The Mechanism of Chromosome Segregation: Genetics and Cytology of the Drosophila Mutants *Dub*, *mei-S332* and *ord*

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ABSTRACT

When a cell divides, its chromosomes must be faithfully partitioned between the daughter cells. This thesis describes three *Drosophila melanogaster* genes that play a role in the mechanism of chromosome segregation. Cohesion between sister chromatids is essential to achieving proper orientation before anaphase in both mitosis and meiosis II. At the onset of anaphase, cohesion must be released. The cloning of two *Drosophila melanogaster* genes required for sister-chromatid cohesion during meiosis, *orientation disruptor* (*ord*) and *mei-S332*, is explained. Both genes predict novel protein products with no homology to proteins from other species. The localization of MEI-S332 protein, principally to meiotic chromosomes in wild type spermatocytes and oocytes, is explored using a hybrid protein of MEI-S332 fused to the autofluorescent protein, GFP. MEI-S332 is seen at the centromere regions of anaphase I chromosomes where sister-chromatid cohesion is believed to be maintained from the first division for the second meiotic division. The protein is first localized to the condensing meiotic chromosomes during prometaphase I, and it is last seen on the meiotic chromosomes at transition from metaphase II to anaphase II, a time when sister-chromatid cohesion must be released. We also examine localization to meiotic chromosomes arrested in meiosis II and meiotic chromosomes in *ord* meiocytes. Segregation of chromosomes during the first meiotic division employs several mechanisms of attachment between homologs. In Drosophila females, exchange and presumably chiasmata mediate attachment between most homologs, but nonexchange chromosomes are also faithfully segregated by what is called the distributive system. Males have no exchange during meiosis I and segregate their homologs using yet a different system from the female distributive system. A conditional dominant mutation, *Double or nothing* (*Dub*), is the only mutation known to disrupt chromosome segregation specifically during meiosis I in both males and females. *Dub* primarily disrupts segregation of the nonexchange chromosomes in females but exchange chromosomes also missegregate to a lesser extent. Additionally, *Dub* homozygotes have temperature-sensitive developmental defects.

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Table of Contents

Acknowledgments 5

Chapter One:
Chromosome segregation during meiosis: Building an unambivalent bivalent 11

Abstract 13
I. Introduction 14
II. Mechanism of chromosome orientation 16
A. Bivalent and dyad structure are critical to orientation 16
B. Reorientation and recognition of bipolar orientation 18
III. Chiasmata 20
A. Chiasmata define points of attachment between homologs 20
B. Crossovers are correlated with chiasmata and disjunction 21
C. Position of crossover can be critical to ensure disjunction 25
D. Why distal crossovers might fail to ensure disjunction 26
E. Proposed means of binding chiasmata 27
1. Terminal binding 27
2. Binding substance near the crossover 27
3. Sister-chromatid cohesion 30
F. Possible mechanisms of sister-chromatid cohesion during metaphase I. 31
1. Catenation as a cohesive factor 33
2. Proteins potentially serving to glue sister-chromatids together 35
a. Mutations disrupting sister-chromatid cohesion early in meiosis .. 35
b. Proteins identified by immunocytology as candidates 36
IV. Homolog attachment and segregation without chiasmata 37
A. Completely achiasmate meiotic divisions 37
B. Non-exchange chromosomes in meiosis with exchange 38
1. The Drosophila distributive system 38
a. Non-exchange homologs associate in prophase I 39
b. Heterologs do not associate in prophase I 39
2. Disjunction without physical attachment: the crowded spindle model .40
V. Sister kinetochore function 41
A. Sister kinetochores must reorganize between meiotic divisions 41
B. Cytological observations of sister kinetochore duplication 42
C. Functional differentiation of sister kinetochores 42
D. Early functional differentiation may be chromosome dependent 44
VI. Maintaining attachment between sister chromatids for meiosis II 45
A. Cytology shows that attachment persists in proximal regions 45
B. Equational nondisjunction resulting from proximal exchange 45
C. Possible mechanisms of cohesion in the centromeric regions 47
D. Mutations that disrupt cohesion for meiosis II 47
VII. Summary 48
Acknowledgments 49
References 50

Chapter Two:
MEI-S332, a Drosophila protein required for sister-chromatid cohesion, can localize to meiotic centromere regions 57

Abstract 59
Introduction 60
Results 62
Isolation of the mei-S332 gene 62
The MEI-S332 protein 65
Nature of the mei-S332 alleles 67
The MEI-S332-GFP fusion protein is localized on meiotic chromosomes ... 68
Does mei-S332 have a role in mitosis? 69
Discussion 76
Localization of MEI-S332-GFP 76
Structure of MEI-S332 protein 78
Mitotic counterpart to MEI-S332? 79
Materials and Methods 80
Acknowledgments 85
References 86

Chapter Three:
Localization of MEI-S332 in oocytes and *ord* meiotic cells 91

Introduction 93
Results 95
MEI-S332 is localized to metaphase I oocyte nuclei 95
MEI-S332 is located on condensed chromosomes after meiosis 96
MEI-S332 remains on condensed chromosomes arrested in meiosis II 103
MEI-S332 is localized to centromere regions in *ord* spermatocytes 104
MEI-S332 is localized to karyosomes in *ord* oocytes 108
Discussion 111
Localization to chromosomes at places other than centromere regions ... 112
Localization without cohesion 112
Localization during mitosis 113
Differentiating between proposed functions of MEI-S332 113
Materials and Methods 115
References 117

Chapter Four:
Identification of ORD, a Drosophila protein essential for sister-chromatid cohesion 119

Abstract 121
Introduction 122
Results 124
Identification of the ORD protein 124

Unusual genetic properties of *ord* alleles suggest a requirement for protein-protein interactions 130
The C-terminal half of ORD is essential for function and mediates negative complementation 134
Discussion 136
Materials and Methods 141
Acknowledgments 146
References 147

Chapter Five:
***Double or nothing*: A Drosophila mutation affecting meiotic chromosome segregation in both males and females** 151

Abstract 153
Introduction 154
Materials and Methods 156
Results 161
Dub is a conditional dominant mutation that causes nondisjunction during meiosis I in females 161
Dub has little effect on recombination 163
Dub primarily affects nonexchange but also exchange chromosomes 166
Cosegregation of chromosomes in *Dub* mutant females 169
Dub dominantly increases nondisjunction during meiosis I in males 171
Dub is a recessive, conditional lethal mutation 174
The nature of the *Dub* mutation 176
Discussion 178
The *Dub* mutation 178
Comparison of *Dub* with other mutations affecting nonexchange chromosomes 179
Possible functions of the *Dub* gene in chromosome segregation 180
Acknowledgments 182
References 183

Chapter Six:
A second look at *Double or nothing* 187

Introduction 189
Materials and Methods 192
Results 198
Dub disrupts organization of the metaphase I karyosome in oocytes 198
The nature of the *Dub* mutation's dominant meiotic phenotype 201
Dub homozygotes and hemizygotes have developmental abnormalities consistent with mitotic defects 203
Dub is located in cytological region 54D-E 204
Putative new alleles of *Dub* 208
Putative second-site suppressors of *Dub* homozygous lethality 213
Discussion 217

Dub disrupts organization of the karyosome in oocytes 217
Nature of the *Dub* mutation 218
Putative new alleles partially revert the *Dub* gain-of-function mutation . . 219
Putative second-site suppressors of *Dub* lethality 219
Initial step toward cloning 220
References 221

Afterword:
Future Considerations 225

Appendix I
Deficiencies in *Drosophila melanogaster* cytological region 59 229

Appendix II
Survey of chromosome *1* for loci that fail to complement *mei-S332* and *ord* 239

Biography of the Author 249

Chapter One

Chromosome segregation during meiosis: Building an unambivalent bivalent

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Abstract

Faithful chromosome segregation during anaphase requires that stable microtubule connections are established between chromosomes and both spindle poles by metaphase. Bipolar orientation follows an active period of transient connections between the kinetochores and poles, and tension mediated through attachments between the chromosomes stabilizes those bivalents that have connections to opposite poles. This review focuses on how the chromatids are tied together in the bivalent to ensure proper segregation in the two meiotic divisions. Homologs are partitioned in meiosis I, and reciprocal crossovers, cytologically defined as chiasmata, usually hold the homologs together for this division. The crossovers themselves must be prevented from migrating off the chromatid arms. Binding substance localized to the crossover and sister-chromatid cohesion distal to the crossover have been proposed to prevent loss of chiasmata. Spontaneous nondisjunction events and mutations that disrupt the maintenance of chiasmata are analyzed in the context of these models. Homologs that segregate in meiosis I without chiasmata are briefly discussed. The bivalent must also be constructed so that four chromatids present only two functional kinetochores prior to anaphase I. Cytology and genetic data suggest that the sister kinetochores are duplicated but constrained to act as a single kinetochore. Additionally, centromeric regions of sister chromatids preserve their cohesion until anaphase II, even as cohesion on the sister-chromatid arms is lost at anaphase I. Mutations that specifically disrupt this process are presented.

I. Introduction

Appropriate partitioning of chromosomes during cell division depends on the arrangement of the chromosomes on the metaphase spindle. Proper segregation of chromosomes is ensured by stable microtubule connections between the chromosomes and opposite poles of the spindle, also called bipolar orientation. The attachments between the chromosomes allow them to resist poleward forces, balancing the connections to opposite poles. Consequently, these paired chromosomes settle at the metaphase plate after a comparatively unstable and active period in prometaphase. The kinetochores of chromosomes and the attachments between chromosomes are vital to achieving bipolar orientation in both mitosis and meiosis.

Chromosomes are segregated differently in meiosis than in mitosis and thus must be attached in different ways. In mitosis, recently replicated chromosomes remain bound together along their lengths until bipolar orientation is achieved at metaphase (Fig. 1A), and all the cohesion is eliminated between the sister chromatids at anaphase (Fig. 1B). Since homologous chromosomes do not segregate from one another in mitosis, they have no need to be attached. Meiosis presents unique requirements for attachment between chromosomes. Following replication, the cell divides twice, reducing the diploid chromosome content to haploid content. The first meiotic division, the reductional division, segregates homologous chromosomes from one another (Fig. 1C-D). In meiosis I, the homologs must be attached to achieve bipolar orientation and segregate reductionally. The second meiotic division, the equational division, segregates sister chromatids (Fig. 1E). Thus, sister chromatids must remain attached in some manner through all of meiosis I, so that they may be oriented and properly partitioned in meiosis II.

The attached meiotic homologs are called bivalents for historical reasons, although four chromatids are in the structure. A pair of sister chromatids in the bivalent is a half-bivalent. If a bivalent dissociates before anaphase I or there is no homolog, the pair of sister chromatids is called a univalent. When the pair of sister chromatids has segregated appropriately from a bivalent at anaphase I, it is referred to as a dyad. The kinetochores on the sister chromatids in a univalent, dyad or half-bivalent are called sister kinetochores.

This review examines what is currently known about the ties between chromosomes during meiosis. Bivalent structure requires that homologs be attached, so the role of reciprocal crossovers between homologs, typically the basis of this

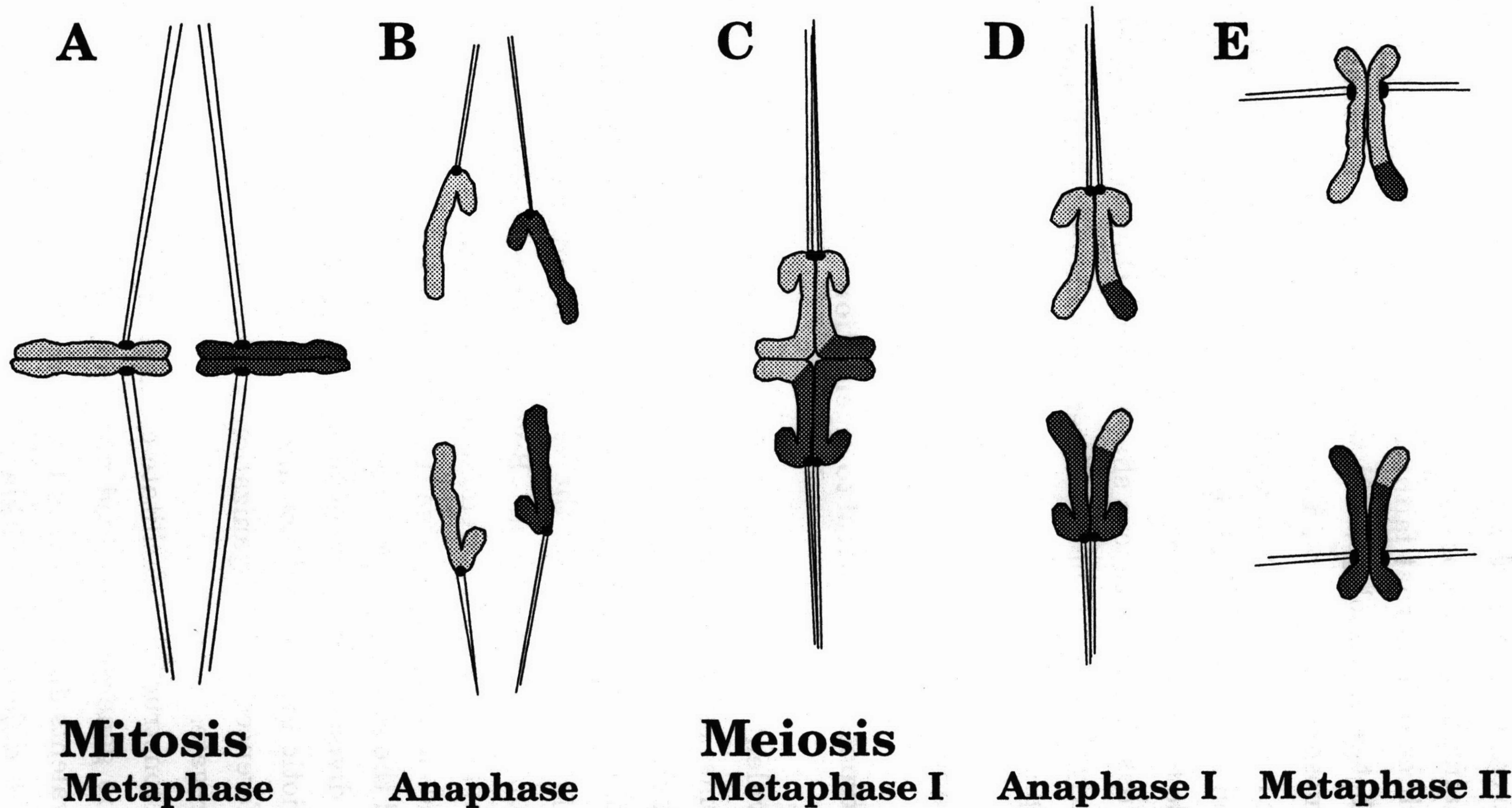

Figure 1: Chromosomes during select phases of mitotic and meiotic cell divisions. Homologous chromosomes are in different shades of gray, kinetochores in black. Thin black lines represent the microtubule fibers connecting kinetochores and spindle poles. (A) During mitotic metaphase, chromosomes align with their kinetochores at the metaphase plate. Sister-chromatid cohesion extends the length of the chromosomes. (B) When mitotic anaphase begins, sister-chromatid cohesion is released along the length of the chromosome. (C) During metaphase I of meiosis, only a portion of the arms of the bivalent is aligned on the metaphase plate, suggesting that chiasmata act as attachments between homologs. Sister-chromatid cohesion extends the length of the chromosomes and may serve to hold the recombinant chromosomes together. The kinetochores of sister chromatids are constrained to face the same direction. (D) During anaphase I of meiosis, cohesion between sister chromatids is released along the arms, but maintained near the centromeres. (E) During metaphase II, the chromosomes align with their kinetochore regions on the metaphase plate. Sister kinetochores are now on opposite sides of the chromatid. Adapted from Luykx (1970).

attachment, will be explored. Reciprocal crossovers by themselves cannot hold homologs together when spindle forces are pulling the chromosomes apart unless a mechanism exists to keep the crossover from sliding off the ends of the chromosomes. Proposed mechanisms for maintenance of crossovers as ties between the homologs will be examined. Not all chromosomes that faithfully segregate in meiosis I use crossovers as attachments between homologs, and we briefly survey alternative methods of holding the chromosomes on the spindle during the reductional division. Attachment between sister chromatids must be preserved through the first meiotic division for proper segregation in the second meiotic division, and we review recently identified proteins responsible for sister-chromatid cohesion during meiosis. Finally, this review will briefly discuss how kinetochore shape affects the attainment of bipolar orientation, particularly the problem of how sister kinetochores function as a single kinetochore before anaphase I.

II. Mechanism of chromosome orientation

A. Bivalent and dyad structure are critical to orientation

Structure within the bivalent and dyad, not factors inherent to the spindle, determine whether chromosomes segregate reductionally or equationally. This was demonstrated by micromanipulation experiments using grasshopper spermatocyte cells fused so that they contained both a meiosis I and meiosis II spindle (Nicklas, 1977). Transfer to another spindle did not alter the behavior of chromosomes with regard to bipolar orientation and segregation. Bivalents transferred from a metaphase I spindle to a metaphase II spindle oriented and segregated as they would in a reductional division. Dyads from prometaphase II spindles were able to orient on prometaphase I spindles, and the sister chromatids segregated from one another as they would in an equational division. Since transfer to a spindle carrying out an entirely different sort of meiotic division did not alter the manner in which the chromosomes segregated, differences in the organization of a bivalent and of a dyad must determine how they segregate.

Bivalents are inherently constructed to facilitate connection to opposite poles. Correct bipolar orientation is generally achieved very quickly. This has been observed, for example, for bivalents during meiosis I in living spermatocytes of the grasshopper species, *Melanoplus differentialis* (Nicklas, 1967). Ostergren (1951) first suggested that initial proper orientation is likely if kinetochores are arranged so that

they face opposite directions. Spindle fibers from a pole connect most readily with a kinetochore facing that pole, so connection to opposite poles is readily accomplished if two kinetochores are constrained to face opposite directions (Nicklas, 1977). In contrast to the general observation that correct orientation is quickly achieved, long flexible bivalents were found to be maloriented more often than smaller bivalents during prometaphase (White, 1961; Nicklas, 1971), presumably because they were less capable of constraining the kinetochores of the bivalent to face opposite poles. The flexibility of these bivalents is thought to be a result of greater distance between the kinetochore and the sites where the homologs are attached, suggesting that the site of attachment is important for the efficiency with which bipolar orientation is achieved (Nicklas, 1971).

The shape of the kinetochore is likely to be another element of bivalent structure important for efficiently establishing connections to opposite poles. Kinetochores are typically cupped by chromatin which may act to hinder access of spindle fibers to the kinetochore itself. Nicklas and Ward (1994) suggest that the cupped shape plays a critical role for the kinetochore that faces neither pole, since the shallower angle of approach of spindle fibers from the more distant pole could favor their attachment over that of fibers from the nearer pole, even though the density of fibers from the nearer pole is greater. The kinetochores of bivalents in *Drosophila melanogaster* spermatocytes are unusually large and protrude such that they are more exposed to spindle fibers from both poles. In studies of living spermatocytes, more than half the bivalents were regularly maloriented and required unusually long times to achieve bipolar orientation, approximately half the period between initial movement at prometaphase and the beginning of anaphase. The unusual shape of the kinetochores has been suggested as an explanation for the lengthy period of reorientation (Church and Lin, 1985).

The unusual bivalents that do not quickly achieve bipolar orientation suggest which elements of the bivalent structure are most important for efficiently establishing connections to opposite poles. The sites of attachment and the shape of kinetochores appear to be critical for the efficiency with which bipolar connections are made. Understanding how orientation is achieved yields further proof that these elements of bivalent and dyad structure are critical for appropriate segregation.

B. Reorientation and recognition of bipolar orientation

Improperly oriented bivalents do reorient and do achieve bipolar orientation. At the beginning of prometaphase I, when interaction with the spindle has just begun, the initial connections between kinetochores and poles are apparently random. All manners of inappropriate microtubule arrangements were observed in electron microscope studies of organisms as diverse as marine worms, insects and plants (Luykx, 1965a; Church and Lin, 1982; Church and Lin, 1985; Jensen, 1982). Reorientation is a lively and active process, and recognition of bipolar orientation is key to attaining the stability ultimately seen at metaphase.

The process of reorientation has been observed in studies of maloriented bivalents artificially produced by their removal from the meiotic spindles of grasshopper spermatocytes. When the bivalent is returned to the spindle, typically a single kinetochore first connected to a pole, and there was movement of the bivalent towards that pole. Subsequently, the other kinetochore connected with the opposite pole, and the bivalent moved to the metaphase plate. Connections to the same pole by kinetochores of both homologs occurred, but these connections were unstable and were quickly lost. The kinetochores made new connections, until eventually a bipolar arrangement was achieved (Nicklas and Staehly, 1967; Nicklas, 1967). Similar initial connection to one pole has been characterized for mitosis as well as meiosis, by observation of both fixed and living cells of several species (see review by Rieder, 1982).

Bivalents are relatively stable at the metaphase plate. Bipolar orientation is recognized in some manner, so that connections between kinetochore and pole do not continue to be lost. Mechanical tension stabilizes the spindle fiber connection. Ordinarily, bipolar orientation provides this tension, since poleward forces pull the kinetochores in opposite directions and are counteracted by the bonds that hold the bivalent together (Fig. 2A). The role of tension in creating stable connections was demonstrated experimentally by providing artificial tension. In one experiment, bivalents with both kinetochores connected to the same pole were stabilized by using micromanipulation to provide an opposing force (Fig. 2B; Nicklas and Koch, 1969). In another type of experiment, micromanipulation or heat shock during prophase I produced bivalents that were tangled or linked with one another, mimicking attachments between chromosomes. These bivalents also achieved a stable position

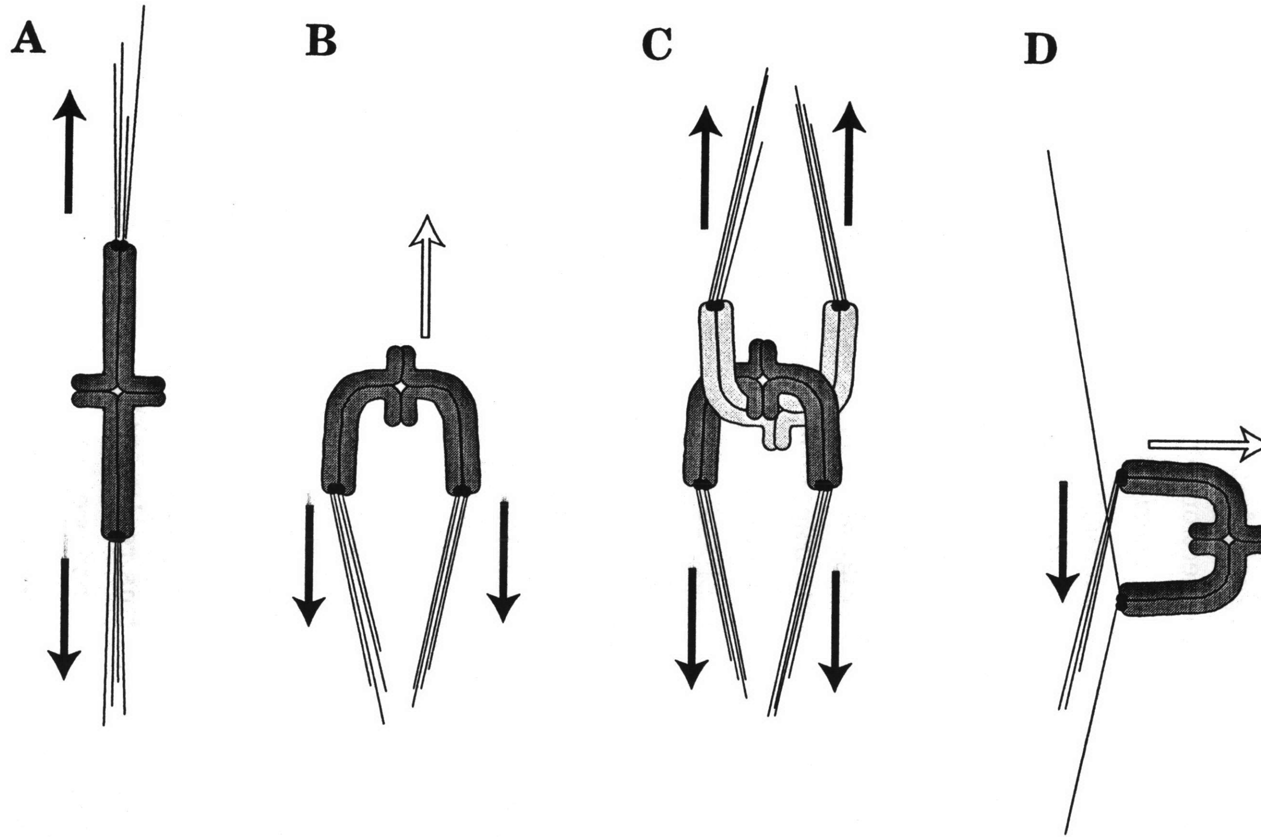

Figure 2: Tension stabilizes spindle fiber connections between kinetochore and pole. (A) In the typical bivalent during metaphase I, spindle fiber connections to the poles provide poleward force (black arrows) and are stabilized by the tension acting through attachments between homologs. (B) A single bivalent during metaphase I has spindle fiber connections between both kinetochores and one pole stabilized by artificial force (white arrow) towards the opposite pole. Artificial force is provided by micromanipulation with a needle. (C) Two bivalents with connections to opposite poles are entangled, mimicking attachments between the homologs, so that tension stabilizes these connections. (D) Artificial force (white arrow) applied perpendicular to the spindle axis stabilizes the connection between the pole and the kinetochore under tension. The kinetochore that is not under tension usually reorients.

on the metaphase plate (Fig. 2C; Henderson and Koch, 1970; Buss and Henderson, 1971). Tension on kinetochores apparently stabilized connections made to spindle poles.

The kinetochores in the above experiments directly faced a single pole. If exposure of a kinetochore to spindle fibers from a pole is critical to making a connection with that pole, it could be argued that tension might not have stabilized the connection. Instead, spindle fiber connections with the other pole may have been hindered by the bulk of the chromosomes, reorientation might have been inhibited, and the kinetochores only seemed to have a stable connection. In recent experiments by Nicklas and Ward (1994), micromanipulation was used to apply a force perpendicular to the spindle axis, towards the cytoplasm rather than towards either pole. In this way, kinetochores that did not directly face a single pole could be studied. Kinetochores under tension maintained a connection to a pole while kinetochores without tension frequently reoriented (Fig. 2D). Mechanical tension, rather than exclusion of spindle fibers from the opposite pole, proved to be the stabilizing factor.

In summary, proper segregation of chromosomes depends on appropriate connections to opposite poles at metaphase. In prometaphase I, the sites of attachments between homologs and the shape of the kinetochores were shown to be important for attaining bipolar orientation efficiently. Bipolar orientation at metaphase is stable, because spindle fiber connections to the poles are stabilized by mechanical tension. Ties between chromosomes within the bivalent are essential for tension.

III. Chiasmata

A. Chiasmata define points of attachment between homologs

Homologs are attached before anaphase I, usually through chiasmata. Exceptions will be addressed later in this review. Chiasmata are observed on the arms of chromosomes in the bivalent in late prophase I. In early prophase I, during pachytene, the homologs have been paired and, in most species, a structure called the synaptonemal complex (SC) is built between them along their length. The SC consists of lateral elements located between the sister chromatids and a central element connecting these lateral elements. Before the central region is in place, the lateral elements are referred to as axial elements. Later in prophase I, at stages termed diplotene and diakinesis, the SC dissolves, and the homologs repulse one

another except at localized points of attachment located on the arms of the chromosomes. The points of attachment are the chiasmata.

The cytology of meiotic cells suggests the role of chiasmata is to hold homologous chromosomes together to provide tension needed for proper orientation. During metaphase I, the arms of the chromosome rather than kinetochores are aligned on the metaphase plate (Fig. 1C), unlike metaphase of mitosis or meiosis II, where kinetochores are aligned on the plate (Fig. 1A, 1E). For bivalents, then, the ties between the chromosomes are not at the kinetochore but are on the arms of the homologs.

B. Crossovers are correlated with chiasmata and disjunction

It is generally accepted that chiasmata are associated with reciprocal crossovers between the homologous chromatids. Experiments by Tease and Jones (1978) using spermatocytes of a locust, *Locusta migratoria*, showed that crossover exchange points within the bivalents, when cytologically detected, were located at the same place as the chiasmata. Exchange events were detected by differentially labeling the sister chromatids with 5-Bromodeoxyuridine incorporated during replication, so that a crossover between dissimilarly labeled chromatids in the bivalent gave a visible exchange point (Fig. 3A-B). Similar experiments in other species gave like results, suggesting that the accordance of crossovers with chiasmata is a general phenomenon (Jones, 1987), although the absolute correspondence of crossovers and chiasmata continues to be questioned (see review of data from plants by Nilsson, et al., 1993).

Crossovers are generally necessary for proper segregation of homologs. A plethora of mutations that reduce or eliminate exchange result in high frequencies of missegregation during the reductional division (Jones, 1987; Hawley, 1988; John, 1990).

More recently, the role of crossovers in ensuring segregation has been examined in organisms without mutations that reduce the overall level of exchange. Missegregation events occur at low frequencies during meiosis in organisms that are otherwise wild type. The origin of spontaneous missegregation events during meiosis I was assessed by reconstructing the recombinational history of chromosomes found in aneuploid progeny of humans and Drosophila. Because the disomic gamete that gave rise to aneuploid progeny could be the result of missegregation in either of the two meiotic divisions, it was critical that errors in disjunction during meiosis I were

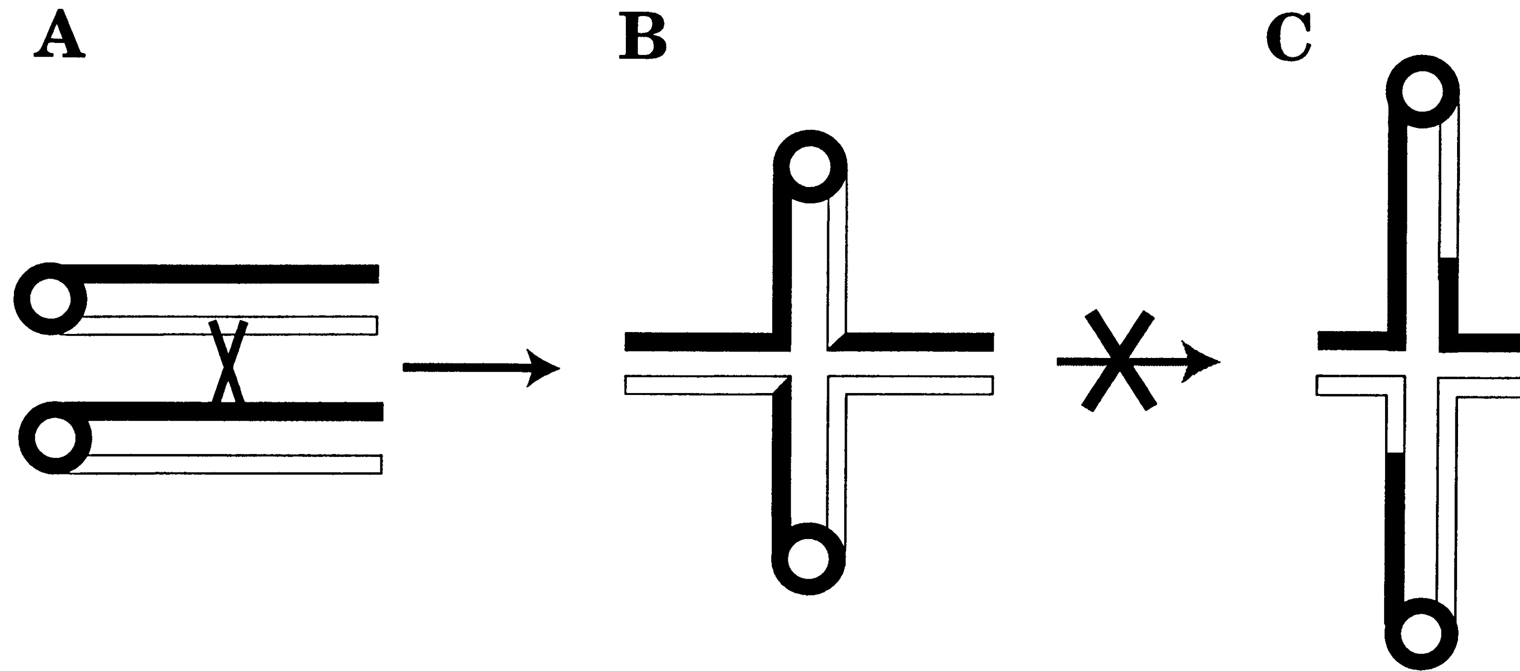

Figure 3: Chiasmata and crossovers are at the same location. Homologs are depicted as white and black lines, and open circles represent centromeres. (A-B) A single crossover between "white" and "black" chromatids in a differentially labeled bivalent yields a visible crossover point in the metaphase bivalent. Note that a single crossover between similarly labeled chromatids would not result in a metaphase bivalent with a visible crossover point. (C) If the crossover migrates towards the ends of the chromatids, regions of similarly labeled chromatid would be seen between the kinetochores and the chiasma. Terminal movement was not observed.

differentiated from errors in meiosis II by using centromere-linked markers (Fig. 4A-C). Chromosomes derived from missegregation during meiosis I had quite different recombinational histories than did chromosomes from meiosis II missegregation events.

The overall frequency of crossovers was reduced in bivalents that underwent spontaneous meiosis I missegregation relative to bivalents carrying out successful meiosis I disjunction. When the recombinational history of chromosomes in human trisomy resulting from meiosis I errors was compared to that of chromosomes from successful meiotic segregation, there was an increased frequency of non-exchange and single-exchange events. This was observed for chromosome 16 (Hassold, et al., 1995), for chromosome 18 (Fisher, et al., 1995) and for chromosome 21 (Sherman, et al., 1994), using DNA markers to analyze the parental origin of the chromosomes and the exchange events that occurred during the meiosis that gave rise to them. The X chromosomes from XXY and XXX individuals similarly experienced reduced amounts of exchange. Notably, most meiosis I nondisjunction occurred in non-exchange bivalents (MacDonald, et al., 1994). Spontaneous meiosis I nondisjunction in *D. melanogaster* females was surveyed by examining visible markers in progeny conceived from ova disomic for the *X* chromosome. The majority of chromosomes were derived from bivalents that had no exchange event. The remainder derived from bivalents with a single exchange event (Koehler, et al., 1996a). The single-exchange bivalents that proved inadequate will be further discussed in the next section. Thus, an appropriate frequency of exchange has been shown to be important for proper segregation of individual bivalents in organisms from normal populations of humans and Drosophila, not just in populations with reduced meiotic exchange due to a mutation.

Crossovers are usually sufficient to segregate chromosomes properly in meiosis I. Indeed, a single crossover in a bivalent has been shown to be sufficient to produce disjunction of chromosomes that are only partly homologous. A small pseudoautosomal region near the telomere of the X and Y chromosomes in humans and mouse has a genetic length consistent with a single crossover, and this appears to ensure disjunction of these mostly nonhomologous chromosomes (Cooke, et al., 1985; Page, et al., 1987). Similarly, rearranged chromosomes in *D. melanogaster* that carried homologous regions resulting from translocation were shown to ensure disjunction of nonhomologous centromeres (Hawley, 1988).

To briefly recapitulate, chiasmata are the cytologically apparent sites of attachment between homologs. Reciprocal crossovers correspond well with

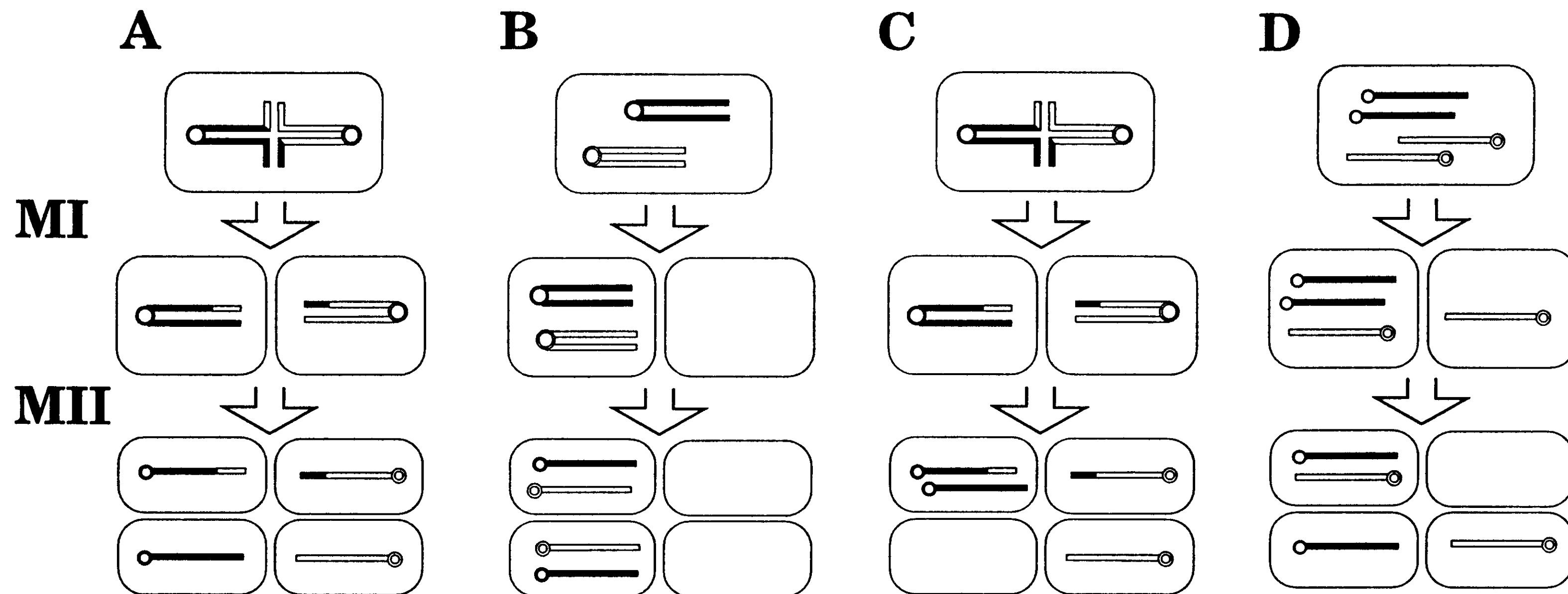

Figure 4: Products of missegregation events during meiosis. Two meiotic divisions partition the chromatids in a single bivalent into four meiotic products. Homologs are depicted as white and black and open circles represent centromeres. Centromere-linked markers allow homologs to be distinguished from sister chromatids even if exchange has occurred. (A) Faithful segregation: appropriate partitioning of chromatids results in one chromatid per meiotic product. (B) Meiosis I error: nondisjunction during the first meiotic division followed by disjunction in the second division yields two nullosomic gametes and two gametes disomic for homologs. (C) Meiosis II error: nondisjunction during meiosis II yields a nullosomic gamete, a gamete disomic for two sister chromatids, and two normal gametes. (D) Precocious separation of sister chromatids: random segregation of sister chromatids through two divisions sometimes yields the unique set of two monosomic gametes, a nullosomic gamete and a gamete disomic for homologs. Random segregation of sister chromatids can also result in four meiotic products that resemble a meiosis I error (B) or a meiosis II error (C). In species where all four products of one meiosis cannot be recovered, random segregation of sister chromatids can be suggested by the relative ratio of gametes disomic for homologs, gametes disomic for sister chromatids and nullosomic gametes (Miyazaki and Orr-Weaver, 1992). With the exception of precocious separation of sister chromatids, these genetic assays do not indicate whether chromosomes disjoined early or maintained attachments too long.

chiasmata, and crossovers are usually necessary and sufficient to ensure proper segregation. The exceptional crossovers that are not sufficient to ensure segregation provide clues to how a bivalent is built.

C. Position of crossover can be critical to ensure disjunction

Exchange is constrained in most organisms such that it is not randomly distributed within the length of the chromosome nor randomly distributed among the chromosomes. Crossovers are usually in the euchromatin and most commonly occur in the medial portion of the chromosome. The terms proximal, medial and distal refer to locations relative to the centromere of the chromosome. Usually, there is at least one exchange event per chromosome, even on chromosomes that are relatively small (Jones, 1987; Hawley, 1988; John, 1990).

In the studies of spontaneous meiosis I errors described earlier, the location of an exchange event was vital to its success at ensuring disjunction. Proximal and medial exchanges were underrepresented among bivalents that led to nondisjunction, while single exchanges in a distal location occurred more frequently in bivalents that experience meiosis I errors than they did in bivalents that disjoin properly. The genetic map lengths of the most distal region of chromosomes from successful segregation events were often smaller relative to that of chromosomes from missegregation. In human ova, this was observed for the short arm of the X chromosome (MacDonald, et al., 1994), for chromosome 16 (Hassold, et al., 1995) and for chromosome 21 (Sherman, et al., 1994). Distal exchanges on the *X* chromosome in Drosophila ova similarly had less ability to ensure disjunction (Koehler, et al., 1996a).

The tendency of non-exchange and distal exchange bivalents to be highly represented in spontaneous missegregation has also been observed for Drosophila chromosome *2* (Carpenter, 1973; Gethmann, 1984). Moreover, in the presence of either of two dominant mutations in Drosophila that primarily disrupt segregation of non-exchange chromosomes in ova, the exchange bivalents that did nondisjoin most frequently had distal exchange events. This has been shown for the *X* chromosome in *Dub* females (Moore, et al., 1994) and for both the *X* and chromosome *2* in *nod*^{DTW} females (Rasooly, et al., 1991).

In *Saccharomyces cerevisiae*, distal exchange events were also observed to be less effective for the segregation of engineered chromosomes not required for viability. Yeast model chromosomes carry elements known to be essential for normal

replication and segregation, namely centromere sequence, telomeres and an origin for replication. Missegregation of test bivalents can be examined without apprehension about progeny inviable due to aneuploidy. Exchange events on the artificial chromosomes usually ensured reductional segregation, but distal exchange events were less effective at ensuring disjunction than were proximal and medial events (Ross, et al., 1992). Evidence from species as diverse as humans, Drosophila and budding yeast demonstrates that distal exchange provides less secure ties between homologs than does medial or proximal exchange.

D. Why distal crossovers might fail to ensure disjunction

While an exchange is usually sufficient to ensure disjunction, the location of the crossover is also quite important. One possible explanation is that distal events do not provide as stiff a linkage between the kinetochores of the homologs as do medial exchange events, and this results in nondisjunction (Rasooly, et al., 1991). At least two arguments may be raised against this hypothesis: 1) The centric heterochromatic regions of homologs have been shown to be paired throughout prophase I in Drosophila oocytes and have been suggested to orient even non-exchange homologs during prometaphase I (Dernburg, et al., 1996), so the location of crossovers on homologs might be expected to have little effect on bivalent organization in the centromeric region of this species; and 2) The distal exchanges on the yeast model chromosomes are closer to their centromeres than many exchanges on the natural yeast chromosomes, yet the natural chromosomes segregate appropriately (Ross, et al., 1996a). Thus, it is proximity of the exchange event to the end of the chromosome, rather than distance from the centromere, that results in occasional missegregation.

Perhaps distal crossovers are inclined to be lost as attachments, since a crossover serves as a connection between the centromeres of the homologous chromosomes only so long as the crossover does not migrate off the ends of the chromosomes. During orientation in prometaphase I, the centromeres of the homologs are pulled in opposing directions, so there must be a mechanism to prevent crossovers from being lost as attachments. Maguire (1974) called this requirement for a contrivance to maintain crossovers "the need for a chiasma binder."

E. Proposed means of binding chiasmata

There are three general proposals for how chiasmata are prevented from migrating off the ends of the chromosomes: (1) migrating chiasmata are stopped by structures at the terminal ends of the bivalent; (2) binding substances at the site of exchanges hold the chiasmata in place; and (3) cohesion along the length of the sister chromatids is not released so migration is not possible. The last two mechanisms are not exclusive of one another and could potentially act redundantly.

1. Terminal binding

In the first model, if the terminal ends of the sister chromatids cannot be separated, exchange crossovers that occur in medial portions of the chromatid may migrate nearly to the chromosome ends and still serve as a bond between the homologs. One specific proposal suggested that the telomeres of the sister chromatids are not duplicated until the metaphase I/anaphase I transition, so that crossovers might move to the ends of the bivalent chromatids and be caught there to act as an attachment between the homologs (Egel, 1979). Chiasmata terminalization was suggested by early cytological studies of fixed meiotic cells, but it is no longer generally accepted (see appropriate sections in reviews by either Carpenter (1988) or by Jones (1987)), although it has been argued that the degree of terminalization may be species-specific (von Wettstein, et al., 1984). In species where chiasmata were directly examined for terminalization in meiosis, it was apparent that the chiasmata were not migrating terminally. Metaphase bivalents with differentially labeled sister chromatids would be expected to display regions of equivalently labeled chromatin if terminalization brought homologous regions together (Fig. 3C), but this was not observed. Within the extent of resolution in each animal system, no chiasmata movement occurred. Terminalized chiasmata usually correlated with a terminal exchange point in locust spermatocytes (Tease and Jones, 1978), in hamster spermatocytes (Allen, 1979), and in mouse spermatocytes (Kanda and Kato, 1980) and ova (Polani, et al., 1981).

2. Binding substance near the crossover

In the second model for a chiasma binder, a binder substance is proposed to hold the exchange event at or near the original site of the exchange. A mutation that

disrupts chiasma binding should result in nondisjunction of exchange bivalents, so such mutations will be reviewed here for evidence to support this model. Analysis of the mutations should be approached with the following general considerations. Exchange should occur at normal levels if the mutated gene product is required only as a binding substance to maintain a chiasma. If a mutant exhibits reduced levels of exchange, it suggests the gene is required for establishment as well as maintenance of functional chiasmata. Cohesion in the proximal region of the half-bivalents would be undisturbed in a mutation that is specific for binding chiasmata, thus cytology should reveal unusual numbers of univalents but should not reveal separated sister chromatids during metaphase I. Separation of sister chromatids before anaphase I suggests a more general loss of sister-chromatid cohesion. In addition to cytology, genetic assays of nondisjunction events can suggest whether complete loss of cohesion between sister chromatids is occurring before metaphase I (Fig. 4D).

The "desynaptic" mutations of *Zea mays* best meet the above criteria for a mutation in chiasma binding. *dy1* and *dsy1* are two desynaptic mutations that have been well characterized, although others have been reported (Golubovskaya, 1989). These mutations have not been tested for complementation.

Consistent with the expectations for a mutation in binding substance function, both univalents and bivalents with one open arm were observed in metaphase I microsporocytes homozygous for the desynaptic mutation, *dy1*. Crossovers occurred at wild-type levels. Heterochromatic knobs that are cytologically visible on the arms of maize chromosomes allowed exchange events to be directly assessed in strains heterozygous for the knob. Strikingly, exchange events had often occurred on the arms of univalents and on the open arms of bivalents, providing graphic evidence of exchange events that were not maintained as chiasmata. Cohesion at the univalent kinetochore was maintained until anaphase I, although some equational segregation was observed during this division. However, more monads (single chromosomes resulting from early separation of a dyad) were seen during prophase II than can be accounted for by these equational segregation events (Maguire, 1978a). Thus, contrary to the ideal expectations for a mutation in chiasma binding substance, *dy1* seems to disrupt sister-chromatid cohesion at some time after the beginning of anaphase I. In addition, the central element of the SC was unusually wide in *dy1* mutants (Maguire, et al., 1991).

Exchange events that failed to bind bivalent arms together were observed cytologically as well, and cohesion at the univalent kinetochore was reported to be maintained until anaphase II (Golubovskaya, 1989; Maguire, et al., 1993). These

observations are consistent with our criteria. However, the frequency of exchange was not reported for *dsy1* homozygotes, and it may be quite reduced. Synapsis was not always complete, and these mutant microsporocytes also had a wide central region in their SC (Maguire, et al., 1993).

The maize desynaptic mutations remain the best candidates for mutations in chiasma binder, although both *dy1* and *dsy1* mutants also have phenotypes that overlap with more general meiotic functions. *dsy1* mutants do not achieve full synapsis and have not been shown to have exchange at normal levels, so this gene may be essential for establishment of chiasmata. The failure of *dy1* mutants to retain cohesion between sister chromatids until metaphase II suggests the gene may be needed for additional functions in sister-chromatid cohesion. It may be difficult to identify a mutation that specifically disrupts chiasma binding using such ideal expectations. Separation of maintenance of chiasmata from establishment of chiasmata may require an unusual allele of a gene required for more than one function. Moreover, binding at the site of a crossover might be redundant with sister-chromatid cohesion in the maintenance of crossovers, so that both mechanisms must be disrupted to result in frequent chiasmata failure.

The wide central element of the SC reported for both the maize desynaptic mutations suggest that mature SC might play a role in establishing or maintaining crossovers that can serve to attach the homologs. Normally, most of the SC dissociates following pachytene. However, remnants of SC were observed to be associated with chiasmata as late as diplotene in diverse organisms (Jones, 1987) including maize (Maguire, 1978b), locust and grasshopper (Moens and Church, 1979) and mouse (Solari, 1970). Disassembly of SC may simply be hindered near sites of attachment, but the possibility exists that SC remnants act to bind chiasmata in place.

Several observations argue against a general requirement for the SC in chiasma maintenance. The Zip1 protein of *S. cerevisiae* was localized to the central region of mature SC and is likely to be a component of the central region. Strains harboring *zip1* mutations have a defect in synapsis; full-length axial elements and paired homologs varied in proximity to each other along their lengths. However, crossovers occurred at approximately wild-type levels, and exchange still ensured disjunction. Sister-chromatid cohesion does not appear to be defective (Sym and Roeder, 1994). Two organisms, *Schizosaccharomyces pombe* and *Aspergillus nidulans*, are unusual in having no SC, although they have structures that look like axial elements. Both species have crossovers in meiosis that ensure disjunction (Olson, et al., 1978; Egel-

Mitani, et al., 1982). Thus, the central region components of the SC are not essential for crossovers that function as attachments between homologs.

Axial SC components, however, could play an important role in establishing and maintaining chiasmata. Exchange bivalents missegregated in *S. cerevisiae* meiotic cells homozygous for *red1,* and these mutants failed to make axial elements (Rockmill and Roeder, 1990). Red1 protein has been suggested to act in initiating the axial element (Roeder, 1995). Recombination was reduced in *red1* meiotic yeast, although the extent to which it was reduced varied widely from region to region. Precocious separation of sister chromatids was seen only at a low level, suggesting that cohesion between the sister chromatids was usually retained until the second meiotic division (Rockmill and Roeder, 1990). The *red1* phenotype suggests that components of the SC might play a part in producing crossovers that can function as attachments between homologs at metaphase I.

Although cytological studies have placed SC remnants near chiasmata late in prophase I, there is no functional evidence that SC remnants play a role in maintenance of chiasmata. No mutations yet exist that specifically meet the ultimate expectations of a chiasma binder (see also review by Carpenter (1994)), although the maize desynaptic mutations meet many of the criteria. It is not yet clear if binding substance exists at the site of crossovers.

3. Sister-chromatid cohesion

Cohesion between the arms of sister chromatids has been proposed as a mechanism to maintain chiasmata. A crossover between two homologous chromosomes cannot migrate to the end of the chromosomes if the distal portion of the recombined chromosome is tightly bound to its sister (Fig. 1C). As a corollary, cohesion along the arms of sister chromatids must necessarily be released during anaphase I beyond the most proximal chiasma, so that the recombined homologs are able to segregate from one another (Fig. 1D).

Sister-chromatid cohesion provides the simplest explanation of why distal chiasmata might be less successful than more proximal chiasmata in ensuring disjunction. The more distal the location of the chiasma, the shorter the length of sister cohesion that would be able to maintain it. If binding substance alone holds chiasmata at sites of exchange, then the length of chromosome distal to the crossover should be irrelevant.

A slow degradation of sister-chromatid cohesion best explains the increase in meiosis I nondisjunction frequency observed as oocytes age in human females. The chromosomes from human trisomy 21 progeny exhibited decreased amounts of recombination and often had single exchange events in the distal regions (Sherman, et al., 1994). Recombination occurs prenatally in human females, followed by a long period of arrest in prophase I until ovulation, a period that can be as long as 50 years. Since oocytes are held in prophase I, well before the meiosis I spindle is built and the bivalents orient, the increase in nondisjunction seems most likely to be due to slow failure of sister-chromatid cohesion over time. The more proximal the crossover, the greater the length of sister chromatid distal to the chiasma, increasing the likelihood that some sister-chromatid cohesion remains to prevent loss of the crossover as an attachment between homologs when the forces of the meiotic spindle begin acting on the bivalent.

In maize microsporocytes, chiasma-like associations often persist after anaphase I for acentric fragments resulting from exchange events on chromosomes heterozygous for paracentric inversions. These have been used by Maguire (1993) to test the three models of mechanisms that maintain chiasmata. In the most easily interpretable case, an acentric fragment and cytologically distinct homologs result from two exchange events involving three strands, one crossover proximal to the paracentric inversion and one within the inversion (Fig. 5A-B). One homolog is a loop dyad and the other homolog is normal. Binding substance localized to the chiasma would give the acentric fragment a tug towards the same pole as the loop dyad. Sister-chromatid cohesion distal to the chiasma would cause the acentric fragment to travel with the normal homolog (Fig 5C-D). The latter event happened frequently and was interpreted as demonstrating that cohesion between sister chromatids is most likely to function as a binder. However, this experiment relies on associations that exist after metaphase I. This persistent association does not exclude the existence of a binder substance at the chiasmata that is weaker than sister-chromatid cohesion or is simply released earlier than sister-chromatid cohesion. Another interpretation of this experiment is discussed in the next section.

F. Possible mechanisms of sister-chromatid cohesion during metaphase I

Sister-chromatid cohesion during meiosis is likely to be even more functionally complex and intricate than during mitosis. Not only must the sister chromatids be held together, but they must be inhibited from interactions that take place in mitotic

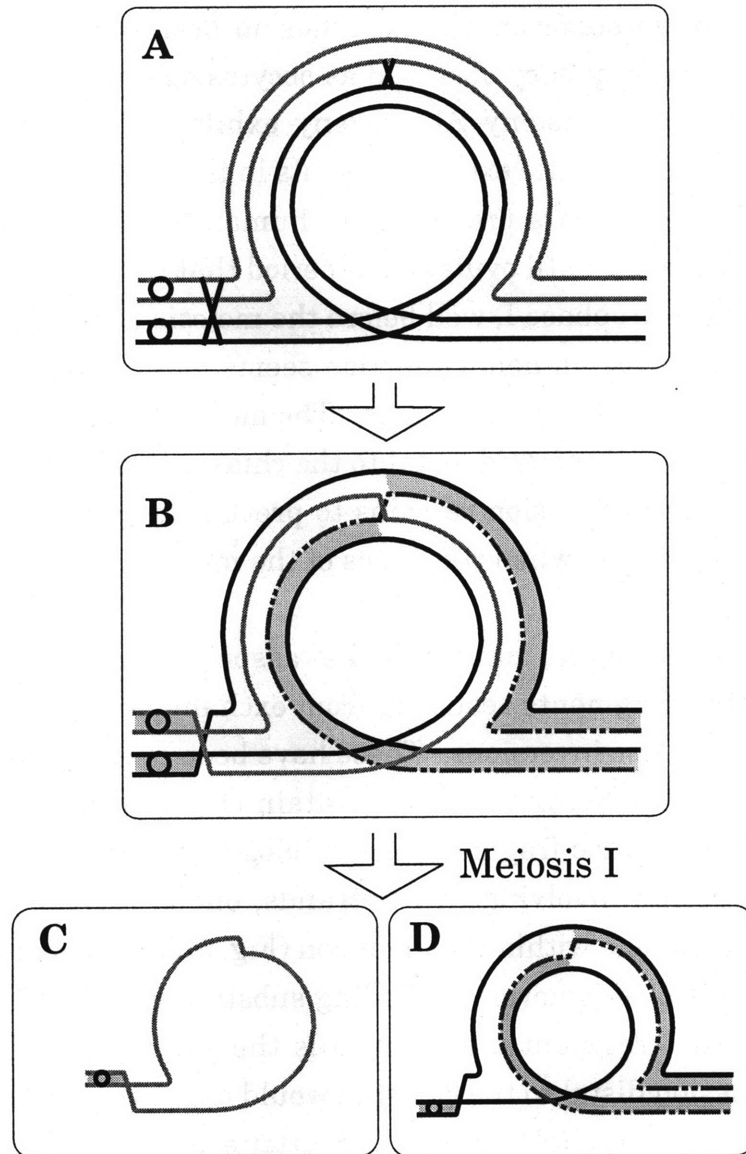

Figure 5: Segregation of a particular three-strand double crossover on a heterozygous paracentric inversion. Small open circles represent centromeres. (A) The aligned homologs are depicted in gray and black. One crossover is proximal to the paracentric inversion while the other is within the inversion. (B) After exchange, one homolog is a loop dyad (gray solid line) while the other homolog is normal (black solid line). An acentric fragment (gray dotted line) also results from exchange. Reductional segregation partitions the loop dyad (C) from the normal dyad (D). The acentric fragment frequently moves with the normal dyad to one pole. Regions of sister-chromatid cohesion that do not experience the spindle forces acting on the dyads are shaded lightly. Adapted from Maguire (1993).

divisions, such as positioning of sister kinetochores to face opposing poles. In meiosis, crossovers between homologs are preferentially formed relative to crossovers between sisters. Recently, recombination intermediates, identified as double Holliday junctions, at a meiotic "hot spot" of recombination in yeast were shown to favor homolog interaction over sister-chromatid interactions (Schwacha and Kleckner, 1994; Schwacha and Kleckner, 1995). Some structural aspect of sister-chromatid cohesion in meiosis may serve to direct interactions away from sister chromatids, favoring homologs, either by making one of the sister chromatids inert or by physically restraining the sister chromatids from interacting.

Proposed mechanisms for cohesion between the sister-chromatid arms include incompletely replicated chromosomes, unresolved intertwinings between chromosomes after replication, and protein structures that act as a glue between the sister chromatids. These mechanisms are not exclusive of one another, and what binds the sister chromatids along their arms may differ from what binds them near the centromeres.

Incomplete replication of short DNA stretches is unlikely to be a mechanism for sister-chromatid cohesion. While heterochromatin of some eukaryotic chromosomes is late-replicating (Lima-de-Faria and Jaworska, 1968), more recently, *S. cerevisiae* chromosomes have been shown to replicate completely during S phase in both mitosis and meiosis (McCarroll and Fangman, 1988; Collins and Newlon, 1994). In addition, pulse labeling in human fibroblasts revealed no replication of DNA in metaphase or early anaphase (Comings, 1966).

1. Catenation as a cohesive factor

Unresolved intertwinings of sister-chromatid strands, also termed catenation, might act as a linkage until anaphase. Control of either access or activity of topoisomerase II to the catenated regions would provide linkage between the sister chromatids until anaphase. Topoisomerase II is required for the metaphase/anaphase transition in mitosis (see review by Holm (1994)). The enzyme is found on pachytene chromosomes in the axial cores of yeast and chicken (Klein, et al., 1992; Moens and Earnshaw, 1989) and has been proposed to be required for formation of the SC, resolving entanglements that arise during this time (von Wettstein, et al., 1984). The necessity of topoisomerase II in yeast meiosis has been directly investigated using a cold sensitive mutation, *top2*^{cs} (Rose and Holm, 1993). Premeiotic replication, chromosome condensation and SC appeared to be unaffected

despite the lack of functional topoisomerase II, but meiosis is blocked and cells arrest prior to anaphase I with a single nucleus. Meiotic recombination can be eliminated with a *rad50* mutation. *rad50 top2cs* double mutants at restrictive temperature were able to pass through anaphase I and produced binucleate cells. Eventually they went on to produce multinucleate cells. Thus, topoisomerase II is required for transition to anaphase I when the homologs have recombined, presumably because entanglements distal to exchange crossovers must be resolved for the recombined chromosomes to segregate (Rose and Holm, 1993).

To resolve catenated molecules rather than generate additional interlocks, topoisomerase II requires directionality provided by other forces. Condensation of sister chromatids provides some directionality to the double-strand passings, and the forces generated by segregation on the spindle at anaphase provide further directionality (see review by Holm (1994)). Regions proximal to all the crossovers on a bivalent arm will not experience this force during anaphase I, since the proximal portions of sister chromatids are being pulled in the same direction. Catenations in these regions may not be resolved in anaphase I. Such sister-chromatid cohesion on the arms after anaphase I has recently been dubbed "adventitious" (Kleckner, 1996).

The persistent association between sister chromatids observed in Maguire's classic experiments using maize paracentric inversions (Maguire, 1993) may be catenation that is not resolved, and thus an example of adventitious cohesion (Kleckner, 1996). Three-stranded double crossovers in this heterozygous paracentric inversion result in a dyad capable of segregation in anaphase I without separation of sister chromatids distal to the two crossovers (Fig. 5). The loop dyad has no distal region, and the acentric fragment does not experience spindle forces in a direction opposing the normal dyad. Since catenation may not be resolved without forces to provide directionality, the acentric fragment would be expected to remain associated with the normal dyad. This adventitious cohesion suggests that catenation in the distal regions of sister chromatids exists, and it does not preclude that catenation could hold chiasma at the site of crossing over, with the provision that topoisomerase II activity is inhibited until the metaphase I/anaphase I transition.

In mitosis, however, mechanisms other than entanglement must also hold sister chromatids together. Circular minichromosomes were observed to be in close proximity during mitosis (Guacci, et al., 1994), although they were not topologically interlocked during metaphase. Despite their lack of catenation, these circular minichromosomes segregated with fidelity (Koshland and Hartwell, 1987). In mitosis,

at least, topoisomerase II may only play a role in disentangling the chromosomes and is unlikely to be the sole mechanism holding sister chromatids together.

2. Proteins potentially serving to glue sister-chromatids together

a. Mutations disrupting sister-chromatid cohesion early in meiosis

In contrast to the phenotypes of the maize desynaptic mutations and of *red1* in budding yeast, mutations in three genes from diverse organisms have phenotypes suggesting complete loss of cohesion between sister chromatids well before metaphase I. These are *ord*, *rec8* and *spo76*. Cytology provides the best evidence of early sister-chromatid separation. Sister-chromatid cohesion is also required for segregation, so precocious separation of sister chromatids can also be ascertained by genetic criteria (Fig. 4D). In mutants in which exchange occurs, it is informative to know whether crossovers are able to ensure disjunction of homologs.

In *D. melanogaster*, mutations in the gene *ord* result in precocious separation of sister chromatids in both sexes. In mutant spermatocytes, separation of sisters is visible during prometaphase I (Mason, 1976; Miyazaki and Orr-Weaver, 1992), and sister kinetochores have been observed to be separated early in prometaphase I (Lin and Church, 1982). Genetic exchange is reduced, and the remaining exchange events do not ensure disjunction of the bivalent in meiosis I (Mason, 1976). ord^1 oocytes had reduced exchange along most of the *X* chromosome, although the reduction was less extreme near the centromere. The few very proximal exchange events that occurred slightly increased the probability of successful reductional disjunction (Mason, 1976). This suggests that ord^1 does not completely obliterate the ability of an exchange to bind homologs. Recently the gene has been cloned, and it is predicted to encode a novel protein (Bickel, et al., 1996).

In *S. pombe* strains that are homozygous for the *rec8-110* mutation, precocious separation of sister chromatids has been detected by both genetic assays and fluorescent in situ hybridization (FISH) (Molnar, et al., 1995). Separation of sister chromatids occurred early in prophase I in 70% of nuclei; more than 20% showed very wide separation. The chromosome ends had the least separation of sister chromatids (Molnar, et al., 1995). Although *S. pombe* lacks SC, there are "linear elements" that are thought to be equivalent to axial elements. Sporadic misalignment in the linear elements was observed in *rec8-110* yeast (Bahler, et al., 1993). Exchange is reduced in a region-specific manner, with as little as a 10-fold reduction at the ends of

chromosome III and with greater reduction at sites examined on the other two chromosomes (DeVeaux and Smith, 1994). The predicted sequence of *rec8* product showed no homology to known proteins (Lin, et al., 1992).

In *spo76* homozygotes of the fungus, *Sordaria macrospora*, precocious separation of sister chromatids was observed cytologically during prophase I, but this cannot be observed genetically, because the meiotic cells arrested and rarely resulted in viable gametes. Regions of the lateral elements appeared split. It is likely, although not yet demonstrated, that recombination is reduced in the homozygous mutant, as it has been shown that recombination was reduced in the heterozygote (Moreau, et al., 1985).

In both *rec8* and *ord* homozygotes, exchange between homologs was inadequate for proper disjunction. This is consistent with the hypothesis that sister-chromatid cohesion is necessary for crossovers to act as an attachment between homologs. However, these mutations may be required for an early function, such as sister-chromatid cohesion immediately after replication, that is a precondition for establishing mature chiasmata without necessarily being required to maintain chiasmata. Moreover, *ord* is required for cohesion of sister chromatids in male meiosis, a meiotic division that has neither exchange nor SC, so it is required to ensure reductional disjunction where chiasmata definitely need not be maintained. Early splitting of sister kinetochores also complicates the conclusion that sister-chromatid cohesion is required to maintain chiasmata, since inability of the bivalent to ensure proper orientation could be a consequence of sister kinetochores orienting independently.

b. Proteins identified by immunocytology as candidates

Immunocytology has been used to identify candidate sister-cohesion proteins during meiosis. COR1 protein is localized to the sister-chromatid core during meiosis in hamster, but it is not seen in somatic cells. This antigen is lost along the arms of the meiotic chromosomes at the metaphase I to anaphase I transition, although it continues to be located near the centromeres, where sister cohesion is retained until anaphase II (Dobson, et al., 1994; Moens and Spyropoulos, 1995). However, the protein has not yet been demonstrated to be essential for cohesion.

In immunocytological studies of mitotic cells, antigens have been found that are specifically localized to the region along the inner surface of the kinetochore, termed the pairing domain (Rattner, 1991), and to the inner surface of the sister-chromatid

arms. This is likely to be the site of interaction between sister chromatids at metaphase. Chromatid linking proteins (CLiPs) are located between the sister chromatids along their length and between the kinetochores during metaphase. Consistent with a role in sister-chromatid cohesion, these proteins are no longer detectable by anaphase. CLiPs were identified by cross reaction with human autoimmune sera (Rattner, et al., 1988). Inner centromere proteins, INCENPs, also have a localization pattern consistent with cohesion between the sister chromatids. Antibodies to the INCENPs were generated to mitotic chromosome scaffold fractions. The INCENPs are located between sister chromatids during metaphase, remain associated with metaphase plate during anaphase, and are focused in the midbody during telophase (Cooke, et al., 1987; MacKay, et al., 1993; Mackay and Earnshaw, 1993; Earnshaw and Mackay, 1994). Recently, an antigen that forms a ring-like structure at the centromere in human and Chinese hamster cells has been proposed to provide sister-chromatid cohesion (Holland, et al., 1995). Although all of these proteins exhibit localization patterns consistent with sister-chromatid cohesion, none of these antigens have been demonstrated to be present on the chromosomes of meiotic cells, nor have they been shown to be required for sister-chromatid cohesion in mitosis.

IV. Homolog attachment and segregation without chiasmata

Although the usual method of holding homologs together for disjunction in meiosis I involves chiasmata, diverse mechanisms have evolved to allow appropriate partitioning of chromosomes. Wolf (1994) recently reviewed a broad range of achiasmate segregational mechanisms. This review will only categorize a few examples for context before focusing on segregation in the best characterized example of non-exchange segregation, the distributive system of *D. melanogaster*.

A. Completely achiasmate meiotic divisions

Homolog segregation in meiotic cells can be carried out without any chiasmata at all. Achiasmate meiosis using cytologically observed SC occurs in oocytes of the silkworm, *Bombyx mori*. Attachment between homologs is achieved by an adaptation of the SC. Late in prophase I, the SC is seen to be augmented rather than dissolved. This "elimination chromatin", a sort of pseudochiasma, is left behind

on the metaphase plate during anaphase I, consistent with a role holding homologs together for reductional division (Rasmussen, 1977).

A well-characterized example of achiasmate meiosis without SC occurs in *D. melanogaster* spermatocytes. The *X* and *Y* chromosome are connected by a threadlike material at metaphase, and molecular analysis has delineated this pairing site to specific sequence in the rDNA locus. Autosomal pairing sites have also been identified. All of these pairing sites carry out attachment between homologs without any exchange. This work has recently been reviewed by McKee (1996) and is reviewed in this volume.

B. Non-exchange chromosomes in meiosis with exchange

In cells that carry out chiasmate meiosis, particular chromosomes may be attached by other means. In particular, heterogametic meiosis involves sex chromosomes that are often largely non-homologous and do not have exchanges, yet these chromosomes segregate faithfully (John, 1990). A variety of mechanisms have been cytologically characterized, ranging from cohesive material that is not SC to chromosomes with microtubules attaching them (Wolf, 1994).

1. The Drosophila distributive system

In *D. melanogaster* oocytes, non-exchange chromosomes have no cytologically obvious physical linkage during metaphase I (Therkauf and Hawley, 1992). While most of the bivalents are bound by crossovers in meiosis I, the fourth chromosome is much smaller and does not undergo exchange, yet it is disjoined faithfully. The fourth chromosomes are often observed to be off the metaphase plate and located on the meiotic spindle midway between the plate and the poles. Moreover, the *X* chromosome is non-exchange 10% of the time, and any of the chromosomes are non-exchange when heterozygous for a homolog carrying multiple inversions; yet the oocyte is able to efficiently segregate any of these non-exchange chromosomes.

Provocatively, a single mutation, *nod*, allows the non-exchange chromosomes to be lost from the spindle. The *nod* gene has been cloned and the N-terminal domain of the predicted protein was shown to share homology with kinesin, a microtubule motor (Zhang, et al., 1990). NOD protein has been localized along the chromosome arms and shown to associate with chromatin and microtubules (Afshar, et al., 1995a; Afshar, et al., 1995b). Thus, a microtubule motor is required to act as an

"attachment" between chromosomes during metaphase I, by maintaining the half-bivalents in position on the metaphase spindle. Interchromosomal microtubules have also been suggested to play a role in linking the chromosomes (Carpenter, 1991).

The requirement for NOD protein to stabilize chromosomes on the spindle still leaves open the question of how orientation of non-exchange chromosomes is achieved in prometaphase I. Bipolar orientation might first be achieved with a transient physical association, or orientation might occur without any physical linkage between the chromosomes. Evidence, reviewed below, suggests that transient linkages may help to orient some, but not all, chromosomes in Drosophila oocytes.

a. Non-exchange homologs associate in prophase I

Recent results suggest that centric heterochromatin plays a part in orienting homologous non-exchange chromosomes during prometaphase I. In genetic experiments, centric heterochromatin was shown to be critical for segregation: rearranged chromosomes segregated from non-exchange partners that shared homology in centric regions (Hawley, et al., 1993); and minichromosomes derived from the *X* chromosome had decreasing ability to segregate from one another as the amount of overlapping centric heterochromatin was decreased (Karpen, et al., 1996). FISH for centric heterochromatin of achiasmate homologs was carried out for the obligatory non-exchange fourth chromosome and, additionally, for *X* chromosomes heterozygous for multiple inversions so that exchange was suppressed. In both cases, the heterochromatic regions of these homologs were tightly associated. In contrast, FISH for regions on the arms of chromosomes showed random distances, suggesting that diplotene is modified in *D. melanogaster* oocytes such that the bivalent is connected primarily at crossovers and at centric heterochromatin (Dernburg, et al., 1996). If this association near the centromeres continues into prometaphase I, it could provide an attachment that facilitates bipolar orientation.

b. Heterologs do not associate in prophase I

Heterologous non-exchange chromatids also segregate from one another, but apparently without the benefit of actual pairing. In flat preparations of Drosophila oocytes, all of the heterochromatic regions were shown to be associated in a "chromocenter" during diplotene (Nokkala and Puro, 1976). However, FISH for

centric heterochromatin of two heterologs, a compound second and compound third chromosome, demonstrated that they do not pair (Dernburg, et al., 1996). Yet, these heterologs segregate from each other, seemingly without any physical linkage.

A difference in the mechanism by which homologs and heterologs orient in Drosophila oocytes is consistent with genetic dissection of the distributive system. Two classes of mutations predominantly result in nondisjunction of non-exchange chromosomes. *Axs*, *mei-S51* and *ald* disrupted segregation of non-exchange homologs only, while *nod* and *Dub* caused missegregation of both heterologs and homologs (Hawley and Theurkauf, 1993; Moore, et al., 1994). Genetics and cytology suggest that transient physical linkage helps to orient homologs. Heterologs do not have this transient attachment, and both mechanisms require functions by NOD protein to segregate appropriately.

2. Disjunction without physical attachment: the crowded spindle model

The "crowded spindle" model has been proposed to explain how chromosomes might be selected to segregate from one another without physical linkage (Hawley, et al., 1993b). This model was derived from observations on heterologous distributive segregation in Drosophila: (1) Disjunction of heterologs occurs in a competitive and preferential manner, so that introduction of a third heterolog disrupts segregation of two heterologs; (2) Chromatids with similar sizes and shapes tend to disjoin (Grell, 1976); and (3) The distributive system has a limited ability to sort out chromosomes. It breaks down when more than four unpaired chromosomes are involved. As an example, mutations that reduce exchange result in nondisjunction of the fourth chromosome (Baker and Hall, 1976).

In the crowded spindle model, a given non-exchange univalent is more likely to connect to whichever pole is not occupied by another univalent. Smaller chromatids have been observed to move poleward more quickly than larger chromosomes, and movement to a pole is impeded by the presence of other chromosomes on the half spindle (Theurkauf and Hawley, 1992). Chromosomes of similar size and shape with connections to the same pole will be in the most direct competition, so these will tend to reorient to balance the crowding at the poles. As the poles become more crowded with non-exchange chromosomes, this system would have less ability to influence other univalents, consistent with the limited ability of the distributive system.

The spindle in Drosophila oocytes is unusually narrow and is organized by the chromatin (Theurkauf and Hawley, 1992), and this may account for some of the

efficiency of the distributive system. Other species have been noted to have meiotic spindles that are organized by chromatin (Vernos and Karsenti, 1995). It will be interesting to discover if these species also have efficient systems of distributive segregation.

The meiotic spindle is organized by spindle pole bodies in *S. cerevisiae*. In this species, the distributive system is less efficient and has been shown to not follow size and shape rules (Ross, et al., 1996b). In situ hybridization studies suggest that pairing occurs between non-exchange chromosomes that lack homologs (Loidl, et al., 1994). Perhaps a difference in the structure of the spindle accounts for the differences between the Drosophila distributive system and that found in budding yeast.

V. Sister kinetochore function

A. Sister kinetochores must reorganize between meiotic divisions

The kinetochores of sister chromatids do not usually connect to opposite poles in the first meiotic division, yet they do in meiosis II, suggesting that there must also be a change in their structure between metaphase I and prometaphase II (Fig. 1). Darlington (1932) proposed that sister chromatids share a single kinetochore during meiosis I and thus must move to the same pole during anaphase I. The other possibility, the existence of differentiated sister kinetochores before prometaphase I, has also been proposed. In this model, the sister kinetochores must somehow be constrained in their behavior to make connections with the same pole (Nicklas, 1977).

Since replication of DNA is completed well before metaphase I (Collins and Newlon, 1994), unreplicated centromeres cannot account for reductional segregation. If both the underlying DNA and the protein structures that make up kinetochores are duplicated by metaphase I, reductional segregation is not a simple consequence of having one functional microtubule attachment site for each half-bivalent. Cytological and functional evidence for sister-kinetochore duplication prior to metaphase I are reviewed here.

B. Cytological observations of sister kinetochore duplication

Kinetochores are cytologically defined structures. By this criterion, duplication of sister kinetochores has been described as occurring before metaphase I for many species. A progressive differentiation of the sister kinetochores of *D. melanogaster* spermatocytes was described during prometaphase I: before microtubule connections are made, there is one structure, and as microtubules attach, there is an amorphous stage and eventually a double disc structure (Goldstein, 1981). A single kinetochore is shared by sister chromatids in early prometaphase I and duplicated by metaphase I in the crane fly, *Pales ferruginea* (Muller, 1972), and in the marine worm, *Urechis caupo* (Luykx, 1965b). Many species have been noted to have observably duplicated sister kinetochores by metaphase I. Lima-de-Faria (1956; 1958) observed distinct sister kinetochores in several plant and insect species during metaphase I. In mouse, paired sister kinetochores were visible in colcemid-arrested metaphase I spermatocytes (Brinkley, et al., 1986). Thus, sister kinetochores appear to duplicate some time before anaphase I.

In a few species, kinetochores have a duplicated appearance as early as prophase I and change to a singular appearance during prometaphase I. Two "spindle spherules" were visible in late diakinesis in cells from the salamander, *Amphiuma tridactylum* (Schrader, 1936; Schrader, 1939). Silver-stained chromosomes of several grasshopper species, *Chorthippus jucundus*, *E. plorans* and *Arcyptera fusca,* had clearly duplicated sister kinetochores in prophase I. They appear as two rounded structures, much like the kinetochores in the relatively relaxed period of anaphase and in contrast to the single conical structure shared by the sister chromatids during early metaphase I (Rufas, et al., 1983; Rufas, et al., 1989; Suja, et al., 1991). These studies suggest there may be duplication of sister kinetochores as early as prophase I, although they appear as a single structure during prometaphase I, a time when they need to act as a single unit.

C. Functional differentiation of sister kinetochores

Does independent function of the sister kinetochores correspond with cytologically observable differentiation? There are instances in which sister kinetochores do function independently in meiosis I. When univalents are present or when mutations disrupt sister-chromatid cohesion, the sister kinetochores are seen to be duplicated and capable of making spindle fiber connections with opposite poles.

Univalents dividing equationally during meiosis I are frequently reported for a broad spectrum of species, from plants to humans (Angell, et al., 1994). In a wheat hybrid with an unpaired chromosome, during prometaphase I, sister kinetochores faced the same spindle pole, but late in metaphase I, sister kinetochores developed connections to opposite spindle poles that allowed congression to the metaphase plate with an equational bipolar orientation (Wagenaar and Bray, 1973). In mouse females carrying a single *X* chromosome that was followed by FISH, the univalent divided equationally in meiosis I about 1/3 of the time (Hunt, et al., 1995). The behavior of univalents suggests that sister kinetochores can sometimes act as independent units and undergo equational division at anaphase I.

Separated sister kinetochores are seen early in prometaphase I in *ord* homozygous Drosophila oocytes (Lin and Church, 1982). Genetic assays suggest that the sister chromatids segregate randomly during this division (Mason, 1976; Miyazaki and Orr-Weaver, 1992). Precocious separation of sister chromatids was observed in *S. pombe* that were homozygous for *rec8-101*, and the centromeric regions of chromosomes appeared to be unusually far apart during prometaphase I (Molnar, et al., 1995). These genes are believed to be required for sister-chromatid cohesion, and both have functional sister kinetochores before metaphase I. The simplest explanation is that sister kinetochores are functionally double before prometaphase I, and sister-chromatid cohesion is required to constrain their shape into a functionally single kinetochore during prometaphase I. Alternatively, the *ord* and *rec8* gene products may be required for at least two functions: providing cohesion of sister chromatids and preventing early functional differentiation of kinetochores.

There is a caveat to these observations that duplicated kinetochores are capable of independent function: in all the examples cited above, anaphase I may be delayed an unusually long time, long enough that the sister kinetochores gain independent function. Univalents are known to delay anaphase I, thereby lengthening metaphase I. Mutations in budding yeast that show low levels of precocious separation of sister chromatids also have been suggested to delay anaphase I past the transition to functionally differentiated sister kinetochores (Carpenter, 1994). For example, *med1*, now known to be an allele of *dmc1* and renamed *dmc1-1*, causes a reduction in recombination, presumably resulting in univalents that might delay anaphase onset, and this results in low levels of tetrads resulting from precocious separation of sister chromatids (Rockmill and Roeder, 1994). Heterozygosity for a ring chromosome III in budding yeast was shown to result in precocious separation of sister chromatids for a normal chromosome VII, perhaps because mechanical problems in orienting the

heterozygous ring chromosome delay anaphase I (Flatters, et al., 1995). It is possible that mutations disrupting sister cohesion similarly delay anaphase I. Nevertheless, functional duplication certainly can occur before anaphase I begins, and cytological observations of sister kinetochore duplication are seen in metaphase I unperturbed by the presence of univalents or mutations.

D. Early functional differentiation may be chromosome dependent

Univalents in the same species show different abilities to orient and segregate equationally. In living spermatocytes of the grasshopper, *Eyprepocnemis plorans*, chromosomes that usually exist as univalents, the X and B chromosomes, oriented with different dynamics and segregational results than did autosomal univalents that were induced by heat shock (Rebollo and Arana, 1995).

In *S. cerevisiae*, chromosomal-dependent segregation behavior has been localized to sequences less than 1.6 kb in length that include the centromere (see review by Simchen and Hugerat, 1993). In certain mutant yeast strains, the majority of meiotic cells yield two-spored asci rather than four-spored asci, and these spores are diploid. This "single-division meiosis" has been characterized for four mutations, two that are meiosis-specific (*spo12* and *spo13*) and two that affect the mitotic cell cycle by arresting late in nuclear division (*cdc5* and *cdc14*). Regardless of the mutation, mixed segregation of chromosomes occurs during the single-division meiosis, and the chromosomes have inherent tendencies towards equational or reductional segregation without regard to the absence or presence of exchange events on the chromosome. The tendency does not correlate with chromosome size. Replacement of the centromere region changed the chromosome's inherent tendency, such that the engineered chromosome segregated with the tendency of the replacement centromere region. Heterocentromeric bivalents often yielded trisomic spores, suggesting mixed segregation even within a single bivalent (Simchen and Hugerat, 1993). Future studies of these centromere sequences may reveal what makes sister centromeres more or less functionally autonomous during the first meiotic division.

VI. Maintaining attachment between sister chromatids for meiosis II

A. Cytology shows that attachment persists in proximal regions

Kinetochores of sister chromatids are aligned on the metaphase plate in meiosis II, suggesting that the attachment between sister chromatids during metaphase II is at or near the kinetochore (Fig. 1E). During anaphase I, the portions of sister-chromatid arms that are distal to a reciprocal crossover segregate away from each other and must lose cohesion distal to the exchange. Lima-de-Faria (1956) observed for a variety of species that "the structures depicted at anaphase I show the kinetochore divided at this stage, the most proximal regions of the arms being also responsible for holding together the sister chromatids."

B. Equational nondisjunction resulting from proximal exchange

Since exchange occurs during meiosis I, it is not obvious that alteration of recombination might result in errors in meiosis II. However, recent studies in humans and Drosophila suggest that exchange occurring in the proximal region, or perhaps an increase in number of exchange events, increases the likelihood of equational nondisjunction. Human trisomy for chromosome 21 resulting from maternal meiosis II errors showed an increase in the overall amount of exchange, and the chromosomes commonly had undergone a proximal exchange (Lamb, et al., 1996). Proximal exchanges were even more common in human X chromosome nondisjunction events, although overall exchange was slightly reduced (MacDonald, et al., 1994). Chromosome 18 derived from human trisomy showed an increase in map length but the locations of the exchanges were not reported (Fisher, et al., 1995). In Drosophila females, the *X* chromosome had a remarkably similar pattern. Meiosis II errors were often correlated with multiple exchanges and exchange in the proximal region (Koehler, et al., 1996a).

Two explanations have been put forward. In the first model (Fig. 6A), the resolution of proximal chiasmata in meiosis I would involve a loss of proximal cohesion, and this might increase the likelihood that sister-chromatid cohesion is lost completely, resulting in nondisjunction during meiosis II. In the second proposal (Fig. 6B), crossovers in the proximal region may result in continued attachment between homologs if sister-chromatid cohesion in the region is not released. The bivalent would be unable to separate and thus segregate in its entirety to one pole. The intact

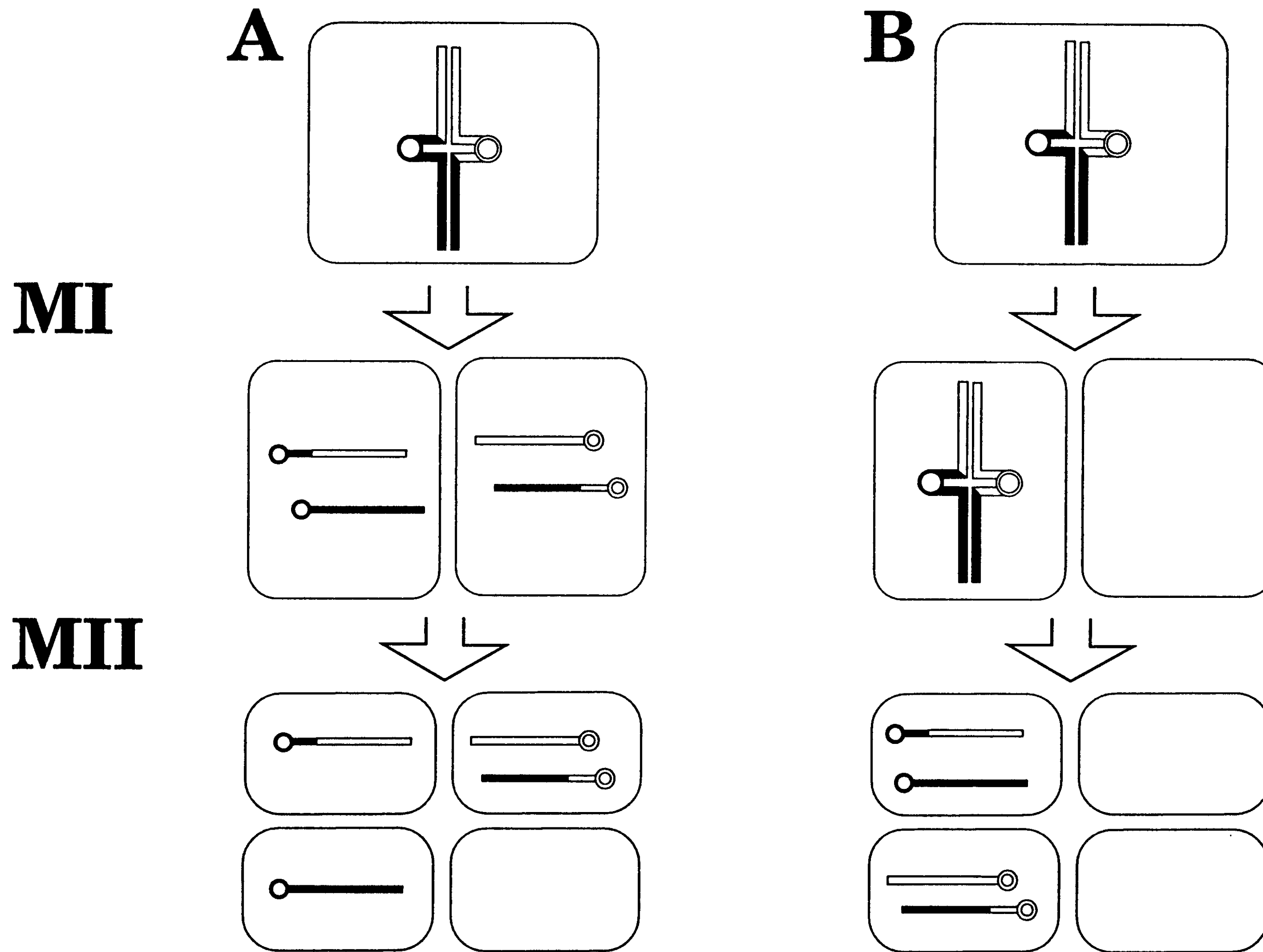

Figure 6: Proposed mechanisms by which proximal exchange might yield gametes disomic for sister chromatids. (A) Sister-chromatid cohesion in proximal regions may be lost when proximal crossovers are resolved during anaphase I. Some sister chromatids may then lack cohesion necessary to segregate properly in meiosis II. (B) Sister-chromatid cohesion in proximal regions may persist so that proximal crossovers cannot be easily resolved during anaphase I. The intact bivalent segregates to one pole, and reductional segregation may occur during meiosis II.

bivalent might segregate reductionally in the second division and yield gametes disomic for sister chromatids. Thus an apparent meiosis II nondisjunction would actually result from meiosis I nondisjunction (Koehler, et al., 1996b).

C. Possible mechanisms of cohesion in the centromeric regions

Attachment between the sister chromatids is maintained through the first meiotic division, although sister chromatids lose cohesion along their arms. Either a mechanism of sister-chromatid cohesion is unique to the centromeric region of half-bivalents, or sister-chromatid cohesion is specifically protected at the centromeric region until anaphase II. Possible mechanisms of cohesion again include catenation of the DNA or structural proteins.

There is not any evidence that catenation binds sister chromatids during metaphase II. *S. cerevisiae* cells that have undergone meiosis I without exchange and without functional topoisomerase II first become cells with two nuclei and then eventually become cells with more than four nuclei (Rose and Holm, 1993). These multinucleate cells suggest that topoisomerase II is important for the second meiotic division. However, topoisomerase II was shown to be required for successful segregation during anaphase I in meiosis with exchange, so it is likely that resolution of catenation on sister-chromatid arms is simply delayed until the second division. There is no evidence that catenation in the centromeric regions provides sister-chromatid cohesion through meiosis I.

D. Mutations that disrupt cohesion for meiosis II

The Drosophila MEI-S332 protein is necessary to maintain the bond between sister chromatids after metaphase I. In *mei-S332* mutants, genetic assays of segregation in both males and females revealed low levels of meiosis I nondisjunction and high levels of meiosis II nondisjunction. In mutant spermatocytes, meiosis appears cytologically normal until the sister chromatids separate prematurely during anaphase I. Segregation in anaphase II is random, the result of the inability to orient in metaphase II (Davis, 1971; Kerrebrock, et al., 1992). The MEI-S332 protein localizes to the chromosomes in a manner consistent with a role in maintaining cohesion after the metaphase I/anaphase I transition. As the chromosomes condense and begin prometaphase I, MEI-S332 localizes at discrete loci on the chromosomes. During anaphase I, the protein is clearly located on centromeric

regions of segregating chromosomes. MEI-S332 remains on the chromosomes until metaphase II, but is dispersed or destroyed at the beginning of anaphase II when sister-chromatid cohesion is released (Kerrebrock, et al., 1995).

The sister chromatids are presumably attached at their kinetochores before MEI-S332 localizes to the chromosomes, so the protein either augments cohesive structures already present in the proximal regions or it acts to protect the cohesive structures until anaphase II. MEI-S332 differentiates the regions near the centromeres from the rest of the chromosome arms. It could supplement, replace or preserve cohesive proteins that extend the length of the sister chromatids, or it could prevent resolution of DNA catenation in the centromeric regions.

A phenotype similar to that of *mei-S332* was observed in the tomato, *Lycopersicon esculentum*. Plants homozygous for the *pc* mutation are infertile, but have no cytologically observable effect on chromosome pairing or chiasmata formation. However, separation of the sister chromatids is visible as early as anaphase I (Clayberg, 1959). *pc* and *mei-S332* are the best candidates for genes encoding cohesive proteins acting at the centromeric regions of dyads.

VII. Summary

The structure of the bivalent is critical for successful segregation of chromosomes in meiosis. In particular, attachments between chromosomes and the arrangement of kinetochores are vital for achieving bipolar orientation on the spindle. Chiasmata usually serve as the attachment between homologs for the first meiotic division. These crossovers between homologs are likely held in place by sister-chromatid cohesion along the arms. Binding substances localized to the crossovers may play a role in chiasma maintenance. Current cytology and genetics does not eliminate either of these models, but failure in maintaining sister-chromatid cohesion best explains why missegregation most often results from distal crossovers. A variety of mechanisms have evolved for the reductional division that do not require exchange between homologs, and in Drosophila females, some partitioning of chromosomes is carried out without physical attachments. After the first meiotic division, sister kinetochores must reorganize, and attachments between homologs must be relinquished while maintaining attachments between sisters. Sister-chromatid cohesion in the centromeric region is preserved for the second division.

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Chapter Two

MEI-S332, a Drosophila protein required for sister-chromatid cohesion, can localize to meiotic centromere regions

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My contribution to this chapter included searches for homology, the MEI-S332-GFP construct and strains, the cytology in Figure 3 and the developmental northern blot in Figure 4.

Abstract

Mutations in the Drosophila *mei-S332* gene cause premature separation of the sister chromatids in late anaphase of meiosis I. Therefore, the MEI-S332 protein was postulated to hold the centromere regions of sister chromatids together until anaphase II. The *mei-S332* gene encodes a novel 44 kDa protein. Mutations in *mei-S332* that differentially affect function in males or females map to distinct domains of the protein. A fusion of MEI-S332 to the green fluorescent protein (GFP) is fully functional and localizes specifically to the centromere region of meiotic chromosomes. When sister-chromatids separate at anaphase II, MEI-S332-GFP disappears from the chromosomes, suggesting that the destruction or release of this protein is required for sister-chromatid separation.

Introduction

Accurate chromosome segregation depends on regulating the linkage between sister chromatids. The sister chromatids must be associated in order to attach to opposite poles of the spindle in metaphase, and sister-chromatid cohesion needs to be completely dissolved to permit segregation in anaphase. In meiosis, two rounds of chromosome segregation occur, and sister-chromatid cohesion is essential for both of these. During meiosis I homologs pair and segregate, thus the sister chromatids must remain associated throughout meiosis I until their segregation in anaphase II. Cytologically it has been observed that during prophase I the sister chromatids are associated along their length, but at the metaphase I/anaphase I transition cohesion on the chromatid arms is lost (Miyazaki and Orr-Weaver, 1994). From late anaphase I until the metaphase II/anaphase II transition, the sister chromatids are attached only at their centromere regions. This behavior contrasts with that of mitotic chromosomes, whose arm and centromere cohesion is dissolved simultaneously at the onset of anaphase, suggesting that meiosis-specific functions must exist to maintain cohesion in the centromere region in meiosis.

The molecular nature of sister-chromatid cohesion is not understood. Replication results in the DNA helices being intertwined (Sundin and Varshavsky, 1980), leading to the proposal that catenation could provide cohesion if topoisomerase II were prevented from acting until the metaphase/ anaphase transition (Murray and Szostak, 1985). This hypothesis has been tested genetically in yeast as well as biochemically in Xenopus in vitro extracts. Nondisjunction and chromosome breakage occur if mitosis is attempted at the nonpermissive temperature in yeast with conditional mutations in topoisomerase II (Holm et al., 1989; Uemura et al., 1987). In extracts from Xenopus, anaphase segregation is blocked by topoisomerase II inhibitors (Shamu and Murray, 1992). Therefore, intertwinings must be removed for separation of sister chromatids. However, catenation is not sufficient to account for sister-chromatid cohesion. In yeast, circular minichromosomes were not found to be interlocked, even though they segregated faithfully (Koshland and Hartwell, 1987).

Several approaches have identified chromosomal proteins that may promote association of the sister chromatids. The inner centromere proteins (INCENPs) were isolated as antigens localized between the sister chromatids in vertebrate mitotic cells (Cooke et al., 1987; Earnshaw and Cooke, 1991; MacKay et al., 1993). Prior to the metaphase/anaphase transition they move off the chromosomes and remain at

the midbody region, thus they may be involved in cytokinesis rather than sister-chromatid cohesion. The centromere-linking protein (CLiP) antigens were identified from autoimmune sera and also are present between sister chromatids in mitosis (Rattner et al., 1988). Some of the components of the synaptonemal complex in hamster persist between the sister chromatids until metaphase II, consistent with a role in maintaining sister associations (Dobson et al., 1994).

Mutations that cause premature separation of the sister chromatids in mitosis or meiosis identify genes needed for sister-chromatid cohesion. Mutations affecting meiotic chromatid linkage have been described in Drosophila, maize, tomato, Sordaria, and yeast; mutations causing premature separation in mitosis exist in Drosophila, yeast, and humans (for review see Miyazaki and Orr-Weaver, 1994).

The product of the Drosophila *mei-S332* gene is likely to control sister-chromatid cohesion in meiosis directly (Davis, 1971; Goldstein, 1980; Kerrebrock et al., 1992). Cytologically, association of the sister chromatids is normal in *mei-S332* mutants early in meiosis I when the sisters are held together along their entire lengths. By late anaphase I the sister chromatids precociously separate in up to 90% of mutant spermatocytes, leading to nondisjunction and chromosome loss in the second meiotic division. Because even in apparent null mutations a defect in cohesion is not detectable until the time at which wild-type sister chromatids are associated only at their centromere regions, *mei-S332* is specifically required to maintain cohesion at the centromere regions in meiosis. As an entry into understanding both the molecular basis of cohesion and its regulation, here we describe the cloning of the *mei-S332* gene, the identification of its novel protein product, and the chromosomal location of a MEI-S332-GFP fusion protein.

Results

Isolation of the *mei-S332* gene

The *mei-S332* gene originally was localized to the cytological region 58A-E on the right arm of chromosome *2* (Davis, 1977). We isolated additional deficiencies in region 58 in order to map *mei-S332* to a smaller cytological interval (see Materials and Methods). We first constructed a chromosome that was deficient for the 58B-D interval ($In(2LR)dpp^{t24L}dpp^{d75R}$) and found that this chromosome failed to complement *mei-S332* in sex chromosome nondisjunction tests, thus placing *mei-S332* in 58B-D. Then we tested twelve cytologically visible deficiencies in 58 isolated from an X-ray screen as well as ethyl methanesulfonate (EMS)-generated deficiency *Df(2R)R1-8* for complementation of *mei-S332*. This analysis localized the *mei-S332* gene to 58B (Table 1).

The 58B genomic region was cloned by chromosome walking. Cosmids obtained at various steps along the walk were checked by quantitative Southern blot hybridization to genomic DNA from flies containing the $In(2LR)dpp^{t24L}dpp^{d75R}$ and *Df(2R)R1-8* deficiencies (data not shown). By cytological and complementation analyses, these deficiencies contained the closest proximal and distal breakpoints, respectively, that define the *mei-S332* locus. These experiments defined a 70 kb region of DNA containing the *mei-S332* gene (Figure 1A).

We identified the *mei-S332* gene in the walk by transforming cosmids and DNA fragments into Drosophila via P elements. Transformed lines were crossed into a *mei-S332* mutant background to test for rescue of chromosome nondisjunction. Two independent inserts each were obtained for the cos1-12 and cos1-5 cosmids. None of these lines rescued *mei-S332* mutants, suggesting that this gene resided in the remaining cosmid cos4-4 (Figure 1A). We transformed restriction fragments from within this cosmid. We obtained more than 10 lines transformed with a 5.6 kb KpnI fragment (P[w^+5.6KK *mei-S332*]) and a single line transformed with a 8.6 kb EcoRI fragment (P[w^+8.6RR *mei-S332*]) (Figure 1A). Both of these constructs complemented the *mei-S332* mutant phenotype in males and females (data not shown); thus, the *mei-S332* gene resides within the 4.2 kb of overlap shared by these constructs.

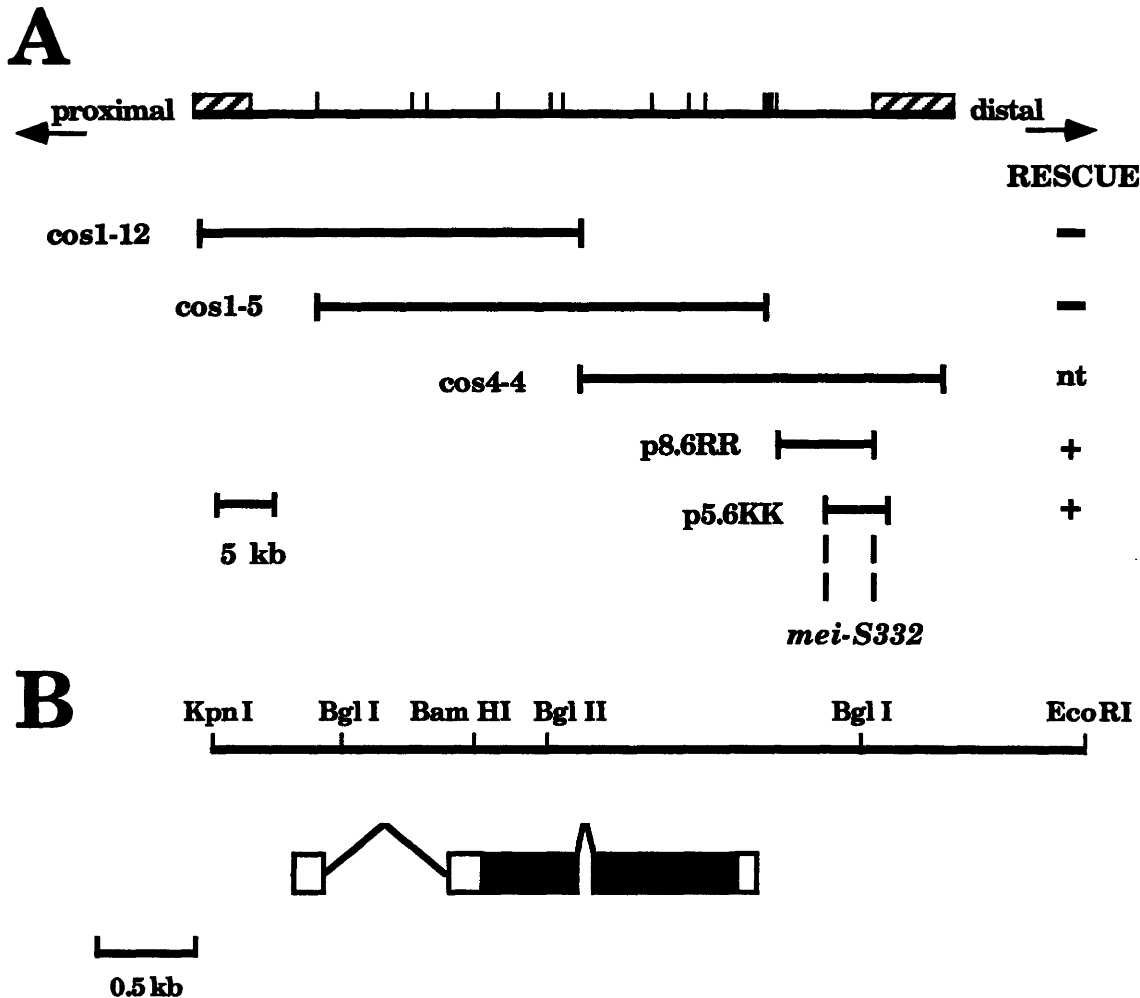

Figure 1: Cloning of *mei-S332*. (A) The top line indicates the *EcoRI* restriction map of the genomic interval containing the gene. This interval is defined by the position of the proximal breakpoint of the deficiency *In(2LR)dpp*^{t24L}*dpp*^{d75R} and the distal breakpoint of *Df(2R)R1-8*. The proximal breakpoint of *In(2LR)dpp*^{t24L}*dpp*^{d75R} mapped to a 4.9 kb EcoRI fragment in the cos1-12 cosmid, and the distal breakpoint of *Df(2R)R1-8* mapped to a 7 kb EcoRI fragment in the cos4-4 cosmid (hatched boxes). The genomic DNA included in cosmids and transposons used for transformation rescue experiments are shown relative to the genomic interval. Ability to complement *mei-S332* mutants is indicated by a plus under rescue. These experiments localized *mei-S332* to a 4.2 kb region defined by the overlap of rescuing transposons P[w+ 8.6RR*mei-S332*] (abbreviated p8.6RR)and P[w+ 5.6KK*mei-S332*] (abbreviated p5.6KK). (B) The position of the *mei-S332* transcription unit is shown relative to the 4.2 kb genomic region. The structure of the most abundant testis transcript is shown; the open reading frame is indicated by a closed box. There are two other testis transcripts and an ovary transcript that differ in the 5' and 3' untranslated regions but contain the same open reading frame.

TABLE 1

Deficiencies in the Cytological Interval 58

Deficiency	Breakpoints	*mei-S332* Phenotype[a]
In(2LR)dpp[t24L]dpp[d75R][b]	58B; 58D	-
Df(2R)R1-8[c]	57F9-11; 58B3-5	-
Df(2R)X58-1	58D6-8; 58F3-5	+
Df(2R)X58-2	58C7-D2; 58F3-5	+
Df(2R)X58-3	58C3-7; 58D6-8	+
Df(2R)X58-5	58A3-B2; 59A	-
Df(2R)X58-6	58A3-B2; 58E3-10	-
Df(2R)X58-7	58A1,2; 58E3-10	-
Df(2R)X58-8	58A1,2; 58F3-5	-
Df(2R)X58-11	58A3,4; 58E3-7	-
Df(2R)X58-12	58D1,2; 59A	+

[a] a minus indicates the deficiency uncovers *mei-S332* and is mutant for the locus; a plus that it does not

[b] This deficiency is the product of an exchange between two pericentric inversion chromosomes.

[c] This deficiency came out of an EMS screen for new alleles of *mei-S332*.

All of the other deficiencies were generated in the X ray screen described in Materials and Methods.

In addition to the transformation rescue, Southern blot analysis of DNA from the *mei-S332* mutants supported the localization of the gene described above. There is a polymorphism in the genomic DNA from *mei-S332*^{1} flies that was likely to be an insertion in the 4.2 kb of DNA that rescued the *mei-S332* mutant phenotype (data not shown). Since the original *mei-S332* allele arose spontaneously in a wild

population (Sandler et al., 1968), it is possible that this allele is due to the insertion of a transposable element into the *mei-S332* gene.

Because the *mei-S332* gene is required for proper meiotic chromosome segregation in Drosophila males and females, we reasoned that its transcript should be present in both testes and ovaries. cDNAs homologous to cosmid 4-4 were isolated from a testis library and mapped to four transcription units. Only one of these is localized entirely within the 4.2 kb of genomic DNA containing *mei-S332* (Figure 1B).

Northern analysis both confirmed that the transcription unit within the 4.2 kb genomic region is that of the *mei-S332* gene and revealed that there are sex-specific forms of the transcript. There are three testis transcripts of 1.55, 1.6, and 1.8 kb as well as a single 1.75 kb ovary transcript (see Figure 4). The transcripts are shortened in *mei-S332*^{1} males and females (data not shown), consistent with the presence of a DNA insertion in *mei-S332*^{1} mutants that causes premature transcript termination. By sequencing testis and male cDNAs, an ovary cDNA, and genomic DNA, we found that the four transcript forms arise by alternative splicing and polyadenylation (data not shown). Despite differences in processing of the 5' and 3' untranslated regions in the different *mei-S332* transcripts, all four cDNAs sequenced share the identical long open reading frame and thus encode the same protein.

The MEI-S332 protein

The *mei-S332* gene contains a single long open reading frame of 1206 nucleotides encoding a 401 amino acid polypeptide (Figure 2), with a predicted molecular weight of 44.4 kDa and a pI of 8.5. The first methionine shown is most likely the true N terminus of the protein because there are stop codons in all three reading frames within 39 amino acids upstream. Using the BLAST database search program (Altschul et al., 1990), we found no significant similarities between MEI-S332 and any other proteins in the existing databases. Thus MEI-S332 is a pioneer protein.

There are several notable features in MEI-S332. First, residues Asn^{13} to Ile^{44} in the MEI-S332 protein are predicted to form a coiled coil (Berger et al., 1995; Lupas et al., 1991). Examination of the predicted coiled-coil region of MEI-S332 reveals that this structure could potentially form a parallel homodimer based on similarities to the GCN4 leucine zipper (Harbury et al., 1993; Lumb and Kim, 1995; O'Shea et al., 1991). Second, there is a striking cluster of acidic residues extending from Asp^{173} to Glu^{198} (14 of 26 residues are Asp or Glu), and a cluster of basic

```
MGSKVEQQYKLLNAELMDQVQKQRLEIGEYRKRVISLEREIMDIREEHVL      50
                           ↓   ↓
                           D   E
                           3   8

QNHRQRMENISIVRSLMLSLNVDSDSLAVRQEPAPAAQINRPSGPRRSSR     100

EICKDMRRTCALARTTRPISPRRSSSVTSTVSSTSRRSSAEVQSEVVTTR     150
           ↓
           M
           4

IPEDRRANKPTPPPRRPAELVFDEDDSDDDFDEAVSPVEETQTEQNEENN     200

RLFSIIEENGSEGESTDSSSSCEAIYCDTTFESSPPNAQVTVTPSGRALR     250

EVDTNIPVAVSLSRGKETGKGSWLAISVAVEDSPQEPSIQCPRLAVTRPS     300
                                          ↓
                                         stop
                                          7

QSSGIFPDVNGLTPRRSLFNGIGKMAGSTSTPKSFLVEEMPSIRTRSRTA     350

ANKKSENTDMSSSFCNNSARPSRSCRPTSLVEPSLKNKLRNGSKGKAKAKK    401
                        ↓ ↓    ↓
                        Y H    R
                        2 4    6
```

Figure 2: Sequence of the MEI-S332 protein. The amino acid sequence is shown together with the position and changes in the sequenced *mei-S332* mutant alleles.

residues at the extreme C-terminus (8 of 16 residues are Lys or Arg). Third, the acidic domain in MEI-S332 is contained within a sequence (residues 167-200) that is a strong candidate PEST sequence (Rogers et al., 1986). A second possible PEST sequence is found from residues 202-242, immediately adjacent to the first. PEST sequences have been proposed to be signals for proteolysis (Rechsteiner, 1988)]. Fourth, the putative MEI-S332 protein also contains several sequences that match the consensus motif S/T-P-X-X, which has been proposed to be a DNA-binding motif (Churchill and Suzuki, 1989; Suzuki, 1989).

Nature of the *mei-S332* alleles

The original *mei-S332*^{1} allele causes high levels of nondisjunction in both males and females and is a possible null allele of the locus (Davis, 1971; Kerrebrock et al., 1992). Two of the EMS-induced alleles (*mei-S332*^{4} and *mei-S332*^{7}) are also strong in both sexes (Kerrebrock et al., 1992). However, hypomorphic alleles of *mei-S332* when homozygous have stronger effects in one sex than in the other: *mei-S332*^{2} and *mei-S332*^{6} are stronger in females than in males, and conversely, *mei-S332*^{3} and *mei-S332*^{8} are stronger in males than in females (Kerrebrock et al., 1992). Results from Northern blots demonstrate that these sex-specific differences are not at the level of mRNA expression (data not shown). We determined the locations of these mutations in the MEI-S332 protein sequence by polymerase (PCR) amplification of mutant genomic DNA to discriminate which regions of MEI-S332 were necessary in both sexes and whether the sex-specific mutations mapped to discrete domains.

The strongest *mei-S332* alleles are predicted to truncate or alter the C-terminal portion of the protein. The *mei-S332*^{7} allele (a potential null) resulted from a stop codon at residue Arg^{293}, producing a polypeptide lacking 109 C-terminal residues (Figure 2). Although we were unable to obtain PCR products using genomic DNA from *mei-S332*^{1} mutants, we mapped the putative insertion in this mutant between two restriction sites corresponding to residues Ser^{300} and Ser^{374} in the protein sequence (data not shown). We found two missense mutations in the third allele that is strong in both sexes (*mei-S332*^{4}). The more dramatic of the two changes is the proline to histidine change at residue 377.

The sex-specific mutations mapped to distinct regions within the MEI-S332 protein. Both female-predominant mutations are missense mutations that mapped very close to the *mei-S332*^{4} mutation in the C terminus (Figure 2). Interestingly, the male-predominant mutations are missense mutations that mapped in the N-terminal

region of MEI-S332, within the predicted coiled coil. The more severe of these two alleles (*mei-S332*^{8}, Kerrebrock et al., 1992) resulted from a Val to Glu substitution at residue 34. This would be predicted to destabilize the coiled coil by introducing a charged residue into the hydrophobic interface at the site of protein-protein interaction (O'Shea et al., 1991).

The MEI-S332-GFP fusion protein is localized on meiotic chromosomes

The phenotype of mutations in the *mei-S332* gene suggested that its product might act during meiosis to hold sister chromatids together at their centromeres. Thus, it was important to determine whether MEI-S332 localized to meiotic chromosomes and, if so, where and when the protein assembled on the chromosomes.

We localized MEI-S332 by fusing its open reading frame to that of the *Aequorea victoria* green fluorescent protein (GFP) (Chalfie et al., 1994; Wang and Hazelrigg, 1994). The GFP sequences were inserted immediately after the N-terminal methionine of MEI-S332. To express the MEI-S332-GFP fusion under the normal *mei-S332* regulatory sequences, we placed it into transposon P[w^+5.6KK *mei-S332*] and produced transformed Drosophila lines. Transformants carrying the MEI-S332-GFP fusion were crossed into a *mei-S332* mutant background to determine whether the fusion protein was functional. The MEI-S332-GFP fusion restored proper sex chromosome segregation in both male and female meiosis, and thus, it was capable of properly ensuring sister-chromatid cohesion (data not shown).

In Drosophila spermatocytes, all stages of meiosis are cytologically well resolved and individual chromosome arms and centromere regions can be seen. We examined the localization of MEI-S332-GFP in spermatocytes from lines with one, two, or four copies of P[w^+ 5.6KK *mei-S332-GFP*]. There was no significant difference in localization. In early prophase I, prior to extensive chromosome condensation, MEI-S332-GFP was not localized on the chromosomes (Figure 3A). There was considerable cytoplasmic MEI-S332-GFP in primary spermatocytes, possibly localized in some type of organelle (Figure 3A). As the chromosomes became condensed later in prometaphase I, MEI-S332-GFP was observed at distinct sites on the chromosomes and the cytoplasmic staining was diminished (Figure 3B).

During anaphase I in primary spermatocytes it was clear that the discrete chromosomal localization sites of MEI-S332-GFP corresponded to the centromere regions. As the chromosomes migrated to the poles, the centromere regions could be identified unambiguously at the leading edge, with the chromosome arms trailing. The

GFP fluorescence was localized specifically to the centromere region (Figure 3C and 3D, arrow). In late anaphase I, MEI-S332-GFP was present at the part of each chromosome closest to the pole, the centromere region (Figure 3C).

The MEI-S332-GFP protein remained on the chromosomes through metaphase II, consistent with the genetic data showing that the gene is required to maintain sister-chromatid cohesion from late in anaphase I until anaphase II. We saw localized fluorescence on the metaphase II chromosomes (Figure 3E). Thus, when the kinetochores of the sister chromatids function independently to attach to opposite poles but are still held together, MEI-S332-GFP remains localized to the centromere region. Strikingly, in early anaphase II, MEI-S332-GFP protein was no longer detected on meiotic chromosomes, consistent with the requirement for release of sister-chromatid cohesion at this time (Figure 3F).

Several controls demonstrate that the pattern of localization observed with MEI-S332-GFP is not due to background fluorescence or an intrinsic affinity of GFP for chromatin, rather it is dependent on the MEI-S332 sequences in the fusion protein. Spermatocytes lacking MEI-S332-GFP showed fluorescence only from the mitochondria during later stages; this can be seen in Figure 3F. There is no chromosomal localization of GFP in spermatocytes from flies transformed with the exuperentia (EXU)-GFP fusion protein (Wang and Hazelrigg, 1994, and data not shown).

Does *mei-S332* have a role in mitosis?

In contrast with the localization of MEI-S332-GFP on meiotic chromosomes, we did not observe fluorescence on mitotic chromosomes in larval neuroblast squashes (D. Moore and T. Orr-Weaver, preliminary results). We had previously ruled out a critical role for *mei-S332* in mitosis by showing that viability was unaffected in flies that had a putative null allele over a deficiency (Kerrebrock et al., 1992). However, we wished to investigate this question more closely by using more sensitive tests to look for effects of *mei-S332* on mitotic divisions. We tested the requirement for *mei-S332* in the mitotic divisions that take place in the larval brain by examining neuroblast squashes of *mei-S332* mutants for defects in mitotic figures. We examined between 800-1000 metaphase figures from *mei-S332*^{7} */ Df(2R)X58-6* and from wild-type Canton S controls as well as 300-400 anaphase figures from each genotype. There were no significant mitotic abnormalities in the *mei-S332* mutants. In wild type, 0.3% of the metaphase figures showed some degree of premature sister

separation, compared to 0.7% in the *mei-S332* mutants. These are not significantly different by a χ^2 test.

As a genetic test for chromosome misbehavior in mitosis, we scored the frequency of somatic clones in the wing in flies heterozygous for the recessive marker *multiple wing hairs* (*mwh*) (Lindsley and Zimm, 1992). Clones were scored in *mei-S332^1/Df(2R)X58-6* flies and control *mei-S332^1/+* flies. The *mei-S332* mutants gave only 0.55 clones per wing, the majority being only the size of a single cell, while the control gave 0.2 clones per wing. The frequency of clones in the *mei-S332* mutants is not significant, because it is less than that seen in wild-type flies by other workers, 0.74 *mwh* clones per wing (Baker et al., 1978). Although the *mei-S332^1* allele was reported previously to lead to a five-fold increase in somatic clones, these experiments were done with the homozygous mutant chromosome, and other mutations on the chromosome may have contributed to the mutant phenotype (Baker et al., 1978). Our results indicate that strong mutations in *mei-S332* have very little, if any, effect on mitosis.

We also looked for the presence of the *mei-S332* transcript in developmental stages during which mitosis is essential (Figure 4). The developmental pattern of *mei-S332* expression was consistent with the gene being essential only for meiosis. The 1.75 kb female transcript was present in embryos until 12 hours after egg laying, when it became barely visible (Figure 4). Since the male transcripts were not

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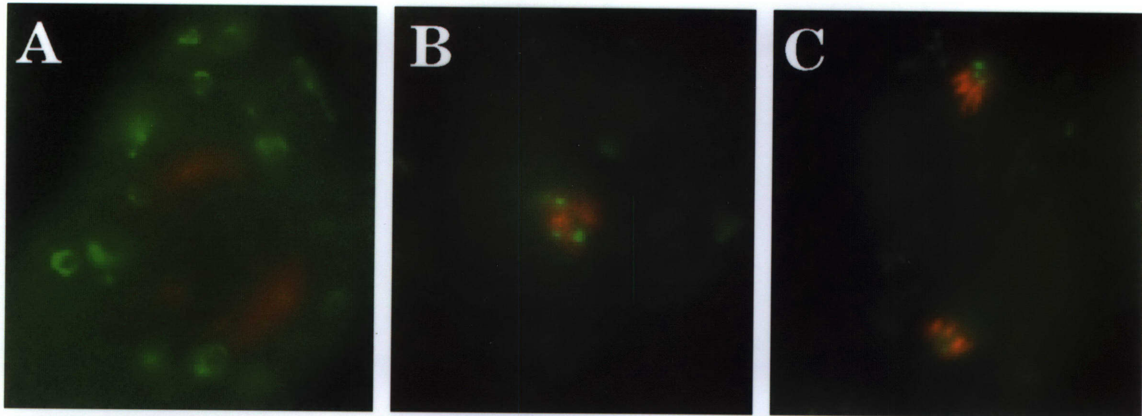
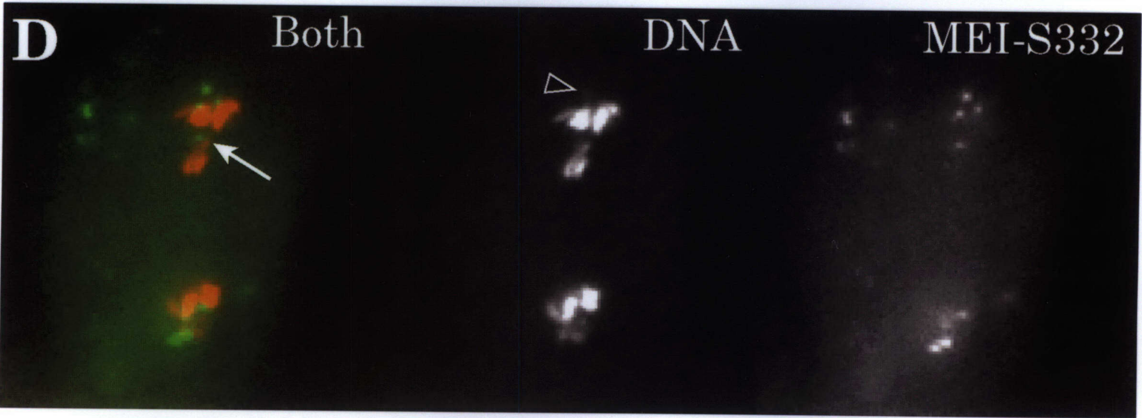
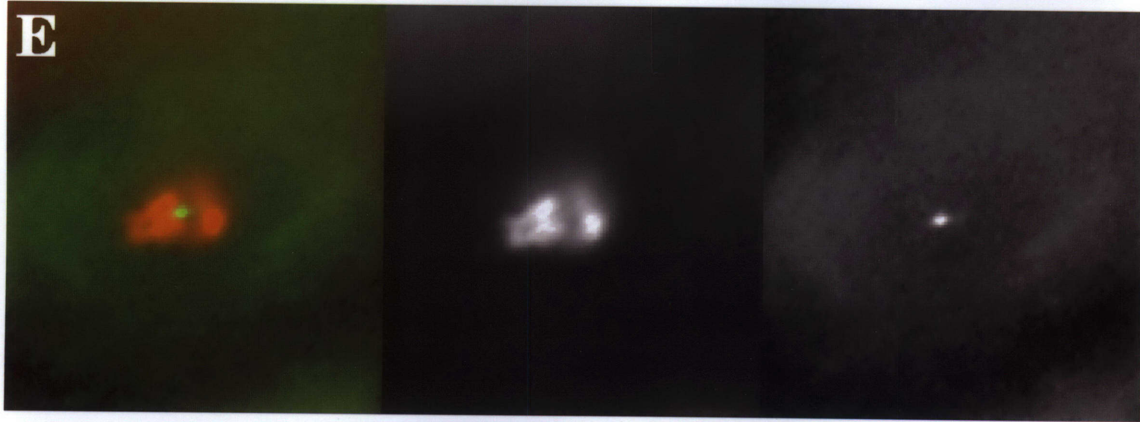
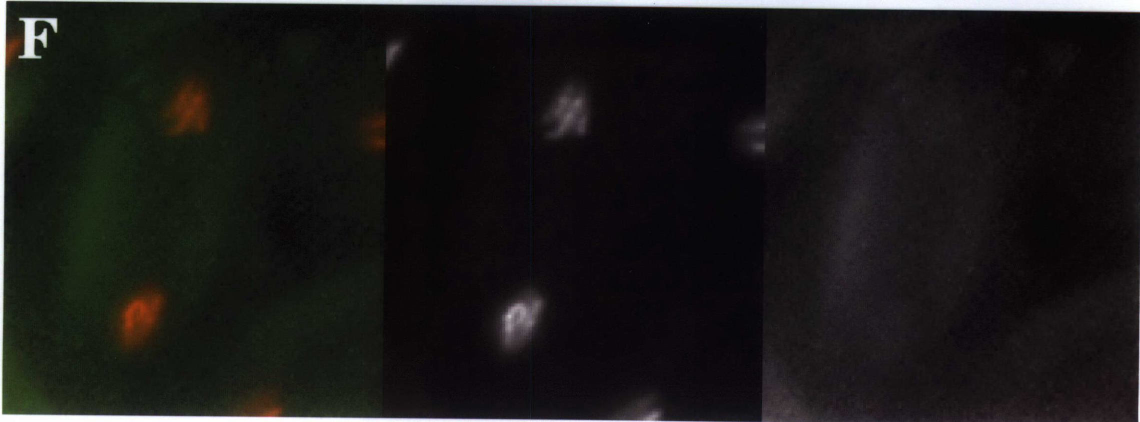
Figure 3: MEI-S332-GFP localization in spermatocytes. Testis were isolated from line *yw* GrM13; GrM1, fixed and stained with 7-AAD. MEI-S332-GFP is shown in green and the DNA in red. (A) In primary spermatocytes early in prophase I, MEI-S332-GFP is not observed on the chromosomes, but there is abundant staining in the cytoplasm. (B) At metaphase I, MEI-S332-GFP is localized to discrete chromosomal sites. The cytoplasmic staining is greatly diminished. (C) In anaphase I, MEI-S332-GFP is localized to the centromere region of each chromosome. (D) Anaphase I figure in which individual chromosomes can be distinguished. Both fluorescence channels are shown in the left panel, DNA staining is shown in the middle, and MEI-S332-GFP on the right. The MEI-S332-GFP is localized to the centromere region of each chromosome, the leading edge towards the pole (arrow). There is no MEI-S332-GFP detectable on the chromosome arms. MEI-S332-GFP is detectable on the tiny dot-like fourth chromosome at the pole (arrowhead). (E) In metaphase II MEI-S332-GFP is still visible at the centromere region. (F) At anaphase II, as cohesion is lost and the sister chromatids separate, MEI-S332-GFP is no longer detectable on the chromosomes. The chromosomal regions in which MEI-S332-GFP is localized stain weakly with 7-AAD. This is most likely because 7-AAD preferentially binds GC-rich DNA, and the centric heterochromatin in Drosophila is AT rich (Ashburner, 1989; Nikitin et al., 1985).


A
B
C


D
Both
DNA
MEI-S332


E


F

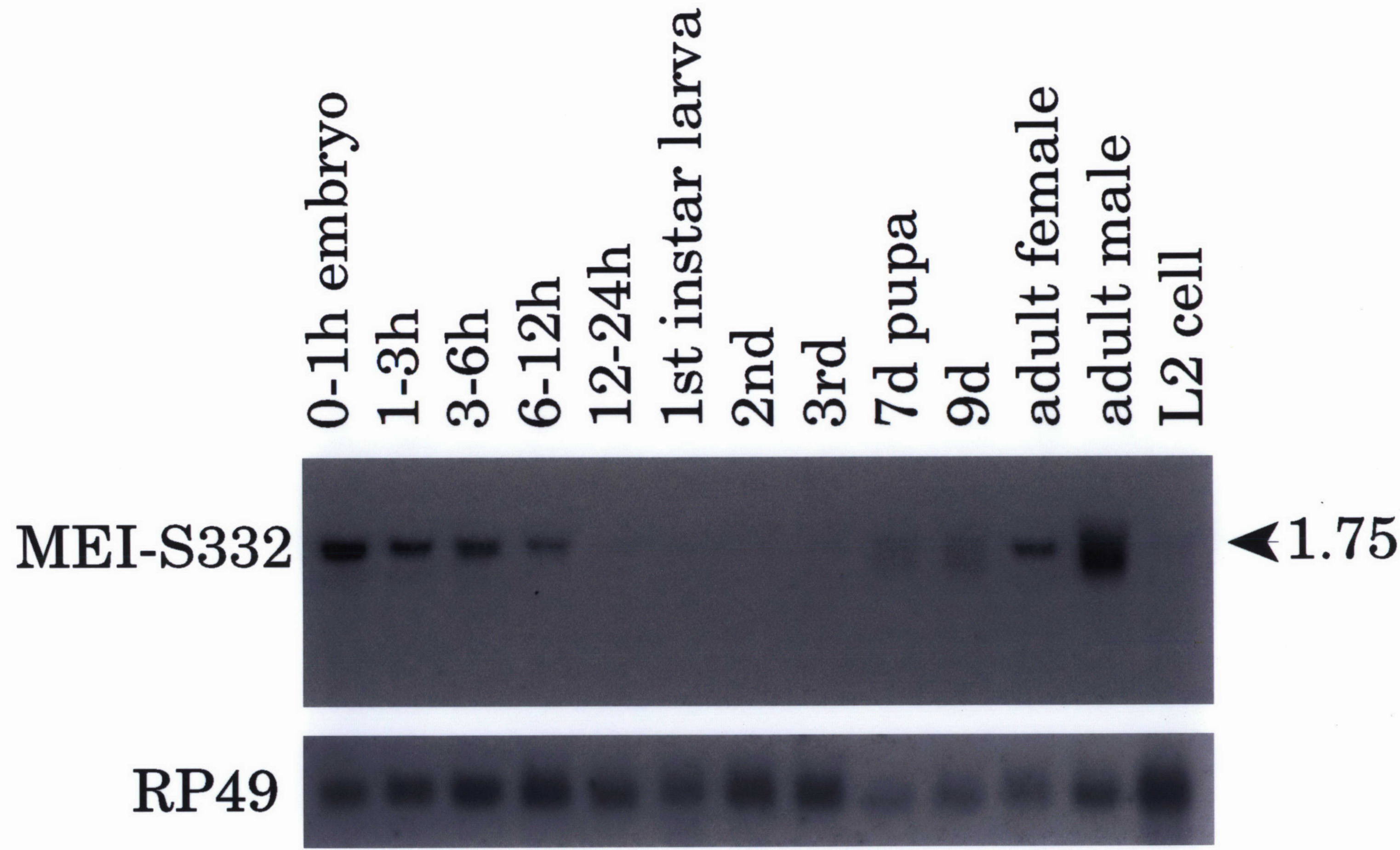


Figure 4: Developmental expression of *mei-S332*. Poly(A)+ RNA was isolated from each of the developmental stages indicated, a Northern blot was prepared, and it was probed with a male cDNA. The ovary form of the transcript is present in the first 12 hours of embryogenesis. Transcripts are not detectable again until the third instar larval stage when all transcript forms are observed. The testis forms of the transcripts are seen in adult males and the ovary form in females. Low levels of the female transcript are present in Schneider tissue culture cells. The ribosomal protein gene *RP49* was used as a standard for the amount of mRNA loaded on the gel.

detectable in embryos, the observed embryonic message was most likely persistence of maternal transcript rather than zygotic expression. Only a trace amount of the *mei-S332* transcript was seen in larvae (Figure 4), a developmental period when many mitotic divisions take place in the imaginal discs and brains. The transcripts were detectable in mature third instar larvae when meiosis begins in the gonads (Figure 4). This is the first developmental stage when we observe the male transcripts, suggesting this is the onset of zygotic expression of the gene.

Discussion

Localization of MEI-S332-GFP

The physical association between sister chromatids observed during mitosis and meiosis raises the possibility that proteins localized between the sister chromatids serve as a glue to hold them together. The time at which premature sister separation is observed in *mei-S332* mutants suggested that the MEI-S332 protein might act at the centromere regions. We isolated the *mei-S332* gene and showed that a MEI-S332-GFP fusion protein is localized to the centromere regions of meiotic chromosomes until the metaphase/ anaphase transition of meiosis II.

Because the MEI-S332-GFP fusion fully complements the *mei-S332* mutant phenotype, the localization of MEI-S332-GFP most likely coincides with that of the MEI-S332 protein. In addition to being localized to the centromere regions in spermatocytes, MEI-S332-GFP shows a localization pattern in oocytes that is consistent with it being on the centromeres (D. Moore and T. Orr-Weaver, unpublished results). Moreover, localization to the centromere region and subsequent disappearance when sister-chromatid cohesion is lost precisely match the genetically derived predictions that this protein is needed to hold sister chromatids together until anaphase II. Chromosomal binding is not an intrinsic property of GFP, since an EXU-GFP fusion does not localize to chromosomes. Consequently, the localization observed is likely to be caused by MEI-S332. Finally, in our experiments the MEI-S332-GFP protein was under the control of the normal MEI-S332 regulatory sequences.

MEI-S332-GFP is associated with the centromere regions before a defect is observed in *mei-S332* mutants. In mutants, premature sister-chromatid separation is not observed until late in anaphase I, yet the MEI-S332-GFP protein assembles onto the centromere regions in late prophase I. There could be a redundant function providing cohesion at the centromere early in meiosis I. In Drosophila *ord* mutants, premature sister-chromatid separation is observed by prometaphase I (Goldstein, 1980; Mason, 1976; Miyazaki and Orr-Weaver, 1992). Thus the ORD protein could promote cohesion both on the arms and at the centromere and compensate for MEI-S332 early in meiosis.

The time of centromere localization also bears on the relationship between MEI-S332 and the behavior of sister kinetochores. During meiosis I, the kinetochores of the sister chromatids must be constrained so that they attach to microtubules from

the same pole. Therefore, the sister kinetochores cannot function independently until meiosis II. In Drosophila male meiosis, the kinetochores of sister chromatids differentiate from a single, shared kinetochore to give rise to a "double-disc" structure between late prometaphase I and early anaphase I (Goldstein, 1981). This morphological doubling of the kinetochore may correspond to a doubling of function and, consequently, the ability to orient independently to the opposite spindle poles. The MEI-S332 protein may be present at the centromere region but not essential until the kinetochore has doubled.

It is a formal possibility that MEI-S332 forces the kinetochores of the sister chromatids to orient to the same pole during meiosis I rather than promoting cohesion at the centromere. If this were the case, then MEI-S332 would have to be inactivated during prometaphase II, when the sister chromatids orient and attach to opposite poles. In contrast, we observe MEI-S332-GFP present on the chromosomes through metaphase II. Moreover, a model in which MEI-S332 controls kinetochore behavior is difficult to reconcile with a phenotype of premature separation in late anaphase I and nondisjunction in meiosis II, since the sisters would be predicted to segregate frequently from each other during meiosis I. The localization of MEI-S332-GFP on the centromere region until anaphase II strongly supports a direct role for the protein in sister-chromatid cohesion.

Our experiments do not distinguish whether MEI-S332-GFP is bound to the kinetochore itself or to the heterochromatin flanking the centromere. MEI-S332 may control cohesion through the centric heterochromatin. Several lines of evidence indicate that during mitosis the centric heterochromatin is important for cohesion. In many organisms, treatment with spindle-disrupting drugs causes the arms of the sister chromatids to separate, but the sister chromatids remain attached at the centromere regions (for review see Miyazaki and Orr-Weaver, 1994). In scanning electron micrographs of human chromosomes, the arms of the sister chromatids are distinct from each other, while the centric heterochromatin does not split until anaphase (Sumner, 1991). In Drosophila, translocations that move centric heterochromatin to distal regions of the arms have been examined cytologically. During anaphase of mitosis, the heterochromatic regions on the arms separate later than the rest of the chromosomes, possibly because there is tighter cohesion in the heterochromatin (Gonzalez et al., 1991).

The disappearance of MEI-S332-GFP from chromosomes in anaphase II could be the consequence of its relocation to a dispersed distribution or its degradation. In mitosis, ubiquitin-mediated degradation of as yet unidentified proteins appears to be

required for the metaphase/anaphase transition (Holloway et al., 1993). The proteins encoded by the *cdc16*, *cdc23*, and *cdc27* genes from *Saccharomyces cerevisiae* and their vertebrate homologs have been demonstrated to be essential for the degradation triggering the mitotic metaphase/anaphase transition (Irniger et al., 1995; Tugendreich et al., 1995). These are part of a 20S complex termed the anaphase-promoting complex (APC) (King et al., 1995). Proteins controlling sister-chromatid cohesion are predicted to be substrates for this proteolytic pathway. It will be interesting to determine whether MEI-S332 is degraded and, if so, what controls its proteolysis. However, in contrast to the B-type cyclins which are known to be substrates of APC, MEI-S332 does not contain a destruction box as defined by the cyclin consensus sequence. It does contain PEST sequences, so it may be degraded by another pathway.

Structure of MEI-S332 protein

MEI-S332 is a novel protein that is not significantly homologous to proteins described in the database. The only other mutation yet isolated with a phenotype similar to *mei-S332* is the *pc* locus of tomato, and no molecular information is available for this gene (Clayberg, 1959). The mammalian Cor1 protein has a localization pattern that suggests it may function analogously to MEI-S332, at least later in meiosis II (Dobson et al., 1994). Early in meiosis I, Cor1, unlike *mei-S332*, is a component of the synaptonemal complex and is localized with the axial elements along the arms of the sister chromatids. After anaphase I, however, Cor1 is localized at the kinetochore and remains there until anaphase II. Despite the similarity in localization after anaphase I and the possibility they have the same function, MEI-S332 does not have homology to Cor1.

The meiosis I division in Drosophila is under different genetic control in males and females, raising the possibility that while providing the same function to promote cohesion MEI-S332 might interact with different proteins in the two sexes (Orr-Weaver, 1995). The four alleles that affect segregation in predominantly one or the other sex are clustered at either end of MEI-S332. The two male predominant alleles are missense changes in a predicted coiled coil, and the strongest of these is predicted to disrupt dimerization. One hypothesis is that the coiled-coil domain may be more critical for function in male meiosis than in female because this domain interacts with a male-specific protein, perhaps by the formation of a heterodimer with the coiled coil. The two female predominant mutations cause amino acid changes in a basic region at

the C-terminus of the protein. This domain cannot be solely required for females, since the *mei-S332*[7] mutation is missing the last third of the protein and is strong in both males and females. Moreover, the *mei-S332*[4] mutation affects both sexes and has two amino acid changes, one of which is in the basic region. The female predominant alleles demonstrate that the basic domain is more important in female meiosis than in males. This may be a region of MEI-S332 that interacts with a female-specific protein.

Mitotic Counterpart to MEI-S332?

All of the evidence indicates that MEI-S332 has no role in mitosis. Apparent null alleles are fully viable and exhibit normal mitotic chromosome segregation in both genetic and cytological tests. The gene is transcribed abundantly at developmental stages when meiosis is occurring, and the transcripts are present at low levels in other stages. Thus far, we have not detected MEI-S332-GFP on mitotic chromosomes.

Is there a need for a function like MEI-S332 to provide cohesion in the centromere regions of mitotic chromosomes? In mitosis, the sister chromatids are closely apposed and appear to be physically associated along their length, but the attachment in the centromere region is more pronounced (for review see Miyazaki and Orr-Weaver, 1994). The cytology of sister chromatids in mitosis implies that cohesion is tighter in the centromere regions, possibly because it is controlled by different functions than those holding the arms in proximity. A protein analogous to MEI-S332 could promote cohesion at the mitotic centromere region. Drosophila mutant for *parallel sister chromatids* (*pasc*) lose cohesion in the centromere region during mitosis (Gatti and Goldberg, 1991). Similarly, in humans, mitotic cells taken from patients with Roberts Syndrome show premature separation of sister chromatids and have aberrant morphology in the centric heterochromatin (German, 1979). These genes are candidates for the mitotic counterparts to *mei-S332*.

The isolation of the *mei-S332* gene and the demonstration that a MEI-S332-GFP fusion protein localizes to meiotic centromeres provide the basis for understanding sister-chromatid cohesion at a molecular level. Determining how MEI-S332 associates with the centromere regions of chromosomes and how it disappears will provide critical insights into proper chromosome segregation and the regulation of the metaphase/anaphase transition.

Materials and Methods

Fly Stocks

The original *mei-S332* allele was isolated from a wild population (Sandler et al., 1968), and the genetic properties of this allele are described by Davis (1971) and Kerrebrock et al. (1992). The isolation and genetic characterization of the EMS-generated alleles *mei-S332*^{2}, *mei-S332*^{3}, *mei-S332*^{4}, *mei-S332*^{6}, *mei-S332*^{7} and *mei-S332*^{8} are described in Kerrebrock et al. (1992); the *Df(2R)R1-8* chromosome was isolated from the same EMS screen. The P[(w)A^{R}]4-043 transformant used for the X-ray screen (see below) was provided by R. Levis at the Fred Hutchinson Cancer Research Center (Levis et al., 1985). Stocks containing the *In(2LR)dpp*^{t24}, *In(2R)dpp*^{d75} and *Tp(2;3)DTD33* chromosomes were provided by W. Gelbart at Harvard University (Lindsley and Zimm, 1992; Spencer et al., 1982). The *Df(1)w*^{67c23} and *iso-1* stock were provided by R. Lehmann at the Whitehead Institute. All genetic markers used are described in Lindsley and Zimm, 1992.

Isolation of deficiencies in region 58

We used two strategies to isolate deficiencies in region 58. We constructed a deletion chromosome by recombination between two pericentric inversion chromosomes, and we performed an X-ray screen to obtain additional deficiencies in the region. The breakpoints of the *In(2LR)dpp*^{t24} and *In(2LR)dpp*^{d75} chromosomes are (22F1-2; 58B) and (22F1-2; 58D), respectively. A single crossover within the inverted regions of these two chromosomes results in two types of recombinants: one is deficient for the 58B-D region and the other is duplicated for the same region. Isolines were set up from the progeny of females that were transheterozygous for the *In(2LR)dpp*^{t24} and *In(2LR)dpp*^{d75} chromosomes. These females also had a duplication for the *dpp* locus on chromosome *3* (*Tp(2;3)DTD33*), which was needed to provide wild-type *dpp* function for viability. We used lactic acid-acetic acid-orcein squashes of salivary gland chromosomes (Ashburner, 1989) to screen the isolines for recombinant chromosomes that were deficient in the 58B-D region. One such recombinant was found and named the *In(2LR)dpp*^{t24L}*dpp*^{d75R} chromosome.

Additional deficiencies in the 58B-D region were generated using X-rays to cause loss of a dominant marker at 58D, a wild-type copy of the *white* gene in a P element transposon inserted into 58D (the P[(w)A^{R}]4-043 transposon (Levis et al.,

1985)). Males homozygous for the P[(w)A^{R}]4-043 transposon were irradiated with either 3000 or 4000 rad using a Torrex 120D X-ray machine (98.9 kV, 5 mamp) and crossed in pools of 25 to 50 *Df(1)w^{67c23}* virgin females. Approximately 350,000 progeny were screened for white eyes, indicative of the loss of the P[(w)A^{R}]4-043 transposon. White-eyed flies were outcrossed to flies from a *y/y^{+}Y; Sco/SM1* stock to make balanced stocks. Putative deficiencies were confirmed by lactic acid-acetic acid-orcein squashes of polytene chromosomes.

All newly isolated deficiencies were tested over the *mei-S332^{1}* allele to determine whether they uncovered the mutant phenotype. Sex chromosome nondisjunction tests in males and females were carried out as described in Kerrebrock et al. (1992).

Isolation of Nucleic Acids, and Southern and Northern blot hybridization

Genomic DNA was isolated from adult females as described in Ashburner (1989). Digestion of DNA with restriction enzymes, electrophoresis on agarose gels, and Southern transfer and hybridization followed standard techniques (Sambrook et al., 1989). Probes for Southern and Northern blots were labeled using the random primed DNA labeling kit (Boehringer Mannheim). Southern blots were exposed to XAR-5 film unless they were to be quantitated, in which case they were exposed to a BAS-III imaging plate (Fuji) and scanned with a Fuji BAS-2000 Bioimager. Controls for DNA loading on quantitative Southern blots were performed by normalizing the signal from the band to be quantitated to the signal from a standard band in the same lane. Standard bands used included the 4.6 EcoRI fragment from the *rosy* locus and the 1.55 kb SalI fragment from the *w* locus.

RNA was isolated as described in Ashburner (1989). Poly(A)$^{+}$ RNA was isolated by batch affinity chromatography on oligo(dT)-cellulose (type 2, Collaborative Research Incorporated) (Sambrook et al., 1989). Electrophoresis of glyoxalated RNA on agarose gels and transfer to Hybond-N membranes (Amersham) were performed as described by Sambrook et al. (1989). Northern blots were hybridized as described in Dombradi et al. (1989). Exposure of Northern blots and controls for quantitation were performed as described for Southern blots, except that the ribosomal protein *RP49* transcript (O'Connell and Rosbash, 1984) was used as a loading control.

Chromosome walk in 58B

Cosmids from the 58B region were isolated from a genomic library constructed by J. Tamkun in the *NotBamNot-CoSpeR* vector using DNA from the *iso-1* strain (Tamkun et al., 1992). This vector has the advantage that it has P element ends, and thus cosmids can be transformed into Drosophila to test for mutant rescue. The starting point for the walk was a 7.3 kb BamHI fragment from the 61D11 cosmid provided by the European Drosophila Genome Mapping Project; this cosmid had been shown by in situ hybridization to contain sequences from the 58B region (I. Siden-Kiamos, personal communication).

Quantitative Southern blots were used to map deficiency breakpoints within the walk. Inserts of representative cosmids were hybridized to Southern blots of EcoRI-restricted genomic DNA from flies homozygous for the *iso-1* chromosome and from flies that had the *iso-1* chromosome transheterozygous to either the $In(2LR)dpp^{t24L}dpp^{d75R}$ or *Df(2R)R1-8* chromosome. The ratio of the normalized signal in each band in the deficiency lanes to that of the corresponding band in the wild-type (*iso-1*) lane was 0.5 if the fragment lay within the deficiency and 1.0 if the fragment was outside of the deficiency.

Transformation Rescue

The P[w^+ 8.6RR*mei-S332*] transposon was constructed by subcloning the 8.6 kb EcoRI fragment from cos4-4 into the EcoRI site of the *CaSpeR4* transformation vector (Pirrotta, 1988), and the P[w^+ 5.6KK*mei-S332*] transposon was constructed by subcloning the 5.6 KpnI fragment from cos4-4 into the KpnI site of *CaSpeR4* (Figure 1). Injections were performed as described in Spradling (1986) using the helper plasmid pIChsπΔ2-3, a wings-clipped derivative of pUChsπΔ2-3 (Mullins et al., 1989). Cosmid DNA at 1 mg/ml or plasmid DNA at 0.5 mg/ml was coinjected with 0.3 mg/ml of helper plasmid into embryos from the $Df(1)yw^{67c23}$ strain, and up to 10 independent lines were established for each construct. Transformed inserts were crossed into flies that were either homozygous or hemizygous for the $mei\text{-}S332^{7}$ allele to assay for sex chromosome nondisjunction. Sibling controls for the nondisjunction tests included flies that were mutant for *mei-S332* but lacked the transposon, and *mei-S332*/+ heterozygotes (with or without the transposon). Quantitative Southern blots were performed on transformed lines to confirm that the insert DNA was intact.

Isolation of cDNA clones and DNA sequencing

The insert from the cos4-4 cosmid was used to screen a testis cDNA library in the λZAPII vector (provided by T. Hazelrigg). A total of 259 positive clones were isolated out of 1.4 x 10^6 clones screened; these clones were assigned to four transcription units based on patterns of cross hybridization on the library filters. cDNAs were isolated also from a male library provided by T. Karr. cDNA phage clones were converted to plasmids using the Exassist/SOLR excision system (Stratagene). Nine female-specific cDNAs were isolated by screening 530,000 clones from a 4-8 hour embryo library in the NB40 vector (Brown and Kafatos, 1988) using a male cDNA insert as a probe.

One testis cDNA clone was sequenced by the Molecular Biology Core Facility at the Dana-Farber Cancer Institute. All other sequencing was carried out with the Sequenase version 2.0 DNA Sequencing kit (United States Biochemicals), using a combination of nested deletions and gene-specific primers. Sequences were assembled and analyzed using DNAStar software. To sequence the *mei-S332* mutant alleles, genomic DNA prepared from homozygous mutant females was digested with EcoRI and sequences from the *mei-S332* gene were amplified by PCR using standard conditions (Sambrook et al., 1989). The PCR products were cloned into the Bluescript vector and sequenced using gene-specific primers. Two independently amplified PCR products were cloned and sequenced for each mutation.

MEI-S332-GFP Construct

We generated a BamHI site at the start of the GFP coding region and a BglII site at the end of the coding region using Tu#65 (Chalfie et al., 1994) as a PCR template with the following 31-mer primers: 5'-CCCCGGGAGATCTTTTGTATAGTTCATCC-AT-3' and 5'-GGAATTCGGATCCAAAGGAGAAGAACTTTTC-3'. The PCR product was ligated into the pCRII vector (Invitrogen), and the plasmid named pTAcsGFP.

The BamHI site in the polylinker of the plasmid carrying transposon P[w^+ 5.6KK*mei-S332*] was eliminated by cutting with StuI and SfiI, filling in the ends and religating. This left a unique BamHI site at the 5' end of the *mei-S332* open reading frame, into which the GFP BamHI-BglII fragment was inserted. The resulting fusion of the GFP and *mei-S332* coding sequences should change the sequence of these two proteins minimally: a glycine is inserted after the initial methionine in GFP, an

arginine is introduced between the fused proteins, and the first two amino acids of *mei-S332* are deleted.

Transformation was carried out as described earlier, using 0.5 mg/ml of plasmid and 0.1 mg/ml of helper. The insertion GrM13 is located on the *X* chromosome, the insertion GrM1 is on the second chromosome, and GrM20 is on the third chromosome. A single copy of the MEI-S332-GFP fusion transposon complemented *mei-*$S332^7$*/Df(2R)X58-6* males and females.

Microscopy

Testes were dissected from adult flies and immediately fixed using the 8% formaldehyde fixative solution described by Theurkauf and Hawley, 1992. The fixed organs were rinsed 10 minutes in 1 x PBS at least twice before staining with 10μg/ml 7-amino-actinomycin D (7-AAD, Molecular Probes, Eugene, OR) for 1/2 hour. After staining, the organs were briefly rinsed twice for 5 minute in 1 x PBS before being mounted on slides in 50% glycerol. Epiflourescence microscopy was done using a Nikon Optiphot-2 microscope equipped with Nikon 60 x and 100 x oil objectives. A Photometrics Image Point cooled CCD video camera was used to photograph the images, and Adobe Photoshop 3.0 run on a Power Macintosh 8100/80 was used to process the images.

Analysis of Mitosis

The cytology of mitotic chromosomes was investigated in larval neuroblasts. Brains were dissected from third instar larvae, fixed in acetic acid, stained with orcein, and squashed as described in Ashburner (1989). Colchicine was not added, and the cells were not hypotonically treated. The cells were examined on a Zeiss Axiophot microscope under phase using a 63 x Plan Apochromat objective. The number of metaphase and anaphase figures per field was scored, as well as their morphology. To score somatic clones, *mei-*$S332^7$*/+; mwh/+* control flies or *mei-*$S332^7$*/Df(2R)X58-6; mwh/+* mutant flies were fixed in 70% ethanol, and their wings were removed and mounted in Hoyers mountant (Ashburner, 1989). Mutant *mwh* clones were scored on a Zeiss Axiophot microscope with a 63 x Plan Apochromat objective.

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Chapter Three

Localization of MEI-S332 in oocytes and *ord* meiotic cells

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Introduction

Mitosis and the second meiotic division share an obligation to partition the sister chromatids during cell division. To orient the sister chromatids on the bipolar metaphase spindle so that they will segregate from one another during anaphase, it is necessary that the sister chromatids stick together until the transition from metaphase to anaphase (see reviews by Bickel and Orr-Weaver (1996) and Miyazaki and Orr-Weaver (1994)). Mitosis consists of one cell division following a single round of replication, thus sister-chromatid cohesion is maintained from synthesis until segregation of the chromosomes. Meiosis consists of two cell divisions following a round of replication, and cohesion between sister chromatids must meet unique requirements. In particular, sister-chromatid cohesion must be maintained through a cell division, through meiosis I. Cytologically, the arms of sister chromatids are observed to lose cohesion during this cell division, while the centromere regions remain tightly associated. Since exchange occurs between homologs during meiosis I of most species, loss of cohesion on the arms of the sister chromatids is likely to be a necessity. An exchange event attaches the arm of one sister chromatid to the kinetochore of a homolog, so that sister-chromatid arms may need to segregate away from one another during anaphase I. At the onset of anaphase I, sister-chromatid cohesion is conserved near the centromere while it is released along the arms.

The product of the *Drosophila melanogaster* gene *mei-S332* is required for maintenance of sister-chromatid cohesion at the centromere regions after the metaphase I/anaphase I transition. In both *mei-S332* males and females, segregation of homologs during meiosis I is infrequently disrupted, but the majority of sister chromatids lose cohesion near the centromere during anaphase I (Davis 1971; Kerrebrock et al. 1992). This loss of cohesion in *mei-S332* flies has been demonstrated by cytology of spermatocytes and by genetic assays of missegregation during both spermatogenesis and oogenesis. The MEI-S332 protein, labeled by fusing it with the autofluorescent protein GFP, was shown to be localized to small foci on the meiotic chromosomes as they condense during prometaphase I in spermatocytes (Kerrebrock et al. 1995). During anaphase I in spermatocytes, MEI-S332 is at the leading edge of the chromosomes, consistent with localization to centromere regions. MEI-S332 was localized to small foci within chromosomes condensed for metaphase II and was not detected on anaphase II chromosomes. The location of MEI-S332 product during meiosis in male Drosophila was consistent with a role providing cohesion between the centromere regions of sister-chromatids or a role protecting

cohesion between the sister-chromatids until the metaphase II/anaphase II transition.

The Drosophila gene *ord* is also required for sister-chromatid cohesion during meiosis in both males and females, and defects in the ord mutants are manifest at an earlier time than those in *mei-S332* (Mason 1976; Miyazaki and Orr-Weaver 1992). Missegregation in genetic assays yielded ratios consistent with random segregation of precociously separated sister chromatids through both meiotic divisions. Cytology of *ord* spermatocytes showed condensation defects as early as prometaphase I and precociously separated sister chromatids in metaphase I (Bickel et. al. 1997).

Mutations in two genes in Drosophila, *grauzone* (*grau*) and *cortex* (*cort*), result in oocytes arrested during meiosis II (Page and Orr-Weaver 1996). The majority of laid eggs have chromatin groupings that appear to be in metaphase II, but with time aberrant anaphase II figures become visible. The possibility that sister-chromatid cohesion could not be released in oocytes mutant for either *grau* or *cort* was shown to be unlikely, because double mutant *grau mei-S332* oocytes were still arrested in meiosis II although the chromatids had scattered.

In this chapter, localization of MEI-S332 is described during the development of normal oocytes. Localization of MEI-S332 in unfertilized eggs and embryos is also examined, as well as localization in eggs arrested in meiosis II by *grau* or *cort*. Finally, localization of MEI-S332 to chromosomes in *ord* spermatocytes and oocytes is considered.

Results

MEI-S332 is localized to metaphase I oocyte nuclei

The bipolar spindle in Drosophila oocytes is organized by the chromatin rather than by centrosomes. Therkauf and Hawley (1992) have divided the development of metaphase I in Drosophila oocytes into four stages. First, the chromatin condenses into a small rounded mass (Fig. 1A). Second, short microtubules are captured by the chromatin (Fig. 1B). Third, the microtubules begin to coalesce into a bipolar arrangement (Fig. 1C). Finally, the bipolar spindle grows in length (Fig. 1D and Fig. 2). In our images, the appearance of the chromatin changed with the development of the spindle, likely reflecting an increase in spindle forces. The dense ball of chromatin (Fig. 1B) took on a more oval shape with a less densely stained center (Fig. 1C) as the bipolar spindle formed. Finally, when the bipolar spindle had lengthened, the chromatin was sometimes found in a bar bell shape, held by a small bridge (Fig. 1D) or held by stretched chromatin (Fig. 2).

No foci of MEI-S332-GFP were observed on the karyosome before stage 13 in oocyte development. As the chromosomes condensed, a small number of foci were seen throughout the karyosome (Fig. 1A). Four were evident in this image, although as many as eight were seen in other nuclei. "Caps" of MEI-S332-GFP were established at opposite ends of the karyosome as the bipolar spindle began to form (Fig. 1B-C). The caps were associated with the stained chromatin but were just outside of it. As the spindle lengthened, brighter dots could sometimes be discerned in the caps (Fig. 1D) and occasionally distinct dots could be observed (Fig. 2).

Individual chromosomes were not visible in these figures. Page and Orr-Weaver (1997) have observed that the chromatin does not become individuated into chromosomes until activation of the egg. However, Hawley et. al. (1993) have described three spindle fiber bundles emanating from each side of the chromatin mass and joining at the poles, and these were suspected to be kinetochore bundles linking chromosomes and the poles. Kinetochores could have some individual character in the metaphase I arrested chromatin mass although the bulk of the chromosomes is not individuated.

MEI-S332-GFP was localized to regions of the karyosome where the centromeres of homologs would be expected. In bivalents, homologs are attached at sites of exchange on their arms, identified as chiasmata in other species. The kinetochores of homologs are usually not at the metaphase plate but are at the end of the

chromosomes nearest the spindle poles. MEI-S332-GFP was located at the ends of the chromosomes nearest the poles, and the bulk of the chromatin lay between these caps.

If discernable kinetochores existed during metaphase I, four dots of MEI-S332-GFP would be expected at each end, corresponding to oriented kinetochores at the polar ends of the four bivalents. The karyosome in Figure 2 was unusual in that the dots in the caps were quite distinct. Surprisingly, more than four foci were observed on each end of the spindle. Four of the dots were brighter and were closer to the leading edge of the chromosomes on the right hand side of the spindle, and brighter dots also were at the chromatin edges on the left side of this spindle. These are likely candidates for the centromere regions, while the less bright and slightly lagging foci of MEI-S332-GFP might be located on other regions of the meiotic chromosomes. Although the metaphase I in Figure 2 was unusual, it was very common to observe dimmer and diffuse foci within the chromatin mass prior to elongation of the bipolar spindle (Fig. 1B-C). These may also be regions of the chromosome that are not near the centromere and so are consistently located with the rest of the chromosome arms.

MEI-S332 is located on condensed chromosomes after meiosis

Meiosis is completed in unfertilized eggs, as well as in embryos, and the chromosomes not involved in mitosis form a bouquet structure that lies at the surface of the egg. This polar body of condensed chromosomes is approximately 10 µm in diameter and is not degraded in embryos until it is displaced by mitotic nuclei, after approximately 10 cycles of mitotic divisions. In both embryos and unfertilized eggs, bright foci of MEI-S332-GFP were observed within the polar body. Figure 3 shows three focal planes, 1.25 µm apart in depth, of a polar body in an

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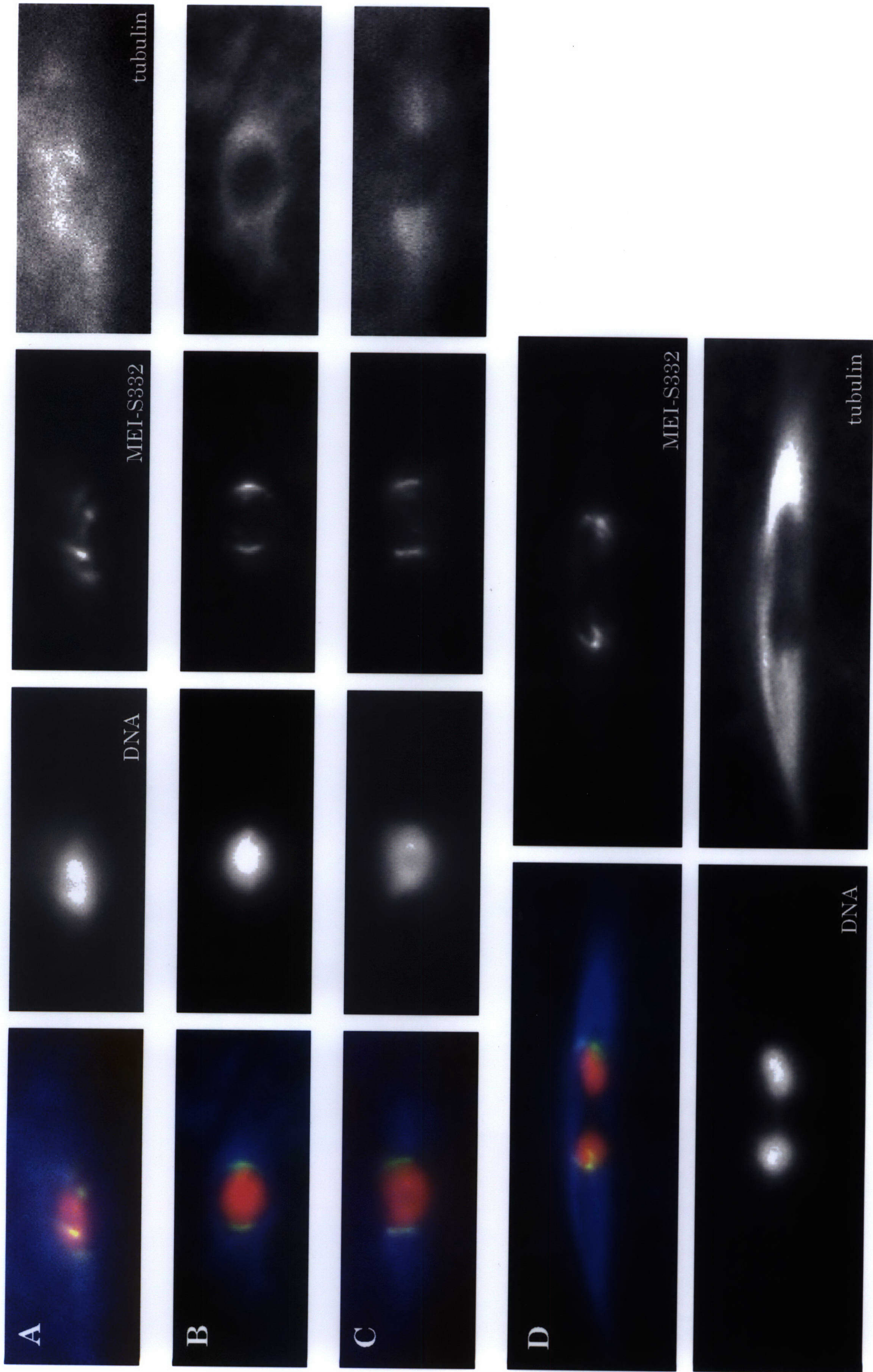
Figure 1: Localization of MEI-S332 on oocyte chromosomes as metaphase I develops. In the color figure, DNA is shown here in the red channel, MEI-S332 in the green channel and tubulin in the blue channel. These channels are also shown independently. (A) MEI-S332-GFP is in four foci associated with the condensing chromosomes. No bipolar spindle has formed but short microtubules are being captured. (B) A bipolar spindle has begun to organize at either end of the condensed chromatin. MEI-S332-GFP is arranged in caps at opposite ends of the karyosome. (C) The bipolar spindle has organized further, and MEI-S332-GFP is still in caps at the polar ends of the karyosome. (D) The spindle is elongated and the chromatin is in a barbell shape with a stretched region of chromatin between the two masses. MEI-S332-GFP is on the ends of the chromatin nearest the spindle poles.


A
B
C
D
DNA
MEI-S332
tubulin
MEI-S332
tubulin
DNA

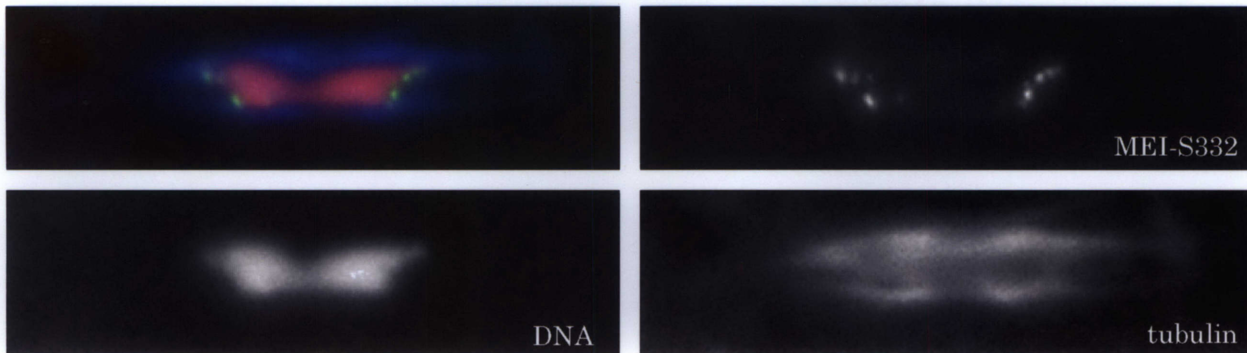


Figure 2: Distinct foci of MEI-S332 at the ends of metaphase I chromosomes in an oocyte. In the color figure, DNA is shown here in the red channel, MEI-S332 in the green channel and tubulin in the blue channel. These channels are also shown independently. Foci of MEI-S332-GFP are discernable as dots that can be counted. More than four foci are observed at either end of the chromatin.

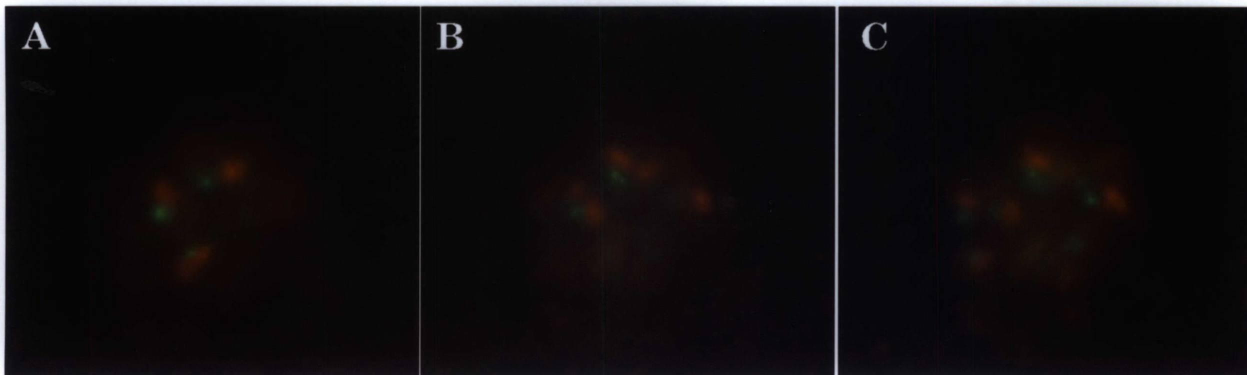


Figure 3: MEI-S332 is localized to foci in the polar bodies of eggs. DNA is shown in red, and MEI-S332-GFP in green. Three different focal planes, representing a total 2.5µm difference in depth, are shown in the panels. This polar body was from an unfertilized egg. MEI-S332-GFP is associated with the chromatin blobs, usually localized just to the left of the chromosomes in these images.

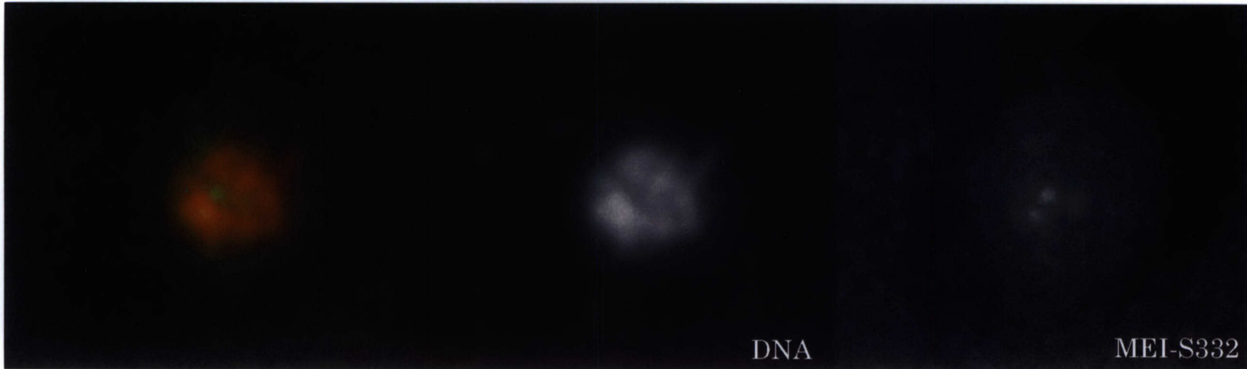


Figure 4: MEI-S332 is located on mitotic metaphase chromosomes in embryos. MEI-S332-GFP is green and DNA is red. Labeling also is shown independently. MEI-S332 is localized to a few foci that are in the middle of the chromatin.

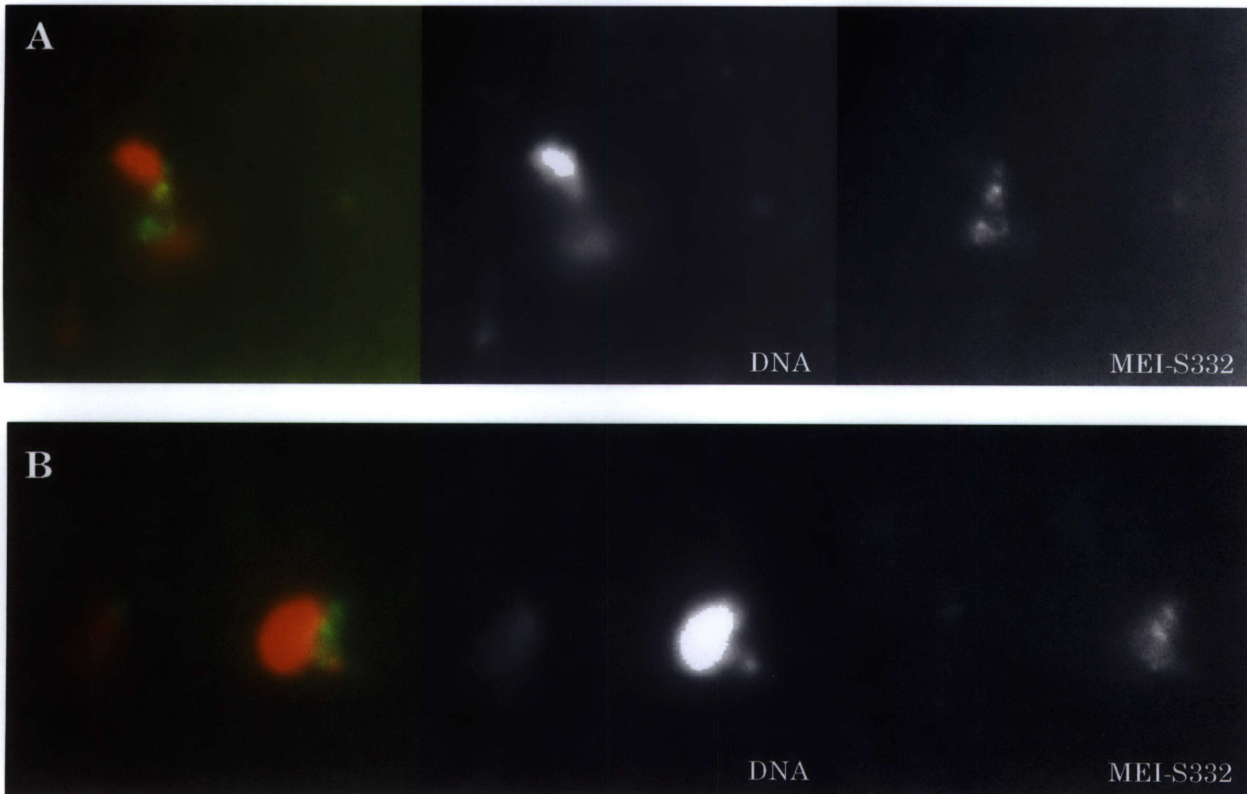


Figure 5: Localization of MEI-S332 to chromatin in an egg laid by *cort* mother. Two different chromatin groups from the same egg are shown in (A) and (B). MEI-S332-GFP is localized to discrete foci associated with condensed chromosomes in both "metaphase II" figures of this egg arrested in meiosis II. Metaphase appears to be breaking down, as some chromosomes are separated from the other chromosomes.

unfertilized egg. As noted for localization on meiotic chromosomes, MEI-S332-GFP foci were clearly associated with chromatin but were found outside the most brightly stained regions of condensed chromatin. As a general observation, MEI-S332-GFP was found on the same side of most chromosomes in any particular polar body (for example, to the left of most of the chromosomes in Figure 3A-C). While the number of foci were difficult to count precisely, the approximate number was consistent with the 12 centromeres anticipated in embryos or the 16 centromeres anticipated in unfertilized eggs.

Mitotic nuclei were examined in five embryos. Interphase chromosomes had no detectable MEI-S332-GFP signal. Metaphase nuclei in a cycle 6-7 embryo had faint foci apparent in the midst of the condensed chromosomes, a signal much reduced in intensity relative to foci on the polar body (Fig. 4). MEI-S332-GFP was localized to less densely stained regions of the chromatin and the foci were located in the center of the condensed chromosomes, reminiscent of localization on metaphase II chromosomes in spermatocytes (see Fig. 3E of Chapter One).

MEI-S332 remains on condensed chromosomes arrested in meiosis II

A large percentage, 80-89%, of the eggs laid by *grau* or *cort* mothers arrest in meiosis II and, with time, aberrant anaphase II figures are visible (Page and Orr-Weaver 1996). We examined the localization of MEI-S332-GFP in such eggs to determine if MEI-S332 is lost from chromosomes that begin to segregate when metaphase II breaks down. More importantly, the arrest in meiosis II allowed us to examine localization to condensed chromosomes in oocytes after metaphase I, although these are chromosomes that are condensed for abnormal lengths of time.

Eggs collected for two hours from $grau^{QE70}$ or $cort^{QW55}$ homozygotes had two chromatin groupings, frequently condensed in metaphase formations. MEI-S332-GFP was localized to the chromosomes in all cases. Chromatin groupings sometimes had chromosomes that fell outside of the bulk of the condensed chromosomes, as observed for the two chromatin groupings from a single egg laid by a *cort* female (Fig. 5A-B). The clarity of the images was dependent on the proximity of the material to the surface of the egg.

One egg from a *grau* mother had both chromatin groupings near the surface and was particularly informative. Both formations had identifiable chromosomes, one of the groupings spread in what might be interpreted as an anaphase II figure (Fig. 6A) and the other in what might be interpreted as early anaphase II (Fig. 6B-C). In both

chromatin groupings, four separate dyads can be distinguished, and the acrocentric *X* chromosomes and small chromosome 4s were identifiable (Fig. 6D). The shapes of the other chromatin blobs were consistent with either metacentric autosome (designated as *a* in Fig. 6D). The brightest foci of MEI-S332-GFP were located at the ends of chromosomes, a position consistent with centromere regions. The *X* chromosome in Figure 6A had less bright staining at its end, but there was a focus where the centromere region was likely to be located. Foci that might correspond to centromere regions are noted in Figure 6D with dark gray ovals. The chromosome *4* in Figure 6A has two less intense foci at either end (arrowheads in Fig. 6D), perhaps because sister kinetochores were no longer together on this particular chromosome. Strikingly, dimmer diffuse foci were seen on the *X* chromosomes in both groupings, midway along the length of the chromosome (arrows in Fig. 6D). The arms of the *X* chromosome had separated in Figure 6A, and both arms had foci at the same approximate location midway along the arms.

MEI-S332 is localized to centromere regions in *ord* spermatocytes

Strong alleles of *ord* disrupt segregation during both meiosis I and meiosis II, yielding nondisjunction frequencies that are consistent with random segregation of separated sister chromatids in both divisions (Miyazaki and Orr-Weaver 1992; Bickel et. al. 1997). We examined spermatocytes of several strong *ord* alleles to determine if MEI-S332 is expressed in *ord* testes and whether the product is appropriately localized to the meiotic chromosomes. Three alleles, ord^1, ord^3 and ord^5, have high frequencies of nondisjunction in male homozygotes and hemizygotes (Miyazaki and

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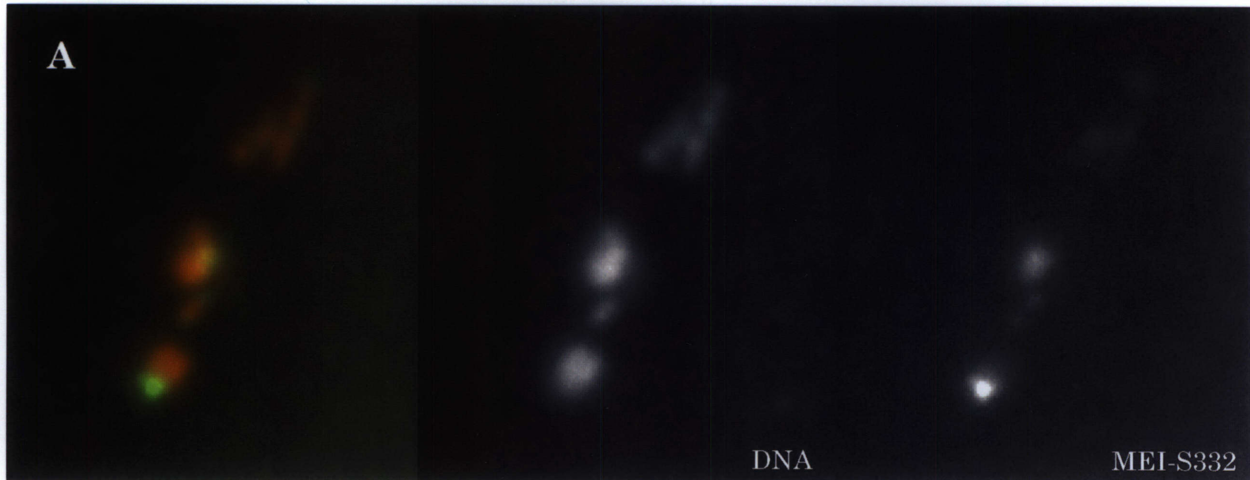
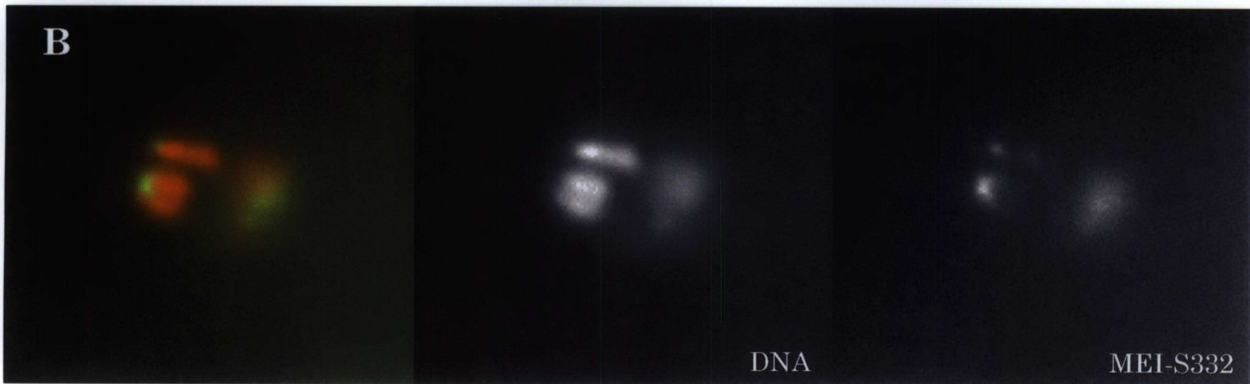
Figure 6: Localization of MEI-S332 to chromatin in an egg laid by a *grau* mother. In the color panel, DNA is shown in the red channel and MEI-S332-GFP in the green channel. (A) One chromatin grouping had condensed chromosomes that were well separated, and in one case, the arms of the chromosome are clearly separated. MEI-S332-GFP is associated with the chromosomes in discrete foci. (B-C) Two focal planes of the other chromatin group in the egg. Again the chromosomes remain condensed but are well separated, and MEI-S332-GFP is associated with each chromosome. (D) The acrocentric *X* chromosome and small chromosome *4* can be identified in the groupings, and they are labeled in this schematic drawing. The pattern of MEI-S332 localization is shown in gray patches, where the darker gray ovals represent localization to likely centromere regions while the lighter gray patches (arrows) are regions midway along the length of the *X* chromosome. One chromosome *4* has MEI-S332-GFP at either end (arrowheads), suggesting that the sister kinetochores may have separated.


A
DNA
MEI-S332


B
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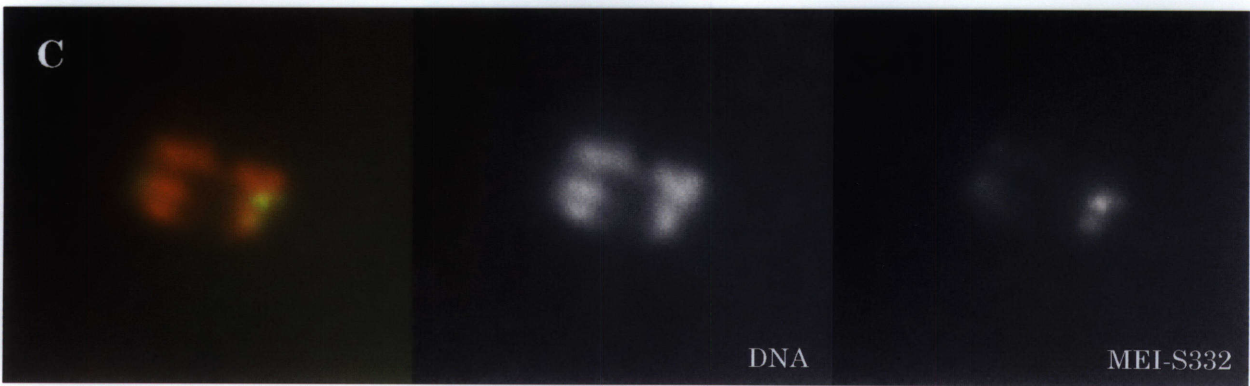
MEI-S332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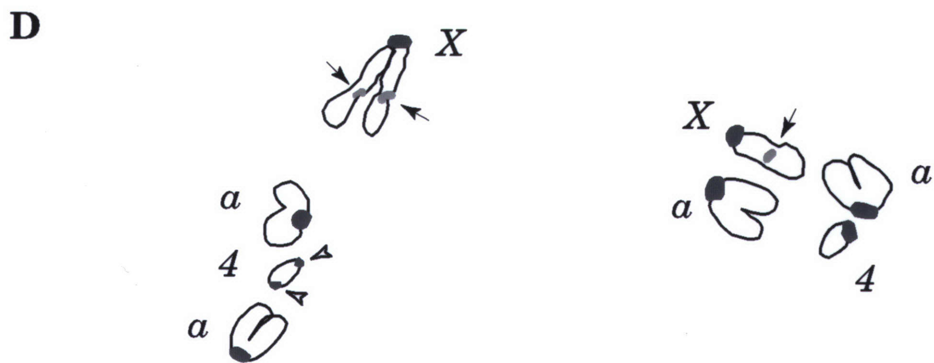
D
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a
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Orr-Weaver 1992). These alleles have been sequenced, and the latter two alleles are predicted to encode truncated products, while *ord*^{1} contains a missense mutation in the C-terminal end of the protein (Bickel et al. 1996). *ord*^{10} is predicted to encode a short peptide of only 25 amino acids and is likely to be a null mutation (Bickel et. al. 1997). All of these alleles were transheterozygous to a deletion of *ord*, *Df(2R)WI370*, in the following experiments.

In *ord* testes, MEI-S332-GFP was expressed at the same time as in wild-type testes, localizing to pockets in the cytoplasm during prophase I with no signal detectable on the chromosomes (Fig. 7A). As the chromosomes condensed and entered prometaphase I and metaphase I, MEI-S332-GFP was located in discrete foci on the condensed chromatin (Fig. 7B-D). This pattern of localization was also reported for *ord*$^+$ meiotic cells (see Fig. 3 in Chapter Two; Kerrebrock et. al. 1995).

In *ord*^{1} spermatocytes fixed in acetic acid, protrusions from the condensed bivalents were noted in prometaphase I figures (Miyazaki and Orr-Weaver 1992). Protrusions also were observed in some of our formaldehyde-fixed spermatocytes during both prometaphase I and during prometaphase II (Fig. 7B, Fig. 7F-G). MEI-S332-GFP localized to the ends of these protrusions, suggesting that these protrusions might be kinetochores pulled from the chromatin mass.

During anaphase I, MEI-S332-GFP was on the leading edges of chromosomes (Fig. 7D-E), providing the best evidence that MEI-S332-GFP is localized to the centromere regions in *ord* spermatocytes, just as it was observed to do in *ord*$^+$ anaphase I figures. Foci were detected on prometaphase II and metaphase II condensed dyads (Figs. 7F-H), but by anaphase II, the foci of MEI-S332-GFP were no longer detectable (Fig. 7I). Two of the cells in these figures had neighboring prophase I cells with pockets of MEI-S332-GFP (Fig. 7F, 7I). Greater contrast was used to visualize the MEI-S332-GFP foci on metaphase II figures, and these neighboring cells give a qualitative sense of the decreased amount of MEI-S332 on metaphase II figures.

The pattern of localization in *ord* testes is generally consistent with that observed in *ord*$^+$ spermatocytes. Lin and Church (1982) did ultrastructural studies of *ord*^{1} spermatocytes and found that the sister kinetochores of bivalents were always distinct, and usually separated, during prometaphase I. Physical separation of sister kinetochores in their micrographs was less than 0.5 μm. It was not apparent whether sister kinetochores are separated at the level of resolution yielded by our cytology, with one exception. On the protrusion from the prometaphase I bivalent in Fig. 7B, there were two foci. All four bivalents were accounted for, and a smaller

protrusion at the other end of the bivalent is also visible with MEI-S332-GFP localized to it These two foci may represent precociously separated sister kinetochores. However, MEI-S332-GFP localization has not been shown to correspond directly to kinetochores, but has only been shown to be at the leading edges of chromosomes in anaphase I. The two foci on a stretched portion of chromatin could as easily be interpreted as representing two divided regions of localization.

MEI-S332 is localized to karyosomes in *ord* oocytes

We examined oocytes isolated from Drosophila females that were either *ord10/Df(2R)WI370* or *ord^{10}/ord^{5}*. Where a karyosome was clearly identified, MEI-S332-GFP was localized to caps at the end of the chromatin mass. Localization on karysomes in oocytes from an *ord^{10}/ord^{5}* transheterozygote (Fig. 8B) had the same pattern of localization as that on karysomes prepared from an *ord^{10}* heterozygote (Fig. 8A). However, spindles were not labeled and nurse cells were removed in these preparations, so the stage of oocyte development could not be determined precisely. In preparations where spindles were labeled, no karyosomes with elongated spindles were observed in *ord^{10}* oocytes, suggesting that metaphase I arrest does not occur in these mutant oocytes (S. E. Bickel, personal communication). We infer from this result that the karyosomes that were identifiable without spindle labeling were those that did not yet have elongated spindles. Thus, our results demonstrate only that MEI-S332-GFP was localized to the karyosomes before the spindle elongated.

Figure 7: MEI-S332 localization in *ord* spermatocytes. MEI-S332-GFP is shown in green and DNA in red. (A) In this *ord^{1}/Df(2R)WI370* prophase I spermatocyte, MEI-S332-GFP cannot be seen on the chromosomes but it is abundant in the cytoplasm. (B) In this *ord^{1}/Df(2R)WI370* spermatocyte in prometaphase I, MEI-S332-GFP is localized to the condensing chromosomes. There are protrusions from one chromatin blob, a phenotype previously reported for *ord* prometaphase figures (Miyazaki and Orr-Weaver 1992), and MEI-S332-GFP is located near the end of the protrusion. One protrusion has two discernable dots of MEI-S332-GFP. (C) MEI-S332-GFP is localized to discrete foci on the congressed chromosomes in this *ord^{3}/Df(2R)WI370* metaphase I spermatocyte. (D-E) Two focal planes of an anaphase I figure from an *ord^{3}/Df(2R)WI370* testis. MEI-S332-GFP is localized to the leading edges of all of the chromosomes. (F-G) Two different prometaphase II cells from *ord^{10}/Df(2R)WI370* testes. There are protrusions from the chromatin with MEI-S332-GFP localized to the end. (H) MEI-S332-GFP is localized to foci in the center of the condensed chromosomes from an *ord^{3}/Df(2R)WI370* father. (I) By anaphase II, MEI-S332-GFP signal is no longer detectable on the chromosomes, here in an *ord^{10}/Df(2R)WI370* secondary spermatocyte.

Figure 7: legend on opposite page.

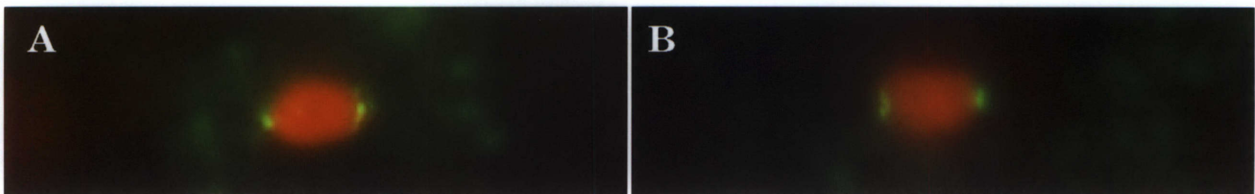

Figure 8: MEI-S332 is localized to chromosomes in *ord* oocytes. (A) A karyosome from an ord^{10} heterozygote shows wild-type localization of MEI-S332-GFP. (B) A karyosome from an ord^{10}/ord^{5} transheterozygote also has MEI-S332-GFP located in caps at the end of the karyosome.

Discussion

The product of the *mei-S332* gene is required for maintenance of sister-chromatid cohesion after the metaphase I transition to anaphase I, at a time when the sister chromatids are held together by only their centromere regions. MEI-S332, when followed by fusing the protein to GFP, localized to small regions of meiotic chromomes in spermatocytes, and the regions are clearly near the centromeres during anaphase I. In all of the cells examined in this chapter, MEI-S332-GFP also localized to chromatin where centromeres might be expected.

Dernburg, Sedat, and Hawley (1996) used in situ hybridization to show that the centromere regions of homologs were paired until prometaphase I. We did not observe MEI-S332 on the chromosomes until they began condensing in prometaphase I, but at this time, the number and position of the foci were consistent with paired centromere regions. At the time that a bipolar spindle is believed to be organized from short captured microtubules, most MEI-S332 was arranged in two caps at opposite ends of the karyosome. The kinetochores of bivalents in meiosis I are usually located at opposite ends of the bulk of their chromosomes, because the sites of attachment between homologs are on the arms of the chromosomes. Thus, MEI-S332 localization was observed where the centromeres of the bivalent were most likely located.

Mitotic chromosomes during metaphase are attached along their length, including cohesion near the kinetochore. Spindle forces align mitotic kinetochores on the metaphase plate. MEI-S332-GFP was localized to foci in the center of the mitotic chromosomes when metaphase nuclei were found in embryos. Sister chromatids during meiosis II similarly have cohesion near their kinetochores, and the pattern of MEI-S332 in mitotic metaphase nuclei was similar to that observed on metaphase II nuclei in spermatocytes.

The condensed chromosomes of the polar body nuclei are reported to be configured like a starburst with most of their centromeres oriented together and towards the embryo surface, and the three polar bodies often fuse together into one rosette structure (Huettner 1924; Doane 1960). The MEI-S332 foci did not group together, but they were always associated with a condensed chromosome and usually had the same general orientation as other chromosomes. The approximate number of foci was consistent with the expected number of centromeres after two meiotic divisions. MEI-S332-GFP was localized to the polar bodies in a pattern that was

consistent with the location centromere regions might assume in the bouquet structure.

Localization to chromosomes at places other than centromere regions

Several pieces of evidence suggest that MEI-S332 also can be localized to regions other than that near the centromere. The brightest foci of MEI-S332-GFP were in locations where centromeres might be expected to be located, but hazier darker foci were also observed. In the metaphase I nucleus in Figure 2, more than four dots were observed at either end of the chromosome mass. In typical stage 13-14 oocytes, the number of foci in the caps at the ends of the karyosome cannot be discerned, but less bright and slightly diffuse foci are seen in the mass of chromatin. Finally, in one clear example of condensed chromosomes arrested in meiosis II (Fig. 6), MEI-S332-GFP was localized to a region on the arm of a chromosome likely to be the *X* chromosome. The meiosis II arrested chromosomes were in a *grau* oocyte and may have been aberrantly condensed. Also, in addition to the normal *mei-S332* loci on chromosome *2*, at least two and as many as four copies of the MEI-S332-GFP construct are present in these Drosophila strains. Localization of MEI-S332-GFP to places other than centromere regions could be an artifact of the high dosage of functional protein, and it may not normally occur. However, MEI-S332 clearly is able to be localized to places other than the centromere, and this localization was to specific regions where signals might exist that are similar to those near the centromere.

Localization without cohesion

MEI-S332 is localized to centromere regions at a time when it would be required to provide cohesion between the sister chromatids, but localization was also observed to chromosomes where there was no sister-chromatid cohesion. First, MEI-S332-GFP was localized to regions on separated arms of a condensed chromosome arrested in meiosis II, the *X* chromosome in a *grau* egg (Fig. 6A, arrows in Fig. 6D). On another chromosome in the same grouping, the chromosome *4*, sister kinetochores appeared to have already separated even as MEI-S332-GFP remained localized to the regions (Fig. 6A, arrowheads in Fig. 6D). These are two cases where cohesion was not occurring, but MEI-S332 continued to be localized.

Genetic and cytological evidence suggests that sister-chromatid cohesion is disrupted in *ord* meiocytes, but MEI-S332-GFP was localized to the same regions of

these chromosomes, likely to be the centromere regions, as was observed in *ord*$^+$ oocytes and spermatocytes. There may be other components required for cohesion, perhaps ORD itself, that also need to be localized in addition to MEI-S332. Alternatively, there might be a threshold amount of MEI-S332 that must be localized for cohesion to be set up or maintained between sister chromatids.

Localization during mitosis

There is no requirement for expression of *mei-S332* for successful segregation of sister chromatids in mitosis. Mitotic missegregation was not observed in squashes of neural tissue from *mei-S332* larvae, and adult *mei-S332* flies were found to be perfectly viable and showed no evidence of somatic clones resulting from missegration (Kerrebrock et al. 1992; Kerrebrock et al. 1995). Thus, although MEI-S332 was localized to mitotic metaphase chromosomes in embryos, it does not seem to be required for chromosome segregation. MEI-S332 in embryos may be from a protein from the maternally-contributed pool rather than a protein that is zygotically expressed. Localization could be occurring without MEI-S332 serving any function in sister-chromatid cohesion. Alternatively, it may be functioning but redundant with other cohesion proteins, so that MEI-S332 does not seem to be required.

Differentiating between proposed functions of MEI-S332

Two models exist for the function of MEI-S332 in meiosis. In one model, MEI-S332 is essential for a mechanism of cohesion that is specific to the centromere regions. MEI-S332 acts as a glue in the centromere regions in this model. In a second model, the mechanism of sister-chromatid cohesion does not differ between the arms and the centromere regions, but MEI-S332 is required in the centromere regions to preserve cohesion near the centromere while it is released along the arms at the onset of anaphase I. MEI-S332 acts as an "anti-solvent" or a preservative that prevents dissolution of the bond between the sister-chromatids in the second model. The localization data in this paper does not prove either model to be correct, but there are implications to consider.

The timing of MEI-S332 localization to meiotic chromosomes in oocytes parallels that observed in spermatocytes. In both cell types, the protein was not localized until the chromosomes began to condense and enter prometaphase I. The sister chromatids are attached at the centromere regions during prophase I (Dernburg et.

al. 1996), so other mechanisms of cohesion must exist to bind sister chromatids near the centromeres before MEI-S332 is localized to the chromosomes. Furthermore, localization of MEI-S332 was observed on chromosomes where cohesion is not occurring. Taken together, the existence of association between centromere regions before MEI-S332 is localized to the chromosomes and localization of MEI-S332 to chromosomes where there is no cohesion, the model of MEI-S332 as a preservative is favored.

Materials and Methods

Stocks

Drosophila stocks and crosses were grown at 25°C on a standard mix of cornmeal, brewer's yeast, molasses and agar. Balancer chromosomes and mutations are described in Lindsley and Zimm (1992) except for the mutations mentioned here. The MEI-S332-GFP construct and strains carrying the GrM13 and GrM1 insertions of the construct were previously described in Kerrebrock et al. (1995). Oocytes examined in this study were isolated from *y w* GrM13; GrM1 mothers. The *grau*^{QE70} and *cort*^{QW55} mutations were generated by Schupbach and Wieschaus (1989), and the meiotic arrest phenotypes were described in Page and Orr-Weaver (1996). Eggs laid by *y w* GrM13; *grau*^{QE70} or by *y w* GrM13; *cort*^{QW55} mothers during a 2 hour collection period were examined. The *ord* alleles and stocks, as well as *Df(2R)WI370*, were described in Miyazaki and Orr-Weaver (1992), Bickel et al. (1996) and Bickel et. al. (1997). *ord* mutants from which ovaries or testes were isolated carried the *y w* GrM13 chromosome.

Microscopy

Stage 14 oocytes were prepared using the protocol described in Theurkauf and Hawley (1992). Tubulin was labeled using two anti-tubulin rat monoclonal antibodies, YL1/2 and YOL1/34 (Harlan Sera-Lab Limited, U.S. distribution by Accurate Chemical & Scientific Corporation), at a dilution of 1:5 in 1 x PBS, along with a secondary antibody, Texas Red-conjugated donkey anti-rat (Jackson ImmunoResearch Laboratories, Incorporated), at a dilution of 1:200. Two hour collections of eggs on apple juice-agar plates were dechorionated in 50% Clorox bleach, followed by a 1/2 hour fixation in 8% formaldehyde and manual devitellinization between two glass slides as described for fixed oocytes in Theurkauf and Hawley (1992). The eggs or embryos were then stained in 1μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) in 1 x PBS for 10 minutes, followed by two 15 minute rinses in 1 x PBS before mounting in 50% glycerol. Testes were dissected and fixed as described in Kerrebrock et al. (1995).

Epifluorescence microscopy was performed using a Nikon Optiphot-2 microscope equipped with a Nikon 60x oil objective. Either a Photometrics Image Point or a Photometrics CE200A cooled CCD video camera was used to photograph the images,

and Adobe Photoshop 3.0 run on a Power Macintosh 8100/80 was used to process the images.

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Chapter Four

Identification of ORD, a Drosophila protein essential for sister-chromatid cohesion

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My contribution to this chapter included the isolation of the deficiencies critical to cloning the gene.

Abstract

Attachment between the sister chromatids is required for proper chromosome segregation in meiosis and mitosis, but its molecular basis is not understood. Mutations in the Drosophila *ord* gene result in premature sister-chromatid separation in meiosis, indicating that the product of this gene is necessary for sister-chromatid cohesion. We isolated the *ord* gene and found that it encodes a novel 55 kDa protein. Some of the *ord* mutations exhibit unusual complementation properties, termed negative complementation, in which particular alleles poison the activity of another allele. Negative complementation predicts that protein-protein interactions are critical for ORD function. The position and nature of these unusual *ord* mutations demonstrate that the C-terminal half of ORD is essential for sister-chromatid cohesion and suggest that it mediates protein binding.

Introduction

Sister chromatids separate and segregate from each other in anaphase of mitosis and anaphase II of meiosis. For proper orientation and segregation in mitosis the sister chromatids must remain attached until the metaphase/anaphase transition. In meiosis, the sister chromatids must remain associated throughout meiosis I as the homologs segregate and retain these associations until the metaphase II/ anaphase II transition. Although mechanisms that ensure sister-chromatid cohesion have been postulated, few candidate genes have been identified (for review see Miyazaki and Orr-Weaver, 1994). There are likely to be structural proteins that hold sister chromatids together, as well as regulatory functions that time the release of cohesion until the metaphase/ anaphase transition. Identifying proteins necessary for sister-chromatid cohesion is critical for an understanding of segregation.

The Drosophila *ord* (*orientation disruptor*) gene is required for sister-chromatid cohesion in meiosis (Mason, 1976; Goldstein, 1980; Miyazaki and Orr-Weaver, 1992). Mutations in the *ord* gene cause chromosome missegregation during female and male meiosis that is consistent with precocious sister-chromatid separation early in meiosis I, followed by random segregation of the sister chromatids during the two meiotic divisions. This interpretation was confirmed cytologically in mutant males; sister chromatids prematurely disjoin as early as prometaphase I (Mason, 1976; Goldstein, 1980; Miyazaki and Orr-Weaver, 1992). Strong *ord* mutations cause missegregation in the mitotic germline divisions in the male, indicating that the *ord* gene product also may promote sister-chromatid cohesion during mitosis (Lin and Church, 1982; Miyazaki and Orr-Weaver, 1992). However, any mitotic requirement for *ord* is likely to be restricted to the germline, because *ord* mutations do not affect mitosis in the somatic tissues (J. Wu, W. Miyazaki, and T. Orr-Weaver, unpublished results).

In addition to causing premature sister-chromatid separation in meiosis, most *ord* mutations reduce recombination in females (Mason, 1976; Miyazaki and Orr-Weaver, 1992). Normally recombination occurs in Drosophila female meiosis, but not in male meiosis (for review see Orr-Weaver, 1995). It is possible that the *ord* gene primarily controls cohesion between sister chromatids and without cohesion the homologs are unable to undergo recombination with normal efficiency, perhaps because they cannot be brought into proper alignment. Alternatively, the *ord* gene could have multiple regulatory roles in meiosis and independently affect recombination and sister-chromatid cohesion. The nondisjunction observed during

female meiosis is not due solely to a failure in exchange however, because both exchange and nonexchange chromosomes segregate improperly in *ord* mutants (Mason, 1976).

The genetic properties of mutations can provide insight into the function of the gene product. For example, intragenic complementation is diagnostic of multiple functional domains within a protein. Another intragenic effect has been termed negative complementation (Fincham, 1966). In negative complementation, the residual activity of one allele can be poisoned by another allele. Negative complementation occurs when two alleles of a locus *in trans* to one another exhibit a more severe phenotype than homozygotes of either allele. Another case of negative complementation is when two alleles *in trans* to each other have a stronger phenotype than one or both *in trans* to a deficiency. A subset of negative complementing interactions is exhibited by mutations known as antimorphs (Muller, 1932), also referred to as dominant negatives (Herskowitz, 1987). Antimorphs are special cases because instead of poisoning the residual activity of a mutant allele they can antagonize the function of the wild-type allele, thus producing a dominant phenotype.

Here we show that some of the mutations in *ord* exhibit negative complementation. We cloned the gene, identified the ORD protein, and mapped the changes the *ord* mutations cause in the protein. The combination of phenotypic and molecular analysis provides critical insight into ORD protein function.

Results

Identification of the ORD protein

We determined the cytological position of *ord* by testing the ability of several deficiencies to complement the *ord* mutation (Table I). *ord* maps proximal to the *brown* (*bw*) locus at 59E. Since the gene was uncovered by *Df(2R)bw-S46* but not by *Df(2R)bw-HB132*, it must lie in the interval 59D5-11 on the right arm of chromosome *2*. To localize *ord* further we generated additional deficiencies in region 59 by irradiating a stock containing a *white*$^+$ P-element insertion at 59C in addition to the normal *bw*$^+$ allele (Levis *et al.*, 1985). This allowed us to score for deficiencies in the region by loss of either visible marker. Although these deficiencies did not refine the location of *ord* further at a cytological level, they were critical in delineating *ord* molecularly. Two important deletions were *Df(2R)bw-WI366* which removes the *bw* locus and *Df(2R)WI370* which removes the *w*$^+$ P element but retains the *bw* gene (Table I). Both of these deficiencies have breakpoints in 59D5-11, but *Df(2R)bw-WI366* is phenotypically ord$^+$, while *Df(2R)WI370* is ord$^-$.

We carried out a molecular walk to clone the genomic region containing *ord*. By quantitative Southern analysis, the proximal *Df(2R)bw-S46* breakpoint mapped within the most distal phage (G21) of the *twist* walk (Thisse *et al.*, 1987). We walked distally from the *twist* walk using a genomic library in lambda phage. Lambda clones from each step were hybridized to genomic DNA from deficiency strains to map the breakpoints. Crossing the proximal breakpoint of *Df(2R)bw-WI366* defined the minimal interval containing *ord* to approximately 25 kb (Figure 1).

To identify the *ord* gene within the chromosomal walk, DNA from this region was transformed into Drosophila to test for rescue of *ord* mutants. Inserts from overlapping lambda clones were subcloned into a P-element transformation vector containing a *white*$^+$ selectable marker (Pirrotta, 1988). Transformed lines were generated for three transposons, crossed into *ord*^{1}/*ord*^{3} transheterozygotes and tested for sex chromosome missegregation (Figure 1). Transposon *P{D39}* restored normal chromosome segregation to *ord* mutant flies (data not shown). Restriction fragments from transposon *P{D39}* were transformed into Drosophila and tested for complementation (Figure 1). The 6.3 kb *Bam*HI fragment in *P{6.3BB}* fully rescues the *ord* missegregation phenotype in both male and female meiosis (Table II). Moreover, recombination levels are restored to normal levels in *ord*^{1}/*ord*^{3} females carrying the *P{6.3BB}* transposon (Table II).

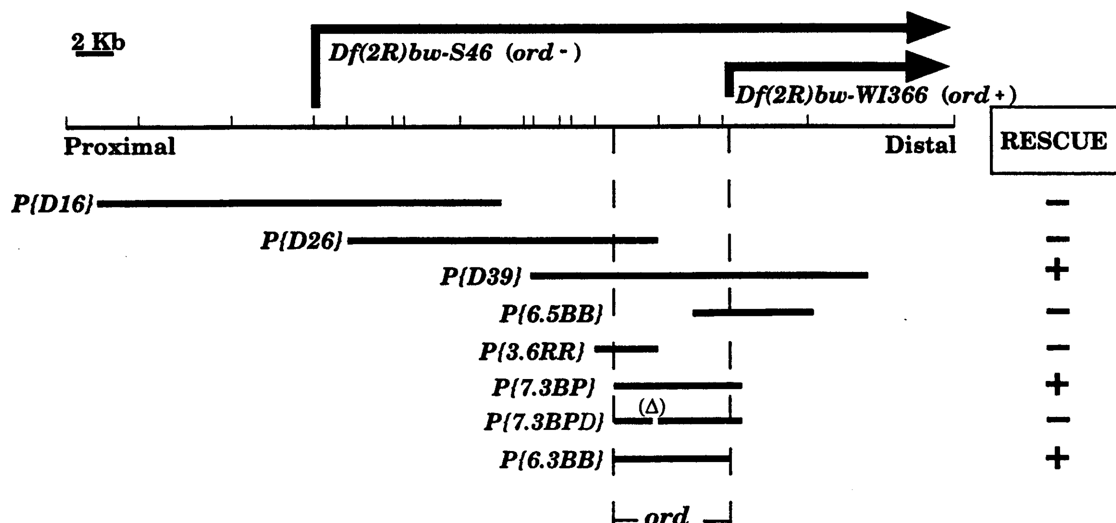

Figure 1: Identification of the *ord* gene. An *Eco*RI restriction map is shown for the genomic interval containing the *ord* gene. The DNA missing in the deficiencies *Df(2R)bw-S46* and *Df(2R)bw-WI366* is indicated by thick lines and arrows. Because *Df(2R)bw-WI366* is ord$^+$ its breakpoint defines the distal border of the region containing the *ord* gene. The DNA fragments transformed into Drosophila are shown relative to the genomic map. A plus indicates that the fragment rescued the chromosome missegregation phenotype of *ord* mutants; a minus indicates it did not. These experiments defined a 6.3 kb region containing the *ord* gene, shown by dashed lines.

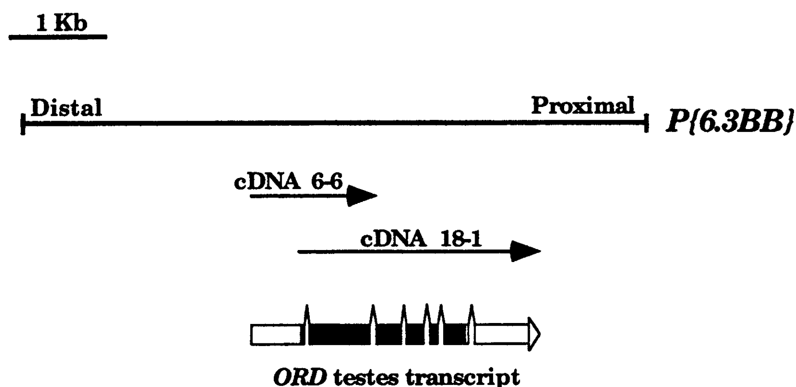

Figure 2: The *ORD* transcription unit. The position of testis cDNAs corresponding to *ord* are shown relative to the 6.3 kb rescuing fragment. The direction of transcription is indicated by arrows. Sequencing of the cDNAs and genomic DNA demonstrated that the *ORD* transcription unit contains six introns. The open reading frame is shown in black.

TABLE I
Deficiencies that define the *ord* interval

Deficiency	Cytological Breakpoints	*ord* Phenotype[a]
Df(2R)bw-S46	59D5-11; 60A7	-
Df(2R)bw-HB132	59D5-11; 59F6-8	+
Df(2R)bw-WI366	59D5-11; 60B1-3	+
Df(2R)WI370	59B3; 59D5-11	-

[a] A minus indicates that the deficiency uncovers *ord* and is mutant for the locus, and a plus indicates that the deficiency does not delete the *ord* gene.

We identified the *ORD* transcription unit by isolating testes cDNAs that hybridized to *P{6.3BB}*, because we knew that the gene is required for proper meiotic chromosome segregation. The cDNAs fell into two groups by cross hybridization experiments. Representative cDNAs from each transcription unit as well as one strand of the genomic DNA were sequenced. This placed one transcription unit completely within *P{3.6RR}*, making it unlikely that it encoded ORD, because this transposon did not complement *ord* mutations. To confirm that the other transcription unit was *ORD*, we generated a 136 bp deletion that only disrupted the second transcription unit (*P{7.3BPΔ}*, Figure 1). When transformed into flies, *P{7.3BPΔ}* failed to rescue *ord* mutants, indicating that this transcription unit is *ORD*.

The *ORD* message is of low abundance, and on Northern blots we detected *ORD* transcripts only in adult ovary and testis and in no other tissue or developmental stage (data not shown). In testes and ovary poly A^+ RNA the predominant message is about 2.7 kb. This agrees with the predicted size of an *ORD* transcript derived from aligning overlapping testes cDNAs (Figure 2). In addition, there are larger transcripts of even lower abundance that differ in size between ovary and testis.

The direction of *ORD* transcription is distal to proximal with respect to the chromosome, and the predominant 2.7 kb transcript contains six introns (Figure 2). The introns are small, ranging in size from 53 to 67 bp. The *ORD* transcription unit has an open reading frame encoding 479 amino acids, predicted to be a 55 kDa protein

TABLE II
Transposon rescue of *ord* phenotypes

A. Male Segregation Tests

genotype[a]	Regular gametes X	Y	Exceptional gametes O	XX	XY	XXY
ord^1 / ord^3	45	65	58	15	26	1
ord^1 / ord^3 ; *P{6.3BB}*	285	225	0	0	3	0

B. Female Segregation Tests

genotype[b]	Regular gametes X	Exceptional gametes O	XX
ord^1 / ord^3	48	7	11
ord^1 / ord^3 ; *P{6.3BB}*	75	0	0

C. Recombination Map Distances

genotype[c]	*y-cv*	*cv-f*	progeny scored
+ / +[d]	10.4 cM	38.7 cM	1536
ord^1 / ord^3	2.1 cM	2.5 cM	591
ord^1 / ord^3 ; *P{6.3BB}*	7.6 cM	36.5 cM	978

[a] y / y^+Y males with the genotype indicated were crossed to attached-*X*, y^2 $su(w^a)$ w^a females. Numbers indicate progeny scored.

[b] *y w* females with genotype indicated were crossed to attached-*XY, v f B males*. Numbers indicate progeny scored.

[c] *y w / w cv v f car* females with the genotype indicated were crossed to *y w* males.

[d] data taken from Miyazaki and Orr-Weaver, 1992.

(Figure 3). There is a 5' untranslated region of 502 bp and a 771 bp 3' untranslated region which includes the last intron. No sequences with significant regions of homology were identified in the databases using several search programs (Altschul *et al.*, 1990; Lipman and Pearson, 1985; Smith and Waterman, 1981).

The sequence immediately upstream of the putative N terminus matches the Cavener consensus sequence for Drosophila translation initiation (Cavener, 1987). Moreover, there are stop codons in all three frames within 55 bp upstream of the N-terminal methionine. Therefore, the methionine indicated in Figure 3 most likely represents the N terminus of the protein encoded by the 2.7 kb predominant testes transcript.

Several results demonstrate that the 55 kDa protein is ORD. The 136 bp deletion described above (P*[7.3BPΔ]*) changes the frame at amino acid 226 and abolishes *ord* rescuing activity. In addition, we showed by DNA sequence analysis that all *ord* mutations characterized thus far result in single nucleotide changes within the open reading frame (see below). In one of the strongest alleles, ord^5, amino acid 245 is mutated to a stop codon, truncating the protein approximately halfway through the open reading frame.

The ORD protein has several notable features. An interval enriched in hydrophobic amino acids (14 out of 20) lies between residues 409-428. Overall the protein is fairly acidic, with a predicted pI of 5.8. The region spanning amino acids 103-116 is quite acidic with 9 out of 14 residues bearing a negative charge. This acidic region lies within a highly significant PEST sequence between amino acids 102-122. PEST regions have been found in proteins known to undergo rapid degradation (Rechsteiner, 1988; Chevaillier, 1993), and it has been proposed that they signal proteolysis by a mechanism distinct from a ubiquitin-dependent system (Rogers *et al.*, 1986). Three out of fourteen potential casein kinase II phosphorylation sites within ORD reside in this PEST interval, perhaps increasing the negative charge of the region.

```
MYGETTLNKN HVIKFIKLKI NNCLGCDEVE INFSKADNVH IIIYSLVTDM       50

AKDLPAVTPV AQAILLLCSL TYPDSDSLET IPQLKIGKGS VSMSFKVYPV      100

NKEEETEPES ESDLDEGPST SKQALERMVQ RAERKAKEAS TRNVHSKGIY      150

VNVERRFDMY FALDTVSYYI NGGKRQSCPL PEFHAKFFVR PQHSINLLRQ      200

LHEKCSGNWL KVIQSDGDGD AFKKFKDPDS PFETFVKLFE SNPIKPNDMM      250
                                                ↓
                                               stop
                                               ord5

GKLAKTCLHV NEAVRLTERE FILEVFNQVR HIFEYITAQE YTVWFLVPCL      300
                                               ↓
                                               R
                                              ord6

GDKDQLRSKT LEDFDLTKVR TSIRRAGDTS NIWWDHTDHN IKDILLVAFQ      350
         ↑
        ord3

LDLATHVNQS VLVISHLETL AEFSTMQYVT AFFMNDFYAK KNTEPKWICH      400
                                 ↓
                                 T
                                ord2

RYLERIIDVA LFLGVIVIIE YPSAFTLLQE GRHLIKCFQK ENAESSRTSQ      450
↓                        ↓
H                        V
ord1                    ord4

WEIFEDVVKE NESDLEFLKE AVGKVQQNV.                            479
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Figure 3: The ORD protein. The 479 amino acid sequence of the ORD protein encoded by the testis transcription unit is shown together with the position and nature of the sequenced *ord* mutations.

Unusual genetic properties of *ord* alleles suggest a requirement for protein-protein interactions

Since ORD is a pioneer protein, the position and nature of mutations within the protein can be useful in identifying important functional domains. This is particularly true for ORD because some of the alleles exhibit unusual genetic properties. Previously, five alleles of *ord* were characterized extensively and shown to be of varying strengths (Mason, 1976; Miyazaki and Orr-Weaver, 1992). In analyzing a sixth weak allele, ord^4, we found that it has striking complementation behavior with some of the other alleles that provides insight into ORD function.

The ord^4 mutation is unusual because it has a large amount of residual activity. However this ability to function is poisoned by some other *ord* mutations. The levels of chromosome missegregation in homozygous ord^4 females were very low (1.1%) (Table III), compared to 60.2% seen in homozygous ord^3 mutants and 0.03% observed in wild-type controls (Miyazaki and Orr-Weaver, 1992). Interestingly, missegregation increased only slightly when ord^4 was placed *in trans* to a deficiency for the *ord* gene (Table III), in contrast to what would have been predicted for a leaky hypomorphic allele. Strikingly, missegregation increased markedly to 20.2% when ord^4 was placed *in trans* to ord^1 (Table III). Levels of missegregation were also significant in ord^4 / ord^2 transheterozygotes compared to ord^4 / Df (Table III).

The ord^1 and ord^2 mutations are recessive for meiotic chromosome segregation. $ord^1/+$ and $ord^2/+$ females gave 0.0% and 0.2% missegregation, respectively, out of approximately 2500 progeny scored. Thus these alleles do not interfere with wild-type function, only the impaired ord^4 function. The intermediate level of aberrant segregation in ord^4/ord^1 mutants compared to that in homozygous ord^4 (Table III) and ord^1 mutants (55.3%) (Miyazaki and Orr-Weaver, 1992) cannot be caused by dominant activity of ord^1.

Mutations in the *ord* gene also exhibit an additional phenotype in female meiosis of reducing recombination (Mason, 1976; Miyazaki and Orr-Weaver, 1992). The frequency of exchange on the *X* chromosome was lower in ord^4/ord^1 or ord^4/ord^2 transheterozygotes than in ord^4 homozygotes or ord^4/Df females (data not shown). Therefore, ord^1 and ord^2 impair the residual activity of ord^4 both for chromosome segregation and recombination.

Not all *ord* mutations interfere with ord^4 function. In females ord^3, ord^5 and ord^6 in combination with ord^4 displayed levels of missegregation very similar to that observed in ord^4 / Df flies (Table III). In addition, no correlation exists between allele

TABLE III
Allele-specific interactions in females

female genotype[a]	exceptional gametes XX	exceptional gametes O	adjusted total[b]	% missegregation
ord⁴/ord⁴	0.7[c] (7)[d]	0.4 (4)	1956	**1.1**
Df[e]/ord⁴	1.1 (41)	1.1 (40)	7389	**2.2**
ord¹/ord⁴	10.5 (209)	9.7 (192)	3971	**20.2**
ord²/ord⁴	3.5 (33)	2.8 (26)	1868	**6.3**
ord³/ord⁴	0.5 (5)	0.3 (3)	2076	**0.8**[f]
ord⁵/ord⁴	1.0 (16)	0.5 (8)	3173	**1.5**[g]
ord⁶/ord⁴	1.5 (15)	1.3 (13)	2030	**2.8**[h]

[a] Females were crossed to attached-*XY, v f B males.*

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

[c] Percentage of gametes in each class

[d] Numbers in parentheses are progeny scored.

[e] *Df(2R)WI370*

[f] The level of missegregation in *ord³/ord⁴* females is less than in *Df/ord⁴* transheterozygotes ($0.001<p<0.01$) but not different from that observed in *ord⁴* homozygotes ($0.30<p<0.50$) by χ^2 contingency analysis (see Materials and Methods).

[g] Missegregation levels in *ord⁵/ord⁴* and *Df/ord⁴* females are not statistically different ($0.10<p<0.20$).

[h] Missegregation levels in *ord⁶/ord⁴* and *Df/ord⁴* females are not statistically different ($0.20<p<0.30$).

strength and ability to interfere with *ord*^{4} function. *ord*^{6} is a moderate allele and did not interact (Miyazaki and Orr-Weaver, 1992). Certain strong alleles, like *ord*^{1} and *ord*^{2}, interacted with *ord*^{4} whereas others, *ord*^{3} and *ord*^{5}, did not.

These data show that if *ord*^{4} was the only form of the protein present (i.e. in *ord*^{4} homozygotes or *ord*^{4}/*Df* transheterozygotes) it functioned fairly well. In addition, certain mutant alleles *in trans* to *ord*^{4} resulted in levels of missegregation similar to *ord*^{4}/*Df*. However, what was striking was that *ord*^{1} and *ord*^{2} *in trans* to *ord*^{4} had a more severe phenotype than *ord*^{4}/*Df*. This behavior previously has been termed negative complementation (Fincham, 1966) .

Negative complementation of *ord* alleles was also observed in male meiosis (Table IV). As in females, *ord*^{4} homozygotes displayed low levels of missegregation (0.56%) compared to the strong allele *ord*^{3} (51%) and wild-type flies (0.25%). Missegregation did not increase when *ord*^{4} was placed over a deficiency. But again *ord*^{1} interfered with *ord*^{4} activity (Table IV). There is negative complementation between *ord*^{6} and *ord*^{4} in males that is statistically significant (Table IV). In contrast to its effect in females, *ord*^{2} did not poison *ord*^{4} function in males (Table IV).

These data demonstrate that the near wild-type activity of *ord*^{4} can be significantly compromised in both males and females by the presence of *ord*^{1}. Therefore, *ord*^{4} responds quite differently than *ord*$^+$ in its sensitivity to the presence of *ord*^{1}, since *ord*^{1}/+ heterozygotes display wild-type levels of chromosome segregation. *ord*^{2} also has the ability to interfere with *ord*^{4} function, but to a lesser extent and only in females. *ord*^{6} weakly poisons *ord*^{4} in males. However, the ability to interfere with *ord*^{4} activity is highly allele specific; *ord*^{3} and *ord*^{5} display no negative complementation effects in combination with the *ord*^{4} mutation. The observation that certain mutant alleles of *ord* act in a more destructive fashion than a deficiency for the locus suggests that protein interactions are essential for *ord* function.

TABLE IV
Allele-specific interactions in males

male genotype[a]	exceptional gametes: XY, XXY	XX	O	total progeny scored	% missegregation
ord^4/ord^4	0.12[b] (4)[c]	0.03 (1)	0.41 (14)	3400	**0.6**
Df^d/ord^4	0.05 (3)	0.10 (6)	0.4 (22)	6132	**0.6**
ord^1/ord^4	2.1 (70)	1.1 (34)	4.4 (144)	3271	**7.6**
ord^2/ord^4	0.5 (3)	0.2 (1)	0.5 (3)	636	**1.2**[e]
ord^3/ord^4	0.3 (2)	0.0 (0)	0.3 (2)	639	**0.6**
ord^5/ord^4	0.4 (3)	0.0 (0)	0.3 (2)	688	**0.7**
ord^6/ord^4	1.5 (10)	0.3 (2)	1.2 (8)	660	**3.0**[f]

[a] y/y^+Y males were crossed to attached-X, y^2 $su(w^a)$ w^a females.

[b] Percentage of gametes in each class.

[c] Numbers in parentheses are progeny scored.

[d] *Df(2R)WI370*

[e] Missegregation levels in ord^2/ord^4 and Df/ord^4 males are not statistically different (0.05<p<0.10).

[f] Missegregation levels in ord^6/ord^4 and Df/ord^4 males are statistically different (p<.001).

The C-terminal half of ORD is essential for function and mediates negative complementation

The phenomenon of negative complementation such as we observed with *ord*^{4} occurs infrequently, and previously identified examples tend to involve multidomain proteins. In all of the *ord* mutants, with the exception of *ord*^{3}, the levels of message on a testis Northern blot were comparable (data not shown), therefore the mutations were likely to affect the coding sequence. Determining the position and nature of *ord* mutations could indicate possible distinct functional domains within the ORD protein.

To sequence the *ord* mutations we amplified genomic DNA by PCR, performing duplicate parallel amplifications and subcloning manipulations to ensure that we would detect any mutations induced during the PCR amplification. The sequence encompassing the open reading frame in each PCR product was compared to the sequence of the isogenic chromosome (*cn bw sp*) on which *ord* mutations 2-6 were generated (Miyazaki and Orr-Weaver, 1992). Each *ord* mutation could be attributed to a single base pair mutation and surprisingly, all six mutations were located within the C-terminal half of the protein (Figure 3).

The *ord*^{4} mutation lies 55 amino acids from the C terminus, within the highly hydrophobic interval, and it changes Ala^{424} to Val. Such a conservative change is consistent with the near normal function of the ORD^{4} protein. However, it is clear that the C-terminal half of ORD is essential for function in both males and females, because one of the strongest alleles, *ord*^{5}, mutates Lys^{245} to a stop codon so that the last 234 amino acids of the protein are missing. In addition, sequence analysis of *ord*^{3} revealed a G to A change that mutates the donor splice site of the third intron. If unspliced and translated, the third intron contains an in-frame stop codon that terminates the open reading frame. Therefore ORD^{3}, like ORD^{5}, is predicted to be missing the C terminus. Moreover, it is striking that *ord*^{3} and *ord*^{5} behave like a deficiency in their interaction with *ord*^{4}, indicating that the negative complementation observed between *ord*^{4} and other alleles must be mediated through the C-terminal half of the protein (Figure 4).

Sequence analysis of *ord*^{1}, *ord*^{2}, and ord^{6} confirmed that negative complementation was a consequence of amino acid changes in the C-terminal half of ORD (Figures 3,4). *ord*^{1} results in an arg to his change at position 401. *ord*^{2}, which interferes less strongly with *ord*^{4} and only in females, changes Ala^{381} to Thr. *ord*^{6}, a third mutation that poisons *ord*^{4} in males, is a tryp to arg substitution at amino acid 294, placing it between the *ord*^{5} and *ord*^{3} truncation mutations (Figures 3,4).

$$\frac{ord^1}{ord^4} \gg \begin{matrix} \frac{ord^2}{ord^4}\ (♀) \\ \frac{ord^6}{ord^4}\ (♂) \end{matrix} > \frac{ord^4}{ord^4} = \frac{Df}{ord^4} = \frac{ord^5}{ord^4} = \frac{ord^3}{ord^4}$$

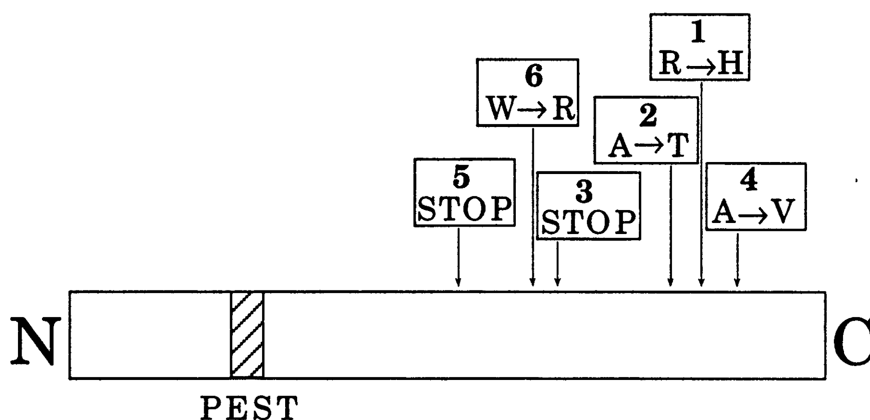

Figure 4: Negative complementation of *ord* mutations. The levels of aberrant chromosome segregation for the *ord* mutant combinations are summarized on the top. The ord^1 mutation poisons the ability of ord^4 to function. ord^1/ord^4 flies exhibit higher levels of missegregation than ord^4 homozygotes or ord^4 *in trans* to either a deficiency, ord^5, or ord^3. In females, ord^2 shows negative complementation with ord^4, although not as strongly as does ord^1. In males, ord^6 shows weak negative complementation with ord^4. The nature and positions of the *ord* mutations are shown relative to a schematic of the protein. The ord^5 and ord^3 mutations cause stop codons that remove the C-terminal half of the protein, implicating this domain in negative complementation. The other mutations, all of which negatively interact, result in missense changes in the C-terminal domain.

Discussion

In this paper we identify the ORD protein which is known to be essential for sister-chromatid cohesion during Drosophila meiosis. Cloning *ord* provides an opportunity to understand the molecular basis of cohesion. Our analysis of several *ord* mutations demonstrates that the C-terminal half of ORD is essential for normal function of the protein. In addition, we describe unusual genetic interactions between specific *ord* alleles that implicate the C-terminal part of the molecule in protein-protein interactions.

The interference between specific *ord* alleles belongs to a unique genetic class termed negative complementation. Such effects are rare, allele specific, and often involve multidomain proteins known to participate in protein-protein interactions. Certain recessive viable *Abruptex* alleles of the *Notch* locus in Drosophila combine to result in lethality (Foster, 1975; Portin, 1975), and specific alleles of the *flb* locus demonstrate negative complementation (Raz, et al., 1991). Both *Notch* and *flb* encode transmembrane proteins containing motifs in the extracellular domain that are thought to mediate homotypic and heterotypic interactions (Muskavitch and Hoffman, 1990; Ullrich and Schlessinger, 1990). The *flb* gene encodes the Drosophila EGF receptor. Of seven *Abruptex* alleles sequenced, all map within six EGF-like repeats in the extracellular domain of NOTCH (Kelley *et al.*, 1987). In *C. elegans*, the body morphology loci *sqt-1*, *sqt-3*, and *rol-8* all display negatively complementing heteroallelic combinations (Kusch and Edgar, 1986). The *sqt-1* locus encodes a collagen molecule (Kramer *et al.*, 1988). Protein-protein interactions are crucial for collagen function because the procollagen polypeptides trimerize to form the collagen fibrils in the *C. elegans* cuticle.

In ORD, the mutations that negatively interact are missense changes in the C terminus, while the mutations that do not poison ORD^4 (ORD^3 and ORD^5) are missing the C-terminal part of the protein. Thus the C-terminal domain appears critical for negative complementation. The negative complementation mediated through the C-terminal domain of ORD is most simply explained by a model in which protein interaction is necessary for ORD activity (Figure 5).

We propose that ORD activity depends on two functions, one required for protein binding and one responsible for promoting sister-chromatid cohesion (Figure 5). Cohesion requires binding, but binding does not insure cohesion. Both functions must lie within the C-terminal half of ORD. The phenotypes can be explained by the binding function being somewhat compromised in ORD^4 protein. Although binding would be

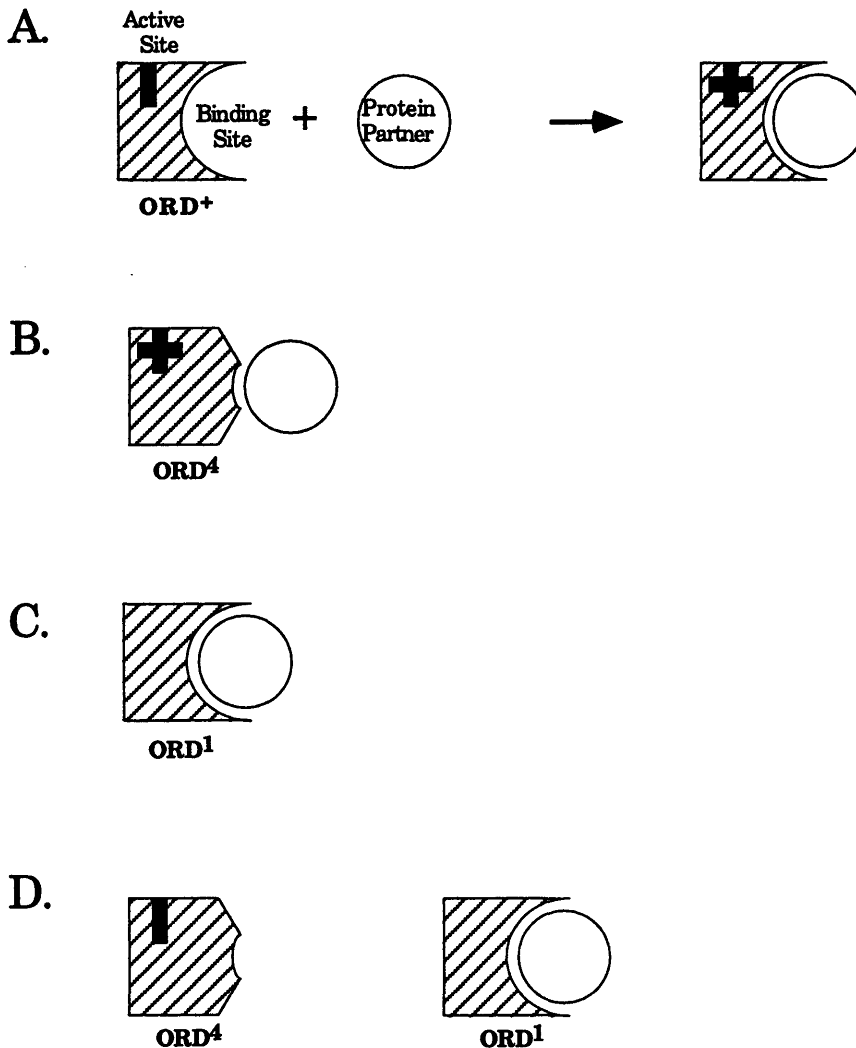

Figure 5: Model to explain the negative complementation observed with *ord* mutations. The negative complementation is explained most simply by the ORD protein requiring two functions for activity. (A) In wild-type ORD protein (stippled) there is a binding site for another protein (or another molecule of ORD) indicated by the open circle. The "active-site", shown in black, promotes sister-chromatid cohesion. The binding site and active site can be separably mutated. Binding is essential but not sufficient for cohesion activity. (B) The ORD^4 protein binds with reduced affinity, but still functions. If ORD^4 is the only form of the ORD protein present, it can act to maintain cohesion. (C) Although the ORD^1 protein binds with normal affinity the active site is altered, as indicated by the absence of the black box. The ord^1 mutation would be recessive to wild-type *ord*, but it would poison ord^4 by competing out the interacting protein as in (D).

weaker than that of ORD+, ORD4 could still interact with its protein partner and promote cohesion, since its cohesion function would be unaffected. The *ord4* mutation lies in a very hydrophobic region of the protein which is consistent with it being involved in protein interaction.

Unlike *ord4*, *ord1* would disrupt the cohesion function but not the binding affinity of the mutant protein. In an *ord1/ord4* transheterozygote, ORD1 protein would out compete ORD4 for binding to the protein partner, but it would form a nonfunctional complex. This would reduce the amount of functional ORD4 complex, thereby reducing the ability of ORD4 to promote cohesion. Since *ord1* is not a dominant negative mutation for segregation and doesn't interfere with wild-type *ord*, the binding ability of ORD1 must not be greater than that of ORD+. The *ord2* and *ord6* mutations would, like *ord1*, affect the cohesion function but still allow binding.

The model we present for ORD protein function relies on interaction between ORD and a protein partner. Such a protein could be another ORD molecule in a scenario whereby dimer or multimer formation is a prerequisite for function at the active site. An ORD4/ORD1 heterodimer could form but it would not be functional. ORD1 would therefore directly poison ORD4 by tying it up in a nonfunctional complex. If this were true it might be possible to detect dimerization of the ORD protein. We have been unable detect ORD dimerization using the yeast two-hybrid system (S. Bickel, L. Young, and T. Orr-Weaver, preliminary results). Considering the complexity of sister-chromatid cohesion, it is likely that ORD binds to another protein, yet to be identified. In transheterozygotes, ORD1 would titrate out available binding sites and make formation of a functional ORD4 complex less likely.

The ORD protein is essential for meiotic sister-chromatid cohesion. ORD could maintain cohesion by structurally holding the sister chromatids together or by regulating the signals that trigger sister-chromatid separation. Another Drosophila protein required for sister-chromatid cohesion in meiosis is MEI-S332 (Davis, 1971; Goldstein, 1980; Kerrebrock *et al.*, 1992). However, MEI-S332 differs from ORD in the time and location at which it appears to be necessary. In *mei-S332* mutants sister-chromatid cohesion is unaffected until late in anaphase I, in contrast to the premature sister separation seen in prometaphase I in *ord* mutants. These phenotypes suggest that MEI-S332 acts only at the centromere to promote cohesion, while ORD is required when cohesion must be maintained along the entire chromatid. A MEI-S332-GFP protein has been shown to localize specifically to the centromere region of meiotic chromosomes and to disappear when cohesion is lost at the metaphase II/ anaphase II transition (Kerrebrock *et al.*, 1995). The localization of

MEI-S332 is consistent with it structurally holding sister chromatids together at the centromere, and its destruction or release being required for separation. We have not detected any significant regions of similarity between ORD and MEI-S332, thus these two proteins may utilize different mechanisms in promoting cohesion at different times of meiosis and at different locations on the sister chromatids.

ord mutations exhibit several phenotypes in addition to premature sister-chromatid separation in meiosis, suggesting that ORD might act as a regulatory protein. Recombination is reduced in *ord* mutant females, and mitotic nondisjunction is observed in the male germline of *ord* mutants (Mason, 1976; Lin and Church, 1982; Miyazaki and Orr-Weaver, 1992). The low abundance of *ORD* message is more consistent with a regulatory rather than a structural role. Moreover, it remains a formal possibility that different forms of the ORD protein may exist, and these could have different regulatory activities. The C-terminal half of ORD must be used for cohesion in both the sexes and for recombination, since all the mutations we have characterized lie within this part of the protein. However, in addition to the predominant transcript, in both ovary and testis there are even rarer larger transcripts. If alternatively spliced, these messages could encode proteins with differing N termini. The effects of *ord* mutations on both sister-chromatid cohesion as well as recombination might be explained by different protein isoforms operating in separate pathways. In addition, a slightly different form of ORD could regulate mitotic cohesion in the germline.

At the sequence level, one of the most striking features of ORD is a very high scoring PEST sequence in the N-terminal half of the protein, suggesting that ORD may be rapidly degraded. Such a mechanism might provide the abrupt loss of arm cohesion that is necessary to allow separation of recombined homologs at the metaphase I/anaphase I transition and/or the timely loss of centromere cohesion that allows the sister chromatids to separate in anaphase II. Recently several labs have implicated the ubiquitin pathway as being instrumental in the mitotic metaphase/anaphase transition, possibly by targeting for proteolysis the proteins holding sisters together (Irniger *et al.*, 1995; King *et al.*, 1995; Tugendreich *et al.*, 1995). The destruction box of mitotic cyclins is known to be necessary for ubiquitination and rapid decay of MPF kinase activity (Glotzer *et al.*, 1991). Because the metaphase/ anaphase transition appears to require ubiquitin-mediated proteolysis of as yet unidentified proteins, the protein(s) responsible for sister association might contain a destruction box (Holloway *et al.*, 1993). There is, however, no destruction box matching the cyclin consensus in the ORD open reading frame. It

remains to be determined whether ORD is degraded at the metaphase/ anaphase transition and the mechanism of proteolysis.

Only a few proteins that are candidates for controlling meiotic sister-chromatid cohesion have been identified. The *Zea mays* mutants *desynaptic* (*dsy* and *dy*) (Maguire, 1978; Maguire *et al.*, 1991; Maguire *et al.*, 1993), the *Sordaria macrospora* mutant *spo76* (Moreau *et al.*, 1985), and mutations in the *S. cerevisiae* gene *RED1* (Rockmill and Roeder, 1988, 1990) cause meiotic defects compatible with these genes having a function in cohesion. Premature separation of the sister chromatids is observed during meiosis in *rec8* mutants of *S. pombe* (Molnar *et al.*, 1995). To date, only the *rec8* and *RED1* genes have been cloned and sequenced (Lin *et al.*, 1992; Thompson and Roeder, 1989). ORD does not share any significant regions of homology that would indicate that it represents the Drosophila homolog of either of these yeast genes.

Other potential cohesion proteins have been identified based on the criterion that they are localized between the sister chromatids, but it is difficult to analyze their function (for review see Miyazaki and Orr-Weaver, 1994). One example is the mammalian protein Cor1, a component of the lateral elements of the synaptonemal complex (Dobson *et al.*, 1994). The localization pattern of Cor1 is consistent with it acting to maintain sister-chromatid cohesion throughout meiosis. However no homology exists between Cor1 and ORD.

The *ord* gene is unusual in being one of the few genes known to be essential for sister-chromatid cohesion. Therefore the identification of the ORD protein provides molecular access to a critical player in cohesion. We have identified regions of ORD necessary for function and protein interaction. This will permit the elucidation of the nature and regulation of cohesion through understanding the basis of ORD function and isolation of its interacting partners.

Materials and methods

Stocks

All Drosophila stocks and crosses were raised at 25°C on standard cornmeal-brewer's yeast-molasses-agar food. *ord^1* was isolated and characterized by Mason (1976). All other *ord* alleles were isolated by their failure to complement *ord^1* and have been previously described (Miyazaki and Orr-Weaver, 1992). They exhibit a wide range in strength, and they are recessive with respect to both chromosome segregation and recombination. The decreased fertility of strong alleles varies in intensity depending on strain background. In addition, the defects in nurse cell morphology described by Miyazaki and Orr-Weaver (1992) also depend on genetic background. The deficiencies *Df(2R)bw-WI366* and *Df(2R)WI370* were isolated in the screen described below. *Df(2R)WI370* was called *Df(2R)3-70* previously (Miyazaki and Orr-Weaver, 1992). *Df(2R)bw-S46* was obtained from R. Lehmann and *Df(2R)bw-HB132* was obtained from R. Nothiger. All other mutations used in these experiments are described in Lindsley and Zimm (1992).

Deficiency screen

We isolated deficiencies in the cytological interval 59 from an X-ray screen. We screened for deletions that removed either *bw$^+$* at 59E or a P-element carrying the *white$^+$* gene inserted into 59C (*P{w$^+$, ry$^+$=A}3-1* transformant, obtained from R Levis) (Levis *et al.*, 1985). *w / Y; cn P{w$^+$}* 59C males were irradiated with 3000-4000 rads, crossed to *w; cn bw If / CyO S^2 cn bw* virgins and their progeny scored for white eyes. Of the 383,000 chromosomes screened, 8 deletions were obtained that removed the P element, while 10 deficiencies deleted the *bw$^+$* locus. Newly isolated deficiencies were tested for their ability to complement the chromosome segregation phenotype of *ord* mutants using the nondisjunction tests described in Kerrebrock *et al.* (1992). The deficiency breakpoints were mapped on the genomic walk by quantitative Southern analysis (see below).

Isolation of the *ord* genomic region

We carried out a chromosomal walk using a l library (gift from Jennifer Mach, R. Lehmann lab) constructed from a partial *Sau*3A digest of genomic DNA from

Df(2R)bw-HB132 / + flies (ord+). The walk was initiated from the most distal clone of the *twist* walk (G21) (Thisse *et al.*, 1987). Two steps distally towards *bw* were taken.

The positions of deficiency breakpoints were mapped on the genomic walk by quantitative Southern blots. Using hybridization probes from each step, we compared genomic DNA isolated from various *Df* / + heterozygotes to wild-type Canton S DNA. A Fuji BAS2000 Bioimager was used to quantify the signal in each band. A probe containing the *rosy* gene was used as an internal control to standardize for the amount of DNA loaded in each lane. The signal for each deficiency band was then expressed as a fraction of the analogous Canton S band using the following formula: (deficiency band "X" / deficiency *rosy* band) / (Canton S band "X" / Canton S *rosy* band). In such a calculation, a value of 0.5 indicated that the fragment of interest was deleted on the deficiency chromosome. Using this strategy, the minimal interval containing *ord* was restricted to 25 kb (Figure 1).

A chromosome walk was also done starting from the most proximal clone of the *bw* walk (Dreesen *et al.*, 1988) and walking proximally. This walk utilized the Tamkun cosmid library (Tamkun *et al.*, 1992). Clones from five overlapping steps (approximately 150 kb) were recovered, but a sixth step proved impossible due to "holes" in this library as well as in several other cosmid libraries tested. Molecular identification of the breakpoint of *Df(2R)bw-WI366* made it unnecessary to continue walking in this direction.

Transformation rescue

For transformation rescue experiments, *Not*I-excised inserts from three overlapping l clones spanning the breakpoints of *Df(2R)bw-S46* and *Df(2R)bw-WI366* were subcloned into pCoSpeR (Pirrotta, 1988) to generate the three transposons *P{*w^{+mC} *ori Amp=D16}*, *P{*w^{+mC} *ori Amp=D26}* and *P{*w^{+mC} *ori Amp=D39}*. *Df(1)*yw^{67c23} embryos were injected with plasmid DNA at 1 mg/ml with 0.3 mg/ml pIChsπD2-3 helper plasmid, a derivative of wings clipped (Mullins *et al.*, 1989). Multiple, independent lines were established for each construct. Crosses were performed to obtain flies carrying the transposon in an ord^1 / ord^3 background (both are strong alleles) and sex chromosome missegregation measured in both males and females (Kerrebrock *et al.*, 1992). In addition, simultaneous sibling tests were performed. ord^1 / ord^3 flies lacking the transposon as well as *ord* heterozygotes with or without the transposon were scored for missegregation frequencies. By testing for

rescue of *ord* transheterozygotes we avoided any phenotypes arising from making other background mutations on the second chromosome homozygous.

Smaller rescue constructs were made as follows. *Bam*HI partial digest fragments from the genomic insert of *P{D39}* were ligated into the *Bam*HI site of pCaSpeR4 (Pirrotta, 1988) to generate the overlapping clones *P{w^{+mC} ori Amp=6.3BB}* and *P{w^{+mC} ori Amp=6.5BB}* (Figure 1). *P{w^{+mC} ori Amp=3.6RR}* has a 3.6 kb *Eco*R1 fragment inserted into pCaSpeR4 (Figure 1). The *P{w^{+mC} ori Amp=7.3BP}* transposon was constructed by inserting a 2.1 kb *Bam*HI-*Pst*I fragment into pCaSpeR4 then opening up the clone with *Pst*I and ligating in a 5.2 kb *Pst*I fragment (Figure 1). Correct orientation was determined by diagnostic restriction digests. To generate *P{w^{+mC} ori Amp=7.3BPΔ}*, a 136 bp deletion was introduced into *P{w^{+mC} ori Amp=7.3BP}* by fusing the *Pst*I and *Eco*RI sites which were made blunt using T4 DNA polymerase. This construct mutates the splice acceptor site of intron 2 which overlaps with the *Pst*I site. If intron 2 is unspliced, a stop codon at the beginning of the intron will terminate the open reading frame. Conversely, if the next consensus acceptor is utilized, the open reading frame changes frames and then truncates prematurely. Transformed lines were generated for these constructs and tested as described above, except that transposon DNA was injected at 0.5 mg/ml and the helper was 0.1 mg/ml.

In order to recover a *w cv v f car* chromosome with which to test recombination frequencies in flies carrying the transposon *P{6.3BB}* , a recombinant chromosome was isolated from w^{1118} / *cv v f car* females. *X* chromosome recombination frequencies in two intervals (*y* - *cv* and *cv* - *f*) were determined in females that were *y* w^{67c23} / w^{1118} *cv v f car; ord^1 / ord^3* and in identical females carrying one copy of the *P{6.3BB}* transposon on the third chromosome.

Isolation of cDNAs

A testes cDNA library (provided by Dr. T. Hazelrigg) was screened with the *ord* genomic insert from the rescuing transposon, *P{6.3BB}*. cDNAs corresponding to two transcription units were isolated from 1.4 x 10^6 clones screened. Phage clones were converted to plasmids using the Exassist/SOLR excision system (Stratagene). Of the four unique cDNAs subsequently demonstrated to encode parts of the *ORD* transcription unit, one was determined by sequence analysis to be a hybrid clone and was not characterized further.

DNA sequencing and computer analysis

Both strands of overlapping *ORD* cDNA clones 6-6 and 18-1 were sequenced. Random cDNA fragments generated by sonication of cDNA insert ligated into circles (Bankier *et al.*, 1987) were subcloned into Bluescript KS^{-}. Sequenase 2.0 (Amersham/USB) chain-termination reactions were performed using T3 and T7 primers and sequences assembled into contigs using SeqMan/DNA Star software. In order to position the cDNA within the rescuing transposon *P{6.3BB}* and to identify intron/exon boundaries, one strand of the 6.3 kb genomic rescue insert was sequenced by generating ExoIII-nested deletions (Pharmacia) of overlapping subclones.

To identify any sequences with homology to ORD, searches of protein, nucleotide and sequence tag data bases were performed using the programs BLAST, FASTA and BLITZ (Altschul *et al.*, 1990; Lipman and Pearson, 1985; Smith and Waterman, 1981). No significant homologies were uncovered, even when utilizing reduced stringency matrix parameters for BLAST such as BLOSUM30. The PEST sequence in ORD (score=29.7) was identified using the program PEST-FIND (Rogers *et al.*, 1986) .

PCR amplification and sequence analysis of mutant *ord* alleles

Genomic DNA was isolated from homozygous mutant females (Ashburner, 1989), digested with *Hin*dIII, precipitated and resuspended in TE. DNA (1 fly equivalent) was amplified using primers outside the *ord* coding sequence. The sense strand primer was 5'CGATAAAGCCCCAACGACTACTGG3' and the antisense strand primer was 5'CGGGCTCTTGGCTTTGCAACTGG3'. The PCR products were restricted with *Nsi*I, gel-purified and cloned into *Pst*I cut Bluescript KS^{-}. To insure that PCR errors were not mistaken for genuine EMS mutations, clones were generated for each mutation from two independent PCR reactions and each completely sequenced using *ord*-specific primers. Only one base pair change that occurred in both PCR products was identified for each mutation. In addition, DNA from the isogenic *cn bw sp* strain (ord^{+}) used for the generation of mutants *ord*^{2}-*ord*^{6} was amplified, sequenced and used for comparison. One polymorphism was uncovered that differed between various wild-type chromosomes, resulting in the conservative substitution of glutamic acid for aspartic acid at position 444. All other polymorphisms identified on different wild-type chromosomes or on the *ord*^{1} mutant chromosome were silent mutations which did not result in amino acid changes.

Analysis of *ord^4* negative complementation

Crosses to analyze the missegregation of sex chromosomes in males and females were performed as described in Kerrebrock *et al.* (1992). By mating mutant $y/y^{+}Y$ males to compound-*X* females or mutant females to compound-*XY* males, gametes bearing all normal and most exceptional sex chromosome constitutions were recoverable and distinguishable. In the male nondisjunction tests, exceptional diplo-*Y* sperm were phenotypically indistinguishable from regular mono-*Y* sperm; therefore, "total missegregation" underestimates the actual level of missegregation. In the female tests, only half of the total number of exceptional gametes were recoverable but all regular X gametes were recovered. To compensate for this, "adjusted total" equals the number of progeny in the normal class plus twice the number of progeny in the exceptional classes. Total missegregation was calculated by doubling the number of exceptional progeny and dividing by the "adjusted total".

In order to minimize any differences in missegregation frequencies due to genetic background, isogenic *X* and *Y* chromosomes (Kerrebrock *et al.*, 1992) were incorporated into all *ord* stocks including *Df(2R)WI370*. In addition, multiple rounds of recombination had been used previously to cross off any extraneous lethal mutations on mutant *ord* chromosomes that might have been induced during EMS mutagenesis (Miyazaki and Orr-Weaver, 1992). To minimize background differences, all tests were performed with the same *ord^4* recombinant chromosome (*ord^4 bw*). In addition, the *ord* alleles tested *in trans* to *ord^4* were from the same round of recombination as *ord^4 bw*.

A 2 X 2 (normal and exceptional gametes) χ^2 contingency analysis (d.f.=1) (Lindren *et al.*, 1978) was used to determine whether differences in missegregation frequencies were statistically significant when comparing different *ord* transheterozygous combinations.

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Chapter Five

Double or nothing: A Drosophila mutation affecting meiotic chromosome segregation in both males and females

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My contribution to this work includes all but original isolation of the mutation and data in Table 7.

Abstract

We describe a novel Drosophila mutation, *Double or nothing* (*Dub*), that causes meiotic nondisjunction in a conditional, dominant manner. Previously isolated mutations in Drosophila specifically affect meiosis either in females or males, with the exception of the *mei-S332* and *ord* genes which are required for proper sister-chromatid cohesion. *Dub* is unusual in that it causes aberrant chromosome segregation almost exclusively in meiosis I in both sexes. In *Dub* mutant females both nonexchange and exchange chromosomes undergo nondisjunction, but the effect of *Dub* on nonexchange chromosomes is more pronounced. *Dub* reduces recombination levels slightly. Multiple nondisjoined chromosomes frequently cosegregate to the same pole. *Dub* results in nondisjunction of all chromosomes in meiosis I of males, although the levels are lower than in females. When homozygous, *Dub* is a conditional lethal allele and exhibits phenotypes consistent with cell death.

Introduction

Meiosis is a specialized cell division that produces haploid gametes, permitting a diploid genome to be restored in the zygote after fertilization. The reduction of the chromosomes to a haploid number during meiosis is accomplished by two rounds of chromosome segregation that follow a single duplication of the DNA. The first meiotic division (meiosis I) differs from mitosis in that the two homologs pair and segregate. In both meiosis II and mitosis the replicated copies of each chromosome, the sister chromatids, segregate.

Organisms utilize several strategies to carry out the specialized aspects of meiosis I (Baker *et al.* 1976). The most common mechanism of homolog pairing and segregation involves the formation of synaptonemal complex and requires recombination for proper segregation (John 1990). Recombination is proposed to lead to the formation of chiasmata that serve as stable attachments between the homologs, persisting after the dissolution of the synaptonemal complex in diplotene until the metaphase I-anaphase I transition. The stable homolog attachments are thought to constrain the kinetochores so that they are oriented in opposite directions and attach to different spindle poles (Nicklas 1974). Mutations that reduce recombination result in nondisjunction in meiosis I.

Although recombination is a widely adopted solution to homolog segregation, alternatives exist. These have been best characterized in *Drosophila melanogaster*, where at least three mechanisms are postulated for segregating chromosomes in the absence of recombination.

Recombination normally occurs in Drosophila females, however the tiny fourth chromosome virtually never recombines yet segregates faithfully. Furthermore, recombination can be reduced or eliminated on the other chromosomes by the presence of multiple inversions (Baker and Hall 1976). Nevertheless, these chromosomes segregate with high fidelity (Grell 1976). Mutations have been isolated that define a pathway for this segregation of nonexchange chromosomes. This pathway, called distributive segregation or more recently achiasmate segregation (Hawley and Theurkauf 1993), is used to segregate heterologous chromosomes as well as achiasmate homologous chromosomes. Separate mechanisms for these two types of events have been proposed based on the behavior of chromosomal rearrangements (Hawley *et al.* 1993). Nonexchange homologs appear to pair and segregate by a homology based mechanism, while the heterologous system segregates chromosomes based on size, shape, and availability (Grell 1976).

Nonexchange chromosomes have been shown to disjoin correctly in the yeast *S. cerevisiae*, implying that this organism also has a mechanism for achiasmate segregation (Dawson, Murray, and Szostak 1986; Guacci and Kaback 1991; Sears, Hegemann, and Hieter 1992).

In Drosophila males there is no detectable recombination, and synaptonemal complex is not formed (Baker and Hall 1976; Meyer 1960; Rasmussen 1973). Mutations affecting distributive segregation in the female have no effect on meiosis I in the male, thus a distinct pathway must exist for homolog segregation in males. This mechanism has been most fully investigated for the sex chromosomes in which specific pairing sites are responsible for pairing and proper segregation (Cooper 1964). The cis-acting pairing site for the *X* and *Y* chromosomes has been localized to part of the rDNA repeat (McKee and Karpen 1990; McKee, Habera, and Vrana 1992). It appears that pairing sites also mediate autosomal segregation (McKee, Lumsden, and Das 1993, Yamamoto 1979).

The specificity of meiotic mutations isolated in Drosophila provides strong evidence for multiple pathways of chromosome segregation in meiosis I. For example, with two exceptions, all of the mutations affect meiosis only in the female or only in the male. The majority of mutations affecting chromosome segregation in the female reduce recombination (Baker and Hall 1976). Other mutations, also female specific, almost exclusively cause nondisjunction of nonexchange chromosomes. Mutations in the *nod*, *Axs*, *ald*, and *mei-S51* genes belong to this class (Carpenter 1973; O'Tousa 1982; Robbins 1971; Zhang and Hawley 1990; Zitron and Hawley 1989). The *ncd* gene is unusual in that mutations in this gene result in aberrant segregation of both exchange and nonexchange chromosomes (Davis 1969). Trans-acting mutations affecting homolog segregation specifically in the male are not well defined.

Mutations in the *mei-S332* and *ord* genes are unique because they result in nondisjunction in both sexes. They also differ from other mutations in exhibiting larger amounts of meiosis II nondisjunction (Davis 1971; Kerrebrock *et al.* 1992; Mason 1976; Miyazaki and Orr-Weaver 1992). *mei-S332* and *ord* mutants show premature sister-chromatid separation in meiosis I, and therefore the products of these genes appear to maintain sister-chromatid cohesion in meiosis.

We describe a novel mutation in Drosophila, *Double or nothing* (*Dub*), that affects meiosis I in both females and males. This conditional dominant mutation causes nondisjunction predominantly of nonexchange chromosomes in female meiosis, but it also significantly disturbs the segregation of exchange chromosomes. When homozygous, *Dub* is a conditional lethal allele.

Materials and Methods

Stocks

All Drosophila stocks and crosses were grown at 25°C (unless otherwise noted) on a standard mix of cornmeal, brewer's yeast, molasses and agar. All balancer chromosomes and all mutations other than *Dub* are described in Lindsley and Zimm (1992). *C(1)RM*, y^2 $su(w^a)$ w^a will be referred to in this paper as compound-*X* or $\hat{XX}$. $Y^SX\bullet Y^L$ y^+, *In(1)EN*, *y v f B* was used as the compound-*XY* chromosome and is referred to as $\hat{XY}$ in this paper. *C(4)EN, ci* ey^R is referred to as $\hat{44}$. These compound chromosomes, the *cv v f car* and the compound autosome stocks are described in Kerrebrock *et al.* (1992). The *FM7c* balancer has the markers y^{31d} sc^8 w^a sn^{X2} v^{of} g^4 *B*. The *c wt px* stock used in mapping was obtained from the Bloomington stock center. The deficiency *Df(2R)PC4* was obtained from R. Lehmann (Whitehead Institute, Cambridge, MA). The *TM3, Sb*/ *T(2;3)CyO, st* Kg^V *red Tb* stock was obtained from W. Saxton.

Isolation of the *Dub* mutation

Double or nothing (*Dub*) is a mutation that was induced on a second chromosome, marked with *J Sco*, using the mutagen, ethyl methanesulfonate (EMS). It was isolated in a screen of 2034 chromosomes for new alleles of *abo* (*abnormal oocyte*) (Sandler 1970; Tomkiel, Pimpinelli, and Sandler 1991), and its isolation number was *1102*. A female-specific meiotic defect as well as a maternal effect lethality are associated with abo^1 (Carpenter and Sandler 1974; Sandler 1970). While the *Dub* mutation complemented the maternal effect, the frequency of nondisjunction in abo^1/*Dub* females was double that of *Dub*/+ females. However, no increase in nondisjunction was observed in abo^2/*Dub* females, suggesting that either the abo^1 interaction is allele specific or due to a locus elsewhere on the chromosome.

Nondisjunction tests, calculation of recombination frequencies and exchange ranks

For simultaneous measurement of *X* and *4* nondisjunction in females, y/y^+Y; *C(4)EN, ci* ey^R males were crossed to *y/y*; spa^{pol} / spa^{pol} females. Regular ova yielded yellow females (*X/X*; $\hat{44}$/*4*) and wild-type males (*X/Y*; $\hat{44}$/*4*). Progeny trisomic for

chromosome *4* were viable, but progeny haploid for chromosome *4* were essentially inviable. Any surviving haplo-*4* Minute progeny were counted and recorded, but they were excluded from any calculations and are not reported in this paper. Exceptional-*X* ova produced yellow$^+$ females ($X/X/y^+Y$) and yellow males (*X*/O). The number of these progeny was doubled for the adjusted total and for calculation of the nondisjunction frequency, because half of the exceptional-*X* ova were not recoverable (those producing *X/X/X* and O/*Y* progeny). Exceptional-*4* ova produced sparkling-poliert progeny (*4/4*) or cubitus-interruptus eyeless-Russian progeny ($\hat{44}$). Although only half of the exceptional-*4* progeny were recovered, it was not necessary to double their number for calculations of nondisjunction frequency because only half of the normal-*4* ova were recoverable.

In the assay of female meiotic nondisjunction for Table 2, compound-*XY*, *v f B* males were crossed to *cv v f car/y* females. Normal ova yielded Bar females ($\hat{XY}/X$) and males wild-type for Bar (*X*/O). Exceptional-*X* ova yielded Bar males ($\hat{XY}$/O) and females wild-type for Bar (*X/X*). The number of exceptional progeny was doubled for the adjusted total and for calculation of the nondisjunction frequencies. The centromere-linked mutation, *carnation*, allowed diplo-*X* ova resulting from meiosis II nondisjunction (carrying two sisters) and those resulting from meiosis I nondisjunction (carrying two homologs) to be distinguished. To calculate map distances, exchange events on the *X* chromosomes were counted. This was done by recording the phenotypes of the *X*O males resulting from normal-*X* ova, and by crossing the F_1 females resulting from diplo-*X* ova to compound-*XY* males and recording the phenotypes of F_2 *X*/O males to determine the markers on the parental chromosomes in the F_1 females. Mapping distances for the diplo-*X* ova were calculated as if the chromosomes had been isolated from independent ova carrying a single *X* chromosome. Exchange rank distributions were calculated by the method of Weinstein (1936) for regular-*X* progeny and by the method of Davis (1969) and Merriam and Frost (1964) for diplo-*X* progeny.

In the assay of female meiotic nondisjunction for Table 5, compound-*XY*, *v f B* males were crossed to *y/FM7c, y B* females. Regular ova yielded yellow$^+$ females ($X/\hat{XY}$ and $FM7c/\hat{XY}$) and yellow males (*X*/O or *FM7c*/O). Exceptional ova yielded yellow females (*FM7c/X* and *X/X*) and yellow$^+$ males ($\hat{XY}$/O). Because particular classes of progeny from regular ova had reduced viability (the *FM7c*/O and $FM7c/\hat{XY}$ progeny), these classes were not used in the adjusted total and calculations. Consequently, the number of exceptional progeny did not need to be doubled.

An unexpected class of progeny was noted in this cross, yellow Bar males with vermilion$^+$ eyes. Although their external appearance was entirely male, these "males" were infertile and their testes had a glittering appearance. This phenotype resembled the crystals observed in *X/O* males that result from overexpression of the Stellate protein in the absence of the *Y* chromosome (Livak 1984). We believe the "males" were actually intersexes (*FM7c/X; 2/2/2; 3/3/3; 4/4* or *4/4/4*) resulting from nondisjunction of autosomes as well as the *X* chromosomes. The ova that produced the intersexes would have produced triploid females if fertilized by $\widehat{XY}$ sperm, but these triploid females had a phenotype not easily distinguishable from the products of normal ova ($X/\widehat{XY}$). To ask if the triploid females were present, we outcrossed approximately 20 of the supposed $X/\widehat{XY}$ females (excluding any vermilion-eyed $FM7c/\widehat{XY}$ females), and we observed male progeny with the phenotype expected of the balancer, *FM7c*. These male progeny revealed the presence of one or more $X/FM7c/\widehat{XY}$ triploid mothers among the 20 supposed $X/\widehat{XY}$ mothers. We estimated that as many triploid females existed as intersexes, and the estimated number of the triploid females was subtracted from the normal ova for the adjusted total and for calculation of nondisjunction frequency. The intersexes were also not included in calculation of the *X* chromosome nondisjunction frequency.

In the nondisjunction assay performed for Table 6, *y* males were mated with compound-X/y^+Y females. Normal ova yielded yellow females ($\widehat{XX}/Y$) and yellow$^+$ males (X/y^+Y). Exceptional ova yielded yellow$^+$ females ($\widehat{XX}/y^+Y/Y$) and yellow males (*X/O*). Only half of the normal ova were recoverable, so doubling of exceptional classes was not necessary. However, females carrying two *Y* chromosomes have reduced viability (Lindsley and Zimm, 1992), so the number of exceptional ova ($\widehat{XX}/Y/Y$ and *X/O*) was estimated as twice the number of yellow males (*X/O*) for the adjusted total and calculation of the nondisjunction frequencies.

For simultaneous measurement of the sex and fourth chromosome nondisjunction in males, *y/y; C(4)EN, ci* ey^R females were mated with y/y^+Y; spa^{pol} males. Normal sperm yielded yellow females (X/X; $\widehat{44}/4$) and yellow$^+$ males (X/Y; $\widehat{44}/4$). As in the female test of *X* and *4* nondisjunction, any surviving haplo-*4* Minute progeny were counted but were excluded from any calculations and are not reported in this paper. Sperm that were diplo or nullo for the sex chromosomes produced yellow$^+$ females ($X/X/y^+Y$) and yellow males (*X/O*). Exceptional-*4* sperm produced sparkling-poliert progeny (*4/4*) or cubitus-interruptus eyeless-Russian progeny ($\widehat{44}$/*/O*).

To determine the meiotic division affected in males, compound-*X*, $y^2 su(w^a)\ w^a$ females were mated with y/y^+Y males. Normal sperm yielded yellow$^+$ females

($\widehat{XX}/y^+Y$) and yellow males (*X*/O). Exceptional sperm yielded yellow or yellow^2 females (*X*/*X* and $\widehat{XX}$/O) and yellow$^+$ males (X/y^+Y). The females resulting from sperm carrying two sister chromosomes (*X*/*X*) were yellow and had a wild-type eye color, whereas exceptional females resulting from nullo-*XY* sperm ($\widehat{XX}$/O) were yellow^2 and had a darker eye color with no pseudo-pupil.

Mapping of *Dub*

The mutation was first mapped to the interval between *nw* and *Pin* in two small scale mappings (15 and 47 recombinants). Females heterozygous for *J Sco Dub* and *S Sp Tft* nw^D *Pin* were mated with abo^1 males, and the female progeny were mated with compound-*XY* males to test for skewed sex ratios or for nondisjunction events in the progeny. No sex ratio skewing was apparent, and nondisjunction events were used to map the mutation. *Dub* was later mapped to the smaller interval between *c* and *wt* . After mating *c wt px* males to *pr cn Dub/c wt px* or *pr cn Dub sp/ c wt px* females, recombinant chromosomes from male progeny were isogenized and tested for three phenotypes: inviability when transheterozygous with the original *pr cn Dub* chromosome, dominant meiotic nondisjunction in females, and dominant meiotic nondisjunction in males. In 33 recombinants between *c* and *wt* all three phenotypes mapped to 2-82.6 cM.

Lethal phase and phenotypes

The lethal phase of *Dub* homozygotes was assessed by mating parents heterozygous for *Dub* (*pr cn Dub/ b pr*). As controls, heterozygous parents were outcrossed to *b pr* mates and, in addition, a mating of *b pr* males and females was set up. The females were allowed to lay their eggs overnight on apple juice-sucrose-agar petri dishes with a wet yeast smear on the surface. The number of clear unfertilized eggs, the number of eggs that hatched, the number of pupal cases and the number of eclosed adults were all recorded. From these counts, a histogram of lethality was constructed.

To examine the pupal lethal phenotype of *Dub*, heterozygous larvae and homozygous larvae were sorted by using the larval mutant phenotypes, Tubby and Kugel (Saxton *et al.* 1991). After *pr cn Dub/SM1* and *TM3, Sb/T(2;3) CyO, st* Kg^V *red Tb* flies were mated, the resulting *pr cn Dub/T(2;3) CyO, st* Kg^V *red Tb* progeny were

crossed *inter se* to give *Dub* homozygotes. The non-Tubby, non-Kugel larvae were moved to new plates and the range of larval and pupal phenotypes was observed.

Neuroblast squashes for mitotic chromosomes

Cytological preparations of larval brains were made by standard methods without colchicine (Gonzalez *et al.* 1991; Sunkel and Glover 1988). These were examined by phase-contrast microscopy using a Zeiss Axiophot equipped with Plan Neofluar 100X and Plan Apochromat 63X objectives.

Results

Dub is a conditional dominant mutation that causes nondisjunction during meiosis I in females

The EMS-induced mutation, *Dub*, was discovered in a screen because it exhibited an increased frequency of *X* chromosome nondisjunction during female meiosis. We have examined meiosis in females carrying *Dub*, using genetic assays to ask whether all chromosomes are affected and which of the meiotic divisions is defective. Nondisjunction produces aneuploid ova, referred to as exceptional ova. By mating mutant females to males carrying compound chromosomes, exceptional gametes could be recovered and the frequency of nondisjunction quantified.

In a cross of heterozygous mutant females to males carrying marked sex chromosomes and a compound-*4* (see Materials and Methods), the frequencies of meiotic nondisjunction of the *X* and fourth chromosomes were measured at two temperatures. *Dub* was found to increase nondisjunction of both chromosomes in a dominant and temperature-sensitive manner (Table 1). We were not able to test homozygous *Dub* females in this assay, because as described below, *Dub* has a recessive, temperature-sensitive lethality. The frequency of fourth chromosome nondisjunction was much higher in *Dub* females than in control females, yielding 34.8% exceptional ova relative to 0.3%. Nullo-*4* ova outnumbered diplo-*4* ova, suggesting that some chromosome loss occurred in addition to nondisjunction. Nondisjunction of the *X* chromosome occurred at a frequency of 16.4%, much higher than the control frequency of 0.5%. Nullo-*X* ova outnumbered diplo-*X* ova.

To assess whether nondisjunction of the large autosomes occurs in *Dub* females, males carrying compound autosomes were mated with mutant and wild-type females in identical numbers, e.g. 10 males and 15 females per vial. This assay gave only a qualitative assessment of autosomal nondisjunction. Ova with the normal autosomal content will not yield viable progeny when fertilized by sperm from a male carrying a compound autosome. The sperm will carry the equivalent of either two or no copies of the autosome, and trisomy or monosomy for either the second or third chromosome is lethal in Drosophila zygotes. However, a female with frequent nondisjunction events will produce exceptional ova, and these may be fertilized by sperm with a compensatory number of autosomes such that viable zygotes are produced. Viable progeny resulted approximately ten-fold more frequently in vials containing mutant females. In crosses to *C(2)EN* the *Dub* females produced on average 27 progeny per

TABLE 1

Dub is a dominant conditional mutation increasing female meioitc nondisjunction frequency

Ova type	25°C		18°C	
	+/+	*Dub*/+	+/+	*Dub*/+
Regular ova				
X; 4	1331	945	1063	822
X nondisjunctional ova				
X/X; 4	0	22	2	6
O; *4*	2	45	0	1
4 nondisjunctional ova				
X; 4/4	1	192	3	39
X; O	2	247	24	41
X,4 nondisjunctional ova				
X/X; 4/4	0	19	2	1
X/X; O	0	5	0	0
O; *4/4*	1	6	0	0
O; O	0	39	0	2
Total progeny scored	1337	1520	1094	912
Adjusted total scored [a]	1340	1656	1098	922
% nondisjunction				
X	0.45	16.43	0.73	2.17
4	0.37	34.84	2.82	9.33

Temperature and maternal genotype (column headings).

y/y; spa$^{(pol)}$ females of the indicated genotype were crossed to *y/y*$^+$*Y; C(4)RM, ci ey*^{R} males.

[a] *X* nondisjunctional progeny were doubled for calculation of nondisjunction frequency (see Materials and Methods).

vial, while the control females produced two. In crosses to *C(3)EN*, *Dub* females produced an average of 55 progeny per vial, but the control siblings produced only three. Therefore, *Dub* affects all four chromosomes.

To ascertain whether chromosome missegregation events were occurring in the first or second meiotic division, we mated *Dub* females to males carrying a compound-*XY* chromosome. The mutant females carried *X* chromosomes heterozygous for a centromere-linked marker, *carnation* (*car*), so that diplo-*X* exceptional progeny carrying two sister chromosomes could be distinguished from those carrying two homologous chromosomes. Nondisjunction occurred almost exclusively during the first meiotic division (Table 2), because essentially all of the exceptional ova carried two homologous chromosomes. The lower percentage of nullo-*X* relative to the number of diplo-*X* ova observed in Table 2 is likely due to cosegregation events of the *X* and *4*, since the nullo-*X* nullo-*4* ova are inviable in this assay. Cosegregation is discussed in further detail below.

In these matings of *Dub* heterozygous mothers there was a low but significant number of gynandromorphs. These result from chromosome instability in the early zygotic cleavages, either due to chromosome loss during the mitotic divisions or recovery by a mitotic spindle of a chromosome lost during a meiotic division. Other meiotic mutations, notably *nod* and *ncd*, show a similar phenotype (Carpenter 1973; Davis 1969).

Dub has little effect on recombination

Since the majority of mutations that affect the first meiotic division in Drosophila females cause a reduction in recombination, we examined the effect of *Dub* on recombination. The *X* chromosomes used in the cross for Table 2 were heterozygous for several recessive mutations, and map distances were calculated from the phenotypes of the regular *X*O male progeny. Surprisingly, although *Dub* causes reductional nondisjunction, it has relatively little effect on recombination. There were slight reductions in all of the intervals, but only one interval showed a significant difference (Table 3, Mono-*X* ova). The *Dub* and control values were significantly different for the *vermillion-forked* (*v-f*) interval (binomial distribution test, $p < 0.01$), but there was no significant difference for the other intervals (Lindren, McElrath, and Berry 1978).

Recombination distances were also assessed in the diplo-*X* exceptional female progeny (Table 3, Diplo-*X* ova), and there was a significant reduction in exchange for

TABLE 2
Dub female meiotic nondisjunction produces reductional exceptions

Ova type	Maternal genotype: $\frac{+}{+}$	Maternal genotype: $\frac{Dub}{+}$
Regular ova [a]		
X	5514	5268
X nondisjunctional ova		
O	3	246
X/X car^{+}/car^{-}	1	335
X/X car^{+}/car^{+} and car^{-}/car^{-} [b]	0	1
Total progeny scored	5518	5850
Adjusted total scored [c]	5522	6432
% nullo-*X*	0.11	7.65
% diplo-*X* (*car+/car-*)	0.04	10.42
% diplo-*X* (*car-/car-* and *car+/car+*)	0.00	0.03
Total % nondisjunction	0.15	18.10

y/cv f v car females of the indicated genotype were mated to compound-*XY*, *v f B* males at 25°C.

[a] The ratio of regular ova fertilized by nullo-*XY* sperm relative to $\hat{XY}$ sperm is 2445/3069 for the control females and 2010/3258 for the *Dub* females.

[b] car^{+}/car^{+} ova were distinguished from car^{+}/car^{-} ova by outcrossing a sample of 100 progeny that were non-carnation. No carnation^{+} homozygotes were observed. Consistent with this observation, the number of car^{+}/car^{+} ova should have been approximately equal to the number of car^{-}/car^{-} ova and only one carnation homozygote was observed.

[c] *X* nondisjunctional progeny were doubled for calculation of nondisjunction frequency (see Materials and Methods).

TABLE 3

Dub has little effect on recombination in females

Mono-*X* ova

Genotype	Mapping interval *y-cv* (cM)	*cv-v* (cM)	*v-f* (cM)	*f-car* (cM)	Total map distance (cM)	Number of progeny scored
+/+	8.4	19.5	21.7	6.5	56.1	2445
Dub/+	7.6	19.2	18.5	5.5	50.8	2010

Diplo-*X* ova

Genotype	Mapping interval *y-cv* (cM)	*cv-v* (cM)	*v-f* (cM)	*f-car* (cM)	Total map distance (cM)	Number of progeny scored
Dub/+	6.6	9.9	12.9	2.1	31.5	167 [a]

[a] Both chromosomes in these progeny came from the mother, so a total of 334 chromosomes were scored for exchange.

all intervals except the most distal (binomial distribution test, $p < 0.001$, except the most distal interval $p > 0.2$). Since we were unable to score the recombination levels in nullo-*X* exceptional gametes, we could not detect whether nonexchange chromosomes were preferentially lost. If this were the case, the effect of *Dub* on recombination would be underestimated. Exchange in the proximal regions appeared to ensure proper disjunction better, because nondisjunction was more likely to be accompanied by exchange in the distal regions. This distribution of exchanges is reminiscent of that in the diplo-*X* and diplo-*2* exceptions of *nod* and nod^{DTW} (Carpenter 1973; Rasooly *et al.* 1991).

Dub primarily affects nonexchange but also exchange chromosomes

Several observations suggested that *Dub* might not affect the exchange-mediated and achiasmate segregation systems equally. The greater nondisjunction of chromosome *4* relative to the *X* chromosome (Table 1) is consistent with disruption of the distributive system, since the fourth chromosomes are achiasmate in Drosophila. The exceptional progeny resulting from diplo-*X* ova showed a reduction in map distances while the normal progeny did not (Table 3), and the reduced amount of exchange was likely to be the result of a bias for nondisjunction of nonexchange chromosomes.

To address the question of whether *Dub* predominantly affects nonexchange chromosomes, we compared the percentage of nonexchange tetrads present in the ova having faithfully segregated chromosomes with the percentage in ova having improperly segregated chromosomes. When there is no exchange in a tetrad, chromosomes are segregated by the distributive system, so the number of nonexchange tetrads reflects the number of chromosomes that must be segregated in the distributive system. Therefore if nonexchange chromosomes were more likely to nondisjoin in *Dub* mutants, a greater proportion of the exceptional ova would be derived from nonexchange tetrads. The frequency of nonexchange, single exchange and multiple exchange tetrads (known as the tetrad or exchange rank) may be estimated from the observed number of no crossover, single crossover and multiple crossover chromosomes. Appropriate equations have been developed for calculating the tetrad ranks from normal and diplo-*X* ova (Davis 1969; Weinstein 1936).

The percentage of nonexchange tetrads in the exceptional ova was much greater than the percentage in the normal ova (Table 4). The normal mono-*X* ova had a tetrad rank similar to the control, however there was a slight decrease in double

TABLE 4
Exchange ranks of normal and exceptional ova

	Mono-*X* ova		Diplo-*X* ova
	Maternal genotype		Maternal genotype
Exchange rank	$\frac{+}{+}$	$\frac{Dub}{+}$	$\frac{Dub}{+}$
E_0	0.13	0.10	0.51
E_1	0.62	0.79	0.40
E_2	0.25	0.10	0.09
E_3	0.0	0.01	0.0
Number scored	(2445)	(2010)	(167)

exchange tetrads and a slight increase in single exchange tetrads. In contrast, the exceptional ova arising from *Dub* females had a decrease in all exchange tetrads and an increase in nonexchange tetrads. Therefore, nonexchange tetrads are more vulnerable to nondisjunction than are exchange tetrads in a heterozygous *Dub* background.

The hypothesis that the distributive system is disrupted in *Dub* females predicts that a chromosome pair that does not undergo exchange will experience higher rates of nondisjunction. To test this, we assayed nondisjunction of a balancer *X* chromosome heterozygous with a normal *X* chromosome. The rearrangements on the balancer *FM7c* have been estimated to suppress recombination completely (Hawley *et al.* 1993). In *Dub* females bearing *FM7c* and a normal *X* chromosome, the nondisjunction frequency dramatically increased to 52.3% compared to 16.4% for the normal *X* chromosome (Table 5). This suggests that the effect of *Dub* on distributive segregation was at least two- to three-fold greater than the effect on exchange-mediated segregation.

We tested the effect of *Dub* on the achiasmate segregation system in one other way. An example of the distributive segregation system in Drosophila is the consistent and faithful segregation of a *Y* chromosome from a compound-*X* chromosome in females (Grell 1976). These chromosomes are segregated by the achiasmate system even though exchange does occur between the two *X*

TABLE 5
Dub females with a balancer *X* chromosome have very high meiotic nondisjunction frequencies

Ova type	Maternal genotype: $\frac{+}{+}$	Maternal genotype: $\frac{Dub}{+}$
Regular ova [a]		
X	2156	1097
FM7c	737	371
X nondisjunctional ova		
O	9	545
X/FM7c	14	483
X/X and *FM7c/FM7c*	0	0
Autosome nondisjunctional ova [b]		
X/FM7c; *2/2*; *3/3*; *4* or *4/4*	0	160
Total progeny scored	2916	2656
Adjusted total scored [c]	2179	1965
% nullo-*X*	0.41	27.74
% diplo-*X*	0.64	24.58
Total % nondisjunction	1.0	52.32

y/FM7c females of the indicated genotype were mated with compound-*XY*, *v f B* males. The control females were *SM1/+*.

[a] The ratio of regular *X* ova fertilized by nullo-*XY* sperm relative to $\hat{XY}$ sperm is 1225/931 for the control females and 457/480 for the *Dub* females (160 triploid female progeny have already been subtracted from the *X/*$\hat{XY}$ progeny for the *Dub* ratio).

[b] These progeny were observed as intersexes, and this number represented only half of the number of such ova (see Materials and Methods).

[c] Calculation of the *X* chromosome nondisjunction frequencies was done using adjustments described in Materials and Methods. These adjustments compensate for the presence of autosomal nondisjunction and the reduced viability of the progeny resulting from regular ova carrying *FM7c*.

chromosome arms of a compound-*X* chromosome. Mutations such as *ncd, ald,* and *Axs* have been shown to interfere with this segregation (Davis 1969; O'Tousa 1982; Zitron and Hawley 1989). In *Dub* females with a compound-*X* chromosome and a *Y*, the nondisjunction frequency was 40.9% compared to 0.6% in the control (Table 6).

These experiments demonstrate that *Dub* affects the segregation of nonexchange chromosomes, but the mutation causes nondisjunction of exchange chromosomes as well. *Dub* did not reduce recombination enough for all of the nondisjoined chromosomes to be nonexchange (Tables 2 and 3), and in the diplo-*X* exceptional gametes almost half of the tetrads have undergone at least one exchange (Table 4).

Cosegregation of chromosomes in *Dub* mutant females

In *Dub* females when more than one chromosome was missegregated in the same ovum, these chromosomes were not segregated independently with respect to each other. By simultaneously following two chromosomes, the *X* and fourth (Table 1), we observed a strong tendency for the missegregating chromosomes to be incorporated into the same meiotic product. The double exceptions seen were not independently distributed among the possible classes: *X/X*; *4/4* and O;O double exceptions were more numerous than were *X/X*; O and O; *4/4* double exceptions. Such a non-random distribution among the double exceptions had been previously observed in the meiotic mutants *nod* and *ncd* for the *X* and fourth chromosomes (Carpenter 1973; Davis 1969; Wright 1974). This "cosegregation" behavior is in marked contrast to the non-random distribution of *X*; *4* double exceptions observed in *Axs* females, where the *X* bivalent is more likely to segregate away from the fourth bivalent, yielding *X/X*; O and O; *4/4* ova (Zitron and Hawley 1989).

Additional evidence indicated that cosegregation of all chromosomes occurred often. When a balancer *X* was introduced into *Dub* heterozygous females, intersexes and triploid females appeared among the progeny at a surprisingly high frequency (Table 5). The intersexes and triploid females resulted from ova carrying two copies of the major autosomes and one or two copies, respectively, of the *X* chromosome. Similarly, when a compound-*X* chromosome and a *Y* chromosome were present in a *Dub* heterozyogous female, many intersexes and triploid females were found in the progeny (Table 6). Thus cosegregation of the sex chromosomes with the autosomes appeared to have occurred, although the number of *X/X* or $\hat{XX}/Y$; *2/2*; *3/3* ova could not be compared to the number of O; *2/2*; *3/3* ova, because the latter were not

TABLE 6

Dub disturbs the segregation of the *Y* chromosome from the compound-*X* in females

Ova type	Maternal genotype $\frac{+}{+}$	Maternal genotype $\frac{Dub}{+}$
Regular ova		
$\widehat{XX}$	1025	673
Y	1280	619
X Nondisjunctional ova		
$\widehat{XX}/Y$ 1	61	
O	7	447
Autosome nondisjunctional ova [a]		
$\widehat{XX}/Y$; *2/2*; *3/3*; *4* or *4/4*	2	39
$\widehat{XX}$; *2/2*; *3/3*; *4* or *4/4*	4	5
Total progeny	2319	1844
Corrected total progeny [b]	2319	2186
% nondisjunction [b]		
$\widehat{XX}/Y$ <-> O	0.60	40.90

Compound-*X*, y^2 $su(w^a)$ w^a / *y+Y* females of the indicated genotype were mated with *y* males. The control females were *Sco/+*.

[a] These ova produced progeny that were either intersexes or triploid females.

[b] See Materials and Methods.

recoverable. It is interesting that in the $\widehat{XX}/Y$ cross, triploid females and intersexes were more likely to have received both the compound-*X* and the *Y* than to receive only the compound-*X* chromosome, as $\widehat{XX}/Y$; *2/2*; *3/3* ova were more frequent than $\widehat{XX}$; *2/2*; *3/3* ova.

Dub dominantly increases nondisjunction during meiosis I in males

The first meiotic division in male Drosophila is distinct from the first division in females (Baker and Hall 1976). There is no recombination, and assembled synaptonemal complex is not observed (Meyer 1960; Rasmussen 1973). Instead, segregation of the homologs employs specific pairing sites. All of the previously isolated Drosophila meiotic mutants are specific in affecting only females or only males, with the exceptions of *ord* and *mei-S332* (Davis 1971; Kerrebrock *et al.* 1992; Mason 1976; Miyazaki and Orr-Weaver 1992). These two mutations cause premature sister-chromatid separation and have significant levels of meiosis II nondisjunction. *Dub* was striking because it caused meiotic chromosome nondisjunction in males and females, and in contrast to *ord* and *mei-S332*, meiosis I segregation was affected almost exclusively.

Meiotic nondisjunction in *Dub* males was characterized by genetic assays to test which chromosomes and which meiotic division were affected by *Dub*. In males, *Dub* acted to increase nondisjunction in a dominant and temperature-sensitive manner (Table 7). Both the sex chromosomes and the fourth chromosome were affected, and the frequency of fourth chromosome nondisjunction was lower than sex chromosome nondisjunction. Sperm that were nullosomic for the sex chromosomes were more common than were *X/X* or *X/Y* sperm, indicating that chromosome loss also occurred. The overall frequency of nondisjunction was lower in males than in females, the difference in fourth chromosome segregation being particularly great.

We have tested qualitatively whether the autosomes have an increased frequency of nondisjunction by crossing *Dub* males to compound autosome stocks by mating 10 males to 15 females in individual vials. The appearance of viable progeny was about ten-fold higher than what was observed when the same number of wild-type males were crossed to compound autosomal females. When *Dub* males were crossed to *C(2)EN* females an average of 26 progeny per vial were recovered, compared to four in wild-type controls. When *Dub* males were crossed to *C(3)EN* females an average of 30 progeny per vial were recovered, while less than one was produced by control males. Therefore, all chromosomes undergo nondisjunction in *Dub* heterozygous males.

By crossing test males to compound-*X* females we were able to assess the meiotic division in which missegregation was occurring (Table 8). The first meiotic division was primarily affected; however, missegregation did not appear to be as exclusive to the reductional division as it was in *Dub* heterozygous females. The

TABLE 7

Dub is a dominant conditional mutation increasing the male meiotic nondisjunction frequency

Sperm type	25°C +/+	25°C *Dub*/+	18°C +/+	18°C *Dub*/+
Regular sperm				
X; *4*	919	962	638	592
Y; *4*	719	1064	421	413
***XY* nondisjunctional sperm**				
X/Y; *4*	8	68	4	9
O; *4*	6	91	5	16
***4* nondisjunctional**				
X; *4/4*	2	9	2	1
X; O	5	10	1	1
Y; *4/4*	3	9	1	4
Y; O	4	13	1	3
***XY*,*4* nondisjunctional**				
X/Y; *4/4*	0	1	0	1
X/Y; O	1	0	0	0
O; *4/4*	0	1	0	0
O; O	1	6	1	1
Total progeny scored	1668	2234	1074	1041
% nondisjunction				
XY	0.96	7.48	0.93	2.59
4	0.96	2.19	0.56	1.06

Column header: Temperature and paternal genotype.

y/y+Y males of the indicated genotype were mated with *y*; *C(4)EN*, *ci* ey^R females.

TABLE 8
Dub male meiotic nondisjunction yields primarily reductional exceptions

Sperm type	Paternal genotype $\frac{+}{+}$	Paternal genotype $\frac{Dub}{+}$
Regular sperm		
X or *Y* [a]	2816	5531
XY nondisjunctional sperm		
O	4	178
X/Y	3	105
X/X	0	15
Total progeny scored	2823	5829
% nullo-*XY*	0.14	3.05
% *X/Y*	0.11	1.80
% diplo-*X*	0.00	0.26
Total observed nondisjunction	0.25	5.11

y/y+Y males of the indicated genotype were mated with compound-*X*, y^2 $su(w^a)$ w^a females. The control in this experiment was not done with siblings of the *Dub*/+ males.

[a] In this assay, *Y/Y* exceptional sperm were indistinguishable from regular mono-*Y* sperm and are therefore included in these numbers.

number of equational exceptions was higher than observed in the control, although the frequency was still less than 1%. Because the progeny from *Y/Y* sperm were indistinguishable from normal progeny, only half of the equational exceptions were scored in this test. Consequently, the true frequency of equational missegregation was probably twice what we measured.

The cosegregation of heterologous chromosomes that nondisjoined was difficult to address in male *Dub* heterozygotes. Since the nondisjunction frequencies in *Dub* males were already low, the number of double exceptions was too low to conclude whether cosegregation of the sex and fourth chromosomes occurred. However, when *Dub* males were outcrossed, triploid females and intersexes appeared more frequently than in wild-type crosses (data not shown). Therefore, it appears that cosegregation of the autosomes occurred.

Dub is a recessive, conditional lethal mutation

The dominant meiotic phenotype of *Dub* is linked closely to a conditional recessive lethality. At 25°C, homozygous *Dub* adults were rare. The rare escapers were very short-lived and had many defects: small rough eyes, etched tergites, crumpled or nicked wings, and bristles either missing or duplicated. At 18°C, homozygous *Dub* progeny were more common, although at most 20% of the expected number of homozygotes eclosed in bottles of the heterozygous stock. Homozygous adults raised at 18°C were more normal in appearance, except for patches of disorder in the eye facets. These flies were infertile.

Recessive lethality and the phenotype of the rare escapers are characteristics observed in mutations affecting mitotic chromosome segregation, such as *rough deal* (*rod*) (Karess and Glover 1989). The presence of gynandromorphs among the progeny of heterozygous *Dub* mothers also suggested that *Dub* product might play a role in mitosis. To test this, we determined the lethal phase and phenotype of *Dub* homozygotes, and we then cytologically examined neural cells of homozygous larvae for mitotic defects. Most known mitotic mutants have late-larval/pupal lethality, although a few embryonic lethal mitotic mutants are known (Edgar and O'Farrell 1989; Gatti and Baker 1989; Hime and Saint 1992).

To determine the lethal phase of *Dub* homozygotes, heterozygous parents were mated and the fate of their eggs was quantitated. One quarter of the progeny should have been homozygous, but about half of the progeny died (Figure 1). Therefore there appeared to be two causes of lethality, homozygous lethal animals and a dominant lethal effect of *Dub*. Control matings of a heterozygous parent and a wild-type parent showed 8-12% embryonic lethality. In contrast, when both parents were *Dub* heterozygotes, there was about 25% pupal lethality in addition to embryonic lethality (Figure 1). *Dub* homozygotes were most likely to account for the pupal lethality.
The embryonic lethality that occurred when either parent was a *Dub* heterozygote appears to have been the consequence of autosomal aneuploidy due to meiotic nondisjunction, rather than a semi-dominant lethal effect of *Dub*, or a maternal-effect lethality. We found that *Dub* had no semi-dominant lethality by crossing *pr cn Dub*/ *pr cn bw* males to *pr cn bw* females and then counting the ratio of Dub^{+} and Dub progeny (data not shown). Maternal lethality seemed unlikely as there was a similar degree of embryonic lethality when either the mother or father was a *Dub* heterozygote (Figure 1).

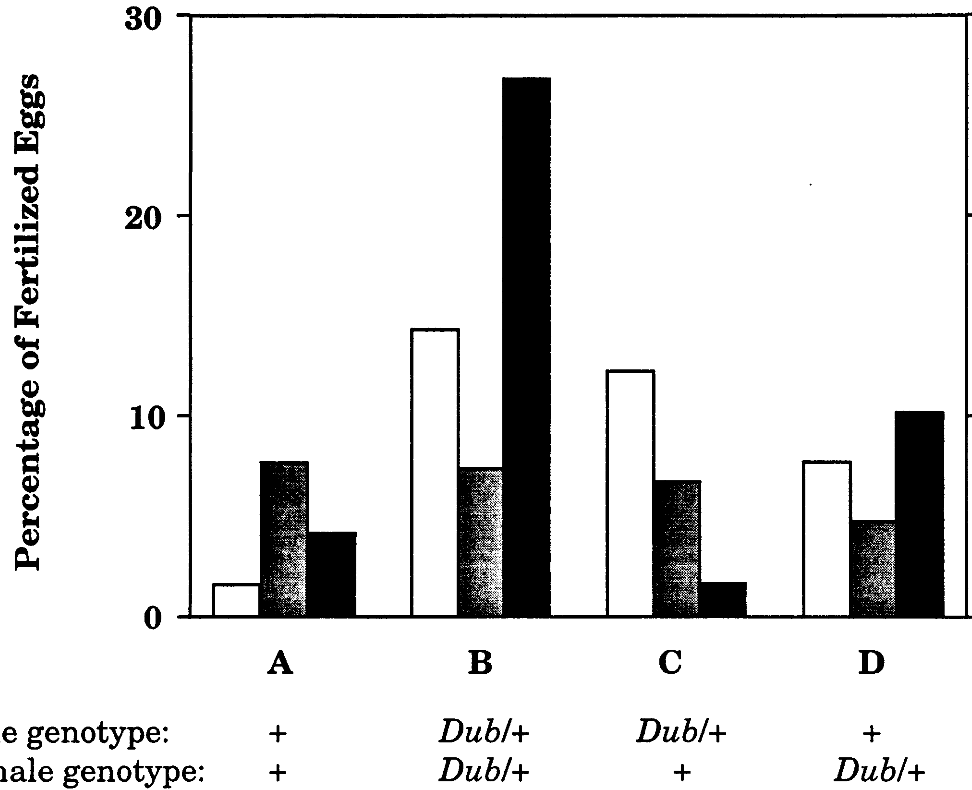

Figure 1: Lethal phase of *Dub* mutants at 25°C. The indicated crosses were performed, eggs were collected, and lethality at the embryonic, larval, and pupal stages was scored. Flies designated here as wild type were *b pr*. The *Dub*/+ flies were *pr cn Dub*/*b pr*. 885 fertilized eggs were examined for cross A, 1468 eggs for cross B, 430 eggs for cross C, and 1016 eggs for cross D.

Pupal lethality produced by heterozygous mothers (Figure 1, cross D) was five-fold greater than the pupal lethality seen in a cross performed in the opposite direction (Figure 1, cross C). This increased lethality was likely due to aneuploidy resulting from meiotic nondisjunction of chromosome *4*. The frequency of nullo-*4* gametes was much higher in females than in males (20.2% relative to 1.3%). The haplo-*4* progeny that would result from such gametes are only rarely viable: many die during the pupal phase, and the rare survivors have a Minute phenotype.

To investigate the lethal phenotype of larval and pupal homozygotes, the dominant mutations *Tubby* and *Kugel*^{V} were used as larval markers for heterozygotes. The homozygous larvae were normal in size but were lethargic; they rarely wandered or pupated outside of the food. The larvae were missing some imaginal discs, and most discs were reduced in size. However, the brains appeared normal in size. The homozygous pupae showed a range of lethal phenotypes such as melanotic tumors, rough eyes, missing or duplicated bristles, and missing body parts (data not shown). We interpret these phenotypes as a result of random cell death.

To ask whether mitotic chromosome missegregation might be yielding aneuploid cells and consequent cell death, we examined larval neuroblast squashes from 10 *Dub* homozygotes. Surprisingly, these squashes did not have any apparent chromosome segregation defects, and aneuploidy was not observed in any of the metaphase figures.

The nature of the *Dub* mutation

We identified a deficiency that uncovers *Dub* in order to determine if the dominant phenotype was due to a haplo-insufficient locus or if the mutation was hypermorphic. *Df(2R)PC4* was semi-viable when heterozygous to *Dub*. Moreover, the cytological location of the deficiency is consistent with the map position of *Dub*.

Many of the deficiency transheterozygotes died during the pupal phase and frequently could only eclose halfway. Adult transheterozygotes that did escape from the pupal case showed phenotypes similar to *Dub* homozygous pupae and to rare adult escapers raised at 25°C. Their eyes had a rough appearance with facets often fused and disorganized overall. The tergites were often etched, and the wings were frequently nicked along the edges or were blistered. Both males and females were sterile. The increased viability of *Dub* heterozygotes relative to *Dub* hemizygotes suggested that the mutation is not hypermorphic, at least with regard to the lethal phenotype.

We examined whether the locus is haplo-insufficient for the meiotic phenotype by mating females heterozygous for the *Df(2R)PC4* deficiency with males carrying the compound-*XY*. This test yielded no exceptional progeny, although approximately 850 progeny were scored (data not shown). Therefore it does not appear that the locus is haplo-insufficient for meiotic chromosome segregation. The mutation is most likely to be either antimorphic or neomorphic.

Discussion

The *Dub* mutation

The dominant *Dub* mutation is the first mutation isolated in *Drosophila melanogaster* that affects the three known pathways of homolog segregation in meiosis I. Both nonexchange and exchange chromosomes in females undergo nondisjunction in *Dub* mutant females, and segregation of homologs is aberrant in mutant males. The segregation of all four chromosomes is disrupted in *Dub* mutant females and males.

Four results demonstrate that *Dub* causes nondisjunction of nonexchange chromosomes in females: 1) the achiasmate chromosome *4* undergoes nondisjunction at high frequencies in females; 2) diplo-*X* ova from *Dub* females show an increased percentage of nonexchange tetrads compared to normal, mono-*X* ova, indicating that nonexchange chromosomes are more likely to nondisjoin in the *Dub* mutant; 3) the segregation of compound-*X* chromosomes from a *Y* chromosome is affected by the *Dub* mutation, a segregation previously shown to be mediated by the distributive system (Grell 1976); and 4) nondisjunction frequencies for the *X* chromosome increase dramatically when it is made nonexchange by making it heterozygous with a balancer chromosome. The fact that both the segregation of chromosome *4* and the disjunction of a compound *X* from a *Y* chromosome are altered indicates that both the homologous and heterologous systems of achiasmate segregation are disrupted by the *Dub* mutation.

Although *Dub* predominantly affects nonexchange chromosomes, it also results in nondisjunction of exchange chromosomes. *Dub* reduces recombination frequencies only slightly, so the frequency of *X* chromosome nondisjunction (16-18%) in the female is too high to be the consequence of failure of only nonexchange chromosomes to segregate. In addition, in diplo-*X* exceptional ova, 49% of the tetrads had one or more exchange.

Dub mutant males also exhibit nondisjunction. The frequencies of nondisjunction in the male are considerably less than in the female. As discussed below, the interpretation of this difference depends on whether the *Dub* mutation is antimorphic or neomorphic. If the mutation is antimorphic, the requirement of the gene product in male meiosis may be lower than in female meiosis, or redundant functions may exist in the male. If the allele is neomorphic, it may not interfere with meiosis in the male to as great an extent as in the female.

Dub differs from mutations in the *ord* and *mei-S332* genes, which also cause nondisjunction in both sexes, in that *Dub* causes nondisjunction in meiosis I almost exclusively. In *ord* mutants, nondisjunction occurs in both meiosis I and II in a ratio suggesting that the four sister chromatids of the bivalent separate prematurely and then segregate randomly through two divisions (Mason 1976; Miyazaki and Orr-Weaver 1992). Indeed, precocious sister-chromatid separation is observed as early as prometaphase I in *ord* mutants (Miyazaki and Orr-Weaver 1992). In contrast, *mei-S332* mutations result primarily in meiosis II nondisjunction (Kerrebrock et al. 1992). Although the sister-chromatids also prematurely disjoin in *mei-S332* mutants, the sister-chromatids do not separate until late in anaphase I (Kerrebrock *et al.* 1992). Thus the *ord* and *mei-S332* genes control the behavior of sister chromatids, whereas the *Dub* mutation causes aberrant segregation of the homologs.

The *Dub* mutation is conditional lethal when homozygous. The homozygous larvae and pupae exhibit phenotypes indicative of extensive cell death such as small or missing imaginal discs, melanotic tumors, rough eyes, etched tergites, and missing bristles. This suggests that when homozygous the *Dub* mutation affects mitotic chromosome segregation. We observed gynandromorphs in the progeny of *Dub* mutant females, consistent with abnormal mitotic chromosome segregation. However, abnormal mitotic figures were not found in neuroblast squashes from homozygous *Dub* larvae at a frequency that could account for the observed cell death. One possibility is that *Dub* affects mitosis in tissues other than the brain. This is consistent with our observation that while the imaginal discs are small or missing in homozygous *Dub* larvae, the brain appears normal in size. An alternative possibility is that the homozygous mutation affects other cell processes in such a manner that results in cell death.

Comparison of *Dub* with other mutations affecting nonexchange chromosomes

Since few Drosophila mutations have been identified that cause nondisjunction of nonexchange chromosomes in the female, the relationship between *Dub* and these genes is of particular interest. Five previously characterized mutations affect achiasmate chromosomes: *ald*, *Axs*, *mei-S51*, *nod*, and *ncd*. *Dub* is most similar to *nod* and *ncd* in its phenotypes.

The *ald*, *Axs*, and *mei-S51* mutants differ from *Dub* in that in a background of normal *X* chromosomes they have low frequencies of chromosome *4* missegregation.

Furthermore, segregation of a compound-*X* chromosome from a *Y* chromosome is more faithful in *ald* and *Axs* than in *Dub* mutants. *ald*, *Axs*, and *mei-S51* show nonhomologous disjunction of the *X* chromosomes from the fourth chromosomes, in contrast to *Dub* (O'Tousa 1982; Robbins 1971; Zitron and Hawley 1989).

Dub is similar to *nod* and *ncd* in showing high chromosome *4* nondisjunction and cosegregation of nondisjoined *X* and fourth chromosomes to the same pole (Davis 1969; Zhang and Hawley 1990). However, there is considerably less loss of chromosome *4* in *Dub* mutants than in *nod* or *ncd*. In terms of its effect on exchange and nonexchange chromosomes, *Dub* can be viewed as being intermediate between *nod* and *ncd*. *nod* causes almost exclusively nonexchange chromosomes to nondisjoin, whereas exchange chromosomes will nondisjoin in *Dub* mutants. *ncd* does not affect nonexchange chromosomes to as great an extent as does *Dub*. *Dub*, *nod*, and *ncd* all produce gynandromorph progeny.

It is interesting that both the *nod* and *ncd* genes encode proteins with homology to the kinesin microtubule motor, and the Ncd protein has been shown to have motor activity *in vitro* (McDonald and Goldstein 1990; McDonald, Stewart, and Goldstein 1990; Walker, Salmon, and Endow 1990; Zhang *et al.* 1990). Aberrant meiotic spindles are present in *nod* and *ncd* mutant oocytes (Hatsumi and Endow 1992; Theurkauf and Hawley 1992). Achiasmate chromosomes are not confined to the spindle in *nod* mutants, while in *ncd* oocytes the spindle structure itself is abnormal. The ends of the spindle do not taper to the pole, suggesting that the Ncd protein may act to bundle microtubules into a functional spindle. The similarities among the phenotypes of *Dub*, *nod*, and *ncd* in females, particularly the cosegregation of nondisjoined chromosomes that occurs in these mutants, raise the possibility that the meiotic spindle is defective in *Dub* mutants as well.

Possible functions of the *Dub* gene in chromosome segregation

The phenotypes of the *Dub* mutation support a role for the gene in an aspect of meiotic chromosome segregation common to female and male meiosis. However, the mutation we have characterized is a dominant allele that may be antimorphic or neomorphic. If *Dub* were antimorphic, its phenotype would be similar to loss-of-function alleles and would reflect the function of the wild-type gene. Antimorphic and neomorphic alleles can be distinguished by the properties of the mutation in the presence of a duplication of the wild-type gene, but unfortunately a duplication covering *Dub* does not exist.

Three other dominant meiotic mutations have been identified in Drosophila, and these provide a precedent in the sense that the alleles have either been shown to be antimorphic or to have meiotic phenotypes similar to loss-of-function alleles. The initial allele of *Axs* was dominant, while $l(1)TW6^{cs}$ was shown to be a dominant mutation in *nod* (now called nod^{DTW}). Revertants of these mutations were isolated and demonstrated to be loss-of-function mutations in the genes (Rasooly *et al.* 1991, Whyte *et al.* 1993). Analysis of the phenotypes of both the dominant and revertant alleles showed that in each case the dominant allele was antimorphic, and its phenotype provided an accurate indication of the role of the gene in meiosis. A third dominant mutation is an allele of *ncd* that initially was dominant but has lost its dominance in the time since its isolation (Komma, Horne, and Endow 1991). Nevertheless, homozygotes for this allele showed the same meiotic effects as loss-of-function alleles.

It is possible that the *Dub* gene regulates a fundamental aspect of homolog separation or spindle function that is used in the segregation of all classes of homologs in female meiosis and also in male meiosis. Since the dominant *Dub* mutation has essentially no effect on meiosis II, it may control properties that are unique to the first meiotic division. Alternatively, redundant functions may exist in meiosis II, or the amount of wild-type *Dub* product required for meiosis II may be lower than that needed for meiosis I.

The other possibility is that the wild-type *Dub* gene controls only one pathway of homolog segregation, and the dominant allele may interfere with segregation systems normally not controlled by the gene. Analogously, as a homozygote or a hemizygote nod^{DTW} affects mitotic chromosome segregation, even though loss-of-function alleles of *nod* affect only the segregation of nonexchange chromosomes in females (Rasooly *et al.* 1991). In addition, the dominant allele in higher dosage or at nonpermissive temperature will affect exchange chromosomes.

Loss-of-function mutations in the *Dub* gene, which can be obtained by reverting the dominant mutation, will reveal whether the wild-type gene is required in all pathways of meiotic chromosome segregation. These mutations will also permit possible functions of the gene in mitosis to be evaluated. Regardless of whether the dominant *Dub* mutation is antimorphic or neomorphic, understanding the manner in which it disrupts meiotic segregation will provide important insights into the mechanism of chromosome segregation in Drosophila meiosis.

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Chapter Six

A second look at *Double or nothing*

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I was assisted in carrying out the screen in Figure 9 by Jen Hahn, Andy Katz and Heidi LeBlanc. Jacqueline Lopez did the in situ hybridizations for the putative alleles isolated from the screen.

Introduction

In mitosis, the newly replicated sister chromosomes are partitioned. During meiosis, a single replication of the chromosomes is followed by two rounds of cell division. Segregation of the homologous chromosomes occurs in the first division, segregation of the sister chromosomes in the second division. The first division of meiosis requires a specialized mechanism to segregate the chromosomes. Typically, organisms use recombination between the homologs and synaptonemal complex to ensure that the homologs are appropriately segregated (John 1990). Reciprocal crossovers are correlated with attachments between homologs called chiasmata that are part of the orientation process before metaphase (Bascom-Slack, Ross, and Dawson 1997). Alternatives to recombination exist, and these are well characterized in *Drosophila melanogaster*.

In *D. melanogaster* females, though crossovers between some of the homologs and synaptonemal complexes do exist, not all of the chromosomes experience an exchange event. The tiny fourth chromosome essentially never recombines and yet is partitioned faithfully. Heterozygosity for multiple inversions can completely suppress exchange between homologs, yet such homologs segregate from each other with great efficiency. Moreover, two heterologous chromosomes are partitioned if neither has an available homolog. Segregation of the nonexchange chromosomes in oocytes, called distributive segregation, has been divided into two systems (Hawley et al. 1993): (1) nonexchange homologous chromosomes are paired at their centromere regions during prophase I (Dernburg, Sedat, and Hawley 1996) and segregate from one another during anaphase I; and (2) available nonexchange heterologs are segregated on the basis of their size and shape. Mutations known to disrupt the segregation of nonexchange chromosomes in females also differentiate between nonexchange homologs and heterologs. *Aberrant X segregation* (*Axs*) and *mei-S51* specifically increase the nondisjunction frequency of chromosomes with homologous centromere regions but segregation of heterologous chromosomes is unperturbed (Zitron and Hawley 1989; Whyte et al. 1993). *No distributive segregation* (*nod*) and *non-claret disjunctional* (*ncd*) disturb segregation of all nonexchange chromosomes during meiosis I (Carpenter 1973; Zhang and Hawley 1990; Davis 1969).

Nonexchange chromosomes in *Saccharomyces cerevisiae* meiotic cells have also been shown to segregate appropriately, suggesting that a similar system exists in yeast (Dawson, Murray, and Szostak 1986; Guacci and Kaback 1991; Sears, Hegemann, and Hieter 1992). However, some of the rules for segregation of

heterologous chromosomes deduced in Drosophila do not apply in *S. cerevisiae* (Ross et al. 1996).

In Drosophila males, exchange normally never occurs (Baker and Hall 1976) and synaptonemal complex is not observed (Meyer 1960; Rasmussen 1973). The *X* and *Y* chromosomes depend on a small homologous region, a site within the rDNA, to pair and segregate (McKee, Habera, and Vrana 1992; McKee 1996). A thin stretched structure, the collochore, has been seen between these chromosomes at metaphase (Cooper 1964). Some pairing sites have also been identified on the other homologs (McKee, Lumsden, and Das 1993). Mutations that specifically disturb segregation of nonexchange homologs in females have no effect on meiotic chromosome segregation in males, thus the achiasmate system of segregating homologs in males is not identical to that found in females.

Two mutations, *ord* and *mei-S332*, disrupt meiotic chromosome segregation in both males and females and have uniquely high degrees of missegregation during the second meiotic division (Mason 1976; Miyazaki and Orr-Weaver 1992; Davis 1971; Kerrebrock et al. 1992; Bickel and Orr-Weaver 1996). These genes are essential for sister-chromatid cohesion. Strikingly, only one mutation is known to primarily cause missegregation in meiosis I in both males and females.

Double or nothing (*Dub*) is a conditional dominant mutation disrupting meiotic segregation of all four chromosomes in both male and female Drosophila (Moore et al. 1994). Missegregation is specific to the first division in females and primarily occurs in meiosis I in males. In females, both exchange and nonexchange chromosomes are affected, but segregation of nonexchange *X* chromosomes in females is essentially random while exchange chromosomes are affected to a lesser extent.

Dub is most similar to *ncd* and nod^{DTW}, a dominant allele of *nod* (Wright 1974; Rasooly et al. 1991). Mutations in these genes disrupt segregation of nonexchange and exchange chromosomes during meiosis I in females, although neither has any effect on meiotic segregation in males. Both have been cloned and their sequences predict that they are members of the kinesin family (Zhang et al. 1990; McDonald and Goldstein 1990). In vitro experiments with NCD have demonstrated that it is a minus-end directed kinesin (McDonald, Stewart, and Goldstein 1990; Walker, Salmon, and Endow 1990).

Dub homozyotes have developmental defects that are suggestive of mitotic missegregation, but examination of neural tissue did not reveal any obvious aneuploidy or unusual anaphase figures. At its restrictive temperature, nod^{DTW} has

developmental defects like those of *Dub* homozygotes, but it clearly disruptssegregation during mitosis (Wright 1974; Rasooly et al. 1991).

In this paper, we examined meiotic cells of parents heterozygous for *Dub*, using MEI-S332-GFP to label the centromere regions of the chromosomes. The nature of the dominant mutation was further investigated by creating a duplication of the region, and *Dub* was mapped using available deficiencies. Finally, two screens for loss-of-function alleles of *Dub* were conducted.

Materials and Methods

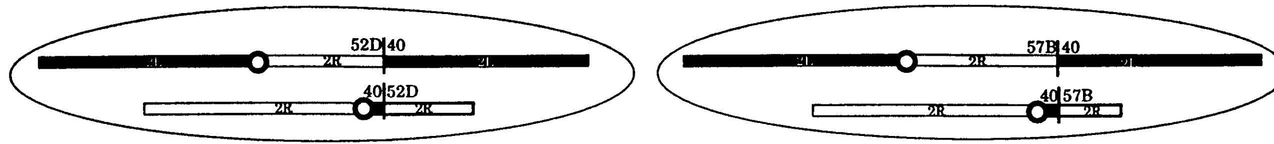
Stocks

All Drosophila stocks and crosses were grown at 25°C on a standard mix of cornmeal, brewer's yeast, molasses and agar. All balancer chromosomes and all mutations are described in (Lindsley and Zimm 1992). *C(1)RM*, y^2 $su(w^a)$ w^a will be referred to in this paper as compound-*X* or $\hat{XX}$. $Y^SX \bullet Y^L$ y^+, *In(1)EN*, *y v f B* was used as the compound-*XY* chromosome and is referred to as $\hat{XY}$ in this paper. These compound chromosomes are described in Kerrebrock et al. (1992). The *CyO DTS-100* chromosome, the *y w* chromosome with multiple P[w^+] elements, the *Sb* P[*ry*+ Δ2-3](99B) tranposase-producing chromosome, and the deficiency *Df(2R)PC4* were obtained from R. Lehmann. *Df(2R)Pcl-XM82* was obtained from S. Bray. *Df(2R)P34* and *Df(2R)P66* were obtained from P. Cherbas. *Df(2R)Pcl7B* and *Df(2R)Pcl11B* were obtained from the Bloomington stock center. The inversion *In(2R)Pcl-W4* was obtained from T. Rizki. *Df(2R)Pcl-W5* was obtained from W. Doane. The autosynaptic stocks $LS(2)Rev^B$, *dp b*, *cn*//$DS(2)$ Rev^B, *bw*, *cn* and $LS(2)Pu^{Ly}$// $DS(2)Pu^{Ly}$ were obtained from B. Reed.

Making a duplication covering region 54 to 56

The duplication was created from the autosynaptic stocks $LS(2)Rev^B$, *dp b*, *cn*//$DS(2)$ Rev^B, *bw*, *cn* and $LS(2)Pu^{Ly}$// $DS(2)Pu^{Ly}$. When these stocks are crossed, the only viable progeny are those carrying $LS(2)Pu^{Ly}$//$DS(2)$ Rev^B (Figure 1A). This autosynaptic stock was briefly established, and virgin females were crossed to y/y^+Y; *cn bw sp* males to select for exchange events that yielded second chromosomes no longer autosynaptic (Figure 1B). Single cinnabar brown male progeny from this cross were crossed to *y*; *Sco/SM1* virgin females, and the resulting speck$^+$ Curly males from this cross were again crossed to *y*; *Sco/SM1* virgin females. Ultimately, the stock is $y/y{+}Y$; $In(2LR)Pu^{Ly^L}Rev^{B^R}$, *cn bw/SM1*, in which the paracentric inversion carries a duplication, *Dp(2R)52D5*; *57C4-6*, and potentially an undefined small deficiency or duplication in region 40, near the centric heterochromatin. Acetic acid-orcein squashes of salivary glands confirmed the presence of the duplication and paracentric inversion (Figure 2).

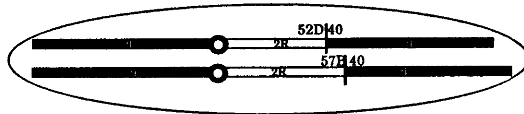
A



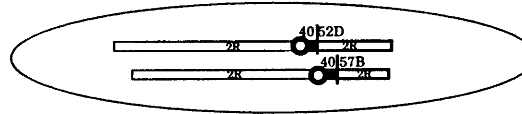
LS(2)RevB, dp b, cn // DS(2)RevB, bw, cn X *LS(2)PuLy // DS(2)PuLy*

$LS(2)Rev^{B}$, *dp b, cn* // $DS(2)Rev^{B}$, *bw, cn* X $LS(2)Pu^{Ly}$ // $DS(2)Pu^{Ly}$

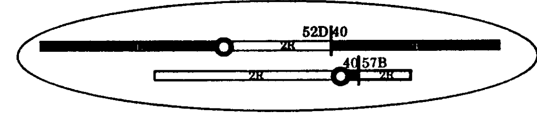
INVIABLE PROGENY



$LS(2)Rev^{B}$, *dp b, cn* // $LS(2)Pu^{L}$

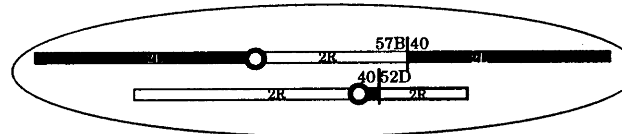


$DS(2)Rev^{B}$, *bw, cn* // $DS(2)Pu^{Ly}$



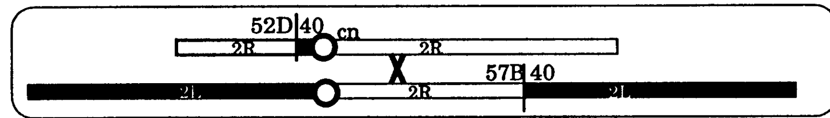
$LS(2)Rev^{B}$, *dp b, cn* // $DS(2)Pu^{Ly}$

VIABLE PROGENY

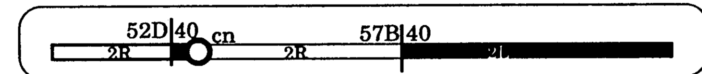


$LS(2)Pu^{Ly}$ // $DS(2)Rev^{B}$, *bw, cn*

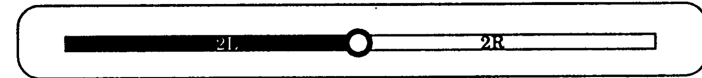
B



$LS(2)Pu^{Ly}$ // $DS(2)Rev^{B}$, *bw, cn*



$In(2LR)Pu^{Ly^{L}}Rev^{B^{R}}$, *cn bw*



bw

Figure 1: (A) Selecting for duplication of region 52D to 57B by crossing two autosynaptic stocks. The autosynaptic stock, $LS(2)Pu^{Ly}//DS(2)Rev^{B}$, is the only viable product of a cross between $LS(2)Rev^{B}$, *dp b, cn*//$DS(2)Rev^{B}$ and $LS(2)Pu^{Ly}//DS(2)Pu^{Ly}$. The other products are deficient for large portions of their genome and are inviable. (B) The paracentric inversion, $In(2LR)Pu^{Ly^{L}}Rev^{B^{R}}$, carries a duplication for regions 52D to 57B and is the product of a crossover on the right arm of chromosome *2* in females that are $LS(2)Pu^{Ly}//DS(2)Rev^{B}$. The inversion is marked with *cn* and *bw* while the normal chromosome *2* produced by this crossover is *bw*.

The *In(2LR)* chromosome does not transmit well through males, likely because the Pu^{Ly} inversion was γ-ray induced on a segregation distorter chromosome, the SD-Roma chromosome (Lindsley and Zimm 1992). This should not disturb the nondisjunction assays in this paper, since we do not measure nondisjunction of the second chromosome at any time.

Nondisjunction assays

For the male tests with results listed in Tables 1, 3 and 5, compound-*X*, y^2 $su(w^a)$ w^a females were mated with y/y^+Y males. Normal sperm yielded yellow+ females ($\hat{XX}/y^+Y$) and yellow males (*X*/O). Exceptional sperm yielded yellow or yellow² females (*X/X* and $\hat{XX}$/O) and yellow+ males (X/y^+Y). The females resulting from sperm carrying two sister chromosomes (*X/X*) were yellow and had a wild-type eye color, whereas exceptional females resulting from nullo-*XY* sperm ($\hat{XX}$/O) were yellow² and had a darker eye color with no pseudo-pupil.

The female tests in Tables 2, 4 and 6 were the result of compound-*XY*, *v f B* males crossed to *y/y* females. Normal ova yielded Bar females ($\hat{XY}/X$) and males wild-type for Bar (*X*/O). Exceptional-*X* ova yielded Bar males ($\hat{XY}$/ O) and females wild-type for Bar (*X/X*). The number of exceptional progeny was doubled for the adjusted total and for calculation of the nondisjunction frequencies.

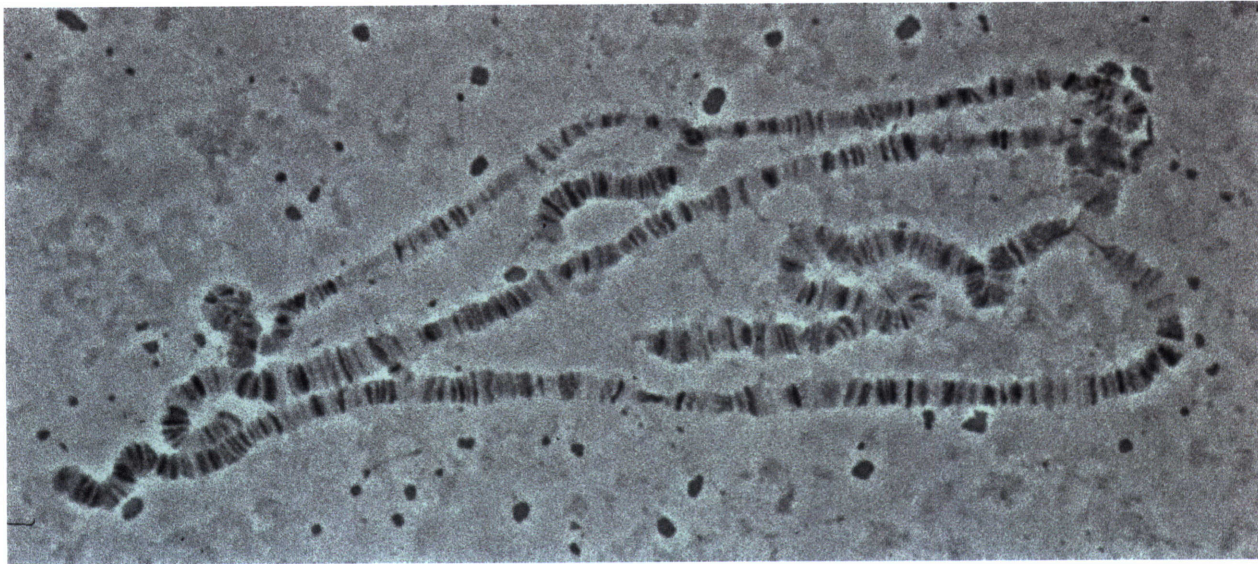
Mutagenesis with ethyl methanesulfonate

Males were mutagenized by feeding overnight on paper tissues saturated with 35mM ethyl methanesulfonate (EMS) in 1% sucrose solution. The protocol is described in Lewis and Bacher (1968).

Cytology

Salivary glands were dissected from larvae in 45% acetic acid and then transferred to 0.1% orcein in 45% acetic acid for 15 minutes. The glands were moved to a drop of 45% acetic acid on a slide, squashed under a cover slip and sealed with nail polish. These were examined by phase-contrast microscopy using a Zeiss Axiophot equipped with Plan Neofluar 100x and Plan Apochromat 63x objectives.

Testes were mounted as described in Kerrebrock et al. (1995) except that they were stained in 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) in 1 x PBS for 10



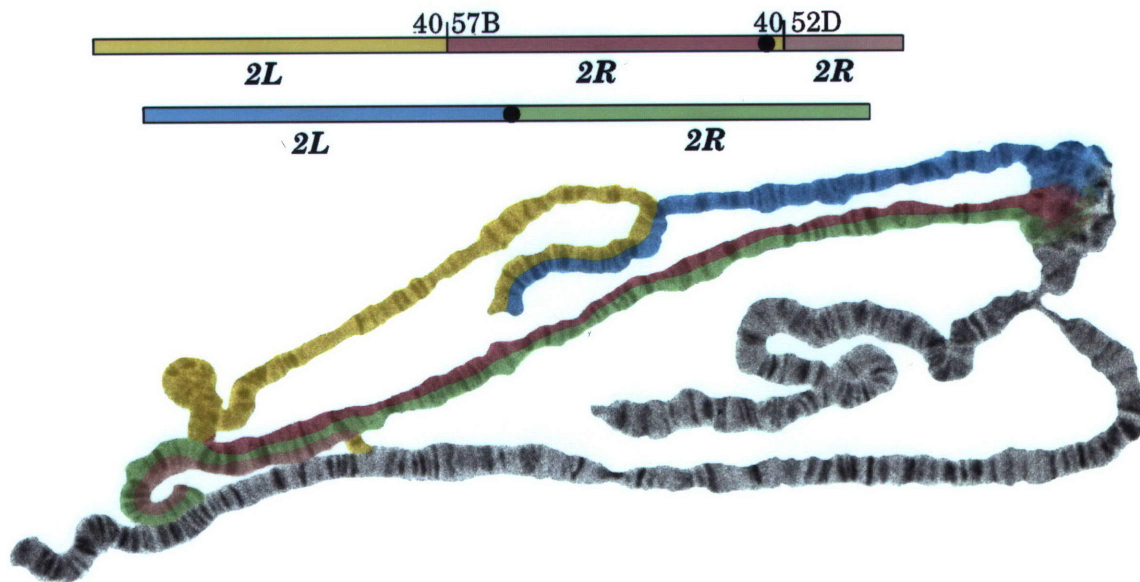


Figure 2: Heterozygous $In(2LR)Pu^{Ly^L}Rev^{B^R}$ chromosome in an acetic acid-orcein squash of a larval salivary gland. The chromosomes in the lower diagram are overlaid with color to match the colored chromosome arms in the schematic diagram of the paracentric inversion. The duplicated region of *2R* is visibly thicker where it is present in three copies. One arm, a small region of *2L*, has torn loose from the centromeric heterochromatin. Most of *2L* arm has divided between the homologs in this figure.

minutes, followed by two 15 minute rinses in 1 x PBS. Ovaries were dissected from adult females and fixed in the same way testes were in Kerrebrock et al. (1995). Epiflourescence microscopy was performed using a Nikon Optiphot-2 microscope equipped with a Nikon 60x oil objective. Either a Photometrics Image Point or a Photometrics CE200A cooled CCD video camera was used to photograph the images, and Adobe Photoshop 3.0 run on a Power Macintosh 8100/80 was used to process the images.

Pupae and eyes and wings of adults were photographed using a Nikon camera mounted on a Zeiss STEMI SR dissecting microscope. Scanning electron microscopy was performed at the MIT electron microscope facility.

Results

Dub disrupts organization of the metaphase I karyosome in oocytes

Missegregation during meiosis I is the primary phenotype of *Dub*, so we examined oocytes from *Dub* heterozygotes for defects during metaphase I. Missegregation of chromosomes during anaphase I cannot be assessed by examining oocytes because they arrest during metaphase I. Defects leading to missegregation might be recognized at the arrest stage.

The metaphase I nucleus of wild-type stage 14 oocyte is a condensed ball of chromosomes, the karyosome, with a bipolar spindle organized by the chromatin (Therkauf and Hawley 1992; see Fig. 1 of Chapter Three, this thesis). Individual chromosomes usually cannot be discerned in these cells, although during metaphase I, two small chromosome *4* dyads sometimes can be seen outside the karyosome on either end of the spindle. Stage 14 oocytes from *nod* mothers often have chromatin well outside of the karyosome, and nonexchange chromosomes, such as chromosome *4*, are frequently lost from the spindle. Because *Dub* primarily disrupts segregation of nonexchange chromosomes, oocytes were examined for such a phenotype.

The *Dub* oocytes that were examined also expressed MEI-S332-GFP, primarily to aid in identifying small chromatids outside of the karyosome but also as an indication of organization within the karyosome. In stage 14 oocytes, MEI-S332-GFP is localized to "caps" at the polar ends of the chromatin mass (Chapter Three, this thesis), presumably because the centromere regions of the bivalents are at the polar ends while the chromosome arms are in the middle of the karyosome. Aneuploidy in progeny of *Dub* parents is usually due to missegregation during the first meiotic division and not the second division (Moore et. al. 1994), so the successful localization of MEI-S332 to centromere regions would be expected to occur. Meiotic cells from GrM13; *Dub*/+ males were examined to determine if *Dub* disrupts localization of MEI-S332-GFP to centromere regions of meiotic chromosomes in the testes. Prometaphase I figures in *Dub*/+ spermatocytes had MEI-S332-GFP localized to the chromosomes (Fig. 3A), and in late anaphase I figures, MEI-S332-GFP appeared on the leading edge of the chromosomes (Figures 3B-C). The *Dub* mutation had no dominant effect on localization of MEI-S332-GFP to the centromere regions of condensed chromosomes in spermatocytes. Since there was no nondisjunction during meiosis II in *Dub*/+ females, MEI-S332-GFP would be expected to be localized in oocytes to the same region of the chromosomes, the centromere regions.

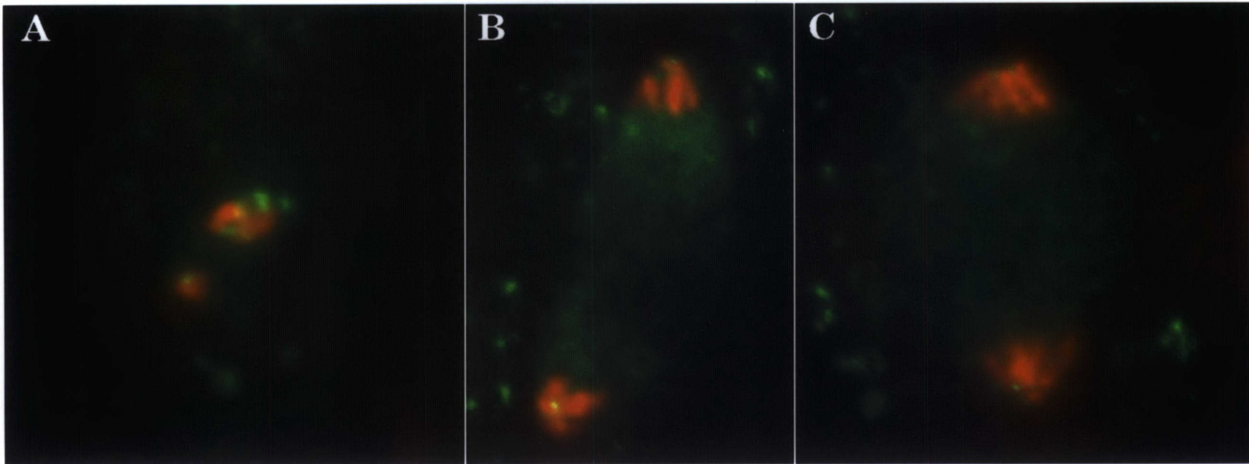


Figure 3: Spermatocytes of *Dub* heterozygotes. DNA in red, MEI-S332-GFP in green. (A) MEI-S332-GFP was localized to the condensed chromosomes in prometaphase I spermatocytes. (B-C) Late anaphase I cells with foci of MEI-S332-GFP visible on the leading edges of the chromosomes, the centromere regions.

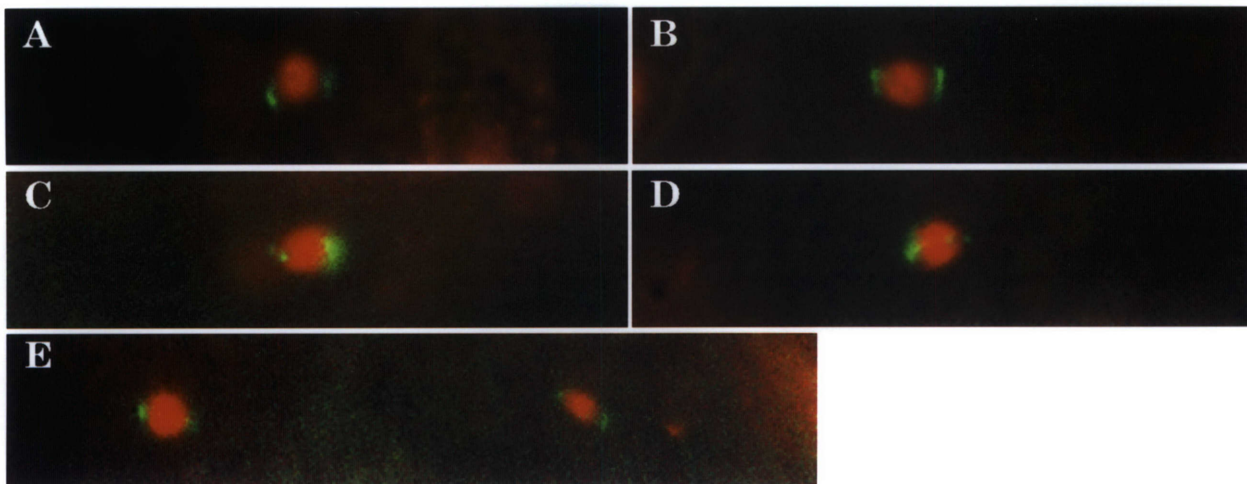


Figure 4: Five examples of karyosomes in stage 14 oocytes from *Dub* heterozygotes. DNA in red, MEI-S332-GFP in green. (A and B) MEI-S332-GFP had the same distribution in these nuclei as seen in wild-type nuclei. (C and D) MEI-S332-GFP was asymmetrically distributed in many of the karyosomes. (E) Smaller chromatin blobs were sometimes observed outside of karyosome. MEI-S332-GFP was observed on the chromatin blobs as well as the karyosome.

Many of the *Dub*/+ oocyte nuclei were normal in appearance (Figure 4A and B), but approximately half clearly had an asymmetric distribution of MEI-S332-GFP (Figure 4C and D). A smaller number of oocytes were found to have chromatin outside of the main chromatin mass (Figure 4E). MEI-S332-GFP was observed in caps at either end of the isolated chromatin.

The unusual distribution of MEI-S332 in the karyosomes of *Dub* heterozygotes provides evidence that they are disorganized before the onset of anaphase I. If MEI-S332-GFP was localized to the centromere regions, the asymmetrical distribution indicates that the normal orientation of bivalents was upset in some *Dub* oocytes.

The nature of the *Dub* mutation's dominant meiotic phenotype

To provide insights into the normal function of the gene, we carried out further investigations of *Dub*'s dominant meiotic phenotype. The *Dub* mutation is not a loss-of-function allele and is not haploinsufficient, since a heterozygous deletion of the region where *Dub* is mapped does not cause nondisjunction (Moore et al. 1994). A dominant gain-of-function mutation can be classed as either a neomorph or an antimorph. A neomorph is a mutation such that the gene product is expressed inappropriately or carries out a new inappropriate function. An antimorph acts by antagonizing the normal function of the gene product, and the phenotype often can be reduced in severity by the addition of normal copies of the gene. Nondisjunction frequency is assayed quantitatively, so introducing a duplication of the region, and hence a second copy of the normal *Dub* locus, should test whether the dominant mutation is an antimorph. If the dominant mutation is due to overexpression of the gene product, the duplication alone might give a phenotype due to the presence of three doses of the wild-type locus.

Recombination between two paracentric inversions on the second chromosome, $In(2LR)Pu^{Ly}$ and $In(2LR)Rev^{B}$, yields a new paracentric inversion, $In(2LR)Pu^{Ly^L}Rev^{B^R}$, with a duplication for the region from 52D5 to 57B13-14 (see Figure 2). Autosynaptic stocks of the paracentric inversions were available, and they were used to select for the chromosome carrying the duplication (see Materials and Methods).

TABLE 1
A duplication of the *Dub* locus does not decrease the frequency of dominant meiotic nondisjunction in males

Sperm type	Paternal genotype $\frac{Dp}{+}$	$\frac{Dub}{+}$	$\frac{Dub}{Dp}$
Regular sperm			
X or *Y* [a]	443	286	419
XY nondisjunctional sperm			
0	0	3	15
X/Y	1	7	20
X/X	1	0	0
Total progeny scored	445	296	454
% nullo-*XY*	0	1.0	3.3
% *X/Y*	0.25	2.3	4.4
% diplo-*X*	0.25	0	0
Total % nondisjunction observed	0.5	3.3	7.7

$y/y^{+}Y$ males of the indicated genotype were mated with compound-*X*, y^{2} $su(w^{a})$ w^{a} females. The + chromosome was actually *SM1* in the above tests. The *Dp* is a paracentric inversion, $In(2LR)Pu^{Ly}y^{L}Rev B^{R}$, *cn bw*, with breakpoints such that the chromosome carries Dp(2*R*)52D5; 57C4-6.

[a] In this assay, *Y/Y* exceptional sperm were indistinguishable from regular mono-*Y* sperm and are therefore included in these numbers.

In males, the duplication alone did not increase the frequency of nondisjunction, nor did the duplication reduce the *Dub* nondisjunction frequency (Table 1). Indeed, the frequency was double that of the control, although it was only slightly higher than the frequency seen in tests with larger numbers of progeny, 5.1% (Moore et al. 1994). Surprisingly small numbers of progeny were obtained in these tests, likely because fertility was reduced by the chromosome carrying the duplication. $In(2LR)Pu^{Ly}y^{L}Rev B^{R}$ was not transmitted well in males, and $In(2LR)Pu^{Ly}$ originated from a segregation distorter chromosome.

The duplication alone also did not increase nondisjunction frequency in females, nor did the duplication suppress the dominant meiotic phenotype of *Dub* (Table 2). The nondisjunction frequency of these *Dub* females was significantly higher than what was reported in an earlier paper (Moore et al. 1994). *Dub* was recombined onto

TABLE 2
A duplication of the *Dub* locus does not decrease the frequency of dominant meiotic nondisjunction in females

Ova type	Maternal genotype		
	Dp/+	*Dub*/+ [a]	*Dub*/*Dp* [a]
Regular ova			
X	842	898	1249
X nondisjunctional ova			
0	0	78	111
X/*X*	2	148	162
Total progeny scored	842	1124	1522
Adjusted total scored	844	1350	1795
% nullo-*X*	0	11.6	12.4
% diplo-*X*	0.5	21.9	18.0
Total % nondisjunction observed	0.5	33.5	30.4

y females of the genotype indicated were mated to compound *XY*, *v f B* males at 25°C. The *Dub* chromosome is marked with *c* and *sp*. The + chromosome is actually a balancer chromosome, *SM1*. The *Dp* is a paracentric inversion, $In(2LR)Pu^{Ly^L}Rev^{B^R}$, *cn bw*, with breakpoints such that the chromosome carries Dp(2*R*)52D5; 57C4-6.

[a] These numbers are the sum of four smaller tests.

a chromosome with different markers, and suppressors of the dominant phenotype may have been recombined away.

Addition of a single extra copy of the *Dub* locus did not suppress the dominant meiotic phenotype. In neither sex did *Dub* meet this criterion for an antimorph, suggesting that *Dub* is a neomorph with regard to meiotic missegregation.

Dub homozygotes and hemizygotes have developmental abnormalities consistent with mitotic defects

Homozygous *Dub* is essentially lethal, and most homozygotes die during the pupal phase. Phenotypes were previously described briefly (Moore et al. 1994). The larvae were lethargic, often failing to climb out of their food to pupate. The inviable progeny ranged in phenotype from histolyzed tissue in a pupal case to pharate adults

with different severity of defects (Figure 5A). Frequently, heads were diminished in size or may never have everted, since eye pigment was found in the thorax of many pupae with tiny head structures. Pharate adults with more developed eyes had rough disorganized surfaces. Bristles were often missing or duplicated. Appendages were sometimes missing. Consistent with these defects, the rare escaper adult had rough eyes, nicked wings and etched tergites.

Dub hemizygotes had the same phenotypes, but their severity was distributed over a different range. Progeny carrying *Dub* over *Df(2R)Pcl-XM82* were semi-viable. The lethal phenotypes included pupae that were merely histolyzed tissue and, more commonly, pharate adults with phenotypes similar to those in *Dub* homozygous pharate adults. Surviving *Dub* hemizygotes had phenotypes like that of the rare *Dub* homozygous adult, although survival to adulthood was much more common and the mitotic defects were usually less severe. Eyes were rough (Figure 5B), and scanning electron micrographs of the *Dub/Df* eyes show fused ommatidia and bristle irregularities (Figure 6). The wings of *Dub/Df* adults had nicking and large bristles along the wing margin (Figure 7).

Although these phenotypes are consistent with a mitotic defect, acetic acid-orcein squashes of larval ganglia revealed no evidence of missegregation (Moore et al. 1994). Mitotic figures were normal in number and in appearance. Metaphase figures were examined closely for aneuploidy that might have resulted from earlier missegregation events. None were observed.

***Dub* is located in cytological region 54D-E**

Dub was previously reported to lie within *Df(2R)PC4* (Moore et al. 1994). An overlapping deficiency, *Df(2R)Pcl-XM82*, heterozygous with *Dub* also resulted in mitotic defects. Other overlapping deficiencies were used to localize *Dub* precisely within the deletion *Df(2R)PC4*, and all were found to complement the *Dub* mitotic defect (Figure 8). The published proximal breakpoint of *Df(2R)PC4* is 55A and the distal breakpoint is 55F (Jurgens 1985), and the deficiencies *Df(2R)Pcl-7B* and *Df(2R)Pcl-11B* have proximal breakpoints in 54E and 54F, respectively (Duncan 1982). This presented us with a paradox, since earlier mapping data placed *Dub* proximal to the recessive mutation *welt* (*wt*) (Moore et al. 1994), and *wt* has been mapped to 55C with *Df(2R)Pcl-11B* and *Df(2R)Pcl-W5* (Deng and Rizki 1988). Moreover, we found that *wt* is complemented by *Df(2R)Pcl-XM82*, providing further evidence that *Dub* must be proximal to the *wt* locus. Another locus, *grainyhead* (*grh*),

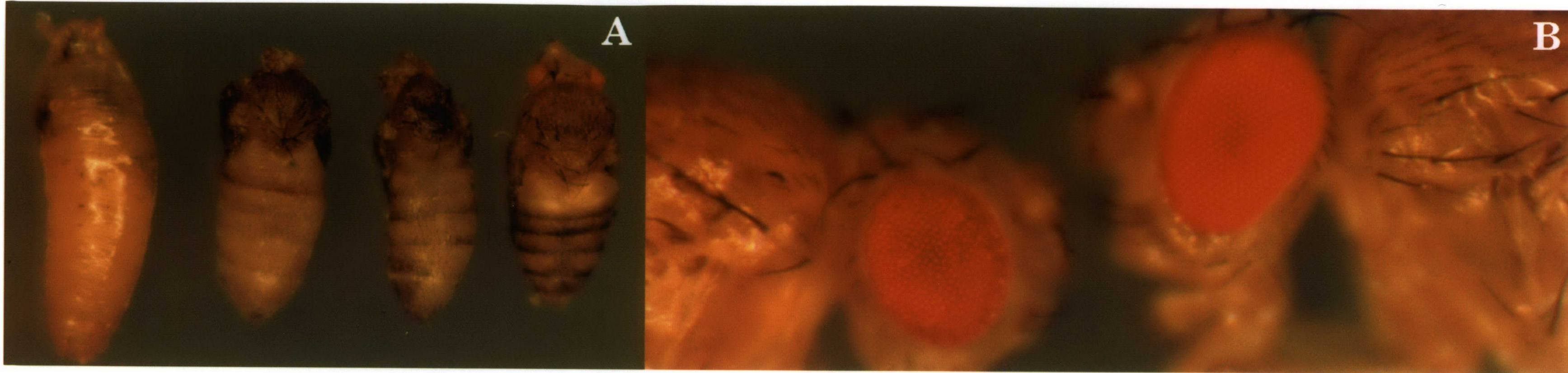
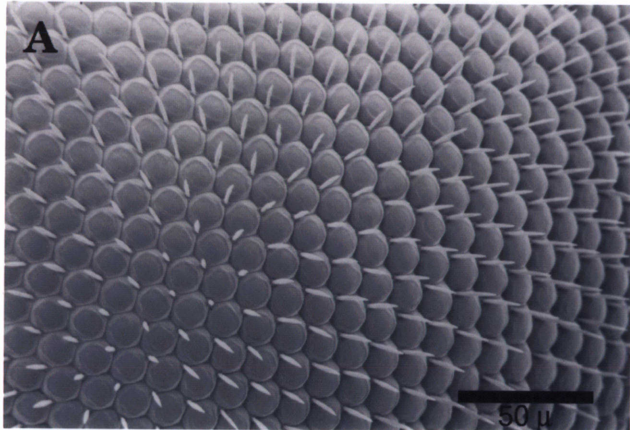
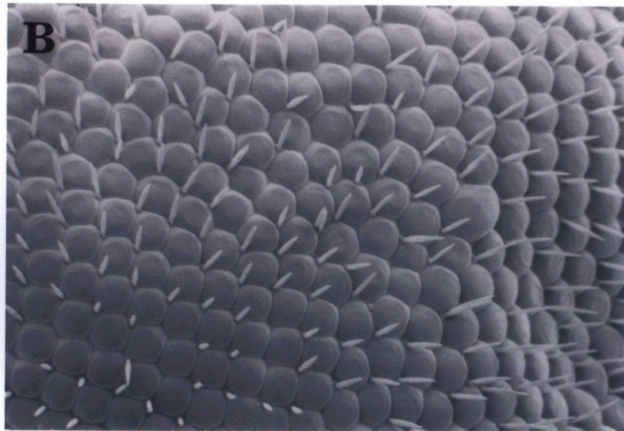


Figure 5: Phenotypes of *Dub* homozygotes and hemizygotes. (A) *Dub* homozygous pupae die with phenotypes ranging from a case of histolyzed tissue to pupae with diminished heads and missing appendages. (B) *Dub* hemizygote at left, and wild type fly at right. The *Dub* hemizygote has rough eyes and bristles in dissarray.





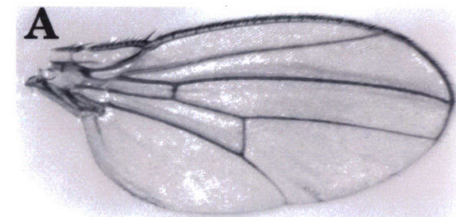


Figure 6: (located above) Scanning electron micrographs of eyes. (A) Wild-type eye has a regular array of ommatidia. (B) The *Dub* hemizygote eye has fused ommatidia in an irregular array, as well as missing and duplicated bristles.

Figure 7: (located at right) *Dub* hemizygotes have nicking along the wing edges. (A) Wild-type wing for comparison. (B and C) *Dub* hemizygote wings with nicking on the margins and bristles that are large and disorganized.

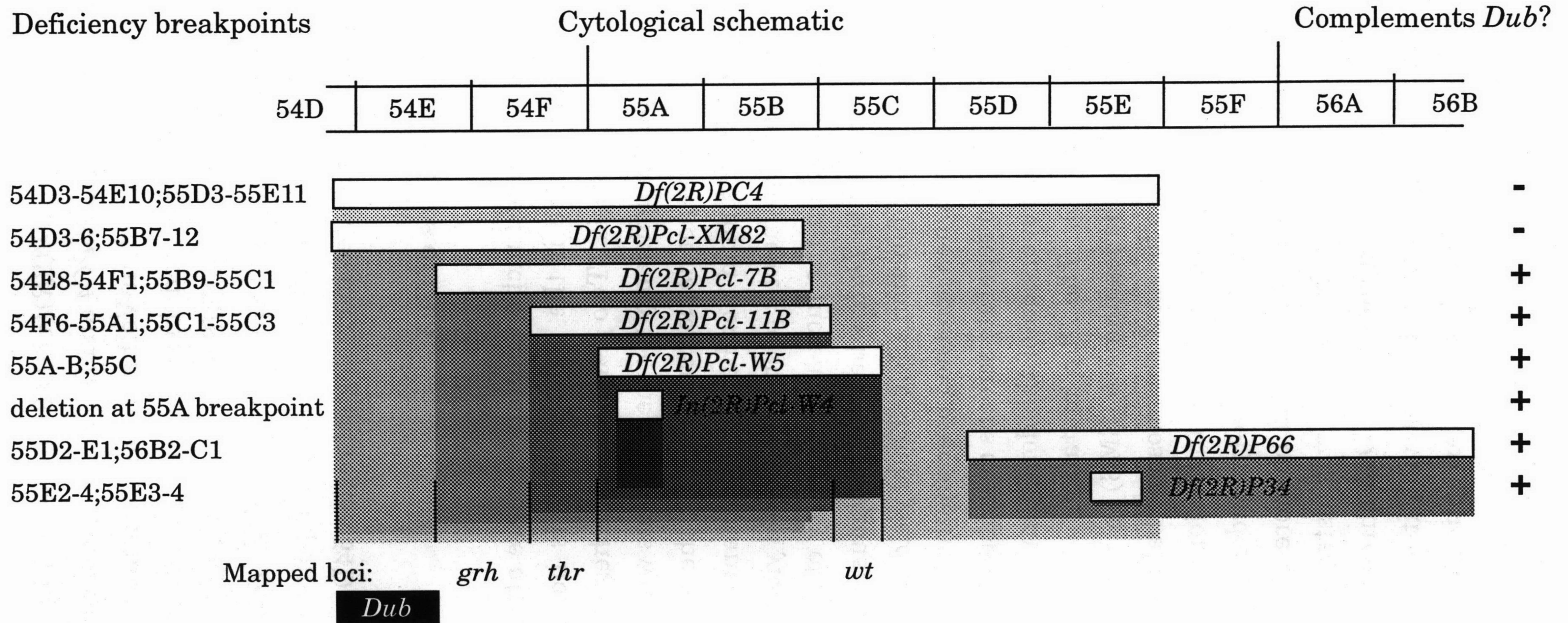


Figure 8: Cytological mapping of *Dub* using deletions. A set of deficiencies that delete portions of 54 and 55 were used to map the *Dub* locus by testing for complementation of *Dub* developmental defects. In the last column, complementation is indicated with a plus, failure to complement with a minus. Complementing deficiencies that overlap *Df(2R)Pcl-XM82* restricted *Dub* to the region 54D-E. Consistent with this cytological mapping, *Dub* was shown to lie proximal of *welt* (*wt*) by recombinational mapping, and *wt* was mapped to 55C with *Df(2R)Pcl-11B* and *Df(2R)Pcl-W5*.

is complemented by *Df(2R)Pcl-11B* but deleted by *Df(2R)Pcl-7B* (Bray and Kafatos 1991), and both *Df(2R)PC4* and *Df(2R)Pcl-XM82* were reported to fail to complement *grh* (Tearle and Nusslein-Volhard 1987; communication by R. Tearle to FlyBase). The previously reported breakpoints of *Df(2R)PC4* must be incorrect. The breakpoints of *Df(2R)Pcl-XM82* and of *Df(2R)PC4* were directly established by acetic acid-orcein squashes of larval salivary glands. *Df(2R)Pcl-XM82* has breakpoints at 54D3-6; 55B7-12. *Df(2R)PC4* has breakpoints at 54D3-54E10; 55D3-55E11. Therefore, *Dub* is located in cytological region 54D or 54E.

Putative new alleles of *Dub*

Dub is a gain-of-function mutation, so we attempted to isolate loss-of-function alleles to determine if the gene product is normally required for chromosome segregation in meiosis. Two general approaches were possible, given that we know the phenotype of a deficiency for the *Dub* locus with regard to two phenotypes. First, because the locus is not haploinsufficient for the dominant meiotic phenotype, a loss-of-function allele should not have a dominant meiotic phenotype. A screen for reversion of the *Dub* meiotic phenotype should yield loss-of-function alleles. A second approach is based on the increased viability of *Dub* when transheterozygous to a deletion compared to *Dub* when homozygous. Reversion of the dominant *Dub* mutation to a loss-of-function allele should yield semi-viable or even completely viable transheterozygotes when screened over another *Dub* chromosome. These strategies were employed in two separate screens described below.

The screen for reversion of the dominant meiotic nondisjunction phenotype is outlined in Figure 9. The *Dub* mutation was on a chromosome exposed to the mutagen ethyl methanesulfonate (EMS) in males, before being crossed into test females heterozygous for an *X* chromosome balancer. The heterozygous presence of an *X* chromosome with multiple inversions in a *Dub* female increases the nondisjunction frequency to essentially random segregation (Moore et al. 1994), so elimination of nondisjunction would be more obvious in females with a balancer. Nondisjunction of the *X* chromosome was tested in individual females that carried a potentially mutagenized copy of the *Dub* chromosome. If no progeny arising from nondisjunction were seen in the test vial, it was retested because a percentage of vials was expected to not have any exceptional progeny even when *Dub* was not reverted.

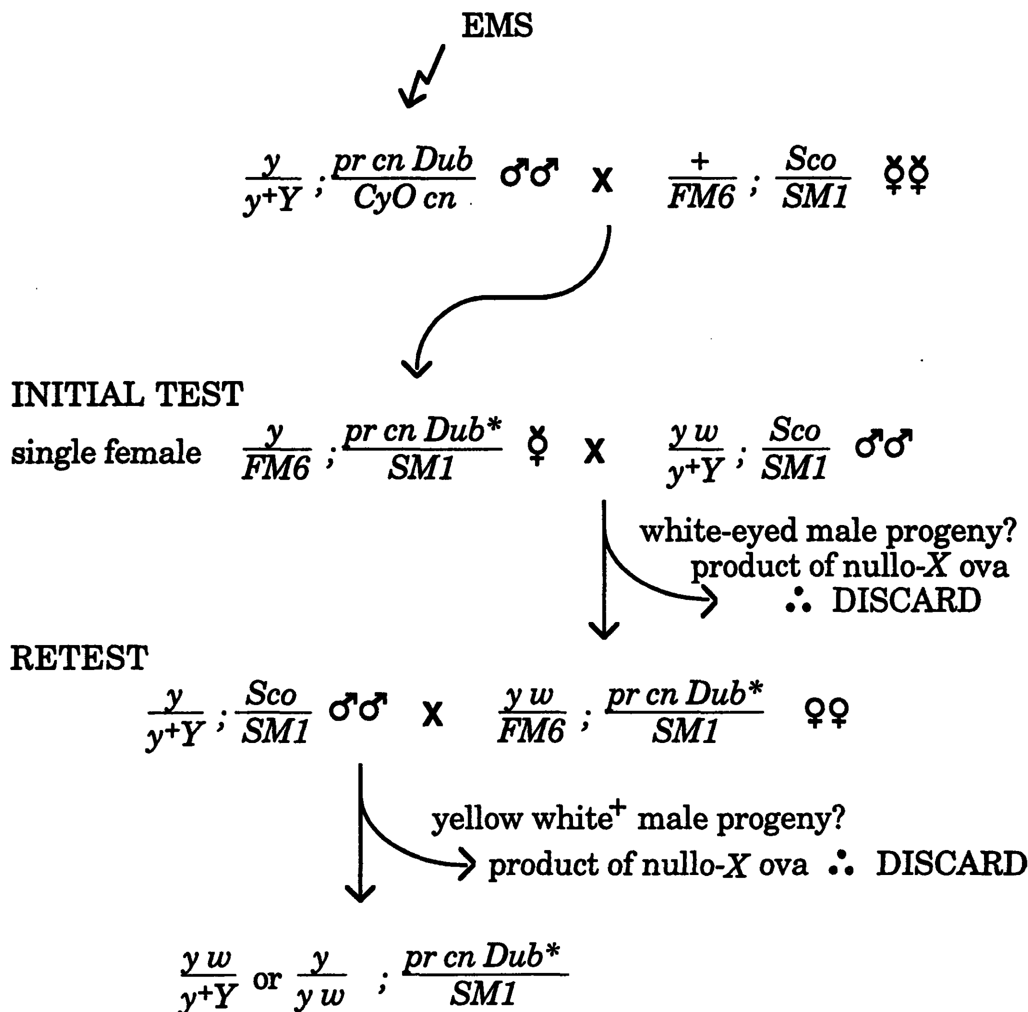


Figure 9: Screen for reversion of the dominant meiotic phenotype. Chromosomes carrying *Dub* were mutagenized in males and crossed so that a balancer chromosome was heterozygous in the test females. Single females that yielded nullo-*X* ova produced white-eyed male progeny in the initial test. These are discarded. Female progeny of females that did not produce white-eyed progeny were retested. In the second test, nullo-*X* ova yielded yellow-bodied male progeny, and these strains were discarded. Any remaining strains were stocked.

Approximately 6000 mutagenized chromosomes were screened, yielding 346 putative new alleles in the first test and 62 putatives after retesting. This number suggested an unusually high reversion frequency of 1%. Other explanations were sought for the large number of flies without a dominant meiotic phenotype. If a rare double recombination event with the balancer chromosome occurred when the *Dub* stock was amplified for mutagenesis, *Dub* could be replaced by Dub^+ from the balancer and the dominant phenotype would seem to have reverted. Stocks

TABLE 3

Nondisjunction frequency in male meiosis:
putative new alleles from screen to revert dominant meiotic phenotype

	Paternal genotype							
Sperm type	*+*/+ [a]	*Dub*/+ [b]	*9A3*/+	*2N1*/+	*10J4*/+	*10J5*/+	*15R1*/+	*11S2*/+
Regular sperm								
X or *Y* [c]	2738	5531	671	292	337	343	250	266
X nondisjunctional sperm								
0	1	178	0	3	0	1	22	17
X/Y	1	105	1	0	0	0	18	15
X/X	0	15	0	0	0	0	0	0
Total progeny scored	2740	5829	672	295	337	344	290	298
% nullo-*XY*	0.03	3.0	0	1.0	0	0.3	7.6	5.7
% *X/Y*	0.03	1.8	0.1	0	0	0	6.2	5.0
% diplo-*X*	0	0.3	0	0	0	0	0	0
Total % nondisjunction	0.1	5.1	0.1	1.0	0	0.3	13.8	10.7

$y/y^{+}Y$ males of the indicated genotype were mated with compound-*X*, y^{2} $su(w^{a})$ w^{a} females.

[a] These numbers are the sum of all the sibling controls in the above tests. The genotype is actually *SM1/+*.

[b] These data are from Table 8 of Moore, et. al. (1995).

[c] In this assay, *Y/Y* exceptional sperm were indistinguishable from regular mono-*Y* sperm and are therefore included in these numbers.

TABLE 4

Nondisjunction frequency in female meiosis:
putative new alleles from screen to revert dominant meiotic phenotype

Ova type	Maternal genotype							
	+[a]/+	*Dub*[b]/+	*9A3*/+	*2N1*/+	*10J4*/+	*10J5*/+	*15R1*/+	*11S2*/+
Regular ova								
X	3644	5268	1201	363	1195	931	603	298
X nondisjunctional ova								
0	0	246	1	0	28	8	41	28
X/X	1	336	4	0	16	9	46	20
Total progeny scored	3645	5850	1206	363	1239	948	690	346
Adjusted total scored	3646	6432	1211	363	1283	965	777	394
% nullo-*X*	0	7.7	0.2	0	4.4	1.7	10.6	14.2
% diplo-*X*	0.1	10.4	0.6	0	2.5	1.9	11.8	10.2
Total % nondisjunction	0.1	18.1	0.8	0	6.9	3.6	22.4	24.2

y females of the genotype indicated were mated to compound-*XY*, *v f B* males at 25°C.

[a] These numbers are the sum of all the sibling controls in the above tests. The genotype is actually *SM1*/+.

[b] These data are from Table 2 of Moore, et. al. (1995). *y/cv f v car* females of the genotype indicated were mated to compound-*XY*, *v f B* males.

previously amplified for the screen had been maintained in anticipation of another round of mutagenesis, and purple cinnabar progeny with no developmental defects were discovered in the stock bottles, consistent with this explanation. Therefore, to sort out *Dub*$^+$ loci from potential new *Dub* loss-of-function alleles, all 62 putatives were tested over unmutagenized *Dub* chromosomes from an unamplified stock. A loss-of-function allele ought to give a phenotype like that of a deletion when transheterozygous to *Dub*, specifically rough eyes and wing nicking. This criterion reduced the number of putatives to 6, all of which showed a phenotype when transheterozygous to *Dub* that was intermediate between that of a deletion and that of an unmutagenized *Dub* chromosome. Six putatives reflects a reversion frequency, 0.1%, that is consistent with typical EMS mutagenesis using our protocol.

The putative new alleles of *Dub* were quantitatively assayed for a dominant meiotic phenotype. Two of the putative new alleles, *9A3* and *2N1*, showed very little dominant meiotic nondisjunction in either males or females (Tables 3 and 4). Two other putative new alleles, *10J4* and *10J5*, had a more moderate reversion of the dominant meiotic phenotype in females and complete reversion in males (Tables 3 and 4). Two other putative new alleles did little to revert the dominant meiotic phenotype (Tables 3 and 4). In the latter two cases, *Dub* may have been suppressed by mutations on other chromosomes. For the quantitative assays, males carrying the mutagenized *Dub* chromosome first were crossed to virgin females with no balancer for the second chromosome, so that siblings could be tested as controls in the nondisjunction assays. Thus, suppressing mutations that existed on other chromosomes were probably crossed away before nondisjunction frequency was measured.

Three of the putative new alleles were examined as hemizygotes, *2N1/Df(2R)Pcl-XM82*, *9A3/Df(2R)Pcl-XM82* and *10J5/Df(2R)Pcl-XM82*, and all had nicked wings and rough eyes. The eyes were less rough than the eyes of *Dub/Df(2R)Pcl-XM82* transheterozygotes. Females were sterile, laying eggs that did not develop. Males were slightly fertile.

Transheterozygotes of the two putative new alleles, *10J5/9A3*, were inviable. However, *2N1/9A3* transheterozygotes were semi-viable and displayed developmental defects similar to those seen in *Dub* hemizygotes. Additionally, the transheterozygotes included a significant number of progeny, equal in number to males and to females, that had no genitalia. This new phenotype is either a developmental defect not typically seen in *Dub* hemizygotes but related to the other defects, or it may have been due to irrelevant mutations existing on the *Dub*

chromosome before mutagenesis that were not observed because of the lethality of *Dub*.

The putative new alleles could not be tested for a loss-of-function meiotic phenotype, and the developmental defects of hemizygous putative new alleles suggested that reversion may have only been partial. However, reversion of the dominant meiotic phenotype did correspond with partial reversion of the developmental defects observed in hemizygotes.

Putative second site suppressors of *Dub* homozygous lethality

A screen for reversion of the homozygous lethal phenotype is outlined in Figure 10. The screen was based on the observation that *Dub/Dub* is rarely viable but *Dub/Df* is semi-viable. Because *Dub* hemizygotes are infertile, siblings were required to recover the new alleles. P elements were used in this mutagenesis so that changes in *Dub* would be marked with known sequence and, if only partial revertants were isolated, there would be the possibility of making imprecise excisions to create a loss-of-function allele.

In the first cross, an immobile source of transposase P[ry^+ Δ2-3] was crossed into flies with a *Dub* chromosome and an *X* chromosome that was loaded with five copies of a P element carrying *lacZ* and a functional but simplified *white* gene (Bier et al. 1989). The potentially mutagenized *Dub* chromosome was isolated in single males mutant for the *white* locus. Males that were entirely white-eyed were discarded, so that insertion of at least one marked P element into chromosome *2*, *3* or *4* was certain. In the test cross, single males with the potentially mutagenized *Dub* chromosome were crossed to females carrying unmutagenized *Dub*. Both *Dub* chromosomes are *pr cn*, so progeny that were viable had the distinctive tangerine eye color of purple cinnabar flies. When more than one such fly was seen in a vial, the speck siblings were used to maintain the stock. Because *Dub* homozygotes sometimes escape the lethal phenotype without mutagenesis, the chromosome was tested twice more for consistent viability when transheterozygous to *Dub*.

From 6890 test crosses, 4 putative new "alleles" were isolated. One of these, *24B*, yielded 10 viable transheterozygotes among approximately 200 progeny (5%), but the P element insertion, followed by expression of the white gene, segregated with the third chromosome. Another of the strains, *51B*, had at least two insertions, because the insertion segregated with both the second chromosome and the third chromosome. The insertion on the second chromosome, *51B2*, was mapped to region

$\frac{\text{P[ry+ }\Delta\text{2-3](99B) } Sb}{TM3\ Ser}$ ♂♂ X $y\ w$ P[w^+] ; $\frac{pr\ cn\ Dub\ sp}{SM1}$ ☿☿

$\frac{y\ w\ \text{P}[w^+]}{Y}$; $\frac{pr\ cn\ Dub\ sp}{+}$; $\frac{\text{P[ry+ }\Delta\text{2-3](99B) } Sb}{+}$ ♂♂ X $y\ w$; $\frac{Sco}{SM1}$ ☿☿

single male $\frac{y\ w}{Y}$; $\frac{pr\ cn\ Dub\ sp^*}{SM1}$ ♂ X y ; $\frac{pr\ cn\ Dub}{SM1}$ ☿☿

any tangerine-eyed (*pr cn*) progeny?

if no *Dub*/Dub* progeny are present, then DISCARD

stock from $\frac{pr\ cn\ Dub\ sp^*}{SM1}$ progeny

Figure 10: P Screen for semi-viable knockouts of *Dub*. P elements on the *X* chromosome were mobilized in the second generation by transposase expressed from the P[ry^+ Δ2-3](99B) element. The *y w* P[w^+] chromosome actually had five P[w^+] elements and is described in Bier et. al. (1989). Single males in the third generation no longer had the transposase source nor the *X* chromosome carrying multiple copies of the P[w^+] element. The presence of a new insertion on a chromosome other than the *X* was assessed by the presence of pigment in the eyes of these single males. The third generation males were crossed to virgin females with unmutagenized *Dub* chromosomes, and suppression or reversion of *Dub* homozygous lethality was quickly assessed by the presence of purple cinnabar progeny. If such progeny were present, the putative new allele can be stocked and retested from the speck siblings.

56 by in situ hybridization to polytene salivary gland chromosomes. The *19A* strain yielded 5 transheterozygotes in approximately 100 progeny (5%). The insertion was mapped to region 48 by in situ hybridization. When transheterozygous to *Df(2R)Pcl-XM82*, *19A* had developmental defects nearly as severe as the original *Dub* hemizygotes. Finally, the *24A* strain yielded 6% viability, 9 transheterozygotes among 140 progeny. This chromosome was semi-viable when homozygous as well as when transheterozygous to *Df(2R)Pcl-XM82*. The hemizygous phenotype was

TABLE 5
Nondisjunction frequency in female meiosis: the effect of
putative second site suppressors from screen to revert recessive lethality

	Maternal genotype						
Ova type	+/+	*Dub* 19A/+	+/+	*Dub* 24A/+	+/+	*Dub* 51B2/+	*Dub*[a]/+
Regular ova							
X	1503	1954	754	907	917	1292	5268
X nondisjunctional ova							
0	1	6	0	35	0	50	246
X/X	1	24	3	106	2	114	336
Total progeny scored	1505	1984	757	1048	919	1456	5850
Adjusted total scored	1507	2014	760	1189	921	1620	6432
% nullo-*X*	0.1	0.6	0	5.9	0	6.2	7.7
% diplo-*X*	0.1	2.4	0.8	17.8	0.4	14.1	10.4
Total % nondisjunction	0.3	3.0	0.8	23.7	0.4	20.2	18.1

y or *y/y w* females of the genotype indicated were mated to compound-*XY*, *v f B* males at 25°C. Where the genotype is indicated as +/+, it is actually *SM1*/+.

[a] These data are from Table 2 of Moore, et. al. (1995). *y*/*cv f v car* females of the genotype indicated were mated to compound-*XY*, *v f B* males.

TABLE 6
Nondisjunction frequency in male meiosis: the effect of putative second site suppressors from screen to revert recessive lethality

	Paternal genotype		
Sperm type	$\frac{+}{+}$	$\frac{Dub\ 19A}{+}$	$\frac{Dub}{+}$ [a]
Regular sperm			
X or *Y* [b]	739	610	5531
X nondisjunctional sperm			
0	1	46	178
X/Y	0	29	105
X/X	0	1	15
Total progeny scored	740	686	5829
% nullo-*XY*	0.1	6.7	3.0
% *X/Y*	0	4.2	1.8
% diplo-*X*	0	0.1	0.3
Total % nondisjunction	0.1	11.1	5.1

y/y^+Y males of the indicated genotype were mated with compound-*X*, y^2 $su(w^a)$ w^a females. Where the genotype is indicated as +/+, it is actually *SM1*/+.

[a] These data are from Table 8 of Moore, et. al. (1995).

[b] In this assay, *Y/Y* exceptional sperm were indistinguishable from regular mono-*Y* sperm and are therefore included in these numbers.

slightly less severe than *Dub/Df* and was semi-fertile. The insertion mapped to 50 by in situ hybridization. All of these strains had insertions at a locus other than *Dub*.

Three of these chromosomes, 19A, 24A and 51B2, were assayed for a decrease of meiotic chromosome missegregation in females. Only one, 19A, suppressed the dominant effect of *Dub* (Table 5). This chromosome was tested in males and did not decrease missegregation in spermatocytes (Table 6).

Homozygous *Dub* lethality was suppressed in these strains, but insertions were not left in *Dub* locus. A reduction in the severity of the visible developmental defects did not accompany increased survival. It seems likely that *Dub* was not mutated in these strains, but loci that compensate for the lethal effect of *Dub* might have been mutated. Only one of these putative second-site suppressors of lethality decreased the dominant meiotic phenotype of *Dub*, and that decrease was in oocytes only.

Discussion

Dub disrupts organization of the karyosome in oocytes

The conditional dominant mutation *Dub* was reported to disrupt segregation of any of the four chromosomes during the first meiotic division (Moore et. al. 1994). In *Dub* females, nonexchange homologs in females were particularly prone to nondisjoin. The *X* chromosome had a 16% nondisjunction frequency when exchange was allowed but had a 52% nondisjunction frequency when exchange was suppressed by the heterozygous presence of multiple inversions. Chromosome *4*, which virtually never has an exchange event, had a nondisjunction frequency of 35%. The *X* chromosome and chromosome *4* typically 'cosegregated' whenever both homologs missegregated, such that diplo-*X* diplo-*4* ova and nullo-*X* nullo-*4* ova outnumbered diplo-*X* nullo-*4* ova and nullo-*X* diplo-*4* ova.

Metaphase I in *Dub* females had two defects visible in our cytology. Approximately half had an asymmetric distribution of MEI-S332-GFP that was particularly striking in that one of the caps appeared to be reduced in labeling. A smaller percentage, roughly 15%, had chromatin outside the karyosome, a phenotype similar to what is observed in *nod* oocytes (Theurkauf and Hawley 1992).

MEI-S332-GFP has been shown in spermatocytes, both wild-type and *Dub* heterozygous, to be located on the centromere regions during anaphase I, and localization during prometaphase I in *Dub* testes had the same appearance as in wild-type testes. Chromosomes in *mei-S332* males and females segregate properly in meiosis I but partition randomly in the second division, suggesting that the product is required to maintain cohesion at the centromeric region in both sexes. The scarcity of meiosis II missegregation in *Dub* females suggests that MEI-S332 is appropriately localized to the centromere regions. It is possible that MEI-S332-GFP was localized to regions other than those near the centromeres to a greater extent in *Dub* oocytes than in Dub^{+} oocytes, resulting in an asymmetric appearance. However, it was the reduced amount of MEI-S332-GFP in one cap that was most apparent, rather than an increase in one cap or an increase of MEI-S332-GFP in the middle of the karyosome where chromosome arms ought to be located. Finally, the cosegregation behavior is consistent with malorientation of kinetochores of several bivalents to one pole, and this would give an asymmetric distribution of centromere regions.

If MEI-S332-GFP is localized to centromeres in *Dub* oocytes, the asymmetry seen in our cytology suggests that orientation of bivalents is not maintained in these

karyosomes, and missegregation during anaphase I is a consequence of maloriented bivalents. The two other mutations with segregation defects most similar to that of *Dub* are unable to maintain aspects of metaphase I arrest. Nonexchange chromosomes are not maintained on the spindle in *nod* oocytes (Theurkauf and Hawley 1992). The bipolar spindle itself is not maintained in *ncd* oocytes. The point of convergence at one or the other end of the spindle breaks down and reassembles again and again (Matthies et. al. 1996). A subset of *Dub* oocytes had a defect resembling that observed in *nod* oocytes. Examination of spindles in *Dub* oocytes, particularly those with asymmetric distributions of MEI-S332-GFP, might reveal similarities to *ncd* oocytes.

Nature of the Dub mutation

Understanding the nature of the dominant *Dub* mutation could provide insight into the normal function of the *Dub* gene product. The dominant meiotic phenotype is due to a gain of function. *Dub* is not haploinsufficient, because a heterozygous deletion did not cause meiotic nondisjunction (Moore et. al. 1994). Adding another copy of the Dub^{+} locus did not reduce the frequency of nondisjunction in *Dub* males or females, so by this criterion, the *Dub* mutation is a neomorph rather than an antimorph. This suggests that *Dub* is disrupting a mechanism in which it normally does not play a role. *Dub* may not normally be expressed in meiotic cells or is perhaps mislocalized in these cells. Alternatively, the *Dub* mutation might be dominant negative in character, but the criterion applied here may be too strict, and more copies of the Dub^{+} gene would reduce the dominant effect of *Dub*.

The *Dub* mutation did not have any dominant developmental defect or any effect on viability, but *Dub* hemizygotes display developmental defects. The presence of wild type *Dub* locus compensates for the *Dub* mutation. However, two doses of the *Dub* mutation are more severe than a single dose of *Dub* in the absence of the wild type locus, so the *Dub* mutation is not simply due to loss of function. With regard to developmental defects, *Dub* is a recessive gain-of-function mutation.

Several lines of evidence suggest that both phenotypes are a result of the same lesion. Each phenotype is due to a temperature-sensitive gain-of-function mutation, so the nature of the lesion is the same for both phenotypes. Attempts to separate the dominant meiotic phenotype and the homozygous lethality of *Dub* by recombination failed during mapping of the locus, in which 33 recombinants were made in an interval of 9.8 centimorgans (Moore et al. 1994). The phenotypes are

tightly linked. Finally, when we reverted the dominant meiotic phenotype, the severity of hemizygous mitotic defects was also partially reverted.

Putative new alleles partially revert the *Dub* gain-of-function mutation

Four putative new alleles of *Dub* were isolated in a screen to revert the dominant meiotic phenotype. Because the mutations in these strains have not been mapped to the same location as the original *Dub* mutation, they remain putative alleles. The putative alleles reduced or eliminated the frequency of nondisjunction observed in both male and female heterozygotes. Furthermore, the severity of the developmental defects displayed by hemizygotes was reduced for the putative new alleles transheterozygous to a deletion relative to *Dub* hemizygotes. Unfortunately, hemizygotes of the new alleles were sterile or only slightly fertile.

All of the putative alleles, when transheterozygous to an unmutagenized *Dub* chromosome, had developmental defects that were more severe than a deletion transheterozygous to an unmutagenized *Dub* chromosome. These putative alleles are not complete loss-of-function alleles but are only partial revertants of the *Dub* mutation. If the relative severity of the developmental defects were ordered for *Dub* and one of the putative alleles, the following rank would be established:

Dub/Dub > *9A3/Dub* > *Dub/Df* > *9A3/Df* > *Dub/+* = *Df/+*

A complete loss-of-function allele, designated here as *l-o-f*, might be anticipated to have the following ranking in terms of severity of developmental defects:

Dub/Dub >> *l-o-f/Dub* = *Dub/Df* > *l-o-f/Df* ≥? *Dub/+* = *Df/+*

It is possible that a true loss-of-function allele of *Dub* might never be fertile as a hemizygote, but hemizygotes of the putative alleles had less severe defects than *Dub* hemizygotes. A true loss-of-function allele might be fertile enough to ask whether meiotic segregation is disrupted.

Putative second site suppressors of *Dub* lethality

Four putative reversion strains were isolated in the screen to revert homozygous lethality using a transposable element. The *Dub* locus is unlikely to have actually been altered in any of these strains, since the P element insertion was mapped by in situ to locations other than 54D-E. Recently, it has been estimated that in screens for lethal mutations using P[w+] elements, only 20-40% of the mutations isolated

actually had an insertion associated with the lethal lesion (personal communication, A. Spradling). It is formally possible that a transposition event moved a P element into and then out of the *Dub* locus, leaving a new lesion but too little of the transposable element to be detected by in situ hybridization. However, a deletion transheterozygous to an unmutagenized *Dub* chromosome was more viable than any of these altered *Dub* chromosomes, so only partial reversion of the *Dub* locus could have occurred in these strains. The screen was highly attenuated, in that even a small shift in the severity of developmental defects can increase the number of viable *Dub* homozygotes. Even before mutagenesis, there were rare escapers. There could be many second site suppressors or enhancers that influence the relative viability of *Dub* homozygotes, and these may or may not have no effect on the dominant meiotic phenotype.

One of the putative second site suppressors of lethality, 19A, did suppress *Dub*'s dominant meiotic missegregation in oocytes but it did not suppress missegregation in spermatocytes. This P element insertion may have mutated a gene with a product involved in chromosome segregation. To test this possibility, the 19A insertion could be recombined off the chromosome it shares with the *Dub* mutation and assayed for meiotic missegregation while transheterozygous to a deficiency.

Initial step toward cloning

Localization of *Dub* to a small region of the genome is a large step towards cloning of the locus. Cosmids known to map to this region could be used to determine the breakpoint of either the *Df(2R)PC4* or *Df(2R)Pcl-XM82* deletions, defining a proximal limit for the location of *Dub*. By similarly determining the breakpoint of *Df(2R)Pcl-7B* or using cosmids known to rescue *grh*, a distal limit could be defined. A chromosome walk then could be carried out to find cosmids that overlap in this region, and many cosmids are already mapped to this region. If the region is reduced to a few cosmids, these could be tested for complementation of the *Dub* hemizygous phenotype.

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Afterword

Future Considerations

Two Drosophila proteins, ORD and MEI-S332, that are essential for cohesion of sister chromatids during meiosis have been identified. ORD is required both prior to and after metaphase I (Bickel et. al. 1997), and MEI-S332 is required only after metaphase I. In the two simplest models for the roles of these products, ORD is considered a glue that holds the sister chromatids together along their entire length, not only along the chromosome arms but also between the centromere regions. In one model, MEI-S332 also acts as a glue in the centromere regions, but this MEI-S332 cohesion is not degraded during the metaphase I/anaphase I transition when the glue between the arms of the sister chromatids is dissolved. Alternatively, MEI-S332 could function during the metaphase I/anaphase I transition to prevent dissolution of the bond that ORD provides in the centromere regions. MEI-S332 acts as a preservative of the glue.

Both models account for the complete lack of cohesion that allows random segregation of sister chromatids through both meiotic divisions when ORD is absent. MEI-S332 is not located on the sister-chromatid centromere regions until the chromatids have begun to condense in late prophase I. The centromere regions of the bivalent are associated throughout prophase I (Dernburg et.al. 1996), so sister chromatids are likely to have been glued together during prophase I by a mechanism that does not require MEI-S332.

Consistent with both of these simple hypotheses, localization of MEI-S332 to centromere regions of sister chromatids should not compensate for the absence of ORD, and MEI-S332 was shown to be localized to the chromatids in *ord* meiotic cells. Furthermore, if ORD is the glue that binds the sister chromatids, *ord* would be epistatic to *mei-S332*. In the double mutant with strong alleles of *ord* and *mei-S332*, the missegregation pattern was shown to be similar to that observed in strong *ord* alleles (S. Bickel, pers. communication).

Localization of ORD in meiotic cells is the most important avenue of investigation to differentiate between the simple models. In both models, ORD would be anticipated along the length of the sister chromatids soon after replication, and after the metaphase I/anaphase I transition, ORD should no longer be localized to the sister chromatid arms. If MEI-S332 acts as a redundant glue, ORD localized to the centromere regions may be "dissolved" during this transition. If MEI-S332 acts as a

preservative of ORD, then ORD will be retained at the centromere regions in *mei-S332*$^+$ meiocytes and "dissolved" in *mei-S332*$^-$ meiocytes.

These models describe functions for ORD and MEI-S332 that may not be carried out simply by these proteins alone. In particular, ORD could play a role in putting a glue in place without actually being a structural component of the glue. Accordingly, one direction of investigation is the identification of other proteins with which ORD and MEI-S332 interact. This has been attempted in two genetic screens for unlinked noncomplementation of *ord* and *mei-S332*: the first surveyed the X chromosome using deletions (Appendix II, this thesis), and the second examined third chromosomes that had been chemically mutagenized (Bickel et. al. 1997).

Now that the products of both *ord* and *mei-S332* have been identified, other means of searching for interacting products are possible. The two-hybrid assay in *S. cerevisiae* can be used to identify Drosophila sequences that produce interacting products. Co-immunoprecipitation using antibodies to ORD or MEI-S332 may also reveal potential interacting proteins.

Because both ORD and MEI-S332 have no known homologs, another direction of investigation should be focused on understanding how these proteins carry out their functions. To define domains, the proteins can be dissected by deleting segments in order to assay for missegregation and examine meiotic cells for localization. Alleles already isolated can be examined for localization. Finding homologs in closely related species and eventually in distantly related species will reveal regions of the protein that are conserved, and mutations engineered in these protein elements may reveal their functional role.

The gain-of-function mutation in the third gene examined in this thesis, *Dub*, primarily disrupts the meiosis I segregation of nonexchange chromatids in oocytes, but it also disturbs reductional segregation of exchange chromatids in oocytes and segregation of chromatids in spermatocytes. This pattern is similar to the effect of *nod* and *ncd* on meiotic segregation in oocytes, particularly the loss of nonexchange chromosomes during meiosis I. Both genes are required for spindle functions, either to hold nonexchange chromatids on the spindle or to maintain the integrity of the bipolar spindle. Based on this similarity, the product of the *Dub* mutation might be anticipated to disrupt some function related to the meiotic spindle.

Much remains to be understood about the role of *Dub* in meiotic chromosome segregation. The structure of the spindle should be examined carefully for defects. To determine if the product of *Dub* is normally required in meiosis, loss-of-function alleles must be isolated. Several screens to identify such new alleles have been attempted,

although only partial revertants were isolated. One screen produced no new putative alleles, although that may have been due to the use of a transposable element for mutagenesis. Transposition to the *Dub* locus may be rare. Another screen suffered from an early recombination event that introduced a Dub^{+} loci from the balancer chromosome onto some of the chromosomes before mutagenesis. The secondary criterion used to sort out these chromosomes assumed that a new loss-of-function allele would have the same phenotype as a deletion does when transheterozygous to the *Dub* mutation, and it is possible that using this criterion may have eliminated reversions of *Dub*. Further genetic screens could be devised and carried out. However, the best strategy may be to clone and characterize the *Dub* locus, so that different screens can be devised to produce new alleles of the gene. Cloning of *Dub* could be carried out by identifying cosmids known to be localized to the region and using these to identify other cosmids. Deletion breakpoints define the interval in which *Dub* must be located, and the interval could be further determined if rearrangements exist from the mutagenesis that produced *Dub*. Genomic fragments can be introduced until a segment is found to complement *Dub/Df(2R)Pcl-XM82* for developmental defects. Once the gene has been identified, it is possible that the gene and alleles may already exist in the literature. Alternatively, screens for the introduction of transposable elements into known sequences can be designed. Even before new alleles of *Dub* are isolated, cloning the gene allows the expression of *Dub* transcripts to be examined. The *Dub* mutation might be gain-of-function simply because the gene product is not normally present in meiotic cells.

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Appendix I

Deficiencies in *Drosophila melanogaster* cytological region 59

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The deficiency screen and all of the complementation tests in this appendix were performed by myself, except for the following: Jennifer Mach assayed complementation of *egalitarian*. Irena Royzman tested complemenation of *minus* and *Df(2R)bw-DRP*. Three of the *ord* complementation tests were earlier reported by Wes Miyazaki in his thesis, and two tests were performed by Sharon Bickel. Bruce Reed contributed the complementation data for *orange* and also did precise mapping of cytological breakpoints for three deficiencies: *Df(2R)WI370*, *Df(2R)bw-WI366* and *Df(2R)HB132*.

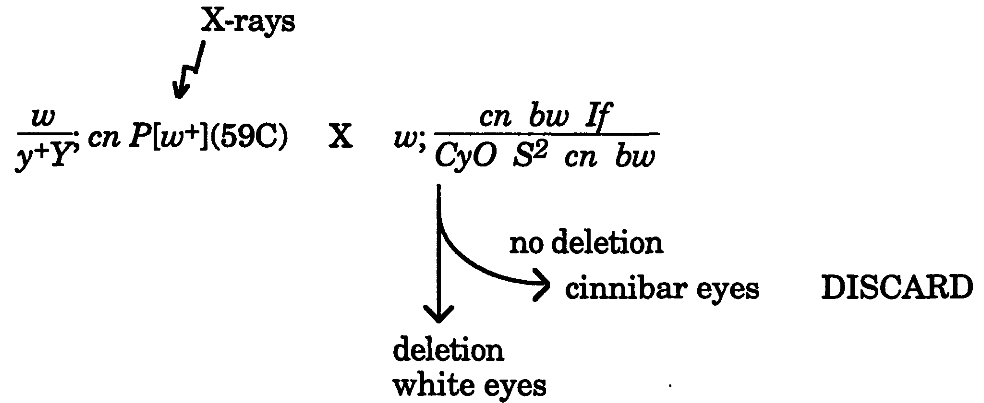
Deficiencies were generated in cytological region 59 on the right arm of chromosome *2*, primarily to physically map the gene *ord* (Bickel, et al., 1996). Subsequently other mutations have been mapped using some of these deficiencies, including *egalitarian* (*egl*) (Mach, et. al. 1997) and *minus* (*mi*). Complementation assays of these mutations and the transheterozygous viability of selected deficiencies have been compiled here so that the deficiencies could be used to create a map of region 59.

To generate the deficiencies, males carrying a second chromosome with a P element insertion into 59C, marked with the *white* (*w*) gene, were mutagenized with X-rays, half at a dosage of 3000 rads and half at 4000 rads (Fig. 1). Their progeny were scored for white eyes. Mutations or deletions at either of two loci were detectable, deletion of the *white* gene in the P element at 59C or deletion of the *brown* (*bw*) locus at 59E. Because the flies were already mutant for *w* at the normal locus, deletion of the P element would have yielded white-eyed flies. The flies were also mutant for *cinnabar* (*cn*), so disruption of the *bw* gene causes the "cinnibar-brown" eye color, namely white eyes. 181 white-eyed progeny were found among an estimated 383,000 progeny. Approximately half of these white-eyed progeny were sterile. Ultimately, 8 deletions of the P element were isolated, and 10 deletions of the *bw* locus were isolated. One of these deletions, *bw-WI3128*, removed both loci.

The ability of the deficiencies to complement several mutations, *ord*, *mi*, *orange (or)* and *egl* (J. Mach and I. Royzman, personal communications), as well as the viability of transheterozygous combinations of deficiencies, are reported in Figure 2. Thirteen deficiencies not isolated in our screen were also tested for viability in transheterozygous combinations. One deficiency chromosome, *Df(2R)bw-HB132*, also contained the *Freckled* (*Frd*) mutation, so inviability over this lesion was also assessed for chromosomes with deletions of 59C.

Where breakpoints were published or were determined directly by squashes of salivary gland polytene chromosomes, they were recorded in Figure 2.

Seven of the deficiency transheterozygotes were semi-viable, eclosing later than their siblings and in small numbers. Several of the combinations included two deficiency chromosomes that deleted *ord*. One such combination, *Df(2R)bw-S46/Df(2R)WI345*, was quantitatively tested for meiotic nondisjunction. Male transheterozygotes were essentially sterile, and female transheterozygotes were nearly sterile. Only 58 progeny were recovered from 45 females, but these revealed a total nondisjunction frequency of 55%, the result of equal numbers of diplo-*X* and nullo-*X* ova.



$$w\ ;\ \frac{cn\ Df}{CyO\ S^2\ cn\ bw}$$

$$w\ ;\ \frac{cn\ P[w^+](59C)\ Df(bw)}{CyO\ S^2\ cn\ bw}$$

$$w\ ;\ \frac{cn\ Df}{cn\ bw\ If}$$

$$w\ ;\ \frac{cn\ P[w^+](59C)\ Df(bw)}{cn\ bw\ If}$$

Figure 1: Screen for deficiencies in regions 59C or in 59E. Males carrying an insertion of the *white* gene in 59C were mutagenized with X-rays and crossed to females that were mutant for *w*, *cinnabar* (*cn*) and *brown* (*bw*). Their progeny were examined for white eyes, since deletion of either the insertion at 59C or the *bw* locus at 59E would yield white eyes. Other markers on the chromosomes include *Curly* (*Cy*), *Star* (*S*), *Irregular facets* (*If*). *CyO* is a chromosome with multiple inversions. All of these mutations are described in Lindsley and Zimm (1992).

A map of the deficiencies has been constructed based on genetic criteria (Fig. 3). Inviability of transheterozygous deficiencies suggests that there is some overlapping region including loci required for viability, and this defines 6 sets of endpoints. Another 5 sets of endpoints are defined by phenotypes observed when deletions are heterozygous to either *ord*, *mi*, *egl*, *bw* or *or*. Viability over the *Frd* locus defines another suite of endpoints. Deletion of the *white* insertion in 59C defines one more set of endpoints, although this criterion is only known for those deficiencies generated in our screen.

All of region 59 can be tested for complementation by as few as four deficiencies. For example, *Df(2R)X58-5*, *Df(2R)59AD*, *Df(2R)WI370*, *Df(2R)bw-WI366* together represent a complete deletion of the region. These deficiencies also break region 59 into smaller genetic units, facilitating the physical placement of genes in this region.

Deficiency or gene

Columns (deficiency or gene): X58-12, X58-5, 59AB, 59AD, WI3117, WI3100, WI3131, twi-S60, Frd-R1, WI345, WI213, WI327, WI370, vir-130, WI362, bw-WI3128, bw-S46, ord, vir-12, bw-WI320, bw-WI366, bw-DRP, or-BR6, bw-HB132, mi, bw-WI3121, bw-WI3140, bw-WI379, bw-WI394, bw-WI3118, bw-WI3129, bw-WI3133, bw, egl, or-BR11, or

Deficiency	ord	mi	bw	egl	or	Breakpoints	Reference
X58-12						58D1-2;59A	Kerrebrock, et.al., 1995
X58-5						58B1-2;59A1	Kerrebrock, et.al., 1995
59AB						59A1-3;59B1-2	Knoblich, et. al., 1993
59AD						59A1-3;59D1-4	Knoblich, et. al., 1993
WI3117	+					59C	
WI3100	+					59C	
WI3131	+					59C	
twi-S60	+					59C3-4;59D1-2	Simpson, 1983
Frd-R1	+					59C1;59C4	Reed, 1992
WI345	-	+				59B; 59C	
WI213	-	+				59B; 59D	
WI327	-	+				59B; 59D	
WI370	-	+				59B3; 59D5-11	Bickel, et. al., 1996
vir-130	-	+					
WI362	-						
bw-WI3128	-	-	-	-			
bw-S46	-	-	-		-	59D5-11; 60A7	Simpson, 1983
vir-12	+	+					
bw-WI320	+	-	-	+			
bw-WI366	+	-	-	-	-	59D5-11; 60B1-3	Bickel, et. al., 1996
bw-DRP		-	-			59D3-4;59F5-6	Reed, 1992
or-BR6					-	59D5-10;60B3-8	Reed, 1992
bw-HB132	+	-		-		59D5-11;59F6-8	
bw-WI3121		-	-	-		59E-F; 60A-B	
bw-WI3140		-	-	-		59E-F; 60A-B	
bw-WI379			-	+			
bw-WI394							
bw-WI3118			-	+			
bw-WI3129			-	+			
bw-WI3133			-	+			
or-BR11				+		59F6-8;60A8-16	Reed, 1992

Figure 2: Tests for transheterozygous complementation. Where transheterozygous deficiencies were assayed for viability, a black box indicates inviability, a gray box indicates viability and a half gray-half black box indicates semi-viability. Deficiencies that complement mutant phenotypes are indicated with a plus, deficencies that failed to complement with a minus. Where cytological breakpoints of deficiencies were observed, they are listed at right.

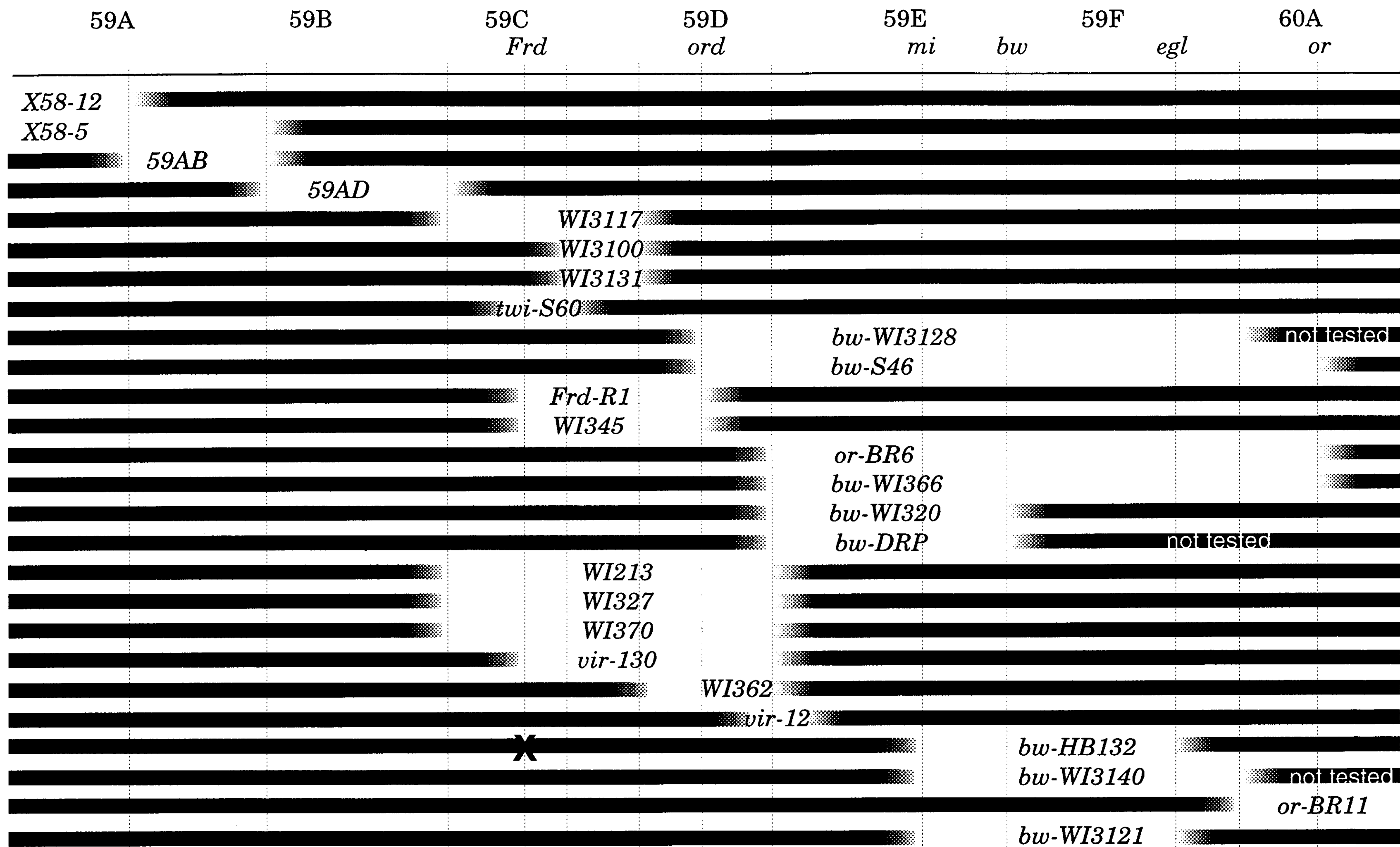


Figure 3: Deficiencies in cytological region 59. Black lines indicate intact chromosomal regions. These deficiencies are mapped as the minimal deleted region based solely on genetic criteria, accounting for transheterozygous inviability with other deficiencies and mutant phenotypes observed when examined over the mutations in the genes at the top of the figure. The **X** on the *bw-HB132* chromosome indicates the presence of a *Frd* mutation. The actual overlap of deficiencies could be greater than that depicted here.

Acknowledgments

Df(2R)bw-S46 was obtained from R. Lehmann. *Df(2R)59AB* and *Df(2R)59AD* from J. Knoblich. *Df(2R)bw-HB132*, *Df(2R)vir-130* and *Df(2R)vir-12* were obtained from R. Nothiger. *Df(2R)bw-DRP*, *Df(2R)or-BR6*, *Df(2R)Frd-R1* and *Df(2R)or-BR11* were obtained from B. Reed. *Df(2R)twi-S60* was obtained from F. Perrin-Schmitt.

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Appendix II

Survey of chromosome *1* for loci that fail to complement *mei-S332* and *ord*

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Interacting gene products can be sometimes be identified by screening for unlinked loci that fail to complement a mutation. This strategy has been used to identify proteins that interact with tubulin and with actin in *S. cerevisiae* and in *D. melanogaster* (Stearns and Botstein 1988, Hays et al. 1989, Vinh et al. 1993, Welch et al. 1993). Loci that may be involved in cell cycle and mitosis have recently been identified using this strategy in Drosophila (White-Cooper et al. 1996). The Drosophila genome can be readily surveyed for unlinked noncomplementation using the deletions of large regions of the Drosophila genome that exist on previously isolated deficiency chromosomes.

We tested deletions of the *X* chromosome for failure to complement *mei-S332* and *ord*. Males carrying a chromosome with *mei-S332*[1] and *ord*[1] were crossed to females carrying deficiencies (Fig. 1). Virgin female progeny carrying both the deletion and the *mei-S332 ord* chromosome were tested for missegregation during meiosis. Females carrying only the *mei-S332 ord* chromosome or carrying only the deficiency served as controls. Meiotic loss or missegregation of the *X* chromosome was measured by mating the females with males that carried *yellow* (*y*) *X* chromosomes and *Y* chromosomes with a *yellow*$^+$ locus, so that a nullo-*X* ova resulted in yellow male progeny. Missegregation of chromosome *4* was measured in two ways: nullo-*4* ova yielded cubitus interruptis eyeless progeny when fertilized by sperm that carried a compound *4* chromosome; and missegregation events during meiosis II that gave diplo-*4* ova yielded sparkling progeny when fertilized by nullo-*4* sperm. Sparkling progeny from haplo-*4* ova rarely survived and had a Minute phenotype in addition to the eye phenotype.

The number of progeny from each cross and the percentage of the total progeny are reported in Table 1. The percentages were typically less than one percent. Because all of the haplo-*X* ova from normal segregation were recovered while only half of the ova from missegregation were recoverable, actual nondisjunction frequencies were approximately double the percentages for nullo-*X* ova reported here. Diplo-*4* ova resulting from meiosis II nondisjunction were also only observable half the time, because the females were heterozygous for *sparkling* (*spa*), so the actual nondisjunction frequencies were approximately twice the percentages reported in Table 1. For most of the deficiencies, nondisjunction frequencies were less than one percent.

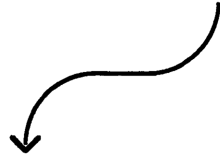
One chromosome carrying a deletion, *Df(1)HA85*, acted in a dominant manner to yield a notable number of exceptional progeny (Table 1). Significant numbers of nullo-*4* ova and nullo-*X* ova were observed whether the chromosome carrying the meiotic

mutants was present or not. Diplo-*4* ova from missegregation during meiosis II were not observed. Chromosomes that independently carry either *ord* or *mei-S332* were tested with *Df(1)HA85* in the same manner that the double mutant chromosome was tested, and the dominant effect of *Df(1)HA85* on missegregation was not enhanced significantly by ord^1 or by $mei\text{-}S332^1$ (Tables 2 and 3). This deficiency has been reported to delete or disrupt the *nod* gene, a gene essential for segregation of nonexchange chromosomes (Voelker et al. 1985). Loss of the nonexchange chromosome *4* and loss of some *X* chromosomes, without missegregation of chromosome *4* during meiosis II, is consistent with disruption of *nod*, although a simple deletion of *nod* would not be expected to have a dominant effect (Zhang et al. 1990). Furthermore, another deletion reported to delete or disrupt *nod*, *Df(1)N71* (Voelker et al. 1985), did not show any unlinked noncomplementation in this screen, so the effect of *Df(1)HA85* on meiotic chromosome segregation cannot be due to simple deletion of *nod*.

Two deletions, *Df(1)HF366* and *Df(1)JA27*, were nearly infertile when combined with chromosome carrying *ord* and *mei-S332*, so these were also tested with *ord* and with *mei-S332* independently (Tables 2 and 3). Neither deletion was infertile or resulted in exceptional progeny in the presence of *mei-S332* or *ord* individually. It is possible that the low fertility was a result of an interaction between three loci, but it is more likely that the *mei-S332 ord* chromosome also carried another mutation that resulted in low fertility in combination with these deletions.

The deletions tested in our survey represented approximately 2/3 of the *X* chromosome (Fig. 2), yet no significant unlinked noncomplementation was observed. Unlinked loci that fail to complement these two genes may be relatively unusual.

$\frac{Df}{FM6\ or\ FM7}$ ♀♀ X $\frac{y}{y^{+}Y}$; $\frac{cn\ mei\text{-}S332^{1}\ ord^{1}}{SM1}$; spa^{pol} ♂♂



TEST CROSSES

A $\frac{Df}{y}$; $\frac{cn\ mei\text{-}S332^{1}\ ord^{1}}{+}$; $\frac{spa^{pol}}{+}$ ♀♀ X $\frac{y}{y^{+}Y}$; $\frac{\hat{44}, ci\ ey^{R}}{O}$ ♂♂

B $\frac{Df}{y}$; $\frac{SM1}{+}$; $\frac{spa^{pol}}{+}$ ♀♀ X $\frac{y}{y^{+}Y}$; $\frac{\hat{44}, ci\ ey^{R}}{O}$ ♂♂

C $\frac{FMx}{y}$; $\frac{cn\ mei\text{-}S332^{1}\ ord^{1}}{+}$; $\frac{spa^{pol}}{+}$ ♀♀ X $\frac{y}{y^{+}Y}$; $\frac{\hat{44}, ci\ ey^{R}}{O}$ ♂♂

Figure 1: Screening for deletions that fail to complement *ord* or *mei-S332*. Females with deficiencies on the *X* chromosome were crossed to males carrying double mutant *mei-S332 ord* chromosome and a chromosome *4* that was *sparkling-poliert* (spa^{pol}). Three classes of progeny were assayed for missegregation: (A) females carrying both the *mei-S332 ord* chromosome and the *X* deficiency; (B) females carrying only the double mutant chromosome; and (C) females carrying only the deficiency. Missegregation was assayed by mating the females to males with a *yellow* (*y*) *X* chromosome and a y^{+} *Y* chromosome and a compound *4* mutant (designated as $\hat{44}$) for *cubitus interruptus* (*ci*) and *eyeless* (ey^{R}). Nullo-*X* ova resulted in y male progeny. Nullo-*4* ova resulted in ci ey progeny. Diplo-*4* ova carrying two spa^{pol} chromatids yielded spa progeny.

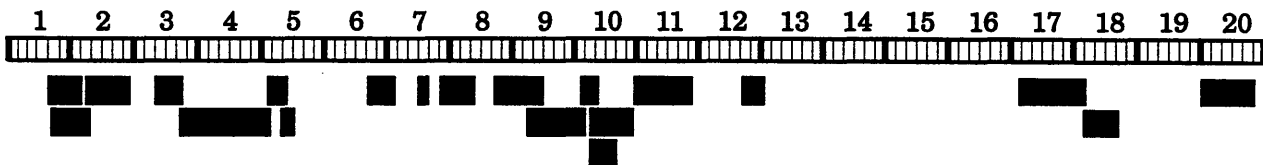


Figure 2: Deficiencies tested in this screen deleted approximately 2/3 of the *X* chromosome. The numbered divisions of salivary gland polytene chromosomes are labeled, the lettered subdivisions shown as simple lines. The black boxes portray the approximate size and location of the deleted segments used in this screen. More exact breakpoints are given in Table 1.

TABLE 1

Missegregation in noncomplementation tests with *ord*^{1} and *mei-S332*^{1}

Deficiency tested [a] name and breakpoints		total progeny	Nullo-*X* ova yellow males number	Nullo-*X* ova yellow males % of total	Nullo-*4* ova ci ey progeny number	Nullo-*4* ova ci ey progeny % of total	Diplo-*4* ova spa progeny number	Diplo-*4* ova spa progeny % of total
Df(1)S39	A	1413	8	0.57	1	0.07	0	< 0.07
	B	1098	0	< 0.09	0	< 0.09	2	0.18
1E1;2B5-6	C	1045	5	0.48	1	< 0.10	0	< 0.10
Df(1)A94	A	156	1	0.64	1	0.64	0	< 0.64
	B	27	0	< 3.70	1	3.70	0	< 3.70
1E3-4;2B11-12	C	964	5	0.52	0	< 0.10	0	< 0.10
Df(1)Pgd35	A	2054	4	0.19	3	0.15	0	< 0.05
	B	1523	4	0.26	1	0.07	0	< 0.07
2C2-4;2E2-F1	C	1028	8	0.78	1	0.10	0	< 0.10
In(1)dm75e19	A	1050	6	0.57	0	< 0.10	0	< 0.10
	B	725	4	0.26	0	< 0.14	0	< 0.14
3C12;3E3	C	554	2	0.36	0	< 0.18	1	0.18
Df(1)HF366	A	18	1		0		0	
	B	703	1	0.14	0	< 0.14	0	< 0.14
3E7-8;5A7	C	1241	8	0.64	0	0.08	1	0.08
Df(1)C149	A	2281	13	0.57	9	0.39	0	< 0.04
	B	2130	5	0.23	3	0.14	0	< 0.05
5A8-9;5C5-6	C	1843	8	0.43	0	< 0.05	0	< 0.05
Df(1)N73	A	2174	7	0.32	2	0.09	1	0.05
	B	1452	5	0.34	0	< 0.07	1	0.07
5C2;5D5-6	C	1678	8	0.48	1	0.06	0	< 0.06
Df(1)HA32	A	916	7	0.76	0	< 0.11	0	< 0.11
	B	881	5	0.57	1	0.11	3	0.34
6E4-5;7A6	C	824	8	0.97	0	< 0.12	0	< 0.12
Df(1)C128	A	2105	14	0.67	5	0.24	0	< 0.05
	B	2189	3	0.14	0	< 0.05	0	< 0.05
7D1;7D5-7D6	C	1997	3	0.15	0	< 0.05	0	< 0.05
Df(1)KA14	A	669	7	1.05	0	< 0.15	0	< 0.15
	B	112	0	< 0.89	0	< 0.89	0	< 0.89
7F1-2;8C5	C	1060	4	0.38	0	< 0.09	0	< 0.09
Df(1)C52	A	705	5	0.71	0	< 0.14	2	0.28
	B	698	1	0.14	0	< 0.14	0	< 0.14
8E;9C-9D	C	817	1	0.12	0	< 0.12	0	< 0.12
Df(1)V-L15	A	1199	2	0.17	1	0.08	0	< 0.08
	B	839	1	0.12	0	< 0.12	0	< 0.12
9B1-2;10A1-2	C	1159	6	2.07	4	0.35	0	< 0.09
Df(1)RA37	A	421	3	0.71	0	< 0.24	0	< 0.24
	B	32	0	< 3.13	1	3.13	0	< 3.13
10A6;10B15-17	C	1578	13	0.82	2	0.13	0	< 0.06

TABLE 1 CONTINUED

Missegregation in noncomplementation tests with *ord*[1] and *mei-S332*[1]

Deficiency tested [a] name and breakpoints		total progeny	Nullo-*X* ova yellow males number	Nullo-*X* ova yellow males % of total	Nullo-*4* ova ci ey progeny number	Nullo-*4* ova ci ey progeny % of total	Diplo-*4* ova spa progeny number	Diplo-*4* ova spa progeny % of total
Df(1)N71	A	3396	11	0.32	0	< 0.03	0	< 0.03
	B	1993	2	0.10	0	< 0.05	0	< 0.05
10B2-8;10D3-8	C	1941	15	0.77	0	< 0.05	0	< 0.05
Df(1)HA85	A	147	3	2.04	8	5.44	0	< 0.68
	B	97	1	1.03	6	6.19	0	< 1.03
10B-10C;10F	C	184	1	0.54	0	< 0.54	0	< 0.54
Df(1)N105	A	2976	19	0.64	1	0.03	5	0.17
	B	1761	4	0.23	0	< 0.06	1	0.06
10F7;11D-11E	C	2365	14	0.59	0	< 0.04	0	< 0.04
Df(1)KA9	A	1682	4	0.24	0	< 0.06	0	< 0.06
	B	1265	8	0.63	1	0.08	1	0.08
12E2-3;12F5-13A1	C	1464	8	0.55	0	< 0.07	1	0.07
Df(1)N19	A	1444	12	0.83	1	0.07	0	< 0.07
	B	1256	3	0.24	5	0.40	0	< 0.08
17A1;18A2	C	1177	18	1.53	0	< 0.09	0	< 0.09
Df(1)JA27	A	8	0		0		0	
	B	448	1	0.22	1	0.22	1	0.22
18A5;18D1-2	C	1647	4	0.24	0	< 0.06	0	< 0.06
Df(1)DCB1-35b	A	1275	2	0.16	0	< 0.08	0	< 0.08
	B	1160	1	0.09	0	< 0.09	0	< 0.09
19F1-2;20E-20F	C	1050	3	0.29	0	< 0.01	0	< 0.01

[a] Three classes of progeny were assayed for missegregation: (A) females carrying both the *mei-S332 ord* chromosome and the *X* deficiency; (B) females carrying only the double mutant chromosome; and (C) females carrying only the deficiency. See Figure 1.

TABLE 2

Missegregation in noncomplementation tests with *mei-S332*^{1}

Deficiency tested [a] name and breakpoints		total progeny	Nullo-*X* ova: yellow males number	% of total	Nullo-*4* ova: ci ey progeny number	% of total	Diplo-*4* ova: spa progeny number	% of total
Df(1)HF366	A	1533	4	0.26	3	0.20	2	0.07
	B	1821	4	0.22	0	< 0.05	1	0.05
3E7-8;5A7	C	2956	7	0.24	3	0.10	1	0.03
Df(1)HA85	A	1432	11	7.68	46	3.21	0	< 0.07
	B	964	7	0.73	61	6.33	9	0.93
10B-10C;10F	C	1779	10	0.56	3	0.17	3	0.17
Df(1)JA27	A	858	1	0.12	0	< 0.12	0	< 0.12
	B	1480	3	0.20	1	0.07	0	< 0.07
18A5;18D1-2	C	1175	3	0.25	0	< 0.09	0	< 0.09

[a] Three classes of progeny were assayed for missegregation: (A) females carrying both a *mei-S332* chromosome and an *X* deficiency; (B) females carrying only the *mei-S332* chromosome; and (C) females carrying only the deficiency.

TABLE 3

Missegregation in noncomplementation tests with *ord*^{1}

Deficiency tested [a] name and breakpoints		total progeny	Nullo-*X* ova: yellow males number	% of total	Nullo-*4* ova: ci ey progeny number	% of total	Diplo-*4* ova: spa progeny number	% of total
Df(1)HF366	A	2382	2	0.08	2	0.08	0	< 0.04
	B	1089	0	< 0.09	2	0.18	0	< 0.09
3E7-8;5A7	C	2543	8	0.31	0	< 0.04	0	< 0.04
Df(1)HA85	A	1329	40	3.01	88	6.62	2	0.15
	B	528	6	1.14	141	26.70	0	< 0.19
10B-10C;10F	C	2505	14	0.56	1	0.04	0	< 0.04
Df(1)JA27	A	1422	7	0.49	0	< 0.07	0	< 0.07
	B	664	0	< 0.15	0	< 0.15	0	< 0.15
18A5;18D1-2	C	1322	4	0.30	0	< 0.08	0	< 0.08

[a] Three classes of progeny were assayed for missegregation: (A) females carrying both an *ord* chromosome and an *X* deficiency; (B) females carrying only the *ord* chromosome; and (C) females carrying only the deficiency.

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Biography of the Author

Daniel P. Moore spent his formative years in Hood River, Oregon, a small town in the Columbia River gorge. He majored in chemistry while studying at the Robert D. Clark Honors College, a liberal arts college within the larger institution of the University of Oregon. While in Eugene, Daniel worked in the laboratory of Professor John Schellman, using linear dichroism to study the orientation of DNA molecules in agarose gels under the influence of pulsed electric fields. For two years, while discussing philosophy at evening seminars of The Graduate Institute of St. Johns College in Annapolis, he spent his daytime hours working in the laboratory of Professor Jef Boeke at The Johns Hopkins University School of Medicine in Baltimore. Studies in this lab focused on Ty*1*, a transposable element in the yeast, *Saccharomyces cerevisiae*. In the autumn of 1989, he began his studies at the Massachusetts Institute of Technology in Cambridge.

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* indicates shared first authorship.