A role for siRNA in X Chromosome Dosage Compensation in Drosophila melanogaster

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Abstract

Sex chromosome dosage compensation requires selective identification of X chromatin. How this occurs is not fully understood. We show that siRNA mutations enhance the lethality of *Drosophila* males deficient in X recognition, and partially rescue females that inappropriately dosage compensate. Our findings are consistent with a role for siRNA in selective recognition of X chromatin.
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 (DENG et al. 2011; GUPTA et al. 2006; NGUYEN and DISTECHE 2006). 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 (DENG and MELLER 2006a). The MSL complex directs H4K16 acetylation to the body of X-linked genes, increasing transcription by enhancing RNA polymerase II processivity (LARSCHAN et al. 2011; SMITH et al. 2001).

Recruitment of the MSL complex is postulated to occur at X-linked Chromatin Entry Sites (CES) (ALEKSEYENKO et al. 2008; KELLEY et al. 1999; 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 (DENG and MELLER 2006b; MELLER 2003). 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 (DENG and MELLER 2006b; MELLER and RATTNER 2002).
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 (reviewed by 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 (JIANG et al. 2010; LEMOS et al. 2008; 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 overexpression of X-linked genes. Our findings are consistent with participation of siRNA in recognition of X chromatin.

Results and Discussion

The roX1\textsuperscript{ex33} roX2Δ X chromosome supports about 20% eclosion of adult male escapers. roX1\textsuperscript{ex33} roX2Δ females were mated to males heterozygous for mutations in the piRNA, siRNA and miRNA pathways (RNAi\textsuperscript{+/+}). The survival of sons with reduced RNAi function (roX1\textsuperscript{ex33} roX2Δ ; RNAi\textsuperscript{+/+}) was divided by that of their brothers with intact RNAi (roX1\textsuperscript{ex33} roX2Δ ; +/+ ) 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\textsuperscript{ex33} roX2Δ males by 30%, 55%, 50% and 70%, respectively (Figure 1A). 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 (Marques et al. 2010; Okamura et al. 2008; Zhou et al. 2009). 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.

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, Figure 1B; Kelley et al. 1995). In contrast, eclosion of sisters not expressing MSL2 peaks on day 2 (gray bars, Figure 1C). Eclosion of [H83M2]6I females with one mutated ago2 allele is advanced by two days, peaking on day 4 (black bars, Figure 1B). Reduction of Ago2 in otherwise wild type females had no discernable effect on eclosion timing (Figure 1C). 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.

The roX1\textsuperscript{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\textsuperscript{ex40} roX2\Delta males is similar to that observed in wild type flies. roX1\textsuperscript{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\textsuperscript{ex40} roX2\Delta males, survival is reduced to 8% (Figure 2A). Loss of Loqs reduces roX1\textsuperscript{ex40} roX2\Delta male survival by over 50% (Figure 2B). roX1\textsuperscript{ex40} roX2\Delta males with reduced D-Elp1 levels have full viability, but D-elp1 lethality precludes
homozygote testing. We took advantage of the synthetic lethality between \textit{roX1}^{ex40} \textit{roX2}\Delta and siRNA mutations to explore how siRNA contributes to dosage compensation.

To address the possibility that siRNA mutations act by modulating the level of \textit{roX} RNA, qRT PCR was used to measure \textit{roX1}^{ex40} transcript in \textit{ago2}^{414} or \textit{D-elp1}^{c00296/+} males. Accumulation of \textit{roX1}^{ex40} RNA was unaffected by these mutations (Figure S1A). 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 (Figure S1C - 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 \textit{roX1}^{ex40} \textit{roX2}\Delta chromosome did not itself affect MSL protein levels (Figure S1C - F). Disruption of dosage compensation in \textit{roX1} \textit{roX2} males with reduced siRNA therefore does not involve reduction in the core components of the MSL complex.

The synthetic lethality between \textit{roX1}^{ex40} \textit{roX2}\Delta 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 (Figure 3A). Reduction of D-Elp1 did not discernibly affect MSL1 localization to the polytene X chromosome of otherwise wild type males (Figure 3B). A slight disruption of X-localization was detected in \textit{ago2} mutants, but this was only marginally higher than that observed in wild type controls (Figure 3B, C and E; Table S1).

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 \textit{roX1}^{ex40} \textit{roX2}\Delta 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 (Figure 3B and C; Table S1). This supports the idea that \textit{roX1}^{ex40} has a defect in function. However, mislocalization of MSL1 was notably more severe in chromosome preparations from \textit{roX1}^{ex40} \textit{roX2}\Delta; \textit{ago2}^{414} and \textit{roX1}^{ex40} \textit{roX2}\Delta; \textit{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 (Figure 3E). These same genotypes
displayed a three-fold increase in ectopic autosomal MSL1 localization (Figure 3D, F and G; Table S1). Despite increased mislocalization of the MSL complex, roX1ex40 roX2Δ; D-elp1c00296/+ male viability appears unaffected, and the viability of roX1ex40 roX2Δ males with reduced levels of Ago2 or Loqs is also high (Figure 2A and B). It is possible that this disparity is because the accumulation of mutated roX1 transcripts, including roX1ex40, is lower in the salivary gland than in other tissues (Figure S1 B, see Figure 3 in (DENG et al. 2005). In spite of reduced transcript in the salivary gland, the roX1ex40A mutant directs considerable X-localization of the MSL complex, in accord with the ability of roX1ex40 roX2Δ 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.

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 (BROWER-TOLAND et al. 2007; PAL-BHADRA et al. 2004; VERDEL et al. 2004; 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 (MOSHKOVICH and LEI 2010; YIN and LIN 2007). 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.
Intriguingly, the male X chromosome displays an interphase conformation distinct from that in females (GRIMAUD and BECKER 2009).

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 1992). roX1 mutations, and a complex roX2 deletion (Df(1)52; [w+4Δ4.3]) have been described (DENG et al. 2005; MELLER and RATTNER 2002; MELLER et al. 1997). A viable deletion of roX2 (roX2Δ) was accomplished by FLP-mediated recombination between CG1169501356 and nod04008. Description of dcr206544, ago2201, ago2414, r2d21, D-elp1c00296, loqs00791, ago1k00281, spn-E1, aubQC42, aubHN, piwi06843 can be found at http://flybase.org. ago2414 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 (LEMS et al. 2008). After rebalancing, all mutations were confirmed by PCR or phenotype. Matings to determine the effect of RNAi pathway mutations on roX1ex33 roX2Δ male and yw female survival are detailed in the supplementary information (Figure S3).

qRT-PCR:
Accumulation of roX1ex40 transcript was measured by qRT PCR as previously described (DENG et al. 2009). 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 TTTTTGTCCACCCGAATAA and CCTTTTAATGCGTTTTCGGA. Expression of roX1ex40 was normalized to autosomal Dmn, amplified with 300 nM of primers GACAAGTTGAGCCGCTTAC and CTTGGTGCTTAGATGACGCA.
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Figure legends

FIGURE 1. siRNA mutations enhance roX1 roX2 male lethality. (A) Eclosing roX1<sup>ex33</sup> roX2Δ 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]<sup>6I</sup> transgene and express MSL2. Black bars represent females heterozygous for ago2<sup>414</sup>; 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<sup>414</sup>; gray bars are their sisters with wild type ago2.

FIGURE 2. roX1<sup>ex40A</sup> roX2Δ is synthetic lethal with siRNA mutations. (A) Loss of Ago2 reduces the survival of roX1<sup>ex40</sup> roX2Δ adult males. The number of males recovered was: ago2<sup>414</sup>, 245; roX1<sup>ex40</sup> roX2Δ, 274; roX1<sup>ex40</sup> roX2Δ; ago2<sup>414</sup>/+, 1356 and roX1<sup>ex40</sup> roX2Δ; ago2<sup>414</sup>, 45. (B) Loss of Loqs reduces roX1<sup>ex40</sup> roX2Δ adult male survival. The total number of males recovered was: loqs<sup><ins>60791</ins></sup>, 230; roX1<sup>ex40</sup> roX2Δ, 274; roX1<sup>ex40</sup> roX2Δ; loqs<sup><ins>60791</ins></sup>/+, 708 and roX1<sup>ex40</sup> roX2Δ; loqs<sup><ins>60791</ins></sup>, 166. Survival of roX1<sup>ex40</sup> roX2Δ; ago2<sup>414</sup> and roX1<sup>ex40</sup> roX2Δ; loqs<sup><ins>60791</ins></sup> males was determined by mating roX1<sup>ex40</sup> roX2Δ; ago2<sup>414</sup> /TM3SbTb males and females, or roX1<sup>ex40</sup> roX2Δ; loqs<sup><ins>60791</ins></sup>/ In(2LR)Bc Gla males and females. Survival of ago2<sup>414</sup> and loqs<sup><ins>60791</ins></sup> males was determined by observation of yw; ago2<sup>414</sup> /TM3SbTb and yw; loqs<sup><ins>60791</ins></sup> / In(2LR)Bc Gla stocks.

FIGURE 3. MSL1 localization is disrupted in roX1<sup>ex40</sup> roX2Δ 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<sup>ex40</sup> roX2Δ; ago2<sup>414</sup> male. (E) Percent nuclei with minimal or no MSL1 recruitment to the X chromosome (Sum of categories "+' and "no MSL recruitment", SI Table 2A). (F) Ectopic autosomal MSL1 binding in a roX1<sup>ex40</sup> roX2Δ; 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<sup>414</sup>, D-elp1<sup>c00296</sup>/+. roX1<sup>ex40</sup> roX2Δ. roX1<sup>ex40</sup> roX2Δ; ago2<sup>414</sup> (white bars). roX1<sup>ex40</sup> roX2Δ; D-elp1<sup>c00296</sup>/+ (white bars). SEM is depicted by error bars. Categories of MSL1 recruitment are detailed in Table S1.