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Investigating X chromosome non-disjunction in *Drosophila melanogaster su(var)3-9* mutants

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ABSTRACT

Meiotic recombination is a highly regulated process necessary for promoting proper chromosome disjunction during the first meiotic division. Notably, reduced levels of meiotic recombination are observed in heterochromatic regions of the chromosome. This study seeks to investigate the molecular mechanisms underlying this observation by examining the effects of reduced heterochromatin on non-disjunction rates in *Drosophila melanogaster*. To accomplish this, we measured non-disjunction in wild-type and reduced heterochromatin mutant *su(var)3-9* flies. To begin, we confirmed the presence of a mutation within *su(var)3-9* via Sanger sequencing. Next, we created allele-specific primers and designed a PCR protocol to more accurately identify mutant flies at the molecular level. Finally, we assayed non-disjunction in wild-type and *su(var)3-9* mutant flies and discovered that *su(var)3-9* mutants have significantly higher levels of non-disjunction than wild-type flies. We also uncovered a striking sex bias in the non-disjunction progeny of *su(var)3-9* mutants.

INTRODUCTION

Chromosomes are thread-like structures of DNA wrapped around histone proteins. This genetic information can be transferred across the generations through meiosis, a type of cellular division resulting in the production of four genetically diverse haploid gametes. During prophase I of meiosis, replicated paternal and maternal homologs pair up and exchange genetic material in a process called meiotic recombination, or crossing over. Notably, this exchange appears to show high levels of regulation. For example, crossover assurance states that every chromosome pair will engage in crossover events during cellular division (Shinohara et al. 2008), resulting in increased genetic variability among offspring. Moreover, crossover events are essential for proper chromosome segregation because they serve as a tether for homologs as they align at the metaphase plate (Youds and Boulton 2011). During normal cellular activity, chromosomes are also subject post-translational modifications to histones. These modifications — referred to as "marks" — regulate gene expression by organizing the genome into two distinct regions: euchromatin and heterochromatin. These distinct regions are characterized by their varying levels of DNA compaction and gene expression. Euchromatin, which comprises a large portion of the genome, functions as the most active region of gene expression. Alternatively, heterochromatin — found in regions proximal to the centromere and telomere — is characterized by tight DNA compaction, low gene density, transcriptional inertness, and strongly reduced levels of meiotic recombination. Studies have also shown that crossovers that occur in heterochromatic regions show strong inhibition of proper chromosome segregation (McKim et al. 2002). Within this study, we seek to examine the role of heterochromatin in the suppression of meiotic recombination and its effect on chromosome segregation.

For this study, we used *Drosophila melanogaster* as a model system. *Drosophila* is an ideal organism for genetic studies because of their fast generation times, large brood sizes, and fully sequenced genome. *Drosophila* is also particularly useful for meiotic studies because flies are able to survive some forms of aneuploidy and moreover, meiotic crossing over was first observed in *Drosophila.* The *Drosophila* genome consists of 4 chromosome pairs including its sex chromosomes. With respect to its composition, chromosome *4* is >70% heterochromatic compared to the other

autosomes comprising ~30% (Hoskins et al. 2002). However, the absence of recombination on chromosome *4* is of primary interest because of its direct contention with crossover assurance. Absence of crossover events on chromosome *4* seems to suggest the activity of strict regulation in place to prevent unfavorable recombination events. Interestingly, one genetic mutant background has recently been discovered, which allows crossing over on chromosome *4* (Hatkevich, Kohl, et al. 2017). This suggests that suppression of recombination events are the result of strict regulatory mechanisms governing chromosome *4*. These findings also suggest that chromosome *4* serves as an ideal model for studies involving meiotic recombination control mechanisms collectively.

This study seeks to elucidate the meiotic regulatory mechanisms functioning on *Drosophila* chromosome *4* by genetically reducing heterochromatin levels using *suppression of variegation (su(var))* mutants. Within this family, the gene *su(var)3-9* was chosen because it encodes a protein responsible for histone H3 lysine 9 (H3K9) methylation — a key agent in the production and maintenance of heterochromatin (Schotta et al. 2002). In a previous study, rates of recombination in *Drosophila su(var)* mutants were assayed in limited centromere proximal intervals not including chromosome *4* (Westphal and Reuter 2002). Our lab seeks to extend this work by investigating the effect of *su(var)3-9* mutation on meiotic recombination and non-disjunction on multiple chromosomes, including chromosome *4*. This study aims to elucidate the molecular mechanisms and epigenetic factors affecting meiotic recombination on chromosome *4* and across the genome. These results may provide us with a molecular explanation of the 80-year old paradigm in *Drosophila* genetics that recombination does not occur on chromosome *4*.

METHODS

Fly stocks

Fly stocks were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN) and included: *ci sv* (wild-type control), $In(1)w^{m4}$; $su(var)3\text{-}9^{2}$ / TM3 (experimental stock) and *y cv v f* / $Dp(1;Y)B^{s}$ (indicator of non-disjunction).

Verification through DNA sequencing

The $su(var)3\text{-}9^{2}$ mutation is a G to A single nucleotide polymorphism (SNP) (G15260859A) causing a missense mutation (C427Y) (Krauss et al. 2006). To verify the presence of the $su(var)3\text{-}9^{2}$ mutant SNP, the region flanking the mutation was amplified from a standard fly prep, excised from a 2.0% agarose gel, and purified using a QIA QuickGel Extraction Kit (Qiagen). Following quantification using a Nanodrop Spectrophotometer (ThermoFisher Scientific), the sample was sent for Sanger sequencing (Eurofins). A heterozygous peak at the predicted location confirmed the presence of the $su(var)3\text{-}9^{2}$ mutation (Figure 1).

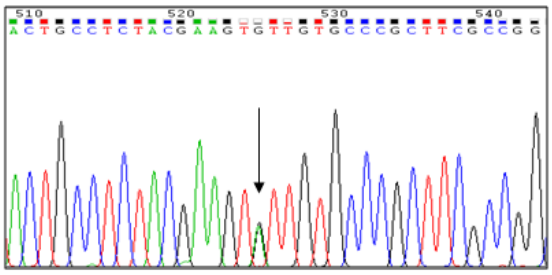

Figure 1. Sanger sequencing chromatogram of su(var)3-9^{2} mutant fly. Low heterozygous peak (overlapping green and black peaks) at the predicted location (indicated by an arrow) confirms the presence of the su(var)3-9^{2} SNP.

Identification through allele-specific PCR

To quickly identify $su(var)3\text{-}9^{2}$ mutants, which do not produce an outward phenotype, we designed allele-specific primers and optimized an allele-specific PCR protocol. Primers were as follows: *asf2* (AGGAGTCTACTGCCTCTACGAATTA) and *2858r* (GCTGCATCGATACTCTACTCG). Reagent concentrations were as follows (Table 1):

Reagents	Amount
dH_20	13.825 µL
10x Standard Taq (Mg^{2+} Free) Reaction Buffer (NEB)	2.0 µL
25 mM $MgCl_2$	1.575 µL
10 mM dNTPs	0.5 µL
50 pmol/µl Primer asf2	0.5 µL

50 pmol/µl Primer 2858r	**0.5 µL**
***Taq* DNA polymerase (NEB)**	**0.1 µL**
Fly prep	**1.0 µL**
Total	**20 µL**

Table 1. Reagent concentrations used in *su(var)3-9²* allele-specific PCR reaction.

Allele-specific PCR reactions were run on a Mastercycler Thermacycler (Eppendorf) using the following conditions (Table 2):

Temperature (C)	Cycle Time	Number of Cycles Repeated
95	3 min.	N/A
95	30 sec.	16 cycles
62*	30 sec.	
72	30 sec.	
95	30 sec.	16 cycles
54	30 sec.	
72	30 sec.	
72	5 min.	N/A
10	∞	N/A

Table 2. Thermalcycler conditions for *su(var)3-9²* allele-specific PCR reaction. *Indicates the annealing temperature decreased by 0.5 °C each cycle for 16 cycles in a "touchdown" procedure.

A 2.0 % agarose gel was run at 120 volts for approximately 45 to 50 minutes to visualize PCR products.

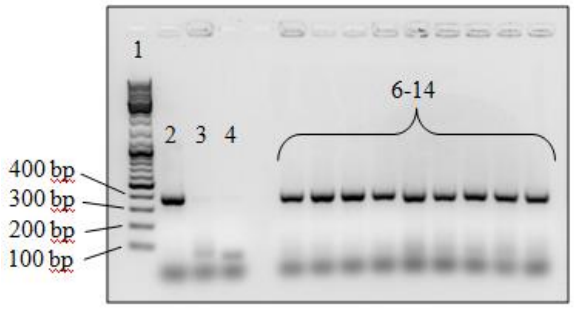

Figure 2. su(var)3-9² **allele-specific PCR with optimized protocol.** Predicted DNA product size was (394 bp). Lane 1: molecular weight marker, Lane 2: product of su(var)3-9² mutant control, Lane 3: absence of product from wild-type control, Lane 4: absence of a product from dH_20-only control. Lanes 6-14: randomly-selected flies from the non-disjunction cross are all su(var)3-9² mutant.

Experimental crosses

A standard *Drosophila* crossing scheme was used for this experiment (Figure 3). All crosses were conducted at 25° C on standard fly media (Genesee Scientific, Bloomington recipe) under a 12:12 light/dark cycle. First, virgin *su(var)3-9²* females were mated to *ci sv* wild-type males in bottles to remove the TM3 balancer. Approximately 16 males and 16 females were mated in 8 individual bottles. Parents were then cleared from these bottles after 3 days. From these bottles, virgin *su(var)3-9²* / + females (lacking the TM3 balancer chromosome) were collected. Next, 3 *su(var)3-9²* females were crossed to 3 *y cv v f* / *Dp(1;Y)B^s* flies per vial for a total of 25 vials. After 3 days, parents were cleared. Progeny were then scored for sex and non-disjunction phenotypes for 8 days post-eclosion (Figure 3). Non-disjunction phenotypes or "exceptionals" include: males with wild-type eyes (XXY) and females with bar-eyes (ØX), normal disjunction phenotypes include: males with bar-eyes (XY) and females with wild-type eyes (XX) and two resulting lethal classes include: XXX and ØY (Figure 4).

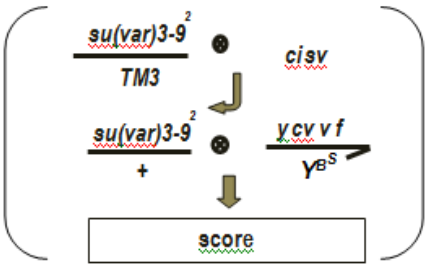

Figure 3. Experimental mating scheme. ci sv males were crossed to su(var)3-9² females to remove the TM3 balancer chromosome. In the next generation, su(var)3-9² / + heterozygous females were crossed with y cv v f / Dp(1;Y)B^s to score non-disjunction.

	X	Y^{B^s} ♂
♀ X^+	X^+X	X^+Y^{Bs}
Ø	Ø X	
X^+X^+		$X^+X^+Y^{Bs}$

Figure 4. Non-disjunction Punnett square. Normal chromosome segregation in females will produce gametes with one X chromosome whereas

non-disjunction will produce XX or Ø gametes. Normal chromosome segregation in males will produce gametes with either an X or Y chromosome. Progeny indicative of female non-disjunction include XØ males and XXY females (exceptionals; circled). Two lethal classes can also result from female non-disjunction (ØY and XXX, crossbones).

Data analysis

Results of non-disjunction scoring were analyzed using a standard percent of non-disjunction equation. The number of exceptional progeny observed was multiplied by 2 (to account for lethal classes) and divided by total number of flies scored plus the number of observed exceptionals. This number was then multiplied by 100 to compute percent non-disjunction. Wild-type non-disjunction rates and *su(var)3-9²* non-disjunction rates were compared via Fisher's exact test (GraphPad QuickCalcs). A chi-square goodness of fit test was used to analyze sex bias in the exceptional male progeny of *su(var)3-9* mutants (GraphPad QuickCalcs).

RESULTS

Su(var)3-9 mutants show statistically higher rates of non-disjunction

Percent non-disjunction was compared between *ci sv* (wild-type) and *su(var)3-9²* mutants. A Fisher's exact test strikingly rejects the null hypothesis that a decrease in the production of heterochromatin in *su(var)3-9²* mutants does not increase rates of non-disjunction ($p = 0.001$). A total of 719 wild-type progeny were scored with no observed non-disjunction events (Bar eyed females and wild-type eyed males). Thus, frequency of non-disjunction for this class was 0.00%. In the *su(var)3-9²* progeny, a total of 4,792 flies were scored including 20 exceptionals. For *su(var)3-9²* mutants, a non-disjunction frequency of 1.13% was calculated (Figure 5).

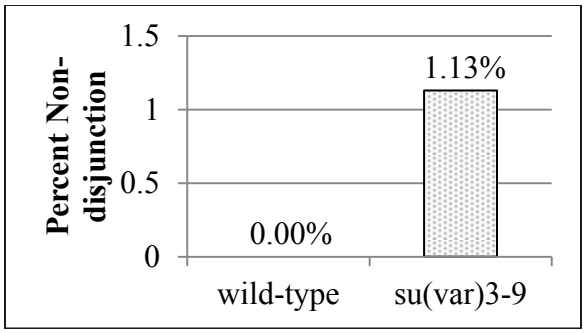

Figure 5. Non-disjunction frequencies. Using a standard percent non-disjunction equation, ci sv wild-type progeny exhibited a 0.00% (n=716) non-disjunction frequency while su(var)3-9² mutants showed statistically higher ($p = 0.001$) rates of non-disjunction with a non-disjunction frequency of 1.13% (n=4792).

Su(var)3-9 mutants show altered sex ratio of non-disjunction offspring

Whereas the Mendelian expectation is an equal proportion of male and female exceptional progeny (Figure 6), the total exceptional mutant progeny from the *su(var)3-9²* heterozygote cross included: 1 female with Bar eyes compared to 19 males with wild-type eyes. A chi-square test rejects the null hypothesis that there exists *no* difference in the distribution of sex within scored progeny ($p < 0.0001$).

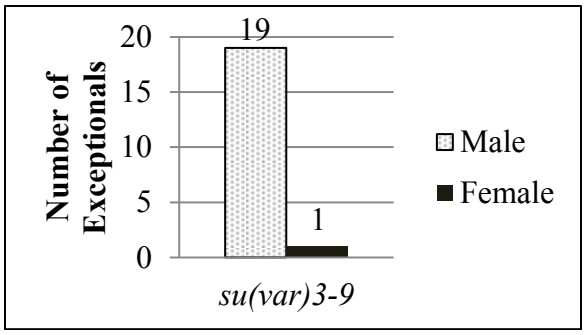

Figure 6. Sex bias to male exceptionals. Male (ØX) exceptional progeny in the offspring of su(var)3-9² / + females represent a significant difference from the expected Mendelian sex distribution ($p < 0.0001$).

DISCUSSION

The molecular function of SU(VAR)3-9 as a H3K9 methyltransferase is well characterized (Schotta et al. 2002), however much less is known about how mutations in *su(var)3-9* affect meiotic recombination. One

previous study tested effects on recombination, but only in centromere-proximal intervals on chromosome *2* in *su(var)3-9²* mutants (Westphal and Reuter 2001). Thus, this project goes further by investigating the role of *su(var)3-9²* on *X* chromosome disjunction using *Drosophila melanogaster*. Flies were scored phenotypically based on sex and eye shape. Amongst progeny, there was a statistically significant difference in the percent of non-disjunction between wild-type flies and *su(var)3-9²* mutants ($p = 0.001$). Progeny of *su(var)3-9²* mutants also exhibited a sex bias towards male exceptional offspring ($p < 0.0001$). This result, combined with the knowledge that SU(VAR)3-9 is a H3K9 methyltransferase necessary for heterochromatin formation, suggests that heterochromatin plays an important role in meiotic chromosome disjunction. However, important data from recombination proclivity (i.e. frequency and location of crossovers) studies are needed to better elucidate the relationship between recombination and chromosome disjunction.

Mutations to H3K9 methyltransferases make heterochromatic DNA sequences more prone to spontaneous or induced damages. This damage may include deletions, insertion of extrachromosomal DNA, or chromosome rearrangements (Peng and Karpen 2008) that may result in recombination errors leading to non-disjunction. Along with the idea that mutations to epigenetic agents may contribute to downstream changes, this observation serves to highlight how changes in chromosome integrity may affect molecular recombination mechanisms that result in increased non-disjunction. This increased frequency of missegregation may also explain the observed sex bias in exceptional progeny. The Y chromosome of *Drosophila* is composed almost entirely of heterochromatin (Wang et al. 2014). Therefore, we hypothesize that XXY females inheriting a heterochromatically "handicapped" paternal Y chromosome may be less viable than those inheriting a highly heterochromatic Y chromosome. Since *su(var)3-9²* is a dominant allele, this may be influencing the occurrence of female *su(var)3-9²* flies within parental stock without any phenotypic indices. With this, it can be assumed that the bias in *su(var)3-9²* exceptional progeny may be due to a compounded inheritance of mutated sex chromosomes or compromised recombination proclivity. Interestingly, XØ males can also arise through spontaneous loss of an *X* chromosome during development. This suggests that normal chromosome segregation that would have resulted in a female (XX) may freely produce a male exceptional (XØ), providing another pathway to the increased observation of male exceptionals. In order to understand this bias more precisely, molecular recombination assays are needed to determine whether weakened *X* chromosome integrity has caused a shift in the loci of crossovers. Furthermore, since the frequency of primary *X* chromosome non-disjunction is usually highest in the first eggs laid by a female (Tokunaga et al. 1970), measuring hatch rates of fertilized eggs may be another option for identifying premature sex biases in *su(var)3-9²* mutants. In this way, we can determine in which stages of development exceptional female progeny are incurring speculated chromosome loss.

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