RECOMBINATION HOTSPOT ACTIVITY IN DROSOPHILA MELANOGASTER

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ABSTRACT OF THE THESIS

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Sexual reproduction depends on the success of faithful chromosome transmission during

meiosis to yield viable gametes. Crucial to proper meiosis is the process of recombination

between paternal and maternal chromosomes which ensures normal homologous chromosome

segregation. Errors in number and location of the recombination events are known to be one of

the leading causes of nondisjunction and aneuploidy. Recombination events tend to cluster in

certain regions of the genome where the frequency of recombination is high compared to the

average recombination rate. These regions are called recombination hotspots. The identification

of these hotspots will bring us closer to understanding the etiology of nondisjunction. We used

transposon insertion at defined sites to test for recombination hotspots in *Drosophila*

melanogaster. The first method employs two transposable element bearing fly strains that have

the insertion at sites flanking the proposed hotspot. The number of recombination events in the

interval between the two transposon insertion sites is used to detect hotspots. The second

strategy is to generate a double strand break (DSB) by mobilizing the excision of the transposon

from a precise location. By monitoring the outcomes of DSB repair event at these loci, sites that

have a higher recombination rates can be detected. Unfortunately, both methods did not reveal hotspot activity.

Studies in yeast and mammals have uncovered a few genes that have a large effect on the distribution and pattern of recombination events. In *Saccharomyces cerevisiae,* Set1 complex (also known as COMPASS complex) has been shown to decreases DSB frequencies at > 80% of DSB sites genome-wide and cause changes in their localization. We want to know if Set1 complex plays a major role in controlling DSB sites in *Drosophila Melanogaster* also. RNAi mediated knockdowns of subunits of the Set1 complex are used to investigate if these flies show a reduction of DSBs. Although Set1 complex knockdowns did not have a significant effect on the DSBs, they may play a role in the repair of DSBs and oocyte development.

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The Origin of Aneuploidy

Aneuploidy is an abnormal number of chromosomes. Improper chromosome segregation during meiosis leads to genetically unbalanced eggs or sperm. If these gametes participate in fertilization, the resulting embryo will be aneuploid. Non-disjunction is the failure of chromosome pairs to separate properly during meiosis stage 1 or stage 2, specifically in the anaphase. Thus, Aneuploidy occurs when the chromosomes do not separate properly during cell division.

An extra or missing chromosome is a common cause of genetic disorders. Cases in which the embryo carries an extra copy of a given chromosome are said to be trisomic, whereas those that carry but one copy are said to be monosomic for that chromosome. Trisomic and monosomic embryos account for at least 10% of human pregnancies and, for women nearing the end of their reproductive lifespan, the incidence may exceed 50%. Most of these terminate in utero, making aneuploidy the leading known cause of miscarriage but some (e.g. trisomy 21) are compatible with live birth, making aneuploidy the leading cause of congenital birth defects and mental retardation(1).

Meiosis

Meiosis generates haploid gametes through a specialized cell division that consists of one round of DNA replication followed by two rounds of cell division. The first division of meiosis (MI)

involves the segregation of homologous chromosomes from each other. The second division of meiosis (MII) involves the segregation of the sister chromatids.

Successful segregation of homologous chromosomes requires the maintenance of physical connections between homologues until anaphase I. This role is fulfilled by sites of recombination, or chiasmata. During MI prophase the homologous chromosomes synapse and undergo recombination resulting in a pair of interlocked chromosomes, known as a bivalent. Formation of chiasmata not only maintains homologue association but also allows genetic exchange. Physical connection between homologue is important for the proper assembly and orientation of the chromosomes on the metaphase plate, where homologues bi–orient (i.e. attach to the opposite sides of the spindle). This allows the spindle apparatus to separate the homologues in anaphase I, when the chiasmata are finally resolved. Therefore, the existence of at least one chiasma per pair of homologues is essential to prevent their premature separation.

The diploid set of chromosomes becomes duplicated during premeiotic S phase and two sister chromatids are generated per chromosome. Thus each homologue is composed of two sister chromatids that must be kept together until anaphase II when the sister chromatids segregate. This role is fulfilled by a complex of proteins called cohesins. Chiasmata serve to link homologous chromosomes together by taking advantage of this strong sister chromatid cohesion maintained along both of the homologs. The sister chromatids must be kept together by this ring like protein structure that embraces the two sister chromatids at their centromeres and along the chromosome arms. Cohesin is required to maintain chiasmata until they are resolved. Thus the resolution of chiasmata and the disjunction of homologues depend on cohesin cleavage. Cohesins along the arms of the chromosome are cleaved at the metaphase I

to anaphase I transition allowing the disjunction of the homologues during MI. But the two sister chromatids within each homologue remain linked because centromeric cohesion is preserved. Protection of centromeric cohesion in meiosis I is therefore another key process that ensures proper chromosome segregation. Centromeric cohesion is only resolved at the metaphase II to anaphase II transition, where the sister chromatids are separated and gametes containing single chromatids are generated (Fig.1). To orchestrate the orderly separation of sister chromatids at MII, cohesion must be released along the chromosome arms at anaphase I (to allow the separation of homologues) but maintained between sister centromeres until anaphase II.

Non-disjunction

Meiosis is a very complex process with a large number of cell cycle events that need to be coordinated. Delay or failure in any the tightly regulate events can lead to non-disjunction of chromosomes and aneuploidy. Failure to resolve chiasmata between homologous chromosomes at anaphase I results in 'true' non-disjunction, whereby the homologues segregate together at MI. In addition, the premature resolution of chiasmata or failure to establish chiasmata between a pair of homologues can result in independent segregation of homologues, which leads to an error if both segregate to the same pole of the MI spindle. Studies show close dependence of nondisjunction on reduced chiasma frequency (1). Reductions arise either from decreased exchange or less often from premature chiasma resolution.

Terminalization or premature loss of connections between homologues due to loss of sister chromatid cohesion has been proposed to be an important contributor. Terminalization is the loss of chiasmata through their movement towards the ends of chromosomes and the eventual slippage off those ends. Terminalization of chiasmata is therefore different from chiasmata resolution, which happens quickly at the end of metaphase I to allow anaphase I to proceed. Terminalization would result in loss of linkage between homologues, failure to properly localize the homologues together on the metaphase plate and therefore missegregation and aneuploidy. Cohesin might physically block chiasmata movement and thus prevent terminalization. Similarly, premature loss of cohesion between sister centromeres can lead to segregation errors at either the first or second meiotic division (4).

Sex-specific differences and Maternal age effect

Over the past fifteen years, more than 1,000 trisomic or monosomic conceptions have been examined to determine the parental origin and meiotic stage of the nondisjunction error (6). Since monosomies are almost always early embryonic lethal, most of the available data is derived from trisomies. These studies indicate remarkable inter-chromosomal variation in nondisjunction. However, regardless of the chromosome involved, most human trisomies originate from errors in maternal meiosis. Maternal errors predominate, accounting for over 90% of cases (see table 2). Maternal origin of the extra chromosomes predominates, ranging from 100% for chromosome 16 to 81% for chromosomes 2-12, with the exception of the major paternal origin (44%) of the extra X in 47, XXY males. The results from the early studies also demonstrated that increasing maternal age is a powerful contributor to the occurrence of aneuploidy. Among women under the age of 25 years ~2% of all clinically recognized

pregnancies are trisomic, but among women over 40 years this value approaches 35%. Since the female is at greater risk, it can be inferred that either more errors are made in the female or these errors are more efficiently culled in the male. Recent studies support both the scenarios.

Although the basic features of meiosis are same for both male and females, there are important sex-specific differences in the time of onset, duration and the outcome of the meiotic process. In human males, meiosis begins with puberty continues for the lifetime of the organism, with largely uninterrupted development from the spermatogonium—a germ stem cell—to the mature sperm. Each cell that enters meiosis progresses through it without delay producing four sperm. Female meiosis on the contrary begins during fetal development, proceed until the end of prophase I and then arrest in a stage known as dictyate arrest. At the end of prophase 1, after the homologous chromosomes undergo synapsis and initiate recombination, the oocytes enter a period of meiotic arrest which could last anywhere from 10 to 50 years (and beyond). Meiosis resumes years later completing M1 and then arresting at metaphase of M2 until fertilization. During this long period of arrest, the physical linkage between oocyte sister chromatids must be preserved to ensure subsequent proper chromosome segregation. With increasing age this seems to fail. Several recent studies provide compelling evidence that the deterioration of sister chromatid cohesion is a crucial reason for age dependent aneuploidy (although certainly not the only one (2).The duration of the division provides ample opportunity for errors to occur and to accumulate, which is a feature that has been the basis of a number of hypotheses to explain the maternal age effect. The incidence of fetal trisomies increases markedly in women aged over 35 with more than one third of aneuploid embryos at this age; therefore this problem is not a trifle (4).

Table 2 | **The origin of human trisomy**

Trisomy	No. of cases	Origin (%)				
		Paternal MI	MII	Maternal MI	MII	Post-zygotic mitosis
2	18	28	–	54	13	6
7	14	–	–	17	26	57
15	34	–	15	76	9	–
16	104	–	–	100	–	–
18	143	–	–	33	56	11
21	642	3	5	65	23	3
22	38	3	–	94	3	–
XXY	142	46	–	38	14	3
XXX	50	–	6	60	16	18

Terry Hassold & Patricia Hunt (April 2001) *Nature Reviews Genetics* 2, 280-291

Aberrant recombination is associated with all MI errors

Recombination in oocytes happens early in meiosis thus it seems unlikely that age effect could occurs because of something that happened prenatally. However, studies of human trisomies suggest that recombination failure is, indeed, an important mechanism of human nondisjunction. Recombination tethers homologous chromosomes, linking them with physical structures known as chiasmata and guiding them through proper segregation during the first meiotic stage (MI). So, it is not surprising that errors in recombination (which takes place in MI) correlate with all human trisomic conditions. Maternal meiosis I errors constitute the most important single class of non-disjunction in humans, but chromosome-specific patterns exist. Maternal MI errors predominate among almost all trisomies, except Trisomy 18 which mostly originates from maternal meiosis II errors (see table 2)**.** A proportion of these trisomies are associated with failure of recombination. The errors arise because the nondisjoining bivalent is "achiasmate." Immunofluorescence methodology has made it possible to examine crossover

associated proteins in spermatocytes and oocytes. Strikingly, in the male, almost all chromosomes are joined by at least one crossover, but the same does not apply to the female. It appears that over 10% of all human oocytes contain at least one 'crossover-less' bivalent that never engages in genetic recombination (3). Consequently, the homologs are left to drift independently across the metaphase plate. This is especially true for the X chromosomes in humans. In studies of sex chromosome trisomy of maternal MI origin approximately 40% of all examined cases showed no evidence of recombination between the nondisjoined Xs (7). As at least three to four exchanges are predicted for the X chromosome bivalent, this represents a significant departure from expectation. However, evidence for a similar effect involving autosomes is not nearly as compelling and in humans the association between absence of recombination and nondisjunction may be largely restricted to the sex chromosomes.

Majority of the cases of human non-disjunction are preceded by an exchange. Reduced recombination is a feature of most, if not all, human trisomies of MI origin (7). Reduced recombination which results in inappropriate location of meiotic exchange has been associated with nondisjunction. Although the magnitude of the effect is variable, significant reductions in recombination have been found for all MI-derived trisomies studied to date, including trisomies 15, 16, 18, 21, Klinefelter syndrome (47,XXY) and 47,XXX of maternal origin (6). Additionally, the location of the exchanges also seems to be important. Altered placement of meiotic recombination has been identified for another subset of maternally derived trisomy 21 and all cases of trisomy 16 (3). Exchanges that are either too near or too far from the centromere are at an increased risk of non-disjunction (7). Sister chromatid cohesion must be maintained proximal to the chiasma and most essentially in the centromeric regions to ensure reductional separation

at MI. Resolution of extremely proximal exchanges would require the release of sister chromatid cohesion in regions too close to the centromere to allow normal centromere function at MI. By limiting most exchanges to more distal regions, the cell also limits the extent of sister chromatid release required for proper separation and chiasma resolution at the onset of anaphase, thus keeping its centromeres out of jeopardy. Similarly, the exchange events that are placed more distally along the chromosome arm appear to be less efficient at proper chromosome segregation, possibly because they are unable to lock homologs together, increasing the likelihood of premature separation of the bivalent allowing independent movement. Also, if the homologues are only joined by a distally located crossover, loss of cohesion past the point of exchange could uncouple the homologues and lead to aneuploidy (4). Impairment in the functioning of sister chromatid cohesion proteins might well preferentially affect this type of bivalent.

Aberrant recombination is associated with most MII errors

A proportion of trisomy also appears to result from errors at meiosis II (MII). As recombination takes place during MI, there was initially little reason to believe it would be altered among the MII cases also. Many meiosis II errors actually originated in meiosis I with the establishment of a susceptible exchange pattern. Crossovers that occur too close to the centromere may predispose to 'chromosome entanglement' at MI, with the bivalent being unable to separate and passing intact to the MII plate; at MII the bivalent divides reductionally, resulting in a disomic gamete. In this situation, the additional chromosomes would have identical

centromeres, and thus be scored as an MII trisomy, even though the origin of the abnormality was at MI. Although these errors appear to occur during meiosis II, the event actually initiated during meiosis I. True MII errors may be relatively rare in humans(7). That is, in studies of trisomies of maternal origin, MII errors are typically reported to account for approximately 20–30% of all cases (table 2). If these errors instead reflect abnormal processing of specific MI chiasmate configurations, it may be that virtually all human nondisjunction is attributable to errors at maternal MI, with errors at maternal MII and paternal MI and MII occurring at low, 'background' levels (7).

Aberrant recombination might render chromosomes more susceptible to later problems, and those might increase with age. One of the more popular models proposed that at least two 'hits' are required for age-dependent trisomy (2). The first involves the establishment of a susceptible bivalent in the in the fetal ovary; this component would be age independent. The second hit involves abnormal processing of the susceptible bivalent at metaphase I, in the adult ovary; this would be the age-dependent component of the process. The 'second hit' occurs during metaphase arrest, when there is an age-related degradation of components necessary for chromosome segregation (5). This increases the likelihood that susceptible exchange defective bivalents will non-disjoin. The meiotic spindle comprises a complex mechanical network that facilitates the proper segregation of chromosomes. This has been proposed to be the target of the 'second hit', resulting in chromosome non-disjunction. This combination of improperly positioned exchange events and an age-impaired spindle seems to yield high frequencies of meiotic errors.

DSB Sites: where Recombination Initiates

Chomosomal regions where recombination occurs are important because recombination mislocalization can be a source of aneuploidies. Recombination events do not occur randomly along chromosomes. The number and position of crossovers are thus strictly regulated. Recombination is initiated by the formation of programmed double strand breaks (DSBs) all along the genome through a conserved mechanism involving Spo11, a homolog of the catalytic subunit of topoisomerase VI with no or little DNA sequence specificity. However, DSBs may not be made randomly throughout the genome, but rather may occur more frequently at places called hotspots. Their localization is highly regulated at several levels to ensure a precise chromosome distribution among gametes. Understanding the mechanisms that promote DSB formation genome-wide as well as their location undoubtedly provide additional clues that move us closer to understanding the connection between altered recombination and nondisjunction.

Recombination patterns in *Drosophila melanogaster*

A half-century of aneuploidy research has yielded a wealth of information on the incidence, origin and etiology of human aneuploidy. However, in order to develop therapies to reduce or eliminate non-disjunction we need to identify the molecular basis of meiotic non-disjunction and the mechanisms responsible for its age-related increases. This step has however proved challenging to carry out in humans. Most analyses of human recombination rely on an indirect approach; i.e., genetic linkage analysis of human pedigrees. Although it is possible to directly measure recombination in human oocytes, considerable hurdles are involved in obtaining the desired object of study - the fully mature, recently ovulated egg. Limited information that is available in this area is mostly based on studies of the spare oocytes that remained unfertilized after attempted in vitro fertilizations. Analysis, especially of earlier stages of meiosis where synapsis and recombination occur in human females, are technically challenging because they require collection of human fetal ovarian tissue which is virtually impossible to obtain. Further, given the many years of separation between prophase and the segregation of chromosomes at the first meiotic division, all stages of meiosis cannot be analyzed. Also, the fact that the female meiotic process is initiated during fetal development but chromosome segregation events occur in the adult female makes ascribing cause and effect difficult.

However, the meiotic pathway is extraordinarily conversed and humans follow the same basic program as do most other organisms. Over the past decades, studies of model organisms—in particular, studies of yeast and *Drosophila*—have yielded remarkable advances in our understanding of how meiotic chromosome pairing, synapsis and segregation occur in lower eukaryotes. *Drosophila melanogaster* is an excellent model organism to study early events of meiosis. The ovary is the largest organ in the female fly and the oocyte the single largest cell; however, the ovary is not essential for survival, allowing for extensive manipulation. The organization of the ovary also makes it an ideal model system to study meiosis in. The female ovary is arranged in accordance with developmental ages. A single ovary contains every stage of development from stem cell to mature egg, allowing easy comparison. A female *Drosophila* has two ovaries made up of approximately 18 ovarioles, each of which can be effectively considered an egg production line (9). Egg chambers bud off and mature as they pass down the ovariole, reaching the posterior as mature eggs competent for fertilization. With the use of genetic and cytological tools available recently many exciting advances have been achieved in our understanding of *Drosophila* meiosis.

 Past studies in *Drosophila melanogaster* have provided compelling evidence that abnormalities in genetic recombination perturb normal meiotic chromosome segregation. Recent studies of Drosophila and humans indicate that aberrant genetic recombination is an important component of nondisjunction in both species (7). Most detectable aneuploidy in Drosophila, as well as in humans, occurs as a consequence of nondisjunction at the first meiotic division. Additionally, in both, a proportion of nondisjunction is associated with failure to pair and/or

recombine and in both, exchanges which are either too distal or too proximal increase the likelihood of mal-segregation (7). Because the underlying causes of aneuploidy are the same, it is reasonable to expect that the origin of nondisjunction in flies will be useful and relevant in elucidating similar mechanisms in humans.

Recombination Hotspots

Recombination events may not occur randomly along chromosomes. Chromosomal regions where recombination occurs are important because recombination mislocalisation can be a source of aneuploidies. The distribution of meiotic exchange along chromosome arms is not proportional to physical length. First, the number of exchanges per bivalent does not fit a Poisson distribution, as there are very few non-exchange bivalents and too few with high numbers of exchange. Thus, recombination events are not distributed randomly between the paired chromosomes. Second, exchange occurs only in euchromatin, and not in heterochromatin, demonstrating that map length is not proportional to DNA content. Third, the frequency of exchange within the euchromatin is lowest near the telomeres and highest in the medial regions of the euchromatic arms. Fourth, specific sites along chromosomes function as hotspots for exchange initiation (5). Crossing over is a tightly regulated process as shown by the nonrandom frequency and distribution of events along each chromosome.

A key to understanding the factors that determine the landscape of recombination is a determination of the chromosome-wide distribution of recombination events at the greatest resolution possible. In humans and many other eukaryotes recombination tends to cluster in recombination hotspots, regions of approximately 2 kb wide in which the rate of recombination

may be one or more orders of magnitude higher than the genome average. However, it is an open question whether hotspots exist in the *D. melanogaster* genome, or to what extent recombination rates vary on a fine scale. Although initially, the classical analysis of crossing over was not able to show the presence of significant hotspots for cross overs (CO) events in D. melanogaster (11), the resolution of such studies may have been insufficient to identify such sites. New sequencing technologies now make this possible. Comeron and colleagues couple the power of classical genetics with next-generation sequencing to provide for the first time a high-resolution recombination map of the *D. melanogaster* genome (8). This study reveals that at a large-scale (100 kb), CO rates exhibit extreme and highly punctuated variation along chromosomes, with hot and coldspots. A low-resolution approximation to the distribution of CO rates along chromosome arms recovers the same general, large-scale distribution as previous maps based on visible markers. As expected, rate of recombination is sharply reduced near telomeres and centromeres, and no CO events were detected in the small fourth chromosome that proceeds to meiotic segregation without chiasmata. This heterogeneity in CO rates is highly punctuated, with intense short-distance variation and several adjacent 100-kb windows differing by 15-to-20-fold thus defining hot- and coldspots for CO in *D. melanogaster (8)*.
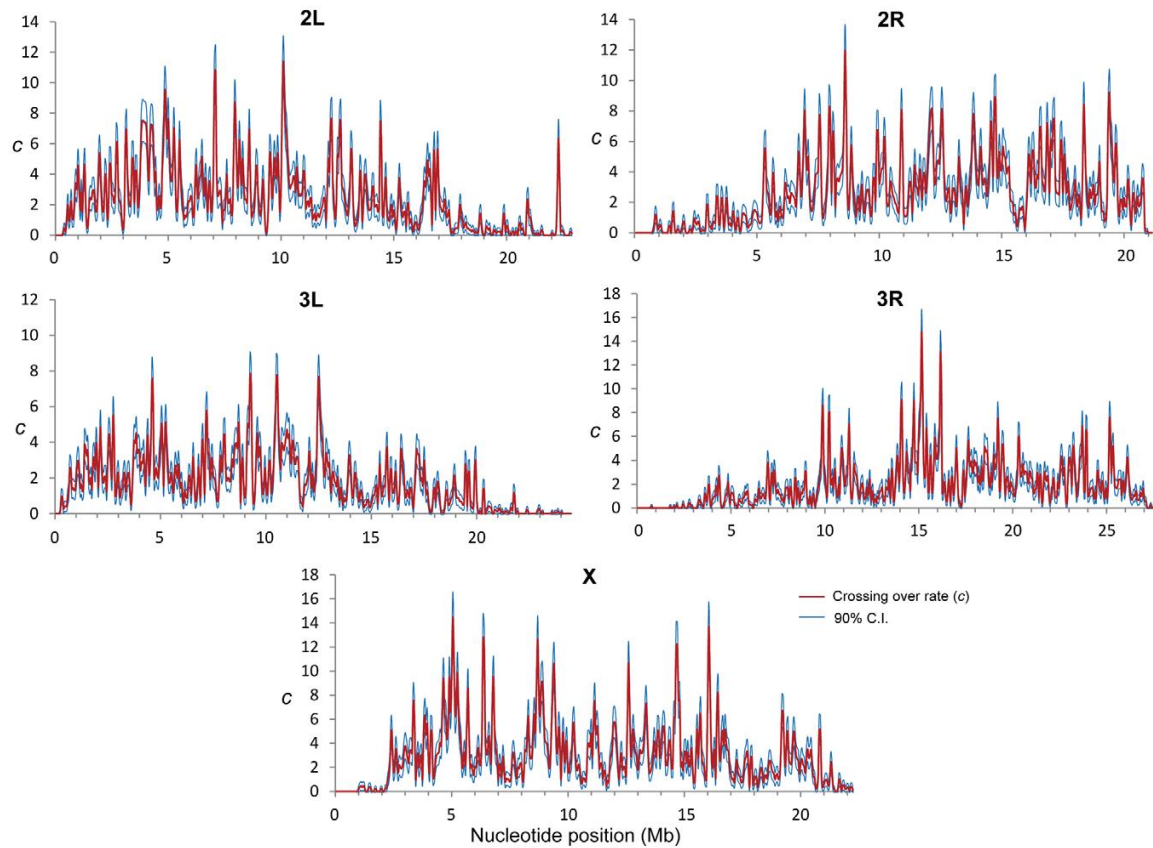
Fig.1 Crossing over rate (c) variation along chromosome arms in D. melanogaster. Rate of crossing over (c) based on data from all crosses and indicated in centimorgans (cM) per megabase (Mb) per female meiosis (red line). c is shown along chromosomes for 100-kb windows and a movement between adjacent windows of 50 kb. Blue lines indicate 90% confidence interval for c at each window. Comeron JM, Ratnappan R, Bailin S (2012) PLoS Genet 8(10): e1002905.

Recombination begins with a DSB

Meiotic recombination can be monitored at two complementary levels: DSBs, which reflect the initiation events, and COs, which reflect one outcome of meiotic repair. In *Drosophila,* meiotic recombination requires a Spo11 homolog, MEI-W68 which is thought to be the enzyme that catalyzes the formation of DSBs (10). The identification of Spo11 homologs in many species

suggests that the formation of DSBs is a conserved mechanism for initiating meiotic recombination.

DSB are either repaired as crossovers or non-crossover. DSBs can be repaired through the formation of a double Holliday junction that can resolve into a cross-over. Alternative means for repairing DSBs do not result in cross-overs (including alternate resolutions of double Holliday junctions or the distinct process of synthesis-dependent strand annealing) and often instead form gene conversion tracts (or non-crossovers). Conversion tract is the region inside the hot-spot interval wherein segment of the chromosome that initiates double-strand breaks are replaced in a non-reciprocal manner by segment from the homologous non-initiating chromosome. Crossovers involve reciprocal exchange of genetic material which is when the two homologs have exchanged large segments of genetic information, while non-crossovers (or gene conversions) result in non-reciprocal exchange. The crossover/non-crossover decision is not random, since in most organisms the number of gene conversions is in significant excess to the number of crossovers. Proper segregation of chromosomes requires a precise number and location of CO per chromosome pair. These requirements for homologous recombination during meiosis highlight a key point: DSB formation and repair have to be tightly regulated to integrate these chromosome-wide controls and to avoid aneuploidy. How sites for DSB formation are

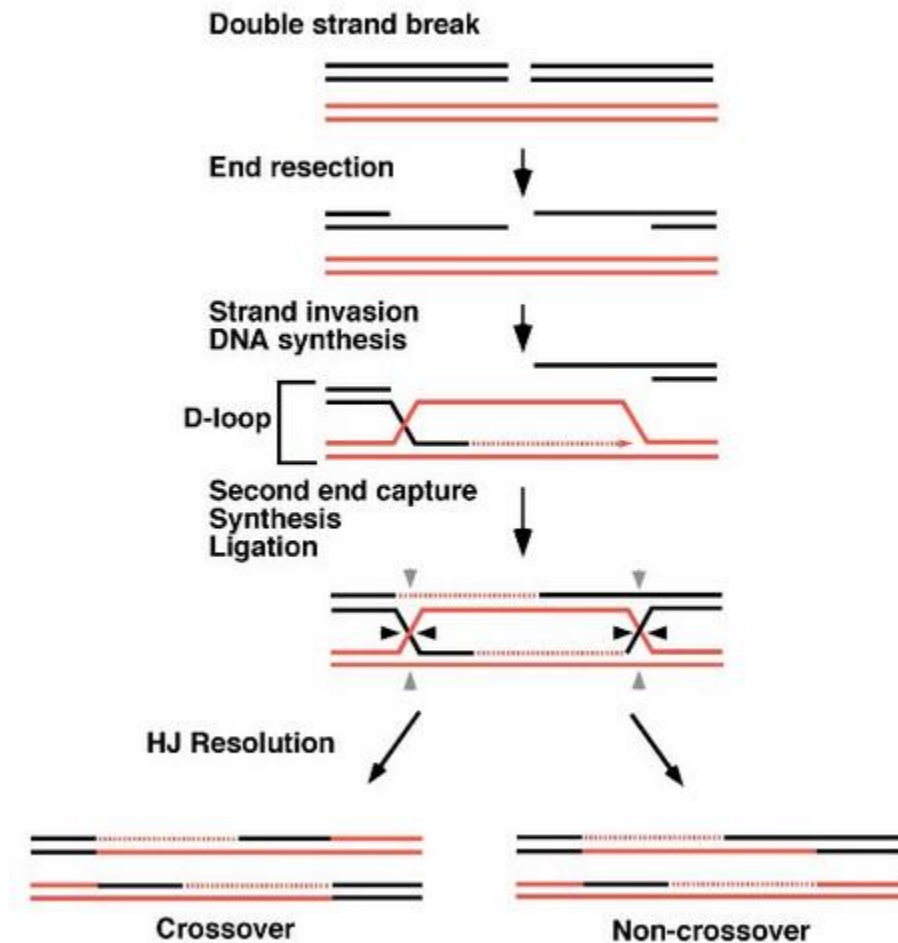selected, however, is poorly understood in Drosophila.

Fig.2 The double-strand break repair model of meiotic recombination. Homologs are indicated in

black (paternal) and red (maternal). Spo11 generates a DSB in one of the parental chromatids. After

Spo11 removal, DSB ends are resected to generate 3'-ended ssDNA tails and one 3'-ended ssDNA tail

invades the duplex homologous DNA sequence (red lines). Capture of the second ssDNA end and DNA

synthesis create a double Holliday junction (dHJ), whose resolution can occur in either plane at both

junctions (triangles) to generate crossover or non-crossover products. Red arrows indicate the 3' ends of

the newly synthesized strands.

M.P. Longhese et al. / DNA Repair 8 (2009) 1127–1138

Gene Conversion Maps can inform about DSB initiation sites

Comeron and colleague have made an attempt to shed light on the DSB landscape by providing

a Gene conversion/ non-crossover map. Because DSBs are generated in excess and only a

fraction of them generate CO, one has to keep in mind they are not necessarily equivalent. GC

can still serve as a good indicator of DSB patterns. The analysis of the distribution of GC rates

along chromosomes reveal a more uniform distribution than that of CO rates, with no reduction

near telomeres or centromeres (8). This suggests there is increased gene conversion in regions

of severely restricted crossing over, consistent with DSBs occurring in these regions but being

preferentially repaired by non-cross-over means. In spite of the uniform distribution, the

location of GC events or DSBs along the chromosome arms are NOT completely random, with

significant heterogeneity along each chromosome. There are sites that have a higher probability

of a DSB. From these maps, it is clear that GC and COs are unevenly distributed along the

genome, defining regions called hotspots, where recombination occurs more frequently than in
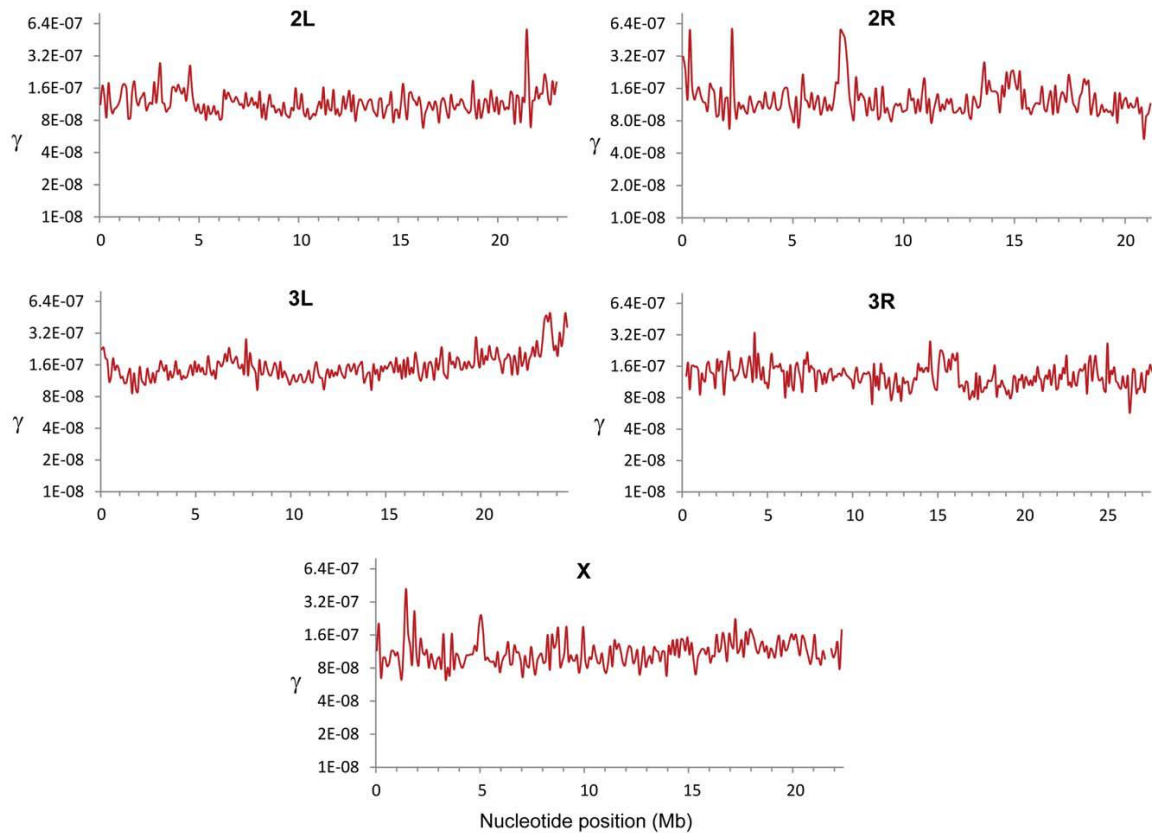
the rest of the genome (8).

Fig.3 Estimates of gene conversion initiation rate (γ) along chromosome arms in *D. melanogaster.* Comeron JM, Ratnappan R, Bailin S (2012) PLoS Genet 8(10): e1002905

Set1 Complex controls DSB initiation sites in Yeast

Along chromosome arms, DSBs appear in domains alternating with regions with less frequent DSBs. Their localization has to be regulated at several levels to ensure a precise chromosome distribution among gametes. The factors responsible for the selecting DSB sites are mostly unknown in Drosophila. Certain histone modifications affect the localization of DSBs; H3K4me3 has been detected at meiotic DSBs in *Saccharomyces cerevisiae* and at active meiotic recombination hotspots in mammals. Set1 complex, (the only known H3K4 methyltransferase activity in yeast), has been shown to be responsible for the hotspot activity. It has been shown that > 80% of DSB sites genome-wide are dependent on Set1 (12). Disruption of Set1 causes

dramatic changes in the pattern of DSB sites across the yeast genome suggesting that

recombination might be regulated by a few genes with large effects. We want to know if Set1

plays a major role in controlling DSB sites in Drosophila also. Identifying such major molecular

components that control the recombination will shed new light on how the placement of

recombination is determined, providing additional clues to the link between altered

recombination and chromosome nondisjunction.

Exploring the role of COMPASS Complex in Recombination Activity in Drosophila

COMPASS Complex (<u>com</u>plex of <u>p</u>roteins <u>as</u>sociated with <u>Set</u> 1) also known as Set1 Complex (Set1-C) is a H3K4 methylase. The Complex is composed of 7 subunits. All subunits show a striking conservation and most subunits are required to efficiently catalyze methylation of H3K4. Set1 is the catalytic subunit of the complex and has been shown to play a role in growth, transcriptional activation, repression and elongation, telomere length regulation, telomeric position effect, rDNA silencing, meiotic differentiation, DNA repair and chromosome segregation. The focus of this project is to explore the role of COMPASS complex in DSB initiation during meiosis.

Role in transcription

Histone H3 lysine 4 methylation (H3K4me) is associated with the transcriptionally active regions of the genome in yeast, flies, and mammals. Genome-wide studies in exponentially growing *Saccharomyces cerevisiae* cells show that H3K4me3 peaks at the start of transcribed portions of genes. H3K4me3 is thought to facilitate transcription through the recruitment of nucleosome remodeling complexes and histone-modifying enzymes, and by preventing repressors from binding to chromatin. H3K4me3 levels are highly dynamic, and affected both by transcription induction and transcription repression (12). Because this modification is required for the regulation of gene expression, absence of Set1p and histone H-3-lysine-4-methylation caused a decrease in the level of expression of about 80% of the genes in *S. cerevisiae* (14). Absence of

histone H3 lysine 4 methylation results in genome-wide gene repression, suggesting that methylation of lysine 4 is required for normal regulation of expression of diverse families of genes.

Set1-C drives H3K4 methylation in a transcription dependent manner due to its indirect association with the RNA polymerase. Set1 has been found to be predominantly associated with the coding regions of highly transcribed RNA polymerase II (Pol II) genes, and the presence of trimethylated H3K4 generally correlates with Set1 occupancy. Set1 associates with the Pol II CTD through the Paf1 complex, although there is no evidence for a direct interaction among any of the subunits of the Paf1 complex with the subunits of the Set1 complex (Set1- C) (13).

The COMPASS complex is responsible for mono-, di-, and trimethylation of H3K4. While trimethylated H3K4 peaks at the beginning of the transcribed portions of genes, dimethylated H3K4 (H3K4me2) is most enriched in the middle of genes, and monomethylated H3K4 (H3K4me) is found predominantly at the end of genes (13). These important results indicate that active transcription is characteristically accompanied by histone H3K4 trimethylation at the beginning of genes, and by H3K4 dimethylation and monomethylation at nucleosomes positioned further downstream in the transcription unit. Set1 associates with newly initiated Pol II when Pol II's carboxy-terminal heptad repeat (CTD) is phosphorylated at Ser5. It has been proposed that *Set1* is released from Pol II after the loss of Ser5 phosphorylation and that the binding of Set1 to Ser5 phosphorylated Pol II restricts H3K4 trimethylation to the 5' end of the genes (13). Although a significant fraction of Set1 may be released from Pol II after the loss of Ser5 phosphorylation, *Set1* can also associate with Pol II, when its CTD is phosphorylated on Ser2 which is how it

carries out the di- and mono-methylations(13). Although a direct correlation has been found between gene transcription activity and H3K4 trimethylation, the function of mono- and di-methylated H3K4 remains unknown. It is not yet clear whether the different methylated states of H3K4 are biologically significant.

There is only one Set1 in yeast; yet in mammalian cells there are multiple H3K4 methylases, including Set1A/B, forming human COMPASS complexes, and MLL1-4, forming human COMPASS-like complexes. In *Drosophila melanogaster*, three homologues of the COMPASS complex, namely, Trithorax (Trx), Trithorax-related (Trr), and dSet1, have been reported to implement H3K4 methylation. Detailed genetic and molecular analyses showed that Trx is required to maintain activation states of its target genes throughout development and counteracts the repressive effects of the Polycomb group proteins. Trr was identified based on sequence similarity to Trx but was shown to function in the regulation of hormone-responsive gene expression (15). dSet1 was identified based on sequence homology to the *Saccharomyces cerevisiae* and mammalian Set1 proteins. RNAi-mediated knockdown studies demonstrated that dSet1 is responsible for bulk H3K4 di- and trimethylation, while the knockdown of Trx or Trr had less pronounced effects on H3K4me2/3. Studies show that dSet1 co-localizes with H3K4me3 and transcribing Pol II on polytene chromosome, and the loss of the dSet1-complex subunit, dCfp1, diminishes dSet1 and H3K4me3 at transcription puffs (16). Set1 mediated H3K4me3 levels are highly dynamic, and affected both by transcription activation and transcription repression.

Role in DSB initiation during meiosis

Meiosis also involves substantial transcriptional reprogramming. Both changes of expression and induction of recombination are expected to involve chromatin structure modifications. H3K4me3 is increasing in the 5'-part of genes that are transcriptionally activated during meiosis.

In *Saccharomyces cerevisiae*, H3K4me3 has been shown to have an essential function in the initiation of meiotic recombination, distinct from its tight association with meiotic gene expression. H3K4Me3 mark the sites of DSB initiation sites (20). The H3K4 trimethylation associated with transcription is very dynamic, which can be removed from chromatin upon transcription repression. However, the level of H3K4me3 is constitutively higher close to DSB, as compared with the DSB-poor sites, independently of local gene expression levels (12). Patterns of meiotic transcription in wild type and spo11Y135F strains are globally similar and similar H3K4me3 profiles are observed in the two strains suggesting that these methyl groups are not deposited after the DSB was made. H3K4me3 mark occurs preferentially near DSB sites in a manner that is independent of DSB. H3K4me3 marks natural DSB regions before entry into meiosis.

Without Set1, the H3K4 methylase, 84% of the DSB sites exhibit a severely reduced DSB frequency (12). The reduction of DSB frequencies at the vast majority of sites in set1 mutant shows that H3K4me3 modification is a common factor involved in DSB formation, irrespective of transcription levels. Although the absence of Set1 decreases DSB frequencies in at least 84% of the natural sites, not all DSB sites are affected to the same extent. Very highly trimethylated sites have a strongly reduced DSB signal in set1mutant, the few sites that were occurring in low trimethylated regions are less affected, and even more, some new sites appear in trimethylation 'deserts'(12). In the absence of H3K4me3, few new DSB sites appear, preferentially in regions

that are poorly trimethylated in wild-type cells. The function of H3K4me3 in DSB formation might be indirect by regulating the accessibility of the chromatin substrate to Spo11 and the other DSB-forming factors, either by recruiting chromatin remodelers or H3K4me3 mark can be direct in recruiting one or several of the factors involved in DSB formation.

The role of Set1-C in DSB initiation during meiosis has not been explored in Drosophila. The focus of this project is to investigate if Set1-C plays a role in choosing DSB initiation sites in Drosophila.

Structure and subunits

Set1 is a large protein bearing a conserved catalytic C-terminal SET domain in addition to its RNA recognition motif. SET domain proteins catalyze the methylation of lysine residues. The SET and RRM domains are conserved in Set1 orthologs from yeast to humans. In addition to the evolutionarily conserved SET domain located at the C terminus of Set1, most associating subunits are also conserved from yeast to human, forming Set1/COMPASS and MLL/COMPASS-like complexes (17).

The *Saccharomyces cerevisiae* Set1-C or COMPASS complex is made up of at least 7 subunits Swd1 (Rbbp5 in Drosophila), Swd2 (Wdr82 in Drosophila), Swd3 (wds in Drosophila), Bre2 (Ash2 in Drosophila), Sdc1 (DPY30-L1 in Drosophila) and Spp1 (Cfp1 in Drosophila) (13). All components of COMPASS also show a striking conservation and most being required to efficiently catalyze methylation of H3K4. Set1 is the catalytic subunit of the protein complex. The core complex is made up of Set1 and the 2 WD40 repeat proteins Swd1 (Rbbp5) and Swd3

(wds). Both proteins, which remain associated with each other in the absence of Set1, are essential for the integrity of COMPASS and the stability of Set1 (13). The integrity of the complex is preserved when one of the other subunits of Set1-C (Spp1, Shg1, Bre2, or Sdc1) is missing. Swd1/Swd3 heterodimer is essential for stability and integrity of the complex, and therefore for any H3K4 methylation. Sdc1 (DYP30-L) and Bre2 (Ash2) form a heterodimer that stimulates the catalytic activity of Set1, but is not required for Set1-C integrity (13). Bre2 interacts directly with Set1 and that this interaction requires the SET domain. Studies show that Bre2 and Sdc1 do not associate with a Set1-C that lacks the SET domain. Spp1 reads the H3K4Methylation and is involved in recruitment of DSB machinery. According to this model, Bre2 (dAsh2)/Sdc1 and Spp1 associate with the Set1/Swd1/Swd3 scaffold to promote H3K4 di- and tri-methylation. Therefore, the Set1 protein possesses very weak enzyme activity by itself and robust activity requires the whole complex. Neither of these subunits (Bre2, Sdc1, and Spp1) are required to target Set1-C to chromatin (13).

Studies have demonstrated that the *Drosophila* complexes are very similar to their yeast mammalian counterparts in subunit composition. One exception is *Hcf*, which does not seem to have a homologue in yeast. Its human homologue is a component of hCOMPASS(11). d*Hcf* has been found to be associates with other chromatin complexes too. For this project we carried out RNAi mediated knockdown of *Set1, Ash2, Wdr82, wds* and *Hcf* to explore their role in the DSB initiation during meiosis.
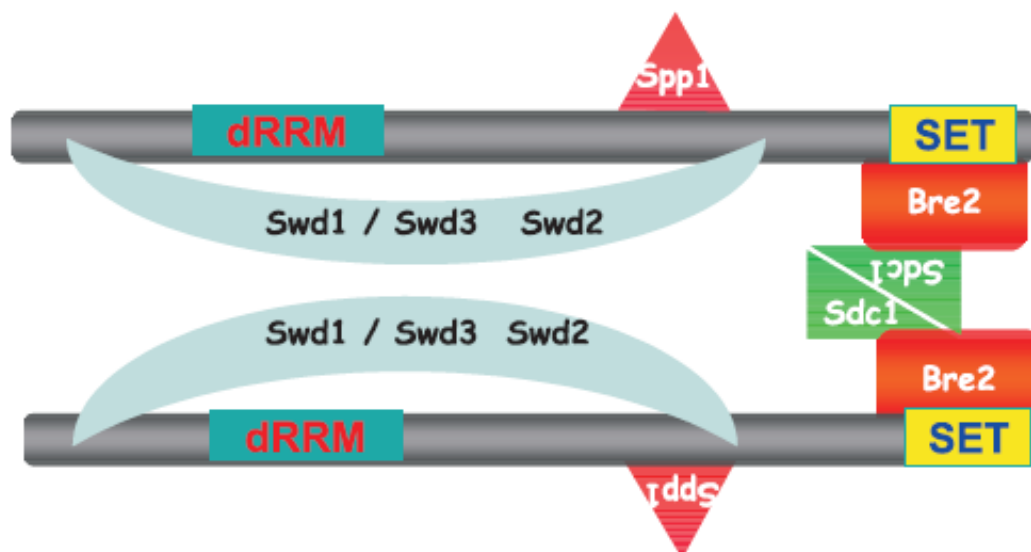
Fig.1 A model for the substructure of the Set1-C. Pierre-Marie Dehe´ and Vincent Ge´ li(2006)

Biochem Cell Biol. 84(4):536-48

Drosophila	#/N	#/C	Human	Yeast
dSet1/CG40351	50	30	hSet1a/b	Set1
dWdr82/CG17293	15	3	Wdr82	Swd2
WDS/CG17437	10	8	WDR5	Swd3
Ash2/CG6677	12	12	hAsh2	Bre2
dCfp1/CG17446	11	8	Cfp1	Spp1
dRbbp5/CG5585	10	8	Rbbp5	Swd1
dHcf/CG1710	20	9	Hcf	-
dDPY30-L1/CG6444	12	11	DPY30	Sdc1

Identified polypeptides from dSet1 complexes compared with human and yeast

complexes. #N, #C: unique peptides from N- or C-terminally tagged dSet1.

M Behfar Ardehali, Amanda Mei, Katie L Zobeck, Matthieu Caron, John T Lis and Thomas Kusch *(2011)*

EMBO J. 30(14): 2817–2828.

AIM

In *Saccharomyces cerevisiae,* Set1 complex has been shown to decreases DSB frequencies at > 80% of DSB sites genome-wide and cause changes in their localization. We want to know if Set1 plays a major role in controlling DSB sites in *Drosophila Melanogaster* also. RNAi mediated knockdowns of subunits of the Set1 complex are used to investigate if these flies show a reduction of DSBs in the absence of a functional COMPASS complex. Cytological analysis was done on germarium of the ovaries where recombination takes place to find out if the COMPASS Complex knockdown have reduced DSBs. DSB are detected using antibodies to γHis2AV which is a very good marker for meiotic DSB formation.

METHOD AND MATERIAL

RNAi mediated silencing of the Set1-C subunits

 RNAi is one of the post-transcriptional gene silencing phenomena, in which double-stranded RNA produced within host cells can effectively inhibit host gene expression in a sequence-specific manner. The transgenic RNAi fly lines carry the transgene on Valium vectors (Valium 20 &22). All the lines used for this experiment have the RNAi gene integrated into the attp2 landing sites on the 3rd chromosome, which permit high levels of inducible expression. All RNAi fly lines are homozygous for the RNAi except for the *Ash2* line. The following are the RNAi lines used in the experiment: *Set1* (GL00307)*, Ash2 (*GL00307)*, Wdr82 (*HMs00718)*, wds* (HMS00746) *and Hcf (*HMS00452)*.

By combining with GAL4-UAS gene expression system, the RNAi can be utilized for knocking down gene expression in a target cell or tissue at a specific developmental stage. The transgenic RNAi flies use the UAS/Gal4 expression system for the expression of the RNAi. The expression of the RNAi is controlled by the UAS element upstream of the transgene. The transcription of the RNAi requires the presence of Gal4 which binds to the UAS element and turns on the expression of the gene lying downstream of the element. To activate the transcription of the RNAi, these flies are mated to flies expressing Gal4 in a particular pattern. The Gal4 expressing fly lines are called the Drivers. A variety of drivers are available which express Gal4 in all or some subset of fly tissues. The availability of a diverse set of GAL4 drivers allows the transgenic RNAi library to be used to target gene inactivation to almost any desired cell type. Tissue specific RNAi knockdown is a very powerful tool for functional studies when it comes to the analysis of genes with pleiotropic effects like the components of compass complex. By knocking down genes in a tissue specific manner, effect of the knockdown in the tissue of interest can be assessed in an otherwise healthy fly.

Test for Viability

In order to find out if these subunits of the COMPASS Complex are required for viability, the RNAi must be expressed in all tissues. To achieve ubiquitous expression of the RNAi the RNAi fly lines are crossed with drivers that have Gal4 under the control of the Tubulin promoter. The tubulin driver leads to ubiquitous expression of Gal4 and the RNAi. The F1 generation of this cross has the gene of interest knocked down in every tissue.

Tubulin Promoter − GAL4   X   UAS - RNAi

$$\downarrow$$

$$\frac{UAS-RNAi}{Tubulin\ Promoter-GAL4}$$

Test for Fertility

As the genes of interest are required for viability, mutant flies cannot be made. RNAi mediated knock down in tissue of interest allows one to bypass the requirement for viability. By silencing these genes only in the germline we can find out if these genes are required for meiosis and fertility. In order to restrict the RNAi mediated only to the germline, driver line that expresses Gal4 only in the female germline was used. When the transgenic RNAi flies are crossed with the Nanos drive, the resulting progeny expresses Gal4 and the RNAi only in the female germline leading to gene silencing only in the germline. If the gene plays a critical role in meiosis, the progeny from this cross will have compromised fertility.

Nanos Promoter − GAL4   X   UAS - RNAi

$$\downarrow$$

$$\frac{UAS-RNAi}{Nanos\ Promoter-GAL4}$$

Genetic Analysis of Non-disjunction

Meiotic nondisjunction is most easily assayed on the X-chromosome. Autosomal nondisjunction generates inviable zygotes. Females that have germline specific knockdown are crossed to males carrying a dominant marker on the Y chromosome. The dominant marker on the Y chromosome

is Bar eye mutation (B^s) which gives them slit eyes. Both males and females carry a recessive (y) marker on the female X-chromosome. When y/yellow females are crossed to y /B^s Y males, the regular progeny are phenotypically yellow-bodied females with normal eyes (y/y) and yellow-bodied males with Bar eyes (y/B^s Y). Because the Bar is a dominant trait all male progeny is expected to have slit eyes and the female progeny, not carrying a Y chromosome are expected to have wild-type eyes. Occasionally, the X chromosomes do not disjoin during oogenesis and this produces eggs with either two X chromosomes (diplo-X) or no X chromosomes (nullo-X). Nondisjunction progeny is yellow-bodied Bar-eyed females (y/y /B^s Y) and yellow-bodied, white-eyed males (y /O) in addition to an equal number of dead embryos due to aneuploidy.

$$\text{Frequency of non-disjunction} = \frac{2 \text{ X number of nondisjunction flies}}{\text{total number of flies}} \text{ X } 100$$

Cytological analysis of the ovaries

To assess the effects of the RNAi mediated silencing of the different components of the COMPASS complex on meiosis, female expressing RNAi in the germline are dissected and the ovaries are examined. The dissected ovaries are further stained with antibodies. The stained ovaries are visualized using confocal microscopy. Cytological studies were carried out on *Set1, Ash2* and *Wdr82* only. *Wds* knockdown flies have very small ovaries which makes handling them difficult. Due to technical difficulty, cytological studies could not be done on *wds* ovaries. As *Hcf* is not requires for fertility, it was not pursued any further.

Drosophila females have two ovaries, each comprised of several ovarioles containing chains of developing oocytes. Each ovariole is divided into the germarium, where early prophase occurs, and the vitellarium, where oocyte growth and differentiation occurs. Because meiotic

recombination occurs in the germarium, we have focused on the germarium which is at the anterior end of each ovariole.
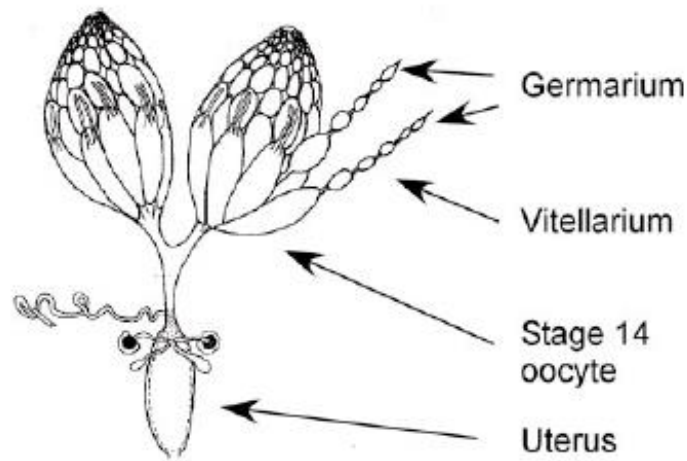

Fig. 2 Schematic of Drosophila Ovary. Kim S. McKim, Janet K. Jang, and Elizabeth A. Manheim (2002) Annual Review of Genetics Vol. 36: 205-232.

Ovaries were dissected and fixed using the 'Buffer A' protocol adapted from reference 29.

1. Prior to dissection of ovaries for immunostaining of germaria, feed females with yeast for 16 h at 25_C (see Note 2). They are not fed yeast for too long because the germarium is relatively small while the later and larger oocyte stages can get in the way. The ovaries from 15 to 20 flies were dissected in 1_Robb's media and moved to a clean well containing fresh media. A tungsten needle was used to remove the ovariolar sheath and to tease the ovaries apart. This procedure should take no more than 20 min from the first dissection. The separated ovaries were moved to the cap of a graduated 1.5mL Eppendorf tube containing 500 mL of Buffer A Fix solution and were left rotating in Fix solution for 9 min at room temperature. The ovaries were then allowed to settle for 1 min and as much liquid as possible was aspirated without removing the ovaries.

2. All washes were performed at room temperature in 1mL of solution unless otherwise noted. The fixed ovaries were washed twice for 15 min each with 1mL of BAT solution and blocked with two washes of BAT-NGS for 15 min each. After the liquid from the second BAT-NGS wash is removed, it was replaced with 100 mL of fresh BAT-NGS solution. Primary antibodies diluted for a volume of 300 mL were added and a graduated tube was filled to 300 mL mark with BAT-NGS. Overnight incubation was done on rotator at 4∘ C.

3. The ovaries were allowed to settle and supernatant containing primary antibody in BAT-NGS was aspirated. Four washes of BATBSA were performed at room temperature for 30 min each. This was followed by one wash with BAT-NGS for 30 min. While the ovaries are in the final BAT-NGS wash, the secondary antibodies/embryos were centrifuged for 10 min at full speed in a microfuge. After the wash, the ovaries were allowed to settle and the liquid was removed. Secondary antibody supernatant was added without any embryos to the ovaries and final volume was brought to 500 mL with BATNGS and incubated in the dark for 2–4 h at room temperature. The ovaries are kept in the dark during all subsequent washes.

4. The ovaries were then washed one time in BAT solution for 30 min. The ovaries were allowed to settle and the liquid was replaced with DNA dye in fresh BAT solution to stain for DNA. If using Hoechst, a 1:5,000 dilution was used and left on rotator for 7 min. One final BAT wash for 15 min and one quick wash in 1X Buffer A were performed. The ovaries are stored in 1mL of 1X Buffer A at 4_C in the dark until ready to mount. The ovaries were mounted in fluorescence mounting media. The ovarioles were completely separated using a tungsten needle before being secured with a coverslip and sealed with nail polish.

Confirm RNAi mediated knockdown

Status of H3K4 methylation was examined by immunofluorescence to ensure RNAi mediated

knockdown. The knockdown of the RNAi is driven by the Nanos promoter which is germline

specific. The efficiency of the knockdown is assessed by staining the ovaries with antibodies to

H3K4me3 and H3K4me2 and comparing the intensity of fluorescent signal of H3K4 di- and

trimethylation of oocyte to that of a follicle cell which is not germline derived. The intensity of

fluorescent signal of H3K4 tri- or di-methylation was qualified using the LICA software. Five

follicle cells and five oocytes were randomly selected from three germaria of wild-type and

knockdown flies, and average of the intensity of H3K4 di- and trimethylation signal in oocytes

and follicle cells was calculated. Relative signal intensity was calculated and graphed which is the

ratio of average signal intensity in the oocytes to that of the follicle cells. Decrease in this ratio is
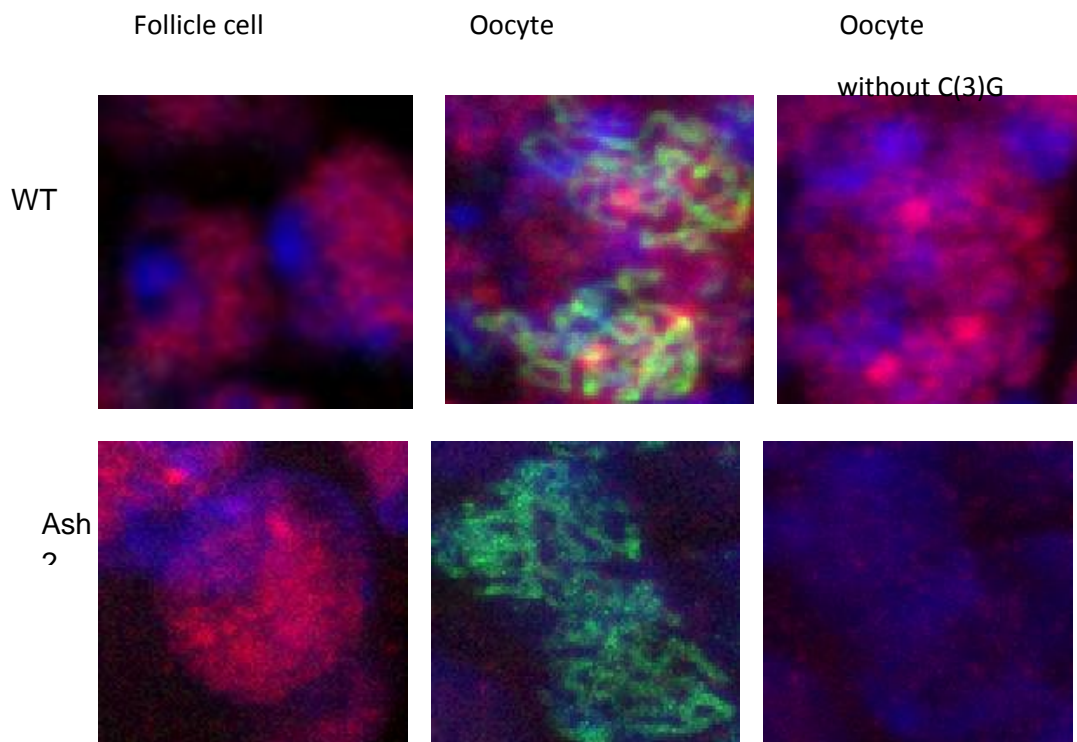
an indication of RNAi mediated knockdown.

Fig. 3 Immunofluorescence Analysis of H3K4 Methylation. Germaria of the indicated genotypes were stained with antibodies against trimethylated or dimethylated H3K4 (red), C(3)G (green) and DNA (blue).

Detecting DSB Sites with an antibody against γHis2AV

DSB are detected with an antibody against phosphorylated form of γHis2AV. In order to characterize DSB formation and repair during meiosis, an antibody specific to γ-His2Av was used. While phosphorylation of γ-His2Av is not itself required for repair of meiotic DSBs, it is an excellent marker for meiotic DSB formation. The phosphorylation response is rapid and appears with sufficient resolution to be counted (9). The number of γ-His2Av foci is used to estimate the number of meiotic DSBs per cell at various time points during prophase of meiosis.

Pro-oocytes are tracked and oocytes are distinguished from the nurse cells by probing for C(3)G which is the component of the Synaptonymal complex(SC). Formation of the SC reflects the pairing or synapsis of homologous chromosomes and can be used to probe the presence of abnormalities in the development of oocyte. DSB repair and oocyte development are linked such that a defect in DSB repair activates a signaling pathway that leads to defects later in oocyte development. Therefore, probing for SC also allows us to visualize the developmental status of the oocyte. The region where the Sc assembly initiates is designated region 2a. In region 2A, up to four cells within the cyst initiate assembly of the SC. However, only two pro-oocytes within the cyst enter into pachytene and form a full-length SC. At this stage the cyst takes on an oval shape and it is designated as region 2b. The two pro-oocytes undergo DSB repair and the decision to select one oocyte simultaneously, and both are completed in mid–late pachytene stage. Eventually, all cells except the oocyte exit the meiotic program and SC is

maintained in only the oocyte. This region with one oocyte with fully assembled SC is designated
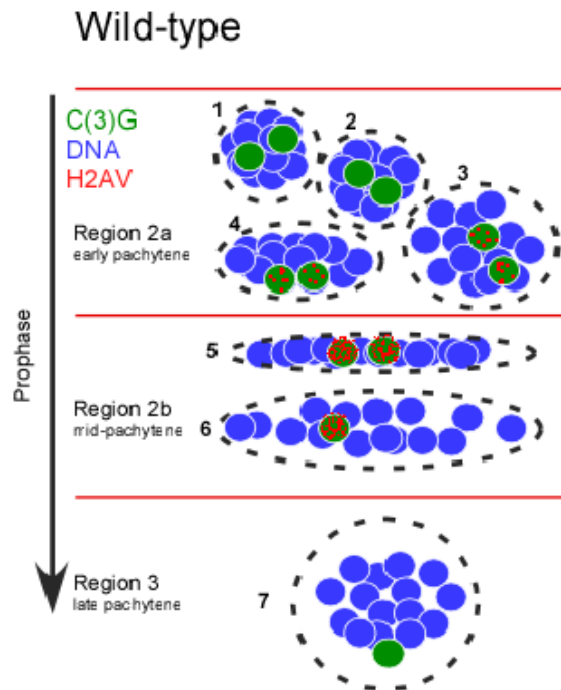
region 3 (21).

Fig. 4 Schematic depiction of the Drosophila germarium. Wild type germaria with SC shown in green, DSBs shown red and DNA or nuclei of cells shown blue.

Eric Joice and Kim McKim (2009) Genetics 181:39-51

γ-H2AV foci were counted only in the pro-oocytes/oocytes i.e. cells that had C(3)G assembly.

The foci were counted in all pro-oocytes/oocytes of each germarium by examining a full series

of optical sections containing a complete oocyte nucleus starting with the youngest cyst at the

anterior most end of the germarium. The germline stem cells reside in the anterior most part of

the germarium. Each germline stem cell divides asymmetrically to produce a cystoblast that

undergoes four rounds of synchronized mitotic cell divisions with incomplete cytokinesis to

produce a 16-cell interconnected cyst (9). The cysts move down the germarium as they mature

such that a cyst in a more posterior position is in a later stage of meiotic prophase than a cyst in

a more anterior position. The arrangement of cyst in the temporal order makes it possible to

track the relative age of the oocytes (and also compare oocytes at different stages of meiosis within a single germarium).

The average number of foci in the pro-oocytes/oocytes from 6 wild type germaria, 6 *Set1* germaria and 3 *Ash2* germaria were calculated and plotted as a function of relative cyst age.

RESULTS AND DISCUSSION

Subunits of Set1-C required for Viability

All the subunits of COMPASS Complex are required for viability as the RNAi mediated knockdown leads to lethality. Some (5%) of the *Hcf* knocked down flies do survive which could be due to inefficient expression of the RNAi.

RNAi	Phenotype
Set1	Lethal
ash2 (absent, small, or homeotic discs 2)	Lethal
Wdr82	Lethal
Wds (will die slowly)	Lethal
Hcf (Host cell factor)	Semi-viable (5% Sb+)

Table 1: Phenotype of flies with ubiquitous expression of the RNAi

Subunits of Set1-C required for Fertility

Knockdown had an impact on the fertility of all the RNAi lines tested. Flies with *Wdr82* deficient germline were fully sterile, where as those of *Set1, Ash2* and *wds* had severely compromised fertility. Hcf does not seem to be absolutely necessary for fertility, although these flies too had reduced fertility. *Hcf* crosses produced around 43 offspring per vial compared to 150 in the wild-type. This is not surprising because Hcf is not conserved in yeast. This suggests that it might not be necessary for the H3K4 methylation as COMPASS complex in yeast, is the only H3K4 methlase and carries out all methylation efficiently. The possibility that the RNAi mediated knockdown is not efficient in this strain cannot be excluded.

RNAi	Phenotype	# of flies/Vials (wt=150)
Set1	Sterile	3
ash2	Sterile	8
Wdr82	Sterile	0
Wds	Sterile	2
Hcf	Low-fertility	43

Table 2: Phenotype of flies expressing RNAi only in the germline. Flies scored are F1 progeny of RNAi strains crossed to nanos driver strains.

Set1 knockdown flies show elevated rated of Nondisjunction

Frequency of nondisjunction could not be tested in *Wdr82* due to complete sterility. However, *Set1, Ash2* and *wds* crosses did produce some progeny that was scored for nondisjuncion. Only *Set1* shows elevated rate of non-disjunction.

RNAi	% Non-disjunction	# of flies scored
Set1	14%	190
ash2	0%	112
Wdr82	0%	0
Wds	0%	15
Hcf	0%	594

Table 3: Percentage of X-chromosome nondisjunction in flies expressing RNAi in the germline. Progeny of flies with RNAi mediated knockdown in the germline are scored.

Successful RNAi mediated knockdown

To find out if the RNAi mediated knockdown was efficient, fly ovaries were stained with antibodies for H3K4 di- and tri-methylation. All three; *Set1, Ash2* and *Wdr82*, show reduced level of H3K4 di- and tri-methylation conforming that RNAi mediated knockdown is working. Although relative intensity was decreased in all three, it was more pronounced in *Set1* knockdown. This is expected as Set1 is the catalytic subunit and possesses some very weak enzyme activity by itself.

There is weak activity of the COMPASS complex in the absence of *Wdr82* and *Ash2*, which is all
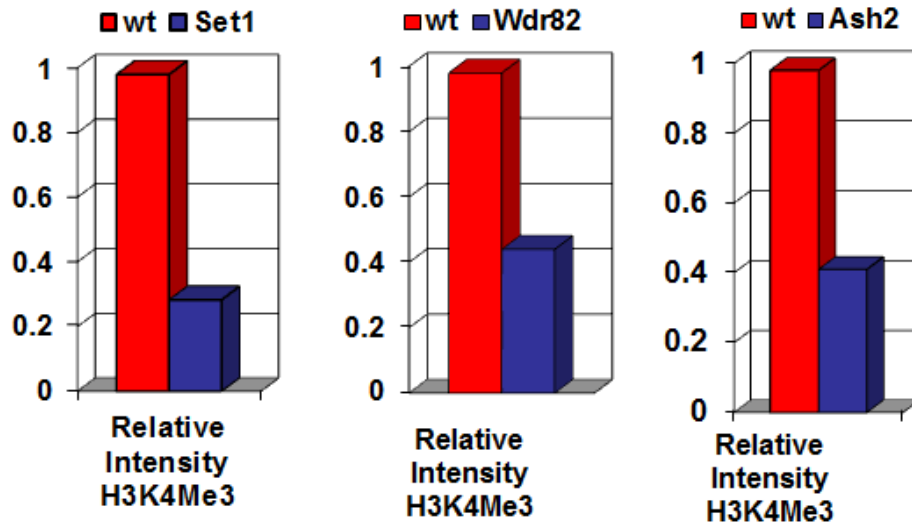
lost in the absence of *Set1*.

Fig.5 Relative signal intensity of H3K4 trimethylation in wild type (red) and RNAi knockdown
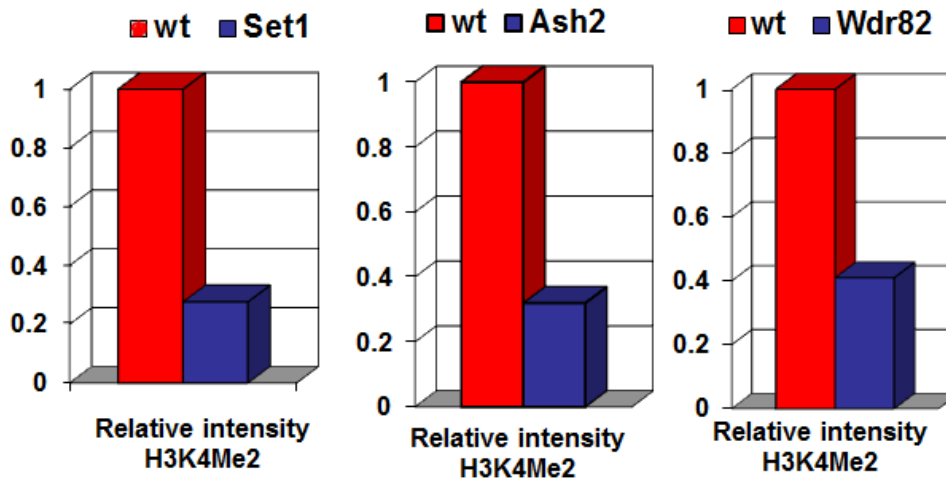
(blue).

Fig. 6 Relative signal intensity of H3K4 dimethylation in wild type (red) and RNAi knockdown

(blue).

No significant Reduction in the number of DSB in RNAi knockdowns

DSBs showed no significant difference between wild-type and the RNAi knockdown flies

oocytes. Number of DSB initiated was similar in wild-type and RNAi knockdowns of *Set1* and

Ash2. Set1 is the catalytic subunit of the COMPASS complex and have been shown to be

indispensible for the function of this complex. *WDRS82* is essential for the stability of the

COMPASS complex and *Ash 2* is essential for its catalytic activity. Thus, the optimal function of

the COMPASS complex requires all three subunits. If normal number of DSB can be initiated in

the absence of these essential components of the COMPASS Complex, this suggests that it might

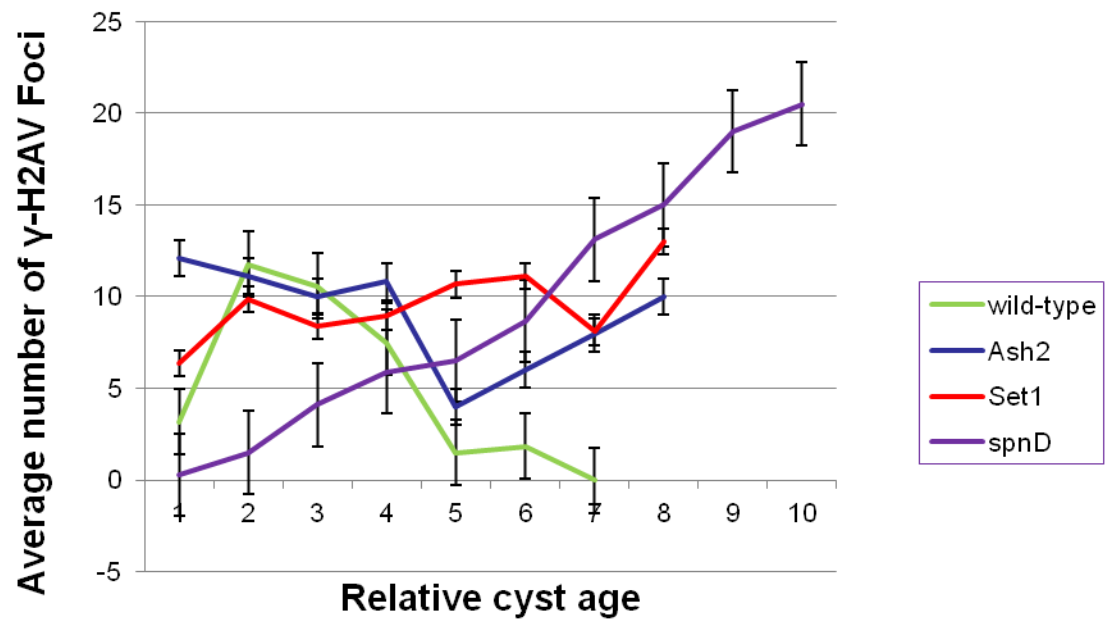not the major player in determining the number (and position) of DSB during meiosis in

Drosophila.

Fig. 7 Plot showing the average number of γ-H2AV foci as a function of relative cyst age in wildtype, *Ash2* and *Set1* RNAi and *spnD* mutant germaria.

DSB are abnormally retained in the RNAi knockdowns

Using γ-H2AV foci as a marker for DSBs, along with the shape and position of the developing cyst within the germarium, allows for visualization of the process of DSB repair over time. All 16 cells of the cyst share the same cytoplasm via intercellular connections or ring canals, the nurse cells as well as the pro-oocytes generate DSBs. In the wild-type, DSBs first appear in early pachytene after SC formation with gradual increase in the number of DSBs as cysts mature in region 2A.This increase is followed by a decline in number as the cyst progresses into early/midpachytene (region 2B). The DSB virtually disappear in the oocyte by the time the cyst reaches mid-pachytene (region 3). The DSBs in *Set1, Ash2* and *Wdr82* knockdowns persist even in region 3. This failure to repair DSB by region 3 indicates there may be a defect in DSB repair pathway. However, the number of unrepaired DSB in region 3 is significantly lower than those found in repair defect mutant. DSB repair-defective mutants *spn-B, spn-A, spn-D* and *okr* have γ-H2AV foci that persist into the late stages of the pachytene stage and are present in larger number than in the wild-type pro-oocytes. These mutants have approximately 20 γ-H2AV foci in region 3 oocyte. Due to the block in DSB repair, the number of foci in these mutants may be the accurate measurement of the total number of DSBs. *Set1* and *Ash2* show around 10-12 γ-H2AV foci on average, which shows that some of the DSB are getting repaired, however the repair process is either very slow or compromised. The COMPASS complex has a major role in controlling expression patterns and it is possible that alteration in expression of repair genes is responsible for the compromised repair response.
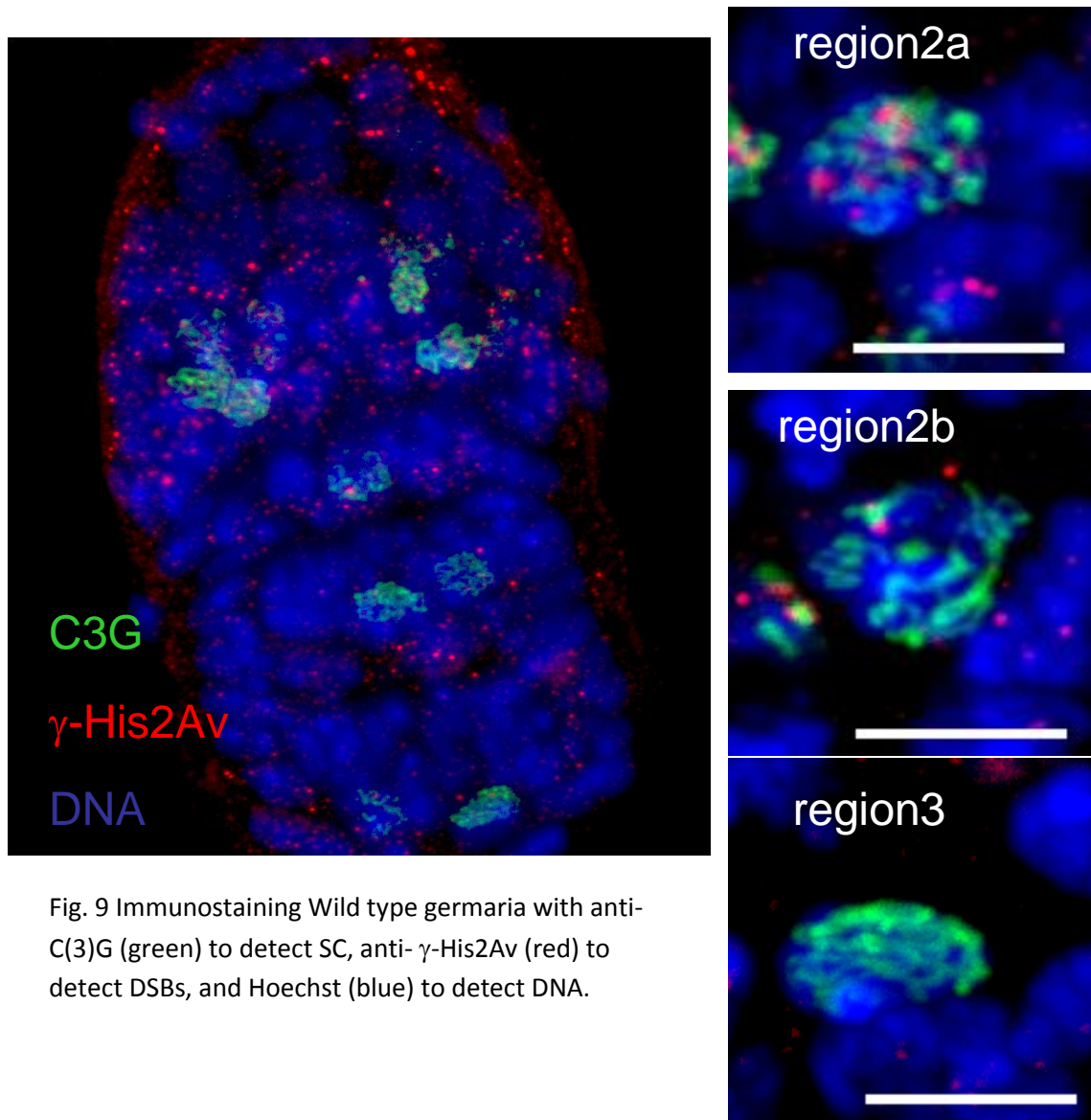
C3G

γ-His2Av

DNA

region2a

region2b

region3

Fig. 9 Immunostaining Wild type germaria with anti-C(3)G (green) to detect SC, anti- γ-His2Av (red) to detect DSBs, and Hoechst (blue) to detect DNA.

Fig. 10 Immunostaining Set1 germaria with anti-C(3)G (green) to detect SC, anti- γ-His2Av (red) to detect DSBs, and Hoechst (blue) to detect DNA

Fig. 11 Immunostaining Ash2 germaria with anti-C(3)G (green) to detect SC, anti- γ-His2Av (red) to detect DSBs, and Hoechst (blue) to detect DNA
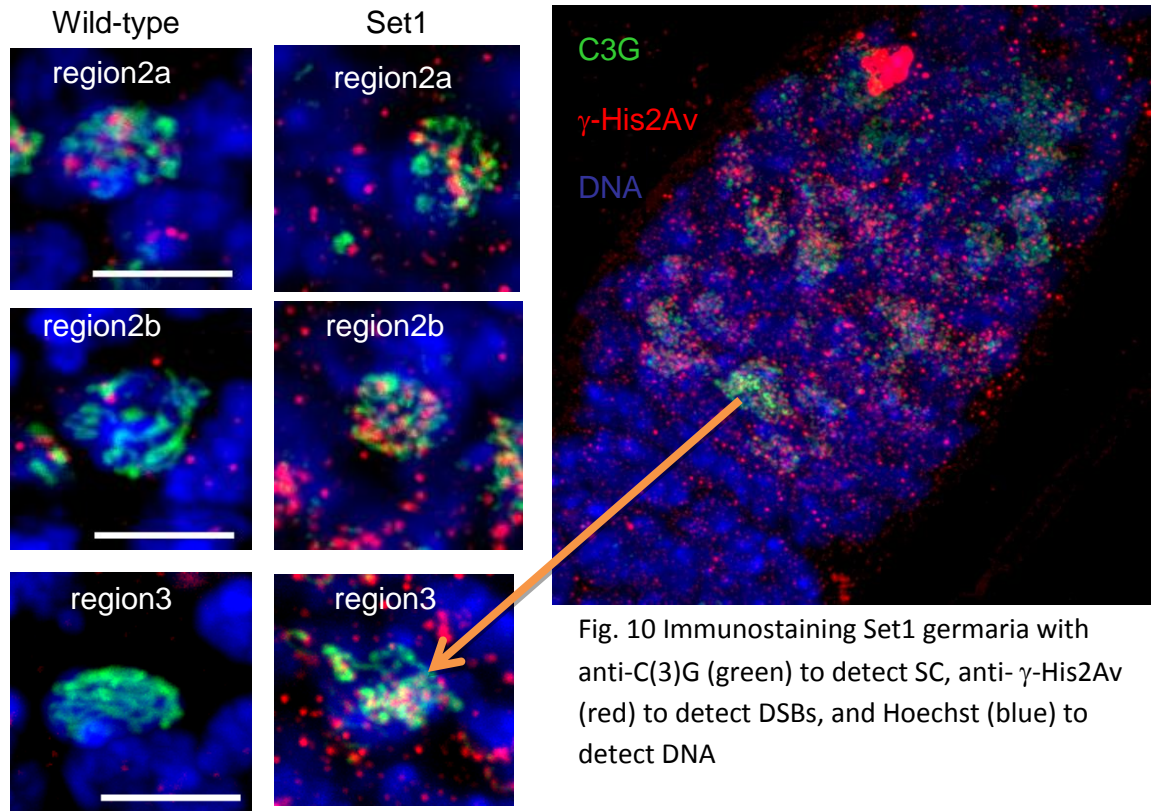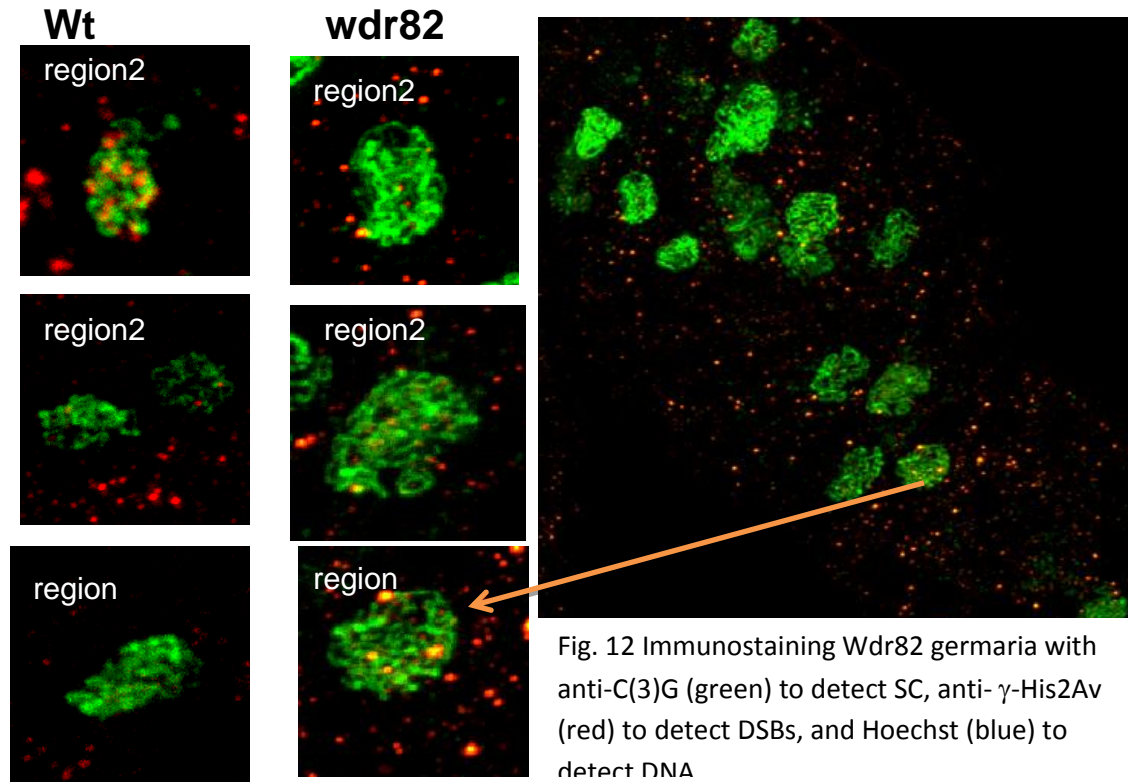
Fig. 12 Immunostaining Wdr82 germaria with anti-C(3)G (green) to detect SC, anti- γ-His2Av (red) to detect DSBs, and Hoechst (blue) to detect DNA
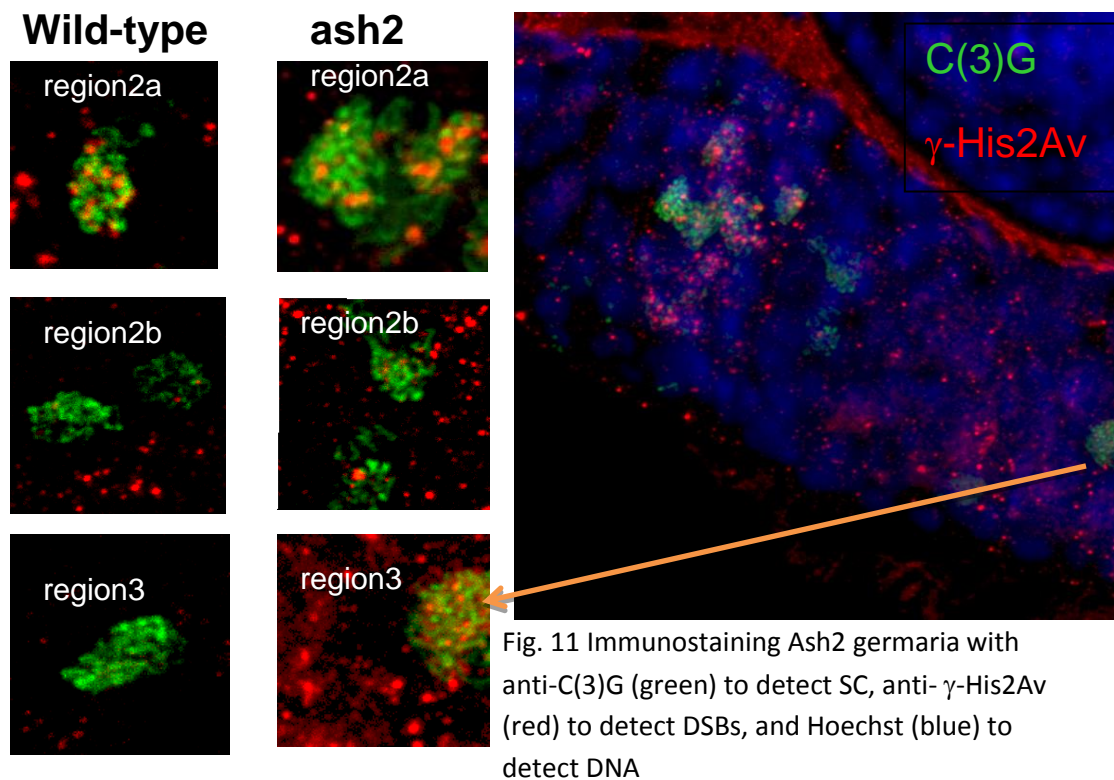
Set 1 shows Synaptonymal complex (SC) defects

Oocytes are identified by probing with the antibody against C(3)G which is an important component of the Synaptonymal complex. Errors with SC assembly are indicative of problems with oocyte differentiation. *Set1* shows defects in SC that are very variable. Wild-type germaria have three distinct regions and in *Set1* these regions are not easily distinguishable. In the wild type up to four cells within the cyst initiate assembly of the SC in region 2A. In some *Set1* germaria all most all cells in region 2a initiate SC assembly (see fig 13c& 14c) while in others none do (fig 13d). Region 2b of the wild-type shows two pro-oocytes within the cyst with full-length SC. Many *Set1* germaria do not show a distinguishable region 2b (13b,c,e). Wild- type region 3, all cells except the oocyte exit the meiotic program and SC is maintained in only the oocyte. Multiple cells with SC in this region is indicative of arrest and developmental errors.*Set1*

shows a variable phenotype in this region too. While some *Set1* germaria have more than one

cell with SC in region 3 (fig. 13b and 14d) others, show none (fig. 13c and 14c).
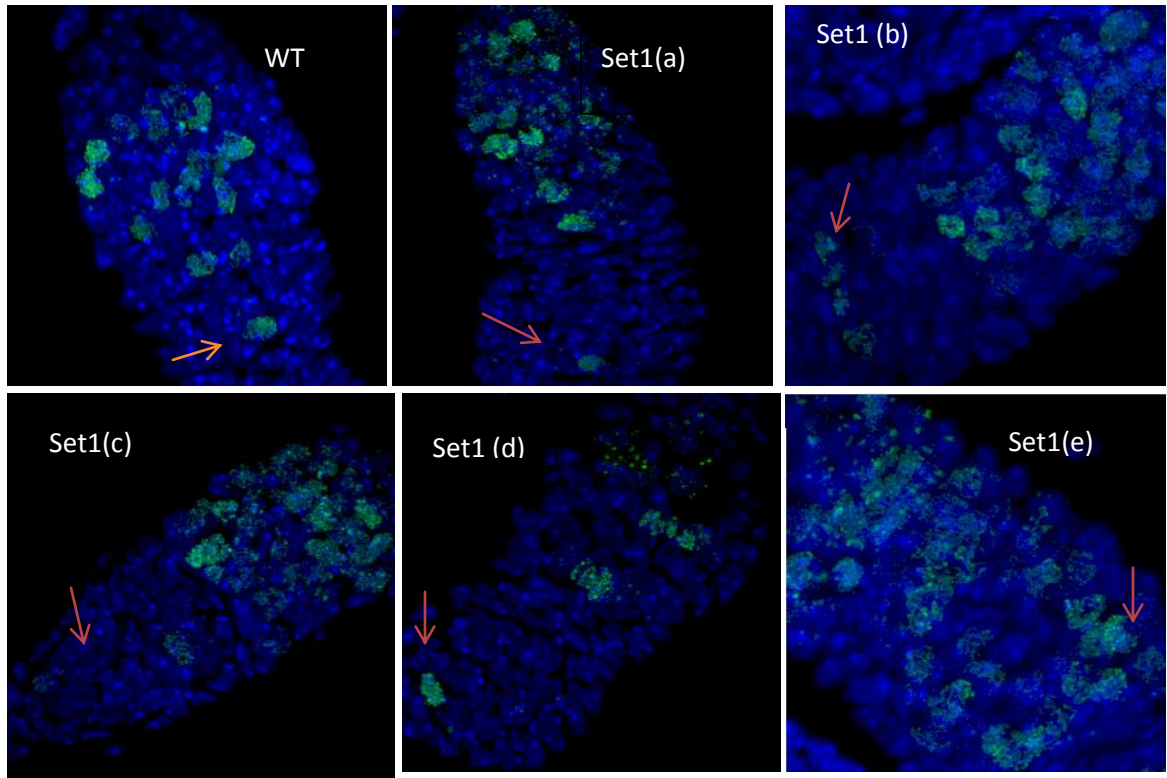

Fig. 13 Immunostained Wild-type and Set1 germaria with anti-C(3)G (green) to detect SC, and

Hoechst (blue) to detect DNA. Arrows mark region 3.

The Oocyte selection can be visualized by probing for Orb. In wild-type, Orb is present uniformly

in all cells of a cyst in region 2a. However, Orb begins to accumulate in only one of the cells by

region 2a- 2b transition, providing the sign that this pro-oocyte has been selected to become the

oocyte. The SC disappears from one pro-oocyte as the cyst enters region 2b, and the cell that

remains in meiosis is always the one that has already accumulated Orb. *Set1* knockdown do not

show this clear pattern and a lot of variation in phenotype is seen. Many *Set1* germaria (fig.14a)

do not show Orb accumulating in any one cell in region 2b or 3 indicating errors with oocyte

selection. While some germaria (Fig 14 c) show accumulation in one region but not in the other

region. Some *Set1* germaria (fig.14d) show higher concentration of Orb in certain cells compared to others, however, this accumulation is not restriction to one cell in region 2b and region 3 as in wild-type. This variability in phenotype could be the result of sensitivity to the amount of knockdown. The normal development of the oocytes at this stage seems to be dependent on the concentration of *Set1* present in the germarium. Slight changes in the amount of *Set1* present, makes the process go wrong.
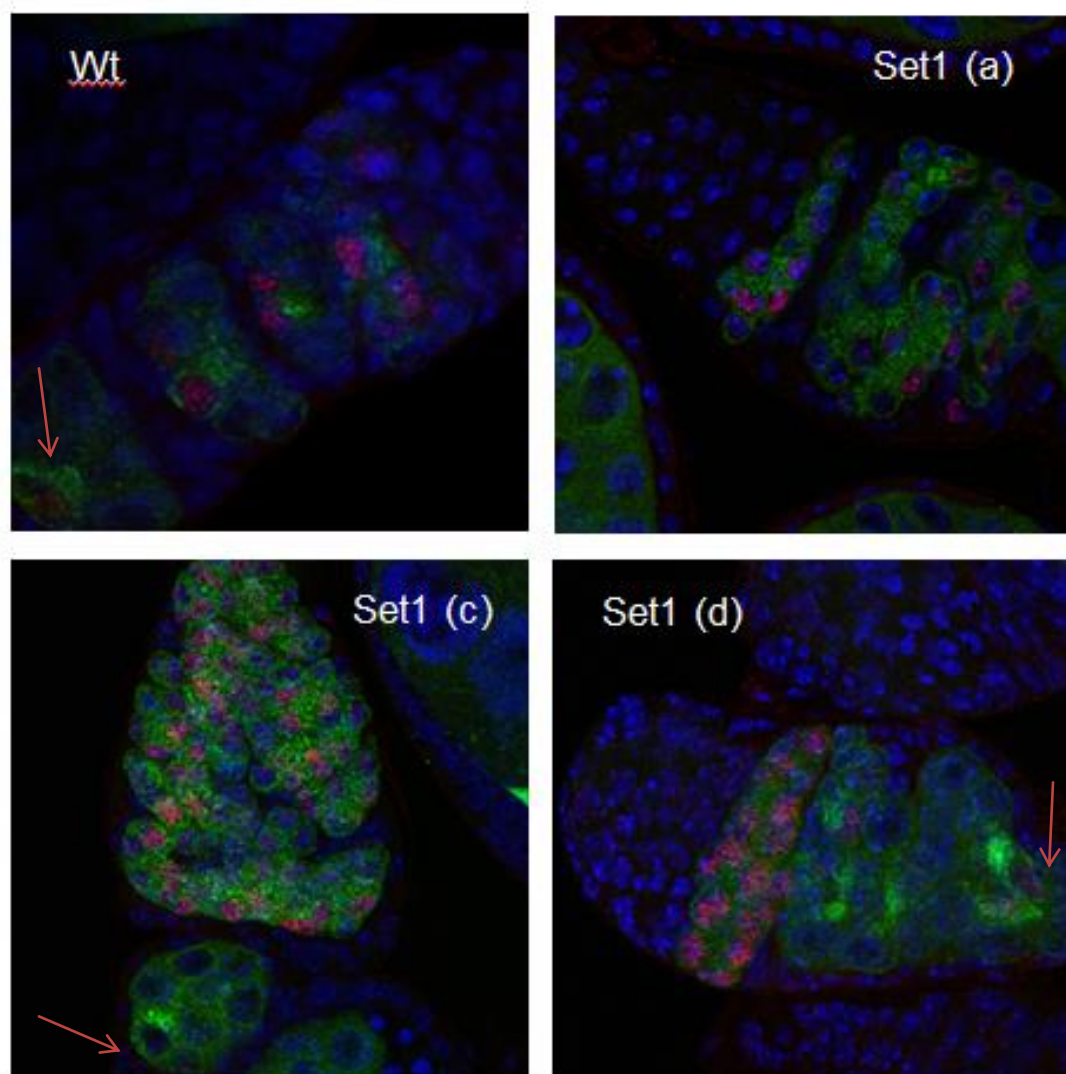
Fig. 14 Immunostained Wild-type and Set1 germaria with anti-orb (green), anti-C(3)G (red) to detect SC, and Hoechst (blue) to detect DNA. Arrows mark region 3 oocytes with Orb accumulation.

Ash2 and *Wdr82* do not show the same abnormalities with the SC. This suggests that the C(3)G phenotypes exhibited by the *Set1* knockdown is independent of the H3K4 methylation activity. It is possible that *Set1* has a role in the development of oocyte independent from its role in the COMPASS complex. This role in the development/differentiation of the oocyte might be responsible for elevated rates of non-disjunction which was seen only in *Set1*. It is also possible that *Set1*knockdown results in fewer number of DSB repaired as cross-overs and this could be a cause for the elevated nondisjunction rates seen in *Set1*.

CONCLUSION

H3K4Methylation mediated by the SET1-C does not seem to be the major player in marking the DSB initiation sites in Drosophila. However, Set1-C seems to other functions in during early meiosis such role in the repair of DSB. Set1 by itself also seems to be involved it the oocyte development.

Detection of Recombination Hotspots in

Drosophila melanogaster

The earlier fine-scale description of recombination hotspots was achieved with the sperm-typing. Hotspots have been characterized in mice and humans by this method in which recombination hotspots are mapped by analysis of crossovers detected in sperm. By typing millions of sperm that contain hundreds of recombination events in the studied region, hotspots can be detected efficiently and relatively easily. However, Drosophila males do not have recombination, therefore, this approach cannot be used.

Llinkage disequilibrium mapping (LD) has been more successfully used to identify hot spots in Drosophila. The availability of fly genome sequence allows the establishment and usage of SNPs. This has made feasible to detect local recombination hotspots from genomic-scale SNP data from different isolates form the wild. Using LD — the nonrandom association of SNPs at different loci -- recombination events can be mapped with high resolution. LD evaluations require genome-wide fine-scale investigations that largely relied on computational analysis of population polymorphism data.

AIM

The focus of this project is to detect recombination hotspots that have been previously detected using linkage disequilibrium (22) in population genetic studies using recombination in Transposons inserted at specific sites.

The strategy relies on classical genetic methods which are easy and quick, and can offer high resolution due to the large collection of publicly available transposon carrying fly strains with the insertions at molecularly defined locations. These transposons are engineered with linked visible markers, such as white+ gene, which makes scoring easy and requires minimal resources.

Two methods were used. The first method employs two transposable element bearing fly strains that have the insertion at sites flanking the proposed hotspot. The number of recombination events in the interval between the two transposon insertion sites is used to detect hotspots. The second strategy is to generate a double strand break (DSB) by mobilizing the excision of the transposon. By monitoring the outcomes of DSB repair event at these loci, sites that have a higher recombination rates can be detected.

Method 1: Detecting hotspots by determining the recombination rate

We investigated three hotspots detected by David J. Begun and colleagues in the wild Drosophila population (22). We wanted to see if these hotspots could be detected using simple classic genetics methods with transposon insertions used as markers. The transposable elements are inserted at molecularly defined sites flanking the proposed hotspot. The number of recombination events in the interval between the two transposon insertion sites was recorded. Since chosen intervals have been shown by other study to be recombination hotspots, we expect a higher rate of recombination events in these intervals. Since the genome average recombination rate is about 100×10^6 bp/300 mu, we expect to see a rate higher than that.
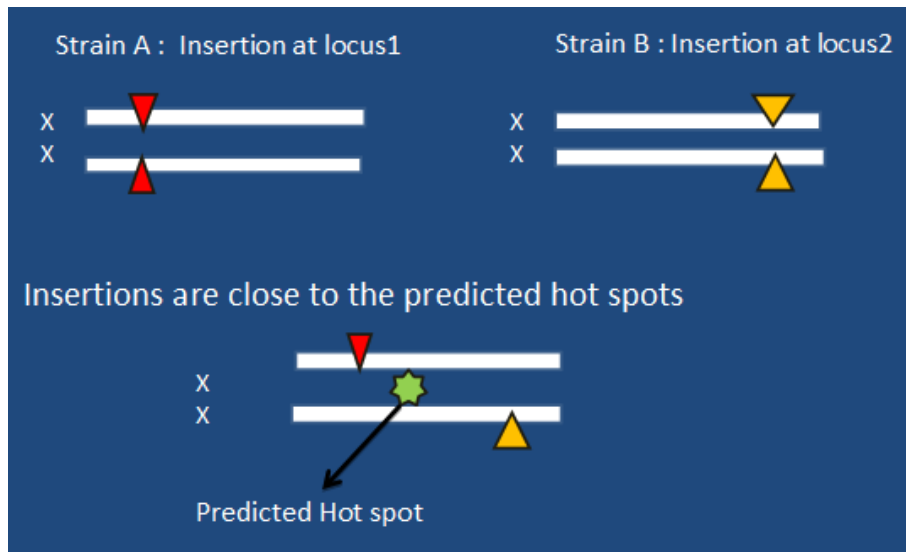
Fig.1 Cross two fly strains that have molecularly mapped transposon inserted such that they are located on either side of the hotspot. The F1 progeny will inherit both the transposon bearing chromosomes.

METHOD AND MATERIAL

Crosses are set up between two strain bearing insertions at sites flanking the predicted hotspot region. Recombination occurs in F1 female that are heterozygous for the transposon insertion-bearing chromosome. These females are crossed to wild-type males. The recombination events are detected in the F2 generation by scoring for eye color. The transposon bearing fly strains are in a white⁻ background and transposable element is the only source of eye color. The F2 progeny are red-eyed as they all carry an insertion with the gene for eye color unless there is recombination event in the interval between the two insertion sites. Recombination event in this interval generates X-chromosome without the insertions resulting in white-eyed flies. Only the male F2 progeny were scored because females will inherit wild-type X –chromosome from the father. Because females are have two copies of X chromosomes, the paternally derived X-chromosome that carries the wild-type gene for eye color will mask the phenotype that results

from maternally derived, recombinant X-chromosome. Therefore, females cannot be used to detect recombinants.

Half of the recombinants will have both the transposon insertions and cannot be distinguished from non-recombinants as they have wild-type eyes. Therefore the number of recombinant progeny is obtained by doubling the number of white-eyed flies. The frequency of recombinant progeny can be calculated as (2 recombinant progeny) / [2recombant progeny)+(non-recombinant progeny)]. Frequency of cross-overs between two transposable element insertion sites can allow us to detect the hotspot activity.

Bloomington Drosophila stock Center has made available thousands of fly strains with molecularly mapped transposon insertions. Three pairs of insertion bearing strains are chosen with each pair flanking each of the three predicted hotspots.
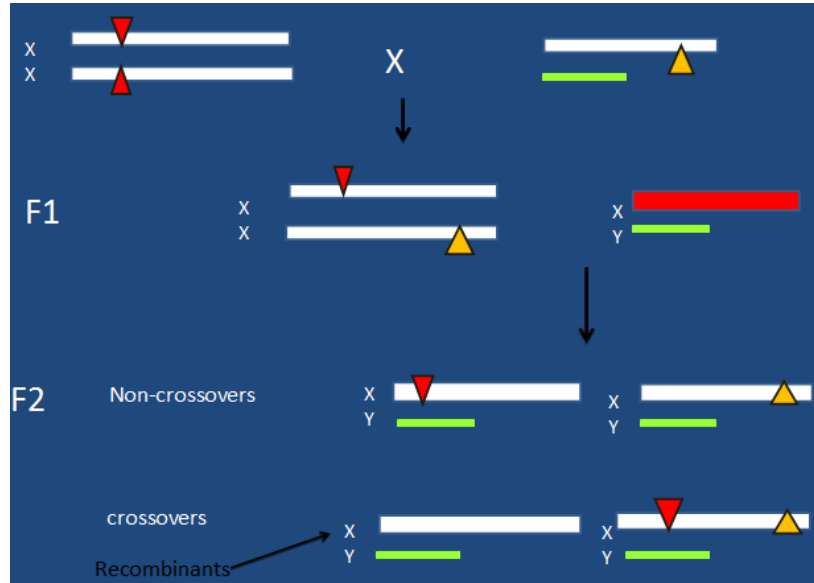

Fig.2 F1 progeny of the insertion bearing strains is heterozygous for the insertions.

Recombination occurs in F1 female generating X-chromosome without the insertions resulting in white-eyed flies

Crossing Scheme 1

The distance between the two insertion sites is around 280,000 bp. Based on average recombination rates, this is 0.9 map units. More than one recombinant is expected in every hundred flies.

$$w, mew\ P\{w^+\} \quad X \quad \frac{w,CG[f03962]\ P\{w^+\}}{Y}$$

↓

$$\frac{w,mew\ P\{w^+\}}{w,CG[f03962]\ P\{w^+\}} \quad X \quad \frac{+}{Y}$$

↓

Non-recombinants

$$\frac{w,mew\ P\{w^+\}}{Y}$$

$$\frac{w,CG[f03962]P\{w^+\}}{Y}$$

Recombinants

$$\frac{w,CG[f03962]P\{w^+\}mew\ P\{w^+\}}{Y}$$

$$\frac{+}{Y}$$

w⁺ (red eyes due to transposon)

w⁻ (white eyes due to loss of transposon)
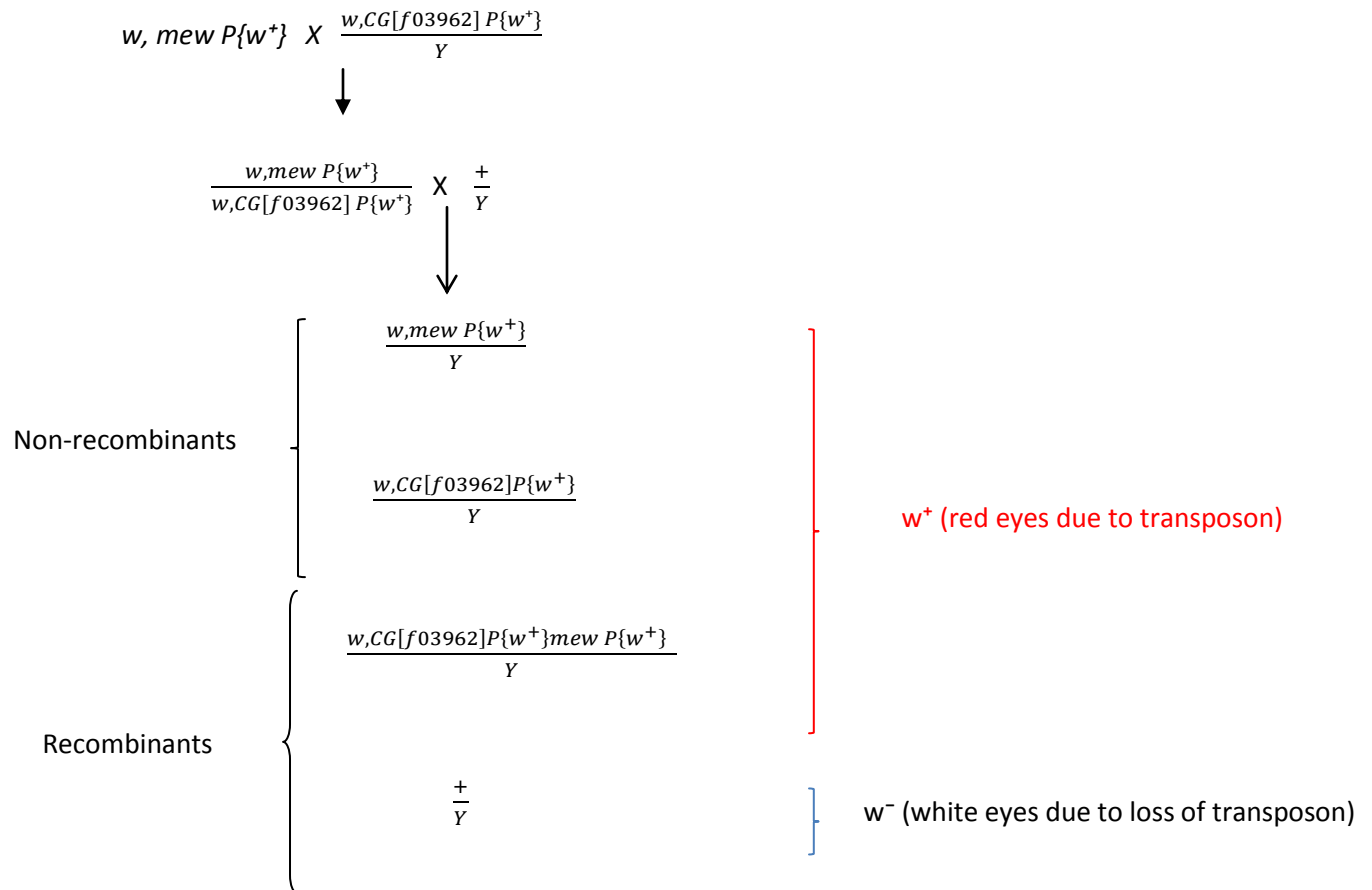
Crossing Scheme 2

The distance between the two insertion sites is around 320, 000bp. Based on average recombination rates, this is 1 map units. More than one recombinant is expected in every hundred flies. The fly line that has the transposon insertion in the *Top1* gene is heterozygous for the insertion. The balancer has a dominant marker that gives kidney shaped eyes. F1 females are picked against this marker to ensure they have inherited both transposon bearing chromosomes.

$$\frac{w,Top1\ P\{w^+\}}{FM7c} \quad X \quad \frac{yw,eag\ P\{w^+\}}{Y}$$

$$\frac{w,Top1\ P\{w\}}{yw,eagP\{w^+\}} \quad X \quad \frac{+}{Y}$$

Non-recombinants

$$\frac{yw,eag\ P\{w^+\}}{Y}$$

$$\frac{w,Top1\ P\{w^+\}}{Y}$$

w^+ (red eyes due to transposon)

Recombinants

$$\frac{yw,Top1\ P\{w^+\}\ eagP\{w^+\}}{Y}$$
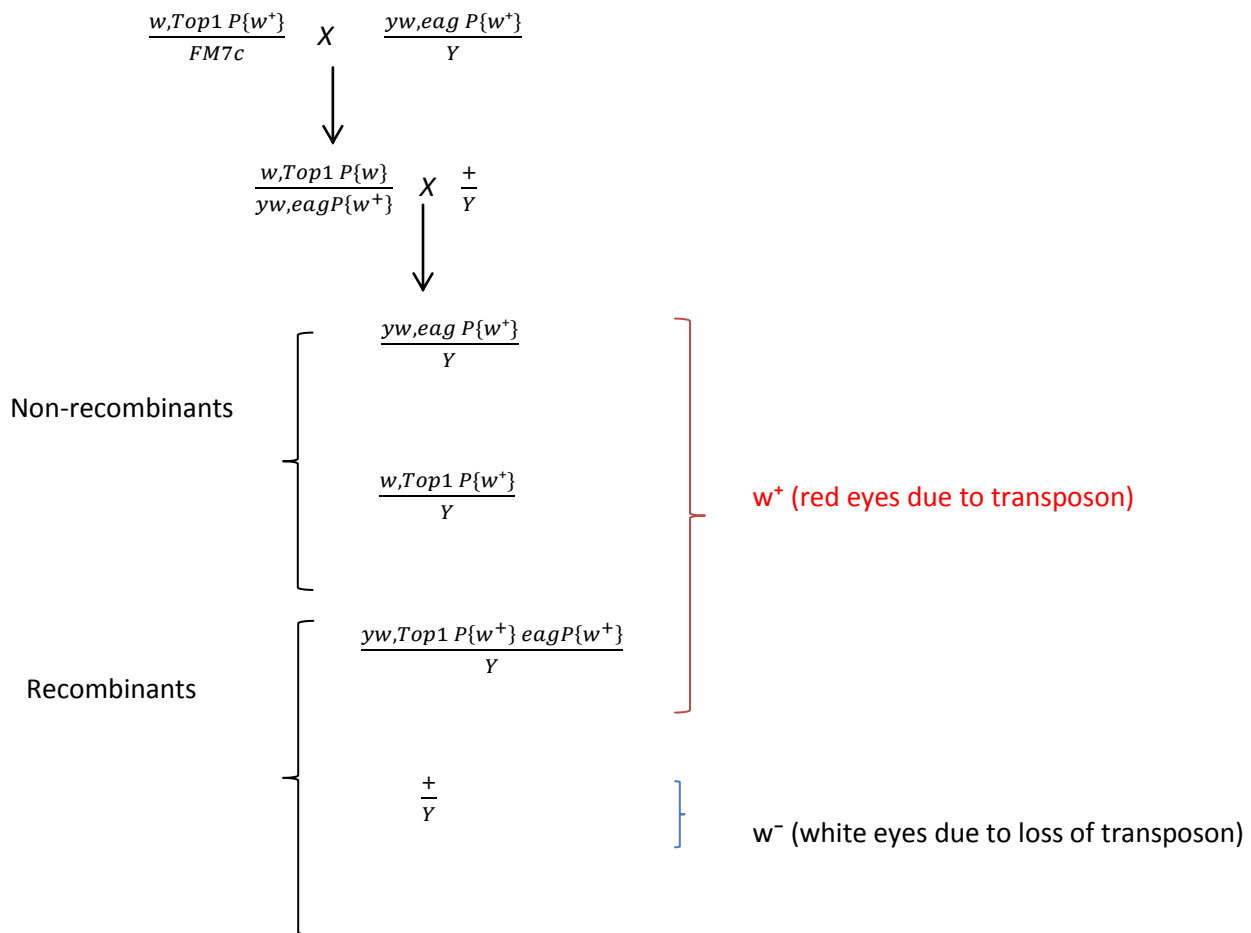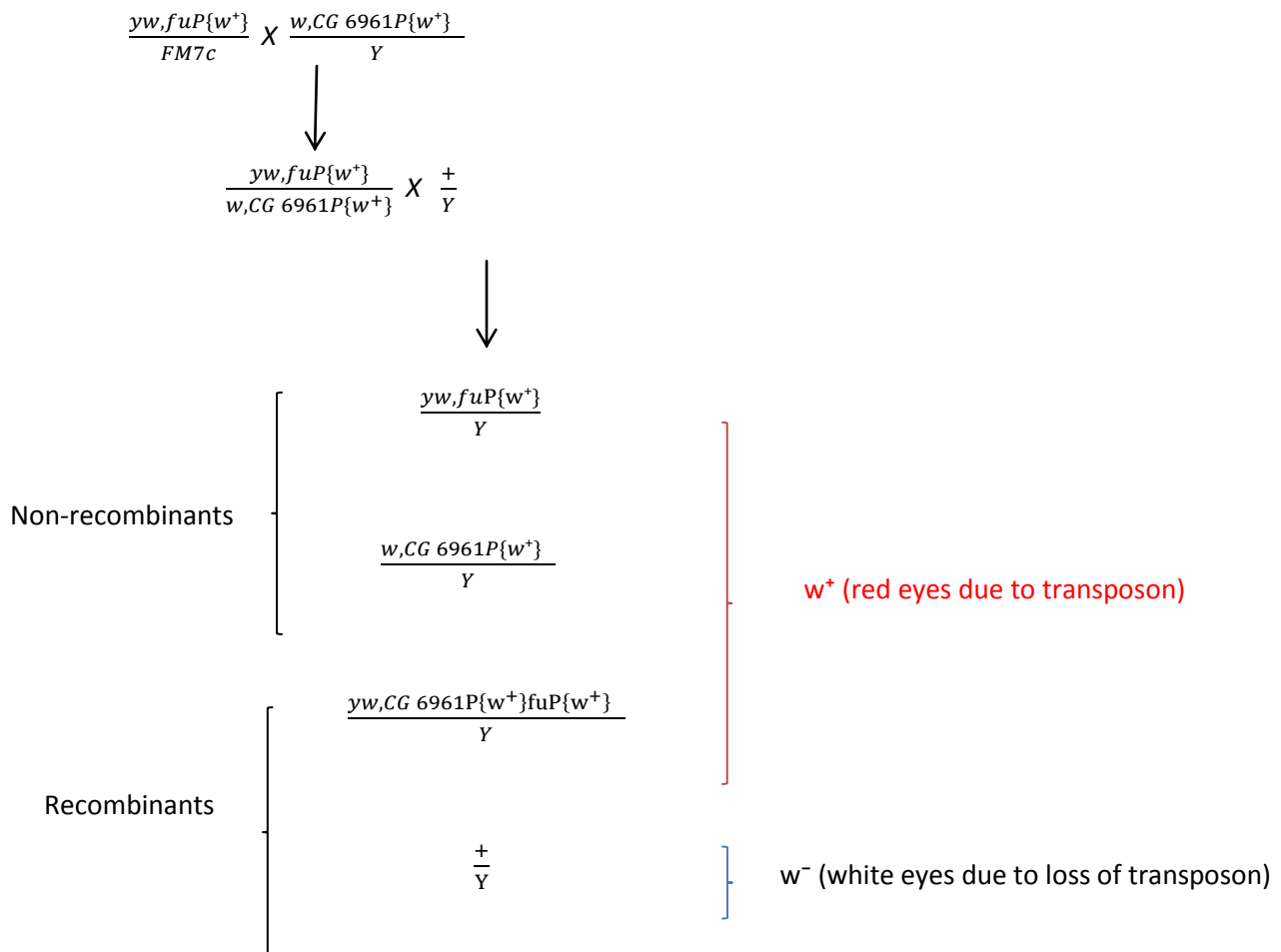
$$\frac{+}{Y}$$

w^- (white eyes due to loss of transposon)

Crossing Scheme 3

The distance between the insertion sites is 127,000 bp. Based on average recombination rates, this is 0.4 map units. More than one recombinant is expected in every two hundred and fifty flies. The fly line that has the transposon insertion in the *fu* gene is heterozygous for the insertion. The balancer has a dominant marker that gives kidney shaped eyes. F1 females are picked against this marker to ensure they have inherited both transposon bearing chromosomes.

$$\frac{yw,fuP\{w^+\}}{FM7c} \quad X \quad \frac{w,CG\ 6961P\{w^+\}}{Y}$$

$$\frac{yw,fuP\{w^+\}}{w,CG\ 6961P\{w^+\}} \quad X \quad \frac{+}{Y}$$

Non-recombinants
$$\frac{yw,fuP\{w^+\}}{Y}$$

$$\frac{w,CG\ 6961P\{w^+\}}{Y}$$

w⁺ (red eyes due to transposon)

Recombinants
$$\frac{yw,CG\ 6961P\{w^+\}fuP\{w^+\}}{Y}$$

$$\frac{+}{Y}$$

w⁻ (white eyes due to loss of transposon)

RESULTS & DISCUSSION

Cross	# of non-recombinants	# of recombinants	% of recombinants
mew X CG[f03962]	8077	1	0
Top1 X eag	3477	0	0
Fu X CG6961	2078	0	0

None of the three predicted hotspots showed elevated rate of recombination. This could be due to difference in genetic background of the flies the hotspots were first identified in and the one used in this experiment. The hotspots studied were first identified in the wild *D. melanogaster* populations (in the study by David Begun and team), whereas the ones used for these experiments were strains that have been used in labs for the last fifty years with very little interbreeding, if any, with the wild populations. This difference is the genetic background could result in different maps of recombination hotspots. Recombination hotspots show high degree of variation in their genomic location even within species. Intra-specific variation is depicted by variable crossing-over rates among different crosses, with particular crosses having regions with exceedingly high rates (>40-fold) relative to either adjacent regions or to other crosses (8). Additionally, crosses sharing one parental strain have more similar maps than crosses not sharing parental strains. The magnitude and the genomic location of this variation are shown in genome-wide study of CO rates in *D. melanogaster* which points at a highly polygenic and polymorphic basis for CO distribution along chromosomes in *Drosophila melanogaster* (8).

Method 2: Detecting hotspots by generating DSBs

DNA transposons move through a 'cut-and-paste' mechanism, which generates a DSB in the chromosome. By mobilization excision of the transposon, a DSB can be generated at specific sites in chromosomes. With thousands of molecularly mapped transposon insertions in fly strains publicly available, one has the flexibility to make a DSB at any chosen loci. By monitoring the outcomes of DSB repair event at these loci, sites that have a higher recombination rates can be detected. The transposon is mobilized by the expression of the transposase which is under the control of a heat-shock-inducible promoter.

Determining the frequency of excision

Parent flies were heat-shocked to induce the excision of transposon from a unique insertion site on the chromosome. Excision can be detected by using a linked marker. All flies are in yellow background. Transposable element carries a gene for yellow$^+$ which gives flies a wild-type body color. Excision causes loss of yellow$^+$ resulting in yellow flies. Phenotypic revertants in the progeny of the heat-shocked flies give the rate of excision of the transposon.
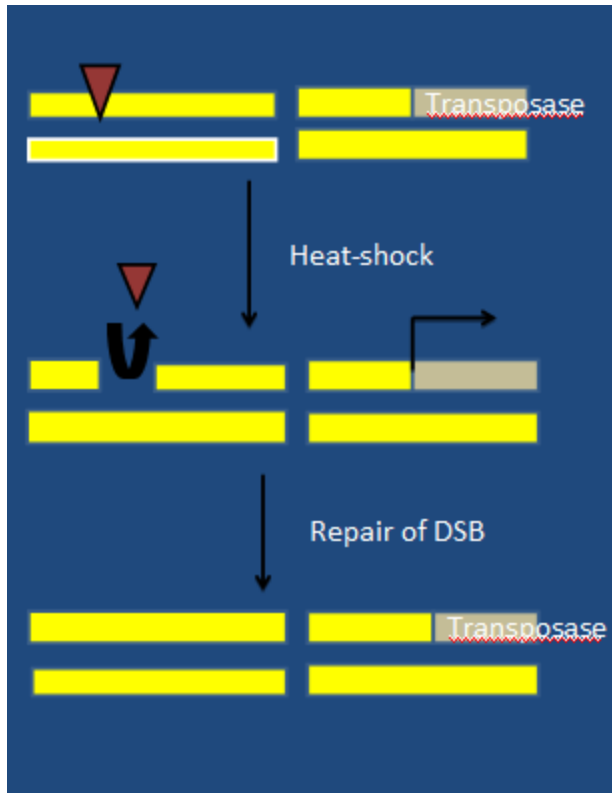
Fig.3 Excision of transposon causes reversion to yellow. Excision assay was performed by giving a heat-shock to the parent flies and screening the progeny for revertants.
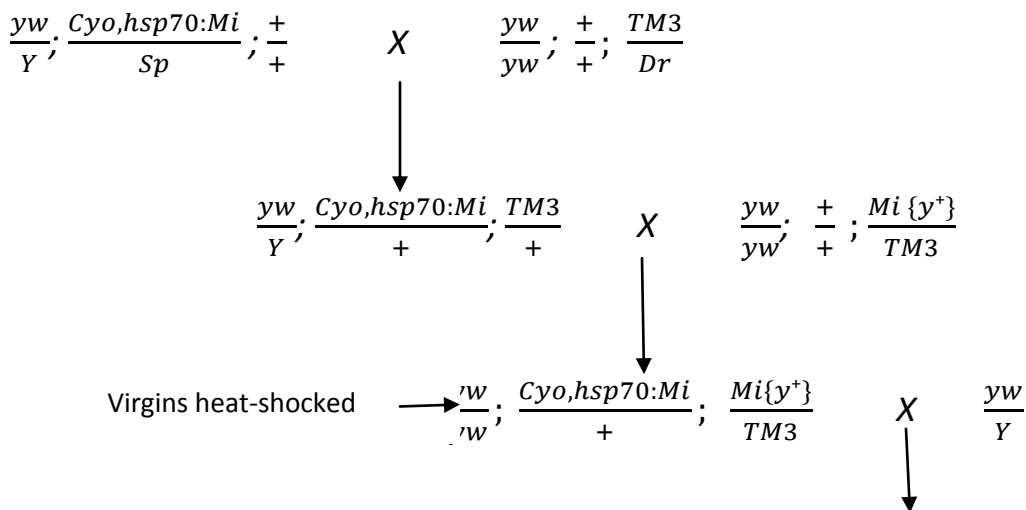
METHODS AND MATERIAL

The production-line organization of the Drosophila germline is exploited to assess the excision rate of the transposon for germ cells at different stages of the meiotic prophase at the time of heat shock. Time-course experiments were conducted in which virgin females were heat-shocked, mated and subsequently transferred to fresh vials every 3 days. Cohorts of progeny arising from eggs laid during each interval were scored for excision events. In order to find out if excision could occur without heat-shock, the same cross was set up without heat-shock and brooded every 3 days. Because of the ubiquitous expression of the heat-shock-inducible

promoter, excision is expected in all four broods. However, we are interested in the 6-9 day

cohort as the eggs laid during this period are expected to be in the germarium at the time of

heat-shock. The rate of excisions in the germarium will determine if this method could be

efficiently used to detect recombination hotspots.

Crossing Scheme

The following scheme is used to test the excision of *minos* element (*Mi{y ⁺}*) inserted on the third

chromosome. The *minos* element carries a yellow ⁺ marker. Flies carrying the *minos* element

(*Mi{y ⁺}*) are crossed to ones with heat-shock-inducible *minos* transposase which catalyzes the

excision. To be able to distinguish between the progeny that had excision from the ones that did

not inherit the *minos* at all, a balancer was introduced. Because the *minos* insertion is on the

third chromosome, balancer for the third chromosome (TM3) is introduced. TM3 carries a

dominant marker (Sb) which allows one to distinguish between yellows that did not inherit the

yellow⁺ marker (which will be Sb) and the ones that had an excision (which will be Sb⁺). The

number of Sb⁺ yellow flies represents progeny that had an excision.

$$\frac{yw}{Y}; \frac{Cyo,hsp70:Mi}{Sp}; \frac{+}{+} \qquad X \qquad \frac{yw}{yw}; \frac{+}{+}; \frac{TM3}{Dr}$$

$$\frac{yw}{Y}; \frac{Cyo,hsp70:Mi}{+}; \frac{TM3}{+} \qquad X \qquad \frac{yw}{yw}; \frac{+}{+}; \frac{Mi\{y^+\}}{TM3}$$

$$\text{Virgins heat-shocked} \longrightarrow \frac{'w}{'w}; \frac{Cyo,hsp70:Mi}{+}; \frac{Mi\{y^+\}}{TM3} \qquad X \qquad \frac{yw}{Y}$$

Sb⁺ yellow⁺ progeny with *minos* insertion

$$\frac{yw}{yw}; \frac{Cyo,hsp70{:}Mi}{+}; \frac{Mi\{y^+\}}{+}$$

Sb⁺ yellow progeny represent excision

$$\frac{yw}{yw}; \frac{+}{+}; \frac{Mi\{y^+\}}{+}$$

Sb progeny without *minos* insertion

$$\frac{yw}{yw}; \frac{Cyo,hsp70{:}Mi}{+}; \frac{TM3}{+}$$

$$\frac{yw}{yw}; \frac{+}{+}; \frac{TM3}{+}$$

RESULTS & DISCUSSION

	Heat-shocked			Control (Not heat-shocked)		
Egg-laying period (days)	Revertant progeny	Total progeny	% of revertant progeny	Revertant progeny	Total progeny	% of revertant progeny
1-3	27	420	6.4	2	204	1.0
3-6	3	104	2.9	0	146	0
6-9	17	184	9.2	8	119	6.7
9-12	5	87	5.7	1	49	2.0

Progeny from eggs laid 6-9 days post heat-shock show the highest rate of excision compared to other broods. The control group (without heat-shock) shows a low rate of excision demonstrating that excision can occur without heat-shock. However, the rate of excision in the heat-shocked flies is not significantly higher that the control group. The 6-9 day cohort, which comes from the eggs that are expected to be in the germarium at the time of heat-shock, shows the highest rate of excision of 9.2%.

CONCLUSION

What can we learn about Hotspots Activity in Drosophila from other Model Organisms?

In sexually reproducing organisms recombination is an essential process needed for the correct segregation of the chromosomes during meiosis. Recombination tends to occur at specific regions called recombination hotspots that are 1–2 kb long, separated from each other by tens of kilobases where recombination is essentially lacking. So far, three types of factors have been suggested to control the location of recombination hotspots: DNA sequence motifs, epigenetic mechanisms and trans-acting loci. A common chromatin feature, the trimethylation of lysine 4 of histone H3 (H3K4me3), defines hotspots for yeast and mouse recombination initiation sites.

Hotpots in Yeast

In budding yeast, DSB hotspots generally map to short 50-200bp regions of open chromatin found almost exclusively adjacent to transcription promoters, but with no obvious DNA sequence motif. Several results in *Saccharomyces cerevisiae* suggest that gene organization and chromatin structure play important roles in the formation of DSBs. Natural DSBs (about 150 DSBs per meiotic cell) are typically in nucleosome-depleted regions (NDR) of promoters (23).

Set1 is the only H3K4me3 methylase in yeast and controls the 'openness' of chromatin which

plays a role in DSB hotspots activity. Levels of DSBs at hotspots are reduced in the *Set1* mutants,

and the most affected DSB sites are those with high H3K4me3 enrichment (12). DSB hotspots in

yeast show no DNA sequence specificity. As expected, Set1-C has no DNA binding domain and is

recruited to the chromatin through its interaction with Pol II at the promoters of highly

transcribed genes (13). The Set1 mediated H3K4me3 modification seems to play a role in the

recruitment of Spo11 to the NDR of the actively transcribed genes (24). Thus, the preferred

hypothesis is that, in yeast DSB formation is opportunistic, occurring where DNA is sufficiently

exposed. It is gene induction that makes the promoter chromatin more accessible and favors its

cleavage by Spo11.

Hotspots in Mammals

H3K4me3 has also been associated in mice with meiotic DSBs and COs, which both occur within

peaks of H3K4me3.Recent studies have identified the histone methyl transferase PRDM9 as an

important determinant of hot spot localization in mammals. PRDM9 is a meiosis-specific protein

containing, among other domains, a conserved SET domain with H3K4 methyltransferase

activity. Hotspots are defined by sites where PRDM9 binds and where it promotes local

H3K4me3 enrichment. In mice and humans, PRDM9 seems to control the activity of a large

fraction of hotspots (25).

Contrary to what is observed in budding yeast, hotspots in mammals are not localized to the

promoters (27). Based on sperm typing in humans and mice, DSB hotspots are found within or

outside genes, and are depleted near promoters or transcription start site (TSS) both in humans and mice. Prdm9 appears not to be essential for DSB formation, but to drive DSB activity away from the TSS. In Prdm9 -/- mice, DSBs still occur, but they are located near TSS and transcription enhancers. In the absence of PRDM9 and PRDM9-introduced H3K4me3 marks, recombination hotspots are re-routed to alternative H3K4me3 sites at the promoter (possibly mediated by Set1). Almost half (44%) of recombination hotspots in the Prdm9-/- mice localize to the promoters of annotated genes compared to just 3% in wild type (27). The distribution of hotspots around transcription start sites (TSSs) in Prdm9-/- mice is reminiscent of that in *Saccharomyces cerevisiae*, where most hotspots are found at promoters. Furthermore, promoter-overlapping hotspots in the PRDM-/- mice are slightly enriched at genes expressed in early meiotic prophase indicating that induction of transcription is making the promoter chromatin more accessible to DSB machinery (27). This indicates that PRDM9 may be directly involved in recruitment of the recombination initiation machinery to DSB hotspots away from TSSs. In the absence of PRDM9 the vast majority of DSB hotspots still coincide with H3K4me3 mark at the promoters possibly mediated by Set1.

PRDM9 has DNA binding specificity, which is determined by a DNA-binding domain with multiple zinc fingers. In mammals the 13-mer CCNCCNTNNCCNC motif which is believed to be targeted by the PRDM9 is associated with crossover activity in 41% of human hotspots (26). The signature of PRDM9 binding sites has been detected at or near DSBs sites strongly suggesting that PRDM9 plays a direct or indirect role in recruiting SPO11 to these sites in mammals.

Hotspot Paradox

Recombination hotspots are thought to be evolutionarily unstable because of a particular

feature of the DSB repair mechanism during meiosis. Recombination is initiated by a double-

strand break in one chromosome, which is then repaired using the homologous chromosome as

a template. Alleles with high recombination-initiation activity are therefore continually being

replaced during their repair by the unbroken, low-activity homologs. As DNA sequences that

influence DSB activity can be located next to the DSB site, strong sites are expected to be

replaced by weaker ones by gene conversion, and in the absence of compensatory effects, this

will ultimately lead to loss of DSB activity. This suggests that hot spots would be short lived on

evolutionary time scales. If hot spot activity is controlled in *cis* by sequences near DSB sites,

then gene conversion should replace ''hot'' alleles with ''cold'' alleles over evolutionary time.

Extending this logic leads to the paradoxical conclusion that DSB hotspots and, thus, meiotic

recombination should no longer exist. It appears that yeast and mammals have found distinct

solutions to this apparent problem.

Mammalian Solution to the hotspot problem

 Mammals control the recombination hotspot activity in *cis* by sequences near DSB sites. These

sites evolve very rapidly and this is supported by the lack of congruence seen in hot-spot activity

and position in closely related species. Hotspot locations are highly polymorphic between

closely related species and even between individuals of the same species suggesting new

hotspots are evolve as old ones turn cold. The highly polymorphic zinc finger array which is

responsible for the sequence specificity of PRDM9 provides an explanation for the high variability in the CO hotspots within mice and human population. This domain is highly variable both within and among species (26). The polymorphic forms of PRDM9 recognize different DNA sequences and therefore can promote crossovers at different chromosomal sites among individuals.

Multiple studies have suggested that the zinc fingers of PRDM9 are evolving under positive selection and concerted evolution across many metazoan species, specifically at positions involved in defining their DNA-binding specificity. The minisatellite structure of the Prdm9 zinc finger encoding region confers a strong potential to generate variability by recombination or replication slippage within the array (25). Specifically, a single–amino acid change within zinc fingers could lead to a PRDM9 variant with novel DNA binding specificity and, thus, could potentially create a new family of hotspots genome-wide.

Yeast solution to hotspot Problem

The feature of the PRDM9 protein described above changes its DNA-binding specificity allowing generation of new hotspots as old ones are lost. Yeast gets around this problem by avoiding cis regulation of hotspot activity. In yeast, diverged *S. cerevisiae* strains and Saccharomyces species show a high degree of conservation of recombination pattern. There is considerable overlap between the recombination hotspot location of the yeast *Saccharomyces paradoxus* and *Saccharomyces cerevisiae* species, despite the fact that they are at least 10 times more divergent than humans and chimpanzees (28). Given that yeast DSB hot spots do not require

specific sequence motifs and are mostly located in transcription promoter regions, maintenance of hot spot activity in yeast may be linked to the maintenance of chromatin structure through the selection for functional promoters.

Hotspots in Drosophila

Recombination hotspots controlled by specific DNA sequences in the vicinity of DSB sites are thought to be short-lived in evolutionary time due to their self-destructive nature. Consistent with this expectation, hotspots in Drosophila are highly dynamic, with little correspondence in location between various Drosophila species. Recombination hotspots may not be conserved and may be transient (short-lived) features within *D. melanogaster* populations (8). So much so that, recombination hotspots show high degree of variation in their genomic location even within the *D. melanogaster* species. Intra-specific variation is depicted by variable crossing-over rates among crosses, with particular crosses having regions with very high rates (>40-fold) relative to either adjacent regions or to other crosses (8). Unlike humans and mice, where histone methyltransferase *PRDM9* is a major determinant of recombination hotspots, Drosophila lacks a functional copy of this gene. Set1-C also does not play as big a role in hotspot activity as it does in yeast. Set1-C is unlikely to be a major player in the activity of cis-regulated hotspots as its role as master regulator of transcription patterns keeps it under strong selective pressures.Set1 and its associating subunits are conserved from yeasts to humans (17).

Studies in Drosophila reveal many motifs significantly enriched in sequences surrounding recombination events indicating cis regulation of hot spots. Cameron and collogues identified 18

and 10 motifs for CO and GC, respectively (8). This is in contrast to human and mice that have

restricted number of DNA motifs. In mammals a degenerate 13-mer motif, which is believed to

be targeted by the PRDM9, plays a role in about 40% of human hotspots. The use of multiple

different DNA sequence motifs to regulate meiotic recombination in Drosophila indicates a

fundamental difference between mammalian and Drosophila DSB hotspots. In Drosophila, DSBs

seem to be controlled by a large number of different sequence motifs suggesting multiple

players involved in the localization of DSB hotspots (8). These players are yet to be identified.

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