Autoregulation of the *Drosophila* Noncoding roX1 RNA Gene

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Abstract

Most genes along the male single X chromosome in *Drosophila* are hypertranscribed about two-fold relative to each of the two female X chromosomes. This is accomplished by the MSL (male-specific lethal) complex that acetylates histone H4 at lysine 16. The MSL complex contains two large noncoding RNAs, roX1 (*RNA on X*) and roX2, that help target chromatin modifying enzymes to the X. The roX RNAs are functionally redundant but differ in size, sequence, and transcriptional control. We wanted to find out how roX1 production is regulated. Ectopic DC can be induced in wild-type (*roX1*−/− *roX2*−/−) females if we provide a heterologous source of MSL2. However, in the absence of roX2, we found that roX1 expression failed to come on reliably. Using an *in situ* hybridization probe that is specific only to endogenous roX1, we found that expression was restored if we introduced either roX2 or a truncated but functional version of roX1. This shows that pre-existing roX RNA is required to positively autoregulate roX1 expression. We also observed massive cis spreading of the MSL complex from the site of roX1 transcription at its endogenous location on the X chromosome. We propose that retention of newly assembled MSL complex around the roX gene is needed to drive sustained transcription and that spreading into flanking chromatin contributes to the X chromosome targeting specificity. Finally, we found that the gene encoding the key male-limited protein subunit, msl2, is transcribed predominantly during DNA replication. This suggests that new MSL complex is made as the chromatin template doubles. We offer a model describing how the production of roX1 and msl2, two key components of the MSL complex, are coordinated to meet the dosage compensation demands of the male cell.

Introduction

Some long noncoding RNAs have the ability to recruit chromatin modifying enzymes to specific genes thereby controlling their expression [1]. Other noncoding RNAs behave as transcriptional enhancers to flanking protein coding genes [2]. The two roX RNAs that participate in dosage compensation of the single male X chromosome in *Drosophila* are some of the best characterized examples of noncoding RNAs that target chromatin remodeling enzymes to large domains [3]. The roX RNAs are enriched in nuclear speckled bodies and move from nucleus to cytoplasm and back [4]. They also form secondary structures [13,14]. Neither roX RNA is maternally deposited in eggs. Zygotic transcription of roX RNA occurs in both male and female embryos beginning at blastoderm [15]. Females lose roX RNA midway through embryogenesis, but males maintain expression through adulthood. By contrast, roX2 RNA first appears a few hours after roX1 but only in male embryos [16]. Despite the vast differences in size, sequence, and regulation, the two roX RNAs are functionally redundant [17].

Little is known about how production of the roX RNAs and MSL protein subunits are coordinated. Unusual *cis* spreading behavior of MSL complex from sites of autosomal roX transgene has been attributed to cotranscriptional assembly of free MSL subunits onto growing nascent roX transcripts [13,18,19], although direct biochemical evidence is lacking. Most MSL protein subunits are made in both males and females, except for MSL2 which is translationally repressed in females by the action of SXL [20,21]. MSL2 is a RING finger protein that binds DNA in a sequence independent manner through a second cysteine-rich motif [22]. The H83M2 transgene removes the 5′ and 3′ UTRs containing SXL binding sites from msl2 mRNA and has been used extensively to drive ectopic MSL2 expression in females [5,11,15,17,23–29]. Forcing females to make MSL2 using the H83M2 transgene induces production of roX RNAs, resulting in ectopic dosage
compensation that is toxic to females [15,30]. These observations led to the idea that MSL2 protein alone, or acting with the other MSL proteins drives transcription of roX1 RNA [31–33].

We reexamined the question of how MSL proteins regulate transcription of the roX1 gene using flies missing the roX2 locus. Deleting roX2 allowed us to study expression of the wild type endogenous roX1 gene without the confounding effects of a second functionally redundant RNA species. In this way we found an RNA-dependent autoregulatory loop controlling roX1 expression. We propose that the early burst of roX1 transcription at blastoderm initiates this cycle. Furthermore, production of the key male-limited MSL2 protein subunit is not only regulated at the translational level as has been extensively documented, but we find msl2 transcription is associated with DNA replication. We propose a model where pre-existing msl2 RNA, assembled in mature MSL complexes, drives bursts of roX1 transcription during S phase when its chromatin target is doubling.

Results

roX1 transcription is dependent on roX2 RNA

Male embryos normally establish dosage compensation by the onset of gastrulation [16,34]. Ectopic expression of MSL2 in females leads to roX1 transcription and dosage compensation [15]. We asked whether dosage compensation can only be initiated during early embryogenesis, or could it be artificially started later during larval development. To achieve that, we used an inducible Flp-out system [35] to create clones of GAL4 expressing cells on day 4 AEL (after egg laying) that in turn drove expression of UAS-GFP and UAS-MSL2 in female larvae (Figure 1A and Methods). The purpose of this was to assay females in which the early burst of roX1 RNA had decayed away leaving cells devoid of any roX1 RNA. When no clones were induced, we never observed GFP or MSL painting in any cell showing that expression was tightly blocked prior to induction (data not shown). Third instar larvae in which late GAL4+ clones were induced displayed overlapping expression of GFP and MSL2 (Figure 1B–1B'). More importantly, MSL2 appeared as subnuclear punctate staining in imaginal disc cells suggesting that it was concentrated on the X chromosome (Figure 1B'). MSL2 immunostaining of polytene squashes confirmed binding along the X (Figure 1C). Unfortunately, we

were unable to perform GFP immunostaining and reliable roX1 FISH (fluorescence in situ hybridization) in the same glands as the proteinase K treatment necessary to expose roX RNA often destroyed protein epitopes. We took comparable salivary glands from GFP positive larvae (Figure 1D) and processed them for roX1 FISH. The results show that roX1 transcription was successfully induced (Figure 1E–1F), consistent with previous reports that MSL2 drives roX1 transcription [15], and confirms an earlier report that dosage compensation can be initiated long after it normally occurs [13]. Figure 1G shows a wild type male X for comparison.

We repeated the experiment in roX1<sup>+</sup> roX2<sup>-</sup> females (Figure 1H). While GFP+ clones were recovered at similar frequencies indicating successful MSL2 induction (Figure 1I, 1K), no MSL2 accumulation was observed in imaginal discs (Figure 1I') or on polytene chromosomes (Figure 1J). We will later demonstrate that the failure to detect MSL2 is due to reduced protein stability in the absence of roX RNA. More importantly, we also could not detect roX1 expression in any cell. Absence of roX1 RNA might be attributed to poor RNA stability or transcription failure. We favor the latter since even minute amounts of roX1 RNA transcription can be readily detected when MSL complex accumulates over the roX1 gene [13]. Moreover, we could not detect nascent transcripts from the roX1 locus in these animals although such nascent roX1 transcripts were easily detected in other genotypes (Figure 1L–1L'). This argues that late MSL2 readily switches on roX2, it is not sufficient alone or with the other MSL subunits to drive expression of the roX1 gene when roX2 is absent. Without any roX RNA, cells rapidly destroy the ectopic MSL2 as well.

To further test our hypothesis that late expression of the endogenous roX1 locus depends on roX2 RNA, we returned to wildtype (roX1<sup>+</sup> roX2<sup>+</sup>) females to perform RNA in situ hybridization for both RNAs in the same nuclei. Late induction of MSL2 results in roX2 RNA painting the length of X chromosomes in many cells (Figure 2A'). By contrast, roX1 painting over the full X chromosome was seen in only a small minority of nuclei (Figure 2A). A much more common pattern was roX2 over the entire X while roX1 expression confined to either several Mbp (Figure 2B–2B') around or just at the endogenous roX1 locus at polytene band 3F (Figure 2C–2C'). This suggests that roX2 expression reliably follows MSL2 induction, but roX1 expression lags. Delayed roX1 expression might be explained if fully functional MSL complex containing roX2 RNA must first assemble before transcription of roX1 can occur. We conclude that roX2 RNA, presumably packaged in mature MSL complexes, is necessary to initiate transcription from the endogenous roX1 gene late in development.

Autoregulation of roX1 expression

Finding unusually late activation of the roX1 gene required preexisting roX2 RNA, we wondered if roX1 RNA could also perform the same role leading to a positive autoregulatory loop. To answer this question, we used a fly stock displaying an unusual mosaic pattern of dosage compensation.

The hH83M2 transgene makes MSL2 constitutively using the hsp<sub>83</sub> promoter [30]. It lacks the regulatory 5' and 3' msl2 UTRs and drives ectopic dosage compensation in 100% of female cells. Females carrying a roX1 deletion also showed MSL X chromosome painting utilizing roX2 RNA in all nuclei (data not shown). However, an entirely different result was obtained in hH83M2 females missing only roX2. Roughly half the nuclei adopted a fully male-like pattern of dosage compensation utilizing roX1 RNA, while the other half lacked dosage compensation (Figure 3A and personal communication Art Alekseyenko). The X chromosomes
of these negative cells showed very weak staining for MSL2, similar to the situation found in roX1 roX2 double mutant animals [17]. Males also exhibited a similar mosaic phenotype when their only source of MSL2 was the H83M2 transgene demonstrating that failed dosage compensation cannot be due to SXL or some other female factor (Figure 3B). It is unclear how two adjacent cells that are genetically identical containing a full set of MSL subunits adopt opposite dosage compensation fates. However, this fortuitous observation allowed us to test whether failure to activate the endogenous roX1 gene might explain lack of dosage compensation in some cells.

In order to test whether pre-existing roX1 RNA is necessary to drive continued transcription of the endogenous roX1 gene, we used a 1.2 kb roX1 minigene, H83roX1-D39, that is able to form partially active MSL complexes (Figure 3C) [13]. We designed a probe that recognizes only the internal sequence of the endogenous roX1 RNA missing from H83roX1-D39. In this way, we could selectively visualize the expression of only the endogenous roX1 RNA in

![Image](image-url)

Figure 1. MSL proteins alone cannot drive roX1 expression late in development. A) 4 day old larvae were heatshocked to induce expression of Fip, resulting in the removal of the blocking sequence from GAL4 and subsequent expression of both MSL2 and GFP. MSL2 is expected to initiate roX transcription and MSL complex assembly. (B) GFP+ clones mark imaginal disc cells that have successfully removed the blocking sequences from GAL4 (B’–B”). Induction of MSL2 results in punctate subnuclear foci in imaginal disc cells. (C) MSL2 immunostaining of polytene chromosome shows late MSL2 paints the entire X chromosome. (D) Whole salivary gland showing GFP induced in some cells. (E–E’) roX1 FISH of whole mount of similar GFP+ salivary glands or (F) polytene squashes shows successful induction of roX1 expression in a subset of cells. (G) roX1 FISH of wildtype males (H) The same experiment was repeated in roX1 roX2− larvae. However, in the absence of roX2, MSL2 fails to drive roX1 expression. (I) Despite the presence of GFP+ (late MSL2 expressing) cells, MSL2 is not detectable over the X in (I’–I”) imaginal disc cells or (J) polytene chromosomes. (K) Whole salivary gland showing successful GFP expression in roX1 roX2− larvae. (L–L”) Expression of roX1 is never observed painting the X or as nascent transcripts at band 3F in separately processed GFP+ glands or on (M) polytene squashes.

doi:10.1371/journal.pgen.1002564.g001

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Figure 2. Late induction of roX1 expression requires roX2 RNA. In nuclei where dosage compensation was successfully turned on after late msl2 induction, extensive roX2 was observed painting the entire X chromosome. (A) However, only 1% of the chromosomes showed extensive roX1 painting. 34% and 59% of chromosomes showed roX1 expression confined to several Mbp around (B) or just at the endogenous roX1 locus (C), respectively. The remaining chromosomes (6%) had no roX1 expression despite the presence of roX2 (data not shown). roX1 and roX2 were detected by biotin (green, A–C) and digoxigenin (red, A’–C’) labeled antisense riboprobes, respectively. The merged figure is shown in A’’–C’’. White arrows denote the endogenous roX1 locus at band 3F.

doi:10.1371/journal.pgen.1002564.g002
animals also making the shorter transgenic \( H83roX1-D39 \) RNA (Figure 3D, 3H and Figure S1C). The \( roX1^{-} \); \( roX2^{-} \) females (\( roX1^{-} \) and \( roX2^{-} \)) displayed the same mosaic \( roX1^{-} \) pattern (Figure 3F) typically seen in polytene squashes (Figure 3A and 3B). When we introduced \( H83roX1-D39 \) into these \( roX2^{-} \) \( H83M2 \) females, the expression of full-length \( roX1^{-} \) RNA made from the endogenous locus was restored to all cells (Figure 3G and Figure S1B, lane 3). In addition, while the distribution of \( roX1^{-} \) RNA was often limited to a single band or several Mbp around 3F in nuclei from the mosaic \( H83M2 \) females (Figure S1E–S1F), the endogenous, full-length \( roX1^{-} \) RNA coated the entire length of the X chromosome in nearly all cells when the constitutively expressed \( H83roX1-D39 \) transgene was also present, (Figure S1G). The \( H83roX1-D39 \) transgene was ineffective in \( H83M2 \) females (Figure S1B, lane 4), perhaps because females have two X chromosomes, depressed MSL1 expression [36] and so require more stimulatory activity than \( H83roX1-D39 \) can supply.

We conclude that the unexpected mosaic pattern found in \( roX1^{-} \) \( H83M2 \) animals results from a failure of the wild-type \( roX1^{-} \) gene to respond to MSL proteins. Providing a constitutive source of \( roX1^{-} \) RNA is sufficient to reliably drive transcription of the endogenous wild-type \( roX1^{-} \) gene. Taken together, these results support an autoregulatory model where new transcription of the wild-type \( roX1^{-} \) gene requires pre-existing RNA, either \( roX1^{-} \) or \( roX2^{-} \), in addition to MSL proteins. We postulate that the early MSL-independent burst of \( roX1^{-} \) RNA made at blastoderm normally assembles the first MSL complexes needed to set up the future maintenance of \( roX1^{-} \) transcription in adult males.

**Figure 3.** \( roX1^{-} \) RNA is needed to sustain endogenous \( roX1^{-} \) transcription in males. X chromosomes from neighboring cells display a mosaic pattern in which the MSL complex either succeeded (arrowhead) or failed (arrow) to paint the X from \( roX2^{-} \); \( msl2^{-} \); \( H83M2 \) female (A) and male (B) salivary glands. (C) Endogenous \( roX1^{-} \) and \( H83roX1-D39 \) transcripts (Orange) and antisense riboprobe recognizing only full length \( roX1^{-} \) (green). Whole mount \( roX1^{-} \) FISH using the internal probe on salivary glands from (D) wild type male, (E) wild type female, (F) \( roX1^{-} \) \( roX2^{-} \) \( /Y; \ msl2^{-} ; \ H83M2 \) mosaic female, (G) \( roX1^{-} \) \( roX2^{-} \) \( /Y; \ msl2^{-} ; \ H83M2 \) \( H83roX1-D39/+ \) male, (H) \( roX1^{-} \) \( roX2^{-} \) \( /Y; msl2^{-} ; H83M2 \) \( H83roX1-D39/+ \) male. The X chromosomes in G are fully painted in all cells with MSL complex relying upon \( roX1^{-} \)-\( D39 \) RNA (Figure S1D), but the truncated \( roX1^{-} \) RNA is not recognized by the internal probe. doi:10.1371/journal.pgen.1002564.g003
The mosaic expression of roX1 is not limited to the polytene chromosomes

The mosaic pattern of dosage compensation described above has been reported before. Certain hypomorphic alleles of Sxl unreliably initiate sex determination and so contain a mixture of XXAA cells that either correctly adopt a female fate repressing dosage compensation, or mistakenly choose a male fate and paint their X chromosomes with MSL complex [37]. The mechanism underlying the mosaic pattern of dosage compensation found in roX2 H83M2 animals studied here must be different, and we set out to understand its basis.

We first tested if the mosaic pattern was a peculiarity of polytene cells or a general feature in most tissues of these animals. When we performed MSL2 immunostaining on diploid imaginal discs, MSL2 protein decorated a subnuclear domain presumed to be the X chromosome in all the imaginal disc cells when both roX RNAs were present in female cells carrying the H83M2 transgene (Figure 4A). Again, roX1 roX2 animals also had no trouble establishing dosage compensation in all the cells (data not shown). However, when roX2 was deleted, only a fraction of the cells displayed dosage compensation utilizing roX1 RNA (Figure 4B–4B′), similar to the spotty pattern observed in salivary glands (Figure 3A, 3B and 3F). When both roX genes were deleted, no MSL2 staining was detected (Figure 4C–4C′). We had expected that removing roX RNA would produce a diffuse nuclear cloud of MSL2 protein unable to bind to the X chromosome. Either diffuse MSL2 protein stains too weakly to detect with our antibodies, or MSL2 protein is unstable without roX RNA. The latter is likely to be the case since MSL2 was previously shown to be unstable when not packaged into MSL complexes [29]. To directly test this interpretation, we incubated the imaginal discs and salivary glands with MG132, a proteasome inhibitor, before performing MSL2 immunostaining. Strong nuclear MSL2 staining reappeared (Figure 4D–4D′) in the imaginal disc, showing that MSL2 is synthesized in the absence of roX RNA, but fails to accumulate due to rapid turnover. MSL2 protein stabilized by MG132 showed a dramatically different staining pattern covering all polytene chromosomes, rather than being restricted to the X (Figure S2). This shows that free MSL2 subunits have a general affinity for all chromatin in agreement with earlier biochemical work [22]. Apparently flies have evolved a mechanism to efficiently destroy any MSL2 subunits that fail to assemble into complexes with roX RNA. A somewhat similar situation was reported for the MSL1 subunit. Massively over expressed MSL1 transiently paints all chromosome arms but is quickly lost [36].

Our data suggest that many cells lacking roX2 RNA were unable to carry out dosage compensation because the remaining wild type roX1 gene failed to switch on. If true, this should affect the viability of the females relying entirely on H83M2. (A–A′) Males and females relying entirely on H83M2 showed subnuclear MSL2 staining in all wing imaginal disc cells when both roX1 and roX2 RNAs were present. (B–B′) Many cells lacked dosage compensation if roX1′ was the only source of roX RNA. (C–C′) MSL2 did not accumulate over the X when both roX1 and roX2 were absent. (D–D′) Nuclear staining of MSL2 was easily detected in the absence of roX RNA after treatment with MG132, a proteasome inhibitor. doi:10.1371/journal.pgen.1002564.g004
of whole animals. As previously reported, ectopic MSL2 produced by \textit{H83M2} is toxic to females (Figure 5A) [30] and toxicity requires \textit{roX} RNA (Figure 5B) [17]. We found that \textit{roX2} alone was almost equally toxic to females as when both RNAs were present (compare Figure 5A–5C). The surviving adults produced very few eggs and were sterile. However, \textit{roX1} alone was much less toxic to females (Figure 5D) as would be expected if many cells lacked dosage compensation and thus escaped the toxic effects of MSL2 production. These surviving females were fertile. We conclude that the mosaic dosage compensation pattern is not limited to polytene tissues, but instead reflects a widespread failure of MSL2 protein made by the constitutive \textit{H83M2} transgene to activate the endogenous \textit{roX1} gene.

**\textbf{H83M2 does not turn on the expression of} \textit{roX1} \textbf{reliably}**

Males that lack \textit{roX2}, but are in all other ways wild type, have normal dosage compensation in all cells utilizing the remaining wild type \textit{roX1} gene [17]. Finding spotty \textit{roX1} activation only in animals relying on \textit{H83M2} prompted us to examine why the transgene did not behave like the endogenous \textit{roX2} locus in only this unusual genetic background. MSL2 made from the endogenous locus must activate \textit{roX1} RNA production more effectively than transgenic MSL2. This was shown by introducing single wild type allele of endogenous \textit{msl2} to \textit{roX2}; \textit{H83M2} males and observing dosage compensation in all cells (Figure S3G and S3I lane 5, Figure S4E and S4G lane 5). Next, we turned to the \textit{NOPU-M2} transgene that also escapes SXL repression in females, but differs from \textit{H83M2} by using the endogenous \textit{msl2} promoter [21]. \textit{NOPU-M2} is not toxic to females because it makes less MSL2 compared to \textit{H83M2}. However, addition of the \textit{NOPU-M2} transgene to \textit{roX2} mutant females makes them sensitive to the toxic effects of \textit{H83M2} (Figure 5E). Females carrying both \textit{msl2} transgenes also drive activation of \textit{roX1} expression in all cells (Figure S3H and S3I lane 6, Figure S4F and G lane 6). This is unlikely to be a simple additive effect of two pools of MSL2 protein since the \textit{H83M2} transgene itself was fully capable of driving \textit{roX1} expression in 100% of the cells when \textit{roX2} was present (Figure S3C–S3D, and S3I lane 1–2 and Figure S4A–S4B and S4G lane 1–2). We propose that \textit{H83M2} differs from the endogenous \textit{msl2} gene in some way needed to reliably drive the \textit{roX1} autoregulatory loop.

The mosaic pattern might arise from the \textit{H83M2} transgene suffering from positional effect so MSL2 protein was not made in some cells. This is not the case since MSL2 expression can be clearly observed in all cells when both \textit{roX} RNAs are present (Figure 4A). A second possibility is that \textit{H83M2} initiates expression too late in embryogenesis to capture the early MSL-independent \textit{roX1} transcripts we postulate are needed to begin the autoregulatory loop. We performed both MSL2 immunostaining and \textit{roX1} FISH on \textit{roX2}, \textit{msl2}, \textit{H83M2} embryos from 2 h to 20 h AEL. These embryos showed \textit{H83M2} expression and subnuclear punctate \textit{roX1} accumulation in essentially all cells at early gastrulation and remained on through at least until 20 hrs AEL when cuticle formation prevents reliable staining of internal tissues (Figure S5 and Figure S6). These results suggest that \textit{H83M2} does come on during early embryogenesis and is effective in driving \textit{roX1} transcription during the rapid cell divisions of embryonic development. However, many cells subsequently lose \textit{roX1} transcription, and thus dosage compensation, later during larval stages.

**Cell cycle regulation of \textit{msl2} expression**

The MSL complex associates with many hundreds of actively transcribed genes along the male \textit{X}. We considered the possibility that like core histone proteins, the MSL complex might need to abruptly increase its abundance following DNA replication. We set out to test if the transcription of \textit{msl2} might be coupled to S phase.

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**Figure 5.** \textit{roX2} mutant females escape the toxic effects of \textit{H83M2}. Adult females eclosing each day were counted from a population of eggs laid on day one. White bars show non-transgenic females without ectopic MSL2 (except E), and black bars show females carrying the \textit{H83M2} transgene. Each experiment varied the \textit{roX} genotype. (A) \textit{roX1}\(^{-}\) \textit{roX2}\(^{+}\), (B) \textit{no roX}, (C) \textit{roX2}\(^{-}\) only, (D) \textit{roX1}\(^{-}\) only, (E) \textit{roX1}\(^{-}\) only. Cumulative viability of \textit{H83M2} females is shown as a percentage of their non-transgenic sisters. doi:10.1371/journal.pgen.1002564.g005
We first used *in situ* hybridization to directly visualize actively growing msl2 transcripts on polytene chromosomes. Most salivary gland cells have completed their endoreplication cycles at the wandering larval stage used in all our experiments, but a few cells are still actively replicating. When males carrying both the wild type endogenous msl2 gene and the *H83M2* transgene were treated with antiense msl2 riboprobes, all nuclei showed strong hybridization to a cloud of msl2 RNA over the *H83M2* transgene inserted at 87A (Figure 6A). By contrast, a weaker hybridization signal was observed over the endogenous msl2 gene at 23F in only about 20% of the nuclei. Most nuclei lacked detectable msl2 transcripts at the endogenous locus. We tested whether the *H83M2* transgene might somehow inhibit expression of endogenous msl2 gene by testing nontransgenic, wild type males. We found that again, only about 20% of male cells actively transcribed msl2 (data not shown). This shows that while wild type males have abundant MSL2 protein painting the X chromosome in all cells, few cells are making new msl2 mRNA.

To test if the sporadic transcription of endogenous msl2 might coincide with the replication phase of the cell cycle, we briefly incubated dissected salivary glands in EdU to label newly replicated DNA, fixed the glands, and processed them for msl2 FISH. We found a strong bias (Fisher’s exact test *p*<0.0001) for replicating cells to also be transcribing msl2, but the overlap was not perfect (Figure 6A). For comparison we also examined histone *H2A* transcription, but even outside S phase every nucleus contained an intense hybridization signal over the endogenous *histone* gene cluster at polytene band 39DE (Figure S7). Only a portion of histone mRNA regulation occurs at transcription initiation. Most regulation is posttranscriptional [38,39].

To directly test whether the sporadic msl2 transcription pattern is associated with S phase, we treated our salivary glands with 1 mM of hydroxyurea (HU) to inhibit replication. EdU incorporation was completely blocked (Figure 6B) but more significantly, transcripts were specifically lost only from the endogenous msl2 locus at 23F while msl2 transcripts made from the *H83M2* transgene at 87A remained strong (Figure 6B, 6B"). Likewise, HU treatment had no effect on bulk nascent transcripts as shown by strong incorporation of BrUTP on all chromosome arms (Figure 6C). These results show that msl2 transcription normally occurs around S phase and is blocked by replication inhibitors. By contrast, *H83M2* does not show any cell cycle regulation. This difference might impact msl2 expression.

To test whether the replication associated msl2 transcription might be limited to only polytene cells with unusual cell cycles, we examined mitotically dividing diploid S2 cell in culture. Actively growing S2 cells were sorted into G1, S, and G2 (Figure 6G) via FACS (Fluorescence Activated Cell Sorting). RNA from these populations was assayed for msl2, histone *H2A*, and *PKA* transcripts by qPCR. As previously reported [38], histone RNA accumulates during S phase but is low in G1 and G2 (Figure 6E). Importantly, msl2 transcripts are most abundant during S while lowest during G2 phase (Figure 6F). These results show that msl2 RNA accumulation and synthesis normally occur predominantly in S phase, but the *H83M2* transgene lacks this coordination with replication.

We attempted to determine whether roX1 transcription was also linked to S phase as might be expected, but were unsuccessful. We used both BrUTP and EU (Ethynyl Uridine) to label all new transcripts followed by a cold chase. We anticipated that the bulk of mRNA would leave the nucleus while newly labeled roX1 RNA remained behind on the X. However, we were unable to detect any labeled RNA localized on the male X after chase (data not shown). We do not know if the signal was too weak to detect, or if the substitutions at the 5 position of uridine interferes with the function of RNA [40], leading to problems in *roX1* RNA folding, stability, MSL protein assembly, and/or targeting to the X.

**Discussion**

Previous studies of *roX1* transcriptional control argued that either MSL2 alone or with a full set of MSL proteins was sufficient to drive male-specific expression [31–33]. Here, we present evidence that the expression of *roX1* gene is instead controlled through an autoregulatory loop. Pre-existing *roX* RNA, presumably in mature MSL complex, is required to drive new transcription. The reason we reached a different conclusion is largely attributed to removing the functionally redundant *roX2* in most of our experiments and assays transcription only from the wild type *roX1* locus at its normal location on the X. The pathway we describe shares some elements with the negative regulatory loop between TFIIIA binding 5S rRNA to drive transcription or 5S rRNA for storage during *Xenopus* meiosis [41].

**A model for autoregulation of roX1**

Figure 7 shows a model for how such an autoregulatory loop might operate. Because *roX* RNA is not maternally deposited into embryos, one problem is how male embryos could build their first MSL complex needed to initiate the cycle. Meller has shown that *roX1* transcription switches on in both sexes around blastoderm, just as general zygotic transcription begins [15,16]. This suggests that an embryonic *roX1* promoter is active without MSL complex and could supply the first *roX1* RNA molecules to males, but these RNAs are eventually degraded in females. Early RNA assembles with MSL proteins and then drive *roX1* transcription from the known male-specific MSL-dependent promoters, setting up a positive autoregulatory loop necessary for the future maintenance of *roX1* expression in males. In our experiments, we showed that *roX2* RNA or truncated *roX1* RNA can also initiate endogenous *roX1* expression late in development after the early *roX1* transcripts are gone.

This model requires that male embryos preferentially sequester newly assembled MSL complex at the *roX1* gene to drive sustained transcription instead of allowing it to diffuse away to the vastly larger pool of ordinary X linked genes that must be dosage compensated. Only after *roX1* transcription is successfully upregulated can MSL complexes be released to the target genes along the X chromosome. Such behavior has been previously documented in cells containing abundant free MSL subunits and low levels of *roX1* transcription, exactly the conditions we believe occur as young male embryos initiate dosage compensation [13]. Examining cells shortly after MSL2 first turned on *roX1* transcription showed the earliest *roX1* transcripts remained near the site of synthesis (Figure 2B and 2C) consistent with the idea that newly formed MSL complexes preferentially act on the *roX1* gene. At later times, such as seen in *H83roX1-A39* animals that have had five days to drive endogenous *roX1* expression, every cell painted the entire X with *roX1* RNA (Figure 3G). While massive local cis spreading of the MSL complex has been reported for *roX1* and *roX2* transgenes inserted into autosomes [13,18,42], the physiological relevance of this is not widely accepted [25,26,43,44]. Here we instead report striking local cis spreading of newly made MSL complex from the wild type *roX1* gene in its normal X chromosome environment (Figure S1F, S1F). Our data agree well with previous reports of local MSL spreading along the X [19] and support a role for cis spreading in the normal process of dosage compensation.

We have not directly determined what region of the *roX1* gene is necessary for autoregulation. However, a strong candidate is the
Figure 6. Transcription of msl2 is correlated with cell cycle. Nascent msl2 transcripts were detected with antisense FISH riboprobe in salivary glands. (A) Transgenic H83M2 expression (bright signal indicated by red triangle) was observed at the 87A insertion site in all nuclei. Hybridization to the endogenous msl2 locus at 23F (faint band indicated by red arrow in inset) was seen in only a minority of nuclei. Because of the difference in signal intensities between the two msl2 loci, the inset is enhanced to show the weaker signal. (A') EdU incorporation shows that this is one of the few nuclei undergoing endoreplication. (B) After treatment with 1 mM of HU (hydroxyurea), no cell transcribed the endogenous msl2 gene (red arrow) but the transcription of H83M2 continued (red triangle). (B') HU blocked EdU incorporation from any cell. (C) Simultaneous treatment of salivary glands with HU, BrUTP, and EdU showed that blocking replication did not inhibit bulk transcription in these cells. (D) Many nuclei without (left table, N = 162) or...
with (right table, N = 170) HU treatment were scored for expression of endogenous msl2 and EdU incorporation. Nascent transcripts were detected at the H83M2 transgene in 100% of the nuclei (data not represented in the table). After sorting growing S2 cells into their respective phase of cell cycle via FACS, qPCR was done to quantify H2A (E) and msl2 (F) transcripts levels normalized to Pax4. (G) The FACS profile of unsorted (U) and sorted S2 cells (G1, S and G2 cell cycle). The sorted cells have a slightly higher content of Vybrant Violet-A dye because the cells are collected into tubes containing Vybrant Violet-A. The Y-axes are drawn on different scales.

doi:10.1371/journal.pgen.1002564.g006

~200 bp male-specific DNase I hypersensitive site (DHS) found about 1.5 kb downstream of the adult roX1 promoters. The DHS is sufficient to recruit the MSL complex to ectopic sites when moved to the autosomes in a sequence specific manner [45,46]. While partial complexes lacking MOF or MSL3 remain bound to the DHS, incomplete complexes lacking either roX RNA or the MLE RNA helicase postulated to fold roX RNA bind the DHS poorly [45,46]. This element stimulates roX1 transcription when MSL complex is bound and represses basal transcription when MSL complex is absent. Also, deleting the DHS greatly reduces transcription of roX1 transgenes [33]. Together, these findings support a model where transcription from the roX1 gene requires pre-existing roX RNA within MSL complexes bound to the internal DHS enhancer. However, this view is likely an oversimplification because very large internal deletions such as roX1Δ39, comparable to our H83roX1-A39 transgene (Figure 3A), remain transcriptionally active despite the loss of the DHS enhancer [32,47].

Cell cycle regulation

Translation of msl2 mRNA is normally subject to elaborate controls acting through the 5' and 3' UTRs [48–52]. Little attention has been given to its transcription control, although recently anti-MSL2 antibodies were found to precipitate msl2 mRNA [53]. We found that msl2 transcription is associated with replication, and this is likely important for its normal control. Without MSL2 protein, naked roX1 RNA is rapidly destroyed [11]. Here we found the converse; cells rapidly clear any MSL2 protein not bound to roX RNA. When free MSL2 subunits are artificially stabilized with proteosome inhibitors, they coat all the chromosomes indiscriminately. This implies that the synthesis of MSL2 and roX RNAs are closely coordinated so each component stabilizes the other ensuring that only correctly targeted molecules survive. Although MSL2 lacks known RNA binding motifs, previous work of others is consistent with an intimate interaction between MSL2 and roX RNA [31]. We suspect the loss of replication-coupled transcription may contribute to the failure of dosage compensation in some cells relying exclusively on H83M2 for MSL2 protein and roX for roX RNA. This defect is corrected when both roX1 and MSL2 are coordinately made from the same hsp83 promoter (Figure 3G). Cells in mosaic animals lose dosage compensation sometime between the end of embryogenesis and third instar larvae. Many tissues undergo significant changes in cell cycle near the end of embryogenesis, particularly the introduction of G1 [54], and we suspect this shift contributes to loss of dosage compensation in our mosaic animals. If MSL complex should ever drop below the level needed to sustain the autoregulatory roX1 loop, it could never recover regardless of later MSL2 production. The details remain unclear because H83M2 is vigorously transcribed at the developmental times we examined, including S phase. We do not know whether the regulatory msl2 UTR sequences removed from H83M2 disrupt additional posttranscriptional controls that might promote efficient translation during replication.

A second issue is that the X is painted with MSL complex throughout the cell cycle. This might drive continuous rather than cyclic roX1 synthesis. While we were unable to directly determine if roX1 transcription is cell cycle regulated, we note that the replication machinery components ORC2 and MCM are bound specifically to the roX1 DHS enhancer only in male cells (Figure S8A). The significance of this is not known, but it is tempting to speculate that components of the pre-initiation complex bound to DHS compete with MSL complex thus inhibiting roX1 transcription in G1. Firing of the replication origins removes ORC2 and MCM possibly allowing MSL complex access to the DHS and so switch on transcription shortly after the onset of S phase. The replication machinery is commonly found near the promoters of many genes [55], including msl2 (Figure S8B), so further experiments will be needed to determine if such binding actually plays any regulatory role here.

**Figure 7. Autoregulation model.** The earliest roX1 transcripts (red) made at blastoderm originate from an uncharacterized MSL-independent promoter. This RNA may assemble with MSL protein subunits to produce the first functional MSL complexes needed to bind the internal DHS enhancer that drives sustained transcription (blue) from the male-specific promoters. When present, roX2 RNA can also drive roX1 transcription. Components of the replication pre-initiation complex also bind the DHS sequence in male cells (Figure S8A). The msl2 transcripts are made predominantly during replication and new MSL2 protein is needed to assemble and stabilize newly made roX1 RNA.

doi:10.1371/journal.pgen.1002564.g007
Regulation of roX2

While we did not specifically address transcriptional control of roX2, it must differ from roX1 in several important ways. Meller has previously shown that roX2 transcription lags roX1 by a few hours during early embryonic development and is always limited to males [16]. Here we found that roX2 transcription differs by not requiring pre-existing roX RNA and can be switched on several days later during larval development simply by ectopically expressing MSL2. MSL2 protein made by the constitutive H83M2 transgene only sporadically activates roX1, but robustly drives roX2. The roX2 gene also carries a DHS enhancer similar to that found in roX1 [45], but if it plays a comparable role in roX2 regulation, it presumably would not require complete MSL complex. The region near the proline rich domain towards the C-terminus of MSL2 is essential for this regulation [51].

Parallels between roX1 and Sxl

The roX1 autoregulation loop described above shares parallels to the SXL autoregulatory loop controlling all aspects of sex determination and dosage compensation in Drosophila. XA counting elements act upon an early establishment Sxl promoter to make SXL in early female embryos. These first SXL proteins stimulate productive splicing of Sxl mRNAs transcribed from a distinct maintenance promoter ensuring further SXL production [56]. Regulation of roX1 of roX2 before abundant SXL represses transcription lags 5' control their production. For instance, the short RepA sequence at the 5' end of mammalian Xist RNA may influence production of full length transcripts [59].

Materials and Methods

Drosophila stocks

Larvae and flies were raised on standard cornmeal-yeast-agar-molasses medium containing propionic acid at 25°C. In all experiments the roX1 mutation is roX1ex6 and the roX2 allele is Df(1) roX23 [17]. The [y roX4.3] transgenic supplies essential adjacent genes lost in the roX2 deletion.

The transgenic flies used have been previously described as follows: [w H83M2-6I] [30], [w NOPU-M2] [21], and [w H83mxoX1-A39] [13]. The H83M2 transgene was recombined with P(SUPor-P)KG02776 which contains y as a marker so larvae of the correct genotype could be recognized by mouth hook color (Flybase). The P(GAL4-Act5C(FRT.CD2);P UASp-MSL2) and MRR5-hTFLP transgenic fly stocks were provided by Graeme Mardon. The UASP-MSL2 transgene was made by digesting the H83M2 transgene with EcoRI and subcloning the MSL2 ORF into the pUASP-P2 plasmid vector (a gift from Pernille Norrell).

Heatshock

The female larvae for the flip-out experiment were kept at 25°C and heatshocked at 37°C for 1 hr each at day 4 and 5 AEL. Their salivary glands were then dissected on day 6 AEL, fixed and immunostained as described below.

The full genotypes of the larvae heatshocked in Figure 1 are as follows:

- B-F) y w; [y y Act-FRT-CDC2-FRT-Gal4] [w UAS-GFP] /+ + [w UASP-MSL2]; MRR5 (Hs-Flp)/+
- H-L) y w roX252 [w A44.3]; [y y Act-FRT-CDC2-FRT-Gal4] [w UAS-GFP] /+ + [w UASP-MSL2]; MRR5 (Hs-Flp)/+

The full genotypes of the larva in Figure 2 are identical to Figure 1H–1L.

The full genotypes of the larvae in Figure 3 are as follows:

- A) y w roX252 [w A44.3]; msl2; [w H83-M2] [w y P(SUPor-P)KG02776]/+ +
- D) y w/+ y w roX1 ex6/; msl2; [w y w w roX252
- B-B') y w roX23 [w A44.3]; msl2; [w y w w roX252
- C-C') y w roX1 ex6 roX252 [w A44.3]/1; msl2/+; [w y w roX252
- D-D') y w roX1 ex6 roX252 [w A44.3]; msl2/+; [w roX1 ex6 roX252
- E) y w roX252 [w A44.3]; msl2; [w y w roX252

Immunohistochemistry and in situ hybridization

Immunostaining and in situ hybridization on third instar salivary glands was performed as described in [11] except for the following modification. The slides were treated with proteinase K (20 µg/ml in PBT) for 3 min. Each slide was hybridized with biotin-labeled single-stranded antisense riboprobes using the T7 high yield Transcription kit (Promega). The slides were treated with proteinase K (20 µg/ml in PBT) for 3 min. Each slide was hybridized with biotin-labeled single-stranded antisense riboprobes using the T7 high yield Transcription kit (Promega).
EdU labeling

To block replication, salivary glands from 3rd instar larvae were first incubated in 1 mM HU for 1 h. If not, they were simply incubated in S2 cell culture media (see below). After which, the glands were transferred and incubated in EdU for 15 minutes followed immediately by fixation. The detection was then performed as instructed by the Click-iT EdU imaging kit #C10337 (Invitrogen). Both HU and EdU were dissolved in S2 cell culture media before use.

Co-labeling replicating DNA and nascent transcripts via EdU and BrUTP respectively

Salivary glands were initially treated the same way as described above. However, after HU treatment, the glands were incubated in a BrUTP/DOTAP/EdU mixture instead for 15 mins. The BrUTP/DOTAP mixture was previously used to allow efficient nucleotide triphosphate uptake by the cell to label nascent transcripts [62]. EdU detection was performed as described above. Rat Anti-BrU monoclonal antibodies #NB500-169 (Novus Biological) and Goat anti-rat Alexa 594 #A11007 (Invitrogen) were then used as primary and secondary antibodies respectively, to detect BrUTP.

Cell culture and FACS

Schneider 2 (S2) cells were cultured in 15-cm plates at a density of 1×10^6 cells/ml in Schneider’s Media #11720034 (Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. After reaching a density of 5×10^6 cells/ml, the cells were re-suspended in new culture media containing 5 μM of Vybrant Dye Cycle Violet #V35003 (Invitrogen) to stain the DNA. The cells were then sorted into their respective phase of cell cycle via FACS/Aria II (BD Biosciences) at the Cytometry and Cell Sorting Facility located in Baylor College of Medicine.

Real-time PCR

Total RNA was extracted from cells using Trizol® reagent 15:596-018 (Invitrogen) as per the manufacturer’s protocol. cDNA was synthesized from 0.5−1 μg of total RNA using random hexamers and MMLV Reverse transcriptase #M1701 (Promega) as per manufacturer’s protocol. The cDNA was purified using MinElute PCR kit #279004 (Qiagen).

Real-time PCR was performed using an Applied Biosystems 7500 Sequence Detection system. The 25 μl PCR included 1 μl of cDNA, 1x SYBR® Green PCR Master Mix #4309155 (Applied Biosystems) and 1 μl of gene specific primers. The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 10 min. The Ct data was determine using default threshold settings. The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. PKA is used as an internal control. Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. PKA is used as an internal control. Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. PKA is used as an internal control. Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. PKA is used as an internal control. Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. PKA is used as an internal control.

Supporting Information

Figure S1 The [H83-mX1A39] transgene turns on endogenous roX1 in males. (A) roX1 transcripts (Orange) and antisense probes (green). (B) Quantification of roX1 hybridization over polytene X chromosomes: entire X (black), only distal X (gray, example E), a single band at the roX1 locus (hatched, example F red arrowhead), or no staining (white). N = nuclei counted. The [H83-A39mX1] transgene does not have an effect on females. (C) The internal probe does not hybridize to A39mX1 RNA. (D) but the roX1A39 probe does. While line delineates the X chromosome. (E) Males display a diverse pattern of roX1 painting, ranging from local spreading from the roX1 locus to (F) just a single band (indicated by the red arrow). (G) A39mX1 RNA (not visualized) helps [H83-M2] strongly activate endogenous roX1 (detected with internal probe) in almost all cells (two nuclei shown) (H) Similar roX1 staining was found in all squashed imaginal disc cells.

Figure S2 MSL immunostaining reveals that MSL2 binds indiscriminately to all the chromosomes in the absence of any roX RNA. (A) In the presence of roX RNA, MSL2 binds and paints the X chromosome only. (B) In the absence of roX RNA, the MSL proteins form incomplete complexes and binds to all the chromosomes, albeit poorly. (C) After treatment with MG132, a proteasome inhibitor, strong MSL2 binding can now be observed to occur throughout the nucleus. roX1- roX2 males are sick and do not survive till adulthood. However, sick and rare 3rd instar larvae can be obtained for salivary squashes although the chromosomes have extremely poor morphology and easily shattered during squashes. (D) The same experiment was repeated in whole mount salivary glands and MSL2 can be seen concentrated on the X chromosome. (E) At low resolution, MSL2 staining becomes undetectable in the absence of roX RNA (compared to B). (F) MSL2 can be seen binding to the entire nucleus when degradation is being inhibited.

Figure S3 MSL immunostaining of polytene chromosomes in [H83-M2] expressing larva reveals mosaic establishment in DC. MSL immunostaining and DAPI is represented by red and blue respectively. (A) The MSL complex is bound along the male single X chromosome at hundreds of bands. (B) Due to the lack of MSL2, female do not have MSL binding to the X chromosome. (C–D) Painting of the X can be restored by the [H83-M2] transgene in m2 mutant animals if both roX1 and roX2 are present. (E) The MSL complex fails to paint the entire X chromosome in males if roX2 is deleted. This cis-spreading phenomenon around the roX1 locus (indicated by red arrow) is similar to the autosomal spreading of roX1 transgene observed under low transcription rate. (F) In females, the X chromosome is either painted (arrowhead) or not painted (arrow). See Figure 1I for quantification. (G–H) Normal painting of the X is re-established when an endogenous copy of roX2 is restored. In females, this is achieved by co-expressing the [NOPU-M2] transgene. (I) The fraction of polytene nuclei displaying complete, partial, or no X MSL1 painting is shown. N = nuclei scored. Genotypes: (1–2) roX1- roX2-;msl2/+ [H83-M2]/+. (3–4) roX1- roX2-;msl2/[H83-M2]/+. (5) roX1+ roX2-;msl2/[H83-M2]/+. (6) roX1+ roX2-;msl2/[NOPU-M2]/ [H83-M2]/+. (PDF)

Figure S4 MSL2 immunostaining of imaginal disc cells in [H83-M2] expressing larva reveals mosaic establishment of DC. (A–B) Painting in all nuclei is observed in animals relying upon the [H83-M2] transgene if both roX1 and roX2 are present. (C–D) Mosaic painting of the X chromosome is observed in both males and females when roX2 is deleted. White arrows indicated unpainted nucleus. (E–F) Normal painting of the X is re-established when an endogenous copy of roX2 is restored. In females, this is achieved by co-expressing the [NOPU-M2] transgene. (G) Cells with (black) and without (white) obvious subnuclear domain MSL2 staining taken
from gently squashed discs were counted (Figure S1). N = cells counted. Genotypes: (1–2) msl2 CA; msl2; [H83-M2]+/+, (3–4) msl2 CA; msl2; [H83-M2]+/+, (5) msl2 CA; msl2; [H83-M2]+/+, (6) msl2 CA; msl2; [H83-M2]+/+, (7) msl2 CA; msl2; [H83-M2]+/+. (PDF)

Figure S5 MSL2 immunostaining of embryos reveals that 100% of the cells expressed [H83-MSL2]. (A–A′) A wildtype embryo at the end of germband extension showing endogenous MSL2 expression detected in 100% of the cells (B–B′) A msl2 CA; msl2; [H83-M2] embryos, expected to display a mosaic pattern of dosage compensation in imaginal discs and salivary glands by 3rd instar, shows no signs of mosaicism during early embryogenesis. (PDF)

Figure S6 msl2 in situ hybridization of embryos reveals that 100% of the cells successfully establish dosage compensation. (A–A′) A wildtype embryo at about 20 hr AEL, has msl2 expression detected in 100% of the cells (B–B′) A similarly stage msl2 CA; msl2; [H83-M2] embryos displaying no signs of mosaicism. (PDF)

Figure S7 Histones transcripts not a good marker of S Phase. (A–A′) Nascent H2A (red arrow) and msl2 transcripts (red arrowhead) can be visualized at cytocolation 23F and 39 respectively via double ISH of polytene chromosomes with antisense msl2 and H2A RNA probes. (B) Although it is well documented that the transcription rate of histones decreases by 5 folds upon Hydroxurea treatment [30], it is hard to quantified using the TSA technique for ISH. (B′–B′′) On the other hand, msl2 transcripts have completely disappeared upon HU treatment. (PDF)

Acknowledgments

We would like to thank Artyom Aleksyenko for sharing unpublished data and Bill Mattos, Herman Dierick, Joel Neilson, and anonymous reviewers for valuable critiques.

Author Contributions

Conceived and designed the experiments: CKL RLK. Performed the experiments: CKL. Analyzed the data: CKL RLK. Contributed reagents/materials/analysis tools: CKL. RJK. Wrote the paper: CKL RLK.

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