A sex-ratio Meiotic Drive System in Drosophila simulans. II: An X-linked Distorter

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The evolution of heteromorphic sex chromosomes creates a genetic condition favoring the invasion of sex-ratio meiotic drive elements, resulting in the biased transmission of one sex chromosome over the other, in violation of Mendel's first law. The molecular mechanisms of sex-ratio meiotic drive may therefore help us to understand the evolutionary forces shaping the meiotic behavior of the sex chromosomes. Here we characterize a sex-ratio distorter on the X chromosome (Dox) in Drosophila simulans by genetic and molecular means. Intriguingly, Dox has very limited coding capacity. It evolved from another X-linked gene, which also evolved de novo. Through retrotransposition, Dox also gave rise to an autosomal suppressor, not much yang (Nmy). An RNA interference mechanism seems to be involved in the suppression of the Dox distorter by the Nmy suppressor. Double mutant males of the genotype dox; nmy are normal for both sex-ratio and spermatogenesis. We postulate that recurrent bouts of sex-ratio meiotic drive and its subsequent suppression might underlie several common features observed in the heterogametic sex, including meiotic sex chromosome inactivation and achiasmy.

Introduction

Sex chromosomes are believed to evolve from a pair of autosomes [1–3]. An incipient Y chromosome, like an autosome, is largely euchromatic and free to recombine, except for a small region determining sex, as exemplified by species such as the papaya plant [4] and the medaka and stickleback fish [5,6]. On an evolutionary time scale, the nonrecombinating region of the Y will generally expand to include most or all of the chromosome, accompanied by an accumulation of transposable elements and other repetitive sequence, as well as mutational inactivation of most of the protein-coding genes. Only a small number of genes remain active in a mature Y chromosome, such as that in humans or Drosophila. Some Y-linked genes are vestiges of the degeneration process, while others have originated from autosomes as a result of recruiting male-specific genes such as those that function in spermatogenesis [7–10]. Accompanying the evolution of sex chromosomes, at least two problems of biological significance arise. One problem is the unequal gene dosage of sex-linked genes between the XY sex and the XX sex. Because of Y degeneration, most genes on the X have only one active copy in the XY sex but two in the XX sex. Myriad strategies to compensate the dosage inequality have been exploited by various species, and some of these mechanisms are now understood in molecular detail in model organisms of fly, worm, and mouse [11].

Another substantial but less obvious problem consists of genetic conflicts over the sex ratio among various parts of a genome, which would allow optimal transmission of their own genes. A corollary to sexual reproduction is Fisher's well-known principle that the sex ratio must be equal for a panmictic population of dioecious species [12]. However, as noted long ago, Fisher's principle applies only to autosomal genes but not to sex-linked genes. Genes linked to one or the other sex chromosome would have a selective advantage were the sex ratio in the population skewed [13]. Because of the genetic isolation between the sex chromosomes, mutations biasing the sex ratio can easily accumulate and enhance each other as long as their deleterious effects are offset by their biased transmission. Thus, the evolution of sex chromosomes leads to an intrinsic conflict among the X, the Y, and the autosomes with regard to sex ratio.

Many cases of sex ratio distortion (sex-ratio hereafter) have been documented, particularly in taxa where intensive laboratory investigation is possible [14]. Because of the biased sex ratio, suppressors unlinked to a distorter are strongly selected to restore the Fisherian sex ratio [15]. The occurrence of sex-ratio in a population can often be transient and easily escape notice. However, recurrent bouts of sex-ratio invasion and suppression can modify the genetic architecture of gametogenesis to such an extent that hybrid incompatibility can be driven to evolve among isolated populations. In other words, genetic conflicts can be a key mechanism for speciation [16–18].

Several cases of sex-ratio have been reported in D. simulans [19–23]. In a companion paper, we reported the cloning of an autosomal sex-ratio suppressor [24]. As Fisher's principle...
Author Summary

Mendel’s first law of genetics states that two alleles of a heterozygote are transmitted to the next generation at an equal ratio. The cornerstone of population genetics, this law states that the evolutionary fate of genetic variants is solely governed by their contribution to the good of their carriers. However, meiotic drive genes—which skew transmission in their own favor—can evolve under certain circumstances, even though they cause harm to the genome as a whole. Meiotic drive elements are often enriched on the two sex chromosomes (i.e., the X and the Y) because of a lack of recombination between them. Here we describe the genetic and molecular characterization of a meiotic drive distorter on the X chromosome in Drosophila simulans. This distorter apparently formed de nova from yet another new gene. To fight back against this harmful distorter, the D. simulans genome has evolved an ingenious mechanism based on DNA sequence homology. We postulate that repeated meiotic drive invasion and its suppression could be a major mechanism for genome evolution, underlying the ultimate cause for the inactivation of sex chromosome during meiosis and the occasional loss of recombination (achiasmy), which is observed only in the heterogametic (XY) sex.

Results

A sex-ratio Distorter on the X Chromosome (Dox)

We previously cloned a D. simulans gene, not much yang (nmy), polytene chromosome position 87F3), in which the homozygous male mutant displays a female-biased sex ratio. This gene belongs to the Winters sex-ratio system, one of three independent sex-ratio systems found in this species [24]. We inferred that the wild-type (Nmy) function is a suppressor of sex-ratio distortion, and that there must be a corresponding X-linked sex-ratio distorter according to Fisher’s principle of sex ratio evolution. By haplotype analysis, we find an X chromosome that did not express the sex-ratio phenotype in homozygous nmy males (Figure S1). This X chromosome was thought to have a loss-of-function mutation in the gene(s) causing sex-ratio. We designate the mutant gene as distorter on the X (dox). Other X chromosomes, including one from the stock y w+ v2 f06, did express sex-ratio when tested in the nmy background and were postulated to carry the distortion allele Dox (Figure S2).

A preliminary mapping of dox was carried out through the scheme described in Figure 1. Recombinant X chromosomes were tested for sex ratio (proportion of females to k) in the nmy background (I in G5 of Figure 1) as well as in the nmy/+ background as control (II in G5). A total of 148 X chromosomes tested could be grouped into eight genotypic classes (Figure 2A–2H). Several inferences can be drawn from the results. First, there are two and one recombinants in classes E and F, respectively, which are exceptional and thus informative in the mapping of dox, allowing it to be placed closely proximal to v at a distance of about 6% (3/51) of the w–v interval. Second, the major sex-ratio distorter shows less strength of distortion when a gene in the vicinity of f is absent. The reduced distortion can be inferred by comparing classes A (k ± standard error of the mean [SEM] = 0.803 ± 0.014, n = 22), D (0.797 ± 0.010, n = 7), and F (0.786 ± 0.020, n = 22, excluding one losing the major distorter) with class G that has a significantly lower sex ratio of 0.655 ± 0.013 (n = 24) (t-test, p = 0.001). The first three classes have similar sex ratios (analysis of variance [ANOVA], p = 0.748). We call the gene near f an enhancer of Dox (E(dox)) because it alone does not cause sex ratio distortion (class H, 0.509 ± 0.004). In light of the above reasoning, the five class B recombinants showing sex-ratio probably have an inferred genotype of w Dox v2 e(dox) (0.686 ± 0.018), the same as class G with respect to sex-ratio distorter and enhancer (t-test, p = 0.157). Finally, based on numerous SNP sites found between the two parental X chromosomes, we genotyped a selected subset of the 148 chromosomes and narrowed the location of dox to a region of 215 kb between CG15316 (8E1–4) and nej (8F7–9), which falls within an interval defined by two visible markers k and v.

Fine Mapping of dox

The fine mapping of dox began with the construction of two X chromosomes of liz3 Dox v2 f06 and y w+ dox, whose phenotype with regard to Dox was confirmed by testing in a stock background and were postulated to carry the distorting allele (independent gene belongs to the Winters system, one of three

Figure 1. Cross Scheme for Mapping dox

One of the parental X chromosomes (yw am v2 f66) has functional distorter(s), whereas the other (w) does not. Single-male matings were set up for all X chromosome recombinants in G3. All recombinants, represented by X in G4 and G5, were tested for sex-ratio in an isogenic background nt; nmy in G5 (I) as well as in a control background of nt; nmy (II). Two classes of recombinants (F and H) with the same visible phenotype were distinguished by progeny from additional crosses with y w+ v2 f06 females in G5. However, class B (+ w v 2) may be a mixture of + w + + and + w v2 +, which were not distinguished. doi:10.1371/journal.pbio.0050293.g001
Dox
the 43 recombinants was unambiguously classified as either
phenotype of Dox
lz
recombinants with crossovers between
þþ
dox
lzs
CG15316
crossovers falling between
cross in G4 was carried out at 18
again using the scheme in Figure 1 (G3 – G5) (Figure 3A). The
homozygous
nmy
Figure 2.

4.1 A y w\textsuperscript{m} v\textsuperscript{2} f\textsuperscript{66} (22) 4.1 B + w + (26)
4.1 C y w + + (6) 4.1 D + w\textsuperscript{m} v\textsuperscript{2} f\textsuperscript{66} (7)
4.1 E y w\textsuperscript{m} + + (28) 4.1 F + w v\textsuperscript{2} f\textsuperscript{66} (23)
4.1 G y w\textsuperscript{m} v\textsuperscript{2} + (24) 4.1 H + w + f\textsuperscript{66} (12)

Recombinants

Figure 2. Sex Ratios (k) Scored for the Preliminary Mapping
Each recombinant X chromosome in classes A–H (Figure 1) was tested
against nmy (filled circle) and the number of recombinants tested for each
class. Also shown are the corresponding sex ratios tested against
each other strain, with an insert of 3,833 bp found within the last 360-bp repeat.

homzygous nmy background through the scheme described
in Figure 1 (G3 through G5). A cross of + + lz\textsuperscript{2} Dox v\textsuperscript{2} f\textsuperscript{66} y w\textsuperscript{m} +
dox + + females to Ub AI D males was set up, and 324 recombinants with crossovers between lz and v were obtained.
We picked 22 lz\textsuperscript{2} and 21 y w\textsuperscript{m} v\textsuperscript{2} f\textsuperscript{66} X chromosomes with
crossovers falling between CG15316 and nge to further test
their sex-ratio phenotype in a homozygous nmy background,
again using the scheme in Figure 1 (G3 – G5) (Figure 3A). The
cross in G4 was carried out at 18 °C so that the sex-ratio
phenotype of Dox can be fully expressed in G5 [24]. Each of
the 43 recombinants was unambiguously classified as either
Dox or dox.

Four SNP markers were found in the CG15316–nje region
(Table S1), and these were used to demarcate the crossover
points for the 43 recombinants. There are two Dox and five
dox lines, with their crossovers falling between the markers
5dox_III and CI4/C17. We sequenced ~31 kb embracing this
region (Figure 3A). The two parental alleles are identical for
the 20,791 bp within the 5dox_III–CI4/C17 interval, except for
a deletion of 105 bp (Δ105) in dox (Figure 3B). We confirmed
the predicted presence or absence of the Δ105 element in the
final seven informative recombinants.

We sequenced six other D. simulans strains in the region
between the primer pair DoxF4-DoxR4 that spans the Δ105
sequence (Figure 3B; Text S3). Two types of haplotypes were
recognized. One is from the SR6 X chromosome that carries the
Paris sex-ratio distorters [24,25]. Three copies of a 360-bp
repeat were found within this haplotype. The other type is
shared by all the other strains, with an insert of 3,833 bp
found within the last 360-bp repeat.

This 3,833-bp fragment has sequences homologous to the
last three exons from the gene CG32702 of D. melanogaster. The
CG32702 ortholog is missing in the current annotation of D. simulans genome (Release 1.0, http://genome.ucsc.edu/). How-
ever, we did obtain a sequence of 18.7 kb covering the
orthologous CG32702 region in D. simulans as well as its full
length cDNA of 11,550 bp (Figure 3B and Text S1). The
transcript consists of 15 exons, largely agreeing with the
computational annotation of this gene in D. melanogaster,
except for differences in two splice sites and one extra exon
at the 5’. end.

Apparently, this 3,833-bp insert (designated Tp3833) was
duplicated and transposed from a sequence of 3,549 bp
(designated Tp3549) in the 3’ region of CG32702. Note that
one copy of the 360-bp repeat is also present next to Tp3549,
suggesting that this repeat may have facilitated the trans-
position. The last two exons and part of exon 13 (Ex13) of
CG32702 are still intact in the Tp3833 region (CG32702d, Figure 3B).

Sequences from the homologous region between DoxF4-
DoxR4 were obtained from one strain of each of the sibling
species D. sechellia, D. mauritiana, as well as D. melanogaster. All
species resemble SR6 in having various copy numbers of the
360-bp repeat (Figure 3C). A phylogenetic analysis of these
360-bp repeats shows a monophyly of the eight copies from D.
melanogaster, but a reticulate relationship among the rest,
suggesting shared evolutionary history of this intergenic
region in the D. simulans clade (Figure S3). However, it
remains to be determined whether a Tp3833-like sequence
on certain chromosome in D. mauritiana. The existence of a functional
Nmy strongly suggests that a corresponding sex-ratio distorter
like Dox may still segregating in this species [24].

Within Tp3549, a fragment of 1,458 bp replaces a fragment
of 1,408 bp downstream of the 3’ end of CG32702 in D.
melanogaster, and these two sequences have no homology
(Figure 3B). Database searches suggest that the 1,458-bp
sequence is absent from D. yakuba, D. yakuba, and D. erecta,
but some similar fragments of 300–600 bp can be found dispersed
in these genomes, often in multiple copies. Transcripts within
Tp3549 were detected, and a new gene, which we designate as
Mother of Dox (MDox), is defined (see below). Tp3549 and
Tp3833 thus represent a fluid portion of the
Drosophila
genome that occasionally gains new functions.
Gene Structure of Dox

Initially the truncated version of CG32702 (CG32702d) appeared to be the best candidate for Dox because of its perfectly conserved open reading frames (ORFs) and intron–exon boundaries (Figure 3B). However, we have not detected transcripts from CG32702. Extensive 5'-rapid amplification of cDNA ends (RACE) experiments using gene-specific primers targeting CG32702d all failed. A 3'-RACE experiment did recover cDNAs, but they could be transcribed from the 3'-end of CG32702, not of CG32702d. There is a divergent site (C/JA) between Tp3549 and Tp3833 in the 1,919-bp region corresponding to the last three exons of CG32702. Using the primers CG32702seqF26 and CG32702seqR26 (F26 and R26 in Figure 4; Text S3), only the CG32702 sequence can be
amplified from cDNA (Figure S4). CG32702d is therefore unlikely to be transcribed or its expression is too low to be detected by reverse-transcription PCR (RT-PCR).

On the other hand, we have detected transcripts that cover the region of Δ105 in the opposite direction of CG32702d (Figure 4). Two transcripts from the allele Dox were recovered with either four or three introns. Their full lengths are 2,781 bp and 2,690 bp, respectively. From the allele dox, we have also recovered two full-length cDNAs identical to those of Dox, except that the exon III, 42 bp in length, is missing because of the deletion Δ105 (Figure 4). This 42-bp element is tandemly repeated in the cDNA of Dox but has only one copy in that of dox. Within Tp3549, we have also recovered a full length cDNA antisense (2,564 bp) to the 3' end of CG32702 (Figure 4). MDox like Dox also has three introns in exactly the same sites, as well as the tandem repeats of 42 bp present in its cDNA (Figure 4).

Surprisingly, all transcripts from the Dox and MDox loci have very limited coding potential. The largest ORFs of MDox in all three frames are shown in Figure 4, and all but one fail to match any known sequences by BLASTX searches through the nr database. The one ORF that was predicted by Genscan encodes 62 amino acids (aa), and this ORF matches with primers that can distinguish between these two genes. The intron–exon boundaries of these two genes are largely the same between Dox and MDox, so they are annotated together. However, the 91-bp intron I of Dox has never been found from MDox. Several alternative splicing forms including earlier termination have been found. In some RT-PCR products, introns III and IV of MDox are not spliced out. Other splice forms may yet be uncovered. The 105-bp deletion (dashed line) in dox causes the loss of exon III in the otherwise identical transcripts as compared to Dox. Identical tandem repeats of a 42-bp element (green and red rectangles) are present in both Dox and MDox transcripts. It is unclear which ORF, if any, is actually translated in each transcript. All potential ORFs larger than 100 bp are shown for all three reading frames (thick black lines on thin red lines). Two ORFs of 157 and 107 aa (located in region 15F7, proximal to the gene f). Similarly, only one of the largest ORFs of Dox has BLASTX hits. This is again the ORF of 69 aa predicted by Genscan and is homologous to the 62 aa ORF of MDox, although only part of it matches to CG8664 (38/44 or 86% identity) due to a frameshift mutation. CG8664 has no known biological functions or phenotypes. In the orthologous position of CG8664 in the current D. simulans genome annotation, a fragment of 2,084 bp, instead of a CG8664 homolog, has been found. Part of this 2,084-bp fragment, approximately equivalent to the 1,458-bp element mentioned above (Figure 3B), is recognized and has a high similarity (99.3% identity) to a region within Dox. If the existence of this partial paralog of Dox in the f region is confirmed by experiment, it would be interesting to test it as the candidate gene for E(Dox).

The pair of tandem 42-bp elements essential for a functional Dox are located within an ORF of 157 aa (Figure 4). A 14-aa domain encoded by this 42-bp element has no known functions. If this ORF is ever translated, the tandem 14-aa domains appear to be required for the wild-type function of Dox as a sex-ratio distorter. Coding or noncoding, the molecular mechanism underlying the effect of Dox in rendering V-bearing sperm dysfunctional awaits further experimental investigation. The wild-type function of MDox is not known, although the presence of the critical tandem repeats of the 42-bp element suggests its biochemical similarity to Dox.
Nmy Suppresses Dox through Homology Effect

The phenotype of Dox as a sex-ratio distorter is uncovered if its suppressor, Nmy, is nonfunctional (Figure 5A) [24]. The Nmy transcript appears to form a stem-loop structure with a double-stranded RNA (dsRNA) stem of 345 bp, and small interfering RNAs (siRNAs) produced from the dsRNA stem could target and suppress Dox [24]. Hence, homology between Dox and Nmy is anticipated. Indeed, sequence comparisons suggest that Nmy originated from Dox through a retrotransposition event [24]. Specifically, the 345-bp dsRNA sequence from Nmy has extensive homology to the potential ORF of both Dox and MDox that contains exon III (Figures 5B). The critical 42-bp element falls within an 85-bp region that has a perfect match with the stem region (positions 264–390 in the alignment of Figure 5C). Whether or how either Dox or MDox is regulated by these hypothetical siRNAs is currently under investigation.

The possibility that Dox evolved solely as a sex-ratio distorter and for no other reasons is supported by the normal phenotype of the double mutant dox; nmy. We have shown previously that the etiology of the Winters sex-ratio is the degeneration of the Y-bearing spermatids during their maturation, as observed both through transmission electron microscopy (TEM) and through light microscopy [24]. We carried out similar observations of the spermatogenesis of dox; nmy males at 16 °C. All stages of spermatid maturation appear to be normal as also found in Dox; Nmy wild type (Figure 6A–6D, in comparison to Figures 4 and 5 in [24]). Quantitatively, 5.7% (n = 1108) of spermatid heads appear to be abnormal under TEM, in a proportion similar to wild-type Dox; Nmy (5.8%, n = 1903). With 4',6-diamidino-2-phenylindole (DAPI) staining, no abnormal spermatid head was observed among the 1,416 heads examined. Consistent with these cytological observations, the sex ratio of progeny from the dox; nmy males at 16 °C was 54%. As a comparison, a dox; Nmy male was similarly examined. No abnormal heads were observed among 1,058 spermatids, and the sex ratio when tested was also 54%. All the evidence together suggests that Dox is not an essential gene and is fully dispensable. Nmy is also dispensable if Dox is absent.

Evolution of sex-ratio in D. simulans

The fate of a sex-ratio system can be loss, fixation, or stable polymorphism. Apparently, the Winters sex-ratio is still segregating in D. simulans [24]. The same is true for the Paris sex-ratio system that has been found in the same species [21]. Evidence from molecular population genetics shows that the Paris SR6 X chromosome has swept through African and Indian Ocean islands only recently (less than 20 thousand years ago [ka]) [25]. The presence of a functional Nmy suppressor in D. mauritiana suggests that the Winters sex-ratio evolved in the ancestor of the D. simulans clade [24]. The following genetic evidence will enforce the above conclusions and help to compare the evolutionary history of these two sex-ratio systems.

We have introgressed the Y chromosome of D. sechellia into D. simulans (D. sim Y[sech]) in a background isogenic to simB (Figure S6). The success of this introgression was confirmed by fingerprinting with a Y-specific probe Y5g (Figure 7A and Figure S2). The D. sechellia Y chromosome was thus tested against the driving effect of either Dox (Winters) or SR6 (Paris). The Dox/Y[sech] male expresses sex-ratio if nmy is homozygous, but does not if one copy of the functional Nmy gene is present (Figure 7B). Hence the D. sechellia Y chromosome is equally sensitive to Dox as is the D. simulans Y chromosome. Intriguingly, SR6/Y[sech] males exhibit male-biased sex ratio distortion (k = 0.33). Unfortunately, similar introgression of the Y chromosome from D. mauritiana cannot be made because D. sim Y[mau] is sterile [26].

The above observations are consistent with the earlier estimate that the origin of Dox predates speciation among D. simulans, D. mauritiana, and D. sechellia about 200–400 ka [27], whereas SR6 arose in D. simulans after the speciation [24,25]. Assuming that an Y-linked distorter causing male-biased sex ratio distortion has little chance of persistence as compared to an X-linked one causing female-biased sex ratio distortion [13,28], we suppose that the Y[sech] still bears sensitive sequence to Dox as in the ancestral Y of the three species. We suggest that the male-biased sex ratio expressed by SR6/Y[sech] is a sign of evolutionary independence between the Y[sech] and the SR6 distorters.

The etiology of SR6 has been attributed to loss or breakage of the D. simulans Y chromosome during meiosis II in SR6/Y[sim] males [29,30]. Our results support earlier findings by showing that male progeny from the SR6/Y[sim] father are sterile at a frequency of 19%. However, the frequency of sterile male progeny from an SR6/Y[sech] father is only 3% (Fisher’s exact test, p < 0.0001), a number that is not different from the control (2%, Fisher’s exact test, p = 0.284) (Figure 7C). It is possible that SR6 does not cause loss or breakage of the Y[sech], hence the etiology of the male-biased sex ratio may be different from similar male sex-ratio (msr) cases reported in D. pseudoobscura [31] and in D. affinis [32], where a large number of nullo XY sperm are produced. The unique cytological mechanism underlying the male-biased sex ratio in SR6/Y[sech] males again suggests that the unequal sex ratio is a neomorph created by a genetic incompatibility between the two chromosomes, rather than a shared evolutionary history of sex-ratio.

Discussion

A sex-ratio meiotic drive distorter, Dox, has been identified. Dox is a new gene that arose from yet another new gene MDox. Intriguingly, both MDox and Dox appear to be transcribed as noncoding RNAs or as mRNAs with very limited coding potential. Dox was also the precursor for the origin of the autosomal suppressor Nmy by a retrotransposition process. Dox functions solely as a sex-ratio distorter and is not essential, because the mutant dox males have normal sex ratio and spermatogenesis. The Dox/Nmy system is the first that has been characterized at the molecular level for sex-ratio meiotic drive, a widespread biological phenomenon that is promoted by the evolution of heteromorphic sex chromosomes.

Nmy Suppresses Dox, Possibly through an RNAi Mechanism

The gene structures of Dox and Nmy strongly suggest that an RNAi mechanism is involved, just as in numerous transgenic studies where inverted repeats (IR) were used to silence target genes in eukaryotes (e.g., [33]). Most likely, the suppression of Dox by Nmy is through a classic RNAi pathway, also known as post-transcriptional gene silencing (PTGS), which has been under intensive genetic and biochemical studies (reviewed in
Figure 5. Homology Among Dox, Mdox, and Nmy

(A) Schematic of the Winters sex-ratio system. An autosomal suppressor, Nmy, is apparently a new gene created by a 2,041-bp insertion (red) in the gene CG14370. The insert contains a pair of almost perfect IRs of 345 bp (red arrows, IR' and IR'') and they are required for the suppression of Dox. Nmy originated through a retrotransposition event from Dox, because all of the 2,041 bp consists of paralogous sequence from cDNA of Dox (red line) and the 5' region upstream of Dox transcription (dashed red line) [24].

(B) A detailed comparison between transcripts of Dox and Nmy. Paralogous sequences are in red. The 5' and 3' ends of Nmy (black line) are from CG14370. IR' might have duplicated from IR'' after the retroposition [24], while some sequences (black between the two small arrow heads) in Dox no longer have paralogs in Nmy. The 42-bp elements are shown in red and green color, as in Figure 4. The dox allele has lost one of the 42-bp elements in the transcript. A dsRNA stem is presumably formed between the two IRs. The critical region marked as ''C'' is detailed in (C).

(C) The critical region ''C'' with a base-by-base comparison among Dox, Nmy, and Mdox. Identical bases or amino acids are represented by a period, and deletion by a dash, and divergent bases are in red. This region starts at position 844 (778) of the Dox (Mdox) transcript and the beginning of IR', respectively. IR' and IR'' are identical except for a 6-bp (TAGGGA) deletion in IR' (cyan). The two tandem 42-bp elements are also shown in red and green, respectively on the Dox sequence. The amino acids encoded by the ORF in this region are shown in the top (Dox) and bottom lines (Mdox), respectively.

These two ORFs have an amino acid identity of 94/107 (88%) and similarity of 100/107 (93%) (underlined).

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In essence, 21–23 nucleotide (nt) siRNAs processed from dsRNA are responsible for guiding the active RNA-induced silencing complex (RISC) to homologous mRNA, resulting in the latter's subsequent cleavage [35]. The PTGS model for Dox/Nmy interactions can be readily tested by comparing the steady-state mRNA levels of Dox between Dox; Nmy and Dox; nmy males, and by detecting the binding of specific siRNAs with the RISC components. Because PTGS happens in the cytoplasm, and spermatid nuclei within a cyst share the same cytoplasmic syncytium, the final gene product of Dox likely has a localized deleterious effect in the Y-bearing spermatid nuclei, whereas the presence of a Y or absence of an X must provide the primary cue that eventually leads to abnormal maturation of the Y-bearing sperm heads.

Alternatively, a different type of RNAi mechanism could be involved in the Dox/Nmy interaction. A class of small RNA in the size range 24–29 nt has been identified as silencing intermediates in the control of repetitive sequences such as retrotransposons [36]. Unlike the classic RNAi machinery, the core proteins do not require DCL-1, DCL-2 and AGO2, and a different type of RNAi pathway (repeat-associated small interfering RNAs or rasiRNAs) has been proposed [36]. The rasiRNA pathway has also been shown to be responsible for silencing a possible cryptic sex-ratio meiotic drive distorter, Ste, in D. melanogaster [37]. The Y-linked Su(Ste) suppresses the deleterious effects of the X-linked Ste, including male sterility and meiotic drive [38]. Both Ste and Su(Ste) consist of repeats that share extensive homology, and rasiRNAs were shown to be the information carrier for the target specificity [36,39,40].

A third possible mechanism for silencing Dox might be at transcriptional level in a manner of co-suppression as first observed in plant transgenics, where the expressions of both

Figure 6. Spermatogenesis of Double-Mutant dox; nmy Is Normal

(A–C) TEM images of sperm maturation. (A) Early post elongation stage of a spermatid head (upper) and tail (lower). Normal nuclear condensation can be seen apposed to rows of microtubules (white arrowheads), while nucleoplasm is eliminated (v). (B) Late post elongation stage of spermatid heads with homogeneously condensed nuclei (upper left and right). Also note the normal head-tail alignment (middle) and the tail (lower right). (C) Post individualization stage of a sperm bundle with 61 normal tails and one degenerated tail in the waste bag (WB). (D) DAPI images of 64 spermatid heads in a bundle at the stage of post elongation. Abbreviations used in annotation: AX (axoneme); BB (basal body); mM (minor mitochondrial derivative); MM (major mitochondrial derivative); mS (minor strip); MS (major strip); MT (microtubule); WB (waste bag). Scale Bars: 500 nm (A–C); 20 μm (D). doi:10.1371/journal.pbio.0050293.g006
an endogenous gene and the homologous transgene were down-regulated [41]. This type of transcriptional gene silencing (TGS) has been demonstrated in *Drosophila* [42,43], and it requires physical contacts between homologous sequences and Polycomb group (PcG) proteins [44]. Note that an intact pair of inverted repeats is not required for an efficient TGS (e.g., [44]). In our case, *nmy* [142] is a loss-of-function mutation that does not have an intact pair of inverted repeats but does have a 1.2-kb sequence paralogous to *Dox*, arguing against this type of TGS as a strong candidate mechanism for silencing *Dox* [24].

**Evolutionary Cause for Meiotic Sex Chromosome Inactivation and Achiasmate Meiosis: The Drive Hypothesis**

The X chromosomes of many species are condensed precociously in prophase of meiosis I when active transcription peaks in the autosomes [45]. The existence of meiotic sex chromosome inactivation (MSCI, also known as X chromosome allecocy) has been well established in several model organisms, either directly through the observation of precocious heterochromatin sex bodies [46–48], or indirectly from genetic analysis of X-autosome translocations [49] as well as with genome-wide gene expression studies [50–55]. Recently, MSCI has been demonstrated in *D. melanogaster* by assaying transgenic expressions in the X chromosome [56]. Though sex bodies are the direct evidence for MSCI, they have not been observed in most species examined so far, including *Drosophila* [57]. The status of MSCI may be assayed with more sensitive methods such as the detection of histone modifications that relate to transcriptional activity (e.g., [48]).

There are several hypotheses for the evolution of MSCI. One hypothesis is that MSCI evolves because of a need to suppress recombination between the two sex chromosomes [58]. Another hypothesis was coined as the SAXI hypothesis (sexual antagonism and X inactivation). Because the X spends 2/3 of its evolutionary history in females, the X will be depleted of male-specific genes, and a feminized X would be under selection to be silenced during male meiosis [59]. A third hypothesis has been suggested in the light of the discovery of the meiotic silencing of unpaired DNA (MSUD) in *Neurospora crassa* [60,61]. MSUD is reasoned to have evolved for defending against invasion of transposons [62–64].
connection between MSCI and MSUD is supported by the silencing of unpaired chromosomal fragments in the mouse and worm [65,66].

Each of the three hypotheses for MSCI captures only some specific features consequent to the evolution of sex chromosomes, and hence provides only a partial and proximate explanation for the evolution of MSCI. The ultimate cause of MSCI, of course, must be the degeneration of the Y or W chromosome. Following this line of reasoning, we propose yet another hypothesis that we call “the drive hypothesis” for the evolution of MSCI: there is a constant requirement for silencing sex-linked genes including potential sex-ratio distorters during meiosis because of an intrinsic conflict over the sex ratio within a genome accompanying sex chromosome evolution.

We believe that the drive hypothesis provides mechanistically superior explanations to the other hypotheses for the evolution of MSCI for the following specific observations: (1) There is a predominant pattern of generating testis-specific genes through retrotransposition between the X chromosomes and autosomes [67–70]. Some of the retrotransposed sequences might be involved in creating new distorters and suppressors. (2) MSCI in the worm and mouse is dependent on a putative RNA-dependent RNA polymerase, suggesting the involvement of an RNAI-like mechanism [71]. The D.odl Nmy case provides a strong mechanistic connection between meiotic drive and the evolution of MSCI. Admittedly, it is not an easy task to test empirically and discriminate among the hypotheses described above. Although the drive hypothesis emphasizes the importance of meiotic drive in the evolution of MSCI, other sex-chromosome–specific features such as suppressed recombination, degeneration, and depletion of sex-specific genes could well be different facets of the same evolutionary process. These features may share biochemical components and have reinforced each other over evolutionary time.

In addition to MSCI, achiasmatic meiosis is another evolutionary oddity that might also be rooted in sex chromosome evolution. An achiasmatic meiosis has no crossovers between homologs, even of autosomes. Between 20 and 30 independent evolutionary occurrences of achiasmatic meiosis have been recorded, and all of them are observed in the heterogametic sex [72], with one possible exception in the caddis fly Glyphotaelius pellulidus [79]. Consistent with this observation, there are only two independent origins of ZW achiasmy [72]. Another explanation for the absence of Z allocyly might be due to a lack of selection pressure to evolve sex-ratio distorters on the Z chromosome, because male-biased sex-ratio distorter cannot persist long in a population [13], therefore it might have left little trace of impact on genomic evolution prior to its disappearance. The question of whether the Z chromosome lacks MSCI deserves special attention because its general absence would be inconsistent with the SAXI or MSUD hypotheses.

Materials and Methods

Fly stocks. The D. simulans stocks are: (1) y w<sup>ex</sup> v<sup>1</sup> f<sup>16</sup> from the Tucson Drosophila Stock Center; (2) w; and simB (w; nt; II) [80]; (3) C(1)RM y w<sup>ex</sup> and UlbdD from J. Coyne; (4) SSR12-2-7 (w; nt; nmy) [24]; and (5) Paris sex-ratio X chromosome SR66 and its standard ST8 as described previously [81]. The ST6 and ST8 X chromosomes are maintained by backcrossing males to females of the stock C(1)RM y w<sup>ex</sup> every generation. The D. sechellia stock 3588 is from A. Clark [23].

The D. mauritiana × D. simulans introgression lines have been described before [18,80]. The following lines were used in this study: heterozygous introgression lines P40–46 nmy, P58–11 nmy, P58H77 Nmy, P40L12 Nmy, and P40B13 Nmy, all having the genotype w; nt; III (Nmy) where P represents the various semi-dominant P-element transgenes P[w] marking the introgressed D. mauritiana material nearby, and III (Nmy) represents the third chromosome III with Nmy. These chromosomes are maintained by backcrossing PII males to simB females every generation. The SSR (skewed sex ratio) line Q15.3 is from a previous D. simulans × D. sechellia hybridization experiment [23]. Several stocks were constructed for this study: C(1)RM y w<sup>ex</sup> and nt; III by backcrossing females of C(1)RM y w<sup>ex</sup> to simB males for >19 generations. C(1)RM y w<sup>ex</sup> dox; nt; nmy was constructed through a scheme described in Figure S5. Another stock, w<sup>ex</sup> y[wach]; nt; III, which is isogenic to simB except that the Y chromosome is from D. sechellia 3588, was constructed through a scheme described in Figure S6. The stock y w<sup>ex</sup> was constructed from the stocks w; e and y w<sup>ex</sup> v<sup>1</sup> f<sup>16</sup>. Another stock, C(1)RM y w<sup>ex</sup> w<sup>ex</sup> f<sup>16</sup>, was constructed from C(1)RM y w<sup>ex</sup> w<sup>ex</sup> f<sup>16</sup>.

Fly work. All flies were reared on cornmeal-molasses-agar medium sprinkled with yeast grains at room temperature (22 ± 1 °C) unless otherwise indicated. The sex ratio of a male was scored by mating this male with three tester virgin females, usually of the stock w<sup>ex</sup>, for 7 d before clearing all adults. The progeny were sexed and counted three times until the 19th day. Sex ratio (k) was calculated as percentage of females.

Molecular biology. SNPs were discovered by sequencing 500–1,000-bp PCR products from relevant X chromosomes. The primers were designed by targeting the D. melanogaster genome (http://www.flybase.org), and the virtual PCR products were compared to the D. simulans sequences (http://genome.wustl.edu/tools/blast) for correcting any mismatches within the primers (Table S1). Genotyping was done...
directly by sequencing. Some other key reagents/kits are: LA Taq long PCR kits (Takara); EZ-Tn5 Insertion Kit for sequencing large DNA fragments (Epipcentric); Lambda ZAP II vector for genomic library (Stratagene); TRIZOL Reagent for RNA isolation, SuperScript II Reverse Transcriptase and 3′ or 5′-RACE kits (Invitrogen).

**Cytology.** Light microscopy and TEM procedures have been described previously [24].

**Supporting Information**

**Figure S1.** How Was the sex-ratio Distorter Discovered? Q15.3 is one of the original SSR (skewed sex ratio) lines reported [23]. This line had been losing the strength of sex-ratio distortion by August 2002 ($k = 0.595; n = 10$). One male, however, sired an all-female brood (109 Ff). In another test, 150 eggs sired by a single male developed into 126 female adults. All-female broods are very rare for any stocks including the wild type. We were curious whether some new sex-ratio mutations had been invading the Q15.3 stock. We crossed P40–36 males (w; P[w+] nmy/Nmy) en masse to females from the latter all-female brood (A, G2). The stock P40–36 nmy was constructed during the positional cloning of nmy [24]. A total of 258 males at G3 were singly mated to three w; e females to score sex ratio, and the genotypes of these males can be readily grouped into 12 phenotypic classes out of 16 possible genotypes (A). Twenty-two males (marked with *) expressed sex-ratio ($k \geq 0.596 \pm 0.004$, which is significantly stronger than a typical nmy male tested at the room temperature with $k$ ranging from 0.47 to 0.56). In total, nine out of 225 males sired all-female broods. This observation suggests that the distorter on the X chromosome from Q15.3 is a hypermorph or there are other new enhancers for sex-ratio. However, the observed numbers of genotypes 1 and 4, and that of genotypes 9 and 12, are significantly smaller than expected (3.955 versus 27.9/55 from the genotypes 1–4, G-test, $p < 0.001$; 19/50 versus. 29.5/59 from the genotypes 9–12, $p < 0.01$, regardless of the magnitude of the cross-over rate, s, between e and nmy). One explanation is that the distorter on the X chromosome from w; e might have lost its wild-type function. This explanation is supported by a tight correlation of the sex-ratio phenotype with the X chromosome from Q15.3 (w”) than that from w; e (w) (19/59 from genotypes 9–12 versus 3.95/55 from genotypes 1–4, $p = 0.003$). Assuming the crossover rate between w and a sex-ratio distorter (D) is r, and there is a loss-of-function mutation (d) from the stock w; e, the current observation can be adequately explained as detailed in (B). Note that the genotypes 5–8 and 13–16 in (A) are not informative. From the genotypes 11.4–2.2, r and s can be estimated as 10.98% and 32.53%, respectively. From genotypes 9.1–12.2, these two estimates are 37.53% and 22.79%, respectively. The values of s are consistent with the position of nmy, which is roughly in the middle of Ubx–pe interval [24]. The two latter mutations are 25.8 cm and 44.9 cm diest from e, respectively. D could be anywhere on the X proximal to cv (1–19.3; 11.1 cm proximal to w) [82].

**Figure S2.** Testing the X Chromosome in the nmy Background This scheme was used to verify that the X from the stock w; e has a loss-of-function mutation for the sex-ratio distorter. In G1, the * symbol represents either P38–11 nmy or P38H77 Nmy and the X symbol represents the X chromosome from any stocks including the following: w; wt, III; w; e, and w; u″ v ″ f ″. In G3, the X chromosomes were tested either in unsuppressing (nmy/nmy) or suppressing (nmy/ Nmy) background. In nmy/nmy background, the X chromosome from w; wt; III (simB) and y u″ v ″ f ″ expressed strong sex-ratio, whereas the X from w; e only expressed very weak sex-ratio (t-test, * $p < 0.05$; *** $p < 0.001$).

**Figure S3.** Phylogenetic Analysis of the 360-bp Repeats among the D. melanogaster Sibling Species The phylogenetic tree was constructed using MEGA 3.1 with Kimura 2-parameter and Neighbor-Joining algorithm, and was evaluated by bootstrap [83]. These repeats are defined in GenBank accessions EF596889–EF596893, and AE014298.4 (957.1245.1246.1604.1605.1963.1978.2393.2681.2682.3040.3041.3399.3400.3758). The eight repeats from D. melanogaster form a single cluster. However, all the repeats from the other three species are intermingled, suggesting a history of gene conversion and/or incomplete lineage sorting.

**Figure S4.** No Transcripts from CG23702d Were Detected The primers CG23702dF26 and CG23702dR26, which flank both the divergent base and intron 13 (Figure 4), were used to amplify a 774-bp fragment from genomic DNA (gDNA) and a 706-bp fragment from cDNA. The PCR products were cut with BsoWI that has a restriction site at position 463 on the CG23702 allele but not on the CG23702d allele. No cDNA band of 706 bp was detected, suggesting there is no transcription across intron 15 in CG23702d. M indicates DNA size marker.

**Figure S5.** Construction of a Double-Mutant Stock for dox and nmy, C21RN y w Doxl w; nt; nmy In G2, P40L12 nmy can have recombination with III (Nmy) to produce P40L12 Nmy, which in turn can have recombination with nmy at G4. This rare possibility was excluded after G5 by Southern blots.

**Figure S6.** Introggression of the Y Chromosome of D. sechellia into Pure D. simulans Background (A) The introgression scheme used here is different from previous ones [84,85]. The D. sechellia Y chromosome (Y[sech]) was substituted into a pure D. simulans background that is isogenic to simB. Y[sech] comes from the line 3588 of D. sechellia. The symbol # represents possible hybrid chromosome between D. simulans and D. sechellia. P58–11 provides a dominant marker.

(B) A schematic of the Y chromosome (modified from [86]). To verify that the Y introgression line D. simulans Y[sech] is authentic, we developed a 3.4-kb Y-specific probe (Y[5g]) that was used to fingerprint the Y chromosomes from D. melanogaster, D. simulans, D. mauritiana, and D. sechellia. D. melanogaster sequences from five Y-specific genes (three from k5, k6, and k2, and two possibly from k1 and k2) were used to design PCR primers [87,88]. The five Y-specific PCR products from D. simulans are as follows (size in bp): k5F-k5R (670), k2F-k2R (990), k13F-k13R (879), k1F-k1R (575), and k2F-k2R (351). A PCR trick was used to join two DNA fragments as follows: In the first PCR, a chimera primer, which consists of the reverse primer of the first fragment and the antisense forward primer of the second fragment, was used to make an anchored fragment. In the second PCR, the forward primer of the first DNA and the reverse primer of the second DNA were used to amplify a product consisting of these two DNA fragments, with a mix of the anchored fragment and the second DNA as template. Y[5g] was made by joining the above five fragments sequentially, and was confirmed by sequencing. Southern blots using Y[5g] show species-specific pattern for the Y-chromosome (Figure 7A).

**Table S1.** Primers Used for SNP Markers

**Text S1.** The Gene CG23702 and Its Transcript in D. simulans

**Text S2.** The Coding Potential of Dox and MDox

**Text S3.** Some Primers Used in This Study

**Accession Numbers**

All sequences have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and have been assigned the accession numbers EF596886–EF596899.

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