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ORIGINAL ARTICLE

Natural variation of the Y chromosome suppresses sex ratio distortion and modulates testis-specific gene expression in *Drosophila simulans*

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X-linked *sex-ratio* distorters that disrupt spermatogenesis can cause a deficiency in functional Y-bearing sperm and a female-biased sex ratio. Y-linked modifiers that restore a normal sex ratio might be abundant and favored when a X-linked distorter is present. Here we investigated natural variation of Y-linked suppressors of *sex-ratio* in the Winters systems and the ability of these chromosomes to modulate gene expression in *Drosophila simulans*. Seventy-eight Y chromosomes of worldwide origin were assayed for their resistance to the X-linked *sex-ratio* distorter gene *Dox*. Y chromosome diversity caused males to sire ~63% to ~98% female progeny. Genome-wide gene expression analysis revealed hundreds of genes differentially expressed between isogenic males with sensitive (high sex ratio) and resistant (low sex ratio) Y chromosomes from the same population. Although the expression of about 75% of all testis-specific genes remained unchanged across Y chromosomes, a subset of post-meiotic genes was upregulated by resistant Y chromosomes. Conversely, a set of accessory gland-specific genes and mitochondrial genes were downregulated in males with resistant Y chromosomes. The *D. simulans* Y chromosome also modulated gene expression in XXY females in which the Y-linked protein-coding genes are not transcribed. The data suggest that the Y chromosome might exert its regulatory functions through epigenetic mechanisms that do not require the expression of protein-coding genes. The gene network that modulates sex ratio distortion by the Y chromosome is poorly understood, other than that it might include interactions with mitochondria and enriched for genes expressed in post-meiotic stages of spermatogenesis.
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INTRODUCTION

The *Drosophila* Y chromosome is an unusual molecule. In *Drosophila melanogaster* the Y chromosome is ~40 megabases but harbors only ~14 protein-coding genes. All of the Y-linked genes with known phenotypes are essential for spermatogenesis and male fertility (Carvalho *et al.*, 2009; Krsticevic *et al.*, 2010). The Y chromosome is otherwise composed of multimegabase-long segments of repetitive sequences, including satellite repeats and transposable elements. Because of this feature, the Y chromosome is maintained throughout most of the cell cycle as highly condensed heterochromatin. Expression of the few protein-coding loci requires major chromatin remodeling in the testis (Ashburner *et al.*, 2005). The *D. melanogaster* Y chromosome also harbors a functional rDNA array that comprises hundreds of active rRNA genes and non-transcribed spacer repeats (Bonaccorsi and Lohe, 1991), the latter constituting the paring sites of the X and Y chromosomes during male meiosis (McKee *et al.*, 1992; Ren *et al.*, 1997). In *Drosophila simulans*, a sibling species of *D. melanogaster*, the rDNA array on the Y chromosome consists only of the non-transcribed spacer repeats (Brosseau, 1960; Lohe and Roberts, 1990), which are presumed to be the X-Y pairing site during meiosis in this species. The content of protein-coding genes is conserved in the Y chromosome of *D. simulans* and *D. melanogaster* (Koerich *et al.*, 2008).

According to the canonical theory, sex chromosomes have evolved from a homologous pair of autosomes. The X and Y chromosomes are therefore expected to share homologous genes, although homology might be limited at advanced stages of X-Y divergence. X-Y chromosome homology is evident in some *Drosophila* species, such as *Drosophila miranda*. In this species, a young pair of sex chromosomes has only recently evolved from autosomes. As there is no recombination in *Drosophila* males, the neo-Y chromosome has lost genetic diversity and accumulated deleterious mutations, in line with the expected evolutionary trajectory of non-recombining chromosome (Bull, 1983; Rice, 1987; Charlesworth and Charlesworth, 2000; Bachtrog *et al.*, 2008; Kaiser *et al.*, 2011). For most *Drosophila* species, however, the homology between the X and Y chromosomes is very limited. For example, the Y chromosome from *D. melanogaster* and *D. simulans* do not share protein-coding genes with their respective

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X chromosomes (Carvalho *et al.*, 2009). Hence, it has been suggested that the Y chromosome from these species might have originated from B chromosomes (Hackstein *et al.*, 1996; Carvalho and Clark, 2005). In any circumstance, a consensus is emerging that the *Drosophila* Y chromosome is not an inert and passive portion of the genome. Instead, recent studies paint a picture of a dynamic chromosome (Carvalho, 2002; Lemos *et al.*, 2008; Carvalho *et al.*, 2009; Kaiser *et al.*, 2011). A survey of the 14 Y-linked genes of *D. melanogaster* across 12 *Drosophila* species found only three genes present in all species and seven of them were acquired within the past 63 million years (Koerich *et al.*, 2008).

The notion that the Y chromosome has an active role in the genome has been further supported by studies demonstrating its contribution to diverse phenotypes, in spite of the very few protein-coding genes residing on this chromosome. The Y chromosome has been shown to regulate organismal traits, such as male fertility (Chippindale and Rice, 2001), sex ratio distortion (Carvalho *et al.*, 1997; Montchamp-Moreau *et al.*, 2001), thermal adaptation (Rohmer *et al.*, 2004) and behaviors (Stoltenberg and Hirsch, 1997; Huttunen and Aspi, 2003). The regulatory bases for these phenotypes have been suggested by genome-wide gene expression studies in *D. melanogaster* (Lemos *et al.*, 2008, 2010; Jiang *et al.*, 2010; Paredes *et al.*, 2011). Transcription of hundreds of genes is altered by Y chromosome variants, which differ in their ability to modulate epigenetic states across the genome (Lemos *et al.*, 2010). These genes are clustered into functional classes related to mitochondria, immunity, as well as chromatin structure and maintenance (Lemos *et al.*, 2008, 2010; Paredes *et al.*, 2011). Variable titration of nuclear proteins by the Y chromosome might cause imbalances in the nucleus and consequently the modulation of gene expression (Platero *et al.*, 1998; Janssen *et al.*, 2000).

Here we studied the ability of polymorphic Y chromosomes of *D. simulans* to modulate genome-wide gene expression levels and the *sex-ratio*. *Sex-ratio* describes a genetic phenomenon in which the offspring sex ratio deviates from the 50% female expectation. At least three sex ratio distortion systems have been uncovered in *D. simulans*, namely the Paris, the Durham and the Winters systems (Tao *et al.*, 2007b). In the Paris *sex-ratio* system, the Y chromosome has high frequency of breakage and non-disjunction during meiosis II (Cazemajor *et al.*, 2000). Consequently, the Y-bearing gametes fail to complete spermatogenesis, thus leading to a functional sperm pool dominated by X-bearing sperm and female-biased sex ratio in the offspring (Montchamp-Moreau and Joly, 1997; Montchamp-Moreau, 2006). In the Durham system, in addition to Y-breakage in meiosis II, the autosomal suppressor also has pleiotropic effect on male fertility (YT, unpublished). In the Winters system, the Y chromosome appears to undergo normal disjunction, but post-meiotic spermiogenesis of the Y-bearing gametes is disrupted (Tao *et al.*, 2007a). Distortion is caused by the X-linked gene *Dox* (*Distorter on the X*; Tao *et al.*, 2007a). In offspring sired by males in which the autosomal suppressor *Nmy* (*not much yang*) is removed, the sex ratio is strongly female biased (Tao *et al.*, 2007b).

In this study we focused on the Winters system. We first identified polymorphic Y chromosomes that vary in their resistance to the Winters *sex-ratio* distorter gene *Dox*, resulting in sex ratio ranging from 63% to 98% females. We then investigated genome-wide gene expression in representative males harboring Y chromosomes that are resistant (low sex ratio) and Y chromosomes that are sensitive (high sex ratio) to *Dox*. A disproportionate number of testis-specific genes were upregulated by the resistant Y, while mitochondria-related and accessory gland-specific genes were downregulated. Similar analyses using XXY females indicated that the expression of protein-coding genes is not required for the Y-linked regulatory functions in *D. simulans*. In conclusion, the *D. simulans* Y chromosome shows abundant polymorphic variation that regulates *sex-ratio* distortion in natural populations. The data raise the prospects that the responsible polymorphisms might not reside exclusively within proteins.

MATERIALS AND METHODS

Drosophila strains

Y chromosomes from 78 isofemale stocks of *D. simulans* were introgressed into a nearly identical background (SR1227: *w Dox*; *nt*; *nmy*[*sim1427*]) (Tao *et al.*, 2007b), using a cross scheme (Supplementary Figure S1) in which all chromosomes except the fourth were tracked with phenotypic markers. These stocks include 44 collected in the Carlson Orchards, Harvard, Massachusetts and 34 of global origin (Supplementary Table S1). For gene expression analyses, flies were grown in incubators maintained at 25 °C, 65% of relative humidity and constant light. Newly emerged adults were collected and aged for 2 days at the same rearing condition before they were flash-frozen in liquid nitrogen and stored at −80 °C. Whenever females were analyzed, virgin females were collected within 7 h after eclosion. All strains were expanded in vials; independent sets of biological replicates were frozen for expression analyses. XXY females were obtained by crossing the SR1227/Y chromosome substitution males to the *C(1)DX, y w* females.

Sex-ratio analysis

The sex ratio of a male genotype was measured by crossing 5 males to 10 virgin SR1227 females at room temperature (∼23 °C), with an average of 2.7 replicates for each of the 78 Y-introgression strains. Offspring were sexed and counted until the nineteenth day after mating. Sex ratio was calculated as the proportion of females.

Gene expression analyses

Microarrays were ∼18 000-feature cDNA arrays spotted with *D. melanogaster* cDNA PCR products (Lemos *et al.*, 2008). We have recently annotated these probes using the *D. simulans* genome. Possible biases introduced by using a *D. melanogaster* platform for a *D. simulans* genome are minimized because the probed genomes are essentially identical except for the Y chromosomes. The platform has been successfully used to profile gene expression variation in *D. simulans* when the probed genomes are isogenic for the sequences assayed (Araripe *et al.*, 2010; Sackton *et al.*, 2011). Here the probed genome is the SR1227 isogenic background. Total RNA was extracted from whole flies using TRIzol (Life Technologies, Carlsbad, CA, USA). The synthesis of cDNA and its labeling with fluorescent dyes (Cy3 and Cy5) as well as hybridization reactions were carried out using 3DNA protocols and reagents (Genisphere, Hatfield, PA, USA). Slides were scanned using Axon 4000B scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) and GenePix Pro 6.0 software (Molecular Devices). We used highly stringent quality control criteria to ensure reliability of foreground intensity reads for both Cy3 and Cy5 channels (Lemos *et al.*, 2008). This procedure screens out poor-quality arrays and ensures that the resulting data are minimally sensitive to the parameters of the normalization method. The final data set consists of 58 high-quality arrays in which >70% of all expressed spots passed our highly stringent quality criteria (Lemos *et al.*, 2008, 2010). We used balanced designs with dye swaps (Supplementary Figure S2). The Y chromosome from *Drosophila sechellia* might have a significant impact on gene expression and was included. Foreground fluorescence of dye intensities was normalized by the Loess method in Bioconductor/Limma (Smyth and Speed, 2003; Smyth, 2005). The significance of variation in gene expression was assessed with the Bayesian Analysis of Gene Expression Levels (Townsend and Hartl, 2002). Results were checked for consistency using Linear Models in Limma. False discovery rates were empirically estimated by permutation of the data set. The microarray gene expression data reported here can be obtained at

the Gene Expression Omnibus database under accession number GSE43665.

Functional categories

Enrichment in Gene Ontology (GO) categories was assessed with modMine (March 2012) (Contrino *et al.*, 2012), and the GO significance was estimated using the Holm–Bonferroni method for multiple hypothesis testing (Holm, 1979). List of genes in various biological categories were obtained from the FlyBase (McQuilton *et al.*, 2012).

Tissue-specific gene expression

As a proxy for the tissue specificity of gene expression in *D. simulans*, we used the *D. melanogaster* data available from the FlyAtlas (downloaded on December 2011) (Chintapalli *et al.*, 2007). We filtered the data to include only a non-redundant set of adult tissues (brain, eye, thoracic abdominal ganglion, crop, midgut, hindgut, tubule, testis, accessory gland, salivary gland, adult fat body, heart and trachea). For each Affymetrix probe in the FlyAtlas data set and for each tissue we set the expression level to 0 unless the probe was called as 'present' in at least two out of four arrays and then averaged over all probes and arrays for each FBgn to calculate an expression level for each gene in each tissue. Genes with expression counts <100 were conservatively set to zero. We calculated a tissue specificity index (TSI_i) for tissue i in a set of n tissues as the expression in tissue i over the total expression values across all tissues $(\text{Expression}(i)/\sum_{i=1}^{i=n}\text{Expression}(i))$. Genes expressed exclusively in a focal tissue have a *TSI* equal to 1. We define a gene as tissue specific if this gene has a $TSI>0.90$ for the focal tissue. We further conservatively require that tissue-specific genes to have expression counts >250 units in the focal tissue.

RESULTS AND DISCUSSION

Natural polymorphism in Y-linked suppressors of the Winters sex-ratio system

Several studies have shown that Y chromosomes from *D. melanogaster* or *D. simulans* are polymorphic for their ability to modulate organismal phenotypes. Here we first investigated whether the Y chromosome in *D. simulans* could modulate sex ratio distortion of the Winters system. We introgressed a collection of 78 independently derived Y chromosomes into the isogenic *D. simulans* stock SR1227 (*w Dox*; *nt*; *nmy*[1427]) (Supplementary Figure S1). The SR1227 genotype expresses the *sex-ratio* distorter *Dox* located on the X chromosome but lacks the functional suppressor *Nmy* on the third chromosome. Hence, SR1227 males with their original Y chromosome sire offspring broods with a biased sex ratio of ~75% (Tao *et al.*, 2007a). Thirty-four Y chromosomes investigated had global origins, while the remaining 44 Y chromosomes were derived from isofemale strains collected in Carlson Orchards, Harvard, Massachusetts (Supplementary Table S1). These Y chromosomes introgressed into the SR1227 genetic background significantly contributed to the large variance of sex ratio in the offspring, which ranged from 63.1% to 98.4% females (analysis of variance with GLM, df = 77, $P<2.2\times10^{-22}$, Figure 1). Even the Y chromosomes originating from a single Carlson Orchards population displayed a similarly large span of variation (two-sample Kolmogorov–Smirnov test, $P=0.7443$) (Figure 1). The sample included males producing a highly skewed female-biased *sex-ratio* (sensitive Y chromosomes) and males displaying a fair rescue of the *sex-ratio* phenotype (resistant Y chromosomes). These observations are in agreement with previous findings in other *sex-ratio* systems where the Y chromosome varied in its resistance to sex ratio distortion (Carvalho *et al.*, 1997; Montchamp-Moreau *et al.*, 2001) and support the theoretical predictions of high genetic variances for *sex-ratio* suppressor on the Y chromosome (Curtsinger and Feldman, 1980; Jaenike, 1999).

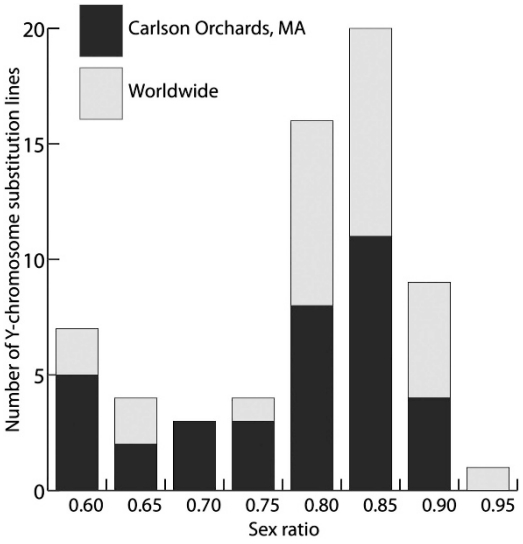

Figure 1 Distribution of sex ratios as regulated by naturally occurring Y chromosomes. Sex ratios were measured in offspring sired by male genotypes isogenic to *w Dox*; *nt*; *nmy* [sim1427], except for the introgressed Y chromosomes. Y chromosomes were independently derived from 78 isofemale *D. simulans* stocks, among which 44 stocks originated from a single locality in Massachusetts and 34 stocks were of global origins.

Regulation of testis-specific genes in males with resistant Y chromosomes

In order to gain insight into the molecular mechanism through which Y chromosomes modulate the Winters *sex-ratio*, we performed genome-wide gene expression analysis in four representative Y chromosome-introgression strains. The four strains carried Y chromosomes derived from the Carlson Orchards population and showed contrasting sex ratio distortion. The resistant Y chromosome strains Y71 and Y76 produced $64.0\pm0.9\%$ (mean ± s.e.m.) and $67.1\pm1.4\%$ females, respectively, while the sensitive Y chromosome strain Y52 and Y56 produced $85.7\pm1.5\%$ and $82.7\pm1.7\%$ females, respectively. The progenitor strain (SR1227; Yct) produced a sex ratio of $73.3\pm2.0\%$ females (Tao *et al.*, 2007a), and was profiled as a control.

We first contrasted genome-wide gene expression differences between the resistant Y chromosome strains and the more sensitive SR1227 control (Yct). We identified 436 genes downregulated and 409 genes upregulated in the resistant Y chromosome strains (Bayesian Posterior Probability (BPP) > 0.99; false discovery rate (FDR) < 0.10). Tissue specificity of differentially expressed genes were analyzed using the FlyAtlas (Chintapalli *et al.*, 2007). Among the 409 upregulated genes, we observed a significant enrichment for testis-specific genes ($P<10^{-10}$, Fisher's exact test; Figure 2a). Conversely, the 436 downregulated genes were not enriched in any single tissue but instead were broadly expressed across multiple tissues (Figure 2b). This result suggests that Y-linked resistance to sex ratio distortion is associated with a general robustness of testis-specific gene expression. Although informative, the contrast between the resistant Y chromosome strains and SR1227 might be confounded by inter-population Y chromosome divergence that might be unrelated to the *sex-ratio* phenotype (Lemos *et al.*, 2008, 2010). We therefore analyzed a smaller set of 521 differentially expressed genes from the statistical contrast between the two resistant and two sensitive Y chromosome introgression lines (BPP > 0.99; FDR < 0.10). Among these, 233 genes were upregulated (Figure 2c) and 288 genes were downregulated (Figure 2d) in the

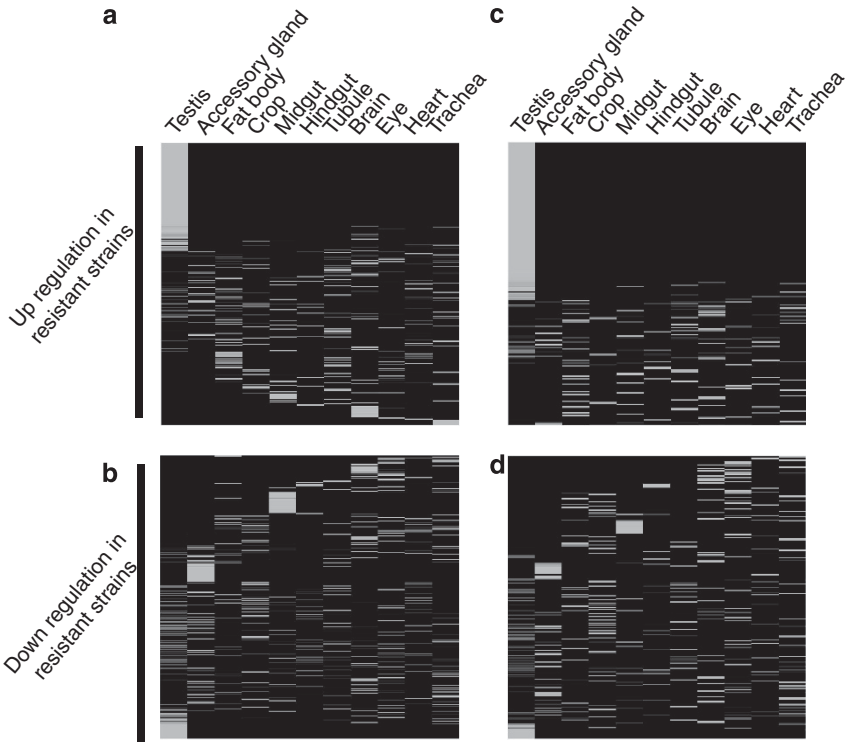

Figure 2 Disproportionate upregulation of testis-specific genes in males with resistant Y chromosomes. Genes differentially expressed in *D. simulans* were clustered based on the *D. melanogaster* FlyAtlas tissue expression data (Chintapalli *et al.*, 2007). Panels show the distribution of differentially expressed genes across 11 adult tissues. Expression levels were normalized for each gene with light and dark denoting high and low transcript abundance, respectively. (**a**) 409 genes that were upregulated and (**b**) 436 genes that were downregulated in the resistant Y chromosome males as contrasted to the control SR1227 males. Similarly, (**c**) 233 genes were upregulated and (**d**) 288 genes were downregulated in the resistant Y males as contrasted to the sensitive Y males.

resistant Y strains relative to sensitive ones, with a modest but significant correlation in fold change between the two resistant strains ($\rho = 0.18$, $P < 0.001$). Tissue-specific analysis of these genes reinforced the results of the initial analysis: genes consistently upregulated in strains with resistant Y chromosomes were enriched for targets with testis-specific expression ($P < 10^{-16}$, Fisher's exact test; Supplementary Table S2). Indeed, nearly 45% of all the upregulated genes were expressed exclusively in this single tissue (Figure 2c). Conversely, among genes downregulated in the Y resistant lines, we observed that only a few showed testis expression (Figure 2d). The lack of gene ontology classification limits the detection of functional clustering among testis-specific genes in *Drosophila* (McQuilton *et al.*, 2012). Altogether, our analyses suggest an association between the suppression of *sex-ratio* by the resistant Y chromosomes and the higher expression of testis-specific genes.

Modulation of mitochondria-associated genes in sex-ratio males

Evolutionary theory predicts a disproportional contribution of sex chromosomes (Hamilton, 1967) and cytoplasmic genes (Cosmides and Tooby, 1981) to the control of sex ratio distortion. Intragenomic conflicts over sex ratio control, which is a perpetual tug-of-war among the X, the Y, the autosomes and the cytoplasmic genes, might cause several well-known genomic patterns uncovered in recent years, including meiotic sex chromosome inactivation and out-of-the-X traffic of testis-specific genes (Meiklejohn and Tao, 2010). Interestingly, many of the mitochondrial genes have evolved to function specifically during spermatogenesis in *Drosophila* (Gallach *et al.*, 2010). This organelle has an essential role in sperm morphogenesis (Noguchi *et al.*, 2011) and motility (Fuller, 1993), although it is not inherited through males in *Drosophila* (DeLuca and O'Farrell, 2012). Altogether, the system sets the stage in which interactions between mitochondria and Y-linked modifiers of *sex-ratio* can evolve.

We observed significant enrichment for several functions related to metabolism and the mitochondria among the 436 genes downregulated in the resistant Y chromosome strains relative to the more sensitive SR1227 (Table 1). Specifically, about 7.5% of all downregulated genes (33 genes out of 436) cluster in the following categories: cellular respiration (GO:0045333; $P = 1.34 \times 10^{-19}$), respiratory electron transport chain (GO:0022904; $P = 8.97 \times 10^{-16}$) and ATP synthesis-coupled electron transport (GO:0042773; $P = 5.79 \times 10^{-13}$). Among the genes in the last category are four encoded by the mitochondrial genome (mitochondrial cytochrome *c* oxidase subunit II (FBgn0013675), mitochondrial cytochrome *c* oxidase subunit III (FBgn0013676), mitochondrial NADH-ubiquinone oxidoreductase chain 5 (FBgn0013684) and mitochondrial cytochrome *b* (FBgn0013678)). We observed similar patterns in the set of 288 genes downregulated in males with the resistant Y chromosome relative to males with the sensitive Y chromosome (Table 2). The signature of Y chromosome by mitochondria interaction is indeed stronger when we subset the analysis to the downregulated genes with the highest expression in the testis (Table 3).

Abnormal mitochondrial degeneration during spermatogenesis is a remarkable feature of the *sex-ratio* males of *D. simulans* (Ramamurthy *et al.*, 1980). Our analyses did not detect the differential regulation of key apoptotic genes, an observation suggesting that the degeneration process is not associated with typical apoptotic pathways. Instead, the

Table 1 Gene Ontology (GO) analysis of the downregulated genes identified in the resistant Y chromosome males relative to SR1227

GO term	*Biological process*	*P-value*[a]	*No. of genes*
GO:0045333	Cellular respiration	1.34E−19	33
GO:0015980	Energy derivation by oxidation of organic compounds	2.91E−18	33
GO:0006091	Generation of precursor metabolites and energy	1.47E−16	35
GO:0022904	Respiratory electron transport chain	8.97E−16	25
GO:0022900	Electron transport chain	5.69E−15	25
GO:0042773	ATP synthesis coupled electron transport	5.79E−13	22
GO:0042775	Mitochondrial ATP synthesis coupled electron transport	2.11E−12	21
GO:0006119	Oxidative phosphorylation	4.40E−12	22
GO:0006120	Mitochondrial electron transport, NADH to ubiquinone	6.54E−08	13
GO:0007602	Phototransduction	6.04E−07	14
GO:0009583	Detection of light stimulus	0.0000028	14
GO:0055114	Oxidation–reduction process	0.0000149	47
GO:0009581	Detection of external stimulus	0.0000454	14
GO:0009060	Aerobic respiration	0.0028969	10
GO:0009416	Response to light stimulus	0.0078173	15
GO:0009314	Response to radiation	0.0089795	16
GO:0006084	Acetyl-CoA metabolic process	0.0341105	9

Abbreviations: ATP, adenosine triphosphate; NADH, nicotinamide adenine dinucleotide.
[a]Corrected with the Holm–Bonferroni Multiple Hypothesis Test.

Table 2 GO analysis of the downregulated genes identified in the resistant Y chromosome males relative to the sensitive ones from the same population

GO term	*Biological process*	P-*value*[a]	*No. of genes*
GO:0009583	Detection of light stimulus	0.000818	10
GO:0045333	Cellular respiration	0.006737	13
GO:0003012	Muscle system process	0.009314	6
GO:0006091	Generation of precursor metabolites and energy	0.012548	15
GO:0015980	Energy derivation by oxidation of organic compounds	0.017841	13

Abbreviation: GO, Gene Ontology.
[a]Corrected with the Holm–Bonferroni Multiple Hypothesis Test.

regulation of genes associated with ATP biosynthesis might point to inefficiencies of the energetic metabolism due to the mitochondrial degeneration in the Y-bearing spermatids that lead the upregulation of bioenergetic genes to compensate the energetic needs of the 'ill' cell (Heddi *et al.*, 1999; Epstein *et al*, 2001). Altogether, the data support the notion that maternally and paternally inherited elements might genetically interact in the etiology of sex ratio distortion.

Genes expressed at post-meiotic stages of spermatogenesis are particularly prone to mis-regulation in sex-ratio males

Disturbance of testis-specific gene expression in *sex-ratio* males might be caused by a general breakdown of spermatogenesis or might be limited only to those genes directly related to the deficiency of the Y-bearing sperm. The testis of SR1227 males does not show any overtly impaired development, but abnormal spermiogenesis of Y-bearing sperm is evident by ultrastructural studies (Tao *et al.*, 2007a), favoring the latter explanation. We narrowed our analyses to testis-specific genes to address this question. We observed 1102 testis-specific genes in the FlyAtlas, 827 of which were detected in our data set. A set of 213 testis-specific genes were differentially expressed across any of the three possible contrasts between the resistant Y, the sensitive Y and the SR1227 control males (Figure 3a). The remaining genes comprise a rather large set (614 out of 827 or 74.3%) of testis-specific genes that were stably expressed across all the three types of strains (Figure 3a; $0.05 < \mathrm{BPP} < 0.95$). Most (93%) of the 213 differentially expressed testis-specific genes have no known functional annotations (Figure 3b). Nevertheless, Vibranovski *et al.* (2009) reported gene expression variation along three time points of spermatogenesis, namely mitotic, meiotic and post-meiotic stages, and identified their peak expression stages. For genes upregulated in males with resistant Y chromosomes, the frequency (12%) of the meiotic genes, which have peak expression levels at meiotic stage, is similar to the frequency (13%) of meiotic genes in the set of background genes that are not differentially expressed ($P = 0.98$). On the other hand, we observed a significant twofold enrichment for post-meiotic genes ($P < 1.1 \times 10^{-5}$), whose expression levels peak at the distal region of the testis that contains mostly spermatids at post-meiotic stages (Vibranovski *et al.*, 2009). This indicates that mis-regulation of testis-specific genes in *sex-ratio* males is not a result of generally abnormal spermatogenesis; rather it appears to be specifically related to the dysgenesis of Y-bearing sperm. This is consistent with morphological observations (Tao *et al.*, 2007a, b).

Table 3 GO analysis of the top 50 highest expressed testis-specific genes identified as downregulated in the resistant Y males contrasted to the sensitive ones

GO term	*Biological process*	P-*value*[a]	*No. of genes*
GO:0045333	Cellular respiration	0.001731	7
GO:0015980	Energy derivation by oxidation of organic compounds	0.003105	7
GO:0006091	Generation of precursor metabolites and energy	0.016984	7
GO:0042775	Mitochondrial ATP synthesis coupled electron transport	0.029857	5
GO:0042773	ATP synthesis coupled electron transport	0.040518	5

Abbreviations: ATP, adenosine triphosphate; GO, Gene Ontology.
[a]Corrected with the Holm–Bonferroni Multiple Hypothesis Test.

Accessory gland expression is downregulated in the resistant Y males

Sex-ratio males are not only weaker in sperm displacement but are also discriminated against by non-virgin females in both *Drosophila pseudoobscura* and *D. simulans* (Wu, 1983a, b; Atlan *et al.*, 2004). Males with the Winters sex-ratio also show reduced fertility (Tao *et al.*, 2007b). Furthermore, the Winters system is polymorphic in natural populations with segregating variation that shows evidence of recent selection in both distorters and suppressors (Kingan *et al.*, 2010). Indeed, the invasion of *sex-ratio* distorters sets the stage for the evolution of genetic suppressors as well as behavioral and physiological mechanisms that might alleviate the fitness cost incurred by a biased *sex-ratio* (Price *et al.*, 2008). We observed that a cluster of

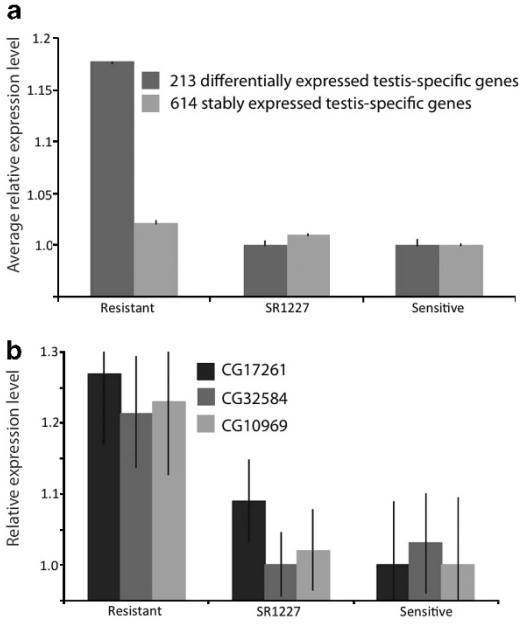

Figure 3 Characterization of the 213 testis-specific genes upregulated in the resistant Y chromosome males. (**a**) The average expression level of the 213 genes is significantly higher in the resistant Y chromosome males as compared with more sensitive Y males ($P<0.01$, dark gray). The other 614 testis-specific genes stably expressed across different Y introgression males ($0.05<P<0.95$, light gray). Error bars denote >99% confidence intervals for the average fold differences (±4 s.e.m.). (**b**) The 213 genes generally lack annotation, as illustrated by three representatives: CG17261 (black), CG32584 (dark gray), and CG10969 (light gray). Error bars denote 95% credible intervals for individual genes.

accessory gland-specific genes were significantly downregulated in males with resistant Y chromosomes ($P=0.002$; Fisher's exact test) (Figures 2b and d). Indeed, the average expression level of 37 accessory gland-specific genes is significantly higher in males expressing more biased sex ratio (Figures 4a and b). Notably, protease inhibitors are accessory gland-specific proteins (Figure 4c) that are able to prevent the premature breakdown of the mating plug and increase sperm vitality (Park and Wolfner, 1995; Chapman, 2001), and that might function to mitigate the lower fitness incurred by *sex-ratio* males (Tao *et al.*, 2007b). Although higher expression of accessory gland proteins (ACPs) might contribute to partly compensate for impaired testis-specific gene expression in males with skewed sex-ratio (Linklater *et al.*, 2007; Dowling and Simmons, 2012), we cannot formally distinguish between adaptive and non-adaptive mechanisms underlying variation in ACP expression. Similarly, although ejaculate composition might be a mechanism to maintain male fitness in response to the disruption of the testis function in males with highly biased sex ratio, we cannot distinguish direct and indirect effects of Y chromosome on ACP expression. Further population-wide analysis and functional studies are required to ascertain the relationship between Y-linked variation, accessory gland expression and sex ratio distortion. Our expectation is that ongoing natural selection might be currently shaping the dynamics of Y-linked resistance in *D. simulans*.

Gene expression in XXY female is modulated by the Y chromosome

Natural variants of the *D. simulans* Y chromosome modulate gene expression and *sex-ratio* in males. What are the polymorphic Y-linked sequences that account for this polymorphism? Because *Drosophila* Y-linked protein-coding genes show little to no variation (Zurovcova

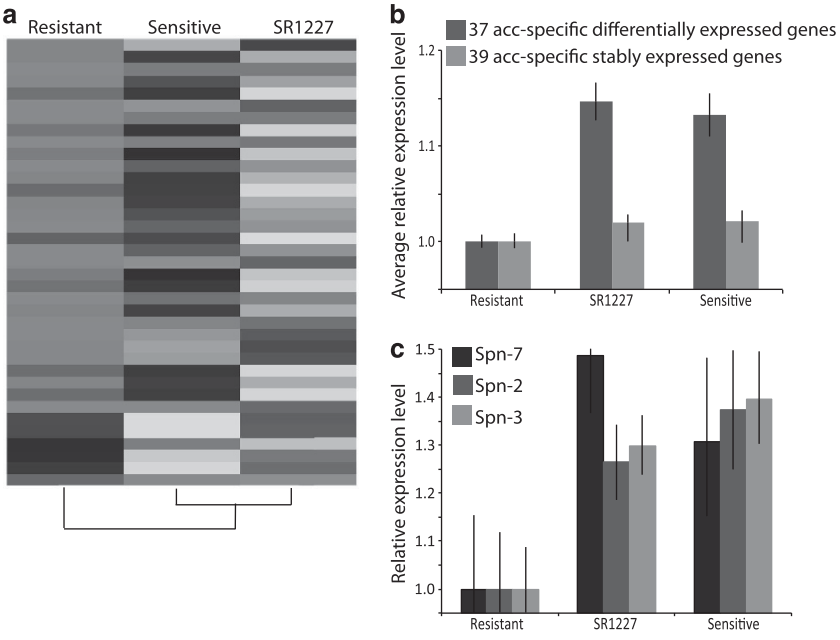

Figure 4 Accessory gland-specific genes were upregulated in the sensitive Y chromosome males. (**a**) Average fold change in the expression of the 37 accessory gland-specific genes that were differentially expressed across lines ($P<0.01$). Darker shades denote lower expression level; lighter shades denote higher expression level (Supplementary Figure S3). (**b**) Average relative expression level of these 37 genes ($P<0.01$, dark gray), as compared with that of the other 39 accessory gland-specific genes that were stably expressed across genotypes ($0.05<P<0.95$, light gray). Error bars denote >99% confidence intervals for the average fold differences. (**c**) Three serine protease inhibitors were upregulated in the sensitive Y chromosome males: serine protease inhibitor 7 (Spn7, black bars), serine protease inhibitor 2 (Spn2, dark gray) and serine protease inhibitor 3 (Spn3, light gray).

and Eanes, 1999), it appears unlikely that these proteins might mediate the resistance to *Dox*. On the other hand, the Y chromosome is mainly composed of non-coding repetitive DNA sequences, and these 'junk' sequences might have an active role in gene expression regulation (Lemos *et al.*, 2008, 2010). To address the issue, we compared genome-wide gene expression levels between XXY female genotypes that are isogenic except for the Y chromosome. Y-linked proteins are known to have no expression levels in female (Ashburner *et al.*, 2005; Lemos *et al.*, 2010). We contrasted a single resistant Y chromosome with a single sensitive Y chromosome for their effects on XXY female genotypes. We found 683 genes that were differentially expressed between XXY(resistant) and XXY(sensitive) females (BPP > 0.99; FDR < 0.10), 94 of which were also differentially expressed in males. This is expected in view of the large proportion of testis-specific genes in the male contrast. The 333 genes upregulated in XXY(resistant) show no functional clustering at $P < 0.01$. On the other hand, the 350 genes downregulated in XXY(resistant) show a strong association to cell cycle (50 genes, $P < 10^{-7}$). In terms of gene ontology categories, these genes are enriched for chromosome organization (GO:0051276; 29 genes, $P = 0.003$), DNA replication (GO:0006260; 12 genes, $P = 0.012$) and chromosome segregation (GO:0007059; 14 genes, $P = 0.034$). Examples of these genes include important chromatin components, such as ORC2, ORC5 and ORC6. Similar observations were made before in *D. melanogaster* (Lemos *et al.*, 2010), which, differ in one important feature from *D. simulans* in that the latter does not have functional rDNA repeats on its Y chromosome. Hence, neither protein-coding genes nor the rRNA genes appear to be the likely candidates for explaining the Y-linked regulatory variation observed in XXY female genotypes. On the other hand, it is possible that the expression of Y-linked protein-coding genes in males might contribute to the modulation of *sex-ratio* during spermatogenesis. Finally, non-coding sequences, including the rDNA untranscribed spacer repeats, might be able to provide a previously underappreciated source of regulatory functions of a myriad of organismal traits, including *sex-ratio*.

Conclusions

Here we have reported abundant Y-linked regulatory variation in *D. simulans*, some of which might be associated with the Y chromosome's ability to regulate the Winters *sex-ratio* system. In males with the resistant Y chromosomes, over 200 testis-specific genes were upregulated relative to their expression levels in males with sensitive Y chromosomes. These genes are over-represented by those expressed during the post-meiotic or spermiogenic stage of sperm development, suggesting that the Y-linked regulatory functions are targeting developing Y-bearing spermatid. Further insights into the mechanisms modulating *sex-ratio* are hindered by the poor functional annotations of testis-specific genes. Our findings nevertheless point to a link between the etiology of *sex-ratio* and post-meiotic genes and mitochondria. Indeed, mitochondria-associated genes were downregulated in strains with the resistant Y chromosome. These observations concur with recent observations that mitochondrial variants differently regulate testis gene expression in *D. melanogaster* (Innocenti *et al.*, 2011). In one similar but more extreme case, the mitochondrial type of Brownsville introgressed into the w^{1118} nuclear background can drastically reduce male fertility (Clancy, 2008). These parallel observations of organismal traits and gene modulations conferred by the Y chromosome and mitochondria might point to a regulatory network of spermatogenesis, including both parties and other nuclear genes. We expect that mitochondria–X chromosome interactions (Rand *et al.*, 2001; Montooth *et al.*, 2010) and mitochondria–Y chromosome interactions may turn out to be critically important in spermatogenesis. Finally, genes specific to the accessory glands were downregulated in males with resistant Y chromosomes, suggesting that this particular tissue might provide protection for spermatogenesis in a stressful environment (that is, sex ratio distortion). The Y chromosome in *D. simulans* also modulates the expression of hundreds of genes that are broadly expressed across various tissues, but the link between these genes and *sex-ratio* regulation is elusive. Lastly, the exact identities of the Y-linked genetic elements that regulate sex ratio distortion remain to be discovered.

DATA ARCHIVING

Expression data deposited in the NCBI's GEO database: GSE43665.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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