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Investigation Of X Chromosome Recognition: The Role Of Small Rna In Drosophila Dosage Compensation

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INVESTIGATION OF X CHROMOSOME RECOGNITION: THE ROLE OF SMALL RNA IN DROSOPHILA DOSAGE COMPENSATION

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DEDICATION

I would like to dedicate this thesis to my Ph.D advisor Dr. Victoria H. Meller, my parents P.unnikrishnan and Rajalakshmi, my aunt Padmaja G. Menon, my wife Shalini Menon and all my teachers. This work would not have been possible without the moral, intellectual and financial support from my advisor. I would like to thank my family for all the care, support, patience and motivation to be a better person. I would especially like to thank my wife for being my best friend and strongest source of comfort and support. I shall be eternally grateful for the valuable service of my teachers whose efforts have inspired and influenced my decision to pursue a career in academia and research.

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Chapter 1

Introduction

In many organisms the X chromosome is gene rich, while the Y chromosome is gene poor or absent all together. This unequal number of X-linked genes between females (XX) and males (XY) results in a potentially lethal imbalance in the X chromosome to autosomal (X:A) gene expression. This imbalance is corrected by a process termed dosage compensation. Different species have evolved diverse strategies to achieve dosage compensation. In mammals a single female X chromosome is inactivated, whereas in the nematode *Caenorhabditis elegans* gene expression from each hermaphrodite X chromosome is reduced by half. By contrast, in the fruit fly *Drosophila melanogaster*, gene expression from the male X chromosome is up regulated two-fold. Although these three strategies differ, the proper execution of each entails the selective recognition and regulation of gene activity from X chromosomes.

Dosage compensation of the *Drosophila* X chromosome

The hyper transcription of X-linked genes in *Drosophila* males is accomplished by the Male Specific Lethal (MSL) complex. The MSL complex consists of five proteins and two non-coding RNAs that are redundant in function. The proteins include the Male-Specific Lethal 1, 2 and 3 (MSL1, MSL2 and MSL3), Maleless (MLE) and Males absent on first (MOF), while the RNAs are RNA on X (*roX1* and *roX2*) (GELBART and KURODA 2009). Analyses of RNA

polymerase II distribution along dosage compensated genes suggest that the MSL complex increases transcriptional output through enhancing RNA polymerase II initiation and transcriptional elongation (LARSCHAN *et al.* 2011; CONRAD *et al.* 2012). MOF is a histone acetyl transferase (HILFIKER *et al.* 1997). The MSL complex targets H4K16 acetlyation to X-linked genes (SMITH *et al.* 2000). This modification is an epigenetic mark usually associated with transcriptionally active chromatin and is likely responsible for increased transcriptional output (SMITH *et al.* 2001; LARSCHAN *et al.* 2011). Critical to this process is the proper recruitment of the MSL complex to the X chromosome by the non-coding *roX1* and *roX2* (RNA on X) RNAs (DENG *et al.* 2005).

Mechanism of X chromosome recognition

A two-step model has been proposed to describe MSL complex binding and spreading on the X chromosome. Following the assembly of the MSL complex, which may occur either co-transcriptionally at the site of *roX* transcription or in the nucleoplasm, the complex binds an estimated 150 high affinity sites, also known as Chromatin Entry Sites (CES) distributed along the X chromosome (KELLEY *et al.* 1999; STRAUB *et al.* 2008). CES on the X chromosome are enriched for 21 bp GA rich motifs termed MSL Recognition Elements (MRE) that mediate MSL binding (ALEKSEYENKO *et al.* 2008). Other motifs found near MSL-bound regions, such as $[G(CG)N]_4$, were identified computationally and are predicted to affect X recognition (GALLACH *et al.* 2010). From the CES the MSL complex spreads to flanking chromatin by recognizing

features of transcriptionally active genes, such as the co-transcriptional H3K36 tri-methyl modification enriched in the body and 3' ends of transcribed genes (LARSCHAN *et al.* 2007). In this two-step model, MSL1 and MSL2 are essential for complex assembly and binding to CES, while MSL3 and MLE regulate spreading (GELBART and KURODA 2009). In addition to the MSL proteins other *trans*-acting factors that facilitate MSL recruitment to dosage compensated genes have been identified. These include a zinc finger protein, CG1832 which binds to CES and MRE sites though out the genome, the Non-Specific Lethal1 (NSL1) complex, that contains MOF and produces H4K16 acetlyation, a H3K36 trimethyl-binding protein, CG4747 and Nup153 and Megator, components of the nuclear pore that define transcriptionally active regions (MENDJAN *et al.* 2006; VAQUERIZAS *et al.* 2010; LARSCHAN *et al.* 2012; WANG *et al.* 2013).

The current model adequately describes the mechanism by which the MSL complex binds transcribed genes, but fails to explain selective recognition of the X chromosome. For example, while MREs are two-fold enriched on the X chromosome, they are also present on autosomes. Hence the MRE sequence motif by itself does not define a CES. In fact, other features such as the chromatin environment flanking the MREs are proposed to influence MSL binding. Functional MREs have been observed to be associated with active chromatin (ALEKSEYENKO *et al.* 2012). Therefore it is highly probable that additional mechanisms involving either accessory factors or *cis*-acting elements regulate X chromosome recognition.

Identifying modifiers of X chromosome recognition

The simultaneous mutation of both the *roX* RNAs reduces the amount of MSL complex bound to the X chromosome and increases ectopic autosomal binding. This defect in X chromosome recognition results in reduced survival of adult males and reduced X-linked gene expression (MELLER and RATTNER 2002; DENG and MELLER 2006b). The central role of the *roX* transcripts in correct MSL localization suggests that mutations that enhance or suppress *roX1* roX2 male lethality may influence X recognition. I used this strategy to identify and characterize novel factors that play a role in dosage compensation. I first demonstrated that the Y chromosome influences dosage compensation. This study comprises Chapter 3 (MENON and MELLER 2009). Surprisingly, germ line imprinting of the Y chromosome is key to its effect on compensation. Chapter 2 is a review that highlights the role of germ line imprinting in genome regulation in *Drosophila* (MENON and MELLER 2010).

In an effort to determine the mechanism by which the Y chromosome influences dosage compensation, I investigated the possibility that small RNA pathways might be involved and I discovered that the siRNA pathway contributes to X chromosome recognition. This study comprises Chapter 4 (MENON and MELLER 2012).

I postulated that sequences unique to the X chromosome might produce siRNA that act through the siRNA pathway and contribute to X recognition. In Chapter 5 I investigate the role of X-linked euchromatic satellite repeats (1.688^{X}) in X chromosome recognition. The ectopic expression of 1.688^{X} repeat siRNA

rescues *roX1 roX2* male survival and MSL localization to the X chromosome, while the expression of long single stranded RNAs from 1.688^{X} repeats reduces *roX1 roX2* male survival. This is the first study to reveal a function for the X-linked euchromatic 1.688^{X} repeats. The striking limitation of 1.688^{X} repeats to the X chromosome suggests that these repeats might serve as *cis*-acting X-identity elements. My findings suggest a role for a siRNA mediated targeting of X-specific repeats in regulating X chromosome recognition.

In Chapter 6 I explore unanswered questions that my work has raised, providing perspective for future studies.

Chapter 2

Germ line imprinting in *Drosophila*: Epigenetics in search of function

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Getting in the last word

Germ line imprinting is often viewed as the parting gift of a meddling parent. Unable to cede control of genetic material, conditions are placed on its use. These instructions come in the form of epigenetic marks that are deposited on chromosomes in the germ line. Allele-specific regulation of individual genes, or differences in the expression or transmission of entire chromosomes, is the result. Because the sex of the parent determines the presence of these marks, imprinting creates functional differences between the maternally and paternally derived copies of the genome. Imprinting was first described in insects, but has subsequently been observed in a wide range of plants and animals (DE LA CASA-ESPERON and SAPIENZA 2003). Imprinted marks in mammals, plants and many insects are necessary for developmentally important processes. While germ line imprinting occurs in flies, a clear understanding of the biological significance of imprinting in *Drosophila* is still lacking.

Imprinted effects in a wide range of organisms include the heterochromatinization or elimination of chromosomes, transcriptional silencing

of a single allele and epigenetic memory. The importance of parental imprints for mammalian embryonic development is illustrated by lethality, and aberrant growth patterns, of diploid androgenetic or gynogenetic zygotes created by pronuclear transfer (SURANI *et al.* 1986). In mammals, imprints regulate gene expression. Transcriptional silencing of one allele, resulting in monoallelic expression, is characteristic of these marks. About 100 mammalian genes are imprinted, and these are clustered around Imprint Control Regions (ICRs) that coordinate the imprinted status of nearby genes.(VERONA *et al.* 2003) Many of these imprints influence genes that regulate embryo and placenta size or developmental processes (COAN *et al.* 2005). For example, methylation at the promoter silences the paternal allele of *Igf2r*, a scavenger receptor for *Igf2* (*Insulin like growth factor 2*) (BARTOLOMEI 2009). Reduction in *Igf2r* increases the concentration of circulating *Igf2*, thus promoting growth (LUDWIG *et al.* 1996). Imprinting of *Igf2r* follows a pattern in which paternal imprints tend to increase embryo size but maternal ones limit growth (WILKINS and HAIG 2003). This has lead to the "parental conflict" hypothesis, which posits that imprinted marks are the means by which parents fight over allocation of resources for their offspring (MOORE and HAIG 1991). An extreme example of clustered imprinted genes is the mammalian X chromosome. The paternal X chromosome is silenced in marsupials and in extraembryonic tissues of rodents (SADO and FERGUSON-SMITH 2005). Silencing of the paternal X chromosome expediently achieves equalization of X-linked gene dosage, known as dosage compensation, between males and females. In the inner cell mass of placental mammals the imprint is

erased, enabling random X inactivation. While the imprinted mark regulates an entire chromosome, the imprint itself need only control the *X inactivation center* (*Xic*), a locus on the X chromosome that directs inactivation. Through regulation of growth, development and X chromosome silencing, mammalian imprints direct multiple essential processes during early embryogenesis. Compelling arguments for the adaptive value of imprinting have consequently focused on its central role in mammalian development (WILKINS and HAIG 2003).

While the ramifications of imprinting are best understood in mammals, imprinting itself was first described in *Sciarid* flies (CROUSE 1960). In contrast to the gene expression effects observed in mammals and plants, imprinting in insects often controls the behavior of entire chromosomes (LLOYD *et al.* 1999; GODAY and ESTEBAN 2001; MAGGERT and GOLIC 2002). For example, in *Sciara*, imprinting directs heterochromatinization of the paternal X chromosome and its elimination in the germ line and soma (CROUSE 1960; GODAY and ESTEBAN 2001). This elimination is attributable to a single controlling element near the centromere, now referred to as the imprinting control region (ICR) (CROUSE 1960). The ICR is a common feature of many imprinted loci, including *Xic* in mammals. Other examples of insect imprinting involve silencing of the entire paternal genome of male mealybugs by heterochromatinization (BONGIORNI *et al.* 2001). In these scale insects, females are diploid and males are pseudohaploid, meaning that somatic cells are functionally haploid as a consequence of silencing. Imprinting thus plays essential roles in sex determination and meiosis in insects. Several examples of imprinting have been documented in *Drosophila*,

but the manifestations of imprinting in flies are markedly different from those in the organisms described above.

Several methods of detection reveal imprinting in *Drosophila*

As in other insects, *Drosophila* imprints are detected through their effects on heterochromatin. Imprints in flies can affect entire chromosomes, but they are usually detected by silencing of a euchromatic reporter that has been moved near heterochromatin by transposition or chromosome rearrangement. This silencing, termed position effect variegation (PEV), produces patches of tissue in which spreading heterochromatin has silenced the reporter. Structurally normal chromosomes presumably have insulators that prevent the spread of heterochromatin into euchromatic regions. While most instances of PEV are not affected by imprinting, in a few the parent of origin dramatically influences the amount of silencing. Examples include the expression of variegating genes on the rearranged *Dp(w^m)264.58a* and *Dp(1;f)LJ9* (mini-X) chromosomes (SPOFFORD 1961; COHEN 1962; LLOYD 2000). *Dp(1;f)LJ9* has been used extensively to explore the mechanism of imprinting in flies. It was created by complex rearrangements that delete most of the X euchromatin and move a group of euchromatic genes, including *garnet* (g^+), close to proximal heterochromatin (chromosome model, Fig. 2.1A) (HARDY *et al.* 1984). As *Dp(1;f)LJ9* is a free duplication of part of the X chromosome, it can be transmitted from either parent. Maternal transmission results in uniform expression of g^+, producing solid red eyes. Transmission from the father

produces orange patches in which g^+ has been silenced. PEV is thus observed only upon paternal transmission.

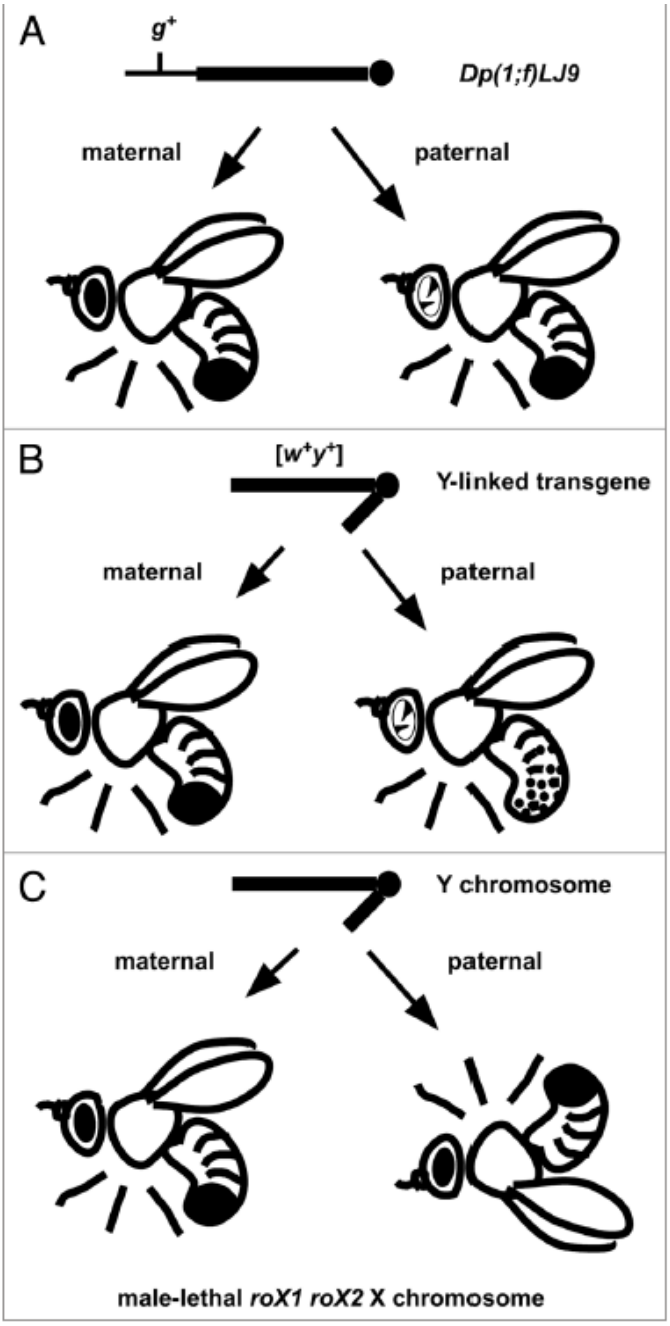

Figure 2.1 Detection of *Drosophila* imprints. A) Imprinting of the *Dp(1;f)L J9* (mini–X chromosome). *Dp(1;f)L J9* is a complex rearrangement that moves *garnet* (g^+) close to proximal heterochromatin of the X chromosome (thick black line). Imprinting is detected through the expression of g^+, which confers dark (wild type) eye color. Maternal inheritance of *Dp(1;f)L J9* produces solid, dark eye color, but paternal transmission results in variegated pigmentation due to silencing of g^+ by neighboring heterochromatin. B) Imprinted transgene insertions on the Y chromosome. Y-linked insertions carrying y^+ and w^+ markers are subject to imprinting.(MAGGERT and GOLIC 2002) Both markers are typically expressed at higher levels when the Y chromosome is inherited maternally. This is illustrated by strong expression of the w^+ and y^+ in the eye and abdomen, respectively (left). Greater variegation is observed when these chromosomes are transmitted from the father, illustrated by patchy expression in the eye and abdomen (right). C) Imprinted Y chromosomes influence dosage compensation. Simultaneous mutation of *roX1* and *roX2* is male-lethal due to disruption of X chromosome dosage compensation (right). A maternal Y chromosome is a potent suppressor of *roX1 roX2* lethality and enables recovery of adult escapers (left) (MENON and MELLER 2009).

Transgene insertions on the heterochromatic Y chromosome are also subject to PEV. Unlike the situation in mammals, the fly Y chromosome does not determine sex, and the known functions of Y-linked genes are limited to spermatogenesis. The Y chromosome of *Drosophila* can thus be transmitted through females. Y-linked insertions typically display greater expression when transmitted through a female (GOLIC *et al.* 1998; HALLER and WOODRUFF 2000a; MAGGERT and GOLIC 2002). This is illustrated by the more uniform expression of the *mini-white* (w^{+mW}) and *yellow* (y^{+}) markers on maternally transmitted Y-linked insertions (Fig. 2.1B).

Other parent-of-origin effects mediated by epigenetic marks deposited in the parental germ lines have also been noted. These include loss of paternal chromosomes in *pal* mutant progeny and defects caused by the *Uab1* inversion of the bithorax complex (BAKER 1975; KUHN and PACKERT 1988). The chromatin structure of the bithorax complex is organized into repressed and active chromatin domains. Imprinting of the *Uab1* inversion may reflect changes in the chromatin organization at this locus, perhaps analogous to the effect of imprinting on PEV.

An interesting imprinting-like effect on the Y chromosome is observed in mutants of *E(var)3-93D,* also known as *mod(mdg4)*. *mod(mdg4)* was one of the first enhancers of PEV identified and is required to maintain an open chromatin conformation (DORN *et al.* 1993; BUCHNER *et al.* 2000). The variegation of eye color in w^{m4h} flies, in which w^{+} has been moved near heterochromatin by inversion, is enhanced in *mod(mdg4)* mutants. A Y chromosome transmitted

through a *mod(mdg4)* male also enhances variegation of the w^{m4h} allele (DORN *et al.* 1993). However, the effect of the Y chromosome is maintained for many generations, even when transmitted through wild type flies. Because this appears to be a permanent change in the Y chromosome, it does not meet the definition of germ line imprints, which are reset every generation as they pass through the germ line.

Surprisingly, imprinting of the Y chromosome can also influence X chromosome dosage compensation (MENON and MELLER 2009). A two-fold increase in expression from the male X chromosome is required to equalize X-linked gene expression between males and females. Two non-coding *roX* RNAs (*roX1* and *roX2*) are components of a ribonucleoprotein complex that achieves this by binding to the X chromosome and modifying chromatin (DENG and MELLER 2006a). The *roX* RNAs are required for recognition of X chromatin.(MELLER and RATTNER 2002; KELLEY *et al.* 2008; PARK *et al.* 2008) Simultaneous mutation of *roX1* and *roX2* leads to reduced X-linked gene expression and low male viability (DENG and MELLER 2006b). Although the Y chromosome has no effect on dosage compensation in otherwise wild type flies, a maternally imprinted Y chromosome dramatically suppresses the lethality of *roX1 roX2* males (Fig. 2.1C) (MENON and MELLER 2009). The mechanism by which this occurs remains under investigation, but expression of X-linked genes is modestly increased in *roX1 roX2* males with a maternal Y chromosome. Male rescue is presumably due to this increase in expression. The Y chromosome imprint is reset each generation, and thus is a true germ line imprint (Menon, unpublished).

How do flies imprint?

The process of establishing and maintaining imprints in flies remains mysterious. In contrast, well-studied mammalian examples reveal that imprint establishment and maintenance each rely on DNA methylation. Mammalian germ line imprints consist of specific methylation patterns established in the gametes by DNMT3A (DNA methyl transferase) and DNMT3L, a non-catalytic co-factor (BOURC'HIS *et al.* 2001; HATA *et al.* 2002; KANEDA *et al.* 2004). After fertilization, these allele-specific patterns are propagated in the soma by the maintenance methyltransferase DNMT1 (HOWELL *et al.* 2001; HIRASAWA *et al.* 2008). During development imprinted marks guide establishment of chromatin organization that reflects the imprinted status of each allele. The best example of this is the *H19/Igf2* locus. Paternal methylation of an Imprint Control Region (ICR) upstream of the H19 gene prevents binding of CTCF (CCCTC binding factor) (FEDORIW *et al.* 2004). The unmethylated maternal ICR is still able to bind CTCF. CTCF binding insulates the upstream *Igf2* gene from enhancers and promotes H19 expression. Recent studies have shown that CTCF binding to the ICR promotes chromosome looping, which contributes to insulation and repression of *Igf2* (KURUKUTI *et al.* 2006; LI *et al.* 2008). Mammalian imprinting centers, characterized by DNA repeats and hypoacetylation, regulate genes clustered within ~ 1Mb (FEIL and KHOSLA 1999; BARTOLOMEI 2009). The clustering of genes affected by imprinting, and potential involvement of repetitive DNA sequence, are features shared with imprinted regions in flies.

In contrast to mammals and plants, the role of DNA methylation in *Drosophila*, and even its existence, remains controversial. Low levels of methylated DNA have been reported during early embryogenesis (KUNERT *et al.* 2003). A subsequent study raised doubts about the existence of methylated DNA in flies (GOLL *et al.* 2006). However, a recent report of DNA methylation restricted to the Long Terminal Repeats (LTRs) of mobile elements supports the presence of limited DNA methylation during early embryogenesis (PHALKE *et al.* 2009). Intriguingly, a role for DNA methylation in retrotransposon silencing and telomere stability was identified by this study. Taken together, these studies suggest a role for DNA methylation in *Drosophila* epigenetic processes. While DNA methylation could be limited to the control of mobile elements, a role in the interpretation of germ line imprints is also possible. A function for DNA methylation in fly imprinting has yet to be tested.

Variegating rearrangements, such as the *Dp(1;f)LJ9* mini-X chromosome, have been used to gather most of the information about imprinting in flies. Imprints appear to reside in heterochromatin of rearranged chromosomes, and genes closest to heterochromatin show the maximum imprinted effect (LLOYD *et al.* 1999; LLOYD 2000; ANAKA *et al.* 2009). Establishment and maintenance of fly imprints are separately regulated. While the establishment of imprints remains mysterious, factors that influence heterochromatin formation have been shown to affect the maintenance of the imprint. Loss of heterochromatic proteins like HP1 and *Su(var)3-9* (H3K9 methyl transferase) suppress expression of the paternal imprint, while mutation of *trithorax* (*trx)* and *Brahma (brm),* proteins that activate

gene expression, suppresses the maternal imprint (JOANIS and LLOYD 2002). Cytological studies have shown that when paternally transmitted, *Dp(1;f)LJ9* undergoes less endoreplication in the salivary gland and has more uniformly compact chromatin than when it is maternally transmitted (ANAKA *et al.* 2009). Taken together, these studies reveal that fly imprints are capable of exerting long-range effects on gene expression, chromosome replication and chromatin structure that are maintained throughout the life of the organism. Surprisingly, given the fact that imprints appear to be placed in heterochromatic regions, factors known to influence heterochromatin do not appear to affect establishment of imprints (LLOYD *et al.* 1999; JOANIS and LLOYD 2002).

Although the nature of the imprint itself remains unknown, it is possible that imprints are placed by transient signals that influence heterochromatin. Maintenance of heterochromatin could then perpetuate the imprinted state throughout the life of the animal. While heterochromatic imprinting is characteristic of flies, a recent study in mice revealed that the establishment of pericentric heterochromatin depends on the parent of origin (PUSCHENDORF *et al.* 2008). Sperm DNA is compacted with protamines. Following fertilization, protamines are removed and the male pronucleus is assembled with maternal proteins. In the zygote, heterochromatin of the maternal genome is enriched for H3K9me3, a mark made by *Suv39h*, and HP1, which binds H3K9me3. Paternal heterochromatin lacks this signature, and instead is enriched for H3K27me3, a mark deposited by the Polycomb-repressive complex 2 (PRC2). This mark recruits the PRC1 complex, necessary for inhibition of transcriptional activation.

Formation of paternal heterochromatin requires the maternal PRC1 complex, and, in its absence, transcription of paternal satellite repeats is derepressed. Asymmetry in heterochromatin establishment has the potential to be a general imprinting mechanism, employed by any organism that restructures a male pronucleus. Imprinting of heterochromatic regions thus could be more widespread, and evolutionarily older, than previously thought.

Given the importance of heterochromatin for fly imprinting, understanding heterochromatin formation is essential. RNAi was first shown to regulate heterochromatin formation in the fission yeast, *Schizosaccharomyces* pombe (VOLPE *et al.* 2002). Mutations affecting RNAi also disrupt heterochromatin formation in *Drosophila* (PAL-BHADRA *et al.* 2004). Multiple RNAi pathways have been shown to regulate heterochromatin formation in the soma and germ line (PAL-BHADRA *et al.* 2004; GRIMAUD *et al.* 2006; BROWER-TOLAND *et al.* 2007; USAKIN *et al.* 2007). Transcripts from repetitive regions are processed into siRNA, which in turn direct silencing chromatin marks to these regions (MOAZED 2009). The role of RNAi in initiation of heterochromatin formation makes it a likely candidate for involvement in imprinting.

Insulators, such as CTCF, that establish higher order chromatin structure by regulating looping and position within the nucleus, are also candidates for a role in imprinting (PHILLIPS and CORCES 2009). Insulators act as barriers, preventing heterochromatin spreading and blocking promoter-enhancer interactions in mammals and *Drosophila* (FEDORIW *et al.* 2004; MOHAN *et al.* 2007). This contributes to CTCF function in imprinting of *Igf2/H19,* and in

organization of chromatin domains in the fly Bithorax complex (KURUKUTI *et al.* 2006; MOHAN *et al.* 2007). It is possible that proteins with insulator function in flies will also affect imprinting. *Drosophila* has several insulator proteins, including CTCF and *SU(Hw)*, which binds to *gypsy* elements and influences looping and nuclear localization (BYRD and CORCES 2003; BUSHEY *et al.* 2009). Despite the fact that these two insulators bind distinct sequences, CTCF and SU(HW) co-localize to insulator bodies, complex nuclear structures that anchor loops to organize multiple, large chromatin domains (GERASIMOVA *et al.* 2007). The ability of insulators to control large chromatin domains, and the central role of CTCF in mammalian imprinting, makes these proteins attractive candidates for a role in establishment or interpretation of imprints in *Drosophila*.

Why do flies imprint?

The presence of germ line imprinting in *Drosophila* is intriguing, but the biological function of these imprints remains mysterious. Studies of imprinting in several other organisms have lead to an understanding of the role of imprinting in these species. Because failure of imprinting in mammals causes a wide range of developmental defects, we now understand the importance of monoallelic expression of imprinted genes for early mammalian development. Imprinting in *Sciara* and scale insects guides the behavior of entire chromosomes, playing a vital role in meiosis and sexual differentiation. In *Drosophila,* imprints are detected by alteration in expression of genes on rearranged chromosomes, but there is little to suggest that expression of any gene in karyotypically normally flies is governed by imprinting. Indeed, genome-wide expression analysis of

progeny from reciprocal crosses of inbred strains suggests that gene expression differences that appear to depend on the parent of origin do not arise from monoallelic expression, but are more likely due to maternal or paternal effects (WITTKOPP *et al.* 2006).

A compelling argument for the origins of imprinting has been made by de La Casa-Esperon and Sapienza (DE LA CASA-ESPERON and SAPIENZA 2003). These authors suggest that imprinting serves to identify homologous chromosomes and sister chromatids, a distinction important during DNA repair and meiotic recombination. Unscheduled double stranded breaks may be fixed by gap repair, using a template from another chromosome. Holliday structures join the damaged and template chromosome. When a homologue is the template, resolution of the Holliday structure can result in mitotic recombination. This has potentially serious effects as it can uncover deleterious recessive mutations. This danger is not present when the template for repair is a sister chromatid. Indeed, cells favor the sister chromatid when undergoing this type of repair (HABER 2000). In contrast, recombination between homologues is usually essential for chromosome segregation during meiosis. Cells thus have compelling reasons to distinguish homologues from sister chromatids. Marks placed on chromosomes in the parental germ line, and maintained throughout the life of the organism, may enable cells to make this distinction. The function of imprints in various types of gene expression might have arisen by taking advantage of existing marks that distinguish homologous chromosomes.

There is also support for a different origin of germ line marks. Imprinting in flies is usually studied in organisms with rearranged chromosomes, raising the possibility that these rearrangements are required for deposition of some imprinted marks. One of the consequences of chromosomal rearrangement is the disruption of normal chromosome pairing. Interestingly, chromatin that is unpaired during meiosis is sometimes modified. This occurs in *Neurospora*, where unpaired DNA creates a signal that silences identical sequences (ARAMAYO and METZENBERG 1996; SHIU *et al.* 2001; SHIU and METZENBERG 2002). In *C. elegans* chromatin that is unpaired in the germ line acquires silencing marks that are retained through early zygotic development (BEAN *et al.* 2004). Silencing of unpaired chromatin in *Neurospora* and the deposition of silencing marks in *C. elegans* may have arisen to inactivate mobile elements. The disruption of pairing by rearrangements might similarly be necessary for deposition of germ line imprints. This idea is supported by investigations of the variegating *In(1)sc*^{8} chromosome. Greater variegation of *y* and *ac* was observed in the offspring of mothers that were heterozygous for the rearrangement, rather than homozygous (SPOFFORD 1976). If marks deposited on unpaired chromosomes establish *Drosophila* imprints, the Y chromosome is an obvious target. In support of this idea, the Y chromosome is imprinted even when it is not rearranged (MAGGERT and GOLIC 2002; MENON and MELLER 2009). As the Y chromosome is entirely heterochromatic, it provides an excellent target for epigenetic marks that require heterochromatin.

These speculations about the origin of imprinted effects in flies raise the possibility that the differences in gene expression that characterize imprinted fly chromosomes may have little relation to the biological function of imprinting in this organism. Even though the origin and molecular basis of imprinted effects in *Drosophila* are not yet understood, it is clear that mechanisms for imprinting exist in flies, and imprinted marks regulate chromatin throughout the life of the organism. *Drosophila* shares epigenetic processes, such as heterochromatin formation, RNAi-directed chromatin regulation, insulation and possibly DNA methylation, with other organisms. Imprinting in flies is a fascinating and potentially powerful system in which to study transgenerational inheritance and propagation of these marks.

Chapter 3

Imprinting of the Y chromosome influences dosage compensation in *roX1 roX2 Drosophila melanogaster*.

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INTRODUCTION

Modification of genetic material in the parental germ line can affect the structure, segregation or expression of chromosomes in the zygote (reviewed by (LLOYD 2000; DE LA CASA-ESPERON and SAPIENZA 2003). Parent-of-origin effects mediated by epigenetic marks on chromosomes are called germ line imprints. The importance of imprints for mammalian embryonic development is illustrated by the early lethality of uniparental diploids (SURANI *et al.* 1986). Unlike mammals, *Drosophila* uniparental diploids are viable and without apparent defect, suggesting that the role of imprinting in flies is minor (LINDSLEY and ZIMM 1992a). In spite of this, imprinting does occur in *Drosophila* and is detected through its effect on gene expression. Euchromatic genes that are moved to heterochromatic environments by inversion or transposition are silenced (WALLRATH and ELGIN 1995). Silencing, detected by variegated expression, is termed position effect variegation (PEV). With few exceptions, imprinting in *Drosophila* is detected through modulation of PEV. Although most variegating

insertions are not affected by transmission, a few are influenced by their parent of origin (GOLIC *et al.* 1998; HALLER and WOODRUFF 2000b; MAGGERT and GOLIC 2002). Rearrangements of the X chromosome that move euchromatic genes into the vicinity of an imprinting center in proximal heterochromatin also display imprinted PEV (ANAKA *et al.* 2009). A common theme in *Drosophila* imprinting is the central role of heterochromatin. Imprinted marks reside in heterochromatin, and formation of heterochromatin in the zygote is required for the maintenance of imprints (LLOYD *et al.* 1999). Because PEV requires rearranged chromosomes or insertion of a transgene into heterochromatin, another recurring motif is that the affected chromosome is structurally abnormal, or a reporter has been moved into an abnormal chromatin environment.

Sex chromosomes are frequent targets of germ line imprints, perhaps because their fate is unusually predictable. Fathers always donate a Y to their sons (Y_P) and an X (X_P) to their daughters. Maternally derived X chromosomes (X_M) are hemizygous when passed to a son. Parents may thus anticipate the genetic and developmental environment that these chromosomes will encounter in the zygote. This is exploited in regulation of several processes. Germ line imprinting of mammalian X-linked genes has been implicated in neural development and determination of sex-specific behaviors (reviewed in (WILKINSON *et al.* 2007). Germ line imprinting directs inactivation of the paternal X chromosome in female marsupials, and in the extra embryonic tissues of female rodents (MIGEON 1998). Inactivation of a single X chromosome equalizes expression between females that carry two copies of the X chromosome and

males that carry single X and Y chromosomes, a process termed dosage compensation.

Drosophila also compensate for unequal X chromosome dosage in males and females, but accomplish this by increasing transcription from genes on the single male X chromosome (LUCCHESI *et al.* 2005). Male flies dosage compensate normally regardless of the origin of their X chromosome. Imprinting therefore does not identify the X chromosome in male *Drosophila*. Both sex determination and dosage compensation in flies is determined by the number of X chromosomes present (BAKER and BELOTE 1983). While the Y chromosome carries genes necessary for male fertility, it is not believed to play a regulatory role in either sex determination or dosage compensation. In flies, dosage compensation is accomplished by the Male Specific Lethal (MSL) complex, composed of proteins and RNA. The MSL complex binds within the body of X-linked genes and alters chromatin to enhance transcription (ALEKSEYENKO *et al.* 2006; GILFILLAN *et al.* 2006; LEGUBE *et al.* 2006). The protein-coding components of the MSL complex were identified by the male-specific lethality of mutations in these genes. *mle* (*maleless*), *msl1, -2, and -3* (*male specific lethals 1, -2* and *-3*), and *mof* (*males absent on first*) together define a set of genes essential for compensation (MENDJAN and AKHTAR 2007). Mutation of any one of these genes causes male lethality as third instar larvae or pupae, but none is essential in females. Elimination of an individual protein not only blocks transcriptional up regulation in males, but also lowers the levels of the remaining MSL proteins and disrupts their association with the X.

The large, non-coding *roX1* and *roX2* RNAs (*RNA on the X*) are essential but redundant components of the MSL complex. Both *roX* RNAs are highly male-preferential in expression. Both genes are X-linked, and their transcripts assemble with the MSL proteins and localize along the male X chromosome (MELLER *et al.* 1997; AKHTAR *et al.* 2000; SMITH *et al.* 2000). Simultaneous mutation of *roX1* and *roX2* causes male-specific lethality, although males have normal survival with a single intact *roX* gene (MELLER and RATTNER 2002). Localization of the MSL complex to the X chromosome is disrupted in *roX1 roX2* males. In polytene preparations from males lacking a wild type *roX* gene, the MSL proteins, no longer exclusive to the X, can be seen binding to heterochromatic regions and autosomal sites (MELLER and RATTNER 2002). MSL binding in nuclei from males carrying partial loss of function *roX1 roX2* chromosomes suggests a direct relationship between the recovery of male escapers and the amount of MSL protein localizing to the X chromosome (DENG *et al.* 2005). A global decrease in X-linked gene expression is detected in *roX1 roX2* males (DENG and MELLER 2006b). These studies indicate that *roX* activity is required for X recognition or stable association of the MSL complex with the X chromosome. Furthermore, integration of *roX* into the MSL complex is required for normal chromatin modification by the complex (PARK *et al.* 2008). In spite of the importance of the *roX* genes in dosage compensation, how *roX* RNA regulates changes in the localization and activity of the MSL complex is poorly understood.

We observed that reversal of sex chromosome inheritance is a potent suppressor of *roX1 roX2* male lethality. Males carrying a paternal *roX1 roX2* chromosome and a maternal Y chromosome have dramatically higher survival than males that inherit identical sex chromosomes conventionally. Surprisingly, this effect can be attributed solely to the presence, and parent of origin, of the Y chromosome. We find that a maternally transmitted Y chromosome suppresses *roX1 roX2* lethality, a paternally transmitted Y chromosome enhances *roX1 roX2* lethality, and absence of the Y chromosome produces an intermediate level of male survival. Males with both maternal and paternal Y chromosomes have very low survival, suggesting that the effect of the paternal Y chromosome is dominant. In spite of the widely held view that the Y chromosome has little genetic information or importance, Y chromosomes from different *Drosophila* strains have unexpectedly large effects on expression throughout the genome, particularly the expression of male-biased genes (LEMOS *et al.* 2008). However, the Y chromosome is not necessary for dosage compensation, and is not believed to influence this process in otherwise normal males (reviewed in (LUCCHESI 1973). The effect we observe thus requires a *roX1 roX2* mutant background. A dose-sensitive X-linked reporter and quantitative reverse transcription PCR (qRT PCR) of X-linked genes reveals higher expression in *roX1 roX2* males with a maternal Y chromosome than with a paternal Y chromosome. We conclude that a maternally imprinted Y chromosome suppresses *roX1 roX2* lethality through a process that culminates in increased expression of X-linked genes.

MATERIALS AND METHODS

Fly culture and genetics

Flies were maintained at 25° on standard cornmeal-agar fly food in a humidified incubator. Unless otherwise noted, all mutations are described in Lindsley and Zimm (LINDSLEY and ZIMM 1992a). The *roX1*^{ex6}, *roX1*^{mb710}, *roX1*^{ex84A}, *roX1*^{SMC17A} and *roX1*^{ex33} mutations have been described (MELLER *et al.* 1997; MELLER and RATTNER 2002; DENG *et al.* 2005). Elimination of *roX2* is accomplished by the lethal deletion, Df(1)52, which removes *roX2* and essential flanking genes. Df(1)52 is combined with insertion of a cosmid carrying essential genes deleted by Df(1)52 but lacking *roX2* ([w^+4Δ4.3]; MELLER and RATTNER 2002). For convenience this combination is referred to as *roX2*. Df(1)52 removes the *nod* gene, immediately proximal to *roX2.* Nod, required for correct disjunction of nonexchange chromosomes in females (ZHANG and HAWLEY 1990), is not restored by [w^+4Δ4.3].

To reverse sex chromosome inheritance, *roX1 roX2* escaper males, or males carrying a rescuing duplication of the *roX2* region on the Y chromosome (Dp(1;Y)$B^s v^+ y^+$) were mated to C(1)DX $y^1 f^1$; [w^+4Δ4.3] females. To obtain males with maternal and paternal *roX1 roX2* chromosomes from the same mothers, *roX1*^{ex6} *roX2* / Df(1)*nod* FM7a; [w^+4Δ4.3] / + females were generated. These females have nonexchange X chromosomes, lack *nod* and display over 50% nondisjunction of their X chromosomes, consistent with previous analysis of *nod* females (ZHANG and HAWLEY 1990). These females were mated to *roX1*^{ex6} *roX2;* [w^+4Δ4.3] male escapers. The maternal and paternal *roX1*^{ex6} *roX2* chromosomes

carry different alleles of *y*, enabling the source of the X chromosome to be determined by body color. Previous studies have reported a high level of gynandromorphs in the progeny of nod females (ZHANG and HAWLEY 1990). We did not recover gynandromorphs, and attribute this to the different *nod* alleles used in our work and in previous studies.

To generate males carrying maternal X and Y chromosomes, females carrying a y^+Y chromosome were selected from a *y roX1*^{mb710} *roX2* / y^+Y; [w^+4Δ4.3] /CyO [w^+ *roX1*$^+$] stock and expanded. *y roX1*^{mb710} *roX2* / y^+Y; [w^+4Δ4.3] virgins were mated to C(1;Y) 6y^2Su(w^a) w^a males to produce *roX1*^{mb710} *roX2* sons lacking a Y chromosome or carrying the maternal y^+Y. Thirty eight percent of the daughters from this mating were y^+ (445 out of 1183). Production of O and X^Y gametes by C(1;Y) 6y^2Su(w^a) w^a males was determined by mating to *yw* virgins. A total of 281 XX^Y daughters and 615 XO sons were obtained, indicating that 31% of gametes are X^Y and 69% are O. Survival of sons from matings between *y roX1*^{mb710} *roX2* / y^+Y; [w^+4Δ4.3] virgins and C(1;Y) 6y^2Su(w^a) w^a males was calculated by dividing the number of males recovered by the number of females derived from the same class of maternal gamete (X or XY). This value was divided by 2.23 to correct for the bias towards production of O gametes by the father. The absence of a free Y chromosome in C(1;Y)6y^2 *Su*(w^a) w^a males was confirmed by examination of mitotic chromosome preparations and by the sterility of sons produced by mating C(1;Y)6y^2 *Su*(w^a) w^a males to wild type females. *y roX1*^{mb710} *roX2* / y^+Y; [w^+4Δ4.3] virgins were mated to *yw* / Y males to produce *y roX1*^{mb710} *roX2* sons with a paternal Y chromosome and with Y

chromosomes from both parents. Survival of these sons was calculated by dividing the number of males recovered by the number of females derived from the same class of maternal gamete (X or XY).

Generation of male larvae and immunostaining

Polytene chromosomes were prepared from salivary glands of 3rd instar *roX1*^{mb710} *roX2* male larvae. Males without a Y chromosome were generated by mating *roX1*^{mb710} *roX2*; [w^+4Δ4.3] females to C(1;Y)6y^2 *Su*(w^a) w^a males. *roX1*^{mb710} *roX2* male larvae with a maternal Y were generated by crossing *y roX1*^{mb710} *roX2* / y^+Y; [w^+4Δ4.3] females to C(1;Y)6y^2 *Su*(w^a) w^a males. Males carrying y^+Y were selected by mouth hook color. Larvae with maternal and paternal Y chromosomes were generated by crossing *y roX1*^{mb710} *roX2* / y^+Y; [w^+4Δ4.3] females to *yw* / Y males and selecting male larvae carrying y^+Y. Polytene chromosomes were squashed and immunostained for MSL1 as previously described (KELLEY *et al.* 1999). Over 100 nuclei of each genotype were scored based for intensity of signal on the X chromosome and in the chromocenter. To avoid bias, the genotype was obscured while slides were processed and scored.

Generation and scoring of *Beadex* flies

To reverse Y chromosome inheritance, *roX1*^{mb710} *roX2 Dp(1;Y)Bx*^{r49k} males were mated to C(1)DX y^1f^1; [w^+4Δ4.3] females. All male offspring carry the paternal *roX1*^{mb710} *roX2 Dp(1;Y)Bx*^{r49k} chromosome and a maternal Y chromosome. *roX1*^{mb710} *roX2 Dp(1;Y)Bx*^{r49k} */ Binsincy* females were mated to *yw* / Y; [w^+4Δ4.3] males to generate *roX1*^{mb710} *roX2 Dp(1;Y)Bx*^{r49k} sons with a

paternal Y chromosome. Wings were mounted in 4:5 lactic acid:ethanol and photographed. Wings were measured using ImageJ software (http://rsb.info.nih.gov/ij/). Anterior and posterior wing notching is expressed as the length of margin lost. To normalize for variation in wing size, this is expressed as a percentage of vein L3 length between the L2 junction and the edge of the wing. The significance of differences in notching was determined using a two-sample unpaired t-test.

Quantitative reverse transcriptase PCR (qRT PCR)

Expression of *Dlmo*, *SkpA* and *Ck-IIβ* was measured by qRT PCR as described previously (DENG *et al.* 2009a). In brief, total RNA was made from 4 groups of 50 larvae of each genotype. One μg of total RNA was reverse transcribed using random hexamers and ImProm-II reverse transcriptase (Promega). Two technical replicates of each biological replicate were amplified. Expression was normalized to the autosomal gene *Dmn*. Primers are: *Dlmo* (F-TGAGATTGTTTGGCAACACG, R-ACGCATCACCATCTCGAAG, 500 nM), *SkpA* (F-CTAAAAGTCGACCAGGGCAC, R-CCAGATAGTTCGCTGCCAAT, 300 nM), *Ck-IIβ* (F-CCTGGTTCTGTGGACTTCGT, R-GTAGTCCTCATCCACCTCGC, 300 nM). The significance of differences attributable to Y chromosome origin was determined by performing a two sample t-test.

RESULTS

The survival of *roX1 roX2* males is increased by reversal of sex chromosome inheritance

The X-linked *roX* genes are essential for exclusive localization of the MSL proteins to the X chromosome. As the *roX* genes are redundant for dosage compensation, the effect of *roX1* mutations is measured in males that are also mutated for *roX2*. All *roX1 roX2* chromosomes are completely deleted for *roX2*. The left column of Table 3.1 presents the survival of males that inherit *roX1 roX2* chromosomes from their mothers, as is conventional. Male survival is dramatically increased when sons are produced by mating *roX1 roX2* males to compound X females (C(1)DX*yf* / Y), reversing the inheritance of the X and Y chromosomes (Table 3.1, right column). This effect was observed for all *roX1 roX2* chromosomes tested. A chi-square test comparing the survival of males with normal or reversed sex chromosome inheritance yields p-values <0.001 for $roX1^{ex6}roX2$, $roX1^{mb710}roX2$ and $roX1^{ex33A}roX2$. In this study, no adult $roX1^{ex84A}roX2$ and $roX1^{SMC17A}roX2$ males were recovered when sex chromosomes were normally inherited. For these genotypes, the confidence intervals for male survival with normal and reversed sex chromosome inheritance were determined and found to be non-overlapping. Reversal of sex chromosome inheritance thus appears to be a potent suppressor of *roX1 roX2* male lethality. Suppression was observed when the father donating the *roX1 roX2* chromosome was an adult escaper, and when the father was rescued by a duplication of the *roX2* region carried on the Y chromosome (Dp(1;Y)$B^s v^+ y^+$). Lack of *roX* in the

father's germ line therefore does not affect the survival of their sons. One interpretation of this is that one or both of the sex chromosomes receives a germ line imprint that influences dosage compensation in *roX1 roX2* males. The following experiments rely on $roX1^{ex6}$ and $roX1^{mb710}$, alleles of comparable severity. *roX1 roX2* males carrying either of these alleles display ~ 5% survival when the sex chromosomes are conventionally inherited and about ~ 40% survival upon reversal of inheritance.

Table 3.1. Reversal of sex chromosome inheritance suppresses lethality in *roX1 roX2* males

Male Genotype	% survival X_MY_P (total adults)	% survival X_PY_M (total adults)
$roX1^{ex6}$ *roX2*	4.8 (1137)	42 (717)
$roX1^{mb710}$ *roX2*	5.3 (1290)	33 (626)
$roX1^{ex33A}$ *roX2*	51 (2323)	86 (680)
$roX1^{ex84A}$*roX2*	0* (2511)	3.3** (497)
$roX1^{SMC17A}$*roX2*	0 (1458)	0.5 (810)

Conventional sex chromosome transmission was accomplished by mating *roX1 roX2* Binsincy females to X/Y; [$w^+4\Delta4.3$] males. Reversal of sex chromosome inheritance was accomplished by mating *roX1 roX2* / Dp(1:Y)$B^sv^+y^+$ males to C(1)DX*yf*; [$w^+4\Delta4.3$] females.

* No living males were recovered, but several eclosed and died in the food, usually without fully expanding their wings. ** Males lived many days and were weakly fertile.

The MOF protein is a histone acetyltransferase that modifies H4 on lysine 16, a mark enriched in the body of X-linked genes (SMITH *et al.* 2001). In spite of a central role in dosage compensation, the *mof*^{1} mutant has a relatively mild phenotype and third instar larvae are quite healthy and abundant. To test whether male escapers carrying *mof*^{1} could be obtained by reversal of sex chromosome inheritance, *mof*^{1}/ Y; *Cy*O [w^{+}*mof*$^{+}$] / + males, with a rescuing *mof* transgene on a *Cy*O balancer, were mated to C(1)DX*yf* / Y virgins. We recovered 1117 C(1)DX*yf* daughters and 234 *mof*^{1}; *Cy*O [w^{+}*mof*$^{+}$] / + sons, but no sons lacking the *Cy*O [w^{+}*mof*$^{+}$] chromosome were recovered. This suggests that suppression of lethality by reversal of sex chromosome inheritance is not applicable to mutations in other members of the MSL complex, and may be specific to *roX1 roX2* mutants.

Wolbachia infection could produce a similar parent of origin affect. *Wolbachia* was detected in some stocks by PCR, but in a manner inconsistent with a role in suppression of *roX1 roX2* male lethality (Fig. 3.1). Furthermore, maintaining stocks on tetracycline for multiple generations did not influence the survival of *roX1 roX2* males with normal or reversed sex chromosome inheritance. We conclude that *Wolbachia* infection is not responsible for the observed differences in male survival.

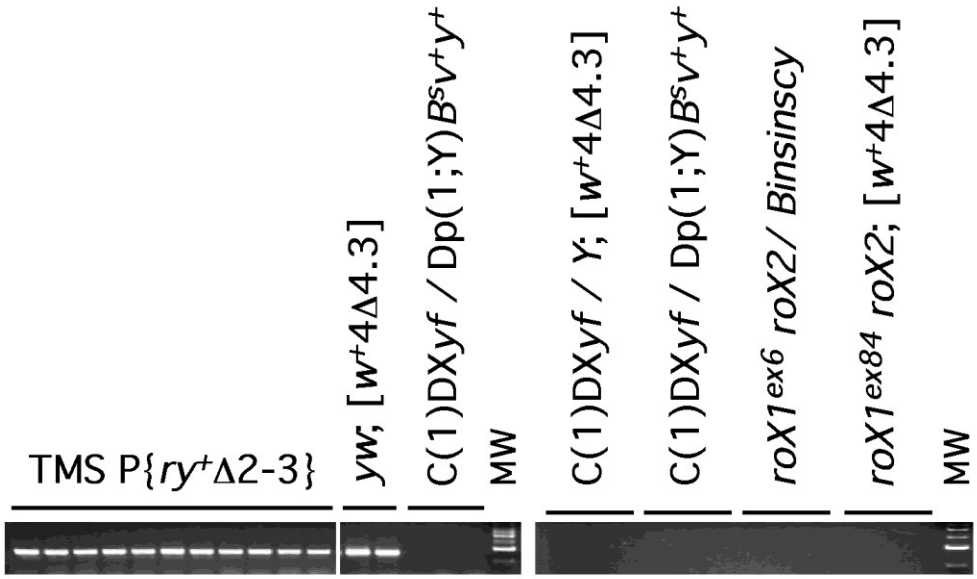

Figure 3.1. *Wolbachia* detection. Adult females from strains used in this study were examined for *Wolbachia* infection by PCR. A strain with the popular transposase source chromosome TMS *P*{*ry*$^{+}$D2-3} was the positive control (left). A cosmid insertion, denoted [*w*$^{+}$4D4.3], carries essential genes removed by the *roX2* deficiency. All *roX1 roX2* flies also carry [*w*$^{+}$4D4.3]. Although the original *yw*; [*w*$^{+}$4D4.3] strain is infected with *Wolbachia*, females from two stocks derived from this insertion, C(1)DX*yf* / Y; [*w*$^{+}$4D4.3] and *roX1*^{ex84A} *roX2* / Dp(1;Y)*B*^{s}*v*$^{+}$*y*$^{+}$; [*w*$^{+}$4D4.3], appear *Wolbachia* free. Females from two stocks that maintain *roX1*^{mb710} *roX2* over C(1)DX*yf* using the *roX2* region duplication Dp(1;Y)*B*^{s}*v*$^{+}$*y*$^{+}$ were also free of *Wolbachia*, as were *roX1*^{ex6}*roX2* / Binsinscy females. Each lane was amplified from template containing three females of the appropriate genotype. Primers specific for the *Wolbachia* 16S rRNA gene were used for amplification (CLARK and KARR 2002). The molecular weight marker is a 1 kb ladder.

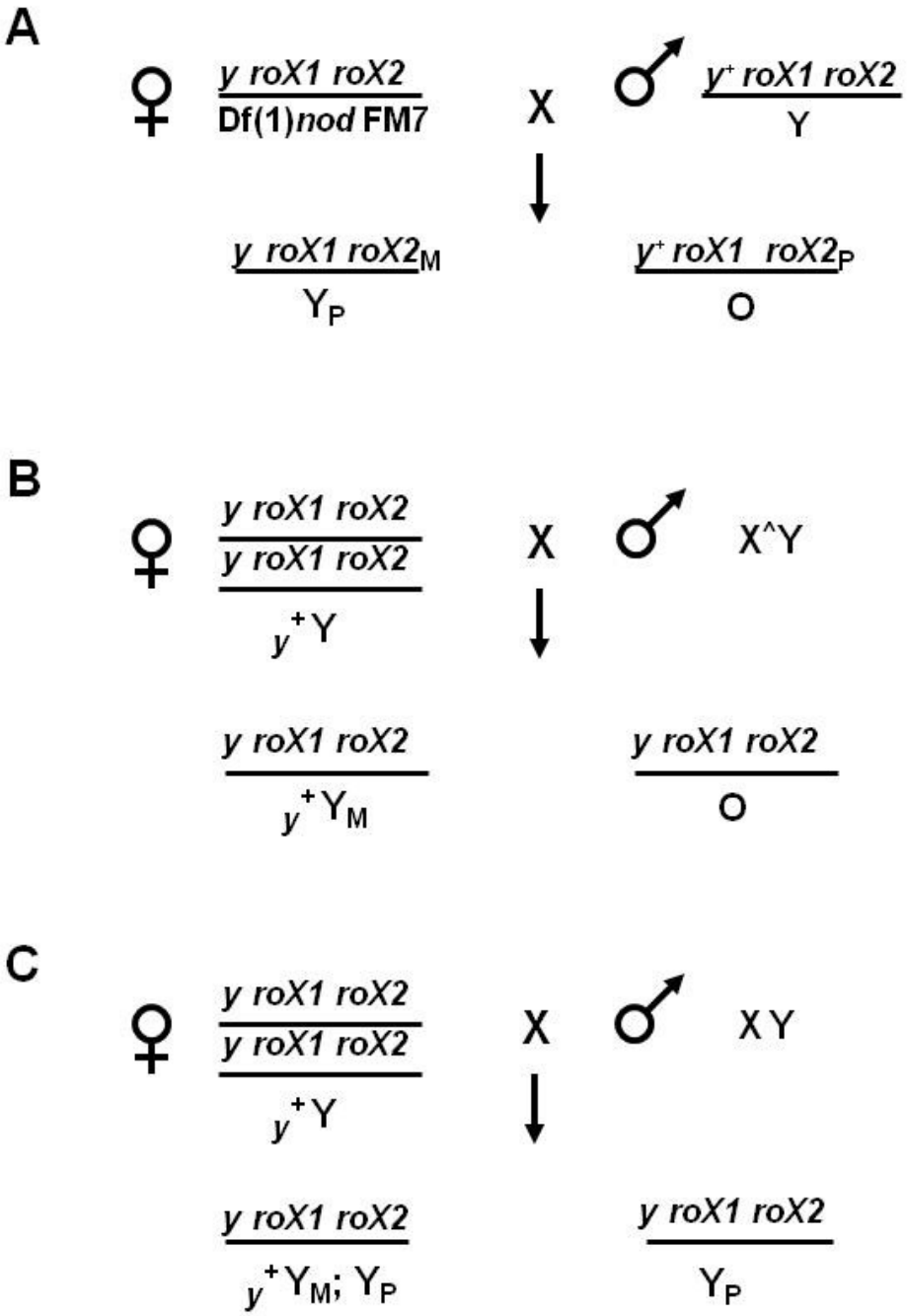

Figure 3.2. Schemes for producing *roX1 roX2* males with reversed sex chromosome inheritance. (A) A mating that produces males with maternal or paternal *roX1*^{ex6} *roX2* chromosomes. Females carrying a *roX1*^{ex6} *roX2* X chromosome and the Df(1)*nod* FM7a balancer produce O, X and XX gametes (Table 3.2). Fertilization of an O gamete with an X-bearing sperm produces XO males. As Df(1)*nod* FM7a is lethal, no sons carrying this chromosome will be recovered. Sons carrying maternal (X_M) or paternal (X_P) X chromosomes are distinguished by the y^+ marker. (B) Scheme for producing *roX1*^{mb710} *roX2* males bearing maternal X and Y chromosomes (Table 3, matings 1 and 2). Females homozygous for *y roX1*^{mb710} *roX2* chromosomes and carrying a y^+Y chromosome are mated to compound X^Y males. All sons inherit a maternal *roX1*^{mb710} *roX2* X chromosome and lack a Y chromosome or carry the maternal Y chromosome. (C) Scheme for producing *roX1*^{mb710} *roX2* males bearing maternal X and Y chromosomes and a paternal Y chromosome (Table 3, matings 3 and 4). Females homozygous for *y roX1*^{mb710} *roX2* chromosomes and carrying a y^+Y chromosome are mated to *yw* males with an unmarked Y chromosome. All sons have a maternal *roX1*^{mb710} *roX2* X chromosome and an unmarked paternal Y chromosome. Sons that inherit the maternal Y chromosome are distinguished by the y^+ marker.

Suppression of the *roX1 roX2* phenotype is not due to a maternal effect

It is possible that the compound X females used to reverse sex chromosome inheritance provide a maternal effect that suppresses the *roX1 roX2* phenotype. To eliminate differences in maternal genotype, a single cross generating sons carrying paternal (X_P) and maternal (X_M) X chromosomes was performed. Females with a high rate of non-disjunction produce gametes with zero, one or two X chromosomes. If a gamete lacking an X chromosome is fertilized by a sperm carrying an X chromosome, the resulting zygote will be a male that carries a paternal X chromosome (X_PO male). The *Drosophila* Y chromosome is necessary for male fertility but does not determine sex. Df(1)52, which deletes *roX2*, is also deleted for *nod* (MELLER and RATTNER 2002). The *nod* gene product is required for faithful segregation of non-exchange chromosomes in females, and *nod* females carrying a balancer X chromosome display over 50% non-disjunction (ZHANG and HAWLEY 1990). *roX1*^{ex6} *roX2* / Df(1)*nod* FM7a females were mated to males carrying a *roX1*^{ex6} *roX2* chromosome that differed at *yellow* (*y*), enabling sons carrying paternal and maternal *roX1*^{ex6} *roX2* chromosomes to be distinguished by body color (Fig. 3.2 A). Because a single female genotype produced both classes of sons, the possibility that differential survival is due solely to a maternal effect can be eliminated. The rate of nondisjunction, calculated from the number of $X_MX_MY_P$ females, was near 50% for each independent mating (Table 3.2). Both parental genotypes suffer reduced fertility, resulting in few progeny and large variation between trials. However, within each trial male survival was improved when the

roX1^{ex6} *roX2* X chromosome was paternal in origin and no Y chromosome was present. On average, the survival of males carrying a maternal *roX1*^{ex6} *roX2* X chromosome and a paternal Y chromosome is 6%, which agrees well with the 4.8% survival of males with maternal X and paternal Y chromosomes from Table 3.1. In contrast, the survival of males carrying a paternal *roX1*^{ex6} *roX2* chromosome and no Y chromosome averages 20%. Comparing these rates of survival using a two sample t-test produces a P-value of 0.026. While this study supports the idea that imprinting of the X chromosome influences dosage compensation, this result is not inconsistent with an imprinted Y chromosome acting as a modifier of *roX1 roX2* lethality. Sons with a maternal X chromosome carry a paternal Y chromosome, but those inheriting with a paternal X chromosome lack a Y chromosome. The Y chromosome has been demonstrated to be subject to a germ line imprint, making this a plausible scenario (MAGGERT and GOLIC 2002).

Table 3.2. Modified *roX1 roX2* lethality is not a maternal effect

Trial	$\underline{roX1\ roX2}_P$ Df(1)*nod* FM7a$_M$	$\underline{roX1\ roX2}_M$ Df(1)*nod* FM7a$_M$ Y$_P$	$\underline{roX1\ roX2}_P$ *roX1 roX2*$_M$	Nondisjunction (%)	% survival (adult males) $\underline{roX1\ roX2}_M$ Y$_p$	% survival (adult males) $\underline{roX1\ roX2}_P$ O
1	186	98	194	52	12 (22)	32 (31)
2	27	17	34	56	10 (3)	18 (3)
3	333	180	335	54	1.8 (6)	11 (20)
4	71	36	96	43	1.2 (1)	31 (11)
Total:	617	331	659	52	5 (32)	20 (65)

Four replicates of the mating depicted in Fig. 3.2 A were conducted. The survival of X_MY_P males is based on the number of X_PX_M sisters obtained. The survival of X_MO males has been corrected using the rate of maternal nondisjunction for each trial. Maternal nondisjunction was determined by the number of $X_MX_MY_p$ daughters obtained. In trials 1,3 and 4 the paternal X chromosome is y^+ and the maternal *roX1 roX2* chromosome is *y*. In trial 2 the paternal X chromosome is *y* and the maternal X chromosome is y^+.

Maternal imprinting of the Y chromosome suppresses *roX1 roX2* male lethality

To determine the effect of a maternally donated Y chromosome on *roX1 roX2* males, females homozygous for a *roX1 roX2* chromosome and carrying a marked Y chromosome (*y roX1*^{mb710} *roX2* / y^+Y) were mated to compound X^Y males lacking a free Y chromosome (C(1;Y)6y^2 *Su(w*^{a}*)* *w*^{a}, Fig. 3.2 B). Females with a Y chromosome also display reduced fertility and produce relatively small numbers of offspring. However, in two replicate experiments transmission of the y^+Y chromosome from the mother partially suppressed *roX1 roX2* lethality (Table 3, matings 1, 2). The overall survival of *roX1*^{mb710} *roX2* sons carrying a maternal

y^+Y was over 44%. This mating also produces X_MO sons, which display an average survival of 15.7%, comparable to the 20% survival of *roX1*^{ex6}*roX2* / O males produced by maternal nondisjunction. The difference in survival of X_MO and X_MY_M males yielded a p-value of < 0.001 by the chi-square test. In spite of the fact that a Y chromosome is present in the maternal germ line, sons that do not inherit this chromosome display little or no suppression of lethality (Table 3.3, column X_MO). The Y chromosome therefore does not achieve its effect through conditioning the oocyte. To confirm that the y^+Y chromosome used in this study was equivalent to the unmarked Y chromosome in our reference lab strain, it was extracted from *roX1*^{mb710} *roX2* / y^+Y into the *yw* lab strain. The survival of *roX1*^{mb710} *roX2* sons receiving the y^+Y chromosome from *yw* / y^+Y fathers was 4.7%. This demonstrates that y^+Y is not genetically unusual with respect to the trait we are measuring.

To determine the effect of multiple Y chromosomes, we mated *roX1*^{mb710} *roX2* / y^+Y females to *yw* / Y males (Fig. 3.2 C; Table 3.3, matings 3, 4). All sons are presumed to receive an unmarked Y chromosome from their father. To our surprise, the paternal Y chromosome blocks the effect of the maternal y^+Y. Two replicate experiments produced survival of *roX1*^{mb710} *roX2* / y^+Y_M / Y_P sons averaging 4.3%, lower than that of *roX1*^{mb710} *roX2* / Y_P brothers from the same mating. The difference in survival of X_MY_P and $X_MY_MY_P$ males yielded a p-value of 0.00002 using the chi-square test.

Table 3.3. Maternal transmission of the Y chromosome suppresses *roX1 roX2* male lethality

	adult daughters		% survival *roX1*^{mb710} *roX2* sons (adult males)			
Mating	**XX^Y**	**XX^YY**	**X_MO**	**X_MY_M**	**X_MY_P**	**X_MY_M Y_P**
1	190	73	12.7 (54)	34.4 (56)	-	-
2	262	85	17.8 (104)	53.3 (101)	-	-
Mating	**XX**	**XXY**	**X_MO**	**X_MY_M**	**X_MY_P**	**X_MY_M Y_P**
3	266	182	-	-	10.9 (29)	2.8 (5)
4	472	263	-	-	11.9 (56)	5.3 (14)

Matings 1 and 2 are between *roX1*^{mb710}*roX2* / *y*$^+$Y females and compound X^Y males (Fig. 3.2 B). Sons lack the Y chromosome (X_MO) or carry a maternal Y chromosome (X_MY_M). Male survival is calculated from the recovery of females derived from the same class of maternal gamete and corrected for a bias in O gametes produced by the fathers (see Material and Methods for details). Matings 3 and 4 (Fig. 3.2 C) are between *roX1*^{mb710}*roX2* / *y*$^+$Y females and *yw* / Y males.

These studies reveal that a maternally transmitted Y chromosome is a potent suppressor of *roX1 roX2* male lethality. The intermediate survival of *roX1 roX2* / O males further suggests that normal inheritance of a paternal Y chromosome enhances the *roX1 roX2* phenotype, and is thus deleterious to males. Most surprising is the observation that when both paternal and maternal Y chromosomes are present, the paternal Y chromosome completely blocks the effect of the maternal Y chromosome.

MSL localization on polytene chromosomes is not influenced by Y chromosome origin

Examination of different *roX1 roX2* chromosomes revealed a direct relationship between male survival and the amount of MSL protein localizing to the X chromosome (DENG *et al.* 2005). To determine if *roX1*^{mb710} *roX2* males

with a maternal Y chromosome have greater MSL localization on the X chromosome, polytene preparations were immunostained to detect MSL1. Wild type males display exclusive localization of MSL proteins along the X chromosome (Fig. 3.3 A, B). All *roX1*^{mb710} *roX2* males have reduced MSL localization to the X chromosome, but display strong and somewhat variable binding at a number of autosomal sites and in the chromocenter (Fig. 3.3 C, D). Disruption of the wild type pattern of MSL localization was seen in all *roX1*^{mb710} *roX2* males, regardless of the presence or origin of their Y chromosome. No relationship between accumulation of MSL1 on the X chromosome and origin of the Y chromosome was apparent, possibly due to the large variation in MSL recruitment in different animals within each group (Fig. 3.3 E). Because deposition of the MSL proteins at the chromocenter is characteristic of *roX1 roX2* males, we considered the possibility that this was attributable to recruitment of the MSL proteins to the Y chromosome. The Y chromosome does not polytenize, but it is present in the chromocenter. If the Y chromosome attracts the MSL proteins in *roX1 roX2* mutants, this might explain its influence on male survival. Chromocenter staining was also highly variable, and a relationship between staining intensity and Y chromosome origin could not be detected (Fig. 3.3 F). However, many XO males retained strong chromocenter staining, eliminating the possibility that chromocenter staining is due to recruitment of MSL proteins to the Y chromosome (Fig. 3.3 C, D).

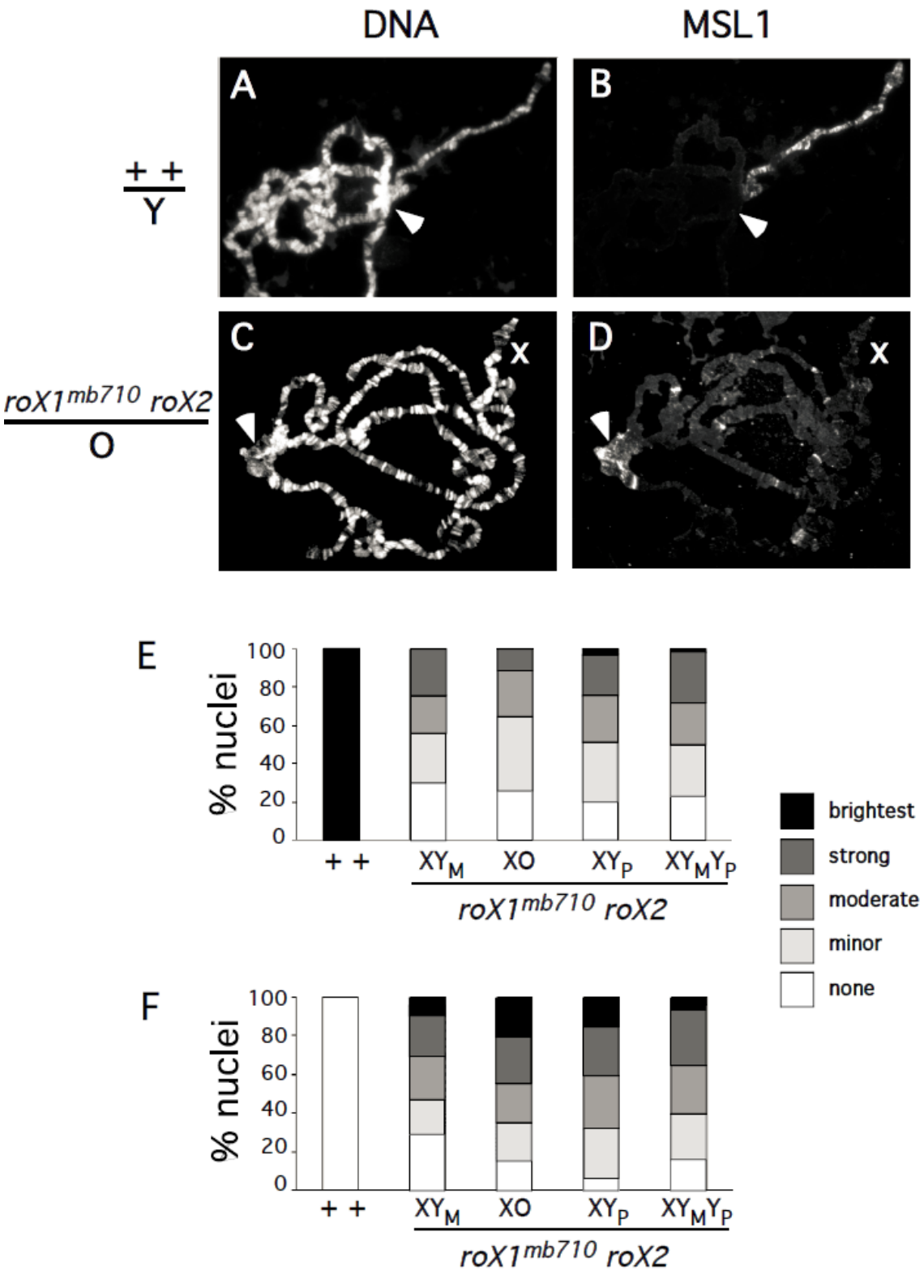

Figure 3.3. MSL1 recruitment to the X chromosome and chromocenter of *roX1*^{mb710} *roX2* males is not influenced by Y chromosome origin. (A, B) Polytene preparation from a wild type male. DNA is detected with DAPI (A) and MSL1 is detected by Texas Red (B). The chromocenter, marked by the arrowhead, has no MSL1 staining. (C, D) Polytene preparation from a *roX1*^{mb710} *roX2* / O male. The X chromosome (X) is scored as having minor MSL1 staining. The chromocenter (arrowhead) has strong MSL1 staining. (E) The intensity of MSL1 signal on the X chromosome was scored in *roX1*^{mb710} *roX2* males carrying a maternal Y chromosome, no Y, a paternal Y chromosome, or both maternal and paternal Y chromosomes. The percentage of nuclei falling into each category is on the Y axis. Over 100 nuclei of each karyotype were scored. (F) The intensity of MSL1 signal at the chromocenter was scored for the same nuclei. All nuclei were scored with labels obscured to prevent bias in scoring.

Preferential disruption of the male polytene X chromosome has been observed for mutations in HP1, *Su(var)3-7*, *ISWI* and a super coiling protein, among others (CORONA *et al.* 2002; DE WIT *et al.* 2005; SPIERER *et al.* 2005; FURUHASHI *et al.* 2006; SPIERER *et al.* 2008b). In mutant males the polytenized X typically appears short, partially decondensed and disruption of banding is readily apparent. In the case of *ISWI* and super coiling factor, disruption depends on a functional dosage compensation system. Intriguingly, normal levels of *Su(var)3-7* are also necessary for establishment of dosage compensation (SPIERER *et al.* 2008b). To determine whether Y chromosome inheritance influences disruption of the male X chromosome in *Su(var)3-7* mutants, we examined the morphology of polytene chromosomes from *Su(var)3-7* males with normal or reversed sex chromosome inheritance. As previously reported, the X chromosome of females is unaffected by the *Su(var)3-7* mutation (Spierer et al., 2005). The degree of disruption detected for male X chromosomes ranges from minor to severe, but no differences attributable to Y chromosome origin were detected (Fig. 3.4).

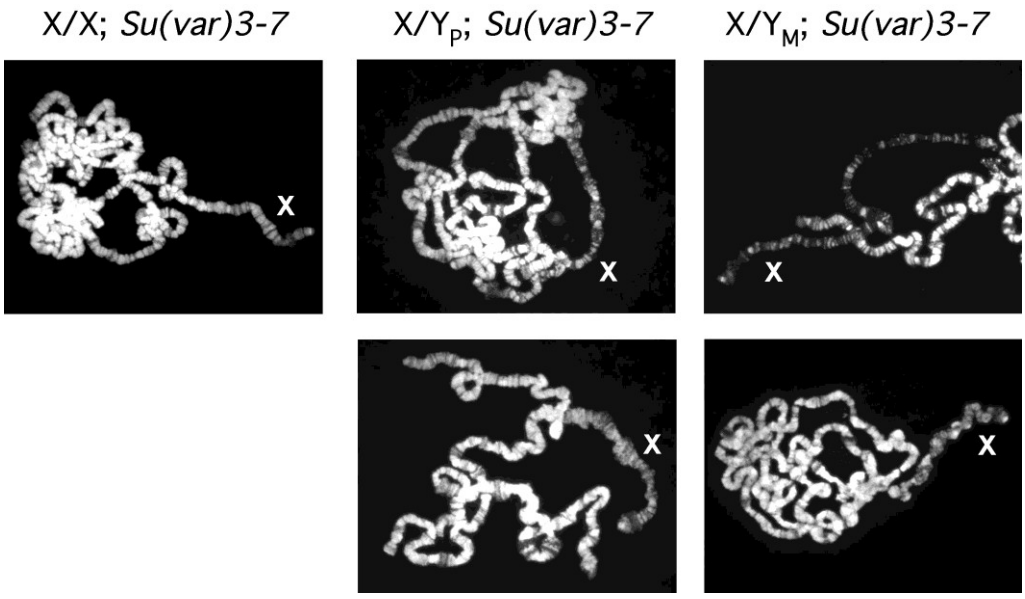

Figure 3.4. Imprinting of the Y chromosome in *Su(var)3-7* males does not influence X chromosome morphology. Salivary glands from larvae homozygous for *Su(var)3-7*^{14} were squashed and stained to detect DNA (SPIERER *et al.* 2005). Consistent with the description of this mutation, the morphology of the female X chromosomes (left panel) appears normal. In contrast, the male X chromosome displays a range of aberrant morphologies, from relatively minor disruption of polytene banding (top) to a more severe disruption of banding coupled with shortening of the X chromosome (bottom). Males with paternal (middle) or maternal (right) Y chromosomes displayed a similar range of abnormal X morphologies.

X linked gene expression is increased by a maternal Y chromosome

We turned to a dose-sensitive X-linked reporter to determine whether the origin of the Y chromosome influences X-linked gene expression. *Beadex* (*Bx*) mutations are dose-sensitive gain of function alleles that increase copy number, or expression, of the *Dlmo* gene (SHORESH *et al.* 1998). The mild *Dp(1;1)Bx*^{r49k} allele is produced by a duplication of *Dlmo*. *Dp(1;1)Bx*^{r49k} males, with two copies of the *Dlmo* gene, display notching of wing margins. Homozygous *Dp(1;1)Bx*^{r49k} females, with four copies, display similar notching. *Dp(1;1)Bx*^{r49k} / + females,

with three copies of *Dlmo*, have normal wings (Fig. 3.5 A). As the phenotype is more severe in males with two copies of *Dlmo* than in females with three copies, *Dp(1;1)Bx^{r49k}* is dosage compensated. To test whether compensation of *Dlmo* depends on the MSL complex, we induced inappropriate formation of this complex in *Dp(1;1)Bx^{r49k}* / + females. The [*w*$^{+}$ H83-M2]6I transgene drives ectopic expression of MSL2, the only member of the MSL complex absent in females. Formation of complexes that bind along both X chromosomes is observed in females carrying [*w*$^{+}$ H83-M2]6I (KELLEY *et al.* 1995). *Dp(1;1)Bx^{r49k}* / +; [*w*$^{+}$ H83-M2]6I / + females display wing notching similar to that observed in *Dp(1;1)Bx^{r49k}* males (Fig. 3.5 B). This suggests that the *Dlmo* gene is regulated by the MSL complex. The [*w*$^{+}$ H83-M2]6I transgene dramatically reduces female survival. Mutating one copy of *msl1* restores the viability of females carrying [*w*$^{+}$ H83-M2]6I (KELLEY *et al.* 1995). Consistent with this, we found that a single *msl1^{1}* allele eliminated wing notching in *Dp(1;1)Bx^{r49k}* / +; *msl1^{1}* / +; [*w*$^{+}$ H83-M2]6I / + females (Fig. 3.5 C). This establishes that the *Dlmo* gene is dosage compensated by the MSL complex.

As *Dp(1;1)Bx^{r49k}* produces a visible, dose-sensitive phenotype that responds to the MSL complex, it was used to report the activity of the MSL complex in males with Y chromosomes from different parents. The extent of notching at anterior and posterior wing margins was measured in *roX1^{mb710} roX2 Dp(1;1)Bx^{r49k}* males with maternal or paternal Y chromosomes. Notching was apparent but minor and usually limited to the posterior margin in males with a paternal Y chromosome (Fig. 3.5 D). Notching was greater when a maternal Y

chromosome was present, and frequently involved the anterior wing margin (Fig. 3.5 E). The difference in marginal notching attributable to Y chromosome origin is both visually apparent and statistically significant, indicating elevated expression of *Dlmo* when a maternal Y chromosome is present (Fig. 3.5 F).

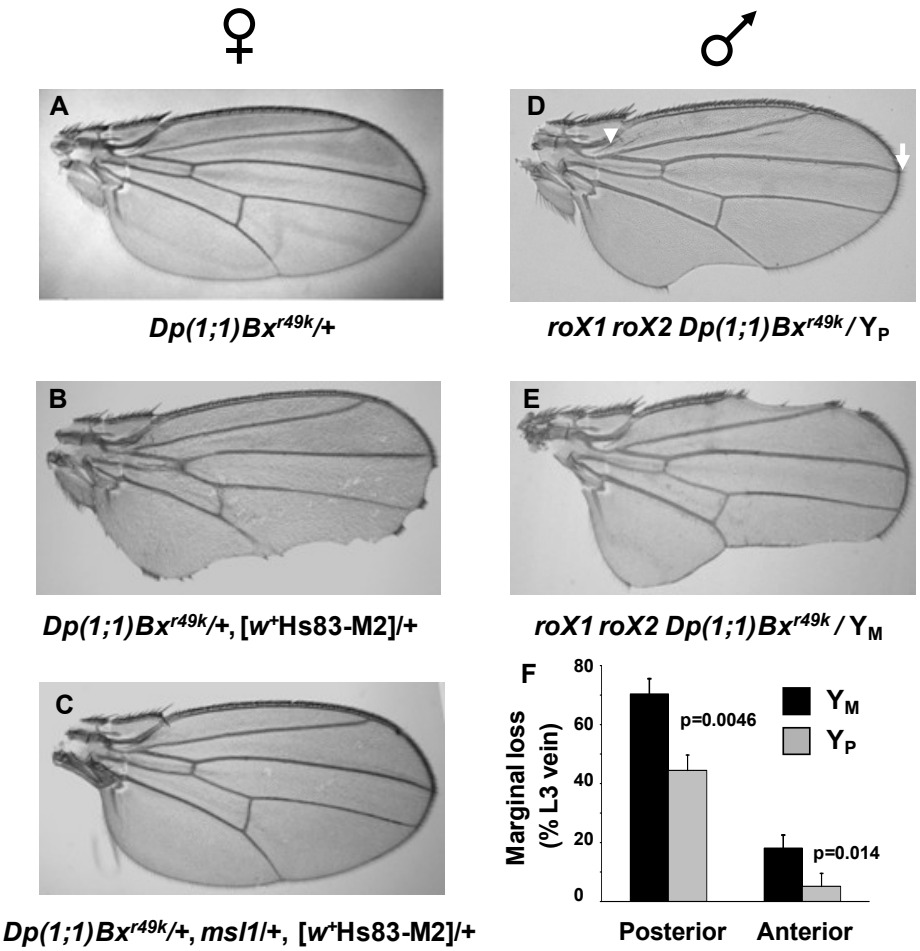

Figure 3.5. *Beadex* responds to MSL complex activity and Y chromosome origin. (A) Wing from Dp(1;1) Bx^{r49k} / + female. (B) Dp(1;1) Bx^{r49k} / + ; [w^{+}Hs83-M2]6I female. (C) Dp(1;1) Bx^{r49k} / + ; $msl1^{1}$/ +;[w^{+}Hs83-M2]6I female. (D) Wing from $roX1^{mb710}$ *roX2* Dp(1;1) Bx^{r49k} male with paternal Y chromosome. (E) Wing from $roX1^{mb710}$ *roX2* Dp(1;1) Bx^{r49k} male with maternal Y chromosome. (F) The amount of wing margin lost is represented as the percentage of L3 vein length (arrowheads in D). Sixteen wings from X_MY_P males and 13 wings from X_PY_M males were measured. P values were determined by a two sample unpaired t-test.

We then used quantitative reverse transcriptase-PCR (qRT PCR) to examine expression of *Dlmo* and two additional X-linked genes, *SkpA* and *Ck-II*, in wild type and *roX1*^{mb710} *roX2* males (Fig. 3.6). Expression in wild type males is set to one. Expression is reduced in all *roX1*^{mb710} *roX2* males, but the reduction is consistently less when the Y chromosome is of maternal origin. However, the difference in expression attributable to Y chromosome origin is slight and statistically significant only for *Ck-II*.

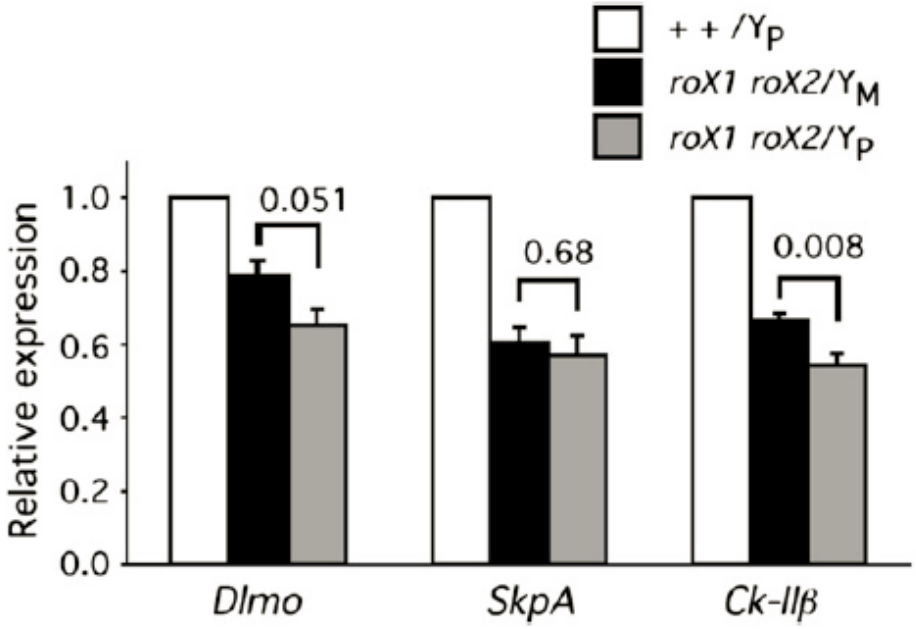

Figure 3.6. X-linked gene expression is increased by a maternal Y chromosome. The expression of the X-linked genes *Dlmo*, *SkpA* and *Ck-IIβ* was measured by qRT PCR in wild type male larvae (open bars) and *roX1*^{mb710} *roX2* males with maternal (black) or paternal (gray) Y chromosomes. Four groups of 50 larvae contributed to each measurement. Expression was normalized using the autosomal gene *Dmn*. The significance of differences in expression was determined by a two sample unpaired t-test.

Enhancement of the *Dp(1;1)Bx*^{r49k} phenotype and direct measurement of gene expression support the idea that the maternal Y chromosome suppresses *roX1 roX2* lethality by increasing X-linked gene expression. The change in expression is sufficiently modest that detection is most conveniently performed using a sensitive phenotypic assay.

DISCUSSION

These studies reveal that imprinting of the *Drosophila* Y chromosome is capable of influencing dosage compensation. Previous work has shown imprinting of the Y chromosome, but our system displays significant differences. Imprinting in flies is usually detected through expression of genes situated close to and on the same chromosome as the imprint. For example, imprinting of the rearranged *DP(1;f)LJ9* mini-X chromosome is detected by expression from euchromatic genes that have been brought into the vicinity of proximal heterochromatin by rearrangement and deletion (ANAKA *et al.* 2009). In contrast, we see that the imprinted Y chromosome modulates the epigenetic process of dosage compensation, targeted to a chromosome different than the one bearing the imprint. Unlike imprinted modulation of PEV, neither the Y chromosome nor the affected X chromosome have suffered major rearrangements that place euchromatic genes in a heterochromatic environment. The relationship of the previously described Y chromosome imprint to the one we have observed, and the biological relevance of Y chromosome imprinting in general, remain unclear. Male flies are fertile with a Y chromosome transmitted by either parent, and thus imprinting is not essential for the known functions of this chromosome.

Our genetic study of Y chromosome imprinting uncovered surprising complexity. *roX1 roX2* / O males display survival intermediate between that of males carrying maternal and paternal Y chromosomes. It is possible that the each parent imprints a different region of the Y chromosome. This issue can not be resolved without knowledge of the location and nature of the imprinted marks, but the dominance off the paternal imprint leads us to speculate that maternal and paternal imprints influence the same step in dosage compensation.

Males that carry a wild type *roX* gene dosage compensate normally, regardless of the presence or origin of the Y chromosome. While all *roX1 roX2* chromosomes tested display a milder phenotype when a maternal Y chromosome is present, reversal of sex chromosome inheritance does not suppress mof^{1} lethality. Larval mof^{1} males are abundant, reasonably healthy and appear less severely affected than $roX1^{SMC17A}$ *roX2* or $roX1^{ex84A}$ *roX2* males, which produce escaping adults upon reversal of sex chromosome inheritance. Suppression may thus be gene-specific, rather than pathway specific. This is particularly interesting as the *roX* RNAs are central to recognition and modification of the X chromosome, but the molecular basis of *roX* activity is poorly understood. Short identity elements that are enriched on the X chromosome are proposed to underlie recognition (ALEKSEYENKO *et al.* 2008). It is possible that integration of *roX* RNA into the MSL complex promotes cooperative binding, favoring the modest enrichment of identity elements on the X chromosome. If this is indeed the case, the maternal Y chromosome might influence the ability of the complex to bind co-operatively. Although polytene

preparations provide no indication of increased X chromosome binding when a maternal Y chromosome is present, it remains possible that a critical difference in MSL localization is undetectable at the level of polytene preparations.

The maternal imprint on the Y chromosome might also act by increasing the enzymatic activity of defective MSL complexes lacking *roX* RNA. This is an attractive idea since increased expression of X-linked genes is detected by functional assay and qRT PCR. Mutational analysis of *roX1* has identified short repeats that are necessary for wild type levels of H4Ac16 modification by the MSL complex, but elimination of these repeats does not prevent selective recognition of the X chromosome (PARK *et al.* 2008). It is therefore possible that a maternally imprinted Y chromosome produces a factor that enables higher activity of the MSL complex in the absence of *roX* RNA.

Imprinting of the Y chromosome could also act by influencing the distribution of general chromatin proteins at a critical time during the establishment of dosage compensation. The Y chromosome is a sink for heterochromatin proteins (WEILER and WAKIMOTO 1995). An imprint on the Y chromosome could modulate its ability to bind these proteins. Mutations in the heterochromatin proteins HP1 and *Su(var)3-7* preferentially affect male survival and selectively disrupt the structure of the polytenized male X chromosome, but the morphology of polytene X chromosomes from *Su(var)3-7* males is not detectably altered by the Y chromosome imprint. This may reflect a limitation of analysis in salivary glands. The Y chromosome is a minor fraction of salivary gland chromatin since it is not polytenized in this tissue. It may be unable to

influence the distribution of heterochromatin proteins in this tissue. The link between heterochromatin and dosage compensation is intriguing. An understanding of the imprinted effect may inform these two epigenetic systems, but will require further study.

The mechanism by which a paternally imprinted Y chromosome enhances *roX1 roX2* lethality remains mysterious. Clues to the function of the paternally imprinted Y chromosome may lie in studies of hybrid lethality between closely related *Drosophila* species. Hybrid lethality prevents fertile offspring of interspecies crosses, and thus has an adaptive benefit (WU and TING 2004). Links between hybrid lethality and heterochromatin, as well as disruption of sex determination in interspecies progeny, have been reported (BRIDEAU *et al.* 2006) (PAL BHADRA *et al.* 2006). It is possible that *roX* mutations mimic a defect in dosage compensation that occurs in interspecies hybrids. In this model, the deleterious effect of a paternally transmitted Y chromosome has adaptive value as it lowers the survival of unfit hybrids. While this idea is highly speculative, it does address the counter intuitive observation that normal inheritance of the Y chromosome is, in a *roX1 roX2* male, quite deleterious to survival. Another possibility is that the paternal imprint enhances the regulatory ability of the Y chromosome. The Y chromosome influences a large number of autosomal and X-linked genes, particularly influencing those contributing to male fitness (LEMOS *et al.* 2008). Interestingly, genes responding to the Y chromosome tend to be divergently expressed between species. An imprint on the Y chromosome could

enhance its regulatory potential, perhaps affecting dosage compensation through an indirect mechanism.

Maternal transmission of the Y chromosome does not normally occur. The influence of a maternally imprinted Y chromosome consequently lacks adaptive significance. In spite of this, the potency of the maternal imprint as a modifier of *roX1 roX2* lethality suggests that it will be of value for dissecting the molecular mechanisms by which *roX* contributes to dosage compensation. *roX* RNA is required for full enzymatic activity of the MSL complex, as well as the exclusive localization of this complex to the X chromosome. Any genetic modifier of the *roX1 roX2* phenotype is thus of great interest.

Chapter 4

A Role for siRNA in X Chromosome Dosage Compensation in *Drosophila melanogaster*

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INTRODUCTION

Males of many species carry a euchromatic, gene-rich X chromosome and a gene-poor, heterochromatic Y chromosome (CHARLESWORTH 1991). This creates a potentially lethal imbalance in the X to autosomal (X:A) ratio in one sex (GUPTA *et al.* 2006; NGUYEN and DISTECHE 2006; DENG *et al.* 2011). Dosage compensation is an essential process that equalizes X-linked gene expression between XY males and XX females, thereby maintaining a constant ratio of X:A gene products. Strategies to accomplish this differ between species, but share the need for coordinated regulation of an entire chromosome (LUCCHESI *et al.* 2005). In flies, the Male-Specific Lethal (MSL) complex, composed of five Male-Specific Lethal (MSL) proteins and non-coding *roX* (*RNA on the X chromosome*) RNA, binds with great selectivity to the X chromosome of males (ZHANG *et al.* 2006). The MSL complex directs H4K16 acetylation to the body of X -linked genes, increasing transcription by enhancing RNA polymerase II processivity (SMITH *et al.* 2001; LARSCHAN *et al.* 2011).

Recruitment of the MSL complex is postulated to occur at X-linked Chromatin Entry Sites (CES) (KELLEY *et al.* 1999; ALEKSEYENKO *et al.* 2008; STRAUB *et al.* 2008). CES contain 21 bp MSL Recognition Elements (MREs), which are modestly enriched on the X chromosome (ALEKSEYENKO *et al.* 2008). The MSL complex then spreads to nearby transcribed genes (LARSCHAN *et al.* 2007; SURAL *et al.* 2008). While this model elegantly describes the local distribution of the MSL complex, it fails to explain the exclusive recognition of X chromatin that is a hallmark of Drosophila dosage compensation.

The initiation of dosage compensation and hypertranscription of X-linked genes is dependent on *roX* RNA (MELLER 2003; DENG and MELLER 2006b). The X-linked *roX* genes, *roX1* and *roX2*, are redundant for these functions (MELLER and RATTNER 2002). Mutation of a single *roX* gene is without phenotype, but simultaneous mutation of *roX1* and *roX2* reduces X-localization of the MSL complex, resulting in a reduction in X-linked gene expression and male-specific lethality (MELLER and RATTNER 2002; DENG and MELLER 2006b).

Because the *roX* RNAs are necessary for exclusive X-localization of the MSL proteins, genetic modifiers of *roX1 roX2* lethality may identify novel pathways that contribute to X-recognition. We previously reported that a maternally imprinted Y chromosome is a potent suppressor of *roX1 roX2* lethality (MENON and MELLER 2009). The expression of Y-linked protein-coding genes is restricted to the germline, making it unlikely that these genes influence the somatic process of dosage compensation. Furthermore, the Y chromosome itself is non-essential for dosage compensation (LUCCHESI 1973). We postulate that in

spite of the fact that Y-linked genes are unnecessary for dosage compensation, the Y chromosome imprint modulates a pathway involved in this process.

Repetitive sequences, which are abundant on the Y chromosome, have been proposed to influence somatic gene expression (LEMOS *et al.* 2008; JIANG *et al.* 2010; LEMOS *et al.* 2010; PIERGENTILI 2010). Small RNA pathways are potential mediators of this effect. To pursue the idea that small RNA might play a role in dosage compensation, we conducted a directed screen of RNAi pathways. Mutations in the siRNA pathway were found to enhance *roX1 roX2* lethality. siRNA mutations disrupt localization of the MSL complex in *roX1 roX2* mutants and partially rescue female flies that inappropriately dosage compensate, leading to toxic over expression of X-linked genes. Our findings are consistent with participation of siRNA in recognition of X chromatin.

MATERIALS AND METHODS

Fly culture and genetics:

Flies were maintained at 25° on standard cornmeal-agar fly food. Unless otherwise noted, mutations are described in Lindsley and Zimm (LINDSLEY and ZIMM 1992a). *roX1* mutations, and a complex *roX2* deletion (Df(1)52; [$w^+4\Delta4.3$]) have been described (MELLER *et al.* 1997; MELLER and RATTNER 2002; DENG *et al.* 2005). A viable deletion of *roX2* (*roX2*Δ) was accomplished by FLP-mediated recombination between CG11695^{f01356} and nod^{f04008}. Description of *dcr2*^{f06544}, *ago2*^{dop1}, *ago2*^{414}, *r2d2*^{1}, *D-elp1*^{c00296}, *loqs*^{f00791}, *ago1*^{k00281}, *spn-E*^{1}, *aub*^{QC42},

aub^{HN}, *piwi*^{06843} can be found at http://flybase.org. *ago2*^{414} was provided by R. Carthew, all other mutations were provided by the Bloomington Drosophila Stock Center.

RNAi mutations were outcrossed for 6 generations to minimize genetic background effects. All stocks were constructed with the Y chromosome from the laboratory reference *yw* strain to eliminate confounding effects attributable to different Y chromosomes that we, and others, have observed (LEMOS *et al.* 2008). After rebalancing, all mutations were confirmed by PCR or phenotype. Matings to determine the effect of RNAi pathway mutations on *roX1*^{ex33} *roX2*Δ male and *yw* female survival are detailed in Fig. 4.1.

Immunostaining:

Polytene chromosome preparations were immunostained for MSL1 as previously described (KELLEY *et al.* 1999)**.** Between 150 and 300 nuclei of each genotype were scored for MSL1 recruitment to the X chromosome and ectopic sites. Genotypes were obscured during scoring to eliminate bias. Categories of MSL1 recruitment are detailed in SI Tables 2 A-C.

Western blot:

Protein blotting was performed on extracts from groups of 10 or 20 adult males homogenized in 100 or 200 µl of Laemmli buffer with 1mM PMSF. Homogenates were boiled and centrifuged at 10,000 rpm for 2 min to obtain crude lysates. Equal volumes of lysate were separated on 7% SDS-polyacrylamide gels and transferred to nitrocellulose (Micron Separations Inc.).

Membranes were blocked with 0.5% fish gelatin and 2-5% BSA in PBST or TBST. Primary and secondary antibodies were diluted in the respective blocking solutions. Primary antibodies to MSL1, MSL2, MSL3 and MLE were a gift from M. Kuroda. Antibodies to β tubulin and dFMR1 are from the Developmental Studies Hybridoma Bank. Alkaline phosphatase conjugated secondary antibodies (Sigma) were used for detection by NBT/BCIP chromogenic system.

qRT-PCR:

Accumulation of *roX1*^{ex40} transcript was measured by qRT PCR as previously described (DENG *et al.* 2009a)**.** Briefly, RNA was prepared from three groups of 50 third instar male larvae. One μg of RNA was reverse transcribed using random hexamers and ImProm-II reverse transcriptase (Promega). Two technical replicates of each biological replicate were amplified with 300 nM of primers TTTTTGTCCCACCCGAATAA and CCTTTTAATGCGTTTTCCGA. Expression of *roX1*^{ex40} was normalized to autosomal *Dmn*, amplified with 300 nM of primers GACAAGTTGAGCCGCCTTAC and CTTGGTGCTTAGATGACGCA.

RESULTS

Genetic interaction of RNAi and *roX1 roX2*

The *roX1*^{ex33} *roX2*Δ X chromosome supports about 20% eclosion of adult male escapers. *roX1*^{ex33} *roX2*Δ females were mated to males heterozygous for mutations in the piRNA, siRNA and miRNA pathways (*RNAi* ¯/+). The survival of sons with reduced RNAi function (*roX1*^{ex33} *roX2*Δ ; *RNAi* ¯/+) was divided by that of their brothers with intact RNAi (*roX1*^{ex33} *roX2*Δ ; +/+) to reveal enhancement or suppression of male lethality.

Mutations in *dcr-2, ago2, loqs* and *D-elp1* were found to lower the survival of *roX1*^{ex33} *roX2*Δ males by 30%, 55%, 50% and 70%, respectively (Fig. 4.2A). Dcr-2 and D-Elp1 play a role in endogenous siRNA (endo-siRNA) production and transposon silencing, and Ago2 is a member of the RNAi-induced silencing complex (RISC) (CARTHEW and SONTHEIMER 2009; LIPARDI and PATERSON 2009; SIOMI and SIOMI 2009). While *loqs* has a prominent role in miRNA biogenesis, an isoform of Loqs has been implicated in the biogenesis of endo-siRNA from structured loci and transposons (OKAMURA *et al.* 2008; ZHOU *et al.* 2009; MARQUES *et al.* 2010). All of the candidate genes therefore affect siRNA production or function. Reduction of the canonical siRNA gene *r2d2* did not enhance *roX1 roX2* male lethality. R2D2 affects strand selection during loading of siRNA onto Ago2 (LIU *et al.* 2003; TOMARI *et al.* 2004). It is possible that this is unnecessary for dosage compensation, or that the level of R2D2 is not limiting when a single copy of the gene is mutated.

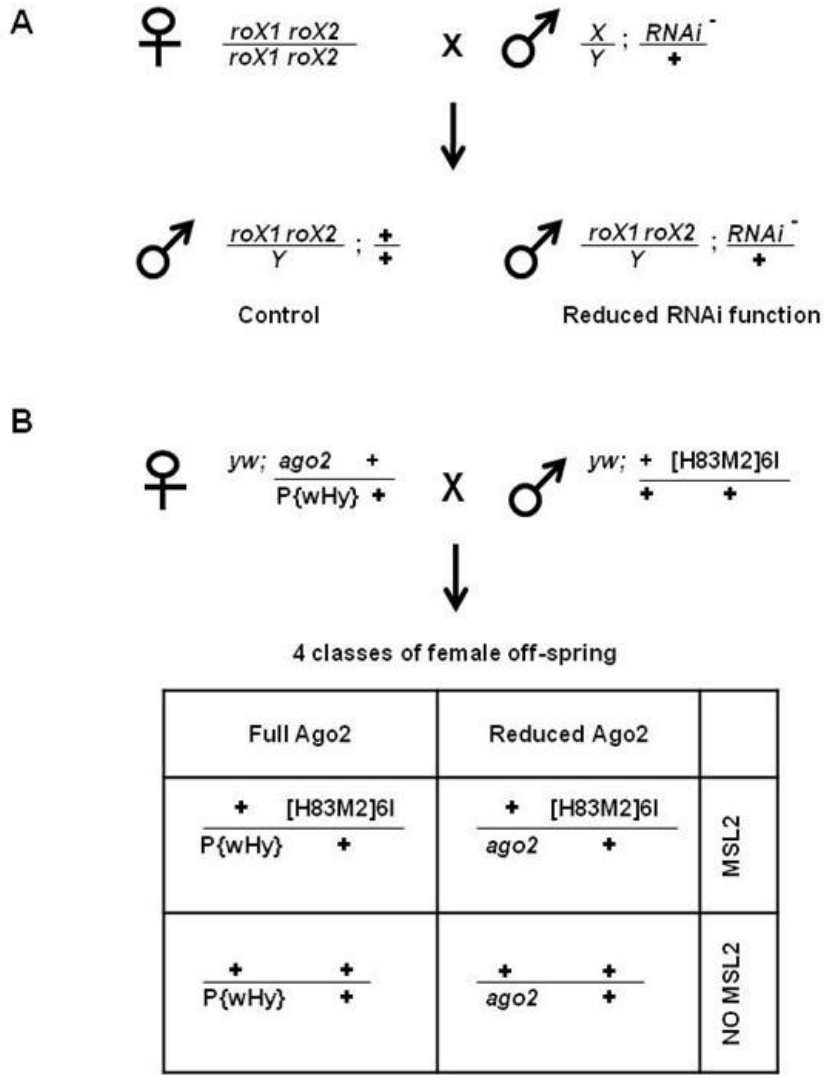


Figure 4.1. Matings to determine effect of RNAi mutations on male and female survival. (A) Screen for genetic interaction between *roX1*^{ex33} *roX2*Δ and RNAi mutants. *roX1*^{ex33} *roX2*Δ females were mated to males heterozygous for RNAi mutations, producing *roX1*^{ex33} *roX2*Δ sons with wild type (control) and reduced RNAi (experimental). (B) Mating performed to determine the effect of reduced Ago2 on female development. Females with a *yw* (wild type) X chromosome and trans-heterozygous for *ago2*^{414} and P{wHy}^{DG23507}, a marker situated 5 kb proximal to *ago2*, were mated to males heterozygous for [H83M2]6I. Equal numbers of the four classes of female zygotes are predicted. Daughters inheriting [H83M2]6I express MSL2, leading to developmental delay (top row); presented in Fig. 1B. Their sisters lacking [H83M2]6I (bottom row) are plotted in Fig. 1C. Daughters with full Ago2 (left) and their sisters with reduced Ago2 (right) were distinguished by *y*$^{+}$, present in P{wHy}.

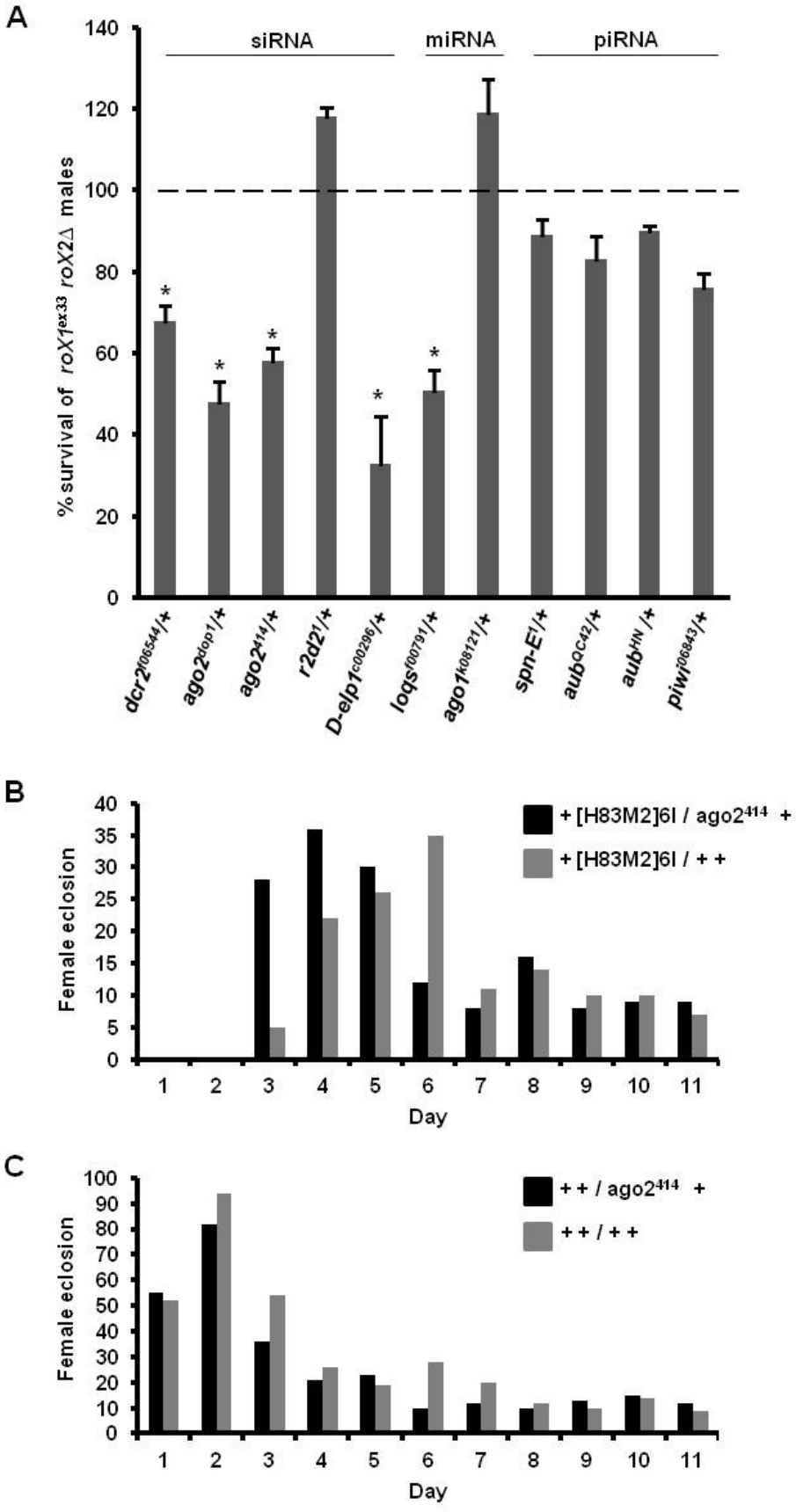


Figure 4.2. siRNA mutations enhance *roX1 roX2* male lethality. (A) Eclosing *roX1*^{ex33} *roX2*Δ males carrying RNAi mutations divided by their brothers with full RNAi function. SEM is represented by error bars. * Students two sample t-test significance ≤ 0.05. (B) Ago2 reduction partially rescues the developmental delay of females expressing MSL2. Females carry the [H83M2]6I transgene and express MSL2. Black bars represent females heterozygous for *ago2*^{414}; gray bars represent females with wild type *ago2*. (C) Ago2 reduction does not influence the eclosion of otherwise wild type females. Black bars depict females heterozygous for *ago2*^{414}; gray bars are their sisters with wild type *ago2*.

To confirm that siRNA selectively affects dosage compensation, we asked whether reduction of Ago2 rescued females that inappropriately deploy the dosage compensation machinery, leading to toxic over expression of both X chromosomes. Ectopic expression of *male-specific lethal 2* (*msl2*) induces dosage compensation in females (KELLEY *et al.* 1995). MSL2 expression, driven by the [H83M2]6I transgene, reduces female survival and delays the peak of eclosion until day 6 (gray bars, Fig. 4.2B;(KELLEY *et al.* 1995)). In contrast, eclosion of sisters not expressing MSL2 peaks on day 2 (gray bars, Fig. 4.2C). Eclosion of [H83M2]6I females with one mutated *ago2* allele is advanced by two days, peaking on day 4 (black bars, Fig. 4.2B). Reduction of Ago2 in otherwise wild type females had no discernable effect on eclosion timing (Fig. 4.2C). The enhancement of *roX1 roX2* male lethality by siRNA mutations and partial rescue of MSL2-expressing females by reduction of Ago2 identifies a role for small RNA in Drosophila dosage compensation.

Mutations in siRNA pathway reduces *roX1*^{ex40A}*roX2*Δ male survival

The *roX1*^{ex40} internal deletion mutant supports full male survival, presumably because it retains essential 5' and 3' *roX1* regions in a transcript of reduced size (DENG *et al.* 2005). Localization of the MSL complex on polytene chromosomes of *roX1*^{ex40} *roX2*Δ males is similar to that observed in wild type flies. *roX1*^{ex40} therefore has a molecularly detectable but sub-phenotypic defect. Loss of Ago2 has no effect on male survival by itself, but when Ago2 is eliminated in *roX1*^{ex40} *roX2*Δ males, survival is reduced to 8% (Fig. 4.3A). Loss of Loqs reduces *roX1*^{ex40} *roX2*Δ male survival by over 50% (Fig. 4.3B). *roX1*^{ex40}

*roX2*Δ males with reduced D-Elp1 levels have full viability, but *D-elp1* lethality precludes homozygote testing. We took advantage of the synthetic lethality between *roX1*^{ex40} *roX2*Δ and siRNA mutations to explore how siRNA contributes to dosage compensation.

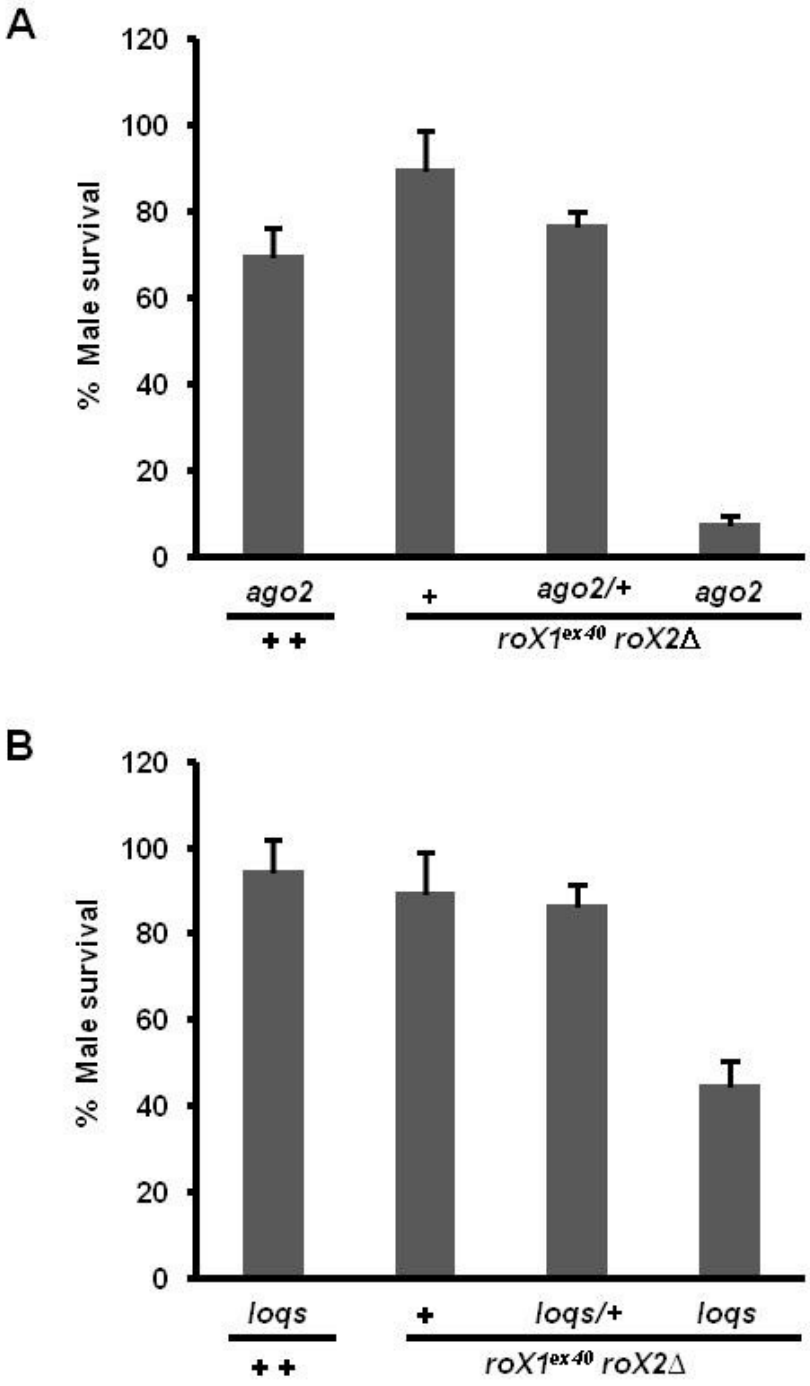


Figure 4.3. *roX1*^{ex40A} *roX2*Δ is synthetic lethal with siRNA mutations. (A) Loss of Ago2 reduces the survival of *roX1*^{ex40} *roX2*Δ adult males. The number of males recovered was: *ago2*^{414}, 245; *roX1*^{ex40} *roX2*Δ, 274; *roX1*^{ex40} *roX2*Δ*; ago2*^{414}/+, 1356 and *roX1*^{ex40} *roX2*Δ*; ago2*^{414}, 45. (B) Loss of Loqs reduces *roX1*^{ex40} *roX2*Δ adult male survival. The total number of males recovered was: *loqs*^{f00791}, 230; *roX1*^{ex40} *roX2*Δ, 274; *roX1*^{ex40} *roX2*Δ*; loqs*^{f00791}/+, 708 and *roX1*^{ex40} *roX2*Δ*; loqs*^{f00791}, 166. Survival of *roX1*^{ex40} *roX2*Δ*; ago2*^{414} and *roX1*^{ex40} *roX2*Δ*; loqs*^{f00791} males was determined by mating *roX1*^{ex40} *roX2*Δ*; ago2*^{414} /TM3*SbTb* males and females, or *roX1*^{ex40} *roX2*Δ*; loqs*^{f00791}/ In*(*2LR*)Bc Gla* males and females. Survival of *ago2*^{414} and *loqs*^{f00791} males was determined by observation of *yw; ago2*^{414} /TM3*SbTb* and *yw; loqs*^{f00791}/ In*(*2LR*)Bc Gla* stocks.

To address the possibility that siRNA mutations act by modulating the level of *roX* RNA, qRT PCR was used to measure *roX1*^{ex40} transcript in *ago2*^{414} or *D-elp1*^{c00296}/+ males. Accumulation of *roX1*^{ex40} RNA was unaffected by these mutations (Fig. 4.4A). We also considered the possibility that siRNA indirectly influences the level of an MSL protein. Protein blotting revealed no reduction in core members of the MSL complex in males lacking Ago2, or reduced for D-elp1 (Fig. 4.4C - F). This conclusion is supported by whole genome expression studies in S2 cells following Ago2 knock down (REHWINKEL *et al.* 2006). As suggested by the lack of a male phenotype, the *roX1*^{ex40} *roX2*Δ chromosome did not itself affect MSL protein levels (Fig. 4.4C - F). Disruption of dosage compensation in *roX1 roX2* males with reduced siRNA therefore does not involve reduction in the core components of the MSL complex.

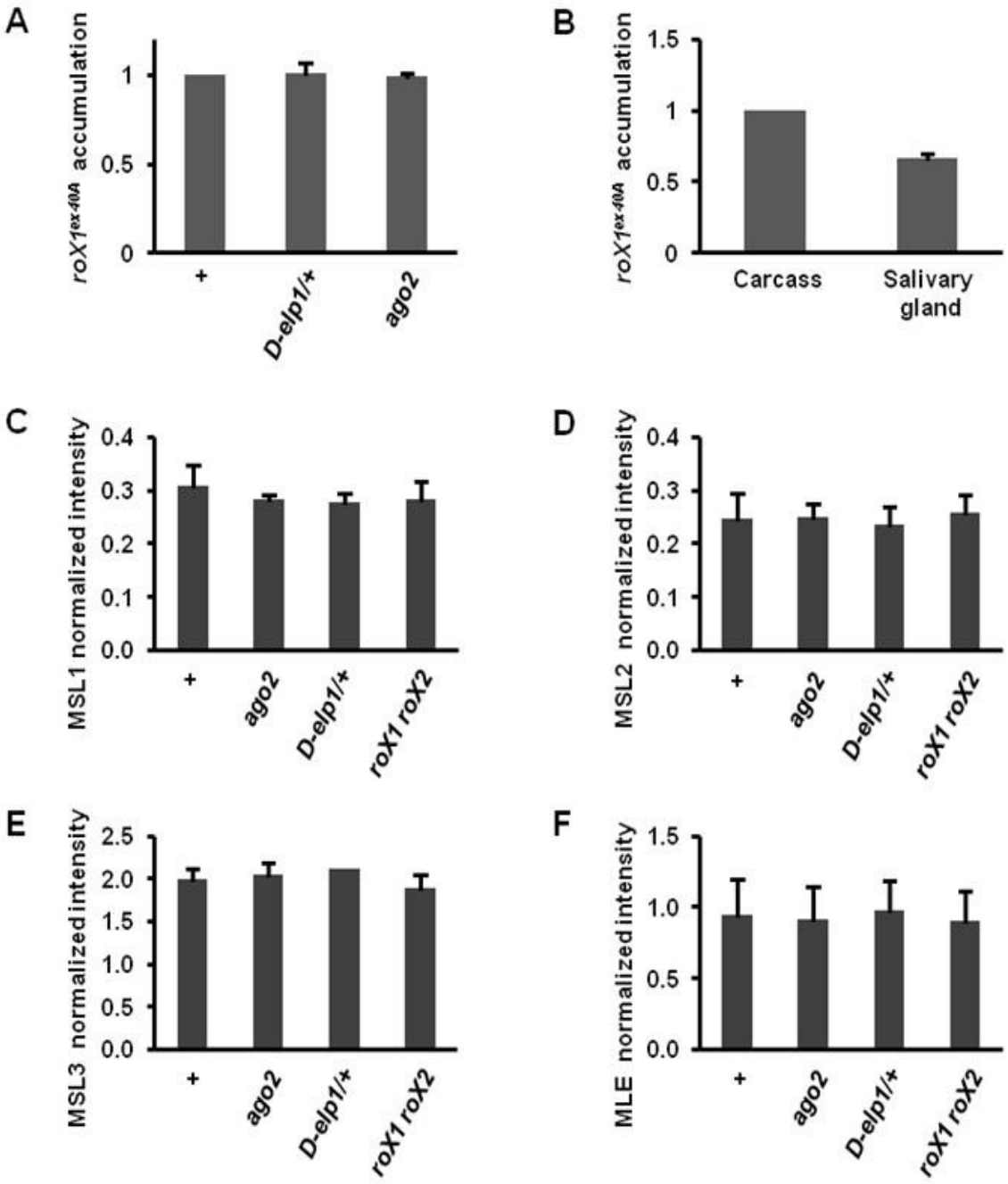


Figure 4.4 Mutation of *ago2* or *D-elp1* does not affect accumulation of molecules in the MSL complex. (A) Accumulation of *roX1*^{ex40} transcript was determined in *roX1*^{ex40} *roX2*Δ (value set to 1), *roX1*^{ex40} *roX2*Δ*; D-elp1*^{c00296}/+ and *roX1*^{ex40} *roX2*Δ*; ago2*^{414} male larvae by quantitative RT-PCR (qRT PCR). (B) Accumulation of *roX1*^{ex40} transcript in salivary glands and carcass (value set to 1) of *roX1*^{ex40} *roX2*Δ male larvae. Expression in A and B is normalized to the autosomal gene *Dmn*. Bars represent the standard error of three biological replicates. (C–F) Quantification of MSL levels from protein blots of (C) MSL1 (n=2), (D) MSL2 (n=4), (E) MSL3 (n=3) and (F) MLE (n=3) in wild type, *ago2*^{414} , *D-elp1*^{c00296}/+ and *roX1*^{ex40A} *roX2*Δ adult males. β-tubulin and d-FMR1 were the loading controls. Quantification was performed by scanning blots and integrating signal density using Image J software (http://rsbweb.nih.gov/ij/). Protein signal was normalized to loading controls. A dilution series established that signal remained within linear range.

Mutations in siRNA affect MSL localization

The synthetic lethality between *roX1*^{ex40} *roX2*Δ and siRNA mutations suggested that siRNA could contribute to X-identification, or to recruitment of the MSL complex to the X chromosome. If this is the case, loss of siRNA alone might disrupt MSL localization, which is exclusive to the X chromosome in wild type males (Fig. 4.5A). Reduction of D-Elp1 did not discernibly affect MSL1 localization to the polytene X chromosome of otherwise wild type males (Fig. 4.5B). A slight disruption of X-localization was detected in *ago2* mutants, but this was only marginally higher than that observed in wild type controls (Fig. 4.5B, C and E; Table 4.1).

Ectopic MSL1 binding on the autosomes, at the chromocenter, and at the telomeres is a sensitive metric for disruption of MSL localization. Although MSL1 recruitment in *roX1*^{ex40} *roX2*Δ males is superficially similar to wild type, examination of a large number of nuclei revealed a reduction of MSL recruitment to the X chromosome in some nuclei, and elevated ectopic localization, particularly at the chromocenter (Fig. 4.5B and C; Table 4.1). This supports the idea that *roX1*^{ex40} has a defect in function. However, mislocalization of MSL1 was notably more severe in chromosome preparations from *roX1*^{ex40} *roX2*Δ*; ago2*^{414} and *roX1*^{ex40} *roX2*Δ*; D-elp1*^{c00296}/+ males. The number of nuclei exhibiting minimal or no recruitment of MSL1 to the X chromosome is enhanced over 3 fold by the loss or reduction of these siRNA proteins (Fig. 4.5E). These same genotypes displayed a three-fold increase in ectopic autosomal MSL1 localization (Fig. 4.5D, F and G; Table 4.1).

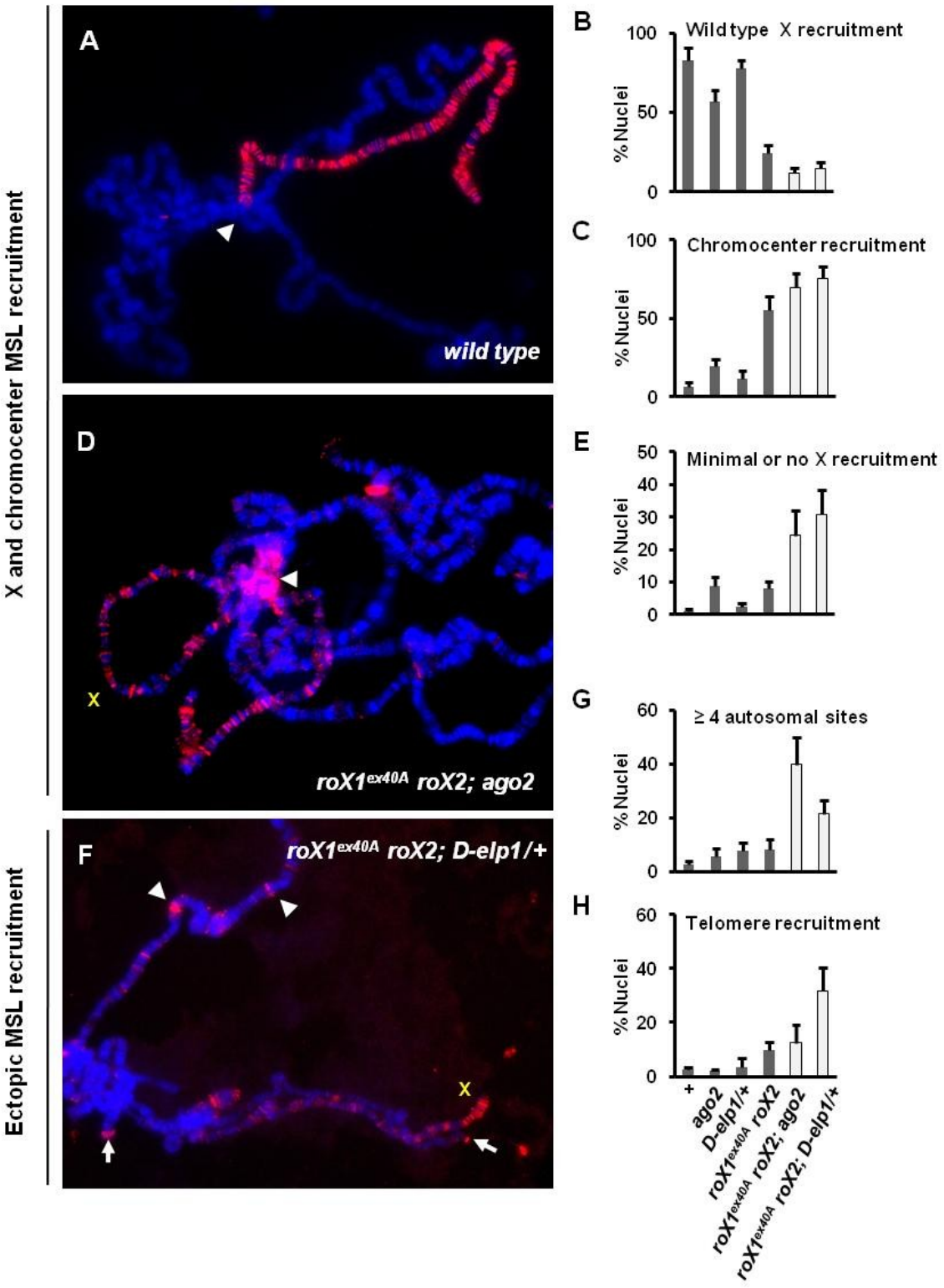


Figure 4.5. MSL1 localization is disrupted in *roX1^{ex40} roX2*Δ males mutated for *ago2* or *D-elp1*. (A) MSL1 localization is exclusive to the X chromosome in a polytene preparation from a wild type male larva. (B) Percentage of nuclei of each genotype that display wild type MSL1 recruitment to the X chromosome. (C) Percentage of nuclei with ectopic MSL1 binding at the chromocenter (compare arrowheads, A, D). (D) Minimal MSL1 recruitment to the X chromosome and strong chromocenter recruitment in a *roX1^{ex40} roX2*Δ*; ago2^{414}* male. (E) Percent nuclei with minimal or no MSL1 recruitment to the X chromosome (Sum of categories "+" and "no MSL recruitment", SI Table 4A). (F) Ectopic autosomal MSL1 binding in a *roX1^{ex40} roX2*Δ*; D-elp1/+* male. (G) Percentage of nuclei with ≥ 4 distinct autosomal MSL1 binding sites (arrowheads in F). (H) Percentage of nuclei with MSL1 recruitment to a telomere (arrows in F). Polytene chromosome preparations were immunostained for MSL1 as previously described (KELLEY *et al.* 1999). MSL1 is detected by Texas Red, DNA is detected by DAPI. One hundred fifty to 300 nuclei of each genotype were scored for MSL1 recruitment. Genotypes were obscured during scoring to eliminate bias. Full genotypes are: *yw* reference strain (wild type). *ago2^{414}*. *D-elp1^{c00296}/+*. *roX1^{ex40} roX2*Δ. *roX1^{ex40} roX2*Δ; *ago2^{414}* (white bars). *roX1^{ex40} roX2*Δ; *D-elp1^{c00296}/+* (white bars). SEM is depicted by error bars. Categories of MSL1 recruitment are detailed in Table 4.1.

Despite increased mislocalization of the MSL complex, *roX1*^{ex40} *roX2*Δ*; D-elp1*^{c00296}/+ male viability appears unaffected, and the viability of *roX1*^{ex40} *roX2*Δ males with reduced levels of Ago2 or Loqs is also high (Fig. 4.3A, B). It is possible that this disparity is because the accumulation of mutated *roX1* transcripts, including *roX1*^{ex40}, is lower in the salivary gland than in other tissues (Fig. 4.4B, see Figure 3 in (DENG *et al.* 2005). In spite of reduced transcript in the salivary gland, the *roX1*^{ex40A} mutant direct considerable X-localization of the MSL complex, in accord with the ability of *roX1*^{ex40} *roX2*Δ males to tolerate a partial, but not a complete, reduction in RNAi. Taken together, these studies reveal a role for siRNA in the process of dosage compensation in *Drosophila*. The genetic interaction between mutations affecting siRNA and *roX1 roX2* chromosomes, as well as enhancement of ectopic MSL mislocalization, suggests that siRNA contributes to X recognition or chromatin binding of the MSL complex.

Table 4.1. MSL1 recruitment to polytene chromosomes

A

X chromosome MSL1 recruitment	+	*ago2*	*D-elp1 /+*	*roX1 roX2*	*roX1 roX2; ago2*	*roX1 roX2 ; D-elp1/+*
++++	82.66 (177)	56.96 (156)	77.76 (138)	24.13 (81)	11.86 (28)	14.73 (22)
+++	16.33 (26)	34.35 (113)	19.84 (36)	65.90 (184)	63.77 (141)	54.49 (84)
+	0.62 (1)	8.07 (18)	2.4 (5)	7.05 (18)	19.47 (36)	26.96 (42)
No stain	0.39 (1)	0.62 (2)	0 (0)	1.03 (4)	4.91 (11)	3.83 (6)
Total nuclei counted	205	289	179	287	216	154

B

Chromocenter MSL1 recruitment	+	*ago2*	*D-elp1 /+*	*roX1 roX2*	*roX1 roX2; ago2*	*roX1 roX2 ; D-elp1/+*
No recruitment	93.54 (192)	80.22 (223)	88.76 (161)	44.93 (119)	30.21 (56)	24.64 (37)
Recruitment	6.46 (15)	19.78 (59)	11.23 (18)	55.07 (163)	69.79 (153)	75.36 (116)
Total nuclei counted	207	282	179	282	209	153

C

Ectopic MSL1 recruitment	+	*ago2*	*D-elp1 /+*	*roX1 roX2*	*roX1 roX2; ago2*	*roX1 roX2 ; D-elp1/+*
No autosomal recruitment	67.83 (134)	73.36 (194)	55.88 (110)	61.48 (168)	35.92 (67)	52.70 (79)
1-2 autosomal bands	14.70 (59)	18.89 (63)	36.49 (58)	30.15 (89)	24.29 (51)	25.88 (40)
≥4 autosomal bands	2.89 (14)	5.81 (17)	7.63 (11)	8.37 (25)	39.80 (91)	21.43 (34)
telomere recruitment	2.59 (8)	1.95 (8)	3.58 (6)	9.70 (29)	12.48 (37)	31.79 (48)
Total nuclei counted	215	282	185	311	246	201

Scoring of polytene nuclei stained for MSL1 from wild type (+), *ago2*^{414}, *roX1*^{ex40} *roX2*Δ, *roX1*^{ex40} *roX2*Δ; *ago2*^{414}/+ and *roX1*^{ex40} *roX2*Δ; *D-elp1*^{c00296} /+ male larvae. (A) MSL1 recruitment to X chromosome is categorized as ++++ (wild type), +++ (moderate) and + (minor). Examples of ++++ and + are presented in Fig. 3. (B) MSL1 recruitment to the chromocenter. (C) MSL1 recruitment to ectopic autosomal sites and telomeres. The percentage of nuclei in each category is represented, followed by the total number of nuclei in parentheses.

DISCUSSION

Small RNA has been implicated in numerous chromatin-based processes, but the present study is the first to link small RNA to *Drosophila* dosage compensation. Small RNA typically acts through gene silencing (PAL-BHADRA *et al.* 2004; VERDEL *et al.* 2004; BROWER-TOLAND *et al.* 2007; WANG and ELGIN 2011). For example, *Ago2* and *Dcr2* mutations suppress position effect variegation (PEV) in flies, suggesting a function in heterochromatic repression (DESHPANDE *et al.* 2005; FAGEGALTIER *et al.* 2009). Ago2 and Dcr2 exert a repressive effect on expression of euchromatic genes by modulating transcriptional elongation (CERNILOGAR *et al.* 2011). In contrast, dosage compensation selectively elevates transcription of a large portion of the fly genome. The siRNA mutations examined in this study dramatically enhance the male-specific lethality of *roX1 roX2* chromosomes and promote delocalization of the MSL complex from the X chromosome. This suggests that siRNA modulates the stability of MSL binding, or contributes to recognition of the X chromosome. While evidence that Ago2, or other siRNA factors, directly activate gene expression is lacking, a few studies have demonstrated increased silencing at some loci upon loss of Ago2 and Piwi (YIN and LIN 2007; MOSHKOVICH and LEI 2010). It is possible that siRNA influences dosage compensation not through direct action at compensated genes, but by contributing to interphase chromosome architecture or organization of the nucleus. This would be consistent with the role of RNAi at insulators (LEI and CORCES 2006; MOSHKOVICH

et al. 2011). Intriguingly, the male X chromosome displays an interphase conformation distinct from that in females (GRIMAUD and BECKER 2009).

Chapter 5

siRNA from 1.688 g/cm^3 satellite-related repeats promote *Drosophila melanogaster* dosage compensation

This chapter is organized as manuscript in preparation (MENON, D. U., et al. in preparation)

INTRODUCTION

Males and females of many species have an unequal number of X chromosomes, producing a potentially fatal imbalance in X-linked gene expression (DISTECHE 2012). The process by which balance is restored is called dosage compensation. In the male fruit fly, *Drosophila melanogaster* the Male-Specific Lethal (MSL) complex modifies the chromatin of X-linked genes to increase expression two-fold, equalizing expression between XX females and XY males. The long non-coding *roX1* and *roX2* RNAs assemble with the MSL proteins to form the intact MSL complex. The *roX* RNAs are required for exclusive X-chromosome binding of the complex, and for increased expression of X-linked genes (MELLER and RATTNER 2002; DENG and MELLER 2006b). How the MSL complex selectively recognizes X chromatin is unclear, but an elegant model for X recognition proposes that the MSL complex binds first to Chromatin Entry Sites (CES) and then spreads to neighboring transcribed genes (GELBART and KURODA 2009). The CES are limited to the X chromosome and defined by their elevated affinity for MSL proteins (KELLEY *et al.* 1999). A 21 bp motif, termed the MSL Recognition Element (MRE), is enriched within the CES

(ALEKSEYENKO *et al.* 2008). However, MREs are only modestly enriched on the X chromosome, indicating that additional factors contribute to X-identification and formation of the CES.

We recently reported that the siRNA pathway contributes to X chromosome binding by the MSL complex (MENON and MELLER 2012). This raises the possibility that siRNA-producing sequences that are limited to the X chromosome might participate in identification of X chromatin. The X-limited distribution of related 1.688 g/cm^3 satellite-related repeats (1.688^X repeats) has previously prompted speculation that they participate in dosage compensation (WARING and POLLACK 1987; DIBARTOLOMEIS *et al.* 1992). These ~359 bp repeats are arranged in short tandem arrays, and, unlike most satellite repeats, favor transcriptionally active regions (KUHN *et al.* 2012). Many 1.688^X repeats are within or flanking genes, and many are transcribed. In this study we investigate the role of long and short RNA from 1.688^X repeats in dosage compensation. Both forms of RNA are detected in wild type larvae. We find that ectopic expression of long single stranded RNA (ssRNA) and hairpin double stranded RNA (hpRNA) from 1.688^X repeats influences dosage compensation, but in opposing manners. Expression of 1.688^X ssRNA lowers the survival of *roX1 roX2* males. In contrast, 1.688^X hpRNA is processed into abundant small RNAs that partially rescue *roX1 roX2* male survival and MSL localization to the X chromosome. We propose that siRNA from the 1.688^X repeats participates in dosage compensation by targeting small RNA effectors to homologous

sequences on the X chromosome. The 1.688^{X} repeats are thus candidates for the elusive X-identity elements that direct dosage compensation in flies.

MATERIALS AND METHODS

Fly culture and genetics:

Flies were maintained at room temperature on standard cornmeal-agar fly food. Unless otherwise noted, mutations are described in Lindsley and Zimm (LINDSLEY and ZIMM 1992b). Descriptions of *dcr2*^{L811fsX}, *ago2*^{414}, *Sco* (*sna*^{Sco}) and *R*^{1} can be found at (www.flybase.org). Expression of hpRNA was driven by the ubiquitous [Gal4-*tub*] driver (Bloomington # 5140). *roX1* mutations and a viable deletion of *roX2* (*roX2*Δ) have been previously described (DENG *et al.* 2005; MENON and MELLER 2012). Matings to determine male survival are detailed in Fig. 5.1 and 5.2.

Generation of 1.688^{X} repeat transgenics:

Satellite repeats were amplified from a *y*^{1} *w*^{1118} laboratory reference strain using primers with BamHI and Sac II linkers (Table 5.1). Transgenes expressing single stranded RNA from 1.688^{X} repeats ([ss 1.688^{X}]) were generated by introducing 2.0kb (1.688^{roX1}) or 800 bp (1.688^{3C}) into pUASTB that had been modified by addition of the hsp83 promoter (GROTH *et al.* 2004). Transgenics were generated by ΦC31-mediated site-specific recombination at 51C (sense-strand constructs) and 58A (anti-sense constructs) (Rainbow Transgenics, Inc., Camarillo CA).

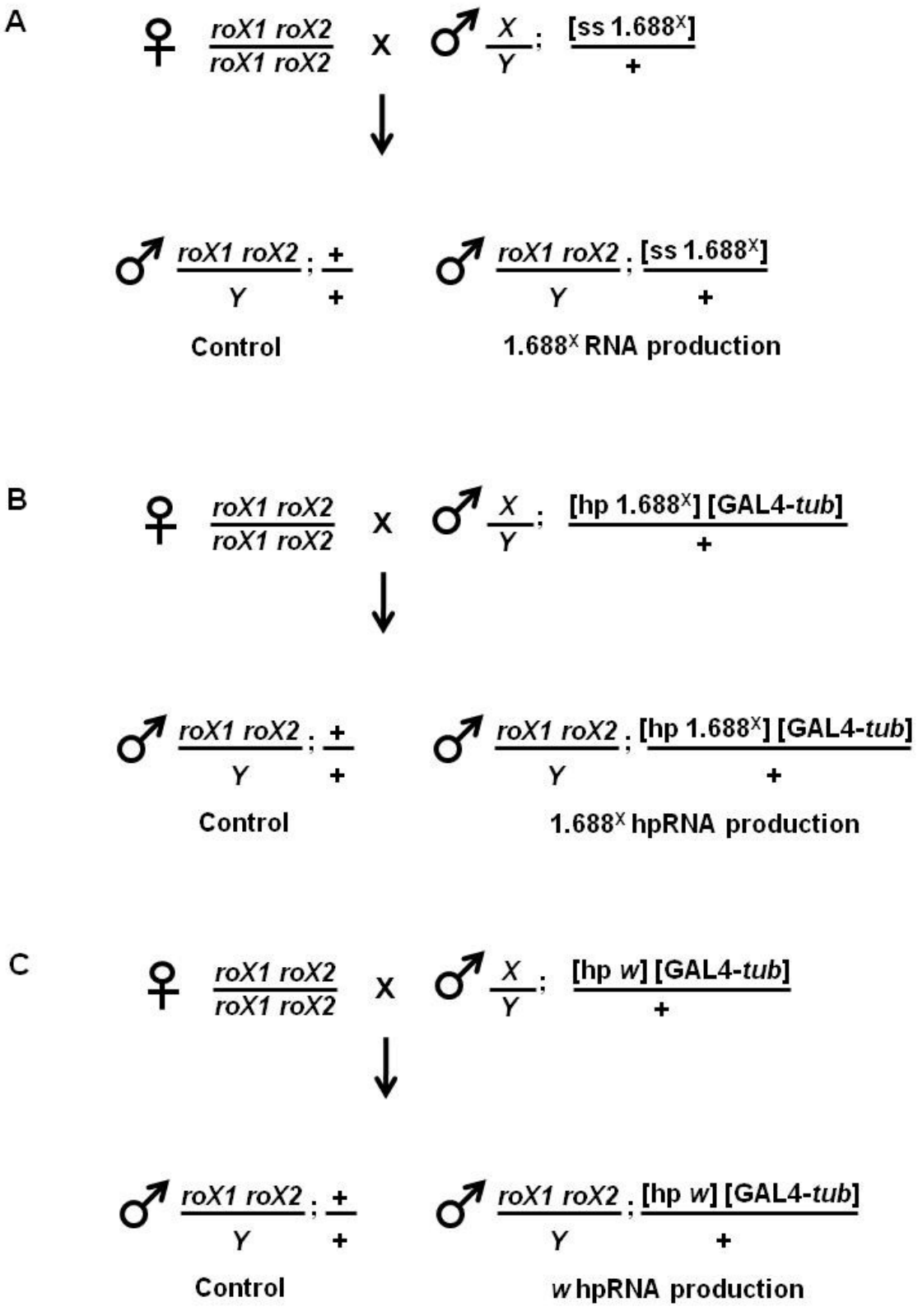


Figure 5.1. Matings to determine effect of 1.688^{X} long and hairpin RNA on *roX1 roX2* male survival (A) Matings to determine the effect of expression of 1.688^{X} sense and anti-sense RNA. Simultaneous expression of sense and anti-sense RNA is achieved by generating recombinant chromosomes with two transgenes. *roX1^{ex33} roX2*Δ females were mated to males heterozygous for [ss 1.688^{X}] insertions. All sons are *roX1^{ex33} roX2*Δ. Those ectopically expressing 1.688^{X} ssRNA are distinguished from brothers lacking the 1.688^{X} transgene (control) by eye color (B) Expression of 1.688^{X} hairpin RNA (hpRNA) under the control of a *tubulin* driver. *roX1^{ex33} roX2*Δ, *roX1^{SMC17A} roX2*Δ, *roX1^{ex6} roX2*Δ, *roX1^{VM18A} roX2*Δ and wild type females were mated to males heterozygous for the [hp 1.688^{x}] [GAL4- tub] chromosome. Sons expressing hp1.688^{X} RNA are distinguished from control brothers by eye color. (C) Expression of *w* hpRNA. [hp *w*] in *roX1 roX2*Δ sons.

A

♀ *roX1 roX2*; *dcr2*^{L811fsx} X ♂ *yw*; $\frac{dcr2^{L811fsx}}{Sco}$; $\frac{[hp\ 1.688^{roX1}]\ [GAL4\text{-}tub]}{+}$

↓

Male and female progeny

1.688^{roX1} hpRNA / Dcr2	-	+
- / -	♀ = 141 ♂ = 0	♀ = 122 ♂ = 29
+ / -	♀ = 135 ♂ = 10	♀ = 126 ♂ = 67

B

♀ *roX1 roX2*; $\frac{[UAS\text{-}Ago2kd]}{+}$ X ♂ *yw*; $\frac{[hp\ 1.688^{roX1}]\ [GAL4\text{-}tub]}{R}$

↓

1.688^{roX1} hpRNA	-	+	+
Adults eclosed	♀ = 654 ♂ = 7	♀ = 284 ♂ = 123	♀ = 417 ♂ = 127
Ago2	Full		Knock down

Figure 5.2. Matings to determine the effect of *dcr2*^{L811fsX} and Ago2 knockdown on the survival of *roX1 roX2* males expressing 1.688^{roX1} hpRNA. (A) *dcr2*^{L811fsX} females with a *roX1*^{ex33}*roX2*Δ X chromosome were mated to males trans-heterozygous for *dcr2*^{L811fsX} and *sna*^{Sco} (*Sco*) and carrying a [hp 1.688^{roX1}][GAL4 *tub*] chromosome. Four classes of offspring are predicted. Survival of *roX1*^{ex33}*roX2*Δ flies not expressing 1.688^{roX1} hpRNA (1st column) or expressing hpRNA (2nd column) are tabulated in table 5.5A. *roX1*^{ex33}*roX2*Δ males lacking Dcr2 (top row) and one copy of Dcr2 (bottom row) were distinguished by *Sco*. (B) Females with a *roX1*^{ex33}*roX2*Δ X chromosome, heterozygous for a [UAS-Ago2] RNAi insertion (Vienna RNAi stock collection) were mated to males trans-heterozygous for [hp 1.688^{roX1}] [GAL4 *tub*] and the dominant marker *R* (*Roughened*). Three classes of *roX1*^{ex33}*roX2*Δ males are expected. The number of offspring from each class are indicated in table 5.5B. Males expressing 1.688^{roX1}hpRNA (+) and those not (-) were distinguished by *R*.

Generation of hairpin (hp) RNA constructs:

A 493 bp amplicon from 1.688^{roX1} and a 800 bp amplicon from 1.688^{3C} bearing Xba I linkers were introduced in inverted orientation into pWIZ (LEE and CARTHEW 2003). Primers used for cloning are listed in table 5.1. Ubiquitous hpRNA expression was achieved by recombining the GAL4 *tubulin* driver with 3rd chromosome insertions.

Table 5.1. Primers used for cloning repeats

Transgene		Primer sequence
[ss 1.688^{roX1}] sense	F	CGGGATCCCCCACCAAAGAGGCTTGACAGAAGA
	R	TCCCCGCGGGTGGCGAAAGGTTATGGAGATGACC
[ss 1.688^{roX1}] anti - sense	F	GAGGGGATCCGTGGCGAAAGGTTATGGAGATGACC
	R	TCCCCGCGGCCCACCAAAGAGGCTTGACAGAAGA
[ss 1.688^{3C}] sense	F	CGGGATCCCCCAATCCAACTGTAACCCCGAA
	R	TCCCCGCGGAAAAAAAAACCGCAGCATCCT
[ss 1.688^{3C}] anti - sense	F	TCCCCGCGGCCCAATCCAACTGTAACCCCGAA
	R	CGGGATCCGACAAGAACAAAACCGCAGCATCCT
[hp 1.688^{roX1}]	F	GCTCTAGAACGAGGTATGGCATTTCCCTTTTGGT
	R	GCTCTAGATGGCCACCTTATAGAGATAACCCCGT
[hp 1.688^{3C}]	F	CGTCTAGACCCAATCCAACTGTAACCCCGAA
	R	TCCTCTAGAGACAAGAACAAAACCGCAGCATCCT

Primers used to amplify 1.688^{roX1} and 1.688^{3C} repeats. Underlined sequences indicate introduced Xba I sites. Forward primers are denoted by F, reverse primers are denoted by R.

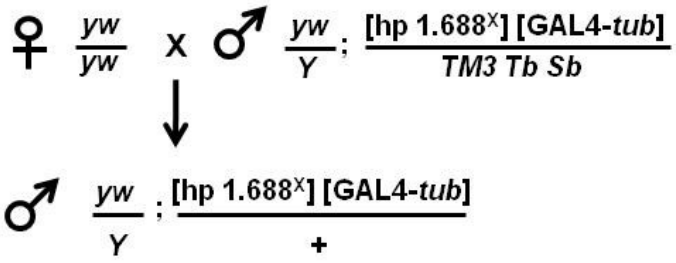
***In-situ* hybridization:**

In-situ hybridizations to salivary gland polytene chromosomes was performed essentially as described in (PARDUE 2011). Chromosomes were hybridized to denatured DIG-11-UTP (Roche) labeled 1.688^{X} riboprobes (1:40) overnight at 42°C. Slides are washed three times in PBST (0.1% Tween 20), blocked 30 min in PBST containing 10 µg/ml BSA and incubated for 2 h with anti-DIG antibody conjugated to alkaline phosphatase (1:200; Roche). Signal was developed with NBT and BCIP, and counterstained with Giemsa (Sigma).

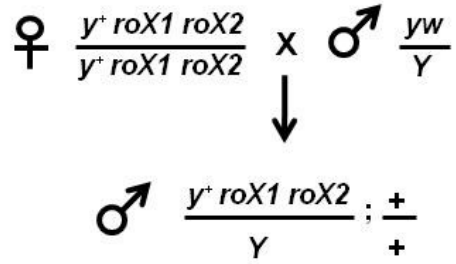
Immunostaining:

Immunodetection of MSL2 on polytene chromosome preparations was as previously described (KELLEY *et al.* 1999). Full male genotypes are: wild type ($y^{1}w^{1118}$), $y^{1}w^{1118}/Y;$ [hp 1.688^{roX1}] [GAL4-*tub*]/+, w^{1118} $roX1^{SMC17A}$ *roX*2Δ and w^{1118} $roX1^{SMC17A}$ *roX*2Δ*;* [hp 1.688^{roX1}] [GAL4-*tub*]/+. Full genotypes of female larvae are: w^{1118} $roX1^{SMC17A}$ *roX*2Δ*;* [H83M2]6I/[hp 1.688^{roX1}] [GAL4-*tub*] and w^{1118} $roX1^{SMC17A}$ *roX*2Δ*;* [H83 M2]6I/+. Following photography and scoring of X localization, the presence of the [hp 1.688^{roX1}] [GAL4-*tub*] chromosome was determined by PCR of genomic DNA from larval carcasses. Matings to obtain desired larval genotypes are detailed in Fig. 5.3.

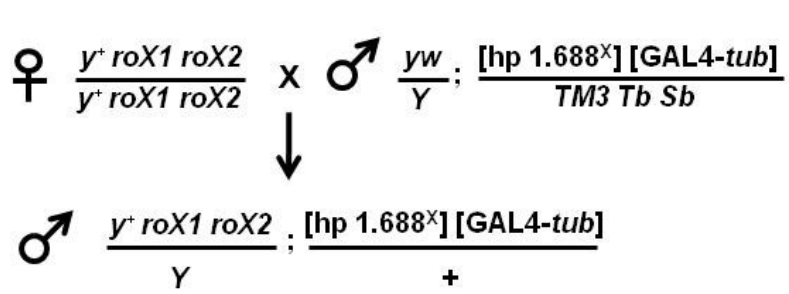
A



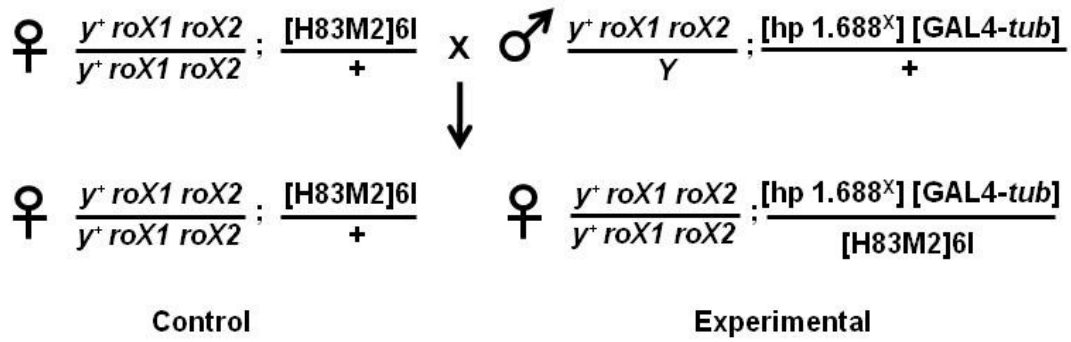
B



C



D



Control Experimental

Figure 5.3. Matings to produce larvae for immunostaining. Scheme to produce (A) wild type male larvae expressing 1.688^{roX1} hpRNA. All non *Tb* male larvae express 1.688^{roX1} hpRNA. (B) Homozygous females bearing a *roX1*^{SMC17A} *roX2*Δ X chromosome marked by y^+ are crossed to wild type *yw* reference males. All sons inherit the y^+ *roX1*^{SMC17A} *roX2*Δ X chromosome. (C) . y^+*roX1*^{SMC17A} *roX2*Δ females are crossed to males bearing a [hp 1.688^{roX1}] [GAL4- *tub*] recombinant chromosome balanced with *TM3 Tb Sb*. All non *Tb* sons inherit the y^+ *roX1*^{SMC17A} *roX2*Δ X chromosome and express 1.688^{roX1} hpRNA. (D) Generation of *roX1*^{SMC17A} *roX2*Δ female larvae expressing MSL2 with or without 1.688^{roX1} hpRNA. y^+*roX1*^{SMC17A} *roX2*Δ females heterozygous for [H83M2]6I are crossed to y^+*roX1*^{SMC17A} *roX2*Δ male escapers heterozygous for the [hp 1.688^{roX1}] [GAL4-*tub*] recombinant chromosome. Daughters inheriting [H83M2]6I express MSL2 (control) and those tans-heterozygous for [hp 1.688^{roX1}] [GAL4- *tub*] and [H83M2]6I express MSL2 in the presence of 1.688^{roX1} hpRNA (experimental).

qRT-PCR:

Accumulation of 1.688^{roX1},1.688^{7F1} and 1.688^{3C} transcript was measured by qRT PCR as previously described (DENG *et al.* 2009b). Briefly, RNA was prepared from two groups of 50 third instar male larvae. One μg of RNA was reverse transcribed using random hexamers and ImProm-II reverse transcriptase (Promega). To distinguish sense and antisense transcription, reverse transcription was done with strand-specific 1.688^{roX1} primers. cDNA was amplified using primers listed in table 5.2. Expression of 1.688^{roX1}, 1.688^{3C} and 1.688^{7F1} transcripts was normalized to autosomal *Dmn*, amplified with 300 nM of primers GACAAGTTGAGCCGCCTTAC and CTTGGTGCTTAGATGACGCA.

Table 5.2. 1.688^{X} repeat specific qRT-PCR primers

1.688^{X}		Primer sequence	Concentration (nM)
1.688^{roX1}	F	TATTTACAAACGGGGTTATCTCTATAAGG	300
	R	AAAACAGTCTTCATTTAAGCGGTAA	300
	R	CGTAACAAAATTTCCTATCGACCT	300
1.688^{7F}	F	GTGCTCCTAATTACCAATACTAATC	300
	R	ATTTTTCAAAGTCCGCC	300
1.688^{3C}	F	GTTTTTTCGGCACAACTT	500
	R	CGAGCTCAACGCGGTATGAC	500

Primers used to measure 1.688^{roX1}, 1.688^{7F} and 1.688^{3C} repeat transcription by qRT-PCR Forward primers are denoted by F, reverse primers are denoted by R.

RNA sequencing:

Small RNA was extracted from two groups of 50 third instar larvae. To avoid differences produced by X or Y polymorphisms, all strains were constructed with sex chromosomes from our y^1w^{1118} laboratory reference strain, used as the wild type control in these studies. Additional male genotypes are: y^1w^{1118}/Y; $ago2^{414}$/+, y^1w^{1118}/Y; $ago2^{414}$ and y^1w^{1118}/Y; [hp 1.688^{roX1}] [GAL4-*tub*]/+. Total RNA was isolated by homogenization in Qiazol reagent (Qiagen) using a Tissue Tearor (BioSpec Products Inc., Bartlesville, OK). RNA was fractionated into small RNA (<200 nt) and large RNA using the miRNeasy kit (Qiagen). RNA quality was assessed on large RNA fractions following clean up (RNeasy MinElute cleanup kit, Qiagen).

Small RNA Sequencing and Analysis:

RNA was prepared using the DGE-Small RNA Sample Prep Kit (Illumina, San Diego, CA) as described previously (CREIGHTON *et al.* 2010). A total of 12 Solexa-ready small RNA libraries were analyzed on an Illumina GA-IIx Genome Analyzer. Initial sequence processing and analysis used the Genboree Small RNA Toolset (http://genboree.org). The Illumina adapter was trimmed, and reads with length between 11 and 30 and a copy number of at least 4 were selected for further processing. Reads were mapped to the Drosophila genome (BDGP R5/dm3) using Pash 3.0 (COARFA *et al.* 2010). Reads mapping at up to 10,000 locations were selected for further analysis. The small RNA definitions from miRBase (GRIFFITHS-JONES *et al.* 2008; KOZOMARA and GRIFFITHS-JONES 2011) were used to construct a profile for each sample. The abundance of small RNAs

was normalized to all usable reads. Repeat definitions were downloaded from UCSC Genome Browser, and repeat coverage was computed based on the read mappings. For reads mapping to multiple locations, the contribution of each read was divided by the total number of genomic mappings.

RESULTS

1.688^{X} repeats are distributed throughout X euchromatin

Different clusters of 1.688^{X} repeats share an average of 73% sequence identity. We performed in-*situ* hybridization to polytene chromosomes with two 1.688^{X} repeats sharing 67% identity (Fig. 5.4). 1.688^{roX1} is flanked by *roX1* and *echinus* (*ec*) and 1.688^{3C} is distal to *white* (*w*). Probes from these repeats hybridize to numerous sites that are most densely distributed around the middle of the X chromosome (Fig. 5.4A, B). However, the relative strength of signals at specific loci differ, and a few distal loci exclusively hybridize to a single probe, emphasizing the sequence diversity in the 1.688^{X} repeat family (Fig. 5.4C) (KUHN *et al.* 2012). Our observations agree with previous reports that demonstrate a similar distribution of homologous 1.688^{X} satellite repeats (WARING and POLLACK 1987; DIBARTOLOMEIS *et al.* 1992).

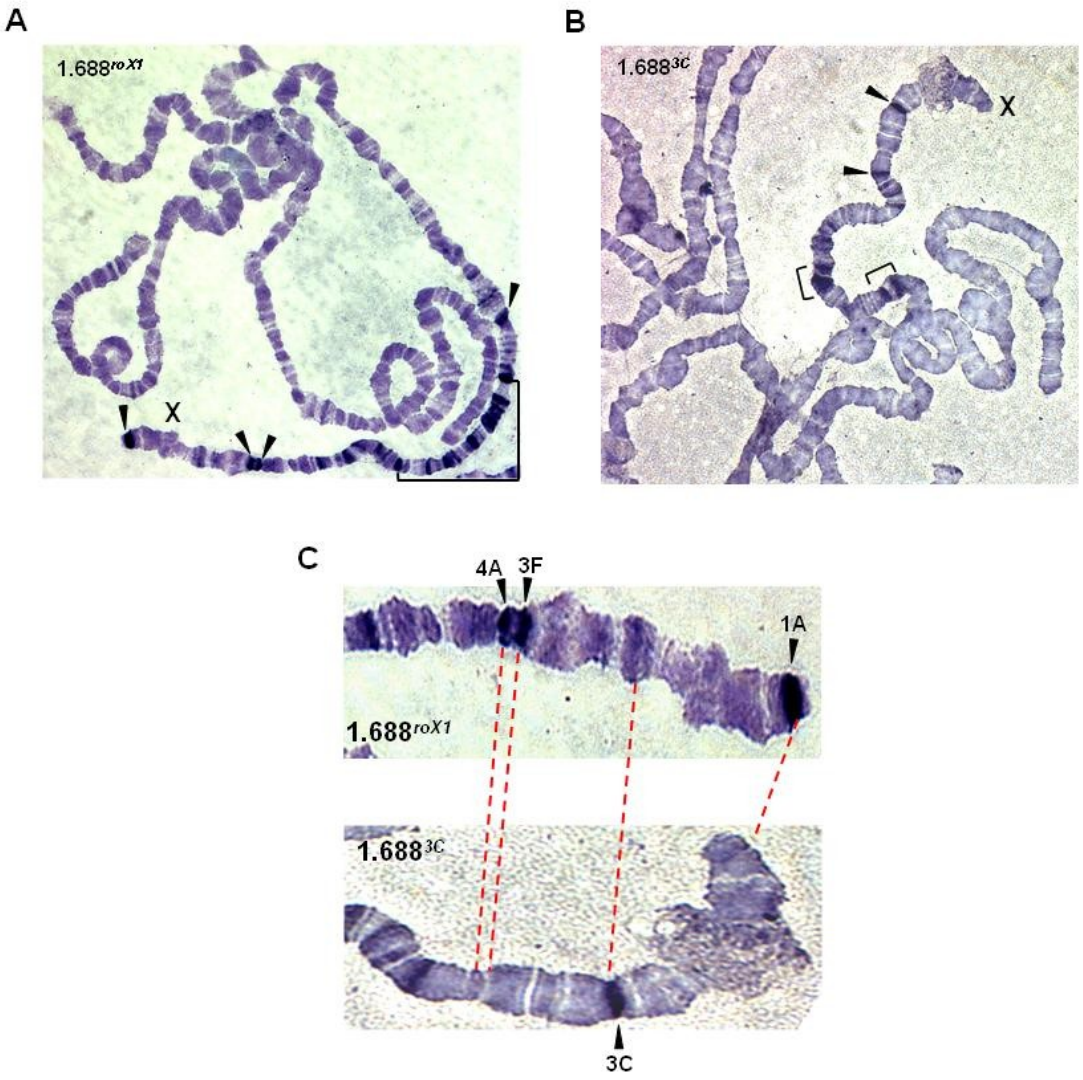


Figure 5.4. 1.688^{X} repeats are enriched on the X chromosome. Probes to the 1.688^{roX1} (A) 1.688^{3C} (B) repeat clusters were hybridized to polytene preparations. (C) Distal X-linked loci exclusive to 1.688^{roX1} and 1.688^{3C} repeat probes. Black arrow heads label the signals detected with alkaline phosphatase (dark blue). DNA is counterstained with Giemsa/ Hoechst.

RNA from 1.688^{X} satellite repeats is present in flies

Expression of many 1.688^{X} repeats has been detected by cDNA sequencing (Flybase). We used quantitative RT-PCR (qRT PCR) to confirm transcription from 1.688^{roX1}, 1.688^{3C} and a cluster of repeats at 7F1 (1.688^{7F1}) in

male larvae (Fig. 5.5A). The specificity of primers that amplify repeat clusters was confirmed on genomic templates from flies deleted for the repeats in question (Fig. 5.6). All three repeats are transcribed. More detailed analysis of transcripts from the 1.688^{roX1} repeats revealed production of sense and antisense strands, suggesting that these regions could produce siRNA (Fig. 5.5B).

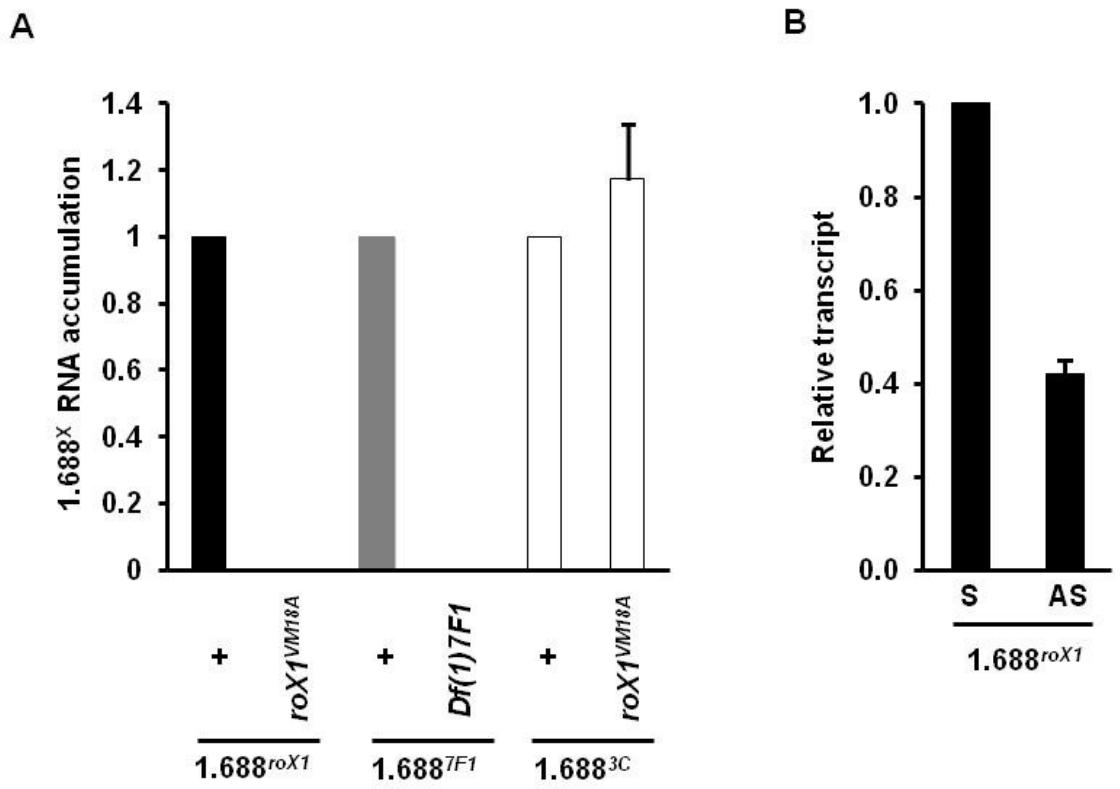


Figure 5.5. 1.688^{X} repeats are transcribed. (A) Accumulation of 1.688^{roX1} (black bars) 1.688^{7F1} (gray bars) and 1.688^{3C} (white bars) repeat transcripts in wild type, *roX1*^{VM18A} and *Df(1)7F1* 3rd instar male larvae. Expression of each repeat is normalized to the autosomal gene *dmn* and is an average of three biological replicates. (B) Relative contribution of 1.688^{roX1} sense (S) and antisense (as) transcripts. The most abundant strand is designated the sense stand. Anti sense RNA constitutes 2/5 the amount of sense RNA. 1.688^{roX1} sense and anti-sense RNA was reverse transcribed from two µg of RNA using 1.688^{roX1} reverse and forward primers respectively.

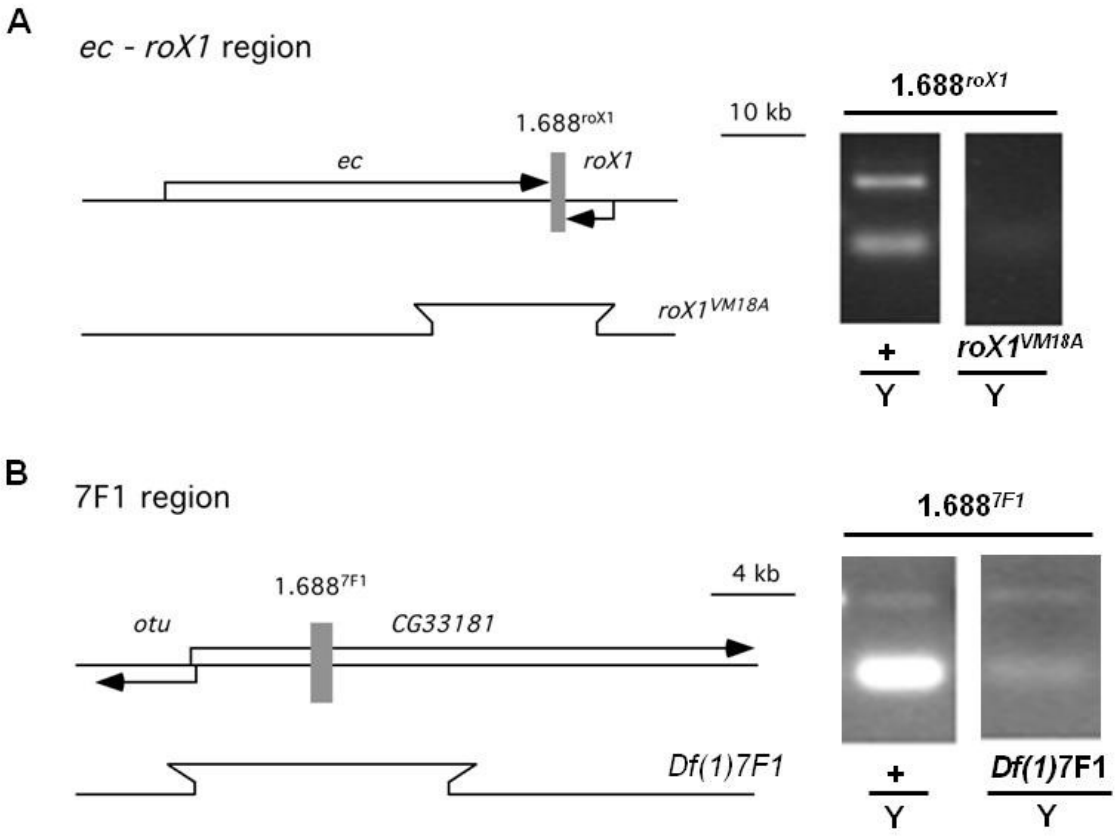


Figure 5.6. Deletions removing the 1.688^{roX1} and 1.688^{7F1} repeats.

A) *roX1*^{VM18A} is a 15.4 kb deletion generated by Hybrid Element Insertion (Preston et al., 1996). Most of *roX1*, almost all translated *echinus* (*ec*) sequence and the 1.688^{roX1} cluster (gray bar) are removed, and a *P*-element is retained at the break site. B) The 12.4 kb deletion removing 1.688^{7F1} (gray bar) was created by Flp-mediated recombination between PBac{WH}otu^{f02343} and P{XP}d01160, creating *Df(1)7F1*. A hybrid PBac/*P*-element remains at the break site. Primers specific for the 1.688^{roX1} and 1.688^{7F1} repeats were tested by amplification of genomic templates from male flies with a wild type X chromosome, *roX1*^{VM18A} or *Df(1)7F1*. DNA from males is used to ensure that unmapped satellites on the Y chromosome are not detectable.

In agreement with this, 18-26 nt RNAs mapping to the 1.688^{roX1}, 1.688^{3C} and 1.688^{7F1} repeats have been identified in embryos, larvae, adults, Kc167 and S2 cells (modENCODE). Representative sequences from embryos, larva and adult stages are listed in table 5.3. Small RNA fractions from wild type male, female and *ago2*^{414} larvae were sequenced to determine whether siRNAs from 1.688^{X} repeats display a sex bias or dependence on Ago2, a member of the siRNA pathway that participates in dosage compensation (MENON and MELLER 2012). To enable selection of sex and genotype, RNA was isolated from sorted larvae. Analysis of the small RNA from larvae did not reveal siRNAs mapping to the 1.688^{X} repeats. The relative lack of abundance of 1.688^{X} siRNA is mirrored by modENCODE studies, where small RNAs are most abundant in embryonic and adult stages. In order to determine whether 1.688^{X} siRNA can be detected at an earlier developmental stage, small RNA preparations from hand sorted male and female embryos are currently being analyzed (Dr. Preethi Gunaratne, University of Houston). I anticipate that the findings of this study will be reported in a subsequent manuscript.

Table 5.3 Small RNA sequences mapping to 1.688^{X} repeat (modENCODE)

Developmental stage	Small RNAs mapping to		
	1.688^{roX1}	1.688^{3C}	1.688^{7F1}
6-10 hr Embryo	TGCCAAAAAGTTGATATTTACAAACG TGCCAAAAAGTTGATATTTACAAA TGCCATACCTCGTTGAATTCGTAAC GCCATACCTCGTTGAATTCGTAACAAAA TACAGGTCGATAGGAAATTTTGTTAC CAAATTTTAATGATGGTACCCCTTAT TTTTAATGATGGTACCCCTTATCAAA TTTAATGATGGTACCCCTTATCAAAAA TAATGATGGTACCCCTTATCAAAAATGC TCTATAAGGTGGCCAAAAAAGATA ATATTTACAAACGGGGTTA	CGCATTTTTTGTAAGGGGTAACATC TTCGCATTTTTTGTAAGGGGTAAC ACCCCTTACAAAAAATGCGAA TCAATTTTCGCATTTTTTGTAAGGG TTTTCGCATTTTTTGTAAGGG GTCAATTTTCGCATTTTTTG TACGAGCTCAACGCGGTA CAATTATTTTTAAAGTTGTGC	TTGGTAATTAGGAGCACAAAA
Larva	TACGAATTCAACGAGGTATGGCA	GTTACCCCTTACAAAAAATG	
Adult male	ATTCAACGAGGTATGGCATTT TACGAATTCAACGAGGTATGGCAT TTACGAATTCAACGAGGTATGGCA GCCATACCTCGTTGAATTCGTAACAA TAACAAAATTTCCTATCGACC TGATAAGGGGTACCATCATTAA TTTTTGATAAGGGGTACCATCATTA ATTTTTGATAAGGGGTACCATCATTA TGTTTTATACTGCCAATAAAC AGGTGGCCAAAAAAGATATT	TTGGCCATTTTTTGCAAATTTT	
Adult female	TGCCAAAAAGTTGATATT TACGAATTCAACGAGGTATGGCATT TACGAATTCAACGAGGTATGGCAT TACGAATTCAACGAGGTATGGC CCATACCTCGTTGAATTCGTAACAA TACGAATTCAACGAGGTATGG TTGTTACGAATTCAACGAGGTATG ATGTTTATTGGCAGTATAAAAC	TTGTAAGGGGTAACATCAT TTTTCGCATTTTTTGTAAGGGGTAACA CGTTGAGCTCGTAATAAAATT GTTGAGCTCGTAATAAAATT TGAGCTCGTAATAAAATT TGAGCTCGTATTAAAATT GCTCGTATTAAAATTTCCAATCAAA TTAAAATTTCCAATCAAA CACAGTTTGATTGGAAAT	

Selected small non coding RNA reads mapping to 1.688^{roX1}, 1.688^{7F1} and 1.688^{3C} repeat clusters were downloaded from modENCODE (E. Lai, Sloan-Kettering Cancer Center). Sequences were obtained from embryos (6–10 hr), larval (1^{st} and 3^{rd} instar) and adults.

Long RNA from 1.688^{X} repeats reduces *roX1 roX2* male survival

To determine if long RNAs from 1.688^{X} repeats affect dosage compensation, transgenic flies were generated that express long single stranded sense or antisense RNA from the 1.688^{roX1} and 1.688^{3C} repeats. These transgenes were tested in males carrying the partial loss of function *roX1*^{ex33} *roX2*Δ chromosome, which allows ~20% adult male escapers (Fig 5.1A). *roX1*^{ex33} *roX2*Δ males display considerable mislocalization of the MSL proteins, making this a sensitive genetic background in which to test factors influencing X recognition (DENG *et al.* 2005; MENON and MELLER 2012). Survival of *roX1*^{ex33} *roX2*Δ males expressing RNA from 1.688^{roX1} or 1.688^{3C} repeats was reduced 40-70%, irrespective of the sense of the strand that was expressed (Fig. 5.7). However, none of these transgenes influenced the survival of otherwise wild type males (Table 5.4). Furthermore *roX1*^{ex33} *roX2*Δ male survival was not influenced by the parent chromosomes bearing attP sites used to generate transgenics, ruling out an effect due to genetic background (data not shown). Surprisingly, recombinant chromosomes with two 1.688^{X} transgenes producing complementary RNA strands had no effect on the survival of *roX1*^{ex33} *roX2*Δ males (Fig. 5.7). We conclude that single stranded RNA from 1.688^{X} repeats interferes with dosage compensation, and speculate that base pairing by complementary 1.688^{X} RNAs neutralizes this effect. To determine if perfect complementarity is required, recombinant chromosomes expressing sense from one repeat cluster and antisense from the other were tested. In spite of the fact that the1.688^{roX1} and 1.688^{3C} repeats have only 67% complementarity, simultaneous expression once again negated the effects of single strand

expression (Fig. 5.7). We conclude that complete base pairing is not essential for the neutralizing effect of simultaneous sense and antisense expression.

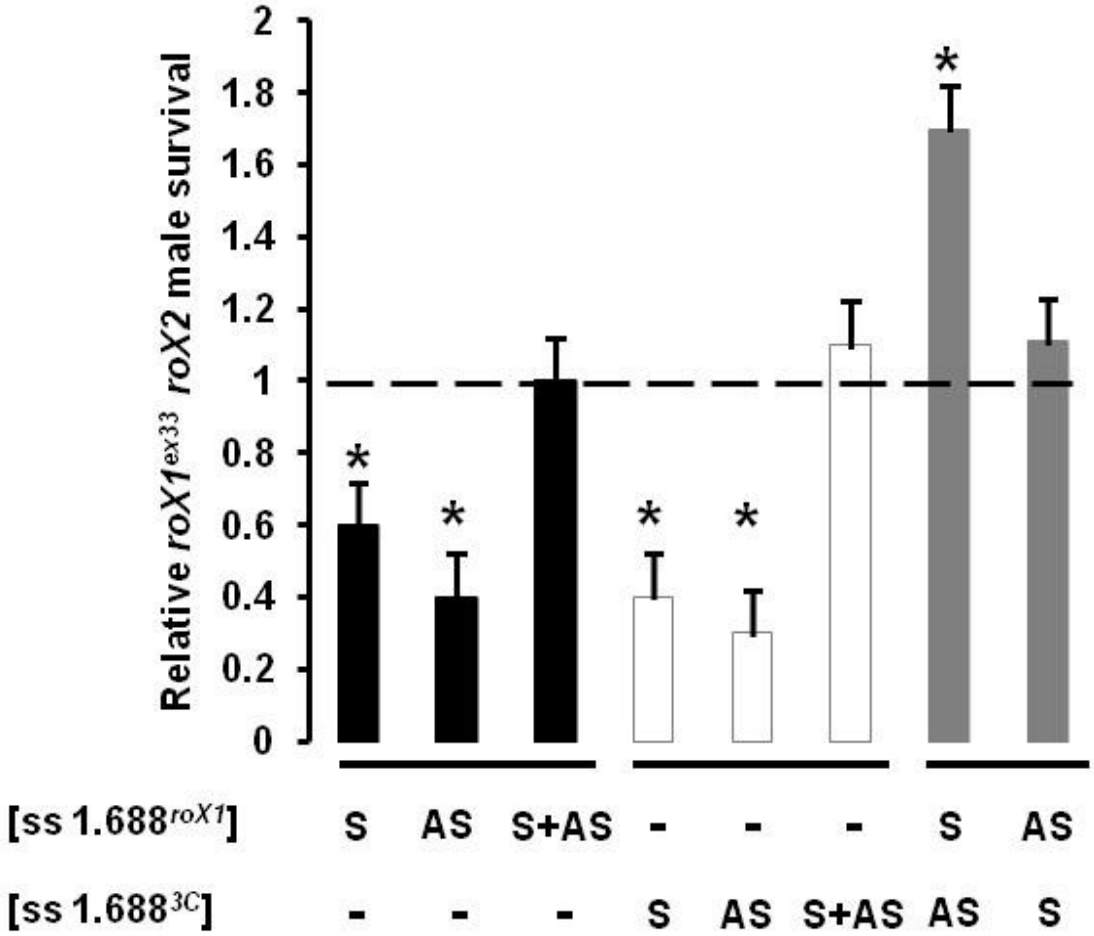


Figure 5.7. Ectopic expression of RNA from 1.688^{X} repeats influences *roX1 roX2* male survival. Expression of single stranded sense or antisense RNA from the 1.688^{roX1} (black) and 1.688^{3C} (white) repeats increases the lethality of *roX1^{ex33} roX2*Δ chromosomes. Male survival is expressed as adult males recovered carrying the 1.688^{X} transgene divided by their brothers lacking this transgene. The effect of single strands is neutralized by simultaneous expression of complementary RNA strands (black, white bars on right) or partially complementary 1.688^{roX1} and 1.688^{3C} RNA strands (gray bars). Error bars represent SEM. * students t-test p<0.05

Table 5.4. 1.688^{X} ssRNA does not affect wild type male survival

[ss 1.688^{X}]	Strand	Wild type % male survival
[ss 1.688^{roX1}]	Sense	100 (119)
	Anti – sense	109 (92)
[ss 1.688^{3C}]	Sense	99 (134)
	Anti - sense	130 (107)

Survival of $y^{1}w^{1118}$ males carrying either [ss 1.688^{roX1}] or [ss 1.688^{3C}], expressing sense or anti-sense long RNA. Male survival was based on recovery of brothers lacking the transgene. To determine survival, $y^{1}w^{1118}$ females were mated to $y^{1}w^{1118}$; [ss 1.688^{X}]/+ males. Total males eclosed for each cross are indicated within parenthesis.

Double stranded hairpin RNA from 1.688^{X} repeats rescues *roX1 roX2* males

Double stranded RNA may be formed upon simultaneous expression of sense and antisense strands, enabling production of small RNA. However, the transgenes expressing single stranded RNA are widely separated on the chromosome, a condition that will limit hybridization. To ensure generation of high levels of double stranded RNA, transgenics that produce hairpin RNA (hpRNA) from the 1.688^{roX1} and 1.688^{3C} repeats were made ([hp 1.688^{roX1}], [hp 1.688^{3C}]). The current inferences are drawn from the expression of 1.688^{roX1} hpRNA (Fig 5.8, 5.9). Examination 1.688^{3C} hpRNA expression is ongoing and will be submitted along with the completed manuscript. Expression of hpRNA from [hp 1.688^{roX1}] has no effect on the survival of wild type males (Fig. 5.8A). In

contrast, 1.688^{roX1} hpRNA increased the survival of *roX1 roX2* males. Eclosion of *roX1*^{ex33} *roX2*Δ males was ~2.5 fold higher when 1.688^{roX1} hpRNA RNA was present. More dramatically, survival of the severely affected *roX1*^{ex6} *roX2*Δ and *roX1*^{SMC17A} *roX2*Δ chromosomes, normally 2% and 0%, was increased to 26% and 31% (Fig. 5.8A). The [hp 1.688^{roX1}] transgenes did not increase *roX1 roX2* male survival without a GAL4 driver, ruling out insertional effects of the transgenes themselves (Fig. 5.8B). Furthermore, expression of hpRNA to the non-essential *w* gene failed to increase the survival of *roX1 roX2* males, ruling out non-specific small RNA production or GAL4 expression as the cause of male rescue (Fig. 5.8A). However, eye color was eliminated in flies expressing *w* hpRNA, demonstrating driver activity and small RNA production.

The effect of 1.688^{X} hpRNA expression on *roX1 roX2* male survival prompted an examination of its effect on another dosage compensation mutant. *Males absent on first* (*mof*) encodes a protein in the MSL complex that acetylates H4 on lysine 16 (H4Ac16), a modification that is dramatically enriched on the male X chromosome and required for enhanced X chromosome expression (SMITH *et al.* 2000; SMITH *et al.* 2001; LARSCHAN *et al.* 2011). The *mof*^{1} mutant is catalytically inactive and male lethal (HILFIKER *et al.* 1997). Expression of 1.688^{roX1} hpRNA did not rescue *mof*^{1} males. This is consistent with 1.688^{X} hpRNA participating in MSL complex recruitment, rather than modifying complex activity (data not shown).

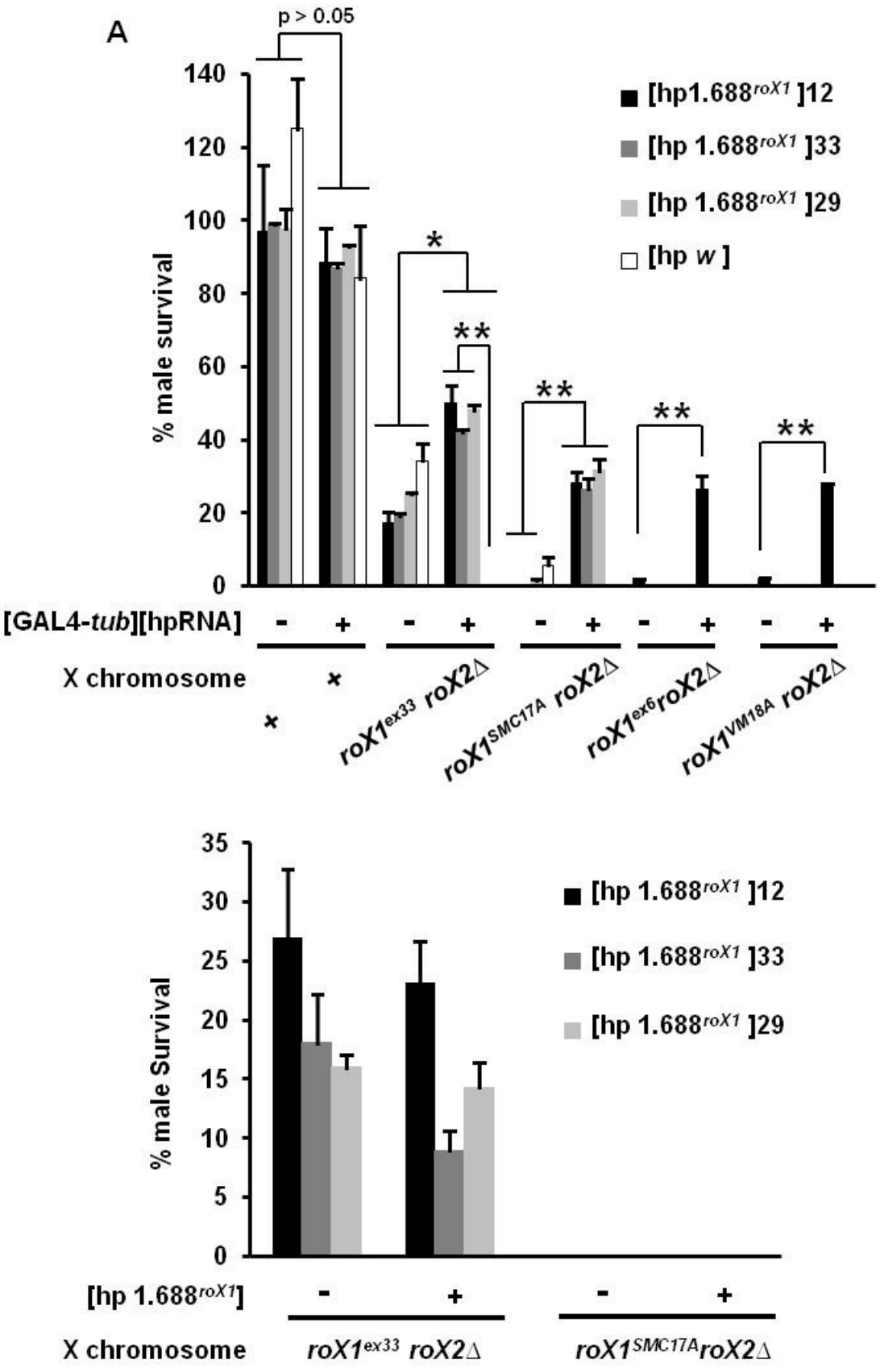


Figure 5.8. Ectopic expression of 1.688^{roX1} hpRNA enhances *roX1 roX2* male survival. (A) Expression of 1.688^{X} hairpin RNA (hpRNA) rescues *roX1 roX2* males. The survival of males wild type for the *roX* genes, or carrying *roX1*^{ex33} *roX2*Δ or *roX1*^{SMC17A} *roX2*Δ chromosomes was determined without (-) or with (+) expression of 1.688^{roX1} hpRNA. Three independent transgenes, [hp 1.688^{roX1}]12 (black), [hp 1.688^{roX1}]33 (dark grey) and [hp 1.688^{roX1}]29 (light grey) were tested, as was a transgene that produces a control hpRNA targeting the *white* gene ([hp w], white bars). Survival of *roX1*^{ex6} *roX2*Δ and *roX1*^{VM18A} *roX2*Δ males were tested with a single transgene, [hp 1.688^{roX1}]12. Expression of hpRNA was regulated by ubiquitous [GAL4-tub] driver. Error bars represent SEM. Students t-test $p<0.05$ and $p<0.001$ are indicated by * and ** respectively. (B) 1.688^{roX1} hpRNA transgenes are ineffective in the absence of a GAL4 driver. The survival of *roX1*^{ex33} *roX2*Δ and *roX1*^{SMC17A} *roX2*Δ males carrying [hp 1.688^{roX1}]12 (black), [hp 1.688^{roX1}]33 (dark grey) and [hp 1.688^{roX1}]29 (light grey) without a GAL4 driver is shown. Error bars represent SEM.

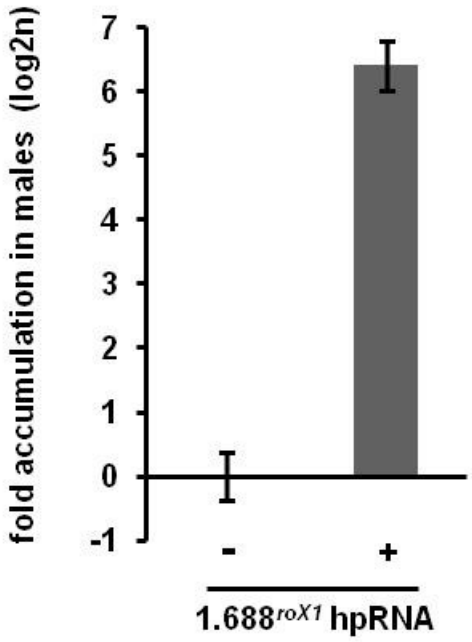


Figure 5.9. Expression of hpRNA from [hp 1.688^{roX1}]12. Fold accumulation of 1.688^{roX1} hpRNA in *y*^{1}*w*^{1118}; [GAL4-*tub*] [hp 1.688^{roX1}]12/+ males. Fold change is represented in log2n scale relative to wild type, *y*^{1}*w*^{1118} males without transgenes. SEM is represented by error bars.

We postulate that 1.688^{X} hpRNA is processed into siRNA. To address this, small RNA fractions from male larvae expressing 1.688^{roX1} hpRNA were sequenced. As expected, these larvae displayed high levels of small RNA with identity to the expressed region (Fig. 5.10A). By far the most abundant of these are 21 nt, which is characteristic of Dcr2 generated siRNA (Fig. 5.10B).

One potential mode of action of 1.688^{X} siRNAs is direction of chromatin changes to cognate loci on the X chromosome, a process that would require homologous sequences on the X chromosome. The presence of hundreds of related 1.688^{X} repeats on the X chromosome suggests that the effectiveness of transgenes producing 1.688^{X} siRNAs is unlikely to require any single cluster of 1.688^{X} repeats, or perfect identity between the hpRNA expressed and repeat clusters on the X chromosome. To examine this, we tested hp 1.688^{roX1} expression in *roX1*^{VM18A} *roX2*Δ males. *roX1*^{VM18A} is a deletion that removes most of *roX1*, the entire 1.688^{roX1} repeat cluster and part of *ec* (Fig.5.6A). Survival of *roX1*^{VM18A} *roX2*Δ males is 2%, but this is increased to 27% upon expression of hp 1.688^{roX1} RNA (Fig. 5.8A). This level of rescue is indistinguishable from that achieved for chromosomes carrying the similarly severe *roX1*^{ex6} and *roX1*^{SMC17A} alleles, retaining the 1.688^{roX1} repeats (Fig. 5.8A,B). We conclude that hp 1.688^{roX1} RNA can achieve its effect even when the cognate DNA is removed from the X chromosome. However, the distribution of hundreds of 1.688^{X} repeats along the X chromosome precludes removal of all similar sequences.

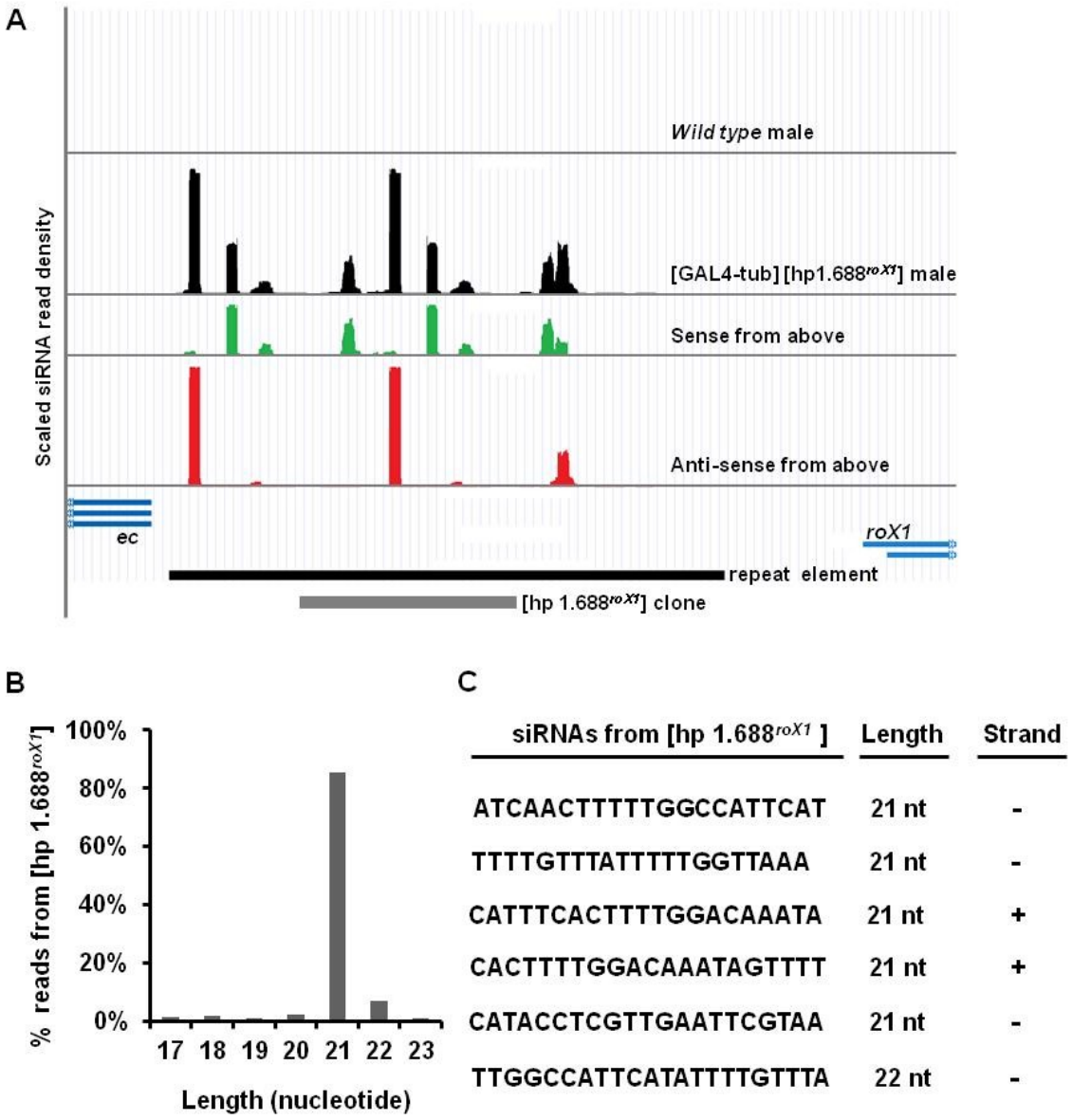


Figure 5.10. Small RNA derived from [hp 1.688^{roX1}]. (A)UCSC browser visualization of small RNA profiles from a wild type (y^1w^{1118}) male (top; no small RNA detected) and y^1w^{1118}; [GAL4-*tub*] [hp 1.688^{roX1}]12/+ male larvae (bottom: black track). Colored tracks depict [hp 1.688^{roX1}]12 strand specific reads: Sense reads (green) and anti-sense (red). Track heights reflect scaled read density. Blue bars depict the annotated *ec* and *roX1* genes. The black bar indicates 1.688^{X} repeat DNA mapping. The 1.688^{roX1} fragment cloned into [hp 1.688^{roX1}] is shown by the gray bar. Size distribution (B), sequence and orientation (C) of the most abundant small RNAs isolated from y^1w^{1118}; [GAL4-*tub*] [hp 1.688^{roX1}]12/+ male larvae. Orientation of siRNAs is with respect to the cloned 1.688^{roX1} fragment.

The effect of 1.688^{X} hpRNA is dependent upon the siRNA pathway

We previously demonstrated that mutations in the siRNA pathway enhance the lethality of *roX1 roX2* mutations and further reduce X chromosome binding by the MSL complex (MENON and MELLER 2012). The observation that 1.688^{X} hpRNA partially rescues *roX1 roX2* males and is processed into small RNA suggests that small RNA from 1.688^{X} repeats acts through the siRNA pathway to promote dosage compensation. To address this, we performed crosses to determine whether key members of the siRNA pathway were necessary for rescue of *roX1 roX2* males by 1.688^{X} hpRNA (Fig.). Dicer2 (Dcr2) is essential for the production of short dsRNA from the precursor hpRNA, and Argonaut 2 (Ago2) binds guide siRNA and directs regulators to cognate sequences (CARTHEW and SONTHEIMER 2009). Reduction in the level of Ago2 or loss of Dcr2 in *roX1*^{ex33} *roX2*Δ males expressing 1.688^{X} hpRNA reduced survival by 30% and 55%, respectively (Table 5.5A,B). We conclude that defects in the siRNA pathway reduce the potency of the 1.688^{X} hpRNA.

Table 5.5. The siRNA pathway is necessary for rescue of *roX1 roX2* mutants by 1.688^{roX1} hpRNA

A

1.688^{roX1} hpRNA	*dcr2*	*roX1*^{ex33} *roX2*Δ % male survival
–	*dcr2*/+	7.32
–	*dcr2*/*dcr2*	0
+	*dcr2*/+	53.20
+	*dcr2*/*dcr2*	23.77

B

1.688^{roX1} hpRNA	Ago2 KD	*roX1*^{ex33} *roX2*Δ % male survival
–	–	1.0
+	–	43.31
+	+	30.46

Mutation of *dicer2* (A) or knock down of *ago2* (B) reduces rescue of *roX1*^{ex33} *roX2*Δ males by 1.688^{roX1} hpRNA. The *dcr2*^{L811fsx} mutation is a null (LEE *et al.* 2004). Male survival is determined from adult eclosion. See Fig. 5.2 for details of crosses.

1.688^{X} hpRNA restores MSL2 localization

The suppression of *roX1 roX2*Δ male-lethality by 1.688^{X} hpRNA prompted an examination of MSL localization in these flies. MSL2 localization is restricted to the X chromosome in polytene preparations from wild type males (Fig. 5.11A). Several studies support the idea that the *roX* genes are themselves *cis*-acting elements that recruit the MSL complex to flanking chromatin (KELLEY and KURODA 2003; KELLEY *et al.* 2008). For example, *roX* transgenes recruit the MSL complex to autosomal insertion sites (KELLEY *et al.* 1999). To determine whether 1.688^{X} transgenes share this property, we examined polytene preparations from otherwise wild type males expressing the 1.688^{roX1} hpRNA. No MSL2 could be detected at the autosomal transgene, ruling out a direct role for 1.688^{X} DNA sequences, or 1.688^{X} hpRNA expression, in MSL recruitment (Fig. 5.11B).

We then examined MSL2 recruitment in *roX* mutants that express 1.688^{X} hpRNA. *roX1*^{SMC17A} *roX2*Δ males display negligible X-localization, but ectopic autosomal binding of the MSL proteins is prominent (Fig. 5.11C) (DENG *et al.* 2005). MSL2 is still observed at ectopic sites in *roX1*^{SMC17A} *roX2*Δ males expressing 1.688^{roX1} hpRNA, but more pronounced X chromosome binding is also apparent (Fig. 5.11C,D). The severely affected *roX1*^{SMC17A} *roX2*Δ males produce chromosome preparations of poor quality. To test 1.688^{roX1} hpRNA in a healthier genotype, we generated females that express MSL2 from the [H83 M2]6I transgene, leading to inappropriate formation of MSL complexes that bind to both X chromosomes (Fig. 5.2D) (KELLEY *et al.* 1995). *roX1*^{SMC17A} *roX2*Δ*;* [H83 M2]6I /+ females produce chromosome preparations of high quality, but display

ectopic MSL mislocalization equivalent to that of *roX1*^{SMC17A} *roX*2Δ males (Fig. 5.11C, E). We compared MSL2 localization in *roX1*^{SMC17A} *roX*2Δ*;* [H83 M2]6I /+ females that expressed 1.688^{roX1} hpRNA to those that did not. Ectopic MSL2 binding appeared similar in these preparations, but increased MSL localization to the X chromosome was clearly apparent in the larvae expressing 1.688^{roX1} hpRNA (Fig. 5.11E, F). The number of nuclei exhibiting strong X chromosome staining was 16-fold higher in 1.688^{roX1} hpRNA larvae, while those with minor X chromosome staining was 4-fold reduced (Fig. 5.11G). We conclude that expression of 1.688^{X} hpRNA dramatically improves MSL localization and male survival. In spite of this, production of hpRNA alone does not completely rescue male survival or the defect in MSL localization that is produced by *roX1 roX2* mutations.

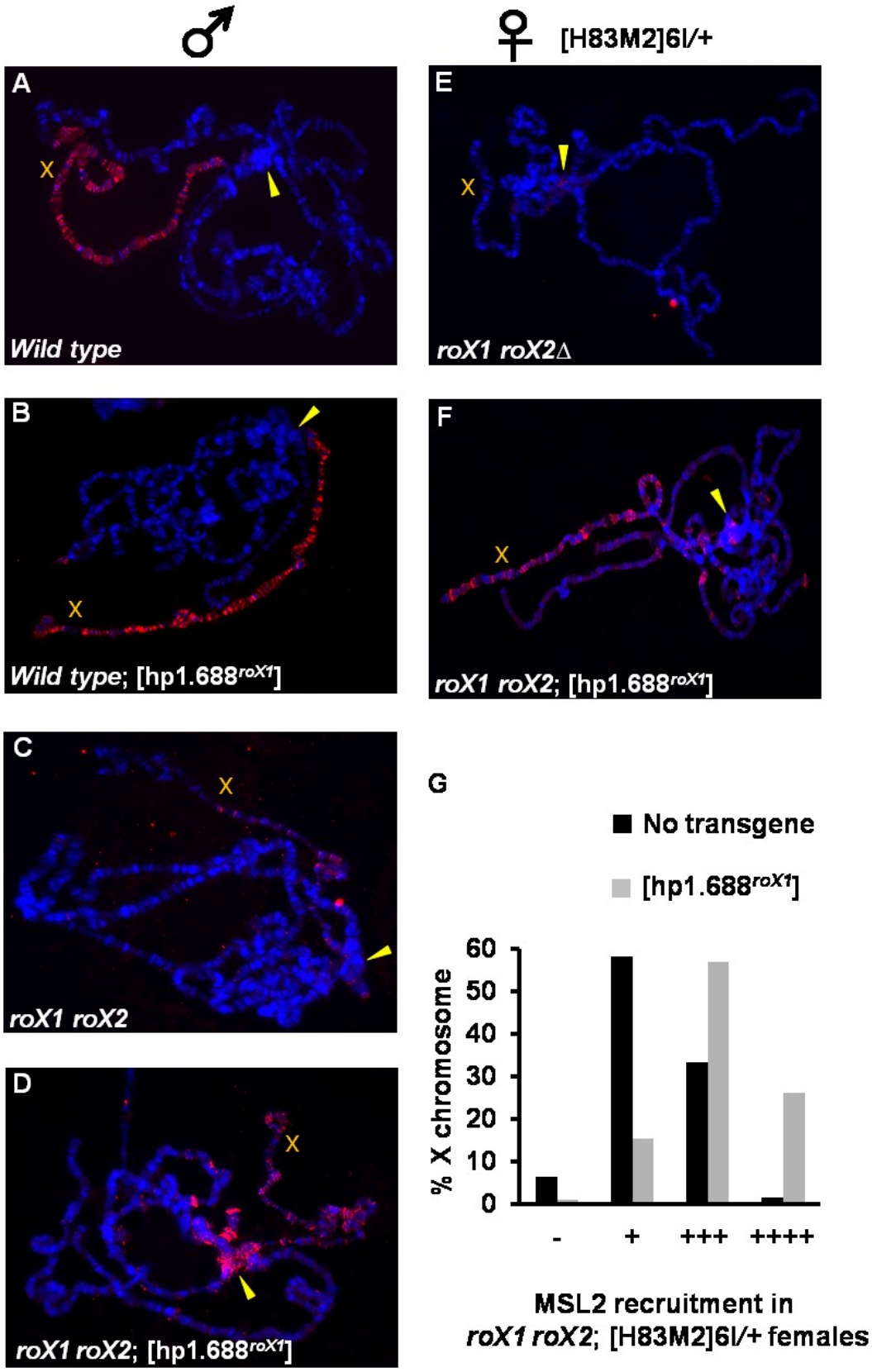


Figure 5.11. 1.688^{X} hpRNA promotes X chromosome recognition. MSL2 localization in (A) wild type males, (B) wild type males expressing 1.688^{roX1} hpRNA, (C) *roX1*^{SMC17A} *roX2*Δ males, (D) *roX1*^{SMC17A} *roX2*Δ males expressing 1.688^{roX1} hpRNA. MSL2 localization in *roX1*^{SMC17A} *roX2*Δ females expressing MSL2 (E) and *roX1*^{SMC17A} *roX2*Δ females expressing MSL2 and 1.688^{roX1} hpRNA (F). MSL2 recruitment to the X chromosome of females from E (black bars, 60 nuclei scored from 9 larvae) and F (grey bars, 84 nuclei scored from 10 larvae) is presented in panel (G). The percent nuclei exhibiting no (-), minor (+), moderate (+++) and strong (++++) is shown. Antibody to MSL2 is detected with Texas Red (red). DNA is counterstained with DAPI (blue). X chromosomes are labeled (X) and arrowheads indicate the chromocenter. Refer to Fig. 5.3 for crosses performed to generate male and female larvae.

DISCUSSION

The discovery that the siRNA pathway contributes to X-localization of the MSL proteins raised the question of what small RNAs are involved, and the mechanism by which siRNAs promote X-recognition (MENON and MELLER 2012). Our current study demonstrates that siRNAs from the 1.688^{X} repeats promote X-localization of the MSL complex in *roX1 roX2* males, and partially rescue the survival of these flies. While these studies implicate the 1.688^{X} repeats in dosage compensation, the molecular details of their function remain speculative. The distribution of 1.688^{X} repeats is strikingly limited to the X chromosome, suggesting a role in establishing X chromosome identity. This is consistent with the finding that siRNA from 1.688^{X} repeats specifically rescued *roX1 roX2* mutants whose signature defect is mislocalization of the MSL proteins.

Long and short RNAs from the 1.688^{X} repeats are detected in flies, and ectopic expression of either type of RNA modifies the male lethality of *roX1 roX2* chromosomes, but in opposing fashion (Fig. 5.7, 5.8A). This prompts the question of what forms of 1.688^{X} RNA are normally biologically active in flies. There are numerous examples of epigenetic modifiers guided to chromatin by complementarity between nascent transcripts and small RNA (PAL-BHADRA *et al.* 2004; VERDEL *et al.* 2004; BURKHART *et al.* 2011; GU *et al.* 2012). We speculate that chromatin at the 1.688^{X} repeats could be the target of a similar mechanism. If this indeed occurs, high levels of single stranded RNA produced from a transgene would compete with nascent transcripts from endogenous 1.688^{X} loci, reducing recruitment to X-linked sites. In support of this idea, the negative

effects of ectopic expression of long single stranded RNA were blocked by simultaneous expression of sense and antisense from separate transgenes. We postulate that complementary strands hybridize to produce dsRNA that is unable to compete with nascent transcripts, but will instead be processed into small RNA. Both of these processes may contribute to neutralization of the negative effects observed when single strands are expressed. Annealing of strands from different transgenes is not expected to be efficient. In contrast, hpRNA will anneal very efficiently, an idea supported by the abundance of small RNA detected in flies expressing 1.688^{roX1} hpRNA (Table 5.5, small RNA analysis in preparation).

An intriguing feature of dosage compensation in flies is the involvement of small RNA in a process that culminates in elevated transcription, rather than silencing. Small RNAs typically destroy target RNA or silence chromatin at cognate loci by recruiting epigenetic modifiers. Small RNAs processed from transcribed repeats direct heterochromatin formation in fission yeast and *Drosophila* (VOLPE *et al.* 2002; PAL-BHADRA *et al.* 2004; USAKIN *et al.* 2007). Indeed, Ago2 and Dcr2 are reported to bind chromatin and repress expression in *Drosophila* (CERNILOGAR *et al.* 2011). Nevertheless, transcriptional up-regulation by small RNA have been documented. These include Piwi activation of telomere associated sequences (TAS) in *Drosophila* and activation of specific genes in human cells transfected with cognate 21nt dsRNAs, a process termed RNA activation (RNAa) (LI *et al.* 2006; YIN and LIN 2007). In contrast to these examples, small RNAs from the 1.688^{X} repeats contribute to a process that

culminates in recruitment of the MSL complex and global up-regulation of an entire chromosome.

Both 1.688^{X} repeats and the CES contribute to X recognition during dosage compensation in flies, but several observations suggest that they act in different fashions. Although the 1.688^{roX1} repeats are located near a CES in *roX1*, close associations between 1.688^{X} repeats and CES is not the rule. In fact, *roX1* and the region near *set2* at 11F5 are the only examples of CES located within 3 kb of 1.688^{X} repeats (C. Coarfa, personal communication). In addition, the CES are distributed evenly along the X chromosome (1B4 – 20B1), but most of the 1.688^{X} repeats cluster around the middle of the X chromosome (7D – 14A) (WARING and POLLACK 1987; ALEKSEYENKO *et al.* 2008; KUHN *et al.* 2012). One interpretation is that both elements act in *cis* to promote X identification, but do so through different mechanisms. Our failure to detect MSL recruitment to 1.688^{X} transgenes further supports the idea that these repeats do not directly recruit the MSL complex, as do the CES. In addition, analyses of proteins that interact with the MSL complex identified chromatin modifiers and DNA binding proteins, but no proteins associated with the siRNA pathway (MENDJAN *et al.* 2006; WANG *et al.* 2013). Based on these observations, we believe that direct recruitment of the MSL complex by an siRNA-directed mechanism is unlikely.

There is increasing interest in the role of nuclear organization and long-range DNA interactions in regulated gene expression. Small RNA has been linked to higher order nuclear organization. For example, Ago2 and RM62E have

been shown to mediate long-range contacts between insulators in flies (LEI and CORCES 2006; MOSHKOVICH *et al.* 2011). Interestingly, the *Drosophila* X chromosome has been reported to assume a male-specific conformation (GRIMAUD and BECKER 2009). Compensated loci on the X chromosome were found to be closer together in male interphase nuclei than in the female nuclei, a difference that depends on dosage compensation. Interactions between the MSL complex and components of the nuclear pore have been reported to affect MSL localization to the X chromosome (MENDJAN *et al.* 2006; VAQUERIZAS *et al.* 2010). These findings support the idea that dosage compensation in flies occurs in the context of a X chromosomal domain with a distinctive chromatin architecture or nuclear position. This idea has parallels in mammals, where the inactive X (Xi) chromosome is organized into a repressive nuclear compartment enriched for *Xist* (CHAUMEIL *et al.* 2006; CLEMSON *et al.* 2006). Interestingly, transcription of repetitive LINE-1 elements, enriched on the X chromosome, coincides with the formation of this compartment (BAILEY *et al.* 2000; LYON 2000; CHOW *et al.* 2010). In humans, Xi-specific interactions between macrosatellite repeats have been observed (HORAKOVA *et al.* 2012). These observations suggest that repetitive elements on the mammalian X chromosome contribute to a nuclear organization characteristic of inactive X chromatin. Taken together, these studies suggest that in spite of the differences between flies and mammals, interphase chromosome architecture participates in X chromosome dosage compensation in both organisms.

The genomes of higher eukaryotes are rich in repetitive elements, but few functions have been attributed to these sequences. Our studies demonstrate that small RNA from the 1.688^{X} repeats promotes dosage compensation, a finding consistent with a role for the siRNA pathway in this process. The remarkable distribution of the 1.688^{X} repeats, which are essentially limited to the X chromosome, makes them strong candidates for *cis*-acting elements that serve to identify X chromatin. Future studies in our laboratories will focus on the mechanism by which the 1.688^{X} repeats promote identification of X chromatin.

Chapter 6

Summary & perspectives

My studies have identified novel regulators of X chromosome recognition such as the imprinted Y chromosome (Chapter 2) and the siRNA pathway (Chapters 4 and 5). Importantly, these are the first studies to implicate small RNA and 1.688^{x} satellite repeats in dosage compensation. I propose that this discovery underlies a novel mechanism in which small RNA from X-linked satellite repeats regulates X-identity. Answering the following questions may yield a new perspective on mechanisms that regulate X chromosome recognition in *Drosophila*.

How do siRNA and 1.688^{x} repeats influence dosage compensation?

The most obvious way that the 1.688^{X} repeats might influence dosage compensation is by facilitating a chromosome-specific organization that promotes recruitment of the MSL complex. Interestingly, the AT rich 1.688^{X} satellite repeats are predicted Matrix Attachment regions (MARs), suggesting a mechanism by which this might occur. MARs mediate the tethering of chromatin to the nuclear matrix, thereby regulating chromosome organization. siRNA from the 1.688^{X} repeats might direct chromosomal modifications at these repeats. These modifications could, in turn, promote the nucleation of X chromatin into an active sub-compartment or enhance chromosomal looping, facilitating MSL targeting of X-linked genes. In support of this idea, previous studies have identified a male-specific conformation of the X chromosome (Grimaud and Becker, 2009). In addition, RNAi factors like Ago2 and Rm62E mediate

chromosomal looping at certain insulator sites (LEI and CORCES 2006; GRIMAUD and BECKER 2009; MOSHKOVICH *et al.* 2011).

The involvement of satellite repeats in *Drosophila* dosage compensation draws an intriguing parallel between this process in flies and mammals. In mammals CTCF-bound macrosatellites repeats and active LINE-1 elements have been implicated in the nuclear organization of the inactive X chromosome (CHAUMEIL *et al.* 2006; CLEMSON *et al.* 2006). AT-binding proteins like SATB1 have been shown to influence the initiation of X inactivation, presumably through regulating chromosome architecture (AGRELO *et al.* 2009). Recent work in Drosophila, including the studies in this dissertation, suggest that similar molecular strategies may regulate dosage compensation in *Drosophila*.

To address these questions, transgenic flies bearing X-linked lacO (lac Operator) sites, combined with lacI-GFP (lac inducer GFP fusion) expression, are being developed to visualize X chromosome sub-nuclear localization. To analyze X chromosome architecture, chromosome conformation capture (3C) can be used to detect inter-chromosomal looping between distant 1.688^{X} repeats. These techniques explore the interphase architecture of the X chromosome, but may also be adapted to reveal the impact of siRNA mutations, 1.688^{x} repeat DNA, 1.688^{x} siRNA and long RNA on higher order organization of the male X chromosome.

Another possibility is that the long 1.688^{x} transcripts directly compete with the *roX* RNA for incorporation into the MSL complex. 1.688^{x} siRNA targeted destruction of these transcripts might enhance MSL recruitment. A RNA

immunoprecipitation (RIP) analysis could be performed to determine the association of long 1.688^{X} RNA with MSL proteins.

Does RNAi regulate chromatin at 1.688^{X} repeats?

Results presented in chapter 5 and Appendix B are consistent with the idea of a siRNA-targeted regulation of 1.688^{X} transcription. I hypothesize that the siRNA pathway, targeted to nascent transcripts, regulates chromatin modifications at these repeats. The role of small RNA in regulation of chromatin structure at specific loci is well-established in *Drosophila* and fission yeast (PAL-BHADRA *et al.* 2004; VERDEL *et al.* 2004; BROWER-TOLAND *et al.* 2007; USAKIN *et al.* 2007; WANG and ELGIN 2011). Targeted modification of 1.688^{X} repeat chromatin may influence the organization of the X chromosome, thereby influencing dosage compensation (CORONA *et al.* 2002; FURUHASHI *et al.* 2006; BAI *et al.* 2007; SPIERER *et al.* 2008b).

MNase sensitivity can reveal differences in chromatin packaging at the 1.688^{x} repeats in wild type, siRNA mutants and males ectopically expressing 1.688^{x} siRNA. Based on the outcome of these studies, Chromatin Immunoprecipitation (ChIP) can be performed to identify changes in specific histone modifications at the repeats. This study may also reveal the role of other epigenetic modifiers in dosage compensation. Known genetic or physical interactors of Ago2 are potential candidates that mediate such chromatin modifications.

What can we learn from the regulation of the Y chromosome imprint?

Results presented in Chapter 2 describe the first example of an imprint manifest in dosage compensation (MENON and MELLER 2009). Pursuing the Y chromosome imprint has the potential to reveal a novel mechanism of trans-generational epigenetic inheritance. Given that *Drosophila* imprints reside in heterochromatin, the Y chromosome is an excellent target for epigenetic marks that regulate imprinting. Genetic screens to identifying factors required for imprint establishment and maintenance will be valuable in understanding the mechanism of X-recognition. Factors such as dCTCF have been implicated in the imprinting of the mini-X chromosome in *Drosophila* (MACDONALD *et al.* 2010).

How does the imprinted locus on the Y chromosome influence X-recognition?

The imprinted locus was mapped to h11-h15 on the Y chromosome, a region that encompasses a small RNA producing locus (Appendix A). Interestingly, the transcription of 1.688^{X} repeats respond to the imprinted status of the Y chromosome (Appendix B). I propose that the imprinted Y chromosome modulates levels of 1.688^{X} siRNA. The technical difficulties encountered when investigating a Y-linked locus may prevent us from ever resolving the molecular function of the imprinted Y chromosome. However, we are currently sequencing small RNA from XXY and XO embryos (females carrying a Y chromosome and males lacking a Y chromosome). We anticipate that this study reveal whether the Y chromosome regulates accumulation of siRNA.

It may be possible to test whether autosomal insertions of BACs carrying h11-h15 sequence mimic the effect of the Y chromosome on dosage compensation. Transgenics can be generated from clones from the CHORI (Childrens Hospital Oakland Research Institute) *Drosophila melanogaster* BAC library, or from a cosmid library as described in (KALMYKOVA *et al.* 1998). Although my mapping studies suggest that *Su(Ste)* is not responsible for the maternal imprinting, an autosomal transgene bearing the *Su(Ste)* sequence from h11 can be directly tested (GVOZDEV *et al.* 2000).

Significant effects on somatic gene expression have been attributed to Y chromosome polymorphisms (LEMOS *et al.* 2010). Y chromosomes that have been evaluated for their effect on genome-wide expression patterns could be tested for their influence on dosage compensation. The influence of these Y chromosomes on *roX1* roX2 male survival may provide insight into the mechanism by which Y chromosomes achieve genome-wide effects. One possibility is that the Y-linked locus that influences dosage compensation affects expression of autosomal genes also. It is theoretically possible that the Y chromosome modulates expression of many somatic genes by modulation of small RNA-dependent systems.

APPENDIX A

MAPPING THE IMPRINTED LOCUS ON THE Y CHROMOSOME

Our studies revealed that parentally imprinted Y chromosomes are potent modifiers of *roX1 roX2* male lethality (MENON and MELLER 2009). When transmitted through the female germline, a wild type Y chromosome from our laboratory reference strain (y^1w^{1118}) can increase *roX1 roX2* male survival as much as nine-fold (Chapter 3). Although once thought to function solely in the male germline, the Y chromosome has been shown to influence expression in the soma, particularly of genes with male-biased expression and those implicated in transcription, chromosome organization and chromatin assembly (LEMOS *et al.* 2008; JIANG *et al.* 2010; LEMOS *et al.* 2010). Mapping the imprinted locus could potentially reveal novel factors or pathways that regulate dosage compensation.

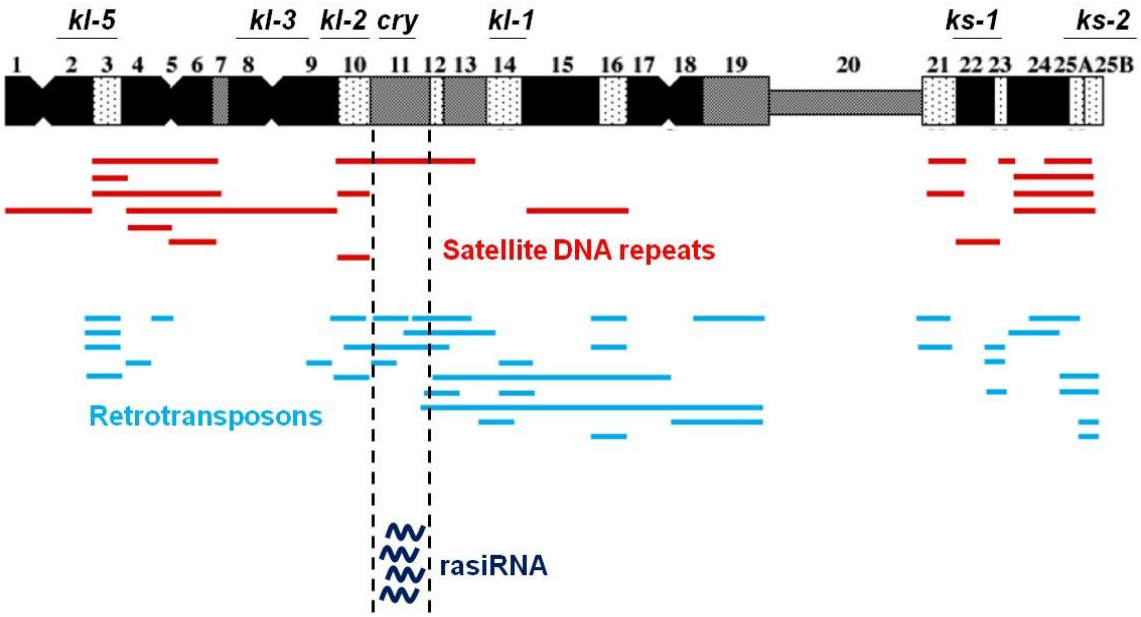


Figure A1. Schematic map of *Drosophila* Y chromosome. The entire Y chromosome is divided into cytological bands h1-h25 based. Positions of Y-linked fertility factors, satellite repeats, retrotransposons and repeat associated small interfering RNA (rasiRNA) producing loci are indicated. The maternally imprinted locus maps to h11 – h15. Adapted from (PIERGENTILI 2010).

Maternal imprinting of the Y chromosome region h11-h15 suppresses *roX1 roX2* male lethality

To identify the region of the Y chromosome that suppresses *roX1 roX2* lethality, translocations [*T(X;Y)*] were used to transmit fragments of the Y chromosome to *roX1 roX2* sons either maternally or paternally (KENNISON 1981; HARDY *et al.* 1984). The region capable of the most pronounced suppression of *roX1 roX2* male lethality lies between h11 and h15 (Table A1, Fig. A1). This indicates that bulk Y chromosome heterochromatin does not mediate the effect of the Y chromosome on *roX1 roX2* survival. The critical maternally imprinted region contains protein coding genes with germline expression, and is enriched

for simple satellite sequences and a wide variety of retrotransposons (PIERGENTILI 2010). It also contains *Su(ste)* at h11, also known as *crystal* (*cry*), a source of small RNA in the male germline (HARDY *et al.* 1984; LIVAK 1984; PALUMBO *et al.* 1994; ARAVIN *et al.* 2001; ARAVIN *et al.* 2004). A deletion of h11 (cry^1) results in a loss of small RNA from this locus (ARAVIN *et al.* 2004). We tested the effect of a maternally transmitted cry^1 Y chromosome on *roX1 roX2* male survival. Unlike the wild type reference Y chromosome, the cry^1 Y chromosome did not suppress *roX1 roX2* lethality, rescuing only 1.4% *roX1 roX2* adult male escapers (Table. A1). This suggested that *cry* might be the imprinted locus influencing dosage compensation. Due to the polymorphic nature of Y chromosomes we considered the possibility that the progenitor of the cry^1 deletion may differ from our reference wild type Y chromosome. Testing the progenitor Y chromosome is limited by uncertainty about the origin of the cry^1 mutant. Nonetheless, the absence of a plausible candidate genes, combined with the presence of a small RNA-producing locus and enrichment for retroelements and satellites, sequences sometimes associated with small RNA production, prompted an examination and the subsequent identification of the role of the siRNA pathway in dosage compensation (chapter4, 5).

Translocation	Y-region	*roX1 roX2* male survival (%) Transmission: Paternal	Transmission: Maternal
Intact Y	h1-h25	5	37
T(1;Y) V24	h1-h3	0.3	0
T(1;Y) P7	h1-h10	2.0	0.7
T(1;Y) E15	h1-h12	8.0	14
T(1;Y) F12	h1-h15	8.0	30
T(1;Y) W19	h21-h25	6.0	3.4
T(1;Y) V8	h24-h25	4.0	0.7
$B^S cry^1 Yy^+$	h11 deletion	2.6	1.4
$B^S Yy^+$ [a]	h1 – h25	ND	0.3
$B^S Yy^+$ [b]	h1 – h25	ND	0
$B^S Yy^+$ [c]	h1 – h25	ND	0

Table A1. The Y chromosome suppressor of *roX1 roX2* lethality resides in region h11-h15. *T(1;Y)* translocations were used to test the effect of different regions of the Y chromosome on $roX1^{mb710}$ *roX2* male survival. The entire Y chromosome (cytological regions h1-h25, (GATTI and PIMPINELLI 1983)), *T(1;Y)* are transmitted paternally or maternally to $roX1^{mb710}$ *roX2* sons. Paternal inheritance of translocations are achieved by mating *T(1;Y)* males to $roX1^{mb710}$ *roX2* females, while maternal inheritance is achieved by mating $C(1)RM y^1 v^1$/ *T(1;Y)* females to *roX1 roX2* / *Dp(1;Y)* $B^s v^+ y^+$ males. Elimination of *roX2* is achieved by the complex deletion (*Df(1)52;* [w^+ 4Δ4.3]) described in (MELLER and RATTNER 2002). To test the effect of the cry^1 deletion within h11 on $roX1^{mb710}$ *roX2* male survival , $B^S cry^1 Yy^+$ and $B^S Yy^+$ (cry^1 progenitor) chromosomes were maternally transmitted by mating *C(1)Dxyf*/$B^S cry^1 Yy^+$ or *C(1)Dxyf*/$B^S Yy^+$ females to *roX1 roX2*/*Dp(1;Y)*$B^s v^+ y^+$ males. Paternal transmission of these Y chromosomes were not determined (ND). Putative cry^1 progenitor chromosomes were identified based on description in (BROSSEAU 1960; BROSSEAU *et al.* 1961; PALUMBO *et al.* 1994). Progenitor $B^S Yy^+$ chromosome derived from (a) *yv*/*Dp(1;Y)*$B^S Yy^+$;*bw* (Bloomington stock#2523), (b) *Df(1)n23*/*Binsn*/*Dp(1;Y)*$B^S Yy^+$ (bloomington stock#7426) and (C) $yw^a ste$/*Dp(1;Y)*$B^S Yy^+$ (bloomington stock# 3707).

The rDNA locus on the Y chromosome does not influence dosage compensation

The Y-linked rDNA is a potent regulator of global heterochromatin in *Drosophila*, and is known to be imprinted (PAREDES and MAGGERT 2009) (Maggert, K. A. per.comm.). The Y -linked rDNA locus consists of 150-225 rDNA cistrons. The rDNA array is smaller in size on a maternally inherited Y chromosome than an identical Y chromosome inherited paternally (Maggert, K. A. per.comm.). The transmission-dependent differences observed at the rDNA locus prompted us to examine the role of Y-linked rDNA on *roX1 roX2* male survival.

Y chromosome	*roX1 roX2* male survival (%)
y^+Y*rDNA*$^+$	18.6
y^+Y*rDNA*Δ	16.8

Table A2. rDNA on the Y chromosome does not modify *roX1 roX2* lethality. The Y chromosome with entire array of rDNA cistrons and an identical Y chromosome deleted for the rDNA array are transmitted paternally to *roX1*^{mb710} *roX2* sons. Three independent Y chromosomes deleted for rDNA were tested. The average survival of *roX1*^{mb710} *roX2* from these three Y chromosomes is represented. Elimination of *roX2* is achieved by the complex deletion (*Df(1)52;* [w^+ 4Δ4.3]) described in (MELLER and RATTNER 2002).

To address this question, I obtained strains of flies with characterized deletions of the Y-linked rDNA genes and determined whether these influenced *roX1 roX2* male lethality. Similar numbers of *roX1 roX2* males inheriting a wild type Y chromosome or a Y chromosome with reduced rDNA copy number were

obtained (Table. A2), suggesting that the rDNA locus on the Y chromosome does not affect dosage compensation.

APPENDIX B

REGULATION OF 1.688^{X} SATELLITE REPEAT TRANSCRIPTION IN *DROSOPHILA*

1.688^{X} satellite transcripts reflect Y chromosome origin

The localization of putative siRNA producing repeats to h11-h15 of the Y chomosome prompted us to ask whether the Y chromosome affects 1.688^{X} repeat transcription (Appendix A). To test this hypothesis I generated males with identical, wild type X ($y^{1}w^{1118}$) and Y chromosomes that differ only in the mode of transmission. I measured the levels of 1.688^{roX1}, 1.688^{7F1} and 1.688^{3C} transcripts in males without a Y chromosome (X_MO), males with a maternally inherited Y chromosome (X_PY_M) and a paternally inherited Y chromosome (X_MY_P) by quantitative RT-PCR using repeat specific primers (Fig. B1). 1.688^{roX1} and 1.688^{7F1} transcript levels were the highest in X_MY_P males, X_MO males had intermediate levels, and X_PY_M males had the lowest levels (Fig. B1.A). The 1.688^{3C} transcript levels were also higher in X_MY_P than in X_PY_M males, but in X_MO males 1.688^{3C} transcript levels were dramatically higher than in either X_MY_P or X_PY_M males (Fig. B1.B). Despite these inconsistencies, the striking effect of the Y chromosome on 1.688^{X} transcript levels lead us to speculate that the Y chromosome might influence dosage compensation by modulating 1.688^{X} siRNA levels from the X chromosome.

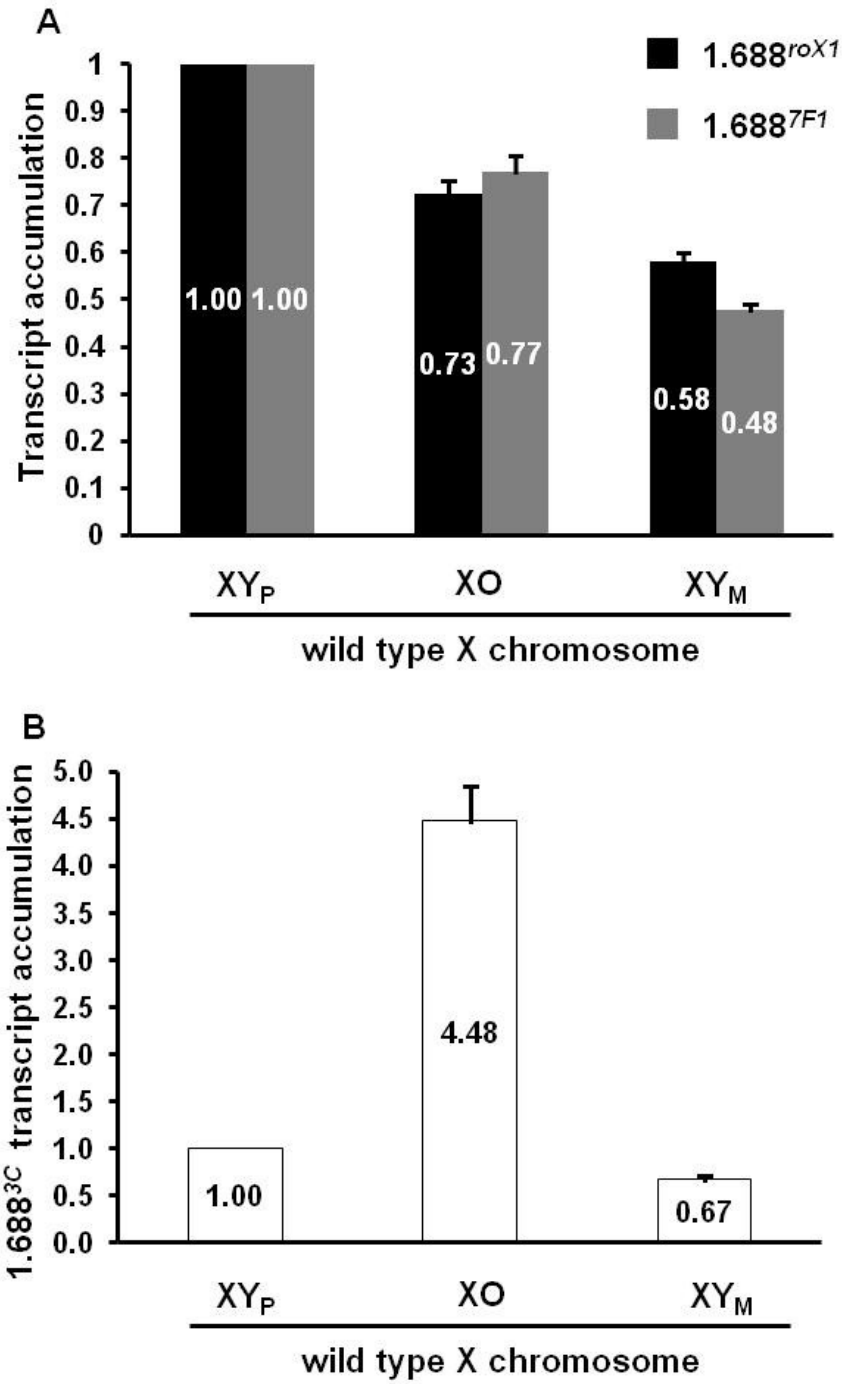


Figure B1. 1.688^{X} repeats respond to Y chromosome origin. Transcription of (A) 1.688^{roX1} (Black bars) and 1.688^{7F1} (grey bars) repeats (B) 1.688^{3C} repeats. RNA was isolated from wild types XY_P, XO and XY_M males and measured by quantitative RT-PCR using repeat specific primers (chapter 5). Numerical values within plotted bars represent the relative expression obtained from two biological replicates of each genotype, normalized to an autosomal gene, *dmn*.

1.688^{X} transcript levels respond to siRNA mutations

Chapter 4 details the role of various siRNA factors in dosage compensation. Chapter 5 contains a study suggesting that the rescue of *roX1 roX2* male survival by 1.688^{roX1} hpRNA is dependent upon the siRNA factors *dcr2* and *ago2*. We postulate that the 1.688^{X} satellite repeats on the X are potential targets and sources of siRNA. To further investigate the regulation of 1.688^{X} repeats by the siRNA pathway, I analyzed expression of 1.688^{roX1},1.688^{7F1} and 1.688^{3C} repeats in *ago2*^{414} , *D-elp1*^{c00296}/+ and *loqs*^{f00791} males. Compared to wild type males, a loss of Ago2 reduces the abundance of transcripts from all three families of repeats, while a loss of Loqs or a reduction in D-Elp1 only decreases the abundance of 1.688^{roX1} transcripts without any significant effect on the 1.688^{3C} transcripts (Fig. B2). Despite these differences, the uniform effect of *ago2*^{414} on 1.688^{X} repeats suggests a model of Ago2 mediated transcriptional activation of repeats rather than repression. I predict that in *ago2*^{414} males a reduction in 1.688^{X} transcription will impact siRNA biogenesis by limiting the availability of precursor sense and anti-sense transcripts. A loss of 1.688^{X} siRNA will reduce recruitment of Ago2 to the repeats. Such a model agrees with the observation that in *roX1 roX2* males both Ago2 and 1.688^{X} siRNA enhance dosage compensation (chapter 4, 5).

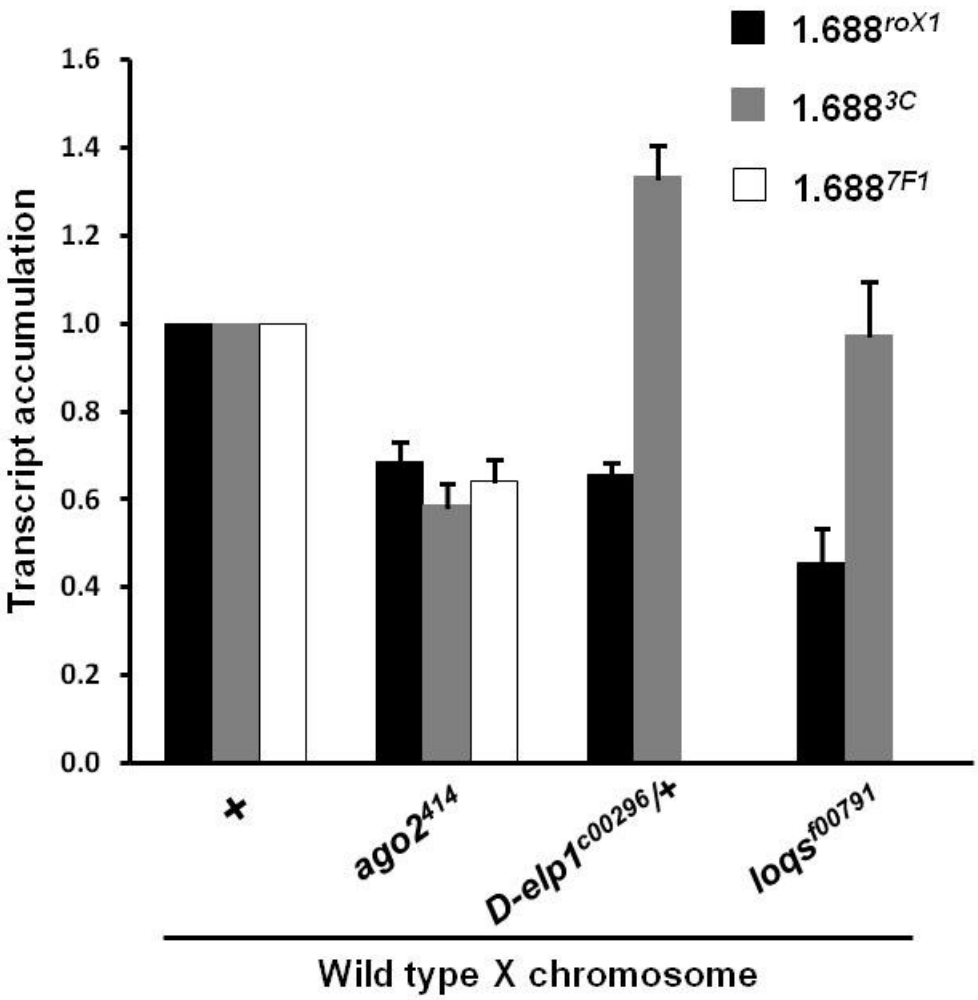


Figure B2. 1.688^{X} transcription responds to siRNA mutations. Accumulation of 1.688^{roX1} (black), 1.688^{3C} (grey) and 1.688^{7F1} repeat (white) transcripts in +, *ago2*^{414}, *D-elp1*^{c00296}/+ and *loqs*^{f00791} males carrying a wild type X chromosome (*y*^{1}*w*^{1118}). RNA was isolated from three biological replicates for each genotype. Expression was determined by quantitiative RT-PCR using repeat specific primers and normalized to an autosomal gene, *dmn*.

APPENDIX C

Ago2 ACTIVITY INFLUENCES *roX1 roX2* MALE SURVIVAL

Ago2 enhances *roX1 roX2* male survival and X chromosome recognition (chapter 4). To test whether the slicing activity of Ago2 is required for its function in dosage compensation, I determined genetic interactions between a RISC (RNAi induced silencing complex) deficient mutant, $ago2^{V966M}$ that is unable to slice target RNA and $roX1^{ex40A}$ *roX2*Δ (KIM *et al.* 2007; MENON and MELLER 2012). $roX1^{ex40A}$ *roX2*Δ males exhibit full viability (chapter 4). Survival of $roX1^{ex40A}$ *roX2*Δ survival is reduced by 40% in the slicing deficient Ago2 (Table C1). A loss of Ago2 catalytic function therefore enhances *roX1 roX2* lethality.

X chromosome	*ago2*	% male survival
$roX1^{ex40A}$ *roX2*Δ	$ago2^{V966M}$/ +	92.16 (658)
$roX1^{ex40A}$ *roX2*Δ	$ago2^{V966M}$	59.40 (297)

Table C1. Ago2 catalytic activity is required for $roX1^{ex40A}$ *roX2*Δ male survival. Loss of a functional Ago2-RISC reduces $roX1^{ex40A}$ *roX2*Δ male survival. Survival of $roX1^{ex40A}$ *roX2*Δ; $ago2^{V966M}$ males was determined by crossing $roX1^{ex40A}$ *roX2*Δ; $ago2^{V966M}$/*TM3SbTb* males and females. Numbers within parenthesis represent total males eclosed.

I similarly tested whether gain of Ago2 function suppressed *roX1 roX2* lethality. To do this I tested the effect of the $ago2^{EY04479}$ on $roX1^{ex33}$ *roX2*Δ male survival. $ago2^{EY04479}$ is generated by a P-element insertion bearing a GAL4 responsive UAS (upstream activating sequence) in the correct orientation to drive expression of *ago2* . A gain of function is achieved by driving the expression of

GAL4 in mutant flies (Table C2). For this study I used the *roX1*^{ex33} *roX2*Δ X chromosome that usually supports ~20 % male survival. The survival of *roX1*^{ex33} *roX2*Δ; *ago2*^{EY04479}/+ males is 20%, suggesting that the P-element insertion by itself does not modify the *roX1*^{ex33} *roX2*Δ male lethality. However in the presence of a [GAL4-tub] driver, *roX1*^{ex33} *roX2*Δ; *ago2*^{EY04479} /+ male survival was increased from 20% to 50%. These studies reinforce our earlier observations and support the involvement of Ago2 in dosage compensation.

Male genotype	Gain of function	% male survival
yw roX1^{ex33} *roX2*Δ; *ago2* ^{EY04479}/ +	-	20 (190)
yw roX1^{ex33} *roX2*Δ; *ago2* ^{EY04479}/ *TM3Sb*	-	37 (364)
yw roX1^{ex33} *roX2*Δ; +/ [GAL4-*tub*]	-	36 (202)
yw roX1^{ex33} *roX2*Δ; *ago2* ^{EY04479} / [GAL4-*tub*]	+	50 (271)

Table C2. Gain of function *ago2* suppresses *roX1*^{ex33} *roX2*Δ lethality. Ago2 gain of function enhances *roX1*^{ex33} *roX2*Δ male survival. The survival of *yw roX1*^{ex33} *roX2*Δ; *ago2* ^{EY04479}/ + males was determined from a cross between yw; *ago2* ^{EY04479}/ + males and *yw roX1*^{ex33} *roX2*Δ females. The survival of *yw roX1*^{ex33} *roX2*Δ; *ago2* ^{EY04479}/ *TM3Sb, yw roX1*^{ex33} *roX2*Δ*; +/ [GAL4-tub]* and *yw roX1*^{ex33} *roX2*Δ; *ago2* ^{EY04479} / [GAL4-*tub*] male siblings were determined by crossing *yw;ago2* ^{EY04479}/ + males to *yw roX1*^{ex33} *roX2*Δ; [Gal4-*tub*]/ *TM3Sb* females. *ago2*^{EY04479} is marked with y^+ and [GAL4-*tub*] contains a w^+ reporter. Numbers within parenthesis represent total males eclosed.

APPENDIX D

EFFECT OF siRNA MUTATIONS ON X-LINKED GENE EXPRESSION

A loss of *roX* function reduces X-linked gene expression by 25 %. siRNA mutations are synthetic lethal with *roX1*^{ex40A} *roX2*Δ in males (chapter 4). To determine whether siRNA mutations affect X –linked gene expression, I examined the expression of 6 candidate genes in wild type, *ago2*^{414}, *roX1*^{ex40A} *roX2*Δ, *roX1*^{ex40A} *roX2*Δ; *ago2*^{414} and *roX1*^{ex40A} *roX2*Δ; *D-elp1*^{c00296}/+ males by qRT-PCR (Fig. D1, Table D1). Although there was a decrease in expression of certain X-linked genes like SkpA, Dlmo and Sgs4 in *roX1*^{ex40A} *roX2*Δ; *ago2*^{414} and *roX1*^{ex40A} *roX2*Δ; *D-elp1*^{c00296}/+ males, It was difficult to identify a stable trend and detect significant changes in gene expression for all genes by qRT- PCR (Fig. D1). Furthermore the sharp decline in certain genes like Sgs4 can also be attributed to its temporal expression profile that transitions from very high levels of expression to a subsequent decay in expression during the 3rd instar larval stage (Flybase temporal expression profile) (GRAVELEY *et al.* 2011).

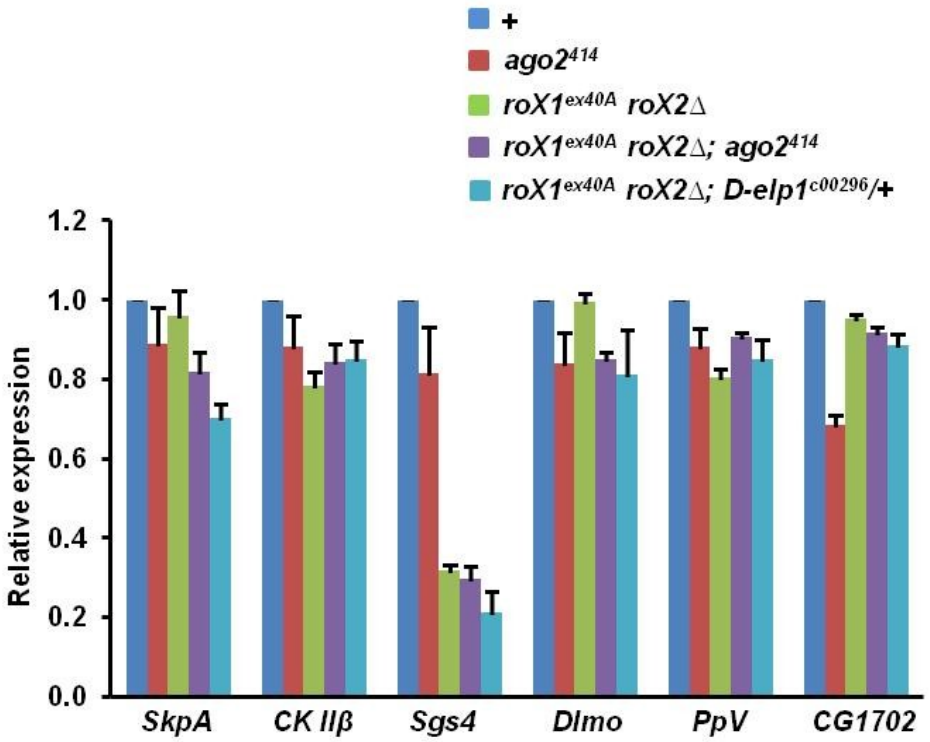


Figure D1. The effect of *ago2*^{414} and *D-elp1*^{c00296}/+ on X-linked gene expression. Expression of the X-linked genes *SkpA*, *Ck- IIβ*, *Sgs4*, *Dlmo*, *PpV* and *CG1702* was measured using qRT-PCR. Male larvae were wild type (blue), *ago2*^{414} (red), *roX1*^{ex40A} *roX2*Δ (green), *roX1*^{ex40A} *roX2*Δ*; ago2*^{414}/+ (purple) and *roX1*^{ex40A} *roX2*Δ*; ago2*^{414} (teal). Expression was normalized to the autosomal genes, *Dmn* and *Ytr*. Error bars represent the standard error of three biological replicates.

Table D1. List of X-linked genes tested by qRT-PCR

Name	Cytological position	Primer sequence	
SkpA	1B14-1B14	F	CTAAAAGTCGACCAGGGCAC
		R	CCAGATAGTTCGCTGCCAAT
Sgs4	3C10-3C10	F	GAAGGACCTGCTAACACCGA
		R	ATTTACACTTGGGTGCAGGC
PpV	5F4-5F4	F	TTGACCACCCATGAACTCAA
		R	GTGTTTGCTATGCTTGGGGT
CK IIβ	10E3-10E3	F	CCTGGTTCTGTGGACTTCGT
		R	GTAGTCCTCATCCACCTCGC
CG1702	19D1-19D1	F	GACATCTTTGCAGCCTGTGA
		R	GCCCTGATCTTGGGGTACTT
Dlmo	17C3-17C4	F	CCAATGTCTATCACTTGGAGTGC
		R	CAGAATCTGTGGTTACACTGCTG

A list of X-linked genes, Cytological position and sequence of primers used to measure transcript levels by qRT-PCR. Forward primers are denoted by F, reverse primers are denoted by R.

APPENDIX E

THE EFFECT OF [hp 1.688^{roX1}]5B ON *roX1*^{ex33} *roX2*Δ MALE SURVIVAL

The expression of double stranded 1.688^{roX1} hpRNA from three independent insertions of an identical snapback transgene resulted in a 2.5 fold increase in *roX1*^{ex33} *roX2*Δ male survival (chapter5). Interestingly an additional insertion, [hp 1.688^{roX1}]5B failed to rescue *roX1*^{ex33} *roX2*Δ male survival (Fig. E1).

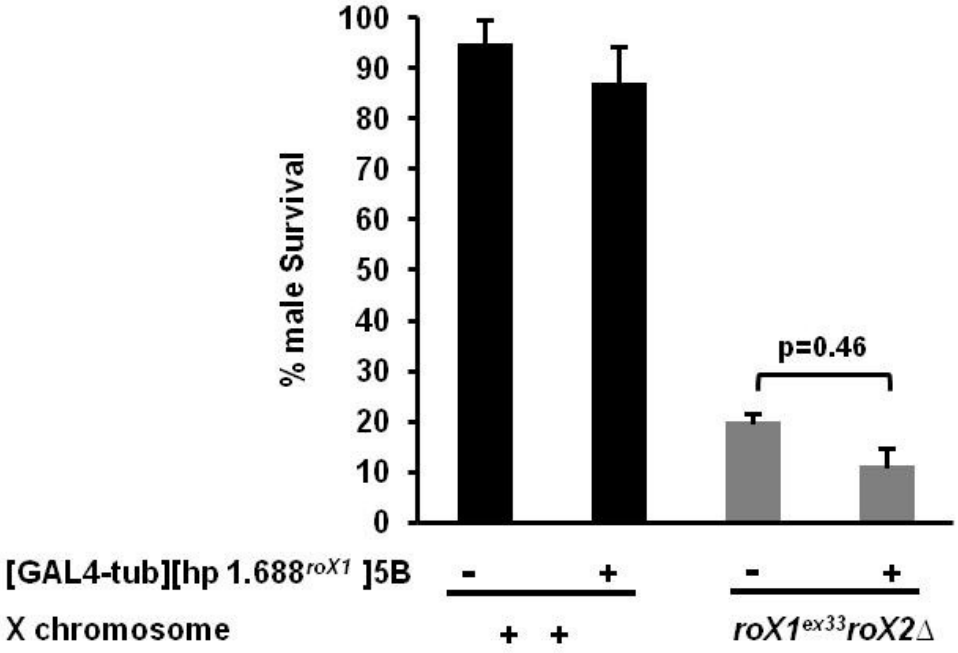


Figure E1. [hp 1.688^{roX1}]5B does not rescue *roX1*^{ex33}*roX2*Δ male survival. The survival of wild type (black) and *roX1*^{ex33} *roX2*Δ (grey) males expressing 1.688^{roX1} hpRNA from the [hp 1.688^{roX1}]5B induced by GAL4. p-value is determined by students unpaired t-test. Error bars represent SEM.

This may be due to the fact that [hp 1.688^{roX1}]5B expresses lower levels of hpRNA . When induced, the [hp 1.688^{roX1}]5B insertion produces 3 fold less hpRNA than the [hp 1.688^{roX1}]12 insertion that rescues *roX1 roX2* male survival (Fig. E2). Based on the lighter eye pigmentation seen in adult [hp 1.688^{roX1}]5B transgenics, we speculate that the transgene may be in a repressive environment. The processing of hairpin RNA from a more heterochromatic site might influence the targeting of the siRNA or shunt it into other pathways.

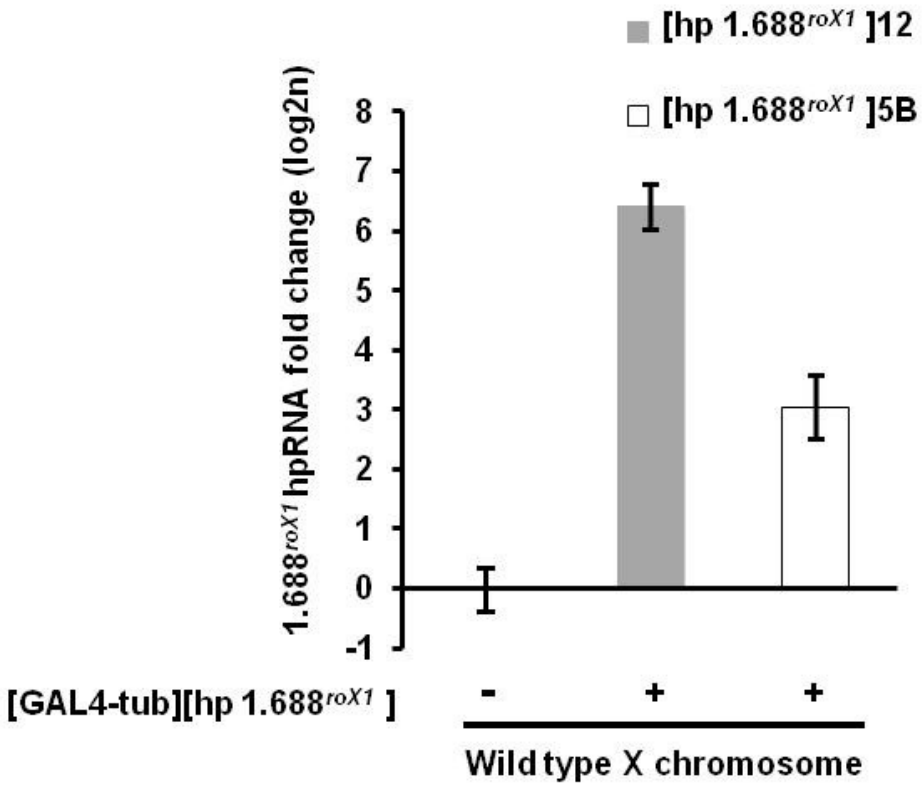


Figure E2. Expression of 1.688^{roX1} hpRNA from [hp 1.688^{roX1}] transgenes. Log fold change in 1.688^{roX1} hpRNA levels from [hp 1.688^{roX1}]12 (grey) and [hp 1.688^{roX1}]5B insertions. Changes in expression are represented in log2n scale relative to wild type males without transgene.SEM is represented by error bars.

APPENDIX F

CHROMATIN MODIFIERS INFLUENCE *roX1 roX2* MALE SURVIVAL

Genetic and physical interactions between the MSL complex and various chromatin modifiers have been previously reported (CORONA *et al.* 2002; SPIERER *et al.* 2005; MENDJAN *et al.* 2006; BAI *et al.* 2007; SPIERER *et al.* 2008a; WANG *et al.* 2013). I screened a few of these factors, Imitation switch protein (ISWI), SU(VAR)2-5 (Heterochromatin protein -1), SU(VAR)3-7 and SU(VAR)3-9 (H3K9 ,methyl transferase) for their ability to modify *roX1 roX2* male lethality. $roX1^{ex33}$ *roX2*Δ females were crossed to males heterozygous for mutations in each of these chromatin modifiers. Survival of $roX1^{ex33}$ *roX2*Δ; *modifier*/+ males relative to their $roX1^{ex33}$ *roX2*Δ ; +/+ brothers was determined. A reduction in the levels of the heterochromatic proteins SU(VAR)3-7 and SU(VAR)3-9 significantly enhanced $roX1^{ex33}$ *roX2* male lethality by 40% and 50%, respectively.

SU(VAR)3-7 , SU(VAR)3-9 are required for chromatin silencing and are known to genetically and physically interact with each other. Interestingly a genetic interaction between the MSL complex and *Su(var)3-7* was revealed through its effect on X chromosome morphology. *Su(var)3-7* mutants exhibit, X chromosome bloating coupled with MSL delocalization. Maintenance of balanced X chromatin structure could influence correct MSL localization. Although a loss of HP1 and ISWI also produce similar effects on X chromosome morphology, I did not detect a significant effect of heterozygous *Su(var)2*-5 (HP1) and *iswi* mutations on $roX1^{ex33}$*roX2*Δ survival. A partial reduction of these factors may not be limiting for dosage compensation.

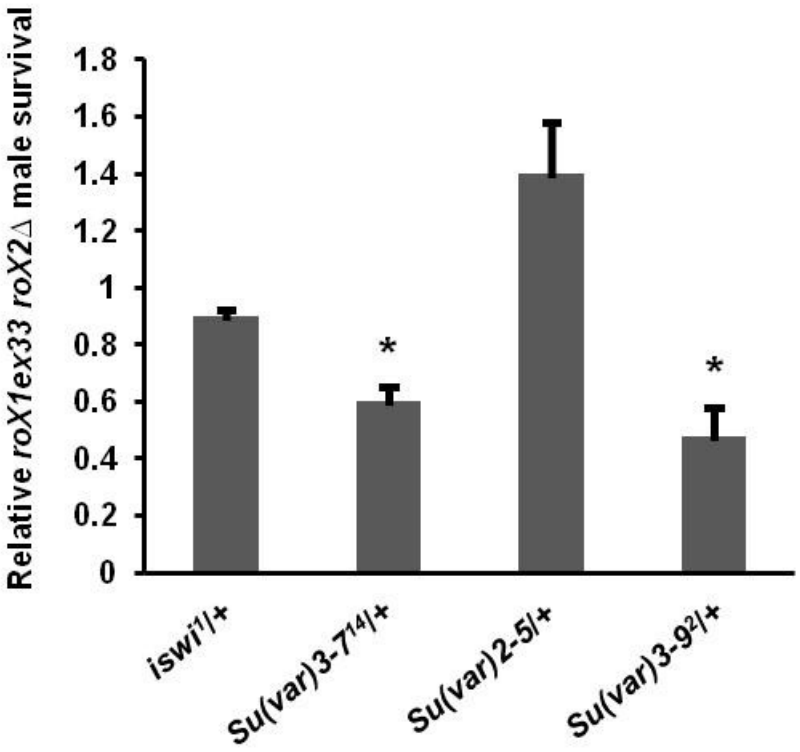


Figure F1. Mutations in chromatin modifiers enhance *roX1 roX2* male lethality. Number of *roX1*^{ex33} *roX2*Δ; *modifier*/+ males divided by their *roX1*^{ex33} *roX2*Δ brother wild type for chromatin modifier. * denotes a significant students unpaired t-test $p<0.05$. Error bars represent SEM.

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ABSTRACT

AN INVESTIGATION OF X CHROMOSOME RECOGNITION: THE ROLE OF SMALL RNA IN DROSOPHILA DOSAGE COMPENSATION

BY

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In humans and flies, females have two X chromosomes but males have one X chromosome and one Y chromosome. This leads to a fatal imbalance in X-linked gene expression in one sex. In mammals and in the fruit fly *Drosophila,* modulation of X chromosome expression is critical for survival. This process is termed dosage compensation. Flies increase expression from the male X chromosome two-fold. This is achieved by the Male Specific Lethal (MSL) complex, which consists of two large, non-coding *RNA on the X* transcripts (*roX1* and *roX2*) and five proteins. The *roX* RNAs have a critical role in complex localization to the X chromosome. Simultaneous mutation of *roX1* and *roX2* reduces X localization of the MSL proteins, lowers X-linked expression and reduces male survival. Using *roX1 roX2* mutants, we performed genetic studies to identify modifiers of X chromosome recognition. In spite of a lack of expression in somatic tissues, the Y chromosome is a potent modifier of the *roX1 roX2* phenotype. I postulated that the Y chromosome could affect dosage compensation through a small RNA-dependent pathway, and performed a screen

of RNAi mutations. This screen identified four siRNA genes that, when mutated, enhance *roX1 roX2* male lethality and disrupt MSL localization to the X chromosome. The role of the siRNA pathway in dosage compensation prompted an investigation of potential sources of siRNA. A class of 1.688g/cm^3 satellite-related repeats is exclusive to the X chromosome (1.688^X). These are transcribed, and thus capable of generating siRNA in animals. Ectopic expression of long single stranded 1.688^X RNA reduced *roX1 roX2* male survival. In contrast, expression of double stranded 1.688^X hairpin RNA produced high levels of corresponding small RNA and dramatically rescued *roX1 roX2* male survival. MSL localization to the X chromosome was partially restored in flies expressing 1.688^X hairpin RNA. Rescue of *roX1 roX2* males was dependent upon the siRNA genes *Dcr2* and *Ago2*. These studies reveal that small RNA from X-linked repeats acts through the siRNA pathway to promote X chromosome recognition. I postulate that the 1.688^X RNA repeats underline X chromosome identity. Future studies exploring this process will help us to understand the molecular basis for exclusive modification of the X chromosome.

AUTOBIOGRAPHICAL STATEMENT

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Educational Qualification

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Publications

Menon, D.U., and Meller, V.H.(2012). A role for siRNA in X Chromosome Dosage Compensation in *Drosophila melanogaster*. Genetics *191(3),1023-1028*.

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