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modifier of mdg4 encodes a protein involved in homologous chromosome pairing in Drosophila melanogaster males

Morvarid Soltani Bejnood
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Morvarid Soltani Bejnood entitled "modifier of mdg4 encodes a protein involved in homologous chromosome pairing in Drosophila melanogaster males." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Bruce D. McKee, Major Professor

We have read this dissertation and recommend its acceptance:

Mariano Labrador, Jae Park, Jeffrey Becker, Mary Ann Handel

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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modifier of mdg4 encodes a protein involved in homologous chromosome pairing in *Drosophila melanogaster* males

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Morvarid Soltani Bejnood

August 2004

Dedication

To my sons, Alborz and Aram, the most precious persons in my life

Acknowledgments

I would like to thank my mentor, Dr. Bruce McKee, for providing me with the opportunity to further my education. I am grateful for all the time you spent teaching me over the last few years. Your belief in me meant a lot and helped me achieve my goals.

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Last, but certainly not least, I would like to thank my husband, Saeed, for his patience and love. Thanks for standing by me and helping me go through difficult times.

Abstract

Our research interest is to uncover mechanisms underlying meiotic chromosome pairing and segregation. *pairing failure 2* (*pf-2*) is a gene involved in this process during meiosis I of male *Drosophila*. The three *pf-2* alleles recovered in a screen for chemically induced (EMS) mutations on chromosome III that cause paternal loss of chromosome IV display strong meiotic phenotypes. Cytological analysis of testes of *pf-2* mutant flies revealed unpaired chromosomes at prophase and metaphase I and "laggard chromosomes" at anaphase I in primary spermatocytes. Meiosis II appears relatively normal. Genetic data confirm that non-disjunction occurs at the first meiotic division and affects the segregation of sex chromosomes as well as autosomes. By deficiency complementation *pf-2* was mapped to region 93D6; 93E1 on chromosome arm 3R and shown to be allelic to *modifier of mdg4* [*mod(mdg4)*], a complex locus that encodes a large family of chromosomal proteins by alternative and *trans*-splicing. The encoded proteins together occupy more than 500 sites on the polytene chromosomes. We show that the *pf-2* mutations disrupt the function of a single isoform, Mod(mdg4)56.3, that is expressed in primary spermatocytes at all stages. Both a GFP-tagged Mod(mdg4)56.3 transgene and the native Mod(mdg4)56.3 protein localize as discrete foci to the major autosomes, and as an intensely fluorescent cluster of foci to the nucleolus throughout prophase. The nucleolar cluster resolves into a sharply defined structure associated with the X-Y bivalent. We conclude that Mod(mdg4)56.3 plays a critical role in homologous chromosome pairing in

Drosophila male meiosis. Transgenic flies with a *pf-2* null genetic background and carrying [*hsp70-pf2* cDNA] fragment on their chromosome II display a complete rescue of the pairing failure phenotype. The expression pattern of the GFP-labeled Mod(mdg4)56.3 in transgenic flies' meiotic cells implies a role for this novel gene in chromosomal cohesion during meiosis.

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List of abbreviations

AE: Axial Element

ATP: Adenosine Tri-Phosphate

bp: Base Pair

C. elegans: *Caenorhabditis elegans*

c.o.c.: Coefficient of Coincidence

c3G: Crossover Suppressor on 3 of Gowen

DAPI: 4'-6-DiAmidino-2-Phenyl Indole dihydrochloride

DCO: Double Cross Over

Df: Deficiency

DNA: Deoxyribo Nucleic Acid

DSB: Double-Strand Break

DSD: Doom Specific Domain

E 1-4: Exon 1-4

GFP: Green Fluorescent Protein

hsp: Heat Shock Protein

IAP: Inhibitor of Apoptosis Protein

IGS: Intergenic Spacer

kb: kilo bases = 1000 bases

Lac O: Lactose Operon

Lac I: Lactose operon Inhibitor

MAR / SAR: Matrix / Scaffold Attachment Region

MD: Map Distance

MI: Meiosis I

MII: Meiosis II

MTOC: MicroTubule Organizing Center

m.u.: Map Unit

NCO: No Cross Over

NDJ: Non-Disjunction

NHD: Non-Homologous Disjunction

NO: Nucleolar Organizer

nt.: Nucleotide

PCR: Polymerase Chain Reaction

pf-2: *pairing failure 2*

PSCS: Premature Separation of Sister Chromatid

RNA: Ribo Nucleic Acid

rRNA: ribosomal RNA

S. (*Cerevisiae* or *Pombe*): *Saccharomyces*

SA-2: Stromalin 2

SC: Synaptonemal Complex

SCO: Single Cross Over

SCP: Synaptonemal Complex Protein

SMC: Structural Maintenance of Chromosomes

SNP: Single Nucleotide Polymorphism

SCC: Sister Chromatid Cohesion

Chapter One

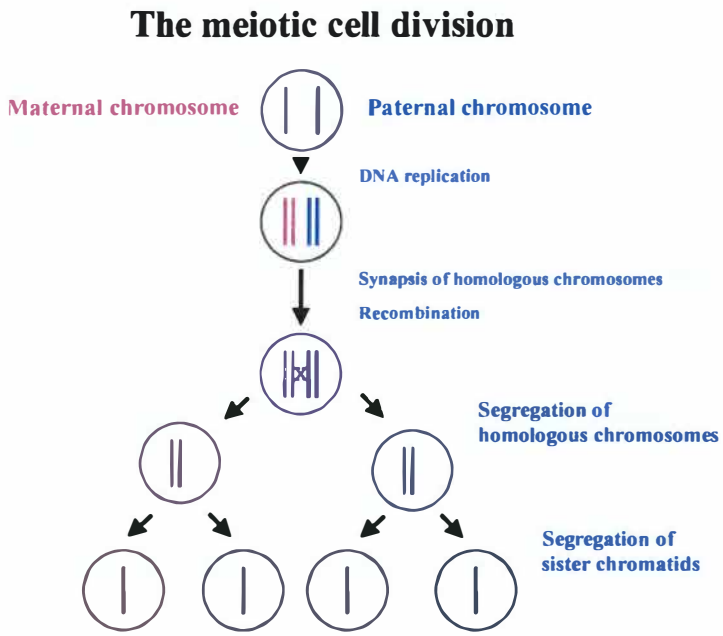
Background and Significance

I- Meiosis and chromosome pairing

I.1- Overview of meiosis

The continuity of life of species that reproduce sexually depends on the formation of genetically balanced gametes. Most sexual species are diploids, or multiples thereof, and must reduce the genome by half to produce gametes that can fuse to regenerate the full complement. Meiosis is the special type of cell division that results in the generation of haploid cells with half of the parental genomic complement. It consists of two cellular divisions preceded by a single round of DNA synthesis. The first division is reductional, meaning that the chromosome number is reduced in half, and the second division is an equational, mitosis-like division (Figure 1). Pairing and accurate segregation of homologous chromosomes during the first division of meiosis are essential for the generation of euploid gametes with a single copy of each pair of chromosomes. Mutations in any of the components of the pairing pathways involved in chromosome cohesion lead to abnormalities such as chromosome non-disjunction (NDJ) and aneuploidy (incorrect number of chromosomes), which are major causes of spontaneous abortions and mental retardation in human populations, or sterility that jeopardize the survival of the species (McKee, 1998; Hawley, 1988). Considering the clinical

a



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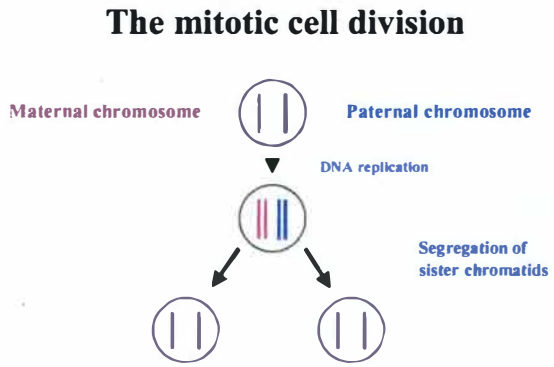


Figure 1: Nuclear divisions. Germ cells undergo meiosis (a), with the separation of homologs at the first, reductional division (MI) and of sister chromatids at the second, equational division (MII) resulting in generation of four haploid cells (gametes) from one original diploid parental cell. MII proceeds similar to mitosis (b), which leads to the production of two daughter cells with the same genetic complement as the parental cell.

significance of aneuploidy and the burden of genetic diseases it causes (e.g. Down, Turner or Klinefelter Syndromes) on society, it is important to uncover the mechanisms underlying chromosome pairing and disjunction during meiosis.

I.2- Sister chromatid cohesion and homologous chromosome pairing

At the first reductional division of meiosis, homologous chromosomes pair and then segregate to opposite poles. Sister chromatids segregate at meiosis II, the "equational" division, which is similar to mitosis. The formation and breakdown of the bonds between homologs and sister chromatids are tightly coordinated in order to lead to an accurate separation of chromosomes.

During replication in both mitosis and meiosis, sister chromatids pair along their entire length. A multi-subunit complex named 'cohesin' that is composed of two members of the Structural Maintenance of Chromosomes (SMC) family, SMC1 and SMC3, and Sister Chromatid Cohesion proteins, SCC1 and SCC3, holds sister chromatids together (Strunnikov, 1999; Hirano, 1999; Hirano, 1998). SMC proteins have DNA binding sites and an ATPase domain at one end. SMC1 and SMC3 each contain two long coiled-coil domains separated by a flexible linker region. In cohesin, SMC1 and SMC3 each folds back on itself to form a long intramolecular antiparallel coiled-coil. The SMC1 / SMC3 heterodimer is a V-shaped structure stabilized by interactions between the linkers. The DNA binding / ATPase domains of each of the SMC1 and SMC3 subunits is composed of N-terminal and C-terminal sequences at the ends opposite the linker. These juxtaposed ends are linked to SCC1. The closed ring that is formed holds the

DNA strands in its center (Petronczki et al., 2003). Cohesin is thought to be loaded on single chromatids prior to or during S phase, and passage of the replication fork through the ring during replication establishes cohesion. The release of cohesion between the arms of sister chromatids at anaphase is mediated by a caspase-like protein called separase, which cleaves the SCC1 subunit (Stoop-Meyer and Amon, 1999; Buonomo et al., 2000). During prophase of mitosis in metazoans, cohesin complexes are removed from the arms of the chromosomes by an unknown mechanism that depends upon phosphorylation, but the chromatids remain attached at the centromeres so that their bipolar attachment to the spindle occurs. At anaphase, the proteolysis of SCC1 results in the opening of the ring and the movement of chromosomes toward opposite poles (Cohen-Fix, 2001).

In meiosis, cohesin is retained on the chromosome arms until anaphase I where it helps to stabilize the linkages between homologs (Michaelis et al., 1997; Klein et al., 1999). Its removal at anaphase I releases the homologs to segregate to the poles. Cohesins then remain only at the centromeres where they persist until anaphase II, at which time a second phase of separase cleavage removes the centromeric cohesin and releases the sister chromatids (Figure 2; for review see van Heemst and Heyting, 2000; Katis et al., 2004).

At early meiotic prophase, chromosomes condense and an "axial element" (AE) forms between the two sister chromatids of each homolog. AEs are unique to meiotic chromosomes and consist of both a cohesin "core" and additional meiosis-specific proteins (reviewed by Scherthan, 2003). Early in meiotic prophase, a homology search results in the alignment of homologs side by side

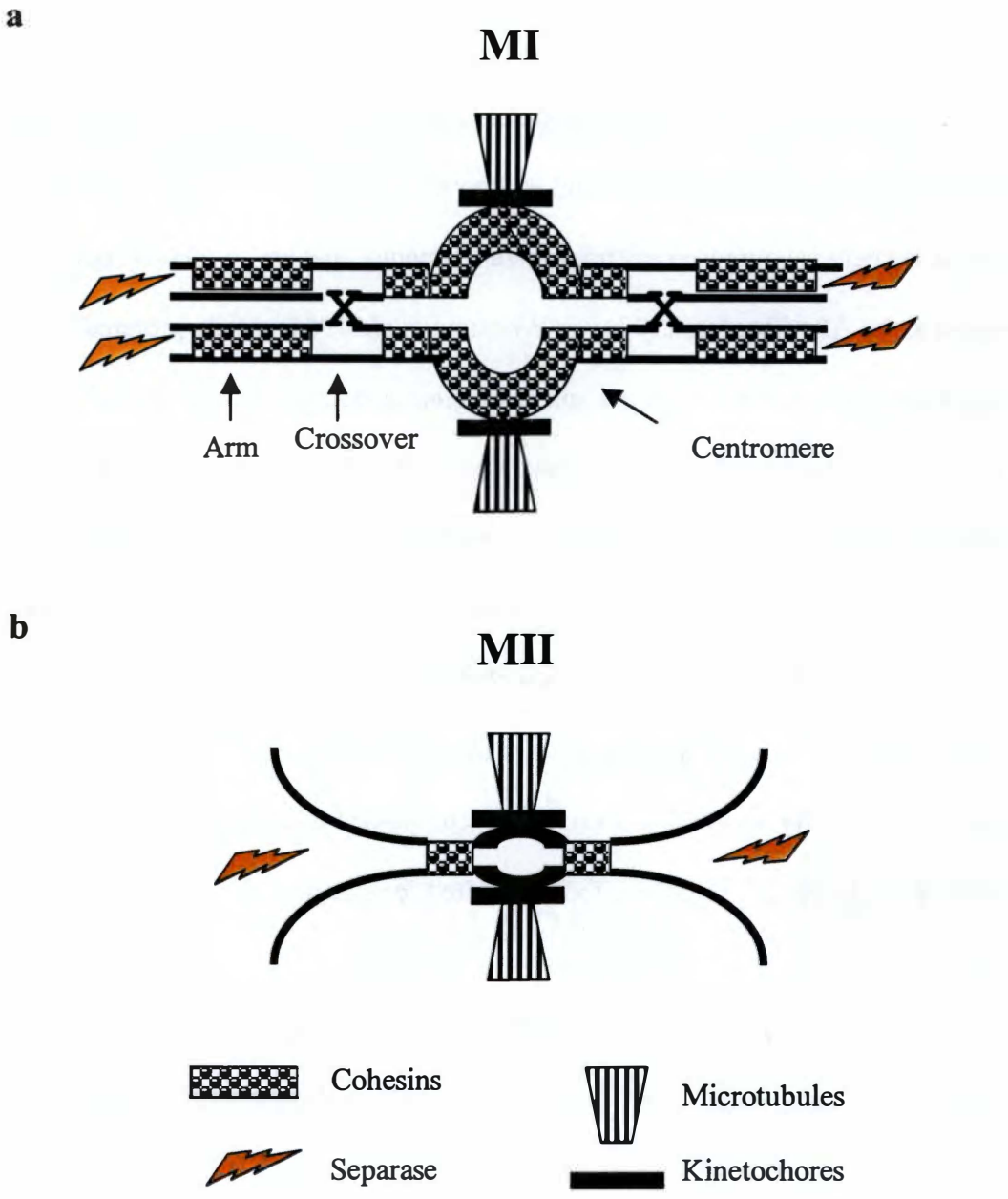


Figure 2: Alternative roles of cohesins during the two meiotic divisions. (a) The proteolytic activity of separase removes sister chromatids' arm cohesion leading to the segregation of homologs to opposite poles at anaphase I. (b) At anaphase II, the centromeric cohesion is released resulting in separation of sister chromatids.

(Roeder, 1997; Weiner and Kleckner, 1994; Loidl, 1990). The weak association of chromosomes is then replaced by a more stable bonding mediated by a proteinaceous structure called synaptonemal complex (SC). The two AEs become connected via a "central element" and will now be called "lateral elements". The central element is composed of "transverse filaments" that are formed at right angles to the AEs (Roeder, 1997; von Wettstein et al., 1984). Many proteins have been identified in yeast, mammals and *C. elegans* as being components of the SC (reviewed by Heyting, 1996; Zickler and Kleckner, 1999). Identified central elements components, such as yeast Zip1 and mammalian SCP3 are long coil-coiled proteins that form the transverse filaments (Sym et al., 1993; Dobson et al., 1994). In *Drosophila*, *c(3)G* encodes a probable component of the central element with a similar structure and is required for synapsis and meiotic exchange (Page and Hawley, 2001). *mei-P22* is a known SC component in *Drosophila*, most likely an axial element protein, it too is required for synapsis and recombination (Liu et al., 2002).

Concomitantly to the formation of SC, meiotic recombination occurs and the sites of crossovers, called chiasmata, are responsible for holding homologous chromosomes together after the removal of SC at late prophase (Walker and Hawley, 2000; Padmore et al., 1991; Alani et al., 1990). The maintenance and stability of chiasmata depends on sister chromatid cohesion distal to the sites of crossovers (for review see Moore and Orr-Weaver, 1998; Buonomo et al., 2000; Bickel et al., 2002).

Cohesins are also required for the meiotic division but some of the mitotic subunits are replaced by meiosis-specific ones. A meiosis-specific variant of Scc1, Rec8, is expressed at the onset of the premeiotic replication and is part of the cohesin complex that maintains sister chromatids together throughout meiosis (Nasmyth, 2001; Klein et al., 1999; Molnar et al., 1995).

At least in spermatocytes, two other meiosis-specific variants of cohesin subunits have been identified in mice and humans: STAG3 replaces STAG1 and STAG2, which are homologs of Scc3 (Prieto et al., 2001; Pezzi et al., 2000) and SMC1β is a meiotic version of Smc1 (Revenkova et al., 2001).

Other proteins that are not part of the cohesin complex but play a role in sister chromatid cohesion have also been identified such as MEI-S332 (Kerrebrock et al., 1992) and orientation disruptor (ORD) (Mason, 1976) in *Drosophila melanogaster*. Mutations in both genes cause premature sister chromatid segregation (PSCS) in both male and female meiosis. MEI-S332 localizes to centromeres from metaphase I until anaphase II and is removed when chromatid cohesion is lost (Kerrebrock et al., 1995). It is thought to function in some way as a protector of centromeric cohesin to prevent its premature removal at anaphase I. Recently, homologs of MEI-S332 have been reported in several eukaryotes including shugoshin 1 (Sgo1) in fission yeast, *Schizosaccharomyces pombe*, and Sgo1-like proteins in *Saccharomyces cerevisiae* and *Neurospora crassa*. Mutations in Sgo1 in these species cause PSCS in meiosis (Kitajima et al., 2004; Rabitsch et al., 2004).

Arm and centromeric cohesion during *Drosophila* meiosis as well as recombination are affected by null mutations in *ord*. ORD is localized on oocyte chromosomes and promotes crossovers between homologs by preventing exchange between sister chromatids (Webber et al., 2004). In males, *ord* mutations cause PSCS and NDJ at both meiosis I and meiosis II (Balicky et al., 2002).

I.3- Recombination and synapsis

In *S. cerevisiae*, meiotic recombination is initiated by induction of DSBs by a putative topoisomerase Spo11 (Keeney et al., 1997; Bergerat et al., 1997). Mei-W68, the fly homolog of Spo11, is also required for meiotic recombination in *Drosophila*, which implies that formation of DSBs is the initiating event in *Drosophila* meiotic recombination as well (McKim and Hayashi-Hagihara, 1998; Keeney et al., 1997). Similar observations have been made in several other organisms; leading to the view that meiotic recombination may be universally initiated by Spo11-induced DSBs (Gadelle et al., 2003).

Supporting evidence for a central role of DSBs in meiotic recombination comes from the finding that meiotic nuclei in several eukaryotes stain strongly during early prophase with antibodies against the phosphorylated form of histone 2AX, a chromatin modification induced by and diagnostic of DSBs (for review see Pilch et al., 2003). Moreover, genes involved in the repair of double-strand DNA damage, especially those involved in the "Rad52" DSB repair pathway,

during mitosis are also required for meiotic recombination in all eukaryotes that have been studied (reviewed by Dudas and Chovanec, 2003).

Of particularly central importance for both homologous repair of DNA damage and meiotic recombination are eukaryotic homologs of the bacterial recombinase RecA enzyme, which is required for homologous DNA pairing and strand exchange. In eucaryotes, Rad51, and other Rad51-like proteins are essential for the homologous repair of DNA DSB damage, and along with the meiosis-specific paralog Dmc1, for repair of meiotic DSB as well. Mutations in several of these genes, as well as in other Rad52 pathway genes lead to accumulation of meiosis-specific double strand breaks, often accompanied by meiotic prophase arrest, thought to be due to a checkpoint sensitive to unrepaired DNA breaks (Zierhut et al., 2004; Wan et al., 2004; Lee et al., 2003; Abdu et al., 2003; Vaze et al., 2002; Klein, 2001; Lydall et al., 1996; Bishop et al., 1992).

In most organisms, such as yeast and mouse, the initiation of DSBs resulting in meiotic recombination precedes synapsis (Roeder, 1995; Kleckner, 1996). In *Drosophila* females, however, chromosome synapsis occurs in the absence of meiotic exchange (McKim et al., 1998). In yeast, the SC is not necessary for meiotic recombination but in mutants for DSB processing and repair pathway synapsis is defective or delayed (Roeder, 1997). However, in *Drosophila* females SC is necessary for the completion of recombination as in mutants lacking C(3)G (Crossover suppressor on 3 of Gowen, a structural component of the SC) meiotic exchange is eliminated (Page and Hawley, 2001). The phenotype associated with the absence of Spo11 in C. *elegans* is different than in *S. cerevisiae*: non-

recombined homologs still synapse and SC is formed (Dernburg et al., 1998). Females of *Bombyx mori* are achiasmatic, and SC is always formed in the absence of recombination (Rasmussen, 1977).

I.4- Proteins required for homologous chromosome pairing

As discussed above, pairing and segregation of chromosomes during cell cycle divisions are of special importance for the inheritance of a complete copy of the genome by daughter cells. Beside all of the subunits of cohesin, condensin and SC complexes, as well as the protein components of the DSB and repair pathways, many other factors function to ensure the accuracy of meiotic events. The movement of chromosomes toward the metaphase plate and their alignment on the plate, the stable bipolar attachment of kinetochores to the spindle fiber made of microtubules, the checkpoint mechanisms ensuring the proper positioning and orientation of the chromosomes, the cohesion and separation of sister chromatids / homologs in the arms or at the centromere, all involve complex protein-protein interactions and enzymatic activities. A defective component could result in inaccurate or failed chromosome recombination or segregation.

Different organisms have developed a variety of pathways to identify homologous partners and to separate them from each other. Numerous proteins are involved and their functions are tightly regulated to ensure the accuracy of these meiotic processes (for reviews see Roeder, 1997; Nasmyth, 2002; Page and Hawley, 2003; McKee, 2004).

Telomeric regions that act as pairing sites may represent target sequences for specific protein aggregates. In many organisms, during early prophase stages, telomeres cluster and form a "bouquet" by attachment to the nuclear envelope and disperse during pachytene (reviewed by Scherthan, 2001). This configuration may facilitate pairing by bringing the chromosome ends within a limited region (Zickler and Kleckner, 1998). In budding yeast, Tam1/Ndj1 was identified as a telomeric protein that might function in the pairing process as mutants displayed a delayed synapsis and a decreased recombination frequency (Chua and Roeder, 1997; Conrad et al., 1997).

In meiotic cells of *S. pombe*, SC is not formed and homologs pair through discontinuous structures called "linear elements" that also promote the exchange events (Yamamoto and Hiraoka, 2001). In *S. pombe*, a pronounced clustering of telomeres near the microtubule organizing center (MTOC) has been observed that oscillates between the two poles during the entire meiotic prophase (Chikashige, 1994; reviewed by Schertan, 2001). Recently, the lacO-lacI-GFP tagged chromosome loci have been studied to demonstrate the movement of chromosomes in meiotic nuclei (Ding et al., 2004). By analyzing the pairing of homologous loci located either at the centromeres or at the telomeres, in wild type and different mutant genetic backgrounds, where telomere clustering is disrupted or a microtubule motor protein is defective, Ding and coworkers show that telomere clustering and oscillation of the chromosomes play an important role in homolog pairing during meiosis. However, mechanisms underlying pairing at the centromeres or in the arms seem to be different.

In *Caenorhabditis elegans* (*C.elegans*), chromosomes enter meiosis unpaired but rapidly align at the onset of leptotene. At the end of each chromosome a single site called the "homolog recognition region" (HRR) promotes and stabilizes homolog pairing in its proximity, even in mutants with no SC (MacQueen et al., 2002). HRRs may act as loading sites for a protein complex involved in chromosome pairing. Several proteins required for pairing in *C. elegans* have been identified. Mutations in high incidence of males (*him*)-3 lead to high frequencies of inviable embryos and surviving adult progeny are mostly males. HIM-3, a meiosis-specific non-cohesin component of chromosome axes and required for synapsis, has recently been shown to be involved in initial homolog alignment, SC assembly and progression of meiotic recombination (Couteau et al., 2004). Chk2, another *C.elegans* protein, belongs to the family of check-point protein kinases that link upstream signaling pathways to specific cell-cycle targets. It was identified in mutants displaying pairing failure of homologous chromosomes and was shown to be involved in spatial nuclear reorganization during early meiotic prophase resulting in the establishment of initial alignment of homologous chromosomes (MacQueen and Villeneuve, 2001).

A unique phenotype associated with the absence of a yeast meiotic gene, *HOP2*, was the formation of SC between non-homologous chromosomes and decreased pairing of homologous ones, as well as synapsis of one chromosome with different partners (Leu et al., 1998). Therefore, the wild type function of Hop2 is to be localized on chromosomes, prevent their rearrangements by excluding ectopic recombination between dispersed repeated sequences and

ensure their proper segregation. In a screen for genes capable of suppressing the *hop2* defect, Rabitsch et al. (2001) identified *MND1*, whose disruption led to the absence of SC formation. Mnd1 and Hop2 form a complex that is involved in homolog pairing and DSB repair during meiosis (Tsubouchi and Roeder, 2002). Both proteins have homologs in other organisms and based on the severe meiotic defects displayed by Hop2 knockout mice (Petukhova et al., 2003) it seems likely that the function of these genes is conserved across species.

In *Drosophila* males, pairing is not mediated by DSB, exchange or SC. Arrays of 240-bp repeats in IGS are pairing sites for X and Y chromosomes (Mckee, 1996) and may be bound by nucleolar proteins in order to hold these two chromosomes together. The lack of identified male-specific meiotic genes with a function in the pairing and segregation of homologous chromosomes during meiosis I has greatly hindered progress in understanding the mechanisms underlying these specific processes. In the following chapters, I describe a novel gene required for male meiotic pairing. Through analysis of this gene, it is likely that our questions regarding the progression of meiotic events will be answered in the near future.

II- *Drosophila* meiosis

II.1- Overview of meiosis in *Drosophila*

Drosophila melanogaster uses a variety of pairing pathways to ensure the accurate pairing and segregation of meiotic chromosomes. Two major systems,

the recombination-based one involving SC and chiasmata, and an entirely non-recombinational pathway exist in germ cells of female and male respectively. Females also have an achiasmate backup system, called distributive segregation, which ensures the disjunction of non-exchange chromosomes. The tiny fourth chromosomes are always achiasmate and do not undergo exchanges, yet they segregate faithfully. Moreover, regular disjunction of chromosomes 1, 2 and 3 occurs even when exchange is suppressed by multiple inversions on balancer chromosomes or by other means. This system is influenced by the availability, size and shape of the chromosomes but also depends upon homology, particularly in heterochromatic regions (for review see Hawley and Theurkauf, 1993). Pairing also occurs during interphase in all somatic cells of *Drosophila* and other Dipterans (McKee, 2004).

An advantage of *Drosophila* for meiotic studies is to facilitate the comparison of various strategies used by males, females or both to ensure the alignment of homologs and their accurate segregation. It is of interest to determine the shared features among these pathways as well as the distinguishing features that discriminate between the male / female, recombinational / non-recombinational and meiotic / somatic specific processes.

II.2- *Drosophila* meiotic genes

II.2.a- Female meiotic genes

Most of the identified meiotic genes in *Drosophila* affect either sister chromatid cohesion and are common to males and females or are specific for pathways such as recombination, synapsis or distributive disjunction that are unique to female meiosis (Orr-Weaver, 1995; Sekelsky, 1999, Mckee, 2004).

In *Drosophila*, mutations in the Rad51 gene *spindle-A* (*spnA*, Staeva-Vieira, 2003), and two of the Rad51-like genes, *spindle-B* (*spnB*), *spindle-D* (*spnD*, Abdu et al., 2003) as well as in a Rad54 homolog, *okra* (*okr*) reduce recombination, increase NDJ and result in defective patterning of the eggshell, called the *spindle* phenotype (Ghabrial et al., 1998; Morris and Lehmann, 1999). *spnA* females are sterile so the evidence for reduction of recombination and increase of NDJ is from RNA interference (RNAi, Yoo and McKee, 2004). The failure of DSB repair in these mutants activates a meiotic checkpoint that leads to decreased levels of a morphogen called Gurken resulting in an altered dorso-ventral patterning of the egg (Ghabrial et al., 1998). *Drosophila* homologs of Spo11 (*mei-W68*; McKim and Hayashi-Hagihara, 1998; reviewed by Carpenter, 2003), a yeast topoisomerase II type endonuclease that creates DSBs required for initiating meiotic recombination, and the cell cycle checkpoint Chk2 kinase (*mei-41*) suppressed the *spindle* phenotype when mutated (Ghabrial and Schupback, 1999; Abdu et al., 2002), thus supporting the idea that the spindle phenotype is triggered by unrepaired DSBs.

In addition to the primary, exchange-mediated system that requires the formation of chiasmata for proper disjunction of homologous chromosomes (Hawley, 1988), another system functions in *Drosophila* females that is specifically involved in segregation of achiasmate chromosomes (Grell, 1962). The first requirement of this secondary means of homologous disjunction, called distributive segregation, is the identification of non-exchange chromosomes. The *altered disjunction* (*ald*; O'Tousa, 1982) mutation disrupts this step as in mutant females, chiasmate X chromosomes undergo non-homologous disjunction at high frequencies. The second step of the distributive system consists of the choice of partners and has been defined by three mutations, *mei-S51* (Robbins, 1971), *ald* (O'Tousa, 1982) and "*Aberrant X segregation*" (*Axs*; Zitron and Hawley, 1989; for review see Kramer and Hawley, 2003), all of which alter the correct segregation events, e. g. the disjunction of the X chromosome from the small fourth chromosome. The orientation and separation of the chromosomes is the third stage of the distributive process and was defined by mutations in "*no distributive disjunction*" (*nod*; Carpenter, 1973), with effects on the disjunction of the always achiasmate fourth chromosome (for reviews see Hawley and Theurkauf, 1993; Orr-Weaver, 1995). *nod* encodes a kinesin-like chromosomal protein (Afshar et al., 1995) with a microtubule-stimulated ATPase activity that might be involved in the attachment of chromosomes to microtubules (Matthies et al., 2001).

None of these mutations that affect distributive disjunction in females by causing high frequencies of meiotic and mitotic chromosome loss and NDJ;

incorrect partner choice; and effects on exchange-mediated disjunction and size recognition, respectively, disrupt male meiotic events.

II.2.b- Male meiotic genes

Drosophila male meiosis appears to be a relatively simple system in which crossing over is absent and SC and chiasmata are not formed. In *Drosophila* males, mutations in the Spo11 homolog *mei-W68* or in the Rad52 pathway genes, *spnA*, *spnB*, *spnD* and *okr* did not result in meiotic phenotype, e.g. altered homologous chromosome segregation. Mutations in the SC genes such as *c(3)G* or *mei-P22*, are similarly without male meiotic phenotypes (for reviews see Walker and Hawley, 2000; McKim et al., 2002; McKee, 2004). These findings are consistent with the well-documented absence of crossing over and SC in male meiosis. More surprising is the failure of mutations that disrupt the distributive segregation system in females, which acts on non-exchange chromosomes to ensure their segregation at anaphase I, to affect male meiotic segregation.

Despite several screens for mutations affecting meiosis in *Drosophila*, few genes have been recovered as being specifically responsible for male meiosis. Many previously identified male meiotic mutations have been lost either by reversion or by careless stewardship (reviewed by Orr-Weaver, 1995; Lindlsey and Zimm, 1992).

Some male-specific mutations that cause meiosis I NDJ affect only certain chromosomes, some affect autosomes only (*teflon*, Tomkiel et al., 2001), or just the 4th chromosome (*mei-S8*, Sandler et al., 1968). Several X chromosomal EMS-

induced mutations thought to be specific for X-Y segregation were recovered but mysteriously reverted before they could be fully characterized (Baker and Carpenter, 1972). However, the *mei-081* and *mei-I1* genes were found to cause high NDJ rates of all the chromosomes (Sandler et al., 1968; Ivy, 1981).

Among the male-specific genes, only *teflon* (*tef*) has been cloned and it encodes a zinc finger protein of unknown function. Mutations in *tef* were shown genetically and cytologically to disrupt the segregation of all of the autosomes, but the disjunction of sex chromosomes remains unperturbed. No effect on female meiosis was detected and mitosis as well as meiosis II divisions proceed normally. Cytological analysis of primary spermatocytes from *tef* mutants showed unpaired chromosomes at late prophase. Based on this observation, the authors speculated that *tef* might play a role in the maintenance rather than the initiation of pairing. However, early stages of meiosis as well as the premeiotic chromosome configuration have to be studied with more sensitive techniques such as GFP-tagging of chromosomal sites in order to determine the exact timing of the pairing defect in *tef* mutants (Tomkiel and Briscoe, 2001).

II.3- Cytological aspects of *Drosophila* male meiosis

Fluorescent dyes such as DAPI or Hoechst 33258 allow the staining of chromosomes and their visualization at different stages of meiosis (Fuller, 1993; Cenci, 1994).

Pre-meiotic S phase occurs immediately after the last gonial mitosis and is followed by a 4-day growth period in which spermatocytes increase 25 fold in

volume. A detailed analysis of early stages of meiosis in young primary spermatocytes (stages S0-S2) by Cenci and his coworkers showed that all the chromosomes are initially clustered in the middle of the nucleus but gradually segregate into discrete chromosomal territories associated with the inside of the nuclear membrane. These separate territories are evident by stage S3 and persist until the onset of chromosome condensation just before prometaphase. The beginning of prometaphase is marked by the breakdown of the nuclear envelope (stage M1) and chromosomes begin moving toward the center of the nucleus while continuing to condense. Condensation allows the visualization of chromosomes with non-fluorescent dyes such as acetic-orcein. Condensed prometaphase chromosomes exhibit an extremely compact, typically spherical morphology.

Prior to this condensation of chromosomes at late prophase, the chromatin is too decondensed to allow an assessment of whether homologs are paired. The demonstration of their pairing at these early stages has been possible by the use of GFP-labeling of individual loci and the use of deconvolution microscopy (Vazquez et al., 2001).

II.4- Meiotic pairing in *Drosophila* males

II.4.a- Cytological evidence

In the early 1900s, it was reported that the pairing of homologous chromosomes occurs in premeiotic cells, as early as anaphase of the last mitotic

gonial division (Metz, 1926; Stevens, 1908). However, significant insights into mechanisms underlying the pairing of chromosomes during interphase and early meiotic prophase were obtained recently by the use of LacI-GFP system that allowed tracking the movement of chromosomes bearing LacO sequences, as targets of the tagged LacI, in several euchromatic regions (Vazquez et al., 2001). The study of live primary spermatocytes by Vazquez and coworkers (2002) showed that euchromatic regions are tightly paired quite early in male meiosis as shown by the unresolved GFP spots at single loci in more than 95% of young primary spermatocytes, compared to about 50% of premeiotic spermatogonia. Their junction persists through the first half of G2 until at mid G2 four distinct spots representing the four sister chromatids forming a bivalent appear. This indicates that both sister chromatids and homologs have fallen apart and they remain separated throughout meiosis I. However, homologous and sister loci remain within a common chromosomal territory throughout the latter half of meiotic G2 phase. Further investigation is required to answer the question of how these meiotic DNA strands remain associated from mid G2 until anaphase I and whether their attachment occurs at specific pairing sites.

By labeling the centromeric regions with a GFP-CID fusion protein, Vazquez et al. (2002) concluded that sister centromeres are tightly paired throughout G2, but homologous centromeres are unpaired except for a brief period in mid-G2, at early S3 stage when chromosomal territories are newly formed, by an unknown mechanism that seems likely to involve components of the nuclear matrix.

In summary, the euchromatic regions are intimately paired in early G2 but separate by mid G2 (stage S3). Chromosomes remain in proximity of each other by virtue of associations as yet uncharacterized (for reviews see McKee, 2004; Hawley, 2002).

II.4.b- Chromosomal pairing sites

It was first demonstrated cytologically that *Drosophila* X and Y chromosomes are linked and their association occurs at one or a few sites on each chromosome, called collochores (Cooper, 1964). Genetically, X chromosomes with a heterochromatin deficiency (Xh) caused X-Y NDJ indicating that pairing sites would be located within these sequences (McKee and Lindsley, 1987). These pairing sites did not include sequences such as satellites and seemed to be composed of more specific regions. The two sex chromosomes have many different types of repeated sequences within their heterochromatin. Of particular importance are the nucleolus organizers (NOs) that are present only on the X and Y chromosomes in *Drosophila*. Each NO consists of ~250 copies of rRNA genes and these arrays are not present in Xh deficiencies that display an X-Y pairing defect (for review see McKee, 1996). Xh deficient flies carrying transgenic insertions of ribosomal DNA sequences were tested and it was found that a single complete rRNA gene including the promoter for RNA polymerase I and the intergenic spacer (IGS) regions of ribosomal rRNA genes, could partially restore the pairing and disjunction of the X and Y chromosomes (McKee and Karpen, 1990). Further studies showed that the 240-bp repeated sequences within the IGS

were sufficient for X-Y pairing. Six to twelve 240-bp units are present within each IGS and the X-Y pairing ability of the Xh deficient flies can be recovered with only 6 copies of the 240-bp units and the presence of additional copies correlated with improved pairing and disjunction of X and Y chromosomes. These IGSs reside within the heterochromatic regions at the base of the X chromosome and the short arm of the entirely heterochromatic Y chromosome (McKee et al., 1992; Merill et al., 1992; Ren et al., 1997), corresponding to the cytological location of the collochores.

Pairing sites of autosomes are distributed much differently than for the X-Y pair. In flies carrying transpositions of euchromatic fragments of chromosome 2 to the Y chromosome, the Y segregates from a normal chromosome 2. Quadrivalents consisting of the X, Y^2, 2^Y and 2 are observed at late prophase / prometaphase and at anaphase. Both of these effects occur at frequencies that are proportional to the size of the transposed region, suggesting that pairing sites are distributed along the entire length of the euchromatic chromosome arms. However, 2-Y transpositions involving only heterochromatin have no effect on segregation or on quadrivalent frequencies (McKee et al., 1993; reviewed by McKee, 1996). This, along with several other observations, argues that in male meiosis, heterochromatic regions do not play a role in pairing of autosomes. This is another difference between male and female meiosis in *Drosophila* as distributive segregation of the homologs in female meiosis has been shown to depend upon pairing within heterochromatic regions of both the X and 4^{th} chromosomes (Hawley et al., 1993; Karpen et al., 1996; Dernburg et al., 1996).

We have a good map of chromosomal pairing sites but no knowledge about the trans-acting factors that are required to mediate pairing. We also have a description of the dynamics of pairing in male meiosis: intimate pairing throughout the euchromatin in early G2, loss of pairing at the mid-G2 transition after the establishment of territories. Key unanswered questions are: what factors mediate the intimate pairing of homologous sequences in early prophase? And how do the homologs remain connected during late G2 despite the loss of pairing, or more precisely, what substitutes for chiasmata in achiasmatic meiosis?

III- Modifier of mdg4 (*mod(mdg4)*)

The primary goal of this research was to identify and characterize novel male meiotic mutations in order to uncover mechanisms underlying the transmission of parental genome to future offspring. The screen for *Drosophila* male-specific mutations led to the identification of a novel meiotic gene, *modifier of mdg4* (*mod(mdg4)*), that encodes a chromosomal protein with a very complex genomic structure. This introduction is intended to provide necessary background regarding the function of this gene for a better interpretation of the collected data (for review see Dorn and Krauss, 2003).

III.1- Structure

mod(mdg4) is a very complex gene, encoding over 33 isoforms generated by alternative and *trans*-splicing, most or all of which are chromosomal proteins (Dorn et al., 1993; Gerasimova et al., 1995; Buchner et al., 2000; Labrador and

Corces, 2003; Krauss and Dorn, 2004). The first four exons (402 amino acids) are common to all isoforms and encode an evolutionarily conserved domain called BTB (Broad complex, Tramtrack, Bric a brac) (Zollman et al., 1994; Ahmad et al., 1998). This 115- residue motif plays a role in protein – protein interactions and mediates the dimerization / multimerization of many transcriptional regulators involved in a wide variety of developmental processes (Bardwell and Treisman, 1994; Buchner et al., 2000; Read et al., 2000).

The second motif identified in the C-terminal sequence of most of the Mod(mdg4) isoforms, consists of 2 Cysteine and 2 Histidine residues along with 4 other hydrophobic amino acids that are also evolutionarily conserved. This motif is called Cys_2His_2 or FLYWCH and forms one zinc finger domain with an unknown function (Buchner et al., 2000; for review see Dorn and Krauss, 2003). The discovery that interactions between two of the isoforms, Mod(mdg4)67.2 and Mod(mdg4)56.3, and their respective partners, Su(Hw) and inhibitor of apoptosis proteins (IAPs), are mediated by the FLYWCH motif, suggests that this domain might play a role in protein-protein interactions (Gause et al., 2001; Ghosh et al., 2001; Harvey et al., 1997). Also, a *Drosophila* transcriptional activator called GAGA factor has a high structural homology to Mod(mdg4) as they both contain the N-terminal BTB domain and the one C-terminal Cys_2-His_2 zinc finger motif (Farkas et al., 1994; for review see Granok et al., 1995). The binding of GAGA to DNA via a single zinc finger domain has been well documented (Pedone et al, 1996; Wilkins and Lis, 1998; Wilkins and Lis, 1999) and the requirement for the N-terminal BTB / POZ domain of the protein has also been reported (Katsani et

al., 1999; Espinas et al., 1999). Based on this finding, a role in DNA-binding cannot be excluded for the Cys_2-His_2 motif.

Seven out of 33 specific exons are encoded by the antiparallel strand of the DNA duplex (Dorn et al., 2001; Labrador et al., 2001). The generation of mature mRNAs could be explained by the *trans*-splicing of two independent pre-mRNAs. Dorn and colleagues (2001) demonstrated experimentally the occurrence of the *trans*-splicing by inserting sequences encoding the two transcription units on different chromosomes. The generation of mature transcripts led to the conclusion that *trans*-splicing occurs not only for exons located on the complementary strand, but also between exons in *cis*, residing on the same coding strand. This hypothesis was supported by the detection of independent endogeneous promoter regions driving the transcription of some of the specific C-terminal exons (Dorn et al., 2001). Further studies by Mongelard et al. (2002) indicated that trans-splicing accounts for the recovery of the wild type function in flies heterozygous for two independent mutations, one within the 5' common region and one in the specific C-terminal exon of Mod(mdg4)67.2 isoform.

III.2- Function

Most of the alleles of *mod(mdg4)* that have been studied bear an alteration within the common region. Most of the alleles that disrupt the coding sequence of the common region are recessive lethals, and several have been shown to cause embryonic lethality. Thus, the locus as a whole is essential for embryonic development. One of the lethal alleles of *mod(mdg4)* proved to have 2 amino acid

changes in the conserved BTB domain, suggesting that this domain is essential for viability (Read et al., 2000). Some of these alleles have also been shown to have dominant effects on position effect variegation and on expression of homeotic genes (Gerasimova et al., 1995; Buchner et al., 2000; Krauss and Dorn, 2004). Thus *mod(mdg4)* is classified as a modifier (enhancer) of PEV and as a member of the *Trithorax group* (*Trx-G*) (Gerasimova et al., 1998; for review see Dorn and Krauss, 2003). Hypomorphic alleles that affect axon growth during embryonic development have also been described (Gorczyca et al., 1999).

It is not surprising that mutations in the common region would exhibit pleiotropic mutant phenotype as all of the isoforms would be affected. Mutations within a specific exon disrupting only one isoform could provide information regarding the specific function of each of these isoforms.

Currently, only two alleles specific for one isoform of *mod(mdg4)* have been identified. $mod(mdg4)^{T6}$ and $mod(mdg4)^{u1}$ disrupt the specific exon of Mod(mdg4)67.2 protein (Gause et al., 2001). This specific isoform is not essential as homozygote flies are viable. Its role in chromatin insulator function has been widely investigated.

Insulators are sequences that prevent the enhancer – promoter interaction when placed between these two elements (Dorsett, 1999). The tissue-specific expression of the *yellow* gene of *Drosophila* is under the control of five different enhancer sequences located upstream of this gene. When *gypsy*, a 7.3-kilobase retrotransposon carrying 350 base-pair insulator sequences, is inserted between the enhancer and promoter of the *yellow* gene, it disrupts their communication and

inactivates the transcription and expression of *yellow* in specific tissues (Geyer et al., 1986; Gdula et al., 1996; for review see Gerasimova and Corces, 1996).

gypsy insulator function depends upon the products of two genes: *su(Hw)* and *mod(mdg4)*. Su(Hw) binds to the 350-bp insulator sequences of *gypsy* (Spana et al., 1988) through its twelve zinc finger motifs (Spana and Corces, 1990). *mod(mdg4)* encodes a large family of chromosomal proteins; one of which, Mod(mdg4)67.2, is involved in the insulator function of *gypsy* retrotransposon (for reviews see Gdula et al., 1996; Bell et al., 2001; Gerasimova and Corces, 2001).

Immunofluorescence experiments using antibodies against Mod(mdg4) and Su(Hw) proteins revealed the presence of Mod(mdg4) at hundreds of sites on polytene chromosomes from salivary glands overlapping all of the Su(Hw) binding sites. The direct interaction between these two proteins has been demonstrated under *in vivo* conditions by yeast two-hybrid assay (Ghosh et al., 2001). Surprisingly, in interphase nuclei of diploid cells of imaginal discs, immunofluorescence reveals only 20-25 foci (Gerasimova and Corces, 2001). The model proposed by these authors involved the juxtaposition of distant insulator sites through interactions between chromosomal proteins and the nuclear matrix, forming large rosette-like structures. This nuclear organization of the chromatin fiber, imposed by *gypsy* insulator sequences, is postulated to be important for the regulation of gene expression.

Another isoform, Mod(mdg4)56.3, also known as Doom, was isolated in a yeast-two-hybrid screen for proteins interacting with IAPs of Baculovirus

(Harvey et al., 1997). The overexpression of Doom induced apoptosis in *Drosophila* S2 cells. The binding of Doom to IAPs, mediated by the FLYWCH domain on the specific C-terminal exon, strongly suggests that each isoform may play a specific role in different cellular pathways.

In this dissertation, I document a novel phenotype of *mod(mdg4)* mutations, namely pairing failure of meiotic homologous chromosomes, and show that these mutations specifically disrupt the Mod(mdg4)56.3 (Doom) isoform.

Chapter Two

A screen for male meiotic mutations reveals a novel gene required for homolog pairing in *Drosophila* males

I- Introduction

Meiosis is an important process in sexually reproducing organisms and results in the production of gametes with reduced chromosome number to yield zygotes with the proper ploidy. Although meiotic events have been extensively studied, many of the mechanisms by which they occur remain obscure. Mistakes that occur during meiosis can have various consequences including sterility and lethality. These problems occur in diverse organisms from yeasts to *Drosophila* to humans.

Drosophila melanogaster males offer an excellent system for the study of chromosome pairing and segregation. As mentioned earlier, the major difference between male and female meiotic pathways is the absence of synaptonemal complexes (Meyer, 1960) and recombination (Cooper, 1964) in males, which must have adopted other primary mechanisms responsible for proper pairing and separation of homologous chromosomes. In order to dissect these pathways, it is necessary to study mutants that are affected at various steps in the meiotic process.

Sandler and coworkers undertook large-scale screens for mutations affecting *Drosophila* meiosis in 1968 followed by Baker & Carpenter in 1972. They have

provided much of the material used in the last 35 years to study *Drosophila* meiosis. Other screens done by Castrillon (1993), Sandler (1971), Gethmann (1974) and Ivy (1981) led to the identification of additional meiotic mutations. Despite these efforts, little progress has been made in the understanding of male meiosis. One problem is that the great majority of identified mutations are specific for female meiosis. Another problem is that although several previous male meiotic mutations were identified, most have been lost (reviewed by Orr-Weaver, 1995; Lindlsey and Zimm, 1992) either by reversion or by careless stewardship. The field is currently lacking *Drosophila* male mutants defective in pairing and segregation and this has greatly hindered progress in understanding the mechanisms underlying homologous chromosome pairing and segregation. The primary focus of our project was to identify and characterize genes involved in this process.

A few meiotic mutations that have been identified in different screens from natural populations (Sandler et al., 1968), EMS-induced mutagenesis (Baker and Carpenter, 1972), P-element insertion (Castrillon et al., 1993; Sekelsky et al., 1999) are reported in Table 1. The only mutation that causes NDJ of all chromosomes at MI in both sexes is *Dub*. Defect in *mei-I3* leads to a phenotype similar to *Dub* but in addition, the sex chromosome disjunction is also disrupted at MII (Ivy, 1981). This suggests that some mechanisms of the first meiotic division are shared between the two sexes.

Table 1: Examples of mutations affecting the meiotic chromosome segregation in *Drosophila*. Only a few mutations are reported in this table in order to compare the two sexes, the meiotic stage at which the defect occurs and the chromosome(s) that is (are) affected. Dots indicate the sex, the division (MI or MII: meiosis I or II) and chromosome(s) that were shown to be affected.

Mutated genes	Symbol	Affected chromosome				Division		Sex specificity		References
		X	2	3	4	MI	MII	Male	Female	
orientation disruptor	*ord*	●	●	●	●	●	●	●	●	Mason, 1976
mei-I3		●	●	●	●	●	●	●	●	Ivy, 1981
Horka		●	●	●	●		●	●	●	Szabad et al., 1995
mei-S332		●	●	●	●		●	●	●	Kerrebrock, 1992
Double or nothing	*Dub*	●	●	●	●	●		●	●	Moore et al., 1994
mei-G17		●	●					●	●	Gethmann, 1974
mei-G87			●			●	●	●	●	Gethmann, 1984
no distributive disjunction	*nod*	●	●	●	●	●			●	Carpenter, 1973
mei-I1		●	●	●	●	●		●		Ivy, 1981
altered disjunction	*ald*	●			●	●			●	O'Tousa, 1982
Aberrant X segregation	*AxsD*	●			●	●			●	Hawley, 1989
equational producer	*eq*	●					●	●		Schultz, 1934
mei-081		●	●	●	●			●		Sandler et al., 1968
mei-S8					●			●		Sandler et al., 1968
teflon	*tef*		●	●	●	●		●		Tomkiel et al., 2001
Suppressor of Stellate	*Su(Ste)*	●	●	●				●		Livak, 1990
homeless	*hls*	●	●					●		Gillespie and Berg, 1995 Stapleton et al., 2001

The distributive system in females underlies the segregation of non-crossover bivalents (Grell 1962). Karpen and colleagues showed that a mostly heterochromatic mini-X chromosome (Dp1187) segregates regularly from a full-length X or from another similar mini-X, suggesting that the necessary information for proper disjunction in females is confined to the pericentromeric heterochromatin. Disjunction was reduced in females bearing further deletions within this heterochromatin region. However, Dp1187 segregated randomly in males, both from an attached-XY or from another mini-X, suggesting that the distributive system in females and achiasmate segregation in males are not the same (Karpen et al. 1996). Consistent with this are findings that mutations disrupting components of the distributive system (e.g. *ald*, *Axs*, and *nod*) do not have any phenotype in males.

Both chiasmate and achiasmate segregation in females and males respectively require proper homolog pairing during meiosis I. However, pairing sites differ between the two sexes. The 240 bp IGS repeats within rDNA sequences, common to the heterochromatin regions of the X and Y chromosomes, have been found to be important in X-Y pairing in males (McKee and Karpen 1990; Merrill et al. 1992). These sequences are not sites of female sex chromosome pairing (Hawley 1988), which involves the region surrounding the X centromere (Karpen et al. 1996). Pairing sites for chiasmate chromosomes are located within euchromatic regions, whereas distributive pairing sites for the 4^{th} and X chromosomes are mainly heterochromatic (Hawley et al. 1993; Dernburg et al. 1996). Autosomal pairing in males appears to involve euchromatic regions exclusively (Yamamoto

et al., 1979; McKee et al., 1993; for reviews see McKee, 1998; McKee 2004). The situation in females is more complex. Recombination is confined to the euchromatic arms, but compound autosomes that share homology limited to the heterochromatin disjoin at high frequencies in females (but randomly in males) due to the distributive system. Other than this, little is known about the mechanism of chromosome pairing and segregation in *Drosophila* males.

Our goal is to characterize genes involved in meiotic chromosome pairing and segregation in *Drosophila melanogaster* by identifying meiotic mutations with defects in homolog or sister chromatid segregation during male meiosis.

As a result of an ethyl methanesulfonate (EMS) mutagenesis to recover non-essential genes in *Drosophila*, a large collection of stocks (12,000 lines) was generated in which flies homozygous for a highly mutagenized autosome were viable (Koundakjian et al., 2004). EMS-treated stocks are currently maintained in the laboratory of Charles Zuker at The University of California, San Diego.

Screening these lines for male sterile mutations and for mutations that disrupt transmission of chromosome 4 led to the identification of >2000 strains, of which 62 bearing mutations on their second or third chromosome displayed a phenotype associated with loss of the paternal fourth chromosome (Wakimoto et al., 2004).

Further analysis of the selected lines revealed the presence of spermatids with unequal nuclear sizes, suggestive of NDJ during meiosis, in forty-eight of the mutants. Cytological and genetic analyses by Bruce McKee led to the identification of 29 strong meiotic mutants on chromosomes 2 and 3 that define 9 complementation groups, seven on chromosome 3 and two on chromosome 2,

named *pairing failure* 1-9 (*pf 1-9*; McKee, personal communication). Mutations in all nine loci were found to disrupt segregation of all four chromosome pairs in male meiosis.

II- Deletion mapping

In order to identify the genes responsible for the observed meiotic phenotypes, the chromosomal locations of the EMS-induced point mutations on the third chromosome had to be determined. To map the *pf* genes on chromosome 3, we used a chromosome three "deficiency kit" available from The *Drosophila* Stock Center at Bloomington, consisting of a collection of stocks, each having a deletion covering a small segment of chromosome three. Collectively, these deficiencies encompass most of the euchromatic regions of chromosome 3. Male flies carrying one representative allele from complementation groups: *pf-1*, *pf-2*, *pf-4*, *pf-5* and *pf-6*, along with a marked Y chromosome ($B^s Y y^+$) were mated to females from each of these kit stocks. F1 progeny males heterozygous for the mutation and each of the various deletions were collected and mated to *y w* females. F2 progeny of these crosses were scored for X-Y NDJ, which results in recovering of XXY (B^s) females and XO (B^+) males (Figure 1).

Display of the NDJ phenotype by hemizygous flies indicates that the mutation lies within the region missing from the deletion chromosome. These experiments succeeded in mapping complementation groups *pf-1, pf- 2* and *pf-6* to chromosome regions 61 F8; 62 A8, 93 D6- E1, and 68 A2-3; 69 A1-3 respectively.

♀ +/+; *Z3, st* / TM6 **x** ♂ +/ B^sYy^+; *Ubx* / TM3, *Sb*

↓

♂ +/ B^sYy^+; *Z3, st* / TM3, *Sb* **x** ♀ +/+; *Df* / TM3, *Sb*

↓

♂ +/ B^sYy^+; *Z3, st* / *Df* **x** ♀ *y w* / *y w*

Sb^+ males

Sperm \ Eggs	$X^{y\,w}$	Phenotype of progeny	
X^{w+y+}	XX	**Wild type: Round, red eyes**	♀
B^s Y y^+	X / B^s Y y^+	**Wild type: Bar, white eyes**	♂
X^{w+y+} B^sY y^+	XX / B^sY y^+	**NDJ: Bar, red eyes**	♀
O	X / O	**NDJ: Round, white eyes**	♂

Figure 1: Crossing scheme for NDJ scoring. Female flies from Zucker stock carrying a mutation on their third chromosome (*Z3*) were crossed to male flies with a marked B^sYy^+ and a balancer chromosome. To generate hemizygote flies, progeny males were mated to females with a deficiency in a fragment of their third chromosome. F2 males, hemizygous for one EMS-induced mutation on their third chromosome, were tested for NDJ by crossing them to *yellow, white* (*y w*) females and scoring their progeny for the NDJ phenotype. *st*: *scarlet*; *Ubx*: *Ultrabithorax*; *Sb*: *Stubble*; B^s: *Bar stone*; TM3 and TM6 are balancer chromosomes.

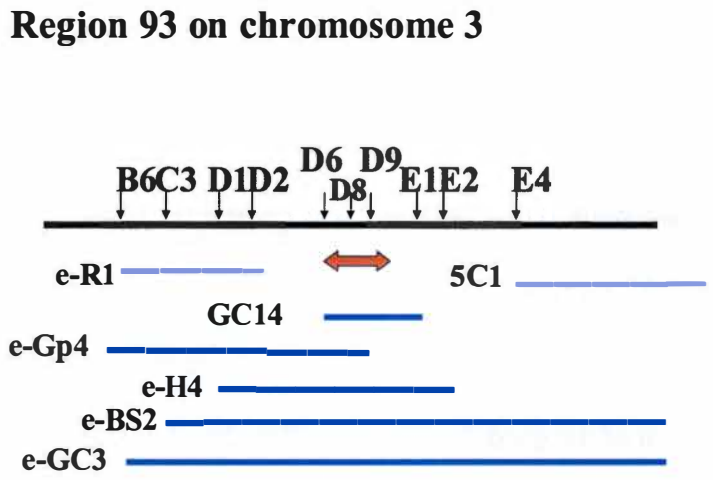
The priority of this research was to study mechanisms underlying homologous chromosome pairing rather than other aspects of meiosis such as sister chromatid cohesion. Based on cytological and genetic data reported later on in this chapter, mutations in *pf-2* disrupt homolog pairing and cause high frequencies of NDJ of all chromosome pairs. Therefore, *pf-2* was chosen for further analysis. Only data for mapping *pf-2* are reported in Table 2; they show that the region of overlap of deficiencies that do not complement *pf-2* mutations is within the 93D8-9 region on the right arm of the third chromosome. Results of deletion mapping are graphically represented in Figure 2a. Figure 2b shows candidate genes identified by searching FlyBase for genes within the region of interest.

Alleles of three candidate genes (e.g. *tinman*, *hsr-omega* and *mod(mdg4)*) were tested. The results of complementation of *pf-2* alleles using the X-Y NDJ assay show that alleles of both *tinman* and *hsr-omega*, tin^{346} and $l(3)05241^{05241}$, fully complemented *pf-2* allele, *Z3-5578*. These data lead to the conclusion that *pf-2* is not allelic to *tinman* and *hsr-omega* and further complementation tests (data reported in chapter 3) revealed that *pf-2* is allelic to *mod(mdg4)*.

III- Cytological analysis of putative meiotic mutants

To determine the phenotype(s) associated with *pf-2* mutations, meiotic cells in mutants were compared to wild type ones at the same stage of division. Testes of mutant flies were dissected, stained with aceto-orcein and squashed to study meiotic chromosomes. Examination of primary spermatocytes of the three *pf- 2* alleles revealed the existence of unpaired chromosomes at prophase and

a



b

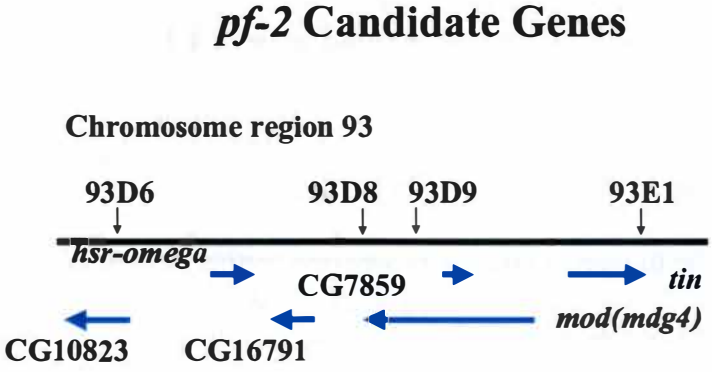


Figure 2: Deletion mapping of *pf-2*. (a) Flies with a chromosome deficient in the illustrated region were crossed to *pf-2* mutants and F1 hemizygote progeny were tested for NDJ. Purple lines represent the deficiencies that are complemented by *pf-2* mutations, whereas blue lines indicate regions that if deleted, cause a NDJ phenotype. (b) Candidate genes located within the critical region. Arrows represent the direction of the transcription.

Table 2: Complementation analysis to identify *pf-2*. Mutant males carrying a marked Y chromosome were crossed to females deficient for a segment of the third chromosome. F1 progeny, hemizygous for *pf-2* mutation and the specified deficiency were mated to *y w* females and the F2 progeny were scored for NDJ.

			% NDJ		
		♂:	*Z3-5578*	*Z3-3298*	*Z3-3401*
♂	♀	Deficiency breakpoints			
Homozygotes			40.13	55.56	29.92
Hemizygotes	***Df(3R)B81***	99C8; 100F5, 99D; 100F	0	0.06	0.07
	Df(3R)5C1	93E-F; 94C-D	0.1	0.1	0.11
	Df(3L)M21	62F;63D,62A;64C(Dp on In)	0.2	0	0
	Df(3R)e-R1	93B6-7; 93D2	0.09	0.2	0
	Df(3R)hh	93F11-14; 94D10-13	0.07	0.33	0.19
	Df(3R)29A6	66F5; 67B1	0.06	0.26	ND
	Df(3R)93F	93F5; 94A8	0.12	0	0
	Df(3R)e-N19	93B; 94	62.35	63.77	sterile
	Df(3R)e-H4	93D1; 93F6-8	45.99	50.08	49.21
	Df(3R)GC14	93D6-7; 93E1	47.66	49.9	47.9
	Df(3R)eGC3	93C6; 94A1-4	40.52	47.28	46.91
	Df(3R)e-BS2	93C3-6; 93F14-94A1	46.38	56.85	54.42

prometaphase (Figure 3), as well as univalents at metaphase I and "laggard chromosomes" at anaphase of meiosis I (Figure 4). Up to eight univalents are seen at metaphase I and these unpaired chromosomes segregate randomly at anaphase I resulting in unequal distribution of the genetic material at opposite poles. The visualization of the chromatin stained with DAPI fluorescent dye revealed the presence of ungrouped mature spermatids with variable length, indicative of aneuploidy (Figure 5). NDJ is restricted to the first meiotic division and no abnormalities were seen during meiosis II.

IV- Genetic analysis of putative meiotic mutants

IV.1- Sex chromosome NDJ

To further characterize the phenotypes of the meiotic mutants, the rate of non-disjunction (NDJ) of sex chromosomes and autosomes were measured genetically. Males homozygous or hemizygous for the *pf-2* mutation and a deletion within the 93D region on the third chromosome (*Df(3R)GC14*), carrying a marked $B^{s}Yy^{+}$ chromosome, were tested for sex chromosome NDJ by mating them with *yellow white* (*y w*) females. These females are chromosomally normal and produce euploid gametes (for the crossing scheme for the generation of *pf-2* mutant flies carrying a marked Y chromosome, see Figure 1). Progeny scores showed elevated numbers of X-Y and nullo gametes relative to wild type controls. Data for X-Y NDJ show 44% - 48% NDJ for mutant flies hemizygous for each of the three *pf-2* alleles (Table 3). *Z3-5578* homozygotes and *Z3-5578 / Z3-3298* heterozygote flies

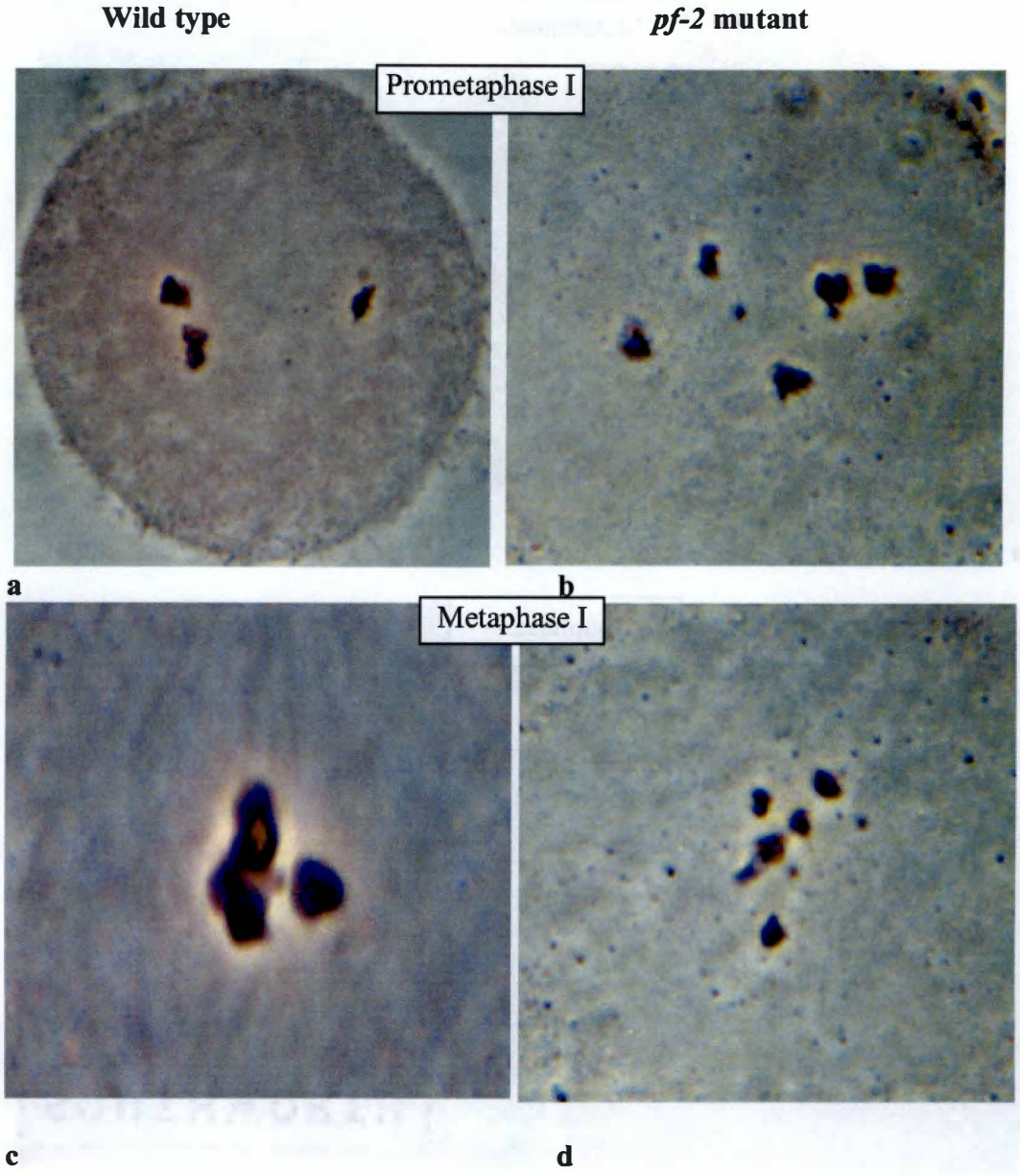


Figure 3: Phase optic visualization of meiotic chromosomes from hand-dissected, squashed and orcein-stained testes. Univalents are seen in *pf-2* mutants' primary spermatocytes (b and d) at prometaphase (a and b) and metaphase I (c and d) compared to condensed bivalents in wild type cells (a and c).

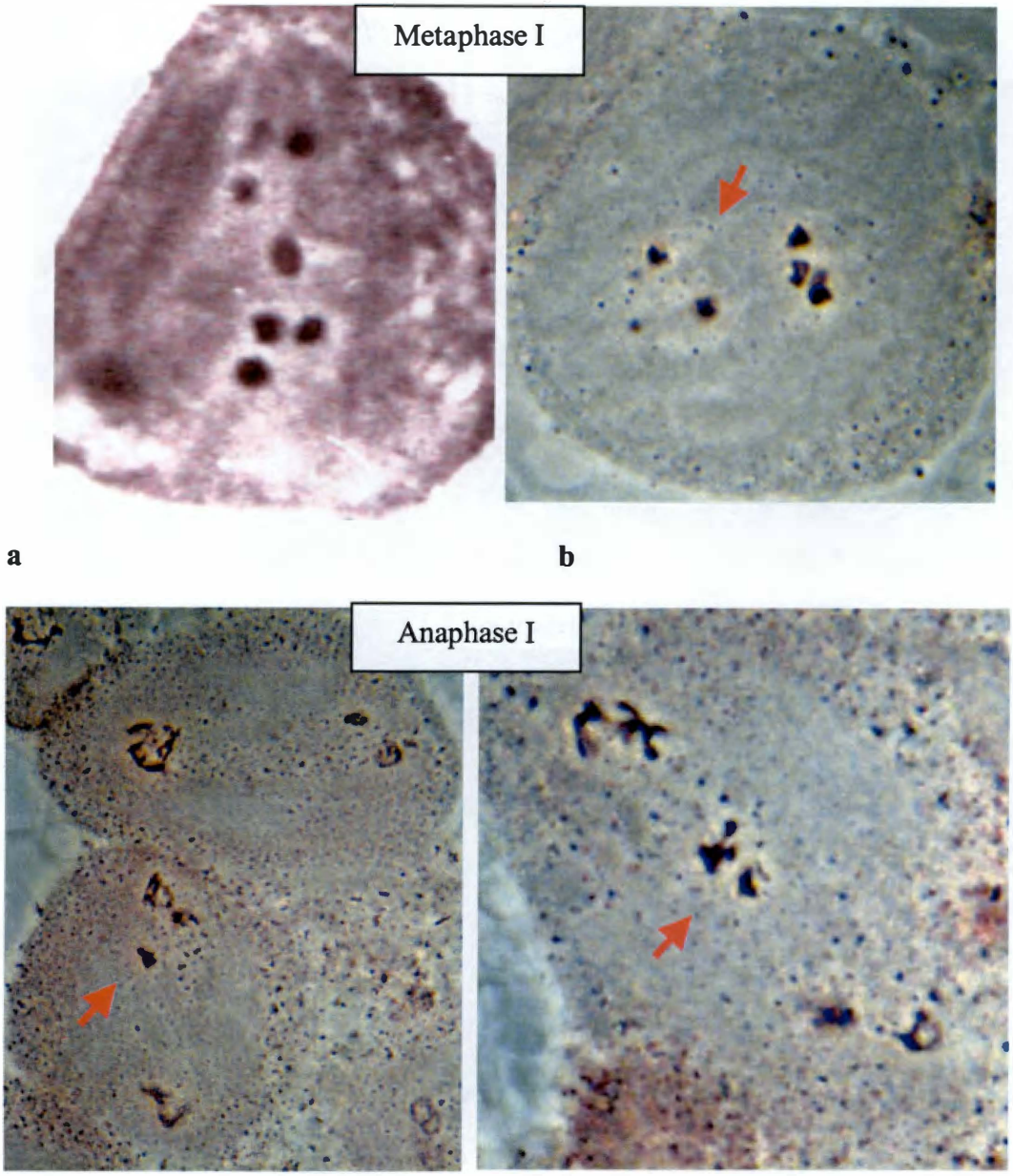
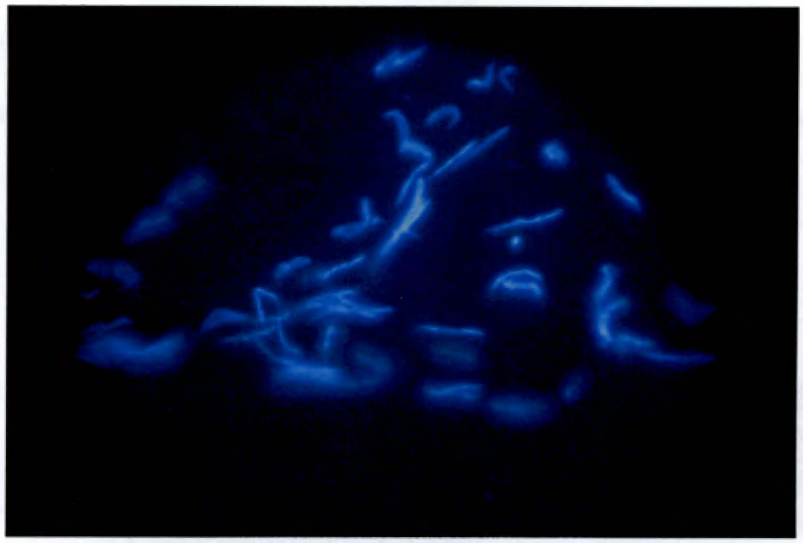
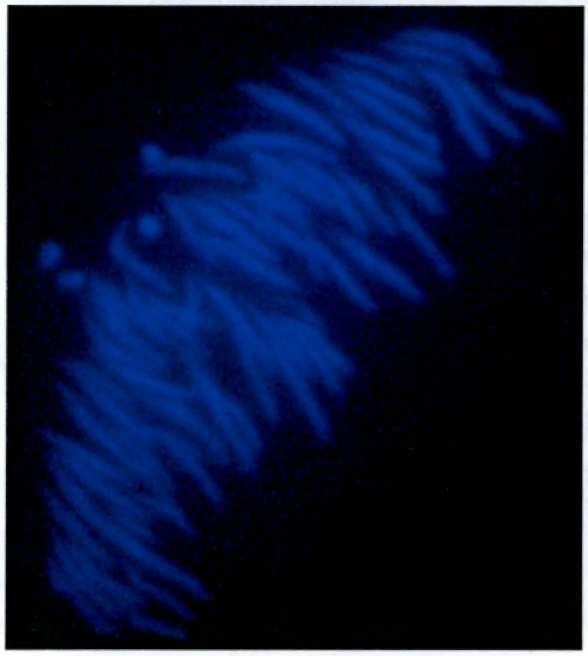


Figure 4: Phase optic visualization of meiotic chromosomes at metaphase and anaphase of the first division of meiosis. Flies' testes were hand-dissected, squashed and stained with orcein. Univalent chromosomes are seen in primary spermatocytes of *pf-2* mutants at metaphase I (a). Unequal distribution of chromosomes and laggard chromosomes are detected at anaphase I (c, d). Arrows point to "laggard chromosomes" that have not reached a pole.



a



b

Figure 5: DAPI-stained sperm from hand-dissected, squashed testes. DAPI fluorescent dye stains condensed regions of the chromatin. Dispersed sperm with variable chromosome sizes are seen in *pf-2* mutants (a) compared to similar and organized chromosomes in wild type sperm. Unequal spermatid sizes are indicative of aneuploidy.

Table 3: Mutations in *pf-2* cause sex chromosome NDJ. Males of the indicated genotype were crossed to *y w* females. *Z3-3298* homozygote males were sterile. % NDJ = NDJ flies / total flies.

	Parental		NDJ		
Sperm-egg genotype:	X-X	Y-X	XY-X	O-X	Y-XX
♂ genotype			**♂ NDJ**		**♀NDJ**
Df(3R)GC14 / Z3-3298	106	100	63	104	3
Df(3R)GC14 / Z3-3401	436	352	179	437	
Df(3R)GC14 / Z3-5578	337	285	228	343	4
Z3-5578 / Z3-3298	203	149	102	136	1
Z3-3401 / Z3-3401	245	207	82	111	
Z3-5578 / Z3-5578	149	227	97	157	3

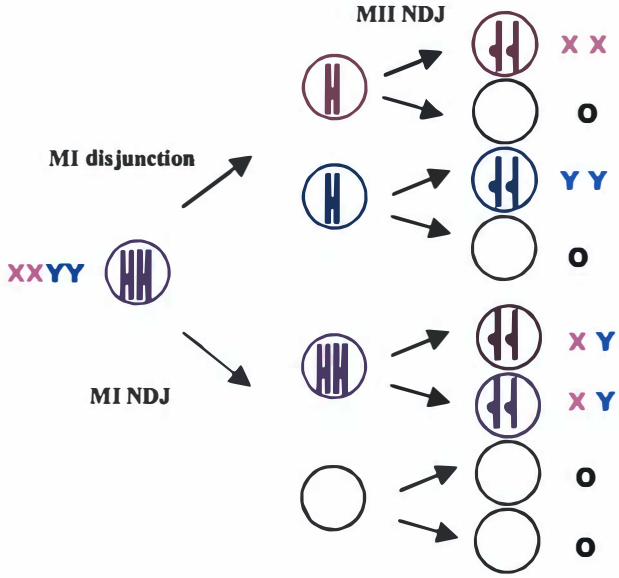
♂ genotype	Total Flies	NDJ Flies	**% NDJ**	Tested males
Df(3R)GC14 / Z3-3298	376	167	**44.41**	22
Df(3R)GC14 / Z3-3401	1404	616	**43.87**	18
Df(3R)GC14 / Z3-5578	1198	571	**47.66**	27
Z3-5578 / Z3-3298	591	238	**40.27**	20
Z3-3401 / Z3-3401	645	193	**29.92**	10
Z3-5578 / Z3-5578	633	254	**40.13**	23

displayed similarly high NDJ rates, suggesting that these may be null mutations. Flies homozygous for *Z3-3401* on the other hand, display a weaker phenotype than the hemizygotes, suggesting that this mutation is hypomorphic.

IV.2- Chromosomal NDJ occurs at early stages of prophase I and is specific to the first division of meiosis

Although the presence of high levels of X-Y NDJ in *pf-2* males is indicative of the occurrence of MI NDJ, it does not address the question of whether missegregation is occurring at both divisions. In order to determine the stage at which the male NDJ occurs, hemizygote *pf-2 / Df(3R)GC14* males carrying a marked Y chromosome ($B^S Y y^+$) were crossed to the females from *C(1)RM/O* stock with attached X chromosomes. Progeny derived from XX and XY sperm were scored to determine the MI / MII NDJ rates (Figure 6). The presence of B^+ *y*, *su*(w^a) w^a females, which result from fertilization of diplo X eggs by nullo-XY sperm, can be explained by the occurrence of either MI or MII NDJ events or by chromosome loss. Since the progeny class that is specifically derived from abnormal segregation at MII (X^X-0, B^+ females) was completely absent, the total NDJ is equal to the amount of 1st division NDJ. Data from these studies show that the sex chromosome segregation defects in *pf-2* males are occurring exclusively at the first meiotic division (Table 4).

a



b

Sperm / Eggs	X^X	O	Phenotype of progeny
X^{w+y+}	⊗	X/O	Parental: B^+ ♂
$B^s Y y^+$	X^X / $B^s Y y^+$	⊗	Parental: B ♀
X^{w+y+} $B^s Y y^+$	⊗	X-$B^s Y y^+$/O	MI NDJ: B ♂
O	X^X / O	⊗	MI or MII NDJ: $B^+ y^2 w^a su(w^a)$ ♀
XX	⊗	XX/O	MII NDJ: $B^+ y^+$ ♀

Figure 6: Non-disjunction of sex chromosomes occurring during meiotic divisions.

(a) Non-disjunction at MI generates XY and O sperm, whereas NDJ at MII generates XX, YY and O sperm.

(b) NDJ scoring in progeny from crosses of *pf* males carrying $B^s Y y^+$ to *C(1)RM* y^2 w^a *su* (w^a) / *O* females (X^X).

MI: meiosis I, MII: meiosis II. ⊗ : Lethal.

Table 4: Non-disjunction caused by *pf-2* mutations is meiosis I-specific. Males of the indicated genotype were crossed to *C(1)RM/O* females with attached X chromosomes. X-X and X-Y progeny were scored to determine the MI vs. MII NDJ rates.

% NDJ = NDJ flies / total flies. No progeny with male MII NDJ phenotype were detected.

				NDJ				%	Tested
Sperm-egg genotype	Y-XX	X-O	O-XX	XY-O	XX-O	Total	NDJ	NDJ	males
♂ genotype			MI/MII	MI	MII				
Df(3R)GC14 / Z3-3298	53	73	69	31	**0**	226	100	**44.25**	20
Df(3R)GC14 / Z3-3401	73	84	126	41	**0**	324	167	**51.54**	19

IV.3- *pf-2* mutations affect the disjunction of the 2nd chromosomes

In order to determine whether the phenotype caused by *pf-2* mutations is chromosome specific or genome wide, stock males were tested for autosomal NDJ. Males hemizygous for each of the *pf-2* alleles and carrying a marked $B^{s}Yy^{+}$ chromosome were crossed with females carrying a compound chromosome 2, *C(2)EN b, pr*. Since all of the eggs from these *C(2)EN* females carry either 0 or 2 copies of chromosome 2, only non-disjunctional sperm that are disomic or nullisomic for chromosome 2 will lead to viable progeny.

Wild type control males produce less than one offspring per tested male in this cross. To estimate the number of sperm that are monosomic for chromosome 2, sibling males were crossed to chromosomally normal (2-2) females. Both experimental and control crosses were carried out under conditions to fully sample sperm from tested males. The calculation of the NDJ ratio takes into account the non-viable progeny and the method used is explained in Figure 7. Table 5 shows the mis-segregation of the 2nd chromosomes based on the number of progeny produced by *pf-2* mutant males. The two *pf-2* alleles, *Z3-5578* and *Z3-3298* previously classified as null alleles, exhibited elevated (~30%) chromosome 2 NDJ frequencies, while the third allele, *Z3-3401* displayed a weaker phenotype (~10% NDJ). Since hemizygotes for this allele exhibit random assortment of the sex chromosomes, these results suggest that *pf-2* may play a more crucial role in sex chromosome than autosomal segregation.

The second chromosome of *C(2)EN* flies is marked with the recessive alleles *black* and *purple* (*b, pr*). *pf-2* flies were heterozygous for the recessive *brown*

	Eggs:	**2^2*b, pr***	**O**	**Eye phenotype**
	Sperm			
Parental	**2*bw* or 2+**	⊗	⊗	
MI NDJ	**2*bw* / 2+**	⊗	2*bw* / 2+	wild type
MII NDJ	**2*bw* / 2*bw***	⊗	2*bw* / 2*bw*	brown
MII NDJ	**2+ / 2+**	⊗	2*b*+ / 2+	wild type
MI or MII NDJ	**O**	2^2*b, pr*	⊗	black, purple

Figure 7: Expected progeny for the cross: $^{+}/B^{s}Yy^{+}$; *bw / +; pf-2 / Df (3R)GC14* x *C(2)EN, b pr* / O. Lethality is indicated by ⊗ . Progeny of the cross to *C(2)EN* indicate the occurrence of a paternal NDJ.

Table 5: *pf-2* mutations affect the disjunction of the 2nd chromosomes. Males of the indicated genotype were crossed to females with attached 2nd chromosomes. Progeny of these crosses are products of gametes with non-disjoined chromosomes. To estimate the number of progeny that were not viable, crosses to *y w* females were set up under similar experimental conditions.

% NDJ = 2 (progeny per male of the cross to *C(2)EN* females) / Total progeny per male

Total progeny = NDJ + Disjunction

Non-disjunction (NDJ) = [progeny of the cross to *C(2) EN*, producing only aneuploid gametes] x 2

Disjunction (DJ) = [Progeny of the cross to *y w* females producing only euploid gametes]

	NDJ progeny of *C(2)EN* ♀		
Sperm-egg genotype:	2+/2*bw*-O	O-22*b,pr*	2*bw*/2*bw*-O
♂ genotype	**MI**	**MI/MII**	**MII**
Df(3R)GC14 / Z3-3298	434	591	4
Df(3R)GC14 / Z3-5578	572	587	1
Df(3R)GC14 / Z3-3401	237	416	1
	C(2)EN ♀		
	Total progeny	Tested males	Progeny per ♂
Df(3R)GC14 / Z3-3298	1029	48	**21.44**
Df(3R)GC14 / Z3-5578	1160	46	**25.22**
Df(3R)GC14 / Z3-3401	664	44	**15.09**
	y w ♀		
	Total progeny	Tested males	Progeny per ♂
Df(3R)GC14 / Z3-3298	2990	27	**110.74**
Df(3R)GC14 / Z3-5578	2322	23	**100.96**
Df(3R)GC14 / Z3-3401	7768	29	**267.86**
	NDJ	NDJ + DJ	**% NDJ**
Df(3R)GC14 / Z3-3298	42.88	153.62	**27.91**
Df(3R)GC14 / Z3-5578	50.43	151.39	**33.31**
Df(3R)GC14 / Z3-3401	30.18	298.04	**10.13**

(*bw*) allele on their second chromosomes. The eye phenotype displayed by the progeny of crosses of *pf-2* to *C(2)EN* flies allows us to distinguish between MI vs. MII NDJ, as *bw*-eyed progeny result only when a fly inherits two *bw* sister chromatids from the *pf-2* father. Table 5 shows that there were 434 progeny with wild type eye color in the Df(3R)*GC14 / Z3-3298* cross, but only four with *bw* eyes. The wild type progeny must have inherited both chromosomes 2 from their father and could be either *bw* / bw^+, reflecting an MI NDJ or bw^+ / bw^+, reflecting an MII NDJ. However, the *bw*-eyed flies can result only from MII NDJ of the other homolog, and since there were only 4 *bw* progeny, it is reasonable to assume that bw^+ / bw^+ progeny were equally low and that virtually all of the wild type progeny are *bw* / bw^+, products of MI NDJ. Based on this ratio of MI / MII progeny, we can assume that the *b, pr* flies derived from nullo-2 sperm that could be generated by a defect at either division are actually produced almost exclusively by an MI NDJ.

Some previously identified NDJ-inducing mutations, such as deletions of the Y chromosomal *Suppressor of Stellate* (Su(Ste), Livak, 1990) locus, also cause other meiotic phenotypes, such as non-homologous disjunction (NHD) in which non-homologs preferentially disjoin to opposite poles.

To assess whether *pf-2* mutations cause non-homologous disjunction, the segregation of X and Y chromosomes was scored in crosses to *C(2) EN* females, taking advantage of the marked Y chromosome in the *pf-2* hemizygote males. Table 6 represents the recovery of gametes with various sex chromosome genotypes relative to nullisomy or disomy for chromosome 2. If *pf-2* mutations

Table 6: Ratio of gametes with more than one non-disjoined chromosomes. The genetic complement of progeny produced by the fertilization of disomic or nullisomic eggs, produced by *C(2)EN* females with attached chromosome 2, by sperm from hemizygote males carrying one *pf-2* allele over the deficiency *Df(3R)GC14* is determined by their eye color (wild type (wt), brown (*bw*) or black, purple (*b, pr*)), indicative of chromosome 2 NDJ; and by the shape of their eye (*Bar* (*B* or B^+) marker on the Y chromosome), indicative of sex chromosome NDJ. The parameters R_x and R_Y used in the calculation of meiotic drive are indicator of the viability of X-bearing or Y-bearing sperm relative to other sperm classes (McKee et al. 1998). $R_x = (X.\ XY / O.\ Y)^{1/2}$ and $R_y = (Y.\ XY / O.\ X)^{1/2}$, where X, Y, XY and O are the number of progeny associate with each class of sperm. These values represent the occurrence of meiotic drive and are equal to 1 in a wild type background. Non-homologous disjunction (NHD) = (XY; 22 + O; O) / (XY; O + O; 22)

	***pf-2* allele:**		***Z3-5578***			***Z3-3298***			***Z3-3401***		
			C(2)EN		*yw*	*C(2)EN*		*yw*	*C(2)EN*		*yw*
	Phenotype		wt + *bw*	*b, pr*		wt + *bw*	*b, pr*		wt + *bw*	*b, pr*	
		Genotype	**2-2**	**O**	**2**	**2-2**	**O**	**2**	**2-2**	**O**	**2**
♀	*B+*	**X**	176	223	337	135	212	203	64	179	436
	B	**Y**	117	136	285	75	135	149	42	89	352
	B	**X-Y**	46	19	228	29	33	102	9	17	179
♂	*B+*	**O**	234	209	343	201	211	136	123	131	437
Rx			0.54	0.39	0.8	0.5	0.5	1.01	0.33	0.51	0.71
Ry			0.47	0.23	0.75	0.27	0.31	0.74	0.22	0.24	0.57
NHD			1			1.02			1		

cause NHD, the sum of XY; 22 and O; O classes should be less than the sum of XY; O and O; 22 sperm classes. However, as shown in Table 6, the NHD ratio [(XY; 22 + O; O) / (XY; O + O; 22)] is approximately equal to 1 for all three alleles of *pf-2*. Therefore, based on these data, *pf-2* mutations do not cause non-homologous disjunction.

A phenomenon associated with some cases of chromosome NDJ is distorted recovery of sperm classes in a genotype-specific manner. This phenomenon is referred to as "meiotic drive" and is observed in *Drosophila* males deficient for XY pairing sites. These males produce a great excess of X-O male progeny compared to X-XY female progeny and a significant excess of X over Y progeny (Sandler et al., 1957; Sandler and Hiraizumi, 1961; Gethmann 1974; McKee and Lindsley 1987). Sperm viability has been shown to be inversely proportional to chromatin content of the spermatids. The cause of meiotic drive is unknown but there is a correlation between the amount of chromosome NDJ and the severity of drive. Moreover, partial rescue of XY pairing by transgenic 240 IGS repeats results in a significant amelioration of meiotic drive.

The data in Table 6 show evidence for very weak meiotic drive, as measured by the drive parameters R_x and R_y, in the crosses of *pf-2* males to chromosomally normal y w females, but moderate levels in the crosses to *C(2)EN* females. However, the drive exhibited by *pf-2* males is different in two respects from that observed in X pairing site-deficient males. First, it is much weaker, typical values of R_x and R_y for Xh^- males are in the range of 0.05 - 0.3, and the males are semi-sterile due to extensive spermatid mortality. Second, there is no evidence in the

pf-2 data for enhanced mortality of 22 relative to nullo sperm, nor for viability interactions between the sex and second chromosomes; R_x and R_y are virtually identical in the 22 and O sperm classes. It is not clear why the *pf-2* hemizygotes exhibit higher X-Y drive in the C(2) vis a vis the 2/2 crosses. In general, drive levels seem to be quite variable in *pf-2* crosses, perhaps reflecting effects of genetic background or environment.

IV.4- Mutations in *pf-2* alter the disjunction of the 4th chromosomes

To determine whether these mutations affect all of the autosomes, *pf-2* mutant males were crossed to females with attached 4th chromosomes. *C(4)EN ci ey* stocks were used to assess by phenotype the amount of 4th chromosome NDJ. Since these *C(4)* flies generate only gametes containing 0 or 2 copies of chromosome 4, normal 4th chromosome disjunction leads to haplo 4 Minute progeny and phenotypically wild-type triplo 4 progeny. The Minute phenotype is caused by hemizygosity for M(4), one of about 40 haplo-insufficient Minute loci in the genome. Minute flies have numerous morphological abnormalities, and their recovery is sporadic; therefore, they are omitted from data collections. Diplo-4 sperm result in progeny indistinguishable from progeny from mono-4 sperm. On the other hand, fertilization of nullo-4 nondisjunctional sperm of *C(4)RM ci ey* gametes leads to *ci ey* progeny which are phenotypically distinguished by reduced eye size and gaps in the wing veins. Note that in this cross, only the nullo sperm will produce progeny phenotypically different from wild-type sperm and, therefore, the actual amount of NDJ could be double the

reported amount due to uncounted diplo-4 sperm (Figure 8). Table 7 shows the high level of 4th chromosome NDJ (44%) in *Z3-3298* males. The normal level of 4th chromosome NDJ is 0.1% (Hawley 1989). The *Z3-3401* mutation causes only 10% of 4th chromosome NDJ and seems to be a weaker allele, consistent with the data for X-Y and 2-2 NDJ.

V- The female meiotic phenotype associated with *pf-2* mutations

V.1- Chromosomal disjunction phenotype associated with *pf-2* mutations in females

Male meiotic chromosomes do not recombine and therefore the study of meiosis can be undertaken without the interference of many overlapping factors. Many meiotic genes that have previously been described are either female specific or affect the process in both genders. It is therefore important to find mutations that alter specifically male meiotic events, as this will increase our understanding of the basis of the chromosomal pairing and disjunction pathways.

The analysis of *pf-2* mutations led to the conclusion that *pairing failure 2* is indeed a meiotic gene, affecting specifically the first division and altering the homologous chromosome segregation. It was then important to analyze the phenotype in *pf-2* mutant females in order to determine whether the disruption of meiosis occurs in both sexes or is male-specific. Based on the deletion mapping results, males hemizygous for any of *pf-2* alleles and seven *Df(3R)* deficiencies displayed elevated rates of NDJ. Females heterozygous for these 7 deficient

Eggs:	**4^4 *ey, ci***	**O**
Sperm		
4	4/4^4	4-0, Minute
4-4	44 / 44	44-O
O	O-44, *ey, ci*	⊗

Figure 8: Expected progeny for the cross: $^{+}/B^{s}Yy^{+}$; *pf-2*$^{-}$*/Df (3R) GC14* x *C(4)RM/O*. Lethality is indicated by ⊗. Progeny of the cross to *C(4)RM/O* indicate the occurrence of a paternal NDJ.

NDJ = [*ey, ci* progeny of the cross to C(4)RM/O] x 2

Disjunction = ey^{+} - *ey*

Total progeny = NDJ + Disjunction = (2 *ey*) + (ey^{+} - *ey*) = ey^{+} + *ey*

% NDJ = NDJ / Total

Table 7: Mutations in *pf-2* alter the 4th chromosome disjunction. Males of the indicated genotype were crossed to *C(4)RM/O* females with attached 4th chromosomes carrying *ci* and *ey*. The presence of eyeless flies indicates the production of paternal nullo gametes. To include the progeny with two paternal 4th chromosomes in the estimate of NDJ, the number of *ey, Ci* flies was doubled. %NDJ = 2 (number of *ey, ci* flies) / total flies, i.e. the sum of *ey* and *ey+* flies.

	***pf-2* allele:**		***Z3-5578***			***Z3-3298***			***Z3-3401***		
			C(2)EN		*yw*	*C(2)EN*		*yw*	*C(2)EN*		*yw*
	Phenotype		wt + *bw*	*b, pr*		wt + *bw*	*b, pr*		wt+*bw*	*b, pr*	
		Genotype	**2-2**	**O**	**2**	**2-2**	**O**	**2**	**2-2**	**O**	**2**
	B+	**X**	176	223	337	135	212	203	64	179	436
♀	*B*	**Y**	117	136	285	75	135	149	42	89	352
	B	**X-Y**	46	19	228	29	33	102	9	17	179
♂	*B+*	**O**	234	209	343	201	211	136	123	131	437
Rx			0.54	0.39	0.8	0.5	0.5	1	0.33	0.51	0.7
Ry			0.47	0.23	0.8	0.27	0.31	0.7	0.22	0.24	0.6
NHD			1			1.02			1		

chromosomes and for the null *pf-2* allele, *Z3-5578*, were generated and tested for X chromosome NDJ by mating them to males carrying attached X-Y*y, B* chromosomes. The occurrence of NDJ is detected by the presence of B^+ females and *B* males among the progeny. Results reported in Table 8 show no sex chromosomal disjunction defect due to the lack of *pf-2*.

V.2- Effect of *pf-2* mutations on recombination events

Recombination is of central importance in female meiosis and it seemed necessary to study the effect of *pf-2* on this meiotic female-specific event. The frequency of recombination along the X chromosome was measured in male progeny of females heterozygous for an X chromosome that is multiply marked with *yellow*, *prune*, *crossveinless*, *miniature* and *forked* (*y, pn, cv, m, f*) (Figure 9). Hemizygous *Z3-3298* or *Z3-3401* females were compared to *Z3-3298* / + or *Z3-3401* / + siblings as controls. The females were crossed with attached-XY, *y, B* / O males, allowing the simultaneous determination of the frequency of crossing over and of NDJ.

The data and analyses are shown in Tables 9 and 10 (for crossing schemes see experimental procedures). It is evident from the data that *pf-2* females have frequencies of X recombination and X-X NDJ similar to those of *pf-2* / + controls. Statistical analysis of the data, using Chi-square test, supported the hypothesis that the difference in map distances seen in control flies (*Z3-3298* / +) vs. *pf-2* mutant ones is non-significant, thus no elevation in NDJ or change in recombination processes can be associated with *pf-2* mutations in females.

Table 8: Test of sex chromosome non-disjunction in female flies hemizygous for *pf-2. Z3-5578* mutant females were crossed to males carrying the attached XY chromosome C(1; Y) Y^S X. Y^L, In (1) EN, *y B* and their progeny were scored for sex chromosome NDJ.

Parents		Progeny								
		♀			♂	Total	NDJ	%	Females	
♂	♀	B^+w^+	Bw^+	B^+w^+	y^+B^+w	flies	flies	NDJ	Tested	Sterile
X^Y*y,B*/O	*Z3-5578/5805*	1	460	661		1122	1	0.09	14	5
	Z3-5578/GC14		794	825		1619		0	15	2
	Z3-5578/5598	3	569	826		1398	3	0.21	14	6
	Z3-5578/1605		549	704		1253		0	16	2
	Z3-5578/3013		942	1242		2184		0	25	1
	Z3-5578/2252	1	431	782		1214	1	0.08	13	2
	Z3-5578/5798		391	402	154	947		0	12	

NDJ

		Total	NDJ	%	Females	
♂	♀	flies	flies	NDJ	Tested	Sterile
X^Y*y,B*/O	*Z3-5578/5805*	1122	1	0.09	14	5
	Z3-5578/GC14	1619		0	15	2
	Z3-5578/5598	1398	3	0.21	14	6
	Z3-5578/1605	1253		0	16	2
	Z3-5578/3013	2184		0	25	1
	Z3-5578/2252	1214	1	0.08	13	2
	Z3-5578/5798	947		0	12	

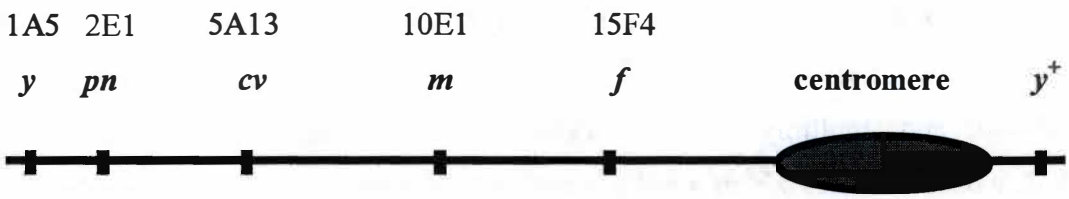


Figure 9: Graphical presentation of the multiply marked X chromosome (mX) carrying *yellow* (*y*), *prune* (*pn*), *crossveinless* (*cv*), *miniature* (*m*) and *forked* (*f*) phenotypic markers allowing the visualization of crossover events. *y+* locus on the short arm allows determining the occurrence of recombination close to the centromere. The cytological location of these loci on the X chromosome is indicated above them.

Table 9: Effect of *pf-2* mutations on recombination rates in females. Trans-heterozygote or hemizygote females bearing one of the 2 *pf-2* alleles, *Z3-3298* or *Z3-3401*, and a multiply marked X chromosome were crossed to Y^{s} X-Y^{L} In(1) EM, *y B* / O to males which produce attached X-Y and nullo-XY gametes, where the attached X-Y chromosome is marked with *yellow* and mild *Bar* alleles. No females with B^{+} eyes were scored among the progeny, indicating that NDJ did not occur during gamete production in these flies.

	♀	*yellow* ♂	
Genotype of tested ♀	***y B***	***y***	***pn cv m f***
Z3-3298, st / +	567	141	104
Z3-3298,st / Df(3R)GC14,st	498	155	71
Z3-3401, st / +	567	157	101
Z3-3401,st / Df(3R)GC14,st	63	24	24
	All Normal no NDJ	**NCO**	

	yellow ♂						
Genotype of tested ♀	***pn cv f***	***pn m f***	***m***	***pn f***	***cv m***	***cv***	***pn m***
Z3-3298, st / +	4	1	5	4	3	3	1
Z3-3298,st / Df(3R)GC14,st	2	3	1	2	2		
Z3-3401, st / +	3		2	3	1	2	
Z3-3401,st / Df(3R)GC14,st	1	1		2	1		
	DCO						**TCO**

	yellow ♂					
Genotype of tested ♀	***cv m f***	***m f***	***f***	***pn***	***pn cv***	***pn cv m***
Z3-3298, st / +	11	51	30	28	56	21
Z3-3298,st / Df(3R)GC14,st	11	34	31	24	48	31
Z3-3401, st / +	21	34	34	22	50	34
Z3-3401,st / Df(3R)GC14,st	2	6	11	2	6	1
	SCO					

Table 10: Statistical analysis to determine the significance of the difference in map distances, measured experimentally, seen between tested and control flies. High values of *P* indicate that the difference in map unit (m. u.) is not significant. ♀s were crossed to X-Y*y B* ♂s; ΣCO = SCO + 2 DCO + 3 TCO; Map distance = ΣCO / NCO + SCO + DCO + TCO. The difference in map distances is statistically not significant. Female flies mutant for *pf-2* do not display any NDJ phenotype or recombination defect.

NCO: non-crossover, SCO: single crossover, DCO: double crossover, TCO: triple crossover, m.u.: map unit (in centimorgan) and Chi2: Chi square statistical test. No B^+ females or *B* males, indicative of NDJ were scored.

Genotype of tested ♀	NCO	SCO	DCO	TCO	ΣCO	Chi2	m. u.	*P* value
Z3-3298, st / +	245	197	20	1	240	0.6424	51.83	**~ 0.5**
Z3-3298,st / Df(3R)GC14,st	226	179	10		199		47.95	
Z3-3401, st / +	258	195	11		217	0.0658	46.76	**~ 0.8**
Z3-3401,st / Df(3R)GC14,st	48	28	5		38		46.91	

In order to detect the recombination events occurring between the centromere and the y^+ locus, and to see the effect of *pf-2* mutations on this process, flies were generated that carried the same marked X chromosome with an additional y^+ locus located close to the centromere on the short arm of X. The control and test flies had the same genotype that was described above. Results of this test are shown in Tables 11 and 12 and lead to the same conclusion as above, that *pf-2* mutations do not disrupt meiosis in females (no NDJ or recombination defect).

Processes by which exchanges interact to control their own distribution are called genetic interference. In general, the occurrence of a meiotic crossover in one interval interferes with the occurrence of a second crossover in an adjacent interval, which results in a deficit of double crossovers in neighboring intervals. To determine whether *pf-2* mutations affect the influence of crossing over between one pair of genes and the adjacent region, the occurrence of recombination between different loci along the arm of the multiply marked X chromosome was analyzed. The formula for calculating interference is: Interference = 1 - coefficient of coincidence (c.o.c.); where c. o. c. is the observed number of double recombinants (DCO) divided by the expected number of double recombinants. Our data, reported in Table 13, show that *pf-2* mutations do not affect the occurrence of DCO in short intervals. For the entire chromosome, a slight decrease in interference is observed, which means that double crossover events seem to occur more often than expected. Females hemizygous for *Z3-3401* were noticeably less fertile than hemizygotes for *Z3-3298* (less than 4 progeny per

Table 11: Effect of *pf-2* mutations on recombination rates in females. Trans-heterozygote or hemizygote females bearing one of the 2 *pf-2* alleles, *Z3-3298* or *Z3-3401*, and a multiply marked X chromosome with an additional y^+ locus close to the centromere on the short arm of X were crossed to males producing attached X-Y and nullo-XY gametes, where the attached X-Y chromosome is marked with *yellow* and mild *Bar* alleles. No females with B^+ eyes were scored among the progeny, indicating that NDJ did not occur during gamete production in these flies.

	♀	*yellow* ♂				
Genotype of ♀Parent	***y B***	***y***	***pn***	***pn cv***	***pn cv m***	***pn cv m f***
Z3-3298, st / +	154	87	16	25	17	3
Z3-3298,st / Df(3R)GC14,st	124	76	7	19	7	5
Z3-3401, st / +	351	187	31	43	35	17
Z3-3401, st / Df(3R)GC14,st	15	25	1	8	5	
	no NDJ	**NCO**	**SCO**	**SCO**	**SCO**	**SCO**
	yellow ♂					
Genotype of ♀Parent	***cv m f***	***m f***	***m***	***f***	***cv m***	***cv***
Z3-3298, st / +			1			
Z3-3298,st / Df(3R)GC14,st		1	2	1		1
Z3-3401, st / +	2	4	5		2	
Z3-3401, st / Df(3R)GC14,st	2				1	
		NCO	**SCO**	**SCO**	**SCO**	**SCO**
	yellow^{+}♂					
	y+ B	***pn cv m f***	***cv m f***	***m f***	***f***	***y +***
Z3-3298, st / +	183	35	3	19	20	6
Z3-3298,st / Df(3R)GC14,st	118	37	3	17	9	9
Z3-3401, st / +	359	123	13	70	49	16
Z3-3401, st / Df(3R)GC14,st	17	11	3	2	2	2
	no NDJ	**NCO**	**SCO**	**SCO**	**SCO**	**SCO**
	yellow^{+}♂					
	pn cv	***pn cv f***	***pn m f***	***pn f***	***pn cv m***	***pn***
Z3-3298, st / +	1	2	1	1		3
Z3-3298,st / Df(3R)GC14,st	1	1		1		
Z3-3401, st / +	3	4	3	4		1
Z3-3401, st / Df(3R)GC14,st						
	DCO	**DCO**	**DCO**	**DCO**	**DCO**	**DCO**

Table 12: Statistical analysis to determine the significance of the difference in map distances, measured experimentally, seen between tested and control flies. High values of *P* indicate that the difference in m. u. is not significant. Females were crossed to X-Y *y, B* males. Due to low fertility of *Z3-3401 / Df* females, counts for *y* and y^+ males, for both the experimental and the control crosses, were pooled together for the statistical analysis. As different markers were employed in the two data sets, the phenotype associated with single, double and no crossover was determined for each test and based on their phenotype, progeny were classified as SCO, DCO or NCO and pooled together. DCO*: y (cv,m,cvm,pnmf,pnf,pncvf)* + y^+*(pncvf,cvm,pnmf,pnf,cv,m)*. ΣCO: sum of crossovers, ΣCO = SCO + 2 DCO, and Chi2: Chi square statistical test. Map distance = ΣCO / NCO + SCO + DCO in map unit (m.u. = centimorgan). The difference in map distances is statistically not significant. Female flies mutant for *pf-2* do not display any NDJ phenotype or recombination defect. NCO: non-crossover, SCO: single crossover, DCO: double-crossover, TCO: triple-crossover. No B^+ females or *B* males, indicative of NDJ were scored.

♀	NCO	SCO	DCO	ΣCO	m. u.	Chi2	*P*
Z3-3298, st / +	122	109	9	127	52.9	1.691	~0.2
Z3-3298,st / Df(3R)GC14,st	113	76	8	92	46.7		
Z3-3401, st / +	601	446	29	504	46.8	0.587	~0.5
Z3-3401, st / Df(3R)GC14,st	86	51	6	63	44.1		

Table 13: *pf-2* mutations cause a mild interference in the distribution of exchange events. Map distance = number of recombinant progeny / total number of progeny. The coefficient of coincidence (c. o. c.) = ratio between the frequency of observed double crossovers (DCO) and the expected frequency of DCOs. The expected frequency = number of DCO / total number of progeny.

♀ Genotype	Map distance (m.u.)						
	pn-cv	*cv-m*	*pn-m*	*m-f*	*f-y+*	*m-y+*	*pn-y+*
Z3-3298 / +	10.7	24.04	34.74	17.1	5.42	22.52	57.26
Z3-3298 / Df(3R)GC14	8.82	20.91	29.73	10.7	8.63	19.33	49.06
Z3-3401 / +	9.57	20.72	30.29	16.18	7.03	23.21	53.5
Z3-3401 / Df(3R)GC14	9.1	16.08	25.18	12.9	6.45	19.35	44.53

	pn-m			
	DCO	Exp	c.o.c.	Total
Z3-3298 / +	6	18.08	0.33	703
Z3-3298 / Df(3R)GC14	4	11.29	0.35	612
Z3-3401 / +	5	21.34	0.23	1076
Z3-3401 / Df(3R)GC14	1	2.09	0.48	143

	pn-y$^+$			
	DCO	Exp	c.o.c.	Total
Z3-3298 / +	9	18.78	0.48	240
Z3-3298 / Df(3R)GC14	8	11.32	0.71	197
Z3-3401 / +	28	43.03	0.65	612
Z3-3401 / Df(3R)GC14	3	3.02	0.99	62

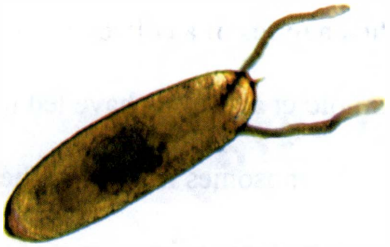
female compared to more than 25-30 for heterozygote *Z3-3401* or *Z3-3298* flies, Table 14). In order to generate recombination results that would be statistically meaningful, a large number of flies had to be tested.

To further investigate the semi-sterility phenotype of these females, we analyzed eggs laid by these females by microscopy. While some eggs laid by these females seemed normal, others displayed abnormalities in dorsal-ventral axis formation, visualized by the detection of missing, fused or unequal dorsal appendages (DAs), indicative of a defect occurring during oogenesis but not related to meiosis (Figure 10). The paired chorionic appendages located asymmetrically along the dorsal / ventral and anterior / posterior axes of the eggshell supply the developing embryo with oxygen. A conserved signaling cascade, involving many activating and inhibiting factors, operates between the oocyte nucleus and its adjacent follicles cells. Briefly, the binding of Gurken, a Transforming Growth Factor (TGF) – alpha like protein concentrated close to the oocyte nucleus, to its receptor, the Epidermal Growth Factor (EGF) receptor, activates the Mitogen-Activated Protein Kinase (MAPK) cascade and other signaling pathways resulting in the specification of the two dorso-laterally positioned respiratory DAs (Peri et al., 1999; for review see Barkai and Shilo, 2002). The *Z3-3401* mutation is located within the common region of *mod(mdg4)* affecting all of the isoforms (see chapter 3). It is therefore likely that at least one of the isoforms plays a role in patterning during the development of the *Drosophila* egg chamber (see chapter 1 for *mod(mdg4)* structure and chapter 3 for further information about *pf-2* alleles).

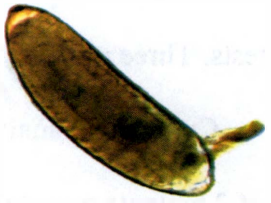
Table 14: Decreased fertility associated with the *Z3-3401* mutation. The number of progeny per female is calculated and shows semi-sterility for hemizygote *Z3-3401* females.

Genotype	Tested ♀	Progeny	Progeny / ♀
Z3-3298 / +	18	463	25.72
Z3-3298 / Df(3R)GC14	15	415	27.67
Z3-3401 / +	15	464	30.93
Z3-3401 / Df(3R)GC14	23	81	3.52

a) Wild type



b) Fused dorsal appendages



c) Unequal sizes of dorsal appendages

d) Missing dorsal appendages

Figure 10: Females hemizygous for one of the *pf-2* alleles, *Z3-3401*, and the deficiency *Df(3R)GC14* displayed semi-sterility. Eggs laid by these females showed abnormalities in dorsoventral axis formation that is indicative of a defect during oogenesis or early embryonic development.

VI- Summary and discussion

Cytological and genetic analyses of a collection of lines that show elevated chromosome 4 loss (Wakimoto et al., 2004) have led to the identification of 29 strong meiotic mutants on chromosomes II and III. These mutations comprise nine complementation groups and are called *pairing failure 1-9 (pf 1-9)*. They all cause high frequencies of NDJ of all chromosomes in cytological and genetic tests. Three *pairing failure-2* (*pf-2*) alleles were recovered and tested in parallel.

Cytological analysis of orcein-stained chromosome preparations from testes of *pf-2* mutants revealed high frequencies of unpaired chromosomes at prophase I and prometaphase I and laggard chromosomes at anaphase I, at which stage the random assortment of chromosomes results in their unequal distribution to each pole and formation of gametes with an inaccurate genomic complement. The phenotype displayed by the progeny produced by fertilization of these gametes is called aneuploidy as it represents the presence of reduced or excessive genomic material. No defect associated with meiosis II was detected by cytological test.

This conclusion was confirmed by genetic analyses. *pf-2* mutant males, carrying a marked Y chromosome, were tested genetically by crossing to tester females of various genotypes and scoring their progeny for NDJ. Results of these tests showed high frequencies (30 – 45%) of NDJ. For the second chromosome data, we were also able to establish that NDJ occurs almost exclusively at MI, by virtue of the virtual absence of progeny derived from 2-2 sperm that were hemizygous for the *bw* marker carried on the paternal homologs.

Autosomal NDJ was also tested genetically by crossing *pf-2* mutant males to females bearing attached second (C(2)EN *b, pr*) or fourth (C(4) EN *ci, ey*) chromosomes. Flies homo- and hemi- zygous for two of the *pf-2* alleles, *Z3-5578* and *Z3-3298*, display similarly high X-Y and autosomal NDJ frequencies (~40%) and are therefore genetically null for *pf-2*. The third allele, *Z3-3401*, is a hypomorph and exhibits weaker NDJ rates (~30% sex chromosome and ~10% autosomal NDJ).

The gamete data supported the cytological evidence that NDJ occurs almost exclusively at MI. Among the progeny produced by mating *pf-2* mutant males to females with attached X chromosomes (C(1)RM / O stock), numerous progeny derived from XY sperm were recovered but no progeny derived from XX sperm (diagnostic of sister chromatid pairing failure) were recovered.

Thus, mutations in *pf-2* affect the pairing and segregation of all of the chromosomes. The elevated NDJ rates are associated with the absence of bivalents at prometaphase and metaphase I and random assortment of chromosomes and presence of laggard ones at anaphase I. Abnormalities seen in *pf-2* mutants occur exclusively at meiosis I. The *pf-2* phenotype is male-specific and no disjunction or recombination defect was detected in females.

Su(Ste) (Livak 1990) is a locus on the Y chromosome that is necessary for the repression of the X-linked *Stellate* locus. Both loci are made up of tandemly repeated sequences containing an ORF that is homologous to the β-subunit of CKII (Livak 1984). The absence of *Su(Ste)* results in high expression of Stellate protein which accumulates in testis as crystals within primary spermatocytes

(Bozzetti et al. 1995). Deletion of the *Su(Ste)* locus or mutations in the *homeless* (*hls*) gene results in nondisjunction of the X-Y and large autosomal pairs, along with chromosome breakage and loss (Hardy et al., 1984; Stapleton et al., 2001). An additional phenotype is the excess recovery of certain sperm classes in a genotype-dependent manner, a phenomenon that is referred to as meiotic drive. A similar phenomenon is also seen in males deficient for the X chromosome pairing site. The progeny of these males show a greater recovery of normal XX females vs. normal XY males and of XO males vs. XXY females has been observed. Our data show that *pf-2* mutations cause high X-Y NDJ frequencies similar to males lacking rDNA (the X-Y pairing sites). Mutations in *Su(Ste)* loci cause high 2-2, 3-3 NDJ but do not affect 4-4 disjunction. Both aberrations result in high meiotic drive. Unlike *Su(Ste)*$^-$ and rDNA$^-$ flies, *pf-2* mutant males display only a mild meiotic drive, and non-homologous disjunction is absent.

Although numerous screens have been undertaken to identify meiotic mutants in *Drosophila*, very few mutants have been recovered with defects in the pairing and segregation of chromosomes in males. The characterization of the majority of male meiotic mutations that have been identified in screens was not possible as a time lapse resulted in loss of the phenotype.

Obviously, meiotic events are significantly different in the two sexes as indicated by the very low rate of recovery of male-specific mutations in several large-scale screens. Both male and female meiosis are affected if processes common to these two sexes, such as sister chromatid cohesion, are disrupted (mutations in *ord*, *mei-S332*, and *mei-G87*).

A careful analysis of meiotic mutations that have been identified leads to the conclusion that two types of mechanisms account for chromosome separation:

- One type of mechanism is chromosome-specific: mutations in *eq*, *mei-G87*, *mei-S8*, *mei-G17* and (*ald* or *Axs*), tested genetically, affect only the disjunction of one or two chromosomes (X, 2, 4, X and 2, X and 4 respectively) chromosomes.

- Another has a global impact on all the chromosomes: *Dub*, *mei-I3*, *mei-081*, *mei-I1* and *nod* alter the disjunction of all chromosomes.

The two male-specific meiotic mutations discovered in the Sandler et al. (1968) screen showed very different phenotypes: *mei-S8* mutants showed high levels of 4th chromosome NDJ and no effect on disjunction of the sex chromosomes while *mei-081* mutation resulted in a genome wide increase in NDJ. These phenotypes are in agreement with the idea that at least two separate types of mechanisms exist for chromosome segregation, a general and a chromosome specific one.

The only male-specific gene that is currently available for further studies and has been cloned was also identified from the Zuker collection of EMS-mutagenized stocks and is called *teflon* (*tef*, Tomkiel et al, 2001). Mutations in *tef* are also meiosis I-specific and affect the segregation of autosomes only without disrupting the X-Y pairing. This finding confirms the existence of pairing pathways that are shared and some that show XY / autosome split.

Our research resulted in identifying a novel factor that functions in the pairing and segregation of meiotic homologous chromosomes. Results of refined

mapping, molecular and genetic analyses, described in the next chapter, demonstrate that *pf-2* is allelic to *modifier of mdg4* [*mod(mdg4)*].

The identification of this novel gene that is involved exclusively in male pathways of pairing and segregation of homologous chromosomes is a great step toward determining how in the absence of SC and chiasmata *Drosophila* male meiosis proceeds normally and what are the specific components of this system. A special feature of *pf-2* is to play a role in pairing and segregation pathways that are shared by all of the chromosomes. Many aspects of meiotic processes can be studied by characterizing *pf-2*, determining its expression pattern and its specific role during meiosis and identifying its cellular counterparts. The further study of such proteins will allow the determination of aspects of female and male meiosis that are conserved and those that differ.

Chapter Three

modifier of (mdg4) encodes a protein required for homolog pairing in *Drosophila melanogaster* male meiosis

* This part will be submitted for publication as Soltani-Bejnood M., Thomas S., Dorn R., Villeneuve L., and McKee B.D. (2004).

My work on this part consists of writing the manuscript and performing all the experiments except the mapping of the breakpoints of the deficiencies.

Abstract

Our research interest is to uncover mechanisms underlying meiotic chromosome pairing and segregation. *pairing failure 2* (*pf-2*) is a gene involved in this process during meiosis I of male *Drosophila*. The three *pf-2* alleles recovered in a screen for chemically induced (EMS) mutations on chromosome III that cause paternal loss of chromosome IV display strong meiotic phenotypes. Cytological analysis of testes of *pf-2* mutant flies revealed unpaired chromosomes at prophase and metaphase I and "laggard chromosomes" at anaphase I in primary spermatocytes. Meiosis II appears relatively normal. Genetic data confirm that non-disjunction (NDJ) occurs at the first meiotic division and affects the segregation of sex chromosomes as well as autosomes. Deficiency complementation showed that *pf-2* was mapped to region 93D6; 93E1 on chromosome arm 3R and shown to be allelic to *modifier of mdg4* [*mod(mdg4)*], a

complex locus that encodes a large family of chromosomal proteins by alternative and *trans*-splicing. The encoded proteins together occupy more than 500 sites on the polytene chromosomes. One isoform, Mod(mdg4)67.2 has previously been implicated in control of chromatin structure. We show that the *pf-2* mutations disrupt the function of a single isoform, Mod(mdg4)56.3, that is expressed in primary spermatocytes at all stages. Both a GFP-tagged Mod(mdg4)56.3 transgene and the native Mod(mdg4)56.3 protein localize as discrete foci to the major autosomes, and as an intensely fluorescent cluster of foci to the nucleolus throughout prophase. The nucleolar cluster resolves into a sharply defined structure associated with the X-Y bivalent. We conclude that Mod(mdg4)56.3 plays a critical role in homologous pairing in *Drosophila* male meiosis. Transgenic flies with a *pf-2* null genetic background and carrying [*hsp70-pf2* cDNA] fragment on their chromosome II display a complete rescue of the pairing failure phenotype. The expression pattern of the GFP-labeled Mod(mdg4)56.3 in transgenic flies' meiotic cells implies a role for this novel gene in chromosomal cohesion during meiosis.

I- Introduction

Meiotic events consist of two cellular divisions that result in the production of haploid gametes with half of the parental genomic complement. Pairing and accurate segregation of homologous chromosomes during the first division of meiosis are essential for the generation of euploid gametes.

Meiosis I is a reductional division in which homologs pair and segregate to opposite poles; sister chromatids then segregate at meiosis II. In most organisms, pairing of homologs is accompanied by formation and processing of double strand breaks (DSBs) resulting in recombination and chiasmata (discrete sites of crossovers) and synapsis.

Meiosis in *Drosophila* males utilizes an "achiasmatic" pathway in which recombination does not occur and neither SC nor chiasmata are detectable, yet homologs pair and segregate with high efficiency. Intimate pairing of homologous loci in early prophase has been demonstrated in *Drosophila* males by single locus fluorescent tagging (Vazquez et al., 2002). Although intimate pairing is lost at mid-prophase, homologs remain connected until anaphase I and this becomes evident in late prophase when the chromatin condenses into four compact bivalents. However, the factors responsible either for the early prophase intimate pairing or the late prophase homolog linkages have remained completely unknown.

The exact mechanism(s) by which chromosomes pair and segregate can be elucidated only by identifying the genes involved in this process and by isolating and characterizing their products. A large number of mutations that disrupt synaptonemal complex formation, recombination or segregation in *Drosophila* have been recovered and have led to the identification of several genes that are central to meiosis I. However, except for two genes involved in meiotic sister chromatid cohesion, *mei-S332* and *ord*, none of those identified thus far has any phenotype in male meiosis. Mutations that disrupt homolog segregation in male

meiosis have been recovered, some cause non-disjunction (NDJ) of only a subset of the chromosome complement, e. g. of autosomes only (*teflon*, Tomkiel et al., 2001) or the 4th chromosome only (*mei-S8*) or the sex chromosomes only (Baker and Carpenter, 1972). Others cause NDJ of both sex chromosomes and autosomes (male-specific mutations in *mei-I1* and *mei-081*). Beside *teflon*, the other mutants have been lost or reverted and currently the field is lacking mutations that disrupt specifically the homolog pairing and segregation in male meiosis.

A collaborative project with C. Zuker, B. Wakimoto and D. Lindsley started with a screen of 12000 highly mutagenized but non-lethal autosomes for mutations that disrupt transmission of chromosome four from homozygous males (Koundakjian et al., 2004; Wakimoto et al., 2004). As a result of the screening, we recovered and identified a novel gene, *pairing failure-2* (*pf-2*), required for homolog pairing and segregation in male meiosis. Cytological examination of primary spermatocytes from flies homozygous or hemizygous for the three *pf-2* alleles revealed the existence of unpaired chromosomes prior to prometaphase of meiosis I and thereafter at metaphase I and anaphase I. Mutant males carrying a marked Y chromosome were tested genetically and found to show 30–50% X-Y (homolog) NDJ, but negligible frequencies of X-X (sister chromatid) NDJ. Other crosses documented high levels of autosome NDJ (44%) for null alleles of *pf-2*, again specific for homologs. Thus, absence of *pf-2* induces pairing failure and random assortment of homologs that occurs exclusively at the first meiotic division.

Females carrying *pf-2* mutations were also tested genetically and did not display any NDJ phenotype or recombination defect. This indicates that *pf-2* is a gene required specifically for male meiosis.

Here we show by deletion mapping, a database search for candidate genes and complementation analysis that *pf-2* is allelic to *modifier of mdg4* (*mod(mdg4)*), a complex gene that encodes a large family of chromosomal proteins (Buchner et al., 2000). At least 33 different isoforms of *mod(mdg4),* each containing a 402 amino acid N-terminal common domain and a variable C-terminus, are generated by alternative and *trans*-splicing (Labrador and Corces, 2003; Krauss and Dorn., 2004). Most of the C-terminal exons contain a Cys_2-His_2 zinc finger motif (Krauss and Dorn, 2004). The first mutations of *mod(mdg4)* were identified by their modifying effect on several *gypsy*-induced mutations (Georgiev and Gerasimova, 1989). The *gypsy* retrotransposon, which prevents enhancer-promoter interactions when inserted between them, contains a 350-bp insulator element. Two mutations specifically disrupt a single isoform of *mod(mdg4)*, Mod(mdg4)67.2 that has been shown to be essential, along with the Su(Hw) protein, for *gypsy* insulator function (Gerasimova et al., 1995).

We show that the Mod(mdg4)56.3 isoform is specifically disrupted by *pf-2* mutations and that lack of this isoform fully accounts for the meiotic phenotypes of *pf-2* mutants. The C-terminal domain of this isoform is encoded by sequences in the same DNA strand as the common N-terminal sequences; however, we show

genetically that the *mod(mdg4)56.3* transcript , like that of *mod(mdg4)67.2* isoform, is generated by *trans*-splicing.

The expression of Mod(mdg4)56.3 was analyzed by fluorescence microscopy using both a GFP-tagged *mod(mdg4)56.3* transcript and an antibody specific for the native Mod(mdg4)56.3 isoform. We show that Mod(mdg4)56.3 is present in primary spermatocytes nuclei from early prophase through metaphase I, on foci clustered in the nucleolus and on linear arrays along the axes of the major autosomes. Intriguingly, both the autosomal and nucleolar foci condense into very compact structures at prometaphase and are associated with the autosomal and sex chromosomal bivalents. Our data thus represent the first evidence for a structural basis for the achiasmatic homolog linkages in *Drosophila* male meiosis.

II- Results

Cytological analysis of primary spermatocytes of *pf-2* mutants revealed the presence of non-disjoined chromosomes

As reported elsewhere (Wakimoto et al., 2004), a screen of 12000 EMS-mutagenized autosomes led to the isolation of 48 mutations with a meiotic NDJ phenotype, as shown by irregular segregation of chromosome 4 in a genetic test, and unequal spermatid nuclei. Further testing using a genetically marked Y chromosome revealed that most of these mutations also caused elevated X-Y NDJ. Three of these mutations (*Z3-5578*, *Z3-3298* and *Z3-3401*) mapped to the

same region of chromosome 3 and were subsequently shown to be allelic (see below). These three mutations define the *pf-2* complementation group.

In order to characterize the defects occurring in *pf-2* males, testis dissections and staining were performed. Acetic orcein staining was used to characterize chromosome behavior during the meiotic divisions. Orcein-stained chromosomes are invisible during the early stages of prophase, but become visible as the chromatin fibers condense at late prophase, which allows the detection of paired chromosomes. In spermatocytes from wild type control males, three distinct masses corresponding to the major bivalents were seen at early stages of the condensation process. They occupy delimited regions on the inside of the nuclear membrane. Tightly paired and condensed homologs were visible at prometaphase. Cytological examination of primary spermatocytes from flies homozygous for each of the three *pf-2* alleles or hemizygous for each of these alleles and the deficiency *Df(3R)GC14*, which covers the entire *mod(mdg4)* locus, revealed the existence of unpaired chromosomes at prometaphase and metaphase and "laggard chromosomes" at anaphase of meiosis I (Chapter 2, Figures 2 and 3). Typical prometaphase spreads in *pf-2* mutant spermatocytes showed four univalents and one bivalent or six univalents, considering only the three major chromosome pairs. Notably, both chromosome condensation and sister chromatid cohesion appeared normal at this stage. Elevated frequencies of unpaired orcein-stained chromosomes (30-50%) were observed as soon as the chromosomes could be resolved at late prophase of the first division.

Testes of hemizygote flies carrying both a *pf-2* mutation (*Z3-3298* or *Z3-3401*) and the deficiency *Df(3R)GC14* were also stained with the fluorescent DNA dye DAPI to allow the examination of earlier stages of prophase I. Chromosomes were more extended and less compact in mutants relative to wild type flies' tissues treated in similar conditions, in which they are closer to the membrane in a delimited region. However, it was impossible to estimate the stage at which unpairing occurs, as chromosomes are not fully condensed at early stages of meiosis (chapter 2, Figure 4).

No obvious abnormalities were observed in germ-line mitotic divisions or in the second division of meiosis. Although the somatic mitoses were not directly examined, *pf-2* hemizygous and *trans*-heterozygous flies are viable and exhibit normal developmental rates and morphology, indicating that *pf-2* mutations are largely specific for the first meiotic division.

pf-2 and *mod(mdg4)* are allelic

We mapped the *pf-2* gene to the region 93D6-E1 on the third chromosome by deletion complementation. FlyBase search led to the identification of candidate genes within the region of interest. We tested alleles of three candidate genes (e.g. *tinman*, *hsr-omega* and *mod(mdg4)*). The results of complementation of *pf-2* alleles using the X-Y NDJ assay (see Experimental Procedures) show that alleles of both *tinman* and *hsr-omega* fully complemented *pf-2* allele *Z3-5578*. However, non-complementation was observed between *mod(mdg4)* and *pf-2* alleles, although the results were complex.

A complex complementation pattern would not be unexpected if *pf-2* and *mod(mdg4)* are allelic. *mod(mdg4)* encodes thirty three different isoforms by alternative splicing (Buchner et al., 2000; Dorn et al., 2001, Labrador and Corces, 2003, Krauss and Dorn, 2004). All the proteins contain a common N-terminus of 402 amino acids that includes a BTB / POZ [(*B*road complex, *T*ramtrack, *B*ric a brac) / (Poxyvirus Zinc Finger)] domain (Zollman et al., 1994; Bardwell and Treisman, 1994), whereas the C-termini are variable. The C-terminal exons are present in several clusters proximal to the common region (Dorn et al., 2001). The structure of *mod(mdg4)* locus and the locations of the mapped mutations and deletions are shown in Figure 1 and the relevant information about allele location is included in Table 1c.

Results of complementation tests of *pf-2 / mod(mdg4)* heterozygotes are summarized in Table 1 (for details, see Appendix). These data showed that all three *pf-2* alleles fail to complement complete deletions of *mod(mdg4)* (Table 1a), but give specific patterns of complementation against partial deletions (Table 1b). Two deletions, $mod(mdg4)^{B2}$ and $mod(mdg4)^{eGp4}$, fail to complement both *Z3-5578* and *Z3-3298*, but complement *Z3-3401* as well as all lethal alleles of *mod(mdg4)* (Table 1b). Conversely, several small deletions *-Δ15*, *-Δ29*, *-Δ32*, *-Δ49* and *-R32* complemented both *Z3-5578* and *Z3-3298* but failed to complement *Z3-3401* (Table 1b). These deletions also fail to complement lethal alleles of *mod(mdg4)* with lesions in the common region. To further evaluate these results, we mapped the breakpoints of *mod(mdg4)* deficiency stocks *-B2*, *-T16*, *-Δ10*, *-Δ33*, Df(3R)*e-Gp4* and Df(3R)*GC14*. Single nucleotide polymorphisms (SNPs),

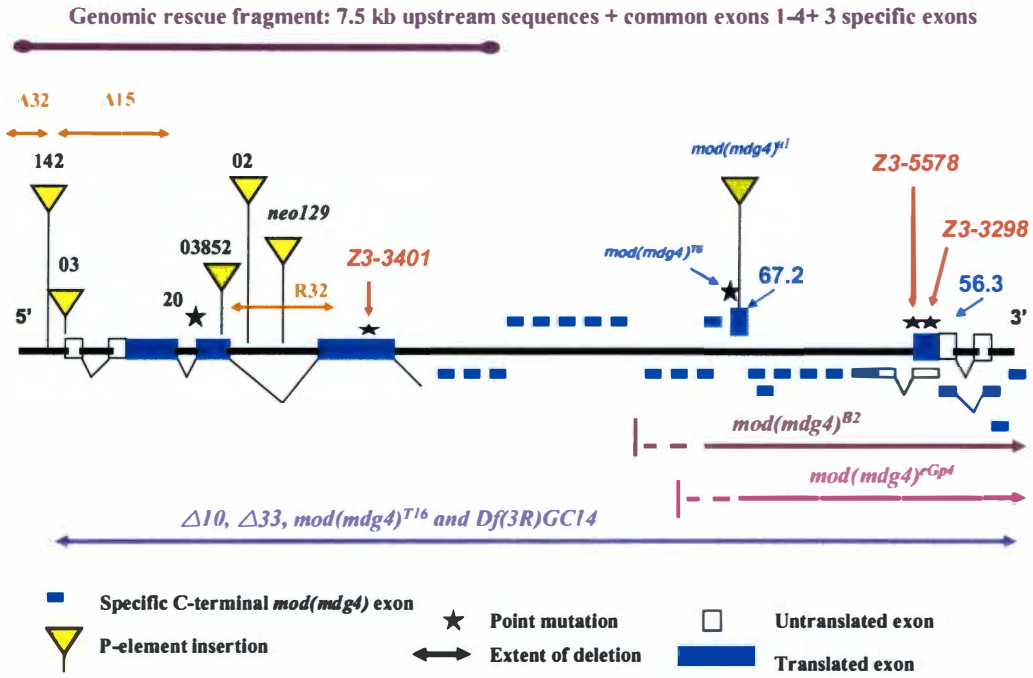


Figure 1: Graphical representation of the structure of *mod(mdg4)* locus. The location of mapped mutations and the extent of deletions are indicated. Also shown is the extent of the genomic fragment inserted on chromosome II of transgenic flies by P-element-mediated transformation. This figure is not drawn to scale.

Table 1: Complementation analysis to identify *pf-2*

(1a) Complementation analysis of males hemizygous for a *pf-2* mutation and a deficiency covering the entire *mod(mdg4)* locus. We have found $mod(mdg4)^{T16}$ is deleted for all of the $mod(mdg4)^{T16}$ locus. Females with the indicated genotype were crossed to males bearing a marked Y chromosome and one of the three *pf-2* alleles. F2 hemizygote males displayed high NDJ frequencies.

	% NDJ		
♀ ♂	***Z3-5578***	***Z3-3298***	***Z3-3401***
Df(3R)e-H4	45.99	50.08	49.21
Df(3R)GC14	47.66	49.9	47.9
Df(3R)e-GC3	40.52	47.28	46.91
Df(3R)e-BS2	46.38	56.85	54.42
$mod(mdg4)^{\Delta 10}$	46.43	43.19	49.32
$mod(mdg4)^{\Delta 33}$	37.25	43.53	49.11
$mod(mdg4)^{T16}$	44.3	44.57	39.55

Table 1 continued

(1b) Differential complementation pattern of males hemizygous for a *pf-2* mutation and partial deletions of *mod(mdg4)*. Based on the extent of the deletion, one, *Z3-3401*, or the two other, *Z3-5578* and *Z3-3298*, allele(s) of *pf-2* were complemented.

		% NDJ	
♀ : ♂	***Z3-5578***	***Z3-3298***	***Z3-3401***
mod(mdg4)$^{\Delta 15}$	0.56	2.34	42.07
mod(mdg4)$^{\Delta 29}$	1.6	1.83	49.36
mod(mdg4)$^{\Delta 32}$	0.95	1.46	28.34
mod(mdg4)$^{\Delta 49}$	0	0.88	54.93
mod(mdg4)^{R32}	2.55	1.93	44.27
mod(mdg4)^{eGp4}	50.31	49	5.79
mod(mdg4)^{B2}	37.31	40.22	0

Table 1 continued

(1c) Complex complementation pattern of males heterozygous for a *pf-2* mutation and alterations within the common N-terminal region of *mod(mdg4)*. These alterations don't complement the *Z3-3401* allele of *pf-2* but partially to fully complement the two other alleles *Z3-5578* and *Z3-3298*.

♀ ♂:	Alteration	% NDJ		
		Z3-5578	*Z3-3298*	*Z3-3401*
mod(mdg)$^{l(3)03852}$	P-element	6.36	9.24	45.74
mod(mdg)^{117}	EMS	0.75	1.55	47.15
mod(mdg)^{269}	EMS	1.44	2.59	49.47
mod(mdg)^{324}	EMS	2.84	5.06	44.38
mod(mdg)^{340}	EMS	1.89	3.18	54.09
mod(mdg)$^{E(var)3-93D}$	P-element	4.46	7.64	41.19
mod(mdg)^{02}	P-element	1.72	2.56	32.11
mod(mdg)^{03}	P-element	0.07	0.12	34.92
mod(mdg)^{04}	Spontaneous	2.96	6.22	43.09
mod(mdg)^{20}	EMS	0.52	0.28	45.81

Table 1 continued

(1d) Complementation analysis between *pf-2* alleles. Males trans-heterozygous for 2 *pf-2* alleles were crossed to *y w* females and their progeny were scored for NDJ. Two of the alleles, *Z3-3298* and *Z3-5578*, complement the third one, *Z3-3401*, but not each other.

Genotype	% NDJ
Z3-3298 / Z3-5578	45.69
Z3-3401 / Z3-3298	1.7
Z3-3401 / Z3-5578	1.07

Table 1 continued

(1e) *pf-2* and ***mod(mdg4)^{T6}*** are not allelic. *mod(mdg4)^{T6}* is a point mutation within a specific C-terminal exon that affects only one isoform, Mod(mdg4)67.2. *pf-2* mutation *Z3-3401*, as well as the deficiency *Df(3R)GC14*, both complemented ***mod(mdg4)^{T6}*** mutation, excluding Mod(mdg4)67.2 as a meiotic isoform.

		Progeny		Total	Tested
Genotype of parents		♀ ***B^{+}w^{+}***	♂ ***B w***	**Flies**	**Males**
♂	♀				
mod(mdg4)^{T6} / Z3-3401	***y w***	560	517	1077	15
mod(mdg4)^{T6} / Df(3R)GC14	***y w***	777	636	1413	18

represented at about one in every 200 nucleotides (Moriyama and Powell, 1996), can be used as markers to compare different *Drosophila* lines (Teeter et al., 2000). The breakpoints of deletions were determined molecularly by comparing the genomic sequences of two lines heterozygous for the same *mod(mdg4)* deficiency and one wild type (either Canton S or Oregon R), nearly isogenic for chromosome 3 (Hoskins et al., 2001), and detecting SNPs within a specific locus (see Experimental Procedures). The map locations of the deficiency breakpoints are displayed in Figure 1. They show that all of the deletions that fail to complement *Z3-3401* remove part or the entire common region; whereas the two deletions that complement *Z3-3401* but fail to complement *Z3-5578* and *Z3-3298* remove part of the variable region but do not disturb the common region.

Taken together, the deficiency complementation results suggest that *Z3-3401* may be located in the common region and *Z3-5578* and *Z3-3298* may be located in the variable region. This conclusion is supported by further complementation analyses. Most of the tested *mod(mdg4)* mutations and deficiencies that affect the N-terminal common region are lethal as homozygotes. All such alleles fail to complement the *pf-2* allele *Z3-3401* but partially or completely complement the other two alleles (Table 1c). Moreover, *Z3-5578* and *Z3-3298* complement the third allele, *Z3-3401* (Table 1d), suggesting that *pf-2* mutants fall into two groups that partially complement each other. These results led to the conclusion that *pf-2* and *mod(mdg4)* are allelic, but the complementation pattern is complex and that the two groups of *pf-2* alleles result from mutations in different regions of the complex *mod(mdg4)* locus. The complex interallelic complementation can be

explained by the occurrence of *trans*-splicing between two pre-mRNAs carrying coding sequences of two separate exons of the same gene, *mod(mdg4)*.

The phenotype seen in *pf-2* mutant flies is not mediated by the insulator proteins Su(Hw) and Mod(mdg4) 67.2

Suppressor of Hairy-wing (Su(Hw)) has been extensively studied as a protein that interacts with Mod(mdg4) and mediates its function in gene expression and chromatin remodeling (Gdula and Corces, 1997; Georgiev and Kozycina, 1996; Geyer and Corces, 1992; Gerasimova and Corces, 2001). We addressed the question whether the role of Mod(mdg4) in homolog pairing during meiosis was mediated by its interaction with Su(Hw). Strong alleles of *su(Hw)* were tested and no NDJ was detected in males lacking a functional Su(Hw) protein. Furthermore, the $mod(mdg4)^{T6}$ allele results from a point mutation in the *mod(mdg4)67.2* specific coding region, producing a truncated protein that lacks the last 32 residues at the C-terminal acidic domain (Gerasimova et al., 1998). Hemizygote $mod(mdg4)^{T6}$ / *Df(3R)GC14* and *trans*-heterozygote $mod(mdg4)^{T6}$ / *pf-2* males did not exhibit any NDJ phenotype (Table 1e). These data suggest that the phenotype seen in *pf-2* mutant flies is not mediated by the insulator proteins Su(Hw) and Mod(mdg4)67.2.

Identification of *mod(mdg4)56.3* as the *mod(mdg4)* isoform causing the NDJ phenotype when mutated

The *pf-2* phenotype is likely to be meiosis-specific, since *pf-2* trans-heterozygotes and hemizygotes are viable and fertile, unlike most *mod(mdg4)* mutations, which are embryonic lethals. Some *mod(mdg4)* mutations have been mapped to the common region and are expected to result in loss or strongly reduced amounts of all the isoforms. Presumably, some of the Mod(mdg4) proteins play a role in early development and their alteration causes lethality. It is therefore expected that the variable C-terminal exons confer specific biological roles to each isoform (Buchner et al., 2000; Dorn et al., 2001). Thus, it seemed likely that the meiosis-specific phenotype of *pf-2* alleles would be caused by mutations in a specific C-terminal exon disrupting only one isoform.

In support of this interpretation, most of the complementation data are consistent with the hypothesis that *Z3-3401* is located in the common region whereas *Z3-5578* and *Z3-3298* are located in the C-terminal exon. Since *Z3-3298* and *Z3-5578* fail to complement each other, we expected that they would disrupt the same C-terminal exon.

To locate the *pf-2* mutations within the *mod(mdg4)* locus, we sequenced all of the exons and some of the introns from the genomic DNA of three *pf-2* mutant alleles and the parental *bw; st* strain used to generate these stocks. Only one mutation was found in each of the three alleles. The mutations in two of the alleles (*Z3-5578* and *Z3-3298*) are located in the C-terminal exon of the *mod(mdg4)56.3* isoform. The third mutation was found to change a residue in the

common region. The encoded protein, designated "Doom", was previously identified in a yeast-two-hybrid screen for *Drosophila* proteins that interact with the baculovirus inhibitor of apoptosis protein (IAP, p35). The Doom protein induces apoptosis when overexpressed in insect cells (Harvey et al., 1997). However, it is not known whether this interaction and apoptosis phenotype are relevant to the normal physiological function of the Mod(mdg4)56.3 isoform. The C-terminus of *mod(mdg4)56.3*, which distinguishes it from other Mod(mdg4) isoforms, is responsible for engagement of IAPs and induction of programmed cell death (Harvey et al., 1997). Like most C-terminal exons of Mod(mdg4), the C-terminal exon of Mod(mdg4)56.3 encodes a single non- canonical Cys_2His_2 zinc-finger which is part of a larger homology domain known as the FLYWCH domain. Interestingly, both of the *mod(mdg4)56.3* exonic mutations disrupt the zinc finger. Mutant DNA from the *Z3-5578* allele contains a G → A transition at the nucleotide position 1498 resulting in a W449 -> stop codon change. The mutation in *Z3-3298* consists of a C → T base change at the 1683 position leading to H511 → Y amino acid substitution. The residue 511 is a highly conserved component of the Cys_2 His_2 motif found in the C-terminal exon of >30 out of 33 *mod(mdg4)* isoforms (Labrador and Corces, 2003; Krauss and Dorn, 2004). The truncated protein expressed in *Z3-5578* flies lacks the Cys_2His_2 finger along with all of the conserved residues in the C-terminal domain. *Z3-5578* is a null allele of *pf-2* based on complementation data. Interestingly, the mutation in the *Z3-3401* allele is not in the *mod(mdg4)56.3* specific domain, but rather in the common region, a C → T transition at position 822, which is located within the 4^{th} exon of

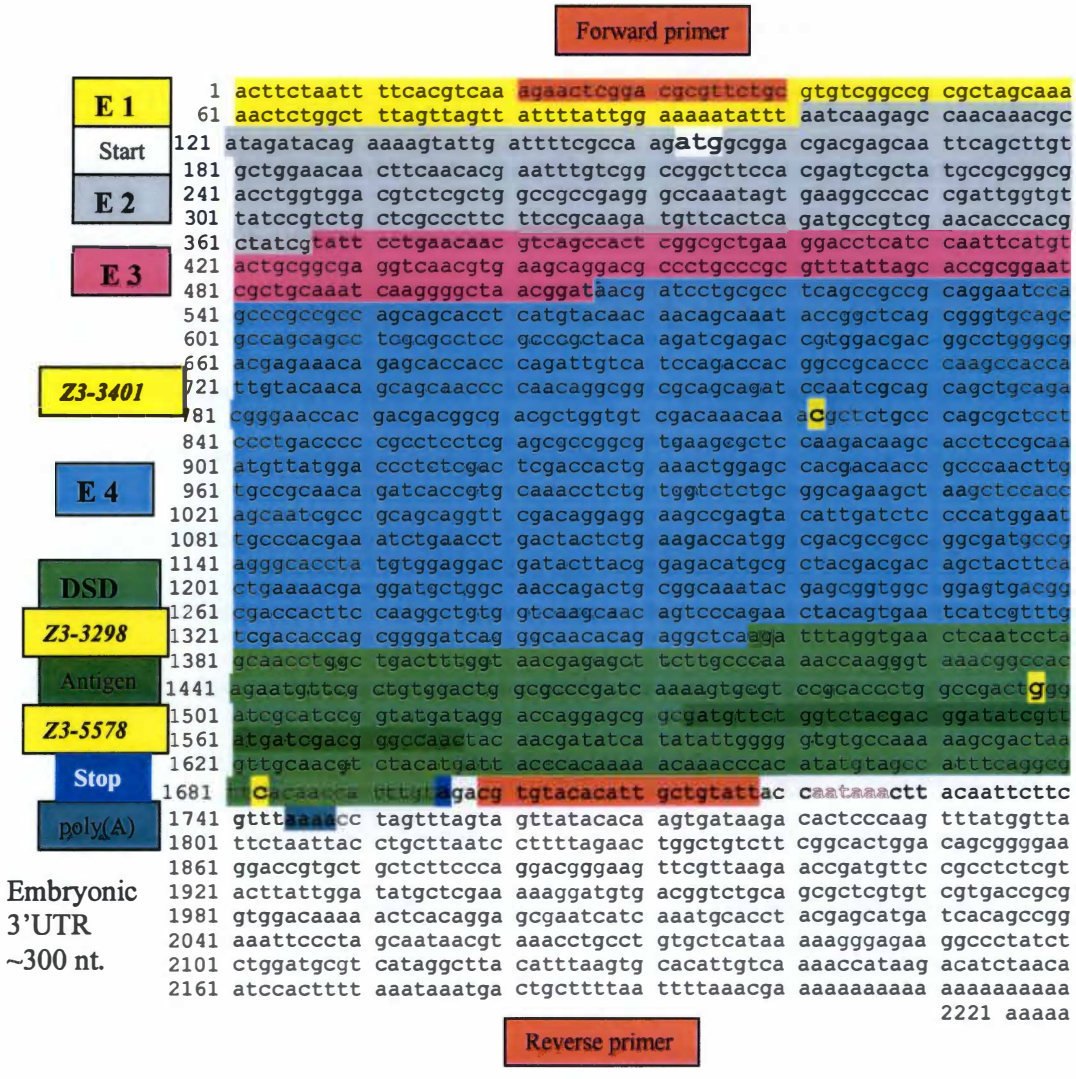
mod(mdg4), and results in R224 → Cys residue change (Figures 2 and 3). No information has been reported about the conservation / putative function of this residue. *Z3-3401* is a hypomorph as hemizygote flies that carry both this *pf-2* mutation and a deletion within the *mod(mdg4)* region display a more pronounced phenotype compared to homozygote flies (chapter 2, Table 1).

Mod(mdg4) 56.3*:* sequence, features and *pf-2* mutations

Based on the genomic complexity of *mod(mdg4)*, and as the protein expressed in this tissue could have been a splicing variant of *mod(mdg4)56.3*, it was important to identify the transcripts present in testis that contained the specific *mod(mdg4)56.3* C-terminal sequence. The full-length cDNA of *mod(mdg4)56.3* was amplified from RT-PCR products prepared from testes of *y w* flies and carried a 3'UTR that was shorter (only 60 nucleotides) than the published sequence (300 nt., Dorn et al., 2001) from embryonic preparations but their predicted protein sequences were identical (Figures 2 and 3).

Computational analyses of the Mod(mdg4)56.3 sequence and specific features include 8 alpha helices and 13 beta strands connected by loops that cover 57% of the structure; many sites for phosphorylation, glycosylation, myristylation and amidation are present. No specific structure could be predicted for Mod(mdg4)56.3 based on its sequence, as all Mod(mdg4) isoforms and many other proteins involved in protein-protein interactions share the well-characterized BTB domain.

Figure 2: Nucleotide sequence of *mod(mdg4)56.3*. All the features of this sequence are indicated. Exons 1-4 are represented as E1, E2, E3 and E4 and are common to all mod(mdg4) isoforms. The Doom specific domain (DSD) consists of the C-terminal variable exon of *mod(mdg4)56.3*. The fragment indicated as Antigen is the short 15-residue sequence used for the generation of polyclonal antibodies against the specific C-terminus of Mod(mdg4)56.3. The three *pf-2* mutations are highlighted single nucleotides (yellow) within the sequence. The reverse primer has been designed close to the 'stop' signal as the 3'UTR of testis mod(mdg4) is only 52 nt. long (**outlined**). The beginning of polyadenylation is marked by the 'Poly(A)' box and indicates where the 3'UTR of the testis *mod(mdg4)56.3* transcript ends. This figure shows the published sequence of this isoform extracted from embryonic preparations (Buchner et al., 2000), which includes a ~300nt. long 3'UTR . The forward and reverse primers were used to amplify by PCR a 1718 nt. long *mod(mdg4)56.3* cDNA.


Forward primer
E 1
Start
E 2
E 3
Z3-3401
E 4
DSD
Z3-3298
Antigen
Z3-5578
Stop
poly(A)
Embryonic
3'UTR
~300 nt.
1 acttctaatt ttcacgtcaa agaactcgga cgcgttctgc gtgtcggccg cgctagcaaa
61 aactctggct ttagttagtt attttattgg aaaaatattt aatcaagagc caacaaacgc
121 atagatacag aaaagtattg attttcgcca agatggcgga cgacgagcaa ttcagcttgt
181 gctggaacaa cttcaacacg aatttgtcgg ccggcttcca cgagtcgcta tgccgcggcg
241 acctggtgga cgtctcgctg gccgccgagg gccaaatagt gaaggcccac cgattggtgt
301 tatccgtctg ctcgcccttc ttccgcaaga tgttcactca gatgccgtcg aacacccacg
361 ctatcgtatt cctgaacaac gtcagccact cggcgctgaa ggacctcatc caattcatgt
421 actgcggcga ggtcaacgtg aagcaggacg ccctgcccgc gtttattagc accgcggaat
481 cgctgcaaat caaggggcta acggataacg atcctgcgcc tcagccgccg caggaatcca
541 gcccgccgcc agcagcacct catgtacaac aacagcaaat accggctcag cgggtgcagc
601 gccagcagcc tcgcgcctcc gcccgctaca agatcgagac cgtggacgac ggcctgggcg
661 acgagaaaca gagcaccacc cagattgtca tccagaccac ggccgcaccc caagccacca
721 ttgtacaaca gcagcaaccc caacaggcgg cgcagcagat ccaatcgcag cagctgcaga
781 cgggaaccac gacgacggcg acgctggtgt cgacaaacaa acgctctgcc cagcgctcct
841 ccctgacccc cgctcctcg agcgccggcg tgaagcgctc caagacaagc acctccgcaa
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961 tgccgcaaca gatcaccgtg caaacctctg tggtctctgc ggcagaagct aagctccacc
1021 agcaatcgcc gcagcaggtt cgacaggagg aagccgagta cattgatctc cccatggaat
1081 tgcccacgaa atctgaacct gactactctg aagaccatgg cgacgccgcc ggcgatgccg
1141 agggcaccta tgtggaggac gatacttacg gagacatgcg ctacgacgac agctacttca
1201 ctgaaaacga ggatgctggc aaccagactg cggcaaatac gagcggtggc ggagtgacgg
1261 cgaccacttc caaggctgtg gtcaagcaac agtcccagaa ctacagtgaa tcatcgtttg
1321 tcgacaccag cggggatcag ggcaacacag aggctcaaga tttaggtgaa ctcaatccta
1381 gcaacctggc tgactttggt aacgagagct tcttgcccaa aaccaaggat aaacggccac
1441 agaatgttcg ctgtggactg gcgcccgatc aaaagtgcgt ccgcaccctg gccgactggg
1501 atcgcatccg gtatgatagg accaggagcg gcgatgttct ggtctacgac ggatatcgtt
1561 atgatcgacg ggccaactac aacgatatca tatattgggg gtgtgccaaa aagcgactaa
1621 gttgcaacgt ctacatgatt acccacaaaa acaaacccac atatgtagcc atttcaggcg
1681 tttcacaacca tttgtagacg tgtacacatt gctgtattac caataaacctt acaattcttc
1741 gtttaaaacc tagtttagta gttatacaca agtgataaga cactcccaag tttatggtta
1801 ttctaattac ctgcttaatc cttttagaac tggctgtctt cggcactgga cagcggggaa
1861 ggaccgtgct gctcttccca ggacgggaag ttcgttaaga accgatgttc cgcctctcgt
1921 acttattgga tatgctcgaa aaaggatgtg acggtctgca gcgctcgtgt cgtgaccgcg
1981 gtggacaaaa actcacagga gcgaatcatc aaatgcacct acgagcatga tcacagccgg
2041 aaattcccta gcaataacgt aaacctgcct gtgctcataa aaagggagaa ggccctatct
2101 ctggatgcgt cataggctta catttaagtg cacattgtca aaaccataag acatctaaca
2161 atccactttt aaataaatga ctgcttttaa ttttaaacga aaaaaaaaaa aaaaaaaaaa
2221 aaaaa
Reverse primer
3'UTR (testis) 52 nt.
Canonical polyA signal

Figure 3: The predicted amino acid sequence of Mod(mdg4)56.3 deduced from the nucleotide sequence of a 3-12 h *D. melanogaster* embryo cDNA library is shown. Different domains predicted by Scan Prosite are indicated. *mod(mdg4)* isoforms are named according to the putative molecular weights of the full length proteins. Residues mutated in *Z3-5578* and *Z3-3298* are highly conserved among several isoforms and are located within the C-terminal specific domain of *mod(mdg4)56.3*. The mutation in *Z3-3401* affects a residue encoded by the 4th exon of *mod(mdg4)* that is common to all isoforms.

```
  1 MADDEQFSLC WNNFNTNLSA GFHESLCRGD LVDVSLAAEG QIVKAHRLVL SVCSPFFRKM
 61 FTQMPSNTHA IVFLNNVSHS ALKDLIQFMY CGEVNVKQDA LPAFISTAES LQIKGLTDND
121 PAPQPPQESS PPPAAPHVQQ QQIPAQRVQR HEPRASARYK IETVDDGLGD EKQSTTQIVI
181 QTTAAPQATI VQQQQPQQAA QQIQSQQLQT GTTTTATLVS TNKRSAQRSS LTPASSSAGV
241 KRSKTSTSAN VMDPLDSTTE TGATTTAQLV PQQITVQTSV VSAAEAKLHQ QSPQQVRQEE
301 AEYIDLPMEL PTKSEPDYSE DHGDAAGDAE GTYVEDDTYG DMRYDDSYFT ENEDAGNQTA
361 ANTSGGGVTA TTSKAVVKQQ SQNYSESSFV DTSGDQGNTE AQDLGELNPS NLADFGNESF
421 LPKTKGKRPQ NVRCGLAPDQ KCVRTLDDWD RIRYDRTRSG DVLVYDGYRY DRRANYNDII
481 YWGCAKKRLS CNVYMITHKN KPTYVAISGV HNHL
```

Z3-3401: R224→C *Z3-5578*: W449→Stop *Z3-3298*: H511→Y

BTB domain **Gln-rich region** **Thr-rich region**

Underlined: FLYWCH zinc finger domain

Bipartite nuclear targeting sequence, identified by the presence of two successive Arginines

Complete rescue of NDJ by heat shock-driven expression of GFP tagged-Mod(mdg4)56.3

The ultimate proof that a gene, when mutated, causes a specific altered phenotype is the rescue of the mutant phenotype in transgenic flies bearing the wild type copy of that gene. We generated flies with a *pf-2* null genetic background carrying the *P{ry*$^{+}$*, hsp70 - mod(mdg4)56.3 - GFP}* transgene (Figure 4). Both second and third chromosome transgenic lines and many different heat shock conditions were tested for the rescue experiments. Control flies were siblings generated in the same crossing scheme not carrying the transgene and treated under the same experimental conditions (for crossing schemes, see Experimental procedures). Data reported in Table 2 indicate that heat shock driven expression of both tested insertions of the *mod(mdg4)56.3* cDNA fully rescues the chromosomal non-disjunction phenotype of *pf-2* males (<1% NDJ in transgenic flies vs. 36-43% in controls). Moreover, significant improvement in disjunction values was observed in the absence of heat shock. This indicates that *hsp70* promoter drives leaky expression of Mod(mdg4)56.3 in primary spermatocytes, even though it was previously reported as being active only in gonial stem cells.

We conclude that the meiotic non-disjunction phenotype of *pf-2* mutations is fully rescued by expression of the Mod(mdg4)56.3 isoform.

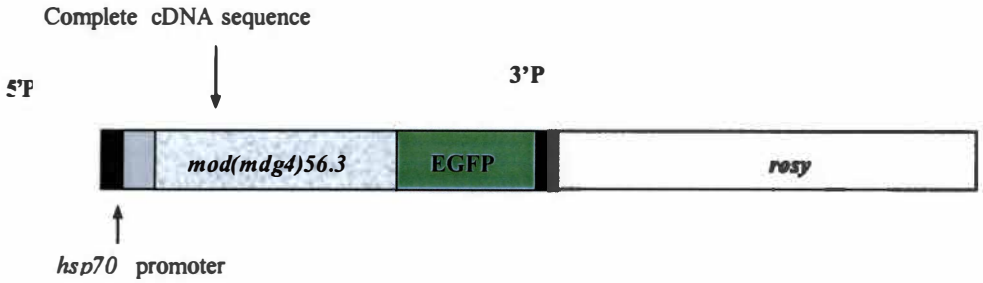


Figure 4: The rescue construct used to transform flies. The *hsp70* promoter sequences drive the expression of the C-terminally GFP-tagged cDNA of *mod(mdg4)56.3* (Schotta and Reuter, 2000). This transgene is carried on chromosomes II or III of flies with a rescued phenotype and is absent in control flies.

Table 2: Complete rescue of the NDJ phenotype by in vivo expression of *mod(mdg4)-gfp* cDNA. Males with the indicated genotype were heat shocked (1 hr. at 39°) at different stages of their life before eclosion. After eclosion, these males were collected every 3 days and crossed to *y w* females. Progeny were scored for NDJ. The transgene, *P* {*ry*$^{+}$; [*hsp70-mod(mdg4)56.3-gfp*], is carried on chromosomes II or III and is absent in non-transgenic, control flies.

	% NDJ (scored progeny)		
	Non-transgenic	**Transgenic**	
P{ry+, hsp70-mod(mdg4)56.3-gfp}	**no hs**		**hs**
Insertion on chromosome II			
Z3-3401 / Z3-3401	**41.77** (79)	**0.13** (783)	**0.17** (576)
Z3-3298 / Z3-5578	**43.46** (237)	**15.79** (95)	**0.76** (263)
Insertion on chromosome III			
mod(mdg4)^{T16} / Z3-5578	**40.83** (169)	**9.62** (499)	**0.92** (109)
mod(mdg4)^{T16} / Z3-3298	**37.12** (132)	**9.01** (577)	**0** (357)
mod(mdg4)^{T16} / Z3-3401	**36.42** (335)	**6.88** (523)	**0.72** (139)

Expression pattern of Mod(mdg4)56.3-GFP

Live primary spermatocytes from endogenous *pf-2* null transgenic lines expressing the GFP-tagged Mod(mdg4)56.3 were analyzed by fluorescent microscopy and revealed nuclear signals representing the *hsp70*-driven expression of the transgene (Figure 5 d-f). In control flies, hemizygous for *pf-2* mutation and *mod(mdg4)^{T16}* deletion, no fluorescent spots were detected (Figure 5 a-c). To show that fluorescent foci were indeed related to GFP expression in these meiotic cells, testis preparations were incubated with anti-GFP as primary and FITC-conjugated IgGs as secondary antibodies and the analysis of primary spermatocytes showed the presence of similar nuclear signals (Figure 6, a and b), represented as foci clustered apparently in the nucleolar regions. The nucleolar localization would not be surprising as the X-Y pairing sites are within the rDNA sequences in proximity of the nucleolus, known as the site of ribosome biosynthesis. The presence of Mod(mdg4)56.3 close to the nucleolus is consistent with its role in pairing of the sex chromosomes.

In order to show that anti-GFP was actually detecting the tagged Mod(mdg4)56.3 protein, testis preparations were also incubated with anti-Mod(mdg4)56.3 antibodies that were raised against the C-terminal specific domain of this isoform (see Experimental Procedure) and that recognize exclusively this protein. The immunofluorescent signals seen in Figure 7 confirm that the expression of the same protein was indeed revealed by the use of either antibody. To exclude any possibility that the localization of the fusion protein Mod(mdg4)56.3-GFP is driven by its GFP moiety, may differ from the one for the

Figure 5: Expression of Mod(mdg4)56.3-GFP in live primary spermatocytes of *Z3-3298 / mod(mdg4)^{T16}* males in the absence (*Top*) or presence (*Bottom*) of a P-element carrying the *mod(mdg4)56.3-gfp* cDNA downstream of *hsp70* promoter sequences. Hand-dissected testes of *pf-2* null males were analyzed with a fluorescent microscope. Meiotic cells are seen with transmitted light (a and d), DNA was visualized by staining live preparations with Hoechst 33342 (c). No GFP fluorescence was detected in control males null for *pf-2* and lacking the transgene (b). The expression of the GFP-tagged Mod(mdg4)56.3 was revealed by the fluorescence associated with the native GFP (e). Meiotic cells (d, shown in red) and GFP fluorescence seen in e were merged in the picture (f) that shows GFP foci, present as single spots or as clusters, within the nucleus of transgenic males' meiotic cells.

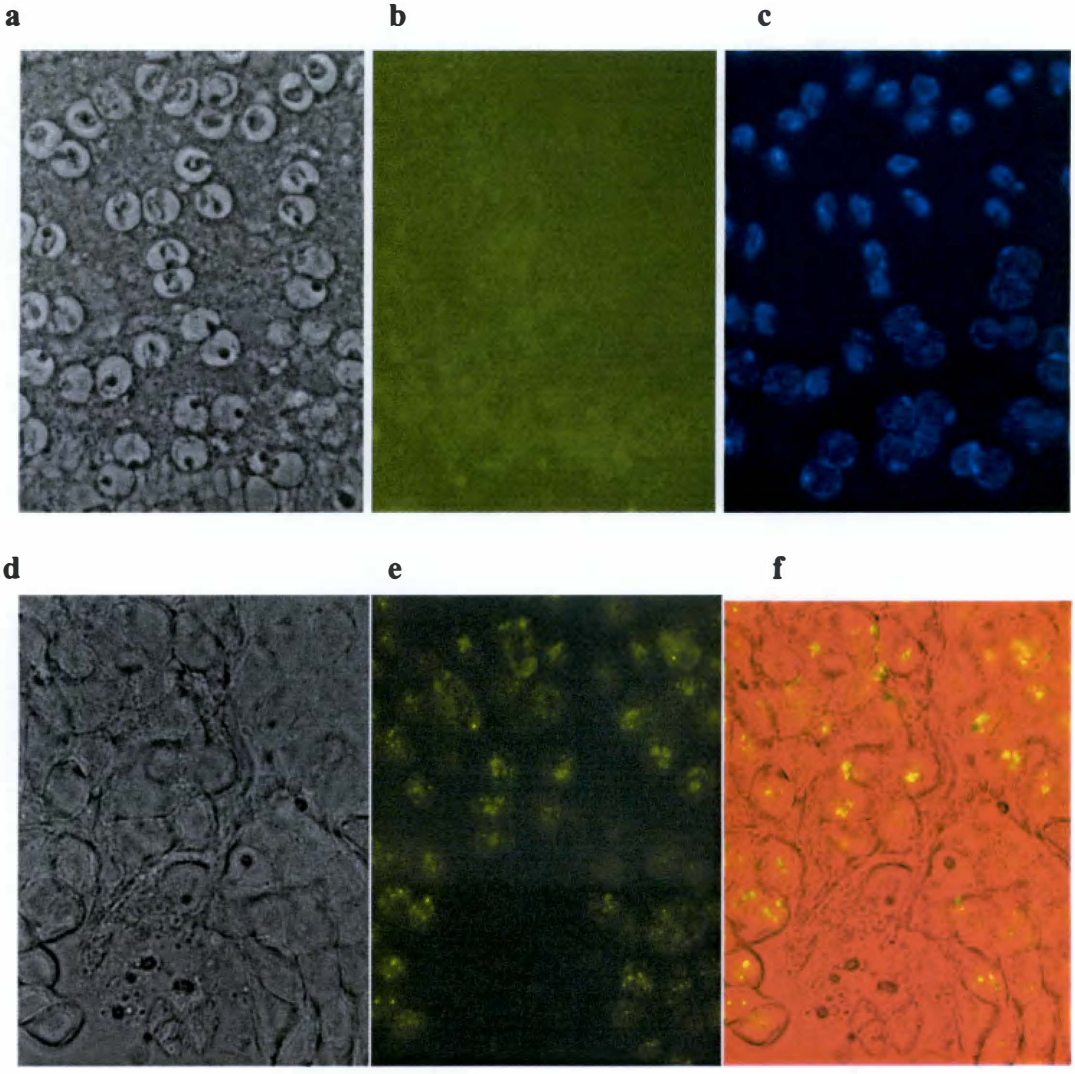

a
b
c
d
e
f

Figure 6: Expression of Mod(mdg4)56.3-GFP detected by anti-GFP antibody staining. (6a): Phase contrast view (a and e) of meiotic cells from males null for endogenous *pf-2* and transgenic for the P-element carrying the GFP-tagged *mod(mdg4)56.3* cDNA reveal the localization of GFP foci stained with rabbit anti-GFP and FITC-conjugated goat anti-rabbit IgGs (c and g) on DAPI-stained chromosomes (b and f). The merged pictures (d and h) show the DNA (b and f respectively, blue) and the GFP foci (c and g respectively, green) localized in or close to the nucleolus. (6b): Primary spermatocytes from these males were stained with anti-GFP antibodies, Cy5-labeled secondary antibodies (red) and DAPI (blue) to visualize Mod(mdg4)56.3-GFP and DNA respectively. Fluorescent foci are seen at prometaphase. FITC-conjugated anti-alpha tubulin antibodies (green) show the dividing meiotic cells.

a

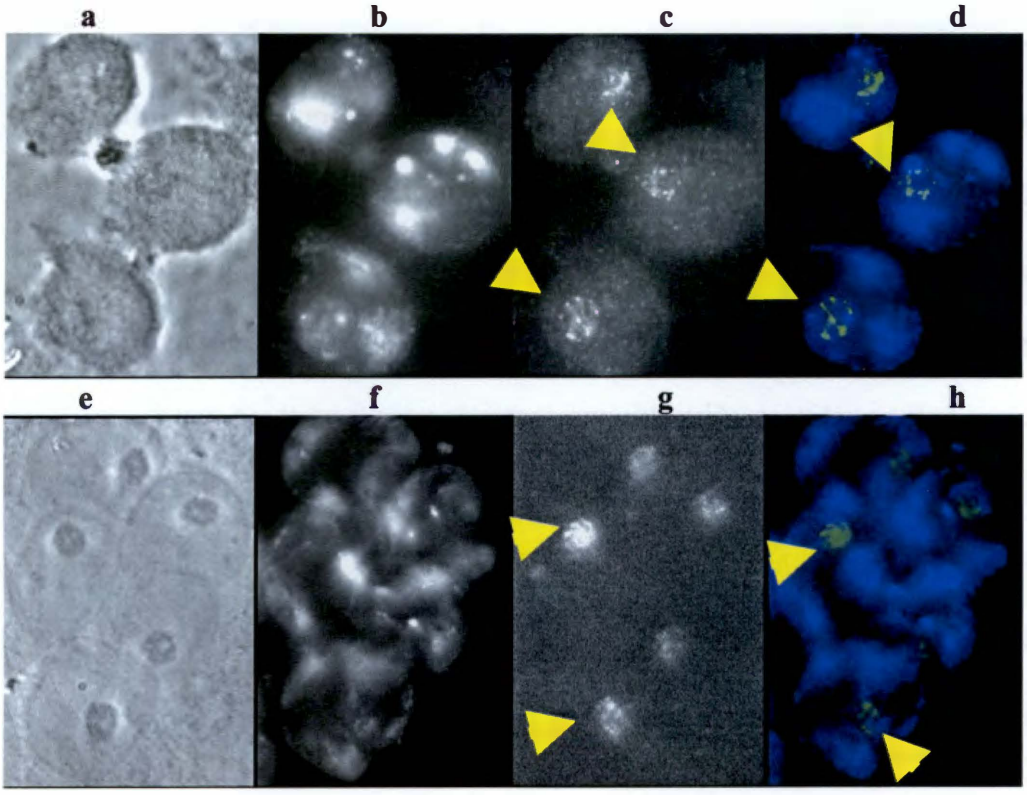


Figure 6 continued

b

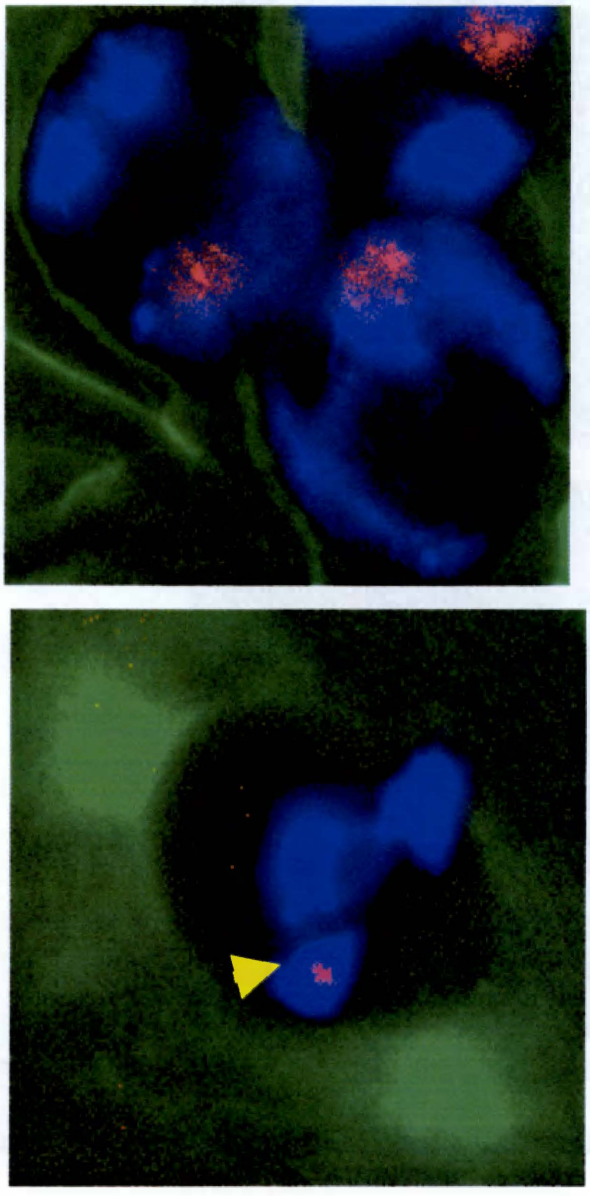
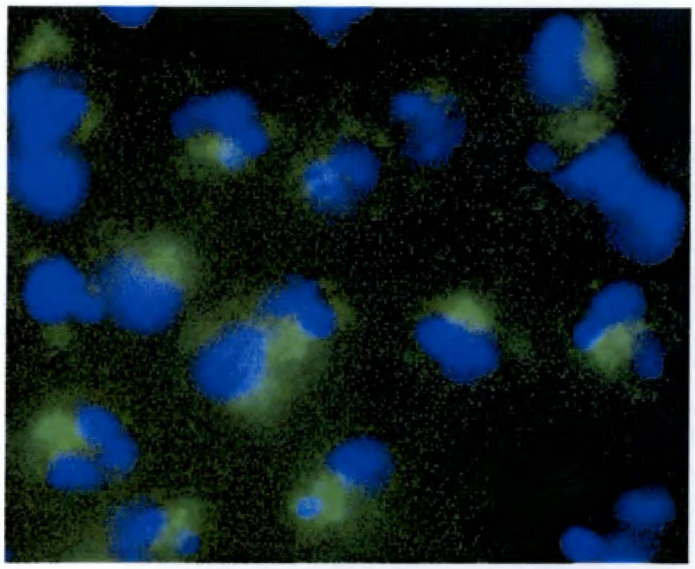


Figure 6 continued

a



b

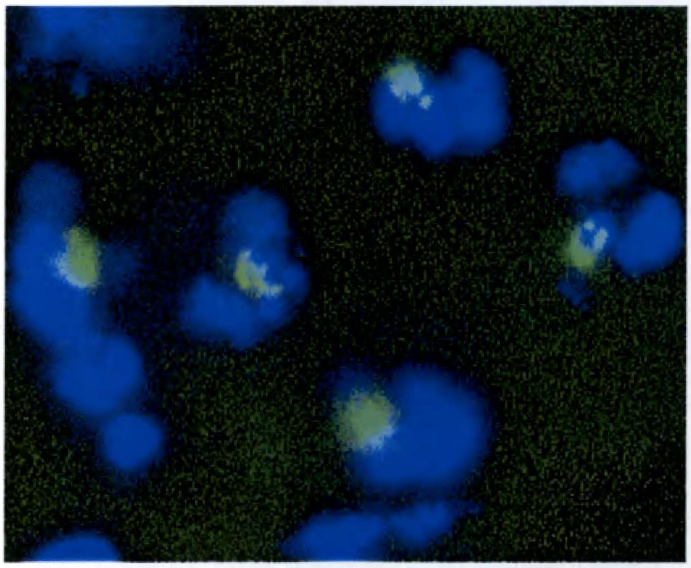


Figure 7: Both antibody staining, with anti- Mod(mdg4)56.3 (a) or with anti-GFP (b), show similar localization of Mod(mdg4)56.3-GFP in spread of meiotic cells from males null for endogenous *pf-2* and transgenic for the P-element carrying the GFP-tagged *mod(mdg4)56.3* cDNA. Hand-dissected testis preparations were incubated with FITC-conjugated secondary antibodies to reveal the expression of Mod(mdg4)56.3-bound primary antibodies (green). DNA is stained with DAPI(blue).).

endogenous protein and that the C-terminally added GFP may interfere with the cellular behavior of this particular isoform of Mod(mdg4), other GFP-labeled proteins were tested as controls. The nuclear expression of histone H2A-GFP in primary spermatocytes of wild type flies showed the expected localization of GFP on the chromosomes (Figure 8 a-d). The analysis of primary spermatocytes from transgenic flies expressing the GFP-labeled Lac I, a protein that binds exclusively to the Lac-operon promoter sequences to inhibit the expression of the beta-galactosidase enzyme in bacteria, showed a general and diffuse nuclear fluorescence (Figure 8e). Thus, the discrete localization pattern detected in Mod(mdg4)56.3-GFP transgenic flies is not an artifact and corresponds to the expression of the only isoform capable of rescuing the chromosomal NDJ phenotype displayed by *pf-2* null males. It was also important to demonstrate that the expression of the fusion GFP-Mod(mdg4)56.3 protein in transgenic lines displayed the same pattern as the endogenous protein in wild type flies. Therefore, anti-Mod(mdg4)56.3 polyclonal antibodies, recognizing only a portion of the specific C-terminal exon of this isoform were used to stain the endogenous protein in meiotic cells prepared from wild type, *y w*, males. Figure 9 shows that Mod(mdg4)56.3 localization in primary spermatocytes of *y w* flies is identical to the one in transgenic flies.

To demonstrate the localization of Mod(mdg4)56.3 in nucleolar regions, fixed meiotic cells were stained for both Fibrillarin, as a marker for the nucleolus, and for Mod(mdg4)56.3. Their colocalization is shown in Figure 10.

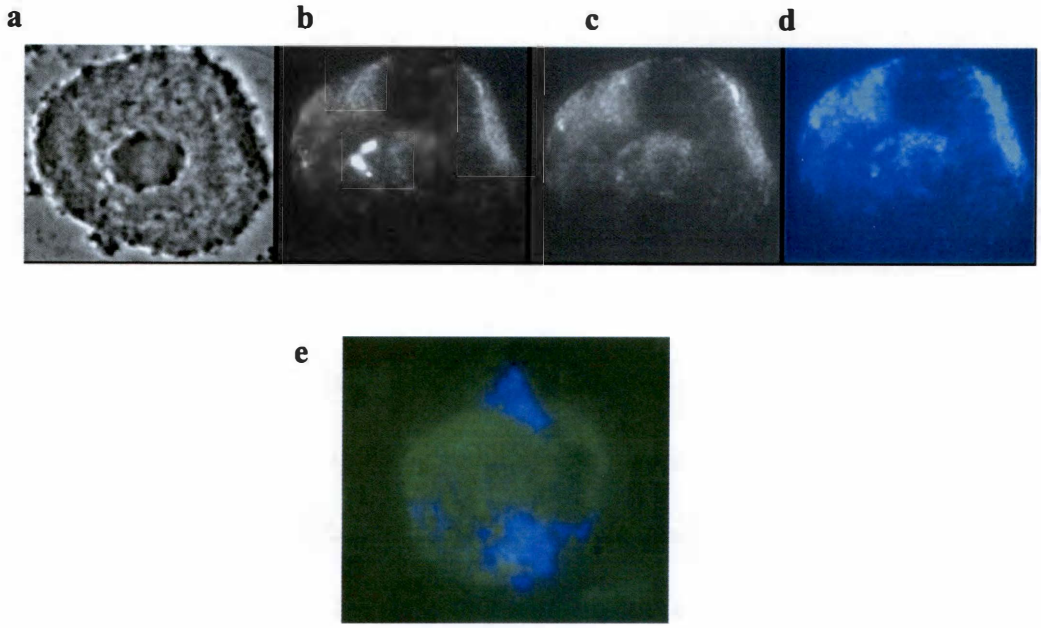


Figure 8: The expression of the tagged fusion protein histone H2A-GFP in live preparations of meiotic cells (a) revealed the localization of the native GFP fluorescence (c, green) on the chromosomes (d, merge). Chromatin was visualized by staining with Hoechst 33342 (b, blue). The expression of the fusion protein Lac I-GFP (e) in live primary spermatocytes revealed a diffuse nuclear distribution of the native GFP fluorescence (green). DNA was visualized by staining with Hoechst 33342 dye (blue).

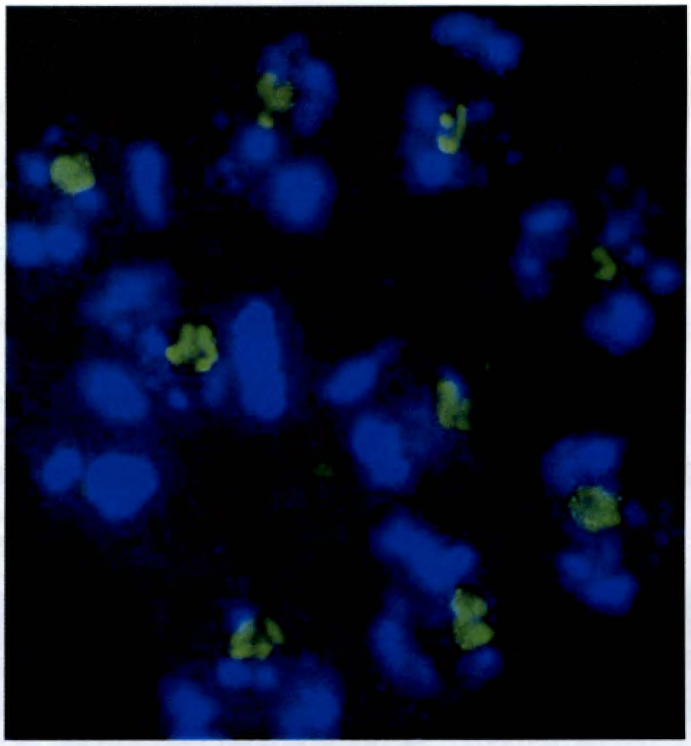
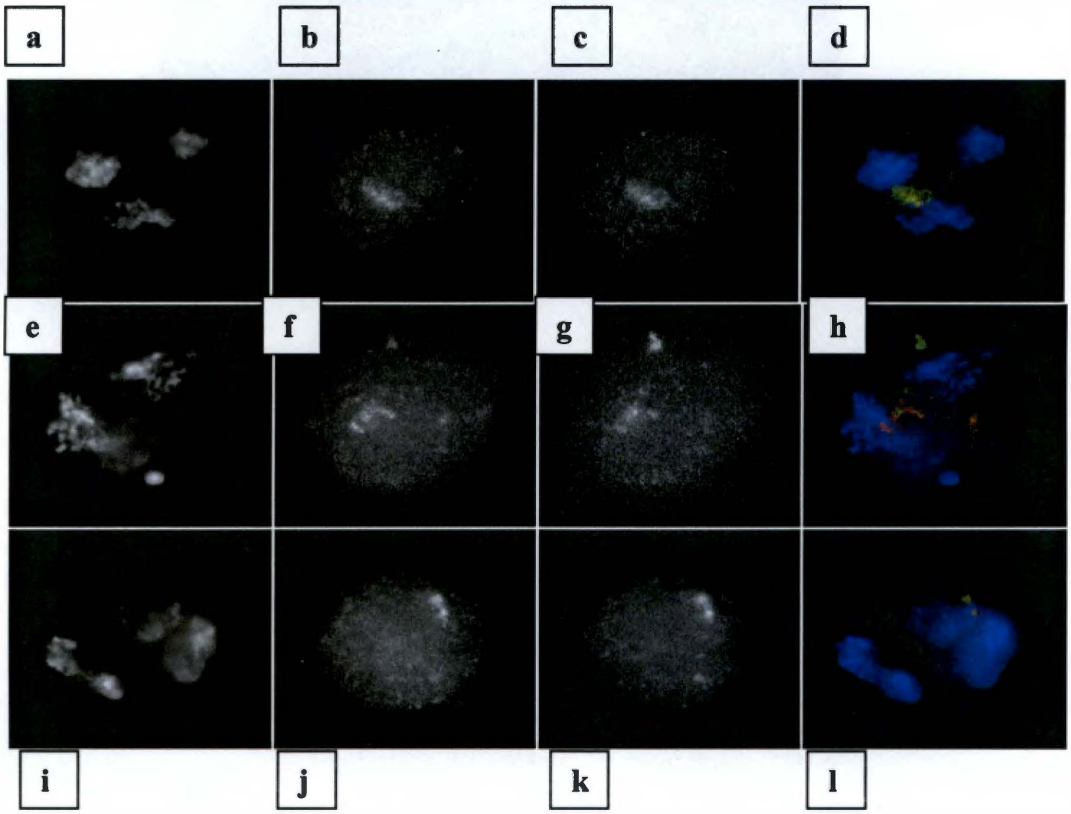


Figure 9: The endogenously expressed Mod(mdg4)56.3 in wild type (*y w*) flies is detected with antibodies raised against the C-terminal specific exon of this isoform. The analysis of spermatocytes with a fluorescent microscope revealed its presence (green foci) concentrated around the nucleolar region. DNA was visualized by staining with DAPI.

Figure 10: Colocalization of Mod(mdg4)56.3-GFP expressed in heat-shocked transgenic line and Fibrillarin, as a marker for nucleolus. Both proteins were stained with antibodies anti-Mod(mdg4)56.3 [(c, g, k), green in merge (d, h, l)] and anti-Fibrillarin [(b, f, j), red in merge]. DNA was visualized by staining with DAPI [(a, e, i), blue in merge]. Meiotic cells were prepared from endogenously null for *pf-2*, transgenic for *mod(mdg4)56.3-gfp*, heat-shocked males.



These data led to the conclusion that *mod(mdg4)*56.3 is indeed the gene involved in chromosome pairing and segregation during meiosis I and no other isoform has a similar function.

In order to uncover the function of Mod(mdg4)56.3 protein during meiosis I, its cellular localization had to be determined. A detailed cytological description of different stages of spermatocyte growth and meiotic divisions has been reported by Cenci and coworkers in 1994. Morphological criteria were used to accurately distinguish each specific stage during spermatogenesis. To determine the expression pattern of Mod(mdg4)56.3, we analyzed the development of primary spermatocytes from the time that they have just completed DNA duplication (stage S1) until the beginning of chromosome condensation (stage S6). The distinction of stages is based on the nuclear size (increasing with cell growth) and position (moving to the center of the cell), as well as the level of compaction of homologous chromosomes and their position within the cell (dispersed chromosomes within the nucleus gradually become organized bivalents seen as clumps in the proximity of the nuclear envelope) and moving toward the center of the cell as meiosis proceeds toward metaphase (Cenci et al., 1994).

The studies using antibodies specifically recognizing Mod(mdg4)56.3 or those binding to the GFP component of the fusion protein expressed in transgenic flies on fixed preparations, as well as the detection of the fluorescence emitted by the native GFP in spreads of live meiotic cells, resulted in determining the stages at which this protein is present in primary spermatocytes and its nuclear / subnuclear localization. Figure 11 shows that this novel meiotic protein is expressed as early

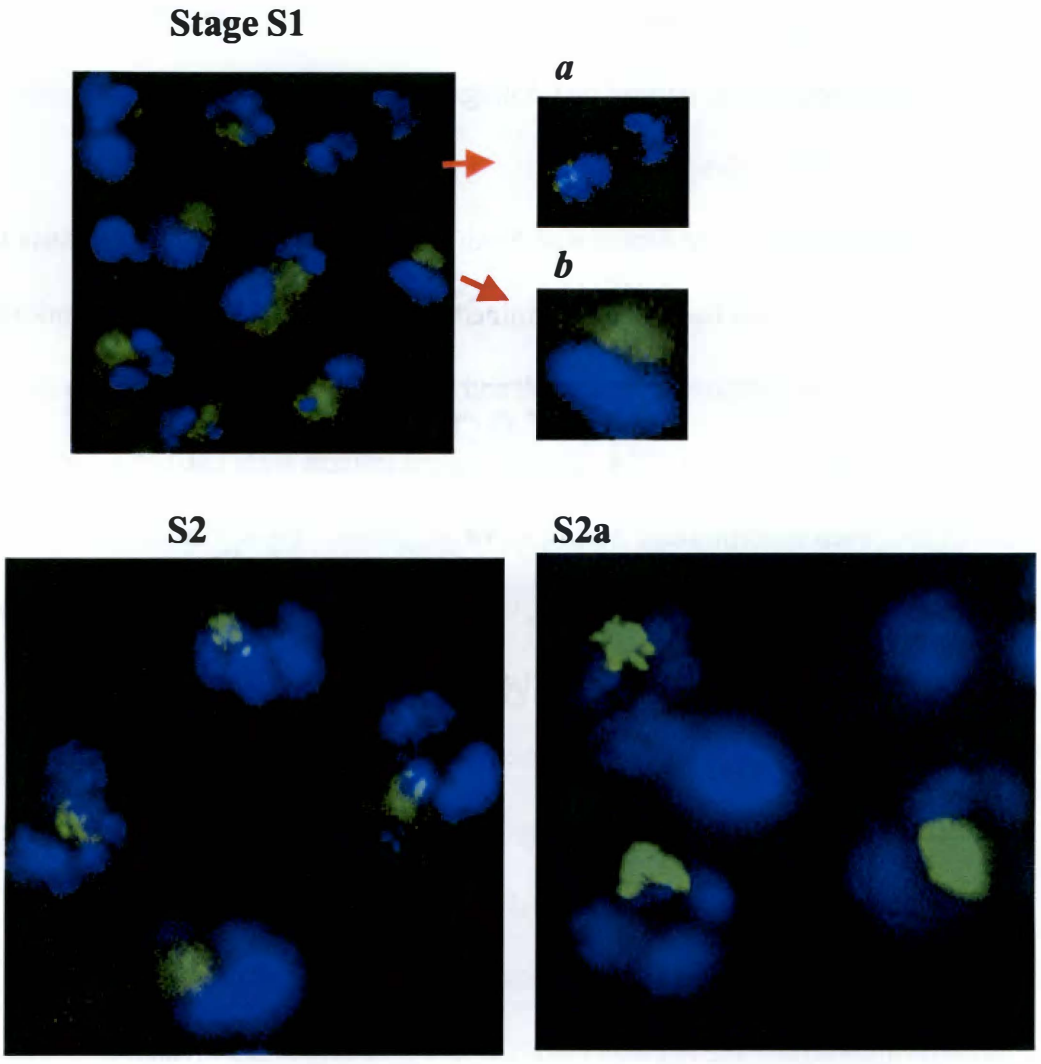


Figure 11: (11a) Expression pattern of the tagged Mod(mdg4)56.3-GFP during early meiotic prophase stages. Hand-dissected testes squashes were incubated with primary antibodies, either anti-Mod(mdg4)56.3 or anti-GFP, and FITC-conjugated secondary antibodies. Primary spermatocytes were analyzed by fluorescent microscopy. Mod(mdg4)56.3-GFP foci (green) are seen as early as stage S1 (clearly seen in b, but some may not be in S1 (seen in a). DAPI-stained DNA is represented in blue.

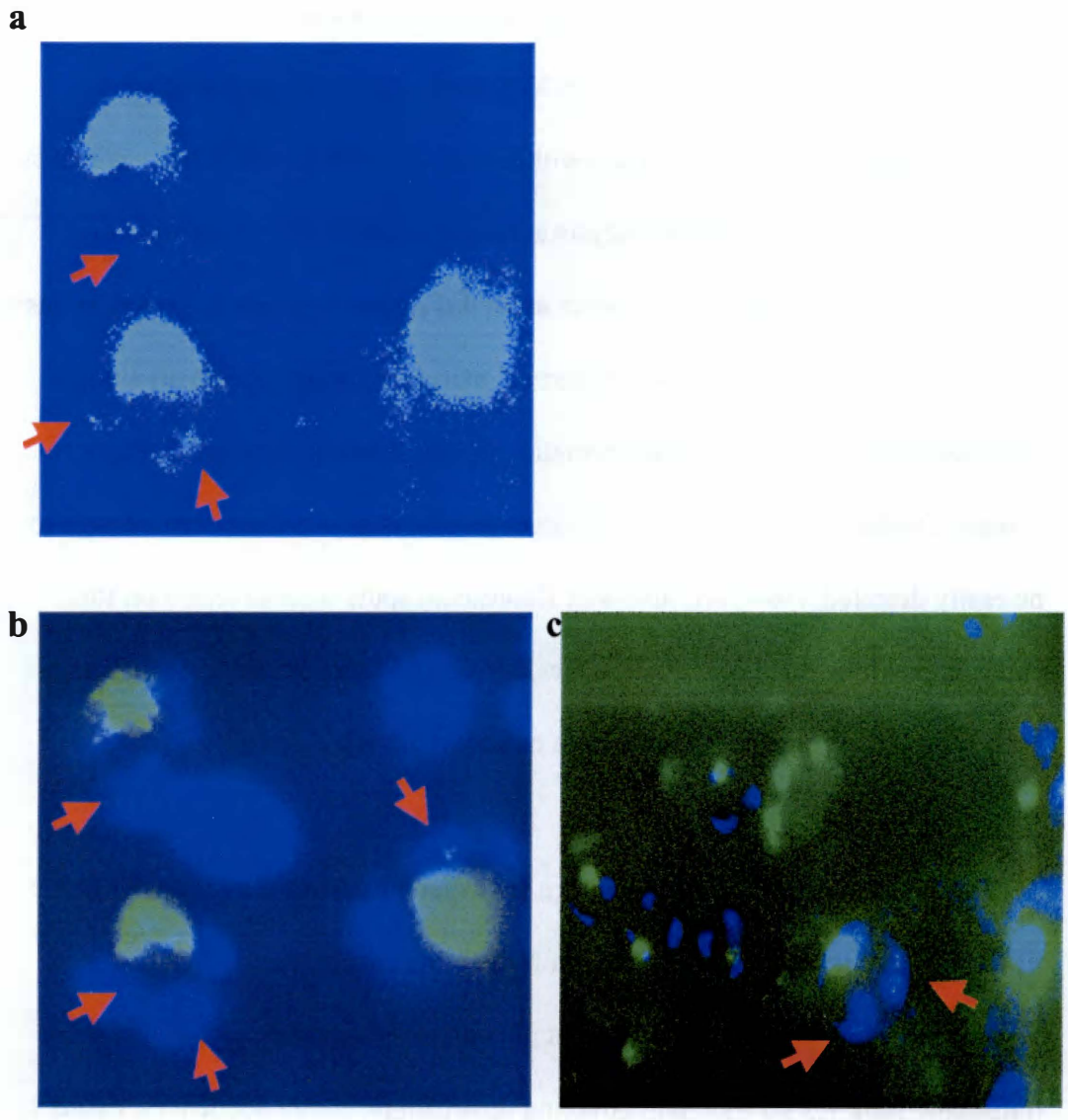


Figure 11 continued

(11b) Localization of Mod(mdg4)56.3-GFP on autosomes during S2 stage (GFP foci: a, and merge: b) and S3 stage (c).

Arrows point to the arrays of autosomal GFP foci.

as stage S1 of prophase, as clustered foci in nucleolar areas. Nucleolar localization is consistent with a role in X-Y pairing, as it has previously been shown that the X-Y pairing sites are within the heterochromatic rDNA sequences close to the nucleolus. Further analysis at longer exposure times revealed the presence of discrete foci on autosomes as well (Figures 12 c and d, 13-16). It has also been demonstrated that the autosomes' pairing sites are located all along chromosomes' length within euchromatic regions. It would be more difficult to visualize these dispersed sites as our target protein is not concentrated enough to be easily detected. However, arrays of fluorescent spots were detected on the entire arms of the meiotic chromosomes, shown in Figure 16, suggesting that Mod(mdg4) 56.3 might play a role as a cohesion protein.

Partial rescue of phenotype with a transgene bearing sequences of the upstream promoter and the common N-terminal exons

We also attempted to rescue the mutant phenotype by generating flies that were transgenic for a 7.5 kb fragment covering upstream promoter sequences, exons 1–4 coding sequences, and extending to the intronic region separating the 3 proximal specific exons from the remaining of the C-terminal sequences. The transgene was carried on the second chromosome. Results of the rescue experiments are reported in Table 3 show a partial (~ 13%) rescue of the NDJ phenotype caused by Z3-3401 mutation, the allele in the common region, which is statistically significant, in the presence of the transgene compared to control mutant flies. The lack of full rescue could be explained by the assumption that

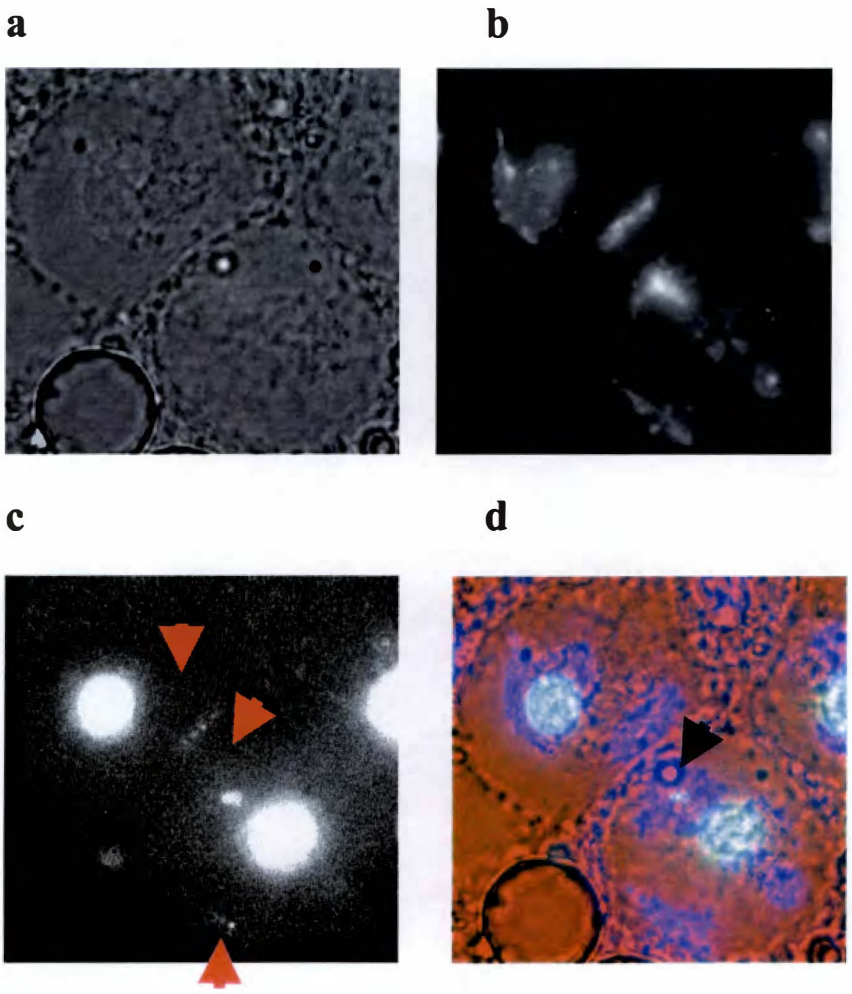


Figure 12: Expression pattern of the tagged Mod(mdg4)56.3-GFP during stage late S5 of meiotic prophase I. Live preparations of primary spermatocytes from transgenic males were analyzed by fluorescent microscopy. Meiotic cells are seen by transmitted light (a). Naturally fluorescent Mod(mdg4)56.3-GFP is seen as foci (c) on Dapi-stained DNA (b), clustered in nucleolar regions (bright signals in b and d) and as single or arrays of spots on autosomes (c and d). Colocalization is visualized by merging all of the fluorescent signals (d). Arrows point to Mod(mdg4)56.3-GFP foci on autosomes.

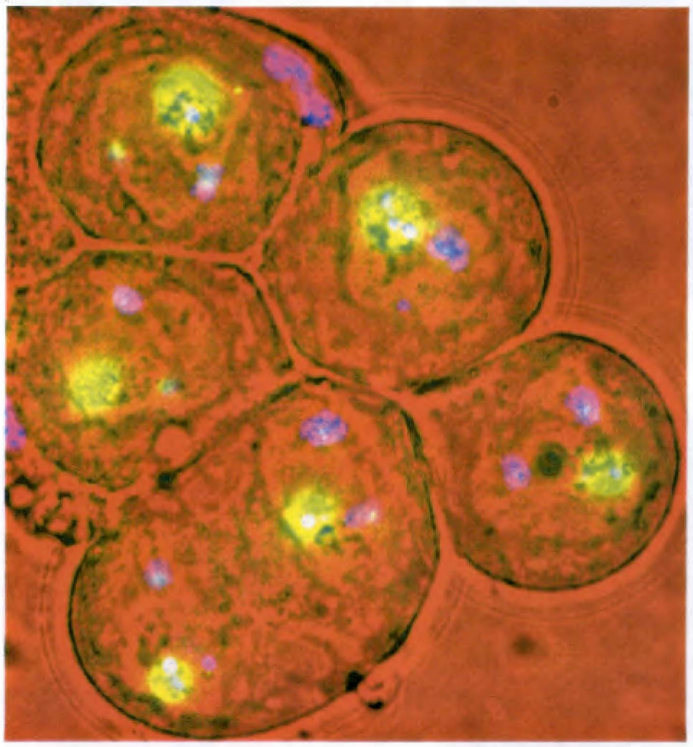
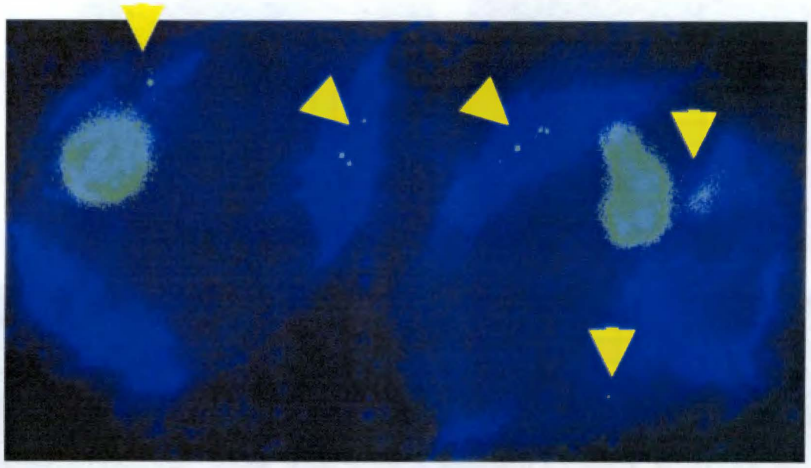


Figure 13: Localization of Mod(mdg4)56.3-GFP in nucleolus and on autosomes at late S5 stage during meiotic prophase I. This merged picture shows native fluorescence of GFP (green) localized on Hoechst 33342-stained DNA (blue) in live primary spermatocytes (red).

a



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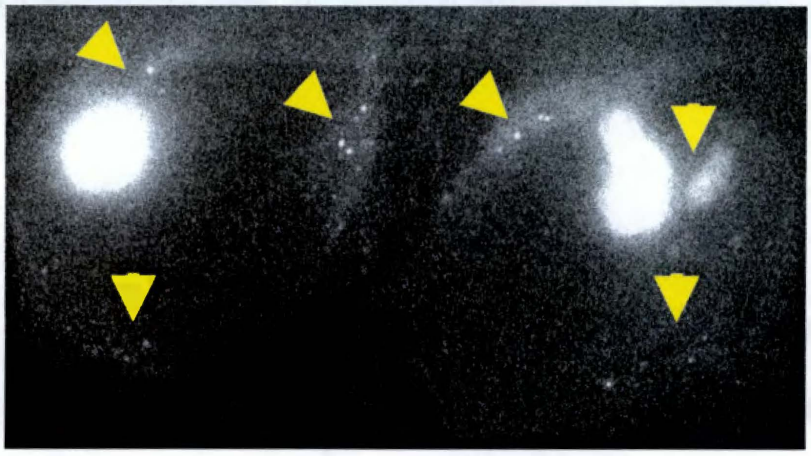

Figure 14: Localization of Mod(mdg4)56.3-GFP in nucleolus and on autosomes during late stage S6 in older primary spermatocytes. The merged picture (a) shows native fluorescence of GFP (green) localized as single or clustered foci on chromosomes stained with Hoechst 33342 (blue) and in the nucleolus.

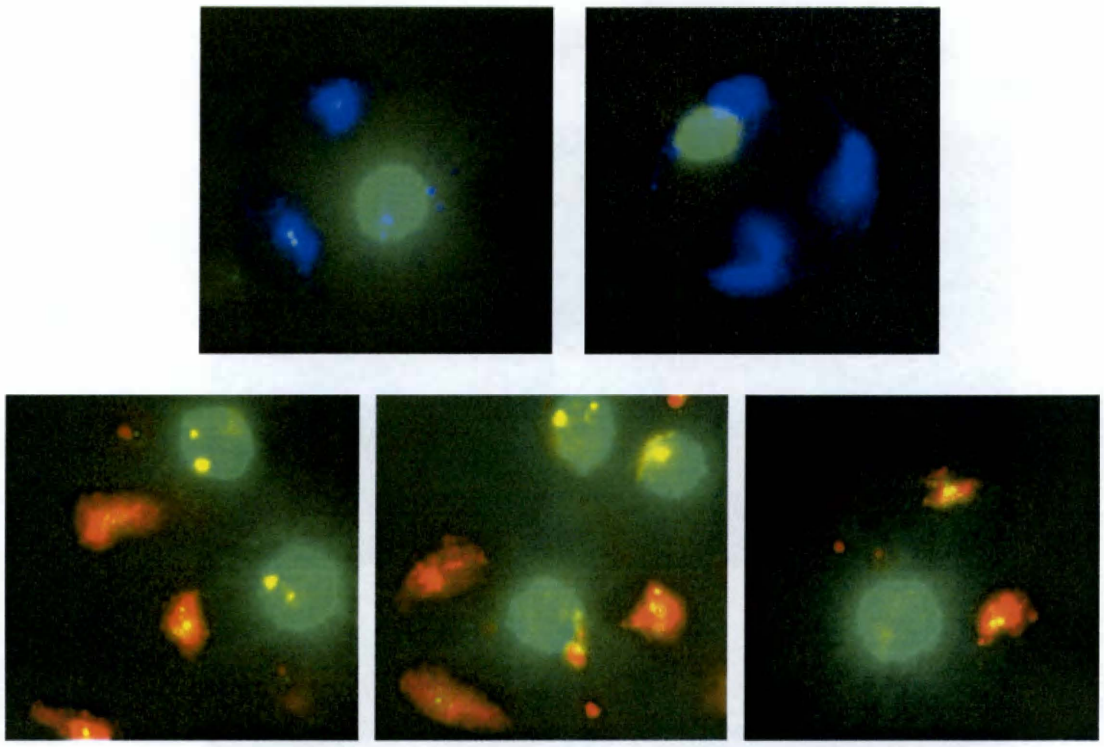

Figure 15: Localization of Mod(mdg4)56.3-GFP in nucleolus and on autosomes at stage S6 during meiotic prophase I. These merged pictures show native fluorescence of GFP (green) localized on all Hoechst 33342-stained chromosomes (represented in red or blue for a better visualization) and clustered in nucleolar region in live preparations of primary spermatocytes (red).

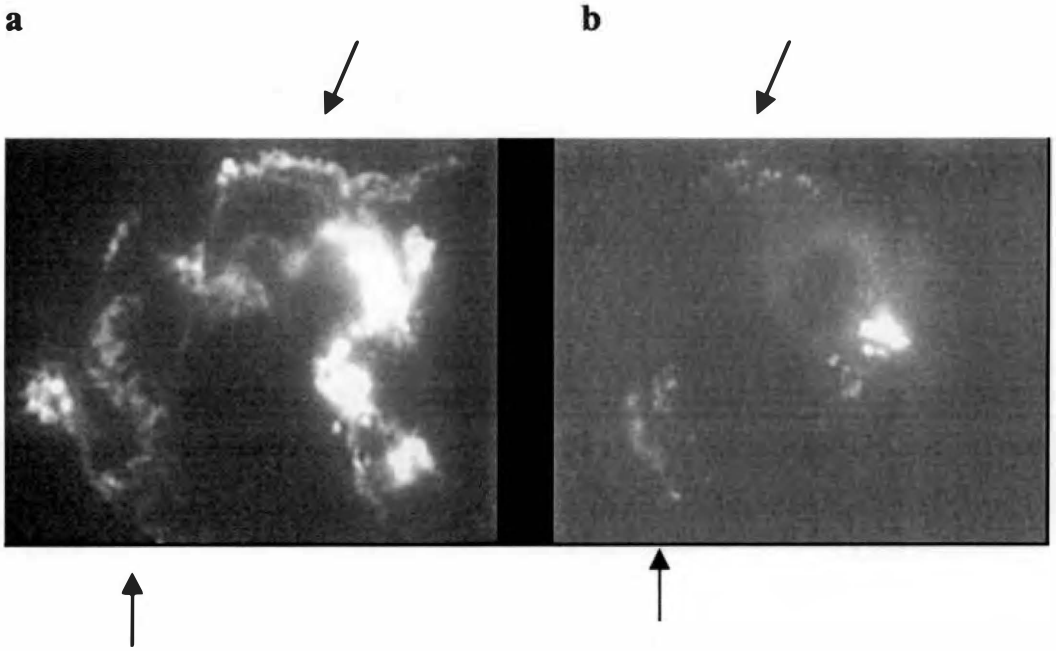

Figure 16: Localization of Mod(mdg4)56.3 on autosomes. Staining of the fusion protein with anti-GFP revealed its presence as an array of single spots (b) along the entire arm of the Dapi-stained chromosomes (a).

Table 3: Partial rescue of the NDJ phenotype of *pf-2* allele *Z3-3401* by transgenic insetion of the common region of *mod(mdg4)*. Males with the indicated genotype were tested for NDJ by mating them with *y w* females. Progeny were scored for NDJ. The transgene, *P {w+, 7.5Kb BamHI genomic sequences}* was carried on chromosomes II and was absent in non-transgenic, control flies.

	% NDJ (scored progeny)	
P{w$^+$, 7.5Kb BamHI genomic sequences}	**Non-transgenic**	**Transgenic**
Insertion on chromosome II		
Z3-3401 / Z3-3401	**45.52** (591)	**31.81** (1028)
Z3-3401 / mod(mdg4)^{T16}	**45.80** (738)	**32.46** (499)
Z3-3401 / mod(mdg4)^{neo129}	**42.42** (943)	**33.42** (1212)

trans-splicing occurs between spatially adjacent chromosomes and the pre-mRNA of the common region, transcribed from the second chromosome might be physically too distant from the independent mRNA of the C-terminal specific exon of Mod(mdg4)56.3, transcribed from the third chromosome.

III- Discussion

Three alleles of *pairing failure-2* (*pf-2*), a novel gene required for meiotic chromosome pairing and segregation in *Drosophila*, were recovered in a screen for EMS-induced mutations on chromosome 3 causing paternal loss of chromosome 4. Chromosomes are normally clustered into three major clumps of DNA that are spatially separated during prophase and prometaphase of male meiosis I (Cenci et al., 1994). Cytological analysis of testes of mutant flies revealed unpaired chromosomes at prophase and metaphase and "laggard chromosomes" at anaphase in primary spermatocytes. Moreover, in *pf-2* mutants, the number and shape of the DNA aggregates detected by staining with fluorescent DNA dyes are different from wild type flies, indicating a lack of spatial nuclear organization of the chromosomes. Genetic data confirm that non-disjunction affects all chromosomes and is male- and meiosis I- specific. Complementation tests showed that *pf-2* maps to chromosome region 93(D6-E1) and is allelic to *modifier of mdg4* gene and might represent a particular isoform of the large family of chromosomal proteins generated by alternative splicing of this complex locus. Two *pf-2* alleles bear a mutation in an exon specific for the *mod(mdg4)56.3* isoform. Transgenic rescue using a heat shock-driven cDNA

expressing only Mod(mdg4)56.3 protein confirmed the identification of *pf-2* as *(mdg4)56.3*. Furthermore, the meiotic phenotypes associated with a third *pf-2* allele, bearing a point mutation in the N-terminal region common to all of the isoforms, were also fully rescued by the expression of only one isoform, the Mod(mdg4)56.3. These experiments led to the conclusion that Mod(mdg4)56.3 isoform is the only one responsible for the male meiotic phenotype of *Z3-3401* but we cannot rule out the possibility that other mutations could disrupt other meiotic isoforms.

Cytological and immunocytological analyses of primary spermatocytes revealed that the GFP-labeled Mod(mdg4)56.3 is localized on meiotic chromosomes and is concentrated in the nucleolus, a dynamic structure that assembles and disassembles repeatedly during each cell cycle. Nucleolar Organizing Regions (NORs) are active ribosomal RNA genes and formation of nucleoli is dependent on the production of ribosomal RNAs. Nucleoli disappear with the cessation of transcription and proteins associated with this subnuclear structure display a rapid turn over (Lamond and Sleeman, 2003). The association of Mod(mdg4)56.3 with these nucleolar bodies that are formed around the tandemly repeated rRNA genes may be suggestive of a function of this protein as a pairing protein. We have a good map of chromosomal pairing sites but no knowledge about the trans-acting factors that are required to mediate pairing.

Pairing sites for X and Y chromosomes consist of arrays of 240-bp repeats in intergenic spacer (IGS) regions (Mckee, 1996) and may be bound by nucleolar proteins in order to hold these two chromosomes together. The autosomal pairing

sites in *Drosophila* males have been reported to be along the entire arm of autosomes, within euchromatic sequences. The presence of Mod(mdg4)56.3 as an array of foci along the chromosomes arm is suggestive of its role as a protein mediating their pairing or involved by direct interaction with the DNA. Signals are often detected in pairs, especially in later stages, representing probably the presence of foci on each homolog. At later stages of prophase, S5 – S6, arrays of single foci are visible on the autosomes as well as the strong nucleolar signal assumed to be associated with the X-Y pairing sites.

Mod(mdg4)56.3 is detected on the chromosomes at early stages of meiotic prophase (S1), and by enhancing the fluorescent signal it is possible to see its association with the autosomes as well at this stage (Figure 11a). Single, faint foci on autosomes are also detected at the S2 stage. Orcein-staining allowed visualizing unpaired chromosomes at prophase / prometaphase stages and was not accurate enough for determining the exact timing of the occurrence of the unpairing in *pf-2* mutants.

As chromosomes condense, these foci appear as a pair of bright signals on all the chromosomes and persist on the chromosomes all through prometaphase and metaphase stages. With the progression of the division toward anaphase, no signal can be detected and this pattern of expression is typical for proteins involved in pairing of chromosomes. Their removal from the chromosomes coincides with the segregation of homologs to opposite poles of the cell.

Here we report the identification of a novel gene that causes chromosomal NDJ when absent. This unique and complex meiotic gene, that we called *pairing*

failure-2, pf-2, is allelic to *modifier of mdg4* gene of *Drosophila*, with over 33 isoforms identified thus far. It is very likely that Mod(mdg4)56.3 encodes a protein involved in pairing and segregation of homologous chromosomes.

Chapter Four

Discussion

Summary

The survival of the species depends on the transmission of the proper amount of genetic material to the offspring. The production of euploid gametes, which carry the correct number of chromosomes, is a critical step in this process. Meiosis is a nuclear division that occurs in sexually reproducing organisms and results in the generation of haploid gametes with half of the parental genomic complement. The fertilization of the oocyte (female gamete) by the sperm (male gamete) generates again a diploid progeny. Aneuploidy is a predominant cause of spontaneous abortions and genetic diseases in human populations. Therefore, it is important to determine the mechanisms underlying meiotic events such as homologous chromosome pairing and separation during the reductional division. The primary goal of our research is to identify and characterize genes functioning in pairing and disjunction pathways during meiosis.

Studies of meiotic homolog pairing, recombination and segregation processes in *Drosophila* females have been quite successful and numerous proteins have been identified that affect female meiosis but very few have been shown to play a role during male meiosis. The differential requirement of proteins presumably reflects different mechanisms used by the two sexes. Without the SC holding homologs in close proximity (Meyer, 1960) or a chiasma providing stability until

anaphase I (Cooper, 1964), some unique mechanism must exist in males to ensure the proper distribution of chromosomes into haploid gametes. Specific sets of proteins might be involved in this process. As emphasized, progress in the field of *Drosophila* male meiosis has greatly been delayed due to the lack of knowledge of genes involved in this process.

Prior to a collaborative project that began in 2000, C. Zuker undertook a large-scale mutagenesis in order to provide to *Drosophila* geneticists a wealth of mutated genes affecting a majority of pathways in the life cycle of this model organism, but viable as homozygote (Koundakjian et al., 2004). 12000 lines bearing a mutation on their second or third chromosome were screened by B. Wakimoto and resulted in identification of 72 stocks that displayed fourth chromosome loss (Wakimoto et al., 2004). D. Lindsley tested mutant males for aneuploidy, represented by unequal nuclear sizes of the spermatids, and recovered 48 lines with an apparent meiotic defect. Cytological analysis of these mutants by B. McKee was carried out by observation of fixed, orcein-stained testis squash preparations using phase optics. Genetic analysis consisted of crossing mutant males carrying a marked Y chromosome both to chromosomally normal females to estimate the X-Y non-disjunction frequency and to C(1)RM/O females with attached-X chromosomes, to estimate the relative frequency of recovery of XY sperm (indicative of only MI NDJ) versus the rate of recovery of XX sperm (produced by NDJ occurring exclusively at MII).

pairing failure-2 (*pf-2*) mutations affect all four chromosome-pairs, and thus differ from *teflon*, which came from the same screen but affects only autosomes

(Tomkiel et al., 2001). However, like *teflon* mutations, *pf-2* mutations disrupt specifically homolog pairing, leading to high frequencies of univalents in late prophase and prometaphase and random assortment in meiosis I, but normal sister chromatid cohesion and segregation at meiosis II, and good fertility.

Also like *teflon*, *pf-2* seems to have no role in female meiosis; females *trans*-heterozygous for the two strong alleles show normal levels of recombination and disjunction suggesting that *pf-2* is specific for male meiotic pairing.

Cytological analysis of meiotic cells from males bearing *pf-2* mutations revealed a defect in chromosome organization in late prophase of the first division prior to chromosome condensation. In wild type cells the three large chromosome pairs appear to occupy delimited and clearly separate regions within the nucleus, in the vicinity of the nuclear envelope. DAPI-staining of primary spermatocytes showed that the integrity of these chromosomal 'territories' is disrupted by *pf-2* mutations. Territories frequently appear to overlap, and DAPI-staining of the nuclear interior is more intense than in wild type. Univalents can be visualized as early as prophase in *pf-2* mutants by orcein-staining. It is difficult to determine by standard methodology the exact time when pairing is lost, as territory formation may still hold chromosomes within a certain distance from each other even though homologs are disjoined. The observation of unorganized and loose chromosomes during prophase supports the idea that pairing might be lost at very early stages of meiosis.

Mapping by deficiency complementation resulted in localizing *pf-2* to the 93D6-E1 region on chromosome 3, an interval defined by the overlap of the non-

complementing deficiencies, *Df(3R)GC14* and *Df(3R)eGp4*. The breakpoints of these deficiencies have been mapped molecularly in our laboratory (Figure 1, chapter 3). Pre-existing alleles of three genes located in the critical region, ***tinman***, ***mod(mdg4)*** and ***hsr-omega*** were tested for complementation against *pf-2* mutants and results excluded ***tin*** and ***hsr-omega*** as candidates for *pf-2*, but many alleles of ***mod(mdg4)*** partially or completely failed to complement *pf-2* mutations, suggesting that these mutations are allelic.

mod(mdg4) is a very complex gene with a broad range of functions including chromatin boundary formation (Gerasimovaet al., 1995; Bell et al., 2001; reviewed by Gerasimova and Corces, 2001), establishment of higher-order organization of chromatin domains (Chen and Corces, 2001), position effect variegation (Dorn et al., 1993; Gerasimova et al., 1995; Gerasimova et al., 1998), programmed cell death (Harvey et al., 1997), regulation of homeotic genes and early development (Buchner et al., 2000), and regulation of synapse development in the nervous system (Gorczyca et al., 1999) and here we report the involvement of one isoform in meiotic chromosome pairing.

This gene is essential as the most severe alleles cause lethality in early embryogenesis (Azpiazu and Frasch, 1993; Zollman et al., 1994). More than 33 isoforms have been identified thus far, most or all of which are chromosomal proteins present at more than 500 sites on polytene chromosomes detected by antibodies against the common N-terminal BTB domain (Buchner et al., 2000). However, antibodies against the specific C-terminal exon of two of the isoforms, Mod(mdg4) 67.2 and Mod(mdg4) 58.0, revealed their localization at differential

sites, with fewer (~50) sites staining for Mod(mdg4) 58.0, and most of the sites but not all for Mod(mdg4) 67.2. Both proteins exclude each other at many of these sites. Most of the Mod(mdg4) binding sites detected by the anti-Mod(mdg4)^{BTB} antibody, but not bound by Mod(mdg4) 67.2, are located at the telomeres of the autosomes and the X chromosome (Buchner et al., 2000). The differential distribution of these isoforms is suggestive of their involvement in specific cellular pathways, without excluding the sites of overlap indicating the possibility of cooperation between some of these isoforms.

Each isoform is generated by differential splicing of four common N-terminal exons encoding amino acids 1-402 to a different C-terminal exon (Krauss and Dorn, 2004; Labrador and Corces, 2003; Dorn et al., 2001). A unique and surprising feature of *mod(mdg4)* is that the C-terminal coding sequences for at least seven of the isoforms are on the antisense strand, not colinearly located within the locus, suggesting the generation of mature mRNAs by *trans*-splicing (Labrador and Corces, 2001 and 2003; Krauss and Dorn, 2004; Dorn et al., 2001), between independent precursor RNA molecules (Agabian, 1990; Sutton and Boothroyd, 1986; Caudevilla et al., 1998; Mongelard et al., 2002). cDNAs from most of the isoforms have been cloned, providing evidence for the occurrence of the predicted splices. RNA encoding the Mod(mdg4)56.3 protein, one *mod(mdg4)* encoded isoform first identified in a yeast-two-hybrid screen of embryonic cDNAs that interact with the baculovirus inhibitor of apoptosis protein (IAP), is a candidate for one of these precursors (Harvey et al., 1997).

Since it was first described in Trypanosomes (Agabian, 1990), many instances of *trans*-splicing have been reported for many organisms from worms to flies to humans (for review see Fedorova and Fedorov, 2003), but mechanisms underlying this process are still unknown. It has been shown by Dorn et al. (2001) that alternative exons of *mod(mdg4)* gene are transcribed independently and *trans*-spliced to the common 5' N-terminal coding sequences, as they identified many promoter sequences within this locus. It seems reasonable that the expression of each isoform is regulated independently and *trans*-splicing provides a good way of controlling the time and tissue specificity of expression of each particular isoform. It also allows complementation between mutations affecting different functional domains of a protein.

The variable C-termini are implicated to specify the function of individual isoforms in different processes. In most of the isoforms a conserved C-terminal Cys_2His_2 protein motif is found, known also as FLYWCH domain and named after its conserved residues (Buchner et al., 2000; for review see Dorn and Krauss, 2003). Mutations in only one C-terminal exon have been identified thus far. Two mutations in the exon specific for the 67.2 isoform (*mod(mdg4)*^{T6} and *mod(mdg4)*^{u1}) are viable but modify the phenotype of the mutations caused by insertion of the gypsy retrotransposon. Extensive analysis has revealed that these mutations modify the activity of the chromatin insulator element located within gypsy (Gerasimova and Corces, 1998; Gerasimova et al., 1995) and that the protein encoded by Mod(mdg4)67.2 interacts directly with Su(Hw) protein which binds to the insulator sequence (Ghosh et al., 2001). To assess whether the 67.2

isoform might be involved in meiotic pairing, we tested the *mod(mdg4)^{T6}* allele, which results from a point mutation in the *mod(mdg4)67.2* specific coding region, producing a truncated protein that lacks the last 32 residues at the C-terminal acidic domain. *Trans*-heterozygotes for *pf-2* alleles and *mod(mdg4)^{T6}*; and for *mod(mdg4)^{T6}* and a deletion encompassing the *mod(mdg4)* locus do not exhibit any NDJ phenotype (chapter 3). We addressed the question whether the role of Mod(mdg4) in homolog pairing during meiosis was mediated by its interaction with Su(Hw). Males homozygous or trans-heterozygous for strong alleles of *su(Hw)* and carrying a marked $B^{s}Y y^{+}$ chromosome were tested and found to have normal X-Y disjunction. Moreover, heterozygous males for both *pf-2* and *su(Hw)* did not display any NDJ phenotype when crossed to *y w* females (Appendix). These data suggested that the phenotype seen in *pf-2* mutant flies was not mediated by the insulator proteins Su(Hw) and Mod(mdg4) 67.2.

Our genetic data showed that two *pf-2* mutations in one of the C-terminal specific exons complement a third *pf-2* mutation in the common region, presumably because *trans*-splicing can occur between a transcript expressing the common region from one homolog and a transcript expressing the C-terminal exon from the other homolog (Mongelard et al., 2002). These data led us to carefully examine the *pf-2 / mod(mdg4)* complementation pattern, taking into account what is known about the locations of *mod(mdg4)* mutations and deficiencies.

The very specific phenotype of *pf-2* alleles suggested to us that *pf-2* mutations would likely be in a C-terminal exon and therefore disrupt only one isoform. By

comparing the sequencing data from PCR-amplified fragments of *mod(mdg4)* locus in *pf-2* homozygote flies to *Z3* parent stock before being mutagenized, we identified one base pair change within exon 4 in the common N-terminal domain (*Z3-3401*), and two point mutations located in the C-terminus specific exon (*Z3-5578* and *Z3-3298*) of one isoform, *mod(mdg4)56.3*.

The specific exon of *mod(mdg4)56.3* is located on the same strand and about 20 kb downstream of the common N-terminal exons. Males trans-heterozygous for two *pf-2* alleles, one mutation located within the common N-terminal region and the other affecting the C-terminal variable exon, almost completely recover the wild type phenotype by displaying less than 2% NDJ frequencies. These intragenic complementation results suggest that at least some of the mature *mod(mdg4)56.3* RNAs are generated by trans-splicing, as *cis*-splicing should generate only mutant RNAs. Although these data could also be explained by "conventional" intragenic complementation in which two mutant proteins can form a functional dimer, this explanation is unlikely because complementation also occurs between *pf-2* mutants in the *mod(mdg4)56.3* exon and deletions that are confined to the N-terminal region of the locus, and between the common region *pf-2* allele and deletions confined to the C-terminal half of the locus. However, the presence of transcripts with a wild type sequence or bearing both mutations, in addition to those carrying either one of the *pf-2* mutations, in heterozygote flies still remains to be demonstrated molecularly. If it can be confirmed, this would be the first demonstration that *trans*-splicing occurs

between two RNA precursors transcribed from two co-linear exons at the *mod(mdg4)* locus.

Based on our genetic data showing complentation of *pf-2* mutations affecting separate domains of this protein and the previously reported *trans*-splicing events (Labrador et al., 2001; Dorn et al., 2001; Mongelard et al., 2002), we expected to recover the wild type phenotype in flies hemi- or homozygous for the mutation that affected the 4th exon common to all *mod(mdg4)* isoforms, *Z3-3401*, by insertion of a 7.5 kb genomic construct, extending from upstream promoter sequences of *mod(mdg4)* to the intronic sequences between the third and fourth variable C-termini, including the four N-terminal common exons. We did observe partial rescue of *pf-2* mutant flies but only 13% rescue when they carried the genomic construct inserted in their second chromosome.

This transgene has also been previously reported to partially rescue the viability of *mod(mdg4)^{neo129}* homozygote flies, an embryonic recessive lethal mutation (Buchner et al., 2000).The fact that the genomic construct did not fully rescue the viability of homozygous lethal *mod(mdg4)^{neo129}* flies (Buchner et al., 2000) or the NDJ phenotype associated with the *Z3-3401* homo- or hemizygote flies reported in this dissertation, could be explained by the location of the transgene on the second chromosome and the mutations are on the third one. Trans-splicing events may occur within spatially restricted intranuclear domains. Precursor RNAs transcribed from the two major autosomes may be too distant to interact at high frequencies; however we can partially but significantly (~13%)

restore the defect, implying that the chance for the two transcripts to recombine is low but not negligeable. Alternatively, the 7.5 kb genomic sequences may lack elements essential for an appropriate level of expression. Beside physical proximity and efficient expression, one might consider the requirement of other factors or features for *trans*-splicing processes as yet unidentified.

On the meiotic role of Mod(mdg4)56.3

Analysis of the expression of a Mod(mdg4)56.3-GFP protein in primary spermatocytes enabled us to show that Mod(mdg4)56.3 localizes to foci within the autosomes and to prominent clusters of foci within the nucleolus throughout meiotic prophase. After chromosome condensation a prominent signal remains associated with one of the three bivalents at prometaphase and metaphase. One alternative is that the loss of the GFP signal from the condensed chromosomes represents the removal of the Mod(mdg4)56.3-GFP protein at this stage. On the other hand, we cannot exclude the possibility that the intense signal might be associated with an increased concentration of the tagged protein, forming larger clusters on condensed chromosomes. Other fluorescent foci might be hidden within the condensed DNA. Although this transgenic Mod(mdg4)56.3 protein was driven by a heat shock promoter, we believe that its expression pattern and localization in primary spermatocytes is a valid indicator of the expression / localization of the native protein for the following reasons:

1- Similar clustered foci (the nucleolar cluster) were detected with an antibody against a peptide from the specific domain of Mod(mdg4)56.3 at two stages of meiotic prophase.

2- Localization to a discrete structure on one of the bivalents at prometaphase and metaphase was confirmed by immuno-staining with an antibody against the common region of Mod(mdg4), which gives an identical pattern at this stage (Thomas and McKee, personal communication).

3- The same staining pattern at prometaphase and metaphase is seen with an antibody against *Stromalin-2* (*SA-2*), product of *pairing failure 1* (*pf-1*) gene. *pf-1* mutants have the same phenotype as *pf-2* mutants and the two proteins co localize (Thomas and McKee, personal communication).

4- The protein reaches maximum abundance by stages S2b-S3, which coincides with establishment of chromosomal territories. The disorder seen at mid-prophase in *pf-2* mutants indicates that this protein is required for the integrity of these territories, thus, it is on the scene at the right time.

5- Mod(mdg4)56.3-GFP signal persists at least until prometaphase I (chapter 3, figure 6b) but is absent at anaphase I (data not shown) and later stages, consistent with its putative role in pairing.

However at this point the conclusion that Mod(mdg4)56.3 is present on autosomal chromatin is based solely on the GFP signal from transgenic Mod(mdg4)56.3-GFP and it remains to be confirmed that the native protein also localizes to autosomes.

Based on our knowledge of the structure of Mod(mdg4)56.3 and its cellular localization in meiotic cells, several putative roles for this protein may be postulated. It is important to determine whether Mod(mdg4)56.3 is directly bound to the DNA or ribosomal RNAs present in the nucleolus, or its interactions are mediated by other proteins. Yeast two hybrid assays may be very useful to identify cellular partners of Mod(mdg4)56.3. Also, immunoprecipitation experiments using antibodies against the fusion protein or specifically binding to Mod(mdg4)56.3 can result in identifying other, probably unknown meiosis-specific, interacting proteins.

Proposed models for participation of Mod(mdg4)56.3 in meiotic pairing

Mod(mdg4)56.3 as a transcription factor

Mod(mdg4) and the GAGA factor, encoded by the *Trithorax-like (Trl)* locus and required for proper expression of many different genes (Farkas et al., 1994; reviewed in Granok et al., 1995; Wilkins and Lis, 1997) have many characteristics in common including their gene structure (presence of the N-terminal BTB domain and the C-terminal C_2H_2 motif), mutant phenotypes (e. g. homeotic transformation, enhancer of PEV, reduced viability, defective female meiotic segregation) and the generation of several transcripts by alternative splicing (Benyajati et al., 1997; Read et al., 2000; Dorn et al., 1993, Gerasimova et al., 1995; for review see Granok et al., 1995). Both GAGA and Mod(mdg4) contain only one C-terminal Cys_2 His_2 finger-like domain and it has been reported that the

DNA binding feature of GAGA is mediated by this motif (Pedone et al., 1996). By similarity, the only zinc finger present in Mod(mdg4) structure may bind to DNA by dimerization with the same or other isoforms of Mod(mdg4) or other partners with a BTB domain or appropriate structural features. A model has been proposed by Dorn and Krauss (2003) suggesting multiple DNA / protein interactions through FLYWCH and BTB domains respectively.

Stromalin-2 (*SA-2*, Thomas and McKee, personal communication) is the product of *pf-1* gene, mutations in which have been shown to cause meiotic phenotypes identical to the ones displayed by *pf-2* mutants. One possibility is that Mod(mdg4)56.3 may be needed for transcription of other genes required for homolog pairing, such as *SA-2*, *teflon* (Tomkiel et al., 2001), or those yet unidentified. Although *SA-2* and *pf-2* meiotic phenotypes are identical, SA-2 is expressed in *pf-2* mutant flies, demonstrated by the presence of its PCR-amplified transcript in RT-PCR products prepared from testis of *pf-2* null mutants (data not shown). Further studies are needed for testing this hypothesis, but our cytological and molecular data are not in favor of this putative role for Mod(mdg4)56.3.

Although a role of transcription in meiotic pairing has already been proposed (McKee, 1998) and also, based on its structure and homology to other transcriptional modulators, it is possible that Mod(mdg4)56.3 plays a role in gene expression. But the specificity of the meiotic phenotype associated with mutations in *mod(mdg4)56.3* gene and our cytological analyses do not support an indirect involvement of this protein in homolog pairing as a transcription factor.

Transcriptional events are less abundant as meiosis progresses and almost do not occur at late stages of prophase when Mod(mdg4)56.3 is still highly expressed. Also, based on the presence of this protein on autosomes, detected as arrays of fluorescent foci associated with the GFP tag of the fusion protein in transgenic lines, it seems unlikely that so many genes would be regulated by Mod(mdg4)56.3, at a time when transcriptional activities are at their lowest levels.

Moreover, there is no reason to think that Mod(mdg4)56.3 has a role in transcribing rRNA genes, yet the nucleolus is the most abundant site of localization for Mod(mdg4)56.3. Therefore, even though a role in transcription cannot be completely excluded, it seems very unlikely that the primary role of Mod(mdg4)56.3 in chromosome pairing is that of a transcription factor.

Mod(mdg4)56.3 as a modifier of chromosome organization

However, the fact that Mod(mdg4)56.3 has the necessary structural features to bind to DNA (or, perhaps, chromosomal RNA) may imply a role for this protein in the modification of chromosome structure in a way that it would facilitate meiotic pairing of homologs.

At least one isoform of *mod(mdg4)* has been shown to have a chromatin-related function, affecting the higher order organization of chromosomes within the nucleus (Chen and Corces, 2001; Dorn et al., 1993; Gerasimova et al., 1995; Cai and Levine, 1997; Gerasimova et al., 1998; Buchner et al., 2000; Gause et al., 2001), suggesting the possibility that Mod(mdg4)56.3 is required to determine a spatial meiotic chromatin structure needed perhaps for proper loading of homolog

pairing proteins. This hypothesis is supported by the fact that both Mod(mdg4)67.2 and Mod(mdg4)56.3 localize to discrete foci, and in both cases multiple binding sites cluster or coalesce to form larger foci. Figure 15 (chapter 3) clearly shows the paired, adjacent foci on partially condensed late prophase autosomes.

Mod(mdg4)56.3 as a cohesion protein

mod(mdg4) contains a BTB domain, a 115 residue-long dimerization / multimerization domain found in many transcriptional regulators (Read et al., 2000; Zollman et al., 1994). The BTB domain forms an extensive dimer interface that is a possible binding site for other proteins (Ghosh et al., 2001). There might be other as yet unidentified partners of Mod(mdg4) that interact with the BTB domain.

A direct role of Mod(mdg4)56.3 as a pairing protein is quite plausible. The BTB domains of both Mod(mdg4)56.3 and of the structurally similar proteins such as GAGA factor have been shown to multimerize and to bring distant DNA sites into close proximity (Ghosh et al., 2001; Katsani et al., 1999). It is not hard to imagine that such properties could be exploited to mediate pairing of homologous chromosomes.

Considering the fact that proteins such as Scc1 are involved in chromosomal cohesion by closing the ring that holds the DNA strands in the middle, and without being physically bound to DNA, we may think about a function of Mod(mdg4)56.3 as part of a multiprotein complex recruiting other nuclear factors

as well, a meiotic form of cohesin being one plausible candidate for such a complex. In support of this suggestion is the finding that *pf-1*, mutations in which give phenotypes virtually identical to mutations in *pf-2*, encodes the SA-2 protein, member of the SCC3 / SA / STAG family of cohesion proteins.

An essential step in meiotic chromosome segregation is the cleavage of the complex in order to release the strands. In meiosis, a stepwise separation of chromosomes occurs: first, cohesion is lost between the two homologs that will migrate to opposite poles and second, homologs that were held together at their centromeric region until anaphase II will segregate into two sister chromatids. This sequential disjunction process is tightly regulated and in yeast, proteins such as Spo13 (Klapholz and Esposito, 1980) and Sgo1 (Rabitsch et al., 2004) have been identified that control the removal of centromeric cohesion proteins (Klein et al., 1999), such as Rec8p (DeVeaux and Smith, 1994). We cannot exclude a possible intervention of Mod(mdg4)56.3 by its specific yet unidentified enzymatic activity. BTB-containing proteins also have roles in ubiquitin conjugation (Furukawa et al., 2003) and this particular isoform of Mod(mdg4) may play a role in targeting proteins for degradation, specifically at the metaphase I – anaphase I transition.

Matrix / Scaffold Attachment Regions (MARs / SARs) are AT-rich short sequences (Grasser and Laemmli, 1986) and have been speculated to be sites of attachment of chromosomes, facilitating the homology searching and promoting the pairing of homologous sequences (McKee, 2004). It is still obscure how MAR / SARs function, and how they might be involved in the pairing of meiotic

chromosomes. The loss of protein complexes that function by connecting chromosomal regions to the nuclear matrix may result in the generation of unpaired homologs, a phenotype detected cytologically in meiotic cells of *pf-2* mutants, where dispersed univalents are seen as early as prophase I. We speculate that Mod(mdg4)56.3 might mediate the binding of chromosomes to the matrix.

Significance of the subnuclear localization of Mod(mdg4)56.3

Many nuclear proteins and / or RNA molecules are organized within the interchromatin spaces of the nucleus in a number of discrete bodies. The nucleolus is a dynamic structure formed around ribosomal DNA repeats and is the site of biosynthesis, processing and assembly of ribosome subunits (Lamond and Earnshaw, 1998). Nucleolus formation is cell cycle- and transcription- dependent and occurs if new ribosome synthesis is required (reviewed by Hernandez and Roussel, 2003).

The finding that Mod(mdg4)56.3 is associated with the nucleolus was not surprising, as it is known that the X and Y chromosomes containing the nucleolus organizer regions are associated with the nucleolar components in meiotic cells in males (Fuller, 1993) and the X-Y pairing sites have been mapped to rDNA sequences within the heterochromatic regions, in the vicinity of the nucleolus (McKee et al., 1992; for review see McKee, 2004). Intriguing was how Mod(mdg4)56.3 played a role in meiotic chromosome pairing as a nucleolar component. Chromatin immunoprecipitation procedure would allow determining whether Mod(mdg4)56.3 binds, directly or indirectly, to the X-Y pairing sites.

Recently, published data report the nucleolar localization of novel proteins and investigators are puzzled about the importance of their subnuclear distribution for their function (for review see Garcia and Pillus, 1999). Nucleolar proteins with meiotic functions have also been identified and their characterization may bring insights into possible roles of Mod(mdg4)56.3 during meiosis (Buonomo et al., 2003; Rabitsch et al., 2001).

In conclusion, it seems highly likely that Mod(mdg4)56.3 acts as a pairing protein. Its localization along the arms of the autosomes supports the idea that it may be involved in cohesion, either by direct DNA-binding or by interactions mediated by yet unidentified partners.

The identification of mutations in a gene disrupting homologous chromosomes pairing and segregation specifically during *Drosophila* male meiosis will set the road to identify components that are essential for proper operation of the meiotic machinery and is a great step toward gaining insights into the mechanism of meiotic pairing in an apparently simple system where structures such as the synaptonemal complex or chiasmata are not functionning. Further molecular analysis could bring insights on the occurrence of trans-splicing, by demonstrating molecularly that mature transcripts produced by flies heterozygous for two *pf-2* mutations, one affecting the common region and one located within the specific C-terminal exon, carry wild type or mutant alleles for both regions.

Chapter Five

Experimental procedures

Fly stocks

The *pf-2* alleles were generated by a large scale EMS-mutagenesis and are maintained by C. Zuker (Koundakjian et al., 2004). *pf-2* alleles were identified in a screen for mutations on chromosome three that cause paternal loss of chromosome four (Wakimoto et al., 2004). *mod(mdg4)* alleles were kindly provided by V. Corces (John Hopkins University, MD) and M. Frasch (Mount Sinai School of Medicine, New York). Transgenic lines with insertion of *mod(mdg4)56.3* on chromosome II or III were generated in the laboratory of R. Dorn. All other stocks used were obtained from the Bloomington Stock Center at the University of Indiana. Stocks were maintained on cornmeal-molasses-yeast-agar medium at 24° C.

Genetic crosses

Unless otherwise specified, the male or female being tested was crossed singly to three flies of the specified genotype for the experimental cross. Parents were removed from the food vial ten days (d10) after the cross was initiated (d0). Progeny were counted between d14 and d21 to avoid the presence of the F2 generation. The marked Y chromosome (Dp(1;Y) B^{S}Yy^{+}) carries two transposed segments of X chromosome carrying the markers B^{S} and y^{+} that are appended to

the ends of the left and right arms, respectively, and that allow us to follow its segregation from the X chromosome in X-Y NDJ tests (Figure 1). In the C(1) RM, y^2 su(w^a) w^a; C(2) EN *b, pr* and C(4) EN *ci, ey* stocks, flies carry attached chromosomes consisting of two genetically complete copies of the chromosome (X, 2 and 4 respectively) attached to a single centromere and produce only diplo and nullo gametes. This feature was exploited in our genetic tests to determine the occurrence of chromosomal NDJ in mutant flies.

TM3, TM6 and TM2 are balancer chromosomes; multiply inverted chromosomes that prevent crossover events. Phenotypic markers were used for the generation and selection of flies with the genotype of interest and are described in Lindsley and Zimm (1982). These markers included:

- Eye phenotype (color or shape): *scarlet* (*st*), *white* (*w*), *prune* (*pn*), *brown* (*bw*), and *eyeless*, *Bar* and *Bar stone* or (*ey*, *B* and B^s, reduced eye size or restricted to narrow vertical bar)

- Bristles: *Stubble* (*Sb*), *forked* (*f*, bristles are short and thick or kinked), *bobbed* (*bb*, thinning and shortening of bristles and etching of the abdomen. The *bb* locus encodes the major (18S and 28S) ribosomal RNAs and is the nucleolus organizer. The rRNA encoding genes form large tandem arrays at both loci, and mutant phenotypes result from complete or partial loss of these. Wings: *Serrate* (*Ser*, dominant wing-nicking phenotype), *cubitus interruptus* (*ci*, the cubital vein L4 is interrupted), *crossveinless* (*cv*, absent crossveins)

- Body color and size: *yellow* (*y*) and *miniature* (*m*).

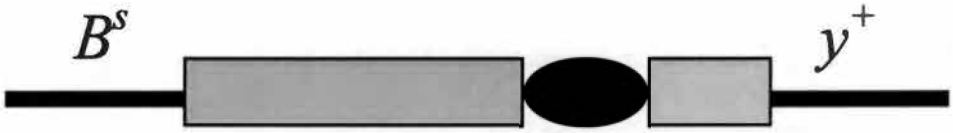

Figure 1: Illustration of the marked Y chromosome ($B^s Y y^+$) used in crosses that allow genetic determination of the occurrence of non-disjunction. The filled oval illustrates the centromere flanked by gray-filled rectangles representing heterochromatic regions. The phenotypic markers, B^s and y^+ are located at each end of the chromosome.

Crossing schemes for genetically testing whether *pf-2* mutations affect NDJ and / or recombination events in females are reported in Figure 2. Figure 3 represents crosses set up for rescue experiments using flies bearing the *P* {ry^+; [*hsp70-mod(mdg4)56.3-gfp*] construct on their second chromosome. Rescue experiments using *P* {ry^+; [*hsp70-mod(mdg4)56.3-gfp*] construct inserted within chromosome 3 of transgenic flies are shown in Figure 4.

To produce flies with a *pf-2* null genetic background carrying the transgene on their third chromosome, females heterozygous for both the transgene and the $mod(mdg4)^{T16}$ deletion (marked phenotypically with *ebony*) were generated and crossed to males bearing a *pf-2* mutation over a balancer chromosome. F1 male progeny of this cross were crossed to *y w* females for the X-Y NDJ assay. 10 days after crosses were set up, genomic DNA of these males was extracted and used as template for amplifying by PCR the entire *mod(mdg4)56.3* sequence (by using primers designed against exon 1 and the 3'UTR sequences) and the entire *gfp* sequence (by using primers designed against both ends of its coding region) to identify those carrying the transgene.

To determine the best heat shock conditions, the water bath temperature was fixed at 37°C, 38°C, or 39°C. Flies were incubated for 15, 30 or 60 minutes. Heat shocks were given at different stages of the embryonic development on d3; d6, d9 or dl 1 after the cross was set up (d0). For some transgenic lines, multiple heat shocks resulted in a more efficient rescue of the mutant phenotype, while for others, even without heat shocking, a leaky expression of Mod(mdg4)56.3 fully rescued the NDJ phenotype. The data showed a better rate of rescue if the

Crossing scheme for testing chromosomal disjunction and recombination rate in females mutant for *pf-2*

Stocks used:

- Tp(1,1) y^+ *y*[1] *pn*[1] *cv*[1] *m*[1] *f*[1]/ C(1)DX, *y,w,f,bb*$^-$ where ♀ and ♂ genotypes are XX^{bb-} / Y *y,w,f* and Tp(1,1) y^+ *y*[1] *pn*[1] *cv*[1] *m*[1] *f*[1] / Y respectively. This multiply marked X chromosome will be referred to as **mX**. The *bobbed* (*bb*$^-$) mutation is complemented with the Y fragment.

- *y / y; Z3-3298, st* / TM6, *Tb, e*
- + / $B^S Y y^+$; *bw / bw; Z3-3401, st* / TM3, *Sb, e*
- *y* / $B^S Y y^+$; *Z3-3401, st* / TM6, *Tb, e*
- C(1)RM / O where ♀ bear attached X chromosomes and ♂ are C(1;Y) Y^S X.Y^L In(1)EN, *y B* (X^Y*y, B* / O)

I- Generation of *y; Z3-3401, st* / TM6, *Tb, e* stock

♀ *y / y; Z3-3298, st* / TM6, *Tb, e* **x** ♂ + / $B^S Y y^+$; *bw / bw; Z3-3401, st* / TM3, *Sb, e*

↓

♂ *y* / $B^S Y y^+$; *Z3-3401, st* / TM6, *Tb, e* **x** ♀ *y* / +; *Z3-3401, st* / TM6, *Tb, e*

↙ ↘

♀ *y / y; Z3-3401, st* / TM6, *Tb, e* **x** ♂ *y* / $B^S Y y^+$; *Z3-3401, st* / TM6, *Tb, e*

II- Generation of males with attached XY chromosomes (X^Y*y, B* / Y)

♀ XX^{bb-} / Y *y,w,f* **x** ♂ X^Y*y, B* / O

↓

Dead ♀ (XX^{bb-} / O) and ♂ X^Y*y, B* / Y

III- Generation of females hemizygous for *pf-2* mutation bearing a multiply-marked X chromosome

♂ mX / Y **x** ♀ *y, w / y, w*

↓

♀ mX / *y, w* **x** ♂ + / Y; Df(3R)*GC14, st* / TM6, *Tb*

↓

♂ mX $^{y \text{ or } y+}$ / Y; Df(3R)*GC14, st* / +

These males were checked for the presence of all the phenotypic markers and crossed to:
♀ *y / y; Z3-3401, st* / TM6, *Tb, e* or ♀ *y / y; Z3-3298, st* / TM6, *Tb, e*

Figure 2: Crossing schemes for the generation of females with the indicated genotype used in NDJ / recombination tests

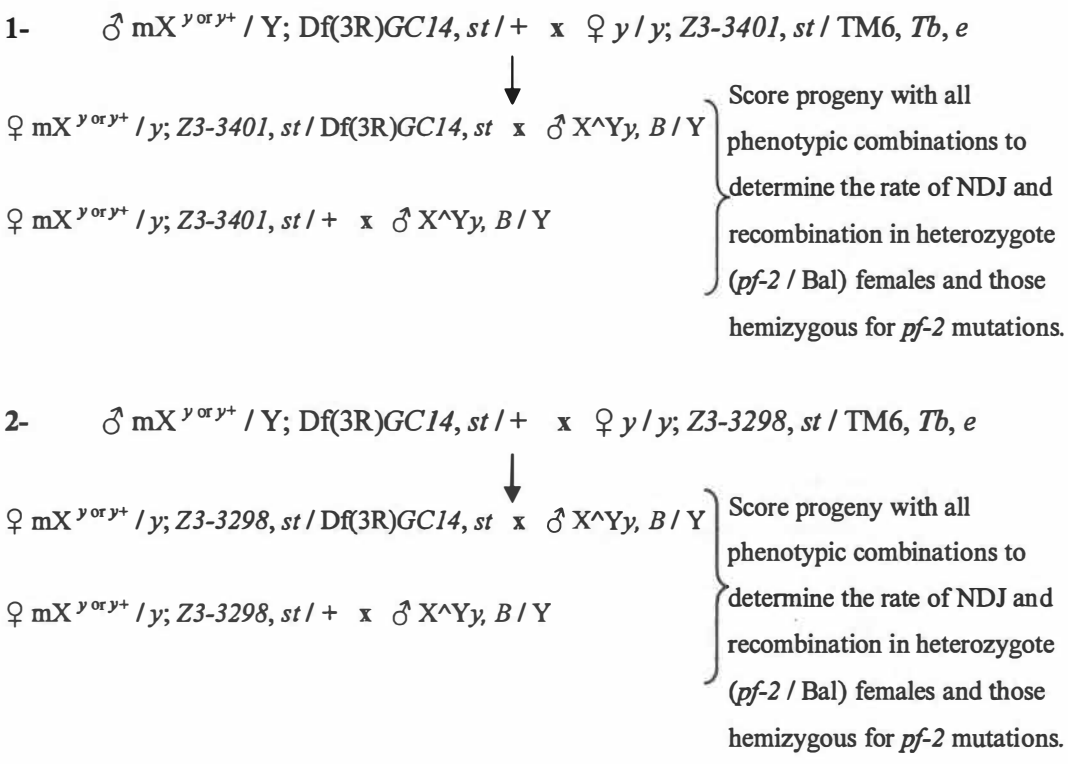

Figure 2 continued.

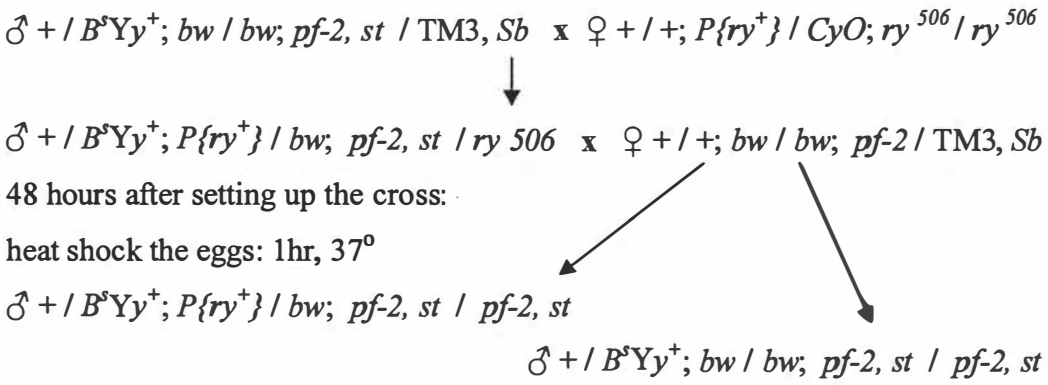

Figure 3: Crossing scheme for the generation of male flies homozygous for *Z3-3401* mutation, and heterozygous for *Z3-5578 / Z3-3298*, bearing a *P* {*ry*$^+$; [*hsp70-mod(mdg4)56.3-gfp*] transgene on their second chromosome that drives the expression of *mod(mdg4)56.3*. Males were crossed to *y w* females and their progeny were scored for NDJ in order to determine whether the expression of the transgene rescues the mutant phenotype.

Figure 4: Crossing schemes for the generation of male flies hemizygous for a *pf-2* allele, *Z3-3401* or *Z3-5578*, and the *mod(mdg4)*^{T16} deletion, bearing a *P* {*ry*$^{+}$; [*hsp70-mod(mdg4)56.3-gfp*] transgene on their third chromosome. Hemizygote males, carrying the recombinant *P{gfp}, mod(mdg4)*^{T16} chromosome or non-recombinant, were crossed to *y w* females and their progeny were scored for NDJ in order to determine whether the expression of the transgene rescues the mutant phenotype. These males were crossed to *y w* females and their progeny were scored for NDJ. After mating, the male parents were individually tested for the presence of the transgene by PCR amplification of their genomic DNA.
⧖ indicates the occurrence of recombination in females.

♀ + / +; + / +; *P {gfp}* / *P {gfp}* x ♂ *w* / Y; + / +; *mod(mdg4)^{T16}*, *e* / TM6, *Tb*, *e*

♀ *w* / +; + / +; *P {gfp}* ⋈ *mod(mdg4)^{T16}*, *e* x ♂ *w* / Y; + / +; *mod(mdg4)^{T16}*, *e* / TM6, *Tb*, *e*

(Occurrence of recombination, collection of progeny carrying the transgene and the *mod(mdg4)^{T16}*, *e* on the same chromosome)

♀ *w* or + / *w*; + / +; *mod(mdg4)^{T16}*, *e* / TM6, *Tb*, *e*

♀ *w* or + / *w*; + / +; *P {gfp}*, *mod(mdg4)^{T16}*, *e* / TM6, *Tb*, *e*

Crossed to: ♂ + / *B^{s}Y y^{+}*; *bw* / *bw*; *pf-2*, *st* / TM3, *Sb*

48 hours after setting up the cross:

heat shock the eggs: 1hr, 39°

To find the best experimental conditions,

various temperature

and number of heat shocks were tested.

♂ *w* or + / *B^{s}Y y^{+}*; *bw* / +; *pf-2*, *st* / *mod(mdg4)^{T16}*, *e*

♂ *w* or + / *B^{s}Y y^{+}*; *bw* / +; *pf-2*, *st* / *P{ gfp}*, *mod(mdg4)^{T16}*, *e*

Collection of *B*, *Tb^{+}*, *Sb^{+}*, *e^{+}* male progeny

Crossed to: ♀ *y w*

Score for NDJ

expression of Mod(mdg4)56.3 was induced during early development stages (at d3). The expression of Mod(mdg4)-GFP was induced in eggs laid by *y w* females that were crossed to a single, yet unidentified, transgenic males. To determine their genetic background, these males were collected at d7 and tested singly for the presence of the transgene. Each male was grinded in 20 ul of PCR reaction mix. Two sets of PCR reactions were set up by adding either primers within exon 1 and DSD, to amplify the entire 1.7 kb fragment of *mod(mdg4)56.3* DNA or primers within the *gfp* coding sequence, to amplify the entire *gfp* DNA, to 10 ul of the mix containing the genomic DNA of these flies. The presence of a PCR fragment in both reactions indicated that the specific fly carried the *gfp*-labeled *mod(mdg4)56.3* transgene within its genome. After eclosion, the F2 *ebony*$^{+}$ male progeny of the molecularly identified transgenic males, which were null for the endogenous *pf-2* but expressed the GFP-tagged gene, were collected and tested for NDJ by mating them with *y w* females. Their progeny were scored for NDJ and rescue was determined by comparison to progeny of non-transgenic males.

Cytological analysis

Orcein-stained meiotic chromosomes from adult testes were prepared according to Lifschytz and Hareven (1977). Briefly, testes were dissected in testis buffer (7% NaCl, Ashburner, 1989) and fixed in 45% acetic acid for thirty seconds. Squashing in a 1:1 mixture of 60% acetic acid and 2% lactic-acetic-orcein followed staining of the testis in 3% orcein-60% acetic acid for five

minutes. Analysis and photographs were carried out using phase-contrast microscopy on a Zeiss Universal Axioplan photomicroscope with a CCD camera.

Live testes were prepared by dissection in testis buffer followed by covering of the tissue with a cover slip and gentle tapping to release cells.

DAPI staining

Hand-dissected testes, 3-4 pairs per slide, were transferred to 8 ul of testis buffer on a poly-L-Lysine coated slide and covered with a siliconized cover slip. Testes were then gently squashed for a better distribution and visualization of meiotic cells. Slides were held in liquid nitrogen until bubbling stops and the cover slip was then rapidly removed with a razor blade. Slides were incubated for at 5 minutes in 95% ethanol, 1 minute in acetone, air dried and transferred to 1% Triton X-100, 0.5% acetic acid in 1X PBS (NaCl, KCl, Na_2HPO_4,, KH_2PO_4) for 15 minutes. After 3 washes of 5 minutes each in 1X PBS, slides were incubated for 5 minutes with 1 ug / ml of 4'-6-DiAmidino-2-Phenyl Indole dihydrochloride (DAPI), washed 2 times in 1X PBS, mounted in Vectashield (Vector Laboratories, Inc.) and covered with a coverslip. Fixed cells were then analyzed with a Zeiss Axio Plan with a CCD camera.

X-Y NDJ test

Male flies to be tested carried a marked Y chromosome (B^sYy^+) and are crossed to *yellow white* (*y w*) females, which produce only euploid gametes and their progeny were scored for NDJ. The presence of XO males (B^+ *w*) and X-XY

females (B, w^+) indicates the occurrence of the paternal NDJ. The ratio of progeny with a NDJ phenotype among total progeny is calculated as the percentage of NDJ.

Molecular analysis

Genomic DNA extractions were performed by grinding 50 flies with a microhomogenizer and using the Wizard^R genomic DNA purification kit (Promega). The polymerase chain reaction (PCR) parameters were 1 minute at 94°C, 35 cycles of 94°C for 1 minute, 55°C for 1.5 minutes, and 72°C for 2 minutes in a Perkin-Elmer thermocycler. Reaction mixtures contained 1 nmol of each primer, 10 ng *Drosophila* genomic DNA (Zuker or *y w*), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix and 2.5 U Taq DNA polymerase (Promega) in a total volume of 50 microliters overlaid with an equal volume of mineral oil. Product size and purity was confirmed by electrophoresis in a 1% agarose gel. The QIA Quick Gel Extraction Kit (Qiagen) was used to purify the PCR products for future use.

RT-PCR products were generated by reverse transcrition of total RNAs isolated from 50 whole flies or pairs of testis using the Superscript^{TM} First-Strand Synthesis System for RT-PCR (Invitrogen).

To amplify *mod(mdg4)56.3* cDNA, PCR was carried out using primers within exon 1 (R153931; 5'AGAACTCGGACGCGTTCTGC3') and 3'UTR (F131753; 5'AATACAGCAATGTGTACACG3'). To determine the 3' end of the testis *mod(mdg4)56.3* cDNA, total RNA was extracted by grinding hand-dissected fly testes in Tri-reagent (Sigma-Aldrich). After 5 minutes at room temperature, the

mixture was spun at maximum speed for 5 minutes at 4°C. The RNA pellet was washed with 75% ethanol and air dried for 1 minute before being resuspended in DEPC-treated water. SMART RACE (Clontech) reactions were carried out using the kit's labeled oligo-dT primers along with various primers within the published nucleotide sequence were used and the amplified product was sequenced using the same primers that generated the amplicon.

Sequencing

Gel-purified PCR product or plasmid DNAs were used as template (50ng – 300ng) along with 5 pM of a primer designed for sequencing the region of interest and the reaction Mix (DNA Sequencing Kit, Big Dye™ Terminator Cycle Sequencing v3.1 Ready Reactions with AmpliTaq DNA Polymerase, *Applied Biosystems*) in a total volume of 10 microliter. The sequencing reaction was carried out at 96° for 4 min. followed by 25 cycles of 10 sec. at 96°, 5 sec. at 50° and 4 min. at 60°.

Immunofluorescence assay

Hand-dissected testes were transferred to poly-L-Lysine-coated slides, squashed under a siliconized cover slip and frozen in liquid nitrogen. The cover slip was then removed with a razor blade and slides were immediately immersed in cold ethanol for at least 10 minutes. After fixing the tissues in 4% para-formaldehyde in 1X PBS, washing 2 x 15 minutes in PBS 1X containing 0.1% Triton-X100 and 0.3% deoxycholate at room temperature, blocking for 1 hr in

Tris-HCl (0.1 M, pH 7.5) NaCl (0.15 M) and BSA (5%) (TNB), the preparation was then incubated overnight at 4°C with the primary antibodies diluted in TNB. Primary antibodies used in this report were anti-Mod(mdg4)56.3, a rabbit affinity-purified polyclonal antibody raised against a 15 residue fragment within the specific C-terminal exon of this isoform and an added residue for conjugation purpose[(C)DVLVYDGYRYDRRAN] (Alpha Diagnostic International), anti-Fibrillarin (kindly provided by M. Fuller), a rabbit anti-green fluorescent protein (GFP) (A111-22, Molecular Probes) and a rabbit FITC-conjugated anti-alpha Tubulin (). The following day, slides were washed 3 x 5 minutes with TNT (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.05% Tween), incubated with the secondary antibodies (FITC- or Cy5- labeled goat anti rabbit immunoglobulins) diluted in TNB for 1 hr at room temperature and washed again before incubating for 5 minutes at room temperature with a fluorescent dye, DAPI (1 ug / ml) or Hoechst 33342 dye (5 ug / ml; Molecular probes), to stain the DNA. 2 x 5 minutes washes were followed by mounting in Vectastain and analyzing meiotic cells with a Zeiss Axioplan microscope linked to a CCD camera.

Determination of the deletions breakpoints

ORiso3 and OSiso3 strains, Oregon R and Canton S wild type lines isogenized by crossing siblings with the third chromosome over a balancer for 10-50 generations (Hoskins et al., 2001), were used to generate flies heterozygous for each inbred line and one *mod(mdg4)* deficiency (*B2*, *T16*, *Δ10*, *Δ33*, Df(3R)*e-Gp4* and Df(3R)*GC14*). Fragments of ~500-800 nt. within and beyond the *mod(mdg4)*

locus were amplified by PCR using the genomic DNA of heterozygote flies as template and primers designed against the coding sequences of the common region and the C-terminal variable exons of this locus. Agarose gel-purified PCR fragments were sequenced directly by using one of the amplification primers. ABI-Big Dye Terminator v3.1 Cycle (Sequencing kit, Applied Biosystems) was used to determine the nucleotide sequence of the fragments. Sequence comparison was performed by using the 'Sequencher software. For each sequenced PCR fragment containing one or more SNPs between ORiso3 and CSiso3, the deletion-bearing chromosome was used as deficient for the tested SNP if the ORiso3 / Df and Csiso3 / Df DNA samples exhibited only a single allele, but was scored as not-deficient for the SNP locus if one or the other sample exhibited both alleles.

Statistical analysis

The parameters R_x and R_Y are used in the calculation of meiotic drive where R_x is an indicator of the viability of X-bearing sperm and R_Y of the viability of Y-bearing sperm relative to otherwise identical sperm classes lacking the X or Y (McKee et al. 1998). Formulas for these parameters are: $R_x = (X.\ XY\ /\ 0.\ Y)^{1/2}$ and $R_y = (Y.\ XY\ /\ O.\ X)^{1/2,}$ where X, Y, XY and O are the numbers of progeny of the indicated genotype. R_X, and R_Y values are equal to 1 in wild-type backgrounds.

Non-homologous disjunction (NHD) = (XY; 22 + O; O) / (XY; O + O; 22), where (XY; 22), (O; O), (XY; O) and (O; 22) are the number of progeny scored

for each class of sperm. The value of NHD is 1.0 if no non-homologous disjunctions occur but less than 1.0 if non-homologous disjunction occurs.

Map distances (MD) and coefficients of coincidence (c.o.c) were calculated by standard formulas. Distance (in map unit or m. u.) = recombinant progeny / total number of progeny. The coefficient of coincidence (c.o.c.) is the observed number of double recombinants divided by the expected number of double recombinants. Interference is equal to 1- c.o.c. and is high when c. o. c. = 0, that means no DCO can occur.

c.o.c. = obs DCO (I, II) / exp DCO (I, II)

c.o.c. = obs DCO (I, II) / [MD (I) / 100] x [MD (II) / 100] x 100

Observed = number of progeny with a phenotype associated with double crossover (DCO) / total number of progeny.

Expected = the product of map distances in 2 adjacent intervals x N.

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Vita

Morvarid Soltani Bejnood is born in Tehran, Iran. She graduated from a French – Iranian high school in Tehran and went to France to further her education. She attended The University of Pierre et Marie Curie (Paris VI) and earned a Bachelor of Science degree in Biochemistry and a Masters degree in Physiology of Reproduction and became a Ph. D. candidate in 1989. Morvarid met her husband in 1991 and moved to The United States of America in August 1991, leaving her Ph. D. research project in France unfinished. Although she enjoyed raising her sons, born in 1992 and 1996, her interest in Science made her decide to enroll in a Ph. D. program at The University of Tennessee in Knoxville. She was accepted by The Graduate School of Genome Science and Technology in 1999 and joined the laboratory of Dr. Bruce McKee to study Genetics. Morvarid earned a Ph. D. degree in Life Sciences in 2004. She has a great interest in teaching and hopes to have a carrier in both Teaching and Research.