The relationship of meiotic checkpoint regulation, synapsis, and crossing over in Drosophila melanogaster

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ABSTRACT OF THE THESIS

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Thesis Director:

Dr. Kim McKim

Proper chromosome segregation is achieved through three important meiotic events. The first is synapsis, where a proteinaceous structure, the synaptonemal complex (SC), forms between homologous chromosomes and juxtaposes them together. Then, recombination, which is initiated by programmed double strand breaks (DSBs) introduced into DNA that leads to a crossover event. Chiasmata, which are physical markers of where an exact exchange of genetic material occurred between homologous chromosomes during crossing over, are important to maintain the genetic integrity and variability of offspring. Zip3, a conserved meiotic protein found in budding yeast to humans, has been found to be required for crossing over. Furthermore, previous studies in budding yeast (Zip3) and *C. elegans* (ZHP-3) have shown the homologs to be crossover markers. However, the exact mechanism as to how Zip3 combines synapsis and recombination to promote crossing over is unknown.

In *Drosophila melanogaster*, there are two homologs of Zip3, Zip3 related protein on the third and X chromosome (Z3rp3 and Z3rpX). Because *Drosophila* currently does not have a way to visualize crossovers, this study involves determining the function of

Z3rp3 and Z3rpX during meiotic recombination and if they are crossover markers. HA-tagged transgenes were constructed to express the protein of each homolog. Z3rp3 HA and Z3rpX HA transgenes revealed an abnormal localization pattern, resulting in them not being good crossover markers. However, the transgenes displayed dominant negative effects on meiosis, indicating they had another important function on the process. Loss of function mutants created in *z3rp3* and *z3rpX* showed that they not only had separate functions on the events of meiosis leading to proper chromosome segregation, but also that they seemed to be redundant, since the *z3rpX z3rp3* double mutant exhibited the most severe phenotypes on synapsis, recombination, and crossing over. Overall, *z3rp* may be playing similar roles in the communication between SC formation and recombination events as in budding yeast and *C. elegans*, but in different contexts. Thus, I have results that provide new insights into the functional features of *z3rp* in *Drosophila* that confirms its role in crossing over.

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DEDICATION

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Meiosis: The key to life

Meiosis I is a specialized cell division in sexual reproduction that takes a diploid number of parental chromosomes and reduces them to haploid number (gametes) (Lynn et al., 2007). There are two rounds of meiotic cell division, each with four distinct stages, which occur to facilitate the proper segregation and establishment of correct chromosome number. Prior to meiotic cell division, maternal and paternal chromosomes are duplicated during DNA replication to create an exact copy of each version so they can become homologs (Lynn et al., 2007) (Figure 1). Each chromosome in the homologous pairs are a sister chromatid and are attached by a region of the chromosome called the centromere. In order for maternal and paternal chromosomes to combine and exchange genetic material, homologous chromosomes must pair the bivalents.

The first round of meiosis, known as the reductional division, starts with homologous chromosomes being paired to undergo meiotic recombination events so the exchange can take place (Figure 1). Then, crossovers are created, which guide spindle fibers to attach to centromeres of each homologous pair and arrange them at the center of the metaphase plate perpendicular to spindle poles (Lynn et al., 2007). Spindle fibers then pull the recombined homologs to the spindle poles, where the cell then divides into two daughter cells that have a haploid number of chromosomes and two chromatids. A second round of meiosis, equational division, then takes place and the two sister chromatids making up each homolog are separated and moved into four resulting gamete cells. Not only does meiosis ensure chromosomes segregate properly but it also results in genetic diversity in a variety of organisms. Thus, there is a variation in physical and behavior

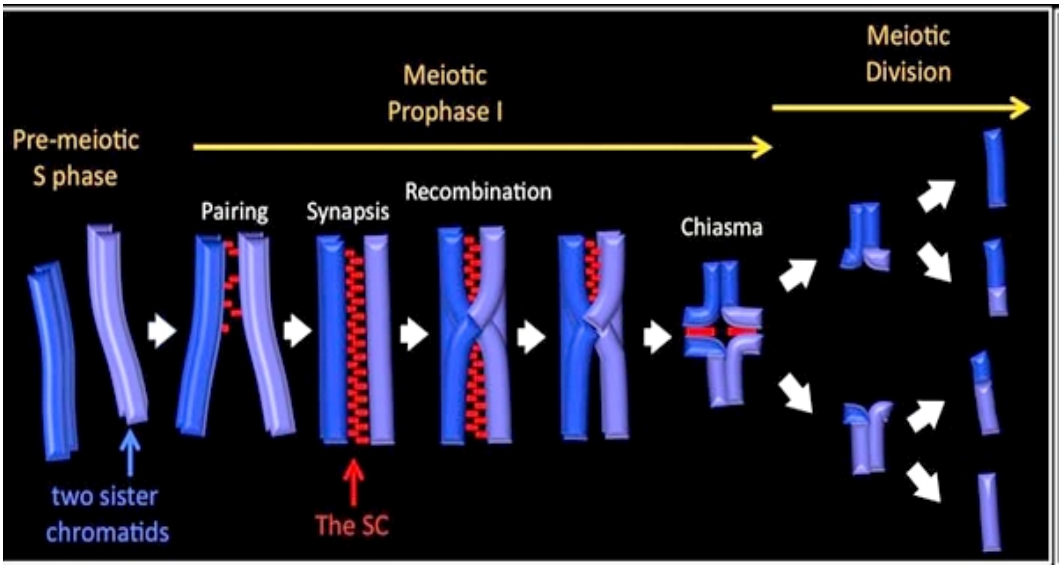

Figure 1: Overview of meiosis.

Before meiosis starts, there is replication of the cell's chromosomes, creating homologous pairs of chromosomes. Each chromosome in the pair is called a sister chromatid, which is an exact copy of its partner. Once diploid cells enter meiosis I (reduction), the homologous chromosomes pair via a proteinaceous structure, called the synaptonemal complex (SC). Double strand breaks (DSBs) form along the lengths of sister chromatids and have to be repaired. Since the homologous chromosome pairs are connected by the SC, they can exchange genetic material and perform meiotic recombination to create crossovers. Chiasma are the physical evidence crossovers occurred and they hold chromosomes stable until they are to separate in anaphase. The recombined homologs then separate into two daughter cells, each containing a haploid number of chromosomes. A second round of division occurs (division), where the two sister chromatids of each daughter cell are separated and move into one of four resulting gamete cells. Figure from http://www.biology.uiowa.edu/smolikove.

characteristics among species that make them very interesting to study in order to discover new roles and functions of genes. Most importantly, studying meiosis helps to understand the mechanisms behind many genetic disorders and cancer, which can lead to better treatments and possible cures.

One of the most important model organisms used to study meiosis is *Drosophila melanogaster*. *Drosophila* is an attractive model system, in which powerful tools in genetics and cytology can be used to identify and characterize the genes required for meiotic recombination (Mehrotra et al., 2007). This is possible due to previous studies in *Drosophila* establishing the first linkage maps and the discovery that crossing over was important for proper chromosome segregation from meiotic mutants and nondisjunction events (Baker et al., 2006; Bridges 1916; Cooper, 1948). These discoveries have led up to revealing genes that function in the meiotic recombination pathway involved with the pairing and segregation of homologous chromosomes. The remainder of this chapter will compare and contrast pairing (synapsis) and crossing over in *Drosophila* and other model organisms and the genes required for proper chromosome segregation. But most importantly, I will focus on my studies with *Drosophila's* unique system to study those genes involved with the mechanisms of meiotic recombination.

Synapsis: Bringing it all together

Chromosome pairing, SC formation, and recombination events occur during the prophase stage of meiosis. One of the two most important features of meiosis is synapsis. Synapsis is a process where homologous chromosomes are aligned and juxtaposed together via a proteinaceous and elaborate structure known as the synaptonemal complex (SC) (Lynn et al., 2007).The SC, which is needed for meiotic exchange, is comprised of two axial elements (C(2)M in *Drosophila*) that run along the sister chromatids, joined to a transverse or central element (C(3)G) (Agarwal & Roeder, 2000) (Figure 2). In other organisms, such as budding yeast, the homolog of C(3)G is Zip1. C(3)G and C(2)M staining appears simultaneously during prophase as thread like structures in the oocyte. The *c(2)M* gene was identified by Manheim and McKim (2003), where mutations in it reduce crossing over and disrupt SC structure by preventing the assembly of C(3)G protein along meiotic chromosomes. Bioinformatic analysis indicates that C(2)M is part of the α-kleisin superfamily of proteins that includes Rec8 (Schleiffer et al. 2003). Rec8 is a meiosis specific, Scc1-like protein that interacts with SMC proteins and provides sister-chromatid cohesion during meiosis in yeast (Jessberger 2002; Petronczki et al. 2003), but C(2)M is not involved in cohesion, since mutants show no defects in sister-chromatid cohesion (Manheim and McKim, 2003).

In *Drosophila*, when DSBs are lacking, there are no delays or defects in SC formation (McKim et al., 1998). Thus, the SC may be required for DSB formation or they are independent of each other. There are also other non SC-specific components, SMC proteins and ORD, which are involved in sister chromatid cohesion (Tanneti et al., 2011). ORD is also required for normal levels of crossing over. The SC forms in different stages

during prophase of meiosis. The first stage is leptotene, where chromosomes appear as long, thin threads. Then, in zygotene, synapsis occurs with homologous chromosomes pairing (bivalents) and the SC is seen in patches. Next during pachytene, there is a shortening and thickening of bivalents, creating thicker threads of SC, and synapsis is complete. The fourth stage, diplotene, occurs when the SC disappears and homologs begin to separate around the centromere. Also, the homologs now have chiasmata at specific crossover sites, indicating a recombination event occurred earlier when they were synapsed. There is at least one chiasma per chromosome arm. The last stage of prophase is diakinesis, where chromosomes are shortened and chiasmata disappear, moving to the ends of chromosomes.

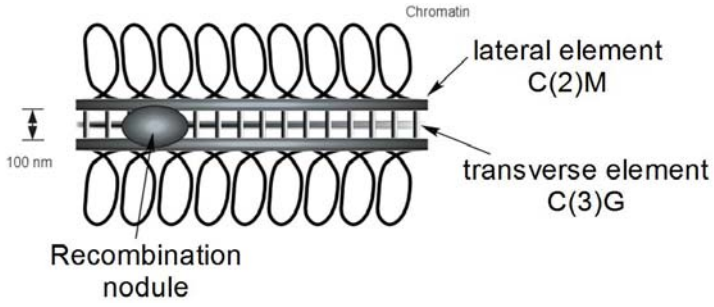

Figure 2: Schematic of synaptonemal complex (SC) structure.

The SC forms between homologous chromosomes during pachytene of meiosis (Page and Hawley, 2001). Components of the SC include C(2)M and ORD (not seen, lateral elements) and C(3)G (transverse or central element). Synapsis is eliminated in *c(3)G* mutant oocytes (Page and Hawley, 2001). Synapsis is defective due to axial structure defects in *c(2)M* and *ord* mutants (Anderson et al. 2005). Meiotic recombination probably occurs within an SC-associated complex called the recombination nodule (Agarwal and Roeder, 2000).

Recombination events are initiated by programmed double strand breaks (DSBs). Thus, the SC is a universal structure required in every organism in order for recombination and crossing over to occur (Roeder, 1997). DSBs also play a key role in meiosis, not only being the driving force for recombination, but also a requirement for proper SC formation, which is true in organisms such as budding yeast (Roeder, 2000).

However, in organisms such as *Drosophila* and *C. elegans*, the SC does not need the presence of DSBs to form (Jang et al., 2003). So it is important to study SC formation along with recombination in *Drosophila*, since this organism displays a different mechanism of how homologous chromosomes are joined together without requiring DSBs. However, the SC is a very important structure that is a conserved meiotic structure needed for crossing over in most organisms (Page and Hawley, 2001; Von Weittstein, 1984). The relationship between proteins involved in SC formation and recombination and their role in crossing over is still not understood. *Drosophila* provides insight into understanding meiosis better, with a well defined meiotic recombination pathway leading to crossovers that will reveal how the proteins involved in synapsis and recombination work together.

Meiotic recombination pathway: Repairing the mistakes, crossing over, and checkpoints

Once homologous chromosomes are fully synapsed in *Drosophila*, several programmed double strand breaks (DSBs) occur, which initiates meiotic recombination to repair the damage. The events of meiotic recombination occur in context of genes operating in four separate functional groups (Figure 3). The proteins in *Drosophila* that are required for meiotic DSB formation are MEI-W68 (Spo11 homolog) and MEI-P22 (unconserved protein) (McKim et al., 1998; Liu et al., 1996). MEI-W68 and MEI-P22 are required for initiation of meiotic recombination and all noncrossovers and crossovers (Liu et al., 1996; McKim et al., 1998). Then, DSB repair genes (Rad52 group) process two DSBs into recombination intermediates that can be resolved into either noncrossover

(gene conversion) or crossover products (Haber, 2000). If there is a mutation in any of the DSB repair genes, DSBs are not repaired.

There are two classes of genes (crossover specific genes) that are needed to make crossovers (Joyce and McKim 2009). Precondition genes are one class of genes responsible for determining which DSBs become crossovers and their distribution. It is unclear how precondition genes promote crossover formation, although there is evidence that it may be via a recombination intermediate known as the Holliday junction (Bhagat et al., 2004). The other class is called exchange genes, which produces crossovers. The exchange class is made up of four proteins: MEI-9, ERCC1, MUS312 and HDM (holdem) (Joyce et al., 2009). The HDM protein of the exchange class was the most recently found and encodes a RPA (Replication Protein-A) like protein (Joyce et al., 2009). RPA is an essential, heterotrimeric protein involved in numerous DNA metabolic pathways including replication, repair, and recombination (Anciano-Granadillo et al., 2010). It binds single-stranded DNA (ssDNA) during homologous recombination through interactions with a series of oligonucleotide/oligosaccharide binding-fold (OB-folds) that display a high affinity for ssDNA to prevent it from winding back on itself so DNA repair and prophase can occur properly (Bochkarev and Bochkareva, 2004; Joyce and McKim, 2009). Mutants that occur within crossover specific genes only affect those proteins that are responsible for DSB repair and making products into crossovers, since they have a high accumulation of noncrossovers. When crossovers are the final products, they have a role in chromosome segregation. During the fourth stage of prophase (diplotene), homologous chromosomes start to separate (at a region surrounding centromere) and the crossovers become chiasmata. The appearance of chiasmata (at least one per chromosome

arm) is proof that recombination occurred when the chromosomes were synapsed and they control holding the homologs stable until they are to segregate on the spindle during anaphase of meiosis I. Mutations in *mei-9*, *Ercc1*, *mus312*, and *hdm* reduce crossing over uniformly along the chromosomes, leaving the nonrandom distribution of crossovers observed in wild type intact (Joyce et al., 2009). MEI-9 is the *Drosophila* homolog of human and yeast nucleotide excision repair (NER) proteins and Rad1p, which contain a highly conserved structure-specific endonuclease domain (Sekelsky et al., 1995; Sijbers et al., 1996). *mei-9* is required for 90% of all meiotic crossovers as well as some types of somatic DNA repair such as NER (Boyd et al., 1976). Thus, according to this data along with the exchange genes being found to interact together via yeast two-hybrid assay, they have been proposed to be directly involved as a complex with endonuclease activity in the reaction that generates crossovers (Joyce et al., 2009).

Also in the meiotic recombination pathway, there are two checkpoint pathways that are activated to monitor delays with different meiotic events. One of the pathways is the ATR/MEI-41-dependent DSB repair checkpoint, which is present when there is a defect in repairing DSBs in *Drosophila* females (Joyce et al., 2011). As a result, there are developmental defects, such as problems with the oocyte establishing dorsal-ventral polarity. Furthermore, when there is a mutation in *mei-41*, there is a reduction in crossing over, indicating it may have a more direct role in DSB repair. A second more recently discovered checkpoint in *Drosophila* females show delays in the chromatin-remodeling response to DSBs and oocyte selection when there are mutations in any of the DSB repair or exchange genes (Joyce and McKim, 2009). This is classified as a meiotic prophase checkpoint since these phenotypes cause delay in pachytene progression. The difference

in the pachytene checkpoint compared to the DSB repair checkpoint is that the delays are not due to DSB formation but requires precondition genes (*mei-218* and *rec*) in order to operate (Joyce and McKim, 2009). Thus, this proves the pachytene checkpoint functions separately from the others and the driving force behind the delays are associated with crossing over. In order for the pachytene checkpoint to function, it requires PCH2, an AAA-adenosine triphosphatase (Joyce and McKim, 2009). Other species such as *Saccharomyces cerevisiae* and *C. elegans* show that PCH2 dependent checkpoints sense synapsis defects that are independent of DSBs (Joyce and McKim, 2009). But in *Drosophila*, the PCH2 dependent delays show no obvious problems with synapsis but there is a defect along the pathway that shows defects in crossover formation as the cause of activating the checkpoint.

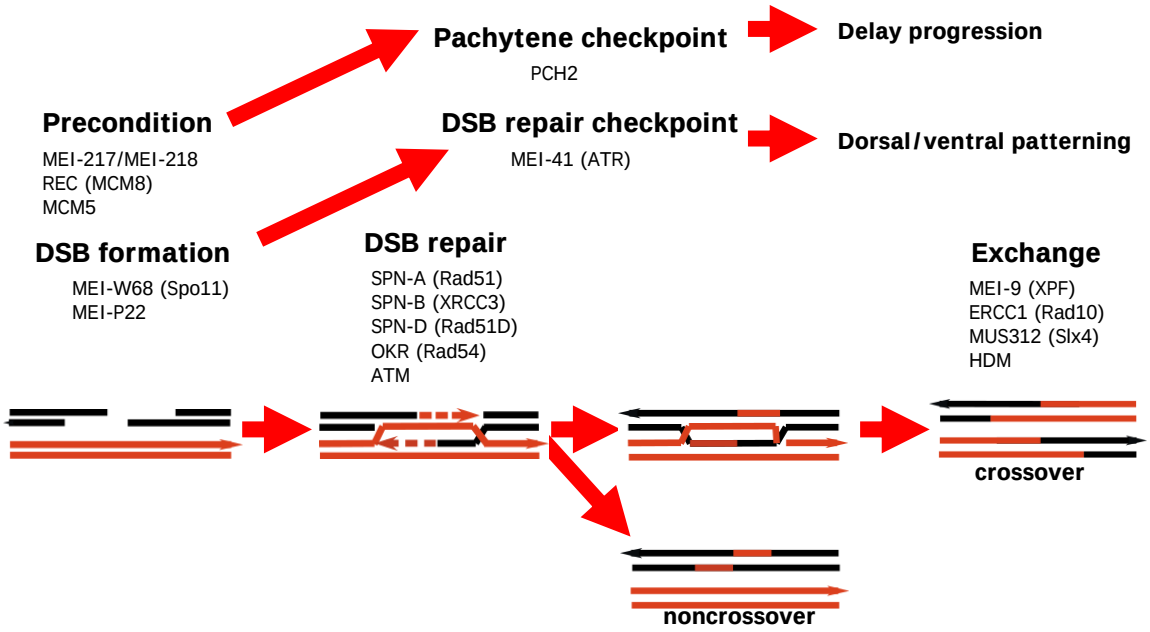

Figure 3: Meiotic recombination pathway.

Genes required for DSB formation, repair, and crossing over in Drosophila that result in crossover or noncrossover products. Also shown are the two checkpoints which regulate this pathway. In parentheses are shown the common names of homologs.

The germarium: Following meiosis in *Drosophila melanogaster* females

Cytologically, the events of prophase can be observed in context of oocyte development within the germarium of the ovary in the *Drosophila* female (Figure 4) (Mehrotra et al., 2007). *Drosophila* females have a pair of ovaries and each is comprised of several tubes (ovarioles) of developing oocytes, at the anterior tip of each ovariole is the germarium (Mehrotra et al., 2007; Mehrotra and McKim, 2006). A stem cell in the germarium generates a cystoblast which undergoes four successive incomplete mitotic divisions to form a 16-cell specialized cyst connected by ring canals, which are cytoplasmic bridge structures where intercellular material can pass from the nurse cells to the oocyte of the developing *Drosophila* embryo (Robinson et al., 1994). After this, prophase and recombination are initiated (Mehrotra et al., 2007). The 16-cell cyst moves toward the posterior end of the germarium, where two pro-oocytes (cells with potential to develop into a mature oocyte) enter pachytene. Eventually, only one of the two proocytes will be selected to become the oocyte and the remaining 15 cells begin to develop to nurse cells (Mehrotra et al., 2007; Mehrotra & McKim, 2006).

The germarium is divided into four stages based on morphology, where there is the16-cell cyst that develops during mitosis (region 1) and three pachytene stages that occur during prophase divided into three regions (2a, 2b, and 3) (Mehrotra et al., 2007) (Figure 4). Throughout regions 2a and 2b, most of the oocytes are in pachytene, with the SC assembled between homologs along their entire lengths. Additionally, structures called recombination nodules (RNs) are seen during this time. The formation of RNs depend on the Spo11 homolog (an enzyme involved in creating DSBs in DNA), MEI-W68, and late RNs may mark the sites of crossing over (Carpenter, 2003; McKim et al.,

1998). By region 3, oocyte determination has been completed since a single oocyte is usually evident and positioned toward the posterior of the cyst (Mehrotra et al., 2007). The absolute position of developmental ages in the germarium does not necessarily equate to their specific meiotic stages, but several successive stages of meiotic recombination and oocyte development can be observed in temporal order (Mehrotra et al., 2007). The germarium allows for studying chromosomal, recombination, and checkpoint proteins to determine how they are interacting together during prophase of meiosis to promote crossing over. Additionally, studying these processes will reveal and classify new genes in the meiotic recombination pathway to better understand the exact mechanisms of how proper chromosome segregation is achieved for the viability and genetic integrity of an organism.

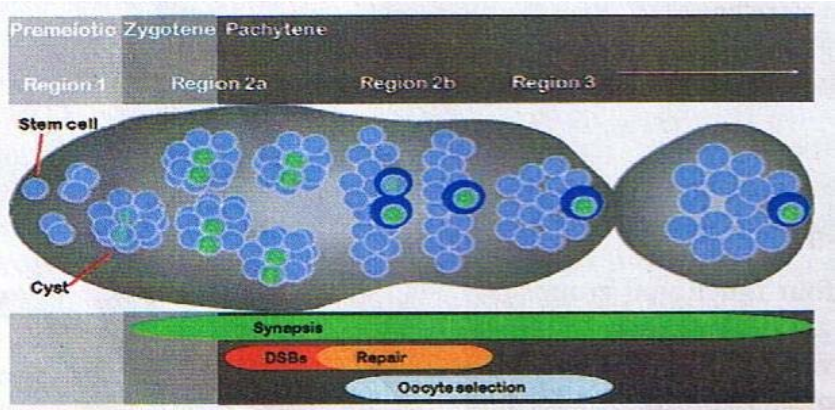

Figure 4: Schematic of the *Drosophila* female germarium: Oocyte development and DSB repair.

Before meiosis, stem cells undergo premeiotic or mitotic (Region 1) divisions in the anterior top end of the germarium to form the 16-cell cyst (14 nurse cells and two pro-ooctyes) (Mehrotra and McKim, 2006). In region 2a, the 16-cell cyst enters meiosis, where the two-proocytes (stained in green with C(3)G) are both potential candidates to become the single mature oocyte. The 16-cell cyst moves rapidly through zygotene, where C(3)G starts to form in the pro-oocytes. Once in early pachytene, C(3)G is fully formed in a thread-like pattern in the pro-oocytes and a accumulation of DSBs are present. The remaining 14 nurse cells stain weakly for SC markers but do experience DSBs. In region 2b, one of the two pro-oocytes transitions to become a nurse cell, where there are few if any DSBs seen, since they are repaired. Then, by late pachytene in region 3, one oocyte is produced along with 15 nurse cells and is defined by the absence of DSBs. The oocytes can be identified by chromosome proteins like C(3)G (green) or cytoplasmic proteins like ORB (blue). The different events are shown color-coded under the germarium that allow for normal progression of pachytene.

CHAPTER 2: An analysis of Zip3 related protein as a requirement for crossing over in *Drosophila melanogaster*

I. Summary

During meiosis, crossing over is essential for the faithful segregation of homologous chromosomes. Zip3 is a meiotic recombination protein that is found to be conserved among organisms ranging from *Saccharomyces cerevisiae* to humans. In budding yeast, Zip3 is needed for proper synaptonemal complex (SC) formation and promoting crossover formation. Additionally, ZHP-3, the *C. elegans* homolog of Zip3, is not required for wild-type SC formation but it does not localize in the absence of the SC. Overall, ZHP-3 is needed for reciprocal recombination and chiasmata formation, thus being a marker for crossovers. However, it is still unknown exactly how Zip3 functions in other multicellular organisms. We report here the role of two Zip3 homologs, *z3rp3* and *z3rpX* (Zip3 related protein on the third and X chromosome), in the *Drosophila melanogaster* meiotic recombination pathway. First Z3rp3- HA and Z3rpX-HA tagged transgenes that were overexpressed resulted in an abnormal localization pattern that was not sufficient enough to represent crossovers. Furthermore, the transgenes exhibited dominant negative phenotypes that affected meiotic events, indicating *z3rp* is an important gene needed during meiosis. A null mutation was made in $z3rp3^{\Delta}$ using FLP-FRT recombination and resulted in showing no chromosome segregation defects and normal levels of crossing over. But $z3rp3^{\Delta}$ was found to activate the pachytene checkpoint (indicating a defect in oocyte selection, crossover formation and axis components) and a SC defect where there was C(3)G (synapsis marker) found in more cells and absent C(2)M formation seen in mid and late pachytene. *P{z3rpX shRNA}attP2*

(transgenic shRNAi (short hairpin RNAi in *z3rpx*) mutants resulted in showing the same phenotypes as $z3rp3^{\Delta}$ except there was a slight chromosome segregation defect and decrease in crossing over. However, when a $P\{z3rpX\ shRNA\}attP2\ z3rp3^{\Delta}$ double mutant was made, there was a severe defect in chromosome segregation and in crossing over, indicating the two homologs are acting redundantly. We propose that *z3rp3* and *z3rpX* are redundant genes that also have different functions in meiosis and are essential for crossing over and possibly for monitoring levels of SC in *Drosophila melanogaster* females.

II. Introduction

During meiosis, it is very important for chromosomes to segregate properly to maintain the genomic integrity of an organism. Two key processes must occur during prophase of meiosis to ensure accurate chromosome segregation: synapsis and formation of crossovers. Once homologous chromosomes align, synapsis is initiated, pairing homologs together by a proteinaceous structure called the synaptonemal complex (SC), which is composed of lateral elements(C(2)M and ORD in flies, Red1 in yeast) connected to a central element (C(3)G in flies, Zip1 in yeast) (Lynn et al., 2007). Then, meiotic recombination events occur in response to programmed DNA double strand breaks (DSBs), induced by Spo11p (MEI-W68 in flies) (McKim et al., 1998). To repair the DSBs, crossovers are formed and mature into chiasmata, which direct the segregation of the homologous chromosomes at anaphase I (Lynn et al., 2007).

There are many proteins that promote crossing over, which includes Zip3. Zip3, a conserved meiosis-specific protein, contains a RING finger domain, which is indicative of ubiquitin and SUMO E3 ligase activity (Watts and Hoffmann, 2011). Thus, the RING

finger domain may be essential for Zip3 function, promoting post-translational modification of recombination and chromosomal proteins in crossing over. Zip3 has been shown in budding yeast to promote the assembly of the SC at DSBs, presumed to be crossover sites and is required for crossover formation (Agarwal & Roeder, 2000; Macqueen & Roeder, 2009). *ZIP3* mutants in budding yeast have a significant reduction in crossovers (Agarwal & Roeder, 2000). In addition, there are numerous DSBs that accumulate and not repaired in *ZIP3* mutants, proving recombination is not progressing normally. Thus, the model in budding yeast proposes that Zip3 marks crossover sites and recruits Zip2 (synapsis initiation sites) and Zip1 (the major central element component of the SC) (Agarwal & Roeder, 2000). Zip3 belongs to a group called the ZMM or SIC (synaptonemal initiation complex) proteins, which are evolutionary conserved and first identified in budding yeast, found to be involved in coordinating SC formation and meiotic recombination events (Lynn et al., 2007).This group of proteins include seven functionally collaborating and structurally diverse proteins: Zip1, Zip2, Zip3, Zip4, Mer3, Msh4, and Msh5 (ZMM). Mer3, Msh4, and Msh5 are involved in mitotic DNA metabolism and promote steps in DNA recombination (Lynn et al., 2007). These proteins are thought to interact together because they colocalize along the meiotic chromosomes and have similar mutant phenotypes. The SIC proteins have been found to play a very significant role in crossover formation, since their absence results in reduced or nonexistent crossover sites, even if recombination is initiated (Agarwal & Roeder, 2000; Lynn et al., 2007).

In contrast, initiation of recombination is not required for SC formation in the *C. elegans zhp-3* mutant because timing of appearance and localization of Rad-51

(recombination protein) is normal (Jantsch et al., 2004). However, GFP-tagged ZHP-3 localization showed it requires the SC for localization and is found between synapsed chromosomes (Bhalla et al., 2008). ZHP-3 is asynchronous in that it localizes along the SC in early pachytene, asymmetrically on the SC in late pachytene, and at foci in late pachytene/diplotene (Bhalla et al., 2008). There are 6 ZHP-3 foci per nucleus in wild-type hermaphrodites and mark the boundary of asymmetric SC disassembly during diplotene/diakinesis. Homozygous *zhp-3* knockout worms showed normal homolog pairing and SC formation. Also, ZHP-3 is essential for crossing over and chiasma formation, since *zhp-3* mutants have a frequent occurrence of univalents and achiasmatic chromosomes, indicating reciprocal recombination is severely decreased (Jantsch, et al., 2004; Bhalla et al., 2008). Overall, studies show that ZHP-3 in *C. elegans* has two different roles during meiosis, promoting crossover formation and mediating the appropriate restructuring of bivalents so that chiasmata ensure proper chromosome segregation (Bhalla et al., 2008). The latter role indicates that it is separate from promoting crossover formation and conserved for *C. elegans,* where in contrast, budding yeast Zip3 couples crossover recombination with SC assembly.

This study involves the first characterization of two Z3rp (Zip3 related protein) homologs, *z3rp3* and *z3rpX*, in *Drosophila melanogaster* during prophase of meiosis and determining if they are crossover markers. The work presented here shows evidence that *z3rp* is needed for promoting crossing over in *Drosophila* females. Z3pr3-HA and Z3rpX-HA tagged transgenes were made to observe if the homologs were crossover markers. During early pachytene of meiosis, both transgenes exhibited an abnormal localization pattern when overexpressed with the germline driver *P(Gal4::VP16-*

*nos.UTR) MVD1.*Thus, dominant negative phenotypes (high levels of aneuploidy, decreased crossing over, and activation of pachytene checkpoint) were observed in overexpressed Z3rp3-HA transgenes but aneuploidy decreased when expressed with a moderate driver, indicating too much of the Z3rp protein causes meiotic defects and therefore has an important role during meiosis. Z3rp3 HA foci were observed to not directly colocalize with recombination associated markers, confirming further with the abnormal localization and dominant effects that the Z3rp3 HA-tagged protein is not functioning properly and not a good crossover marker. But the meiotic defects were explored further to reveal other functions of *z3rp3* and *z3rpX* during meiosis.

Loss of function mutants were made in both homologs, utilizing FLP-FRT recombination to make a null $z3rp3^{\Delta}$ mutant and transgenic shRNAi (short hairpin RNAi) to generate a *z3rpX* knockdown mutant. Individually, there were some defects seen in SC assembly, chromosome segregation, DSB repair, pachytene checkpoint activation and crossing over in both mutants, but most were mild phenotypes. However, based on *z3rpX z3rp3* double mutant analysis, there was a reduction in crossing over, increased levels of aneuploidy, the presence of C(3)G in more cells, high accumulation and persistence of DSBs and the presence of the pachytene checkpoint observed.

Overall, *z3rp* seems to be playing similar roles in the communication between SC formation and recombination events as in budding yeast and *C. elegans*, but in different contexts. Like *C. elegans*, SC formation is independent of DSBs in *Drosophila.* Thus, *z3rp* in *Drosophila* may be acting in an asynchronous fashion, promoting the ubiquitinylation of SC, chromosomal, and recombination proteins at different time points. Based on the *z3rpX z3rp3* double mutant activating the pachytene checkpoint and

decrease in crossing over, *z3rp* may have a role in the meiotic recombination pathway to control synapsis levels and promote crossing over, functioning as a central protein of all the processes needed for proper chromosome segregation. This work reveals new structural and functional features of *z3rp* based on the powerful genetic and cytological analysis of *Drosophila* female meiosis and provides the foundation for determining the exact mechanism of how *z3rp* promotes crossing over. Most importantly, this work displays how *z3rp* may mark chiasmata in *Drosophila.*

III. Materials and Methods

Fly Stocks: *Drosophila* stocks and crosses were maintained on standard medium at 25^{o}C. *PBac{WH}CG5508^{f01088}* and *PBac{WH}CG5508^{f04927}*(PiggyBacs are transposons) FRT sites of the z3rp3(CG31053) gene were identified in flybase.org (Drysdale et al. 2005) on the right third chromosome arm at 98B6, upstream and downstream of the gene, respectively. The *PBac* stocks previously mentioned were obtained from Exelixis Harvard Medical School and Bloomington *Drosophila* Stock Center, respectively. The deficiency stock (*Df(3R)ED6280/TM6C*) deletes cytological band 98B6. The following mutant stocks were utilized: *PBac{WH}CG5508^{f01088} -PBac{WH}CG5508^{f04927}*/TM6C (*z3rp3$^{\Delta}$*), *z3rp3$^{\Delta}$ pch2^{EY01788a}* (*pch2*$^{\text{EY01788a}}$ allele from Joyce and McKim, 2008), *yv;P{z3rpX shRNA}attP2, y+ /TM3* and *P{z3rpX shRNA}attP2, y+ z3rp3$^{\Delta}$/TM3*.

Generation of *z3rpX* RNAi: A mutant of *z3rpX* was generated using RNAi knockdown in the *Drosophila* germline, utilizing the construction of short hairpin microRNAs (shmiRNA). The "Cloning hairpins into pVALIUM20 and pVALIUM22" protocol developed by the Transgenic RNAi Project (TRIP) at the Harvard Medical School was used (written by Jian-Quan Ni and Norbert Perrimon). First, the transcript sequence of

z3rpX was obtained from FlyBase and put in the FASTA format. The sequence was then placed into the DSIR (Designer of Small Interfering RNA) website (http://biodev.extra.cea.fr/DSIR/DSIR.html) to select 21 nucleotide siRNAs and run the analysis. The results were sorted based on the highest corrected score. The single stranded sequences that were selected were checked to make sure there was no more than 15 nucleotide complimentary elsewhere in the *Drosophila* genome by using BLAST at FLYBase. Next, 71 nucleotide top and bottom strand oligos were designed so each contained the chosen single stranded sequence and reverse complement to make a *z3rpX* hairpin. The top and bottom oligos were then annealed by adding them both to annealing buffer (10mM Tris-HCl, pH 7.5, 0.1M NaCl, 1mM EDTA). The mix was then incubated at 95ºC for 5 min, then slowly cooled down to room temperature, using a program in the thermocycler. The resulting DNA fragment had overhangs for *NheI* and *EcoRI*. The DNA fragment was directly cloned into a pVALIUM22 (Vermilion-AttB-Loxp-Intron-UAS-MCS) vector, which was linearized by *NheI* and *EcoRI.* The ligation was incubated for 1 hour at 16^{o} C in the thermocycler. The ligation mixture was then added to competent cells, following standard transformation protocol. The competent cells were plated on ampicillin plates (pVALIUM22 has ampicillin resistance gene) and put in the 37^{o} C incubator overnight for 18 hours. Colonies were selected from the plates and mini prepped. The possible clones were checked via PCR using forward and reverse pVALIUM22 specific primers, where the correct clones produced a 350 bp band. The correct *z3rpX* shRNA construct was confirmed by sequencing, using a pVALIUM22 specific primer. The final plasmid, pVALIUM22 *z3rpX* shRNA, was sent off to Model System Genomics at Duke University for injection into an *yv; attP2* stock. After

receiving injected flies, they were crossed to *yv* flies in order to identify *vermillion*$^{+}$ transformants (RNAi is present). Once single *vermillion*$^{+}$ males (*yv*$^{-}$*/Y; P{z3rpX shRNA}attP2, v*$^{+}$ *y*$^{+}$ were identified, they were crossed to *yv/yv; Dr/TM3* females. Then *yv/yv; P{z3rpx shRNA}attP2, v*$^{+}$ *y*$^{+}$ */TM3* males and females were crossed together to make a stock. Thus, flies from this stock, *yv/yv; P{z3rpx shRNA}attP2, v*$^{+}$ *y*$^{+}$ */TM3,* were crossed to *P(Gal4::VP16-nos.UTR) MVD1* to express the RNAi.

Genetic Techniques: To generate a transposon-induced mutation of the *z3rp3* gene, the FLP-FRT recombination method (Golic, 1991) was used. Heat activated FLP (Flipase) acted upon FRTs (Flipase recombinase targets, *PBacs* mentioned above), stimulating recombination and causing the FRT sites to combine and delete out the *z3rp3* gene.

In the first cross, males from the *PBac{WH}CG5508*^{f01088} FRT stock were crossed to *ywhsFLP; Dr/TM3, Sb* females. Then, *ywhsFLP/Y; PBac{WH}CG5508*^{f01088} */TM3, Sb* males were picked , with the FLP (X chromosome) and FRT (third chromosome), and crossed back to females from the same FLP stock in the first cross in order to make the X chromosome homozygous for FLP. This is done so males instead of females can be chosen for the *z3rp3* deletion, since the heat shock affects the females' fertility and they undergo recombination. Next, *ywhsFLP/ywhsFLP; PBac{WH}CG5508*^{f01088} */TM3, Sb* females homozygous for FLP (X chromosome) and with the first FRT (third chromosome) were crossed to males from the other FRT stock, *PBac{WH}CG5508*^{f04927}*/TM6B, Tb*. This cross was done in vials that were transferred to fresh vials every two days and the larvae were heat shocked on day three or four for one hour to activate the FLP, which causes recombination between the FRT sites and have them replace the *z3rp3* gene. From the heat shock cross, *ywhsFLP/Y;*

PBac{WH}CG5508^{f01088}-PBac{WH}CG5508^{f04927} males were chosen with both FRTs present on the third chromosome, which were possible recombinants with *z3rp3* deletions. These males were crossed to *yw; Dr/TM3*, Sb females, to balance the third chromosome to prevent recombination. Then, *yw/Y; PBac{WH}CG5508^{f01088}-PBac{WH}*CG5508f04927/*Dr* single males were chosen with possible *z3rp3* deletions and crossed to females from *z3rp3* deficiency stock, *Df(3R)ED6280/TM6C.* From this cross, *PBac{WH}CG5508^{f01088}-PBac{WH}*CG5508f04927/ *Df(3R)ED6280* (females with the *z3rp3* deletion over the deficiency) were chosen to test homozygous lethality. Since the *z3rp3* deletion survived over the deficiency, their total genomic DNA was used for PCR. Gene specific primers designed in the deleted region were used to determine if there was a *z3rp3* deletion. Additionally, a balanced stock was made, where the same males and females were chosen from the last mentioned cross, resulting in *PBac{WH}CG5508^{f01088}-PBac{WH}CG5508f04927/ TM6C (z3rp3$^{\Delta}$/TM6C),* which will be referred to as *z3rp3$^{\Delta}$*.

Third chromosome nondisjunction was assayed by crossing females to *yw/Y^{BS}* males. The frequency of third-chromosome nondisjunction is calculated as 2 (Bar females + Bar^{+} males)/[2(Bar females + Bar^{+} males]. Second chromosome crossing over was assayed in *yv/Y*; P{*z3rpX shRNA}attP2, y+/TM3* and *+/Y*; P{*z3rpX shRNA}attP2 z3rp3$^{\Delta}$/TM3* mutants by crossing males from both genotypes to *y; al dp b Sp pr cn bw/CyO* females. *y/Y; al dp b Sp pr cn bw; P{z3rpX shRNA}attP2*, *y+* and *+/Y; al dp b Sp pr cn bw; P{z3rpX shRN}attP2 e z3rp3$^{\Delta}$* males were picked and crossed to *yw; P(Gal4::VP16-nos.UTR) MVD1 and yw; P(Gal4::VP16-nos.UTR) MVD1e z3rp3$^{\Delta}$*, respectively to make sure the P{*z3rpX shRNA}attP2* was expressed on the third

chromosome and to distinguish flies that are *y (yw/y; al dp b Sp pr cn bw; P(Gal4::VP16-nos.UTR) MVD1,* which are flies that served as the control and crossed to *al dp b pr cn cpx sp/CyO and scored for Cy+ and Sp)* or *y+*, indicating the flies that had the driver and did not. Then *yw/y; al dp b Sp pr cn bw; P{z3rpX shRNA}attP2, y+/ P(Gal4::VP16-nos.UTR) MVD1* and *yw/y; al dp b Sp pr cn bw; P{z3rpX shRNA}attP2, y+ e z3rp3$^{\Delta}$/ P(Gal4::VP16-nos.UTR) MVD1* females were crossed to *al dp b pr cn cpx sp/CyO* males to score crossing over in recombinants among Cy^{+} progeny. Third chromosome crossing over was assayed in *z3rp3$^{\Delta}$* mutants by crossing females from *z3rp3$^{\Delta}$* recombinant stock *th st cu sr e z3rp3$^{\Delta}$/TM3* back to males from the original *z3rp3$^{\Delta}$/TM6C* mutant stock to make *z3rp3$^{\Delta}$* homozygous on the third chromosome. Then *th st cu sr e z3rp3$^{\Delta}$/z3rp3$^{\Delta}$* females were picked and crossed to *ru th st cu sr e Pr ca/TM6B, Bsb Tb* males in order to score third crossing over among the *Pr* progeny males. Third chromosome crossing over was assayed in the *P{Z3rp3 HA}93* transgene on the second chromosome. *P{Z3rp3 HA}93/+; P(Gal4::VP16-nos.UTR) MVD1 /ru th st cu sr e Pr ca* females were crossed to *ru h th st cu e ca/TM6B, Tb* males in order to score the crossing over among the Pr progeny. As a control, crossing over on the third chromosome was scored with *P{Z3rp3 HA}93* on the second chromosome the same as described previously except *P{Z3rp3 HA}93/+; + /ru th st cu sr e Pr ca* females were used without the MVD1 driver.

Counting two oocytes and calculating *P*-values: The oocytes were observed using an anti-C(3)G antibody. A cell was scored as an oocyte if complete SC filaments were clear and distinct. *P*-values were calculated using the Fisher's exact test. The *P*-value from the test compares the ratio of one-oocyte to two-oocyte cysts that were observed in two genotypes.

Counting of γ-HIS2AV foci: γ-HIS2AV foci were counted from germaria, where the foci were clear and distinct in pro-oocytes stained with C(3)G antibody. Foci numbers in wild type were at a maximum in region 2a (early pachytene). Fewer foci were visible by region 2b (mid pachytene). In region 3 (late pachytene), all foci were absent.

To compare foci numbers in different genotypes, I used a method that includes all cysts with γ-HIS2AV foci, averaging the number in each pair of pro-oocytes. I compared the average number of foci in all the pro-oocytes or oocytes of each germarium, starting with the youngest cysts at the anterior end, by examining a full series of optical sections. The foci were counted manually by examining each section in a full series of optical sections containing a complete pro-oocyte nucleus.

Plotting γ-HIS2AV as a function of relative cyst age: Since the position of a cyst in the germarium is only a rough estimate of its meiotic stage, the foci were first counted in all the pro-oocytes/oocytes (identified by C(3)G staining) in the germarium. The meiotic stage of each pro-oocyte was then normalized according to the relative position of the entire cyst within the germarium since the relative position is more important than absolute position. The pro-oocytes from 6 wild-type germaria, 10 z3r*p3*$^{\Delta}$, 7 *P{z3rpX shRNA}attP2,* 8 P{*z3rpx shRNA}attP2 z3rp3*$^{\Delta}$*,* and 6 *P{Z3rp3 HA}93*, and 5 *P{Z3rpX HA}98* were arranged according to their relative age. The average number of γ-HIS2AV foci per pro-oocyte at each stage was then calculated and plotted as a function of relative cyst age.

Cytology and Immunofluorescence: For immunolocalization experiments, females were yeasted and aged at room temperature for 16 hours. Ovaries were then dissected and fixed using the "Buffer A" protocol (Belmont et al. 1989, McKim et al. 2008). The

antibody to γ-HIS2AV was described by Mehrotra et al. (2006) and used at a 1:500 dilution. Additional primary antibodies included mouse anti-C(3)G antibody used at 1:500 (Page and Hawley, 2001), rabbit anti-C(2)M antibody used at 1:400 (Mannheim et al. 2003), rat anti-HA antibody used at 1:15, rabbit anti-GFP used at 1:200, rabbit anti-CID used at 1:400, and rabbit anti-H3K9 trimethylation used at 1:500 .

The secondary antibodies were Cy3 labeled goat anti-rabbit (Jackson labs) used at 1:165, Alexa fluor 488 goat anti-mouse (Invitrogen) used at 1:100, and Cy3 labeled goat anti-rat (Jackson labs) used at 1:100, and Alexa fluor 488 goat anti-rabbit (Invitrogen) used at 1:200. Chromosomes were stained with Hoechst at 1:50,000 (10mg/ml solution) for seven minutes at room temperature. Images were collected using a Leica TCS SP2 confocal microscope with a 63X, N.A. 1.3 lens. In most cases, whole germaria were imaged by collecting optical sections through the entire tissue. These data sets are shown as maximum projections. The analysis of the images, however, was performed by examining one section at a time.

Construction of *P{Z3rp3 HA}* and *P{Z3rpX HA}* transgenes: The annotated coding region of *z3rp3* and *z3rpX* was obtained from Flybase and amplified off the cDNA clones IP08077 and LD30246 by PCR, respectively. The coding region of *z3rp3* and *z3rpX* was then cloned into the Gateway® pENTR™4 vector (Invitrogen). A LR 'clonase' reaction was then performed to recombine *z3rp3* and *z3rpX* into the ppHW destination vector (Invitrogen) that contains 3 copies of an N-terminus HA-tag under the control of an inducible UASP promoter that is GAL4 driven and for female germline expression. The construct was injected into fly embryos by Model System Genomics at Duke University. To express the transgenic lines, they were crossed to flies expressing Gal4 using either

the *P(Gal4-nos.NGT)40) NGT* (Tracey et al. 2000) or *P(Gal4::VP16-nos.UTR) MVD1* (Van Doren et al. 1998) drivers, which result in presumed moderate expression and overexpression in the germline and vitellarium, respectively. The drivers will be abbreviated as NGT and MVD1, respectively.

Counting of z3rp foci: Z3rp3 HA foci were counted in germaria where they were clear and distinct. The average foci was counted in pro-oocytes and oocytes, only with C(3)G staining in regions 2a, 2b, and 3 of each germarium by examining a full series of optical sections.

Western blot for detecting protein expression: Ovaries were dissected in 1X PBS from *Drosophila* females of the genotypes: *P{Z3rp3 HA}/ P(Gal4::VP16-nos.UTR)MVD1*, *P{Z3rpX HA}/ P(Gal4::VP16-nos.UTR) MVD1)* and *P{Z3rpX HA}98;P{ Z3rpX shRNA}attP2/ P(Gal4::VP16-nos.UTR) MVD1.* The ovaries were then boiled and sonicated in SDS sample buffer prior to loading onto a 12% polyacrylamide gel at the equivalent of one pair of ovaries per lane. After separation by electrophoresis, proteins were transferred to polyvinyl difluoride (PVDF) membrane. Membrane was blocked with PBST+ 0.1% nonfat milk and was rinsed in numerous PBST washes. Primary antibodies used were rat anti-HA at 1:4000 and rat α-tubulin at 1:4000. The secondary antibody used was rat horseradish peroxidase (HRP) at 1:5000. *P{Z3rp3 HA}*, *P{Z3rpX HA}* and *P{Z3rpX HA}; P{Z3rpX shRNA}attP2* (RNAi knockdown of transgene on the second chromosome) expression was detected using the ECL detection kit (Amersham, Arlington Heights, IL). The germline specific driver *P(Gal4::VP16-nos.UTR) MVD1* (Van Doren et al. 1998) is known to drive high levels of expression in the germarium.

IV. Results

CG31053 and CG12200 are homologs of budding yeast Zip3 and *C. elegans* ZHP-3

A BLAST search of the *D. melanogaster* genome was performed with budding yeast (*S. cerevisiae*) Zip3 (Cst9) and *C. elegans* ZHP-3 proteins (Figure 5). Surprisingly, two homologs were revealed in *Drosophila*, CG31053 and CG12200 (see also www.flybase.org). Related proteins are also found in mouse and humans (Figure 5). Both CG31053 and CG12200 are introns in the genes, CG5508 and CG32533, respectively. CG31053 and CG12200 are 219 and 211 amino acids in length, respectively. In contrast, Zip3 and ZHP-3 proteins are 482 and 387 amino acids in length, respectively. Both *Drosophila* homologs share similar domain structure to Zip3 and ZHP-3, with an N-terminal RING Finger, a short 50-amino acid middle region that forms a coiled coil, and a C-terminal S-rich region. The RING Finger domain indicates there is ubiquitin or SUMO E3 ligase function, deeming Zip3 (all homologs) to be involved in post-translational modification of proteins. Zip3 has an exclusive 50-amino acid N-terminal extension, which could be indicative of a different function in budding yeast (Figure 5). *Drosophila* and human homologs are both missing a significant amount of amino acids from their C-terminal sequence (Figure 5). This is interesting, considering the fact that *Drosophila* and humans are not closely related species. Since the identity and similarity percentages of aligned protein sequences between *Drosophila* and other species are significantly close based on meiotic expression (Table S1), CG31053 and CG1200 will be referred to as *Drosophila z3rp3* (Zip3-related protein 3) and *z3rpX* (Zip3-related protein X), respectively. *z3rp3* and *z3rpX* are located at 98B6 on chromosome 3R and 18C7 on chromosome X, respectively. RT-PCR confirmed that *z3rp3* and *z3rpX*

transcripts were expressed in the ovaries, consistent with a meiotic function (Figure S1). I hypothesized that both *Drosophila* homologs are redundant genes and therefore both have to be characterized in order to obtain the most severe phenotypes and determine the function and localization of *z3rp*.

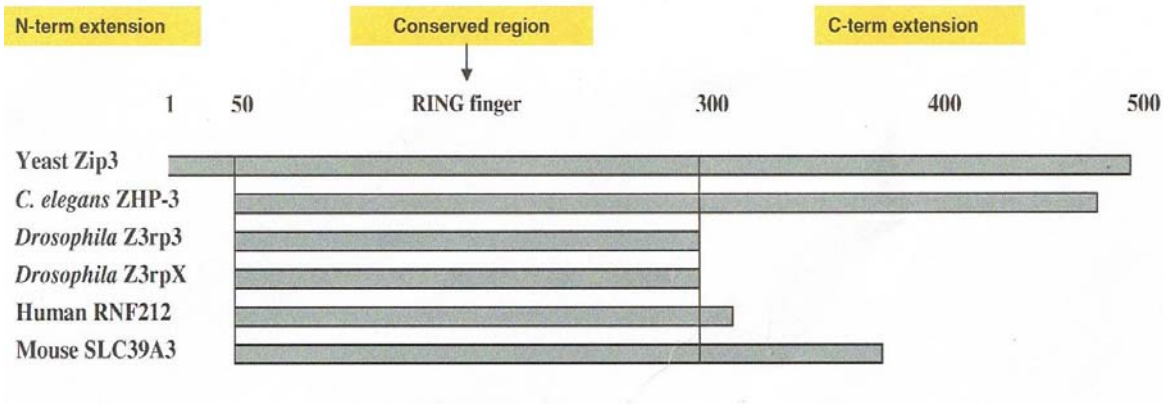

Figure 5: Comparison of protein sequence alignments of Zip3 and homologs in various model organisms.

Schematic of protein alignment sequences of Zip3 homologs. In budding yeast, Zip3 has an exclusive 50 amino acid N-terminal extension. Then all homologs have amino acid sequence from 50-300, where the conserved region of the protein, the RING finger domain, is coded. The two *Drosophila* homologs (Z3rp3 and Z3rpX) and human RNF212 end their sequence at 300. *Drosophila* and human then are missing significant parts of their C-termini sequence. Mouse sequence continues on through most of its C-terminus. Last, budding yeast and *C. elegans* have exclusive C-terminal extensions of the protein. The conserved region is not making any indication of identical or similar amino acids but rather just the sequence length that encodes a RING finger domain.

Z3rp3 HA foci localize to synapsed meiotic chromosomes during early pachytene

To establish the localization of Z3rp3 and Z3rpX and determine if they are crossover markers, the germaria of Z3rpX and Z3rp3 transgenes tagged with a HA epitope and overexpressed (Figure S2) were analyzed via cytology. *P{Z3rp3 HA}* transgenes were expressed using a driver that expresses in the germarium and vitellarium (results in high level of expression), *P(Gal4::VP16-nos.UTR)MVD1* (will be referred to as MVD1), and it localized as single foci on meiotic chromosomes in early pachytene (region 2A) (Figure 6). Z3rp3 HA foci were found in cells with C(3)G and at times colocalized. There were no Z3rp3 HA foci in cells at the zygotene stage. When going through sections of a germarium to observe when the Z3rp3 HA foci were appearing, there was the appearance at times of two Z3rp3 HA foci localizing in the nuclei of one cell (Figure 6). This was surprising since it was expected that five-six foci would appear, a representation that a crossover occurred on each of the four chromosome arms. There were no Z3rp3 HA foci localizing in region 3 of the oocyte or in any of the follicle cells, which would have been expected to be seen if the foci represent crossover sites. Some of the Z3rp3 HA foci localize in the nuclei with no C(3)G and in random areas other than the oocyte. Additionally, *P{Z3rp3 HA}* expression was observed in the germarium with a weaker driver, *P(Gal4-nos.NGT)40) NGT* (will be referred to as *NGT*), and these germaria showed fewer Z3rp3 HA foci than with MVD1, where there was almost never any foci seen in a cell (data not shown). *P(z3rpX HA}* transgenes driven with *MVD1* did not show any significant localization as far as foci but a cloud of HA over the germarium in early pachytene (Figure 6). Because of the abnormal localization pattern, these results indicate the Z3rpX and Z3rp3 transgenes are not good crossover markers.

In the experiment described above, *P{Z3rp3 HA}* transgenes were expressed at low and high levels using a weak (*NGT*) and strong (*MVD1*) driver, respectively. It is possible the appearance of the abundant foci using the *MVD1* driver may be a result of overexpression of the protein. These data are consistent with Zip3 and ZHP-3 localization in budding yeast and *C. elegans*, where it is associated with SC assembly and acting on homologous chromosomes that are synapsed. However, it was expected that there would be greater than one or two Z3rp3 HA foci per nuclei. Additionally, Z3rp3 HA foci did not colocalize with any recombination related markers, such as DSBs and centromeres (data not shown). Furthermore, this data does not necessarily show the true localization and expression of Z3rp3 and Z3rpX. Because Z3rp3 HA foci in *Drosophila* appear during pachytene and not in zygotene, Z3rp3 is not likely to be functioning at synapsis initiation sites, like in budding yeast. This correlates with the differences of Z3rp and ZHP-3 localization corresponding to the overall difference of regulation of synapsis in *Drosophila* and *C. elegans*, respectively. The difference between the number of crossover sites and Z3rp3 containing foci may simply reflect the asynchrony of recombination events. Z3rp3 and its partners might localize to every crossover site, but to different sites at different points in time.

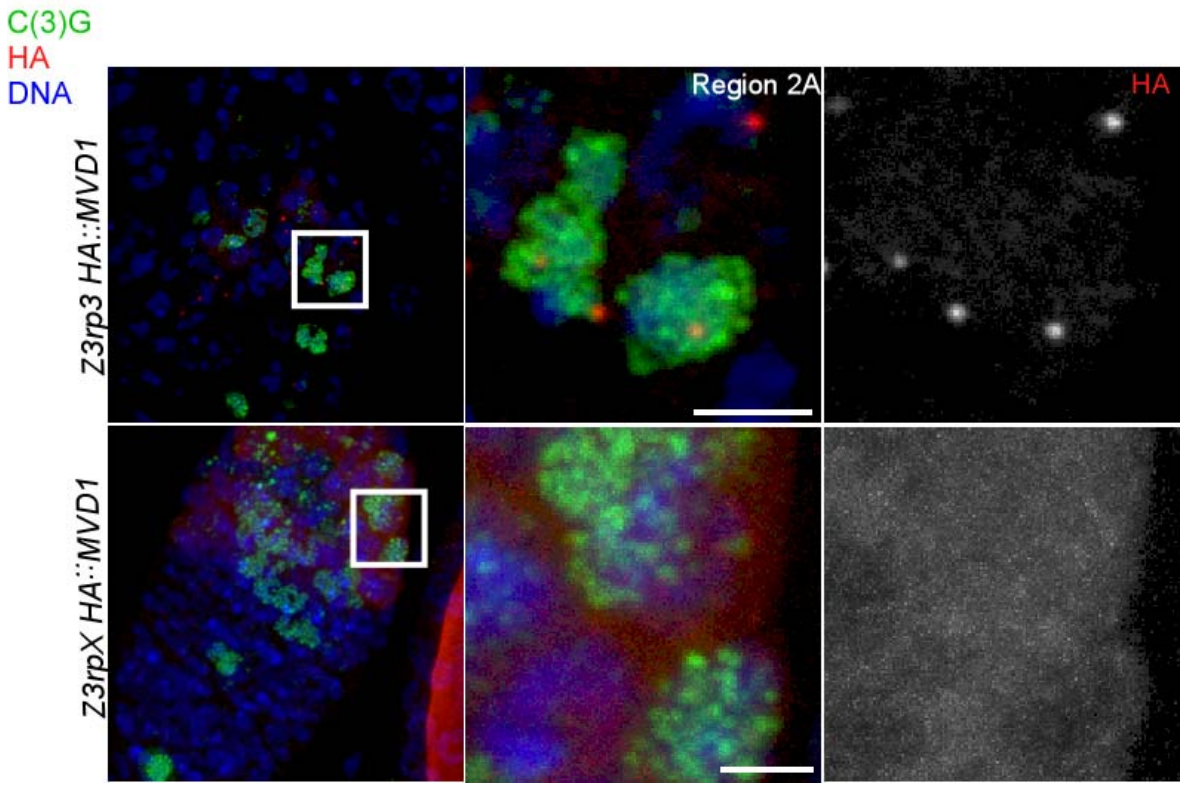

Figure 6: Z3rp3 HA foci have abnormal localization pattern in early pachytene within synapsed cells.

Germaria of *P{Z3rp3 HA} and P{Z3rpX HA}* transgenes expressed with germline specific drivers to determine their localization pattern. *P{Z3rp3 HA}* transgenes expressed at high levels with MVD1 (*P(Gal4::VP16-nos.UTR) MVD1)* show a localization pattern of one and two Z3rp3 foci (HA stained in red) in cells with fully formed SC (C(3)G stained in green). *P(Z3rpX HA}* transgenes expressed with MVD1 showed a cloud of HA staining in early pachytene, but no distinct Z3rpX foci seen. MVD1 is the driver that shows the most distinct localization pattern of Z3rp3. White boxes outlined in the whole germaria represent the larger confocal image sections of the pro-oocytes. Bars, 5 µm.

Dominant negative phenotypes are observed in *P{Z3rp3 HA}* transgenes expressed with *MVD1*

To test if the expression of *P{Z3rp3 HA}* and *P{Z3rpX HA}* transgenes affects meiosis, nondisjunction (ND) (abnormal chromosome segregation) frequency was scored using *NGT* and *MVD1* drivers, where the expression yielded 1.2% and 29.0 % ND, respectively (Table 1). In contrast, *P{Z3rpX HA}* transgenes expressed with *MVD1* yielded only 1.3% . Thus, the contrasting levels of ND seen within the *P{Z3rp3 HA}* construct indicates ND frequency depends on dosage of the protein.

When analyzing the germaria for the expression and localization of Z3rp3 HA foci, it was noticed that there was a high frequency of the two-oocyte (delay in oocyte specification) phenotype in late pachytene (region 3). There was 68%and 55% frequency of two oocytes seen in two different *P{Z3rp3 HA}* transgenic lines driven by *MVD1*, respectively, which is a 4-fold increase compared to WT (15%) (data not shown). Even when *P{Z3rp3 HA}* transgenes were expressed with the NGT driver, the frequency of two-oocytes did not change and levels were similar to those with the *MVD1* driver (data not shown). This is consistent with a meiotic checkpoint that is activated and triggers a delay in prophase progression independent of DSBs in response to a defect that is associated with crossover formation. *P{Z3prX HA}* transgenes expressed with MVD1 had a lower frequency of two-oocytes (20%), comparable to wild-type at 15% (data not shown). *P{Z3rp3 HA}* expression could be causing defects in recombination and the repair/formation of DSBs no matter what expression pattern is used. However, the lower levels of ND observed when using the NGT driver indicates that the sensitivity of

chromosome segregation and recombination does depend on the level of expression.

Because *P{Z3rp3 HA}* transgenes driven with *MVD1* exhibited high frequencies of both nondisjunction and two oocytes, it was expected that crossing over would be severely affected. Thus, crossing over on the third chromosome was scored in a *P{Z3rp3 HA}* transgene and as expected, the overall frequency was 58.8% of wild-type crossing over, reduced by almost 50% (Table 3). Thus, the high nondisjunction and two-ooycte phenotypes seen in *P{Z3rp3 HA}* transgenes is most likely due to a decrease in crossing over. Overall the transgenes may not be good crossover markers because the HA tag could be dysfunctional and causing an abnormal localization of Z3rp3 and Z3rpX. However, the dominant negative affects the transgenes exhibit on meiosis is evidence that that they are playing another important role in meiosis and loss of function mutants will help to reveal what those roles may be.

Construction of null *z3rp3$^{\Delta}$* mutants using FLP-FRT recombination

There are two transgenic insertion sites located upstream (*PBac{WH}CG5508^{f01088}*) and downstream (*PBac{WH}CG5508^{f04927})* of the *z3rp3* gene (Figure 7). These sites are transposable elements that have Flipase recognition targets (FRTs). Thus, the FLP-FRT recombination system (Golic, 1991) works by utilizing a heat activated Flipase (FLP) recombination enzyme (derived from a 2µm plasmid in budding yeast) to cause recombination between the FRT sites and delete the *z3rp3* gene, creating a null mutation (Figure 7). To confirm the *z3rp3*$^{\Delta}$ deletion (null), genomic PCR of females heterozygous for the *z3rp3$^{\Delta}$* deletion and chromosome 3R deficiency, *Df(3R)ED6280,* was carried out using gene specific primers. PCR revealed that there was

indeed a true knockout of the *z3rp3* gene in all three mutant lines, due to the absence of the 832 bp band that is seen in wild-type (Figure 7).

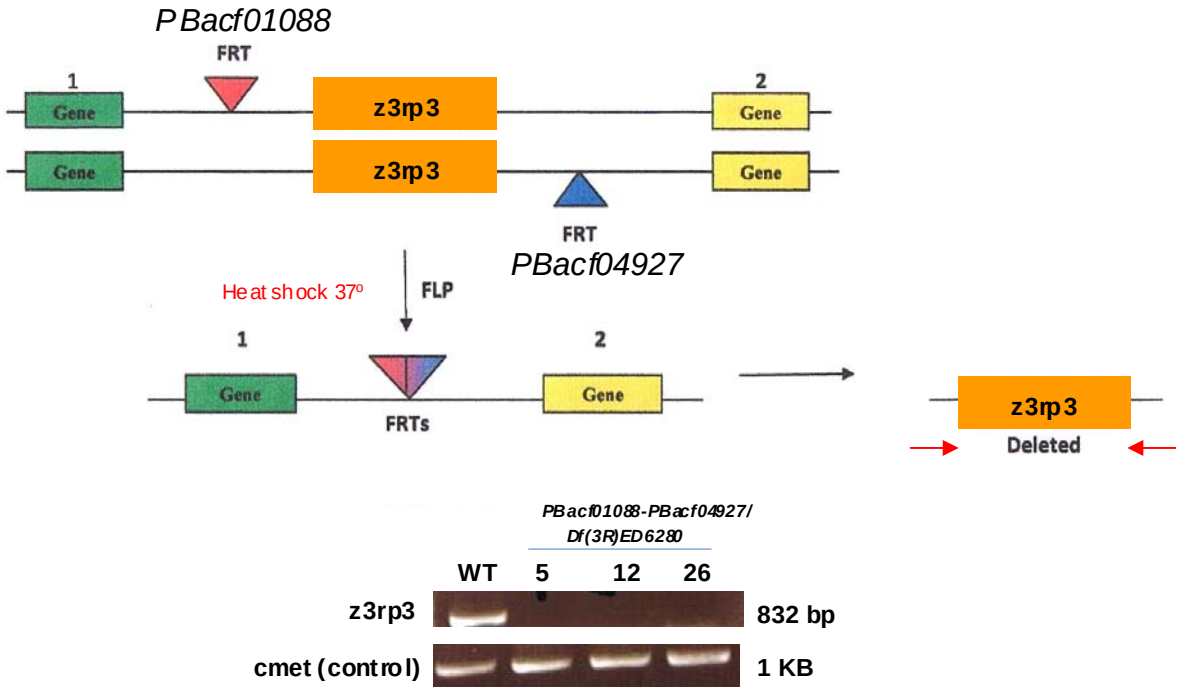

Figure 7: PCR verifies *z3rp3*$^{\Delta}$ deletion mutants.

FLP-FRT recombination method for making transposon-induced mutation to delete the *z3rp3* gene. Heat shock activated flipase (FLP), which acted on the transposase of the upstream and downstream FRTs PBacf01088 and PBacf04927, caused recombination to occur between the two and combine them, resulting in deletion of the *z3rp3* gene. PCR was performed utilizing the genomic DNA of the deletion mutant over the deficiency. Gene specific primers (red arrows represent location of *z3rp3* forward and reverse primers used to amplify gene) designed upstream and downstream were used to determine if there was a *z3rp3*$^{\Delta}$ deletion. The same was done for *cmet* (primer location not shown). The *z3rp3*$^{\Delta}$ PCR line shows the expected presence of the gene in WT with an 832 bp band and the three different *z3rp3*$^{\Delta}$ mutant lines (5, 12, 26) show no band, indicating a deletion. The second line was another positive control, indicating the expected presence of *cmet*, with a resulting1 KB band in WT and *z3rp3*$^{\Delta}$ lines.

Knockdown of z3rpX using short hairpin microRNA

To obtain a mutant in the *z3rpX* homolog, short hairpin microRNA (shmiRNA) was cloned into the pVALIUM22 vector and the transgene was inserted via site-specific recombination by PhiC-31 integrase between attB and attP pseudo sites, which triggered effective RNAi knockdown and corresponding phenotypes in the female *Drosophila* germline (Ni et al., 2010). To express the *z3rpX* RNAi hairpin in the transgenic animals, the UAS-GAL4 system was used for driving its expression under the control of heterologous promoters (Brand and Perrimon, 1993). The two component system involves crossing flies with the transcriptional activator protein, GAL4, to flies with the UAS (Upstream activation sequence) and target gene. Thus, when GAL4 binds to the UAS, the target gene will be activated and expressed in a tissue specific manner. The *z3rpX* knockdown mutant will be referred to as either *P{z3rpX shRNA}attP2* or *z3rpX shRNA or RNAi.*

To determine how efficient the *z3rpX* shRNA knockdown was, it was expressed in the *P{z3rpX HA}98* transgenic background using the overexpression driver *P(Gal4::VP16-nos.UTR) MVD1* (referred to as MVD1), that promotes expression of the protein in the germarium and the vitellarium. Western blot analysis showed that the *z3rpX shRNA* knockdown was partial, relative to the expression of the transgene expressed on its own (Figure 8). Because some protein is still present, the *z3rpX shRNA* may not exhibit the most severe phenotype when crossed with the $z3rp3^{\Delta}$ mutant. Additionally, the expression could be follicle cell protein.

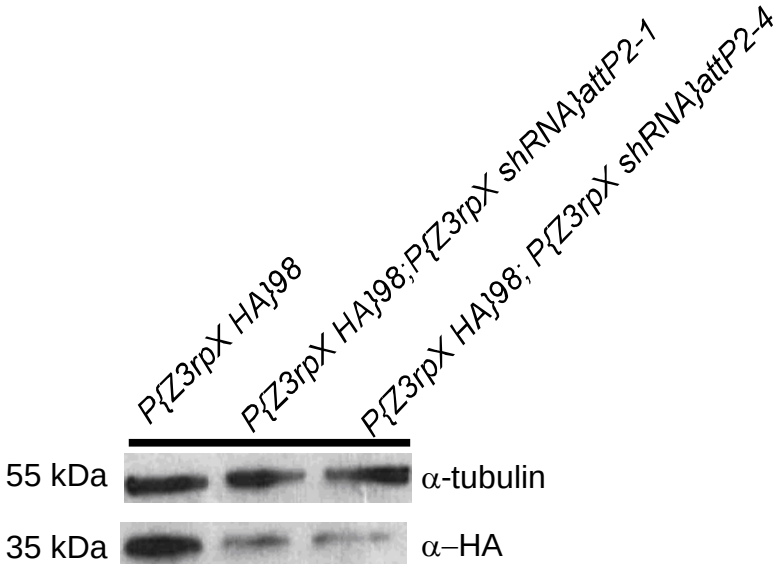

Figure 8: *z3rpX* short hairpin RNAi construct is a partial knockdown when expressed in a *P{Z3rpX HA}* transgenic line.

To test the knockdown efficiency of *z3rpX* short hairpin transgenic constructs (*P{Z3rpX shRNA}attP2*), they were analyzed in a Z3rpX HA-tagged transgene (*P{Z3rpX HA}98*) background. Western blot analysis shows the first column is the transgene alone, showing the presence of the protein with an HA antibody at 35 kDa. Columns 2 and 3 are two different lines generated from the same short hairpin construct, crossed to the Z3rpX HA transgene. Observed is a partial knockdown of the protein with the HA antibody in both lines at 35 kDa, compared to the transgene alone. Thus, some function of the protein is still present. The tubulin antibody is a loading control. All protein expression seen was due to using the MVD1 driver.

***P{z3rpX shRNA}attP2 z3rp3*$^{\Delta}$ double mutants undergo meiosis I nondisjunction and have decreased levels of crossing over**

Since Zip3 and ZHP-3 is known to promote meiotic crossing over in budding yeast and *C. elegans*, nondisjunction and crossing over was measured in *Drosophila* females. First, these tests were measured in *z3rp3*$^{\Delta}$ and *P{z3rpX shRNA}attP2* mutants individually. There was no significant levels of nondisjunction in *z3rp3*$^{\Delta}$ (0.9%) mutants but *P{z3rpX shRNA}attP2* mutants had 3.4% (more significant than the latter), when both were compared to wild-type at 0% (Table 1). When crossing over was scored on the third chromosome in *z3rp3*$^{\Delta}$ mutants, they exhibited 82.1% of wild-type crossing over, which was close to normal levels (Table 3). However, when crossing over was scored on the second chromosome in *P{z3rpX shRNA}attP2* mutants, there was a slight reduction to 66.9% of wild-type crossing over, due to an approximate half reduction of crossing over in *al-dp*, *dp-b*, and *pr-cn* intervals (Table 2). This was definitely an indication that the *shRNA* was affecting the flies' ability to undergo crossing over. But this slight reduction still was not significant compared to the other organisms that show an even more extreme reduction and delay of crossing over like in yeast *zip3* mutants. These are clues that the *z3rp* homologs may be redundant genes.

Due to the predicted redundancy of the *z3rp3* and *z3rpX* homologs, it was expected that the double mutant would have an elevated frequency of meiosis I nondisjunction and decrease in crossing over compared to the single mutants. When scoring nondisjunction in the *P{z3rpX shRNA}attP2 z3rp3*$^{\Delta}$ double mutant, amazingly the frequency was 36.5% compared to wild-type (0%) and those of the single mutants mentioned previously (Table 1). Additionally, the double mutant was partially sterile.

Given this data, it was expected that crossing over would be severely affected in the *P{z3rpX shRNA}attP2 z3rp3*$^{\Delta}$ double mutant because of the high level of nondisjunction. Thus, a significant decrease in second chromosome crossing over was observed at 52.5% of wild-type crossing over, compared to the single mutants (Table 2). However, even though the frequency of crossing over in the double mutant was reduced compared to wild-type, it still exhibited a normal pattern and the levels of crossing over were similar to the *P{z3rpX shRNA}attP2* single mutant. Overall, this is evidence that *z3rp3* and *z3rpX* are indeed acting redundantly. Additionally, because the *P{z3rpx shRNA}attP2* is only a partial knockdown, it is functioning as a hypermorph to *z3rp3*$^{\Delta}$. Last, this data may indicate that crossing over is not sufficient for proper chromosome segregation and *z3rp* has another function.

Table 1: Scoring Nondisjunction on the third chromosome in *z3rp* mutants and *P{Z3rp HA}* transgenes

Genotype[a]	**% ND**[b]	**N=# WT flies**	**N=# ND flies**	**Total Progeny**
Wild-type	0	1055	0	1055
z3rp3$^{\Delta}$	0.9	1344	6	1350
P{z3rpX shRNA}attP2/MVD1	3.4	1321	23	1344
P{z3rpX shRNA}attP2 z3rp3$^{\Delta}$*/MVD1 z3rp3*$^{\Delta}$	36.5	265	76	341
P{Z3rp3 HA}93/MVD1	29.0	1210	248	1458
P{Z3rp3HA}93/NGT	1.2	4972	29	5001
P(Z3rpX HA}98/MVD1	1.3	1216	8	1224

[a]MVD1= *P(Gal4::VP16-nos.UTR)MVD1*
[b]Nondisjunction (ND) was scored by crossing females from each genotype to *yw/Y*^{BS} males. The frequency of third-chromosome nondisjunction is calculated as 2 (Bar females + Bar^{+} males)/[2(Bar females + Bar^{+} males].

Table 2: Crossing over in *z3rp* mutants

	Crossing over on the Second Chromosome (cM)[a]					
Genotype[b]	***al-dp***	***dp-b***	***b-pr***	***pr-cn***	**Total *al-cn***	**N[c]**
Wild-type	12.7	28.2	4.8	1.3	45.3	606
P{z3rpX shRNA}attP2/MVD1	7.4 (58.3)	18.2 (64.5)	4.0 (83.3)	.66 (50.8)	30.3(66.9)	557
P{z3rpX shRNA}attP2 z3rp3$^{\Delta}$/MVD1 z3rp3$^{\Delta}$	3.3 (26.0)	15.9 (55.2)	3.3 (68.8)	1.3 (68.8)	23.8 (52.5)	454

[a]Second chromosome crossing over was assayed by crossing *al dp b pr cn/+* females to *al dp b pr cn/CyO* males in the indicated backgrounds. The *Cy+* progeny were scored for recombinants. Crossing over is expressed as cM across the intervals shown. Numbers in parentheses denote the percentage of wild-type recombination frequency.
[b]MVD1= *P(Gal4::VP16-nos.UTR)MVD1*
[c]N= total flies counted.

Table 3: Crossing over in the z3rp3$^{\Delta}$ mutant and *P{Z3rp3 HA}* transgene

	Crossing over on the Third Chromosome (cM)[a]				
Genotype[b]	***st-cu***	***cu-e***	***e-ca***	**Total *st-ca***	**N[c]**
Wild-type	8.0	25.1	38.9	72.0	311
z3rp3$^{\Delta}$	6.7 (83.8)	22.6 (90)	29.8 (76.6)	59.1 (82.1)	988
P{Z3rp3 HA}93/+; MVD1	4.8 (60)	5.0 (19.9)	32.5 (83.5)	42.3 (58.8)	400

[a]Third chromosome crossing over was assayed by crossing *th st cu sr e z3rp3$^{\Delta}$/z3rp3$^{\Delta}$ and P{Z3rp3 HA}93/+; P(Gal4::VP16-nos.UTR) MVD1 /ru th st cu sr e Pr ca* females to *ru h th st cu sr e Pr ca/TM6B, Bsb Tb and ru h th st cu e ca/TM6B, Tb* males, respectively. The *Pr*$^{+}$ and *Tb*$^{+}$ progeny were scored for recombinants. Crossing over is expressed as cM across the intervals shown. Numbers in parentheses denote the percentage of wild-type recombination frequency.
[b]MVD1=P(Gal4::VP16-nos.UTR)MVD1
[c]N=total flies counted.

P{z3rpx shRNA}attP2 z3rp3$^{\Delta}$ mutants have C(3)G in more cells and DSB repair defects

zip3 mutants in budding yeast were shown to have delayed and incomplete SC formation, whereas the SC in *C. elegans zhp-3* mutants is normal, due to them not requiring recombination to initiate synapsis. Due to the defects seen in nondisjunction and crossing over, the SC (C(3)G) was examined in *Drosophila P{z3rpx shRNA}attP2 z3rp3*$^{\Delta}$ double mutants to determine its affect on synapsis. SC formation is observed in the germarium during pachytene using C(3)G (central element) staining, which appear as complete thread-like filaments and represent pro-oocytes within a 16-cell cyst progressing through the germarium temporally. Wild-type germaria have two pro-oocytes that enter meiosis and initially appear equivalent in early pachytene (region 2a) cysts with fully formed C(3)G (Figure 10). Once late pachytene (region 3) cysts are in the posterior of the germarium, only a single oocyte with SC should be present (Figure 10). Wild-type germaria have an average of 6 cysts that progress through pachytene.

The formation of the SC was normal in the double mutant, having the complete filament structures of C(3)G staining during pachytene, but there was more levels of C(3)G observed in early pachytene (region 2A), an indication that there was either C(3)G seen in more cells or there were more than 6 cysts than in wild-type (Figure 10). This observation was also seen in the *z3rp3*$^{\Delta}$ mutant and *P{z3rpX shRNA}attP2* single mutants. Another mutant known to have high levels of SC, are *sir2* mutants. The function of Sir2 is an active deacetylase of H3K9 during early pachytene and prevents SC from polymerizing in acetylated regions of the chromosome (Das et al., 2009). In other words, *sir2* promotes a low acetylated state of chromosomes to possibly limit how much SC is

incorporated to create an environment more suitable for crossover formation. These results could indicate that *z3rp* is performing a role in controlling the levels of SC in early pachytene by turning on *sir2*. Furthermore, there is no apparent effect on the initiation of recombination but there may be a delay in producing crossovers, which could explain a compensation mechanism that is activated making more SC in order to make up for breaks not being repaired via crossing over. Therefore, there is a relationship between SC formation and crossing over. This is further supported by the presence of two-oocytes seen in late pachytene of the *z3rp* single and double mutants.

To explain as to why there is more SC than normal observed in the double mutant, other delayed aspects of meiotic progression were analyzed, including the dynamics of DSB formation and repair by staining for γ-HIS2AV. In wild-type germaria, most γ-HIS2AV foci are seen in early pachytene (region 2A) and absent by late pachytene (region 3) (Figure 9). Observed in *P{z3rpx shRNA}attP2 z3rp3*$^{\Delta}$ double mutants was a high accumulation of DSBs persisting in region 3 cysts compared to wild-type (Figure 9). Interestingly, the amounts of breaks accumulated are high at the beginning of early pachytene ranging from an average of 10-13 in each cyst (Figure 9). Thus, there is no obvious delay in DSB formation but there is a delay in the response to DSBs. *z3rp3*$^{\Delta}$ mutants only have an average of 2 breaks persisting in region 3 but have a lower amount of breaks (5-7 breaks in cysts 3 and 4) than wild-type (10 breaks) (Figure 9). *P{z3rpx shRNA}attP2* mutants have an elevated amount of breaks, even higher sometimes (14-15 breaks in cysts 4-9) than the double mutant (10-12 breaks), but break persistence levels out and decreases in late pachytene to an average of 2 breaks like in *z3rp3*$^{\Delta}$ mutants (Figure 9). Overall, γH2AV staining indicates there is a delay in the response to repairing

the DSBs. It was expected that because double mutants had a decreased level of crossing over because there may be less breaks. However, that was not the case since there was a large accumulation of breaks. The accumulation of a large amount of breaks in the double mutant cannot be repaired fast enough via crossing over and there is the possibility that the breaks are repaired via a different pathway or are just not repaired at all.

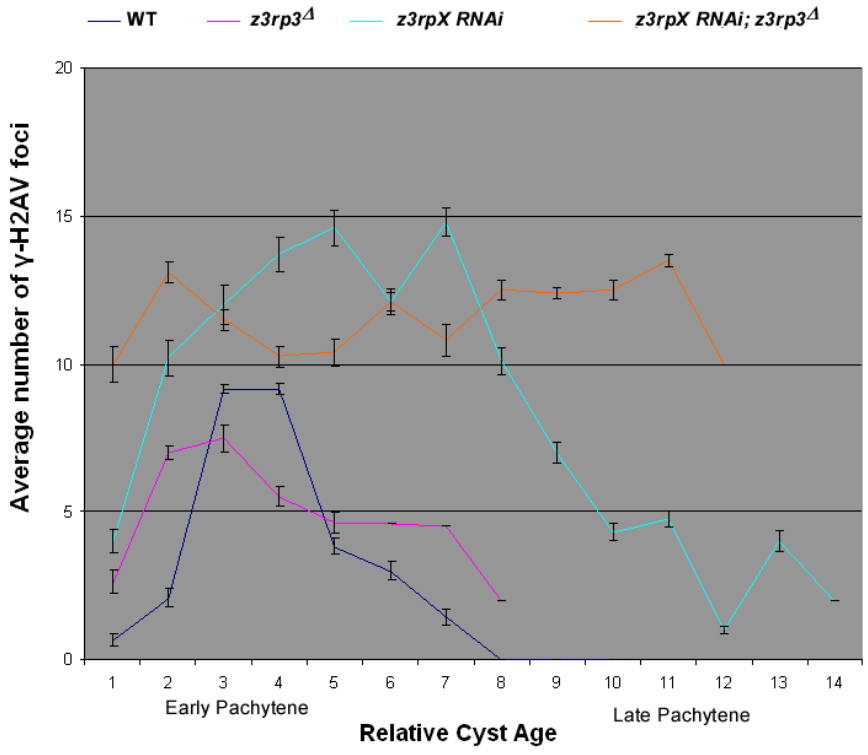

Figure 9: Pattern of γ-HIS2AVstaining in wild-type and *z3rp* mutants.

The average number of γ-HIS2AV foci is plotted relative to cyst age. Oocytes are arranged in temporal order, where the lowest numbers are in cyst 1 and are the first to have complete SC. Cysts 10-14 are in late pachytene (region 3). *z3rpX z3rp3* double mutants show the most severe affect on DSB repair, where there is a high accumulation of breaks in early pachytene and an average of ten breaks persisting in late pachytene. *z3rpX RNAi* mutants have a large accumulation of breaks in mid-pachytene, but most are repaired by late pachytene. Error bars denote the standard error of the mean.

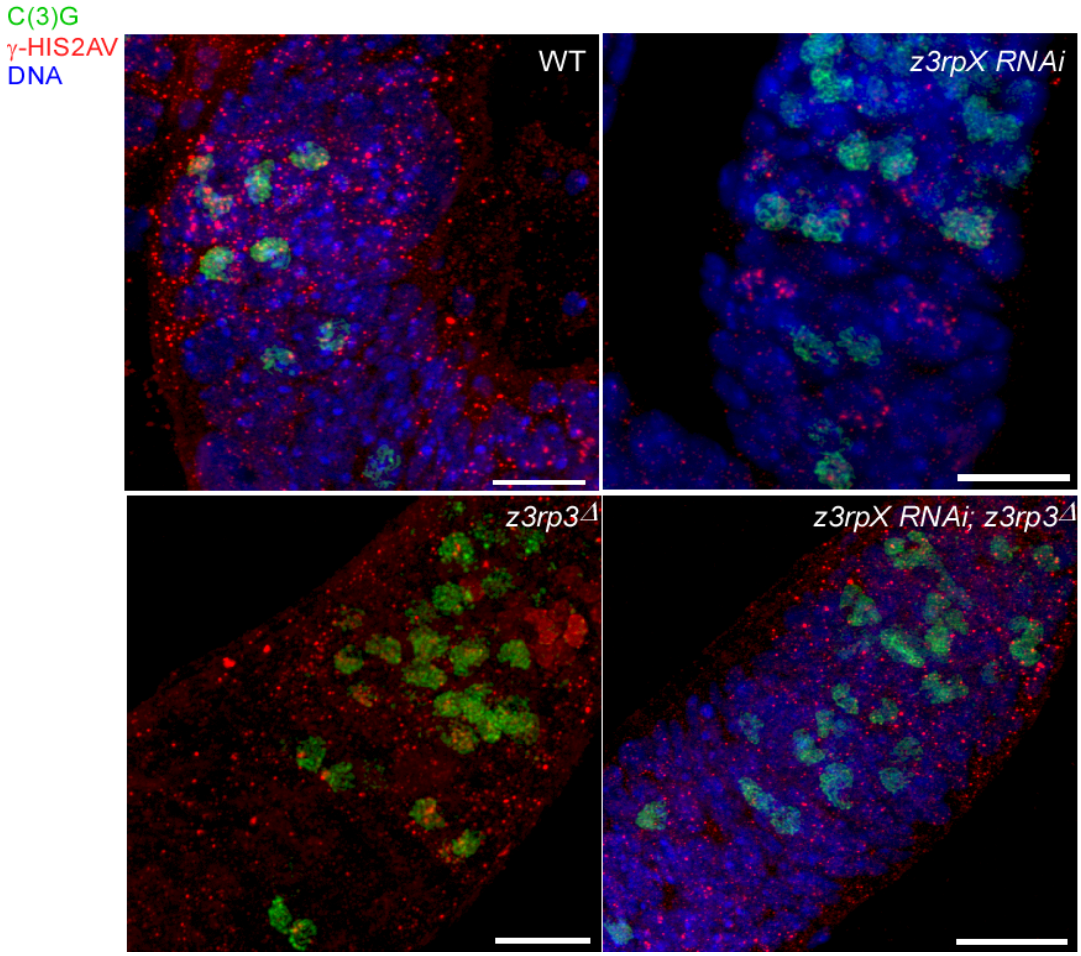

Figure 10: *z3rp* mutants have two-oocytes and more cells with C(3)G

Wild-type germarium showing progression of pro-oocytes (stained with C(3)G in green) through early, mid- and late pachytene. DSBs (stained with γ-HIS2AV in red) accumulate in pro-oocytes and nurse cells (DNA stain in blue) in early pachytene (region 2A) and are absent in late pachytene (region 3), indicating they were repaired. *z3rp3*$^{\Delta}$ mutants have more cells with levels of C(3)G in early pachytene, compared to wild-type and most breaks are repaired in late pachytene. *z3rpX RNAi* mutants also have more SC levels in early pachytene. *z3rpX z3rp3*$^{\Delta}$ double mutants have an even higher level of SC in early pachytene. (note: double mutants are seen to have a high frequency of two-oocytes, even though this germarium has one oocyte in region 3, thus this image is mainly more to focus on the elevated levels of C(3)G seen in early pachytene. Arrows are showing the number of oocytes in region 3. Two-oocytes are determined if there are two cells with fully formed C(3)G or one with fully formed C(3)G and the other loosing C(3)G in region 3. Images are confocal max projections. Bars, 5 μm.

$z3rp3^{\Delta}$, *P{z3rpX shRNA}attP2*, and *P{z3rpX shRNA}* $z3rp3^{\Delta}$ mutants all activate the pachytene checkpoint

Checkpoints often function to slow progression through the cell cycle so that a problem can be corrected. Previous studies have shown evidence for a new meiotic prophase checkpoint in *Drosophila* females, where mutations in DSB repair genes and exchange genes cause delays in the chromatin remodeling response to DSBS and oocyte selection (two-oocyte phenotype) (Joyce and McKim, 2009). These phenotypes may be a consequence of a general delay in pachytene progression, which could be associated with the pathway leading to crossovers. The pachytene checkpoint requires the gene *pch2*. When analyzing the progression (early pachytene (region 2A), mid-pachytene (region 2B), and late pachytene (region 3)) of cysts containing 2 pro-oocytes marked by C(3)G staining in the germaria of $z3rp3^{\Delta}$, *P{z3rpX shRNA}attP2,* and *P{z3rpX shRNA}attP2* $z3rp3^{\Delta}$ mutants, a high frequency of two oocytes was observed at a range between 77%-95% (each $P<0.001$ compared to wild-type at 15%) (Figure 11).

Double mutants were made with $z3rp3^{\Delta}$ and the pachytene checkpoint gene, $pch2^{EY01788a}$ (null allele with 8% two-oocytes) (Joyce and McKim, 2009) to determine if the delays are dependent on the pachytene checkpoint. In $z3rp3^{\Delta}$ $pch2^{EY}$ mutants, the frequency of two-oocytes is reduced to 22% (Figure 11), an indication $z3rp3^{\Delta}$ is causing a *pch2* dependent delay. Previous studies have shown that the pachytene checkpoint is also sensitive to defects in chromosome axes (Joyce and McKim, 2010). Mutations in genes that encode structural axis components, C(2)M and ORD, cause *pch2*-dependent pachytene delays. Thus, we determined if the pachytene checkpoint delays seen in the *z3rp* single and double mutants is also because of a chromosome axis defect in C(2)M.

C(2)M staining is seen with C(3)G simultaneously in a thread-like pattern in pro-oocytes in early-late pachytene, where the loosing oocyte does not have C(2)M. *z3rp3*$^{\Delta}$ mutants have strong C(2)M staining in region 2A of the germarium, but it deteriorates in region 2B and completely gone in region 3 oocytes (Figure 12). This is consistent with *z3rp3*$^{\Delta}$ mutants having a pachytene delay because of a defect in C(2)M structure, which is needed for proper synapsis and for crossing over to occur. It is possible that *pch2* responds to the C(2)M defect (activating the checkpoint) caused by *z3rp3*$^{\Delta}$, thus there could be a pathway in which *z3rp3* is a positive regulator that promotes (through ubiquitinylation or sumoylation) the monitoring of proper C(2)M assembly. However, when C(2)M was analyzed in *P{z3rpX shRNA}attP2* and *P{z3rpX shRNA} z3rp3*$^{\Delta}$ mutants, surprisingly, C(2)M staining remained present in all regions of the germarium (Figure 12). This could indicate that *z3rpX* has an opposite role on C(2)M structure, being a negative regulator, since the double mutant does not exhibit the phenotype. Or it could be because *P{z3rpX shRNA}attP2* is a partial knockdown and hypermorphic, C(2)M levels are not affected and having the function supercedes the deterioration of C(2)M in *z3rp3*$^{\Delta}$ mutants in an epistatic pathway. Overall, the C(2)M phenotype could just be exclusive to the *z3rp3*$^{\Delta}$ mutant, meaning that particular homolog is required for the stability of the C(2)M component of the SC. Because of more C(3)G seen in cells of the *z3rp* single and double mutants, this could correlate with having elevated levels of C(2)M or the defect seen in z3rp3 mutants is due to the presence having so much C(3)G that C(2)M became dysfunctional.

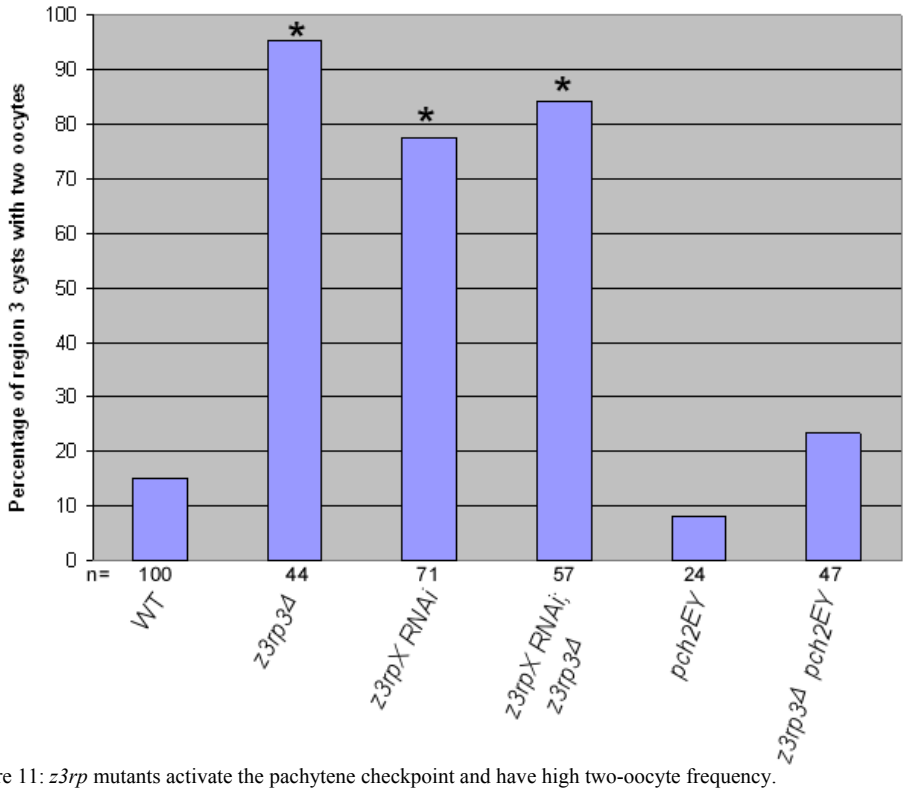

Figure 11: *z3rp* mutants activate the pachytene checkpoint and have high two-oocyte frequency.

Two-oocyte phenotype in region 3 cysts of wild-type and single and double *z3rp* mutant females. Also two-oocytes were scored in $z3rp3^{\Delta}$ mutants with $pch2^{EY}$ (pachytene checkpoint). The percentage of region 3 cysts with two-oocytes is based on C(3)G staining. Asterisks located above each bar correspond to a genotype that gave a *P*-value <0.001 when compared to wild-type. The number of cysts (which is equivalent to the number of germaria) counted is shown at the bottom of each bar. $z3rp3^{\Delta}$, *z3rpX RNAi,* and $z3rpX\ z3rp3^{\Delta}$ mutants all have a very high frequency of two-oocytes compared to wild-type.

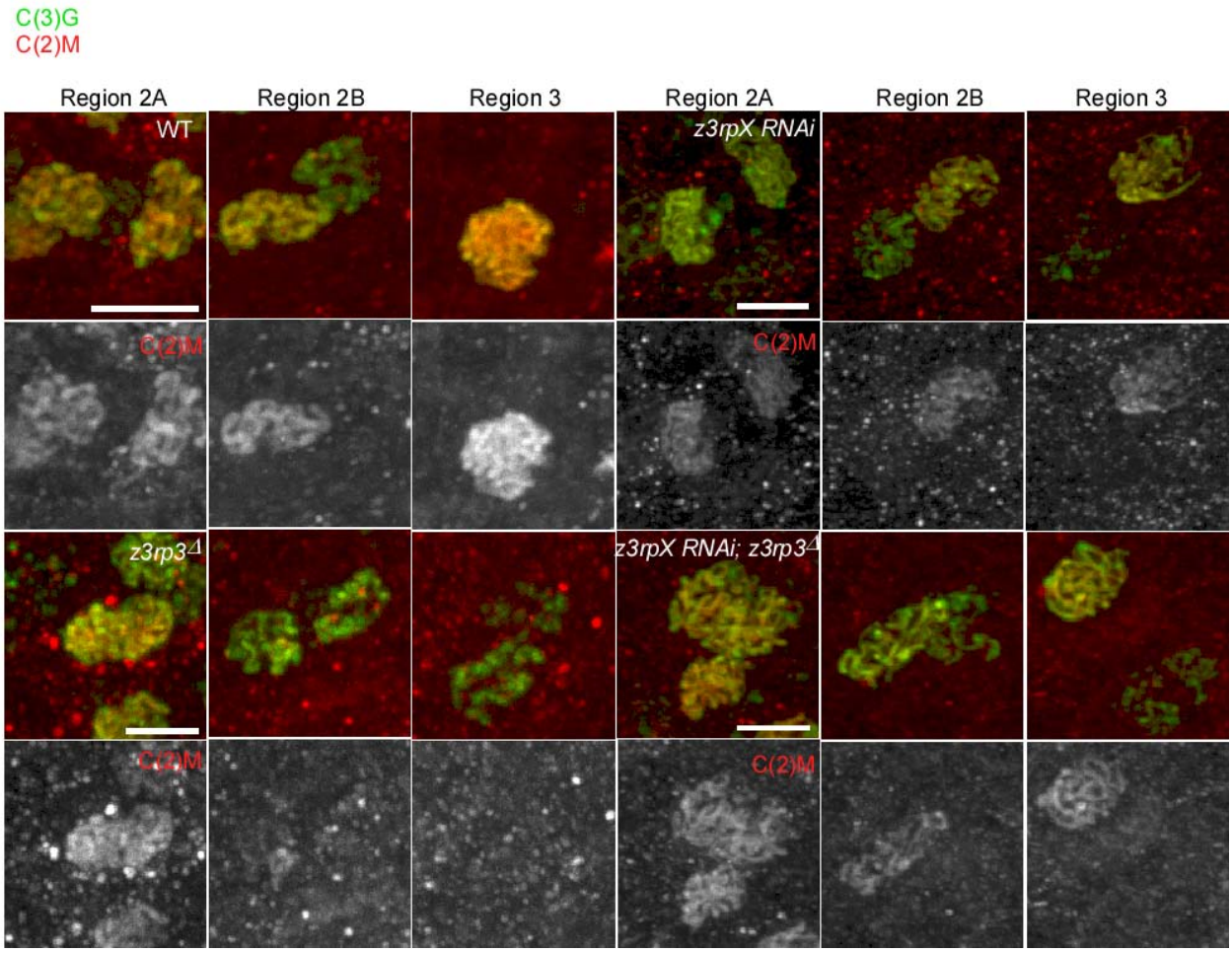

Figure 12: *z3rp3*$^{\Delta}$ and z3rpX RNAi mutants have different affects on C(2)M.

Since *z3rp3*$^{\Delta}$ mutants (also *z3rpX RNAi* and double mutant) activate the pachytene checkpoint, which is known to be activated because of a defect in chromosome axis components, C(2)M was analyzed in single and double mutants of *z3rp3* and *z3rpX* and with *pch2*^{EY} (pachytene checkpoint protein). Wild-type pro-oocytes have a consistent pattern of C(2)M staining all the way up to region 3 with the winning oocyte. *z3rp3*$^{\Delta}$ mutants loose C(2)M in regions 2B and 3, compared to *z3rpX RNAi* and the double mutants, where C(2)M staining is observed to still be present. Green staining represents the central SC element, C(3)G. Red staining is the SC lateral element, C(2)M. Each image shows a max projection of all confocal sections through the oocytes. C(2)M is only seen in winning oocytes in region 3. Bars, 5µm.

V. Conclusion

Here we have presented the first characterization of two *Drosophila melanogaster* Zip3 homologs, revealing their redundant and different functions in the meiotic recombination pathway, including roles in synapsis, crossing over, and activating the pachytene checkpoint. *Drosophila* is an excellent model organism to understand these functions because of it exclusively being the only organism with two Zip3 homologs (*z3rp3* and *z3rpX*) and having a visualization of meiotic events of pro-oocytes via the female germarium, including DSB formation to their repair into crossovers. Thus, *Drosophila* has provided new insights into how synapsis and recombination events are connected.

First, when it was discovered *Drosophila* had two homologs of *z3rp*, we realized immediately that they would have to be characterized separately and together via transgenes and mutants because they are most likely redundant. When Z3rp3 was tagged with the epitope marker HA and overexpressed, Z3rp3 localized as single foci (sometimes two) to cells (pro-oocytes) with fully formed C(3)G in early pachytene of the germarium. Because Z3rp foci are presumed to be possible crossover sites, this indicated that there were only one or two crossover sites present, where there should have been five-six in all, one per chromosome arm. This abnormal localization pattern was not suitable for a good crossover marker and it could be due to the HA tag on the protein acting as a poison and is altering Z3rp's true expression or it is indeed a unique pattern of only a certain amount of crossovers being seen at a time cytologically with asynchrony occurring. The rest of the breaks could be localized somewhere else and not being seen. Furthermore, when the *P{Z3rp3 HA}* transgene was analyzed, it was found that it

exhibited high levels of nondisjunction, a decrease in crossing over, and high frequency of two-oocytes. Z3rpX HA-tagged transgenes showed no distinct localization (localization possibly in follicle cells), thus further localization experiments (construct new transgene and/or antibody) have to be carried out to confirm the true localization of the protein. Because of the transgenes exhibiting dominant effects, that showed Z3rp3 and Z3rpX had other important functions in meiosis.

Mutants were made in *z3rp3* (null made via FLP-FRT) and *z3rpX* (partial knockdown via transgenic short hairpin RNA). *z3rp3*$^{\Delta}$ mutants alone had no effect on nondisjunction, DSB repair or crossing over. However, they did activate the pachytene checkpoint (two-oocytes in late pachytene) and had deterioration of the chromosomal axis protein C(2)M in mid- and late pachytene. The other two mutants (*z3rpX* RNAi and double mutant) had normal C(2)M levels. This indicates that *z3rpX* is a negative regulator in an epistatic pathway with *z3rpX*, not promoting C(2)M formation. Or it could be *z3rpX RNAi* mutants still expressed a normal copy of C(2)M because of it being a partial knockdown. The pachytene checkpoint, which is *pch2* dependent, is thought to be activated because of chromosome axis defects, where C(2)M is an axis component. *pch2* may activate *z3rp3*, which then controls the formation of C(2)M possibly by ubiquitinylation. Furthermore, in budding yeast, it has been found Zip3 sumoylates the substrate Red1, a lateral element of the SC, along with Zip1 (Watts and Hoffmann, 2011), which could be equivalent to the relationship of *z3rp3* and C(2)M in *Drosophila*.

Additionally, there were more cells seen with C(3)G in *z3rp* single and double mutants, indicating that *z3rp* has a role in monitoring SC levels in early pachytene (Figure 13). *P{z3rpX shRNA}attP2* mutants also had high levels of C(3)G in early

pachytene and there was an observation of a slight decrease in crossing over. However, the double mutant showed the most severe phenotypes, including very high nondisjunction, accumulation of numerous breaks in late pachytene, activation of the pachytene checkpoint (two-oocytes in late pachytene), and a decrease in crossing over. When crossing over was scored on the second chromosome in the double mutant, it was lower than wild-type in all intervals but there still was a consistent normal pattern of an increase and decrease. However, for the high level of nondisjunction (36.5%) that was seen in the double mutant, the levels of crossing over should have been extremely low (at least 15% or lower of wild-type) to be considered a true crossing over mutant (which none were). This may be an indication that crossing over is not enough for proper chromosome segregation and *z3rp* has another function beyond just promoting crossover formation. Overall, even though *z3rp3* and *z3rpX* are redundant genes, it is interesting how there are some exclusive phenotypes exhibited among the single and double mutant (Table 4), more evidence of the dynamic roles *z3rp* may have in meiosis.

As expected, the results indicate that Z3rp is functioning in a different context, compared to Zip3 and ZHP-3 in budding yeast and *C. elegans*, respectively, despite sequence homology. In budding yeast, the SC depends on the presence of DSBs, where Zip3 promotes formation of SC (Zip1) at synapsis initiation sites (Zip2) at the centromeres (which also is marking DSBs). Additionally, these sites are where Zip3 interacts with other recombination proteins and therefore localizes to crossover-destined recombination intermediates. Also it is thought Zip3 is promoting crossing over by sumoylation of chromosomal/recombination proteins. Zip3 is proposed to link synapsis to meiotic recombination. In *C. elegans* and *Drosophila*, DSB formation is not required for

SC formation and there is no Zip2 homolog, thus being pieces of evidence that ZHP-3 and Z3rp are uniquely functioning within each organism, respectively. Studies show that ZHP-3 in *C. elegans* requires the SC for localization and has two different roles during meiosis, promoting crossover formation and mediating the appropriate restructuring of bivalents so that chiasmata ensure proper chromosome segregation. Thus, *Drosophila z3rp* also may have more than one role during meiosis based on the crossing over data in the double mutant. Sequence alignments of Z3rp show it is more likely to promote ubiquitinylation of its substrates to promote synapsis/crossing over (Figure 13). Thus, future studies will include determining what other genes *z3rp* interacts and complexes with to reveal those it is actually modifying or degrading. This will involve also studying and characterizing proteins in the ubiquitinylation or sumoylation pathway (i.e. E2 conjugating enzymes since Z3rp is an E3 ligase). Thus, preliminary data shows the E2 conjugating enzyme, *lesswright*, may be a good candidate (Appendix 2, Figure 14). To determine true wild-type localization of Z3rp, antibodies are currently being made in both *z3rp* homologs, which could be the first markers of chiasmata in *Drosophila*. Visualizing the crossover sites will hopefully help to reveal *z3rp's* mechanism of action in relation to synapsis, DSB formation, DSB repair, and how many stable bivalents are present. Overall, *z3rp* may be playing similar roles in the communication between SC formation and recombination events as in budding yeast and *C. elegans*, but in different contexts.

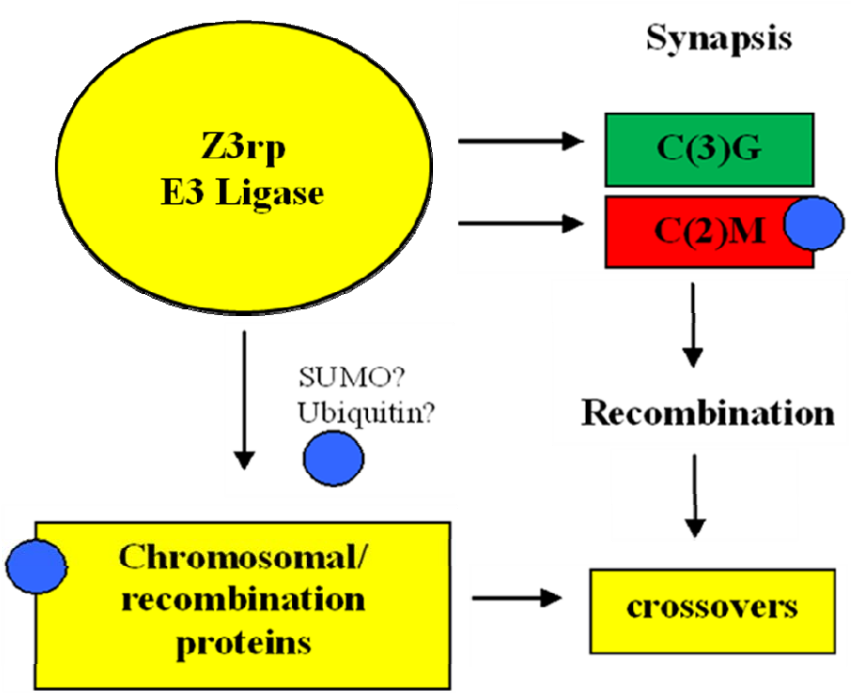

Figure 13: Model for Z3rp activity during pachytene.

This model reflects Z3rp as being an E3 ligase in the sumoylation or ubiqutinylation pathway (more the latter) to modify the function or degradation of chromosomal/recombination proteins to ensure proper crossover formation, respectively. From these studies, it seems Z3rp is in the center of monitoring and structuring synapsis component levels at DSBs for making an environment more prone for crossing over, by a mechanism that is unknown. Pch2, required for the pachytene checkpoint to delay oocyte selection to increase the chance of DSBs becoming crossovers, senses axis defect and activates and modulates Z3rp to ubiqutinate C(2)M (possible substrate) and have it polymerize along chromosomes. Additionally, checkpoint-mediated effects require Sir2 (histone deacytelase), which has a connection with Pch2 and then activates Z3rp to monitor the levels of SC in early pachytene. These assumptions are made based on the *z3rp* mutant showing severe phenotypes in chromosome segregation, DSB repair, and crossing over. Having proper synapsis ensures the proper functioning of the rest of the events in the meiotic program. Boxes are color coded to show the connection of the proteins and events they control or are a part of.

Table 4: Comparison of *z3rp* mutant phenotypes

						Pachytene progression defects			
Mutant	**Nondisjunction**	**C(3)G Elevated**	**C(2)M defect**	**Crossover levels**	**Crossover distribution**	**Delayed γ-HIS2AV foci**	**Persistence γ-HIS2AV foci[a]**	**Two-oocytes**	**Pachytene Delays suppressed by *pch2***
z3rp3$^{\Delta}$	No	Yes	Yes	Normal	Normal	No	No	Yes	Yes
P{z3rpX shRNA} attP2	Yes	Yes	No	Reduction	Abnormal	No	No	Yes	ND
P{z3rpX shRNA} attP2 z3rp3$^{\Delta}$	Yes	Yes	No	Reduction	Abnormal	No	Yes	Yes	ND

ND, not determined

[a]Average no. of foci that persist into late pachytene (region 3) oocytes

APPENDIX 1: Supplementary figures and tables

Table S 1: Percentage of Identity/Similarities of Z3rp homologs in model organisms.

Organism	Z3rp(3)	Z3rp(x)
***D. melanogaster* Z3rp3**	**----**	**48/66**
Mouse	**36/57**	**40/53**
Human	**29/51**	**29/50**
C. elegans	**26/48**	**27/50**
S. Cerevisiae	**25/44**	**28/44**

A BLAST search was performed of the *Drosophila* genome to the model organisms shown for Z3rp homologs. The first number represents the percentage of identical amino acids in the protein alignment sequences compared to the two *Drosophila* homologs, Z3rp3 and Z3rpX. The second number is the percentage of how similar the entire sequences are to one another. Z3rp3 is also shown compared to Z3rpX.

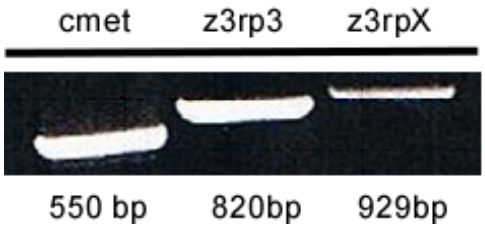

Figure S 1: RT-PCR shows z3rpX and z3rp3 both function in meiosis.

mRNA was extracted from wild-type female ovaries (prepared by Dr. Sarah Radford). The SuperScript™ One-Step RT-PCR with PlatinumR *Taq* System was used to detect and analyze RNA molecules by RT-PCR. Components for both cDNA synthesis and PCR amplification were combined and the reaction was ran in a thermal cycler. Gene-specific primers for cmet, z3rp3, and z3rpX target sequences were also used . Samples were ran on a 0.7% agarose gel. The resulting and expected band sizes are shown under each lane.

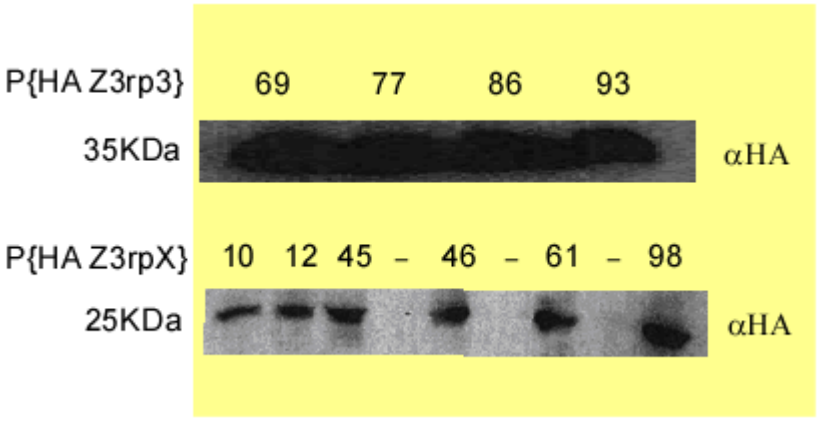

Figure S 2: Western blotting from ovaries expressing transgenic Z3rp3 and Z3rpX, detected using anti-HA antibody. The transgenic lines were expressed with the MVD1 overexpression driver. The presence of the Z3rp3 and Z3rpX protein was detected at 35 kDa and 25 kDa, respectively. – Represented nothing was loaded those lanes.

APPENDIX 2: lesswright (lwr) is an E2 conjugating enzyme in the SUMOylation pathway that promotes disjunction of chromosome

UBC9 was first identified in *S. cerevisiae* as a 157 amino acid protein that is one of the 13 ubiquitin conjugating (E2) enzymes, most of which have been shown to carry out the second step in ubiquitin-mediated proteolysis, conjugating ubiquitin to proteins (Hochstrasser, 1995). However, it has been shown that UBC9 does not carry out the same role as other E2 proteins, where it does not use ubiquitin as a substrate, but rather an ubiquitin-like molecule known as SUMO (Small ubiquitin-like modifier). LWR, the *Drosophila melanogaster* UBC9 homolog, mediates the dissociation of heterochromatic regions of homologs at the end of meiotic prophase I (Apionishev et al., 2001). A previous proposed model shows LWR playing a role in freeing "glue" that holds chromosomes together as the spindle forms (Apionishev et al., 2001). The "glue" behaves like the synaptonemal complex (SC) protein Cor1, which interacts with LWR in a two-hybrid assay, indicating it could be the target of LWR. Cor1 associates with homologs as they begin to synapse to form a mature SC and as prophase ends, it does not dissociate from chromosomes. Thus, Cor1 distribution becomes discontinuous as it moves to heterochromatic centromeric regions (Dobson et al. 1994; Moens & Spyropoulos 1995). Thus, it is speculated that Cor1 is modified by SUMO-1 conjugation mediated by LWR. The modification might promote the prompt redistribution of Cor1 to the centromere.

Since Z3rp is an E3 ligase, possibly in the same pathway as the E2 conjugating enzyme LWR, experiments were started to first characterize LWR and eventually determine its relationship with Z3rp. *P{lwr shRNA}* mutant (from the Transgenic RNAi Project of Harvard University) *Drosophila* females were driven with the MVD1

overexpression driver and scored for nondisjunction. When scored, the females were found to be sterile and did not have ovaries (data not shown). Thus, germline clones were made, which is a tool to produce homozygous mutant cells in an otherwise heterozygous animal to determine if the same phenotype would be observed. FLP-FRT (Flipase-Flipase Recognition target) mitotic recombination is stimulated in the germline, where clones are marked using visible mutations that act cell-autonomously. *lwr* germline clones (were not sterile and had ovaries) were analyzed in the germarium, where GFP FRT (visible marker) chromosomes were paired with *lwr* FRT chromosomes (gene of interest). Once flipase acts on the FRTs, recombination occurs between the homologous FRT sites and centromere, creating progeny that will have green (non-mutant) and non-green (mutant) cells. We screened for clones that had visible holes in the GFP staining of the germarium, indicating a germline clone of *lwr*. Clones were found in regions 2B and 3 of the germarium, and we observed pro-oocytes with an accumulation of breaks that are not repaired and the two-oocyte phenotype (Figure 14A,B). It also seems as though there could be three oocytes in region 3, indicative of a possible orientation defect where the extra oocyte seen should have been in region 2B (Figure 14A). These preliminary results show that *lwr* is an important gene in meiosis and a good candidate for studying its relationship with *z3rp* in the sumoylation pathway, both having a role in promoting synapsis for proper crossover formation and chromosome segregation through modification of chromosomal/recombination proteins.

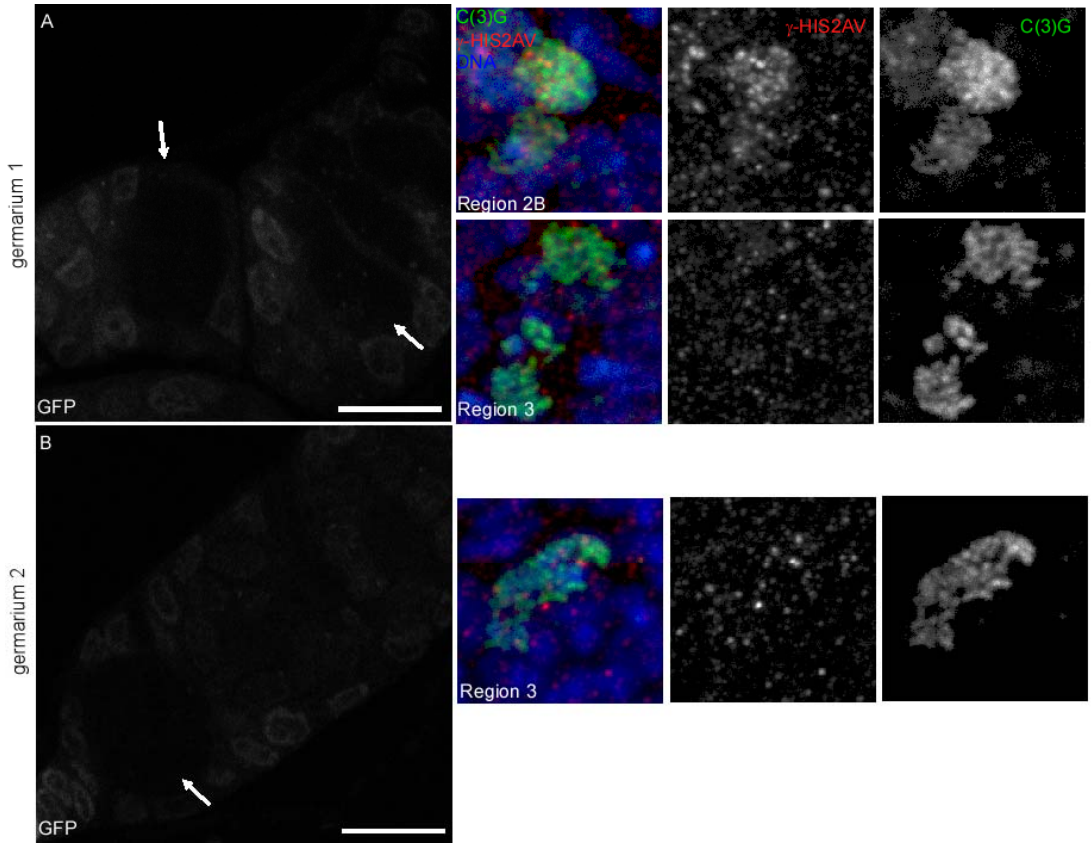

Figure 14: *lwr* germline clones have a DSB repair defect and delayed oocyte selection.

A) Germarium of *lwr* mutant clone stained with C(3)G in green (pro-oocytes and oocytes), γ-HIS2AV in red (DSBs) and DNA in blue. Pro-oocytes (region 2B) and oocytes (region 3) shown are from the indicated cysts where GFP staining was missing (arrows pointing to holes). Large amount of DSBs persist in both regions. Two-oocyte phenotype is observed in a different pattern than the typical phenotype usually shows. B) Same as A, just observed in a different germarium. Bars, 5 μm.

APPENDIX 3: Gene targeting to create a *z3rpX* knockout mutant

Because *z3rpX* (CG12200) does not have another PBac downstream to make a null mutant via FLP-FRT recombination or the presence of a P-element to make an excision, a short-hairpin RNA construct was made to knockdown *z3rpX* in the germline as a quick alternative to determine its phenotype. RNAi is a convenient method to analyze the effects of the loss of function of a gene but only a true mutant will reveal exactly how important the gene is in meiosis. Thus, the gene targeting method developed by Rong and Golic (2000) will be used in the future to knock out *z3prX* and create a null mutant. The development of homologous recombination based gene targeting is a landmark breakthrough in *Drosophila* genetics (Rong and Golic 2000, Rong and Golic 2001). Gene targeting includes an "ends out" method or replacement-type gene targeting that offers a straightforward approach for generating knockout alleles (Huang et al. 2008). Overall, there is a modification of an endogenous gene sequence by recombination between an introduced DNA fragment and homologous target gene (Figure 15) (Huang et al. 2008). The process involves creating a donor molecule homologous to the genomic sequences to be replaced but with an introduced mutation (white gene sequence).

So far, some cloning experiments for *z3rpX* gene targeting were completed. Because a DNA fragment has to be large (3-4 kb) for efficient targeting, the *z3rpX* genomic DNA was cloned off of the bacterial artificial chromosome (BAC, a very large DNA construct plasmid) (used genomic clone BACR27L16) using specific primers engineered with restriction enzymes at the 3'(XhoI and SpeI) and 5'(KpnI and NheI) ends analogous to the destination vector (pGX-attP). Both the 5' and 3' fragments at 3 kb were then cloned separately into the linearized pJET1.2/blunt cloning vector (Fermentas), where cloning into the vector is fast, efficient, and yields more than 99% positive clones

because it has a lethal gene disrupted by ligation of the DNA insert into the cloning site, avoiding the previous method of blue/white screening of colonies (Fermentas). The 5' PCR product fragment was cloned successfully into pJET1.2 using blunt-end protocol (fragment contained both *Taq* and vent polymerases). However, the 3' PCR product fragment would not clone into the pJET1.2 vector properly so the fragment was amplified in two pieces ((Xho1-EcoRI (1.6 kb) and EcoRI-SpeI (1.5 kb)) by cloning each fragment separately off of the BAC using two sets of primers designed for each fragment and placing one at a time in pJET1.2. The next step involves cutting the 3' and 5' fragments out of pJET1.2 cloning them into the pGX-attP vector.

The *Drosophila* marker, *white* gene (w^+), is also on the donor construct and marks the mutation via eye color. The donor sequence has two FRT sites introduced at each end. In addition, a target site for a very rare cutting restriction endonuclease, I-SceI will be introduced at each end of the donor DNA (Huang et al. 2008). The donor sequence will be cloned into the ends out targeting and I-CreI (endonuclease induces homologous recombination) vector, pGX-attP, which carries an attP-50 site that mediates efficient DNA integration in *Drosophila* (Huang et al. 2008). Once the construct is injected into embryos and the flies come out, a series of two crosses are done, including the targeting cross (heat shock to activate FLP and I-SCEI to act on FRT sites for recombination and linearize circular DNA, respectively) and the screening cross (for non-mosaic progeny that have w^+ gene and are possible mutants).

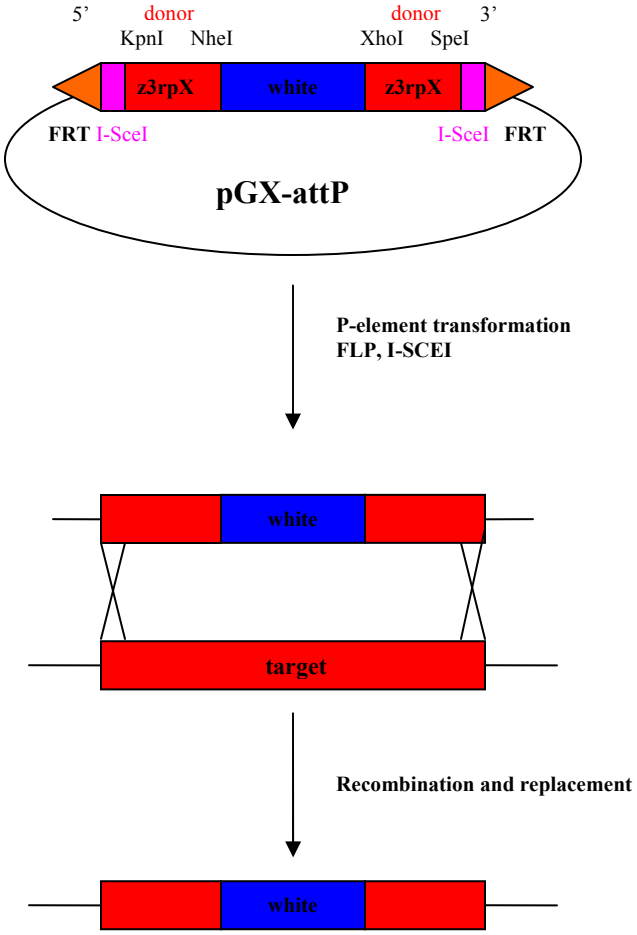

Figure 15: Ends-out gene targeting method to knockout *z3rpX*

The transgenic donor construct is diagrammed at the top within the P-element transformation pGX-attP vector that carries recognition sites, FRTs (flipase recombinase targets) (orange arrows), for a site specific recombinase (FLP), site specific endonuclease (I-SceI) (purple rectangles) to cut and make a linear fragment, z3rpX donor DNA (red boxes that are 5' and 3' fragments engineered with the shown restriction enzymes) from the locus to be targeted and the *Drosophila* mini-white (blue box) gene sequence that will interrupt the z3rpX gene and knock it out. Once the donor construct is introduced to the flies by transformation, targeting is induced by crossing transformants to flies with FLP and I-SceI, which will be activated by heat shock. The FLP-mediated excision and I-SceI-mediated cutting to produce the extrachromosomal targeting molecule shown. DSBs are present at the ends of the donor fragment because of the FLP excision, so that stimulates homologous recombination between the donor fragment and z3rpX target DNA to repair the breaks. As a result, the mini-white gene will integrate into the target gene, interrupting the function of the z3rpX gene.

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EDUCATION

- M.S. Cell and Developmental Biology, May 2012, Rutgers The State University of New Jersey

- B.S. Biology, Chemistry minor, May 2008, Delaware State University

RESEARCH EXPERIENCE

- May 2009-May 2012: Masters research with Dr. Kim McKim, Waksman Institute, Rutgers University, New Jersey

- September 2007-May 2008: Undergraduate research with Dr. Fatma Helmy, Department of Biology, Delaware State University, Delaware

- June 2007-August 2007: Summer Undergraduate research with Dr. Jennifer Zarcone, Strong Children's Research Center, University of Rochester, New York

- June 2006-August 2006: Summer Undergraduate research with Dr. Prabhas Moghe, Department of Biochemistry/Biomedical Engineering, Rutgers University, New Jersey

- June 2005-August 2005: Summer Undergraduate research with Dr. Constance Noguchi, NIH, NIDDK, Maryland

PUBLICATIONS

- Joyce EF, Pedersen M, Tiong S, **White-Brown SK**, Paul A, Campbell SD, McKim KS. (2011). Drosophila ATM and ATR have distinct activities in the regulation of meiotic DNA damage and Repair. J Cell Biol **195**(3): 359-67.

- Helmy F, **White S**, Amiri S, Amiri R, and Saliu A. (2010). On the different lipolytic capability of diverse organs from young adult guinea pigs. A chromatographic study. J. Plan Chromat **23**(4): 277-81.