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Satellite repeats identify X chromatin for dosage compensation in *Drosophila melanogaster* males

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Summary

A common feature of sex chromosomes is coordinated regulation of X-linked genes in one sex. *Drosophila melanogaster* males have one X chromosome, while females have two. The resulting imbalance in gene dosage is corrected by increased expression from the single X chromosome of males, a process known as dosage compensation. In flies, compensation involves recruitment of the Male Specific Lethal (MSL) complex to X-linked genes and modification of chromatin to increase expression. The extraordinary selectivity of the MSL complex for the X chromosome has never been explained. We previously demonstrated that the siRNA pathway, and siRNA from a family of X-linked satellite repeats (1.688^{X} repeats), promote X-recognition. Now we test the ability of 1.688^{X} DNA to attract compensation to genes nearby, and report that autosomal integration of 1.688^{X} repeats increases MSL recruitment and gene expression in surrounding regions. Placement of 1.688^{X} repeats opposite a lethal autosomal deletion achieves partial rescue of males, demonstrating functional compensation of autosomal chromatin. Females block formation of the MSL complex and are not rescued. The 1.688^{X} repeats are therefore *cis*-acting elements that guide dosage compensation. Furthermore, 1.688^{X} siRNA enhances rescue of males with a lethal deletion, but only when repeat DNA is present on the intact homolog. We propose that the siRNA pathway promotes X recognition by enhancing the ability of 1.688^{X} DNA to attract compensation in *cis*. The dense and near-exclusive distribution of 1.688^{X} sequences along the X chromosome suggests that they play a primary role in determining X identity during dosage compensation.

Keywords

Dosage compensation; sex chromosomes; X recognition; satellite repeats; 1.688^{X} repeats; *roX* RNA; siRNA; *Drosophila*

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Introduction

Males of many species carry a gene-rich X chromosome and a gene-poor, heterochromatic Y chromosome. Dosage compensation equalizes X-linked gene expression between XY males and XX females, thus maintaining a constant ratio of X:A gene products [1]. Strategies to accomplish this differ, but a unifying theme is coordinated regulation of an entire chromosome. *Drosophila melanogaster* males increase expression from most X-linked genes approximately twofold [2]. This is achieved in part by the Male Specific Lethal (MSL) complex, consisting of five proteins and one of two redundant, long non-coding RNAs called *RNA on the X 1* and -2 (*roX1* and *roX2*) [3]. The *roX* RNAs are essential for proper identification and compensation of X-linked genes [3, 4]. The MSL complex is recruited into the bodies of transcribed genes on the X, where it modifies chromatin through deposition of the activating H4K16ac mark [5]. How the MSL complex selectively recognizes X chromatin has never been explained.

Co-transcriptional assembly of the MSL complex is thought to occur at sites of *roX* RNA transcription [6, 7]. This is followed by binding of the MSL complex to X-linked loci called Chromatin Entry Sites (CESs) that are functionally defined by their ability to retain core MSL proteins in the absence of the complete complex [8, 9]. CESs contain the MSL Recognition Element (MRE), a 21 base pair, GA-rich motif required for MSL complex recruitment. The CLAMP protein is bound at MREs and may recruit the assembled MSL complex [10]. The MSL complex then spreads into nearby transcribed genes by recognition of active chromatin marks [11-13]. This elegant model suffers from major drawbacks. For example, X-identification cannot be attributed solely to MREs, since they are only ~2 fold enriched on the X chromosome [8]. The discovery that the CLAMP protein enables MREs to recruit the MSL complex was a significant finding, but CLAMP-bound MRE motifs are present on all chromosomes. Modified MREs that also contact one of the MSL proteins are enriched on the X, but this motif is similarly found throughout the genome [14]. Although MREs play an important role in recruiting the MSL complex, their genome-wide distribution suggests that other factors uniquely specify X chromatin.

Both *roX* genes overlap CES and are X-linked, suggesting a role in marking the X for compensation. Indeed, when inserted on an autosome the *roX* genes recruit the MSL complex to the insertion site, and spreading to polytene bands hundreds of kb from the transgene is sometimes observed [15]. Subsequent studies confirmed up to two-fold activation of reporters and genes close to autosomal *roX* transgenes [16, 17]. The action of *roX* could be considered analogous to that of the long non-coding *X-inactive specific transcript* (*Xist*) that inactivates an X chromosome in mammalian dosage compensation. *Xist* is a part of the X inactivation center (*Xic*), a region that is necessary and sufficient to silence the entire chromosome [18]. Silencing is limited to chromatin in *cis* to the *Xic*, thus sparing the active X chromosome [19]. However, when *roX* genes are deleted from the X and placed on an autosome, *roX* RNA is incorporated into the MSL complex, which then travels to the X chromosome and rescues compensation [3]. Furthermore, MSL recruitment near autosomal *roX* insertions is considerably weaker than recruitment to the X chromosome. These observations indicate that the *roX* genes do not specify X identity by themselves.

Exclusive X-recognition by the MSL complex must therefore involve additional mechanisms.

Our lab previously demonstrated a role for the siRNA pathway in X chromosome recognition [20]. This led to the discovery that siRNA from a repetitive element that is strikingly enriched on the X chromosome enhanced X-identification by the MSL complex and rescued the lethality of *roX1 roX2* mutant males [21]. These repetitive sequences, the 1.688 g/cm^3 satellite related repeats (hereafter 1.688^X; superscript denotes cytological position) are ~359 bp, AT-rich and found in short, tandem clusters in X euchromatin [22, 23]. Related repeats are found on other chromosomes, but clades that are near-exclusive to the X chromosome have been noted for 30 years [24, 25]. The 1.688^X repeats are dissimilar in sequence to MREs within the CES, and display low recruitment of MSL proteins, suggesting that any role in recruitment of the MSL complex is indirect [26].

We postulated that the 1.688^X repeats might act cooperatively with *roX* genes to identify X chromatin. The presence of hundreds of dispersed 1.688^X repeats along the X chromosome makes evaluation of the effect of individual repeats impractical. Instead, we devised a functional approach that tested the ability of *roX1,* and three different 1.688^X repeats, to recruit compensation to an autosome. A transgene carrying *roX1* and 1.688^X was integrated on an autosome. *roX1* or 1.688^X was then excised from the transgene, allowing testing of individual elements. As expected, the full transgene and *roX1* alone recruit the MSL complex in *cis*. To our surprise, all 1.688^X repeats tested were also able to recruit compensation to surrounding genes by themselves. All transgenes partially rescued a lethal autosomal deficiency of the homologous chromosome in males, but not in females, demonstrating functional compensation of autosomal chromatin. Finally, we demonstrate that ectopic production of siRNA from 1.688^{3F}, previously shown to promote X recognition, enhances rescue of deficiency males if 1.688^X sequences are present on the intact homolog. These findings suggest that recruitment of dosage compensation to nearby genes is a general property of 1.688^X repeats. We postulate that the siRNA pathway normally acts at the dispersed, X-linked 1.688^X sequences to promote identification of X chromatin.

Results

To test the idea that 1.688^X act cooperatively with *roX* to identify X chromatin we generated transgenes with *roX1* and 1.688^X repeats [>*roX1*> w^{+mC}>1.688^X >]. LoxP and FRT sites (>) enable excision of *roX1* or 1.688^X to permit testing of individual elements (Fig. S1). Transgenes with 1.688^X repeats from 1A, 3C and 3F (1.688^{1A}, 1.688^{3C} and 1.688^{3F}) were integrated on 2L at cytological position 22A3, and the transgene carrying 1.688^{3F} was also integrated at 24A2 and 25C7 (see STAR methods for details). Full transgenes are henceforth denoted as [*roX1*+1.688^X] and reduced transgenes as [*roX1*] or [1.688^X]. These repeats were selected for examination because ectopic 1.688^{3F} siRNA production from a hairpin transgene ([hp 1.688^{3F}]) achieved rescue of *roX1 roX2* males, while similar transgenes producing hairpin RNA from 1.688^{1A} (89 % identity to 1.688^{3F}) and 1.688^{3C} (68% identity to 1.688^{3F}) afforded little or no rescue [21]. This suggested that 1.688^{3F}, located on the X chromosome immediately distal to *roX1*, could have an unusual function, perhaps related to its situation near *roX1*. However, if 1.688^X sequences generally identify X chromatin for

compensation, we expect that many different repeat clusters will attract compensation to nearby genes.

1.688^{X} and *roX1* transgenes recruit MSL proteins to autosomal sites

Polytene chromosome preparations were made from a laboratory reference strain, and from males with full or reduced integrations at 22A3 ([*roX1*+1.688^{3F}]^{22A3}, [*roX1*]^{22A3}, [1.688^{3F}]^{22A3}). These were immunostained for the core MSL complex component Male Specific Lethal 2 (MSL2). As expected, all preparations displayed strong recruitment to the X chromosome. No autosomal recruitment of MSL2 to 2L was detected in the laboratory reference strain (Fig. 1A, B). In contrast, males carrying complete or reduced transgenes displayed recruitment near the tip of 2L at cytological position 22A3 (yellow arrows, Fig. 1C-F). Surprisingly, recruitment of MSL2 by [1.688^{3F}]^{22A3} was comparable to [*roX1*+1.688^{3F}]^{22A3}, and more robust than [*roX1*]^{22A3} (Table 1, Fig. S2). Both [1.688^{3F}]^{22A3} and [*roX1*+1.688^{3F}]^{22A3} support easily discernable spreading of MSL2 into the chromatin of 2L, most strikingly to a strong, subtelomeric band observed in most preparations with these transgenes (white arrows, Fig. 1D, F; Table 1). Interestingly, about half the preparations of [1.688^{3F}]^{22A3} and [*roX1*+1.688^{3F}]^{22A3} also showed MSL2 recruitment to the 3L telomere (white arrowheads, Fig. S2B, C). Although MSL2 did not spread into subtelomeric regions of [*roX1*]^{22A3} preparations, some proximal spreading was observed in about 20% of samples (Table 1). To determine if additional members of the MSL complex are similarly recruited, polytene preparations from [1.688^{3F}]^{22A3} males were probed with antibodies to Male Specific Lethal 3 (MSL3) and Maleless (MLE). A partial complex capable of binding to the CES forms in the absence of MSL3, but spreading to nearby genes is reduced [12]. MLE colocalizes with MSL2, but is not considered part of the complex core [26]. Both antibodies detect minor signal at 22A3, as well as spreading into the subtelomeric region (Figure S3A-D). The 1.688^{3F} repeats by themselves thus appear capable of recruiting intact MSL complexes.

To determine if recruitment is a general property of 1.688^{X} repeats, we generated *roX1* transgenes with 1.688^{1A} and 1.688^{3C} repeats, sharing 89% and 68% identity with 1.688^{3F}, respectively. MSL2 was not detected on 2L in [1.688^{1A}]^{22A3} or [1.688^{3C}]^{22A3}, and may be below the level detectable by antibody staining (Fig. 1G). However, [*roX1*+1.688^{1A}]^{22A3} and [*roX1*+1.688^{3C}]^{22A3} display recruitment of MSL2 to 2L and robust spreading into the sub-telomeric region (yellow and white arrows, Fig. 1 H). As recruitment is considerably stronger than that achieved by *roX1* alone, 1.688^{3C} and 1.688^{1A} act synergistically with *roX1* to elevate recruitment and spreading. We conclude that all three 1.688^{X} repeats facilitate MSL2 recruitment and spreading, either independently or in cooperation with *roX1*.

We examined 1.688 ^{X} sequences for similarity to the CES consensus and found none (Fig. S4)[27]. To determine whether a group of closely related 359 bp repeats that comprise 10 Mb of pericentromeric X heterochromatin recruit MSL2, we performed immunostaining of mitotic embryo preparations. No convincing signal could be detected within X heterochromatin, largely composed of this repeat (Fig. S5). These findings are consistent with the fact that 1.688^{X} repeats do not generally display MSL enrichment [26]. Taken

together, these observations suggest that 1.688^{X} repeats do not directly recruit MSL proteins, and likely act indirectly to promote MSL recognition of nearby chromatin.

Increased autosomal gene expression near transgene integrations

Recruitment of MSL2 by *roX1* and 1.688^{3F} integrations suggested a possible increase in expression of nearby genes in males. To test this, we generated chromosomes with three complete integrations on 2L, increasing the number of genes near integration sites ([*roX1*+1.688^{3F}]$^{22A3+24A2+25C7}$). Chromosomes with *roX1* only ([*roX1*]$^{22A3+24A2+25C7}$) or 1.688^{3F} only ([1.688^{3F}]$^{22A3+24A2+25C7}$) were produced by Cre or FLP expression. Quantitative RT-PCR was used to measure mRNA from test genes situated near integration sites (Fig. 2A) and control genes on different chromosome arms. Control genes showed no expression differences between males of the laboratory reference strain and males with complete or reduced transgenes (white boxes, Fig. 2B). In contrast, genes near integration sites (gray boxes, Fig. 2B) displayed slightly elevated expression in [*roX1*]$^{22A3+24A2+25C7}$ males. A greater, statistically significant increase of 30% was observed in [1.688^{3F}]$^{22A3+24A2+25C7}$ males, and [*roX1*+1.688^{3F}]$^{22A3+24A2+25C7}$ animals displayed a 50% increase in expression of nearby genes.

Since local MSL complex recruitment is cotranscriptional, highly expressed genes should be more strongly compensated [28, 29]. Indeed, the most highly expressed gene tested, *RpL37A*, displayed a 2-fold increase in expression, indicative of full compensation (Table S1). As a group, the test genes revealed a positive correlation between expression in the control and the relative increase achieved when *roX1* and 1.688^{3F} transgenes were present (Fig. 3A-C). The correlation was strongest when both *roX1* and 1.688^{3F} were present on the transgene, reflecting the presence of two recruiting elements. In contrast, no correlation between increased expression and distance to the nearest transgene was detected up to 142 kb (Fig. 3D-F). We conclude that local recruitment of the MSL complex by *roX1* and 1.688^{3F} transgenes displays the properties of dosage compensation as it favors active genes and achieves up to a two-fold increase in expression. The ability of *roX1* and 1.688^{3F} to attract compensation to genes over 100 kb away is consistent with the long-range effects of transgenes containing only *roX* [17].

1.688^{X} and *roX1* transgenes functionally compensate a 2L deficiency

To determine if increased gene expression near *roX1* and 1.688^{X} transgenes constituted a functional dosage compensation system, we attempted rescue of the lethality produced by hemizygosity of distal 2L. Males were generated with a lethal 2nd chromosome deficiency and a single [*roX1*+1.688^{3F}]^{22A3} transgene on the intact homolog, close to the deficiency break point. As the dosage compensation complex is only assembled in males, females served as a control (Fig. 4A). Significantly, this study was performed in flies that were completely wild type for the canonical dosage compensation machinery. Chromatin recognition and spreading will therefore occur in the context of a fully functional MSL complex.

We generated translocation T(2;Y)22A2 by moving the distal 1.5 Mb of 2L onto the Y chromosome [30]. T(2;Y)22A2 can be separated into a terminal deficiency that is

completely lethal (Df(2)22A2) and a reciprocal duplication on the Y chromosome (Dp(2:Y)22A2) by mating (Fig. S6A). Transgenes at the integration site closest to the deficiency break point, 22A3, were tested first. Neither [*roX1*]^{22A3}, [1.688^{3F}]^{22A3} or [*roX1*+1.688^{3F}]^{22A3} rescued Df(2)22A2 males to adulthood. Df(2)22A2 adults could not be recovered when three copies of full or reduced transgenes were present at 22A3, 24A2 and 25C7. This was not surprising as the 24A2 and 25C7 integration sites are several Mb proximal to the deficiency. However, examination of vials producing Df(2)22A2 males with full or reduced transgenes at 22A3 revealed some adult Df(2)22A2 males within the pupal cuticle (pharate males), as well as a few dead but partially eclosed males (Fig. 4B). Dissection of pupal cases revealed adult males with eye color indicative of the Df(2)22A2 chromosome. The presence of Df(2)22A2 and lack of Dp(2:Y)22A2 was confirmed by PCR (Fig. S6B). In contrast, no pharate Df(2)22A2 males were observed from matings that lack transgene integrations, and no pharate females could be recovered with or without transgenes. *roX1* alone enabled recovery of 6% pharate males, based on the survival of brothers with two intact 2nd chromosomes. [*roX1*+1.688^{3F}]^{22A3} supported 18% pharate males. Intriguingly, the repeats alone, [1.688^{3F}]^{22A3}, supported 11% male pharate males, exceeding rescue with *roX1* (Fig. 4B). While this suggests that *roX1* and 1.688^{3F} transgenes do achieve partial compensation of 2L, the difficulty in scoring pharate animals prompted a switch to examination of 3rd instar larvae.

Male larvae show functional compensation of autosomal chromatin

Late 3rd instar larvae from matings that produce Df(2)22A2 males and females were sexed and genotyped using visible markers, and a subset of Df(2)22A2 larvae were genotyped by PCR to confirm the deficiency (see mating strategies in Fig. S7A, B). Third instar Df(2)22A2 females were recovered at 3% the anticipated number in control matings in which no transgene is present on 2L, and female recovery is unchanged by the full [*roX1*+1.688^{3F}]^{22A3} transgene (Fig. 4C). Df(2)22A2 males with no transgene are recovered at 4% (hatched bars, Fig. 4D). However, when [*roX1*]^{22A3} was present on the intact 2nd chromosome, 20% of Df(2)22A2 3rd instar males were recovered. This increased to 36.5% with [1.688^{3F}]^{22A3}, and to 51% when [*roX1*+1.688^{3F}]^{22A3} was present (Fig. 4D). This confirms that 1.688^{3F} repeats are more effective in recruiting compensation to nearby genes than *roX1* itself, and suggests that *roX1* and 1.688^{3F} act in a cooperative manner.

The 22A3 landing site is marked with y^+, and the intact [*roX1*+1.688^{3F}]^{22A3} transgene also carries the w^{+mC} marker. Both *y* and *w* are X-linked, and w^{+mC} is partially compensated at many autosomal insertion sites. This raised the concern that the genetic markers might contribute to local recruitment. To address this, we tested the P{EPgy2}haf^{EY08668} insertion, 8 kb distal to the 22A3 landing site, marked with y^+ and w^{+mC} (Control TG, Fig. 4D). No rescue of 3rd instar Df(2)22A2 male larvae was detected, eliminating the possibility that the genetic markers alone are effective recruiting elements.

The absence of visible MSL2 recruitment by [1.688^{1A}]^{22A3} and [1.688^{3C}]^{22A3} suggested that these repeats might be ineffective by themselves. To test this we measured the survival of Df(2)22A2 male larvae with insertions of 1.688^{1A} and 1.688^{3C} repeats alone or with *roX1* (hatched bars, Fig. 5). Surprisingly, [1.688^{1A}]^{22A3} and [1.688^{3C}]^{22A3} partially rescued

Df(2)22A2 males, supporting 13% recovery of 3rd instar larvae. Recovery increased to 33-34% when *roX1* was also present, exceeding rescue by *roX1* alone. We conclude that even though [1.688^{1A}]^{22A3} and [1.688^{3C}]^{22A3} do not recruit visibly detectable levels of MSL2 on polytene preparations, they do in fact recruit dosage compensation and act cooperatively with *roX1* to enhance compensation of nearby genes.

Ectopic expression of 1.688^{3F} siRNA enhances rescue of male larvae when cognate sequence is present on 2L

Ectopic expression of hairpin RNA from 1.688^{3F} produces abundant siRNA and partially rescues the lethality of *roX1 roX2* males [21]. It is possible that the siRNA pathway acts upon chromatin at related repeats throughout the X, and that this facilitates recruitment of the MSL complex to nearby genes. If this is indeed the case, 1.688^{3F} siRNA may also enhance recruitment of compensation by autosomal 1.688^{X} transgenes. Ectopic expression of 1.688^{3F} siRNA had no effect on the recovery of Df(2)22A2 male larvae with no transgene, or with [*roX1*]^{22A3} on the intact homolog (black bars, Fig. 4D; mating strategy presented Fig. S7C). However, expression of hp 1.688^{3F} increased recovery of larval males carrying [1.688^{3F}]^{22A3} or [*roX1*+1.688^{3F}]^{22A3} by 8% (black bars, Fig. 4D). In contrast, expression of hp RNA to the non-essential *white* gene did not influence rescue by any transgene (gray bars, Fig. 4D). Enhanced rescue is therefore not the result of non-specific small RNA production.

To determine if 1.688^{3F} siRNA also modulates the recruitment of compensation by 1.688^{1A} and 1.688^{3C} transgenes, we expressed hp 1.688^{3F} in Df(2)22A2 males carrying [1.688^{1A}]^{22A3}, [*roX1*+1.688^{1A}]^{22A3}, [1.688^{3C}]^{22A3} or [*roX1*+1.688^{3C}]^{22A3} on the intact homolog. Expression of hp 1.688^{3F} enhanced male survival when 1.688^{1A} or 1.688^{3C} was present on 2L, either alone or with *roX1* (black bars, Fig. 5). The increase in survival, 7-9%, is comparable to that achieved in transgenes carrying 1.688^{3F}. Taken together, these studies demonstrate that 1.688^{3F} siRNA does not modulate the intrinsic activity of the MSL complex or influence recruitment by *roX1*, but acts through cognate sequence to elevate compensation at nearby genes. Complete sequence identity is not necessary, as the increase in survival achieved by production of 1.688^{3F} siRNA in flies carrying [1.688^{3C}]^{22A3} or [1.688^{3F}]^{22A3} transgenes, containing repeats that share only 69% identity, was essentially identical. We propose that 1.688^{3F} siRNA acts at numerous 1.688^{X} repeats along the X chromosome, enabling these to more effectively recruit compensation to nearby genes.

Discussion

Previous studies have identified GA-rich MRE elements, as well as variations on this sequence, that directly recruit the CLAMP adapter protein and MSL complex [10, 14]. The enrichment of these motifs on the X chromosome is modest, suggesting that additional features contribute to X recognition [8]. Our prior studies led to the surprising conclusion that the siRNA pathway, and siRNA from a 1.688^{X} repeat, participated in X recognition [20, 21, 31]. Many of the 1.688^{X} repeats are transcribed and produce siRNA, making them attractive candidates for involvement in an siRNA-mediated process. The current study now demonstrates that 1.688^{X} DNA itself is capable of attracting compensation to nearby

transcribed genes, an effect that is enhanced by cognate siRNA. The mechanism by which 1.688^{X} sequences on the X contribute to MSL recruitment is under investigation, but the repeats are not themselves sites of strong MSL recruitment, and thus anticipated to act indirectly. One hypothesis is that 1.688^{X} sequences influence the architecture of the X chromosome to facilitate spreading of MSL complex along the chromosome [32]. Interestingly, the male X chromosome assumes a distinct interphase organization with compensated genes close together [33]. Parallel ideas have been proposed in mammalian dosage compensation, where the L1 (LINE-1) elements, and small RNA have been implicated in formation of a silencing domain [34].

Regardless of the mode of action, our study demonstrates that different 1.688^{X} repeats share a remarkable ability to recruit compensation to nearby transcribed genes. Although visible recruitment of MSL2 by repeat-only transgenes was only observed for 1.688^{3F}, 1.688^{1A} and 1.688^{3C} enhanced visible recruitment when *roX1* was also present. More importantly, all three repeats alone supported detectable levels of autosomal compensation, as revealed by partial rescue of males with a lethal deficiency opposite a chromosome with a single transgene insertion. Functional compensation is thus achieved by MSL protein recruitment that is below the threshold for visual detection. It is important to note that, in all cases, partial rescue of Df(2)22A2 males was achieved by insertion of a single transgene with less than 2 kb of repeat DNA. In contrast, hundreds of 1.688^{X} repeat clusters are broadly distributed across the X chromosome, an arrangement that provides redundancy of function.

The finding that ectopic production of 1.688^{3F} siRNA increased autosomal compensation when 1.688^{1A}, 1.688^{3C} or 1.688^{3F} was present on the autosome links the compensation function of the siRNA pathway to the 1.688^{X} repeats. Although the mechanism by which 1.688^{3F} siRNA acts at disparate 1.688^{X} sequences is unknown, siRNA has previously been found to regulate genes and mobile elements in fly somatic cells [35, 36]. It is plausible that 1.688^{3F} siRNA enables recruitment of chromatin modifying activities to cognate loci, as in other organisms, and that these modifications alter properties of 1.688^{X} chromatin (Fig. 6) [37, 38].

The unusual properties of 1.688^{3F} siRNA, and the situation of this cluster immediately distal to *roX1*, raised suspicions that 1.688^{3F} harbored a novel function related to *roX1* [21]. For example, special properties of 1.688^{3F} DNA could facilitate MSL complex spreading from *roX1*. In accord with this idea, 1.688^{3F} does appear to recruit compensation more vigorously than 1.688^{1A} and 1.688^{3C}. One intriguing possibility is that the physical linkage of 1.688^{3F} and *roX1* coordinates two pathways that cooperate to identify X chromatin (Fig. 6). While the significance of this is unclear, it is interesting that *roX1* is one of the earliest zygotic transcripts, and supports initial X recognition at 3 h of development [39, 40]. In contrast, *roX2* is first expressed several hours later. It is possible that 1.688^{3F} and *roX1* collaborate in initial X recognition, and their close localization coordinates these activities.

Fly X chromosomes display the evolutionary signature of adaptation to compensation. Mutations, and expansions of GA on the fly X chromosome that contributed to the rise of MREs have been reported, and MREs have propagated across the *D. miranda* X chromosome by their inclusion in a mobile element [41-43]. More broadly, Drosophilid X

chromosomes are strikingly enriched for chromosome-specific repeats, which account for 45 times more coverage on the *D. melanogaster* X chromosome than the autosomes [22]. This raises the possibility that additional families of repeats might function in a manner similar to the 1.688^{X} repeats. Interestingly, a newly evolved X chromosome arm in *D. pseudoobscura* rapidly acquired satellite repeats that are present on the ancestral X [22]. How these repeats proliferate is unknown, but the current study suggests a mechanism that restricts 1.688^{X} repeats to the X chromosome. The demonstration that autosomal insertions of 1.688^{X} repeats induce misregulation of nearby genes in males reveals that autosomal 1.688^{X} repeats would be targeted for elimination by natural selection. In contrast, 1.688^{X} sequences on the X chromosome would enhance compensation of nearby genes, making them subject to positive natural selection.

STAR Methods

Contact For Reagent And Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Victoria H. Meller (vmeller@biology.biosci.wayne.edu).

Experimental Model And Subject Details

Fly culture and strains—Flies were maintained at 25°C on standard cornmeal agar diet in a humidified incubator. Strains are provided in the Star Methods section. Survival of male and female larvae to the end of pupation (pharate pupal stage) and to late 3rd instar (wandering stage) was measured. Animal sex was determined by gonad morphology (larvae) or adult genitalia (pharate pupae).

Method Details

Cloning and transgene integration—The [>*roX1*> w^{+mC}>1.688^{3F} >], [>*roX1*> w^{+mC}>1.688^{1A} >] and [>*roX1*> w^{+mC}>1.688^{3C} >] transgenes were assembled in the pUASTB vector by traditional cloning techniques[44]. Details of assembly and primer sequences are available upon request. 1.688^{3F} repeats were contained in a 2 kb genomic fragment.1.688^{1A} and 1.688^{3C} repeats were introduced as 1.4 and 1.6 kb of amplified DNA, respectively. Construction was verified by restriction mapping, sequencing and PCR at each stage. Injections were performed by Genetic Services Inc. (Sudbury, MA) and Rainbow Transgenics (Camarillo, CA) in stocks containing 22A3 [VK00037], 24A2 [su(HW)attP6] and 25C7 [attP40] landing sites. In-situ hybridization to polytene preparations verified integration.

Scoring of larval rescue—Matings to generate Df(2)22A2 male and female larvae are presented in Fig. S7A, B. The intact 2nd chromosome was marked with p[Sqh-mCherry.M] to allow visual identification of Df(2)22A2 larvae. All third instar larvae were sexed and scored 3 times daily. PCR was performed on a subset of non-fluorescent larvae to confirm Df(2)22A2. Primers used for genotyping are available on request.

Immunodetection—Immunodetection of MSL2, MSL3 and MLE on polytene chromosomes was done as previously described [7, 45]. Briefly, transgenic larvae were

grown at 18°C in uncrowded vials on standard cornmeal molasses food. Larvae were dissected in PBS, 4% formaldehyde, 1% Triton X100, and fixed for 45 sec. The fixative was replaced with 50% acetic acid, 4% formaldehyde for 2 min and then the glands were placed in lactoacetic acid (lactic acid:water:acetic acid, 1:2:3) and spread under a coverslip. The slides were blocked with 0.2% BSA and treated with affinity-purified primary antibodies (for antibody descriptions please refer the Key Resources Table). Visualization and photography were performed with an Olympus IX81 inverted microscope fitted with a Photometrics CoolSNAP EZ CCD camera.

Mitotic chromosome preparations—Timed collections of early embryos were dechorionated, homogenized in PBST with 2% formaldehyde by two strokes with a tight pestle and filtered through Nytex (Millipore). Nuclei were collected by centrifugation at 2 K RPM for 5 min, fixative removed and nuclei suspended in 60 ml hexylene glycol fixative (1 mM HEPES pH 6.8, 1 mM $CaCl_2$, 3.7% freshly made paraformaldehyde with 26% hexylene glycol). Five ml drops are squashed between cover slip and slide. Slides were plunged into liquid nitrogen, cover slips removed and slides stored at -20°C in 95% EtOH. After rehydration in PBST, prep arations were refixed in 4% formaldehyde in PBST, washed, blocked and incubated with antibody as described for polytene chromosome preparations.

Quantitative RT-PCR—Total RNA was prepared from two biological replicates of 50 third instar males using Trizol reagent (Invitrogen). One microgram of total RNA was reverse transcribed using ImProm-II reverse transcriptase following manufacturer recommendations (Promega). Duplicate reactions were amplified using iTaq Universal SYBR Green Supermix (Bio-Rad) with an Mx3000P Real-Time PCR system (Stratagene). The genes analyzed were stably expressed in late third instar larvae. Gene and primer information is available upon request. Values were normalized to *Dmn* and expression calculated using the efficiency corrected comparative quantification method [46].

Quantification And Statistical Analysis

After measuring gene expression using quantitative RT-PCR, box and whiskers plots of control and test genes were generated with SPSS. One-way ANOVA followed by Tukey's HSD post-hoc tests were used to determine the significance of gene expression. The Pearson correlation coefficients of relationships between change in gene expression and the distance from the transgene or basal expression were generated using SPSS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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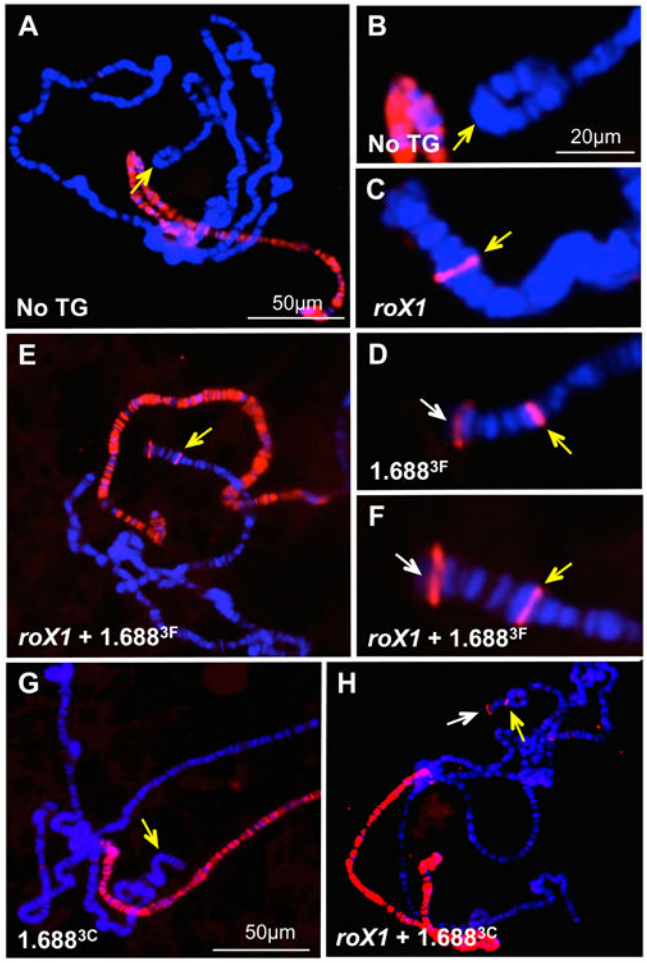

Fig. 1. Autosomal insertions of *roX1* or 1.688^{3F} recruit MSL2

A) MSL2 recruitment (red) is limited to the X chromosome in a control male. **B)** Enlargement of cytological position 22A3 (yellow arrow) from A. **C)** [*roX1*]^{22A3} recruits MSL2 to a single band near 22A3. **D)** [1.688^{3F}]^{22A3} recruits MSL2 robustly near the integration site (yellow arrow). Spreading to a sub-telomeric region is observed (white arrow). **E)** MSL2 recruitment by [*roX1*+1.688^{3F}]^{22A3} is robust and similar to [1.688^{3F}]^{22A3}. Yellow arrow marks the 22A3 integration site. **F)** Detail from E. White arrow marks sub-telomeric spreading. Scoring of MSL2 recruitment and spreading is presented in Table 1. **G)** Visible MSL2 recruitment (red) in a [1.688^{3C}]^{22A3} male larvae is limited to the X chromosome. Yellow arrow marks the insertion site at 22A3. **H)** Robust MSL2 recruitment (yellow arrow) and spreading to a subtelomeric region (white arrow) is observed in [*roX1*+1.688^{3C}]^{22A3} males. Chromosome preparations were probed with anti-MSL2 antibody and detected by Texas Red. DNA is counterstained with DAPI (blue). See also Figures S1-S3 and Figure S5.

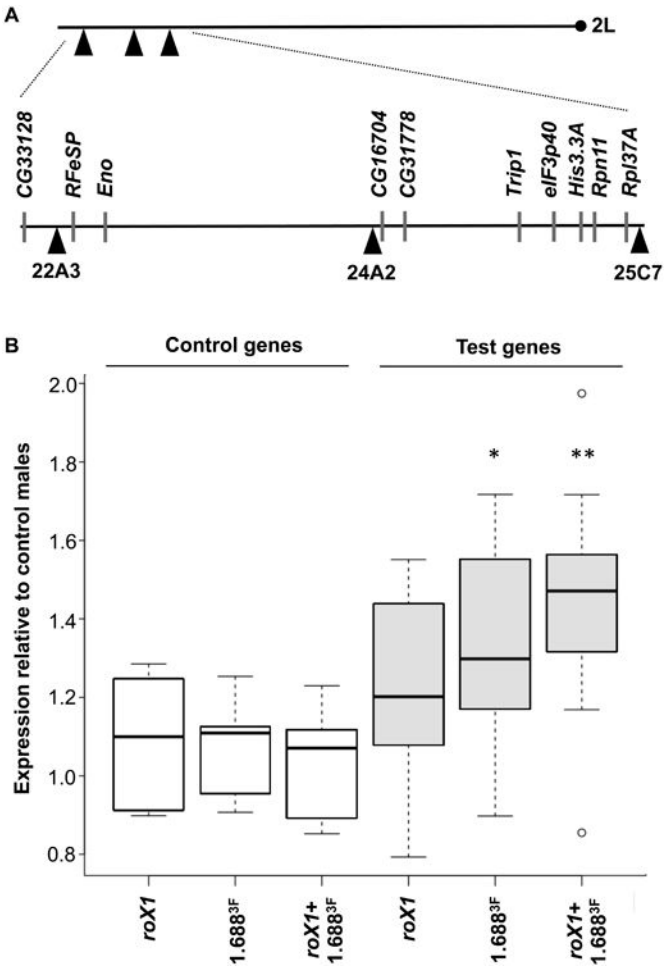

Fig. 2. Increased expression of autosomal genes near *roX1* and 1.688^{3F} transgenes

A) Three transgene integration sites on distal 2L are depicted by black triangles. The centromere is shown by a black circle. Positions of test genes and integration sites are depicted below. **B)** Relative expression of control genes (located on 2R or 3) and test genes was measured by quantitative RT-PCR. The ratio of expression in larval males with the indicated transgenes to control males (no transgenes) is depicted for 5 control genes (white) and 10 test genes (gray). Control genes show no changes in expression, but test genes display significant increases when $[1.688^{3F}]^{22A3+24A2+25C7}$ or $[roX1+1.688^{3F}]^{22A3+24A2+25C7}$ transgenes are present. * $p<0.05$, ** $p<0.01$. See also Figure S1 and Table S1.

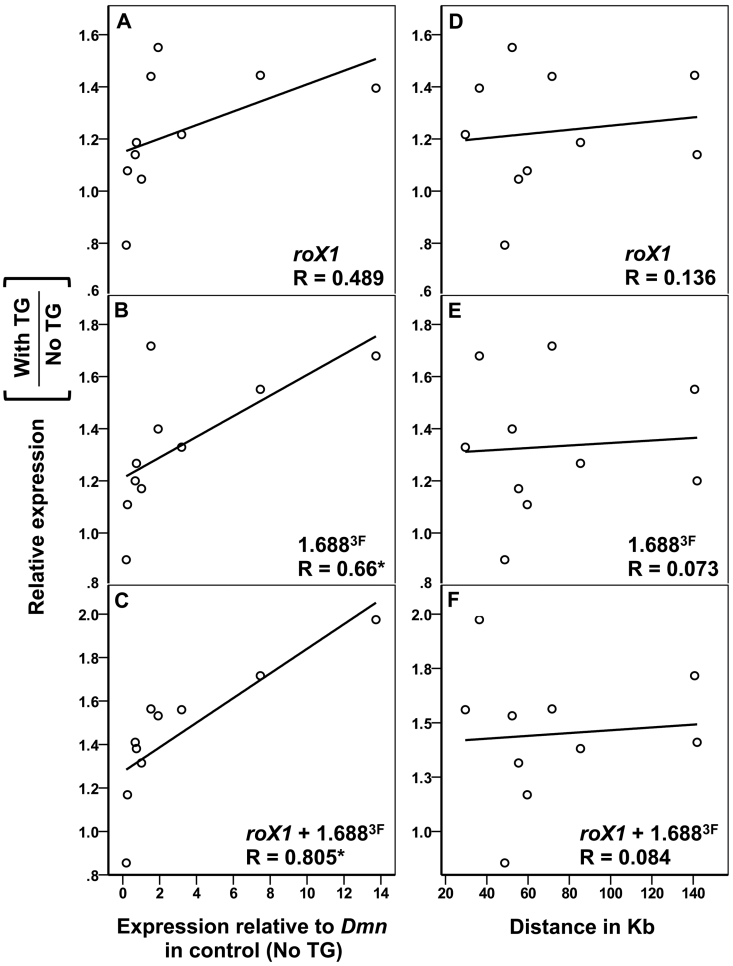

Fig. 3. Increased expression near *roX1* and 1.688^{3F} correlates with gene activity. A-C) The fold change in expression of individual genes near 2L transgenes (Y-axis) is plotted against expression in control males (no transgenes; X-axis). Each gene was internally normalized to *Dmn* before calculating fold change. Dots represent the average of two biological replicates. A significant correlation is observed for [1.688^{3F}]$^{22A3+24A2+25C7}$ (B) and [*roX1*+1.688^{3F}]$^{22A3+24A2+25C7}$ (C) but not [*roX1*]$^{22A3+24A2+25C7}$ (A). **D-F)** The fold change in expression of individual genes near 2L transgenes (Y-axis) is plotted against the distance between each gene and the nearest transgene (X-axis). No significant correlation was detected in these analyses. Pairwise correlation was performed using SPSS, the Pearson Correlation Coefficient (R) is shown (* $p < 0.05$). See also Figure S1 and Table S1.

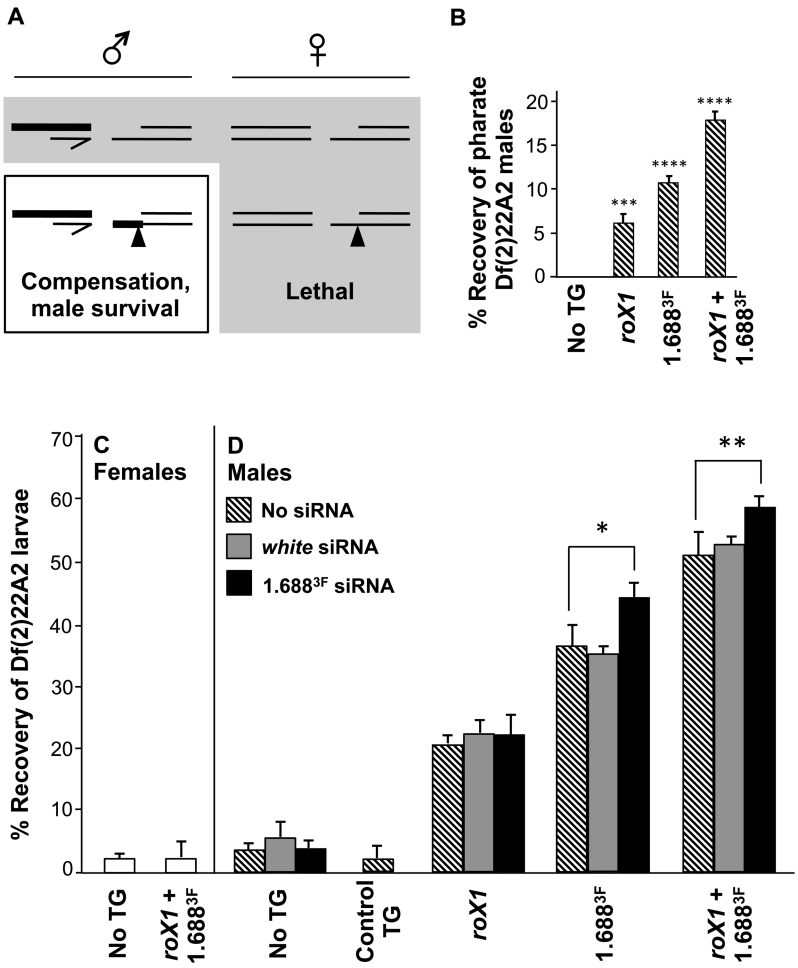

Fig. 4. Insertions of *roX1* and 1.688^{3F} on 2L partially rescue males with a lethal 2L deficiency

A) Df(2)22A2 is lethal in males (top left) and females (top right). If a transgene on the homolog recruits compensation, male-limited rescue will occur (bottom left). Thick lines indicate compensated chromatin. Females block formation of the MSL complex, preventing rescue (bottom right). **B)** Pharate Df(2)22A2 males are observed only when [*roX1*]^{22A3}, [1.688^{3F}]^{22A3} or [*roX1*+1.688^{3F}]^{22A3} is present on the homolog. The recovery of pharate males is calculated from brothers that emerged with an intact 2nd chromosome (See Fig. S6A for mating strategy). **C)** Recovery of Df(2)22A2 female larvae is unaffected by [*roX1*+1.688^{3F}]^{22A3} on the homolog (See Fig. S7B for mating strategy). **D)** Recovery of Df(2)22A2 male larvae increases when [*roX1*]^{22A3}, [1.688^{3F}]^{22A3} or [*roX1*+1.688^{3F}]^{22A3} is present on the intact homolog (hatched bars), but not when a control P{EPgy2}^{hafEY08668} transgene marked with y^+ and w^+ is inserted at 22A3 (Control TG). Expression of siRNA to *white* (gray) has no effect on recovery of male larvae, but expression of 1.688^{3F} siRNA (black) enhances recovery when transgenes containing 1.688^{3F} DNA are present on the intact homolog (mating strategy in Fig. S7A, C). See Figure S1 for transgene composition and Figure S4 for 1.688^{3F} sequence. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

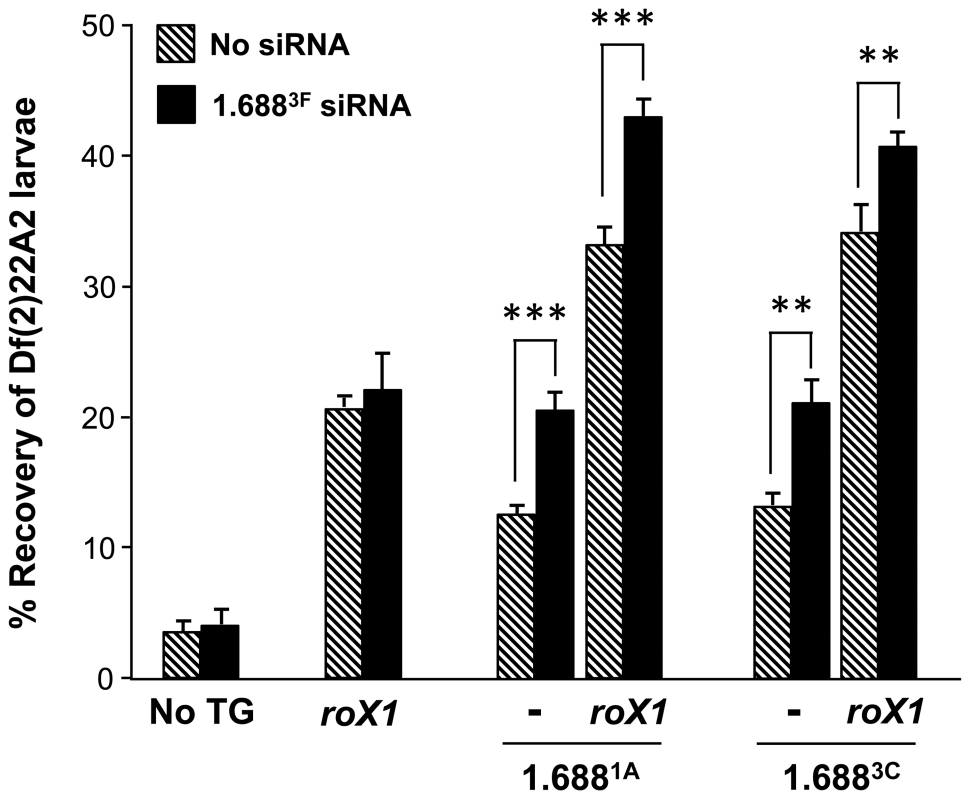

Fig. 5. Insertion of 1.688^{1A} and 1.688^{3C} on 2L partially rescue males with a lethal 2L deficiency

Recovery of Df(2)22A2 male larvae increases when $[1.688^{1A}]^{22A3}$, $[roX1^{+}1.688^{1A}]^{22A3}$, $[1.688^{3C}]^{22A3}$ or $[roX1^{+}1.688^{3C}]^{22A3}$ is present on the intact homolog (hatched bars). The ratio of male larvae carrying Df(2)22A2 to brothers with an intact 2nd chromosome is presented. Recovery of males with no transgene or $[roX1]^{22A3}$ is redrawn from Fig. 4D. Ectopic expression of 1.688^{3F} siRNA (black) enhances recovery when 1.688^{1A} or 1.688^{3C} DNA is present on the homolog. The mating strategy is presented in Figure S7A, C. See Figure S1 for transgene composition and Figure S4 for comparison of 1.688^{X} sequences used. Error bars represent SEM. ** $p < 0.01$; *** $p < 0.001$.

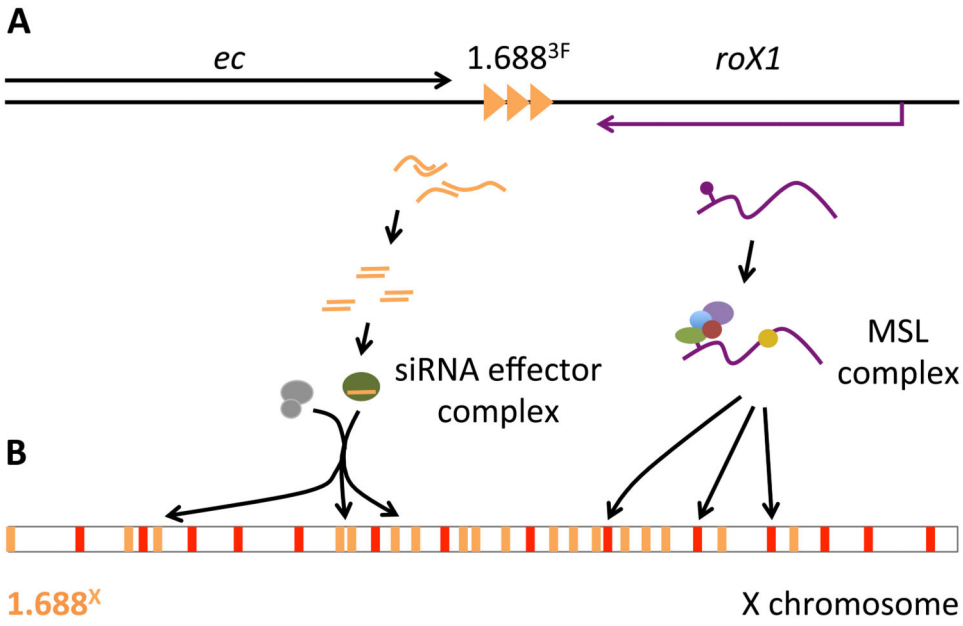

Fig. 6. Linkage of 1.688^{3F} and *roX1* could coordinate function

A) 1.688^{3F} is located between *echinus* (*ec*) and *roX1*. Bidirectional transcription of 1.688^{3F} may generate siRNA that is loaded onto an effector complex (left). Ago2, and other genes in the siRNA pathway, participate in X recognition [20]. The *roX1* transcript is assembled into the MSL complex (right). *roX1* and 1.688^{3F} produce different classes of noncoding RNA, and each element retains biological activity when separated from the other. It is possible that the proximity of 1.688^{3F} and *roX1* coordinates different pathways that cooperate to identify X chromatin. **B)** A siRNA-containing effector complex may recruit chromatin modifiers (gray) to 1.688^{X} repeats (gold) across the euchromatic X chromosome. We hypothesize that this produces epigenetic or architectural changes that facilitate MSL recruitment, or spreading of the MSL complex along the X. The MSL complex is initially recruited to Chromatin Entry Sites (CES, red), and spreads into active genes nearby. No direct association between proteins of the siRNA pathway and the MSL complex has been reported, suggesting that the siRNA pathway influences MSL recruitment indirectly.

Table 1

Autosomal MSL2 recruitment by [*roX1*]^{22A3}; [1.688^{3F}]^{22A3} and [*roX1*+1.688^{3F}]^{22A3}

Recruitment close to the integration site is ranked from none (-) to very strong (++++). Additional autosomal signals are categorized by cytological position. The percentage of nuclei in each category is followed by the number of nuclei (parentheses). Counts were derived from 3 individuals with no transgene (*yw* laboratory reference strain) and groups of 5 individuals with [*roX1*]^{22A3}, [1.688^{3F}]^{22A3} or [*roX1*+1.688^{3F}]^{22A3}. Scoring was done on coded samples to prevent bias. Representative images depicting weak (+) to very strong (++++) recruitment are presented in Figure S2A.

		Transgene			
		None	***roX1***	**1.688^{3F}**	***roX1* + 1.688^{3F}**
22A3 Recruitment	-	100 (63)	0 (0)	0 (0)	1.01 (1)
	+	0 (0)	10.78 (11)	0 (0)	0 (0)
	++	0 (0)	65.68 (67)	15.17 (17)	30.3 (30)
	+++	0 (0)	23.52 (24)	55.35 (62)	54.54 (54)
	++++	0 (0)	0 (0)	29.46 (33)	14.14 (14)
Additional signal on 2L	None	98.41 (62)	75.49 (77)	9.82 (11)	15.15 (15)
	Sub-telomeric	0 (0)	6.86 (7)	89.3 (100)	78.78 (78)
	Proximal to 22A3	1.5 (1)	19.6 (20)	8.03 (9)	4.04 (4)
3L telomere	None	98.41 (62)	95.09 (97)	49.1 (55)	45.45 (45)
	One Band	1.58 (1)	4.90 (5)	50.89 (57)	54.54 (54)
Other	Ectopic bands on other chr	3.17 (2)	36.27 (37)	7.14 (8)	9.09 (9)
	X-staining normal	100 (63)	100 (102)	100 (112)	100 (99)
Total nuclei counted		**63**	**102**	**112**	**99**

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-MSL3	[45]	N/A
Rabbit polyclonal anti-MLE	[45]	N/A
Rabbit polyclonal anti-MSL2	[45]	N/A
Experimental Models: Organisms/Strains		
D. melanogaster y^1 w^{1118};*PBac{*y^+-attP-3B}VK00037	Bloomington Drosophila Stock Center	BDSC: 9752, Flybase ID: FBst000972
D. melanogaster y^1 *w*;P{CaryIP}su(Hw)attP6*	Bloomington Drosophila Stock Center	BDSC: 34767, Flybase ID: FBst0034767
D. melanogaster y^1 v^1; *P{*$y^{+t7.7}$*CaryP}*attP40	Bloomington Drosophila Stock Center	BDSC: 36304; Flybase ID: FBst0036304
D. melanogaster w;P{sqh-mCherry.M}3*	Bloomington Drosophila Stock Center	BDSC:59024, Flybase ID: FBst0059024
D. melanogaster P{sqh-GAL4}2	Bloomington Drosophila Stock Center	Flybase ID: FBrf0191737
D. melanogaster y^1 w^{67c23}; ;*P* {w^{+mC} $y^{+\ mDint2}$ EPgy2}haf^{EY08668}	Bloomington Drosophila Stock Center	BDSC: 17484 Flybase ID: FBst0017484
D. melanogaster T(2;Y)22A2	[30]	N/A
*D. melanogaster y*w**; P{w^{+mC} hp-1.6883F}	[21]	N/A
Recombinant DNA		
pUASTB	[44]	N/A
Software and Algorithms		
T-Coffee	[27]	N/A