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Exploring the role of *solo*, in *drosophila* females

Jessica Michelle Adams
University of Tennessee-Knoxville

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Exploring the role of *solo* in *Drosophila* females

Abstract

Meiosis is an important process in all sexually reproducing animals, and the mechanism has been found to be highly conserved across many species. However, mystery still surrounds many of the specifics of the process, such as pairing of homologous chromosomes and cohesion of the sister chromatids. A new gene, *solo*, has been identified which plays a key role in meiotic cohesion. *Solo* mutant gametes experience significantly decreased levels of recombination and a high percentage of nondisjunction of chromosomes. This project aims to discover at exactly which step of meiosis *solo* is active and what role it plays. To do this, I examined synaptonemal complex and DSBs in $solo^{-/-}$ $spnB^{-/-}$ female germaria. I found significant differences in SC formation and ovariole development. However, my γ-H2Av antibody did not work, so I could come to no conclusions about DSBs in the mutant flies.

Introduction

Although the entire genome of *Drosophila melanogaster* has been recently sequenced, there are many genes whose function remains a mystery, and indeed many genes whose existence has yet to be identified. Identification of such genes that are involved in physiological functions but are not required for viability has been the goal of the Zuker lab. They treated thousands of cultures of flies with a strong dose of ethyl methanesulfonate (EMS), a well-known mutagen that produces point mutations in DNA.

The resulting vials were then screened for the presence of a homozygous viable mutation. According to calculation, each of the 12,000 resulting lines contains five to six point mutations in nonessential genes on the second and third chromosomes (Koundakjian et al, 2004).

The Zuker collection is available for use by other labs, and the Wakimoto lab screened the entire collection for male sterile (*ms*) and male chromosome loss (*mcl*) vials. Chromosome loss was identified phenotypically in the progeny, and almost always consisted of loss of the paternal fourth chromosome. Sixty-two *mcl* lines were isolated and cytologically categorized as premeiotic, meiotic, or post-fertilization mutations (Wakimoto et al, 2004). The meiotic *mcl* lines are being analyzed by the McKee lab and were identified by variation in spermatid nuclei. Twenty-eight mutations were recovered and have been categorized into nine different genes, called pairing failure (*pf*) 1-9. These pairing failure mutations affect both meiosis I and meiosis II, involving the cohesion of homologous chromosomes and sister chromatids.

This paper focuses on *pf-7*, now called *solo*. *Solo* is located on the second chromosome and results from alternative splicing of the *Vasa* gene. To determine at what stage pairing failure was occurring, mutant males were crossed and their sperm were scored into classes. The presence of XY sperm indicates homologous nondisjunction, whereas XX sperm indicates premature sister chromatid separation (PSCS). In *Solo* mutants, about 10% of sperm were XY and about 4% were XX. This indicates that cohesion is failing both in meiosis I and meiosis II. However, chromosome distribution in mutant spermatocytes following anaphase I appears normal, and it is not until after meiosis II that clear differences in nucleus size are apparent. Also, *solo* mutants

experience a much lower rate of recombination, which is common in cells that contain nondisjunction, a relationship that will be explained below (McKee, unpublished results).

To understand what might be occurring in *solo* meiotic nuclei, a clear understanding of the meiotic mechanism is needed. This experiment focused on the *solo* mutation in female oocytes, as opposed to earlier studies in male spermatocytes. Male and female meiosis in *Drosophila* is quite different. Male chromosomes separate without synapsis and crossing over at a synaptonemal complex, whereas female chromosomes' separation relies on this synapsis and the resultant crossing over of genes, as described below. The meiotic mechanism is highly conserved in many multicellular organisms, and female *Drosophila* meiosis is often studied as a model for more complex life forms, especially humans.

Drosophila ovaries are an ideal palette in which to study meiosis, because the successive stages of development are laid out linearly along a single ovariole, about fifteen of which make up one ovary. The ovariole itself can be divided into fourteen stages, although at stage 3 the chromosomes are condensed into a spherical mass called the karyosome, bypassing the traditional prophase stages of diplotene and diakinesis. At stage 13, prometaphase begins, but metaphase I is arrested at stage 14 and resumes at passage through the oviduct (Hawley et al, 1993). Synapsis and recombination occur in stage 1, which is further subdivided into regions 1, 2a, 2b, and 3, and collectively this area of the ovariole is called the germarium (see figure 1).
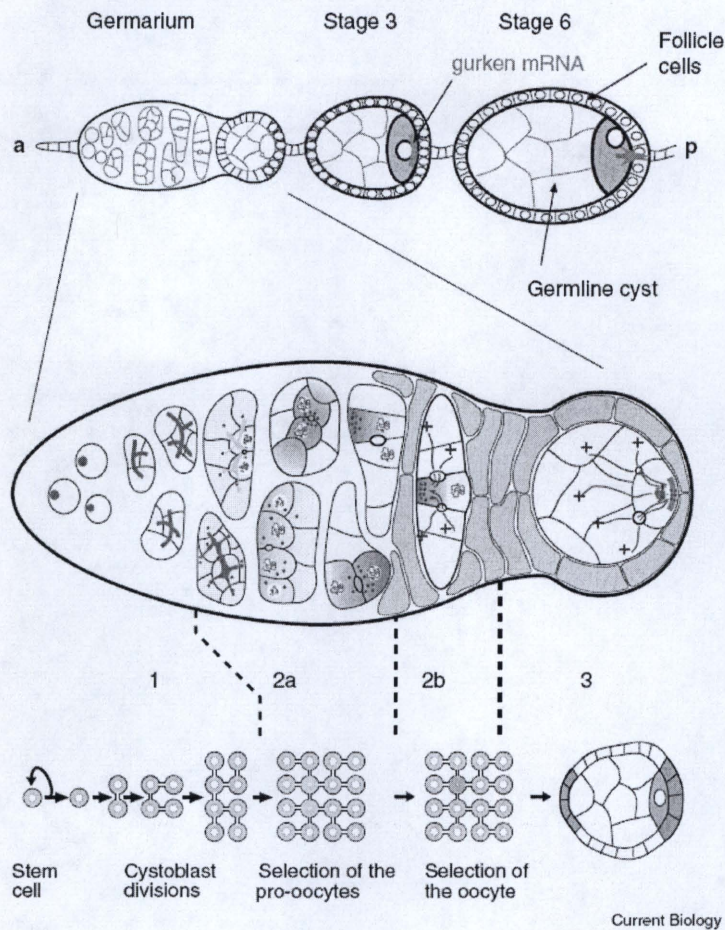
Figure 1. The germarium is at the earliest stage of development. Cystoblasts divide four times to produce 16-cell cysts. The oocyte is chosen by region 2b, and the remaining 15 cells are nurse cells. Here, the synaptonemal complex is shown in red.

From Huynh and St. Johnston, *Current Biology,* 2004.

Synapsis along the synaptonemal complex (SC) and the formation of chiasmata is essential in the correct segregation of chromosomes. The SC is formed in the pro-oocytes and later in several other cells, but by region 2b it is only found in the oocyte, which is the only cell undergoing meiosis (see figure 1). It is a proteinaceous structure that is composed of lateral and axial elements which connect homologous chromosomes in meiosis. Although clearly visible only with an electron microscope, the synaptonemal complex can be visualized by antibodies to its components, especially c(3)g (Zickler and Kleckner, 1999). According to the currently accepted model, homologous chromosomes align along the synaptonemal complex, although alignment occurs to a certain degree before the SC is formed and is fairly accurate in the event of an SC mutation, suggesting

a separate non-SC alignment (Sherizen et al, 2005). A double-stranded break (DSB) then

initiates the process of recombination in region 2a. In many other species the SC forms

after DSBs have been made, but in *Drosophila,* the two events appear to be somewhat

independent and the SC usually forms first (Jang et al, 2003). Special enzymes repair the

DSB from the homologous chromosome, linking the two chromosomes by two chiasmata

(see figure 2). The successful repair of the DSB occurs by region 3 in wild type flies

(McKim et al, 2002). Without synapsis and the resultant crossover, the homologous

chromosomes cannot separate properly during anaphase, because the tension created by

the chiasmata is crucial for correct attachment of the spindle poles to the kinetochores

and subsequent chromosomal migration (Miyazaki and Orr-Weaver, 1994).

Figure 2.
Diagram of the synapsis of
homologous chromosomes
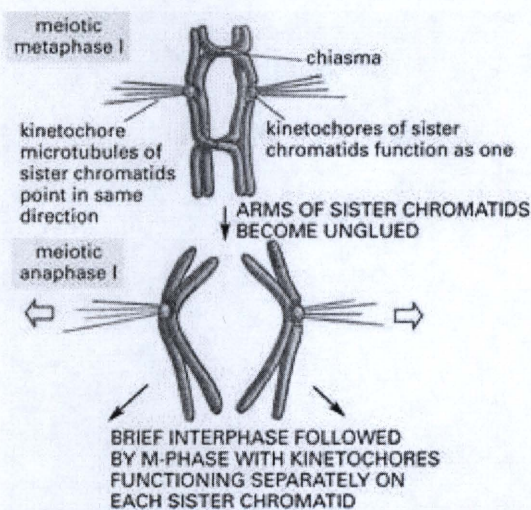and the resulting formation
of chiasmata in meiosis I.

Figure 20–11 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Cohesion between the sister chromatids is an important part of making this model

work. The cohesion between sister chromatids in mitotic cells has been well-

characterized and is enabled by cohesin, a protein complex that literally surrounds the

two chromatids in a ring. However, its role in meiosis must be more specialized and

cohesion must involve other protein complexes. In anaphase I, cohesion of the chromatid

arms is lost but remains tight around the centromeres, which don't dissociate until anaphase II (Roeder, 1997). In addition to simply keeping the sisters together, sister cohesion plays the same role in meiosis II that chiasmata play in meiosis I, creating tension so that spindle poles attach properly (Miyazaki, 1994). Several additional proteins that aid in centromeric cohesion have been identified, including *mei-S332* and *ord*. *Mei-S332* mutants experience normal synapsis and recombination, but the sisters segregate randomly in meiosis II (Roeder, 1997). On the other hand, *ord* mutants exhibit DSBs that are incorrectly repaired from the sister chromatid because of lack of sister cohesion, resulting in high levels of nondisjunction (Webber et al, 2004).

In *solo* mutants, some part of this chain of events does not function correctly. The synaptonemal complex may not form completely, or recombination and chiasmata cannot form because there are no DSBs being made. Conversely, the problem could lie in the cohesion of the sister chromatids. In order to discover the function of the *solo* protein, we plan to investigate each of these possibilities. This experiment focuses on determining whether DSBs are being formed in pachytene by immunostaining mutant ovaries with an antibody to γ-H2Av, a modified histone known to form around DSBs. I have created flies that are homozygous *solo* and homozygous *spnB*, which is a protein important in the correct repair of DSBs during recombination. Thus, if DSBs are being formed, they should have severely delayed repair, and the γ-H2Av foci should be visible in many stages of the ovariole.

Materials and Methods

Fly cross. For the P generation, I crossed heterozygous *solo* over the TM3 balancer (Sb) with heterozygous *spnB* over a Cy balancer. *Solo* is located on the second chromosome

while *spnB* is on the third chromosomes. From the F1 generation I collected all Sb+Cy+ males and females and brother-sister mated them. These flies are heterozygous *solo* and *spnB* over wild type. Because *spnB* is physically very close to the *ebony* gene on chromosome III, all of the ebony progeny are homozygotes for *spnB*. To determine the genotypic state of *solo*, I examined the eye color. Cinnabar (*cn*) is similarly linked to *solo*, so all of the flies with *cn* eyes are homozygotes for *solo*. Brown (*bw*) is an eye color gene located on chromosome III, and when both it and *cn* are expressed, eye color is white. This means that white eyed flies are homozygous for *cn* and thus also for *solo*. Wild type eye color indicates either heterozygous *solo* or homozygous wild type, and these flies were collected as a negative control.

Ovary dissection. Female ebony flies were collected and incubated with males for 4-5 days to ensure that they were not virgins. I then dissected the ovaries according to the protocol from Hawley's lab. I dissected the flies under a light microscope in 1X PBS solution. I left the ovaries in the PBS solution for up to a week in a normal refrigerator. These ovaries were then fixed with 200 uL fixative (the fixative is 100 uL 10X PBS, 5 uL nonidet P40, 770 uL H_2O, and 125 uL 16% formaldehyde) and 600 uL heptane with rocking for 20 minutes. They were then rinsed three times and washed three times in PBST. I tweezed the ovarioles away from each other to allow for more efficient binding of the antibodies. At this point, I left the fixed ovarioles in the refrigerator for up to a week.

Immunolocalization. The ovarioles were blocked in 1% BSA in PBST for one hour. I then incubated them for one hour with the primary antibodies, mouse α-c(3)g at 1:200 and rabbit α-γH2Av (Upstate) at 1:100. These were washed three times with PBST and

incubated for one hour with the secondary antibodies, Cy5-labeled α-rabbit and FITC-labeled α-mouse at 1:1000. I then washed the ovarioles in PBST with DAPI, tweezed each ovariole completely away from each other and mounted them on a slide with the ProLong Antifade kit (Invitrogen).

Microscopy. These slides were examined using a Zeiss microscope and the Metamorph imaging software. Sections were taken every 0.3 microns and stacked to create a 3-D representation of the foci.

Results

The ovaries collected from the homozygous *solo* mutants were noticeably different in size than those of wild-type or homozygous *spnB* mutants (see figure 3A). Often, they were so small that they were difficult to spot in dissection, and attempting to tweeze them apart in preparation for immunostaining and slide mounting was difficult and sometimes destroyed the ovarioles. The reason for the small size is probably due to the lack of development in the ovaries – none of the examined ovarioles progressed past stage 8 (see figure 3B).
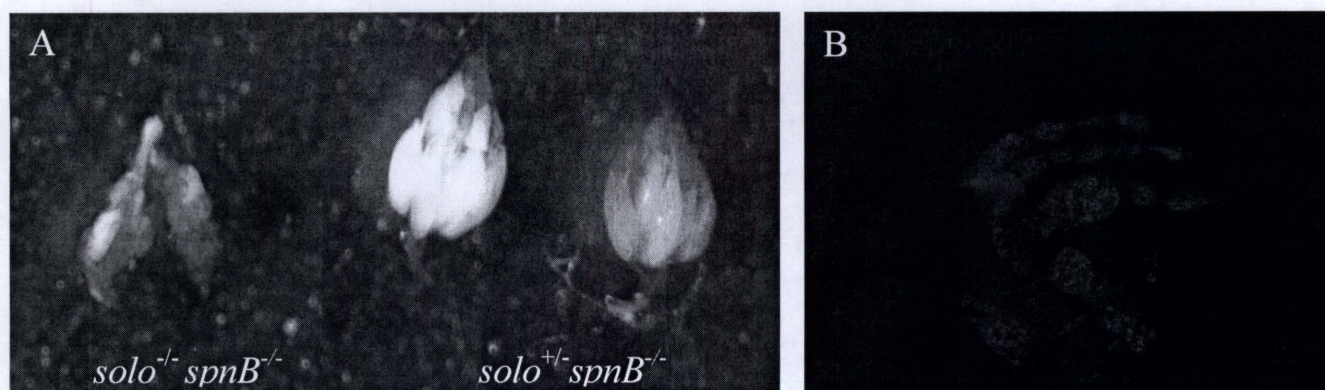

Figure 3. View of dissected ovaries. A, light microscope image of full ovaries. B, DAPI staining of individual ovarioles of *solo*$^{-/-}$ *spnB*$^{-/-}$. Notice lack of development.

Unfortunately, my immunostaining was not very successful. The γ-H2Av antibody did not work correctly and I could not observe any foci in any ovarioles. The *spnB* mutant was used as a control because DSBs should be abundant and visible for several stages, but these ovarioles did not show any γ-H2Av foci in my experiment. The c(3)g staining did work, however, and showed a marked difference in SC formation in the negative control flies and in *solo* mutants (see figure 4). Whereas c(3)g normally stains multiple cells in regions 2a and 2b and stains only the oocyte in region 3 through stage 6, I could only observe staining of one cell in region 2a in *solo* mutants, and then c(3)g dissolved.
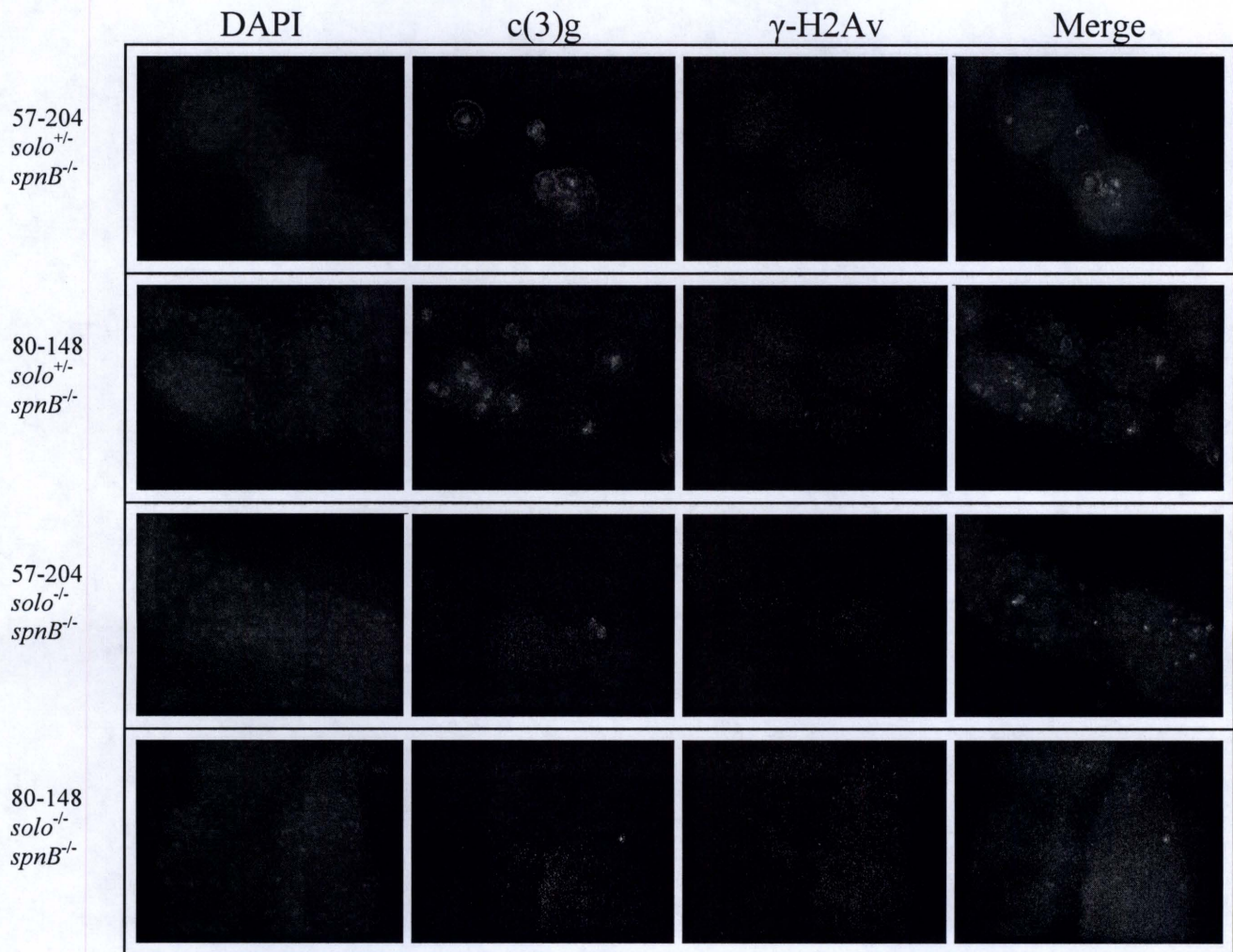

Figure 4. DAPI staining shows the DNA in the germarium, and c(3)g localizes to the SC in both pro-oocytes and oocytes. Here you can see that the SC is faulty in *solo* mutants,

appearing in only one cell in region 2a. γ-H2Av staining should show the DSBs, but hasn't worked in this experiment.

Discussion

There are several reasons why my γ-H2Av could have not worked in this experiment. Due to time constraints, I had to modify Hawley's original protocol and leave the ovaries in the refrigerator for long periods of time, extending a normal one-or two-day procedure to that of several weeks. This could have had a deleterious effect on the protein integrity within the ovarioles. There are also several other γ-H2Av antibodies available. The Upstate antibody had the added advantage that it was monoclonal and commercially available, but perhaps another antibody would work better on these ovarioles. I could also experiment with which secondary antibody to use, although preliminary tests showed that Cy5 gave the best results and the lowest background levels.

However, I can still make some conclusions based on the staining pattern of c(3)g, and speculation on what DSB pattern I might have seen will be educational and could still elucidate *solo* function in the future. As stated above, c(3)g only shows up in one cell during region 2a in *solo*$^{-/-}$, although in *spnB*$^{-/-}$ it shows a normal SC pattern (see figure 4). This leads me to believe that the SC only forms in one of the pro-oocytes for a brief period before it breaks down. Thus, c(3)g staining cannot be used to determine the oocyte in *solo*$^{-/-}$, because the oocyte has not been established by the germaria until region 3, at which point c(3)g staining is not visible. It might be helpful to use another antibody to the SC, such as c(2)m, to affirm that the SC is degraded shortly after its formation. Other methods of visualizing the oocyte can be used, such as an antibody to Orb, a protein which localizes to the oocyte shortly after the formation of the 16-cell cyst (Lantz et al, 1994).

Since evidence points to a role for sister chromatid cohesion in *solo*, investigation of other such proteins is warranted. Several have been identified, including *mei-S332* and *ord*. It is believed that *mei-S332* does not even appear until anaphase I, at which point it acts to keep sisters together. Thus, meiosis I can run smoothly in its absence and synapsis and crossing over are not affected (Kerrebrock et al, 1992). *Ord* plays a much earlier role, protecting sister cohesion from the beginning of prophase I. In *ord* mutants, the standard number of DSBs occur and the SC forms normally, but the SC degrades prematurely and DSBs are repaired off of the sister instead of the homologous chromosome, resulting in decreased recombination and high nondisjunction levels (Webber, 2004).

Even without an SC, DSBs can form, as shown in the c(3)g mutant by Jang et al (2003), although the quantity of γ-H2Av foci is severely reduced. Also, as stated above, the SC forms but deteriorates early in the absence of *ord*; however, DSBs are formed and repaired at the correct rate. Thus, if DSB foci can be observed in *solo* mutants, it could indicate that *solo* plays a similar role as *ord* and that sisters are being repaired off each other, which would explain the low rate of recombination.

However, the lack of a long-term SC could also be indicative of a failure of the chromosomes to synapse correctly and form chiasmata. This can be confirmed if we observe an absence of γ-H2Av foci in *solo* mutant germaria. Such an absence of c(3)g and γ-H2Av foci could implicate *solo* in a role either in sister chromatid cohesion or SC stabilization. It is likely that these two roles go hand in hand, and that SC cannot be maintained without sister chromatid cohesion, as has been found in *ord* mutants (Webber, 2004).

Without proper cohesion, synapsis cannot occur and crossovers do not occur, which means that meiosis I should be disrupted and the resulting nuclei should have high levels of nondisjunction. However, in observed *solo* spermatid, anaphase I appears normal and the nuclei are the same size. This is probably due to achiasmate segregation and the absence of disparate nuclei does not rule out sister chromatid cohesion as the role of *solo*. There are two types of achiasmate segregation, homologous and heterologous, which segregate chromosomes based on homology and size and shape, respectively. Homologous segregation is the mechanism used to segregate the tiny fourth chromosome in female *Drosophila*. Studies have also shown segregation of chiasma-deficient chromosomes, such as attached-X and attached-4th chromosomes, based on relative size and shape (Hawley and Therkauf, 1993).

My proposed model is that *solo* is important in the cohesion of sister chromatids during prophase I. Without it and the resulting cohesion, the synaptonemal complex deteriorates prematurely, DSBs do not occur, chiasma do not form and the normal segregation mechanism reliant on crossing over does not happen. However, chromosomes are allocated to separate spindles based on homology or based roughly on size and shape, so that the products of meiosis I look normal. In meiosis II, sisters segregate completely randomly, resulting in high nondisjunction in the oocytes. This model does not explain the small size and stunted development of the double mutant ovaries – a phenomenon that does not occur in either of the single mutants. This suggests that an interaction of some sort is occurring, and it merits further investigation.

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