

Sequence-specific targeting of MSL complex regulates transcription of the *roX* RNA genes

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In *Drosophila*, dosage compensation is controlled by the male-specific lethal (MSL) complex consisting of at least five proteins and two noncoding RNAs, *roX1* and *roX2*. The *roX* RNAs function in targeting MSL complex to the X chromosome, and *roX* transgenes can nucleate spreading of the MSL complex into flanking chromatin when inserted on an autosome. An MSL-binding site (DHS, DNaseI hypersensitive site) has been identified in each *roX* gene. Here, we investigate the functions of the DHS using transgenic deletion analyses and reporter assays. We find that MSL interaction with the DHS counteracts constitutive repression at *roX1*, resulting in male-specific expression of *roX1* RNA. Surprisingly, the DHS is not required for initiation of *cis* spreading of MSL complex, instead local transcription of *roX* RNAs correlates with extensive spreading.

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Introduction

Transcription in eukaryotes utilizes at least two levels of regulation: gene-specific control that operates locally on individual genes and global modulation of larger domains by chromatin composition or remodeling. Dosage compensation is an example of interplay between these two regulatory mechanisms that has evolved to make X-linked gene expression equivalent in males with one X chromosome and females with two. In *Drosophila*, dosage compensation is achieved by increasing the transcription of most X-linked genes two-fold in males (Lucchesi, 1998; Meller and Kuroda, 2002). This requires at least five proteins: MLE (*maleless*), MSL1, MSL2 and MSL3 (*male-specific lethal 1, 2 and 3*, respectively), and MOF (*males absent on the first*), and one of two *roX* (RNA on X) RNAs. MLE and MOF have enzymatic activities that are essential for dosage compensation: MLE is a

DExH RNA helicase (Kuroda *et al*, 1991; Lee *et al*, 1997) and MOF is a MYST family histone acetyltransferase (Hilfiker *et al*, 1997; Akhtar and Becker, 2000; Smith *et al*, 2000). JIL-1, a histone H3 kinase, also associates with the MSL proteins (Jin *et al*, 2000). The MSL proteins, JIL-1 and *roX* RNAs bind in a precise pattern along the length of the male X chromosome, resulting in enrichment of chromatin modifications associated with hypertranscription, such as histone H4 acetylated at lysine 16 and H3 phosphorylated at serine 10 (Turner *et al*, 1992; Wang *et al*, 2001).

The two noncoding RNAs, *roX1* and *roX2*, are functionally redundant (Meller and Rattner, 2002) even though they have very little sequence homology and are distinct in size (3.7 kb for *roX1* RNA versus 0.5–1.2 kb for *roX2* RNA) (Amrein and Axel, 1997; Smith *et al*, 2000). Deletion of either *roX* gene has no effect on males. Missing both of them, however, results in male lethality (Meller *et al*, 1997; Meller and Rattner, 2002). The MSL-binding pattern on the X chromosome is drastically disrupted in these *roX1roX2* double mutant males, suggesting that *roX* RNAs are important for correctly targeting MSL complex to the X chromosome. Both *roX* genes are located on X and overlap two of ~35 chromatin entry sites (CESs), which are proposed to be high-affinity sites for MSL complexes due to their ability to recruit partial MSL complex in some *msl* mutant backgrounds (Palmer *et al*, 1994; Lyman *et al*, 1997; Gu *et al*, 2000). Remarkably, when either *roX* gene is moved to an autosome as a transgene, it can recruit the MSL complex to the insertion site, from which the complex can extensively spread into flanking autosomal DNA (Kelley *et al*, 1999). This spreading from *roX* transgenes is most prominently seen if the endogenous *roX* genes on the X chromosome are deleted (Park *et al*, 2002). Recent data have shown that spreading can also occur on the X chromosome and suggested that *roX* genes may function as the major nucleation sites for MSL complex spreading on the X chromosome (Oh *et al*, 2003). However, spreading *in cis* from *roX* genes or the ~35 CESs cannot be the only mechanism for MSL targeting to the X chromosome, as X to autosome transpositions that lack a mapped CES can still attract MSL complexes (Oh *et al*, 2004). DNaseI hypersensitivity and transgenic deletion mapping have identified an ~200 bp MSL-binding site in each *roX* gene, designated here as DHS (DNaseI hypersensitive site), initially proposed to be the site from which MSL complexes can spread (Kageyama *et al*, 2001). Sequence alignments reveal short stretches of evolutionarily conserved consensus elements in both DHSs and mutagenesis data have suggested that they are essential for MSL binding (Park *et al*, 2003).

roX RNAs are male specific (Amrein and Axel, 1997; Meller *et al*, 1997). Female flies carrying a *roX* transgene driven by a constitutive promoter fail to accumulate *roX* RNA unless the complete MSL complex is also ectopically expressed, indicating that the male-specific expression of *roX* RNA is at least partially caused by the MSL-dependent stabilization of *roX* RNAs (Meller *et al*, 2000). Little is known about the

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regulation of *roX* genes at the transcriptional level. A recent model proposes that the rate of *roX* RNA transcription needs to match the rate of MSL complex assembly to regulate the distribution of complexes on the X chromosome (Oh *et al*, 2003), implying that *roX* RNA transcription should be finely regulated. The finding that the DHSs in *roX* genes attract MSL complex raises the question: can MSL complex regulate *roX* transcription through interaction with the DHS? In this case, the end result would be much greater than two-fold regulation since *roX* RNAs are male specific.

In the present paper, we dissect the functions of the *roX*-DHS and study the regulation of *roX* RNA transcription. We find evidence that the *roX*-DHS regulates male-specific *roX* RNA expression by enhancing transcription in males through interaction with the MSL complex, and by repressing transcription in females utilizing unknown repressors. Surprisingly, the *roX*-DHS is not essential for spreading of the MSL complex; instead, a low level of local *roX* RNA transcription is sufficient for the ability to spread extensively *in cis* from *roX* genes. We propose a model for regulation of *roX* sex specificity during development in which *roX* RNA transcription is locked into the male mode by the proper assembly of the MSL complex on the male X chromosome.

Results

DNase I hypersensitive sites in roX genes positively regulate roX RNA levels in males

The *roX1*-DHS is located near the middle of the *roX1* transcription unit, while the *roX2*-DHS is located downstream of the major *roX2* 3' end. Both DHSs are less than 300 bp and share short stretches of conserved sequences. Although transcribed, the DHS sequences are not required for *roX* RNA stability or function (Park *et al*, 2003; Stuckenholz *et al*, 2003), allowing us to make DHS deletions in genomic *roX* constructs to determine their roles as DNA elements.

GMroX1 and *GMroX2* are transgenes that contain full-length *roX1* or *roX2* genes and flanking sequences (Figure 1A). Both transgenes express *roX* RNAs at a level comparable to the endogenous *roX* genes (Figure 1B–D) and rescue *roX1roX2* double mutant males (Table I). We deleted ~300 bp encompassing the DHS from each *GMroX* transgene to make *GMroX1-ΔDHS* and *GMroX2-ΔDHS* (Figure 1A). By performing Northern analyses in either a *roX1*⁻ or a *roX2*⁻ background, we found that the level of *roX* RNA expressed from *GMroX1-ΔDHS* or *GMroX2-ΔDHS* was dramatically decreased in most transgenic lines. The average expression in *GMroX1-ΔDHS* lines was 16.5% of wild type, with a range of 1–70% (Figure 1B), while *roX2* expression was reduced to 5% of wild type, with a range of 0.1–20%, and was barely detectable by Northern analysis (Figure 1C). Complementation experiments showed a marked decrease in rescue of *roX1roX2* double mutants by *GMroX-ΔDHS* transgenes, and the rescue frequency correlated with *roX* RNA levels (Table I legend).

roX1-DHS and *roX2*-DHS share evolutionarily conserved sequences that are essential for attracting MSL complex (Park *et al*, 2003). To test if these sequences are important for *roX* RNA expression, we mutagenized the three most conserved elements in *roX2*-DHS to make the *GMroX2*-DHS-

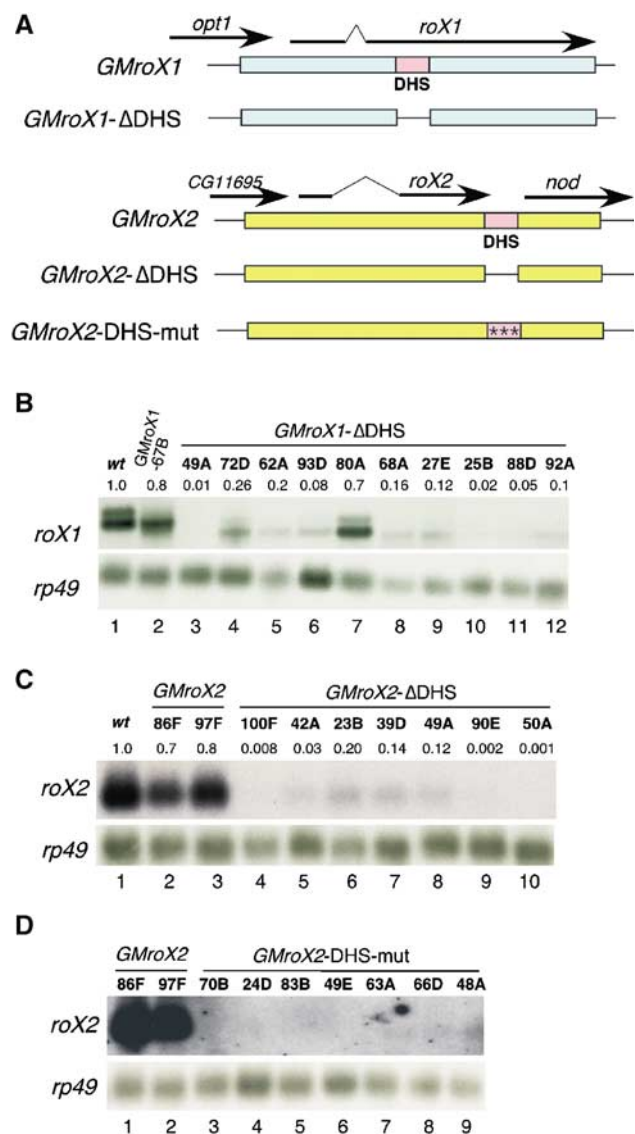


Figure 1 DHS positively regulates *roX* RNA levels in males. (A) Structures of the transgenes. *GMroX1* and *GMroX2* contain full-length *roX* genomic sequences along with partial segments from flanking genes as indicated by an arrow. In both Δ DHS transgenes, ~300 bp encompassing the DHS are deleted. In *GMroX2*-DHS-mut, three blocks of consensus sequences are mutagenized, as indicated by ***. (B–D) Northern blots from adult males to compare RNA level among *GMroX*, *GMroX-ΔDHS* and *GMroX2*-DHS-mut. All transgenic lines carry either a mutant allele (*roX1^{exc6}*) at their endogenous *roX1* locus (B), or are deleted for endogenous *roX2* (C, D). Hybridization for *rp49* is the loading control in all Northern blots. (B) *roX1* Northern. Lane 1: wild-type males; lane 2: *GMroX1-67B*; lanes 3–12: different *GMroX1-ΔDHS* lines with the site of insertion indicated. Quantification of *roX1* RNA by a phosphoimager is shown as relative to the wild-type level (designated as 1.0), after normalization to *rp49* levels. The *roX1-ΔDHS* transcript is ~300 nt shorter than wild-type *roX1* RNA. (C) *roX2* Northern. Lane 1: wild-type males; lanes 2 and 3: *GMroX2*-86F and 97F, respectively; lanes 4–10: different *roX2-ΔDHS* transgenic lines with the site of insertion and RNA quantification indicated. (D) *roX2* Northern. Lanes 1 and 2: *GMroX2*-86F and 97F, respectively; lanes 3–9: *GMroX2*-DHS-mut transgenic lines.

mut transgene (Figure 1A; see Materials and methods). Consistent with the Δ DHS data, mutagenesis of the DHS in *roX2* also abolished *roX2* expression in most transgenic lines (Figure 1D).

DHS-dependent MSL binding is not essential for MSL spreading from *roX* genes

MSL complex not only binds to *roX* genes but also can spread *in cis* from these loci up to ~1 Mbp into flanking sequences. Based on this observation, our lab previously proposed that MSL complexes spread from ~35 CESs to paint the entire 21 Mbp of X euchromatin (Kelley *et al*, 1999). The discovery of each *roX1* or *roX2* genes prompted us to test if deletion of the DHS would abolish spreading from *GMroX1* and *GMroX2* transgenes.

We assayed Δ DHS transgenes for spreading in *roX1roX2* mutant males, in which extensive spreading from wild-type *GMroX1* or *GMroX2* is very consistent due to lack of competition from endogenous *roX* genes (Figure 2A and E, and

Table II; Park *et al*, 2002). To our surprise, three out of nine *GMroX2*- Δ DHS and seven out of 12 *GMroX1*- Δ DHS lines showed variable but extensive MSL spreading in the absence of the DHS (Figure 2B–D and F–H, and Table II). Unlike wild-type *GMroX* transgenes, spreading was mosaic, seen in some nuclei but not others. In fact, many nuclei showed no MSL association with the transgene, demonstrating that no robust MSL-binding sites remain in these constructs. Transcription of *roX* RNAs is thought to be required for extensive spreading (Park *et al*, 2002), and we found that whether a transgenic line showed spreading correlated with its *roX* RNA level seen by Northern analysis (Figure 1C and Table II legend). These results indicate that *cis* spreading of the MSL complex from *roX* transgenes does not require high-affinity DNA sequences for MSL binding

Table I Rescue of *roX1roX2* mutants

| Transgene | No. of lines tested ^a | Rescue range (%) ^b | Average of rescue frequency (%) ^c |
|------------------------------|----------------------------------|-------------------------------|--|
| <i>GMroX1</i> | 5 | 60–92 | 86 |
| <i>GMroX1</i> - Δ DHS | 12 | 1–57 | 26 |
| <i>GMroX2</i> | 4 | 65–85 | 82 |
| <i>GMroX2</i> - Δ DHS | 9 | 0.2–15 | 3.5 |
| <i>GMroX2</i> -DHS-mut | 6 | 0.1–5 | 1.2 |

yw roX1^{ex6} Df(1)roX2⁵² [w + 4 Δ 4.3] virgins were crossed with males carrying each transgene. The rescue frequency was calculated as the percentage of expected by comparing the number of male and female progeny.

^aThe number of independent lines that were tested for each transgene.

^bNumbers summarize the range of rescue frequency, from the lowest to the highest. For *GMroX1*-DHS, the lines expressing more *roX1* RNA generally had higher rescue. For *GMroX2*- Δ DHS, three lines (39D, 23B and 49A) showed >10% rescue, while all the other lines showed <3% rescue.

^cNumbers represent the average rescue frequency for each transgene.

Table II MSL spreading at transgene loci in *roX1roX2* mutants

| Transgene | No. of lines tested ^a | No. of lines showing spreading | % of nuclei showing spreading ^b |
|------------------------------|----------------------------------|--------------------------------|--|
| <i>GMroX1</i> | 5 | 5 | ~95 |
| <i>GMroX2</i> | 6 | 6 | ~98 |
| <i>GMroX1</i> - Δ DHS | 12 | 7 | 5–82 |
| <i>GMroX2</i> - Δ DHS | 9 | 3 | 3–23 |
| <i>GMroX2</i> -DHS-mut | 6 | 4 | 7–55 |

^aThe number of independent lines that were tested for spreading for each transgene.

^bFor *GMroX1* and *GMroX2*, every line showed spreading. The numbers represent the average percentage of nuclei showing spreading for each of these transgenes. For the mutant transgenes, spreading was only seen in a few lines and the percentage of nuclei showing spreading varied from line to line. The number indicates the range of this variation. For *GMroX1*- Δ DHS, the seven lines with spreading are 72D, 62A, 80A, 68A, 27E, 92A and 93D. The three *GMroX2*- Δ DHS lines that showed spreading are 39D, 23B and 49A. In *GMroX2*-DHS-mut flies, the spreading was more frequently seen than in *GMroX2*- Δ DHS flies, but was usually very limited (2–3 bands). A total of 120–250 nuclei were counted for each line.

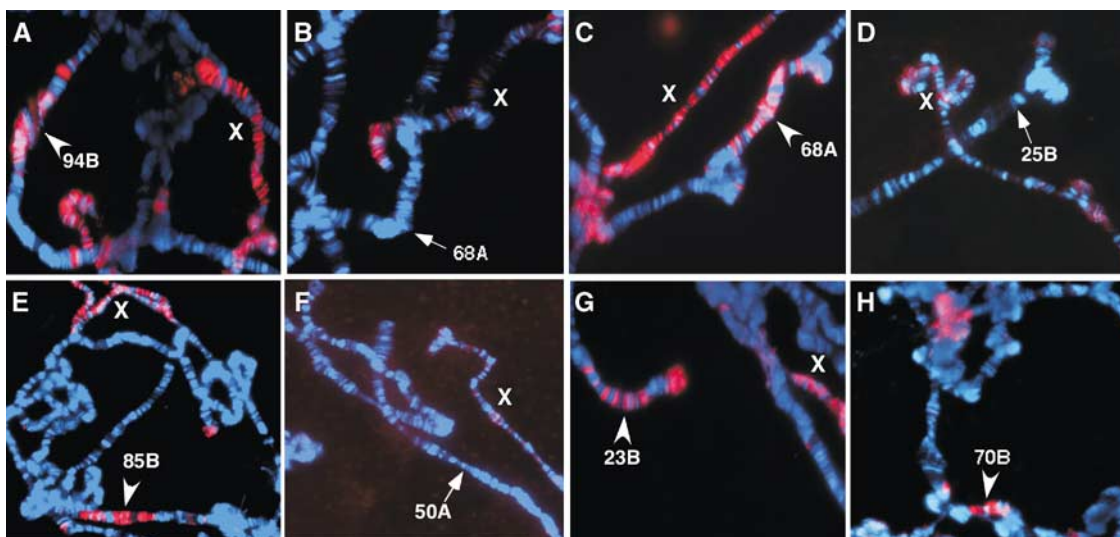


Figure 2 DHS is not essential for spreading of the MSL complex from *roX* loci. Polytene chromosome immunostaining by anti-MSL1 antibody (red). DNA is stained with DAPI (blue). *GMroX1* (A) and *GMroX2* (E) transgenes provide nucleation sites for extensive spreading (arrowhead) of the MSL complex in ~100% of nuclei in *roX1roX2* mutants. (B) *GMroX1*- Δ DHS-68A, no binding and spreading at the transgene (arrow). (C) The same transgenic line as in (B) but a different nucleus showing extensive spreading (arrowhead). (D) *GMroX1*- Δ DHS-25B with no binding and spreading detected (arrow). (F) *GMroX2*- Δ DHS-50A with no binding and spreading at the transgene (arrow). (G) *GMroX2*- Δ DHS-23B shows extensive spreading in some nuclei (arrowhead). (H) *GMroX2*-DHS-mut-70B with limited spreading (arrowhead).

within the transgenes themselves. It is important to note, however, that an isolated multimer of *roX1*-DHS occasionally nucleates limited spreading of the MSL complex (Kageyama *et al*, 2001). We propose that although DHS is not essential for spreading, it may facilitate the process by recruiting a high concentration of MSL complexes to a specific location (see Discussion).

***roX1* reporter constructs recapitulate DHS-mediated sex-specific regulation**

Our analysis of *GmroX*- Δ DHS transgenes suggested that MSL binding at the DHS upregulates expression of *roX* RNAs. To study regulation of the *roX1* promoter directly, we employed a reporter assay. We identified three distinct 5' ends of *roX1* RNA (see Supplementary information) and therefore chose a 750bp region that encompassed all three for promoter analysis (Figure 3A). The *ProX1* construct was made by inserting this segment upstream of a promoterless eGFP reporter. A second construct, *ProX1*-DHS, was made by adding the *roX1*-DHS downstream of the SV40 polyA site of the eGFP reporter (Figure 3A). To minimize possible position effects, both constructs were made in vectors that contain *gypsy* insulators flanking the reporter cassette (Barolo *et al*, 2000). eGFP fluorescence was not detected in *ProX1* or *ProX1*-DHS transgenic flies, but this was not unexpected, as our constructs were predicted to produce *roX1*-eGFP fusion transcripts that contained nonproductive ATG codons from the *roX1* 5' region. Therefore, we directly measured eGFP RNA levels by Northern analysis and real-time RT-PCR, normalized to *rp49* or PKA mRNA levels. As shown in Figure 3B, no transcription was detected from promoterless eGFP (lanes 2 and 3). In the absence of the DHS, we detected transcription from *ProX1* in both sexes, with males showing a slightly higher level of transcription than females (~2-fold) (Figure 3B, lanes 4–7). When the DHS was added downstream of the 3' end of eGFP, reporter transcription increased in males (Figure 3B, lanes 8 and 10 versus lanes 4 and 6), but also decreased in females (Figure 3B, lanes 9 and 11 versus lanes 5 and 7), resulting in male-specific expression. Quantitative real-time RT-PCR showed that by adding the DHS, reporter transcription increased 2- to 2.5-fold in males and decreased ~3-fold in females (Figure 3D). We tested four independent insertion sites for each reporter and got consistent results with *P*-values <0.05, as evaluated by the Student's *t*-test.

DHS-mediated regulation requires MSL proteins

MSL complex is assembled only in males, due to translational repression of the key subunit, MSL2, by Sex Lethal protein in females (Bashaw and Baker, 1997; Kelley *et al*, 1997). When MSL2 is ectopically expressed in females, functional MSL complex is formed in these animals (Kelley *et al*, 1995). To determine if the difference in reporter expression between males and females was due to the male-specific MSL complex, we asked whether MSL2 expression in *ProX1* and *ProX1*-DHS females would alter their reporter transcription. Northern analysis and quantitative RT-PCR showed no effect on *ProX1* females by ectopic expression of MSL2 protein (Figure 3C and D); therefore, the different level of basal expression between *ProX1* males and females is not MSL dependent. In contrast, a robust increase of transcription in *ProX1*-DHS females was seen in the presence of MSL com-

plexes (Figure 3C and D), suggesting that MSL complexes act through the DHS to mediate positive regulation of *roX1* RNA expression. Although the DHS normally lies within the *roX1* transcription unit, it is not required for *roX1* RNA function or stability (Stuckenholz *et al*, 2003). Here, the DHS functions downstream of the transcription unit in the *ProX1*-DHS construct and is not incorporated into the resulting *roX1*-eGFP reporter RNA (see Supplementary information), strongly suggesting that it functions as a DNA element and therefore is unlikely to influence RNA stability. Our results favor a model in which MSL complexes positively regulate *roX1* transcription by interacting with the DHS DNA element.

MSL complex consists of at least five proteins, MSL1, MSL2, MSL3, MLE and MOF, and two *roX* RNAs. MSL1 and MSL2 proteins are proposed to form the 'core' of the complex, which remains at ~35 CESs in the absence of other MSL proteins (Lyman *et al*, 1997; Copps *et al*, 1998). MSL3, MLE and MOF, on the other hand, are not essential for binding to most CESs. To test which MSL proteins are required for *roX1* transcription, we assayed the *ProX1*-DHS reporter in Hsp83-MSL2 females mutated for *msl1*, *msl3*, *mle* or *mof*. Quantitative real-time RT-PCR assays showed that reporter transcription was dramatically decreased in *msl1*, *mle* and *msl3* mutants (Figure 4A). *msl1* and *mle* had the most severe effect in that reporter transcription was decreased to wild-type female level. We tested two different *msl1* alleles with similar results: *msl1*^{L60} is a 2 kb deletion removing most of the coding region (Chang and Kuroda, 1998); *msl1*^{L183} is a point mutation that disrupts MSL1 function without changing the protein level (R Kelley, personal communication). In contrast, high-level transcription is maintained in *mof*^{fl} mutants (Figure 4A) carrying a point mutation that disrupts the acetyltransferase function of MOF (Hilfiker *et al*, 1997). These results suggest that *roX1* transcription requires MSL1, 2, 3 and MLE, but not the function of MOF. However, recent observations have suggested that *mof*^{fl} retains sufficient enzymatic activity to acetylate histone H4 at CESs (Sass *et al*, 2003), raising the possibility that residual enzymatic activity may contribute to *roX1* transcription.

In a previous study, females expressing MSL2 but mutant for *msl3* failed to incorporate *roX1* RNA at the ~35 binding sites for partial MSL complexes, although *roX2* RNA was consistently detected (Meller *et al*, 2000). The interpretation at the time was that *roX2* RNA might be assembled into MSL complex earlier than *roX1* RNA, and that incorporation of *roX1* RNA required MSL3. However, the results of our studies raised the strong possibility that the *roX* RNAs have the same ability to assemble into incomplete complex, but that *roX1* transcription is repressed when lacking MSL3. To test this idea, we performed *in situ* hybridization for *roX1* RNA in *msl3* [Hsp83-MSL2] females with or without an Hsp83-*roX1*cDNA transgene on an autosome. We found that *roX1* RNA was not detected on female X chromosomes when lacking MSL3 (Figure 4B, left panel). However, when *roX1* RNA was expressed constitutively from the Hsp83 promoter, it was detected at many CESs (Figure 4B, right panel). This strongly suggests that endogenous *roX1* transcription is indeed repressed in the absence of MSL3 and this deficiency in *roX1* expression can be overcome by an actively transcribed *roX1* transgene.

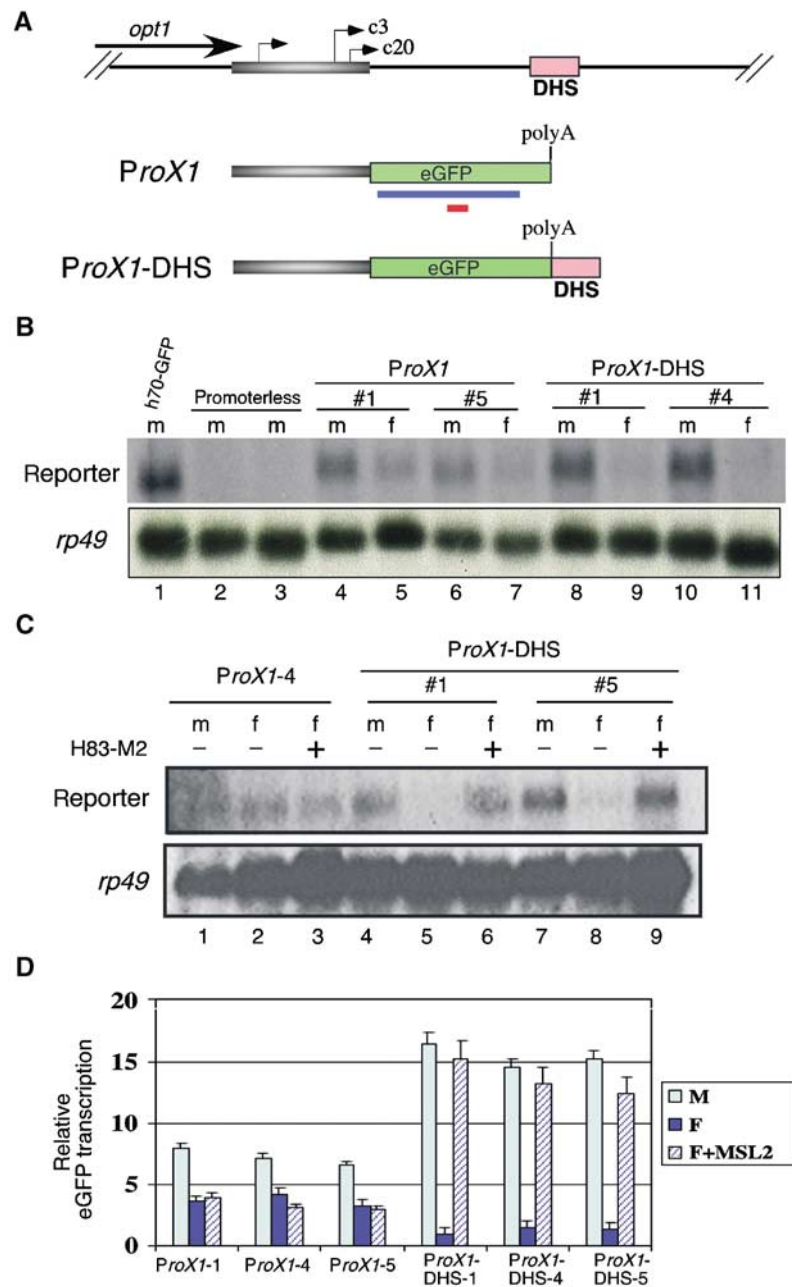


Figure 3 DHS directly regulates *roX1* promoter activity. **(A)** Reporter constructs. *ProX1* contains 750 bp sequences (gray) from the *roX1*-5' region inserted upstream of a promoterless eGFP reporter (green). *ProX1-DHS* is the same as *ProX1* except that the DHS (pink) is added downstream of the SV40 polyA site at the 3' end of eGFP. The blue bar under the reporter construct represents the probe used in Northern experiments and the red bar indicates the amplified region in real-time PCR. The 750 bp *roX1*-5' region contains three *roX1* 5' ends mapped by RNase protection assay (see Supplementary information), indicated by arrows, two of which correspond to the start sites of *roX1* cDNAs *c3* and *c20*, respectively. **(B)** Northern blot of reporter RNA from transgenic males (m) or females (f). Lane 1: Hsp70-eGFP; lanes 2 and 3: promoterless eGFP; lanes 4 and 5: *ProX1*-1; lanes 6 and 7: *ProX1*-5; lanes 8 and 9: *ProX1*-DHS-1; lanes 10 and 11: *ProX1*-DHS-4. Note: The reporters express a *roX1*-eGFP fusion RNA that is slightly larger (see Supplementary information) than eGFP mRNA in lane 1. **(C)** Northern blot of reporter RNA from males (m) or females (f), in the presence (+) or absence (-) of an Hsp83-MSL2 transgene. Lanes 1–3: *ProX1*-4; lanes 4–6: *ProX1*-DHS-1; lanes 7–9: *ProX1*-DHS-5; hybridization for *rp49* acts as the loading control. **(D)** Quantitative real-time RT-PCR of *ProX1* and *ProX1*-DHS males (M), females (F) and females with ectopic expression of MSL2 (F + MSL2). For each sample, eGFP transcription has been normalized to the RNA level of the internal control gene *pka*. Relative eGFP transcription is presented as a ratio to the RNA level of the calibrator, which is the sample with the lowest expression level of eGFP. Here, *ProX1*-DHS-1 has the lowest expression and is chosen as the calibrator (designated as 1). For each sample, data represent the mean \pm s.d. from three independent experiments.

***roX* RNAs are not required for positive regulation of *ProX1*-DHS**

We next tested whether *roX* RNAs autoregulate *roX1* transcription. Our previous study showed that MSL complex was

not detectable at the DHS in the absence of *roX* RNAs (Park *et al*, 2003), suggesting that we would see a decrease of *ProX1*-DHS reporter transcription in *roX1roX2* double mutant males. In contrast, we found that the *ProX1*-DHS reporter still

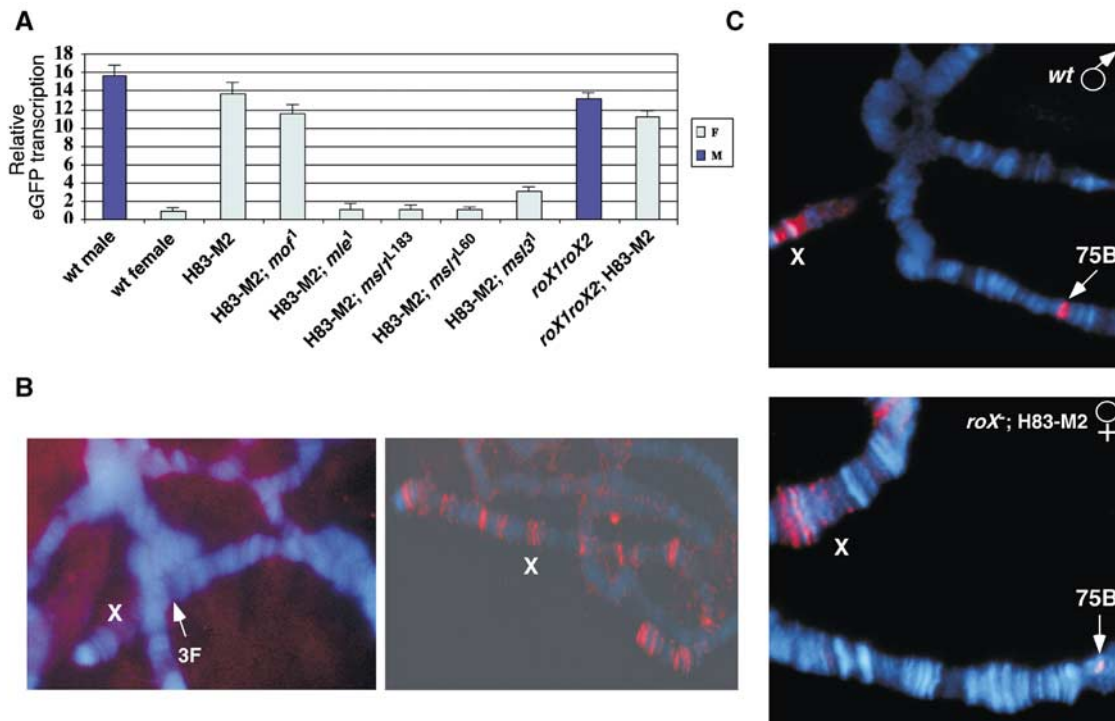


Figure 4 DHS-mediated regulation requires MSL proteins but not *roX* RNA. (A) Quantitative real-time RT-PCR of *ProX1*-DHS males (M) or females (F) in wild-type or different *msl* or *roX1roX2* mutant backgrounds (as indicated at the bottom). Results are represented as in Figure 3D, with the transcription level of wild-type female designated as 1. (B) Left panel: *in situ* hybridization of *roX1* RNA (red) in *msl3* [Hsp83-MSL2] females. No *roX1* RNA was detected on X. We also failed to detect transcription at the endogenous *roX1* locus (3F), as indicated by the arrow. Right panel: in the presence of an Hsp83-*roX1* transgene on an autosome, *roX1* RNA was detected at many CESs on the X chromosome. (C) Polytene chromosomes from *ProX1*-DHS-1 stained with anti-MSL1 antibody (red). The top panel shows the male chromosomes in a wild-type background; the bottom panel shows the chromosomes from females with ectopic MSL2 protein but mutant for *roX1* and *roX2*. The arrows indicate the transgene loci. X: X chromosome. DNA was stained with DAPI (blue) in (B, C).

retained a high level of transcription in *roX*⁻ males (Figure 4A). To verify this result, we tested females that ectopically express MSL complex but are mutant for *roX* genes. Consistent with the result in males, *ProX1*-DHS reporter transcription remained high in females expressing a full set of MSL proteins but no *roX* RNAs (Figure 4A). To re-examine if a weak MSL-DHS interaction could occur in the absence of *roX* RNA, we performed chromosome immunostaining in these female third-instar larvae, which are healthy and have much better chromosomal morphology than *roX1roX2* mutant males assayed in our earlier work (Park *et al*, 2003). Weak MSL-binding signals at the *ProX1*-DHS transgene insertion sites were detected in some nuclei (Figure 4C), suggesting that MSL complex without RNA components can interact with DHS in a weak and/or transient way. Surprisingly, *roX* RNAs stabilize this interaction, apparently without having a strong effect on *roX1* transcription.

MSL proteins counteract constitutive repression at the *roX1* promoter

Our reporter assay showed that in the presence of the DHS, transcription from the *roX1* promoter was repressed in females compared to basal transcription in the absence of the DHS (Figure 3B and D), raising the question of how *roX1* transcription is regulated in females. *roX1* RNA is expressed in both males and females during early embryonic development, but it disappears in females and remains in males as the MSL complex becomes established (Meller *et al*, 1997).

This suggests that early *roX1* expression is MSL independent. Consistent with this, our results indicate that the isolated *roX1* promoter has constitutive basal activity in both sexes. The lack of *roX1* expression in females at later stages could be explained by unknown repressor proteins that may interact with DHS and be normally counteracted by MSL complexes in males. To determine whether repression is truly female specific or it can happen in males that lack MSL complexes, we analyzed *msl* mutant males.

All MSL proteins are required for male viability, but some *msl* mutant males survive to the late third-instar larval stage, making it possible to measure their RNA levels. We have shown that MSL complex increases *roX1* reporter transcription about two-fold in males (Figure 3B and C). If there were no repression in males, we would expect to see the transcription decreased by half in *msl* mutant males. However, as shown by quantitative real-time RT-PCR, we found that in *mle1/mle1⁷³⁸* male larvae, *ProX1*-DHS reporter transcription is significantly decreased (more than 10-fold on average) compared to their *mle1/CyOy⁺* brothers (Figure 5A), suggesting that repression can occur in males as well as in females. *msl3¹/msl3^{mak1}* males also have more than a four-fold decrease of transcription, although not as low as *mle* males (Figure 5B). In contrast, *ProX1* reporter transcription is not affected in either *msl* mutant male (Figure 5A and B), confirming that the decrease of transcription is caused by the DHS and not other sequences. Taken together, our results from *msl* mutant males suggest that *roX1* transcription is

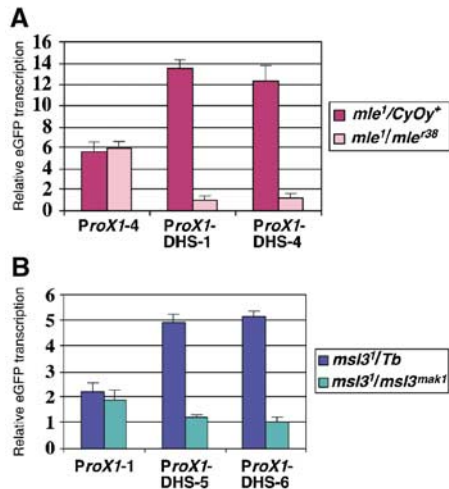


Figure 5 The *roX1* promoter is repressed in *msl* mutant males. Real-time RT-PCR of *ProX1* or *ProX1*-DHS reporter transcription from *mle* (A) and *msl3* (B) mutant male larvae. Results are represented as in Figures 3 and 4, with the transcription level of *ProX1*-DHS-1 and *ProX1*-DHS-6 designated as 1 in (A) and (B), respectively.

regulated by both positive and negative factors, through interaction with a shared *cis* element, the DHS.

Discussion

roX RNAs are male specific and play essential functions in dosage compensation. They require MSL complex for RNA stabilization, which contributes at least partially to their male-specific expression pattern (Meller *et al*, 2000). In this paper, we analyzed regulation of *roX* RNA transcription by assessing the function of DHS, the primary MSL-binding sites in *roX* genes. Using transgenic deletion analyses and reporter assays, we found that *roX1* transcription is directly regulated by DHS. Genetic data demonstrated that this regulation requires MSL1, 2, 3 and MLE, but not *roX* RNAs. We further verified this regulation at the endogenous *roX1* locus. We propose that the DHS regulates *roX1* transcription in both sexes. It acts as a derepressor/enhancer in males to attract MSL complex to activate *roX1* transcription, and as a silencer in females to downregulate *roX1* RNA transcription. At this point, we have not identified potential repressors. The fact that repression can happen in both sexes in the absence of MSL complex suggests that it may represent the default state. Interestingly, a recent study has shown that MSL complex can overcome silencing of *mini-white*-containing transgenes inserted into various silent chromatin environments, allowing male-specific activation of *mini-white* through binding to an adjacent *roX1* segment (Kelley and Kuroda, 2003). The underlying mechanism of this antagonism of repression may be similar to what we propose here for the regulation of *roX1* transcription.

The finding that the *roX1* promoter has MSL-independent basal activity is consistent with previous results that *roX1* RNA is expressed in both sexes at the early embryonic stage before MSL complexes are established (Meller *et al*, 1997). Surprisingly, we found that transcription from the *roX1* promoter was only increased two-fold by MSL-DHS interaction, suggesting that MSL complex may use the same strategy

to upregulate *roX* genes as to modulate chromosome-wide transcription. However, we cannot exclude the possibility that, at a functional *roX1* locus, MSL complex may act more robustly. For example, nascent *roX1* RNA may continually attract MSL proteins to assemble locally to provide a strong positive feedback loop on local transcription. This may explain why we see a large difference in *roX1* RNA levels between *GMroX1* and *GMroX1*- Δ DHS flies. That the DHS can regulate *roX* expression and is required for genomic transgene function does not rule out additional levels of regulation at the endogenous *roX1* locus on the X chromosome. Rattner and Meller (2004) have recently proposed that MSL2 alone can upregulate transcription of *roX1* in females and that this is DHS independent. The magnitude of this regulation by MSL2 and its site and mechanism of action remain to be determined.

As shown in Figure 6, we propose a model in which transcription of *roX1* RNA is developmentally regulated by antagonism between repression and activation. In the early embryonic stages, before MSL complex is established, *roX1* RNA is transcribed in both sexes from an MSL-independent basal promoter. Later, in females unknown repressors downregulate *roX1* transcription by interacting with DHS, while in males *roX1* transcription is maintained and enhanced as MSL complex becomes established. This could occur by successful competition of MSL complexes with repressors for binding to the DHS. Alternatively, both MSL complex and repressors could bind to DHS simultaneously, with the outcome being a finely tuned regulation of *roX1* RNA transcription. A question raised by this model is why *roX1* transcription needs to be repressed in the absence of MSL complex. One possibility is that *roX1* transcription is harmful for female development and thus has to be shut down. Although females carrying a *roX1* cDNA transgene driven by a constitutive promoter have no obvious phenotype (Meller *et al*, 2000), this could be a form of redundant control to ensure fidelity of dosage compensation. Another possibility is that repression is a default mechanism, which is primarily utilized in males to adjust *roX1* transcription to an appropriate level. This adjustment might be achieved by maintaining a balance between enhancement and repression.

roX loci overlap two of ~ 35 CESs, which were proposed to be nucleation sites for MSL spreading. The identification of DHS as the primary MSL-binding site prompted a hypothesis that DHS carries the CES function of *roX* loci. Surprisingly, we found that *roX* genes can still nucleate spreading in the absence of DHS, as long as transcription occurred at these loci. Perhaps initial RNA binding can lead to a histone-binding/modification cycle in the absence of a specific DNA interaction, as proposed for HP1 spreading to form heterochromatin (Bannister *et al*, 2001; Lachner *et al*, 2001).

Finally, although our data suggest that DHS does not function as a spreading initiation site, it may facilitate the efficient spreading from *roX* loci due to its high binding affinity for the MSL complex. This may explain why we only saw spreading in some nuclei in the *GMroX*- Δ DHS stocks. The local concentration of the MSL complex is suggested to be important for *cis* spreading (Oh *et al*, 2003). Nascent *roX* RNA may be the most important attractant to concentrate the MSL complex at the site of assembly (Park *et al*, 2002), but DHS may also help recruit and maintain this local MSL pool. The previous observation that a multimer of

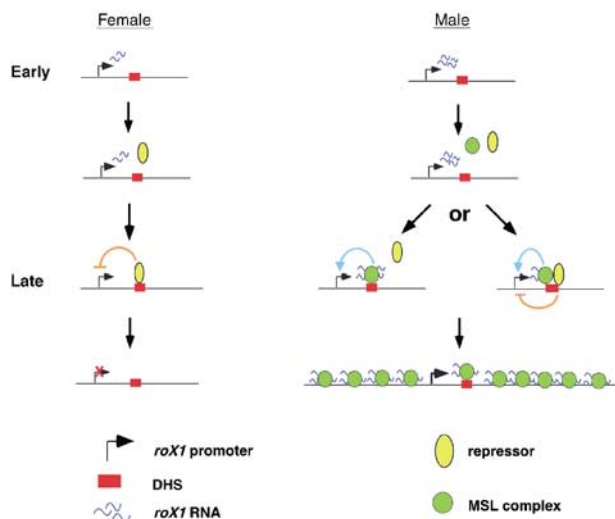


Figure 6 Model for regulation of *roX1* transcription. See text for details.

roX1-DHS can occasionally nucleate limited spreading (Kageyama *et al*, 2001) may reflect this function.

Materials and methods

Transgene construction and transformation

GMroX1-ADHS was constructed by two-step subcloning. The sequences upstream of DHS (2.0 kb) were amplified from pCaSpeR-*GMroX1* using primers Δ DHS-3 (5'-CGGAATTCAAGTTCACC AGCTC-3') and Δ DHS-10 (5'-AAAAAGCGGCGCC TTCTCGAAACG CAAG-3'), sequenced and subcloned into *EcoRI/NotI*-digested pCaSpeR3. The sequences downstream of DHS (2.6 kb) were amplified by primers Δ DHS-9 (5'-AGCAAGGCCTTTCCTATCGAA CTG-3') and Δ DHS-11 (5'-AAAGCGGCGC CTCTGGAAAGACC-3'), sequenced and then subcloned into the *NotI* site in the former construct to make the final pCaSpeR-*GMroX1*-ADHS transgene construct. To construct *GMroX2*-ADHS, an *XbaI-SpeI GMroX2* fragment linked to a PCR fragment (forward primer 5'-ACTAGTTAT GACAAATAAGAC-3' and reverse primer 5'-GATATCGCA GATTGAA GAATTGAAG-3') containing the sequences 3' of DHS was subcloned into pCaSpeR3. To make *GMroX2*-DHS-mut, PCR-mediated mutagenesis was performed as described previously to mutagenize blocks 1, 2 and 4 (Park *et al*, 2003). The mutant *roX2*-DHS was confirmed by sequencing and then subcloned into *GMroX2*-ADHS at the *SpeI* site. *ProX1* was made by subcloning a 750 bp *EcoRI* fragment containing the *roX1* 5' region amplified from pCaSpeR-*GMroX1* (forward primer 5'-TGTAGTTGGCTGTAAA TACG-3' and reverse primer 5'-TACATCTTGCCAGAGATTTCCG-3') into pGreen-Pelican (Barolo *et al*, 2000). *ProX1*-DHS was made by subcloning DHS into the *SpeI* site of *ProX1*.

Transgenic flies were made by P element-mediated transformation (Spradling and Rubin, 1982).

Northern analysis

Fly total RNA was isolated using the TRIzol Reagent (Invitrogen), and 20 μ g of RNA was used per lane. Hybridization was performed as described (Church and Gilbert, 1984), using PCR fragments amplified from *roX1* c20 or pCaSpeR-*GMroX2* as the probes. eGFP probe was prepared by PCR amplification of eGFP coding region (forward primer 5'-ATGTTGA GCAAGGGCGAGGAG-3' and reverse primer 5'-CTTGACAGCTCGTCCATGCC-3'). Quantification of Northern blots was performed using a Phosphorimager (Molecular Dynamics).

Polytene chromosome immunostaining and in situ hybridization

Preparation of polytene chromosomes and immunostaining using anti-MSL1 antibody were as previously described (Kelley *et al*, 1999).

In situ hybridization was performed as described previously (Meller *et al*, 2000) except that hybridization was at 46°C rather than 42°C. A digoxigenin-labeled antisense RNA from the 3' part of *roX1* (1.8 kb *EcoRI/BglII* fragment) was used as the probe.

Quantitative real-time RT-PCR

DNaseI-treated total RNA from *ProX1* or *ProX1*-DHS flies was subjected to first-strand cDNA synthesis using SuperScriptII reverse transcriptase (Invitrogen) and primed by oligo dT. Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The *pka* gene was used as the internal reference for normalizing variance in the quality of RNA and the amount of input cDNA. The primer sequences for eGFP were as follows: forward 5'-AGCAGACTTCTCAAGTCCG-3' and reverse 5'-GTGTCGCCCTCGAAGTTCAC-3'. The primer sequences for *pka* were as follows: forward 5'-TTCTCGGAGCCGCACTCG CGTCTAC-3' and reverse 5'-CAATCAGCAGATTCTC CGGCT-3'. PCR amplification was performed in triplicate in a 25 μ l final volume containing 1 \times SYBR green PCR buffer, 5.5 mM MgCl₂, 200 μ M dNTP, 200 nM of each primer, 0.6 U platinum *Taq* DNA polymerase (Invitrogen) and 1 μ l of cDNA. The PCR protocol used an initial denaturing step at 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min. Dissociation curve analysis was run at the end of 40 cycles to verify PCR product identity. Relative quantification of eGFP transcription was determined by comparative C_T method based on the manufacturer's instructions (ABI Prism 7700 sequence detection system User Bulletin #2, Applied Biosystems). Standard curve for each set of primers was constructed using a serial dilution of cDNA to verify equal amplification efficiency of the two systems.

Fly stocks and genetic crosses

GMroX1-ADHS, *GMroX2*-ADHS or *GMroX2*-DHS-mut males were crossed with *yw roX1^{ex6}*, *w Df(1)roX2⁵²*; [*w⁺ 4A4.3*] or *yw roX1^{ex6}Df(1)roX2⁵²* [*w⁺ 4A4.3*] females. The resulting male progeny carrying an autosomal transgene and mutant alleles of *roX1*, *roX2* or both were collected for Northern analyses or chromosomal immunostaining.

To generate *mSl1^{L183}*, *mSl1^{L60}*, *mle* or *mSl3* mutant females with ectopic MSL2 expression, [Hsp83-MSL2] females homozygous for each mutant allele were crossed to [ProX1] or [ProX1-DHS] males heterozygous for *mSl1^{L183}*, *mSl1^{L60}*, *mle* or *mSl3*. The resulting homozygous mutant females were identified by the absence of CyO or *Tb* phenotype. To generate *mof^f* mutant females, *w cv mof^f*; [Hsp83-MSL2] females were crossed to *w cv mof^f*; CyO [*mof⁺18H1*]/[ProX1] or [ProX1-DHS] and *mof^f* female adults were selected by the absence of CyO. To generate *roX1roX2* females with ectopic MSL2 expression, *yw roX1^{ex6}Df(1)roX2⁵²* [*w⁺ 4A4.3*]; *mSl3* [Hsp83-MSL2] females were crossed with *yw roX1^{ex6}Df(1)roX2⁵²* [*w⁺ 4A4.3*]; +/CyO[*GMroX1*-39A]; [ProX1-DHS] males. The resulting *yw roX1^{ex6}Df(1)roX2⁵²* [*w⁺ 4A4.3*]; +/+; *mSl3* [Hsp83-MSL2]/[ProX1-DHS] females were identified by the absence of CyO. *mle^f/mle³⁸* male larvae were generated by crossing *yw; pr mle^f*; [ProX1] or [ProX1-DHS] females with *yw; mle³⁸/CyOy⁺* males. For *mSl3* mutant males, *yw; [ProX1] or [ProX1-DHS]; mSl3^f* females were crossed to *yw; mSl3^{mak1red e/TM6 Tb}* males and the resulting non-Tb male larvae were selected. *yw; mSl3^f [Hsp83-MSL2]/mSl3^f [H83-roX1cDNA20]* females were generated by crossing *yw; mSl3^f [H83-roX1cDNA20]* females with *yw; mSl3^f [Hsp83-MSL2]/TM6 Tb* males and non-Tb females were selected in the next generation.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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