Requirement of Male-Specific Dosage Compensation in Drosophila Females—Implications of Early X Chromosome Gene Expression

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Abstract
Dosage compensation equates between the sexes the gene dose of sex chromosomes that carry substantially different gene content. In Drosophila, the single male X chromosome is hypertranscribed by approximately two-fold to effect this correction. The key genes are male lethal and appear not to be required in females, or affect their viability. Here, we show these male lethals do in fact have a role in females, and they participate in the very process which will eventually shut down their function—female determination. We find the male dosage compensation complex is required for upregulating transcription of the sex determination master switch, Sex-lethal, an X-linked gene which is specifically activated in females in response to their two X chromosomes. The levels of some X-linked genes are also affected, and some of these genes are used in the process of counting the number of X chromosomes early in development. Our data suggest that before the female state is set, the ground state is male and female X chromosome expression is elevated. Females thus utilize the male dosage compensation process to amplify the signal which determines their fate.

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Introduction

When the sex chromosomes carry substantially different gene numbers, dosage compensation is necessary to equalize gene expression between the two sexes. In the three best studied model systems Drosophila, C. elegans and mammals where males are XY and females XX, this involves targeting X-specific components which modify the chromatin and transcription of X-linked genes. In each of these cases the end result is different; Drosophila upregulates transcription of the male X by about two-fold, C. elegans downregulates transcription of both X chromosomes in the hermaphrodite by approximately half, and mammals generally shut down transcription of one of the two female X chromosomes (reviewed in [1]).

As it is the Drosophila male which requires dosage compensation, mutation of genes strictly dedicated to this process results in male lethality. The first male specific lethal identified, maleless (mle, [2]), is indeed involved in dosage compensation as are the next identified male lethals, msl-1 and msl-2 [3]. msl-3 identified by Uchida et al. [4] and males absent on the first (mof, [5]) complete the proteins collectively known as the male specific lethals (msls; reviewed in [1,6,7]). In addition to these proteins, two RNAs on the X chromosome (the roX RNAs), which are not present in females, are also essential for dosage compensation [8]. Although roX1 and roX2 show no sequence similarity and do not have an open reading frame that could encode a significantly sized protein, they function redundantly; either roX is adequate for function, while loss of both RNAs is required for a failure in dosage compensation and male lethality [9]. The MSL proteins and roX RNAs function as a complex, coating the male X chromosome; the X chromosome is hypertranscribed and MOF acetylates histone H4 on lysine 16. Finally, a protein that appears to be part of the dosage compensation complex (DCC) but is required by both sexes is the JIL histone H3 kinase. JIL is also enriched on the male X chromosome but its loss leads to lethality in both sexes [10].

In 1980, Skrijpke and Lacceci [11] reported that females heterozygous for a Sex-lethal (Sxl) null allele, Sxl0, and homoygous for mle showed morphological characteristics indicative of sex transformations. Sxl is the Drosophila sex determination master switch which is on in females but off in males. The Sxl0+/+; mle/mle sex transformation result was confirmed and extended by Uenooyama et al. [12] who observed similar effects with two different mle alleles as well as msl-2 and msl-3. This argued that this phenomenon was not unique to mle, but likely a general property of the msls.

These results, a requirement of male specific genes in females, present a paradox. First, homoygous msl− females show no sex transformations and are fully viable [3]. Second, besides controlling differentiation, a key function of Sxl is to turn off the male dosage compensation system to prevent hypertranscription of the two female X chromosomes, which would otherwise lead to female lethality. As a splicing and translation regulator, Sxl alters the splicing and inhibits translation of msl-2 mRNA so preventing assembly of the DCC [13–17]. The absence of MSL-2 also destabilizes MSL-1 and MSL-3 assuring inactivation of the dosage compensation machinery.
Author Summary

When substantially different, sex chromosomes present the challenge of not only gene dose iniquity between the sexes, in the heterogametic sex where one chromosome (frequently the Y) carries few genes, but also an iniquity relative to the autosomes which are diploid. Dosage compensation refers to the process which equates gene dose between the sexes. Recent results, however, indicate that the mammalian X chromosome avoids monosomy and has a level of expression that is two-fold relative to the autosomes. Hyperactive X chromosome expression in Caenorhabditis elegans has also been suggested, and dosage compensation in the hermaphrodite appears to lower expression of the X chromosomes to match autosomal levels. We find that, before the female state is set in Drosophila, the X chromosomes may also express their genes at the two-fold male level and that this level of expression is used to female advantage to consolidate their sex determination. Together, the results suggest that elevated X chromosome expression may be the norm, and that the various dosage compensation processes different organisms utilize reflect a mechanism to counteract an initial hyperactive X chromosome state.

The initial activation of Sxl is transcriptional, at the Sxl ‘establishment’ promoter, SxlPm [18]. In cycle 12 of embryogenesis, SxlPm responds to activating X linked genes (known members: sisterless-a (sis-a), sisterless-b (sis-b), runt (run) and unpaired (upd)), in conjunction with positive maternal factors such as Daughterless, balancing their dose against the negative effect of genes on the autosomes (deadpan (dpn), the only identified member) and maternal factors such as Groucho (Gro) and Extramacrochetae (hereafter collectively referred to as the X-A ratio; reviewed in [19]). Protein from SxlPm transcripts alters the splicing of transcripts from the ‘maintenance’ promoter, SxlPm, first transcribed in cycle 14 in both sexes. In the absence of Sxl protein, default splicing includes a translation terminating exon into the transcripts from SxlPm. As male embryos do not activate SxlPm, Sxl protein is absent and a splicing change on SxlPm transcripts is only effected in females. Females thus set in motion a splicing autoregulatory feedback loop which serves to maintain Sxl expression, and sexual identity, through the rest of the life cycle [20].

Returning to the paradox of a female requirement of male specific genes, one explanation is that XX embryos with only one copy of Sxl fail to reliably activate the gene. These XX cells would be male and are presumably eliminated, due to the gene imbalance from inappropriate dosage compensation. However, when one or more of the msls is mutant, these masculinized XX cells might survive since assembly of the male DCC is prevented. The resulting clones grow but are sexually transformed, so accounting for the observed sex transformations.

Results

While plausible, the above explanation does not account for the recessive nature of Sxl null alleles, which have very high viability (Figure 1A). This high viability requires these females survive the removal of those pockets of male tissue with inappropriate dosage compensation, as Sxl hemizygous females show no male differentiation. Figure 1A also shows that the viability of females with only one Sxl+ allele is badly compromised if maternal MSL-3 is removed. Maternal MSL-1 was next most effective followed by MSL-2, while the effect of MLE was negligible relative to wild type. These data demonstrate a synergism of these msls with Sxl for female viability, as one wild type copy of both Sxl and the msl is present in these animals.
greater effect of the sis genes is not surprising, given they function in a dose sensitive process to activate Sxl and so determine female sex. What is surprising is that the msls interact with the numerators to promote female viability.

To test whether the loss of a single numerator gene could also affect females, we performed crosses with reduced dose of either sis-a or sis-b. Since msl-3 showed the strongest overall interaction, this msl was examined. Figure 2A shows that sis-a as well as sis-b alone affected females, with sis-b having the stronger effect. The sis gene interactions suggest that very early steps in the female sex determination process are compromised. Testing two Sxl alleles, an early (SxlP) versus a late (SxlP,sb) defective allele, indicated that the early defective allele had an effect, almost as strong as sis-a alone, while the late defective allele did not. These data are consistent with the view that early, dose sensitive events in female sex determination are influenced by the msls. The late Sxl transcripts may not turnover or be as dose sensitive as the early transcripts, so a 50% reduction may not be sufficient to sensitize the females.

sis-a and sis-b are zygotic in their role in female sex determination. To determine whether the effect observed with the msls was maternal or zygotic, reciprocal crosses to Figure 1B were performed. Under these conditions, halving the dose of each of the four msls, including msl-2, reduced female viability (Figure 2B). The zygotic effect was generally weaker than the maternal.

A maternal effect of msl-2 is surprising given that the protein is not detected in females [13,15,17]. We note that a maternal effect of msl-2 was also described by Uenoyma et al. [12]. msl-2 RNA is deposited into the egg (Flybase microarray data; http://www.flybase.org/), so the strength of the zygotic effect is presumably influenced by the amount of maternal protein/RNA of each of the msls.

As the msls, particularly MSL-3 and MOF, have been shown to bind to both autosomal and X-linked genes where they might perform an unknown role, we wondered whether the entire male DCC, including MOF and the roX RNAs, influenced female viability. With the numerator gene dose compromised, halving mof dose had an effect, as did roX1 which was much stronger in effect than roX2 (Figure 2B). Since the roX RNAs function redundantly, the impact of roX1 and the weaker interaction of roX2 can be explained by the fact that first expression during embryogenesis is later for roX2 than for roX1 [9]. Combined, these results indicate that the msls affect an early event and that the entire male DCC is required for promoting female viability.

Transcription of Sxl is affected by the msls

The foregoing suggests an event early in Sxl expression is altered by the DCC. To directly assess the effect of the DCC on Sxl transcription, in situ were performed with Sxl probes specific for either the early or late transcripts. Embryos from homozygous

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**Figure 2.** *msls* act early in the female sex determination process, and all key components of the dosage compensation complex, affect female viability. (A) msl-3 homozygous mothers mated to males mutant for single numerator gene (sis-a or sis-b) or Sxl early phase (f9) or late phase defective (M1, f12) allele (test class total n = 1135, 1047, 840, 1129, 696, 915, 924, respectively). sis-b<sup>sa</sup> cross was done at 29°C, the non-permissive temperature for this temperature-sensitive allele. Percent viability of females related to their brother is shown with percentage standard error. Relevant genotype of fathers shown on x-axis. (B) w, sis-b<sup>sa</sup>-1, sis-a<sup>1</sup>, mof<sup>-1</sup>/CyO females were mated to either Ore R, mle<sup>/CyO</sup>, msl-1<sup>18D8</sup>/CyO, msl-2<sup>21</sup>/CyO, msl-3<sup>TM3</sup>, mof<sup>/Y</sup>; 18H1[mof]<sup>+</sup>, roX1<sup>res</sup>/Y or roX2<sup>res</sup>/Y males (test class total n = 420, 241, 285, 363, 447, 408, 418, respectively). Percent viability of sis-a, b<sup>+</sup> females with the msl mutation relative to their FM7/+ sisters also carrying the msl mutation is shown, with percentage standard error. For mof it is for females that did not receive the 18H1[mof]<sup>+</sup> transgene. Relevant msl genotype of fathers shown on x-axis. doi:10.1371/journal.pgen.1001041.g002
A constitutive Sxl allele rescues females from msl-1-promoted lethality

If, as the data suggest, the primary reason for female lethality is the failure to activate Sxl, a constitutive allele (such as SxlM1) which bypasses the X:A ratio should rescue them. Since msl-1 showed the strongest interaction in the genetic tests, we determined whether the presence of SxlM1 could rescue the lethality of sis-a, b or Sxl dose reduction in embryos from mothers homoygous for msl-3. The rescue (Figure 4) of 72.8% and 98.7% of the females by SxlM1 for sis-a, b or Sxl dose reduction, respectively, demonstrates that female lethality is primarily caused by the inadequate expression of Sxl.

Both Sxl promoters are affected by the DCC

We next examined whether transcripts from the maintenance promoter, SxlM1, were affected. As shown in Figure 5, this promoter was also affected by loss of DCC components. For msl-1, msl-2 and msl-3 about 50% of the embryos, presumably the homozygotes, showed weaker expression. For mle and the roX2 double mutants almost all the females (2-dots/cell embryos) showed weaker than normal expression. As noted for SxlM1, most of the females are mutant for roX1 and roX2 excepting the few non-disjunction embryos that also receive the Y with a duplicated roX2.

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mutant mle, msl-1FM7, msl-2FM7, msl-3FM7 or roX1(+), roX2− double mutant mothers, mated to heterozygous msl males were analyzed. For the roX1, roX2 double mutant embryos, the roX1−, roX2− males have a duplication of roX2 on their Y chromosome so only the females are roX1−, roX2−.

In wild type embryos, SxlM1 is not activated until cycle 12, its expression becomes stronger in cycles 13 and 14 before it rapidly ceases expression early in cycle 14. For all the msls about half the embryos showed weaker than normal expression of SxlM1, as judged by the size and intensity of the in situ dots on their X chromosomes (Figure 3). The fraction was higher in the roX1−, roX2− cross where all the females are expected to be mutant. These data indicate that the entire DCC complex is used to upregulate transcription from SxlM1.

Figure 4. A constitutive Sxl allele rescues the female lethality from loss of msl-3, y, cm, SxlM1/FM7; msl-3− females mated to w, sis-bFp7BS, sis-a, mlfY or y, w, cm, SxlM1Y males (test class total n = 555, 851, 1065, 681 respectively). Percent viability of females relative to their brothers is shown with percentage standard error. Genotype of fathers shown on x-axis. As the reference males were the balancer FM7/Y, correction for their reduced viability (48.7%) was determined relative to SxlM1+/+ females from a mating of y, cm, SxlM1/FM7, msl-3− to Ore R males. doi:10.1371/journal.pgen.1001041.g004

Figure 5. Transcription from the Sxl maintenance promoter, SxlM1, is also reduced in embryos homozygous for the msls. In situ on embryos with an early transcript specific probe during early and mid cycle 14. As cellularization proceeds during cycle 14, the membranes between the nuclei drop into the embryo, the cell volume changes allowing embryo staging. Only females are shown. Male embryos also transcribe SxlM1 – single X chromosome dots – not shown. All embryos photographed at 40× mag.

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Figure 3. Transcription from the X chromosome dose sensitive promoter of Sxl, SxlM1, is reduced in embryos homozygous for the msls. In situ on embryos with an early transcript specific probe reveals two dots/nucleus at the sites of transcription on the X chromosomes during cycles 13 and 14 in development. The roX double mutants had a reduction in expression in most embryos as all the females are mutant, the msl showed reduced expression in about 50% of the embryos (crosses described in text). The 'sibs' panels show the signal from normal looking cycle 14 embryos in the same collection, presumably the heterozygous siblings. NA – not applicable. All embryos photographed at 40× mag.

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the same developmental stage as Ore R embryos have comparable transcription from both transcription elongation and not initiation is altered by the DCC susceptible to the DCC and suggest that MLE, the data together indicate that both Sxl and msl-31 affected. This may be an outcome of the DCC having slightly greater than a two-fold effect. Alternatively, because MLE also affects the stability of roX1 RNA, which has a larger role in sex determination than roX2 (Figure 2B), the effect of mutating MLE may be amplified as it not only eliminates the maternal MLE but also reduces the levels of roX1 RNA, acting as a double mutation. Despite this unexplained effect on SxlPe by maternal MLE, the data together indicate that both Sxl promoters are susceptible to the DCC and suggest that Sxl, which resides on the X, is a dosage compensated gene. Consistent with the idea that transcription elongation and not initiation is altered by the DCC [21], transcription from both Sxl promoters, which are regulated by different factors, is affected. Although the in situ of Ore R embryos did not show the distinctly different classes we observed with the msl embryos, to control for the possibility that the quality of the in situ was responsible for generating a poor signal in half the embryos from msl mutant mothers, in situ for SxlPe transcripts were simultaneously performed with a distinguishable second probe - the segmentation gene hairy which has a striped pattern of expression. As seen in Figure 6, embryos from msl mutant mothers that are at the same developmental stage as Ore R embryos have comparable hairy stripes but poor SxlPe signal, indicating that the poor signal is not an artifact of the in situ but an effect of the msls on Sxl transcription.

Quantitation of Sxl expression in embryos from msl mothers

The in situ are qualitative and the nuclear dots detect transcription directly off the chromosomes, indicating only high levels of transcription. For a better measure, we performed quantitative RT-PCR analysis on 2-3 h and 2.5-3.5 h embryos for SxlPe and SxlPm expression, respectively. Embryos were from homozygous mutant mothers for msl-2-1, msl-3-1 or roX1+6, roX2-2 double mutants, mated to heterozygous msl males. As for the in situ, in the roX1-2, roX2-2 embryos only the females are doubly mutant as males have a duplication of roX2 on their Y. RNA levels were normalized to tubulin levels and compared to Ore R embryos which were set to 1.

Figure 7 shows that SxlPe is expressed at lower levels than Ore R embryos in all three msl genotypes. The median for msl-2-1 embryos was slightly above, for msl-3-1 and roX1-2, roX2-2 embryos the median slightly below half of Ore R. The medians for SxlPe were also close to half, except for the roX1-2, roX2-2 genotype which was closer to 0.7. SxlPe is transcribed in both sexes and all the males have a functional mX2 gene in the roX1-2, roX2-2 embryos. In these embryos, SxlPe gave a value of 0.7, suggesting males are transcribing SxlPe at close to normal levels while the females express SxlPe at close to half. This would suggest that functional mX2 RNA is present mid-way through cycle 14, a little earlier than in situ can detect [9].

A value close to 0.5 for both promoters (excepting SxlPm for roX1-2, roX2-2) was a little surprising given the in situ results which show about half the embryos have close to normal levels of transcription. It suggests that the DCC may be upregulating the expression of Sxl by a little more than two-fold, not unlike the mX genes [22]. Alternatively, and not mutually exclusive, it may also indicate that at 2-3 h of development most of the DCC is assembled primarily from maternal reserves and the presence of one wild type chromosome in half the embryos (from the heterozygous fathers) makes a small contribution. With respect to SxlPm the qRT-PCR score embryos whose average age is slightly younger than the in situ, at cycles 13 and 14 (2.75-3.25 h). Close examination of those in situ shows few embryos in early cycle 13 with uniform, wild type levels of SxlPe expression. However, when the membranes begin to drop between the nuclei later in cycle 13, the class with more uniform expression resembling wild type, is more readily observed (data not shown). By late cycle 13 and cycle 14, the zygotic contribution of the wild type chromosome from the heterozygous fathers must begin, and the two different classes are more readily apparent in cycle 14 embryos (Figure 3). For SxlPm the data are more consistent with the DCC having slightly greater than a two-fold effect.

Effects of the DCC were also scored for some of the sex determination genes in the 2-3 h collections from homozygous msl-2-1 or msl-3-1 mothers. msl-3-1 embryos show sis-a, like Sxl, with a median expression close to 0.5, while run and dpm gave medians close to 1 as expected for non-dosage compensated genes (sis-b could not be reliably scored as it has an anti-sense transcript, CG32816). upd appeared reduced to ~0.7 but this was not statistically significant and the data showed greater variability than for the other genes. This may be because upd begins expression later (cyc 13; [23]), and half the embryos are beginning to perform normal dosage compensation. Also, there are 2 DCC high-affinity sites (see Discussion below) relatively close to upd. These are predicted to make upd less sensitive to the loss of MSL-3, since MSL-3 is required for spreading of the DCC from its initial entry sites.

For msl-2-1 embryos, the upd median did drop to ~0.5, consistent with the loss of MSL-2 having a greater effect than MSL-3 for genes with close DCC entry sites. run did not show a significant change from wild type. However, unlike for the msl-3-1 embryos, sis-a was slightly elevated relative to wild type, while dpm mRNA, at a low level of significance, showed a small decrease. As the msl-3-1 embryos show that sis-a is dependent on the DCC, these latter data suggest that besides dosage compensation, MSL-2 may have an additional role, one that perhaps affects mRNA stability. MSL-2 affects the steady state levels of the mX RNAs [24]; such an activity could explain the greater variability in the values we measured for
msl-21 embryos. To test this, in situ of sis-a mRNA were performed to determine if over time, the mRNA levels would show a change consistent with accumulation. Indeed, we found this to be the case (Figure S1), suggesting that in the case of sis-a MSL-2 may serve to destabilize its RNA. During the early cycles, embryos from msl-21 mothers had signal which was generally weaker than wild type, but by cycle 12 when the message has its highest accumulation in wild type [25], the accumulated levels in the msl-21 embryos were even higher. While alternative explanations, e.g. repression of the sis-a promoter by MSL-2 are also plausible, this effect would have to occur at some but not all stages of sis-a transcription and be independent of the DCC, as loss of MSL-3 shows the predicted 2-fold drop in sis-a mRNA levels.

Despite the suggestion of an additional role beyond dosage compensation for MSL-2, the qRT-PCR data show that the 2 Sxl promoters are expressed at approximately half their normal levels by the loss of the DCC. Expression of other X-linked genes also appears to be similarly affected, very clearly evident in the msl-31 embryos. This indicates the DCC functions relatively early, and may also affect the handful of genes known to be expressed during these early stages of embryonic development [26,27].

**Transient expression of the DCC in females**

The data argue for a role of the male DCC in females, a function not ascribed to it, and the complex has not been detected in female embryos [28–30]. Our data suggest that prior to the full activation of Sxl there is a brief window of male dosage compensation in females, after which Sxl protein is predicted to shut down MSL-2 expression, and destabilize the entire DCC. Not all anti-MSL antibodies have been reported to detect the complex at this early stage, even in males (see [30]). Given this limitation, we used an anti-MSL-1 antibody from the Lucchesi lab which has high sensitivity and enhanced the signal with an M3TAP construct [31]. These embryos were co-stained with anti-Sxl antibodies and closely examined around the cellular blastoderm stage. Figure 8 shows that there is indeed a very brief stage, in mid cycle 14, when it is possible to simultaneously detect both Sxl and the DCC in females. The ant-MSL-1 signal in the female nuclei is not as bright and generally covers an area of DNA larger than in males, presumably the two X chromosomes.

**Discussion**

The effect of the DCC on Sxl transcription early in embryogenesis explains the contradiction of why genes that are

![Figure 7. Change in mRNA expression for dosage compensated and control genes compared to Ore R. All replicates are plotted and display the 25 and 75 percentiles (boxed), median (line in boxes), max and min (whiskers) of the data set. Significance is measured using an unpaired t-test with a Welch’s variance correction, levels indicated above whiskers as *** for p<0.001, ** for 0.001<p<0.01, * for 0.01<p<0.05, and ‘ns’ for p>0.05. (A) SxlPe and SxlPm transcripts show a highly significant drop in expression when DCC function is compromised by mutation in msl-2, msl-3 or the roX genes. Embryo genotype on the x-axis. As noted in the text, the lower significance in the change in SxlPm levels for the roX- genotype is most likely the influence of males which have a wild type roX2 gene. (B) dpn and run, show almost no significant change in expression, with the slight exception of dpn in the msl-21 mutants. sis-a and upd show significant change in a DCC-compromised backgrounds, with specific differences between mutant genotypes. sis-a shows the expected drop in expression for msl-3 embryos but shows a slight increase in expression for msl-2 embryos. This appears to arise from an additional role of MSL-2, affecting mRNA levels (see Figure S1). upd is affected by the loss of MSL-2 but is essentially unaffected by the loss of MSL-3, as would be expected for a gene with close DCC entry sites. doi:10.1371/journal.pgen.1001041.g007

![Figure 8. Sxl and the DCC are transiently co-expressed in female embryos. During mid cycle 14, Sxl (green) and the DCC (red - mostly from anti-MSL-1 antibody) can be simultaneously detected in females. MSL-1 overlaid on DNA (blue) signal (Merge). Top set of images from males, as determined by the lack of specific Sxl signal, lower set is of females. The ant-MSL-1 signal in the female nuclei is less intense and generally covers a larger area than in males, as might be expected for their double dose of X chromosomes. As previously noted [46], the signal in males is more frequently at the nuclear periphery. doi:10.1371/journal.pgen.1001041.g008

Male Lethals for Females
normally off in females are required to promote their viability. In
the absence of the *msl* and a functional male DCC, transcription of
some of the genes on the X chromosome as well as *Sxl* is not
elevated by two-fold. This effect weakens the X:A ratio and
lowers the levels of the *Sxl* early as well as late transcripts, which
when low enough leads to female lethality. With respect to *SxlPm*,
insufficient levels of early protein are produced and splicing of
*SxlPe* transcripts into the female mode is compromised. With
respect to *SxlPe*, a reduction may compromise establishment of the
female state as the autoregulatory splicing feedback loop would
have to rely on reduced mRNA and protein levels.

In the absence of mutations in feminizing genes, lowering of *Sxl*
expression by the *msl* is not detrimental, presumably as the very
process which would lead to female lethality - male dosage
compensation - is no longer functional, while the *Sxl* positive
autoregulatory feedback loop slowly establishes itself into the
female state. Without the DCC, however, reduced dose of
feminizing genes, particularly the dose sensing X-linked genes or
numerators, lowers *SxlPm* transcript levels further, and has
deleterious consequences for females. *Sxl* dose also has an effect,
but unlike the numerators *Sxl* is not strictly dose sensitive, and not
unexpectedly, when its copy number is halved, has a less profound
effect on female viability. It should be noted that extremely low
levels of *Sxl* in females, even in the absence of the male DCC is
lethal [32]. *Sxl* protein directly performs a female dosage
compensation role, reducing the levels of X-linked genes such as
*num* [33]; the latter is not upregulated by the male DCC [34; Figure
7].

Early X chromosome expression is elevated in females

Our data indicate that some of the earliest expressed genes on
the X, the numerators as well as *Sxl*, are dosage compensated.
Dosage compensation is a chromosome wide phenomenon, and, at
the least, the effect of the *msl* can be detected as early as cycle 13.
Previous work timed the DCC in males at cellular blastoderm
(Stage 5, [30]) and early gastrulation (Stage 6, [29]). Our data
(qRT-PCRs and in situ) suggest dosage compensation sets in
earlier, by 2–3 h in development and appears to initially rely on
maternal stores and the zygotic expression of the *nX* RNAs (*nXI*
primarily). As discussed by McDowell et al. [30] antibody
sensitivity sets the limit for the prior studies. The present studies
relied on different assays, which may account for the difference.
Indeed, we were also unable to detect convincing signal in males,
which is normally stronger, much before blastoderm by antibody
staining (Figure 8). It is also possible that early in development the
DCC is harder to detect directly as there are fewer genes being
transcribed, so less of the complex may have assembled onto the X
chromosome before cycle 14, when the mid-blastula transition
occurs and there is a large transcriptional burst. The zygotic
expression of *nX* RNA has been placed at around 2 h of
embryogenesis [9], consistent with the effects we observe.

Targeting of the DCC to the X chromosome, rather than the
autosomes, is thought to rely on transcription marks, sequence
elements (~150 MREs – MSL recognition elements and ~130
HAS – high affinity sites), and other unknown elements [35,36].
The identified sequence element set is still incomplete since the
two data sets show an overlap of 69%; it is predicted that the X
chromosome may have as many as 240–300 elements [reviewed in
[7]]. Examination of the published MRE and HAS shows the
closest element to *Sxl* ~139 Kbp 5′ of the gene. This distance is on
the large side, although it should be noted that all elements which
target the DCC to genes on the X remain to be identified; as an
example, the white gene has its closest known MRE/HAS 93 Kbp
away but its mini form in transgenes, which does not include this
site, is clearly dosage compensated. Finally, ChIP data (mod-
ENCODE, Flybase) show *Sxl* with strong H4K16 acetylation
marks, a modification dependent on the DCC. ChIP data for *Jil-
1* kinase also suggest the DCC is at *Sxl*.

None of the other sex determination genes, other than *upd* (two
3′ elements at ~5.6 and 6.8 Kbp away) had an element within
10 Kbp (sis-a ~26 Kbp, sis-b ~38 Kbp), consistent with the
observation that the *msl* involved in spreading the DCC from its
entry sites on the X (MSL-3, MOF and the *nX* RNAs), are
required for their elevated expression. *upd*, the exception, showed
greater sensitivity to the loss of MSL-2 than MSL-3, as might be
expected for dosage compensation which is less dependent on
spreading. An interesting correlation is that *num* which is not
compensated, had its closest elements ~343 (5′) and ~373 Kbp
(3′) away, further than the rest of the other known key sex
determination genes.

Default mode is male

By using the DCC before the female state is established, *Sxl*
capitalizes on the default male state. Transcription from *SxlPm*
is amplified, an effect unique to females as males do not transcribe
from *SxlPm*. Determination of female identity is thus consolidated.
As expression of *Sxl* protein levels is established, *Sxl* protein
subsequently shuts down the DCC and eliminates the very
difference in gene dose between the sexes which set in motion, as
well as augmented, its own activation. Implicit, is that before the
establishment of *Sxl* expression, each X-chromosome in females is
transcribed at 2X levels, as in males, and our qRT-PCR data of
some of the key sex determination genes would support this view.
The conventional X: A ratio would then be 4:2 rather than 2:2, and in
males 2:2 rather than 1:2 (Figure 9).

In that there is a 2-fold difference between the sexes, this
scenario is mathematically the same. However, there are practical
and functional implications. An X: A ratio that is transiently 4:2
rather than 2:2 in females, would have some of the X-linked genes
which activate *SxlPm* at twice the level of their putative
counteracting autosomal or denominator genes. In a screen which
seeks suppression of a female-specific lethal condition due to a
decrease in numerator elements, it would require the equivalent of
two autosomal genes to be mutated to reestablish an X: A ratio
favorable for female survival. Obtaining two mutations in genes
functioning in the same process at once is unlikely, which may
have skewed the outcome of screens which sought to identify
zygotic autosomal genes. It may not be a coincidence that the only
autosomal acting component identified is *dna* [37,38]. As both *Dpn*
and *Run* bind the co-repressor *Gro* [39,40] but have opposing
effects on *SxlPm*, it has been speculated they may antagonize each
other [39,41]. Screens may have repeatedly identified *dna* as it
would be counteracting a gene expressed at its chromosomal
equivalent, since *num* is not upregulated by the male DCC.

Elevated X chromosome transcription

On a more general level, our data suggest an upregulation of
transcription of the Drosophila X, and may reflect a universal
requirement of elevated *X* chromosome expression to avoid
monosomy. Recent microarray analysis of mouse ES cells indicates
that mammalian dosage compensation is more complex than
previously thought: there is higher expression of the X
chromosome relative to the autosomes giving them equivalence,
i.e. chromosome per chromosome the X is overexpressed by about
two-fold relative to each autosome [42–44]. As differentiation
proceeds, females lose expression of one of their *X* chromosomes,
silencing it through inactivation. Put in other words, the
mammalian X chromosome is not monosomic in expression but
rather is hyperactive, and the process of dosage compensation appears to shut down elevated X chromosome transcription in females. (Hyperactive X chromosome expression in C. elegans has also been suggested [42], so dosage compensation in the hermaphrodite would then serve to lower the X chromosomes to match autosome levels).

In this regard, Drosophila would not be very different from mammals except that rather than inactivating one of the female X’s, Sxl inactivates the mechanism which upregulates X chromosome specific expression. In all cases, dosage compensation avoids tetrasomy of the X. What the components are which specifically upregulate the mammalian or C. elegans X chromosome—the Drosophila male DCC counterpart—remain to be determined.

Materials and Methods

Fly crosses

Flies were reared under uncrowded conditions on standard cornmeal medium. All crosses were done at 25 C; Ore R was the wild type control. Progeny were counted out to 8 days from the first day of eclosion. Description of genes can be found in Flybase (http://www.flybase.org/).

In situ and immunofluorescence

These were done as in Erickson and Cline [25]. The Sxl early (407 nt) and late (1039 nt) transcript specific probes were generated by the primers, respectively: 5’ GTTCCAGCTCGTGA-

\[ \text{CAAGTCC 3'} \text{and 5'} \text{ GTTTCTAAGCGATCCCCG 3'}; \text{ 5'} \text{ GGGAACCGTACACTGC 3'} \text{ and 5'} \text{ GGCCGATGCGGAT} \text{CATGTTGC 3'} (\text{17 promoter sequence removed}) \]

For hairy, the primers 5’ CCAGAACCTTGCTGCTCAT TCG 3’ and 5’ GGGAAGCGCGCTA ACCTCGTTC 3’; for sis-a the primers 5’ CAAAATGCACTACGCCGACG 3’ and 5’ GCATCGTGCTCCAACATGAG 3’ were used. All in situ were repeated at least once. Each batch was done simultaneously with an Ore R control, and had sufficient embryos so that several representatives of each cycle could be examined. M3TAP embryos [31] were stained for Sxl (mouse) and MSL-1 (rabbit) as previously described [45]. To enhance the MSL signal, the M3TAP was first bound (blocked) by the same anti-rabbit fluorescent secondary used for the anti-MSL-1 primary before addition of the primary antibody.

qRT–PCRs

Embryos were collected on apple juice agar plates for one hour and aged for the appropriate time. They were washed off the plate, dechorionated with 30% chlorox, washed extensively with 1x PBST and frozen at −80°C. RNA was extracted from the frozen embryos using tri-reagent as per manufacturer’s protocol. An additional phenol extraction was performed on the purified RNA, followed by DNase treatment. A PCR test was performed on the RNA to confirm the lack of DNA, after which 4 ug of the RNA was reverse transcribed (RT) with AMV RT at 50°C for 15 min followed by 1.5 h at 42°C. A small amount (2 ng) of Sxl primer (5’ CGT GTC CAG GTG ATC CAG GG 3’) was added to the oligo-dT mix (100 ng) per RT, as the stage specific 5’ exons of Sxl

Figure 9. Model depicting X chromosome expression levels in the two sexes. At 25°C cycle 13 is around 2 h 15 min and cycle 14 concludes around 3 h 15 min. Reduction of the DCC effect is shown as being gradual as the female mode of Sxl splicing is established, and repression of MSL-2 expression is more complete.

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Supporting Information

Figure S1 MSL-2 affects accumulation of sis-a mRNA. At sites of embryos using a sis-a probe shows early expression (cycles 9 and 10) to be slightly lower in embryos from homozygous msl-2 mothers. By cycle 12, however, the levels accumulated in embryos from msl-2 mothers were higher than wild type. Found at: doi:10.1371/journal.pgen.1001041.s001 (1.00 MB TIF)

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Author Contributions

Conceived and designed the experiments: JIH. Performed the experiments: NG MNN JIH. Analyzed the data: NG MNN JIH. Wrote the paper: JIH.
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