Sex And Heterochromatin: An Investigation Of Sexual Dimorphism In Drosophila Melanogaster

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SEX AND HETEROCHROMATIN: AN INVESTIGATION OF
SEXUAL DIMORPHISM IN DROSOPHILA MELANOGASTER

by

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DISSERTATION

Submitted to the Graduate School
of Wayne State University,
Detroit, Michigan
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for the degree of

DOCTOR OF PHILOSOPHY

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Approved by:

________________________________
Advisor

Date
DEDICATION

I would like to dedicate this dissertation to my great-grandmother Indira V. Gondhalekar, my grandparents Vinayak and Sulochana Apte and Sadanand and late Shailaja Lele, my parents Sadanand and Meenal Apte, my sister Sai Apte and my husband Amit Joshi. They all have always inspired me to dream big and taught me to live in pursuit of excellence! I am eternally grateful to all my teachers, whose passion and efforts have inspired me to pursue a career in scientific research.
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Chapter 1
Introduction

Eukaryotic genomes are assembled into different types of chromatin with distinct properties. Heitz (1928-29) first suggested this based on the differential compaction of interphase chromosomes. Over the years, researchers have gathered a wealth of information about many cytological and molecular differences between transcriptionally active, gene-rich ‘eu’-chromatin and relatively silent, gene-poor ‘hetero’-chromatin. These differ not only in their transcriptional status, but also in the relative gene density, chromosome organization and histone and DNA modification patterns (reviewed by ELGIN AND REUTER, 2013). Unlike euchromatin, heterochromatin is less accessible and highly condensed (GREWAL AND JIA 2007). Heterochromatin is characterized by histone hypoacetylation (mainly at H3K9Ac) and hypomethylation (H3K4Me and H3K79Me) (JENUWEIN AND ALLIS 2001; GREWAL AND JIA 2007). Heterochromatin is also enriched for repetitive DNA sequences, including satellite repeats and transposable elements (MARTENS et al. 2005; SCHUELER AND SULLIVAN 2006; SLOTKIN AND MARTIENSSEN 2007). Although heterochromatin is relatively gene-poor, there are several hundred Drosophila genes in heterochromatic regions (SMITH et al. 2007). Interestingly, endogenous heterochromatic genes require a heterochromatic environment for full expression, and may be silenced when moved into an environment that would be permissive for an expression of a euchromatic gene (YASUHARA AND WAKIMOTO, 2006).
Heterochromatin displays a unique ability to influence gene expression in a sequence-independent manner. This is exemplified by the phenomenon of ‘Position Effect Variegation’ (PEV), extensively studied in *Drosophila melanogaster* but also present in plants, mammals and yeast (reviewed in MARTEINSSEN AND COLOT 2001). When chromosome rearrangement or transgene insertion juxtaposes a euchromatic gene next to a heterochromatic region, heterochromatin can spread into the formerly euchromatic region, resulting in stochastic transcriptional silencing. This produces a characteristic mottled expression known as Position Effect Variegation, or PEV (Fig. 1). The role of heterochromatin in PEV is illustrated by the fact that the amount of variegation is sensitive to the levels of proteins that comprise heterochromatin (reviewed in ELGIN AND REUTER 2013). PEV is thus generally considered an indirect measure of proper heterochromatin structure and function.

![Diagram of PEV](image)

**Fig. 1.1: Schematic illustrating position effect variegation (PEV).** P-element carrying *White* (*w*⁺) within euchromatin gives rise to red eye phenotype (top) while the same P-element when inserted in proximity of heterochromatin undergoes stochastic silencing, resulting in variegated expression of *white* gene, phenomenon termed as position effect variegation (bottom).
The fly Y chromosome is entirely heterochromatic, as are large blocks of pericentromeric chromatin on the X and autosomes (ADAMS et al. 2000). Altogether, heterochromatin makes up 1/3 of the fly genome. Autosomal heterochromatin is generally not considered to be sexually dimorphic, and PEV of autosomal insertions is thought to behave similarly in males and females. However, our laboratory identified a potent modifier of PEV with an effect that is limited to males (DENG et al. 2009). Intriguingly, this modifier of PEV is part of the fly system of dosage compensation, a process that is also limited to males.

Dosage compensation equalizes X-linked gene expression between the sexes. Being a heterogametic organism, Drosophila melanogaster males and females differ in their sex chromosome content. Drosophila females have two gene-rich X chromosomes while males carry a single X and a gene-poor Y-chromosome. Dosage compensation produces a two-fold up-regulation of virtually all X-linked genes in males, correcting the gene dosage imbalance. Dosage compensation is brought about by the Male Specific Lethal (MSL) complex. MSL complex consists of 5 proteins namely - Male Specific Lethal-1, 2 and 3, Maleless (MLE) and Males absent on first (MOF) and one of two functionally redundant, non-coding roX RNAs (RNA on the X 1 and -2) (reviewed in MELLER AND RATTNER 2002). The MSL complex also contains one of two functionally redundant, non-coding roX RNAs (RNA on the X 1 and -2). The intact MSL complex localizes exclusively to X chromatin of males. Interestingly, we discovered that the roX RNAs are not only required for normal dosage compensation, but are also required for full expression of autosomal
heterochromatic genes in males, but not in females (DENG et al. 2009). Loss of both \textit{roX} RNAs reduces the expression of hundreds of autosomal heterochromatic genes. Furthermore, variegating heterochromatic insertions showed a dramatic increase in expression, known as “suppression of PEV” upon loss of \textit{roX} RNAs in males, but not in females (Figure 2). Both the reduced expression of endogenous heterochromatic genes and increased expression of variegating euchromatic transgenes in heterochromatic environments are symptomatic of disruption of heterochromatin. Identification of a condition (i.e. loss of \textit{roX} RNA) that differentially affects heterochromatin in male and female flies reveals that heterochromatin differs in the sexes. We proposed that \textit{roX} RNA is required for heterochromatic integrity in males, but not in females. Further studies showed that some, but not all, MSL proteins are also necessary for full expression of heterochromatic genes in males (DENG et al. 2009; Koya and Meller, Submitted). Interestingly, MSL2, the only male-limited member of MSL complex, is unnecessary for full expression of autosomal heterochromatic genes in males (DENG et al. 2009). This reveals that the full MSL complex is not required for heterochromatin. But the finding that the only male limited member of the MSL complex was not involved in this process raised the question of how the sex- specificitiy of this process is achieved. The objective of my dissertation is to determine how the sex of the organism regulates heterochromatin.

I first wanted to know if \textit{roX} RNAs regulate heterochromatin directly or indirectly. We hypothesized that \textit{roX} RNA might participate in initial formation of heterochromatin, which occurs 1-3 h after embryo deposition (AEL) (ELGIN AND
To accomplish this, I developed a gene engineering technique named Targeted Gene Conversion (TGC), and used it to tag the endogenous roX allele with six MS2 loops (roX<sup>1MS2-6</sup>). roX<sup>1MS2-6</sup> localization is visualized in vivo when an MCP-GFP fusion protein, capable of binding to MS2 loops, is present (BERTRAND et al. 1998). My studies revealed localization of roX RNAs on the male X-chromosome after 3 hr AEL, but the GFP signal was too weak to be useful in younger embryos. Testing of TGC and generation of roX<sup>1MS2-6</sup> is described in Chapter 3 (APTE et al. 2014).

Next, I initiated a search for the genetic basis of the sexual dimorphism of heterochromatin. I hypothesized that either the somatic sex determination pathway, or direct sensing of karyotype, could be the signal that regulates heterochromatin. Interestingly, flies pair homologous chromosomes in somatic tissues throughout life. As the non-homologous X and Y chromosomes do not pair, unpaired chromatin could signal the male karyotype. Chapter 2 is a review highlighting the role of chromosome pairing in regulation of gene expression (APTE AND MELLER 2012).

To identify the genetic pathway that leads to sexually dimorphic heterochromatin, I performed systematic analysis with sex determination mutants, as well as number of genes implicated in chromosome pairing. This required development of an assay that reliably identifies heterochromatin that requires roX (masculine heterochromatin). I developed a PEV assay (described in Chapter 4) that enabled me to distinguish masculine and feminine heterochromatin. Using this assay, I found that mutation of Topoisomerase II
(Top2), a general chromatin organizer that is necessary for homolog pairing, masculinizes XX heterochromatin. While this is provocative, and consistent with the idea that full pairing signals an XX karyotype, Top2 was the only pairing modulator tested that disrupted heterochromatic sex. Coincidentally, in interphase nuclei Top2 is enriched on a large (~10 Mb) block of pericentromeric satellite repeats, known as the 359 bp repeats or 1.688g/cm$^3$ repeats, that are exclusive to the X chromosome. Translocations that remove almost all pericentromeric heterochromatin from the X enabled me to test the idea that the interaction of Top2 and X-heterochromatin could underlie a karyotype sensing mechanism that regulates the sexual differentiation of heterochromatin. These studies, described in Chapter 4, reveal a novel sex-determination signal that that links fly karyotype to heterochromatin (Apte and Meller, Submitted).

Chapter 5 is a summary of my findings and a discussion of the questions raised by my research.
Chapter 2

Homologue pairing in flies and mammals: gene regulation when two are involved

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INTRODUCTION

One of the most intriguing aspects of somatic homologue pairing is that such a basic condition has enormous variability between species. Homologues pair vigorously in *Drosophila*, as illustrated by the remarkable alignment of polytene chromosomes. In fact, homologue pairing is pervasive throughout the Diptera, but in other organisms, homologue pairing is often uncertain (STEVENS, 1908; METZ, 1916). Close association of homologous chromosomes in vegetative diploid budding yeast has been reported, but a careful reexamination suggested that little, if any, pairing occurs (LORENZ *et al.* 2003). In diploid fission yeast both homologues occupy the same chromosome territory and centromeric pairing is observed in most cells (SCHERTHAN *et al.* 1994). Early studies suggested somatic homologue pairing in numerous plant species (Reviewed in METZ, 1916). Recent work supports the idea of homologue pairing in some grains and fungi, but also casts doubt on other reports of pairing in plants (ARAMAYO AND METZENBERG 1996; HOLLICK *et al.* 1997; BENDER 1998;
MAMMALS: PAIRING TO SHARE INFORMATION

Mammals have perhaps the most elaborate manifestation of homologue pairing. While complete pairing of the mammalian genome is not reported outside of the germ line, somatic pairing of specific chromosomal regions does occur, but is tightly regulated. For example, homologous association of pericentromeric regions of human chromosome 1 is detected in cerebellar, but not cerebral, tissue (ARNOLDUS et al. 1989). Heterochromatic regions of chromosomes 8 and 17 also pair in parts of the brain (Fig. 2.1 A) (ARNOLDUS et al. 1991; DALRYMPLE et al. 1994). Chromosome-specific pairing of chromosome 7 and 10 is also seen in case of cell line derived from follicular lymphoma (ATKIN and JACKSON 1996). Several cell lines derived from renal carcinomas display an abnormal pairing of one arm of chromosome 19, and mis-express genes within the paired region. (Fig. 2.1 B) (KOEMAN et al. 2008). This suggests that modulation of homologue associations may be necessary for normal gene regulation. The mechanism of pairing in these examples has not been investigated. However, this type of pairing is very tissue-specific and limited to portions of particular chromosomes. It therefore must depend on chromosome-specific features, as well as developmental cues.

The best understood somatic homologue associations in mammalian cells are transient and occur at individual loci, rather than encompassing extensive chromosomal regions. These contacts appear to be a subset of long-range interactions between chromosomes, which includes looping and interactions
between non-homologous regions (Fig. 2.1 C) (CREMER AND CREMER 2001; BARTKUHN AND RENKAWITZ 2008). One notable function of these interactions is their role in controlling monoallelic expression of imprinted genes, and from the female X chromosome.

The long-range contacts made by mammalian homologues overlay a general nuclear organization that seems designed to discourage interaction. Mammalian chromosomes occupy non-overlapping regions, termed chromosome territories, in the nucleus. These territories are organized by specific rules (Reviewed by SPECTOR 2003). For example, gene-poor regions tend to be close to the nuclear membrane, while gene-dense chromosomes localize in interior of the nucleus (CROFT et al. 1999; CREMER AND CREMER 2001). The territories of small and early replicating chromosomes also tend to be interior. Interestingly, in human epithelial cancer cell lines and mouse primary lymphocytes the territories occupied by the homologues are more widely separated than expected from a random distribution (CADDLE et al. 2007; HERIDE et al. 2010). One function of chromosome territories may be to keep the homologues apart.

The properties of the molecules that mediate long-range contacts between allelic and non-allelic loci suggest strategies that facilitate specific interactions. One of these molecules is CTCF (CCCTC-binding factor), a highly conserved, DNA-binding protein with a multitude of seemingly disparate regulatory functions (Reviewed by PHILLIPS AND CORCES 2009). Depending on context and binding partners, CTCF can be a transcriptional repressor or an activator
Adjacent CTCF binding sites are often drawn into chromatin loops, insulating promoters from nearby regulatory regions (MURRELL et al. 2004; KURUKUTI et al. 2006; SPLINTER et al. 2006; HOU et al. 2008; LI et al. 2008; MAJUMDER et al. 2008). One of the best-understood examples is found at the imprinted Igf2/H19 locus. Imprinting, established in the parental germ line, produces an allele-specific difference in genetic properties (Reviewed by VERONA et al. 2003). The Igf2/H19 locus has a CTCF-binding site that is differentially methylated in the parental germ lines (TREMBLAY et al. 1995; HARK et al. 2000; FEDORIW et al. 2004). Methylation of the paternal allele blocks CTCF binding, preventing formation of an insulator that would otherwise separate Igf2 from an enhancer (BELL AND FELSENFELD 2000; HARK et al. 2000; KANDURI et al. 2000; SZABO et al. 2000). On the maternal allele, CTCF binds between Igf2 and this enhancer, silencing Igf2 by insulation and through recruitment SUZ12, a member of the Polycomb Repressive Complex 2 (PRC2) (LI et al. 2008). On the maternal chromosome CTCF binding adjacent to H19 is necessary to induce expression of this transcript (SCHOENHERR et al. 2003).

CTCF also mediates interactions between Igf2/H19, on chromosome 7, and other regions throughout the genome. Igf2/H19 contacts the Wsb1/Nf1 locus on chromosome 11 (KURUKUTI et al. 2006; LING et al. 2006). This interaction is dependent upon binding of CTCF to the maternal Igf2/H19 allele, and is required for mono-allelic expression from Wsb1/Nf1. Additional interactions between
Igf2/H19 and several other imprinted loci have been identified, and these findings are consistent with the idea that Igf2/H19 coordinates the epigenetic status of imprinted regions throughout the genome (SANDHU et al. 2009).

Some imprinted homologues pair transiently, an activity that may be necessary for normal developmental regulation. In lymphocytes, transient association at 15q11-q13 occurs in late S phase (LASALLE AND LALANDE 1996). This region contains imprinted loci containing several monoallelically expressed genes (Reviewed by LALANDE 1997). Loss of expression, or lack of normal imprinting at this locus, causes Prader-Willi and Angelman syndromes, both of which display developmental and neurological abnormalities. Interestingly, lymphocytes from Prader-Willi and Angelman syndrome patients do not pair (LASALLE AND LALANDE 1996). Homologue communication at 15q11-q13 may be a factor in normal brain development, as this locus pairs persistently in normal brain, but not in brains from patients with some autism-spectrum disorders (THATCHER et al. 2005).

Homologue pairing also plays a central role in orchestration of X inactivation in mammalian females. Mammalian females randomly inactivate one X chromosome, thus maintaining an equivalent ratio of X to autosomal gene products in both sexes (GUPTA et al. 2006; NGUYEN AND DISTECHE 2006). Each cell of the early embryo counts the number of X chromosomes and inactivates all but one (Reviewed by ROYCE-TOLLAND AND PANNING 2008). Counting, and choice of the inactive X, relies on a transient pairing of the X inactivation center (Xic), a locus on the X chromosome (Fig. 2.1 D). Pairing is
believed to enable XX cells to coordinate inactivation of a single X chromosome. Deletion of regions engaged in pairing lead to skewed or chaotic X inactivation (LEE 2002). The process of pairing is complex, involving multiple elements within the Xic. The X-pairing region (Xpr) may support initial interactions, and its deletion diminishes Xic pairing (BACHER et al. 2006; AUGUI et al. 2007). Several genes within the Xic produce non-coding RNAs that participate in counting and inactivation of the X chromosome. Xist, a long non-coding RNA, initiates the process of X inactivation and coats the inactive X (Reviewed by CHOW AND HEARD 2009). Tsix, transcribed antisense to Xist, and a nearby gene Xite, contribute to pairing of the Xic and also produce non-coding RNAs (Reviewed by LEE 2009). Following pairing, transcription of Tsix and Xite is necessary for orderly X inactivation, suggesting that communication might occur by an RNA-protein bridge between two X-chromosomes (XU et al. 2007). CTCF plays a central role in pairing at the Xic. The Tsix promoter contains numerous CTCF binding sites (Fig. 2.1 D) (CHAO et al. 2002; XU et al. 2006; XU et al. 2007; XU and COOK 2008). Pairing at the Xic is disrupted upon the loss of CTCF (DONOHoe et al. 2009). Initiation of inactivation occurs during a narrow window in early development (WUTZ AND JAENISCH 2000). Oct4, a transcription factor key to the maintenance of stem cells, forms a complex with CTCF at Tsix, and is required for transient association of Xics (DONOHoe et al. 2009). After this transient pairing, the X chromosomes separate, assume different fates and localize to distinct nuclear compartments.
Figure 2.1. Modes of somatic homolog pairing in mammalian tissues. A) Pericentromeric homologue pairing in parts of the brain. Centromeres are depicted by black dots. B) Abnormal pairing of chromosome 19q in renal carcinoma. C) Looping between two sites on a chromosome (left) and interchromosomal contacts (right) are mediated by sequence-specific DNA binding proteins such as CTCF (triangle) and cohesin (brown circle). D) Pairing of the X inactivation center (Xic) initiates X chromosome inactivation in females. Sequences that participate in Xic pairing are depicted. The X-pairing region (Xpr, yellow) initiates Xic pairing. Tsix (light blue) and Xite (pink) pair transiently, enabling counting and choice to occur. Oct4 and CTCF are necessary for contact and communication at the Xic. Oct4 binding sites (green ovals) and CTCF binding sites (triangles) within the Tsix and Xite regions of the mouse Xic are depicted.
The examples above illustrate the idea that CTCF fulfills disparate functions in a developmental and cell type-specific manner. The proteins mentioned above, Oct4 and SUZ12, are among many CTCF partners that enable modulation of CTCF effects (WALLACE AND FELSENFELD 2007). An additional CTCF binding protein that contributes to its localization and function is nucleophosmin, a component of the nucleolus (YUSUFZAI et al. 2004). Some loci that bind CTCF are anchored at the nucleolus, leading to the idea that the nucleolus functions as a hub where long-range interactions occur. While this appears to be a factor for some CTCF-bound loci, it does not contribute to X chromosome pairing (YUSUFZAI et al. 2004; MASUI et al. 2011).

Another protein that contributes to CTCF function is cohesin, a multi-subunit complex that regulates sister chromatid cohesion during meiosis and mitosis. Cohesin, consisting of SMC1, SMC3, Scc1 and Scc3 subunits, is believed to encircle sister chromatids to maintain their association (IVANOV AND NASMYTH 2007; NASMYTH AND HAERING 2009). The C-terminus of CTCF interacts with the cohesin subunit Scc3, and cohesin and CTCF are often colocalized on mammalian chromosomes (PARELHO et al. 2008; WENDT et al. 2008; XIAO et al. 2011). Depletion of CTCF results in loss of cohesin binding but, at most sites, loss of cohesin does not affect CTCF binding to DNA (HADJUR et al. 2009; NATIVIO et al. 2009). CTCF thus appears to recruit cohesin to specific DNA sequences. This facilitates long-range interactions, either by securing aligned regions or by inducing looping. For example, cohesin plays a regulatory role in CTCF-mediated intra-chromosomal contacts between sites in the
interferon-γ locus (XIAO et al.; HADJUR et al. 2009). Loss of cohesin or CTCF also leads to misregulation of expression from Igf2/H19 (LING et al. 2006; WENDT et al. 2008).

While cohesin colocalizes with CTCF on mammalian chromosomes, the association of these molecules is not universal. In Drosophila, cohesin and CTCF have not yet been shown to colocalize. In spite of this, in flies CTCF performs many functions similar to those in mammals. For example, it localizes to insulators and contributes to looping between boundary elements (HOLOHAN et al. 2007; KYRCHANOVA et al. 2011). Drosophila CTCF also plays a role in imprinting in flies (MACDONALD et al. 2010).

**FLIES: ALWAYS IN TOUCH**

In contrast to the carefully orchestrated pairing of specific loci in mammals, complete homologue pairing is the default condition in Drosophila. Pairing is evident from the mitotic cycle 13 of embryogenesis onwards (FUNG et al. 1998; HIRAOKA et al. 1993). Cellularization occurs during cycle 14, which marks a dramatic reorganization of the nucleus (FOE AND ALBERTS 1983). Heterochromatin becomes detectable at cycle 14, and transcription of zygotic genes begins in earnest (LU et al. 1998). While pairing is persistent throughout the cell cycle from this point onwards, it is relaxed, but still apparent, during replication and mitosis (CSINK AND HENIKOFF 1998; WILLIAMS et al. 2007).

Homologues might encounter each other by directed movement, or by random diffusion (COOK 1997). Analysis of chromosomal movements preceding pairing in embryos supports the idea that random motion leads to homologue
encounters, and suggests independent initiation at numerous sites, rather than a processive zippering along the length of the chromosome (CSINK AND HENIKOFF 1998; FUNG et al. 1998). Space constraints within a chromosome territory, or an underlying chromosome arrangement could speed the search. Early studies by Rabl and Boveri revealed the non-random organization of the interphase nucleus. The centromeres cluster at one pole of the nucleus, while the chromosome arms extend across the nucleus towards the other pole. This polarized pattern of chromosomal arrangement, known as Rabl configuration, is not apparent in some species (rice, maize, mouse and humans) but is observed in a wide range of organisms (S. cerevisiae, S. Pombe, Drosophila and several grains) (Reviewed by SPECTOR 2003; SANTOS AND SHAW 2004). The Rabl configuration is reminiscent of the arrangement of chromosomes following mitosis, where the centromeres lead the chromosomes into the daughter cells. While the anaphase movement of chromosomes does promote this arrangement, cell division is not essential for the Rabl conformation in yeast (JIN et al. 2000). Regardless of how formed, homologous chromosomes in the Rabl configuration are roughly aligned, more or less parallel, placing alleles closer together than predicted by chance distribution.

While pairing of imprinted loci and the Xic is necessary for correct regulation of developmentally important genes in mammals, there are no examples of flies utilizing chromosome pairing to count X chromosomes or to regulate monoallelic gene expression. However, homologue pairing in flies does affect gene expression through a mechanism known as transvection (LEWIS
Pioneering work by Lewis on the Ultrabithorax (Ubx) gene showed that the mutant phenotype was stronger when pairing between two loss of function Ubx alleles was disrupted by chromosomal re-arrangements. When paired, Ubx expression was elevated, enabling complementation between the two mutations. A well-supported model for transvection is that pairing enables regulatory elements on one chromosome to drive (or silence) expression from an intact promoter on the other chromosome (GEYER et al. 1990). Confirmation of transvection is obtained when the phenotype is sensitive to disruption of pairing, for example, by inversion of one chromosome (LEWIS 1954; OU et al. 2009). Transvection has been demonstrated for numerous genes in Drosophila, and it appears able to operate throughout the genome (CHEN et al. 2002). Transvection has also been observed in the diploid stages of Neurospora (ARAMAYO AND METZENBERG 1996). A few examples of transvection have been described in mammals, and the term is often used to describe non-allelic regulatory interactions in trans, such as the CTCF-mediated long-range interactions that were described in preceding sections (RASSOULZADEGAN et al. 2002; LIU et al. 2008).

A limitation of our understanding of transvection is how alleles communicate, a mechanism that may differ from gene to gene. For example, transvection at Ubx is disrupted by breaks anywhere within a large critical region between Ubx and the centromere, but transvection at the yellow gene is only sensitive to breaks very close to the gene. This is consistent with different mechanisms of pairing or communication at these loci, but could also reflect the
length of the cell cycle, and thus the time available for homologue association, at the time of gene expression (GOLIC AND GOLIC 1996). For example, expression of *Ubx* is required in rapidly cycling embryonic cells. In contrast, the critical period for *yellow* expression is in pupal cells that have ceased dividing. In accordance with this idea, extension of the cell cycle in *Ubx* mutants with inversions reduces phenotypic severity, presumably by allowing extended time for chromosome pairing (GOLIC AND GOLIC 1996).

One molecule that affects pairing-dependent gene regulation is encoded by *zeste* (*z*). Zeste is a DNA-binding protein that affects pairing-dependent expression at many genes that display transvection (Reviewed by PIRROTTA 1991; DUNCAN 2002). The Zeste protein polymerizes, leading to the suggestion that it might bridge homologues, but loss of Zeste does not affect homologue pairing (GEMKOW et al. 1998). Zeste binding sites are found in promoters, and the Zeste protein interacts with the activating *Trithorax* chromatin regulatory complex, as well as the repressing *Polycomb* PRC1 complex (KAL et al. 2000; SAURIN et al. 2001). Thus it appears likely that Zeste is a transcription factor able to interpret the state of homologue pairing.

An RNAi screen in tissue culture cells identified *Topoisomerase II (Top2)* as necessary player in homologue pairing (WILLIAMS et al. 2007). Topoisomerases play pivotal roles by solving topological problems associated with DNA replication, transcription, recombination, repair and chromosome segregation (Reviewed by NITISS 2009). Type II topoisomerases introduce double strand breaks, pass an intact DNA duplex through the cut, and rejoin the
cut ends. Top2 also makes up a large fraction of the insoluble nuclear matrix and contributes to chromosome architecture. It preferentially binds scaffold-associated regions, which anchor chromatin loops during interphase (GASSER et al. 1986; ADACHI et al. 1989). There are several potential mechanisms through which Top2 might contribute to pairing. Because it plays a central role in chromosome organization, loss of Top2 could lead to a general disruption that abrogates homologue association. It is also possible that Top2 engages in protein/protein interactions that stabilize pairing.


In spite of the dependent interactions between condensin and Top2, condensin acts to antagonize homologue pairing in Drosophila (HARTL et al.}
2008). Most dramatically, ectopic expression of Cap-H2 in salivary glands separates the aligned polytene chromosomes. Increased condensin reduces transvection at two loci, revealing the dissociation of paired homologues in diploid cells. The involvement of Top2 and condensin reveals that homologue pairing in flies is regulated by conserved proteins necessary for the maintenance of chromosomal architecture and stability in all eukaryotic organisms. It will be fascinating to see if Top2 or condensin levels affect pairing in other organisms.

PAIRING AND SEX CHROMOSOMES

An unanswered question is whether pairing-dependent regulation contributes to the expression of wild type genes in *Drosophila*. Analysis of *Ubx* revealed that expression from a wild type allele was increased when it could pair with a gain of function mutation (GOLDSBOROUGH AND KORNBERG 1996). Homologue pairing might also contribute to expression of other unmutated genes in a wild type context. The phenotypic normality of flies with inverted chromosomes would suggest that transvection makes little contribution to expression, but a functional assay for homologue association demonstrated that alleles on inverted chromosomes can pair surprisingly efficiently, when given sufficient time (GOLIC AND GOLIC 1996). But there are situations in which homologue pairing cannot occur, including the single male X chromosome and regions made hemizygous by deficiency. If pairing influences expression of wild type genes, the regulation of the entire X chromosome might differ between the sexes. This could contribute to sexually dimorphic expression, or influence the biology of the X chromosome.
Flies have a dedicated regulatory system that accommodates hemizygosity of the X chromosome in males. Males produce the chromatin-modifying Male Specific Lethal (MSL) complex, which is recruited to the X chromosome at 3 h after fertilization (LUCCHESI 1996). The result is increased expression of virtually every X-linked gene. Surprisingly, RNA sequencing of single, sexed embryos has identified partial dosage compensation at mitotic cycle 13, an hour before the MSL complex localizes to the X chromosome (LOTT et al. 2011). One mechanism proposed to explain this is that pairing of X chromatin in females inhibits transcription from X-linked genes. This idea deserves to be tested, as it could explain several situations in which dosage compensation occurs in the absence of the MSL complex. For example, X-linked genes are dosage compensated in the male germ line, where the MSL complex is not formed (RASTELLI AND KURODA 1998; GUPTA et al. 2006). Autosomal deficiencies are partially compensated by an unknown mechanism (STENBERG AND LARSSON 2011). In addition, considerable evidence supports the idea that the MSL complex does not fully compensate X-linked genes in somatic cells. If formation of the MSL complex is blocked, expression of X-linked genes is reduced by 25-30%, rather than the predicted 50% (HAMADA et al. 2005; DENG AND MELLER 2006). These observations support the idea that differences in gene copy number are buffered by mechanisms that operate throughout the genome (Reviewed by STENBERG AND LARSSON 2011)

A striking feature of the X chromosome is the difference in gene distribution between the X chromosome and the autosomes in many species
(Reviewed by VICOSO AND CHARLESWORTH 2006; GURBICH AND BACHTROG 2008). For example, the mammalian X chromosome appears enriched for genes with a male-biased expression, including those expressed in the premeiotic testes (LERCHER et al. 2003). This is postulated to reflect the fact that hemizygosity of the male X chromosome enables rapid selection for beneficial recessive alleles. The same argument should apply to other species with XY males, including flies. However, the X chromosomes of Drosophila melanogaster and related species are depleted for genes with male-biased expression in somatic tissues and testes, and enriched for genes with female-biased expression (STURGILL et al. 2007). These notable differences in the distributions of sex-biased genes in mammals and flies have yet to be adequately explained. A recent study revealed that the fly X chromosome was also depleted for developmentally regulated genes, with the notable exception of those expressed in the ovary (MIKHAYLOVA AND NURMINSKY 2011). The authors propose that demasculinization of the X chromosome was due in part to the fact that male-biased genes tend to be developmentally regulated, and suggest that chromatin modification by the MSL complex may be incompatible with developmental regulation, making the X chromosome an unfavorable environment. However, a genome-wide buffering system that contributes to X chromosome dosage compensation could also influence the distribution of developmentally regulated genes. Analysis of expression in flies with autosomal deficiencies and duplications lends support to the idea that such a system exists, but constitutively expressed genes and those with highly regulated expression
respond differently (STENBERG et al. 2009). A speculative model for the role of homologue pairing in buffering gene dose is presented (in Fig. 2.2). A key feature of our model is that homologue pairing is repressive. The absence of pairing of the male X chromosome, and autosomal deficiencies, leads to a modest increase in expression from these regions.

Figure 2.2. Hypothetical model for pairing-dependent buffering of gene dosage in flies. A) The unpaired X chromosome of males escapes repression. B) Paired female X chromosomes are subject to repression. C) Paired regions of an autosome are repressed, but an unpaired region created by deficiency escapes repression.
CONCLUSIONS

Somatic chromosome pairing obeys strikingly different rules in mammals and flies. Mammals sharply limit contacts between homologues. When homologues do make contact it often serves to coordinate regulatory mechanisms, such as imprinting and X inactivation, that are essential for normal development. It seems ironic that mammals use pairing to communicate critical information, yet flies, with constant homologue pairing, appear to make little use of this feature of genome organization. Recent studies of early dosage compensation and buffering of copy number variation in flies suggest that additional regulatory mechanisms exist to accommodate variation in gene dosage. A pairing-based regulation of gene expression could account for many of the findings of these studies. A broader question is why homologue pairing exists in some species, but not in others. The precise control of homologue association in mammals, and inappropriate pairing in some cancers, suggests that homologue association can be dangerous. What this danger is, and how flies evade it, remains to be discovered.
Chapter 3

Generation of a useful roX1 allele by Targeted Gene Conversion


INTRODUCTION

roX1 and roX2 (RNA on the X -1 and 2) are non-coding transcripts that play a central role in sex chromosome dosage compensation in flies. This process ensures a constant ratio of X-linked to autosomal gene products in males, which have a single X chromosome. A complex of proteins and roX RNA (the Male-specific lethal, or MSL complex) is recruited to X-linked genes. This complex directs chromatin modifications that result in increased expression from X-linked genes (SMITH et al. 2001; DENG AND MELLER 2006; CONRAD AND AKHTAR 2011; LARSCHAN et al. 2012). The roX RNAs are essential for X localization of the intact complex, and, in spite of their lack of sequence similarity, are functionally redundant (MELLER AND RATTNER 2002). Expression of roX RNA from an autosomal transgene will rescue roX1 roX2 males. However, both roX genes are X-linked, and both can recruit the MSL complex to chromatin adjacent to sites of roX transcription (KELLEY et al. 1999; KAGEYAMA et al. 2001; PARK et al. 2003; OH et al. 2004). This suggests that the function of the
*roX* genes depends, in part, on their situation on the X chromosome.

During P-element induced mutagenesis of *roX1* we observed numerous identical rearrangements. These appear to be produced by a highly favored gene conversion that replaces over 1 kb of *roX1* with sequence contained within a P-element inserted in *roX1*. Replacement is driven by homology between genomic sequence flanking the insertion site and within the P-element. We tested this as a general strategy for gene engineering by introducing RNA loops from the MS2 virus (MS2 loops) into the endogenous *roX1* gene, creating *roX1*\(^{MS2-6}\). RNAs that contain MS2 loops can be visualized in vivo when a fusion of GFP to the MS2 loop binding protein (MCP-GFP) is expressed (BERTRAND et al. 1998). The *roX1*\(^{MS2-6}\) allele preserves the normal chromatin context of *roX1* and lacks all P-element sequence. *roX1*\(^{MS2-6}\) activity in dosage compensation is indistinguishable from that of wild type *roX1*. We have named the replacement strategy ‘Targeted Gene Conversion’ (TGC). TGC is technically simple and capable of introducing large blocks of non-homologous sequence. It is able to replace sequences that are over 1 kb from a P-element insertion. The strategy that we tested relies on a P-element near the site to be mutated. However, recently developed methods for directed mutagenesis may enable a modified form of TGC in regions that lack P-elements.
MATERIALS AND METHODS

Fly culture

Flies were raised on a yeast, molasses and corn meal diet at room temperature. Mutations are described in citations or LINDSLEY and ZIMM 1992.

Gene conversion using an autosomal template

The p[w^{+mC} GM roX1^{MS2-6/12}] transgenes were generated by inserting 6 or 12 MS2 loops into a BglII site in a 4.9 kb genomic EcoR1 fragment containing roX1. Males with autosomal insertions of these transgenes were mated to w roX1\Delta^{891}\text{Df(1)52/}\text{Binsincy virgins to generate w roX1\Delta^{891}\text{Df(1)52/ Dp(1;Y) B}^{S} v^{+} y^{+}; p[w^{+mC} GM roX1^{MS2-6/12}]/+ males. Df(1)52 removes roX2 and nearby essential genes. Males are rescued by a duplication of the roX2 region on the Y chromosome. These males were mated to C(1)DX y^{1} f^{1}/ Dp(1;Y) B^{S} v^{+} y^{+}; p[y^{+}\Delta2-3]99B/+ females to produce w roX1\Delta^{891}\text{Df(1)52; p[w^{+mC} GM roX1^{MS2-6/12}]}/ p[y^{+}\Delta2-3]99 dysgenic sons, that were mated to C(1)DX y^{1} f^{1}; p[4\Delta4.3] females. The cosmid p[4\Delta4.3] restores all essential genes removed by Df(1)52, but is deleted for roX2 and w^{+mC} (MELLER AND RATTNER 2002). If the break created by mobilization of roX1\Delta^{891} was repaired by copying roX1 sequence within p[w^{+mC} GM roX1^{MS2-6/12}], this would result in loss of the w^{+} marker, restoration of roX1 activity and incorporation of MS2 loops into roX1. White eyed sons were mated individually to C(1)DX y^{1} f^{1}; [4\Delta4.3] females and MS2 loop incorporation determined by PCR of single fly squashes.
Targeted transposition

The p[w+mc GM roX1^{MS2-6}] transgene was moved into roX1 by targeted transposition, using the roX1^{mb710} plArB element as the target site. Dysgenic males (y w-roX1^{mb710}; p[w+mc GM roX1^{MS2-6}] / Sb p[rY^Δ2-3]99B) were mated to C(1)DX y¹ f¹ females. Hops (w+mc Sb sons) were collected and individually mated to C(1)DX y¹ f¹ females. X-linked insertions were mapped by in situ hybridization. Insertions close to roX1 (3F) were characterized by single fly PCR to verify the presence, and orientation, of p[w+mc GM roX1^{MS2-6}]. Outward facing primers (plac1(+), pry4(+) and pry2) in P-ends were paired with each other, or with primers in roX1 (BPR10, BPR15) to determine the arrangement of tandem insertions. Primers are presented in Table 3.1. Targeted transpositions are designated as roX1^{[MS2-6]TXX} (Tandem insertion) or roX1^{[MS2-6]RXX} (Replacement of plArB), followed by the transposition number.

Table 3.1 Primer sequences used for characterization of roX1 rearrangements (5’-3’)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pry2</td>
<td>CTTGCCGACGGGACCACCTTATGTTATT</td>
</tr>
<tr>
<td>Pry4(+)</td>
<td>TAATCAACAATCATATCGCTGTCTCACTCAG</td>
</tr>
<tr>
<td>pLac1(+)</td>
<td>CCAAGGCTGCACCCAAGGCTGCTGCTCCAC</td>
</tr>
<tr>
<td>BPR10</td>
<td>GAGGACCCGGGTAGAGCGCATAGCTCTTG</td>
</tr>
<tr>
<td>BPR15</td>
<td>CGGAACGAAAGAGACAAATG</td>
</tr>
<tr>
<td>roX1^{ex8}F</td>
<td>GCTCTAGAATTCCGAAGTGGTTGCGTATAACGG</td>
</tr>
<tr>
<td>BPR19</td>
<td>GATGGCCTTCAGTTGGTG</td>
</tr>
<tr>
<td>roX1 F8</td>
<td>TCAGTGTTACGACCTCGTC</td>
</tr>
<tr>
<td>roX1 R8</td>
<td>TTTTGGGCACCTTGGTGAAG</td>
</tr>
</tbody>
</table>
Gene conversion in males

Three independent targeted transpositions of \( p[w^{+mC}\text{ GM } roX1^{MS2-6}] \) in \( roX1 \) were remobilized with \( p[\gamma^+\Delta2-3]99 \). Lines \( roX1^{[MS2-6]T2A} \) and \( roX1^{[MS2-6]T4B} \) retain \( plArB \) in tandem, and \( roX1^{[MS2-6]R36A} \) has replaced \( plArB \) with \( p[w^{+mC}\text{ GM } roX1^{MS2-6}] \). Dysgenic males were mated to \( C(1)DX y^I f^I \) females. White-eyed sons were individually mated to \( C(1)DX y^I f^d \) females. Introduction of MS2 loops and retention of P-element sequences was determined by PCR. \( roX1 \) primers flanking the MS2 loops (\( roX1^{ex6}F \) and BPR19) amplify 547 bp from \( roX1^+ \) and 869 bp when MS2 loops are inserted (\( roX1^{MS2-6} \)).

Gene conversion in females

The targeted transposition \( roX1^{[MS2-6]T2A} \) was mobilized in females. A total of 244 dysgenic females (\( roX1^{[MS2-6]T2A} / \text{ Binsincy; Sb p[\gamma^+\Delta2-3]99/+} \)) were mated to \( yw \) males, with about 10 females per vial. 25 out of 26 vials produced at least one white-eyed, non-balancer son, indicating excision. Two hundred and sixty-nine excisions were mated individually to \( C(1)DX y^I f^d \) females. A randomly selected subset of these was analyzed by PCR for MS2 loop incorporation and loss of P-element sequences.

DNA blotting

DNA from 100 flies was extracted as described previously (http://www.fruitfly.org/about/methods/ inverse.pcr.html). DNA was suspended in 300 µl of DEPC water and treated with RNAse A and Proteinase K. Fifteen µg of
DNA was digested overnight with EcoRI, concentrated, electrophoresed and transferred to a charged nylon membrane. Blots were probed with a P\(^{32}\)-labelled, 2.03 kb EcoR1-Mlu1 fragment spanning the promoter and 5’ end of roX1 using described methods (CHURCH AND GILBERT 1984). Restriction digests of a 4.9 kb roX1 genomic clone served as a molecular weight marker.

**Visualization, photography and image processing**

Immunodetection of MSL1 on polytene preparations was performed as previously described (KELLEY et al. 1999). MCP-GFP is removed by acetic acid fixation, preventing visualization on polytene chromosomes. To visualize MCP-GFP recruitment in embryo nuclei, homozygous roX1\(^{MS2-6} \)roX2\(^{\Delta} \) [\(w^+mC\) MCP-GFP] females were mated to males carrying a p[\(w^+mC\ sqh-mCherry\)] insertion on the X chromosome. Male embryos are distinguished by lack of mCherry signal. Three to 12 h embryo collections were dechorionated, fixed in 4% paraformaldehyde with 0.1 % Tween-20, DAPI stained and mounted with DABCO anti-fade agent in 50% glycerol. Z-stacks were recorded for individual embryos using an Olympus Fluoview FV10i scanning confocal microscope with a 60X water/oil immersion lens. Images were processed by converting to 8 bit format and importing individual Z stacks into ImageJ. As mCherry signal was weak and diffuse, the brightness of this channel was uniformly enhanced for reproduction (Fig. 3.4 C, H and M). Consistent patterns of GFP localization were observed in images of over 30 embryos from 3 collections.
RESULTS

An autosomal $\text{roX1}^{\text{MS2-6}}$ transgene restores X-chromosomal MSL1 localization

RNA accumulation can be visualized in tissues or chromosome preparations by \textit{in situ} hybridization. Although useful, this method is time consuming and incompatible with living tissue. RNAs that contain stem loops from the MS2 virus can be visualized \textit{in vivo} when a fusion of GFP to the MS2 loop binding protein (MCP-GFP) is expressed (Fig. 3.1 A) (BERTRAND \textit{et al.} 1998). A $\text{roX1}$ transgene was constructed with six MS2 loops ($\text{roX1}^{\text{MS2-6}}$) inserted in a region previously shown to be non-essential (Fig. 3.1 B) (STUCKENHOLZ \textit{et al.} 2003; DENG \textit{et al.} 2005). An autosomal copy of this transgene, p[$w^{+mC} \text{GM roX1}^{\text{MS2-6}}$], rescues X-localization of a key member of the MSL complex, Male Specific Lethal 1 (MSL1) in $\text{roX1 \text{roX2}\Delta}$ males (Fig. 3.1 C). However, ectopic recruitment surrounding the site of transgene insertion is also observed (arrow, Fig. 3.1 C). Fully wild type behavior of $\text{roX1}$ is consequently expected to require expression from the X chromosome, possibly from the $\text{roX1}$ locus itself.
Figure 3.1. *roX1*$_{MS2-6}$ restores X chromosome MSL localization  

A) MS2 loops in RNA enable transcript visualization with MS2 coat protein (MCP) fused to GFP. 

B) Structure of the p[*w*$_{+mC}$ GM *roX1*$_{MS2-6}$] transgene. Six tandem MS2 loops (322 bp) are inserted in a 4.9 kb genomic *roX1* clone. 

C) Polytene chromosomes from a male *roX1*$_{ex6}$*roX2*Δ/Y; p[*w*$_{+mC}$ GM *roX1*$_{MS2-6}$] /+ larva were immunostained with MSL1 antibody detected by Texas Red. DNA is counterstained with DAPI. Restoration of X localization and spreading of MSL1 into the autosome flanking the p[*w*$_{+mC}$ GM *roX1*$_{MS2-6}$] insertion site (arrow) is observed.
Gene conversion by repair using a sister chromatid template

During P-element mutagenesis of roX1 we obtained a series of mutations that suggested a strategy for inducing precise changes in target genes. A reporter construct containing the roX1 promoter fused to LacZ (p[w^mC roX1P-βgal]) was moved into roX1 in an effort to capture enhancers in the vicinity. This was accomplished by targeted transposition to the plArB element in roX1<sub>′<sub>mb710</sub></sub> (Fig. 3.2 B) (GLOOR et al. 1991; HESLIP and HODGETTS 1994). The resulting insertion, roX1<sub>′</sub>wtandem, retained plArB and is marked with w<sup>+mC</sup>, facilitating subsequent mutagenesis. Hybrid Element Insertion was used to generate roX1Δ<sub>891</sub>, deleted for the plArB element and 891 bp flanking the insertion site, but retaining p[w<sup>+mC</sup> roX1P-βgal] (PRESTON AND ENGELS 1996; PRESTON et al. 1996)(Fig. 3.2 C). Remobilization of roX1Δ<sub>891</sub> produced numerous white-eyed offspring from virtually every dysgenic parent. However, only a few imprecise excisions were identified (Fig. 3.3 A and 3.4). Instead, almost 70% of excisions carried molecularly identical rearrangements exemplified by the severe roX1<sup>SMC17A</sup> allele (Fig. 3.2 D). These appear to be produced by a gene conversion that occurs when the double stranded break produced by P- element mobilization undergoes gap repair using a sister chromatid template (Fig. 3.3 B).
Figure 3.2. Overview of roX1<sup>SMC17A</sup> creation  A) roX1<sup>mb710</sup> is created by insertion of pArB. B) p[w<sup>matC</sup> roX1P-bgal], containing the roX1 promoter (white arrow) fused to LacZ, was moved into roX1 by targeted transposition. The resulting tandem insertion (roX1<sup>w+tandem</sup>) was the starting point for Hybrid Element Insertion mutagenesis that removed pArB and deleted 891 bp flanking the insertion site, producing roX1<sup>Δ891</sup> C). Mobilization of p[w<sup>matC</sup> roX1P-bgal] produced roX1<sup>SMC17A</sup> D), and numerous identical rearrangements. The roX1<sup>SMC17A</sup> chromosome carries the fusion of LacZ with the roX1 promoter that is present in p[w<sup>matC</sup> roX1P-bgal]. All roX1 sequences between the promoter and the 5' P-end have been replaced with a full length LacZ gene. The 5' P-end has been replaced precisely with the 3' end. A complete list of the rearrangement classes produced by roX1<sup>Δ891</sup> mobilization, and a model for the homology-dependent gene conversion event that likely produced roX1<sup>SMC17A</sup>, is presented in Figure 3.3.
Figure 3.3.  Rearrangements produced by roX1Δ891 mobilization  A) Four classes of rearrangements were present in white eyed offspring of dysgenic roX1Δ891 flies (top). The roX1 promoter is depicted by a white arrow. Imprecise excisions that remove all (class 1) or the 3' end (class 2) of p[w+mc roX1P-bgal] occurred in 4 flies. Rearrangements identical to roX1SMC17A (class 3) were recovered 38 times. Rearrangements similar to roX1SMC17A, but with the 3' P-end missing, or inserted at a different location, account for 14 flies (class 4). A hypothetical mechanism for generating class 4 is presented in Figure 3.4.  B) Excision followed by resection reveals homology between the roX1 promoters on the chromosome and in p[w+mc roX1P-bgal] (red arrow). Homology is also present at the 3' P-end on the sister chromatid and at the site where the 5' P-end excised (blue arrow). We postulate that these homologies support gap repair using a sister chromatid template. This will insert the full length LacZ gene into roX1 and substitute the 3' P-end for the original 5' end, the precise rearrangement found in roX1SMC17A (bottom). Drawings not to scale.
Figure 3.4. Proposed mechanism capable of producing class 4 rearrangements. A) Hybrid Element Insertion (HEI) creates an inverted duplication of p[w^mcrOX1P-bgal] on one chromatid, depicted in B. Red and blue arrows show the insertion sites of the 3' and 5' P-ends participating in HEI. Proximal (1,2) and distal (3,4) chromatid ends are labeled. HEI places a 3' P-end downstream from the 5' end in roX1Δ^891. A green genomic fragment from roX1 now appears in inverted orientation between the p[w^mcrOX1P-bgal] elements in B. B) Chromatid arm 1 is resected to reveal homology to the roX1 promoter. Broken arm 1 initiates recombinational repair with the roX1 promoter in p[w^mcrOX1P-bgal] (red arrow). C) Resolution produces a chromosome carrying the roX1 promoter fused to LacZ. The 5' P-end has been replaced by a 3' P-end that is downstream from the insertion sites in roX1Δ^891 and roX1^SMC17A. This model is consistent with the structure of roX1^SMC20A,B, identical to roX1^SMC17A but with the 3' P-end moved 350 bp, creating a deletion of 1.25 kb. Twelve additional flies in this class also had the roX1 promoter fused to LacZ, but no P-end could be detected using primers in roX1. We postulate that these rearrangements were similarly produced, but that the HEI insertion occurred distal to roX1.
The rearrangement generated is consistent with repair driven by homology between the roX1 promoter on the broken chromosome over 1 kb from the break site and in p[w^{+mC} roX1P-βgal]. Homology is also shared by terminal inverted repeats at the 5' and 3' P- element ends (P-ends). In all 38 flies recovered with this rearrangement, the 3' P-end has been precisely replaced by the 5' P-end, a structure consistent with the proposed mechanism of repair. These chromosomes have lost 1.2 kb of roX1 sequence flanking the p[w^{+mC} roX1P-βgal] insertion site and replaced it with over 3 kb of LacZ sequence fused to the roX1 promoter. This mechanism is thus capable of efficiently replacing large regions close to P-elements.

**Lack of repair utilizing a template on a different chromosome**

To determine if efficient gene conversion was an intrinsic property of the roX1 locus that is independent of template location, we attempted to generate a useful allele of roX1 by introducing sequence from an engineered roX1<sup>MS2-6</sup> transgene situated on an autosome. Gene conversion at white (w) occurs in a few percent of excisions when a P-element is mobilized from w and a template with homology to insertion site is present in the genome (BANGA AND BOYD 1992; JOHNSON-SCHLITZ AND ENGELS 1993; NASSIF et al. 1994; LANKENAU et al. 1996). We attempted to introduce MS2 loops into roX1 from an autosomal p[w^{+mC} GM roX1<sup>MS2-6</sup>] template. Dysgenic males with a p[w^{+mC} GM roX1<sup>MS2-6</sup>] donor on the 3rd chromosome and the roX1<sup>A891</sup> target site on the X chromosome were generated. To enable phenotypic detection of gene
conversion, the target X chromosome was also deleted for roX2 (see Materials and Methods for full description of genotypes and matings). roX1Δ891 is a severe loss of function mutant. Conversion to roX1<sup>MS2-6</sup> will restore male viability and eliminate the w<sup>+mc</sup> marker in roX1Δ891. Approximately 100 white-eyed sons were recovered and tested by PCR for incorporation of MS2 loops, but only wild type roX1 sequences were detected. While a gene conversion strategy utilizing a template situated on another chromosome may be productive in some situations, it was not useful in this instance.

**Targeted transposition of p[w<sup>+mc</sup> GM roX1<sup>MS2-6</sup>]**

To determine if p[w<sup>+mc</sup> GM roX1<sup>MS2-6</sup>] would be utilized for gap repair if situated in roX1, targeted transposition was used to move it to the plArB insertion site in roX1<sup>mb710</sup> (Fig. 3.5). Mobilization produced abundant hops to the X-chromosome, 68% of which (34/50 insertions) were in roX1. The reason for the unusually high efficiency of targeting is unknown, but an interaction of roX genes in the male germ line, where transposition occurred, is suggested. Insertions on the X-chromosome were characterized by *in situ* hybridization and PCR. plArB was retained in tandem with 32 of the insertions. However, two precise replacements of plArB with p[w<sup>+mc</sup> GM roX1<sup>MS2-6</sup>] were recovered.
Figure 3.5. **Strategy for targeted transposition into roX1** Top. A p[w+mc GM roX1[MS2-6]] insertion on the third chromosome was mobilized in roX1[mb710] males with plArB (rY+) in roX1. Bottom. Tandem insertions (roX1[MS2-6]T2A or roX1[MS2-6]T4B) retain plArB. roX1[MS2-6]R36A is a precise replacement of plArB by p[w+mc GM roX1[MS2-6]].
Mobilization of targeted insertions to create $roX1^{MS2-6}$

Three targeted insertions in $roX1$ were remobilized. A replacement line ($roX1^{[MS2-6]R36A}$) and two tandem insertions with different orientations ($roX1^{[MS2-6]T2A}$ and $roX1^{[MS2-6]T4B}$, Fig. 3.5). Dysgenic males ($roX1^{XX}; Sb p[y^+\Delta2-3]99/+\text{; C(1)DX }y^f f^f$) were mated to C(1)DX $y^f f^f$ females. Mobilization is very frequent, with over 90% of dysgenic males producing white-eyed sons, which make up ~20% of male offspring. White-eyed sons were mated individually to C(1)DX $y^f f^f$ females and analyzed by PCR for repair of $roX1$ and inclusion of MS2 loops. Amplicons spanning the MS2 loop insertion site produce products characteristic of both wild type $roX1$ (547 bp) and $roX1^{MS2-6}$ (869 bp) from targeted transpositions, but almost 99% of white-eyed offspring produced a single amplicon. A total of 352 excisions were analyzed (169 for $roX1^{[MS2-6]T2A}$, 103 for $roX1^{[MS2-6]T4B}$, 80 for $roX1^{[MS2-6]R36A}$). Regardless of the starting line, over 10% of white-eyed sons had incorporated MS2 loops into the repaired chromosome (Fig. 3.6 B-E; Table 3.2). Amplicons from representative flies containing MS2 loops were sequenced, confirming faithful copying. The MS2 loops are 322 bp of non-homologous sequence situated 430 bp from the point of P-element insertion (Fig. 3.6 A). Incorporation of MS2 loops therefore requires a gene conversion tract over 750 bp in length. However, three flies generated by mobilization of $roX1^{[MS2-6]R36A}$ produced 800 bp PCR amplicons, consistent with contraction of the MS2 loop array during gene conversion (Fig. 3.6 E).
Figure 3.6. All predicted products of homology-dependent gene conversion are recovered A) roX1^{[MS2-6]T2A} is a tandem insertion of p[w^{+mC} GM roX1^{MS2-6}] at the 3' end of plArB. Alignment of the engineered roX1^{[MS2-6]} (gray line) is shown collinear to and below the corresponding genomic sequence. The MS2 loops are 430 bp from the plArB insertion site. B) and C) Predicted products of homology dependent gap repair and gene conversion. Left panels depict short repair tracts that do not incorporate MS2 loops; right panels depict longer tracts incorporating MS2 loops into the repaired chromosome. B) Homology in roX1 precisely substitutes a portion of roX1^{[MS2-6]T2A} (heavy gray line) at the plArB insertion site. C) Homology in roX1 and at P-ends leads to retention of the 3'P-end and duplication of 5' roX1 sequence. D) An imprecise excision removing w^{+mC} from roX1^{[MS2-6]T2A}. E) MS2 loop incorporation was detected by PCR using primers (arrows) flanking the MS2 loop insertion site (top). roX1^{MS2-6} produces an 869 bp amplicon and roX1^{+} produces a 547 bp amplicon. Three representative excisions in each category are shown. Contraction of the MS2 loop array in excision 36A.1 was detected by a reduction of the amplicon to 800 bp (right). F) Blot of EcoR1 digested DNA probed with the roX1 promoter (black bar, E). Hybridization to a single 4.9 kb roX1 fragment is seen in wild type flies (WT), and in a gene conversion that did not incorporate MS2 loops or retain a P-end (roX1^{+}). A single 5.2 kb fragment is detected in two precise conversions incorporating MS2 loops (lines 2A.1, 4B.1). Hybridization to a single, 5.1 kb band is observed in excision 36A.1, consistent with the reduced MS2 loop array observed by PCR. Line 2.5 is the imprecise excision depicted in D. A 5.2 kb band from p[w^{+mC} GM roX1^{MS2-6}] and a 2.5 kb band produced by disruption of genomic roX1 by insertion of plArB are both present.
Our aim was to engineer roX1 without leaving vector or P-element sequence behind. However, homology at P-ends can support gap repair, leading to predictable rearrangements. When the tandem insertion roX1\^[MS2-6]T2A is mobilized, homology-dependent gap repair can restore roX1 with no P-element sequences, or with a 3’ P-end retained (Fig. 3.6 B, C). Flies that retain the 3’ P-end also duplicate the 5’ end of roX1 and, depending on the length of repair tract, have full-length wild type roX1 (roX1\^P3) or roX1 with MS2-loops (roX1\^P3\^[MS2-6], Fig. 3.6 C). Retention of the 3’ P-end is also possible following mobilization of the replacement line roX1\^[MS2-6]R36A (Fig. 3.8). When the tandem insertion roX1\^[MS2-6]T4B is mobilized, the 3’ end of p[w\(^{+mC}\) GM roX1\^[MS2-6]] as well as the entire p\[ArB element may be retained (Fig. 3.7). All of these alternative outcomes were readily identified by PCR (Table 3.3). Eight out of 18 MS2 loop-containing excisions of roX1\^[MS2-6]T2 retained a 3’ P-end. However, one of these is an imprecise excision that is mutated for w\(^{+mC}\) but retains both P-elements in tandem (Fig. 3.6 D). In agreement with the structure determined by PCR, this line also produced both 547 and 869 bp PCR amplicons when tested for presence of MS2 loops in roX1.
Two out of 12 excisions of roX1<sup>[MS2-6]T4B</sup> retained the 3’ P-end and plArB (Fig. 3.7). No residual P-element sequences were detected in the 8 excisions of roX1<sup>[MS2-6]R36A</sup> examined (Table 3.2). We conclude that the overwhelming majority of excisions are repaired by a mechanism consistent with template directed gap repair. Sixty one percent of these had eliminated all vector sequences.

Table 3.3. Retention of P-element sequences

<table>
<thead>
<tr>
<th>PARENT LINE</th>
<th>FLIES WITH MS2 LOOPS</th>
<th>3’ P-END</th>
<th>P-ELEMENT JUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>roX1&lt;sup&gt;[MS2-6]T2A&lt;/sup&gt;</td>
<td>18</td>
<td>8</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>roX1&lt;sup&gt;[MS2-6]T4B&lt;/sup&gt;</td>
<td>12</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>roX1&lt;sup&gt;[MS2-6]R36A&lt;/sup&gt;</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PARENT LINE</th>
<th>FLIES WITHOUT MS2 LOOPS</th>
<th>3’ P-END</th>
<th>P-ELEMENT JUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>roX1&lt;sup&gt;[MS2-6]T2A&lt;/sup&gt;</td>
<td>13 (out of 150)</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>roX1&lt;sup&gt;[MS2-6]T4B&lt;/sup&gt;</td>
<td>10 (out of 90)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>roX1&lt;sup&gt;[MS2-6]R36A&lt;/sup&gt;</td>
<td>12 (out of 71)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>b</sup> Imprecise excision.

<sup>c</sup> Two rearrangements retained plArB and the 3’ P-end of p[w<sup>+mC</sup> GM roX1<sup>MS2-6</sup>].
Figure 3.7. Products of gap repair generated by mobilization of tandem insertion $\text{roX1}^{\text{MS2-6}T4B}$. A) $\text{roX1}^{\text{MS2-6}T4B}$. The $\text{roX1}^{\text{MS2-6}}$ insert (heavy gray line) is shown collinear to and below the corresponding genomic sequence. The MS2 loops are 430 bp from the plArB insertion site in $\text{roX1}$. Predicted products of homology-dependent gap repair presented in (B-D). Left panels depict short repair tracts (gray) that do not incorporate MS2 loops, right panels are longer tracts incorporating MS2 loops into the chromosome. B) Precise replacement by $\text{roX1}^{\text{MS2-6}}$ sequences. C) Repair is supported by homology in $\text{roX1}$ and at the 3' P-end, leading to retention of a P-end and duplication of the 5' $\text{roX1}$. D) Retention of plArB. E) Imprecise excision mutates mini-white, but leaves both P-elements in place.
Figure 3.8 Predicted products of gap repair upon mobilization of tandem insertion \( \text{roX1}^{[\text{MS2-6}]R36A} \). A) \( \text{roX1}^{[\text{MS2-6}]R36A} \) has replaced plArB with \( \text{p[w}^+\text{mc GM roX1}^{\text{MS2-6}} \). \( \text{roX1}^{\text{MS2-6}} \) (heavy gray line) is shown collinear to and below the corresponding genomic sequence. The MS2 loops are 430 bp from the plArB insertion site in the roX1. Predicted products of homology dependent gap repair are depicted in B) (precise repair of roX1) and C) (retention of the 3' P-end and duplication of 5' roX1 sequences). Left panels depict short repair tracts that do not incorporate MS2 loops, right panels describe longer tracts incorporating MS2 loops into the chromosome.
To confirm the structure of rearranged chromosomes, representative lines were analyzed by DNA blotting using the *roX1* promoter region as probe (Fig. 3.6 F). Excisions 2A.1 and 4B.1 are conversions to *roX1*<sup>MS2-6</sup> that retain no P-ends. Each produces a single 5.2 kb hybridizing EcoR1 fragment, consistent with introduction of 322 bp MS2 loops into the 4.9 kb genomic EcoR1 fragment. Line 36A.1, which displayed contraction of the MS2 loop array, shows a single hybridizing band at 5.1 kb (Fig. 3.6 E, F). Line 2.1 retains no P-element sequences and has repaired *roX1* without incorporating MS2 loops. As expected, a single 4.9 kb band is detected in this line. In contrast, the imprecise excision line 2.5, described above, has two hybridizing bands. The EcoR1 fragment present in p[w<sup>mc</sup> GM *roX1*<sup>MS2-6</sup>] is 5.2 kb, and a 2.5 kb band, consistent with insertional disruption of the chromosomal *roX1* gene, is also present.

*roX1*<sup>MS2-6</sup> is functional in dosage compensation

*roX1* is functionally redundant with *roX2* for dosage compensation. We tested the engineered *roX1*<sup>MS2-6</sup> allele for *roX* activity by determining adult male survival after recombination with *roX2Δ*, a deletion of *roX2* (MENON AND MELLER 2012). Male flies inheriting *roX1*<sup>MS2-6</sup> *roX2Δ* chromosomes derived from three independent gene conversions were fully viable (Table 3.3).
Table 3.4. $roX1^{MS2-6}$ retains $roX1$ activity

<table>
<thead>
<tr>
<th>$roX1^{MS2-6}$ LINE</th>
<th>MOTHER</th>
<th>FATHER</th>
<th>DAUGHTERS</th>
<th>SONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A.1</td>
<td>$roX2\Delta$</td>
<td>++/Y</td>
<td>100% (1048)</td>
<td>96% (1001)</td>
</tr>
<tr>
<td>4B.1</td>
<td>$roX1^{MS2-6}$ $roX2\Delta$</td>
<td>++/Y</td>
<td>100% (480)</td>
<td>99% (474)</td>
</tr>
<tr>
<td>36A.1</td>
<td>$roX1^{MS2-6}$ $roX2\Delta$</td>
<td>++/Y</td>
<td>100% (661)</td>
<td>99% (654)</td>
</tr>
</tbody>
</table>

Note: Male survival is based on the number of females emerging from each mating. The total number of flies recovered is in parentheses.

Mobilization of targeted insertions in females

While $roX1^{MS2-6}$ was produced with high efficiency, excision was performed in males. As $roX1$ is X-linked, no alternative template for repair is present. It is possible that mobilization in females would be less efficient due to selection of the homolog, rather than the sister chromatid, as the repair template. To test this idea, we mobilized the tandem insertion $roX1^{MS2-6}T2A$ in females. Only 3 out of 131 white-eyed sons incorporated MS2 loops into the $roX1$ locus. This efficiency, 2.3%, contrasts with over 10% MS2 loop incorporation in the offspring of dysgenic males. Two of the 3 lines contained a 3' P-end, and thus represent an alternative rearrangement.

Reduced efficiency of MS2 loop incorporation could result from use of $roX1^{+}$ on the balancer chromosome as the repair template. Alternatively, it could reflect differences in the repair process in the male and female germ lines. For example, if repair tracts tend to be shorter in females, inclusion of MS2 loops would be less frequent. To address these possibilities, we searched for P-element sequences on the repaired chromosomes. Retention of P-ends is
expected when a sister chromatid template is utilized. We examined 125 randomly selected white-eyed offspring (including 3 with MS2 loops) for the presence of a 3' end. One hundred three out of 125 (82.4%) retained the 3' end. We then selected 29 flies at random (out of 125) and tested for the junction between the 3' end of p[w+mC GM roX1^{MS2-6}] and plArB. Twenty one (72.4%) retained the junction. These findings are consistent with the idea that template directed gap repair in females strongly favors copying of the sister chromatid.

**Visualization of roX1 localization in roX1^{MS2-6} embryos**

To visualize roX1 distribution in embryos, roX1^{MS2-6} roX2Δ stocks carrying p[w+mC MCP- GFP] were generated. Females (roX1^{MS2-6} roX2Δ; [w+mC MCP-GFP]) were mated to males carrying an X-linked p[w+mC Sqh-mCherry] insertion. All embryos from this mating carry the roX1^{MS2-6} roX2Δ X chromosome and a single copy of p[w+mC MCP-GFP], but females display weak mCherry expression throughout (Fig. 3.9, compare panels C and H). MCP-GFP is recruited to a single large, subnuclear domain in male (panels A-E) but not female (panels F-J) embryos. MCP-GFP in males overlaps the nuclear DAPI signal, and the domain occupied is a size consistent with X chromosome painting (Fig. 3.9 E). Examination of confocal Z-stacks from individual embryos reveals a single GFP foci in virtually every nucleus.
Figure 3.9 *roX1<sup>MS2-6</sup> supports focal recruitment of MCP-GFP in male embryonic nuclei* Embryos were generated by mating *roX1<sup>MS2-6</sup> roX2Δ* [w<sup>+</sup>mC MCP-GFP] females to males carrying an X-linked [w<sup>+</sup>mC Sqh-mCherry] transgene. Sons (*roX1<sup>MS2-6</sup> roX2Δ/Y; [w<sup>+</sup>mC MCP- GFP]/+) lack [w<sup>+</sup>mC Sqh-mCherry] (Panels A-E). Females express mCherry (Panels F-J). A wild type embryo reveals auto fluorescence limited to the vitelline membrane (Panels K-N). Detail in Panel E reveals MCP-GFP recruitment to a single domain within the male nucleus, consistent with X chromosome painting. MCP-GFP recruitment is absent in the female nucleus (Panels I, J). Each set of panels is derived from a single Z-plane image. The brightness of mCherry signals was uniformly enhanced for reproduction (panels C, H and M). See materials and methods for details of photography and image processing.
DISCUSSION

The roX RNAs occupy a central position in fly dosage compensation. Full up-regulation of X-linked genes does not occur in male roX1 roX2 mutants, and the MSL proteins mislocalize to ectopic autosomal sites (MELLER AND RATTNER 2002; DENG AND MELLER 2006). Although autosomal roX transgenes rescue roX1 roX2 males, these transgenes also recruit MSL proteins to flanking autosomal chromatin, which is then modified in a manner similar to that at compensated X-linked genes (KELLEY et al. 1999; HENRY et al. 2001; KELLEY AND KURODA 2003; OH et al. 2004; LARSCHAN et al. 2007). These observations suggest that position of roX genes on the X chromosome contributes to their normal function. More generally, the presence of complex or distant regulatory elements, or a requirement for a specific chromatin context, may contribute to deficiencies in the function of transgenics. Our objective was to generate an allele of roX1 that would function normally, yet be readily visualized by GFP. The engineered allele roX1<sup>MS2-6</sup> supports full male viability in a roX2Δ background. Visualization of roX1<sup>MS2-6</sup> RNA with MCP-GFP reveals punctate labeling of a subnuclear domain in male embryos, and does not require lengthy histological protocols, making roX1<sup>MS2-6</sup> a new resource for detection of roX1 localization.

The absence of readily accomplished homologous recombination in Drosophila is a notable drawback in a powerful model organism. Ground breaking studies over a decade ago established a technique for homologous recombination in flies, but this process remains labor intensive (RONG AND
GOLIC 2000; GAO et al. 2008; HUANG et al. 2009; WESOLOWSKA AND RONG 2010). More recently, a strategy for reinsertion of large clones that have been modified by recombineering has been shown to be quite efficient (BATEMAN et al. 2013). This, and similar strategies that employ site-specific recombination, leave vector remnants or recombination sites within the genome (CROWN AND SEKELSKY 2013). In contrast, we have introduced an engineered change with no residual vector sequences. Alternative rearrangements that retain a P-end can be predicted and easily detected by PCR.

We have named this new strategy ‘Targeted Gene Conversion’ (TGC) to reflect the two-step process required: targeted transposition followed by gene conversion. TGC is a variation of older techniques that utilized repair-mediated gene conversion to engineer Drosophila genes. These relied on transposon mobilization to generate double stranded breaks that were then repaired using a template supplied by the homolog (GLOOR et al. 1991; JOHNSON-SCHLITZ AND ENGELS 1993), by a transposon at another position in the genome (NASSIF et al. 1994; LANKENAU et al. 1996; MERLI et al. 1996), or by DNA injected into dysgenic embryos (BANGA AND BOYD 1992). The efficiency of this process, typically not exceeding a few percent of excised chromosomes, has limited its use. In contrast, almost all excisions of targeted insertions containing the template are repaired using the template, and 10% of these incorporated MS2-6 loops into roX1.

Directed mutagenesis has been improved by the use of zinc finger
nucleases (ZFN), and more recently, TALENs and CRISPR/Cas9 nucleases, to introduce double stranded breaks at specific sites (BIBIKOVA et al. 2002; CHRISTIAN et al. 2010; BASSETT et al. 2013; GRATZ et al. 2013). When repair templates with the desired changes are present, these sequences may be introduced by gene conversion (GAJ et al. 2013). The ability to rapidly generate mutations at a specific site is a clear strength of these methods. However, the efficiency of gene conversion decreases sharply with an increased distance from the break site (MOEHLE et al. 2007).

The potential for efficient replacement of longer sequences is anticipated to be a primary strength of TGC. Generation of roX1^{SMC17A} required resection of over 1.2 kb from the break site, followed by copying over 3 kb of sequence, including the entire LacZ gene, into the break. As this precise rearrangement accounted for 68% of excisions, TGC readily replaces large blocks of sequence. Gene conversion was also easily achieved upon mobilization of roX1^{TM2-6T} or roX1^{TM2-6R}, but, in this instance, no resection of broken ends is necessary to uncover homology with p[w^{+mC} GM roX1^{MS2}]. Instead, incorporation of MS2 loops requires a repair tract to extend at least 750 bp from the break and accommodate 322 bp of non-homologous sequence. Ten percent of excisions incorporate MS2 loops, consistent with a previous study that documented conversion tracts extending almost 2 kb (NASSIF AND ENGELS 1993).

roX1Δ^{891} and three targeted roX1^{MS2} insertions are readily mobilized by transposase, with over 90% of dysgenic males producing white-eyed offspring. This is not a general feature of P-element insertions in roX1, as only 20% of
dysgenic $roX1^{mb710}$ males produce $ry$ offspring (V.H.M, unpublished). In spite of high mobility, recovery of imprecise excisions was remarkably low. Four out of 56 excisions of $roX1\Delta^{891}$ and 4 out of 352 excisions of targeted $roX1^{[MS2-6]}$ insertions were imprecise. The apparent high mobility and bias against imprecise excision are likely both attributable to the presence of an alternative template for gap repair that excludes $w^{+mc}$.

A clear limitation of our strategy is the need to move the template sequence close to the target site. We have accomplished this by targeted transposition, but targeted transpositions are typically a few percent of new insertions and requires a P-element at the target site. The exceptionally rich coverage of P-element insertions in $Drosophila$ makes this feasible in many instances. Alternatively, recently developed techniques that use engineered nucleases, such as TALENs, CRISPR/Cas9 or zinc finger nucleases, could be used to introduce a landing site, such as $attP$, at the desired location (GROTH et al. 2004; GAJ et al. 2013). Integration of a selectable marker and template flanked by P-ends would generate a mutagenic precursor for TGC without the need for a preexisting P-element (Fig. 3.10)
Figure 3.10 Directing transposase-mediated gene conversion to a region lacking a P-element  A) A double stranded break is introduced in a gene of interest by an engineered nuclease. An oligonucleotide containing a landing site, such as attP, and homologous arms is introduced as a repair template. B) A longer construct with engineered changes to the target gene (thick line), a visible marker (w*) and P-ends (black and gray arrowheads) is integrated into the landing site C). D) Mobilization with transposase creates a double stranded break. Homology is revealed by resection of broken ends. Gap repair using a sister chromatid template produces engineered chromosomes lacking the w* marker.
Chapter 4

Novel sex determination signal in *Drosophila melanogaster* is revealed by functional studies of chromatin biology

This chapter is a manuscript is ready to be submitted as: Novel sex determination signal in Drosophila melanogaster revealed by functional studies of chromatin biology. Manasi S. Apte and Victoria H. Meller.

Introduction

Approximately 30% of the *Drosophila* genome is heterochromatic (Smith et al. 2007). Many cytological and molecular features distinguish the relatively gene-poor heterochromatin from gene-rich euchromatin. Heterochromatin forms a compact, relatively inaccessible domain with ordered nucleosome arrays (Huisinga et al. 2006). Heterochromatic loci tend to be near the nuclear periphery during interphase. Heterochromatin is characterized by repetitive DNA sequences, low levels of histone acetylation, hypomethylation of H3K4 and H3K79 and enrichment for Heterochromatin Protein 1 (HP1) (Elgin and Reuter 2013). Although relatively gene-poor, *Drosophila* heterochromatin harbors hundreds of protein coding genes (heterochromatic genes) (Gatti and Pimpinelli 1992; Smith et al. 2007). The heterochromatic environment has been shown essential for full expression of some of these genes and disruption of heterochromatin lowers their expression (Lu et al. 2000; Schulze et al. 2006; Yasuhara and Wakimoto 2006).
Euchromatic genes also rely on their native chromatin context, and stochastic silencing is observed when a euchromatic gene is placed in a heterochromatic environment, a phenomenon known as ‘Position Effect Variegation’ (PEV). PEV represents variable spreading of heterochromatin over the euchromatic gene, producing irregular silencing (Elgin and Reuter 2013). PEV is extraordinarily sensitive to the heterochromatin integrity. For example, mutation of a single copy of Su(Var)2-5, encoding HP1, elevates expression of variegating reporters inserted in heterochromatic regions. This effect, called suppression of PEV, enables identification of genes involved in heterochromatin formation and silencing.

Drosophila heterochromatin is typically not thought to be sexually dimorphic. However, two recent studies suggest that heterochromatin differs in male and female flies. Reduction in HP1 results in preferential male lethality and gene misregulation (Liu et al. 2005). Loss of the Drosophila roX1 and roX2 RNAs (RNA on the X-1 and 2) is a potent suppressor of PEV for autosomal insertions in male flies, but not in females (Deng et al. 2009). A general reduction in the expression of autosomal heterochromatic genes is also observed in roX1 roX2 males (Deng et al. 2009). These findings indicate a general disruption of autosomal heterochromatin that is limited to males. The male-limited requirement for roX RNA reveals that heterochromatin is itself sexually dimorphic. Interestingly, the roX RNAs are essential for the male-limited process of X chromosome dosage compensation (Meller and Rattner 2002). roX RNAs
assemble with the Male Specific Lethal (MSL) proteins to form a complex that is targeted to X-linked genes. Enzymatic activities within the MSL complex modify chromatin to increase expression of X-linked genes, doubling transcription in male flies. Most of the MSL proteins are also required for full expression of autosomal heterochromatic genes in males (DENG et al. 2009). The only member of the MSL complex that is unnecessary for heterochromatic genes is the Male Specific Lethal 2 (MSL2) protein. This is surprising as MSL2, the key regulator of X chromosome dosage compensation in males, is the sole member of the MSL complex with strictly male-limited expression. This raises intriguing questions about how heterochromatic dimorphism is determined. We hypothesized that heterochromatin exists in a ‘masculine’ form, which requires roX RNA for normal PEV and heterochromatic gene expression, and a ‘feminine’ form, which does not. We postulated that heterochromatic sex is under genetic control, and conducted experiments aimed at determining the signal that establishes sex-specific heterochromatin in flies.

Using a PEV reporter assay we demonstrated that feminization of heterochromatin is independent of female-limited components of the Drosophila sex determination pathway. Furthermore, neither MSL2 nor the Y chromosome directs heterochromatin masculinization. We postulated that a novel signal, perhaps direct sensing of karyotype, could be involved. Karyotype detection could occur by X chromosome counting, or by detection of unpaired chromatin in XY or XO flies. We did not detect XY feminization in flies overexpressing numerator elements, the known X chromosome counting elements in cell. Next,
we screened viable mutations that influence chromosome organization and homologue pairing, and discovered that Topoisomerase II (Top2), is necessary for feminization of autosomal heterochromatin in XX flies. Top2 promotes homologue pairing in flies, consistent with pairing-dependent detection of karyotype. However, Top2 also binds X chromosome-specific satellite repeats that make up >10 Mb of pericentric heterochromatin (Ferree and Barbash 2009). Interestingly, loss of X-heterochromatin partially masculinizes autosomal heterochromatin in XX flies. We propose that Top2 and pericentromeric X heterochromatin comprise a mechanism that distinguishes XX from XY and XO by direct karyotype sensing. Our findings reveal the presence of a novel sex determination signal contributes to the sexual differentiation of heterochromatin in Drosophila melanogaster.

**Materials and Methods**

**Fly strains:**

Flies were maintained at 25°C on standard cornmeal–agar fly food. Unless otherwise noted, mutations are described in (Lindsley and Zimm 1992). roX1 mutations have been described (Meller et al. 1997; Meller and Rattner 2002; Deng et al. 2005). Elimination of roX2 was accomplished by a viable deletion of roX2 (roX2Δ) or a lethal deletion complemented by a cosmid carrying essential deleted genes but lacking roX2 (Meller and Rattner 2002; Menon and Meller 2012). Variegating transgene insertions used in this study have been described (Sun et al. 2000; Yan et al. 2002). Variegating insertions were selected
to facilitate stock construction, but key findings were validated with multiple reporters. Top2<sup>17-1</sup> and Top2<sup>17-3</sup> mutations were generously provided by C. T. Wu and P. Geyer (Hohl et al. 2012). Additional mutations used are as follows: Cap-D3<sup>07081</sup> (Longworth et al. 2008), Cap-H2<sup>20019</sup> (Hartl et al. 2008), MCPH1<sup>0978</sup> (Rickmyre et al. 2007), Dhc64c<sup>8-1</sup> (Gepner et al. 1996), [w<sup>+</sup>-hsp83::MLE] ([H83 MLE]) (Morra et al. 2008). [w<sup>+</sup>-hsp83::MSL2]6I ([H83M2]6I) and [w<sup>+</sup>-hsp83::MSL1]Z1 ([H83M1]Z1) (Kelley et al. 1995; Chang and Kuroda 1998).

2XP(w<sup>+</sup>mC,sisA<sup>+</sup>)+2XP(w<sup>+</sup>mc,sc<sup>sisB+</sup>) (Cline 1988); Gonzalez et al. 2008).

Descriptions of Sxl<sup>2593</sup>, Sxl<sup>M1F3</sup>, Tra2<sup>B</sup>, Tra2<sup>fs1</sup>, Tra2<sup>fs2</sup>, Dsx<sup>+</sup>, Dsx<sup>D</sup>, Top2<sup>17-1</sup>, Top2<sup>17-3</sup>, Cap-D3<sup>07081</sup>, Cap-H2<sup>20019</sup>, MCPH1<sup>0978</sup>, Dhc64c<sup>6-10</sup>, Dhc64c<sup>8-1</sup>, fs(1)h<sup>1</sup>, and Zhr<sup>+</sup> are available on Flybase (http://www.flybase.org). All other mutations used in this study were obtained from the Bloomington Drosophila Stock Center.

**Transvection and insulator assays:**

Restoration of yellow pigmentation by transvection is a standard measure of homolog pairing (Geyer et al. 1990; Morris et al. 1998; Morris et al. 1999). Pigmentation was scored in 1-2 days old flies on a scale of 1-4, where 1 is the no pigmentation and 4 is wild type levels. At least 100 flies of each genotype were scored for transvection. <sup>y</sup><sup>2</sup> allele has a gypsy insulator insertion that disrupts the communication between enhancers and promoter, causing yellow body and cut wing phenotypes when insulator proteins bind to the gypsy sequence (Geyer et al. 1990). Flies were aged for 24 h before scoring on the pigmentation scale described above. At least 25-30 flies from two independent
crosses were scored. Statistical significance was determined by a Student’s T-test. Representative images were obtained using a Zeiss Discovery V8 stereo microscope.

Results

Two metrics of autosomal heterochromatin integrity reveal disruption in \textit{roX1 roX2 (roX)} males, but not in females. Expression of heterochromatic genes on autosomes decreases in male larvae carrying the severely affected \textit{roX1^{SMC17}roX2Δ} chromosome (Deng et al. 2009). Adult male escapers with the partial loss of function \textit{roX1^{ex33}roX2Δ} chromosome display a dramatic suppression of PEV at autosomal insertions (Deng et al. 2009). However, no suppression of PEV or reduction in heterochromatic gene expression is detected in \textit{roX1 roX2} females, revealing an autosomal \textit{roX} requirement that is limited to males. These observations were surprising because the \textit{roX} RNAs were not thought to play a role outside of \textit{X} chromosome dosage compensation. In addition, autosomal heterochromatin is not overtly sexually dimorphic. Variegating insertions typically behave similarly in males and females, and the autosomal heterochromatic genes that are misregulated in \textit{roX1 roX2} males rarely display sex-biased expression. The genetic regulation of the differences in male and female heterochromatin is completely unknown. In this study, we used a genetic approach to examine this question.

Suppression of PEV increases black abdominal pigmentation from \textit{y}^+ reporters (Fig. 4.1 A, Fig. 4.2 A) and red eye pigmentation from \textit{w}^{^+\text{mw hs}}
reporters (Fig. 4.2 B). The 3rd chromosomal insertion KV24 displays $y^+$ PEV in both sexes and the 2nd chromosome KV20 displays PEV in males but typically produces $<1$ $y^+$ spot/female abdomen. Suppression of PEV of all the variegating insertions we tested occurs in $roX^1ex33roX2\Delta$ males, but not in $roX^1ex33roX2\Delta$ females, revealing an effect that is not unique to a specific insertion or reporter (DENG et al. 2009).

We refer to heterochromatin as masculine if loss of $roX$ dramatically suppresses PEV of an autosomal reporter, and feminine if little or no suppression of PEV occurs upon loss of $roX$. This distinction was the basis for a search for the genetic basis of heterochromatic sexual dimorphism. *Drosophila* sex determination is triggered by the X to autosome ratio (X:A, Fig. 4.1 B). The Y chromosome is believed to have no role in *Drosophila* sex determination. An X:A ratio of 1.0 in XX embryos activates transient, early *Sexlethal* (*Sxl*) expression (SALZ AND ERICKSON 2010). *SXL* regulates productive *transformer* (*tra*) splicing in XX embryos (BOGGS et al. 1987). *tra* and *transformer 2* (*tra2*) direct splicing of the female isoform of the *doublesex* transcription factor (*dsxF*). Conversely, in XY embryos *Sxl* is not expressed (CLINE 1983; SALZ et al. 1987). *SXL* represses MSL2 translation (BASHAW AND BAKER 1997; KELLEY et al. 1997; GEBAUER et al. 1998). As MSL2 is a key protein in X chromosome dosage compensation, this limits dosage compensation to males. The absence of *Sxl* in males also prevents *tra* expression, resulting in the production of default male isoform of *dsx* (*dsx^M*). We hypothesized that components in this pathway could drive the masculinization (or feminization) of heterochromatin in one sex.
**Figure 1.** Heterochromatin masculinization is revealed by position effect variegation (PEV).  

**A)** PEV of a $y^+$ marker in the KV20 insertion produces black abdominal spots. Few spots are visible in $yw$; KV20/+ males but suppression of PEV in $yw\;roX1^{ex33}\;roX2\Delta/Y$; KV20/+ males produces increased pigmentation. Females (bottom) typically produce less than one spot per female, and no suppression of PEV is detected in $yw\;roX1^{ex33}\;roX2\Delta/Y$; KV20/+ females (right).  

**B)** Somatic sex determination in flies is controlled by the number of X chromosomes. Two copies of X-linked numerator elements ($sisA$, $sisB$, runt and $upd$) turn on early Sexlethal ($Sxl$) expression in XX embryos. $Sxl$ blocks dosage compensation by preventing translation of MSL2 in XX embryos. $Sxl$ is also necessary for productive splicing of transformer ($tra$) mRNA. $tra$ works with transformer2 ($tra2$) to produce a female-specific isoform of doublesex ($dsx^F$). In males only ($dsx^M$) is produced. The $dsx$ transcription factors coordinate visible somatic differentiation. Additional $tra$ and $tra2$ targets (not shown) regulate differentiation of the nervous system.
Figure 4.2. Suppression of PEV in roX1 roX2 males is independent of reporter or insertion site. PEV of y+ in KV24 (3rd chromosome), visible as black abdominal spots in both sexes, is suppressed in roX1 roX2 males, but not in females (left). PEV of w+mw.hs in 118E-10 (4th chromosome) is detected by eye pigmentation. roX1 roX2 males (top), but not females (bottom), suppress PEV of 118E-10. 118E-10 is examined in the roX1sex33 Df(1)52;[4Δ4.3]/+, background as it lacks additional w markers, enabling visualization w+mw.hs expression.
The Y chromosome does not masculinize heterochromatin

We first considered the possibility that a male-limited signal masculinizes heterochromatin. Although the Y chromosome is unnecessary for the masculinization of somatic tissues, it is thought to act as a sink for heterochromatin proteins, and thus has epigenetic effects throughout the genome (WEILER AND WAKIMOTO 1995; LEMOS et al. 2008). We generated males with the $w^{*mw.hs}$ reporter 118E-10 that were either wild type for the roX genes, or carried the partial loss of function roX$^{1\text{ex33}}$ allele and a deletion of roX2, a combination that allows over 20% escaper males. Control ($yw/Y; 118E-10/+)$ males have variegating eyes with an average of 20% pigmented facets (black, Fig. 4.3 A), but roX$^{\text{ex33}}$/roX2 males display over 90% pigmentation (gray), representing a dramatic suppression of PEV. In XO males, lack of the chromosome frees heterochromatic proteins to reinforce silencing and enhance PEV at other loci (WEILER AND WAKIMOTO 1995)). We generated control and roX mutant XO males lacking a Y chromosome ($yw/O; 118E-10/+ \text{ and } yw \text{ roX}^{1\text{ex33}} \text{ Df(1)52; [w}^4\Delta4.3]/O; 118E-10/+; \text{ Fig. 4.3 A}$. As expected, PEV was enhanced in control XO males, almost 90% of which have no detectable eye pigmentation (striped, Fig. 4.3 A). However, all roX mutant XO males display some eye pigmentation (patterned, Fig. 4.3 A). We conclude that the loss of roX still suppresses PEV in XO males. Although the Y chromosome modulates heterochromatic silencing, the presence of the Y chromosome does not masculinize heterochromatin.
**MSL2 does not masculinize heterochromatin**

The protein Male Specific Lethal-2 (MSL2) binds the roX RNAs and is the only male-limited member of the dosage compensation complex (KELLEY et al. 1995; ILIK et al. 2013; MAENNER et al. 2013). To determine if MSL2 plays a role in heterochromatin masculinization, we expressed MSL2 from the [H83M2]6I transgene in XX females with the variegating $y^+$ reporter KV20, and compared females that were either wild type or mutated for roX RNAs ((KELLEY et al. 1995; KONEV et al. 2003; BELLEN et al. 2004). This, and following studies utilize the simple deletion roX2Δ that facilitates stock construction (MENON AND MELLER 2012). We found that expression of MSL2 does not masculinize XX heterochromatin. PEV in females expressing MSL2 is not influenced by roX mutations (Fig. 4.3 B bottom; $yw\ roX^{1ex33}\ roX2\Delta; KV20/+; [H83M2]6I /+\ and\ yw; KV20/+; [H83M2]6I /+). In contrast, roX mutations suppress PEV in males carrying the [H83M2]6I transgene (Fig. 4.3 B, top). These observations are consistent with a study finding that MSL2 is not required for full expression of autosomal heterochromatic genes in males (DENG et al. 2009). As MSL2 appears to have no role in either measure of sexually dimorphic heterochromatin, we conclude that it is not the signal that masculinizes heterochromatin.
Figure 4.3. Neither the Y chromosome nor MSL2 direct heterochromatin masculinization. A) Eye pigmentation was examined in flies with a variegating \( w^{+mw.\text{hs}} \) marker in 118E-10. Wild type males with and without a Y chromosome (XY, XO; black and striped bars) and mutated for \( \text{roX} \) (\( \text{roX1 roX2/Y} \) or \( \text{roX1 roX2/O} \); gray or patterned bars) were compared. Full genotypes and number of individuals scored: \( \text{yw}/Y; 118E-10/+ \), 110, \( \text{yw}/O; 118E-10/+ \), 21, \( \text{yw roX}^{\text{ex33}}\text{roX2}^\Delta/Y; 118E-10/+ \), 83, \( \text{yw roX}^{\text{ex33}}\text{roX2}^\Delta/O; 118E-10/+ \), 30. B) MSL2 does not masculinize XX heterochromatin. Ectopic MSL2 expression was driven by the [H83M2]6I transgene. The \( y^+ \) marker is KV20. Representative male (top) and female (bottom) adults are presented. PEV of KV20 is suppressed in \( \text{roX}^{\text{ex33}}\text{roX2}^\Delta \) males, and this is unchanged by the MSL2 transgene. Expression of MSL2 does not lead to suppression of PEV in \( \text{roX}^{\text{ex33}}\text{roX2}^\Delta \) females.
The somatic sex determination pathway does not feminize heterochromatin

We then addressed the possibility that female-limited proteins in the somatic sex determination pathway feminize heterochromatin in XX flies. If this occurs, mutation of the feminizing gene would masculinize XX heterochromatin (Fig. 4.4 A). We tested Sex-lethal (Sxl), transformer2 (tra2) and Doublesex (dsx), representing different levels of the pathway (Fig 4.1 B, left). As these genes directly female somatic differentiation, mutations produce XX pseudomales, or intersexes with male-like body pigmentation and altered genital morphology.

dsx\textsuperscript{1} is an amorph, while dsx\textsuperscript{D} produces the male splice form. XX; dsx\textsuperscript{1}/dsx\textsuperscript{D} flies were visibly masculinized. We generated XY; dsx\textsuperscript{1}/dsx\textsuperscript{D} and XX; dsx\textsuperscript{1}/dsx\textsuperscript{D} flies with the \textit{y}\textsuperscript{+} PEV reporter KV20 and carrying \textit{roX1\textsuperscript{ex33}roX2\Delta} chromosome. Masculinized XX; dsx\textsuperscript{1}/dsx\textsuperscript{D} flies were distinguished from XY flies by the absence of a marked Y chromosome (\textit{B\textsuperscript{2}Y}). Sexual transformation increased abdominal pigmentation, allowing detection of a few \textit{y}\textsuperscript{+} spots in XX flies. Although \textit{roX1\textsuperscript{ex33}roX2\Delta / B\textsuperscript{2}Y; KV20/+; dsx\textsuperscript{1}/dsx\textsuperscript{D}} males displayed strong suppression of PEV, XX pseudomales of the same genotype did not (\textit{roX1\textsuperscript{ex33}roX2\Delta; KV20/+; dsx\textsuperscript{1}/dsx\textsuperscript{D}}, Fig. 4.4 B).

We next tested the \textit{tra2\textsuperscript{ts1}} and \textit{tra2\textsuperscript{B}} mutations. \textit{tra2\textsuperscript{ts1}} is a temperature sensitive hypomorph and \textit{tra2\textsuperscript{B}} is a null allele. Loss of \textit{tra2} had no effect on XY flies, but produced XX intersexes or pseudomales. We generated XX and XY \textit{tra2\textsuperscript{m}} mutants carrying the KV20 \textit{y}\textsuperscript{+} reporter and \textit{roX1\textsuperscript{ex33}roX2\Delta}. Loss of \textit{roX} suppressed PEV in \textit{tra2\textsuperscript{m}/ tra2\textsuperscript{m}} males (\textit{roX1\textsuperscript{ex33}roX2\Delta / Y; tra2\textsuperscript{m} KV20/ tra2\textsuperscript{m}}) compared to \textit{yw / Y; tra2\textsuperscript{m} KV20/ tra2\textsuperscript{m}} males (Fig. 4.4 B). In contrast, XX
tra2 pseudomales mutated for roX displayed no suppression of PEV in comparison to XX pseudomales with wild type roX (roX1\textsuperscript{ex33}roX2\Delta; tra2\textsuperscript{m}KV20/tra2\textsuperscript{m} and yw; tra2\textsuperscript{m}KV20/tra2\textsuperscript{m}; Fig. 4.4 B).

Although dsx and tra2 do not regulate heterochromatic sexual differentiation, it remained possible that Sxl, the master regulator of sexual determination, acted through a different pathway. Because null Sxl mutations are embryonic lethal in XX zygotes, we tested a heteroallelic combination, Sxl\textsuperscript{M1,f3}/Sxl\textsuperscript{2593}, that produces a few masculinized XX adult escapers. Both the roX genes and Sxl are X-linked, necessitating generation of two roX1\textsuperscript{ex33} Sxl roX2\Delta chromosomes to test in a roX background. Control masculinized XX adult escapers (Sxl\textsuperscript{M1,f3}/Sxl\textsuperscript{2593}; KV20/+ ) emerged late and displayed developmental defects and partial sexual transformation (Fig. 4.4 C). Similar to XX flies masculinized by tra2 and dsx, a few abdominal spots were present. However, similar numbers of spots were present in XX roX mutants (roX1\textsuperscript{ex33}Sxl\textsuperscript{M1,f3}roX2\Delta / roX1\textsuperscript{ex33}Sxl\textsuperscript{2593}roX2\Delta; KV20/+ ; Fig. 4.4 D, hatched bars). In contrast, XY males with either recombinant chromosome (roX1\textsuperscript{ex33}Sxl\textsuperscript{M1,f3}roX2\Delta /Y; KV20/+ and roX1\textsuperscript{ex33}Sxl\textsuperscript{2593}roX2\Delta /Y; KV20/+ ) displayed dramatic suppression of PEV when compared to males with wild type roX genes (Fig. 4.4 C and D). This supports the idea that sexual differentiation of heterochromatin is independent of the somatic sex determination pathway. One caveat to this test of Sxl is that it requires adult escapers; preventing testing of null Sxl alleles. It remains possible that a novel Sxl function is retained in the heteroallelic combination tested. Nevertheless, the stability of heterochromatic sex in genetic backgrounds
mutated for *tra* and *dsx* suggests the involvement of a novel pathway that operates at the level of *Sxl* or above.

**Direct sensing of the fly karyotype: A possible genetic signal for heterochromatic sex**

A mechanism that detects sex chromosome karyotype could bypass the sex determination pathway altogether. One way this could occur is if the X chromosome counting mechanism that turns on *Sxl* in XX embryos also controls a second pathway that leads to heterochromatin feminization. Proteins from the X-linked *Sisterless A and B* (*sisA* and *sisB*), *unpaired* (*upd*) and *runt* (*runt*) genes, collectively known as numerator elements, promote early *Sxl* expression in XX embryos (Van Doren *et al.* 1991; Younger-Shepherd *et al.* 1992; Erickson and Cline 1993; Erickson and Cline 1998). Elevated *sisA* and *sisB* expression is benign in XX flies, but turns on *Sxl* expression in XY flies, a lethal situation that can be overcome by mutating *Sxl* (Sefton *et al.* 2000). It is possible that higher levels of these proteins in XX embryos activate a pathway leading to heterochromatin feminization. We examined heterochromatic sexual differentiation in XY flies with multiple *sisA* and *sisB* transgenes and the *Sxl*[^1] mutation (Cline 1988) (Fig. 4.4 E). Both the *roX* genes and *Sxl* are X-linked, necessitating generation of a *roX1*[^3] *Sxl*[^1] *roX2Δ*
A

X chromosome

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Expected heterochromatic sex

Masculine

Feminine

Masculine

B

Abdominal spots

***

n.s.

C

roX1 roX2

roX1 roX2

Sx

Sx

roX1 Sx

roX1 Sx

roX1 Sx

roX1 Sx

D

Abdominal spots

***

n.s.

E

roX1 roX2

SisA+B

roX

roX

SisA+B

SisA+B
Figure 4.4. The somatic sex determination pathway as well as numerator elements do not control heterochromatin feminization. A) Scheme for identification of genetic regulators of heterochromatic sex. Heterochromatin is designated as ‘masculine’ if loss of roX suppresses PEV of an autosomal reporter. If a gene in the sex determination cascade feminizes heterochromatin in XX flies, mutation of that gene will masculinize XX heterochromatin, leading to suppression of PEV in roX1\textsuperscript{ex33}roX2Δ females. B) tra2 and dsx do not feminize heterochromatin. roX1\textsuperscript{ex33}roX2Δ / B\textsuperscript{3}Y; KV20/+ males carrying tra2\textsuperscript{B}, tra2\textsuperscript{ls1} or dsx\textsuperscript{1}/dsx\textsuperscript{D} display suppression of PEV, detected by an increased number of abdominal spots. XX pseudomales and intersexes generated with these mutations display a minor increase in spots, consistent with masculinization of pigmentation patterns. However, no suppression of PEV is observed in roX1\textsuperscript{ex33}roX2Δ XX pseudomales (full genotypes: yw roX1\textsuperscript{ex33}roX2Δ; tra2\textsuperscript{B} KV20/ tra2\textsuperscript{B}, yw roX1\textsuperscript{ex33}roX2Δ; tra2\textsuperscript{Ts1} KV20/ tra2\textsuperscript{Ts1} and yw roX1\textsuperscript{ex33}roX2Δ; KV20/+; dsx\textsuperscript{1}/dsx\textsuperscript{D}). C) Sxl mutations do not masculinize XX heterochromatin. Representative XY (top) and XX (bottom) flies are shown. XY flies with Sxl mutations suppress PEV upon loss of roX function (right two panels). XX Sxl\textsuperscript{M1,f3}/Sxl\textsuperscript{2593} pseudomales display masculinization of abdominal structures and pigmentation pattern, but no suppression of PEV is observed in pseudomales mutated for roX. D) Abdominal spots in adults with Sxl mutations. Full genotypes of XY flies: yw/Y; KV20/+, 75 flies, yw Sxl\textsuperscript{M1,f3}/Y; KV20/+, 64 flies, yw Sxl\textsuperscript{2593}/Y; KV20/+, 75 flies, roX1\textsuperscript{ex33}Sxl\textsuperscript{M1,f3}roX2Δ/Y; KV20/+, 17 flies, roX1\textsuperscript{ex33}Sxl\textsuperscript{2593}roX2Δ/Y; KV20/+, 37 flies. Full genotypes of XX flies yw Sxl\textsuperscript{M1,f3}/yw Sxl\textsuperscript{2593}, KV20/+, 21 flies, yw roX1\textsuperscript{ex33}Sxl\textsuperscript{M1,f3}roX2Δ/yw roX1\textsuperscript{ex33}Sxl\textsuperscript{2593}roX2Δ; KV20/+, 10 flies. Unless otherwise noted, average spot counts were derived from 20-50 individuals. (p-value ***<0.00001). E) Numerator elements do not feminize XX heterochromatin. Overexpressing transgene for numerator elements is indicated by ++. roX1\textsuperscript{ex33}Sxl\textsuperscript{f1}roX2Δ/Y; 2XP(w\textsuperscript{+mc},sisA\textsuperscript{+})+2XP(iw\textsuperscript{+mc},sc\textsuperscript{sisB\textsuperscript{+}})/KV20 flies over-expressing numerator elements- sisA and sisB show strong suppression of PEV but ywSxl\textsuperscript{f1}/Y; 2XP(w\textsuperscript{+mc},sisA\textsuperscript{+})+2XP(w\textsuperscript{+mc},sc\textsuperscript{sisB\textsuperscript{+}})/KV20 do not. Average spot counts were derived from 20-50 flies. (p-value ***<0.00001).
chromosomes to test in a roX background. We found suppression of PEV in roX mutant males that overexpress sisA and sisB, and normal PEV in control males with wild type roX (Fig. 3E; genotypes yw roX1 ex33 Sxf f roX2 Δ Y; 2XP(w+mC, sisA) + 2XP(w+mC, sc sisB+) / KV20 and yw Sxf f Y; 2XP(w+mC, sisA)+2XP(w+mC, sc sisB+) / KV20). This reveals stable heterochromatin masculinization in the male genotypes tested. Although SisA and SisB are key components of a well-studied X chromosome counting mechanism, we conclude that they do not feminize heterochromatin.

Other method of karyotype detection

Another possible mechanism for detection of karyotype involves chromosome pairing. Interphase chromosomes of Drosophila are paired throughout development (Stevens 1908; Williams et al. 2007; Apte and Meller 2012). All chromosomes pair in females, but the structurally dissimilar X and Y chromosomes of males remain unpaired. In theory, unpaired chromatin in the cells of XY and XO flies could signal the male karyotype.

Mutations in Topoisomerase II affect determination of heterochromatic sex

To investigate this possibility, we performed a targeted screening of genes that regulate homolog pairing in Drosophila (Williams et al. 2007; Joyce et al. 2012). Three pairing promoters, Topoisomerase II (Top2), Dynein Heavy chain-64c (Dhc64c) and Microcephalin-1 (MCPH1), and three anti-pairers, condensin II subunits Cap-H2 and Cap-D3, and Female sterile (1) homeotic (fs(1)h) were examined. Some of these are essential, requiring testing of partial
loss of function mutations, or heteroallelic combinations that produce adult escapers. If a fully paired genome signals the XX karyotype, and this in turn regulates heterochromatic sex, mutations in anti-pairers will increase pairing, leading to feminization of XY heterochromatin. We generated XX and XY flies with viable mutations in individual anti-pairers, the \( y^+ \) KV20 reporter and mutated or wild type or \( roX \). PEV was minimal, but unchanged, in \( roX \) mutant females. In contrast, Males with \( Cap-H2^{20019} \), \( Cap-D3^{c07081} \) or \( fs(1)h^1 \) mutations continued to suppress PEV when mutated for \( roX \) (Fig. 4.5, compare grey and black bars).

We then tested mutations in candidate pairing promoters. These mutations increase unpaired chromatin, a condition that could mimic unpaired chromatin in XY flies. We postulate that if unpaired chromatin signals the XY karyotype, reduced pairing in XX flies could lead to inappropriately masculinized heterochromatin. We first generated individual XX and XY flies with loss of function mutations in \( Dhc64c \) or \( MCPH1 \), the \( y^+ \) KV20 reporter and wild type or mutated for \( roX \). XY flies mutated for \( Dhc64c \) or \( MCPH1 \) continued to show suppression of PEV when mutated for \( roX \) (\( roX1^{ex33}roX2\Delta/Y; \ MCPH1^{0978} \) KV20/\( MCPH1^{0978} \) and \( roX1^{ex33}roX2\Delta/Y; \ KV20/+; \ dhc64c^{6-10}/dhc64c^{8-1} \) (Fig. 4.5, grey bars). However, no masculinization of heterochromatin was apparent in females mutated for \( Dhc64c \) or \( MCPH1 \) (Fig. 4.5, hatched bars).
Figure 4.5. Pairing regulators that do not affect heterochromatic sex.
Heterochromatic sex was determined in flies mutated for anti-pairers (Cap-H2, Cap-D3 and fs(1)h) and pairing promoters (MCPH1 and Dhc64c). All flies carried the y+ KV20 reporter. Flies mutated for each pairing regulator were generated in control and roX mutant backgrounds. Almost no abdominal pigmentation was observed in XX flies wild type (white) or mutated (hatched) for both roX genes. In contrast, PEV in XY flies (black) is suppressed in roX mutants (gray). A slight enhancement of PEV is detected in Cap-D3 mutant flies, consistent with previous reports of condensin mutations as PEV enhancers (Dej et al. 2004; Cobbe et al. 2006). Fifteen-50 flies were counted for each genotype.
We then tested Top2, a pairing promoter with critical roles in chromosome organization, mitosis, meiosis and DNA repair. Since loss of Top2 is lethal, the complementing heteroallelic Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} combination was used (HOHL et al. 2012). Each mutation is individually lethal, but Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} adults display >50% viability. Both mutations are missense. Top2\textsuperscript{17-1} (S791F) in the WHD domain reduces protein accumulation, but Top2\textsuperscript{17-3} (L471Q) in the TOPRIM domain produces stable, full-length protein (Fig. 4.7 A, A. Hohl, Personal communication). We generated Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} XX and XY flies with the y\textsuperscript{+}KV24 reporter that were either wild type or mutated for roX. The switch to KV24 (3\textsuperscript{rd} chromosome) reporter was necessitated by our inability to recover a recombinant second chromosome with KV20 and Top2 mutation. Interestingly, we observed that loss of Top2 itself suppressed PEV in males but not in females. This result suggests that perhaps, Top2 and roX RNAs both are required for maintaining normal heterochromatin in both sexes but males are more sensitive to their loss than females. Further, as expected, roX\textsuperscript{1\textsubscript{ex33}}roX2\Delta Y; Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3}; KV24/+ males showed strong suppression of PEV when compared with control males with wild type roX function (yw/Y; Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3}; KV24/+ , Fig. 4.6 B, dark gray bars). Surprisingly, ywroX\textsuperscript{1\textsubscript{ex33}}roX2\Delta; Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3}; KV24/+ females also displayed suppression of PEV, suggesting masculinization of XX heterochromatin (p-value** = <0.00001, Fig. 4 A, hatched bars). All XX; Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} mutant flies showed characteristic female morphology, pigmentation and the absence of sex combs on forelegs.
Figure 4.6 Mutation of Topoisomerase II (Top2) masculinizes XX heterochromatin. A, B) Wild type (+) and mutant (-) genes, as well as overexpressing transgenes (+++) are indicated on the X axis. A) Females mutated for Top2 and roX suppress PEV. Pigmentation is not increased significantly in XX flies mutated for roX or Top2 alone (left three bars). However, simultaneous loss of roX and Top2 produces a significant increase in pigmentation. Over expression of MLE, but not MSL1, partially restores PEV in roX and Top2 females (right two bars). Full genotypes (left to right) are wv; KV24/+ , roX1<sup>ex33</sup> roX2Δ; KV24/+ , Top2<sup>17-1</sup>/Top2<sup>17-3</sup>; KV24/+ , roX1<sup>ex33</sup> roX2Δ; [H83MLE]+/+ , KV24/+ , roX1<sup>ex33</sup> roX2Δ; +/+; KV24/[H83M1]Z1 , roX1<sup>ex33</sup> roX2Δ; Top2<sup>17-1</sup>/Top2<sup>17-3</sup>; KV24/+ , roX1<sup>ex33</sup> roX2Δ; Top2<sup>17-1</sup>/Top2<sup>17-3</sup> [H83MLE]; KV24/+ , roX1<sup>ex33</sup> roX2Δ; Top2<sup>17-1</sup>/Top2<sup>17-3</sup> ; KV24/[H83M1]Z1. p-value **=<0.001, ***=<0.00001, n.s = non-significant. B) XY flies suppress PEV in roX mutants, but PEV is not rescues by MLE or MSL1 overexpression. Unless noted, data is derived from 20-50 flies of each genotype. p-values as in A. C) Overexpression of MLE rescues Top2 lethality in both sexes. wv; Top2<sup>17-1</sup>/CyO y<sup>+</sup> females were mated to wv; Top2<sup>17-1</sup>/CyO y<sup>+</sup> or wv; Top2<sup>17-3</sup>[H83 MLE] /CyO y<sup>+</sup> males. Survival of wv;Top2<sup>17-1</sup>/Top2<sup>17-3</sup> (black) and wv; Top2<sup>17-1</sup>/Top2<sup>17-3</sup> [H83 MLE] (gray) was calculated by setting recovery of flies with CyO<sup>+</sup> to 100%. Averages of least 3 replicate matings are shown. D) Overexpression of MSL1 does not rescue Top2<sup>17-1</sup>/Top2<sup>17-3</sup> survival. wv; Top2<sup>17-1</sup>/In(2LR)GlaBc female flies were mated to wv/Y; Top2<sup>17-3</sup>/In(2LR)GlaBc; [H83M1]Z1/+ males. Survival was calculated by setting recovery of flies with In(2LR)GlaBc to 100%. Adult survival is derived 5 from 5 replicate matings.
Characterization of Top2 mutants

Top2 was the sole pairing promoter that disrupted the sexual differentiation of heterochromatin. We examined Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} flies for evidence of defects in specific processes. Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} males are fertile, but embryos deposited by Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} females fail to hatch (Fig. 4.7 B). No evidence of DNA replication could be detected in these embryos by DAPI staining (not shown). We conclude that either successful fertilization or early embryonic development in Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} mutants requires maternally provided wild type Top2.

We then examined polytene preparations from wild type or Top2\textsuperscript{17-1}/Top2\textsuperscript{17-3} larvae to determine if there was a visible effect on chromosome organization. Similar heteroallelic Top2 mutants have been shown to specifically disrupt the male X-chromosome (A. Hohl, personal communication). We scored chromosome morphology, as 'abnormal' if banding was diffuse and 'puffy' if the chromosome was enlarged and bloated along its entire length. Top2 mutant chromosomes are more susceptible to breaking during polytene preparations, suggesting fragility. Seventy percent of nuclei from male Top2 mutants had abnormal or puffy X chromosomes (Fig. 4.7 C, black arrows), but only 14% abnormality was detected in wild-type male larvae. Top2 mutant females and wild type females display similar levels of X chromosome abnormality (10-15%). We also observed that 50% of Top2 mutant nuclei of both sexes had partially unpaired homologs, in contrast to 15% of wild type larvae (Table 4.1, Fig. 4.7 C, white arrows). The size, position and extent of unpairing varied between nuclei.
As most homologues in the polytene preparations continue to pair, this suggests that $Top2^{17-1}/Top2^{17-3}$ larvae have a relatively minor defect in homolog pairing. In summary, examination of chromosomes suggests selective disruption of polytenization of the male X-chromosome and homolog pairing that is largely intact in $Top2^{17-1}/Top2^{17-3}$ larvae.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Karyotype</th>
<th>Normal X</th>
<th>Abnormal or puffy X</th>
<th>Unpairing</th>
</tr>
</thead>
<tbody>
<tr>
<td>++</td>
<td>XX</td>
<td>95.26% (161/169)</td>
<td>4.74% (8/169)</td>
<td>13.01% (22/169)</td>
</tr>
<tr>
<td></td>
<td>XY</td>
<td>85.53% (136/159)</td>
<td>14.47% (24/159)</td>
<td>15.72% (25/159)</td>
</tr>
<tr>
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<td>XX</td>
<td>87.02% (208/239)</td>
<td>12.97% (31/239)</td>
<td>47.69% (114/239)</td>
</tr>
<tr>
<td>$Top2^{17-3}$</td>
<td>XY</td>
<td>29.63% (72/243)</td>
<td>70.37% (171/243)</td>
<td>54.73% (133/243)</td>
</tr>
</tbody>
</table>

Table 4.1. Polytene preparations from Top2 mutants display altered X-chromosome morphology and disrupted pairing. Polytene chromosomes from control (+/+, laboratory reference yw strain) and $Top2^{17-1}/Top2^{17-3}$ larvae were examined for disrupted morphology and local unpairing. The incidence of abnormality, and total nuclei scored, is in parentheses. Chromosomes with a diffuse banding pattern and those bloated along the entire chromosome length were scored as abnormal. Any nuclei with visible unpairing of homologs was scored as positive for unpairing.

We then examined homolog pairing by a pairing-dependent transvection assay at the yellow ($y$) gene. Homolog pairing enables enhancers
from one mutant allele to drive the promoter of a different allele, restoring gene expression. Expression is detected by increased adult pigmentation. While $y^{82f29}$ is a deletion of upstream enhancer elements, $y^{1\#8}$ retains enhancers but lacks a promoter. Transvection in $y^{82f29}/y^{1\#8}$ flies restores body, wing and bristle color (Fig. 4.7 D). $y^{3c3}$ lacks a bristle enhancer and the $y$ promoter, but retains a wing enhancer. Transvection in $y^{82f29}/y^{3c3}$ flies restores wing pigmentation (Fig. 4.7 D). Flies homozygous for any one of these alleles have light bodies, wings and bristles. Heterallelic $y^{82f29}/y^{1\#8}$ and $y^{82f29}/y^{3c3}$ flies were generated in wild type and $Top2^{17-1}/Top2^{17-3}$ mutant backgrounds. We found no evidence that transvection at $y$ is affected in $Top2^{17-1}/Top2^{17-3}$ animals, as the levels of pigmentation were indistinguishable between flies with wild type or mutant $Top2$ (Fig. 4.7 D, E). We conclude that homolog pairing activity sufficient to support transvection at $y$ is retained in $Top2^{17-1}/Top2^{17-3}$ mutants.

Top2 is also necessary for insulation in Gypsy transposons (RAMOS et al. 2011). The $y^2$ allele is created by a Gypsy insertion that prevents wing and body enhancers from contacting the promoter. Disruption of insulator function allows these enhancers to contact the promoter, restoring pigmentation in wing and body. We tested insulator function by comparing pigmentation in $y^2$ males that are wild type for Top2 or carry the $Top2^{17-1}/Top2^{17-3}$ heteroallelic mutation. No increase in body or wing color could be detected in $y^2/Y;Top2^{17-1}/Top2^{17-3}$ flies (Fig. 4.7 F). We conclude that the $Top2^{17-1}/Top2^{17-3}$ flies retain insulator function at Gypsy elements. This is consistent with analyses of other viable $top2$ heteroallelic combinations (A. Hohl, personal communication).
**Figure 4.7: Top2^{17-1}/Top2^{17-3} mutants are deficient in specific functions.**

A) The missense Top2 mutations used disrupt different domains. Top2^{17-1} is in the WHD domain while Top2^{17-3} is in the TOPRIM domain.  

B) Top2^{17-1}/Top2^{17-3} males are fertile but Top2^{17-1}/Top2^{17-3} females are sterile. Both mutations are homozygous lethal.  

C) Characteristic abnormalities in a polytene preparation from a male Top2^{17-1}/Top2^{17-3} larvae. A puffy X chromosome (black arrow) and homolog unpairing (white arrows) are visible. One hundred-250 nuclei from at
least 5 larvae were scored for each genotype. D) Transvection at yellow. $y^{82f29}$ is a deletion of upstream enhancer elements. $y^{1#8}$ retains enhancers but lacks a promoter. $y^{3c3}$ lacks a bristle enhancer and the promoter, but retains a wing enhancer. Pairing between $y^{82f29}$ and $y^{1#8}$ or $y^{3c3}$ enables enhancers on the homolog to drive the $y^{82f29}$ promoter, restoring $y$ expression. Wing and body pigmentation was ranked from 1 (no pigmentation) to 4 (wild type). Flies were aged 1-2 days before scoring and photography. Flies homozygous for each allele have light body and wing color (1,1). Transvection in $y^{82f29}/y^{1#8}$ flies restores wing and body color near wild-type levels (3, 3). Transvection in $y^{82f29}/y^{3c3}$ flies restores wing pigmentation only (3, 1). Transvection is not disrupted in $Top2^{17^-1}/Top2^{17^-3}$ mutants (shaded). At least 100 flies were scored for each genotype. E) Representative abdomens showing $y$ transvection. F) The $Top2$ mutations tested do not disrupt Gypsy insulation. Loss of pigmentation in $y^{2}$ requires $Top2$-dependent insulation. Loss of insulation enhances body pigmentation. Full genotypes are: $y^{2}/Y; +/+$, $y^{2}/Y; Top2^{17^-}/CyO$ and $y^{2}/Y; Top2^{17^-1}/Top2^{17^-3}$. At least 25-30 flies of each genotype were aged for 24 h before scoring.
Top2 interacts with MLE

Top2 has recently been reported to participate in dosage compensation (Cugusi et al. 2013). In support of this idea, a physical interaction between Top2 and the MLE RNA helicase, a member of the dosage compensation complex, was detected. Based on this, and the male-limited disruption of the X chromosome morphology in Top2^{17-1}/Top2^{17-3} mutants, we asked whether Top2^{17-1}/Top2^{17-3} genetically interacts with dosage compensation mutations. Interestingly, Top2^{17-1}/Top2^{17-3} flies do not display male-preferential lethality, suggesting that dosage compensation may not be affected (Fig. 4.6 C, black). The association between Top2 and MLE prompted us to test whether overexpression of MLE from a heat shock-driven transgene influenced the survival of Top2^{17-1}/Top2^{17-3} flies. MLE overexpression dramatically rescued Top2^{17-1}/Top2^{17-3} mutants of both sexes (Fig. 4.6 C, gray). Taken together, our findings support a genetic interaction between Top2 and MLE occurring in both sexes. Interestingly, we did not detect rescue of Top2 mutants by overexpression of another dosage compensation complex member, male-specific lethal 1 (MSL1) (Fig. 4.6 D).

Increased survival of Top2^{17-1}/Top2^{17-3} mutants upon MLE overexpression next prompted us to ask if MLE could also support the function of Top2 in regulation of heterochromatic sex. To address this we generated Top2^{17-1}/Top2^{17-3} mutants that overexpress MLE, carry the KV24 reporter and are either wild type or roX mutant. We found that roX^{1 ex33}roX2Δ males carrying Top2^{17-1}/Top2^{17-3} and overexpressing MLE (roX1^{ex33}roX2Δ/Y ; Top2^{17-1}/ Top2^{17-3} [H83
MLE]; KV24/+), continued to suppress PEV comparable to the \( \text{roX}^1\text{ex}33\text{roX}2\Delta/Y \); Top2^{17-1}/Top2^{17-3}; KV24/+. Notably, \( \text{roX}^1\text{ex}33\text{roX}2\Delta \); Top2^{17-1}/Top2^{17-3} [H83 MLE]; KV24/+ females largely restored the PEV comparable to the \( \text{roX}1^{\text{ex}33}\text{roX}2\Delta/+ \)[H83 MLE]; KV24/+ females (Fig. 4.6 A). However, overexpression of MSL1 did not restore PEV in females mutated for \( \text{roX} \) and \( \text{Top2} \) (Fig. 4.6 A). Taken together, these findings support the idea that a \( \text{Top2} \)-MLE interaction is necessary for a process other than compensation, but the basis for the sex-specific effect of MLE on restoration of female PEV is speculative at present.

**Loss of X heterochromatin disrupts autosomal heterochromatic sex**

The involvement of \( \text{Top2} \) in a process triggered by chromosomal content suggested an alternative mechanism of karyotype detection. At least 10 Mb of X heterochromatin is composed of satellite repeats (359 bp repeats) that are unique to the X chromosome (Lohe et al. 1993; Williams et al. 2007). Interestingly, the 359 bp repeats bind TOP2 in interphase nuclei (Kas and Laemmli 1992; Ferree and Barbash 2009). This suggested the possibility that X heterochromatin interacts with Top2 to signal karyotype. If this is the case, deletion of X heterochromatin will disrupt karyotype sensing. The X;Y translocation \( \text{Zhr}^7 \) replaces X heterochromatin, consisting primarily of 359 bp repeats, with a part of the Y chromosome (Sawamura and Yamamoto 1993; Sawamura et al. 1993). We generated \( \text{roX} \) mutant females that were heterozygous for \( \text{Zhr}^7 \) and carry the \( \varphi^+\)KV20 reporter (\( \text{yw}\text{roX}1^{\text{ex}33}\text{roX}2\Delta\text{Zhr}^7/\text{yw}\text{roX}1^{\text{ex}33}\text{roX}2\Delta +; \text{KV20/+} \)). Interestingly, we observed weak suppression of PEV
in roX females with a single Zhr\textsuperscript{1} mutation, but not in females with wild type roX and Zhr\textsuperscript{1} (yw+/yw Zhr\textsuperscript{1}; KV20/+; Fig. 4.8 A). As removal of one copy of X heterochromatin generates XX females that now require roX for normal autosomal PEV, we conclude that loss of X heterochromatin partially masculinizes autosomal heterochromatin in these flies.

The involvement of Top2 in homolog pairing, and its localization at the 359 bp repeats, suggested the possibility that a large block of unpaired 359 repeats itself could signal the XY karyotype. If unpaired 359 repeats generate a signal, we reasoned that autosomal heterochromatin in Zhr\textsuperscript{1}/Zhr\textsuperscript{1} females would be feminine, as there are no 359 bp repeats present (Fig. 4.8 A). In contrast to these expectations, we found increased suppression of PEV in homozygous Zhr\textsuperscript{1} females that lack roX (yw roX\textsuperscript{1}ex33 roX2\Delta Zhr\textsuperscript{1}; KV20/+). No suppression of PEV was observed in homozygous ywZhr\textsuperscript{1}; KV20/+ females, indicating that suppression of PEV is not caused by the differing chromatin content of the Zhr\textsuperscript{1} chromosome. These findings are consistent with Top2 and X heterochromatin acting together to signal karyotype, but do not support the idea that the signal is generated by unpaired chromatin.

The suppression of PEV in roX females with one or two Zhr\textsuperscript{1} alleles is weak (contrast suppression of PEV in roX\textsuperscript{1} roX\textsuperscript{2} males, Fig. 4.6 A). To determine if the effects of Top2 and Zhr\textsuperscript{1} mutations are additive, we generated Zhr\textsuperscript{1}/+ females mutated for Top2 and compared PEV in the presence and absence of roX (yw + + Zhr\textsuperscript{1} / yw + + +; Top\textsuperscript{217-1}/Top\textsuperscript{217-3}; KV24/+ compared to yw roX\textsuperscript{1}ex33 roX2\Delta Zhr\textsuperscript{1}/yw roX\textsuperscript{1}ex33 roX2\Delta +; Top\textsuperscript{217-1}/Top\textsuperscript{217-3}; KV24/+). In this
study we used the KV24 reporter, which produces a low number of $y^+$ spots in females. Females mutated for Top2 with a single $Zhr^1$ chromosome displayed greater suppression of PEV suppression upon loss of $roX$ than females mutated for just Top2 or $Zhr^1$ alone, supporting the idea that Top2 and pericentric X heterochromatin act together (Fig. 4.8 B).

If the dose of X-heterochromatin acts as a signal for karyotype, duplication of this region on the Y in XY flies should feminize their heterochromatin. We attempted to generate XY flies with a duplication of X heterochromatin on the Y chromosome ($Zhr^+ Y$) to test this idea (FERREE AND BARBASH 2009). Unfortunately, no $roX1 roX2/ Zhr^+ Y$ males were recovered, suggesting a genetic incompatibility between some of the contributing chromosomes.
**Figure 4.8.** Pericentromeric X heterochromatin contributes to feminization of autosomal heterochromatin in XX flies. The X;Y translocation Zhr\(^1\) lacks almost all X-heterochromatin. roX1 roX2 is indicated by (-), Top2\(^{17-1}/\)Top2\(^{17-3}\) is indicated by (-) and removal of X heterochromatin by Zhr\(^1\) is (heterozygous; +/-, homozygous; -/-). A) Females with one or two Zhr\(^1\) chromosomes suppress PEV upon loss of roX. The KV20 reporter, which normally produces <1 spot/abdomen, was used. Full genotypes (left to right): yw; KV20/+ , yw/yw Zhr\(^1\); KV20/+ and yw Zhr\(^1\)/yw Zhr\(^1\); KV20/+ , yw roX\(^{1ex33}\) roX2Δ; KV20/+ , yw roX\(^{1ex33}\) roX2Δ / yw roX\(^{1ex33}\) roX2Δ Zhr\(^1\); KV20/+, yw roX\(^{1ex33}\) roX2Δ Zhr\(^1\) / yw roX\(^{1ex33}\) roX2Δ Zhr\(^1\) / yw roX\(^{1ex33}\) roX2Δ Zhr\(^1\); KV20/+. Average values are derived from 20-50 flies of each genotype. p-value ***= <0.00001. B) Loss of Top2 further masculinizes heterochromatin in Zhr\(^1\)/+ females. Greater suppression of PEV is observed in roX females mutated for Top2 and with Zhr\(^1\). This study uses the KV24 reporter, producing about 30 spots/female in a wild type background. Top2\(^{17-1}/\) Top2\(^{17-3}\) is indicated by (-). Full genotypes (left to right): yw; KV24 /+, yw +/-yw Zhr\(^1\); KV24 /+, yw Top2\(^{17-1}/\)Top2\(^{17-3}\); KV24 /+, yw roX\(^{1ex33}\) roX2Δ; KV24 /+, yw roX\(^{1ex33}\) roX2Δ; Top2\(^{17-1}/\) Top2\(^{17-3}\); KV24 /+, yw roX\(^{1ex33}\) roX2Δ Zhr\(^1\) / yw roX\(^{1ex33}\) roX2Δ Zhr\(^1\); KV24/+. Underlined genotypes (coarse hatched bars) are reproduced from Fig. 4 for comparison. p-value ***= <0.00001.
Discussion

Sexual dimorphism in heterochromatin

Autosomal heterochromatin is typically not thought of as being sexually dimorphic, but studies have documented sexual dimorphism in fly and mammalian heterochromatin. Knock down of HP1, a major heterochromatin protein, produces preferential lethality and higher gene misregulation in male flies (LIU et al. 2005). Localization of HP1 by Dam-ID revealed some sex-specific differences in HP1 binding in male and female genomes (DE WIT et al. 2005). Recently, sex-specific heterochromatic silencing has been observed in mice, where a variegating transgene is more highly expressed in females than in males (WIJCHERS et al. 2010). Interestingly, this study found that the sex chromosome karyotype and Sry, the Y-linked sex determining locus, determines silencing. More importantly, this study reveals that the sexual dimorphism of autosomal heterochromatin is not limited to Drosophila.

Karyotype sensing as a signal for sex determination

Many recent studies have highlighted the complexity of gene regulation at the base of the fly sex determination cascade (SANDERS AND ARBEITMAN 2008; ITO et al. 2012; HOXHA et al. 2013; FAGEGALTIER et al. 2014). In contrast, for close to 30 years the chromosomal counting mechanism that triggers sexual differentiation at the top of sex determination cascade was thought to be reasonably well understood (ERICKSON AND CLINE 1998; ROBINETT
et al. 2010; Salz and Erickson 2010). Our current findings are most easily interpreted as evidence for a second mechanism that detects sex chromosome karyotype. Interestingly, this mechanism is responsible for establishing a difference in the autosomal heterochromatin of males and females. The presence of the large, heterochromatic Y chromosome means that XY males have considerably more total heterochromatin than females. While we do not yet understand the rationale for the sexual dimorphism of autosomal heterochromatin in flies, one possibility is that the different chromatin content of XY and XX cells drove a compensatory adaptation in males (Liu et al. 2005; Deng et al. 2009).

We used a candidate gene approach to rule out the conventional sex determination pathway or numerator elements as regulators of heterochromatic sex. We also eliminated the male-limited Y chromosome itself, and the key dosage compensation protein MSL2, as determinants of heterochromatin masculinization. Targeted genetic tests focused on chromatin regulators with roles in homolog pairing revealed masculinization of XX heterochromatin in Top2 mutants. This suggested that maintenance of normal chromatin organization, and perhaps homolog pairing, was important for karyotype sensing and sexual dimorphism. The involvement of Top2 in various aspects of chromatin biology complicates interpretations of these studies. The heteroallelic Top2 combination (Top2\(^{17-1}/Top2^{17-3}\)) retains partial function as it supports about 50% adult escapers. However, the complete inviability of embryos deposited by Top2\(^{17-1}/Top2^{17-3}\) mothers indicates that the heteroallelic
combination is incapable of supporting development in the absence of maternally deposited wild type Top2. Nevertheless, we were able to determine that transvection and insulation appear intact in \( \text{Top2}^{17-1}/\text{Top2}^{17-3} \) flies, even though defects in chromosome organization and homolog pairing were also detected.

\( \text{Top2} \) is enriched on the X-chromosome specific satellite repeats and interestingly, our study established that the reduction in the amount of X-heterochromatin affects the sexual differentiation of autosomal heterochromatin. Reduction in \( \text{Top2} \) function and deletion of X heterochromatin in XX flies additively enhanced heterochromatin masculinization. These findings reinforce the idea that a sequence within X-heterochromatin, possibly the 359 bp repeats, and \( \text{Top2} \) are central elements of a karyotype sensing mechanism. Several scenarios for how this might occur are possible. XX flies have double the X-heterochromatin of XY flies. An absolute difference in the amount of \( \text{Top2} \)-bound 359 bp repeats could be the signal for karyotype (Fig. 4.9 A, left). Alternatively, it is possible that the 359 bp repeats act as a sink for \( \text{Top2} \), leading to higher levels of free \( \text{Top2} \) in XY nuclei, with only one copy of X-heterochromatin (Fig. 4.9 A, right).

The identification of \( \text{Top2} \) as a pairing promoter initially suggested that pairing of X-heterochromatin in XX cells but not XY cells, signals karyotype (Fig. 4.9 B). However, studies with a similar heteroallelic \( \text{Top2} \) combination found no defect in pairing of 359 bp repeats (A. Hohl, Ph.D Dissertation). However, this study used flies that had wild type maternal supplies of \( \text{Top2} \), a factor that might obscure a requirement for \( \text{Top2} \). Alternatively, association of \( \text{Top2} \) with the 359
bp repeats might be necessary for sensing paired X-heterochromatin (Fig. 4.9 C). These possibilities remain to be investigated to deduce the exact mechanism of karyotype sensing.

It is also possible that multifunctional Top2 participates both in detection of karyotype and as an effector that modulates autosomal heterochromatin. In fact, loss of Top2 in an otherwise wild type female fly does not influence PEV, but loss of Top2 in an otherwise wild type male suppresses PEV (Fig. 4.6 A and B). This emphasizes the differences in heterochromatin in the sexes. Our study does not rule out involvement of additional regulators in generation of a signal, or in the sexual differentiation of heterochromatin. Our requirement for adult viability, and the complexity of stock generation, allows testing a limited number of candidate genes.
Figure 4.9: Hypothetical strategies for detection of XX karyotype. The absolute amount of X heterochromatin (A) or pairing of X heterochromatin (B, C) could generate a signal specifying XX karyotype. XX flies have two copies of X heterochromatin (thick lines) but XY has only one. Top2 (red) binds the 359 bp repeats (gray). Non-359 bp X-heterochromatin is shown in white. A) The absolute amount of Top2-bound 359 bp chromatin (top, left) or free Top2 in males (top, right) could generate a karyotype-specific signal. Mutant Top2 (red and white, bottom) is deficient in a function necessary for generating the signal. B) Top2-dependent pairing of X heterochromatin could signal the XX karyotype. Mutant Top2 fails to support normal pairing. C) Top2-independent pairing requires Top2 to generate or transmit a signal.
**Top2 functions in dosage compensation and sex determination**

Top2 has been isolated with chromatin-bound MSL components in S2 cells (WANG et al. 2013). As Top2 is an abundant component of chromatin, this in itself is unsurprising. However, Top2 is also reported to participate in *Drosophila* dosage compensation (CUGUSI et al. 2013). Either chemical inhibition or RNAi knockdown of Top2 produced a two-fold down regulation of a *luciferase* reporter in a plasmid-based model for dosage compensation. Physical interaction between Top2 and a single member of the MSL complex, MLE detected in this study echoed an *in vitro* interaction of Top2α with the mammalian ortholog of MLE, RNA helicase A (ZHOU et al. 2003). Curiously, while our studies confirm the genetic interaction between MLE and Top2, this interaction appears equally important in males and females, and thus is not limited to dosage compensation. Our result is consistent with the previous observation that loss of Top2 does not alter recruitment of MSL proteins on the male X- chromosome (HOHL et al. 2012). Further, loss of function Top2 mutant (*Top2*<sup>17-1</sup>*/*Top2<sup>17-3</sup>) used in this study did not preferentially affect males. We propose that the involvement of MLE in X chromosome dosage compensation in males reduces the availability of overexpressed MLE for interaction with Top2.

A remaining question is what the nature of the Top2-MLE interaction might be. Proteins with helicase/ATPase domains, such as Top2, are suggested to participate in multi-protein complexes that require ATP hydrolysis to produce chromatin remodeling (KINGSTON et al. 1996). MLE, an RNA/DNA helicase, is also ATP-dependent. Perhaps, overexpression of MLE could support the activity
of Top2 mutants. Another DEAD/H box RNA helicase P68 is known to associate with mouse centromeric repeats in vitro (ENUKASHVILY et al. 2005). This suggests the possibility that MLE associates with Top2 on X-heterochromatin.

A novel sex determination signal in *Drosophila*

Numerous sex determination strategies have arisen in the heterogametic organisms. Each utilizes a primary signal that orchestrates the process of being a female or a male. The sex determination pathway in *Drosophila* relies on the titer of X-linked gene products as a surrogate for X chromosome number resulting in activation of *Sxl* in XX cells. *Sxl* controls two essential pathways in female somatic development, feminization of tissues and suppression of dosage compensation. Since the discovery of numerator elements in early 80’s, the mystery of the primary signal for somatic fly sex determination was considered solved. To the best of our knowledge, the current study is the first example of a sexually dimorphic feature of *Drosophila* that is specified independent of the numerator elements and *Sxl*. Our findings suggest that chromosome-specific DNA sequences, combined with proteins that interact with these sequences, constitute a second mechanism for karyotype sensing in flies. While details of this mechanism remain to be discovered, universality of Top2 and repetitive sequences in higher eukaryotes suggests a general mechanism for karyotype sensing can also occur in other heterogametic organisms.
Our lab previously reported a role for the non-coding roX RNAs in autosomal heterochromatin in male *Drosophila*. It was not known if roX RNAs participate in this process directly or indirectly. My first project, described in Chapter 3, generated a tool to address questions about the role of roX1 in this process and resulted in creation of roX1<sup>MS2-6</sup> (APTE et al. 2014). In future, this allele can be used to perform roX1 localization studies in living cells. The technique used to generate roX1<sup>MS2-6</sup>, Targeted Gene Conversion (TGC), is capable of precise replacement of large sequences. We predict that this methodology could be combined with rapid gene engineering techniques like CRISPR to improve the speed of TGC, increase the range of possible targets and increase the size of engineered regions beyond that currently achieved through CRISPR alone.

Although roX RNA participates in regulation of autosomal heterochromatic regions only in male flies, the genetic basis for the sex-specificity of this was completely unknown. In Chapter 4, I described studies to determine the genetic basis of this sex specificity. My findings indicate that sexual differentiation of heterochromatin in *Drosophila* is independent of the conventional sex-determination pathway. My studies identified a previously undescribed signal for sexual differentiation that likely involves a novel karyotype sensing mechanism. Components of this system are the chromatin protein Topoisomerase II (Top2)
and pericentric X-heterochromatin. The primary component of X-heterochromatin is a massive array of 359 bp repeats that bind Top2, suggesting potential mechanisms for detection of sex chromosome karyotype. These observations raise several interesting questions about the precise nature and function of the signal. In addition, the mechanism by which autosomal heterochromatin is modulated in males, and in XX flies with masculinized heterochromatin, remains speculative.

**How is the karyotype detected?**

While Top2 and 359 bp repeats on X-heterochromatin both influence the sexual differentiation of autosomal heterochromatin, the mechanism by which these elements act is unknown. Top2 promotes chromosome pairing (Williams *et al.* 2007; Joyce *et al.* 2012), raising the possibility that pairing of 359 bp repeats in female nuclei signal the XX karyotype. However, preliminary data from the Geyer lab has suggested that the loss of Top2 in eye and wing discs does not influence pairing of the X-linked 359 bp repeats (A. Hohl, unpublished). This observation hints that pairing at the 359 bp repeats is perhaps maintained by other pairing regulators and an interaction between Top2 and satellite repeats is independent of the Top2’s function in somatic pairing.

Another potential mechanism that these elements could act to detect karyotype relies on the fundamental role of Top2 in nuclear architecture. Top2 is a major component of nuclear matrix and known to bind to Scaffold Attachment Regions (SARs) (Adachi *et al.* 1989; Tang 2011b; Tang 2011a). SARs are *cis-*
acting elements that regulate interphase chromatin architecture. According to the recently proposed CORE model (TANG 2011a), repetitive DNA regions on different chromosomes form a higher order meshwork that creates distinct structural domains during interphase. It is formally possible that the large arrays of 359 bp repeats on the X-chromosome bind Top2 to form a distinct region in the nucleus. The size of this region, which depends on the number of X chromosomes, would generate a signal for animal karyotype (scenario described in Fig. 4.9 A). Additionally, nuclear lamins also interact with AT-rich satellite repeats (BARICHEVA et al. 1996; RZEPECKI et al. 1998; MEULEMAN et al. 2013). Lamins interact with HP1 and D1, known interactors of Top2 (MELLER AND FISHER 1995; MELLER et al. 1995; BLATTES et al. 2006). D1 has been shown to interact with AT-rich sequences in the nuclear envelope/lamina associated chromatin fraction (MONOD et al. 2002). The co-incidental presence of Top2 along with lamins, D1 and HP1 on the 359 bp repeats suggests a possible role for these factors in karyotype sensing. It is possible that HP1, D1 and lamins influence clustering of satellite repeats in sex-specific manner and help Top2 in sensing the amount of X-heterochromatin to generate a signal for karyotype. DNA FISH (For 359 bp repeats) with Immuno-staining (for Lamins/HP1/D1/Top2) can detect possible alterations in interactions between these candidate proteins and 359 bp repeats in XX masculinized animals compared to XX females. In addition, Chromatin immuno-precipitation (ChIP)-MS in wild type and Zhr7 cells could identify additional candidates that specifically interact with Top2 in masculinized cells.
Limitation of using PEV assay as a read-out for heterochromatin silencing

Using PEV as readout for heterochromatin function, we found that a block of X-heterochromatin can regulate autosomal heterochromatin elsewhere in the genome in sex-specific manner. Our study also highlights the role for Top2 as well as roX RNAs in this process. Use of adult PEV assay allowed us to test limited number of possible candidates. Other possible regulators in this process might include HP1 and D1. HP1, a major heterochromatin protein enriched on the pericentric heterochromatin is an anti-pairer (JOYCE et al. 2012). 359 bp repeats have been reported to associate with HP1 along with Top2 (BLATTES et al. 2006). Earlier evidences also suggest sex-specific effects in HP1 conditional mutants. On the other hand, D1 is little less known AT-hook protein that is dispensable for viability in flies (WEILER AND CHATTERJEE 2009) and it is known to interact with 359 bp repeats with Top2. We did not include D1 or HP1 in our initial targeted screen as they themselves modify PEV in dose dependent manner and would have confounded our interpretations (AULNER et al. 2002, ELGIN AND REUTER, 2013) but the role of D1 and HP1 in this sex-specific regulation can be tested by looking for masculinization of XX heterochromatin by gene expression analysis.

Do Top2 and X-heterochromatin regulate other sexually dimorphic features of flies?

We have demonstrated masculinization of autosomal heterochromatin in XX flies with reduced Top2 and 359 bp satellite repeats. While these animals
remain phenotypically female, there may be additional sexually dimorphic features regulated by Top2 and X-heterochromatin. To identify these, one could perform genome-wide gene expression analysis in XX flies with or without heterochromatin masculinization. Genes showing significant changes in gene expression in XX flies with masculinized heterochromatin could be a starting point to identify additional sexually dimorphic features regulated by direct sensing of fly karyotype.

**Do autosomal heterochromatic genes require roX RNA in XX flies with masculinized heterochromatin?**

While we detected heterochromatin masculinization by the PEV assay, associated changes in heterochromatic gene expression have not been investigated. Our initial attempts of determining gene expression changes by quantitative real-time PCR using XX Top2 mutant masculinized flies have revealed that gene expression changes are very slight. Additionally, loss of an essential gene product like Top2 is expected to produce generalized genome-wide effects not relevant to heterochromatin masculinization. In future, genome wide gene expression analysis using XX flies masculinized by Zhr1 would be a better experimental strategy. Genome-wide analysis has better normalization, experimental power, and sensitivity than quantitative real time PCR. However, given the need to generate three biological replicates of six genotypes, this would require a major investment of laboratory resources.
APPENDIX A

The $\text{roX1}^{\text{MS2-6}}$ allele is wild type for heterochromatic silencing

Previous studies indicated that levels of $\text{roX1}$ that were too low to support dosage compensation were able to restore heterochromatic PEV (S. K. Koya, unpublished). Additional studies suggested that some regions of $\text{roX1}$ are necessary for heterochromatic silencing, but not for dosage compensation. Various $\text{roX1}$ deletions were tested for restoration of PEV in $\text{roX1}^{\text{ex33}} \text{roX2}\Delta$ mutant males. These experiments identified distinct regions required either for dosage compensation, heterochromatic silencing, or both. The 3' and 5' ends of $\text{roX1}$ are essential for both dosage compensation (STUCKENHOLZ et al. 2003) and heterochromatic silencing (S.K. Koya, unpublished). However, removal of the central portion of $\text{roX1}$ blocked heterochromatic silencing, but not dosage compensation.

The allele $\text{roX1}^{\text{MS2-6}}$ has a 322 bp insertion within a region that appears necessary for heterochromatic silencing. This allele, generated by a targeted gene conversion, is fully functional in dosage compensation (APTE et al. 2014). The inserted sequence can form six tandem stem loops in vivo. The insertion site lies within the $\text{roX1}^{\text{ex33}}$ and $\text{roX1}\Delta6$ deletions; alleles spanning a region that appears important for heterochromatic silencing (Fig. A1.A). It is possible that the 322 bp insertion in $\text{roX1}^{\text{MS2-6}}$ disrupts an element within this region that is necessary for heterochromatic regulation. To test this idea, I generated $\text{roX1}^{\text{MS2-6}} \text{roX2}\Delta; \text{KV20/+}$ male and female flies carrying $y^+ \text{KV20}$ reporter. PEV of the $y^+$
marker was compared with \( \text{roX1}^{\text{MS2-6}} \text{roX2}^+ \); KV20 \(+\) males and females (Fig. A1.B).

There was no suppression of PEV in \( \text{roX1}^{\text{MS2-6}} \text{roX2}\Delta \); KV20/\(+\) males, as the amount of pigmentation was similar in \( \text{roX1}^{\text{MS2-6}} \text{roX2}\Delta \) males, and in the control males with a wild type \( \text{roX2} \) gene. This indicates that although this insertion is in a region necessary for the heterochromatic function of \( \text{roX1} \), it does not disrupt the function of this region.

Figure A1: The \( \text{roX1}^{\text{MS2-6}} \) insertion allele supports heterochromatic silencing. A) Schematic representation of the \( \text{roX1} \) deletion allele \( \text{roX1}^{\text{ex33}} \), the \( \text{roX1}\Delta^6 \) transgene and the \( \text{roX1}^{\text{MS2-6}} \) insertional mutation. B) Suppression of PEV in \( \text{roX1}^{\text{ex33}} \text{roX2}\Delta \); KV20/\(+\) males produces an increased number of abdominal spots. No suppression of PEV was detected in \( \text{roX1}^{\text{MS2-6}} \text{roX2}\Delta \); KV20/\(+\) males, or in control males with wild type \( \text{roX2} \) (two right bars). Twenty to fifty male (gray) and female (hatched) flies from each genotype were scored.
APPENDIX B

Targeted gene conversion at an autosomal gene, CTCF

To determine the generality of Targeted Gene Conversion (TGC; APTE AND MELLER 2014) gene engineering was attempted at the autosomal CTCF gene. I generated a FLAG-tagged CTCF repair template, as well as a template with a frame-shift at the N-terminus. These were engineered within a ~1.5Kb genomic fragment spanning the CTCF transcription start site. These repair templates also carried a phenotypic marker, mini-white+. Fig. B1 depicts these transgenes. Flies were generated carrying each of these transgenes, and the insertions were mapped to chromosomes.

TGC requires a P-element insertion near to the target gene. An insertion at the 5’ end of CTCF, p(EPgy2)CTCF\textsuperscript{EY15833}, was selected for this purpose. I attempted to use targeted transposition to move a 2\textsuperscript{nd} chromosome insertion of the frame-shift template (T4F) and an X chromosome insertion of the FLAG tagged template (T15A) onto 3\textsuperscript{rd} chromosome carrying endogenous CTCF gene. I was unable to recover targeted transpositions in the CTCF gene on the 3\textsuperscript{rd} chromosome.
Generation of CTCF repair templates for TGC

1. CTCF genomic fragments were amplified with primers CTCF-271 and CTCF flgR (500 bp) and CTCF-R3 and CTCF flgF (~1Kb). The CTCF flgF primer introduces a Nde1 site, while CTCF flgR introduces Nde1 and Bcl1 RE sites.

2. Amplified fragments were cloned in pCR4-TOPO (Invitrogen). Transformants were identified by colony PCR, and confirmed by restriction digestion with Nde1-Pst1 and Bcl1-Not1.

3. Both fragments were sequentially moved into pBluescript(+)KS, reconstructing a 1.5 kb CTCF fragment with Nde1 and Bcl1 restriction sites introduced very near to the transcription start site. The 1Kb fragment was cloned between Not1 and Spe1 in pBluescript(+)KS while the 500bp fragment was cloned at the Eco R1 site. Construction was confirmed by restriction digestion and sequencing.

4. Once both CTCF fragments were cloned in pBluescript(+)KS in the appropriate orientations, Nde1 digestion and re-ligation was performed to remove the intervening region. Reduction at Nde1 generated a 1.5 Kb CTCF template with a frame shift introduced due to Nde1 and Bcl1 restriction site insertion (1.5 mCTCF).

5. An Eco R1 fragment containing 1.5 mCTCF was moved into pCaSpeR 4 and confirmed by PCR, restriction digestion and sequencing. The confirmed plasmid was sent for embryo injection (23.6.6 was the specific transformant confirmed by sequencing and sent for embryo injection).
6. For generating a FLAG-CTCF repair template, a FLAG linker (DYKDDDDK) was inserted between the Nde1 and Bcl1 restriction sites in the 1.5 kb CTCF construct in pBluescript(+)KS. Transformants were screened by colony PCR. As introduction of the FLAG tag will destroy the Nde1 restriction site, colonies were also screened by digestion with Nde1 and Kpn1.

7. An EcoR1 fragment containing the FLAG-CTCF construct was moved into pCaSpeR 4 and confirmed by PCR, restriction digestion and sequencing. (23.1.1.3 was the specific transformant confirmed by sequencing and sent for embryo injection).

8. Transgenic insertions of these plasmids were generated by embryo injection (Rainbow Transgenics). Transgenes were mapped to chromosome.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLAG-F (New)</td>
<td>GAT CGA TTA CAA GGA TGA TGA TGA TAA GGG</td>
</tr>
<tr>
<td>FLAG-R (New)</td>
<td>TAC CCT TAT CAT CAT CAT CCT TGT AAT C</td>
</tr>
<tr>
<td>CTCF flgF</td>
<td>CCC CCA TAT GCC AAG GAG GAC AAA AAA GGA CGA GG</td>
</tr>
<tr>
<td>CTCF flgR</td>
<td>GGC ATA TGG GGG GGT GAT CAT TCC TAT GGA CAA ATT GGA TTT G</td>
</tr>
<tr>
<td>MA_frameCTCF F</td>
<td>GTC CAT AGG AAT GAT CAC CCC</td>
</tr>
<tr>
<td>MA_frameCTCF R</td>
<td>TGT CCT CCT TGG CAT ATG GGG G</td>
</tr>
<tr>
<td>CT F-271</td>
<td>TAC CCA TGA GCG ATC CAT CCA CTC AAG AG</td>
</tr>
<tr>
<td>CT F 98</td>
<td>TAT TGG CAA CCA AGT GTC GGT AGG TG</td>
</tr>
<tr>
<td>CTCF R1</td>
<td>CTG CAG ATC CTC GGG GTC CTC GTC C</td>
</tr>
<tr>
<td>CTCF R2</td>
<td>TGG CGG TGG CAT CGC CGA TTG CTT CG</td>
</tr>
<tr>
<td>CTCF R3</td>
<td>TGT GGG CAT GAG TAC TTA TGT CCC G</td>
</tr>
<tr>
<td>1.5CTCF-F (not used)</td>
<td>GAA TGA TCA CCC CCC CAT ATG CCA AG</td>
</tr>
<tr>
<td>1.5CTCF-R (not used)</td>
<td>GAA CCG TAT ACC CCC CCA CTA GTA AG</td>
</tr>
</tbody>
</table>
Figure B1. Repair template structure for 1.5mCTCF and FLAG-CTCF constructs in pCaSpeR4. An engineered 1.5 Kb CTCF genomic fragment from pBluescript(+)KS was introduced into the pCaSpeR4 EcoR1 site to create (pCaSper41.5mCTCF). A frame-shift is created by insertion of Nde1 and Bcl1 restriction sites into the CTCF fragment. The authentic AUG is to the immediate right of the Bcl1 site. 23.6.6 indicates a specific transformant confirmed by sequencing and sent for embryo injection. pCaSper4-FLAG CTCF was created by introducing the 1.5 Kb CTCF fragment with the FLAG tag into pCaSpeR4. The FLAG linker is inserted between Nde1 and Bcl1 sites. FLAG insertion destroys Nde1 and Bcl1 sites but maintains the reading frame. 23.1.1.3 indicates a specific transformant confirmed by sequencing and sent for embryo injection.
APPENDIX C

Effect of Wolbachia on heterochromatic silencing

Wolbachia is a maternally inherited intracellular endosymbiotic bacterium. Wolbachia can have diverse effects on fly development (CLARK et al. 2002, CLARK et al. 2005, IKEYA et al. 2009, MERCOT AND CHARLAT 2004). Effects of persistent Wolbachia infection include feminization of genetic males as well as selective male killing at an early developmental stage, a phenomenon widely observed in lepidopteran and arthropod species (KAGEYAMA AND TRAUT 2003). This sex-specific effect of Wolbachia on host physiology prompted me to test the effect of Wolbachia on position effect variegation (PEV). Wolbachia infection is widespread within laboratory strains of Drosophila melanogaster. Multiple stocks in the Meller Lab were found to have Wolbachia infection. Wolbachia curing was done by maintaining fly stocks for multiple generations on food containing tetracycline (200 µg/ml tetracycline in 70% ethanol, 0.1ml of tetracycline stock/10 ml of fly food). Curing was performed for several stocks including yw, yw; KV20, yw; KV24 and roX1\textsuperscript{ex33} roX2\Delta stocks. Wolbachia was detected by PCR using primers specific for the Wolbachia 16S rRNA gene (CLARK and KARR 2002) (Figure C1 A). Genetic crosses were performed with flies carrying y\textsuperscript{+} insertion KV20, before and after Wolbachia curing and male and female progeny was scored for number of abdominal spots. I did not see any effect of Wolbachia curing on the PEV expression of KV20 reporter regardless of parental transmission. Further, I hypothesized that, if the presence of Wolbachia influences male- specific PEV, Wolbachia curing should only affect the PEV in
male flies. Female \( roX1^{ex33} \) \( roX2\Delta \) flies were crossed to \( roX1^{ex33} roX2\Delta/Y; \) KV24 males before and after \textit{Wolbachia} curing and progeny was scored for number of abdominal spots. Number of abdominal spots remained unchanged in male flies before and after \textit{Wolbachia} curing. There was no effect on the PEV expression in female flies (data not shown). This enabled me to conclude that suppression of PEV in \( roX1^{ex33} roX2\Delta \) males does not depend on \textit{Wolbachia} infection (Fig. C1 B and C).
**Figure C1. Wolbachia does not alter Position Effect Variegation (PEV).**

**A.** The presence of Wolbachia was determined by PCR before and after antibiotic curing. Female yw; SbJsΔ2-3/TMS flies, known to have persistent Wolbachia infection, were used as a positive control. Curing was done by maintenance on food containing tetracycline for at least 3 generations. The absence of PCR product (right) indicates curing. PCR (initial denaturation at 95°C for 3 min, followed by 30 cycles of denaturation for 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 2 min and final extension for 5 min). Primers are (Wolb1F–TTGTAGCCTGCTATGGTATAACT and Wolb1R-GAATAGGTATGATTCTGT).

**B.** PEV is not affected by Wolbachia. yw females were crossed to yw/Y;KV20 flies before and after Wolbachia curing for paternal transmission of KV20 (right) while yw/Y flies were crossed to yw/yw; KV20 females before and after Wolbachia curing for maternal transmission of KV20, (left). 20-50 individual males (gray bars) were scored for number of abdominal spots. Male progeny continued to show comparable levels of PEV before and after Wolbachia curing indicating that the Wolbachia does not influence PEV phenotype.

**C.** Suppression of PEV in roX1<sup>ex33</sup> roX2Δ males is not affected by Wolbachia. roX1<sup>ex33</sup> roX2Δ/Y; KV24 males were crossed to roX1<sup>ex33</sup> roX2Δ female flies before and after Wolbachia curing. 15-20 individual male offspring were scored abdominal spots. Males continue to show suppression of PEV after Wolbachia curing, suggesting suppression of PEV in roX1<sup>ex33</sup> roX2Δ males is not influenced by Wolbachia.
Prior to systematic EMS mutagenesis screen to generate Top2 mutations (HOHL et al. 2012), very few molecularly defined Top2 deletion alleles were available. I used FLP/FRT recombination to generate a molecularly defined deletion that mutates Top2. FLP-mediated recombination was induced between P(XP)-CG10026-[do2517] ([do2517]) in CG10026, next to Top2, and P(XP)-Top2-[do5357] ([do5357]) in the first intron of Top2. The detailed crossing scheme is presented (Fig. D2). Potential deletions were tested by PCR for the presence of appropriate P-ends (3’ end of [do5357] and 5’ end of [do2517]). Primary PCR screen was performed to detect presence of PCR product for primer pair - pry2 and Top2 R1 and absence of PCR product for– pLac(+) and Top2 F4. Candidates that showed the expected PCR product pattern were further screened by secondary PCR screen using pLac4 and Top2 F8 or Top2 F9 primer pairs. (Fig. D1 A describes the location and orientations for P-ends and PCR primers) Top2 mutations (Top2Δ) were balanced over CyOy+ to maintain a stable stock. Further characterization revealed that Top2Δ is homozygous lethal during pupal stages. Quantitative reverse transcription PCR (qRT PCR) with homozygous and heterozygous Top2Δ larvae revealed that Top2Δ is near-null or a severe hypomorph (Fig. D1 B). Top2Δ homozygous larvae had abnormal mitotic chromosomes (Fig. D1 C). Moreover, brain dissection and subsequent
experiments suggested that Top2 mutant shows severely compromised brain development that results in near-complete loss of mushroom body in CNS (data not shown).
**Figure D1. Generation and characterization of Top2Δ.**

**A.** Schematic of the FLP-FRT recombination that generated Top2Δ. This produces reciprocal products with deletion or duplication of the intervening sequence. PCR primers used for screening the candidate recombinants are designated. FRT sites are shown by open triangles.

**B.** Quantitative reverse transcription PCR (qRT-PCR) analysis of Top2 mRNA in Top2Δ heterozygous and homozygous larvae (performed by Ferzin Sethna).

**C.** Mitotic chromosome preparations from Top2Δ/Top2Δ larval brains show abnormalities suggesting mitotic defects.
Figure D2. Crossing scheme used to generate Top2Δ. Thirty five candidate recombinant males and females were used to establish balanced stocks. Loss of the essential Top2 gene was detected by the absence of any offspring without a balancer chromosome. Both starting FRT insertions are viable.
APPENDIX E

Does reduction in Topoisomerase II influence survival of roX mutants?

Topoisomerase II (Top2) has been previously reported to participate in the process of dosage compensation (CUGUSI et al. 2013). This suggests that there might be a genetic interaction between partial loss of function mutations in the roX noncoding RNAs and Top2. The Top2<sup>17-1</sup>/ Top2<sup>17-3</sup> heteroallelic combination reduces Top2 function, but allows recovery of escaper adults.

The roX<sub>1</sub><sup>ex40</sup> roX<sub>Δ</sub> chromosome has reduced accumulation of roX<sub>1</sub> but supports 100% adult male survival due to the presence of essential 3' and 5' regions within the transcript (DENG AND MELLER 2005). If adult eclosion of Top2<sup>17-1</sup>/In(2LR)Bc Gla or Top2<sup>17-3</sup>/In(2LR)Bc Gla (Top2<sup>m</sup>/In(2LR)) is set to 100%, the Top2<sup>17-1</sup>/ Top2<sup>17-3</sup> heteroallelic combination results in decrease in adult viability in both sexes (Chapter 4). To detect a possible genetic interaction between roX and Top2, roX<sub>1</sub><sup>ex40</sup> roX2<sub>Δ</sub>; Top2<sup>17-1</sup>/In(2LR)Bc Gla females were mated to roX<sub>1</sub><sup>ex40</sup> roX2<sub>Δ</sub> /Y ; Top2<sup>17-3</sup>/In(2LR)Bc Gla males. Survival of adult progeny with reduced Top2 function (XX and XY roX<sub>1</sub><sup>ex40</sup> roX2<sub>Δ</sub>; Top2<sup>17-1</sup>/ Top2<sup>17-3</sup>) was compared to the survival of roX<sub>1</sub><sup>ex40</sup> roX2<sub>Δ</sub>; Top2<sup>m</sup>/In(2LR)Bc Gla flies. Recovery of roX<sub>1</sub><sup>ex40</sup> roX2<sub>Δ</sub>; Top2<sup>17-1</sup>/ Top2<sup>17-3</sup> adults was equivalent for both sexes (70% as compared to full viability, Fig. E1). The lack of male-preferential lethality when a roX1 roX2 chromosome is combined with Top2 reduction argues against these Top2 mutations influencing the male-limited process of dosage compensation.
Figure E1: No male-specific genetic interaction between Top2 and roX$^{ex40}$ roX2Δ was detected. Loss of Top2 reduces survival of roX$^{ex40}$ roX2Δ male (gray) and females (hatched) equivalently, suggesting a non-sex specific genetic interaction. Total adults recovered from five independent biological replicates were pooled to calculate adult survival. Total adults recovered were: roX$^{1\text{ex}40}$ roX2Δ; Top2$^{m}$/In(2LR), 1820; roX$^{1\text{ex}40}$ roX2Δ/Y; Top2$^{m}$/In(2LR), 1508; roX$^{1\text{ex}40}$ roX2Δ; Top2$^{17-1}$/Top2$^{17-3}$, 660; roX$^{1\text{ex}40}$ roX2Δ/Y; Top2$^{17-1}$/Top2$^{17-3}$, 545. The survival of males and females of genotype roX$^{1\text{ex}40}$ roX2Δ; Top2$^{m}$/In(2LR) was set to 100%.
APPENDIX F

Does chromosome pairing influence female-specific expression of LacZ in roX1mb710?

roX1mb710 was produced by an insertion of pLArB within the roX1 gene. pLArB is an “enhancer trap” that contains a LacZ gene that is sensitive to nearby enhancers (WILSON et al. 1989). The LacZ reporter in roX1mb710 is strongly expressed in a specific region of the brains of females called the mushroom body but nearly silent in the male mushroom body (Fig.F1). This is intriguing since P-element is inserted into a gene (roX1) that has strong male-preferential expression. What regulates the sex specific mushroom body expression of LacZ in roX1mb710 remains unknown.

![XX and XY images of mushroom bodies](image)

**Figure F1: Female specific LacZ expression in roX1mb710 mushroom bodies.**

The roX gene is on the X chromosome, and thus this gene will be paired in females and unpaired in males. It is possible that pairing of this locus is the signal that activates LacZ expression in roX1mb710. Several strategies were used to determine if chromosome pairing controls LacZ expression in this insertion.
1. An X chromosome balancer does not disrupt female-specific LacZ expression in mushroom bodies.

Female $\text{roX1}^{mb710}$ flies were mated to males carrying an X-chromosome balancer ($\text{FM7a}$ or $\text{Binsinscy}$) or a second chromosome balancer ($\text{CyOy}^+$). Since the balancer chromosomes contain multiple inversions they disrupt homolog pairing. We hypothesized that reduced pairing of the X chromosomes, or lack of pairing at the $\text{roX1}$ locus, might disrupt the female-specific LacZ expression in $\text{roX1}^{mb710}$. I performed X-Gal staining of larval brains from $\text{roX1}^{mb710}/\text{FM7a}$, $\text{roX1}^{mb710}/\text{Binsinscy}$ and $\text{roX1}^{mb710}/\text{CyOy}^+/+$ females. These showed comparable LacZ expression to the $\text{roX1}^{mb710}/+$ female brains. Male brains did not show LacZ staining.

Next, female $\text{roX1}^{mb710}$ flies were crossed to $\text{roX1}^{ex6}$ and $\text{roX1}^{ex33}$ males, which are deleted for a key element within $\text{roX1}$ called the DNAse hypersensitivity site. This site contains an MSL recruitment element (MRE), and is also thought to bind GAGA factor (GAF), a protein linked to long-range nuclear interactions, including pairing. I found that LacZ staining was equivalent in $\text{roX1}^{mb710}/\text{roX1}^{ex6}$ and $\text{roX1}^{mb710}/\text{roX1}^{ex33}$ female larval brains compared to $\text{roX1}^{mb710}$ female brains but no staining was visible in the male larval brains.

These results indicate that sex-specific LacZ expression is possibly regulated by some other signal that differentiates XX versus XY karyotype.
2. Role for Topoisomerase II as a regulator of female-specific LacZ expression in mushroom bodies.

A targeted genetic screen to identify potential regulators of heterochromatic sex identified topoisomerase II (Top2) (Chapter 4, Apte and Meller, In preparation). The rationale for testing Top2 was its known role in homolog pairing (WILLIAMS et al. 2007). Thus, it is possible that the mechanism by which the cell senses heterochromatic sex also works to regulate sex specificity of the LacZ expression in roX1mb710.

To determine if reduced Top2 activity influences the sex-specificity of the roX1mb710 mushroom body staining, first we tested a Top2Δ deletion generated by FLP-FRT recombination. Homozygous Top2Δ flies do not survive to adulthood but 3rd instar Top2Δ/Top2Δ larvae can be recovered. yw roX1mb710; Top2Δ/CyO y+ females were mated to roX1mb710/Y; Top2Δ/CyO y+ males and third instar y larvae were dissected and their CNS stained with X-Gal. While y+ female larvae showed mushroom body staining as expected, but brains from y females (Top2Δ/Top2Δ) did not stain, even upon prolonged incubation. Male larvae of either genotype did not show staining. This suggested that Top2 might play role in determining sex-specific LacZ expression pattern in roX1mb710, but further observations confounded this result. Brains dissected from Top2Δ/Top2Δ larvae were smaller than those from Top2Δ/CyO y+ larvae. Prolonged incubation in X-Gal staining buffer normally results in non-specific staining in few non-sex specific cells present near the mushroom body. This non-sex-specific staining was completely absent from Top2Δ/Top2Δ brains. This suggested that Top2 is
necessary for normal brain development, and perhaps mushroom body development. The loss of mushroom body staining in Top2Δ/Top2Δ larvae could thus be due to the absence of mushroom body. To test this, I generated Top2Δ/Top2Δ larvae carrying a UAS-LacZ and with strong, mushroom body-specific driver [GAL4-OK107] to drive expression of LacZ in both sexes. Male and female homozygous Top2Δ larvae carrying UAS-LacZ and GAL4-OK107 were identified by the absence of y+ expression in mouth hooks as compared to the Top2Δ/CyOy+ larvae with UAS-LacZ and GAL4-OK107. While y+ larvae were abundant, very few and skinny y− larvae were isolated. X-gal staining of y+ larval brains showed β-gal expression within 5-10 min while even after 30 min incubation y− larval brains did not show any mushroom body specific X-Gal staining. This study further provided support for the idea that loss of Top2 is affecting normal brain and especially mushroom body development. At this point, we concluded that Top2Δ is not an ideal allele to test role of Top2 in the regulation of mushroom body staining.

I then tested the Top217-1/ Top217-3 heteroallelic combination. The 50% decrease in adult viability suggested reduced function of Top2 in this mutant. roX1mb710; Top217-1/In(2)LR females were crossed to roX1mb710; Top217-3/In(2)LR males and third instar larvae were collected. All the larvae lacking In(2)LR balancer are Top217-1/ Top217-3. All larvae carrying In(2)LR (Top2m/ In(2)LR) will have a wild type copy of Top2. Brains dissected from both male and female Top217-1/ Top217-3 larvae were comparable in size to the control Top2m/ In(2)LR larvae, suggesting no gross abnormalities in brain development. X-Gal staining of
female roX1mb710; Top217-1/ Top217-3 larvae compared to the control roX1mb710; Top217-1 or 3/ In(2)LR was interesting. Compared to controls, the Top217-1/ Top217-3 larvae showed less initial X-Gal staining (Fig. F2, compare intensity of X-Gal staining at 1 hr and 2.5 hr in Top2m/ In(2LR) and Top217-1/ Top217-3 brains. The difference in staining was only apparent during the first few hours. Overnight incubation produced similar patterns of non-specific β-gal expression in Top2m/ In(2LR) and Top217-1/ Top217-3 brains. Male larval brains did not show LacZ expression upon loss of Top2. This suggested that the Top2 function required for mushroom body development is intact in the Top217-1/ Top217-3 mutant. The difference in LacZ expression in Top2m/ In(2LR) and Top217-1/ Top217-3 females suggests a possible role for Top2 in regulating sex-specific LacZ expression.

3. Deletion of the X-linked Zygotic hybrid rescue (Zhr)

Zygotic hybrid rescue (Zhr) is an unusual genetic element situated at the base of the X chromosome. It is composed of ~10Mb of 1.688 g/cm³ satellite repeats (359 bp repeats) that compose most of the pericentric heterochromatin of the X (SAWAMURA AND YAMAMOTO 1993, FERREE AND BARBASH 2009). Mutations in Zhr rescue XX hybrid lethality in mating between D. melanogaster males and D. simulans females (SAWAMURA, YAMAMOTO AND WATANABE, 1993). The Zhr1 mutation, deleted for almost all pericentromeric 359 bp repeats, was produced by an X:Y translocation that joins X euchromatin to the Y chromosome centromere. I found that a single copy of Zhr1 masculinized XX heterochromatin, suggesting a role in karyotype sensing. To determine if the
female-specific mushroom body staining of \( \text{ro}X1^{mb710} \), was also disrupted, I dissected and X-Gal stained brains from \( \text{ro}X1^{mb710} + + / \text{ro}X1^{ex33} \text{ro}X2 \Delta \text{Zhr}^1 \) female larvae. \( \text{ro}X1^{mb710} / + \) female brains were used as a positive control for LacZ staining. Similar levels of X-Gal staining were visible in female brains of both genotypes. As expected, \( \text{ro}X1^{mb710} / \text{Y} \) male brains did not show any LacZ expression.
Figure F2: Top2 modulates sex-specific LacZ expression in \textit{roX1}^{mb710}.

Time-lapse images of the male (XY) and female (XX) brains stained with X-Gal are shown. \textit{roX1}^{mb710} \textit{Top2}^{17-1} / \textit{Top2}^{17-3} female brains showed less initial staining than \textit{roX1}^{mb710}; \textit{Top2}^{m} / \textit{In(2)LR} females at 1hr and 2.5 hr. This difference was obscured by increased incubation time (>10 hr). The experiment was performed 5 times with \~10-15 brains in each class. Difference in the X-Gal staining pattern was consistently observed in all the replicates.
Figure F3: Loss of one copy of Zhr does not influence LacZ expression in \(roX1^{mb710}\) females.
Time-lapse images of male (XY) and female (XX) brains stained with X-Gal. \(roX1^{mb710}+ + \) / \(roX1^{ex33}roX2\Delta Zhr^i\) and \(roX1^{mb710}+ + + + +\) female brains showed comparable staining at 1 and 2 hr. The experiment was performed 2 times with ~5-10 brains of each genotype.
APPENDIX G

Primer design for allele specific PCR and List of primers

Allele specific PCR relies on specific PCR product generation by using a forward primer with 3’ nucleotide matching the point mutation and a mismatched nucleotide at third to last position in the 3’ end of primer sequence (BUI AND LIU, 2009). I generated several mutant specific forward primers and tested them to validate point mutations in Top2 and Cap-H2 used throughout the screening to identify the genetic regulator of sex-specificity of heterochromatin gene regulation (Table G1). All forward primers are designed to indicated mutations (ex., Top 35.1.1 and Top235.1.2). Primers with a single 3’ base matching a mutant are designated by suffix “m F or mut F”. Those with an additional mismatch at the third base from the 3’ end have suffix “ASm F or ASmut F”. Bases that mismatch wild type sequence are bold.

Table G1:

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<th>Sequence</th>
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<tr>
<td>Cap-H2-19 R4</td>
<td>ACG TTT CCG TGG TTC GTC TGC</td>
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Note: Cap-H2 primers need further PCR standardization. They do not give reliable PCR products using TDVM PCR program.
Mutant specific PCR primers were also generated to confirm mutations in the alleles of Cap-D3, MCPH1 and fs(1)h. 3’ base matching to the mutant allele in all the primers is designated in bold (Table G2).

**Table G2:**

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APPENDIX H

Measurement of heterochromatic gene expression by Quantitative RT–PCR

Heterochromatic gene expression analysis and position effect variegation (PEV) assay are distinct but linked matrices for the integrity of heterochromatin silencing. Disruption in the heterochromatin integrity not only results in suppression of PEV but also negatively affects native heterochromatic gene expression resulting in down-regulation. Our PEV reporter assay revealed that XX flies mutated for Top2 display masculinized heterochromatin (Chapter 4) but we were also curious about the expression of native heterochromatic genes in females mutated for Top2 and roX. Our prediction is that heterochromatic genes will decrease in expression in these flies, as they do in roX mutant males relative to wild type flies. However, we also predict that heterochromatic genes should not decrease in expression in XX flies mutated only for roX or Top2 alone.

To determine if roX1 roX2 females with masculinized heterochromatin also show reduced heterochromatic gene expression, quantitative reverse transcription PCR (qRT-PCR) was used to measure gene expression in the appropriate genotypes. Total RNA was prepared from two groups of at least 50 larvae per genotype. One microgram of total RNA was reverse transcribed using random hexamers and ImProm-II reverse transcriptase (Promega). Quantitative PCR was performed as previously described (DENG et al. 2005). A total of 5 genes were selected from four different gene groups (2nd and 3rd chromosome heterochromatic, 4th chromosome, and X-linked). dmn, an autosomal
euchromatic gene, was used for normalization. All primers and primer efficiencies are presented in Table H1. Wild type control (yw) and roX1<sup>ex6</sup> roX2Δ male larvae served as controls for full and reduced expression of X-linked and heterochromatic genes (DENG et al. 2009). As expected, roX1<sup>ex6</sup> roX2Δ male larvae showed down regulation of X-linked and autosomal heterochromatic genes (Fig. H1 A, green bars). We also tested gene expression in XX flies that were mutated for Top2, mutated for roX1 roX2 or mutated for both. Top2 mutant females showed down-regulation of X-linked as well as heterochromatic genes, an observation possibly attributable to the multi-functionality of Top2 within the cell (Fig. H1 B, pink bars). Large variability in the gene expression profile was observed for roX1<sup>ex6</sup> roX2Δ females tested for X-linked or autosomal heterochromatic genes (Fig.H1 B, green bars). Importantly, roX1<sup>ex6</sup>roX2Δ, Top2<sup>17-1</sup>/Top2<sup>17-3</sup> females showed a trend towards down regulation of X-linked as well as autosomal heterochromatic genes tested (Fig. H1 B, purple bars). It appears that it will be challenging to obtain significant data using this particular method. At present four heterochromatic genes have been examined, but the large number of genotypes (6) that need to be tested in parallel makes it particularly challenging to expand the number of genes tested. Nonetheless, this preliminary finding suggests that reduced expression of heterochromatic genes might occur in roX1<sup>ex6</sup>roX2Δ, Top2<sup>17-1</sup>/Top2<sup>17-3</sup> females. During data analysis we noted that the normalizing genes themselves might be responding to genotype. This underscores the need to identify better normalizing genes. In addition we expect the changes in heterochromatic gene expression to be very slight. This
will make changes difficult to detect by qRT PCR examination of a handful of genes. Microarray or RNA sequencing expression studies would better address both the problems with normalization and sample size. One limitation here is that the large number of genotypes makes an adequately replicated study of this type prohibitively expensive at the present time.

Table H1: Primers used for real-time PCR

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<th>Primer Name</th>
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<td>2&amp;3 Eu</td>
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**Figure H1: Measurement of heterochromatic gene expression in females with masculinized heterochromatin.**

Expression of 4 autosomal heterochromatic genes (Eph, Rad23, CG40439 and MED21) as well as one X-linked gene (SkpA) was measured in males (A) and females (B) using quantitative RT-PCR. Male larvae are control (yw; blue) and roX1ex6roX2Δ (green). Female larvae are control (yw; blue), Top2^{17-1}/Top2^{17-3} (pink), roX1^{ex6}roX2Δ (green) and roX1^{ex6}roX2Δ;Top2^{17-1}/Top2^{17-3} (purple). Expression was normalized to the autosomal gene dmn. Error bars represent the standard error of two biological replicates for each genotype.
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ABSTRACT

SEX AND HETEROCHROMATIN: AN INVESTIGATION OF SEXUAL DIMORPHISM IN DROSOPHILA MELANOGASTER

by

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Over 30% of Drosophila genome is assembled into heterochromatin. Heterochromatin is relatively gene poor, transcriptionally less active and remains condensed during interphase. Previous studies established that roX RNA and some of the Male Specific Lethal (MSL) proteins, all components of the dosage compensation complex, are required for full expression of autosomal heterochromatic genes in male flies but not in females. This was surprising since heterochromatin is generally not thought to be sexually dimorphic. The genetic basis for the regulation of sex-specific heterochromatin was completely unknown.

To determine if roX RNAs localize directly at the heterochromatic regions that they regulate, I generated an MS2-tagged roX1 allele (roX1<sup>MS2-6</sup>) using a novel gene engineering technique named ‘Targeted Gene Conversion’ (TGC). roX1<sup>MS2-6</sup> was used to visualize in vivo roX1 localization in early Drosophila embryos, but subnuclear localization was only detectable on the X chromosome of males after the onset of dosage compensation (3hr AEL).
I then performed genetic screens to determine the signal that dictates differentiation of male and female heterochromatin. I hypothesized that either the sex determination pathway, or direct karyotype sensing, could act as a signal. To determine the signal, I conducted targeted genetic screens using a reporter that responds differently to the loss of roX RNAs in males and females. I found that heterochromatic sex is independent of the female-specific components of the somatic sex determination pathway, as well as the male-limited Y-chromosome and MSL2, a dosage compensation protein that is only present in males. I then explored the possibility that direct sensing of sex chromosome karyotype bypasses the somatic sex determination pathway to determine heterochromatic sex. Examination of various chromatin regulators with known functions in homolog pairing identified Topoisomerase II (Top2) as an essential factor for feminization of XX heterochromatin. Intriguingly, Top2 also binds to a large block of satellite repeats present exclusively on the X chromosome (359bp repeats). I then discovered that deletion of X heterochromatin, which removes one copy of these satellite repeats, masculinizes heterochromatin in XX flies. Simultaneous loss of Top2 and deletion of X heterochromatin enhances masculinization of XX heterochromatin, but has no effect on somatic sexual differentiation. I postulate that the X-exclusive 359 bp heterochromatic satellite repeats and Top2 act together as a mechanism of direct karyotype sensing. This in turn regulates heterochromatin differentiation independent of all known sex determination pathways. My studies thus reveal a novel sex determination signal in Drosophila melanogaster that links fly karyotype to one aspect of sexual differentiation.
AUTOBIOGRAPHICAL STATEMENT

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