siRNAs from an X-linked satellite repeat promote X-chromosome recognition in *Drosophila melanogaster*

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Highly differentiated sex chromosomes create a lethal imbalance in gene expression in one sex. To accommodate hemizygosity of the X chromosome in male fruit flies, expression of X-linked genes increases twofold. This is achieved by the male- specific lethal (MSL) complex, which modifies chromatin to increase expression. Mutations that disrupt the X localization of this complex decrease the expression of X-linked genes and reduce male survival. The mechanism that restricts the MSL complex to X chromatin is not understood. We recently reported that the siRNA pathway contributes to localization of the MSL complex, raising questions about the source of the siRNAs involved. The X-linked 1.688 g/cm^3 satellite related repeats (1.688^X repeats) are restricted to the X chromosome and produce small RNA, making them an attractive candidate. We tested RNA from these repeats for a role in dosage compensation and found that ectopic expression of single-stranded RNAs from 1.688^X repeats enhanced the male lethality of mutants with defective X recognition. In contrast, expression of double-stranded hairpin RNA from a 1.688^X repeat generated abundant siRNA and dramatically increased male survival. Consistent with improved survival, X localization of the MSL complex was largely restored in these males. The striking distribution of 1.688^X repeats, which are nearly exclusive to the X chromosome, suggests that these are *cis*-acting elements contributing to identification of X chromatin.

dosage compensation | siRNA | *roX* RNA | X chromosome recognition | epigenetics

Males and females of many species have an unequal number of X chromosomes, producing a potentially fatal imbalance in X-linked gene expression (1). The process by which balance is restored is called dosage compensation. In the male fruit fly, *Drosophila melanogaster,* the male-specific lethal (MSL) complex modifies the chromatin of X-linked genes to increase expression by twofold, equalizing expression between XX females and XY males (2). The long noncoding *roX* RNAs assemble with the MSL proteins to form the intact MSL complex. *roX* RNA is required for exclusive X-chromosome binding of the complex and for increased expression of X-linked genes (3, 4).

How the MSL complex selectively recognizes X chromatin is not fully understood, but an elegant model for X recognition proposes that the complex is first recruited to chromatin entry sites (CESs), then spreads into nearby active genes through a cotranscriptional mechanism (5). The CESs, defined by elevated affinity for the MSL proteins, are limited to the X chromosome (6). A 21-bp motif, termed the MSL recognition element (MRE), is enriched within the CES and binds CLAMP, a protein essential for MSL recruitment (7, 8); however, MREs are only modestly enriched on the X chromosome, and CLAMP binds autosomal MREs without recruiting the MSL complex. These observations indicate that additional factors must contribute to X identification.

The siRNA pathway contributes to X chromosome recognition during dosage compensation (9). This suggests that siRNA-producing sequences on the X chromosome might participate in the identification of X chromatin. The X-limited distribution of 1.688 g/cm^3 satellite-related repeats (1.688^X repeats) has prompted speculation that they function in dosage compensation, an idea supported by the remarkable enrichment of rapidly evolving repeats on the X chromosomes of related species (10–13). The *D. melanogaster* 1.688^X repeats are arranged in short tandem arrays and, unlike most satellite repeats, are associated with transcriptionally active regions (14). Many 1.688^X repeats are located within or flanking coding genes, and some are transcribed from both strands, suggesting the potential for small RNA production.

In this study, we investigated the role of long and short RNA from 1.688^X repeats in dosage compensation. Both forms of RNA are found in wild-type (WT) flies. Ectopic expression of long single-stranded RNA (ss RNA) and double-stranded (ds) hairpin RNA (hp RNA) from 1.688^X repeats influences dosage compensation in a sensitized genetic background, but in opposing fashions. Expression of single-stranded (ss) 1.688^X RNA lowers the survival of *roX1 roX2* males. In contrast, hp 1.688^X RNA is processed into abundant small RNAs, rescues *roX1 roX2* male survival, and significantly restores MSL localization to the X chromosome. We propose that siRNA from the 1.688^X repeats participates in dosage compensation by targeting small RNA effectors to similar sequences on the X chromosome. Thus, the 1.688^X repeats are candidates for the elusive *cis*-acting elements that distinguish X and autosomal chromatin.

Significance

Modulation of X-linked gene expression is essential in organisms with XX females and XY males. Various strategies for global regulation of X chromosomes have been proposed, but all require highly selective recognition of X chromatin. How this is achieved is not understood. The siRNA pathway contributes to X recognition in a well-studied *Drosophila* model. We now show that ectopic production of siRNA from a repetitive sequence that is limited to the X chromosome also promotes X recognition. Differential activities of X-linked repeats suggest a control region model, in which siRNA produced by a few repeats acts on widely distributed X-linked target sequences to promote selective recognition, and modification, of a single chromosome.

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Results

The 1.688^{X} Repeats Are Distributed Throughout Euchromatin of the X Chromosome. On average, clusters of 1.688^{X} repeats share 73% sequence identity (14). We performed in situ hybridization to polytene chromosomes with probes from clusters sharing 67% identity (Fig. 1). 1.688^{3C} is distal to *white* (*w*), and 1.688^{3F} is flanked by *roX1* and *echinus* (*ec*). Superscripts denote cytological position. As reported previously, 1.688^{X} probes hybridize to numerous sites that are distributed most densely around the middle of the X chromosome (Fig. 1 *A* and *B*) (10, 11). The relative strength of 1.688^{3C} and 1.688^{3F} signals differs at individual loci, emphasizing the diversity in this family of repeats (Fig. 1 *C* and *D*) (14). Only 1.688^{3C} probes hybridize to related repeats in pericentric X heterochromatin (arrows in Fig. 1 *A* and *B*).

RNA from 1.688^{X} Satellite Repeats Is Present in Flies. Quantitative RT-PCR (qRT-PCR) was used to examine transcription from 1.688^{3F}, 1.688^{3C}, and a cluster of repeats at 7F1 (1.688^{7F1}) in male larvae (Fig. S1). The specificity of primers was confirmed by amplification of genomic DNA from flies deleted for clusters at 3F and 7F (Fig. S1 *A* and *B*). 1.688^{3F} is transcribed from both strands, and thus is a potential source of siRNA (Fig. S1*D*). In agreement with this, 18- to 26-nt RNAs mapping to various 1.688^{X} repeats have been identified in embryos, larvae, adults, Kc167, and S2 cells (15, 16).

Long RNA from 1.688^{X} Repeats Reduces *roX1 roX2* Male Survival. To determine whether ss 1.688^{X} RNA influences dosage compensation, we generated males carrying the partial loss-of-function *roX1^{ex33} roX2*Δ chromosome and transgenes expressing ss RNA from the 1.688^{3C} and 1.688^{3F} repeats. Although *roX1^{ex33} roX2*Δ females are fully viable, male survival is only 20%. *roX1^{ex33} roX2*Δ males exhibit considerable mislocalization of the MSL proteins, making it a sensitive background in which to test factors influencing X recognition (9, 17). Survival of *roX1^{ex33} roX2*Δ males expressing ss 1.688^{3C} or 1.688^{3F} RNA was reduced by 40–70%, regardless of the strand expressed (Fig. 2*A*), whereas otherwise WT males expressing ss 1.688^{X} RNA were fully viable (99–130% eclosion; Table S1), demonstrating the need for a sensitized genetic background. Surprisingly, the effect on *roX1^{ex33} roX2*Δ males was neutralized when complementary RNA (cRNA) strands were expressed simultaneously (S,AS; Fig. 2*A*).

To determine whether complete base pairing is required, we tested recombinant chromosomes expressing partially complementary strands from 1.688^{3C} and 1.688^{3F}. Sense is defined with respect to the genomic scaffold. In this instance, sense strands from 1.688^{3C} and 1.688^{3F} are complementary. Although 1.688^{3C} and 1.688^{3F} share only 67% identity, coexpression of partially cRNA strands blocked the effect of ss RNA. We conclude that ss RNA from 1.688^{X} repeats interferes with dosage compensation, an effect neutralized by partial or full base pairing.

Double-Stranded hp RNA from 1.688^{3F} Repeats Rescues *roX1 roX2* Males. The transgenes expressing complementary ss RNA are widely separated on the chromosome, a condition that may limit hybridization. To produce high levels of ds RNA, we generated UAS$_{gal}$-driven transgenes producing hp RNA from repeats at 1.688^{1A}, 1.688^{3C}, and 1.688^{3F} (hp 1.688^{XX}). Ubiquitous expression of hp 1.688^{X} RNAs had no detectable effect on WT males (Table S1). We tested three independent insertions of each transgene using the severe *roX1^{SMC17A} roX2*Δ chromosome (< 1% adult male escapers; Fig. 2*B* and Fig. S2). Expression of hp 1.688^{1A} (89% identity to 1.688^{3F}) had little or no effect on male survival, and hp 1.688^{3C} had no effect. In contrast, hp 1.688^{3F} dramatically increased recovery of adult *roX1^{SMC17A} roX2* males to 25–30% (Fig. 2*B*). Using hp 1.688^{3F} insertion 12 as a reference, we tested various *roX1 roX2* chromosomes (Fig. 2*C* and Fig. S2). These chromosomes carried a complete deletion of *roX2* and either *roX1^{ex33}* (internal excision), *roX1^{ex6}* (deleted for major transcription start sites), or *roX1^{ex84A}* (lacking all start sites). We also tested *roX1^{VM18A}*, deleted for the 3′ end of *roX1*, 1.688^{3F} repeats, and part of *ec*. We found partial rescue of all *roX1 roX2* chromosomes, regardless of the *roX1* allele present. Rescue required a GAL4 driver (Fig. S3). Expression of hp RNA to the nonessential *white* (*w*) gene failed to rescue *roX1 roX2* males, but eye color was eliminated, confirming driver activity (Fig. 2*B*). These observations rule out insertional effects, nonspecific small RNA production, or GAL4 expression as the basis of male rescue.

In composite, the *roX1* alleles tested removed all *roX1* sequences, eliminating the possibility that specific *roX1* DNA or RNA sequences are essential for rescue. Rescue by hp 1.688^{3F} did not require the cognate X-linked sequence, given that survival of *roX1^{VM18A} roX2Δ* males, lacking 1.688^{3F} repeats, was increased from 2% to 27% on expression of hp 1.688^{3F} RNA. This was indistinguishable from the rescue achieved for chromosomes carrying the similarly severe *roX1^{ex6}* and *roX1^{ex84A}* mutations that retain the 1.688^{3F} repeats (Fig. 2*C* and Fig. S2). Male survival was increased by only 25–30%, regardless of the severity of the *roX1 roX2* chromosome tested. Thus, ectopic 1.688^{3F} hp RNA production ameliorates a dosage compensation defect, rather than overcoming it entirely. This is consistent with the central role of *roX* RNA in the MSL complex (3).

We sequenced small RNA from male larvae expressing hp 1.688^{3F} RNA to determine whether processing into small RNA occurred. No siRNA to the 1.688^{3F} region was detected in WT male larvae, consistent with low levels of small RNAs in animals from this stage (Fig. 3). Small RNAs from the 1.688^{3F} region have been detected from several stages in WT animals, however

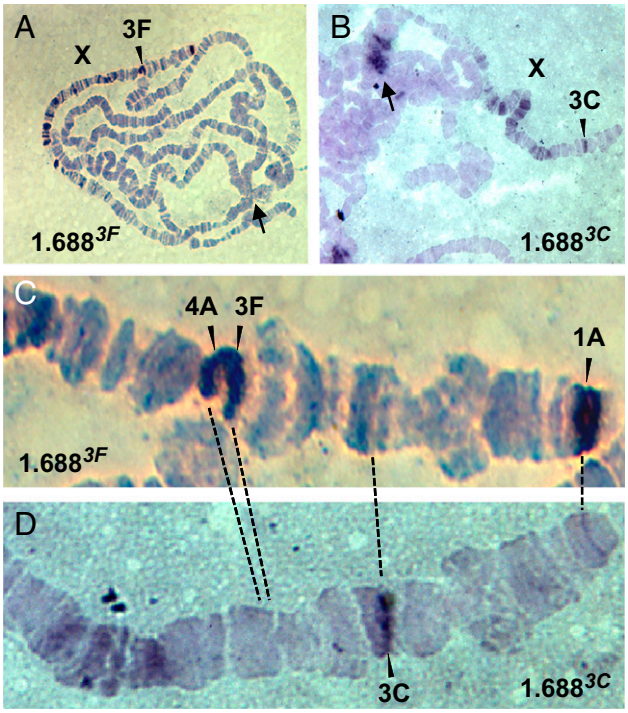

Fig. 1. 1.688^{X} repeats are enriched on the X chromosome. Probes to the 1.688^{3F} (*A* and *C*) and 1.688^{3C} (*B* and *D*) repeat clusters were hybridized to polytene preparations. Black arrowheads indicate cytological positions; arrows mark the chromocenter. Higher magnification of the distal X reveals hybridization exclusive to 1.688^{3F} (*C*) or 1.688^{3C} (*D*) probes. Hybridization is indicated by dark purple. DNA was counterstained with Giemsa.

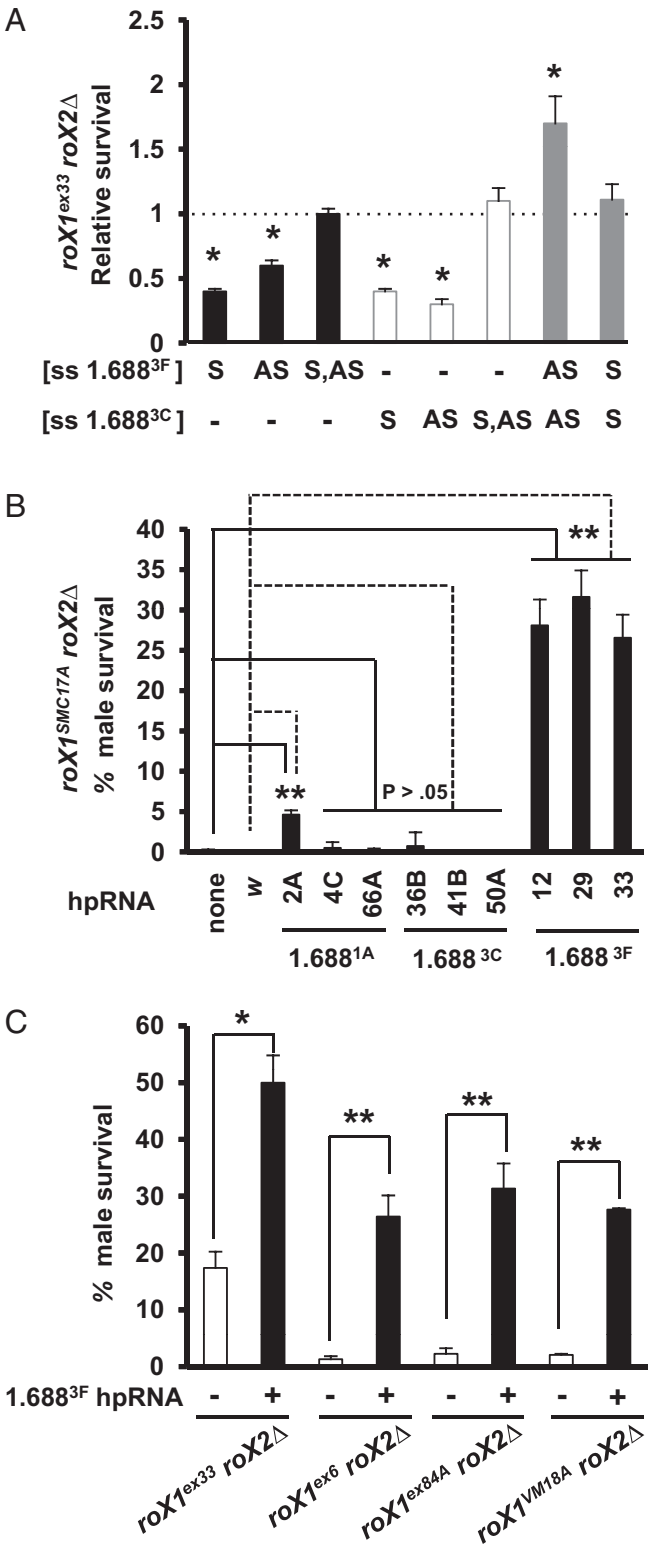

Fig. 2. RNA from 1.688^{X} repeats influences *roX1 roX2* male survival. (*A*) Expression of sense (S) or antisense (AS) RNA from 1.688^{3F} (black) and 1.688^{3C} (white) repeats reduces the survival of *roX1^{ex33} roX2*Δ males. The ratio of adult male escapers with the 1.688^{X} transgene to their brothers lacking the transgene is presented. Simultaneous expression of fully complementary RNAs neutralizes this effect (S, AS), as does expression of partially complementary strands from 1.688^{3F} and 1.688^{3C} (gray bars). Sense strands of 1.688^{3C} and 1.688^{3F} are complementary (sense is with respect to the genomic scaffold). Error bars represent SEM. (*B*) Expression of 1.688^{3F} hp RNA rescues *roX1^{SMC17A} roX2* males. The survival of *roX1^{SMC17A} roX2*Δ males was determined without (*Left*) or with expression (*Right*) of hp RNA from the *white* gene (*w*) or insertions producing hp 1.688^{1A} RNA, hp 1.688^{3C} RNA, and hp 1.688^{3F} RNA. (*C*) Expression of hp 1.688^{3F} RNA (insertion 12) rescues males carrying the *roX1^{ex33} roX2*Δ chromosome, as well as the severely affected *roX1^{ex6} roX2*Δ, *roX1^{84A} roX2*Δ and *roX1^{VM18A} roX2*Δ chromosomes. Fig. S2 provides a description of *roX1* mutations. Error bars represent SEM. *$P < 0.05$; **$P < 0.001$, Student *t* test.

(15, 16). Abundant short RNAs were found in male larvae expressing hp 1.688^{3F} RNA, and were strikingly similar in distribution to those seen in WT embryos (Fig. 3). Interestingly, individual peaks from hp 1.688^{3F} larvae were composed almost exclusively of reads with identity to a single strand (sense and antisense tracks; Fig. 3). Dicer2 (Dcr2), previously shown to play a role in dosage compensation, processes ds RNA into 21-bp siRNA (9, 18). More than 85% of the short RNAs from hp 1.688^{3F} RNA males were 21-nt long, consistent with Dcr2 processing (Fig. 3, *Inset*). As predicted by in situ hybridization, the 1.688^{3F} siRNAs share similarity with numerous 1.688^{X} repeats along the X chromosome, several examples of which are presented in Fig. S4. However, a comparison of 1.688^{1A} and 1.688^{3F} revealed numerous differences that overlap peaks of 1.688^{3F} siRNA accumulation (Fig. S5). How these differences prevent 1.688^{1A} hpRNA from rescuing *roX1 roX2* males is cur rently unknown.

One potential mode of action is that expression of hp 1.688^{3F} RNA enhances the accumulation of residual mutated *roX1* transcripts. Identity between *roX1* and 1.688^{3F} is limited to 11 bp of an AT-rich sequence that is deleted in *roX1^{VM18A}*, arguing against a mechanism requiring sequence identity (green bar in Fig. S2); however, 1.688^{3F} is immediately adjacent to *roX1,* and long transcripts spanning *roX1* and 1.688^{3F} have been annotated (19). These transcripts were not apparent on RNA blots, suggesting that they are rare or unstable (17). Significantly, we found that *roX1* accumulation was essentially unaltered in flies expressing hp 1.688^{3F} or ss RNA (Fig. S6 *A* and *C*). Given that expression of hp 1.688^{3F} RNA rescues *roX1 roX2* males deleted for all *roX1* transcription start sites (*roX1^{ex84}*) or lacking the entire 1.688^{3F} repeat cluster (*roX1^{VM18A}*), we conclude that rescue does not require *roX1* expression or transcripts that span *roX1* and 1.688^{3F}. The remarkably close association of two genetic elements that participate in dosage compensation is provocative, and we return to this topic later (*Discussion*).

hp 1.688^{3F} RNA Restores MSL2 Localization. The suppression of *roX1 roX2* male lethality by hp 1.688^{3F} RNA prompted an examination of MSL protein localization in these flies. A key member of the complex, male-specific lethal 2 (MSL2), is restricted to the X chromosome of WT males (Fig. 4*A*). Several studies support the idea that the *roX* genes are themselves *cis*-acting elements that recruit the MSL complex to flanking chromatin (20, 21); for example, *roX* transgenes recruit the MSL complex to autosomal insertion sites (6). To determine whether 1.688^{3F} transgenes share this property, we examined polytene preparations from otherwise WT males expressing hp 1.688^{3F} RNA. No MSL2 could be detected at the site of the transgene insertion (arrowhead in Fig. 4*B*), ruling out a direct role for 1.688^{3F} DNA sequences in recruitment of the MSLs. In addition, the AT-rich 1.688^{X} repeats share no detectable similarity to the GA-rich CESs that are believed to initially bind the MSL complex (7).

We then examined MSL2 levels and recruitment in *roX1 roX2* mutants expressing hp 1.688^{3F} RNA, and found that MSL2 levels were not increased by hp 1.688^{3F} expression (Fig. S6*B*). *roX1^{SMC17A} roX2*Δ males displayed negligible X-localization and prominent ectopic autosomal binding of the MSL proteins (Fig. 4*C*) (17). MSL2 still bound to autosomal sites in *roX1^{SMC17A} roX2*Δ males

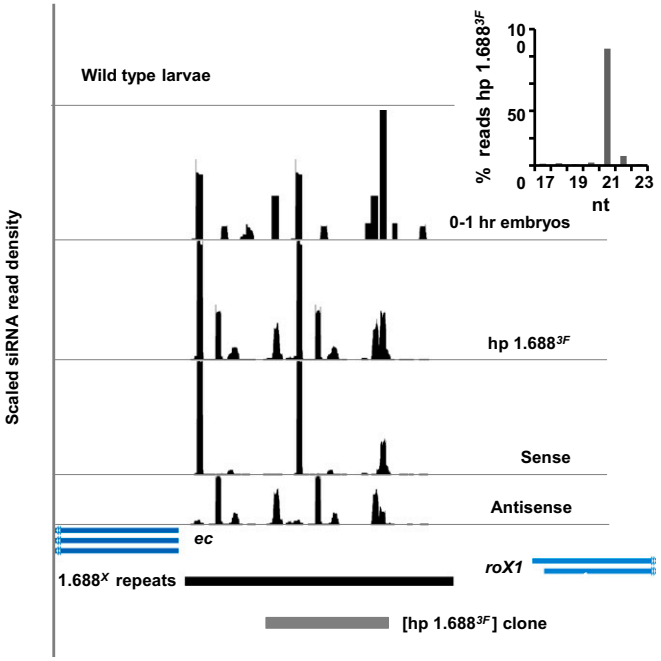

Fig. 3. 1.688^{3F} hp RNA is processed into small RNA. Small RNA profiles in WT male larvae (top track; no small RNA detected), embryos (second track), and male larvae expressing hp 1.688^{3F} RNA (bottom three tracks). Total siRNA reads from hp 1.688^{3F} larvae are shown, with sense (S) and antisense (AS) reads, with respect to the genomic scaffold depicted below. (*Inset*) Small RNAs derived from hp 1.688^{3F} RNA are predominantly 21 nt. The 3′ ends of *echinus* (*ec*) and *roX1* are in blue. The 1.688^{3F} repeat cluster (black bar) and the sequence used to generate hp 1.688^{3F} RNA (gray bar) are shown.

expressing hp 1.688^{3F} RNA, but more pronounced X chromosome binding was apparent as well (Fig. 4*D*). This analysis was complicated by the fact that the *roX1*^{SMC17A} *roX2*Δ males were sick, producing chromosome preparations of poor quality. To test hp 1.688^{3F} RNA in healthier animals, we generated females expressing MSL2 from the [H83 M2]6I transgene, leading to inappropriate formation of MSL complexes that bind to both X chromosomes (22). *roX1*^{SMC17A} *roX2*Δ; [H83 M2]6I/+ females produced good chromosome preparations and displayed autosomal MSL mislocalization equivalent to that of *roX1*^{SMC17A} *roX2*Δ males (Fig. 4*E*). Elevated MSL2 localization to the X chromosome was clearly apparent in *roX1*^{SMC17A} *roX2*Δ; [H83 M2]6I/+ females expressing hp 1.688^{3F} RNA, although autosomal binding was still observed (Fig. 4*F*). The number of nuclei exhibiting strong X chromosome staining was increased by 16-fold, and minor X chromosome staining was reduced by fourfold (Fig. 4*G*). We conclude that expression of hp 1.688^{3F} RNA dramatically improves X-localization but does not rescue all defects in *roX1 roX2* mutants.

The suppression of *roX1 roX2* male lethality by hp 1.688^{3F} RNA prompted examination of another dosage compensation mutant. *Males absent on first* (*mof*) encodes a member of the MSL complex that acetylates H4 on lysine 16 (H4AcK16), a modification that is enriched on the male X chromosome and required for increased X chromosome expression (23–25). The catalytically inactive *mof*^{1} mutant allows no adult male escapers (26). Despite this, late third instar *mof*^{1} males appear healthy, suggesting that a minor enhancement of dosage compensation might permit escapers. No adult males were recovered when hp 1.688^{3F} RNA was expressed in *mof*^{1} males, however. This finding is consistent with 1.688^{3F} siRNA participating in MSL recruitment, rather than modifying the activities of this complex.

Conservation of *roX1*-Adjacent Repeats in Other *Drosophila* Species. Enrichment of X chromosome-specific satellite repeats in other species prompted an examination of regions flanked by *roX1* and *ec* in related *Drosophila* species (13). An extensive array of 359-bp repeats sharing 69% identity to 1.688^{3F} is found in *D. sechellia* (Fig. S7). *D. simulans* and *D. erecta* have shorter, *roX1*-adjacent repeats that share 81% identity with each other, but have no similarity to 1.688^{3F} or *D. sechellia* repeats. Thus, it appears that the presence of tandem repeats adjacent to *roX1*, but not the repeat sequence itself, is conserved.

Discussion

The discovery that the siRNA pathway contributes to X-localization of the MSL proteins raised questions about the small RNAs involved and their mode of action. The 1.688^{X} repeats produce small RNA and are strikingly enriched on the X chromosome. The remarkable conservation of X chromosome-specific

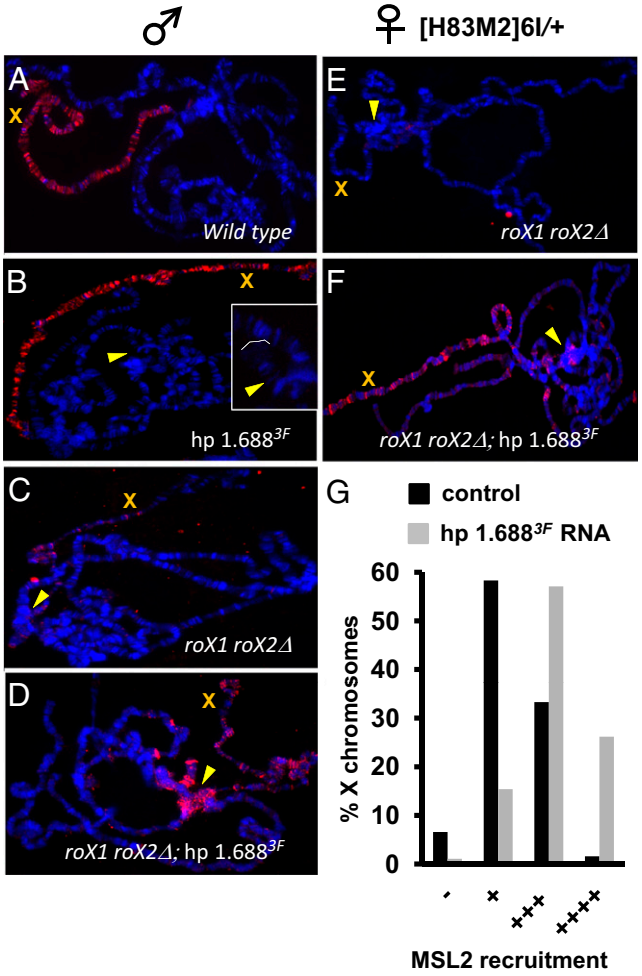

Fig. 4. 1.688^{3F} hp RNA promotes X chromosome localization of MSL2. MSL2 was immunolocalized in WT males (*A*), males expressing 1.688^{3F} hp RNA (*B*), *roX1*^{SMC17A} *roX2*Δ males (*C*), *roX1*^{SMC17A} *roX2*Δ males expressing 1.688^{3F} hp RNA (*D*), *roX1*^{SMC17A} *roX2*Δ; [h83M2]6I/+ females expressing MSL2 (*E*), and *roX1*^{SMC17A} *roX2*Δ; [h83M2]6I females expressing MSL2 and 1.688^{3F} hp RNA (*F*). (*G*) MSL2 recruitment to the X chromosome of *roX1*^{SMC17A} *roX2*Δ; [h83M2]6I/+ females (black, 60 nuclei from nine larvae) and *roX1*^{SMC17A} *roX2*Δ; [h83M2]6I females expressing MSL2 and 1.688^{3F} hp RNA (gray, 84 nuclei from 10 larvae). X chromosome recruitment: –, none. +, minor; +++, moderate; ++++, strong. Antibody to MSL2 was detected with Texas red (red), and DNA was counterstained with DAPI (blue). Arrowheads in *B* indicate the transgene insertion site at 61C7. Telomere 3L is delineated by a white line in the magnified *Inset* in *B*. Arrowheads in *C*–*F* indicate the chromocenter.

repeats also hints at function. Intriguingly, the recently evolved X chromosome of *Drosophila pseudoobscura* has acquired repeats from the ancestral X chromosome, suggesting coordination with the acquisition of dosage compensation (13). The present study demonstrates that ectopic production of 1.688^{3F} siRNA promotes X-localization of the MSL complex in *roX1 roX2* males, dramatically improving the survival of these flies. This is consistent with the signature defect of *roX1 roX2* mutants, the failure of X chromosome recognition.

Long and short RNAs from the 1.688^{X} repeats are detected in WT animals. However, ectopic expression of long and short 1.688^{3F} RNA modifies *roX1 roX2* male lethality in opposing fashions, prompting the question of what forms of RNA are normally biologically active in flies. There are numerous examples of epigenetic modifiers guided to chromatin by complementarity between nascent transcripts and small RNA (27–30). We speculate that chromatin at the 1.688^{X} repeats could be the target of a similar mechanism. If this were the case, then ss RNA from a highly expressed transgene would compete with nascent transcripts from endogenous 1.688^{X} loci, reducing recruitment to these sites. In support of this idea, the effect of ectopic ss 1.688^{X} RNA expression was neutralized by expression of complementary RNAs. We postulate that hybridization produces ds RNA unable to compete with nascent transcripts, but capable of being processed into small RNA. Although both of these processes may contribute to overcoming the negative effects of single-strand expression, the rescue of *roX1 roX2* males requires a high levels of siRNA generated by the hp 1.688^{3F} construct.

An intriguing feature of dosage compensation in flies is the involvement of small RNA in a process that culminates in elevated transcription, rather than silencing. Small RNAs typically destroy target RNA, or silence chromatin at cognate loci by recruiting epigenetic modifiers; for example, small RNAs processed from transcribed repeats direct heterochromatin formation in fission yeast and *Drosophila*, and Piwi plays a role in heterochromatic silencing in *Drosophila* (27, 31–33). Small RNA pathways also regulate euchromatic genes, as demonstrated by Ago2 and Dcr2 repression of heat shock-induced genes in *Drosophila* (34). A few examples of transcriptional up-regulation by small RNAs have been documented as well; these include Piwi activation of telomere-associated sequences in *Drosophila* and activation of specific genes in human cells transfected with cognate 21-nt RNAs (35, 36).

In contrast to these gene-specific examples, small RNAs from 1.688^{3F} contribute to a process that culminates in recruitment of the MSL complex and global up-regulation of an entire chromosome. How this occurs remains an intriguing question. The failure to detect MSL2 at a 1.688^{3F} transgene supports the idea that the 1.688^{X} repeats themselves do not recruit the MSL complex. Analyses of proteins interacting with the MSL complex have identified chromatin modifiers and DNA-binding proteins, but no components of the siRNA pathway (37, 38). Thus, the MSL complex is unlikely to be recruited directly through an siRNA-dependent mechanism.

If the 1.688^{X} repeats do not recruit the MSL complex, how do they promote X recognition? An attractive model is that 1.688^{X} repeats underlie an X chromosome-specific nuclear architecture modulated by siRNA (9, 13). In fact, 1.688^{X} repeats have been implicated in the organization of X chromatin (39). Small RNA pathways also have been linked to higher-order nuclear organization in flies; for example, Ago2 is involved in long-range contacts between insulators (40, 41). Interestingly, the *Drosophila* X chromosome assumes a male-specific conformation in which compensated loci are closer together in the male interphase nucleus (42). Interactions between the MSL complex and components of the nuclear pore also have been reported to promote MSL recruitment to the X chromosome (38, 43). We postulate that an X chromosome-specific organization, or subnuclear localization, facilitates MSL complex recruitment or spreading. This is consistent with the idea that both the CESs and 1.688^{X} repeats act in *cis* to promote X identification, but do so through different mechanisms.

Our findings raise questions about the differential activities of hp 1.688^{X} RNAs. Whereas hp 1.688^{3F} achieved remarkable rescue of every *roX1 roX2* chromosome tested, 1.688^{1A} and 1.688^{3C} hp RNAs were ineffective. A likely mode of action of 1.688^{X} siRNAs is modification of chromatin at cognate loci on the X chromosome, but it is possible that only a few of the 1.688^{X} repeats produce active siRNA. The striking similarity in small RNA profiles from WT embryos and larvae expressing hp 1.688^{3F} RNA reveals that processing into small RNA, or accumulation, is determined by RNA sequence. We are currently working to understand the genetic architecture of the 1.688^{X} repeat family with respect to dosage compensation. The strikingly different results obtained with repeats that share 89% identity demonstrate that the activity of 1.688^{X} siRNAs is determined by minor sequence variations.

An intriguing possibility is raised by the remarkably close localization of *roX1* and 1.688^{3F} on the X chromosome. Both *roX* genes overlap CESs, placing the 1.688^{3F} repeats near the *roX1* CES; however, close association between 1.688^{X} repeats and CESs is not the rule, and the *roX1* locus is the sole instance of a CES within 2 kb of a 1.688^{X} repeat. Furthermore, the distribution of 1.688^{X} repeats and CESs are qualitatively different; whereas CESs are distributed rather evenly along the length of the X chromosome, the 1.688^{X} repeats are distributed more densely at the middle of the chromosome (7).

The adjacent situation of *roX1* and the 1.688^{3F} repeats suggests a spatial coordination of X recognition. *roX1*, first expressed in blastoderm embryos, normally functions during the initiation of dosage compensation at 3 h after egg laying (AEL) (44). *roX1* mutants delay compensation until *roX2* is expressed at 6 h AEL. MSL complexes are thought to assemble on nascent *roX* RNAs before moving to CESs and transcribed genes (5). If 1.688^{X} repeats underlie an X chromosome-specific nuclear organization—for example, by anchoring chromosome loops—then the situation of *roX1* adjacent to 1.688^{3F} would enable newly assembled MSL complexes to access distant sites. The spatial proximity of *roX1* and 1.688^{3F} thus could coordinate two distinct pathways that cooperate during the initiation of X chromosome compensation, an idea supported by the conservation of tandem repeats adjacent to *roX1*. Therefore, the 3F region, containing *roX1* and 1.688^{3F}, might act as a "locus control region" for initiation of X chromosome recognition. The abundance of small 1.688^{X} RNAs in WT embryos but their rarity in older stages also suggests a role during the initiation of dosage compensation. Analysis of siRNA in hp 1.688^{3F} animals relied on third instar larvae that could be sorted by sex and genotype; however, the critical time for siRNA action in X recognition likely is much earlier.

The genomes of higher eukaryotes are rich in repetitive elements, but few functions have been attributed to these sequences. Our studies demonstrate that small RNAs from the 1.688^{3F} repeat promote dosage compensation, a finding consistent with the role of the siRNA pathway in this process. The involvement of this family of repeats in a well-studied epigenetic process provides an unusual opportunity to explore the molecular mechanisms involved. The remarkable distribution of the 1.688^{X} repeats, which are essentially limited to the X chromosome, makes them strong candidates for *cis*-acting elements that uniquely identify X chromatin.

Materials and Methods

qRT-PCR. Quantification of 1.688^{X} transcript accumulation is described in *SI Materials and Methods*.

Genetics and Immunodetection. Unless noted otherwise, mutations were as described by Lindsley and Zimm (45). Descriptions of *dcr2*^{L811fsX}, *Sco* (*sna*^{sco}), and *R*^{1} can be found at www.flybase.org. hp RNA expression was driven by Gal4-*tub* (BDSC # 5140). *roX1* mutations and a viable deletion of *roX2* (*roX2*Δ) have been described previously (9, 17). Creation of 1.688^{X} transgenics is described in *SI Materials and Methods*. Matings to measure male survival and to generate flies for immunodetection of MSL2 are described in *SI Materials and Methods*. Immunodetection of MSL2 on polytene chromosome preparations was performed as described previously (6).

In Situ Hybridization. In situ hybridization to salivary gland polytene chromosomes was performed as described previously (46). Chromosomes were hybridized to DIG-11-UTP–labeled (Roche) 1.688^{3F} (1:20) and 1.688^{3C} (1:10) probes. Slides were washed, blocked with 10 μg/mL BSA, and incubated with anti-digitoxigenin antibody conjugated to alkaline phosphatase (1:200; Roche). After color development, DNA was counterstained with Giemsa (Sigma-Aldrich).

RNA Preparation, Small RNA Sequencing, and Analysis. All strains were constructed with sex chromosomes from a *y*^{1}*w*^{1118} laboratory reference strain. Total RNA was isolated from two replicates of 3rd instar larvae by homogenization in Qiazol (Qiagen) using a Tissue Tearor (BioSpec Products). RNA was fractionated into small RNA (<200 nt) and large RNA using the miRNeasy Kit (Qiagen). RNA quality was assessed on large RNA fractions after cleanup (RNeasy MinElute Cleanup Kit; Qiagen). Details of sequencing and analysis are provided in *SI Materials and Methods*.

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