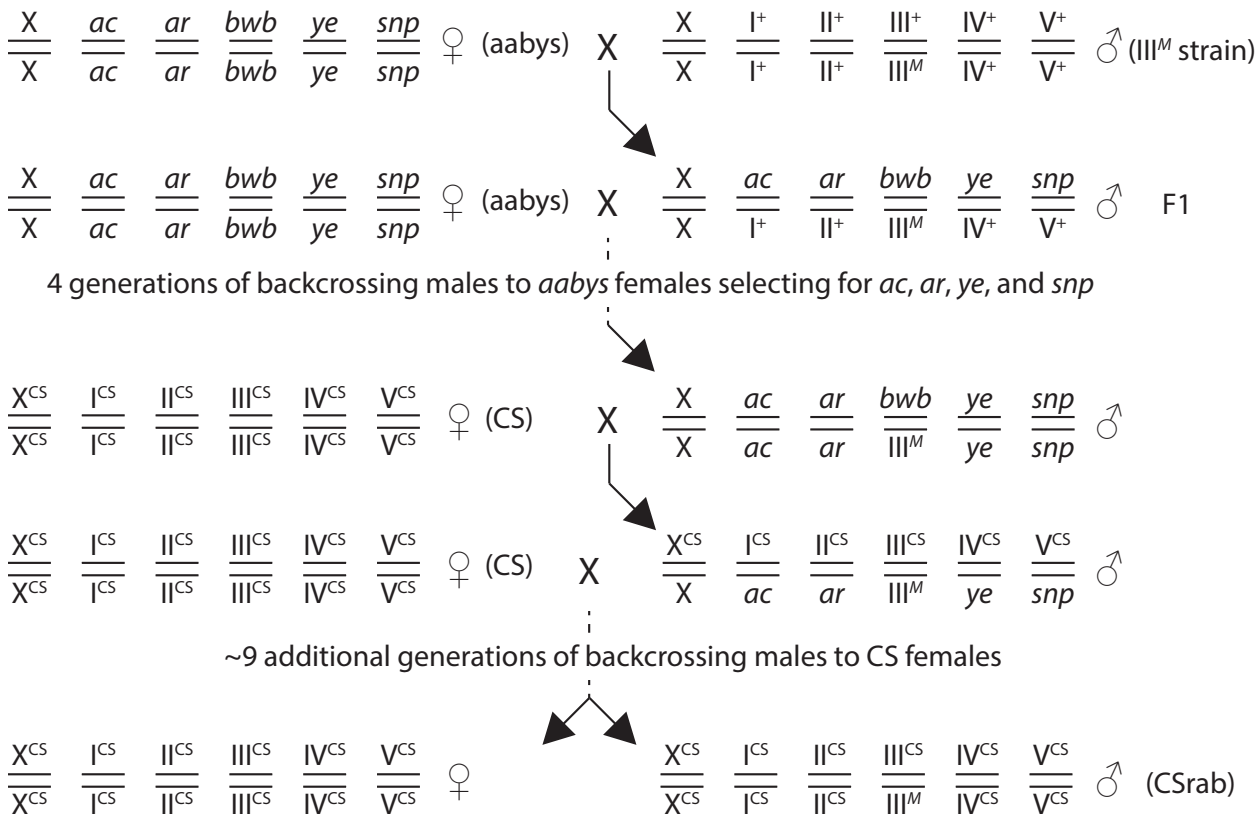
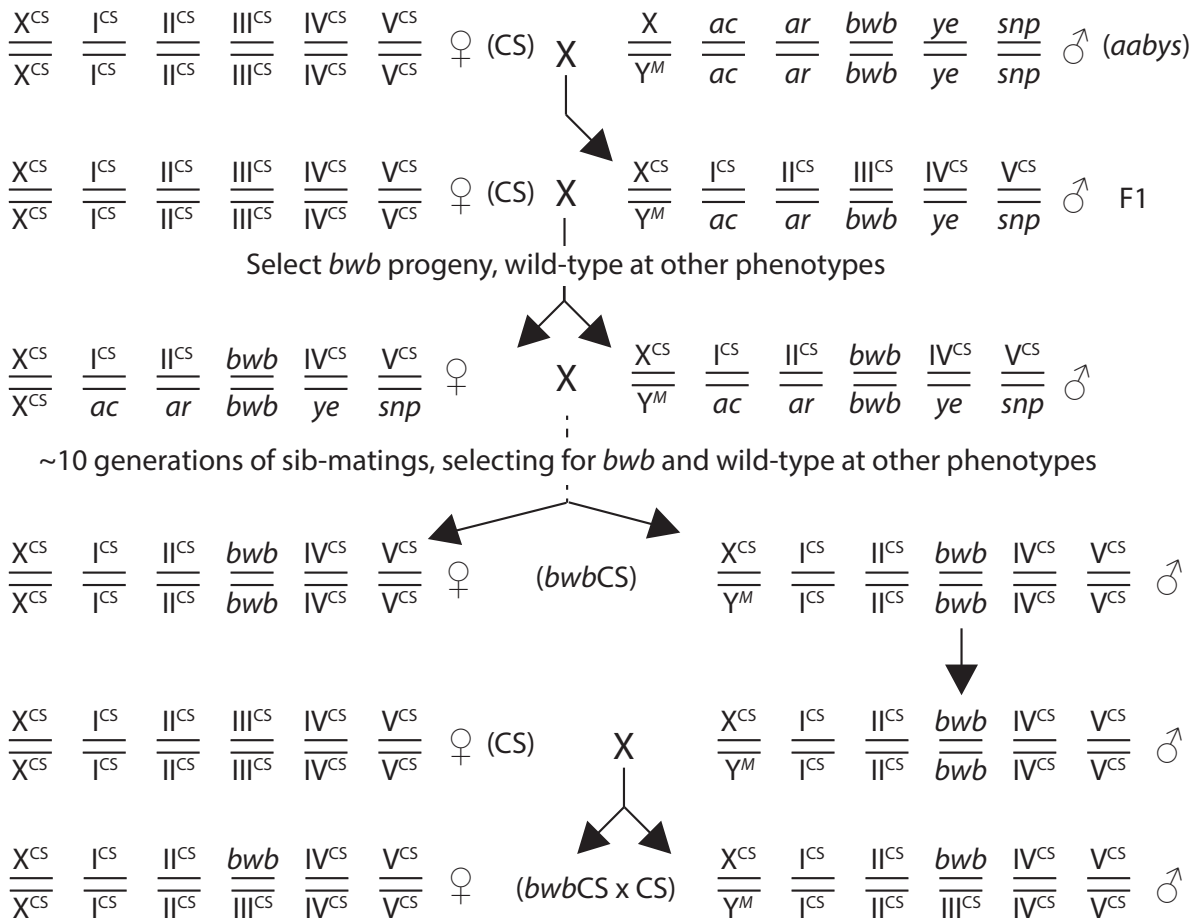
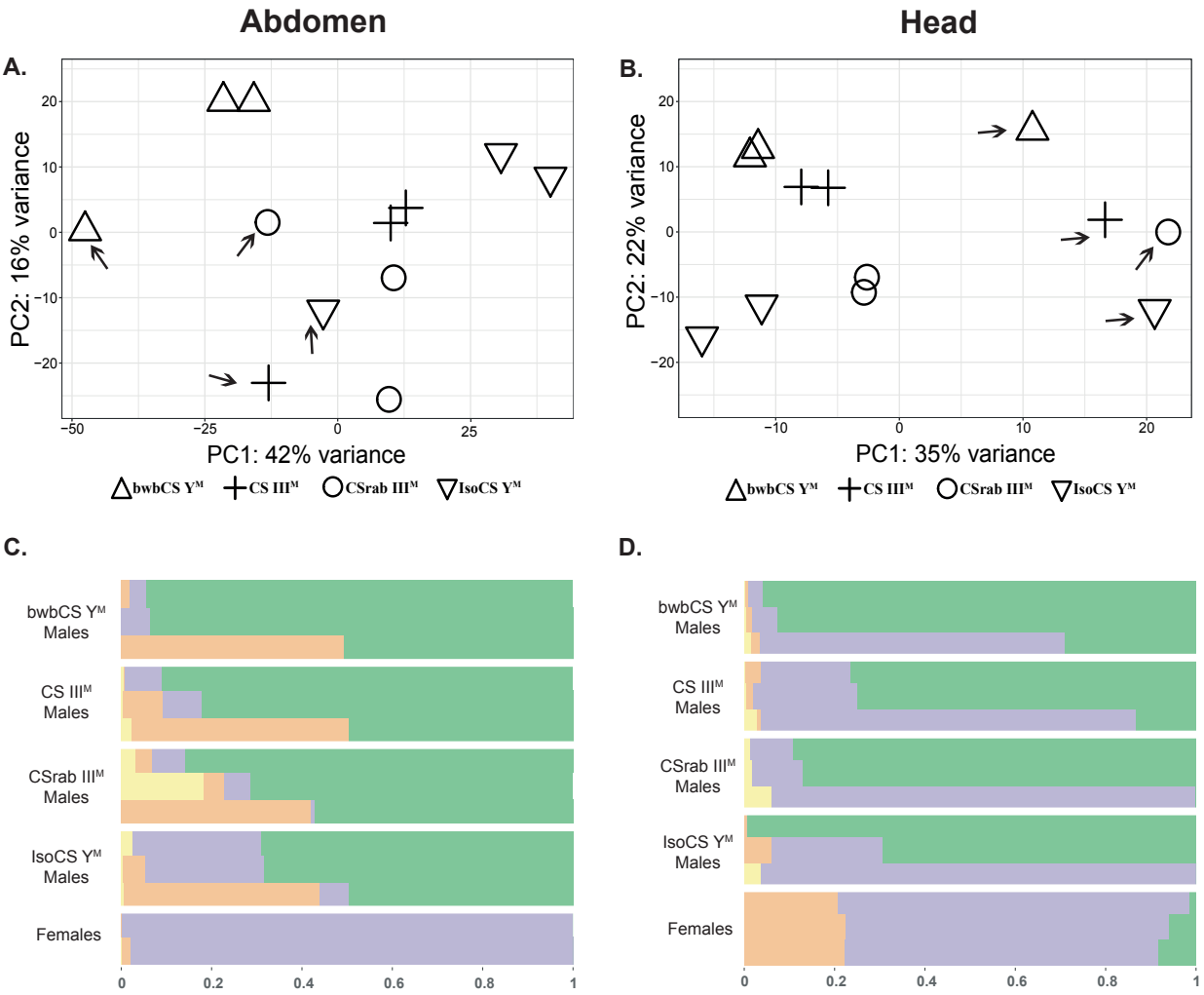
Supplementary Material



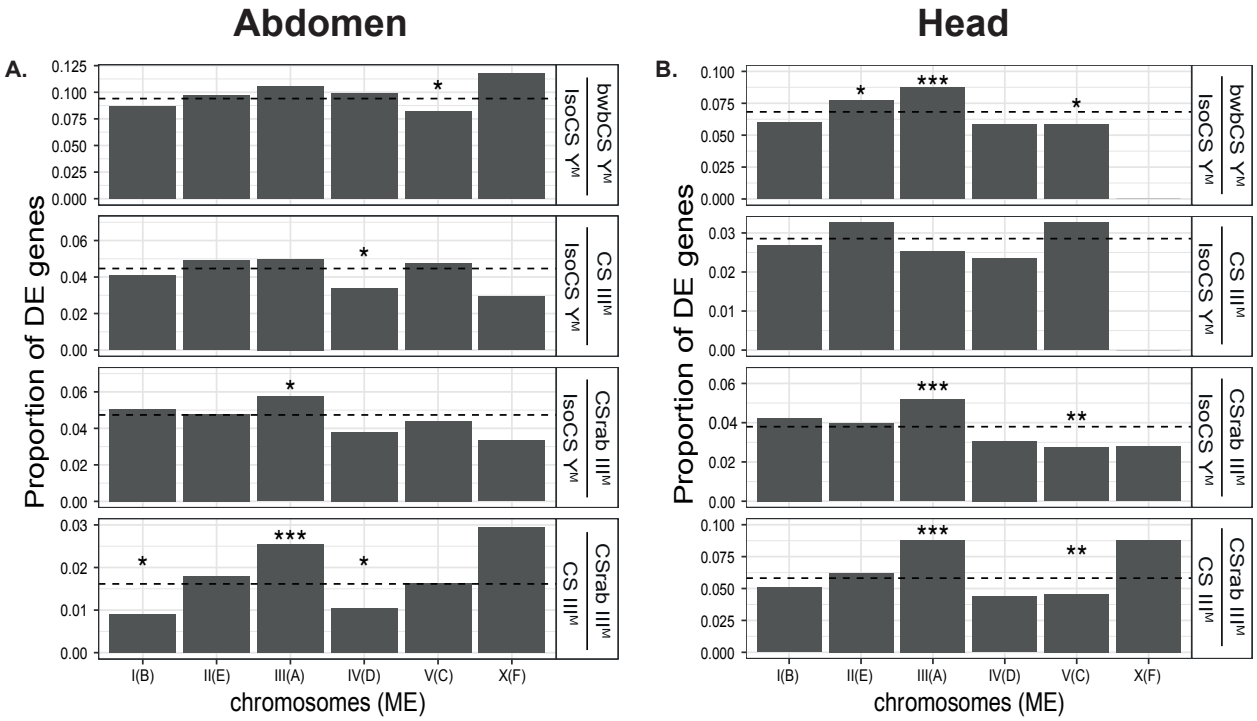
Supplementary Figure 1. Crossing scheme used to create the CSrab strain. Each pair of parallel horizonal bars represents homologous chromosomes; there are six pairs of chromosomes (X/Y, I, II, III, IV, V). The aabys strain has a recessive phenotypic marker on each autosome.



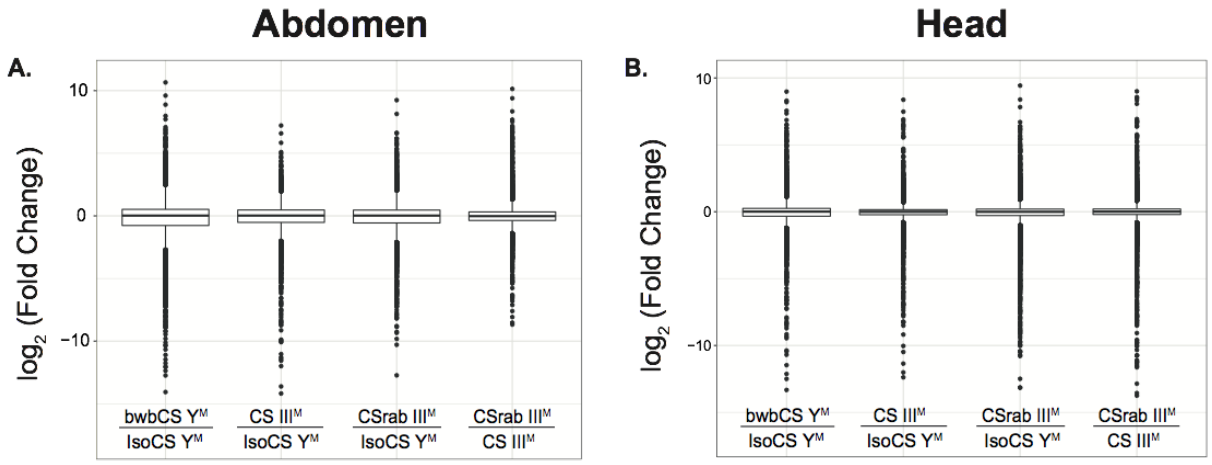
Supplementary Figure 2. Crossing scheme to create the *bwb*CS strain and *bwb*CS × CS line used in this experiment. Each pair of parallel horizonal bars represents homologous chromosomes; there are six pairs of chromosomes (X/Y, I, II, III, IV, V). The aabys strain has a recessive phenotypic marker on each autosome.



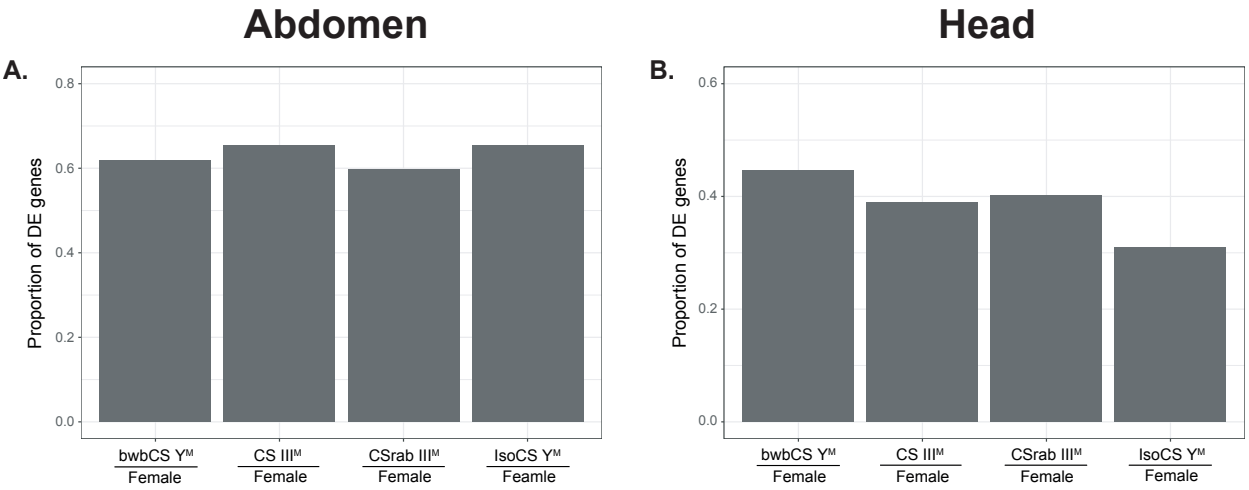
Supplementary Figure 3. (**A, B**) Principal component (PC) analysis of four strains that have different naturally occurring proto-Y chromosomes on a common genetic background in abdomens (**A**) and heads (**B**). Arrows point to outlier samples, one for each of the four strains. Female abdomens are excluded from the PCA (**A**) to show the outliers. (**C, D**) Grade of membership model ($K = 4$) for gene expression patterns of four strains that have different naturally occurring proto-Y chromosomes on a common genetic background in abdomens (**C**) and heads (**D**). Each row represents one replicate of a genotype, with the outliers excluded. Each color represents the proportion of each replicate assigned to each of the three clusters.



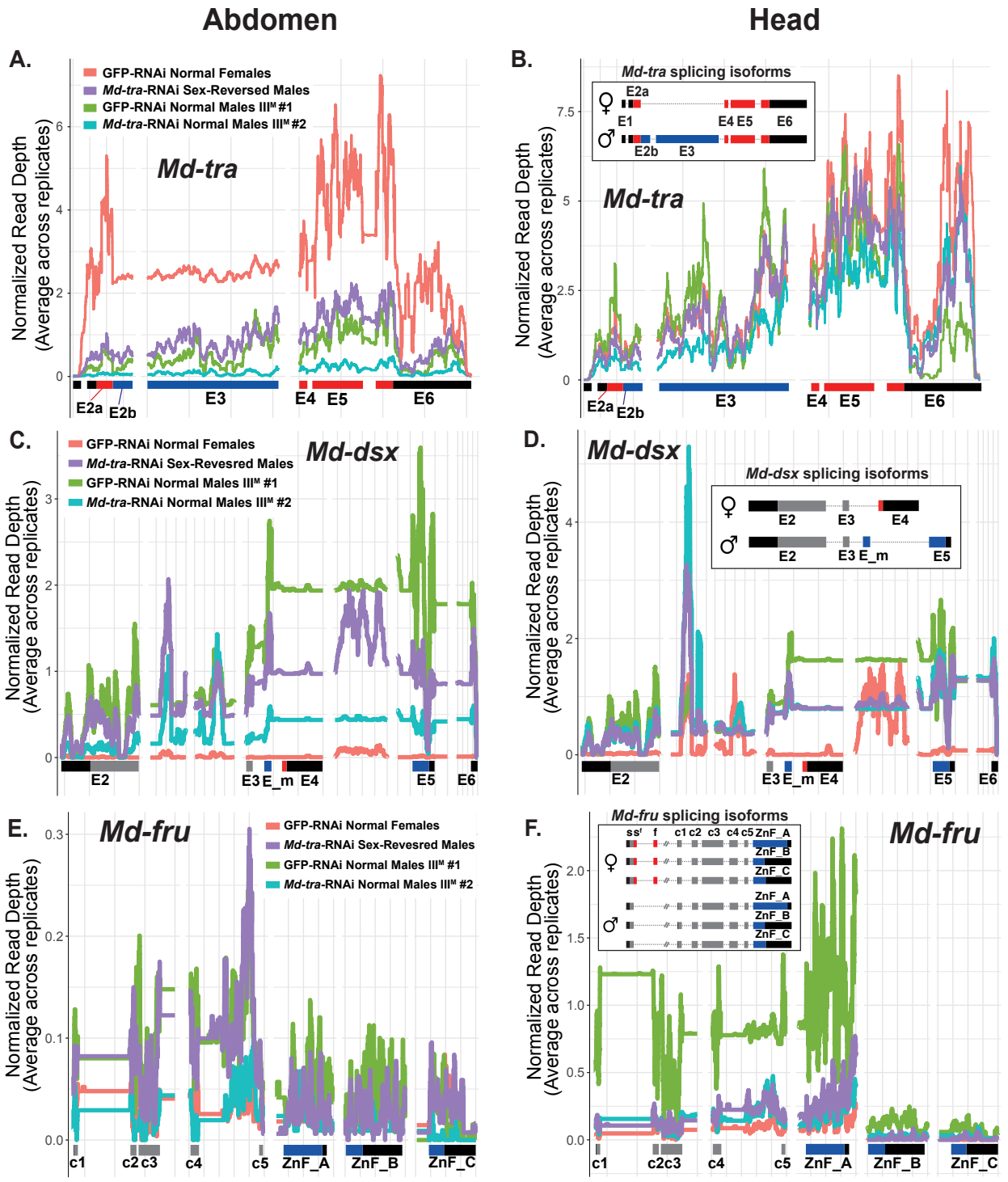
Supplementary Figure 4. Bar graphs indicate the proportions of genes on each chromosome (*Drosophila* Muller element in parentheses) that are differentially expressed (DE) between different male genotypes in abdomen (**A**) and head (**B**). Asterisks indicate significant differences based on Fisher's exact test comparing the number of DE genes on a chromosome against the number of DE genes in the rest of the genome (**P*<0.05, ***P*<0.01, ****P* < 0.001).



Supplementary Figure 5. Boxplots show fold changes of gene expression between males with different *Mdmd*-bearing chromosomes in abdomens (**A**) and heads (**B**). Outliers are included as points.



Supplementary Figure 6. Bar graphs show the proportions of differentially expressed (DE) genes between females and males with different Y^M or III^M proto-Y chromosomes in abdomens (**A**) and heads (**B**). bwbCS Y^M stands for the strain bwbCS×CS.



Supplementary Figure 7. The graphs show read depth coverage of *Md-tra* (**A, B**), *Md-dsx* (**C, D**) and *Md-fru* (**E, F**) in abdomens (**A, C, E**) and heads (**B, D, F**) of flies with different RNAi treatments. Exons of *Md-tra*, *Md-dsx*, and *Md-fru* are presented along the X-axis. The names of the *Md-tra*, *Md-dsx*, and *Md-fru* exons follow published nomenclature (Hediger *et al.* 2004, 2010; Meier *et al.* 2013). Insets show female and male isoforms of *Md-tra*, *Md-dsx* and *Md-fru*, respectively. In *Md-tra*, Blue exons (E2b, E3) that contain premature stop codons are included in

the male isoforms of *Md-tra* but excluded from the female isoforms. In *Md-fru*, red exons (s^f and f) that are contained in female isoforms have premature stop codons, but are excluded from the male isoforms. Exons (s,s^f,f) upstream from an exon 'c1' of *Md-fru* are not included in the read depth coverage because they are not on the same scaffold in the genome assembly.

To confirm that the *Md-tra*-RNAi treatment knocks down *Md-tra* expression, we examined the expression of *Md-tra* using RNA-seq coverage data collected from the abdomen and head of each of our four sample types (**A, B**). We expect the expression of *Md-tra* in females to be higher than in males because males produce a splice variant with a premature stop codon that is likely to be processed by the nonsense-mediated decay (NMD) pathway (Hediger *et al.* 2010; Kervestin and Jacobson 2012). In addition, the ovaries are expected to produce large amounts of *Md-tra* transcripts because *Md-tra* activity is necessary for maternal establishment of zygotic splicing of *Md-tra* via an auto-regulatory loop (Dübendorfer and Hediger 1998). In abdomen, normal females (GFP-RNAi treated genotypic females) do indeed express *Md-tra* approximately three times higher than normal males (genotypic males with either the GFP-RNAi or *Md-tra*-RNAi treatment). This high *Md-tra* expression in female abdomens might reflect the outcomes of strong ovarian expression. Importantly, *Md-tra* expression in sex-reversed males (genotypic females that are phenotypic males because of *Md-tra*-RNAi) was comparable to that of the genotypic males, not the normal females (**A**). This is likely because knock down of *Md-tra* by RNAi produces sex-reversed males that have functioning testes instead of ovaries. The *Md-tra* exons that are included in the functional, female-determining transcript were the highest expressed exons in phenotypic females (**A**), consistent with the production of the female-determining transcript in female ovaries (Hediger *et al.* 2010).

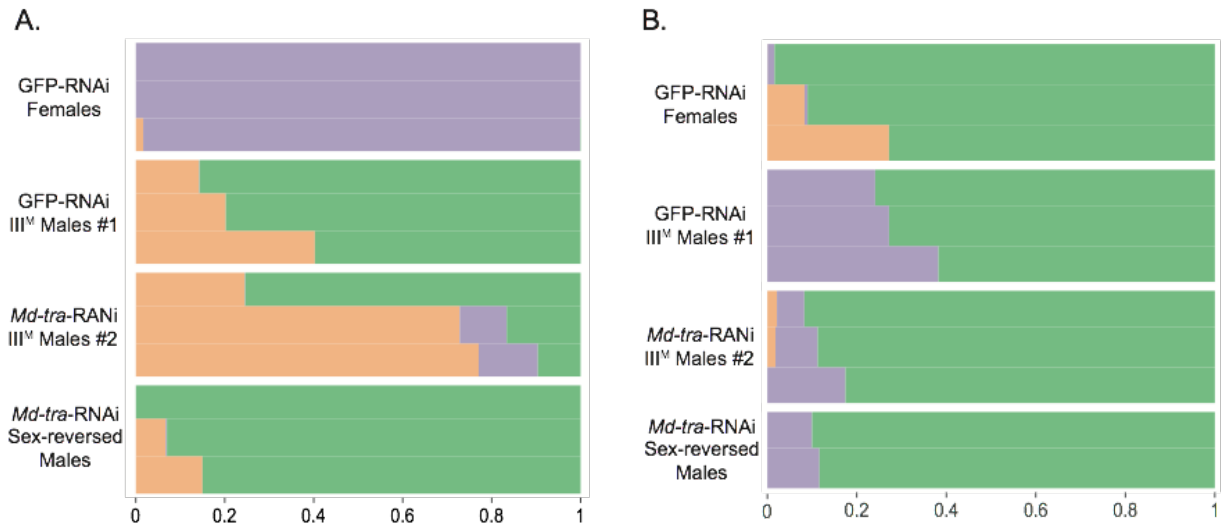
We find that *Md-tra* is also differentially expressed between females and males in head, but the difference is much smaller than in abdomen (**B**). Notably, when we analyze the read mapping to *Md-tra* using DESeq2, expression is significantly higher in normal females than in genotypic (III^M #1) males. However, there is not a significant difference in *Md-tra* expression between sex-reversed males and either normal males or normal females. These results were observed after we excluded a sex-reversed male head sample that had an outlier expression profile (see Main Text). The lack of sexually dimorphic expression of *Md-tra* in head is consistent with minimal sex-biased expression in *Drosophila* and house fly heads (Goldman and Arbeitman 2007; Meisel *et al.* 2015). In addition, most somatic cells in *Drosophila* are sexually monomorphic as a result of cell autonomous sex determination in *Drosophila* (Robinett *et al.* 2010), suggesting that the same may be true for most cells in house fly heads.

Md-tra regulates the splicing of at least two downstream genes, *Md-dsx* and *Md-fru*, which are both differentially spliced between females and males (Hediger *et al.* 2004, 2010; Meier *et al.* 2013). Only the female isoform of *Md-tra* is translated into a functional protein. In the presence of Md-Tra, *Md-dsx* is spliced into an isoform that promotes female morphological development. *Md-dsx* is spliced into an isoform that initiates male morphological development in the absence of Md-Tra (Hediger *et al.* 2004, 2010). *Md-fru* is spliced into a male-specific behavioral regulator in the absence of Md-Tra (Meier *et al.* 2013).

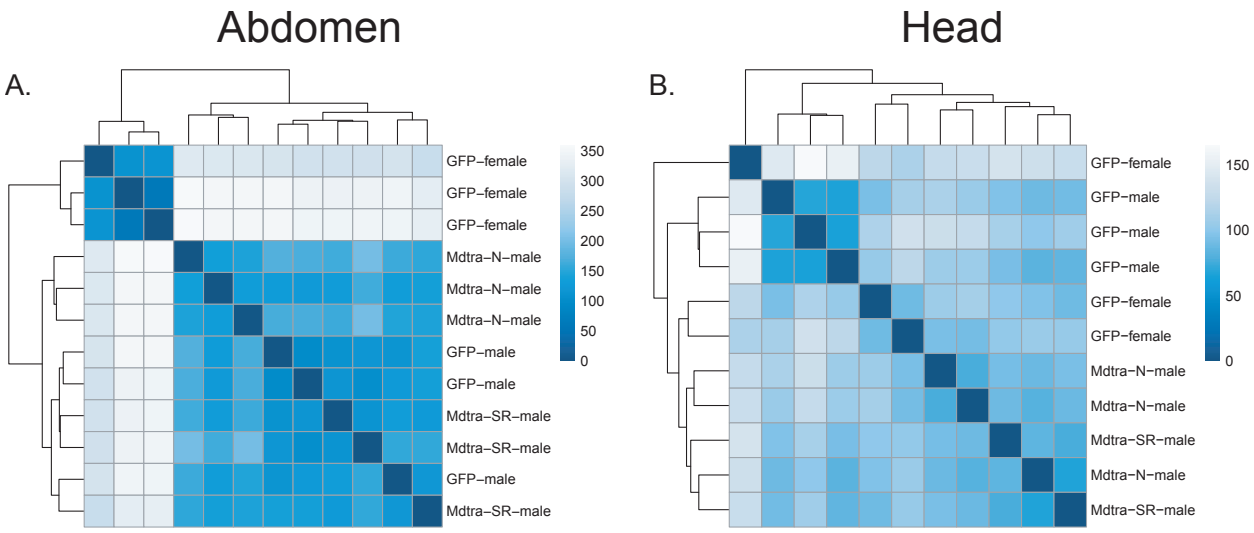
The expression of *Md-dsx* and *Md-fru* in sex-reversed males was more similar to that of normal (genotypic) males (especially *Md-tra*-RNAi treated III^M males #2) than phenotypic females (**C-**

F), confirming that *Md-tra* knock down affects the downstream genes in the sex determination pathway (Hediger *et al.* 2010; Meier *et al.* 2013). For example, *Md-dsx* expression in phenotypic males was higher than in phenotypic females, especially across male-specific exons (**C-D**), consistent with the expected effect of Md-TRA on *Md-dsx* splicing in females (Hediger *et al.* 2004, 2010).

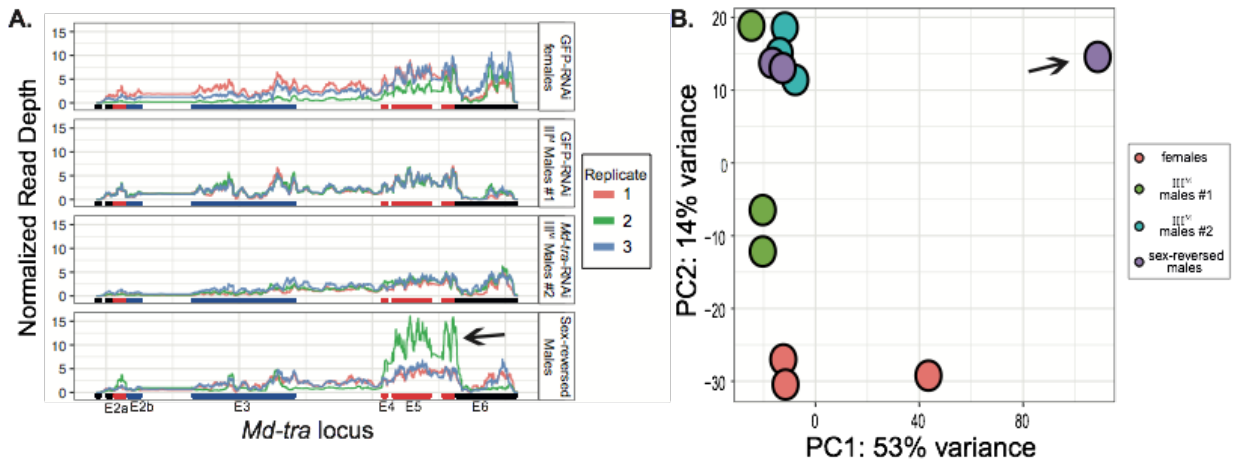
The expression of *Md-fru* was higher in head than in abdomen (**E-F**), consistent with its role as a behavioral regulator (Meier *et al.* 2013). Md-TRA regulates the splicing of *Md-fru* by promoting the production of splice variants with premature stop codons in females (Heinrichs *et al.* 1998; Meier *et al.* 2013). Sex-specific splicing of *Md-fru* occurs at the 5' end of the transcript (Meier *et al.* 2013), but the 5' end of *Md-fru* was not completely assembled and annotated in the reference genome. We therefore cannot test for differential splicing of *Md-fru* between males and females. However, we expect expression of *Md-fru* to be higher in males than females because the female splice variants will be removed by the NMD pathway. We indeed observe that *Md-fru* expression was much higher in the heads of GFP-RNAi treated genotypic males (III^M males #1; see Table 1) than GFP-RNAi treated normal females (**E-F**). However, in *Md-tra*-RNAi treated genotypic males (III^M males #2; see Table 1) and sex-reversed males, the expression of *Md-fru* is intermediate between females and GFP-RNAi treated genotypic males (**F**). A possible explanation is that RNAi knock down of *Md-tra* affects the expression or splicing of *Md-fru* in these flies (sex-reversed males and III^M males #2), but testing this hypothesis is beyond the scope of the work presented here.



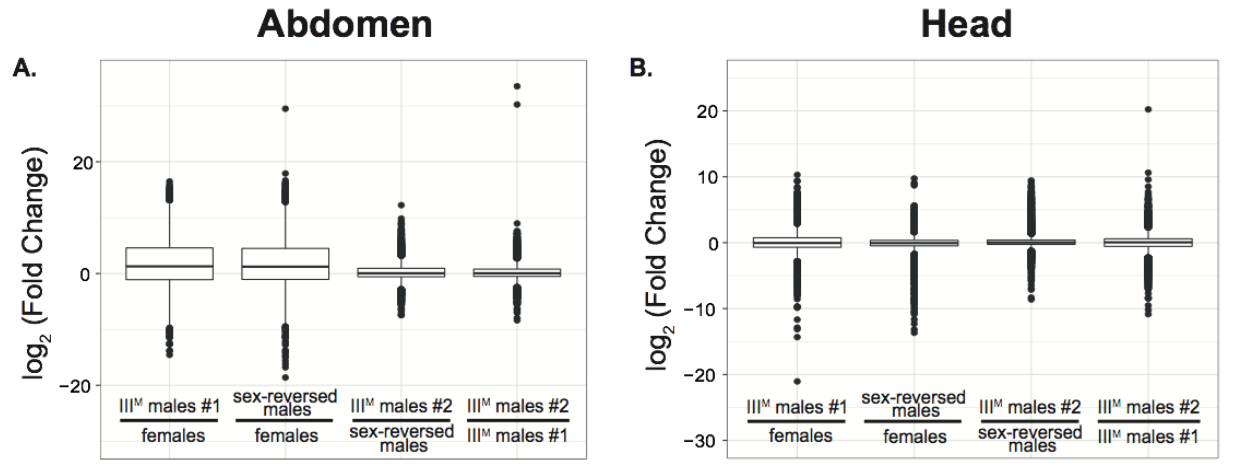
Supplementary Figure 8. Grade of membership model ($K = 3$) for gene expression patterns of four types of dsRNA injected flies in abdomens (**A**) and heads (**B**). Each row is one replicate of each genotype-by-treatment combination. Each color represents the proportion of each replicate assigned to each of the three clusters.



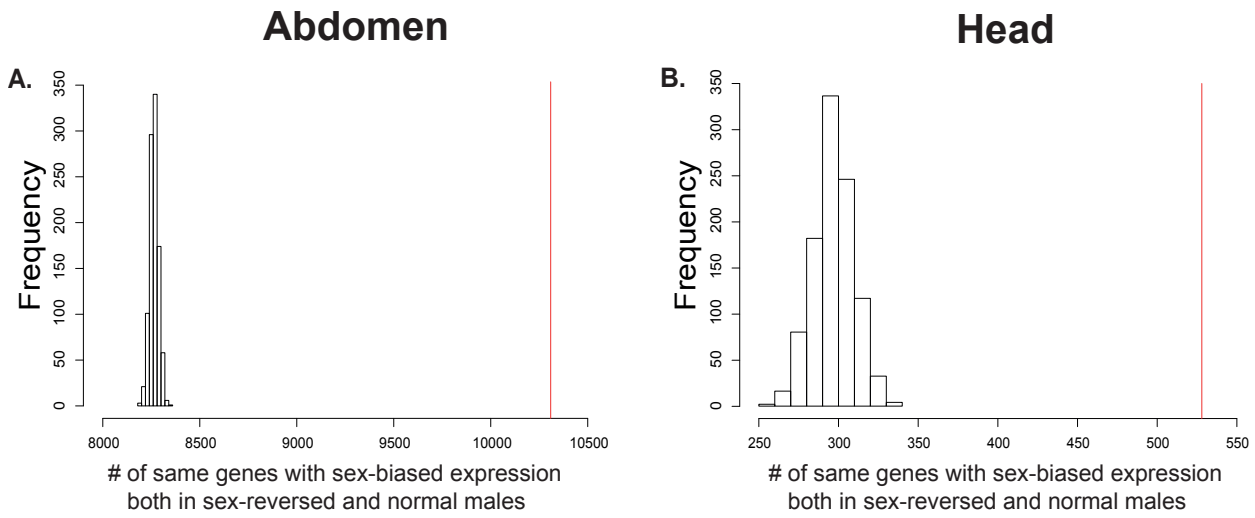
Supplementary Figure 9. Hierarchical clustering of RNAi-treated flies in abdomen (**A**) and head (**B**). GFP-female stands for GFP-RNAi normal females, GFP-male for GFP-RNAi normal males #1, Mdtra-N-male for *Md-tra*-RNAi normal males #2, and Mdtra-SR-male for *Md-tra*-RNAi sex-reversed males.



Supplementary Figure 10. *Md-tra* expression (**A**) and PCA of global expression (**B**) of GPF-RNAi and *Md-tra*-RNAi individuals in heads. Arrows indicate the sex-reversed male head sample that we excluded from our analysis because of its outlier expression profile. Females are GFP-RNAi Normal Females; III^M males #1 are GFP-RNAi Normal Males; III^M males #2 are *Md-tra*-RNAi Normal Males; sex-reversed males are *Md-tra*-RNAi Sex-Reversed Males. SR stands for sex-reversed.



Supplementary Figure 11. Boxplots show fold changes of gene expression among comparisons in abdomens (**A**) and heads (**B**). Outliers are included as points.



Supplementary Figure 12. Permutation tests for whether the same genes have sex-biased expression both in sex-reversed males and normal males (III^M males #1) in abdomens (**A**) and heads (**B**). Histograms represent null distribution and red lines indicate the observed number of genes with the same sex-biased expression both in sex-reversed and normal males.

strain	sex	tissue	Reads mapped	Total reads	% mapped reads
bwbCS	male	abdomen	28,797,051	53,216,164	54.11
bwbCS	male	abdomen	73,634,683	117,292,281	62.78
bwbCS	male	abdomen	34,387,641	46,794,091	73.49
CS	male	abdomen	23,436,748	43,892,080	53.40
CS	male	abdomen	47,225,783	73,027,117	64.67
CS	male	abdomen	29,656,467	39,569,021	74.95
CSrab	male	abdomen	26,931,208	44,702,325	60.25
CSrab	male	abdomen	10,639,480	17,154,576	62.02
CSrab	male	abdomen	39,105,284	51,590,908	75.80
IsoCS	male	abdomen	20,125,936	32,675,559	61.59
IsoCS	male	abdomen	5,422,571	8,475,350	63.98
IsoCS	male	abdomen	65,535,221	85,353,585	76.78
IsoCS	female	abdomen	49,248,555	61,591,761	79.96
CSrab	female	abdomen	24,268,638	33,301,977	72.87
bwbCS	male	head	41,466,785	55,597,781	74.58
bwbCS	male	head	36,041,171	50,840,587	70.89
bwbCS	male	head	62,840,805	80,615,028	77.95
CS	male	head	38,149,107	50,861,349	75.01
CS	male	head	19,919,990	27,227,824	73.16
CS	male	head	51,341,822	67,253,493	76.34
CSrab	male	head	56,191,834	75,651,390	74.28
CSrab	male	head	10,481,663	17,377,586	60.32
CSrab	male	head	55,120,600	73,965,574	74.52
IsoCS	male	head	17,332,681	27,276,701	63.54
IsoCS	male	head	28,841,223	39,570,265	72.89
IsoCS	male	head	62,213,672	82,095,892	75.78
IsoCS	female	head	49,089,361	63,708,772	77.05
CSrab	female	head	45,789,698	61,740,463	74.16
CS	female	head	45,947,520	57,865,112	79.40

Supplementary Table 1. Number and percent of RNA-seq reads mapping to the house fly reference genome for females and each male with a different naturally occurring proto-Y chromosome.

Sample Name	Reads mapped	Total reads	% mapped reads
Abdomen samples			
GFP-female-A2	91,396,275	106,578,289	85.76
GFP-female-A3	79,790,013	93,362,685	85.46
GFP-female-A4	67,507,547	78,662,688	85.82
GFP-male-A1	30,825,624	41,147,995	74.91
GFP-male-A2	43,102,152	57,473,417	74.99
GFP-male-A3	43,544,801	59,178,052	73.58
tra-N-A2	36,982,401	52,453,415	70.51
tra-N-A3	68,463,509	96,288,121	71.10
tra-N-A4	34,147,545	48,784,891	70.00
tra-SR-A1	45,637,174	60,334,091	75.64
tra-SR-A2	54,578,379	72,306,455	75.48
tra-SR-A3	35,999,042	47,579,279	75.66
Head samples			
GFP-female-H2	41,898,942	53,096,665	78.91
GFP-female-H3	54,190,305	69,827,731	77.61
GFP-female-H4	68,366,760	90,002,468	75.96
GFP-male-H1	86,270,347	106,498,553	81.01
GFP-male-H2	76,486,314	96,639,044	79.15
GFP-male-H3	105,491,479	132,492,819	79.62
tra-N-H2	42,279,891	55,339,089	76.40
tra-N-H3	22,602,895	29,743,740	75.99
tra-N-H4	31,937,609	41,479,997	77.00
tra-SR-H1	47,090,731	60,413,724	77.95
tra-SR-H2	82,077,805	101,558,122	80.82
tra-SR-H3	25,046,702	31,679,494	79.06

Supplementary Table 2. Number and percent of RNA-seq reads mapping to the house fly reference genome for each sample from the RNAi experiment.

Tissue	Comparison	#Diff	#Genes	Freq Diff
Abdomen	Y^M vs Y^M with new chr III	1159	11533	0.100
	Y^M vs III^M (CS)	511	10344	0.049
	Y^M vs III^M (CSrab)	479	9346	0.051
	III^M (CS) vs III^M (CSrab)	196	10460	0.19
Head	Y^M vs Y^M with new chr III	878	11909	0.074
	Y^M vs III^M (CS)	377	11845	0.032
	Y^M vs III^M (CSrab)	525	12390	0.042
	III^M (CS) vs III^M (CSrab)	739	12409	0.060

Supplementary Table 3. Differential expression between males with different genotypes. Counts of the number of genes that are expressed differentially (# Diff) and total genes expressed (#Genes) are shown, as well as the frequency of genes that are expressed differentially (Freq Diff). Y^M males are from the IsoCS strain; bwbCS Y^M males have a standard chromosome III from CS (bwbCS×CS males); III^M males are from either the CS or CSrab strain.

Tissue	Comparison	#Diff	#Genes	Freq Diff
Abdomen	III^M males #1 vs females	11030	14993	0.736
	sex-reversed males vs females	11005	14686	0.749
	III^M males #2 vs sex-reversed males	2867	13769	0.208
	III^M males #2 vs III^M males #1	2243	13162	0.170
Head	III^M males #1 vs females	5077	13558	0.374
	sex-reversed males vs females	735	12360	0.059
	III^M males #2 vs sex-reversed males	204	12959	0.016
	III^M males #2 vs III^M males #1	3260	13258	0.246

Supplementary Table 4. Differential expression between genotypic males and females with different RNAi treatments. Counts of the number of genes that are expressed differentially (#Diff) and total genes expressed (#Genes) are shown, as well as the frequency of genes that are expressed differentially (Freq Diff). Females are GFP-RNAi treated normal females; sex-reversed males are *Md-tra*-RNAi treated genotypic females; III^M males #1 are GFP-RNAi treated genotypic males; III^M males #2 are *Md-tra*-RNAi treated genotypic males.

Abdomen (# genes)		sex-reversed males vs females			
		male-biased	not female-biased	not male-biased	female-biased
genotypic male (III^M males #1) vs females	male-biased	6136	407	19	1
	not female-biased	483	1574	271	5
	not male-biased	26	274	841	182
	female-biased	5	18	277	4167
Head (# genes)		sex-reversed males vs females			
		male-biased	not female-biased	not male-biased	female-biased
genotypic male (III^M males #1) vs females	male-biased	254	1897	380	4
	not female-biased	50	2221	1177	34
	not male-biased	13	1091	2660	110
	female-biased	3	154	2045	267

Supplementary Table 5. Genes with sex-biased expression in sex-reversed or genotypic males. Counts are the number of genes that belong to each column and row combination. Columns compare sex-reversed males and normal females. Rows compare genotypic (normal) males and normal females. Genes with male-biased (female-biased) expression are expressed at significantly different levels between the sexes. Genes with not female-biased (not male-biased) have $\log_2$M/F not greater (less) than zero.

		Abdomen					
		normal-up-discordant (n-u-d)			sex-reversed-up-discordant (sr-u-d)		
Chromosomes (Muller elements)	# genes on chr	# genes	Odds ratio	95% CI	# genes	Odds ratio	95% CI
1(B)	2000	1	0.270	0.006 - 1.709	2	0.270	0.031 - 1.048
2(E)	2910	4	0.806	0.194 - 2.532	11	1.231	0.550 - 2.568
3(A)	**2094**	**9**	**4.129**	**1.482 - 11.322**	**13**	**2.386**	**1.119 - 4.853**
4(D)	2184	3	0.817	0.152 - 2.58	5	0.660	0.201 - 1.705
5(C)	2469	2	0.440	0.049 - 1.855	7	0.844	0.313 - 1.958
X(F)	45	0	0	0 - 57.539	0	0	0 - 27.459
Total	11702	19			38		

Supplementary Table 6. Chromosomal distribution of discordant sex-biased genes in abdomen. The chromosomal distribution of discordant genes was compared to all genes in the genome. Genes that were not assigned to a chromosome were excluded. A Fisher's exact test was performed to test for an excess of discordant sex-biased genes on each chromosome relative to genes on all other chromosomes.

		Head					
		normal-up-discordant (n-u-d)			sex-reversed-up-discordant (sr-u-d)		
Chromosomes (Muller elements)	# genes on chr	# genes	Odds ratio	95% CI	# genes	Odds ratio	95% CI
1(B)	1750	67	1.145	0.859 - 1.507	18	0.862	0.490 - 1.436
2(E)	2550	73	0.792	0.601 - 1.032	24	0.759	0.463 - 1.201
3(A)	**1842**	**108**	**2.028**	**1.592 - 2.569**	**44**	**2.665**	**1.787 - 3.933**
4(D)	1888	50	0.735	0.531 - 0.999	12	0.494	0.247 - 0.902
5(C)	2147	51	0.641	0.465 - 0.868	21	0.805	0.476 - 1.303
X(F)	34	1	0.858	0.021 - 5.144	0	0	0 - 9.947
Total	10211	350			119		

Supplementary Table 7. Chromosomal distribution of discordant sex-biased genes in head. The chromosomal distribution of discordant genes was compared to all genes in the genome. Genes that were not assigned to a chromosome were excluded. A Fisher's exact test was performed to test for an excess of discordant sex-biased genes on each chromosome relative to genes on the other chromosomes.

References cited

Dübendorfer A., and M. Hediger, 1998 The female-determining gene *F* of the housefly, *Musca domestica*, acts maternally to regulate its own zygotic activity. Genetics 150: 221–226.

Goldman T. D., and M. N. Arbeitman, 2007 Genomic and functional studies of Drosophila sex hierarchy regulated gene expression in adult head and nervous system tissues. PLoS Genet. 3: e216.

Hediger M., G. Burghardt, C. Siegenthaler, N. Buser, D. Hilfiker-Kleiner, *et al.*, 2004 Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator *doublesex*. Dev. Genes Evol. 214: 29–42.

Hediger M., C. Henggeler, N. Meier, R. Perez, G. Saccone, *et al.*, 2010 Molecular characterization of the key switch *F* provides a basis for understanding the rapid divergence of the sex-determining pathway in the housefly. Genetics 184: 155–170.

Heinrichs V., L. C. Ryner, and B. S. Baker, 1998 Regulation of sex-specific selection of fruitless 5' splice sites by transformer and transformer-2. Mol. Cell. Biol. 18: 450–458.

Kervestin S., and A. Jacobson, 2012 NMD: a multifaceted response to premature translational termination. Nat. Rev. Mol. Cell Biol. 13: 700–712.

Meier N., S. C. Käppeli, M. Hediger Niessen, J.-C. Billeter, S. F. Goodwin, *et al.*, 2013 Genetic control of courtship behavior in the housefly: evidence for a conserved bifurcation of the sex-determining pathway. PLoS One 8: e62476.

Meisel R. P., J. G. Scott, and A. G. Clark, 2015 Transcriptome Differences between Alternative Sex Determining Genotypes in the House Fly, Musca domestica. Genome Biol. Evol. 7: 2051–2061.

Robinett C. C., A. G. Vaughan, J.-M. Knapp, and B. S. Baker, 2010 Sex and the single cell. II. There is a

time and place for sex. PLoS Biol. 8: e1000365.