survivin, and Borealin/Dasra (Cooke et al. 1987; Schumacher et al. 1998; Terada et al. 1998; Adams et al. 2000; Skoufias et al. 2000; Uren et al. 2000; Adams et al. 2001a; Carvalho et al. 2003; Gassmann et al. 2004; Sampath et al. 2004). These proteins function as a complex, and the members of the complex mutually require each other for their localization, both to the centromere and to the spindle midzone (Adams et al. 2000; Kaitna et al. 2000; Wheatley et al. 2001a; Bolton et al. 2002; Honda et al. 2003). INCENP has been shown to bind microtubules *in vitro* and is speculated to mediate the interaction with the mitotic spindle (Wheatley et al. 2001b).

Passenger proteins play important roles in regulating not only the localization, but also the enzymatic activity of Aurora B kinase. INCENP binds Aurora B through its C-terminal "IN-BOX," the best-conserved region of the INCENP protein, and is phosphorylated by Aurora B in this same domain (Terada et al. 1998; Bishop and Schumacher 2002; Honda et al. 2003). The binding and phosphorylation of INCENP greatly enhances Aurora B's kinase activity (Kang et al. 2001; Bishop and Schumacher 2002; Honda et al. 2003). Aurora B also phosphorylates itself and further stimulates its own kinase activity, in a positive feedback loop (Bolton et al. 2002; Chen et al. 2003;

Honda et al. 2003). In addition, survivin may stimulate Aurora B's kinase activity, and Borealin/Dasra is a substrate of the kinase (Gassmann et al. 2004).

Several targets of Aurora B kinase have been identified, in addition to those within the passenger complex itself. Aurora B is required for phosphorylation of histone H3 on serine 10 (Hsu et al. 2000; Adams et al. 2001b; Giet and Glover 2001; Crosio et al. 2002). This modification is often correlated with mitotic chromosome condensation, and indeed passenger protein disruption also leads to defects in condensation (Adams et al. 2001b; Giet and Glover 2001), though the precise relationship between H3 phosphorylation and condensation is not well understood (Gurley et al. 1978; Adams et al. 2001b). Aurora B also phosphorylates the H3 centromere variant CENP-A (Zeitlin et al. 2001), as well as other kinetochore proteins including Ndc10 and Dam1, which is important for kinetochore-microtubule attachments, in budding yeast (Biggins et al. 1999; Cheeseman et al. 2002).

One of the best characterized roles of Aurora B is its function in destabilizing unproductive kinetochore-microtubule attachments (Tanaka et al. 2002; Lampson et al. 2004). The kinase was shown, first in budding yeast and then in metazoan systems, to be required for release of kinetochores inappropriately oriented on the mitotic spindle, in order to allow additional attempts at proper biorientation. In the absence of this activity, an increased rate of sister chromatids associated with the same spindle pole (syntelic attachments) and single sister kinteochores associated with both spindle poles (merotelic attachments) are observed (Ditchfield et al. 2003; Hauf et al. 2003; Lampson et al. 2004). These defects in attachment results in errors in mitotic chromosome segregation. By producing unattached kinetochores, the passenger complex also activates the spindle

checkpoint and blocks entry into anaphase before stable bipolar attachments are achieved (Hauf et al. 2003; Lens et al. 2003; Pinsky et al. 2006).

One intriguing substrate of Aurora B kinase that may be involved in this process is Mitotic Centromere-Associated Kinesin (MCAK). This kinesin family member does not behave like a typical motor protein, rather it catalyzes microtubule disassembly (Desai et al. 1999; Tournebize et al. 2000), suggesting a possible role in turning over unproductive kinetochore-microtubule attachments. Indeed, in mitosis, MCAK localizes to the inner centromere and kinetochore in an Aurora B-dependent manner, Aurora B phosphorylates MCAK, and depletion of MCAK results in failure of chromosomes to congress to an organized metaphase plate (Andrews et al. 2004; Lan et al. 2004; Ohi et al. 2004). Somewhat confoundingly, however, phosphorylation of MCAK by Aurora B inhibits its microtubule destabilizing activity (Andrews et al. 2004; Ohi et al. 2004). Given this inhibitory relationship, a clear model for how Aurora B and MCAK both promote release of unproductive kinetochore-microtubule associations remains to be elucidated.

In addition to its roles in chromosome dynamics, the passenger complex also functions in anaphase and telophase spindle stability, and in cytokinesis. Aurora B is required for phosphorylation or localization of a number of central spindle and cleavage furrow components including Pavarotti-KLP and intermediate filament proteins. Depletion of passenger proteins or expression of non-phosphorylatable substrates results in failure to complete cytokinesis (for a review see (Carmena and Earnshaw 2003; Vagnarelli and Earnshaw 2004)).

The various roles played by the chromosomal passenger complex in ensuring faithful chromosome segregation suggests that disruption of the complex might lead to aneuploidy and ultimately tumorigenesis (for a review see (Giet et al. 2005)). The importance of these proteins in cancer progression is underscored by the fact that Aurora B is overexpressed in many cancer cells, particularly in advanced stages of colorectal cancers. Additionally, overexpression of this kinase in cells injected into nude mice enhances aggressive tumor formation and development of metastases. As a result, Aurora B kinase has become an attractive candidate for chemotherapy.

B. The condensin complex and its roles in chromosome resolution and compaction

The condensin complex is named as such because of its function in mitotic chromosome condensation, however suggestions about the exact nature of the complex's roles in condensation vary among systems. Early work in Xenopus showed that the condensin complex is required for condensed chromosome architecture of sperm DNA in mitotic extracts (Hirano and Mitchison 1994; Hirano et al. 1997), and experiments in budding yeast demonstrated that loci on a chromosome arm are not held as close together in the absence of condensins (Strunnikov et al. 1995; Freeman et al. 2000; Lavoie et al. 2000; Ouspenski et al. 2000). Work *in vivo* in metazoan systems has suggested that even in the absence of condensin function, chromosomes will reach a highly condensed conformation (Steffensen et al. 2001; Hagstrom et al. 2002; Dej et al. 2004). One possible explanation for the differing observations is that condensin functions in chromosome compaction, but does so redundantly with other pathways to condensation. As such, without condensin function, chromosomes may experience delays and defects in condensation, but given enough time these errors can be righted by other mechanisms.

An essential role for the condensin complex has been characterized in resolution of sister chromatids (Saka et al. 1994; Bhat et al. 1996; Sutani et al. 1999; Steffensen et al. 2001; Hagstrom et al. 2002; Dej et al. 2004). Condensin mutants display fuzzy, poorly-individualized chromosomes in prometaphase and chromosome bridging in anaphase. Here we discuss the condensin complex in mitosis, and we return, below, to characterization of the condensin proteins in meiosis.

The condensin complex is composed of two SMC (Structural Maintenance of Chromosomes) components, SMC2 and SMC4, as well as three non-SMC components, CAP-D2/D3, CAP-G/G2, and CAP-H/H2, classified as Chromosome Associated Proteins when they were purified from Xenopus extracts (Hirano and Mitchison 1994; Hirano et al. 1997). The SMC components each include two globular head domains at the N- and C-termini and a hinge region in the middle. The proteins fold at the hinge and an intramolecular coiled coil brings together the globular domains at the termini (Haering et al. 2002; Hirano and Hirano 2002). The N- and C-terminal domains contain Walker A and B motifs, respectively, that together have ATPase activity (Strunnikov et al. 1993; Saitoh et al. 1994; Lowe et al. 2001; Hopfner and Tainer 2003). To form the condensin complex, SMC2 and SMC4 interact directly with each other through their hinge regions, and the head domains are joined by the non-SMC components (Figure 1-3. (Anderson et al. 2002; Yoshimura et al. 2002)).

Purified SMC2/4 dimers have been shown to generate double-stranded DNAs from complimentary single-stranded DNAs. The condensin complex has been shown, *in vitro*, to physically compact DNA and to introduce positive superhelical tension into DNA, dependent on phosphorylation by Cyclin B-Cdk1 (for a review see (Hirano 2005)).

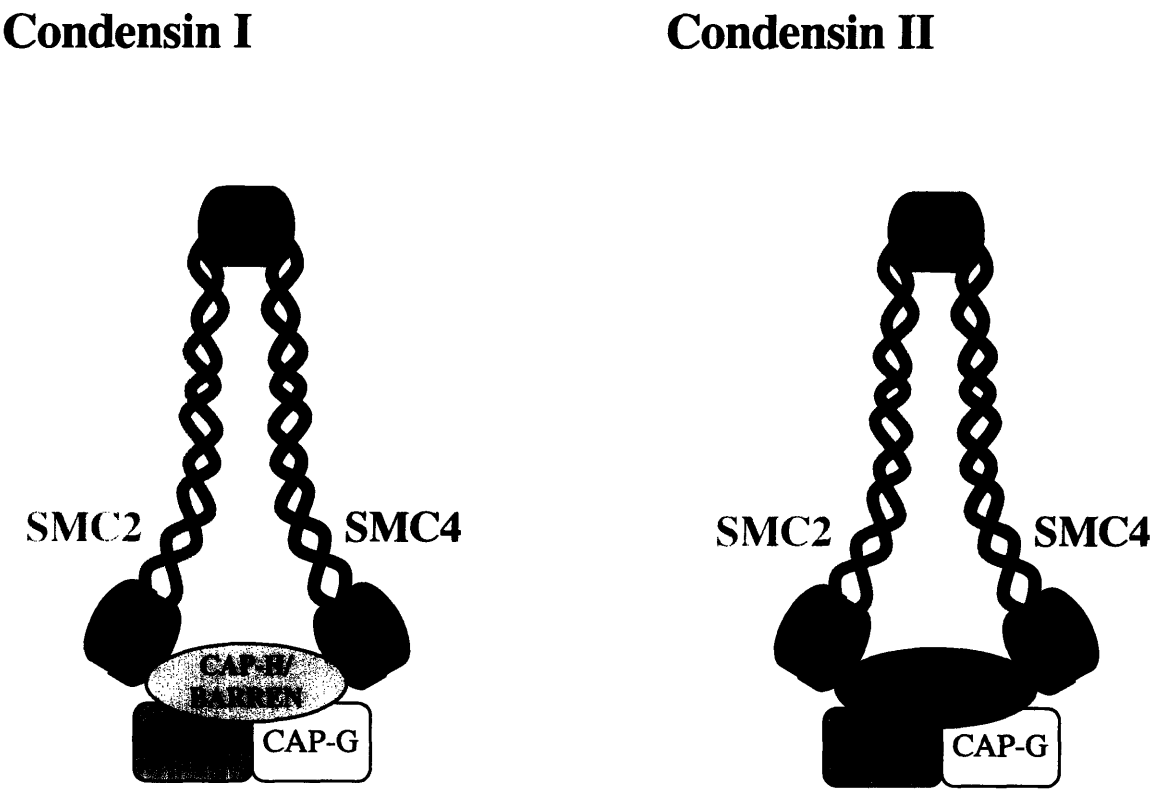

Figure 1-3. Structure of the condensin complexes in Drosophila

In many metazoan systems, two condensin complexes have been identified, both containing the same SMC molecules, but differing in their non-SMC components (Ono et al. 2003; Yeong et al. 2003). In Drosophila the condensin I complex includes non-SMC proteins CAP-D2 and CAP-H (BARREN), and the condensin II complex contains CAP-D3 and CAP-H2. Only one CAP-G subunit has been identified in Drosophila, and therefore it is presumed to function in both complexes (Dej et al. 2004; Jager et al. 2005). Work in mammalian tissue culture systems suggests that both condensin complexes are involved in chromosome condensation and sister-chromatid resolution (Ono et al. 2003; Hirota et al. 2004; Ono et al. 2004). These experiments showed that the condensin II complex is nuclear from the beginning of mitosis and acts early in chromosome condensation, whereas the condensin I complex is cytoplasmic in prophase and requires nuclear envelope breakdown to access the chromosomes. Once chromosomes are fully condensed later in mitosis, the two types of condensin complexes appear to alternate along the length of the chromosome.

Several results suggest that the roles of the two condensin complexes may be different in Drosophila. Depletion of SMC4, which is essential in both complexes, and mutation of the condensin I-specific component *cap-h/barren* result in similar defects in mitotic chromosome dynamics (Coelho et al. 2003; Oliveira et al. 2005), and condensin II component CAP-D3 was shown not to localize across mitotic chromosomes but rather was restricted specifically to centromeres (Savvidou et al. 2005). Furthermore, recent experiments examining BARREN dynamics by live imaging showed this condensin I subunit associating with the chromosomes early in prophase I, as soon as chromosome condensation was morphologically evident (Oliveira et al. 2007).

In *C. elegans* only one condensin complex is involved in chromosome structure, another complex related to condensins at the sequence level has a more diverged function and plays important roles in dosage compensation (for a review see (Hagstrom and Meyer 2003)).

C. Interactions between the Condensin and Passenger complexes in mitosis

The relationship between the condensin complex and the chromosome passenger complex is of particular interest because of the overlapping roles of these two complexes in mitotic chromosome condensation. This involvement in related processes raises the question of whether the two complexes coordinate chromosome morphology by separate parallel pathways or whether they regulate each other in some manner.

A suggestion that these complexes may regulate each other comes from a series of localization studies. In a variety of systems including *D. melanogaster, S. pombe,* and *C. elegans*, condensin complex components fail to localize onto mitotic chromosomes in the absence of Aurora B or other members of the passenger complex(Giet and Glover 2001; Morishita et al. 2001; Hagstrom et al. 2002; Kaitna et al. 2002). In HeLa cells Aurora B is required for localization and maintenance of the condensin I complex on the chromosomes, and it also may phosphorylate the non-SMC compoments of condensin I, but it is not required for localization of the condensin II complex (Lipp et al. 2007). However, experiments in *X. laevis* and *S. cerevisiae* have indicated that the condensin complex does not require the passenger proteins in order to load onto the chromatin (Losada et al. 2002; Lavoie et al. 2004). Although in *S. cerevisiae*, Ipl1, the Aurora

kinase, is required for phosphorylation of Ycg1, the CAP-G condensin subunit (Lavoie et al. 2004).

In addition, work from mammalian tissue culture showed that depletion of ScII, the condensin component SMC2, disrupts localization of the passenger protein INCENP. In these depleted cells, INCENP is localized across the chromatin in prophase but fails to restrict to the centromere in metaphase (Hudson et al. 2003). This maintenance across the chromatin is similar to INCENP localization when the passenger complex is disrupted by depleting Aurora B kinase (Adams et al. 2001a). In the case of the ScII depletion, however, at the onset of anaphase INCENP transfers to the spindle midzone as normal, suggesting that the defects in localization may be limited to its roles in chromosome dynamics.

Although both the condensin and passenger complexes have been implicated in chromosome condensation, important differences in the phenotypes arising from mutations in each complex have also been characterized. In *C. elegans* embryos, chromosomes normally condense in prophase, prior to nuclear envelope breakdown. Embryos mutant for *Aurora B* (called *air-2* in *C. elegans*) complete this early condensation stage normally, whereas embryos depleted of SMC-4 fail to individualize chromosomes (Kaitna et al. 2002). Upon nuclear envelope breakdown, however, *air-2* mutants display defects in metaphase plate formation and completely fail to separate chromosome masses in anaphase. Embryos depleted of condensin subunits behave quite differently; with little delay they form an organized metaphase plate, and upon entry into anaphase the majority of the chromatin separates into two distinct masses, though these masses are joined by robust bridges. Intriguingly, despite these differences in

chromosome behavior, depletion of AIR-2 in these embryos disrupts localization of condensin subunits to the chromosomes, as assayed by immunfluorescence (Kaitna et al. 2002). The metaphase disorganization and severe anaphase separation defect that result from AIR-2 depletion suggest that the passenger complex has additional roles in chromosome dynamics beyond a role in localizing the condensin complex. The prophase defects seen when the condensin complex, but not the passenger complex, is disrupted are more confounding. One likely explanation is that a low level of condensin complex may localize to the chromosomes in the *air-2* mutant, though it is not seen by immunofluorescence, and that this is sufficient for prophase chromosome individualization.

Work in *S. cerevisiae* also supports a role for the passenger complex in chromosome condensation in anaphase. As mentioned above, Ipl1, the budding yeast Aurora kinase, is not required for association of the condensin complex with the chromatin, but it is required for a phosphovariant of Ycg1, the condensin CAP-G subunit (Lavoie et al. 2004). This modification is not found early in mitosis, but becomes prominent in later stages. Furthermore, *ipl1* mutants do not display condensation defects, assayed in budding yeast by rDNA morphology, in early mitosis, but condensation defects are seen in late mitosis. These results suggest a model in which the passenger complex phosphorylates the condensin complex in the late stages of mitosis and that this modification may be required for function, but not localization, of the condensin complex.

D. Specialized roles for the passenger complex in meiosis

The roles of the condensin and passenger complexes in meiosis are much less understood, and the relationship between the two complexes is even murkier. Passenger complex localization has been analyzed in a number of meiotic systems and in many of these it is generally seen to have a similar localization pattern to that in mitosis. In mouse spermatocytes, INCENP localizes along the axis of the chromosomes early in prophase I, and then reorganizes to the centromere and the pericentric heterochromatin (Parra et al. 2003). By metaphase I, INCENP is found predominantly at the centromere, and during the course of anaphase I, INCENP disappears from the kinetochore and accumulates at the spindle midzone. By telophase, no visible INCENP remains at the centromere. Upon entry into meiosis II, INCENP reaccumulates at the chromocenter and again focuses to the kinetochores by metaphase II. In anaphase II, some INCENP transfers to the spindle midzone, although an additional pool is maintained at the centromere through the completion of meiosis. Aurora B localization coincides almost entirely with this INCENP pattern (Parra et al. 2003). The similiarites in the meiotic and mitotic localization patterns suggest that the passenger complex may likely be involved in many of the same regulatory processes in both types of cell cycle.

Recent work in *S. cerevisiae* also supports a meiotic role for the passenger complex that is similar to its mitotic role. Ipl1 (Aurora kinase) was found to localize to the nucleus in metaphase and the spindle in anaphase of both meiotic divisions, and to associate specifically with kinetochores at metaphase I (Monje-Casas et al. 2007). In the absence of Ipl1, homologs frequently moved together to the same pole in meiosis I, and this defect was partially rescued by transient destabilization of the microtubules. These results suggest that, just as in mitosis Ipl1 destabilizes kinetochore-microtubule

interactions when sister chromatids are inappropriately attached to the same pole, so too it destabilizes the monopolar attachment of homologs in meiosis I. Segregation of sister chromatids in meiosis II was also disrupted in the absence of Ipl1. The same study and another also showed a role for Ipl1 in maintaining cohesin protein Rec8 at the centromere after separation of homologs in meiosis I, and a role in localization of MEI-S332 homolog Sgo1 to the centromere (Monje-Casas et al. 2007; Yu and Koshland 2007).

A meiosis-specific role for the passenger complex has been described in *C. elegans* oogenesis. In metaphase I, the passenger proteins localized along the axes of the cohesed sister chromatids, but were specifically restricted to the region of the chromosome distal to the chiasma. In the absence of passenger proteins, the meiosis-specific cohesin protein Rec-8 was not removed from this distal part of the chromosomes in anaphase I and homologs failed to separate in the first meiotic division. Conversely, depletion of a phosphatase that antagonizes Aurora B kinase, *Ceglc-7α/β*, resulted in removal of cohesin along the entire chromosome in meiosis I, rather than just the distal portion. In this case, sister chromatids lost all physical attachment in the first meiotic division and separated from each other prematurely (Kaitna et al. 2002; Rogers et al. 2002).

Intriguingly, the localization pattern of Aurora B and INCENP in Drosophila oocytes is quite distinct from these other systems. In metaphase I arrested oocytes, the passenger proteins are not visible on the chromosomes, as they are at metaphase in most systems, but rather the passenger proteins are localized to the midspindle region (Jang et al. 2005). Female meiosis in many systems, including Drosophila, utilizes a spindle organized by the chromosomes themselves rather than by centrosomes. The absence of a

centrosome in the oocyte allows the zygote to enter the first mitotic division with only one centrosome, which is contributed by the sperm. Formation of the acentrosomal meiotic spindle is initiated by microtubule nucleation orchestrated by the chromosomes, then the microtubules are bundled and further organized, generating tapered poles, by microtubule motors and other proteins (Theurkauf and Hawley 1992; McKim and Hawley 1995; Matthies et al. 1996; Walczak et al. 1998). In the midspindle region, microtubules from both spindle poles overlap, and proteins localized to this site may be important for stability and bipolarity of the spindle (Jang et al. 2005).

INCENP's localization to the midspindle region in metaphase I does not rule out a role for the passenger complex in chromosome dynamics in this system, but it may suggest that the timing of the switch from chromosome-predominant localization to spindle-predominant localization is earlier than in most cell types, where this transition typically happens at anaphase. A role for the passenger complex in meiotic spindle organization has also been suggested in work from Xenopus extracts, in which chromosome-mediated microtubule nucleation was shown to require the chromosomal passenger complex, apparently through its role in inhibiting the microtubule destabilizing activity of MCAK (Sampath et al. 2004; Kelly et al. 2007).

E. The condensin complex and chromosome resolution in meiosis

The failure to separate homologs in meiosis I of *C. elegans* oogenesis upon depletion of passenger proteins is strikingly different from the effects of condensin depletion in the same meiotic system. When the condensin SMC-4 is depleted in these oocytes, homologs separate from each other without delay or defect, and the first polar

body is extruded normally. However, extensive chromosome bridging results from attempted sister-chromatid separation in meiosis II. This lagging chromatin is robust and sometimes results in interference of the second polar body in embryogenesis, due to a failure to separate the maternal pronucleus and second polar body (Hagstrom et al. 2002; Kaitna et al. 2002). Combination of a temperature sensitive allele and RNAi depletion of condensin subunit HCP-6 (homologous to CAP-D3) reveals lesser lagging-chromatin defects in anaphase I (Chan et al. 2004). The possibility that this lagging chromatin might arise due to failures in sister-chromatid resolution was supported by the finding that depletion of condensins suppressed premature sister-chromatid separation in the absence of the cohesin protein Rec-8. The role of the condensin complex in chromosome dynamics is not limited to inter-sister interactions, because depletion of condensin also suppressed premature separation of homologs in a *spo-11* mutant. SPO-11 introduces the double-strand breaks that are required for recombination, and therefore this result implicates condensin in homolog resolution independent of recombination and chiasma formation (Chan et al. 2004).

In addition, in *C. elegans*, meiotic chromosomes were normally compacted in pachytene, and the the synaptonemal complex assembled and disassembled normally when condensin was depleted. In diplotene and diakinesis, however, chromosomes were elongated and formation of discrete bivalents was delayed. In metaphase I, bivalents did not form an organized cruciform structure (Chan et al. 2004).

Just as different systems make different suggestions about the mitotic roles for condensin, implications about condensin's function in meiosis vary as well. In contrast to the observations in worms, the condensin complex is required in *S. cerevisiae* for length-

wise compaction and chromosome resolution in pachytene, and it is also required for proper formation of the synaptonemal complex (Yu and Koshland 2003). Anaphase bridging was seen, in this system, in both meiotic divisions, but meiosis I lagging chromosome defects were eliminated in a *spo11* mutant (Yu and Koshland 2003), suggesting that in this system condensin is only important for sister-chromatid resolution and not for other types of homolog interactions. This failure to separate sister chromatids is due, at least in part, to a requirement for condensin to recruit Polo kinase, Cdc5, and thereby properly remove the cohesin complex from meiotic chromosomes (Yu and Koshland 2005).

IV. DROSOPHILA AS A MODEL SYSTEM FOR UNDERSTANDING MEIOSIS

Drosophila melanogaster provides a wonderful model for exploring the regulation and progression of meiosis (Maines and Wasserman 1998; McKim et al. 2002). A broad and powerful set of genetic tools have been developed through many decades of work in the system. Combined with a relatively short lifecycle, these tools allow for robust experimentation *in vivo*. The tissues in which meiosis takes place are easily accessible and manipulable; and the meiotic cells themselves are large and conducive to informative imaging. This allows meiosis to be examined in its developmental context, surrounded by other cell types that are frequently key players in developmental regulation. In addition, mechanisms of meiosis are highly conserved and therefore many of the insights into meiotic regulation that have been made in Drosophila are highly relevant in mammalian systems. Finally, certain aspects of meiotic regulation and progression are approached differently in female and male Drosophila, providing two

complimentary systems for analyzing meiotic events. Similar to many vertebrate systems, male meiosis proceeds from start to finish with little delay, whereas female meiosis is arrested at certain points to coordinate cell cycle progression and oocyte development. In addition, male Drosophila do not undergo synaptonemal complex formation, double-strand break formation, or homologous recombination; this provides an opportunity to separate effects of proteins with multiple roles during the course of meiosis.

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Chapter Two

A Screen for Regulators of the Completion of Meiosis and Restart of the Cell Cycle in Drosophila embryos

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ABSTRACT

Successful sexual reproduction requires not only the production of haploid gametes, but also the fusion of these cells and reentry of the diploid zygote into the mitotic cell cycle. Coordination of the completion of meiosis and restart of mitosis necessitates modification of the cell cycle in ways that are poorly understood. In many organisms, the early mitotic divisions are also regulated to achieve particular developmental goals. We conducted a screen to identify mutations that disrupt these specialized cell cycles. Using the model system *Drosophila melanogaster*, we took advantage of the powerful genetic and cytological tools that allow investigation of developmentally regulated cellular events. We identified nineteen mutants that disrupt the processes of completion of meiosis and early embryonic mitosis. These mutations were organized into five classes including mutants that fail in fertilization, pronuclear fusion, embryonic mitosis, or both DNA synthesis and mitosis. One mutation was mapped and molecularly characterized as an allele of an important mitotic regulator, providing strong support for the potential value of mutations characterized through this screen.

INTRODUCTION

The formation of an embryo from a sperm and an egg relies on a series of carefully regulated cell cycle transitions and non-canonical cell cycles, each with a critical developmental role (for a review see (Foe 1993)). Study of these cell cycle variants not only helps us to understand the mechanisms of embryogenesis, but also provides insight into the ways the cell cycle can be adapted, and therefore reveals much about the fundamental requirements of cell division. Remarkably, in Drosophila many distinct cell cycle programs are coordinated by the single cytoplasm of the egg, supplied with mRNAs and proteins by the female during oogenesis (Zalokar 1976; Edgar and Schubiger 1986).

Before ovulation and fertilization, the mature oocyte is arrested in metaphase I of meiosis (first described in Huettner 1924, reviewed in Foe 1993). Upon ovulation, re-entry into meiosis is triggered, homologs separate in anapase I, and then chromosomes proceed through meiosis II without further delay (Mahowald et al. 1983). These meiotic divisions are coordinated by a spindle that is organized by the chromosomes themselves, as the oocyte lacks centrosomes (Theurkauf and Hawley 1992). In addition, Drosophila female meiosis proceeds in the absence of cytokinesis, with all four haploid products remaining in a common cytoplasm. One of them then fuses with the male pronucleus and contributes to the developing embryo, while simultaneously the other three transition through an interphase state and then condense their chromosomes and come together into a characteristic rosette structure. These unused polar bodies are held inactive in this configuration but remain in the common cytoplasm of the embryo through approximately the first ten rounds of mitotic cell cycling, until they are displaced from their position at the cortex and eventually degraded. Very little is understood about the way in

which these polar bodies are held inactive, but maintenance of this state is critical so that the meiotic products do not interfere with ongoing embryogenesis.

When an egg is fertilized, the sperm enters the oocyte cytoplasm entirely and here it undergoes reprogramming to re-enter the mitotic cell cycle (Foe 1993). The DNA in the sperm head is packaged with sperm-specific protamines, and upon entry into the egg it discards these and replaces them with histones and other chromatin components (Loppin et al. 2005). The product of female meiosis that will become the female pronucleus is brought to the male pronucleus by a large microtubule array, coordinated by the centrosome brought in with the sperm (Huettner 1924). Together, the pronuclei enter the first zygotic mitosis on a spindle organized by a paternally-contributed centrosome. Male and female pronuclei come together to a common metaphase plate, but because the pronuclear envelopes have not completely broken down, they remain as separate masses until telophase, when pronuclear envelopes fuse and chromosomes finally commingle (Stafstrom and Staehelin 1984).

The first thirteen mitotic divisions also take place in a common cytoplasm, called a syncytium, and they proceed extremely rapidly, oscillating between DNA synthesis (S) and mitosis (M) without gap phases in between, to allow the embryo to quickly increase its nuclear number (Foe and Alberts 1983). These earliest embryonic nuclear divisions are each completed in approximately nine minutes and therefore must be extremely efficient at transitioning between S and M phases.

The ability of the single cytoplasm of the oocyte and embryo to coordinate this diverse panoply of developmental and cell-cycle programs is truly striking. In addition, these processes are controlled by maternally-deposited stores of mRNA and protein, without contribution from new zygotic transcription (Edgar and Schubiger 1986). Furthermore, because this system lacks

cytokinesis, the embryo must employ a special mechanism for directing these programs simultaneously without the benefit of a plasma membrane between them to partition regulatory proteins.

Finally, the rapid oscillation between DNA synthesis and mitosis in the early embryo provides a unique and powerful context for studying regulation of cell cycle transitions and the elements required for tight and irreversible progression from one stage of the cell cycle to the next. Failure to coordinate S and M phases properly is catastrophic, leading to broken chromosomes, aneuploidy, and death of the organism. Improper regulation of these very same processes in other tissues is thought to promote the accumulation of the mutations that lead to cancer. Aneuploidy is a common feature of many human tumors (Pellman 2007). A better understanding of the essential elements of cell cycle progression will directly contribute to our ability to characterize and combat the development of cancerous tissues.

Although these processes have been well described through careful observation of wild-type embryos, little is understood about the processes that control this developmental window. By addressing this cornucopia of fundamental cell cycle questions in this context we can take advantage of the robust genetic system available in *Drosophila melanogaster*. In addition, the large tractable Drosophila embryos allow for clear visualization of cellular events as well as phenotypic analyses using many techniques of cell and molecular biology. To begin unraveling the regulatory mechanisms of these non-canonical cell cycles, we sought mutants that disrupt these processes. Because this developmental program is controlled by maternal stockpiles of mRNA and protein, these mutants can be found among maternal-effect mutants, in which homozygous mutant females are viable, but the embryos they lay fail to develop. We screened a

collection of female-sterile alleles and classified the mutations based on the developmental processes in which they are defective.

RESULTS

Characterization of mutations affecting early embryogenesis

The unique factors required for regulation of early embryogenesis have remained poorly characterized due to the extreme rarity of mutations that specifically affect this developmental program. A recent screen in the lab of Charles Zuker generated 12,000 viable EMS alleles (Koundakjian et al. 2004), of which 2,400 were female sterile. This subset was screened by members of the Orr-Weaver lab and characterized by stage of developmental arrest. Of these, fewer than twenty were identified as completing meiosis, but failing to progress through the embryonic syncytial divisions. Complementation tests were performed by crossing each of the nineteen lines to the other stocks containing a mutation on the same chromosome. Transheterozygous females were tested for fertility. The nineteen lines were placed into eighteen complementation groups.

Class 1 mutants: Eggs laid by mutant mothers appear unfertilized

In Drosophila, eggs complete meiosis even in the absence of fertilization (Doane 1960). All four haploid products of meiosis transition through a post-meiotic interphase, condense, and come together to form a rosette structure. This rosette is the only DNA present throughout the cytoplasm of the oocyte. It is positioned near the cortex and remains in this inactive state, similar to the behavior of the polar body rosette in fertilized embryos. A small percentage of unfertilized eggs are recovered among embryos laid even by wild-type females mated to wild-type males.

In the case of four mutants from our screen, eggs with solely a rosette structure were the only or the majority morphology recovered in embryo collections from mutant females (Fig. 2-1). This pattern is consistent with defects in fertilization of these oocytes. Embryos were

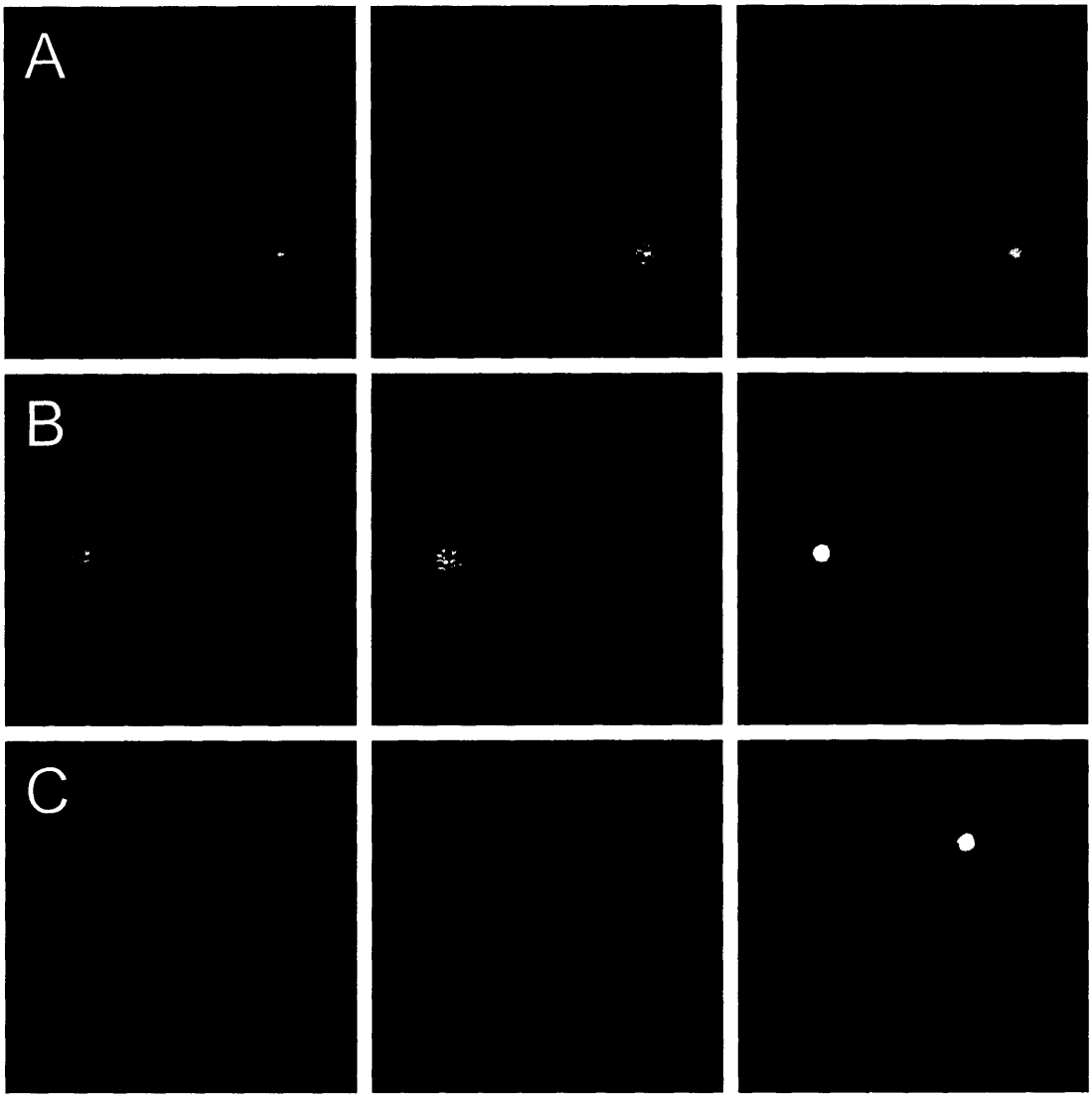

Figure 2-1. Class 1 mutants suggest defects in fertilization Embryos laid by mutant mothers, stained with YOYO-1 (green) and anti-tubulin antibodies (red). A) *Z2-1790*, B) *Z2-1867*, C) *Z3-4152*

collected from mutant females mated to wild-type males, eliminating the possibility that absence of fertilization resulted from defects in the sperm.

The mutants in this class were *Z2-1790* (Fig. 2-1A), *Z2-1844*, *Z2-1867* (Fig. 2-1B), and *Z3-4152* (Fig. 2-1C). The mutations *Z2-1790* and *Z2-1844* are likely lesions in the same gene because they fail to complement each other. Failure to complement is also consistent with mutations in two genes that interact functionally.

Class 2 mutants: Embryos from mutant mothers display phenotypes consistent with defects in pronuclear fusion

Two mutant lines produced phenotypes suggestive of defects in pronuclear fusion. In embryos laid by *Z3-1373* females, embryos progressed through a number of mitotic cycles, but the chromosomes on the mitotic spindle appeared to be haploid in number (Fig. 2-2B). This observation suggested that one pronucleus entered the mitotic cell cycle successfully and the other did not. The mutations *maternal haploid* and *Hira* (also called *sesame*) both display such a phenotype, which has been shown in both cases to be due to failure to reprogram the paternal chromosomes to successfully enter mitotic cycling (Loppin et al. 2000; Loppin et al. 2001; Loppin et al. 2005). Both of these characterized mutations are on the *X* chromosome, and therefore *Z3-1373*, which maps to chromosome *3*, is not allelic with either of them.

Embryos laid by homozygous *Z2-0706* females arrested with a polar body and one other focus of DNA. Frequently the rosette appeared small, likely containing less than the three haploid complements that should remain from the female meiosis (Fig. 2-2A'), leading to the hypothesis that too many of the meiotic products were drawn toward the male pronucleus. The other focus of DNA was generally located deep within the embryo, where the earliest zygotic

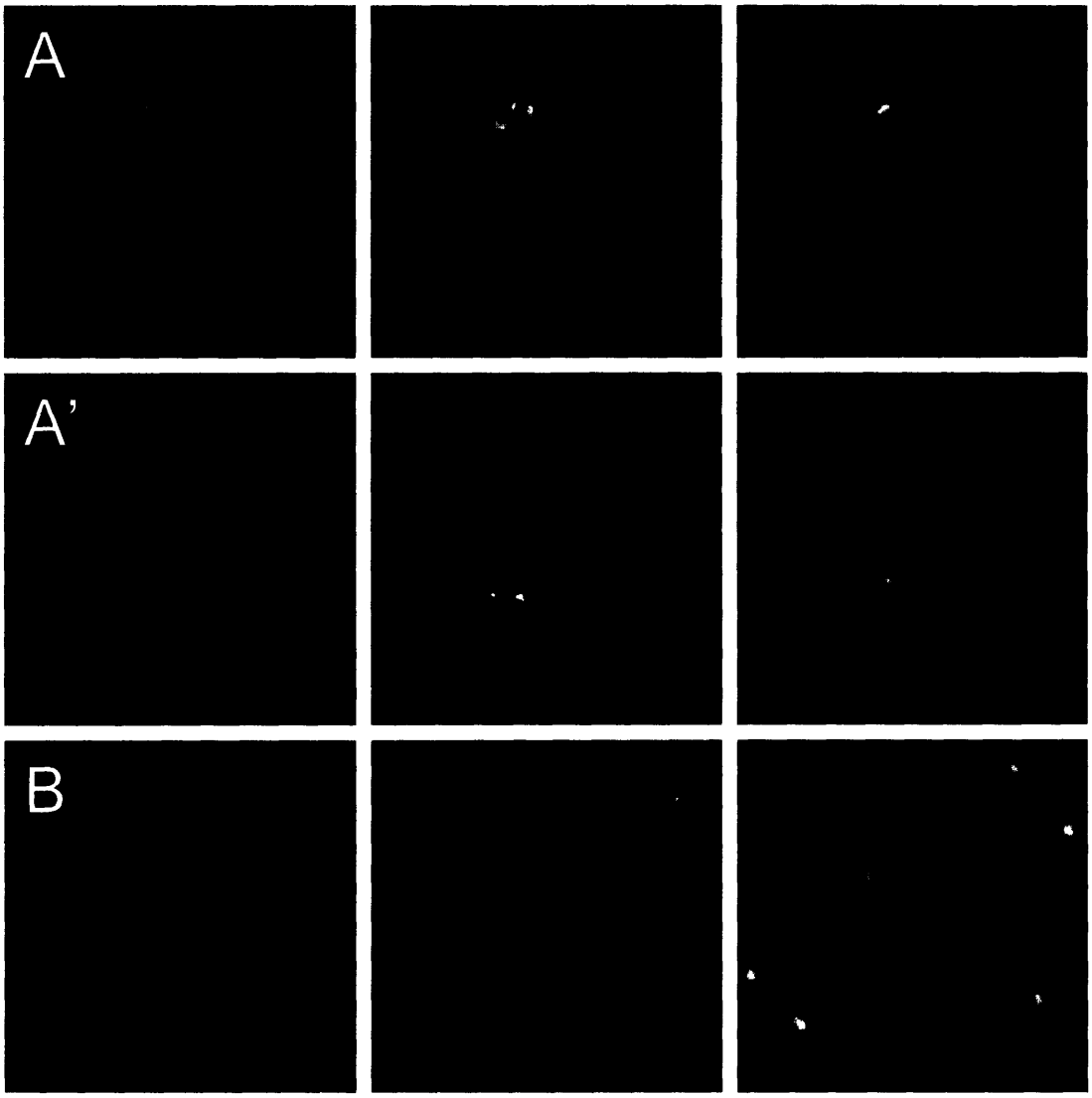

Figure 2-2. Class 2 mutants suggest defects in pronuclear fusion Embryos laid by mutant mothers, stained with YOYO-1 (green) and anti-tubulin antibodies (red). A, A') Two focal planes in the same embryo, *Z2-0706,* B) *Z3-I373*

divisions occur. In some embryos, this DNA could be seen in three or four masses, held close together (Fig. 2-2A). No spindle or other indication of attempted mitosis was seen associated with this DNA. Taken together with the reduced amount of DNA in the polar body structure, the chromosomes deep within the embryo likely include the male pronucleus and more than one of the female meiotic products.

The early-arrest phenotype of *Z2-0706* is uncovered by the deficiency *Df(2R)PC4*, which removes cytological region 55A1;55F1-2 on the right arm of the second chromosome. In addition, the mutation *IR28* fails to complement *Z2-0706*, with the transheterozygous females and males both being sterile; and the lethal *IR28* phenotype has also been shown to be uncovered by *Df(2R)PC4*. The mutation *IR28* was generated in a screen for lethal mutations in genes affecting the cell cycle later in embryogenesis, and it causes a mitotic arrest in the post-blastoderm embryo after maternal stockpiles have been used up or degraded. During these post-blastoderm divisions, *IR28* homozygous embryos showed an aberrantly high level of metaphase-arrested cells.

Repetition of the complementation tests revealed only male sterility in *Z2-0706/IR28* flies, and female and male sterility in *IR28/Df(2R)PC4* flies. This is consistent with the generation of a suppressor mutation on the *IR28* chromosome. Several other mutations from the Zuker collection also failed to complement *IR28*. These included *Z2-3883*, *Z2-4385*, *Z2-0674*, *Z2-3456*, and *Z2-3822*. Retesting of these mutations revealed male semi-sterility with *Z2-0706*. In addition, preliminary meiotic mapping suggested that the lesion in *Z2-0706* was to the right of *purple* (cytological position 38B) and to the left of *curved* (at 52D). Although these results are preliminary, they raise the important possibility that the interaction with *Df(2R)PC4* is a second-site effect. The potential to characterize an allelic series makes these putative genetic

relationships worth further study, but uncovering the true relationships may require removal of extraneous mutations on the chromosomes by recombination.

The identification of a lethal mutation putatively in the same complementation group as *Z2-0706* compels reconsideration of the previously characterized pronuclear fusion defect. The mutated gene may have a specific role in pronuclear fusion as well as other distinct roles in canonical mitosis, in which case *Z2-0706* may be a special allele in which only the pronuclear fusion function is affected. Alternatively, the true defect in *Z2-0706* embryos may be in a process that also takes place in other cell cycles. In this case *Z2-0706* may be a weak allele that possesses enough activity to progress through cellularized divisions, but not through the rapid syncytial stage. If *Z2-0706* disrupts a process common to cellularized divisions, it may still provide a powerful tool to understand regulation of the mechanism by which the number of female meiotic products brought toward the male pronucleus is regulated.

Class 3 mutants: Embryos from mutant mothers arrest DNA synthesis and mitosis in the earliest zygotic mitoses

Mutants in the third class arrest both DNA synthesis and mitotic division within the first two or three attempted cell cycles. Both mutants in this class were recovered from screens other than the Zuker screen described above. *fib* was generated in a P-element insertion screen performed by M. Goldberg (Cornell University, personal communication) and *QA26* was recovered in an EMS screen performed by T. Schüpbach and E. Wieschaus (Schupbach and Wieschaus 1989).

Five-ball (*fib*) derives its name from its typical arrest point, with four mitotic nuclei and the polar body rosette forming "five balls" of DNA in the embryo. The mitotic nuclei often

contain DNA fragments rather than complete chromosomes, and foci of tubulin rather than organized spindles (Fig. 2-3B). Weak spindles are occasionally organized around the DNA and these usually appear to lack asters. DNA fragments frequently appear tightly condensed. Premature condensation of chromosomes that have not completed replication could lead to the observed chromosomal fragments and cell cycle breakdown. The extremely early point of cell cycle and developmental arrest in *fib* mutants raises the possibility that the disrupted gene might function specifically in the syncytial S-M cycles of embryogenesis. One important caveat in the mapping of *fib* arises from the fact that it was recovered from a P-element mutagenesis, but the female sterility maps away from any P-element present. The nature of the lesion, therefore, is difficult to predict, but may be a deletion or P-element footprint.

Embryos laid by *QA26* homozygous mutant mothers form robust spindles with chromatin stretched aberrantly between spindle poles (Fig. 2-3A). We have mapped and molecularly characterized the *QA26* mutation and found an aspartate to valine change in the conserved gene *incenp*, a member of the passenger protein complex, along with Aurora B, Survivin, and Borealin/Dasra. RNAi and mutant analyses of *incenp* and the other passenger proteins in a variety of organisms have revealed mitotic defects including failed chromosome segregation and chromosome bridging in anaphase, similar to those seen in *QA26* embryos (Adams et al. 2001; Giet and Glover 2001). Additional work characterizing the *QA26* mutant in embryogenesis and in male and female meiosis is described elsewhere (Resnick et al. 2006, Chapters 3 and 4 of this thesis). Identification of one of the mutations in this screen as affecting a critical cell cycle regulatory gene, and ensuing work demonstrating it to be a valuable allele for studies of meiosis,

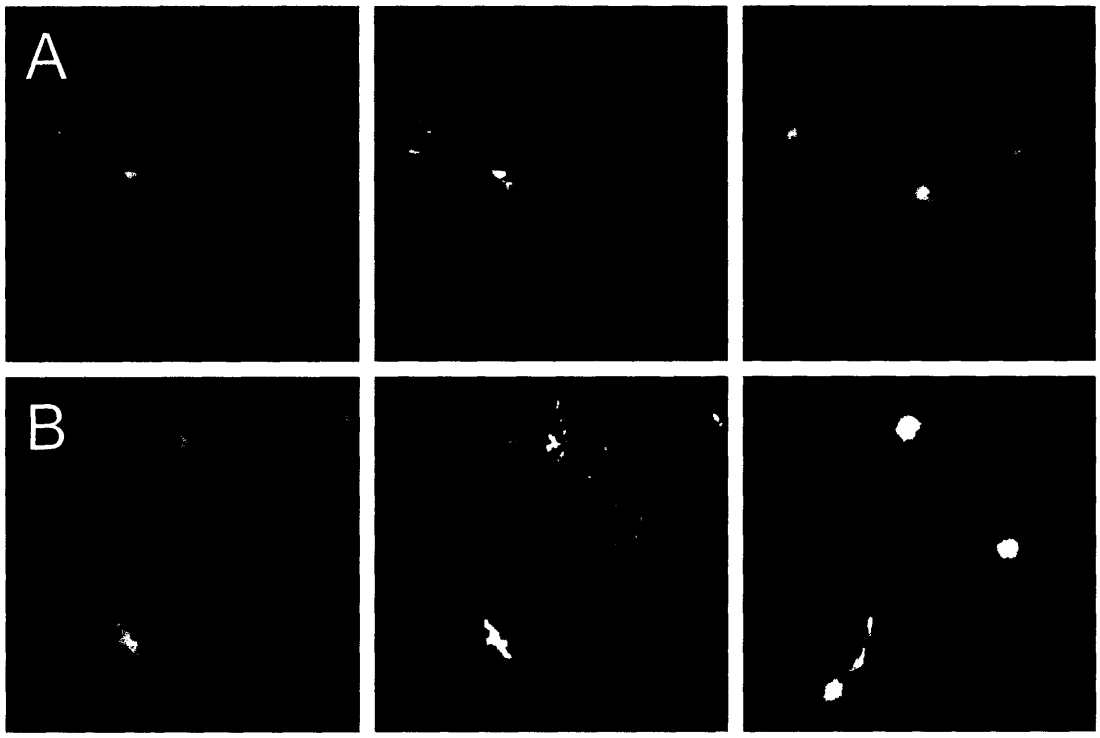

Figure 2-3. Class 3 mutants arrest DNA synthesis and mitosis in earliest zygotic cycles
Embryos laid by mutant mothers, stained with YOYO-1(green) and anti-tubulin antibodies (red). A) *QA26*, B) *fib*

provide strong support for the potential importance of mutations characterized through this screen.

Class 4 mutants: Embryos from mutant mothers arrest mitosis and become polyploid in the earliest zygotic divisions

Mutations in Class 4 are similar to those in Class 3 in their early arrest of mitosis. In Class 4, however, mutants continue synthesizing DNA in the absence of division, resulting in a small number of large polyploid masses of DNA (Figure 2-4). The three mutants in this class are Zuker mutations *Z2-1596* (Fig. 2-4A), *Z3-5130* (Fig. 2-4B), and *Z3-5711* (Fig. 2-4C). In each of these mutants, the large masses of DNA were frequently surrounded by lattices of microtubules (Fig. 2-4A, C). Some of the DNA in the masses appeared highly condensed (Fig. 2-4C), other times it appeared to be less compacted (Fig. 2-4A), and sometimes the DNA appeared fragmented (Fig. 2-4B, C). In addition, smaller masses of DNA were sometimes seen on barrel-like spindles (Fig. 2-4B, arrows).

This range of defects was observed in each of the Class 4 mutants, suggesting that these morphologies are likely non-specific or degradative phenotypes. Therefore an important step in understanding the true phenotypes of these mutants is capturing the specific defects, perhaps by shortening the embryo collection time. However, because these mutants become polyploid, they are likely to disrupt processes required for separation of chromosomes or processes required to coordinate an alternation between DNA synthesis and mitosis.

Preliminary mapping of *Z2-1596* suggests that it is likely located to the right of *dumpy* (at 25A) and to the left of *curved* (at 52D).

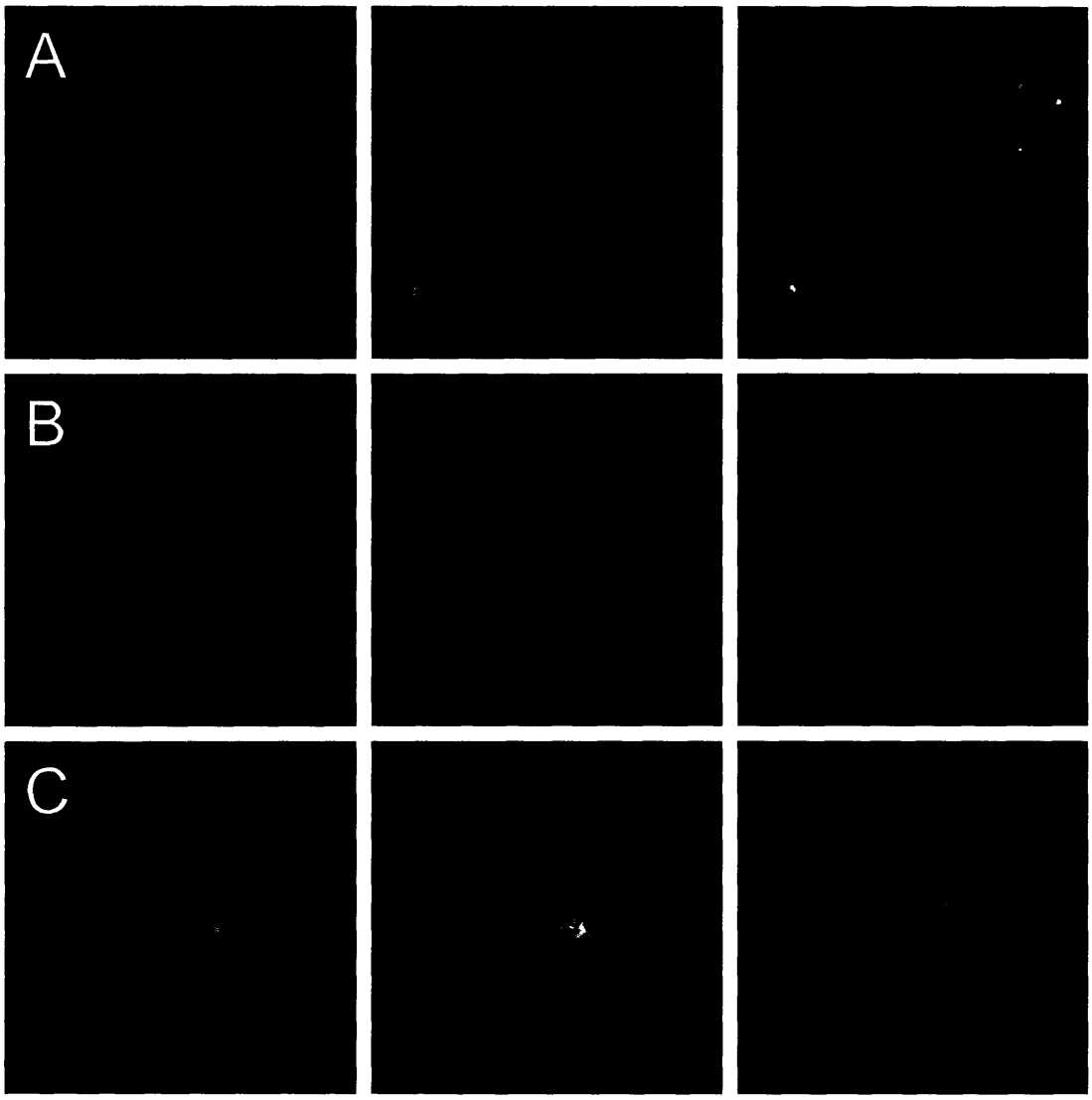

Figure 2-4. Class 4 mutants arrest mitosis in earliest zygotic cycles Embryos laid by mutant mothers, stained with YOYO-1 (green) and anti-tubulin antibodies (red). A) *Z2-1596,* B) *Z2-5130*, arrows indicate barrel-like spindles, C) *Z3-5711*

Class 5 mutants: Embryos from mutant mothers complete several successful divisions before experiencing mitotic defects

Class 5 mutants undergo several rounds of successful cell division before exhibiting defects in mitosis (Fig. 2-5). Mutants in this class include *Z3-3111* (Fig. 2-5A), *Z3-0435* (Fig. 2-5B), and *Z2-0040* (Fig. 2-5C). Mitotic defects observed in each of these mutants included lagging chromatin in anaphase and nuclei of differing sizes, suggesting that some had become polyploid. Because these phenotypes arose only after cell division was completed without error several times, these mutations are less likely to disrupt genes critical for this specific developmental window. They are most likely weak alleles, probably affecting genes required for cellularized divisions as well as the syncytial cycles. These mutations may still prove very valuable, as demonstrated by *QA26*, however they are less likely to provide insight into the specific developmental processes discussed here.

Defects in post-meiotic rosette structure

Many of the mutations described in the classes above also disrupted proper formation of the polar body rosette (Fig. 2-6). In wild-type embryos, the rosette structure is formed by tightly condensed chromosome arms of equal length (Huettner 1924). In some embryos, all three unused products of female meiosis come together to form a single rosette (Fig. 2-6A). In others, two haploid meiotic products come together and the third remains separate, thus forming one larger (diploid) and one smaller (haploid) rosette structure (Fig. 2-6A'). The chromosomes of the *QA26* polar body rosette frequently appeared elongated, and broken chromosomes were often located at the edges of the rosette (Fig. 2-6B). The *fib* polar bodies were stretched out and wispy in appearance and typically also included DNA fragments near to the rosette (Fig. 2-6C). Other

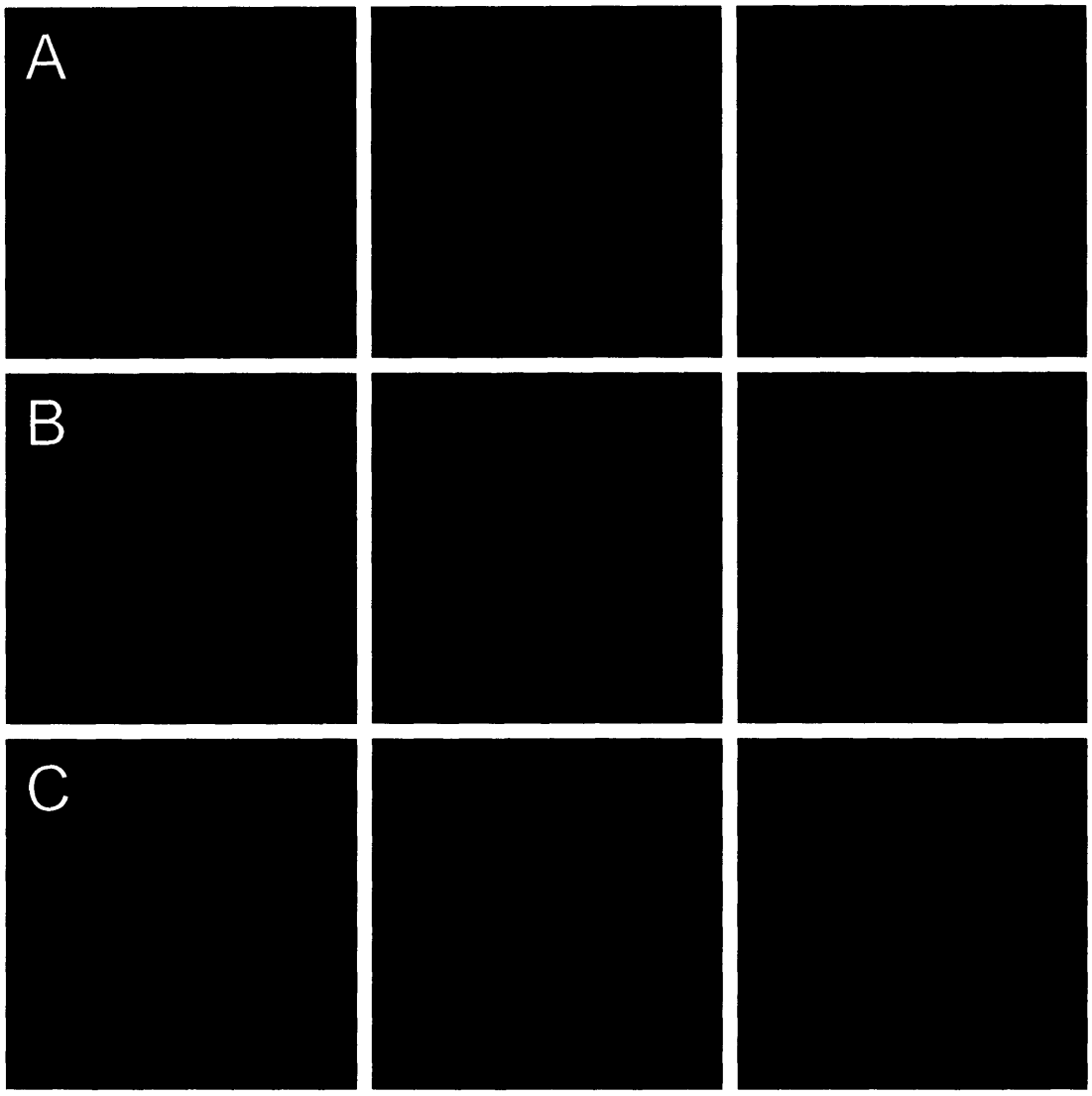

Figure 2-5. Class 5 mutants display mitotic defects in syncytial S-M cycles Embryos laid by mutant mothers, stained with YOYO-1 (green) and anti-tubulin antibodies (red). A) *Z3-3111,* B) *Z3-0435,* C) *Z2-0040*

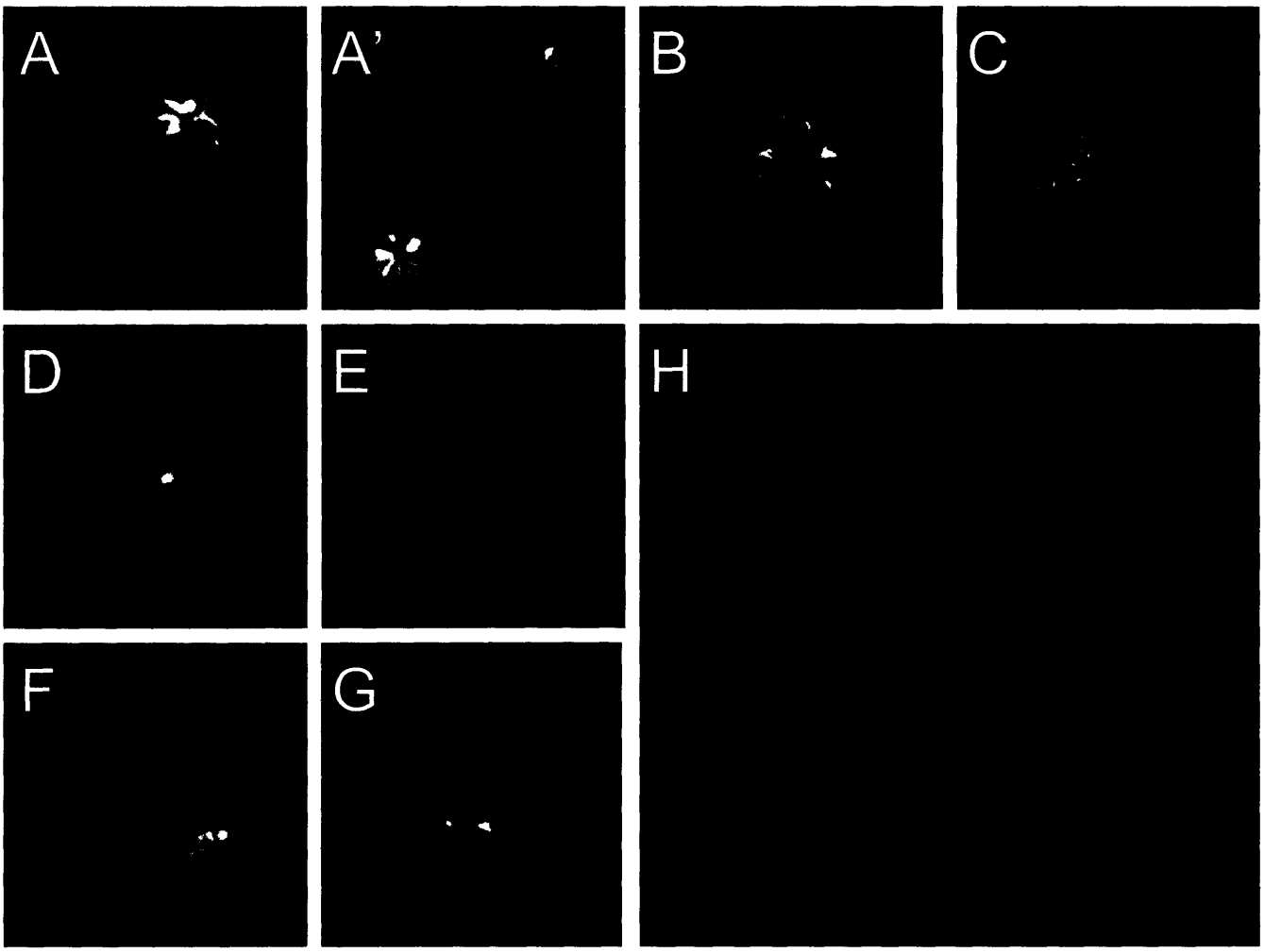

Figure 2-6. Maternal effect mutants display defects in polar body rosette structure
Embryos laid by mutant mothers, stained with YOYO-1. A, A') *yw* - wild-type rosettes display condensed arms of uniform length. Some embryos contain one rosette structure (A), others contain two rosettes (A'), B) *fib* - rosette arms are elongated and wispy, C) *QA26* - rosette arms are elongated and fragmented, D) *Z2-1867* - post-meiotic chromosomes pulverized, E) *Z3-4152* - post-meiotic chromosomes hypercondensed, F) *Z3-5130* - polar body chromosomes arranged on spindle, G) *Z3-0706* - too few polar body chromosomes, H) *Z3-5711* - too many polar body chromosomes

defects were more common among the mutants. Several mutants, including *Z2-1867*, sometimes displayed pulverized polar bodies (Fig. 2-6D). Another common defect was hypercondensed chromosome arms, as shown for *Z3-4152* (Fig. 2-5E). Polar bodies that appeared to be arranged on a spindle were also seen in several mutants, including *Z3-5130* (Fig. 2-6F). The mechanisms leading to these disruptions require further examination. In addition, *Z2-0706* frequently displayed too few chromosomes in the polar body rosette (Fig. 2-6G), as described above. Finally, one mutant, *Z3-5711,* sometimes exhibited too many chromosomes arranged in rosette structures (Fig. 2-6H), suggesting that either the unused products of female meiosis inappropriately replicated or that other chromosomes aberrantly displayed polar-body behavior.

DISCUSSION

This newly isolated collection of rare mutations affecting the S-M cycles of Drosophila embryogenesis provides great promise of identifying new cell cycle regulators. We have characterized these mutants into several classes: 1) eggs from mutant mothers cannot be fertilized, 2) male and female pronuclei fail to fuse, 3) embryos attempt fewer than three divisions before arresting DNA synthesis and mitosis, 4) embryos complete fewer than three mitoses but DNA synthesis continues resulting in polyploidy, and 5) several successful S-M cycles are followed by mitotic defects and polyploidy. In particular, studies of the mutations in classes 3 through 5 may identify novel regulatory mechanisms critical to coordinating the progression of S and M phases. They may also reveal structural components of the cell division machinery that are essential for packaging and partitioning the genetic material.

In pursuing these lines for further analysis, several considerations are important for prioritizing the mutations and for understanding the biology of the processes they disrupt. Some of these considerations have been mentioned throughout, a few additional points are discussed further below.

Class 1 mutants, which appear to fail in fertilization, could be disrupted in a number of different biological processes. This phenotype could arise due to defects in mating, in sperm entry into the female reproductive tract, in storage of sperm in the specialized organs of the female reproductive tract, in entry of the sperm into the oocyte through the micropyle, or in reception of the sperm by the egg. From a cell cycle regulatory perspective, this last class would be the most intriguing, though some of the other classes may be important developmentally. Distinguishing among these possibilities will be important in pursuing these mutants. One important tool for dissecting this phenotype will be *don juan-GFP* males, which express GFP

throughout the spermtail (Santel et al. 1998). By mating Class 1 mutant females to these males, laid eggs can be examined directly for presence of sperm, in order to confirm that these eggs remain unfertilized. In addition, sperm storage organs in the female can be examined to determine whether sperm is properly entering and being held in the female reproductive tract.

The early embryo utilizes a distinct set of processes leading to developmental arrest and degradation of defective nuclei (Raff and Glover 1988). To that end, distinguishing between primary defects, due to the effects of the mutation, and secondary defects that arise from downstream or degradative processes is extremely important. This issue is relevant in clarifying the phenotypes of many of the mutants in this screen. For example, do *Z2-0706* embryos fail in the mitotic cell cycle at a stage when the male pronucleus is meant to be drawing in a female pronucleus, such that the cell cycle arrest results in the pronuclear defect? Or do these embryos fail to regulate pronuclear apposition and fusion, and thereby cause a downstream defect in cell cycle progression? Another example comes from the *fib* mutant. We have suggested, above, that chromosome condensation prior to completion of DNA replication could lead to tightly condensed chromosome fragments. This is supported by the observation that *fib* embryos displayed pulverized chromosomes after very short collection times. However, another possibility is that the mutation causes some other sort of arrest and that the DNA fragments are breakdown products.

Distinguishing between primary and downstream defects is most important for Class 4 mutants. Similarities among the mutant embryos suggest that many features of chromosome morphology and microtubule networks observed in these mutants are likely downstream effects. An important distinction can be made, however, between this class of mutants and *pan gu* complex mutants (Shamanski and Orr-Weaver 1991). The *pan gu* family mutants also become

polyploid in the earliest stages of embryogenesis, but the DNA appears completely decondensed, centrosomes depart from the nuclei, and microtubule structures do not form around the polyploid masses. Thus, the observation that Class 4 mutants do display partially condensed DNA and microtubule organization suggests that they likely retain more mitotic character than *pan gu* mutants. Unraveling the true primary phenotypes of these mutations, however, will most likely require catching these nuclei earlier, just as they are beginning to become aberrant and polyploid.

One additional common feature in Class 4 mutants, also mentioned above, was the presence of barrel-like spindles (Fig. 2-4B, arrows). These broad-ended, acentriolar spindles frequently associated with tightly condensed or abnormal chromosomes. This morphology has been seen in many mutants, including the Class 4 mutants described here as well as other mutations including *morula*, a member of the Anaphase Promoting Complex/Cyclosome (Reed and Orr-Weaver 1997; Kashevsky et al. 2002). The possibility that these barrel-like spindles are a downstream effect of some other aberration is supported by several observations. First, they are common to a number of different mutants that arrest in these stages. Second, experiments in later-stage syncytial Drosophila embryos have shown that nuclei that experience DNA damage lose association with their centrosomes in a manner that leads to degradation of the damaged nuclei (Takada et al 2003, cell). This response is mediated by the checkpoint kinase *DmChk2*. Third, work from the laboratory of Laurie Lee (Vanderbilt University Medical Center) has shown that two different mutants that arrest in the early syncytial cycles with condensed chromosomes on barrel-like spindles are suppressed by mutation of *DmChk2*. This suppression allows both mutants to progress much farther into embryogenesis and completely restores spindle morphology (L. Lee, personal communcation). This suppression supports the conclusion that early Drosophila embryos employ robust systems for arresting division of damaged nuclei

long before the primary defects themselves would lead to catastrophic conditions, and emphasizes the importance of viewing early-arrest phenotypes with this consideration in mind.

MATERIALS AND METHODS

Fly stocks

Mutants for this screen were obtained as described above. *IR28* was recovered in a screen for mutants that displayed embryonic lethality and arrested cell-cycle progression (Royzman et al. 1997). Other stocks were obtained from the Bloomington Stock Center. Flies were raised on standard Drosophila medium at 25°C.

Embryo collections and cytology

Embryos were collected for 1 or 2 hours on apple juice plates, dechorionated in 50% bleach, devitellinized in methanol and heptane, and fixed in methanol for 3 hours at room temperature. Embryos were stained for DNA with YOYO-1 (Molecular Probes) and with antibodies to α-tubulin (YL 1/2 and YOL 1/34, Axyll), each at 1:40. Tubulin antibodies were detected using Cy3-conjugated fluorescent anti-rat secondary antibodies (Jackson Immunoresesarch). Imaging of stained ovaries was performed using a Zeiss microscope with LSM510 confocal imaging software (Keck Imaging Facility) or a Zeiss Axiophot microscope with a Spot CCD camera and software. Images were processed using Adobe Photoshop.

ACKNOWLEDGEMENTS

We thank B. Wakimoto, D. Lindsley, and M. McKeown who screened the Zuker mutant collection for female-sterile lines. With much gratitude, we thank members of the Orr-Weaver lab who screened the female sterile collection: K. Dej (currently of McMaster University, Hamilton, ON, Canada), G. Bosco (currently of University of Arizona, Tucson, AZ), L. Lee (currently of Vanderbilt University Medical Center, Nashville, TN), I. Ivanovska (currently of Rosetta Inpharmatics, Seattle, WA), H. Kashevsky, and D. Epstein. We also thank K. Dej for her observation that *Z2-0706* fails to complement *IR28*. Some of the microscope images were collected in the Keck Imaging Facility of the Whitehead Institute.

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Chapter Three

INCENP and Aurora B Promote Meiotic Sister Chromatid Cohesion through Localization of the Shugoshin MEI-S332 in Drosophila

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This chapter was published in Developmental Cell *11*, 57-68, July, 2006.

SUMMARY

The chromosomal passenger complex protein INCENP is required in mitosis for chromosome condensation, spindle attachment and function, and cytokinesis. Here we show that INCENP has an essential function in the specialized behavior of centromeres in meiosis. Mutations in Drosophila *incenp* profoundly affect chromosome segregation in both meiosis I and II, at least in part due to premature sister chromatid separation in meiosis I. INCENP binds to the cohesion protector protein MEI-S332, which is also an excellent *in vitro* substrate for Aurora B kinase. A MEI-S332 mutant that is only poorly phosphorylated by Aurora B is defective in localization to centromeres. These results implicate the chromosomal passenger complex in directly regulating MEI-S332 localization and therefore the control of sister-chromatid cohesion in meiosis.

INTRODUCTION

Sexually reproducing organisms need a specialized cell division, meiosis, to generate haploid cells to maintain diploidy after fertilization. During meiosis two divisions give rise to four haploid products. In the first meiotic division -the reductional division- homologous chromosomes pair and then segregate from each other. Without an intermediate S-phase, the second meiotic division proceeds similarly to mitosis. The success of meiosis depends on specific regulation of the cell division machinery. Some components are common to mitosis and meiosis, but are regulated differently in the two types of division. Other components function only in meiosis (McKee, 2004).

In both mitosis and meiosis, sister chromatids must physically associate with each other to biorient on the spindle. The sister chromatids are attached by the cohesin complex, and in mitosis this cohesion is released at the metaphase-anaphase transition following cleavage of the Scc1/Rad21 subunit (Uhlmann et al. 2000).

Specialized features are required in meiosis I to facilitate homologue segregation and to ensure that sister-chromatid segregation is deferred until meiosis II (Petronczki et al., 2003). In most organisms homologues are linked by chiasmata, the sites at which homologues recombined. The sister kinetochores of each chromosome act as a unit, attaching to the same spindle pole and ensuring that both sister chromatids of each homologue migrate to the same pole in anaphase I. To coordinate proper segregation, cohesion is lost in a step-wise manner. Cohesion distal to the chiasmata is lost in anaphase I, allowing homologues to separate (Buonomo et al., 2000), but cohesion between the centromeres of the sister chromatids is preserved until the onset of anaphase II to guarantee accurate segregation of sister chromatids (for a review see (Petronczki et al., 2003)).

Retention of cohesion at the centromere requires the Drosophila MEI-S332 protein, founding member of a class of protective proteins, now known as Shugoshins (Kerrebrock et al., 1995). Yeast Shugoshin proteins appear to act by preventing cleavage of the Rad21 meiotic paralog, Rec8, at the metaphase I-anaphase I transition (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). Similarly, human Shugoshin ensures that cohesin does not prematurely dissociate from mitotic centromeres (McGuinness et al., 2005).

The chromosomal passenger complex plays essential roles in mitosis and cytokinesis (Carmena and Earnshaw, 2003; Vagnarelli and Earnshaw, 2004), including chromosome condensation, biorientation of kinetochores, stability of the bipolar spindle and central spindle formation. Four members of the complex have been identified: Aurora B (Adams et al., 2001a; Adams et al., 2000; Schumacher et al., 1998; Terada et al., 1998) INCENP (Inner Centromere Protein, (Cooke et al., 1987)), Survivin (Carvalho et al., 2003; Skoufias et al., 2000; Uren et al., 2000), and Borealin/Dasra-B (Gassmann et al., 2004; Sampath et al., 2004). Aurora B is a member of a highly conserved family of Ser-Thr kinases that are key mitotic regulators (Carmena and Earnshaw, 2003). The other members of the complex regulate the kinase activity and target it to its different cellular substrates. INCENP binds Aurora B (Adams et al., 2000) through a highly conserved domain called the IN-BOX (Adams et al., 2001a; Adams et al., 2000; Honda et al., 2003). INCENP is phosphorylated by Aurora B and activates the kinase in a positive feedback loop (Bishop and Schumacher, 2002; Honda et al., 2003; Kang et al., 2001). Loss of INCENP function leads to mis-targeting and loss of kinase activity (Adams et al., 2001b). INCENP binds microtubules *in vitro* (Wheatley et al., 2001) and has a defined centromere-targeting

domain, thus it has been suggested to target Aurora kinase to subcellular locations at which its activity is required. In yeast, dephosphorylation of the INCENP homologue, Sli15, by Cdc14 is required for transfer of the complex to the central spindle (Pereira and Schiebel, 2003).

Much less is known about the roles of the chromosomal passenger proteins in meiosis. In *C. elegans* AIR-2/Aurora B localizes on chromatin distal to chiasmata in meiosis I , and in *air-2* RNAi embryos, the REC-8 on the distal region of the chromosomes remains undegraded and homologues cannot separate (Kaitna et al., 2002; Rogers et al., 2002). Because phosphorylation of Scc1 increases the efficiency of separase cleavage (Uhlmann et al., 2000), phosphorylation by AIR-2 was proposed to promote Rec8 degradation distal to chiasmata. Aurora B may also contribute, together with Plk1, to the release of cohesion between sister-chromatid arms in mitotic prophase/prometaphase (Gimenez-Abian et al., 2004; Losada et al., 2002).

We have used two Drosophila mutants in the INCENP protein to define the role of the chromosomal passenger complex in the specialized behavior of sister centromeres during meiosis, employing a system in which meiosis I chromosomes are naturally achiasmatic. During Drosophila male meiosis, homologous chromosomes pair but no synaptonemal complex is detected (Ault et al., 1982) and recombination is absent (Morgan, 1912). The effects of *incenp* mutants on meiosis, the localization of INCENP protein, and its effect on MEI-S332 localization, indicate that one function of the chromosomal passenger complex during Drosophila meiosis is to regulate MEI-S332 localization and protect centromeric chromatid cohesion during meiosis.

RESULTS

DmINCENP remains at the centromeres after the metaphase-anaphase transition in male meiosis I

The chromosomal passenger complex shows a characteristic distribution in mitosis (for a review see (Carmena and Earnshaw, 2003; Vagnarelli and Earnshaw, 2004). It associates with chromatin during prophase, concentrates at centromeres in prometaphase, then transfers to the central spindle at anaphase onset.

INCENP behavior in Drosophila male meiosis exhibits several notable features. During meiotic prometaphase I and metaphase I INCENP associated with chromatin and concentrated at centromeres as it does in mitosis (Figure 3-1A), however, at the transition to anaphase I INCENP remained primarily associated with the centromeres (Figure 3-1B). In early anaphase I, only low levels of INCENP were detected on the central spindle microtubules; later the centromeric signals became weaker and the protein spread over the chromosome arms. At this time, a subset of INCENP became associated with the central spindle (Figure 3-1B, arrow). INCENP remained associated with chromosomes through telophase I (data not shown).

During the second meiotic division INCENP again concentrated at centromeres through metaphase II, but then dispersed across the segregating chromatin at the onset of anaphase II (Figure 3-2A). The diffuse association with the chromosome arms in anaphase II was prominent relative to anaphase I. In addition, low levels of INCENP were associated with central spindle microtubules and with the cell cortex (Figure 3-2A, arrow).

Figure 3-1. INCENP protein localization and mutant defects in meiosis I.

(A) Wild-type metaphase I: INCENP concentrated on centromeres (arrow); (B) Wild-type anaphase I: INCENP on centromeres and some protein transfers to the central spindle (arrow); (C) *P(EP)2340* prometaphase I: abnormally condensed bivalents, an abnormally long and wavy spindle, decreased levels of centromeric INCENP; (D) Same as (C) but INCENP staining is undetectable; (E) *QA26* meiosis I: INCENP on centromeres and small segments of chromatin, the result of chromosome fragmentation or aberrant condensation. Scale bars are 5 μm.

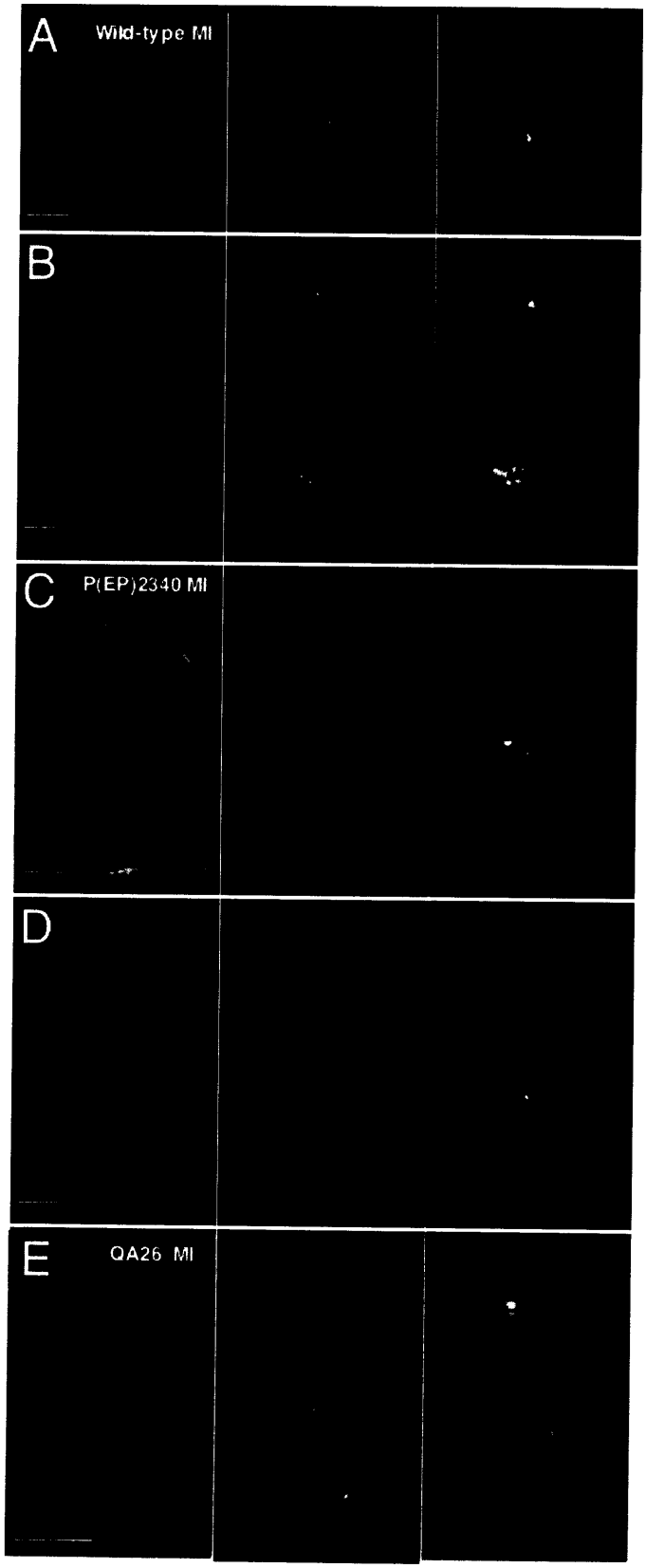

Figure 3-1.

Figure 3-2. INCENP protein localization and mutant defects in meiosis II.

(A) Wild-type anaphase II: INCENP associated with chromatin, central spindle and cell cortex (arrow); (B) *P(EP)2340* prometaphase II-like figure: elongated spindle, decreased levels of centromeric INCENP and chromatin masses aligned along the spindle; (C) Meiosis II spindle showing absence of INCENP staining and missegregation of chromosome *4*; (D) *QA26* prometaphase II-like figure showing absence of INCENP from some chromosomes and both copies of chromosome *4* at one pole. Scale bars are 5 μm.

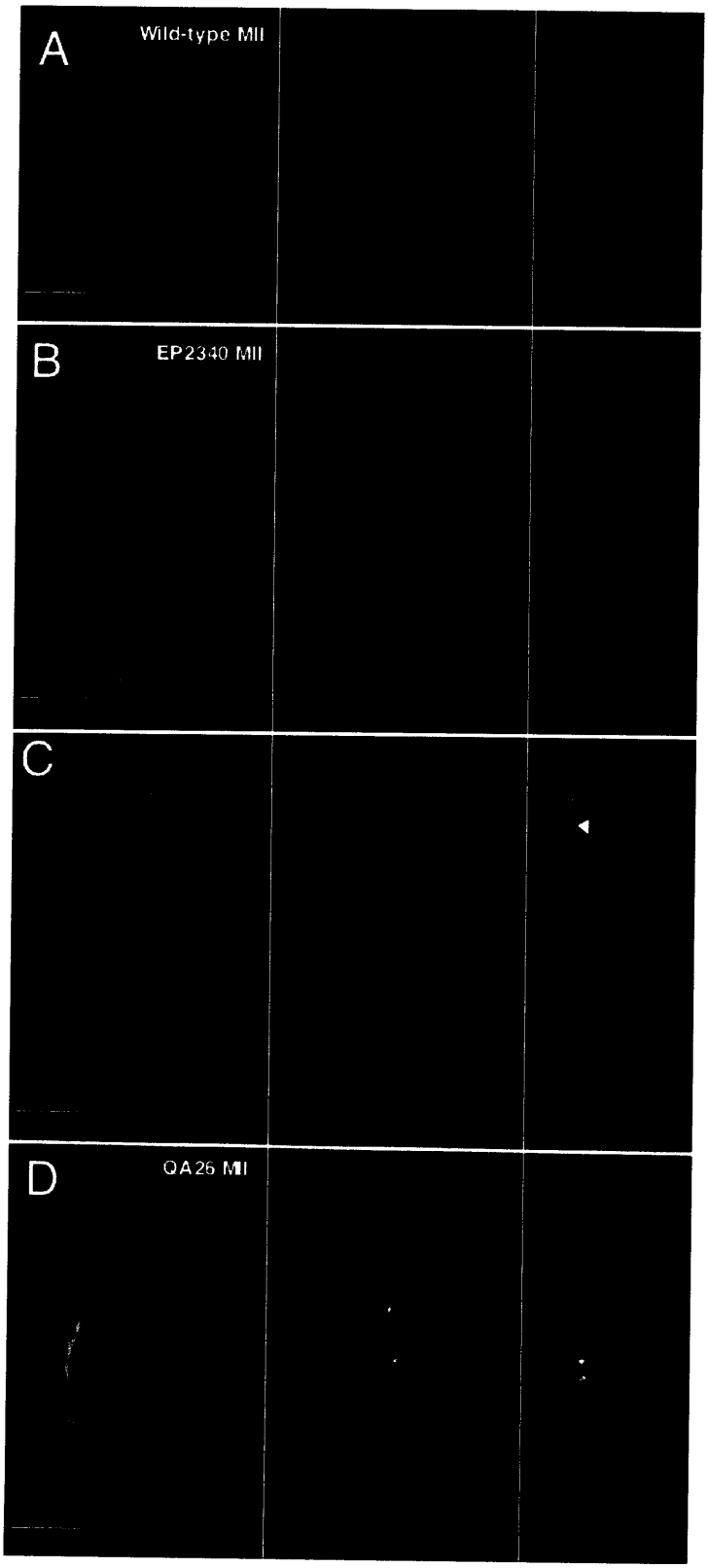

Figure 3-2.

INCENP persistence on centromeres after the metaphase-anaphase I transition parallels the preservation of centromeric sister-chromatid cohesion, consistent with a possible role for the chromosomal passenger complex in this process. Maintenance of INCENP at centromeres through anaphase I is also seen in mouse spermatocytes (Parra et al., 2003).

The female-sterile mutation *QA26* is located in *Dm-incenp*

The *QA26* allele was generated in a screen for female-sterile mutations and characterized as causing defects prior to cellularization of the embryo (Schupbach and Wieschaus, 1989). A combination of deficiency (see Materials and Methods) and P-element-induced male recombination mapping strategies (Chen et al., 1998) localized *QA26* to a region including 43 genes, one of which was *incenp* (Adams et al., 2001b). PCR-amplification and DNA sequencing from homozygous mutant genomic DNA revealed that *QA26* is a point mutation that converts aspartic acid 675 to valine in the highly conserved IN-BOX, which is essential for the interaction between INCENP and Aurora B (Adams et al., 2001a; Adams et al., 2000; Honda et al., 2003).

QA26 homozygotes are viable, likely because INCENP retains some function. *QA26* therefore provides an opportunity to examine the role of *incenp* in meiosis, which is not possible with stronger *incenp* alleles that prohibit development of adult flies (Chang et al., 2006). *QA26* mutant females completed meiosis without detectable defects, but there were aberrations in embryonic mitoses (data not shown). Homozygous mutant *QA26* males had reduced fertility, suggestive of defects in male meiosis.

In addition to the *QA26* allele, we made use of *P(EP)2340*, a P element insertion in the third exon of the *incenp* gene (Chang et al., 2006; Rorth, 1996; Tseng and Hariharan, 2002). Homozygous *P(EP)2340* individuals die late in embryogenesis (Chang et al., 2006; Tseng and Hariharan, 2002). When overexpressed in dividing cell populations in the eye or posterior of the wing, *P(EP)2340* results in a decrease in cell number and overall organ size (Tseng and Hariharan, 2002). Heterozygotes are viable but show reduced fertility; thus this allele had a potential dominant defect in meiosis. Genetic and molecular assays demonstrated that the P element specifically affects *incenp* and that its effects are not due solely to dosage reduction (Chang et al. 2006).

The *incenp* mutants show phenotypes consistent with disruption of chromosomal passenger function

In many *P(EP)2340* heterozygous or *QA26* homozygous spermatocytes, chromatin-associated INCENP protein was low or undetectable (Figure 3-1C, D). Quantification of the fluorescence intensity of centromeric INCENP signals revealed a four-fold decrease in *P(EP)2340* mutants relative to controls. Although INCENP localized normally in many *QA26* homozygotes meiosis I cells (Figure 3-1E), in other cells it was distributed along the chromosome arms in prometaphase I rather than being restricted to the centromeres (see Figure 3-5D). These effects were observed using two antibodies recognizing opposite ends of INCENP.

During meiosis I in both *incenp* mutants, we observed a variety of defects, including cells in which unaligned chromosomes were distributed along the spindle (Figure 3-1C-E), and others in which the four bivalents were not distinguishable or the chromosome

morphology was abnormal (Figure 3-1D). In *QA26* we saw small bits of chromatin that could be fragmented or aberrantly hypocondensed chromosomes (Figure 3-1E). In addition, we observed cells with more than four chromosomal masses in prometaphase I in both mutants. These extra chromosomal masses are likely due to failure in chromosome pairing or sister-chromatid cohesion in meiosis. Only a low level of premeiotic defects were observed by orcein stain and phase contrast in *QA26* mutants and therefore premeiotic disruption is not sufficient to explain the meiotic phenotypes (see below and data not shown). In meiosis II of both mutants we observed chromatids randomly distributed along the spindle and unequal chromatid segregation (Figure 3-2B-D), including asymmetric segregation of chromosome four as shown in Figure 3-2C.

Spermatocytes from both mutants also showed a range of defects in central spindle formation in anaphase and telophase of meiosis I and II (data not shown). Pavarotti-KLP (Pav), a kinesin-like protein related to MKLP1 and required for central spindle stability (Adams et al., 1998), was absent or present at low levels on the central spindle in the mutant spermatocytes (data not shown).

These results demonstrate that INCENP function is required during Drosophila male meiosis for chromosome condensation, chromosome segregation, and central spindle organization. These phenotypes in *incenp* mutant spermatocytes are consistent with the mitotic phenotypes from depletion of INCENP or Aurora B in Drosophila S2 cells (Adams et al., 2001b; Giet and Glover, 2001). We pursued the possible role of INCENP in meiotic cohesion by further quantitative cytological and genetic analyses of the chromosomal phenotype in *QA26* male meiosis.

Disruption of *incenp* function leads to premature loss of sister-chromatid cohesion in meiosis

Quantitative genetic analysis revealed that both *incenp* mutants underwent significantly increased levels of chromosome nondisjunction during meiosis. By crossing *QA26* males to attached-*X* females we detected progeny generated by nondisjunction in meiosis I (both *X* and *Y* chromosomes from the father) and those from nondisjunction during meiosis II (with two paternal *X* chromosomes). We also recovered progeny from sperm lacking sex chromosomes, which could have experienced nondisjunction in either meiotic division, as well as progeny from *XXY* sperm, which must have undergone nondisjunction in both divisions.

For 1,516 progeny from *QA26* males the rate of total nondisjunction was 15.8%, a 26-fold increase over the 0.6% nondisjunction measured for *QA26/SM1* heterozygous siblings (Figure 3-3A). The recovery of exceptional sperm could result either from a failure of the sex chromosomes to disjoin or from premature separation of the sister chromatids followed by random segregation to the poles. The cytology of *QA26* chromosomes supports precocious separation as one mechanism by which nondisjunction arises in this mutant (see below), but other effects may also be present.

The *P(EP)2340* allele was tested for dominant effects on meiotic segregation. We performed crosses and counted progeny to score nondisjunction of the sex, second, fourth chromosomes. Each of these tests revealed significantly higher rates of nondisjunction in *P(EP)2340* heterozygotes than in controls (data not shown).

Figure 3-3. Meiotic chromosome cohesion and condensation defects in *incenp* mutants. (A) Mutations in *incenp* cause elevated nondisjunction during meiotic chromosome segregation. (B) defective prophase I chromosome condensation in a *QA26* mutant spermatocyte; (C) *QA26* mutant prophase I: chromatid arms protrude from loosely packed bivalents (arrow). Inset shows wild-type prophase I bivalent configuration; (D) wild-type anaphase I: at one pole, one major autosome (arrow) and the *X* chromosome (arrowhead) attached at their centromeres are indicated. This cohesion configuration is retained into prometaphase II; (E) anaphase I *QA26* mutant: sister chromatids of all dyads at one pole (arrow) are precociously separated. (F) prometaphase II *QA26* spermatocyte: sister chromatids of one dyad have lost cohesion (arrow) rather than retaining cohesion at the centromere as seen in the adjacent dyad (arrowhead); (G) prometaphase II *QA26* mutant cell with aneuploid number of chromosomes, most likely the result of meiosis I nondisjunction. Scale bars are 10 μm.

A

Sex chromosome nondisjunction in *QA26* males

	Regular sperm		Exceptional sperm				Total exceptional progeny (% exceptional progeny)	Total progeny
	X	Y(Y)	O (% Nullo-XY)	XY(Y) (% XY(Y))	XX (% XX)	XXY(Y) (% XXY(Y))		
QA26	734	543	179 (11.8%)	41 (2.7%)	17 (1.1%)	2 (0.1%)	241 (15.9%)	1516
QA26/SM1	405	201	3 (0.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	3 (0.6%)	509

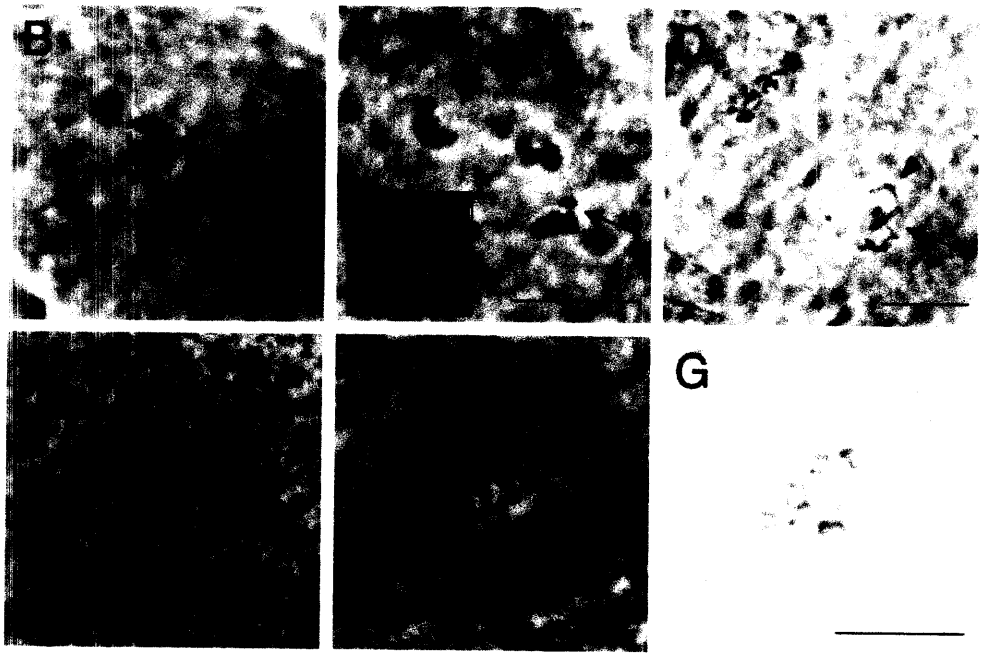

Figure 3-3.

Genetic evidence of nondisjunction in *incenp* mutants was confirmed cytologically by orcein staining of *QA26* homozygous spermatocytes. This staining revealed abnormalities in both chromosome morphology and number (Figure 3-3). Prophase I figures with loosely packed and minimally condensed chromosomes were observed in 34% of *QA26* spermatocytes as compared to 14% of wild type (Figure 3-3B, C; 380 *QA26* and 130 wild-type spermatocytes scored).

In addition to defects in chromosome condensation, *QA26* spermatocytes displayed premature loss of sister-chromatid cohesion. This was suggested by prometaphase I bivalents compacted into blobs but with protruding arms with the appearance of single sister chromatids (Figure 3-3C). These figures were strikingly reminiscent of those present in *ord* mutants in which sister-chromatid cohesion is prematurely released in prophase I (Miyazaki and Orr-Weaver, 1992) and contrast with the tight packing of the bivalents normally seen in wild type (Figure 3-3C, inset).

Premature loss of cohesion was unambiguous in anaphase I *QA26* spermatocytes, where completely separated sisters were present at the poles (Figure 3-3D, E). Prometaphase II cells with separated sister chromatids were also observed (Figure 3-3F). Of those spermatocytes in which the chromosome arrangement permitted cohesion to be assessed, 34% of *QA26* mutants had precociously separated sister chromatids (Figure 3-3F), compared to 7.8% in wild type (90 *QA26* and 65 wild type scored). These data reveal that the centromeric cohesion that normally holds sisters together until the onset of anaphase II is lost prematurely in the *QA26* mutant. The defects in chromosome

condensation and sister-chromatid cohesion likely led to missegregation, as aneuploid meiosis II spermatocytes were present (Figure 3-3G).

Together, this genetic and cytological analysis demonstrates that *incenp* plays a critical role in Drosophila male meiosis, and is required for proper chromosome segregation in both the reductional and equational divisions.

INCENP/Aurora B functions are required for normal centromeric MEI-S332 localization in mitosis

The cytological and genetic analyses together reveal a requirement for INCENP function for cohesion at sister-chromatid centromeres. We explored whether INCENP might affect the localization or function of MEI-S332, a member of the Shugoshin family of proteins required for the maintenance of sister-chromatid cohesion in meiosis I (Lee et al. 2005). In both mitotic and meiotic chromosomes MEI-S332 localizes within the functional centromere, (Blower and Karpen, 2001; Lee et al., 2004; Lopez et al., 2000), where it contributes to sister-chromatid cohesion. In mitosis, this role of MEI-S332 is not essential (LeBlanc et al., 1999; Lee et al., 2005).

On mitotic chromosomes, INCENP/Aurora B and MEI-S332 exhibited an overlapping distribution, but did not coincide completely (Figure 3-4C, F). INCENP/Aurora B were enriched in the heterochromatin beneath kinetochores, whereas MEI-S332 appeared closer to the kinetochores.

To test whether INCENP is required for localization of MEI-S332 in mitosis, we used RNAi to deplete INCENP in S2 cells. 48 hours after addition of INCENP dsRNA, cultures exhibited a prometaphase delay, as described following depletion of INCENP or

Figure 3-4. Loss of INCENP/Aurora B in mitosis correlates with delocalization of MEI-S332.

(A) Distribution of mitotic phases in DmINCENP RNAi (red bar), DmAurora B RNAi (yellow bar), and control (blue bar) shows a reduction of the percentage of metaphases and an increase in the percentage of prometaphases (t=48 hours after addition of dsRNA); the percentage of abnormal anaphase cells is shown separately (right). (B) Analysis of the colocalization of MEI-S332 with INCENP in control and INCENP RNAi treated cells (upper panel) and with Aurora B in AuroraB RNAi treated cells (lower panel) (t=48 hours) n>300. (C) Localization of MEI-S332 and INCENP in S2 cells. Zoomed image at right shows both proteins overlap partially but INCENP extends beneath MEI-S332. (D) INCENP dsRNA-treated S2 cells showing unaligned chromosomes. INCENP and MEI-S332 are present on most centromeres. Arrow points to a chromosome in which both proteins are absent; (E) INCENP dsRNA treated S2 cells showing absence of both INCENP and MEI-S332 (arrow). Scale bars are 5 μm. (F-I) Aurora B dsRNA treated S2 cells. (F) Control metaphase cell; zoomed image shows partial colocalization of Aurora B and MEI-S332 on centromeres. (G) Prometaphase cell with decondensed chromosomes showing absence of both Aurora B and MEI-S332 from centromeres. (H) Prometaphase cell showing INCENP and MEI-S332 on some centromeres; arrow points to unaligned chromosome showing low levels of INCENP and undetectable MEI-S332. (I) Prometaphase cell showing abnormal INCENP localization on chromatin and dispersed MEI-S332 staining.

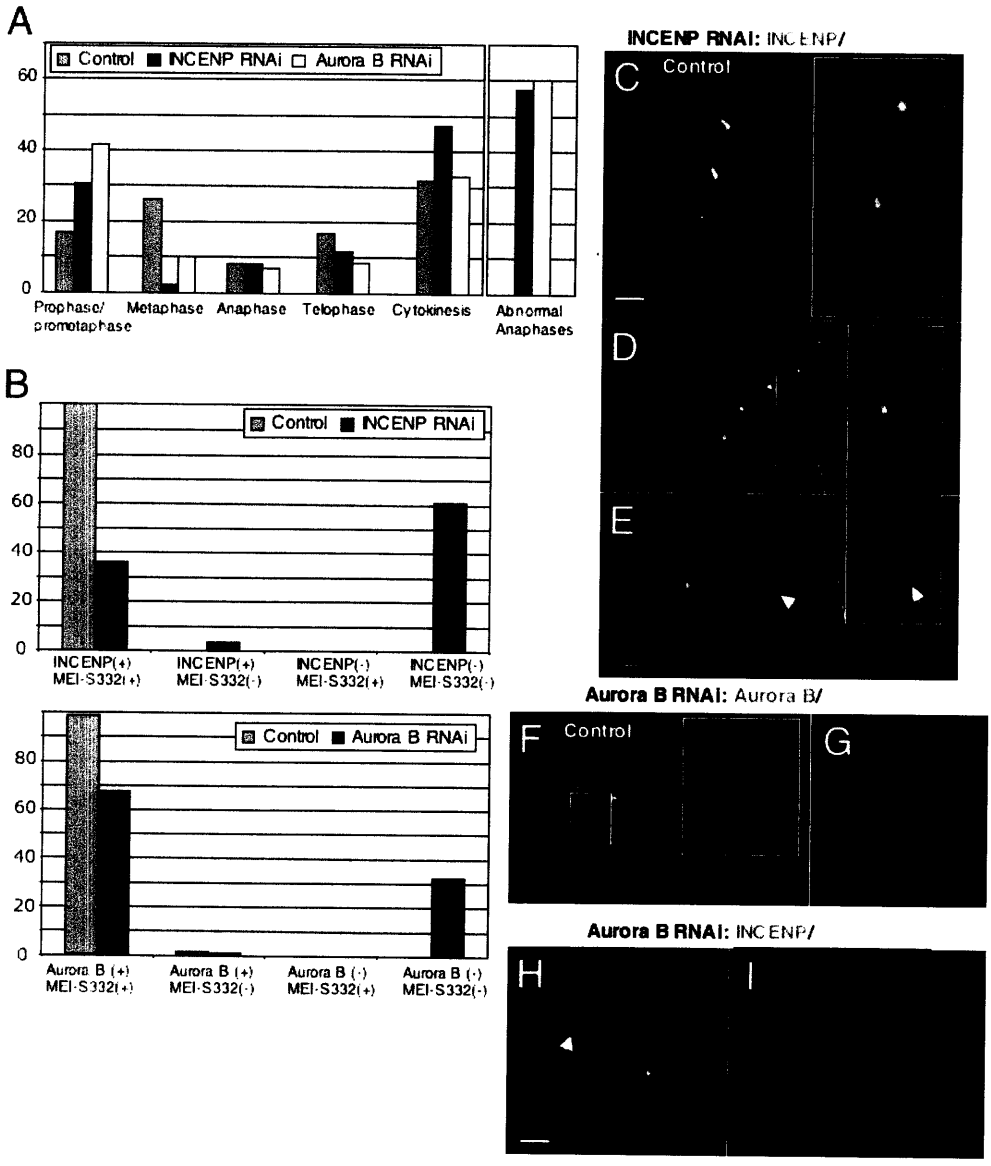
A
Control
INCENP RNAi
Aurora B RNAi
60
40
20
0
Prophase/ prometaphase
Metaphase
Anaphase
Telophase
Cytokinesis
Abnormal Anaphases
B
Control
INCENP RNAi
80
60
40
20
0
INCENP(+) MEI-S332(+)
INCENP(+) MEI-S332(-)
INCENP(-) MEI-S332(+)
INCENP(-) MEI-S332(-)
Control
Aurora B RNAi
80
60
40
20
0
Aurora B (+) MEI-S332(+)
Aurora B (+) MEI-S332(-)
Aurora B (-) MEI-S332(+)
Aurora B (-) MEI-S332(-)
INCENP RNAi: INCENP/
C
Control
D
E
Aurora B RNAi: Aurora B/
F
Control
G
Aurora B RNAi: INCENP/
H
I

Figure 3-4.

Aurora B (Figure 3-4A (Adams et al., 2001b). At this time point 61% of mitotic cells in prometaphase/metaphase showed no INCENP staining, while 39% retained some INCENP staining (Figure 3-4B, upper panel).

We exploited the variable penetrance of the RNAi phenotype to examine the dependency of MEI-S332 centromeric localization on INCENP. We found that every mitotic figure with normal INCENP staining at centromeres was also positive for MEI-S332 staining, and every cell without INCENP staining at centromeres also lacked MEI-S332 (Figure 3-4B, upper panel; Figure 3-4E). A small percentage of mitoses (3.6%) showed extremely decondensed chromosomes with very low levels of INCENP and undetectable MEI-S332 (Figure 3-4B, upper panel). Occasionally an INCENP-positive cell contained individual chromosomes with low or undetectable INCENP signal at centromeres. These chromosomes showed low or undetectable levels of MEI-S332 (Figure 3-4D).

To test further for roles of the passenger complex in MEI-S332 localization we examined Aurora B-depleted cells. Again, in cells with normal Aurora B kinetochore staining, MEI-S332 was localized on centromeres. In cells with undetectable levels of Aurora B, MEI-S332 was aberrantly dispersed around the chromatin and in the cytoplasm (Figure 3-4B lower panel, 3-4G). Similar to the INCENP depletion, we observed cells in which the levels of both INCENP and MEI-S332 (Figure 3-4H) or Aurora B and MEI-S332 (data not shown) were low or undetectable only in a subset of chromosomes. As we reported previously (Adams et al, 2001b), in S2 cells in which Aurora B is depleted INCENP associates with chromatin on entry into mitosis but fails to concentrate on the

centromeres during prometaphase/metaphase (Figure 3-4I). In these cells, MEI-S332 also failed to localize normally to centromeres (Figure 3-4I).

These experiments indicate that INCENP and/or Aurora B function is required for the stable localization of MEI-S332 at centromeres in mitosis. They also suggest that Aurora B phosphorylation of INCENP or MEI-S332 could contribute to maintaining MEI-S332 on centromeres.

INCENP is required for normal MEI-S332 localization at centromeres in meiosis

MEI-S332 is essential for proper chromosome segregation in meiosis, thus we next investigated the distribution of INCENP and MEI-S332 during meiosis in wild-type and *incenp* mutant flies. INCENP and MEI-S332 colocalized at centromeres during wild-type male meiosis (Figure 3-5A-C). In metaphase I, INCENP partially overlapped and linked the two sister kinetochore-associated MEI-S332 dots. Early in anaphase I the two proteins appeared largely to overlap, but late in anaphase I INCENP was concentrated in the heterochromatin linking sister kinetochores, and only partially overlapping with MEI-S332 (Figure 3-5C, inset). This is reminiscent of the relative distributions of INCENP and the Aurora B substrate MCAK during mitosis in mammalian cells.

In *QA26* homozygous males we observed prometaphase I-like figures in which both MEI-S332 and INCENP proteins were not restricted to the centromere, but were dispersed along the chromosome arms (Figure 3-5D). This phenotype is consistent with defective interactions between INCENP and Aurora B (Adams et al., 2001b). In addition, we commonly observed meiotic figures in both *QA26* homozygotes and *P(EP)2340* heterozygous males in which MEI-S332 and INCENP were absent or reduced on one or

Figure 3-5. Localization of INCENP and MEI-S332 is disrupted in *QA26* meiosis.

(A) Wild-type metaphase I: INCENP and MEI-S332 on centromeres. Inset shows INCENP staining partially overlapping and linking the two sister kinetochore-associated MEI-S332 dots. (B) Wild-type early anaphase I; INCENP and MEI-S332 remain associated with the centromeres. Inset shows the overlap in localization of the proteins. Arrow points to the absence of INCENP staining on central spindle. (C) Wild-type late anaphase I: INCENP remains associated with centromeres, but some protein is associated with chromatin and central spindle. (D) *QA26* meiosis I spermatocyte in which INCENP and MEI-S332 were distributed diffusely on the chromosomes. (E) *QA26* anaphase I in which both INCENP and MEI-S332 were absent from the chromosomes. Scale bars are 5 μm.

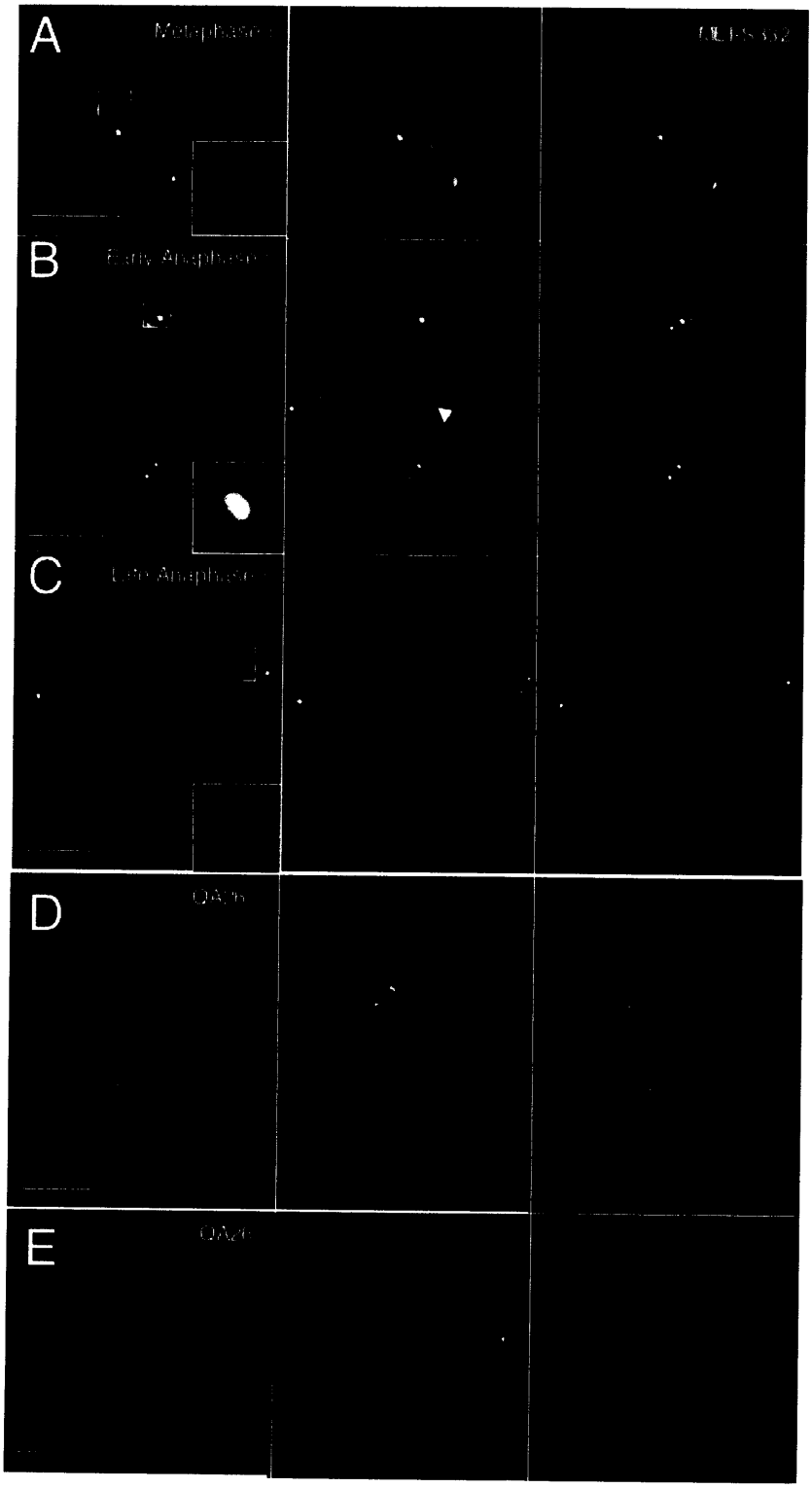

Figure 3-5.

more chromosomes (Figure 3-5E). This was particularly evident in mutant anaphase I cells (Figure 3-5E), and contrasts with wild-type cells, in which both proteins persist at centromeres until anaphase II (data not shown). Together, these observations are consistent with INCENP being necessary for stable MEI-S332 localization at centromeres.

To determine whether INCENP and MEI-S332 have a mutual requirement for proper localization, we examined INCENP distribution in *mei-S332*^{4} spermatocytes. In each meiotic division INCENP was found specifically at the centromeres in metaphase, and in anaphase it spread across the chromosomes and a pool transferred to the spindle (data not shown). Because *mei-S332*^{4} flies do not have detectable levels of MEI-S332 protein (Tang et al., 1998), we conclude that INCENP localization does not require MEI-S332.

MEI-S332 associates directly with DmINCENP *in vitro*

To elucidate the mechanism underlying the interaction between INCENP and MEI-S332, we next investigated whether MEI-S332 was able to bind DmINCENP *in vitro*. Bacterially expressed GST-INCENP was assayed for binding to *in vitro*-translated MEI-S332, DmAurora B, or a mixture of both proteins (Figure 3-6 A-B). GST-DmINCENP interacted directly with MEI-S332 (Figure 3-6A) and binding of MEI-S332 was increased in the presence of DmAurora B (i.e. active kinase complex) (Figure 3-6B).

MEI-S332 is phosphorylated by Aurora-B *in vitro*

MEI-S332 is an excellent *in vitro* substrate of Aurora B kinase. When GST-MEI-S332 was incubated with recombinant bacterially-expressed Xenopus Aurora B/INCENP, it was phosphorylated at levels comparable to a strong test substrate, myelin basic protein

Figure 3-6. INCENP binds MEI-S332 *in vitro*. (A) Proteins were translated in the presence of [^{35}S]-methionine and incubated with bacterially expressed GST-DmINCENP or GST bound to glutathione sepharose beads. Bound ("B") and unbound ("U") fractions were separated by SDS-PAGE, and proteins were visualized using a phosphorimager. (B) Quantification of the binding experiment shown in A. The bars represent the percentage of total protein bound to or GST (black) or GST-DmINCENP (grey).

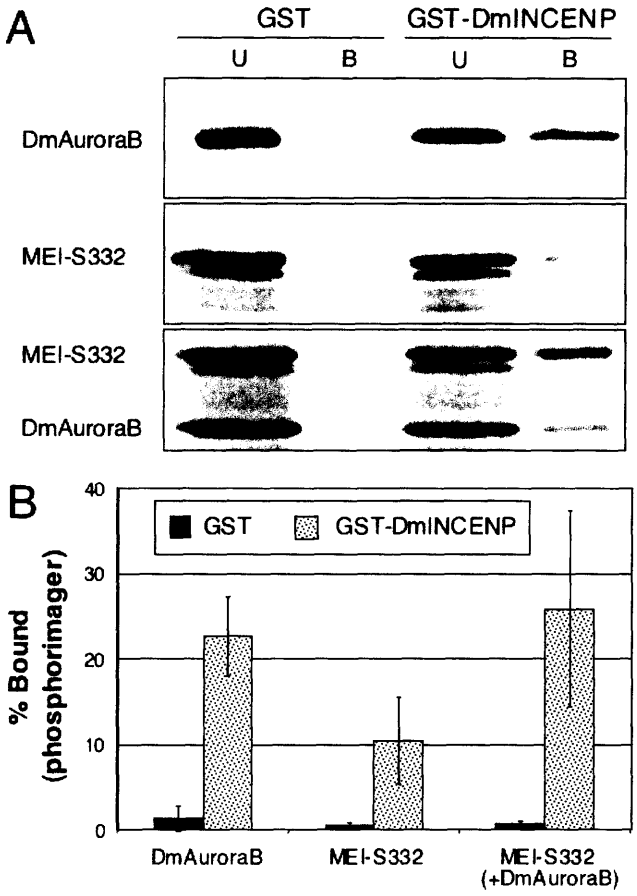
A
GST
GST-DmINCENP
U
B
U
B
DmAuroraB
MEI-S332
MEI-S332
DmAuroraB
B
GST
GST-DmINCENP
% Bound
(phosphorimager)
40
30
20
10
0
DmAuroraB
MEI-S332
MEI-S332
(+DmAuroraB)

Figure 3-6.

(MBP) (Figure 3-7A). Moreover, at similar concentrations MEI-S332 could compete label away from MBP (Figure 3-7A). In general, *bona fide in vivo* substrates such as MCAK, MKLP1 and INCENP, are phosphorylated by Aurora B/INCENP as efficiently as MBP (PTS data not shown).

Three regions of MEI-S332 contain putative Aurora B consensus sites (RX S/T). Each site includes two or more consecutive serine residues (98S 99S, 124S 125S 126S, 138S 139S). Three mutant MEI-S332 proteins in which the consecutive serines in each putative site were mutated to alanines were engineered and purified from *E. coli* . Of these, MEI-S332^{S124,125,126A} was a poor substrate for Aurora B kinase *in vitro* when compared to MEI-S332^{WT}, MEI-S332^{S98,99A} or MEI-S332^{S138,139A} (Figure 3-7B). Because residues 124-126 constitute the only Aurora B target site that diminishes phosphorylation when mutated, we conclude that Aurora B most likely phosphorylates MEI-S332 within these residues.

MEI-S332-124AAA phosphorylation mutant does not stably associate with centromeres in mitosis.

To analyze the role of Aurora B phosphorylation of MEI-S332 *in vivo*, we studied the behavior of the GFP-tagged MEI-S332^{S124,125,126A} phosphorylation mutant (MEI-S332-124AAA) in transiently transfected S2 cells. We found high levels of centromeric wild-type GFP-MEI-S332 (Figure 3-7C) in 94% of prometaphase/metaphase cells (Figure 3-7F; n >400 per experiment). In contrast, only 33.3% of prometaphase/metaphase cells showed high levels of GFP- MEI-S332-124AAA mutant protein at centromeres. 66% of cells expressing this mutant version showed reduced signal at centromeres (Figure 3-7D-E, arrow; Figure 3-7F). Quantification of fluorescence intensity showed that kinetochores in

Figure 3-7. Aurora B phosphorylates MEI-S332 and regulates its stable association with centromeres in mitosis.

(A-B) Aurora B/INCENP phosphorylates MEI-S332 *in vitro* within residues 124-126. (A) Recombinant Aurora B/INCENP complex was incubated with ^{32}P-ATP and the indicated substrate for 1 minute and incorporation of phosphate onto the proteins was visualized by autoradiography (right) and protein loading analyzed by Coomassie Blue (left). MBP-Myelin Basic Protein, GST-Glutathione S-transferase. (B) Time course of Aurora B/INCENP kinase activity (assayed as in A) using WT MEI-S332 or the indicated phospho-site mutant.

(C-F) MEI-S332-124AAA phosphorylation mutant does not stably associate with centromeres in mitosis. (C) High level of centromeric GFP-MEI-S332 in metaphase. (D)Reduced level of the phosphorylation mutant GFP-MEI-S332-124AAA on metaphase centromeres (arrow). (E) Microscope field showing a prometaphase cell with high levels of centromeric GFP- MEI-S332-124AAA and a metaphase cell with very reduced levels of mutant protein in most centromeres (arrow). In C-E the GFP staining alone is shown in gray. (F) Percentage of cells transfected with GFP-MEI-S332 or GFP-MEI-S332-124AAA showing normal levels of GFP signal on kinetochores (HIGH), lower than normal (LOW) or no signal (NEGATIVE). (G) Model of the role of INCENP in the regulation of MEI-S332 in meiosis.

a) Meiotic chromosome dynamics and the localization patterns of key regulatory proteins INCENP/Aurora B (blue), MEI-S332 (green), and POLO (red). b) Protein interactions at the kinetochore. In prophase I, CDK phosphorylates INCENP at the POLO binding site, promoting the targeting of POLO kinase to the kinetochore; INCENP targets Aurora B to

the kinetochore; MEI-S332 is recruited to the kinetochore. Before the metaphase-anaphase I transition, Aurora B initiates its autoactivation backloop, phosphorylating INCENP and itself. The INCENP/Aurora B complex stabilizes centromeric MEI-S332 through direct binding and phosphorylation. At the onset of anaphase I, INCENP stays on the centromere, stopping MEI-S332 from being phosphorylated by POLO, and POLO transfers to the central spindle. During the metaphase-anaphase II transition INCENP transitions off the centromere, redistributing over chromatin and transferring to the central spindle. POLO is free to phosphorylate MEI-S332, promoting its release from centromeres. POLO then transfers to the central spindle.

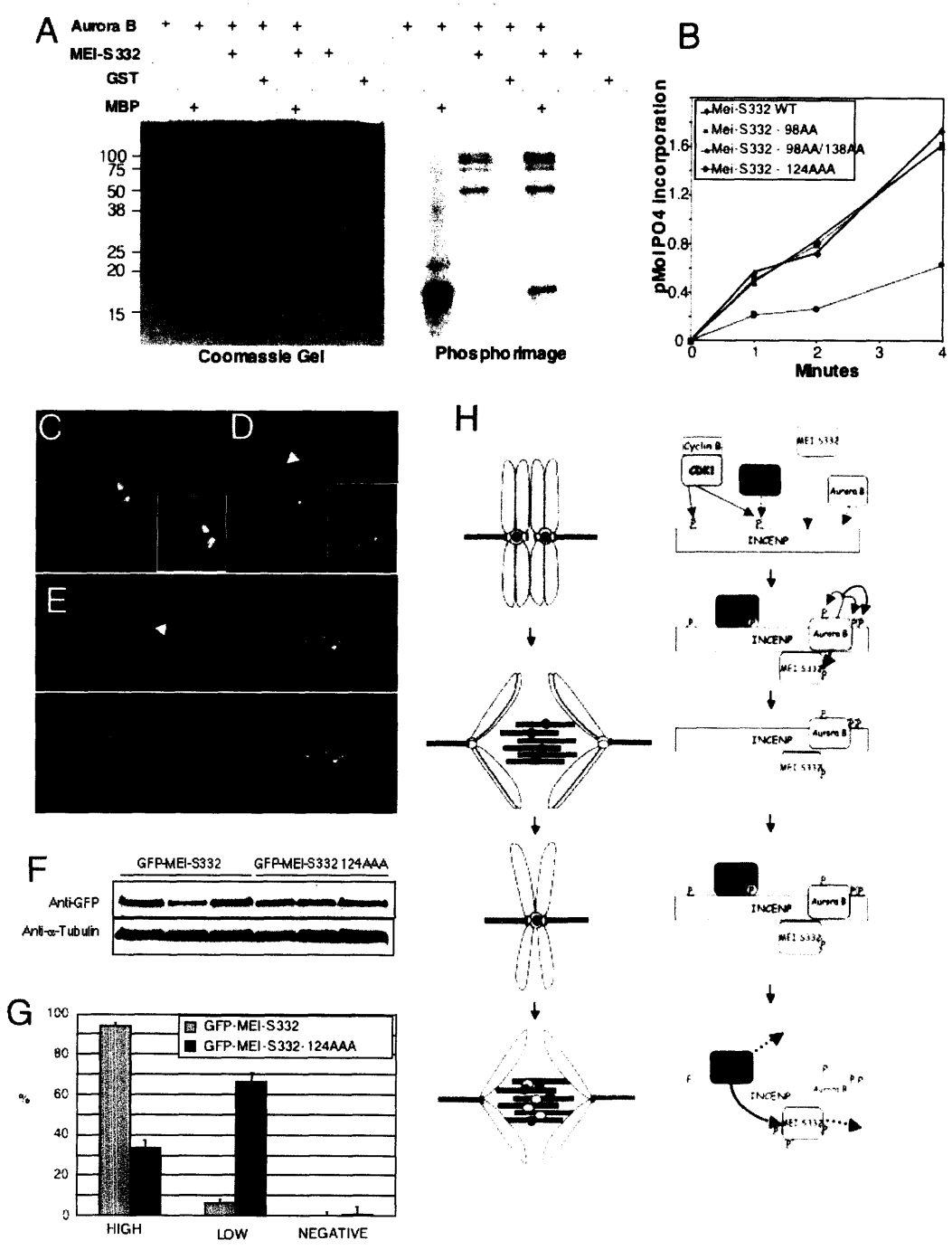
A
Aurora B
MEI-S332
GST
MBP
100
75
50
38
25
20
15
Coomassie Gel
Phosphorimage
B
Mei-S332 WT
Mei-S332 - 98AA
Mei-S332 - 98AA/138AA
Mei-S332 - 124AAA
pMol PO4 incorporation
Minutes
C
D
E
F
GFP-MEI-S332
GFP-MEI-S332 124AAA
Anti-GFP
Anti-α-Tubulin
G
GFP-MEI-S332
GFP-MEI-S332-124AAA
%
HIGH
LOW
NEGATIVE
H
INCENP

Figure 3-7.

cells with high levels of mutant MEI-S332 have a similar level to wild type, whereas kinetochores with lower levels of mutant protein show up to a fifteen-fold reduction in fluorescence (data not shown).

DISCUSSION

This analysis of Drosophila *incenp* mutants reveals for the first time a crucial role for INCENP in regulating centromeric cohesion during the reductional division of meiosis. INCENP influences the localization and/or function of MEI-S332: precocious sister-chromatid separation is observed at the centromeres in the mutants, the distribution of MEI-S332 is abnormal when INCENP levels are decreased, INCENP can bind MEI-S332 *in vitro*, the protein is phosphorylated *in vitro* by Aurora B, and MEI-S332 localization to centromeres in mitosis is perturbed when its preferred Aurora B phosphorylation site is mutated.

incenp mutations affect sister-chromatid cohesion, chromosome condensation and cause nondisjunction in Drosophila male meiosis

The *QA26 incenp* mutation perturbs chromosome condensation and causes precocious separation of the sister chromatids in spermatocytes. Quantitative genetic nondisjunction tests showed that chromosome segregation fails in both meiosis I and II, and these nondisjunction events are consistent with premature separation of sister chromatids and random segregation in both meiotic divisions. This genetic analysis is likely to underestimate the true rates of nondisjunction because many of the defects caused by loss of passenger function (e.g. defective spindle organization or cytokinesis) would not yield functional gametes, thereby preventing us from scoring all of the nondisjunction events. Although the aberrant condensation in prophase and prometaphase I made direct visualization of the onset of loss of cohesion difficult, completely separated sister

chromatids could unambiguously be seen in mutant anaphase I cells, confirming one mechanism that contributes to the genetic nondisjunction phenotype.

In *C. elegans* meiosis, the chromosome passenger complex is necessary for chiasma resolution (Kaitna et al., 2002; Rogers et al., 2002). If chromosomal passengers were to participate both in regulation of centromeric cohesion as well as processing chiasmata in *C. elegans*, essential roles in the latter might obscure roles in the former. In Drosophila male meiosis there is no synapsis of homologues or recombination. Rather, segregation of homologous chromosomes is regulated via specific pairing sites (McKee, 2004). The analysis of passenger function was therefore simplified in Drosophila males, where chiasmata do not form.

INCENP is required for the stable localization of MEI-S332 protein to centromeres in mitosis and meiosis

The MEI-S332-related yeast Shugoshin proteins are critical for maintenance of the meiotic-specific cohesin subunit Rec8 at centromeres during anaphase I (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004).
Interestingly, no Rec8 homologue has yet been found in Drosophila. The only Drosophila meiotic kleisin, C(2)M, is a component of the synaptonemal complex (Anderson et al., 2005; Manheim and McKim, 2003) and has been shown to have an earlier role in female (Heidmann et al., 2004) and male meiosis (MC, unpublished observations). Thus, what MEI-S332 protects at centromeres in meiosis remains unclear. In mitosis, ablation of MEI-S332 does not lead to premature loss of the mitotic cohesin Rad21 from centromeres (Lee et al., 2005).

In both *incenp* mutants, impaired INCENP function results in failure of MEI-S332 localization to centromeres in meiosis. This presumably leads to defects in the protection of cohesion at sister centromeres and contributes to the observed increase in meiotic nondisjunction. The failure to localize MEI-S332 in the *incenp* mutants is not a general secondary effect of prophase I condensation defects or of premature sister-chromatid separation prior to the onset of anaphase I: *ord* mutants, which display both of those phenotypes, localize MEI-S332 normally (Bickel et al 1998). Although our data support a role for MEI-S332 in the increased nondisjunction in *incenp* mutants, *mei-S332* mutants give predominantly meiosis II nondisjunction whereas the *incenp* alleles show defects in both meiotic divisions. Thus INCENP must be required for additional functions beyond its role in MEI-S332 regulation described here.

Potential roles for INCENP in regulating MEI-S332 function

One mechanism by which INCENP could promote MEI-S332 function is through its role in establishing or maintaining the specialized chromatin structure around centromeres. The chromosomal passenger complex is involved in regulation of chromatin remodelling complexes like ISWI, (MacCallum et al., 2002) and interacts with histone and non-histone proteins from the pericentric heterochromatin (Ainsztein et al., 1998; Rangasamy et al., 2003). Recent studies show a direct link between Aurora B activity and regulation of HP1 localization in mitosis (Fischle et al., 2005; Hirota et al., 2005), suggesting a possible role in the regulation of heterochromatin structure. Since heterochromatin is important for cohesin binding to centromeres (Bernard et al., 2001; Nonaka et al., 2002), it is possible

that modifications of both MEI-S332 and the underlying heterochromatin are important for stabilizing centromeric cohesion during meiosis I.

Alternatively, INCENP could act as a platform for regulation of MEI-S332 at centromeres. The direct binding between INCENP and MEI-S332 could target MEI-S332 to heterochromatin, or it could help to direct its regulation by protein kinases. MEI-S332 binds better *in vitro* to a mixture of INCENP and Aurora B than to INCENP alone, suggesting that the interaction is strengthened by phosphorylation of either INCENP or MEI-S332. In addition to its role in binding and activating Aurora B, INCENP that has been phosphorylated by CDK1 can bind to Plk1, the human homologue of POLO kinase. This binding is required to target Plk1 to centromeres in mitosis. (Goto et al., 2005) Thus, INCENP is suitably placed to coordinate the functions of POLO and Aurora B. The relationship of the regulation of these kinases is of particular interest as both Aurora B and POLO regulate cohesin and MEI-S332. These kinases have been shown to cooperate in the release of arm cohesion in chromosomes assembled in Xenopus extracts (Losada et al., 2002).In contrast to Aurora B, however, POLO is needed for the dissociation of MEI-S332 from centromeres during mitosis and meiosis (Clarke et al., 2005). In *polo* mutants MEI-S332 persists on the centromere and mutation of two POLO box domains disrupts POLO binding and phosphorylation of MEI-S332 *in vitro*, as well as MEI-S332 dissociation from the centromeres.

Together, these observations suggest that INCENP may act to integrate the various pathways controlling MEI-S332 function in meiosis I (Figure 3-7G). Early in meiosis I, INCENP/Aurora B complexes may stabilize centromeric MEI-S332 through direct binding or modification of the underlying chromatin as described above. Similar to what happens in

mitosis, CDK1 could phosphorylate INCENP at the POLO binding site and phosphorylation-dependent binding of POLO to INCENP could target the kinase to the centromere (Goto et al., 2005). This binding might also sequester the kinase so that it is unavailable to phosphorylate MEI-S332. During the metaphase-anaphase I transition, INCENP remains on the centromeres and might stop MEI-S332 from being phosphorylated by POLO. At the onset of anaphase II, however, as INCENP transitions off the centromere POLO may be free to phosphorylate MEI-S332, thereby releasing it from centromeres. This would then allow the release of sister-chromatid cohesion.

INCENP is emerging as a key regulator of kinase signalling pathways in mitosis. The present study reveals that this versatile protein may have a similar role in meiosis, using its interactions with Aurora B and POLO to coordinate the specialized behavior of sister chromatids in meiosis I.

MATERIALS AND METHODS

Drosophila Strains and *QA26* mapping

All flies were reared in standard Drosophila medium. Unless otherwise stated, crosses were performed at 25°C, and parental flies transferred every 3-4 days to account for any age-related effect on the phenotype studied. Many Drosophila strains were obtained from the Bloomington Stock Centre. The deficiencies *Df(2R)Drl-rv25, Df(2R)Drl-rv17, Df(2R)Drl-rv3, Df(2R)Drl-rv18, D(2R)pk78k, Df(2R)cn87e* failed to complement *QA26*. We used EP insertion elements to map *QA26* more precisely and found the mutation *QA26* is located to the right of *EP(2)2052* and *EP(2)2336* and to the left of *EP(2)2475*.

Meiotic Chromosome Nondisjunction Tests with *QA26*

Sex chromosome nondisjunction in *incenp* mutant males was quantified by crossing to females bearing attached *X* chromosomes, as described previously (Kerrebrock et al., 1992). *yw/y⁺Y; QA26/QA26* (experiment) or *yw/y⁺Y; QA26/SM1* (control) males were crossed to attached-*X* females, of genotype *C(1)RM, y^2 su(w^a)w^a Y^SX•Y^L, In(1)EN y^+ v f B.* The following progeny were scored as resulting from nondisjunction of the sex chromosomes: y^2 su(w^a)w^a females (resulting from nondisjunction in either meiosis I or meiosis II), yw females (resulting from nondisjunction in meiosis II), y^+w males (resulting from nondisjunction in meiosis I), y^+w females (resulting from nondisjunction in both meiosis I and II). Percent nondisjunction was calculated by dividing the number of progeny resulting from sperm having undergone nondisjunction by the total number of progeny.

Meiotic Chromosome Nondisjunction Tests with *P(EP)2340*

Attached second chromosome stocks were used to determine whether autosomal nondisjunction occurred in *incenp* mutants. *w; P(EP)2340/CyO7.1* (experiment) or Canton-S (control) males were crossed to *C(2)EN, bw sp* females. These female produce either diplo-2 or nullo-2 eggs; therefore viable progeny can only be produced if nondisjunction in male meiosis results in nullo-2 or diplo-2 sperm. From a total of 155 females crossed, 14 individuals were obtained for the experiment and 3 for the control.

Fourth chromosome nondisjunction was measured by crossing *w; P(EP)2340/CyO7.1* (experiment) or Canton-S (control) males to *y; C(4)RM, spa*^{pol} females, a standard test. Nondisjunction will produce sperm lacking fourth chromosomes or sperm with two copies of the fourth chromosome. Only the nullo-*4* sperm could be distinguished in this cross, and they produced progeny with solely the *C(4)RM, spa*^{pol} chromosome. The nullo-*4* sperm that fertilized nullo-*4* ova were not recovered. Diplo-*4* sperm that fertilized a nullo-*4* ova produced progeny wild type for the fourth chromosome markers, and these could not be distinguished from those in which a normal sperm fertilized a *C(4)RM, spa*^{pol} ova. The other class of progeny arose from normal sperm fertilizing a nullo-*4* ova, but these haplo-*4* individuals were Minute and had reduced viability, so they were not scored. The calculation of nondisjunction frequency factored in the missing exceptional classes (assuming equal frequencies of diplo and nullo-*4* sperm) and appropriately corrected total progeny number for the exceptional classes and haplo-*4* progeny from normal disjunction. A total of 1,178 flies were scored in the experiment and 1,071 in the control. The *P(EP)2340* mutants showed 2.55% nondisjunction versus 0.37% in controls.

To estimate the rate of nondisjunction in the second meiotic division, *y/B*^{s}*Y; P(EP)2340/SM6; spa*^{pol}/+ males were crossed with *C(1)RM, y v/0; C(4)RM, ci ey* ^{R} females

(Kerrebrock et al. 1992). This cross was set up also using males Canton-S (control); males *y/B^{s}Y; P(EP)2340/cn mei-S332^1 ord^1; spa^{pol}/+* and males *y/B^{s}Y; cn mei-S332^1 ord^1/+; spa^{pol}/+*. In this case *spa^{pol}* individuals that were not Minute, and thus have two fourth chromosomes, were the result of nondisjunction of chromosome *4* in the second meiotic division. Nullo-*4* sperm, arising either from meiosis I or II nondisjunction, produced ci ey^R progeny. The already poor fertility of *P(EP)2340* males was further reduced by the introduction of the marker chromosomes. Although the number of progeny obtained from these tests was low (about 700) there was a significant increase in meiosis II nondisjunction in *P(EP)2340* males: 5.08% exceptional progeny versus 0.33% in the controls. This result was similar for *y/B^{s}Y; P(EP)2340/cn mei-S332^1 ord^1; spa^{pol}/+*males (4.8%). Thus mutation of one copy of each of the two sister-chromatid cohesion genes *mei-S332* and *ord* did not enhance the *P(EP)2340* phenotype.

The cross of *y/B^{s}Y; P(EP)2340/SM6; spa^{pol}/+* males to *C(1)RM, y v/0; C(4)RM, ci ey ^{R}* females was also used to measure nondisjunction of the sex chromosomes in meiosis I, as *XY* sperm yielded y B male progeny. The frequency of *XY* sperm was 2.54% *-P(EP2340)-* or 2.7% *-P(EP2340/mei-S332^1 ord^1)* versus 0.33% in controls. Progeny resulting from meiosis II nondisjunction of the *X* chromosome were not scored in this experiment.

Phenotypic analysis of mutant meiosis and immunofluorescence

To analyze chromosome structure the testis were squashed, fixed, and stained with orcein as described previously (Bickel et al., 1997) and examined on a Zeiss Axiophot microscope with a 63X Apochromat objective. Testis were processed for immunofluorescence analysis as described previously (Carmena et al., 1998; Bonaccorsi et al., 2000). Primary antibodies and concentrations used included: α-INCENP rabbit

polyclonal serums Rb801 and Rb803,1:500 (Adams et al., 2001); α-alpha-tubulin mouse monoclonal B512, 1:2000 (Sigma); α-MEI-S332 guinea pig serum 1:5000 (Tang et al., 1998); α-Pavarotti KLP rabbit polyclonal serum Rb-3301, 1:500 (Adams et al., 1998), gift from R. Adams); α-Cyclin B rabbit polyclonal serum Rb271 1:500 (gift from David M. Glover (Whitfield et al., 1990).

Imaging was performed using Olympus IX-70 and IX-71 microscopes controlled by Delta Vision SoftWorx (Applied Precision, Issequa, WA, USA). Image stacks were deconvolved, quick-projected and saved as tiff images to be processed using Adobe Photoshop.

In order to quantify the INCENP and MEI-S332 staining density on centromeres stacks of images were projected using an averaging algorithm. The total integrated intensity of a square box containing the centromeric signal was measured at the appropriate wavelength in as many chromosomes as possible using the Data Inspector tool. For each cell, corresponding measures were taken of the background outside the cell, and values were corrected by subtracting this background measurement.

INCENP/Aurora B dsRNAi experiments

Drosophila S2 cells were treated with dsRNA to deplete the INCENP or Aurora B proteins as described before (Adams et al., 2001). 48 hours after the addition of dsRNA we determined the distribution of mitotic phases, the proportion of cells with no detectable levels of INCENP or Aurora B protein and the presence or absence of either INCENP or Aurora B and MEI-S332 on mitotic chromosomes.

In vitro binding assays

Full-length proteins labeled with [^{35}S]-methionine were expressed from cDNAs in pOT2 using a reticulocyte lysate coupled transcription/translation system (Promega). For each binding reaction *in vitro* translated DmAuroraB or MEI-S332 was added to binding buffer (PBS, 5mM EGTA, 0.1% triton, 0.5mM PMSF, and 1μg/ml CLAP [chymostatin, leupeptin, antipain, and pepstatin A]) containing GST-DmINCENP or GST alone bound to glutathione sepharose beads. Samples were incubated for 90 minutes at 4°C. The supernatant was precipitated with TCA and the beads were washed three times with binding buffer. Bound (B) and unbound fractions were separated by SDS-PAGE, and the proteins were visualized using a phosphorimager (STORM 860) with ImageQuant software (Amersham biosciences).

Aurora-B Kinase assays

A bicistronic vector expressing *Xenopus* INCENP and *Xenopus* Aurora B-6His was constructed in pET28. The Bicistronic was assembled by digesting pET28 Aurora B with XbaI and NotI (Bolton et al., 2002), pCS2+Myc INCENP (Stukenberg et al., 1997) with NcoI and XbaI, and pET28 with NcoI and Not1. These products were trimolecularly ligated producing a C-terminal 6 His on Aurora-B and a single N-terminal Myc on INCENP. The proteins were expressed in BL21 (pLysS) and purified using Ni^{2+}-NTA agarose (Qiagen) according to manufacturer instructions. These proteins were subsequently run over a Superose6 gel filtration column (Amersham Pharmacia). Kinase reactions were performed as described previously (Bolton et al., 2002). 100ng Aurora-B-INCENP was used for each kinase reaction containing 2.2uM of each substrate. MEI-S332

was cloned by PCR into the EcoRI and XhoI sites of pGEX-4T-3 (Amersham). MEI-S332 phospho-site mutants were generated using PCR mutagenesis and confirmed by sequence analysis. These proteins were expressed in BL21(pLysS) and purified using GST-Agarose (Amersham Pharmacia) following the manufacturer instructions. Following purification, proteins were dialyzed into kinase buffer (20mM Tris pH7.7, 1mM $MgCl_2$, 25mM KCl, 1mM DTT).

Analysis of the localization of GFP-tagged wild type and mutant proteins in S2 cells

The S124A, S125A, S126A triple mutation was subcloned using SpeI and BlpI into pJL9 -a vector expressing *mgfp6,* fused to the N-terminus of *mei-S332* under a constitutive *armadillo* promoter (Lee et al., 2004). Exponentially growing S2 were transiently transfected with GFP-MEI-S332 or GFP MEI-S332 124AAA using a Nucleofector (Amaxa biosystems) following the manufacturer instructions. Three independent transfections where done for control and experiment plasmid, using two different sets of transfection conditions. Transfection efficiencies ranged between 40 and 70%. Nucleofected cells were plated onto poly-LYS coated coverslips and cultured for 24 hours. Cells were then fixed for 10 minutes in 4% paraformaldehyde solution, rinsed in PBS and mounted as described above. GFP fluorescence was used for scoring prometaphase and metaphase figures, which were classified visually according to the level of GFP signal on the kinetochores into high level (normal), reduced level and negative. Representative individual kinetochores showing high level GFP fluorescence in control (n=23) or experiment (n=19) or low level (n=36) iwere selected in order to obtain a quantitatively estimate of the signal reduction.

ACKNOWLEDGEMENTS

We thank Richard R. Adams and David M. Glover for gifts of antisera and Daniel Roth for technical assistance to MC. Laura Lee carried out the initial mapping studies on *QA26*. Some fly strains were obtained from the Bloomington Stock Center. TDR and TO-W were supported by NSF grant MCB0132237 and NIH grant GM39341. TDR was supported by an Anna Fuller graduate fellowship. DLS was supported by training grant HD07528 for Developmental Biology at the University of Virginia. PTS was supported by a grant from the National Institutes of Health, GM63045 and by a grant from the Pew Charitable Trust. MC and WCE were supported by the Wellcome Trust, of which WCE is a principal investigator.

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Chapter Four

The Chromosomal Passenger Complex and the Condensin Complex Differentially Affect Synaptonemal Complex Disassembly and Metaphase I Configuration in Drosophila Female Meiosis

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*T.D.R. characterized and imaged *incenp*^{QA26} embryonic and polar body defects, imaged *dcap-g* embryonic defect, performed MEI-S332 Western blot, quantified and imaged *dcap-g* karyosome phenotype, recombined *incenp*^{QA26} chromosomes with *ord*^{10} and *Df(2L)Exel7049*, performed C(3)G immunofluorescence and quantified mutant phenotypes, quantified metaphase I configuration defects, performed and analyzed immunofluorescence experiments in stage 12-14 oocytes, characterized and imaged uneven meiotic products in *incenp*^{QA26}.

ABSTRACT

Production of haploid gametes relies on the specially regulated meiotic cell cycle. Analyses of the role of essential mitotic regulators in meiosis have been hampered by a shortage of appropriate alleles in metazoans. We characterized female-sterile alleles of the condensin complex component *dcap-g* and used them to define roles for condensin in Drosophila female meiosis. The condensin complex is required for sister-chromatid resolution in mitosis and contributes to chromosome condensation. In meiosis, we demonstrate a requirement for *dcap-g* for proper disassembly of the synaptonemal complex and for proper configuration of the metaphase I-arrested chromosomes. The chromosomal passenger complex is also known to have mitotic roles in chromosome condensation and is required in some systems for localization of the condensin complex. We used the *QA26* allele of passenger component *incenp* to compare the roles of the condensin and passenger complexes in meiosis. Strikingly, in $incenp^{QA26}$ mutants, maintenance of the synaptonemal complex is disrupted, and the metaphase I configuration is also defective, but in a manner distinct from the *dcap-g* disruption. We show that *incenp* interacts genetically with *ord*, suggesting an important functional relationship between them in meiotic chromosome dynamics.

INTRODUCTION

Organisms that undergo sexual reproduction utilize a specialized cell cycle, meiosis, to generate haploid gametes. Precise partitioning of the genome in meiosis is essential so that diploidy is reestablished upon fertilization. This is extremely important, since zygotic aneuploidy most often results in developmental arrest in the embryo (Hassold and Hunt 2001). To facilitate accurate reduction of the genetic material, meiosis employs regulatory mechanisms that are distinct from those of mitotic division. In meiotic cells, the DNA is replicated exactly once and then divided twice without an additional intervening round of DNA replication.

In preparation for the meiotic divisons, homologs find each other and pair. In many systems, a proteinaceous structure, the synaptonemal complex, forms an axis between homologs and regulates meiotic recombination (Page and Hawley 2003). Through generation of crossover events, covalent linkages are formed between homologs. These, in combination with sister-chromatid cohesion distal to the chiasmata (the physical structures resulting from crossing over), allow homologs to remain physically attached after synaptonemal complex disassembly and to thereby coordinate their movements.

In meiosis I, homologs biorient on the spindle while sister chromatids co-orient toward a single spindle pole (for a review see (Petronczki et al. 2003)). Release of cohesion distal to the chiasmata at the onset of anaphase I allows homologs to separate from each other and move toward opposite poles; maintenance of centromere cohesion holds sister chromatids together as they travel toward a single spindle pole. The enduring physical attachment between sister chromatids is essential for them to biorient on the

spindle in the second meiotic division. Centromere cohesion is severed at the onset of anaphase II and sister chromatids segregate to opposite poles, in a manner more similar to mitosis.

Proper progression through the meiotic program requires activity of meiosis-specific factors as well as critical contributions from proteins that are also essential in mitosis. Indeed, many chromosomal behaviors are common between these two cell cycles: chromosomes must become tightly condensed, they must orient stably on a bipolar spindle, and they must disentangle from each other in order to segregate. Study of the meiotic roles of proteins also required in mitosis has been experimentally complicated in metazoans by a shortage of alleles, for genetic analysis, that retain sufficient function to allow development of a whole organism, but compromise activity enough to reveal meiotic phenotypes. The condensin complex and the chromosomal passenger complex are two important regulators of chromosome dynamics in mitosis, and their roles in meiosis remain much less characterized (Vagnarelli and Earnshaw 2004; Hirano 2005).

The conserved condensin complex is required in mitosis for resolution of sister chromatids, and in the absence of condensin proteins, chromosomes appear fuzzy in prometaphase and lagging chromatin is observed in anaphase (Steffensen et al. 2001; Hagstrom et al. 2002; Hagstrom and Meyer 2003; Dej et al. 2004). Condensins also play roles in chromosome condensation, though when condensin subunits are mutated *in vivo* in metazoans, chromosomes generally reach a fully compacted conformation after some delay. Five conserved subunits together form the condensin complex. These include two SMC (Structural Maintenance of Chromosomes) components, SMC2 and SMC4; and three non-SMC components, CAP-D2/3, CAP-G/G2, and CAP-H/H2 (Hirano and

Mitchison 1994; Hirano et al. 1997). In many metazoan systems two condensin complexes have been identified, both of which contain the same SMC subunits but vary in their non-SMC components (Ono et al. 2003). In Drosophila, only one CAP-G protein has been identified, and thus it likely functions in both complexes (Dej et al. 2004; Jager et al. 2005). In addition, the condensin I complex includes non-SMC subunits CAP-D2 and CAP-H, and the condensin II complex includes CAP-D3 and CAP-H2.

Some meiotic roles have been described for the condensin complex in *C. elegans* and *S. cerevisiae*, but many suggestions about condensin's function vary between the two systems. In worms, SMC4 depletion resulted in robust chromosome bridging in the second meiotic division, and less severe lagging chromatin in the first division (Hagstrom et al. 2002; Kaitna et al. 2002; Chan et al. 2004). The possibility that the bridging resulted from failure to resolve sister chromatids and also homologs was supported by the observation that condensin depletion suppressed premature separation of sister chromatids in the absence of cohesin protein REC-8 and premature separation of homologs in the absence of SPO-11, the enzyme that introduces double-strand breaks to initiate recombination (Chan et al. 2004). In condensin mutants in yeast, anaphase bridging arose in both meiotic divisions, but these defects were eliminated when *spo-11* was mutated (Yu and Koshland 2003), suggesting that separation defects resulted specifically from sister-chromatid interactions.

In addition in *C. elegans*, when condensin subunits were depleted the synaptonemal complex was properly formed and properly disassembled, and the meiotic chromosomes displayed no defect in compaction in pachytene in prophase I, though delays in condensation were apparent later in prophase I (Chan et al. 2004). In contrast, in

condensin mutants in *S. cerevisiae*, longitudinal compaction and chromosome resolution were both disrupted in pachytene, and the synaptonemal complex failed to assemble properly (Yu and Koshland 2003).

The chromosomal passenger complex includes Aurora B kinase, INCENP, Survivin, and Borealin/Dasra. Complex members require each other for their stereotypic mitotic localization pattern in which they are found across the chromosomes in prophase, specifically at the centromere in metaphase, and on the spindle midzone during anaphase and telophase (for reviews see (Carmena and Earnshaw 2003; Vagnarelli and Earnshaw 2004)). The passenger complex plays important mitotic roles in each of these locations, including functions in chromosome condensation, biorientation of sister-chromatids on the spindle, chromosome separation, spindle stability, and cytokinesis. Aurora B phosphorylates many important cell cycle regulatory proteins, and the other passenger complex members are important for its kinase activity (Kang et al. 2001; Bishop and Schumacher 2002; Honda et al. 2003). Among its characterized substrates is serine 10 of histone H3 (Adams et al. 2001; Giet and Glover 2001). Presence of this modification often correlates with a condensed chromosome state, and the passenger complex has been implicated in chromosome condensation, though the relationship between H3 phosphorylation and condensation is not well understood (Gurley et al. 1978; Adams et al. 2001).

Meiotic studies of the passenger complex components have shown that in some systems, including budding yeast and spermatogenesis in mice and flies, localization of the complex resembles the mitotic pattern in many respects (Parra et al. 2003; Resnick et al. 2006; Monje-Casas et al. 2007; Yu and Koshland 2007). Furthermore, in budding

yeast a functional similarity has been shown for the passenger complex at mitotic and meiotic centromeres. In mitosis the passenger complex destabilizes unproductive kinetochore-microtubule attachment when sister chromatids fail to biorient. In meiosis I, the passenger complex similarly severs unproductive attachments when homologs fail to biorient (Monje-Casas et al. 2007).

In addition, the passenger complex has been shown to play a role in preserving centromere cohesion in meiosis I and in localizing MEI-S332/Sgo1, a protector of centromere cohesion, to the centromere in both Drosophila and *S. cerevisiae* (Resnick et al. 2006; Monje-Casas et al. 2007; Yu and Koshland 2007). This activity is important for facilitating segregation of sister-chromatids in the second meiotic division. In contrast, in meiosis I in *C. elegans* oogenesis, AIR-2 (Aurora B) localizes specifically to the region of the chromosome distal to the chiasma and is required for release of arm cohesion and separation of homologs in the first meiotic division (Kaitna et al. 2002; Rogers et al. 2002).

The observation that the condensin and passenger complexes both play roles in chromosome condensation and resolution raises the question of whether they act independently in these processes or whether one of them regulates the other. In support of a possible interrelated function, in some mitotic systems the passenger complex is required for proper localization (Giet and Glover 2001; Morishita et al. 2001; Hagstrom et al. 2002; Kaitna et al. 2002; Lipp et al. 2007) and for phosphorylation (Lavoie et al. 2004; Lipp et al. 2007) of the condensin proteins, though in other systems condensins localize independently of the passenger complex (Losada et al. 2002; Lavoie et al. 2004).

The condensin complex has also been suggested to be required for centromere localization of INCENP (Hudson et al. 2003).

In this paper we explore the meiotic roles of the condensin and passenger proteins in Drosophila oogenesis. We find that both these complexes play important roles at several points in meiotic progression, but that their effects are distinct from each other, in processes including synaptonemal complex disassembly and metaphase I chromosome configuration. We also investigate the relationship of the passenger complex and ORD, a meiotic protein with roles in chromosome condensation and cohesion (Miyazaki and Orr-Weaver 1992), and find that *incenp* and *ord* interact genetically in their regulation of metaphase I chromosome behavior.

RESULTS

Identification of female-sterile alleles of the condensin *cap-g*

Alleles of the condensin *dcap-g* that disrupt mitotic divisions of embryogenesis and result in embryonic lethality have been previously characterized (Dej et al. 2004). We sought to identify female-sterile alleles of *dcap-g* that would allow us to analyze the roles of the condensin complex in the specialized cell cycles utilized in oogenesis. We screened the Zuker collection of EMS-generated, female-sterile mutations (Koundakjian et al. 2004) for mutations failing to complement the embryonic lethal allele $dcap\text{-}g^{K1}$. We identified three mutations that were female sterile as transheterozygotes with $dcap\text{-}g^{K1}$.

Sequencing identified aberrations in the *dcap-g* sequence in each of these lines. $dcap\text{-}g^{Z1}$ (Zuker mutant *Z2-5052*) is a point mutation predicted to change amino acid 157 from valine to glutamic acid. $dcap\text{-}g^{Z2}$ (Zuker mutant *Z2-4027*) would convert amino acid 1210 from glutamine to a premature stop in the DCAP-G-PB protein variant. $dcap\text{-}g^{Z3}$ (Zuker mutant *Z2-5019*) contains a deletion within intron 4, following the last common exon shared by all the DCAP-G isoforms. In addition, the $dcap\text{-}g^{K3}$ larval lethal allele (Dej et al. 2004) allows a few viable adult escapers, and these are also female sterile. We performed most of our analyses of condensin function, below, using the $dcap\text{-}g^{Z1}$ allele transheterozygous to the $dcap\text{-}g^{K4}$ allele, a putative null allele that deletes a portion of the 5' end of the *dcap-g* gene (Dej et al. 2004), because this combination provided the strongest female-sterile phenotypes (data not shown).

dcap-g and *incenp* mutants arrest early embryonic mitoses and *incenp* disrupts embryonic MEI-S332 phosphorylation

To determine the cause of the female sterility of the *dcap-g* mutations, we collected embryos from mutant mothers and examined the DNA morphology. These embryos displayed aberrant polyploid, fragmented nuclei and arrested in the earliest stages of embryogenesis (Fig. 4-1B), which are controlled by maternally contributed mRNAs and proteins. The presence of polyploid nuclei is consistent with failure to separate chromosomes in mitosis, followed by entry into DNA synthesis in subsequent cell cycles.

Given the relationships between condensin and the chromosome passenger complex in other systems (Giet and Glover 2001; Morishita et al. 2001; Hagstrom et al. 2002; Kaitna et al. 2002; Lavoie et al. 2004; Lipp et al. 2007), we took advantage of the *incenp*^{QA26} female-sterile allele to compare the functions of the passenger and condensin complexes in these developmentally-regulated cell cycles in Drosophila. We collected and stained embryos from *incenp*^{QA26} mothers and found that they, too, arrest in the earliest stages of embryogenesis. In these embryos we detected a failure to separate sister chromatids, resulting in chromatin bridging. This occurred as early as the first zygotic anaphase (Fig. 4-1C), in which male and female pronuclei can be distinguished as separate masses on the first mitotic spindle. Embryos from *incenp*^{QA26} mutant mothers arrested with lagging, poorly-condensed chromosomes after attempting no more than three cell division cycles, presumably due to repeated failure to separate the chromosomes (Fig. 4-1D). The lagging chromatin was frequently stretched along multi-polar spindles, generating a web-like DNA morphology. This phenotype is reminiscent of the aberrant anaphases seen in Dmel2 cells treated with RNAi constructs to *incenp* or *Aurora B* (Adams et al. 2001; Giet and Glover 2001).

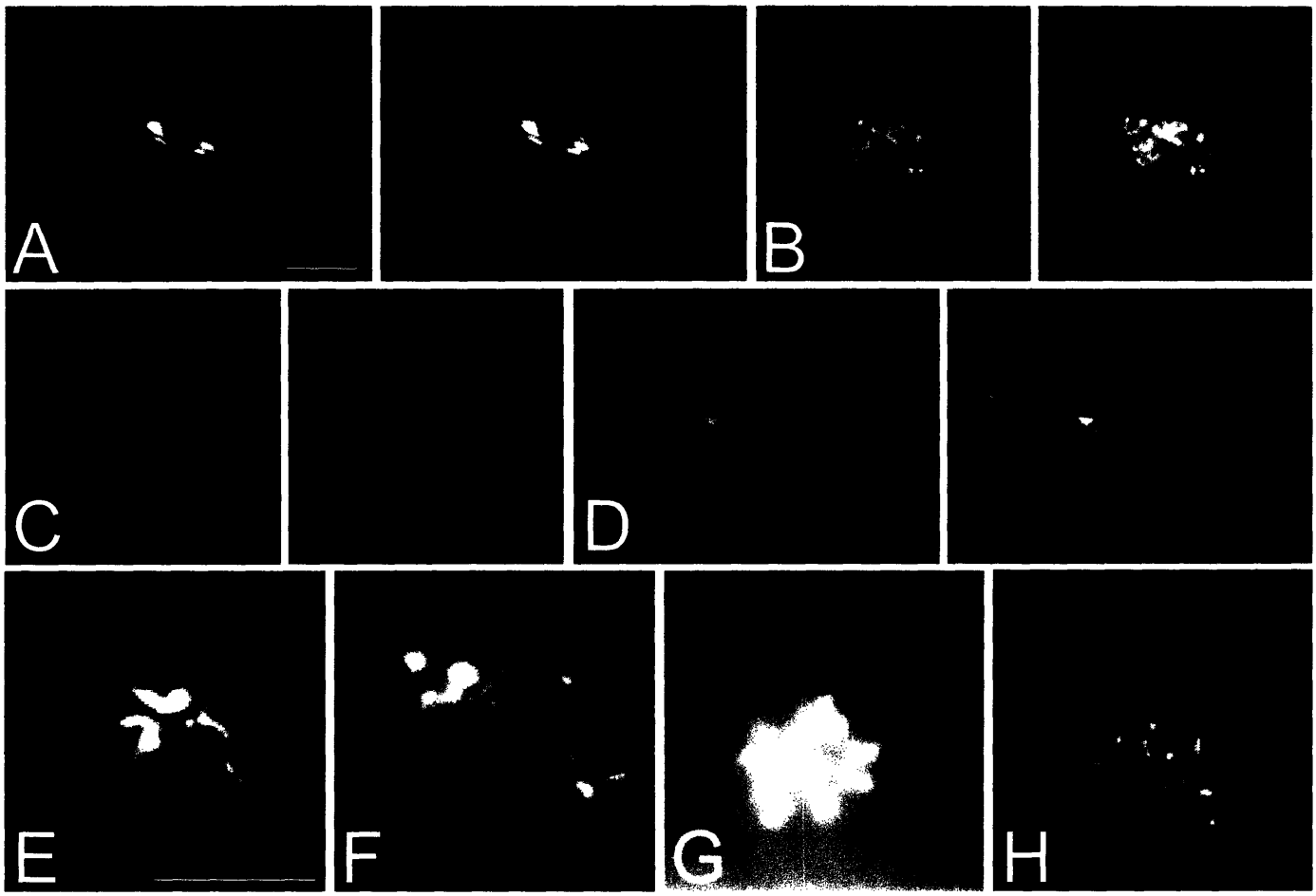


Figure 4-1. *dcap-g* and *incenp* mutants display defects in mitosis and post-meiotic chromosome structure Early embryos stained for DNA (green, and gray panels) and tubulin (red) A) *Oregon R*, anaphase in first mitotic division B) *dcap-g*^{Z1} / *dcap-g*^{K4}, polyploid mass in early arrested embryo C) *incenp*^{QA26}, lagging chromosomes in first anaphase D) *incenp*^{QA26}, chromatin bridging in early arrested embryo E-H) polar body rosette structures E) *Oregon R*, displays condensed arms of even length F) *dcap-g*^{K3}, pulverized G) *dcap-g*^{Z1}, hypercondensed H) *incenp*^{QA26}, elongated and fragmented. Scale bars are 5 μm.

Kinase assays *in vitro* have shown that Aurora B/INCENP can phosphorylate the centromere cohesion protein MEI-S332, and INCENP is required for proper localization of MEI-S332 in tissue culture cells and in meiotic spermatocytes (Resnick et al. 2006). We asked whether the passenger complex might phosphorylate MEI-S332 *in vivo* in early embryos. We collected 0-2 hour embryos from $incenp^{QA26}$ females, prepared extracts for Western blotting, and probed with an antibody to MEI-S332. Previous work has shown that MEI-S332 can be visualized in multiple bands on Western blots and that less phosphorylated forms run more slowly on a polyacrylamide gel (Clarke et al. 2005).

We compared MEI-S332 from $incenp^{QA26}$ and wild-type embryos and found that a slower migrating form of the protein was present only in the mutant extracts (Fig. 4-2A). To determine whether this extra band might be a hypophosphorylated form of MEI-S332, we incubated wild-type extracts with lambda phosphatase and ran them alongside the untreated wild-type and $incenp^{QA26}$ extracts. In the phosphatase treated lane, a slower-migrating band, presumably representing dephosphorylated MEI-S332, was seen at the same position as the extra MEI-S332 form in the $incenp^{QA26}$ extracts (Fig. 4-2B). This result is consistent with a model in which the passenger complex regulates MEI-S332 by phosphorylation and that this interaction is disrupted in $incenp^{QA26}$ mutants.

dcap-g and *incenp* mutants display defects in the highly condensed post-meiotic chromosome structure

Because both the condensin and passenger complexes have characterized roles in chromosome condensation, we examined the condensed chromosomes of the polar body

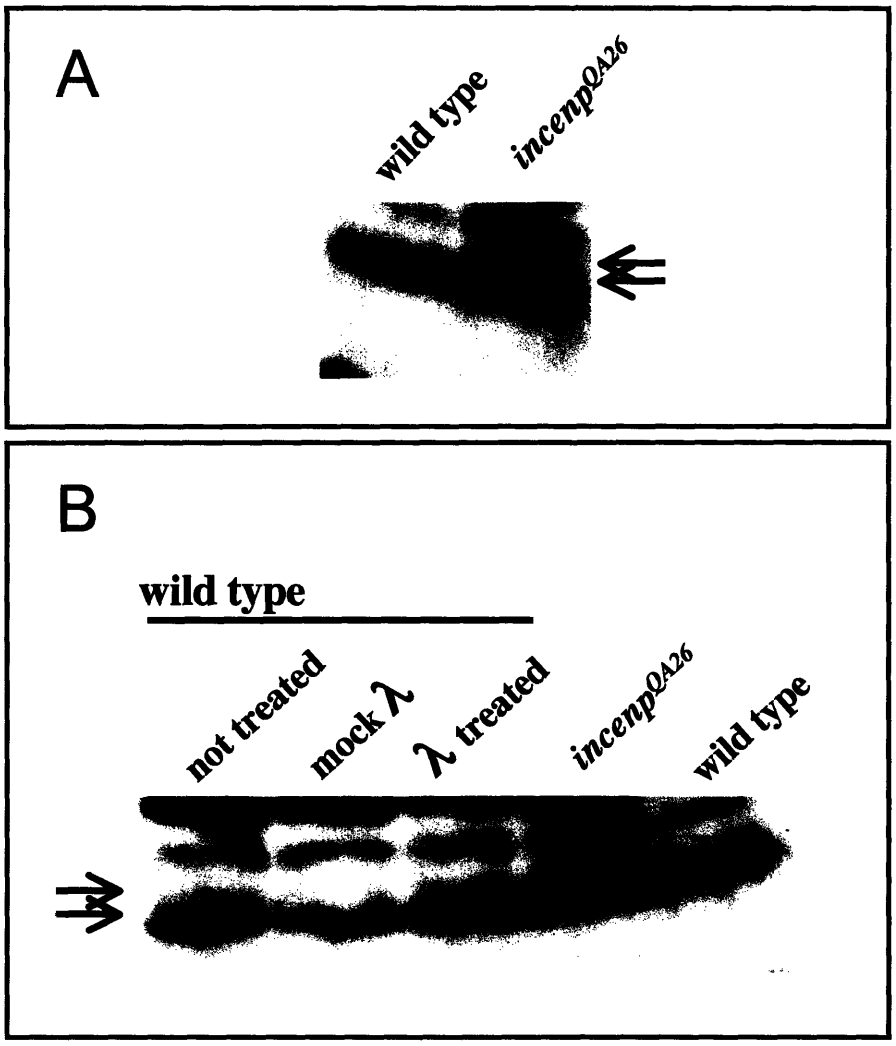


Figure 4-2. MEI-S332 phosphorylation state is disrupted in *incenp*^{QA26} mutants. Immunoblots of protein extracts from embryos derived from wild-type or mutant mothers. A) A slower migrating MEI-S332 band is present in embryo extracts from *incenp*^{QA26} females, B) λ-phosphatase treatment of wild-type extracts generates a band with similar mobility to the extra band in *incenp*^{QA26} mutant extracts.

rosette to explore whether these alleles affected chromosome morphology. This structure forms from the unused products of female meiosis, which remain in the common cytoplasm of the embryo and transition through an uncondensed, interphase state into an arrested, tightly compacted body (Foe 1993). In wild-type rosette structures, the chromosome arms are evenly condensed and uniform in length (Fig. 4-1E). Eggs laid by *dcap-g*^{K3} mutant females contained pulverized polar body structures, displaying regions of condensed DNA and uncondensed, stretched DNA (Fig. 4-1F). In *dcap-g*^{Z1}, *dcap-g*^{Z2}, and *dcap-g*^{Z3} eggs, most of the rosettes contained chromosomes that appeared hypercondensed and formed small rounded pellets (Fig. 4-1G). In embryos laid by *incenp*^{QA26} mutant mothers rosettes formed in which chromosome arms were elongated rather than uniform in length, and the overall structure of the rosette was neither as tight nor as organized as in wild-type embryos (Fig. 4-1H). In addition, small fragments of DNA frequently surrounded the rosette structure; this was very rarely seen in wild-type embryos. These defects in the polar body rosette structure suggest that these hypomorphic alleles of *dcap-g* and *incenp* provide a powerful opportunity to explore the roles of the condensin and passenger complexes in developmentally-regulated chromosome dynamics.

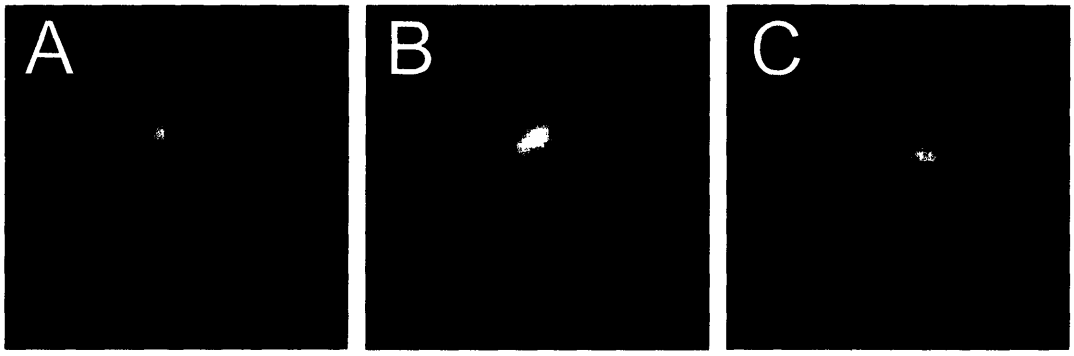
dcap-g mutation disrupts tightly condensed karyosome structure in meiosis

The requirement for the passenger and condensin complexes in mitosis creates experimental obstacles to studying the role of these proteins in meiosis in metazoans, because strong alleles of these essential genes prevent development of viable adults in which meiosis takes place. We took advantage of the female-sterile alleles described

above to investigate regulation of chromosome condensation in meiosis. In Drosophila female meiosis, chromosomes form a tightly condensed mass, called the karyosome, during prophase I and are held in this structure for a prolonged period during which oocyte development proceeds (Spradling 1993). In wild-type flies, the karyosome is seen as a compact spherical body that takes up only a small portion of the oocyte nucleus (Fig. 4-3A). We dissected ovaries from mutant females, stained for DNA, and examined the meiotic chromosome morphology during this prophase I arrest. In *dcap-g*^{Z1} females, the karyosome appears tightly condensed in some egg chambers (Fig. 4-3B), but stretched and distorted in others (Fig. 4-3C, D), suggesting that the condensin complex is involved in formation or maintenance of this condensed structure. Although we did not see a disruption of the karyosome in *incenp*^{QA26} females, this does not rule out a role for the passenger proteins during this meiotic stage because *incenp*^{QA26} is a weak allele and the passenger complex may retain sufficient activity for karyosome formation.

Regulators of chromosome condensation differentially affect synaptonemal complex maintenance

In addition to roles in chromosome condensation, the condensin complex has also been implicated in other important aspects of meiotic chromosome regulation. Loading of the condensin complex in meiotic prophase I correlates temporally with the disassembly of the synaptonemal complex. A suggestion that there is an important regulatory link between these two processes, as well as the temporal correlation, comes from mutation of *nucleosomal histone kinase-1* (*nhk-1*), which disrupts both condensin loading and synaptonemal complex unloading (Ivanovska et al. 2005). We used the *dcap-g* alleles to



D Prophase I karyosome condensation disrupted in condensin mutants

	% dispersed karyosomes	n
w^{1118}	**0**	20
***dcap-g*^{Z1}/*dcap-g*^{Z1}**	**27**	22
***dcap-g*^{Z1}/*dcap-g*^{K4}**	**8**	24
***dcap-g*^{Z2}/*dcap-g*^{K4}**	**20**	10

Figure 4-3. *dcap-g* mutations disrupt condensed karyosome structure A) w^{1118} oocytes stained with DAPI to show a karyosome with a round, compact structure B) *dcap-g*^{Z1} karyosome displaying wild-type morphology C) *dcap-g*^{Z1} karyosome displaying stretched morphology D) Quantification of karyosome defects in wild-type and *dcap-g* mutant oocytes.

examine whether loading of the condensin complex onto the chromsomes is required for unloading of synaptonemal complex proteins.

We dissected ovaries from *dcap-g*^{Z1} /*dcap-g*^{K4} females and stained with an antibody to C(3)G, a protein in the transverse filaments of the synaptonemal complex. In both wild-type and *dcap-g* ovaries, this synaptonemal complex protein can be visualized in the earliest oocytes, located in the germarium, in a ribbon-like structure, corresponding to the axes that have formed between the paired homologs. As oocyte development continues, egg chambers leave the germarium and the meiotic chromosomes are held in an extend prophase I arrest as the oocyte grows in size and undergoes important morphological changes (reviewed in Spradling 1993). Developing egg chambers progress through distinguishable stages that have been defined by their morphological characteristics. These stages begin at stage 2, after the egg chamber has left the germarium, and continue until stage 14, at which point the egg is mature. The synaptonemal complex disassembles gradually as development progresses and meiotic chromosomes are arrested in prophase I. We examined the dynamics of synaptonemal complex unloading over developmental time in wild-type and mutant females. We saw a significant difference between synaptonemal comlex disassembly in wild-type and *dcap-g* ovaries (Table 4-1).

In wild-type ovaries, C(3)G was strongly associated with the chromosome axis in only 9% of stage 6 egg chambers, compared with 33% of *dcap-g* ovaries at the same stage. By stage 7, 31% of wild-type oocytes displayed C(3)G fully or mostly associated with the chromosomes, whereas 69% of *dcap-g* oocytes showed much or all of the C(3)G to be chromosome-localized. Furthermore, in wild-type oocytes, most had no

Table 4-1. Synaptonemal complex assembly in condensin and passenger mutants

		C(3)G localized to chromosome axis	C(3)G dispersed on axis and spreading through nucleus	Trace amounts C(3)G on chromosomes	No C(3)G associated with chromosomes	n
Oregon R	Stage 4	**83%**	13%	3%	0%	30
	Stage 5	**69%**	22%	6%	3%	32
	Stage 6	9%	**46%**	**43%**	3%	35
	Stage 7	0%	**31%**	**43%**	25%	32
	Stage 8	0%	0%	**38%**	**63%**	32
	Stage 9	0%	0%	11%	**89%**	18
dcap-g^{Z1}/dcap-g^{K4}	Stage 4	**94%**	6%	0%	0%	17
	Stage 5	**71%**	29%	0%	0%	14
	Stage 6	**33%**	**47%**	20%	0%	15
	Stage 7	16%	**53%**	26%	5%	19
	Stage 8	0%	13%	**73%**	13%	15
	Stage 9	0%	0%	**92%**	8%	12
incenp^{QA26}, Df(2L)Exel7049/ incenp^{QA26}	Stage 4	**59%**	**35%**	6%	0%	17
	Stage 5	21%	**36%**	**36%**	7%	14
	Stage 6	7%	**40%**	**47%**	7%	15
	Stage 7	0%	0%	**32%**	**68%**	19
	Stage 8	0%	13%	**33%**	**53%**	15
	Stage 9	0%	0%	14%	**86%**	7
incenp^{QA26}, ord^{10}/ incenp^{QA26}	Stage 4	**47%**	**47%**	0%	7%	15
	Stage 5	21%	**36%**	**43%**	0%	14
	Stage 6	0%	25%	**55%**	20%	20
	Stage 7	0%	0%	**53%**	**47%**	17
	Stage 8	0%	0%	29%	**71%**	17
	Stage 9	0%	0%	22%	**78%**	9

Numbers over 30% are are shaded in gray as a visual aid to highlight the behavior of the preponderance of the oocytes.
All ovaries were dissected from females fattened at 18°C.
Quantification of C(3)G localization was performed blind.

remaining C(3)G on the chromosomes by stage 8 and the vast majority showed no chromosome-specific C(3)G in stage 9. *dcap-g* mutants retained at least trace levels of C(3)G on the chromosomes in nearly all oocytes at these stages. From this staining pattern, we conclude that the condensin complex is, indeed, required for proper disassembly of the synaptonemal complex. Furthermore, because the *dcap-g* mutations are weak alleles, the condensin complex likely plays an even more important role in synaptonemal complex disassembly than is evidenced by the delay seen here.

The passenger complex also has characterized roles in chromosome condensation, so we asked whether it also is required for synaptonemal complex unloading. Because the $incenp^{QA26}$ allele is weak, we sought to enhance its effects in female meiosis. $incenp^{QA26}$ generates a single amino acid change in the C-terminal IN-BOX of the INCENP protein, the region through which INCENP interacts with Aurora B kinase (Adams et al. 2000; Resnick et al. 2006). We reasoned that the mutation likely weakens the interaction between these two proteins, and that reducing the amount of Aurora B protein might enhance the meiotic phenotype. We crossed one copy of the small chromosomal deficiency *Df(2L)Exel7049*, which removes 18 genes including *Aurora B* (Parks et al. 2004), into the $incenp^{QA26}$ background.

We dissected ovaries from $incenp^{QA26}$, *Df(2L)Exel7049/* $incenp^{QA26}$ and wild-type flies, and we stained for C(3)G. In these mutants as well, assembly of the synaptonemal complex was unaffected. Strikingly, we found that, rather than prolonged maintenance of the synaptonemal complex, these mutants displayed premature disassembly of this structure (Table 4-1). In the wild-type control, C(3)G was localized to the chromosome axes in 69% of stage 5 oocytes, and only 6% of oocytes showed C(3)G spread throughout

the nucleus with just trace amounts of C(3)G associated specifically with the DNA at the same stage. In *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} ovaries, disassembly of the synaptonemal complex was well underway in stage 5, with only 21% of oocytes displaying C(3)G strongly associated with the chromosomes and 36% showing only trace amounts of C(3)G remaining specifically localized to the DNA. Thus, the mutation of two protein complexes that are both involved in chromosome condensation has different effects on synaptonemal complex disassembly in meiosis: the chromosome passenger complex is required for maintenance of the synaptonemal complex and the condensin complex plays an important role in its disassembly.

The condensin and passenger complexes are required for metaphase I arrest

One aspect of meiotic chromosome dynamics that is notably different from mitotic behaviors is the stable biorientation of homologs in metaphase I, which is critical for proper segregation of the homologs in anaphase I (Petronczki et al. 2003). In many systems, including Drosophila female meiosis, this metaphase I configuration is further distinguished from mitosis because the spindle lacks centrosomes (Theurkauf and Hawley 1992). The female meiotic spindle is organized by the chromosomes themselves, as they capture microtubules that are bundled into poles by kinesin-like motor activity. In Drosophila female meiosis, the metaphase I configuration is organized as the final stages of egg maturation are completed (stages 12 and 13), and then the chromosomes are arrested in this configuration until the mature egg (stage 14) is ovulated, at which time meiosis continues to completion (Mahowald et al. 1983).

To ask whether the passenger and condensin complexes are involved in the establishment or maintenance of the metaphase I configuration, we stained ovaries to visualize the DNA, and we examined the chromosome morphoplogy in late-stage mutant oocytes. We distinguished between oocytes that had not yet completely matured (stages 12 and 13) and those that had (stage 14) by the presence or absence of nurse cell debris. Nurse cells are large, polyploid cells that produce vast quantities of mRNA and protein, then undergo apoptosis and dump these products into the oocyte in stage 11 of development. By stage 14, nurse cell debris is eliminated (Spradling 1993).

In wild-type oocytes that had exited the prophase I arrest, the meiotic chromosomes were predominantly seen in a single, round, compact mass. Only rarely were the chromosomes elongated or separated into multiple chromosome masses. This was true in stage 12 and 13 oocytes, and in stage 14 oocytes (Table 4-2). In *dcap-g*^{Z1} */dcap-g*^{K4} mutants, stage 12 and 13 oocytes showed a modest increase in the number of aberrant metaphase I arrest configurations, but stage 14 oocytes revealed a dramatic increase in metaphase I defects, with nearly half the oocytes disrupted. The most commonly observed metaphase I aberration was multiple chromosome masses (data not shown). These results suggest a failure to maintain chromosomes stably at the metaphase I plate, a phenotype consistent with various failures in chromosome dynamics including premature loss of cohesion.

We explored whether the passenger complex is involved in organizing chromosomes at metaphase I, and whether its involvement is similar to that of the condensin complex. We stained *incenp*^{QA26} and *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} ovaries, dissected from females fattened at 18°, and examined late-stage oocytes.

Table 4-2. Mutations in *dcap-g* and *incenp* prevent proper organization of metaphase I configuration

	Stage 12 and 13 oocytes (Nurse cell debris visible)		Stage 14 oocytes (No nurse cell debris present)	
	% with aberrant prometaphase I configurations	n	% with aberrant metaphase I configurations	n
w^{1118}	**17**	29	**9**	78
$dcap\text{-}g^{Z1}/dcap\text{-}g^{K4}$	**26**	15	**45**	20
$incenp^{QA26}/\ incenp^{QA26}$	**55**	49	**9**	46
$incenp^{QA26}, Df(2L)Exel7049/\ incenp^{QA26}$	**69**	49	**17**	115
$incenp^{QA26}, ord^{10}/\ incenp^{QA26}$	**85**	20	**53**	43

Quantification of metaphase I configuration was performed blind.
All ovaries were dissected from females fattened at 18°C except for *dcap-g* mutants, which were fattened at 25°C.

Intriguingly, we found that over half the stage 12 and 13 *incenp*^{QA26} oocytes displayed aberrant metaphase I configurations, but that stage 14 oocytes of the same genotype exhibited defects comparable to those seen in wild-type mature oocytes (Table 4-2). *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} oocytes showed a similar pattern: 69% of stage 12 and 13 oocytes contained disrupted metaphase I configurations, whereas only 17% of stage 14 oocytes displayed this defect. In all cases, multiple chromosome masses were the most commonly observed metaphase I defect (data not shown). These results are striking because premature loss of cohesion, as observed in *incenp*^{QA26} male meiosis, would not explain a defect in which chromosome masses separated from each other transiently and then congressed to a single, stable chromosome mass.

From these data we draw three important conclusions. First, the passenger complex, as well as the condensin complex, has an important function in chromosome dynamics in metaphase I. Second, the roles played by the condensin and passenger complexes in this process are distinct from each other. This is evidenced by the observation that condensin mutants revealed a defect that worsened as maturation continued, whereas passenger mutants gave rise to a clear defect in stage 12 and 13 oocytes that mostly recovered, at least at the level of DNA morphology, by stage 14. Third, the metaphase I aberrations were more severe in *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} mutants than in *incenp*^{QA26} mutants, supporting our hypothesis that removal of one copy of *Aurora B* dominantly enhances the *incenp*^{QA26} mutation.

Mutation of the meiotic gene *ord* dominantly enhances *incenp*^{QA26}

The gene *ord* has also been shown to have an important role in meiotic chromosome condensation. In male meiosis, mutation of *ord* results in defects in packing and pairing of the prophase I bivalents that are remarkably similar to the phenotypes observed in *incenp*^{QA26} spermatocytes (Miyazaki and Orr-Weaver 1992; Resnick et al. 2006). Furthermore, just as the passenger complex is required for synaptonemal complex maintenance, so too is ORD (Webber et al. 2004). Therefore, we asked whether *ord* and *incenp* interact genetically. We introduced a single copy of the *ord*^{10} allele into an *incenp*^{QA26} background. *ord*^{10} generates an early stop codon, and therefore is presumed to be a null allele (Bickel et al. 1997).

We stained the DNA of *incenp*^{QA26}, *ord*^{10} / *incenp*^{QA26} ovaries, from females fattened at 18°, and examined late-stage oocytes. 85% of stage 12 and 13 oocytes displayed aberrant metaphase I configurations, a dramatic increase over the 55% seen in *incenp*^{QA26} mutants (Table 4-2). Even more strikingly, over half the stage 14 *incenp*^{QA26}, *ord*^{10} / *incenp*^{QA26} oocytes also showed metaphase I chromosome defects, in contrast to resolution of these defects seen by this stage in *incenp*^{QA26} alone. These observations raise the possibility that there may be two different types of defects that arise from this mutant combination: one type of defect that is unable to resolve during maturation, accounting for the defects observed in stage 14, and a second type of defect that can recover to form a normal metaphase I configuration, accounting for the higher percentage of defective oocytes in stages 12 and 13 than in stage 14. The aberrations that are resolved by stage 14 are likely caused by the same mechanism as the abnormalities seen in the *incenp*^{QA26} and *incenp*^{QA26}, *Df(2L)Exel7049*/ *incenp*^{QA26} oocytes. However, the defects that persist in stage

14 oocytes when levels of ORD are reduced may be generated by a different underlying mechanism.

In addition to the metaphase I defect, *incenp*^{QA26}, *ord*^{10} / *incenp*^{QA26} females also exhibited premature disassembly of the synaptonemal complex (Table 4-1). In conclusion, the enhanced phenotype suggests that *ord* and *incenp* interact genetically and that both are involved in metaphase I chromosome dynamics. Mutation of a single copy of *ord* has not been shown to result in aberrant phenotypes on its own and reduction of *ord* copy number in a *mei-S332* mutant background does not enhance chromosome segregation defects (Bickel et al. 1998), suggesting that this dominant enhancement of *incenp* by *ord* is likely due to a direct functional interaction between the two rather than simply parallel roles in the same biological process.

Centromere orientation reveals distinct roles for condensin and passenger protein complexes in metaphase I chromosome dynamics

For more insight into the mechanisms underlying the metaphase I defects resulting from disruption of the condensin and passenger complexes, we examined the spindle morphology and centromere orientation of meiotic chromosomes in stage 12-14 oocytes. We mechanically removed the thick eggshell from these oocytes, and stained with antibodies to tubulin and MEI-S332, a cohesion protein that localizes to meiotic centromeres from prophase I until anaphase II (Kerrebrock et al. 1995). In wild-type oocytes, most metaphase I figures displayed the normal morphology in which a single chromosome mass was located at the center of a thin, tapered spindle, and centromeres were positioned toward each spindle pole, reflecting stable biorientation on the spindle

(Fig 4-4A) (Moore et al. 1998). In cases where multiple chromosome masses were present, these were almost always seen as two masses of equal size, each with centromeres located only on one side, the face oriented toward the nearer spindle pole (data not shown). This configuration is consistent with anaphase I behavior, which is triggered at low frequency during preparation of oocyte samples (Theurkauf and Hawley 1992). Because this morphology is seen in wild-type oocytes and the mechanism by which it arises is understood, we focused on mutant oocytes displaying behaviors distinct from this.

In *dcap-g*^{Z1} */dcap-g*^{K4} oocytes, when multiple chromosome masses were present, the predominant phenotype consisted of two chromosome masses, one that localized the centromere marker MEI-S332 on both poleward faces and a second, frequently smaller, mass that had MEI-S332 only on the side toward the nearer pole (Fig. 4-4B). This organization is consistent with a single homolog or chromatid departing from the metaphase plate due to premature loss of cohesion. That this defect might arise from loss of cohesion is also consistent with the observation, described above, that as maturation continues the metaphase I disruption in *dcap-g*^{Z1} */dcap-g*^{K4} oocytes worsens (Table 4-2). Defects in condensation or resolution in the *dcap-g*^{Z1} */dcap-g*^{K4} mutant are also suggested by lagging chromatin between separating chromosomes masses in oocytes activated to undergo anaphase I movements (Fig. 4-4C).

The orientation of separated chromosome masses in passenger complex mutants was distinct from the *dcap-g* phenotype. Despite a characterized role for the passenger complex in MEI-S332 localization (Resnick et al. 2006), the centromere protein localized properly in these oocytes, presumably due to sufficient passenger complex activity in this

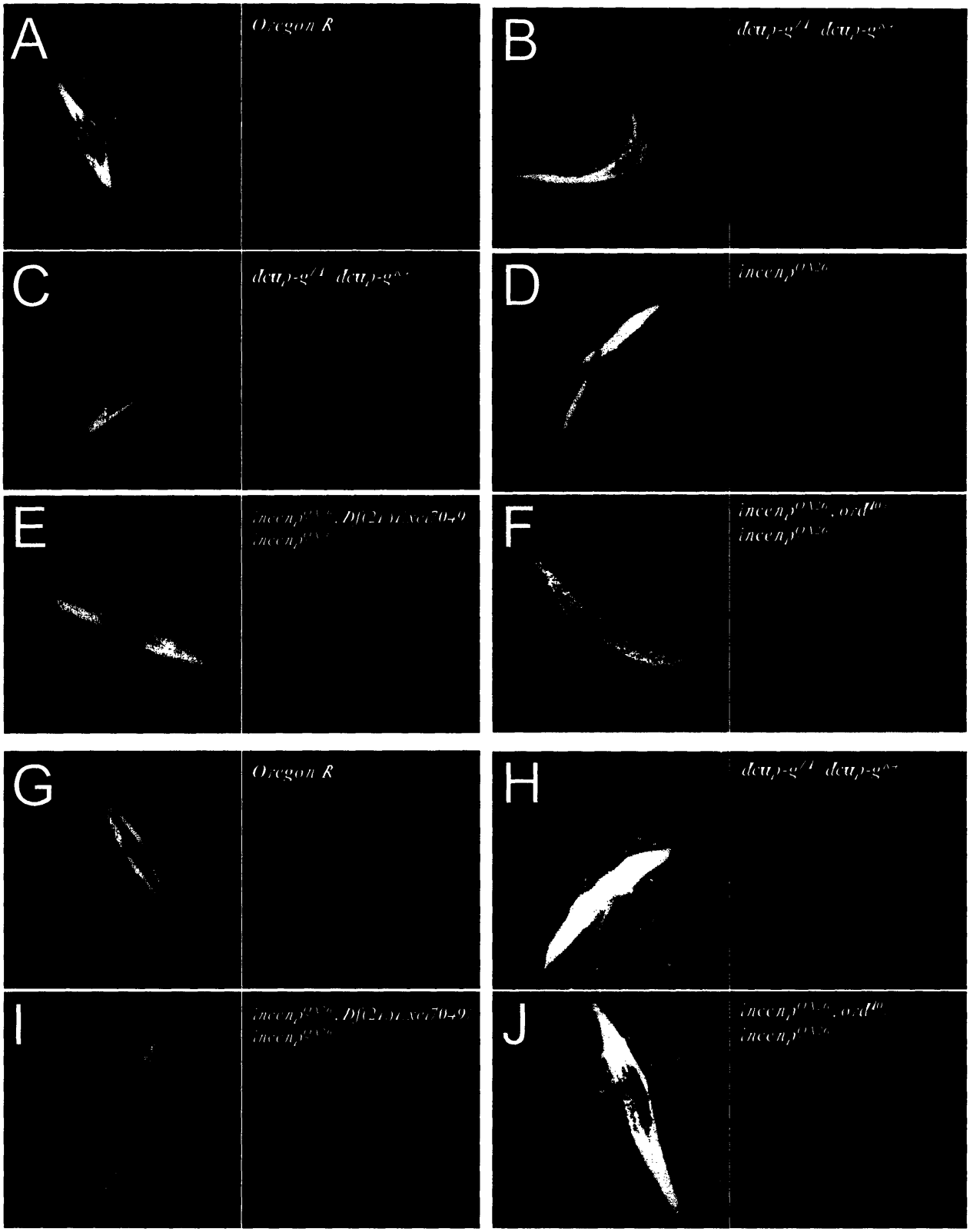


Figure 4-4. Centromere localization reveals differences between mutants in metaphase I configurations and suggests loss of centromere cohesion. Stage 12-14 oocytes stained for DNA (blue), tubulin (green), MEI-S332 (red).

tissue, therefore we were able to use it as a centromere marker. In *incenp*^{QA26}and *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} oocytes with multiple chromosome masses, the predominant pattern was that each chromosome mass appeared bioriented on the spindle, with centromeres on both poleward faces (Fig. 4-4D, E). Taken together with the observation, above, that the multiple masses are chiefly seen in nearly-mature oocytes and not in fully-mature oocytes (Table 4-2), the presence of multiple bioriented masses may suggest bivalents in flux, having not yet congressed to the metaphase I plate. As this organization was never seen in wild-type oocytes, these results are consistent with a role for the passenger complex in the process of chromosome congression in prometaphase I.

In *incenp*^{QA26}, *ord*^{10} / *incenp*^{QA26} mutants, oocytes with multiple bioriented masses, as described for *incenp*^{QA26} alone, were also observed. However, a new category of oocytes was present as well. In these oocytes, more than two chromosome masses were present on the spindle and each had centromere/s oriented only toward the nearer pole (Fig. 4-4F). This organization is consistent with premature loss of cohesion leading to uncoordinated movement toward the poles, and this possibility is also supported by *ord*'s characterized role in cohesion (Miyazaki and Orr-Weaver 1992; Bickel et al. 2002). This configuration was not seen in wild-type oocytes, and can be distinguished from the normal anaphase I movements described above, in which chromosomes move together with only one mass oriented toward each pole. The presence of this class of oocytes with mono-oriented chromosomes in *ord* enhanced *incenp*^{QA26} oocytes but not in *incenp*^{QA26} or *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} oocytes, suggests that these may constitute the class that retains metaphase I defects in stage 14 oocytes (Table 4-2).

Sister centromeres appear separated in condensin and passenger mutants in prometaphase I

In addition to the oocytes described above, some oocytes displayed centromeres scattered across the chromosome mass, in both wild-type and mutant flies. Because in these oocytes the centromeres were not closely positioned together at the poleward face, we were able to examine the number of individual MEI-S332 spots. Wild-type oocytes displayed not more than 8 round, identifiable spots, each presumably corresponding to the centromere of one univalent, with sister chromatids tightly held together by cohesion (Fig. 4-4G). In each of the mutants, we observed examples in which more than 8 round spots were apparent, which likely corresponded to either separated sister centromeres or ectopic MEI-S332 localization. MEI-S332 does not localize ectopically across the chromosomes in *dcap-g* mutants in mitosis (Dej et al. 2004), supporting the explanation that the extra spots in *dcap-g*^{Z1} */dcap-g*^{K4} oocytes are separated centromeres (Fig. 4-4H). *incenp*^{QA26} mutants do display MEI-S332 across the chromatin in spermatocytes (Resnick et al. 2006), however the ectopic localization is seen in a diffuse pattern, not clear, round foci. Therefore, we limited counting of MEI-S332 spots to clear, round foci that are likely to represent centromeres. In the *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} and *incenp*^{QA26}, *ord*^{10}*/ incenp*^{QA26} mutants, these oocytes still revealed at least 11 of these round foci (Fig. 4-4I, J). This is likely an underestimate of the number of separate centromeres, as some of the other bright spots may also represent centromeres. The extra MEI-S332 foci in these mutants support a role for both the condensin and passenger complexes in cohesion of sister-centromeres in meiosis I.

Meiotic chromosomes fail to segregate properly in passenger complex and condensin complex mutants

Failure to coordinate chromosome dynamics in meiosis can disrupt proper partitioning of the DNA and can thereby result in meiotic products that are not haploid, a consequence that is catastrophic for a zygote. To assay whether the genetic material was equally divided in meiosis in *incenp*^{QA26} mutants, we collected embryos laid by mutant mothers at 18°, fixed them less than 30 minutes after egg laying, and stained for DNA. Briefly within this time window, the unused products of meiosis can be visualized individually before they condense and come together to form the rosette structure (Foe 1993). In wild-type embryos these unused products are of equal size (Fig. 4-5A), reflecting the equal number of chromosomes and therefore DNA they contain. In *incenp*^{QA26} embryos, we saw many examples in which the meiotic products were dramatically different in size (Fig. 4-5B, B'). The variation in size of the meiotic products provides strong evidence that meiotic chromosomes were not precisely partitioned, and that the passenger complex plays roles in chromosome dynamics that are ultimately critical for properly coordinating segregation.

To assay the effects of condensin mutation on meiotic chromosome segregation, we took advantage of the observation that the weak allele *dcap-g*^{Z3} allowed recovery of some viable progeny. *dcap-g*^{Z3} transheterozygotes with either the presumptive null *dcap-g*^{K4} or a deficiency that removes *dcap-g*, *Df(2R)vg56*, produce a small number of adult progeny. We used these escapers to assay meiotic chromosome segregation using a genetic assay for nondisjunction of the sex chromosomes. *dcap-g*^{Z3}/*dcap-g*^{K4} females gave rise to 9.2% exceptional progeny and *dcap-g*^{Z3}/*Df(2R)vg56* produced 10.0% exceptional

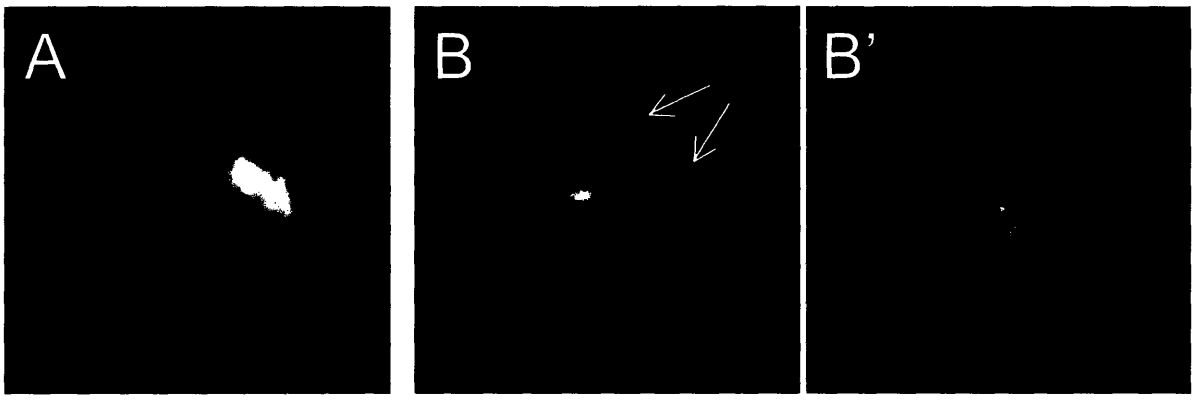


Figure 4-5. Uneven-sized meiotic products reveal chromosome segregation defects in *incenp*^{QA26} female meiosis A) wild-type morphology, meiotic products are equivalent sizes B, B') Two focal planes in the same embryo, laid by *incenp*^{QA26} female at 18°C, uneven sizes (arrows) suggest different chromosome content.

progeny, compared with 2.1% exceptional progeny from *dcap*-g^{Z3}/*CyO* females and 0.0% exceptional progeny from *yw* mothers (n=382, 90, 571, 1620 respectively). This genetic assay for defects in meiotic chromosome segregation shows that the condensin complex, as well as the passenger complex, plays an important role in partitioning meiotic chromosomes.

DISCUSSION

The condensin and chromosomal passenger complexes have both been characterized in mitosis as having important roles in chromosome condensation, and furthermore the passenger complex has been shown in many systems to be required for localization or phosphorylation of condensin proteins (Giet and Glover 2001; Morishita et al. 2001; Hagstrom et al. 2002; Kaitna et al. 2002; Lavoie et al. 2004; Lipp et al. 2007). Strikingly however, mutations in *dcap-g* and *incenp* in Drosophila female meiosis resulted in defects in many of the same processes, but the defects themselves were very different from each other. Polar body rosette structures were elongated and fragmented in the *incenp* mutant, but were hypercondensed or pulverized in *dcap-g* mutants; synaptonemal complex disassembly was premature in *incenp* mutants but delayed in *dcap-g* mutants; metaphase I configurations were disrupted in both mutants, but in clearly distinguishable ways.

The results that both the condensin and passenger complexes affect synaptonemal complex disassembly is intriguing because relatively little is known about the regulation of this process. A suggestion that condensin might be required for synaptonemal complex disassembly arose from the observation that mutation of *nhk-1* disrupts both condensin loading and C(3)G unloading from the chromosomes, within the same developmental window (Ivanovska et al. 2005). Condensin seems not to be required for synaptonemal complex assembly or disassembly in *C. elegans*, whereas it is required for proper synaptonemal complex assembly in *S. cerevisiae* (Yu and Koshland 2003; Chan et al. 2004). Understanding the differences among these systems and the manner in which

condensin is required for synaptonemal complex disassembly in Drosophila remain important questions for future study.

A requirement for the passenger complex in maintenance of the synaptonemal complex is striking in combination with the result that *incenp* and *ord* interact genetically, at least in later stages of meiosis. *ord* is required, as well, for synaptonemal complex maintenance (Webber et al. 2004), raising the possibility that *incenp* regulates *ord* and that the synaptonemal complex phenotype seen in the *incenp* mutant is due to defects in ORD localization or activity. In addition, synaptonemal complex disassembly in *C. elegans* has been suggested to play a role in positioning passenger protein AIR-2 (Aurora B) for its role in releasing cohesion at the onset of anaphase I (Nabeshima et al. 2005), suggesting the interesting possibility that these two complexes dynamically regulate each other's localization.

In our analysis of the metaphase I defects characterized in the $incenp^{QA26}$ mutants, we have suggested that the meiotic defects are not seen in stage 14 because they are resolved as developmental time passes. One alternative explanation for the presence of metaphase I aberrations in stage 12 and 13 oocytes but not in stage 14 oocytes is that defects in the meiotic chromosome behavior might signal to the nurse cells to arrest developmental progression, such that only those egg chambers with unperturbed metaphase configurations reach stage 14. We favor the former explanation for two reasons. First, *spindle* mutants fail to repair double-strand breaks in the meiotic chromosomes and in these mutants no defect in nurse cell apoptosis and cytoplasmic dumping has been observed, and mature eggs are formed normally (Ghabrial et al. 1998). This suggests that defects in meiotic progression do not trigger an arrest in developmental

progression. Second, the proportion of oocytes counted in stages 12 and 13 versus stage 14 is similar in *incenp*^{QA26}, *Df(2L)Exel7049/ incenp*^{QA26} and wild-type ovaries. If a developmental arrest were occurring, a significant increase in stage 12 and 13 oocytes should be apparent. Indeed, the increase in number of stage 12 and 13 oocytes would have to be dramatic to compensate for nearly 70% of oocytes arresting at this stage. An increase in the proportion of stage 12 and 13 oocytes is seen in *incenp*^{QA26} homozygotes, but because this effect is not present in the enhanced genetic combinations, it is more likely a non-specific effect due to a lesion elsewhere on the homozygous chromosome.

The defects in metaphase I configuration lead to several important conclusions about the roles of the passenger and condensin complexes at this stage of meiosis. First, mutations in both complexes resulted in clear abnormalities, strongly supporting a role for both complexes in stable, bipolar attachment of homologs on the metaphase I spindle. Second, the defects from the *dcap-g* mutation and the *incenp* mutation were distinct from each other, suggesting that the roles played by the complexes are different and that the phenotypes observed in the *incenp* mutants are not mediated by defects in condensin localization or activity. Third, *ord* dominantly enhances the *incenp*^{QA26} mutation. Taken together with the observations that *ord*^{10} does not display dominant behavior alone or in a *mei-S332* background, this enhancement of *incenp* may reveal an important functional relationship between the two proteins.

Based on the defects seen for the various mutant combinations and characterized functions of these proteins, we propose the following model that would explain many of the results described above. The metaphase I defects described for the *dcap-g* mutant are consistent with a premature loss of cohesion. Loss of cohesion leads to chromatids or

homologs associated with only a single pole, as seen in the *dcap-g* mutant. Interestingly, in these mutants, only one of the separated homologs or chromatids can be visualized apart from the major chromosome mass, raising the question of where the other homolog or chromatid is. If this model is correct, then the other chromatids must be retained in the major chromosome mass. Retention of a chromatid in the major chromosome mass could be due to defects in condensation or chromosome resolution. Indeed failure to resolve both chromatids and homologs in the absence of condensin subunits has been described in *C. elegans* meiosis (Chan et al. 2004), and we see lagging chromatin between separating chromosome masses in some oocytes. Premature loss of cohesion, however, has not been seen in condensin mutants in other contexts; indeed condensin is required to facilitate cohesin removal in *S. cerevisiae* (Yu and Koshland 2005).

Intriguingly, the defects observed in $incenp^{QA26}$ and in $incenp^{QA26}$, *Df(2L)Exel7049/* $incenp^{QA26}$ mutants are not consistent with loss of cohesion. Once cohesion is lost, chromatids can no longer biorient on the spindle, and therefore defects resulting from loss of cohesion should not resolve over developmental time. In addition, chromosome masses that have separated due to loss of cohesion would not be likely to appear to be oriented in a bipolar manner on the spindle. More consistent would be a defect in interactions between the chromosomes and the meiotic spindle, leading to a delay in congression to the metaphase plate. Such a model would account for both the biorientation and the recovery by stage 14. This explanation is also supported by the role for the passenger complex in meiotic spindle assembly (Sampath et al. 2004; Kelly et al. 2007) and the localization of INCENP to the midspindle region of the metaphase I spindle in Drosophila oocytes ((Jang et al. 2005) and data not shown). Importantly, the

premature separation of centromeres suggested by the increased number of MEI-S332 foci is not inconsistent with this model because cohesion between chromosomes may be retained due to persisting arm cohesion.

In *incenp*^{QA26}, *ord*^{10} / *incenp*^{QA26} oocytes, defects similar to *incenp*^{QA26} are observed as are two additional phenotypes: by DNA stain a significant proportion of stage 14 oocytes are disrupted, and by immunofluorescence some oocytes contain more than two masses each oriented only toward the nearer spindle pole. We consider two possible explanations for these defects that are present in *incenp*^{QA26}, *ord*^{10} / *incenp*^{QA26} but not in *incenp*^{QA26}. First, these defects are consistent with a loss of cohesion, for the same reasons described for the *cap-g* phenotype: separated masses are oriented only toward one pole and do not ultimately congress to a single mass. In addition, *ord* has established roles in cohesion, and so this phenotype may, in a sense, result from reduction of passenger activity enhancing the *ord* mutation. Second, the multiple masses each oriented toward only one pole could also arise from an inability of bivalents to biorient. Such a defect would be supported by the passenger complex's characterized role in destabilizing unproductive kinetochore-microtubule attachments. In this case, the persistence of aberrant configurations in stage 14 might be explained by the severity of the defects in chromosome orientation on the spindle, thereby preventing recovery. However, because these defects were apparent in the *ord* enhanced oocytes, but not in the *Df(2L)Exel7049* enhanced oocytes, and because *ord* has characterized roles in cohesion but not in spindle attachment, we favor the former explanation.

MATERIALS AND METHODS

Fly stocks:

The *dcap-g*^{K3} and *dcap-g*^{K4} alleles and deficiency *Df(2R)vg56* have been described previously (Dej et al. 2004). *dcap-g*^{Z1}, *dcap-g*^{Z2}, and *dcap-g*^{Z3} (*Z2-5052*, *Z2-4027*, and *Z2-5019* respectively) were isolated through a genetic complementation screen with a collection of female-sterile alleles selected from a collection of non-lethal mutations (Koundakjian et al. 2004). The *QA26* allele of *incenp* and the *ord*^{10} allele have been described previously (Bickel et al. 1997; Resnick et al. 2006). *Df(2L)Exel7049* and other stocks were obtained from the Bloomington Stock Center. Flies were raised on standard Drosophila medium at 25°C or 18°C. For *incenp*^{QA26} mutants, meiotic phenotypes were analyzed at 18°C and defects were dependent on this cooler temperature. *incenp*^{QA26} embryonic phenotypes were characterized at 25°C. *dcap-g* analyses were conducted at 25°C unless otherwise noted in the text.

Analysis of cytology and immunofluorescence:

Ovaries were fixed and stained as described (de Cuevas et al. 1996) with DAPI or propidium iodide for DNA, and with antibodies to C(3)G (generously provided by M. Lilly, S. Hawley).

Late-stage oocytes were dissected, fixed, and dechorionated between glass slides as described (Bickel et al. 2002). Oocytes were stained with TOTO-3 (Molecular Probes) and DAPI to detect DNA, and with two rat antibodies to α-tubulin (YL 1/2 and YOL 1/34, Axyll), each at 1:40 overnight. Oocytes were also stained with anti-INCENP rabbit polyclonal serum Rb803 (generously provided by W. Earnshaw) at 1:250 overnight, or

with anti-MEI-S332 guinea pig polyclonal serum (Tang et al. 1998) at 1:1000 over three nights.

Embryos were collected for 30 minutes or 2 hours, dechorionated in 50% bleach, devitellinized in methanol and heptane, and fixed in methanol for 3 hours. Embryos were then RNase treated for 1 hour and stained with YOYO-1 (Molecular Probes) and with antibodies to α-tubulin (YL 1/2 and YOL 1/34, Axyll), each at 1:40.

In all tissues, antibodies were detected using fluorescent secondary antibodies (Jackson Immunoresesarch). Imaging of stained ovaries was performed using a Zeiss microscope with LSM510 confocal imaging software (Keck Imaging Facility), a Zeiss Axiophot microscope with a Spot CCD camera and software, or a Zeiss Axioskop with an AxioCam HRm camera and AxioVision AC software. Images were processed using Adobe Photoshop.

Quantification of metaphase I configuration defects and C(3)G localization was performed blind, on slides for which the genotype of the tissue had been masked.

Phosphatase Treatment of Embryo Extracts and Immunoblotting:

Embryos were collected for 2 hours, homogenized, and treated with lambda protein phosphatase as described (Clarke et al. 2005). Embryo extracts were resolved by SDS-PAGE, blotted to immobilon-P membranes (Millipore), probed with anti-MEI-S332 guinea pig serum at 1:20,000 and with anti-guinea pig alkaline phosphatase-conjugated secondary antibody at 1:5000, and developed with Tropix reagent (Applied Biosystems) as previously described (Clarke et al. 2005).

Meiotic Chromosome Nondisjunction tests:

dcap-g mutant or wild-type control females were crossed at 18°C to *attached-XY*, *v f B* males. Exceptional progeny were distinguishable from progeny generated by normal meiotic chromosome segregation in the female. *Bar* males resulted only from *nullo-X* ova that were fertilized by *X^Y* sperm. Bar^+ females resulted only from *diplo-X* ova fertilized by *nullo-X* sperm. Because only half the exceptional progeny were viable but all the normal progeny were viable, the number of exceptional progeny recovered was doubled to calculate the percentage of exceptional progeny. The number of recovered progeny was doubled and added to the number of normal progeny to generate the total number of progeny.

ACKNOWLEDGEMENTS

We thank B. Wakimoto, D. Lindsley, and M. McKeown who screened the Zuker mutant collection for female sterile lines. Thank you to Janice Lee for a careful reading of this chapter. Some of the microscope images were collected in the Keck Imaging Facility of the Whitehead Institute. TDR was supported by an Anna Fuller graduate fellowship. This research was supported by NSF grant MCB0132237 and NIH grant GM39341 to TO-W.

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Conclusions and Perspectives

Drosophila provide an excellent system in which to study meiosis in part because meiosis is regulated differently in males and females, thereby facilitating examination of different aspects of chromosome dynamics in spermatogenesis and oogenesis. In prophase I in Drosophila females, double-strand breaks are introduced to the DNA, homologs recombine, and the syanptonemal complex forms between homologs (reviewed in McKim et al. 2002). These meiotic events are shared by many meiotic systems, but do not occur in Drosophila males. Instead, pairing and physical attachment of homologs occur by mechanisms that do not depend on recombination and involve proteins specific to male meiosis, including SNM and MNM (Thomas et al. 2005, for a review see Hawley 2002). In addition, meiosis in the female employs a non-canonical spindle that is organized by the chromosomes and lacks centrosomes, whereas the meiotic spindle in the male is organized by centrosomes. Finally, female meiosis is arrested at two points in order to coordinate cell-cycle progression and developmental progression. Meiosis is arrested first in prophase I, while chromosomes are held in a tightly condensed structure called the karyosome, as the oocyte grows and develops. This arrest is released in the final stages of oogenesis. Meiosis is arrested a second time in metaphase I until the mature egg is ovulated (reviewed in Spradling 1993). In contrast, meiosis in Drosophila males proceeds from start to finish without arrests.

These differences in biology between male and female meiosis partially dictate the aspects of chromosome dynamics that are best studied in each system. In this thesis we address the role of the passenger and condensin complexes in synaptonemal complex formation in the female, because in Drosophila it is a female-specific event. In addition, because, in the female, progression from prometaphase I to metaphase I is tied to

morphologically-distinguishable developmental events and because meiosis is then arrested in metaphase I for a prolonged period, oogenesis provides a powerful system in which to examine the process of formation of the metaphase I configuration. Whether the effects shown for the passenger and condensin complex mutants in formation of the metaphase I configuration in the female would also be present in the male remains an open question.

The condensin complex likely plays a similar role in metaphase I establishment in male meiosis, as the condensin components are presumed to function in both systems and the requirements for chromosome condensation and resolution are likely similar. However, if indeed the separated chromosome masses are due, as we have suggested, to premature loss of cohesion, then there may be differences in male and female meiosis, as some cohesion proteins utilized in male meiosis are specific to this system, including SNM which is a homolog of cohesin component SA (Thomas et al. 2005).

The role of the passenger complex in metaphase I assembly may be different in male and female meiosis, as the passenger complex seems to play an important role in assembly of the acentrosomal spindle in female meiosis and localizes to the spindle midregion in metaphase I; INCENP is not seen by antibody staining on the chromosomes at this time (data not shown, Jang et al. 2005). In contrast, INCENP is localized to centromeres in metaphase I in male meiosis (Chapter 3 of this thesis, Resnick et al. 2006).

The role of the passenger complex in centromere cohesion and MEI-S332 localization that we characterized in male meiosis (Chapter 3, Resnick et al. 2006) is likely also important in female meiosis. Both male and female meiosis rely on loss of arm

cohesion in the first division and loss of centromere cohesion in the second division, and both require MEI-S332 for maintenance of centromere cohesion. MEI-S332 localizes normally to centromeres in the passenger complex mutants in female meiosis (Chapter 4), but we presume this is because the effect of the *QA26* allele is weaker in female meiosis than in male meiosis, and not because MEI-S332 localization is independent of the passenger complex in this system. The possibility that the effect of the *QA26* allele is weaker in female meiosis is supported by the observations that characterizable defects in female meiosis were only seen in flies raised at 18°C, whereas defects in male meiosis were apparent at 25°C (Chapters 3 and 4), and that we did not detect defects in INCENP localization in female meiosis even at 18°C, though defects in INCENP localization were apparent in male meiosis (data not shown, Chapter 3).

Finally, the relationship between *incenp* and *ord* is likely important in male meiosis as well as female meiosis. ORD plays important roles in cohesion in both meiotic systems. Indeed, two of the important phenotypic similarities that suggested exploration of a relationship between the two, the prophase I condensation defect and the genetic nondisjunction in both meiotic divisions, were observed in male meiosis. We showed a genetic interaction between *incenp* and *ord* in female meiosis simply for the pragmatic reason that this is the system we were examining at the time we had generated and received the appropriate tools to look for an interaction. Analysis of the relationship between these two proteins in both meiotic systems is an important area for future study.

In addition to providing many new insights into chromosome dynamics in meiosis, the work described in this thesis also raises many important questions for further analysis. Some of these questions are described below.

I. Further analysis of female meiosis in condensin and passenger complex mutants

A. Roles of the complexes in prophase I

Our work has demonstrated that the condensin and passenger proteins have important functions in synaptonemal complex disassembly, however additional roles of these complexes in meiotic prophase I remain uncharacterized. In Drosophila, the synaptonemal complex forms even in the absence of double-strand breaks. One interesting set of questions is whether double-strand breaks are formed and repaired normally in these mutants and whether the proper number of breaks is made. These questions can be addressed using an antibody to γ-H2Av (Madigan et al. 2002), which localizes to sites of double-strand breaks, and examining the stages at which foci appear and disappear, and whether the number of foci is the same in mutant and wild-type oocytes.

In a related question, disruption of double-strand break behavior might affect recombination in meiosis. This can be assayed using a multiply marked chromosome and scoring crossover events in different intervals across the chromosome (Moore et al. 1994). This experiment cannot be performed for *incenp*^{QA26} mutants because the developmental arrest of embryos laid by mutant females prevents recovery of the progeny required for this assay. However, recombination can be scored for *dcap-g* females, using the same allele combination that revealed chromosome nondisjunction.

Additionally, the cohesin components have been shown in other systems to contribute to the axial elements of the synaptonemal complex (Klein et al. 1999; Eijpe et al. 2003). Work from the laboratory of Sharon Bickel (Dartmouth College) has shown

that cohesin subunits SMC1/3 localize along the chromosome axes in prophase I, with similar timing to C(3)G localization (S. Bickel, personal communication). By staining *dcap-g* and *incenp* mutant ovaries for SMC1/3, we can analyze whether this localization, like C(3)G, is disrupted.

This set of experiments will help to define which prophase I processes require the condensin and passenger complexes and thereby to narrow in on the specific mechanisms by which these complexes affect chromosome dynamics at this early stage of meiosis.

B. Defining the disruptions to metaphase I dynamics

In addition to these prophase I events, many questions remain unanswered about the disruptions of the metaphase I configuration that we have described for these mutants. Some of the experiments that might help support or refute our speculative models are not possible in this tissue. Prominent among these, an antibody to a cohesin subunit that is effective in late-stage oocytes has not been identified, preventing us from examining directly whether the cohesin complex is prematurely removed.

Fluorescent *in-situ* hybridization (FISH) has been used successfully to explore cohesion (Dernburg et al. 1996; Bickel et al. 2002). By probing for a repeated element near the *X* chromosome centromere, not more than two spots were seen in wild-type metaphase I configurations whereas four spots were typically seen in *ord* mutants. We could use this technique in *dcap-g* and *incenp* mutants to look directly for loss of cohesion at the centromere and also to explore how many chromatids are present in the various separated masses.

Visualization of more than two separated centromeric FISH signals would support the suggestion that centromeres have likely lost cohesion that was made by the observation of more than eight round MEI-S332 foci, and would eliminate the concern about ectopic MEI-S332 localization. Furthermore, our model that the multiple bioriented masses in *incenp*^{QA26} mutants represent bivalents delayed in congression would be refuted by a finding that *X*-chromosome loci are present in each of the masses, but would be supported by an observation that all observable FISH signals were present in the same chromosome mass. Similarly, in both *dcap-g* oocytes and *incenp*^{QA26}, *ord*^{10} / *incenp*^{QA26} oocytes, the suggestion that premature loss of cohesion may explain the separation of chromosome masses would be supported by visualization of *X*-chromosome loci in more than one mass. Importantly, in this case we would also expect to see examples in which both/all FISH signals were in the same chromosome mass, consistent with the multiple masses being generated by separation of one or both of the major autosomes.

In addition, recent work in the laboratory of Scott Hawley (Stowers Institute for Medical Research) has led to development of a protocol for live visualization of chromosome and spindle dynamics in late-stage Drosophila oocytes as they assemble the metaphase I configuration (S. Hawley, as presented at public meetings). Using this technique could provide more specific information in the *incenp*^{QA26} mutant about the dynamics of the chromosome masses that we speculate to be delayed in congression. Watching these masses in real time could reveal if indeed there is a delay in congression and how long that delay is, whether and how the masses are oscillating between both spindle poles, how spindle dynamics may be involved in this congression phenotype, and whether the *Df(2L)Exel7049* and *ord* enhancements lead to defects that are worse but

similar or that are different in kind from *incenp*^{QA26}. Similarly, live visualization of this process in *dcap-g* mutants could demonstrate the dynamics of chromosome mass separation, answering questions including whether the chromosome masses ever recongress after separation and what behaviors lead to the worsening phenotype seen over developmental time.

II. Interaction between *ord* and *incenp*

The relationship between *incenp* and *ord* is intriguing for several reasons. First, the dominant enhancement by *ord* of the *incenp*^{QA26} metaphase I phenotype suggests that they interact functionally. Second, several strikingly similar phenotypes arise from mutation of each gene: in spermatogenesis prophase I bivalents are poorly compacted, chromosomes undergo nondisjunction in both divisions of male meiosis, and in female meiosis the synaptonemal complex disassembles prematurely (Miyazaki and Orr-Weaver 1992; Webber et al. 2004). Importantly, these phenotypes do not result from disruption of *mei-S332*.

These observations raise the possibility that INCENP is required for ORD localization or function. Imaging of ORD localization has proven difficult and the best results have come from mutating endogenous *ord*, expressing a GFP-ORD transgene, and using antibodies to detect GFP (Balicky et al. 2002). We were unable to detect disruption of ORD localization in the germarium in *ord*, *incenp*^{QA26}; P{GFP-ORD } or in *ord*, *incenp*^{QA26}; P{GFP-ORD } / + flies. However, in this tissue GFP-ORD is expressed at high levels and distinguishing chromosome-specific localization over background levels

of fluorescence in whole-mounted germaria is difficult (Webber et al. 2004), therefore subtle effects may have been missed.

For two reasons, possible defects in ORD localization may be easier to see in male meiosis in these flies. First, the localization pattern is easier to visualize, with clear centromere spots on meiotic chromosomes (Balicky et al. 2002). Second, male meiosis may be more disrupted than female meiosis in *incenp*^{QA26} mutants. This is suggested by the observation that female meiosis was characterizably aberrant only at 18°C, whereas male meiosis was disrupted at 25°C, and by the finding that MEI-S332 localization is aberrant in *incenp*^{QA26} spermatocytes but seems to be normal in *incenp*^{QA26} stage 12-14 oocytes. Therefore characterizing a role for *incenp* in ORD localization may be possible in spermatocytes even though we were not able to see such an effect in oocytes.

If the passenger complex does regulate ORD, it may do so directly by phosphorylation. The consensus target site for Aurora B kinase, RXS/T is found four times within the sequence of ORD: at Ser177, Thr267, Ser322, and Ser449. A first step in determining whether ORD might be a phosphorylation target of Aurora B/INCENP could be to perform *in vitro* kinase assays with these proteins, and if ORD is a substrate *in vitro*, to use targeted mutagenesis to determine which of these sites are important for the modification.

III. A screen for additional genes that interact with *incenp* in meiosis

The finding that *incenp*^{QA26} mutants display moderate phenotypes in both male and female meiosis raised the possibility of genetically enhancing or suppressing these phenotypes. Indeed, we have shown that removal of one copy of *Aurora B* or mutation of

one copy of *ord* dominantly enhances the metaphase I configuration defects observed in the *incenp*^{QA26} mutant. Therefore, a screen for dominant enhancers of *incenp*^{QA26} may be instrumental in identifying additional targets or regulators of INCENP and the other passenger proteins in meiosis. In such a screen, suppressors of the *incenp*^{QA26} defects may also be recovered. These would likely represent inhibitors of passenger complex activity.

One straightforward approach to such a screen would be to use a set of deficiencies on the third chromosome. Because *incenp* is located on the second chromosome, using third chromosome deficiencies prevents the need to make recombinant chromosomes. Ovaries from *incenp*^{QA26} ; *Df* / + females could be dissected, stained with DAPI, and examined for increases in metaphase I configuration defects. Given the observed temporal dynamics of metaphase I aberrations in *incenp*^{QA26} mutants enhanced by a deficiency that removes *Aurora B* and by mutation of *ord*, attention should be paid to stage of oocytes examined in such a screen. If only stage 14 oocytes were examined, important interactors might be missed.

Additionally or alternatively, a screen for enhancement of the nondisjunction phenotype could be performed in males. *incenp*^{QA26} ; *Df* / + males could be crossed to *attached-X* females and assayed for missegregation of the sex chromosomes. Because *incenp*^{QA26} alone results in approximately 16% total exceptional progeny, both enhancers and suppressors of this defect can be recovered. In addition, this assay allows distinction between defects in meiosis-I and meiosis-II segregation. Enhancers or suppressors of *incenp*^{QA26}'s nondisjunction phenotype might affect one of the meiotic divisions more than the other, and such information could be recovered from this screen.

In addition to screening a set of deficiencies across the third chromosome, exploring possible genetic interactions with candidate genes may also provide intriguing new insights. Among these, assaying for genetic interaction between *incenp*^{QA26} and *dcap-g* may provide a valuable new way of exploring the relationship between these two chromosome regulators. Although our analysis of these two genes in female meiosis revealed phenotypes that were strikingly different from each other, we cannot rule out the possibility that the passenger complex regulates the condensin complex. If the passenger complex were required for condensin localization or function, two important factors might confound our ability to detect this interaction by examining the *incenp*^{QA26} mutation individually. First, the *incenp*^{QA26} allele might retain sufficient activity to regulate the condensin complex, at least well enough to prevent a visible defect. Indeed, we know that *incenp*^{QA26} is a weak allele that retains significant function; if it were not then homozygous flies would not be viable (Chang et al. 2006). In addition, INCENP regulates many proteins and the *incenp*^{QA26} allele might disrupt function of one or more of these other targets in a way that is epistatic to defects arising from condensin disruption. In either case, reducing the amount of DCAP-G, or another condensin subunit, present in the fly might draw out phenotypes that suggest the passenger proteins regulate the condensin complex. In exploring a possible dominant genetic interaction, using one of the weak maternal-effect alleles of *dcap-g* described in this thesis is much less likely to show an interaction than using a stronger allele. The putative null allele, *dcap-g*^{K4}, is a more suitable candidate for such an experiment (Dej et al. 2004).

Intriguingly, preliminary experiments in which a single copy of a deficiency that removes *borealin*, *Df(2L)30A-C,* was introduced into an *incenp*^{QA26} background suggested

that this combination of mutations is lethal. Since these experiments were initiated, an allele of Drosophila *borealin* has been characterized (Hanson et al. 2005). Performing the genetic analysis with this allele rather than with the deficiency may reveal a different phenotype, due to contributing effects from other genes disrupted within the deleted region or elsewhere on the deficiency chromosome. This caveat is greater with respect to *Df(2L)30A-C* than the deficiency used to remove *Aurora B* because the former aberration removes a larger number of genes and also because it was generated by X-ray mutagenesis, making endpoints of the deletion difficult to pinpoint and other abnormalities elsewhere on the chromosome likely. The deficiency used to delete *Aurora B* was generated by molecular recombination techniques that result in much less disrupted chromosomes (Parks et al. 2004). Examining the defects from $incenp^{QA26}$ mutants dominantly enhanced by *borealin*, may reveal additional roles of the passenger complex in meiosis.

IV. MEI-S332 phosphorylation and INCENP's role in male meiosis

We have shown that *incenp* is required for proper localization of MEI-S332 in male meiosis, and have also shown that Aurora B/INCENP phosphorylates MEI-S332 *in vitro* and that this modification is required for localization of MEI-S332 in tissue culture cells. One important follow-up experiment in this analysis is introduction of the mutant MEI-S332, which cannot be phosphorlyated at the relevant Aurora B target site, into flies lacking endogenous MEI-S332 to examine whether localization of the mutant is disrupted in meiosis and whether defects in chromosome segregation ensue. Pursuing this *in vivo* analysis may reveal new insights into MEI-S332 localization and activity. Indeed, *in vivo*

analysis of POLO binding-site mutants showed differences from tissue culture analysis of the same mutants (A. Clarke, personal communication).

In addition to examining, *in vivo*, a mutant form of MEI-S332 that cannot be phosphorylated at the most important site by Aurora B/INCENP, there may also be much to learn from flies expressing a form of MEI-S332 with a phosphomimetic mutation at this site. Based on our current understanding of the system, we might hypothesize that this modification could drive MEI-S332 to the centromere even in an *incenp* mutant background. The phosphomimetic mutation might thereby alleviate chromosome segregation defects in an *incenp* mutant, or it might generate different segregation defects due to a failure to release cohesion. Expression of a such a MEI-S332 mutant would allow us to test these hypotheses. In addition, we often speculate that $incenp^{QA26}$ phenotypes likely arise due to reduction in Aurora B kinase activity. Indeed, this is a likely scenario, but INCENP may also have separate roles that are also important. Therefore, expression of a phosphomimetic MEI-S332 may allow us to begin separating effects arising from disruption of the kinase activity of the passenger complex and defects resulting from other functions of INCENP.

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