The Y Chromosome Modulates Splicing and Sex-Biased Intron Retention Rates in *Drosophila*

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ABSTRACT The *Drosophila* Y chromosome is a 40-Mb segment of mostly repetitive DNA; it harbors a handful of protein-coding genes and a disproportionate amount of satellite repeats, transposable elements, and multicopy DNA arrays. Intron retention (IR) is a type of alternative splicing (AS) event by which one or more introns remain within the mature transcript. IR recently emerged as a deliberate cellular mechanism to modulate gene expression levels and has been implicated in multiple biological processes. However, the extent of sex differences in IR and the contribution of the Y chromosome to the modulation of AS and IR rates has not been addressed. Here we showed pervasive IR in the fruit fly *Drosophila melanogaster* with thousands of novel IR events, hundreds of which displayed extensive sex bias. The data also revealed an unsuspected role for the Y chromosome in the modulation of AS and IR. The majority of sex-biased IR events introduced premature termination codons and the magnitude of sex bias was associated with gene expression differences between the sexes. Surprisingly, an extra Y chromosome in males (X^{YY} genotype) or the presence of a Y chromosome in females (X^{XY} genotype) significantly modulated IR and recapitulated natural differences in IR between the sexes. Our results highlight the significance of sex-biased IR in tuning sex differences and the role of the Y chromosome as a source of variable IR rates between the sexes. Modulation of splicing and IR rates across the genome represent new and unexpected outcomes of the *Drosophila* Y chromosome.

KEYWORDS sex-biased splicing; intron retention; Y chromosome; *Drosophila*

MALES and females share an identical set of autosomes, yet display extensive phenotypic variation in behavior, morphology, and physiology. These sex-specific and sex-biased phenotypes partially emerge from regulatory differences in gene expression. While sex differences in gene expression are observed in several tissues, they are especially manifested in the gonads (Meiklejohn *et al.* 2003; Parisi *et al.* 2003; Ellegren and Parsch 2007; Zhang *et al.* 2007; Assis *et al.* 2012; Parsch and Ellegren 2013; Perry *et al.* 2014) and are often traced to variation in the sex chromosomes (Gibson *et al.* 2002; Lemos *et al.* 2008; Sackton *et al.* 2011; Coolon *et al.* 2015). In *Drosophila*, the X chromosome

is relatively large with thousands of protein-coding genes, with experimental evidence confirming population genetic predictions about the consequences of X-linked variation. For instance, the X chromosome is a hotspot for sexually antagonistic variation in *Drosophila* (Gibson *et al.* 2002) and harbors disproportionate, nonadditive variation for gene expression diversity in females (Wayne *et al.* 2007). In contrast to the X chromosome, the Y chromosome carries a handful of protein-coding genes, which are mostly monomorphic within populations (Zurovcova and Eanes 1999). Despite the lack of diversity in coding regions (CDS), the 40-Mb chromosome is loaded with low-complexity DNA arrays (*e.g.*, microsatellites) and transposable elements (Dimitri and Pisano 1989; Carvalho 2002; Piergentili 2010; Bachtrog 2013). These repetitive elements are variable within populations (Lyckegaard and Clark 1989) and affect gene expression diversity in males (Lemos *et al.* 2008, 2010; Sackton *et al.* 2011). The mechanism through which Y-linked repeats modulate gene expression have been a matter of recent discussion (Francisco and Lemos 2014).

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Sex-biased alternative splicing (AS) can contribute to sex-specific adaptation (Parsch and Ellegren 2013). Accordingly, studies focusing on *Drosophila* and primates have described AS events that are differentially regulated between the sexes in both somatic and gonadal tissues (McIntyre *et al.* 2006; Telonis-Scott *et al.* 2009; Blekhman *et al.* 2010; Gan *et al.* 2010; Chang *et al.* 2011; Hartmann *et al.* 2011; Sturgill *et al.* 2013; Trabzuni *et al.* 2013). In *Drosophila*, the mechanism of sex determination hinges on the post-transcriptional regulation of the *sex-lethal* (*Sxl*) gene (Bell *et al.* 1988; Salz and Erickson 2010); *Sxl* encodes 21 protein isoforms, with all male-specific isoforms containing a translation-terminating exon. Proper splicing of *Sxl* is necessary to orchestrate the expression of genes that control sex-specific development and behavior (Salz and Erickson 2010). Similarly, *doublesex* (*dsx*) and *transformer* (*tra*), two members of the core pathway of sex determination, also encode male- or female-specific isoforms (Christiansen *et al.* 2002; Pomiankowski *et al.* 2004; Venables *et al.* 2012). Intron retention (IR) is a type of AS event in which one or more introns are retained in the messenger RNA (mRNA) molecule. IR is the dominant AS type in *Drosophila* (Graveley *et al.* 2011) and affects about half of all introns in mammals (Wong *et al.* 2013; Braunschweig *et al.* 2014; Edwards *et al.* 2016; Pimentel *et al.* 2016). The “anomalous” mRNA generated by the retention of an intron may contain a premature termination codon (PTC) (Jaillon *et al.* 2008), which can sometimes result in the degradation of the mRNA by the nonsense-mediated mRNA decay (NMD) pathway. Because of this, mRNAs generated through IR events could be viewed as failures of the splicing process and void of biological function (Jaillon *et al.* 2008; Roy and Irimia 2008). However, recent studies suggested that IR events are involved in multiple cellular and physiological processes, including hematogenesis (Wong *et al.* 2013; Edwards *et al.* 2016; Pimentel *et al.* 2016), T cell activation (Ni *et al.* 2016), neuronal differentiation (Braunschweig *et al.* 2014), and carcinogenesis (Dvinge and Bradley 2015; Jung *et al.* 2015). Despite these advances, questions remain regarding the extent to which introns are differentially retained between sexes and how sex-biased IR rates are regulated. It is possible, for instance, that the Y chromosome might contribute to sex-biased IR in addition to its ability to modulate gene expression across the genome.

Here we documented sex-biased IR in *Drosophila melanogaster* and investigated the role of the Y chromosome in the modulation of AS and IR rates. We first analyzed multiple RNA sequencing (RNA-seq) samples to discover IR events and identify tissue and sex differences in IR rates. We then evaluated the relevance of the Y chromosome for IR rates using recently developed strains that differ exclusively on the origin and number of the Y chromosome. Analysis of IR in those genotypes uncovered a novel contribution of the Y chromosome in the regulation of splicing and the emergence of sex-biased IR rates.

Materials and Methods

Data sets and genotypes

RNA-seq data were obtained from two sources (Supplemental Material, Table S1). First, ovaries, testes, and accessory glands from 4-day-old flies, as well as whole body of adult flies of different ages (1, 5, and 30 days old) were downloaded from the modENCODE project (Graveley *et al.* 2011; Brown *et al.* 2014). Second, gonad tissues (ovaries and testes) of genotypes with wild-type chromosome numbers and gonad tissues of genotypes with Y-chromosome aneuploidy (females: $X^{\wedge}XY^{ohio}$ or $X^{\wedge}XY^{congo}$; males: $X^{\wedge}YY^{ohio}$ or $X^{\wedge}YY^{congo}$) (see also Lemos *et al.* 2010; Branco *et al.* 2017). These genotypes were recently constructed in our laboratory and are identical within each sex: (i) $X^{\wedge}X$ and $X^{\wedge}XY$ females differ exclusively by the presence of the Y chromosome in $X^{\wedge}XY$, and (ii) $X^{\wedge}Y$ and $X^{\wedge}YY$ males differ exclusively in the number of Y chromosomes (Table S1) (Branco *et al.* 2017). Splicing factors were downloaded using FlyBase QueryBuilder (<http://flybase.org/cgi-bin/qbgui.fr.html>) (dos Santos *et al.* 2015); 278 genes under gene ontology (GO) biological processes terms GO:0008380 (RNA splicing) and GO:0000398 (mRNA splicing, via spliceosome) were selected. The 15 exon junction complex (EJC) factors were as defined in Hayashi *et al.* (2014).

Processing of RNA-seq reads

For our sequenced 150×2 paired-end reads, trim_galore! was used to trim the 3' adaptor, and the trimmed reads with only one end longer than 119 bp were treated as single end. The remaining reads were further clipped from the 3' end to exactly 120 bp. For the downloaded reads, the last 25 bp of each end were also removed. Trimmed reads were mapped onto the *D. melanogaster* genome r6 using TopHat version 2.0.13 (Trapnell *et al.* 2009), with “dmel-all-r6.08.gtf” downloaded from FlyBase as guidance (-G option). To detect exon-exon splicing junctions from the mapping files (“BAM” format), we examined uniquely mapped reads that skipped particular regions from the reference (the mapping quality equals 50, and the CIGAR is “N”). From these reads, we extracted the coordinates of the skipped region (which corresponds to the intron regions, also see below). The strand origins of these junctions were confirmed by allowing three types of introns, i.e., “GT-AG,” “GC-AG,” and “AT-AC.” We filtered those junctions that were not included in FlyBase by requiring a Shannon entropy (Graveley *et al.* 2011; Brooks *et al.* 2015) higher than two in both data sets. Altogether, we detected 66,724 junctions (61,056 of which were annotated in FlyBase r6.08, and 6588 were novel).

Identification of IR events and calculation of IR rates

For each intron, we counted the number of reads that support the IR isoform (reads mapped to exon-intron junctions and within the intron region), as well as the number of reads that support the intron-splicing isoform (mapped to exon-exon junction)

(Figure 1A). We excluded introns that overlap exons of any other genes. Introns fulfilling the following two criteria in any sample were regarded as IR events: first, the intron region was mapped with at least 10 reads (each read overlapped at least 6 bp with the intron); second, the intron region had 100% coverage. To quantify IR rates, we used the metric percent IR (PIR) as calculated in previous studies (Braunschweig *et al.* 2014; Pimentel *et al.* 2016) and briefly described here with minor modifications (Figure 1A). For ease of description, suppose that the length of the target intron and the trimmed reads were L_1 and L_2 , respectively. By requiring at least 6-bp overhang for reads at both sides of the exon–intron junction (for the IR isoform) we could observe $L_1 + L_2 - 11$ mappable positions that are specific to the retention isoform. If the number of reads mapped to these positions is I , we then could calculate N_r , the normalized abundance of the retention isoform, as: $N_r = I / (L_1 + L_2 - 11)$. Similarly, under the requirement of at least 6-bp overhang for reads at both sides of the exon–exon junction (for the intron-splicing isoform), we could observe $L_2 - 11$ mappable positions that are specific to the intron-splicing isoform. If the number of reads mapped to these positions is J , we then could calculate N_s , the normalized abundance of the intron-splicing isoform, as: $N_s = J / (L_2 - 11)$. Therefore, for a specific IR event, its IR rate is defined by the PIR metric: $PIR = N_r / (N_r + N_s) \times 100$.

Identification of differentially retained introns

For each IR event we summed the reads from biological replicates that were specifically mapped to either the IR isoform or the intron-splicing isoform. When comparing a pair of tissues, we required $PIR > 0$ in either tissue and at least 15 total mapped reads to the IR isoform and intron-splicing isoform-specific regions [$(I + J) \geq 15$] in both tissues. We then used Fisher's exact test to detect significant events. The resulting P -values were further adjusted by false discovery rate (FDR). When identifying IR events that are sensitive to the Y chromosome, we required that (i) events show consistent change for the two Y introgressions in each tissue ($X^{\wedge}XYohio$ and $X^{\wedge}XYcongo$ for ovary; or $X^{\wedge}YYohio$ and $X^{\wedge}YYcongo$ for testis) compared with their corresponding wild type ($X^{\wedge}X$ ovary or $X^{\wedge}Y$ testis), (ii) the IR level changed in the same direction and by >5 , and (iii) FDR of Fisher's combined P -values was <0.05 .

Identification of differentially expressed genes

HTSeq version 0.6.1 (Anders *et al.* 2015) was used to count the reads mapping to exon regions of each gene, which was followed by applying the edgeR package (Robinson *et al.* 2010) to obtain the differentially expressed genes between groups. During the differential expression analysis, genes were filtered by requiring a minimum of 1 count per million in at least half of the samples.

GO analysis

We used PANTHER (Mi *et al.* 2016) (<http://www.pantherdb.org/>) for GO analysis. Each analysis requires a test list and a reference list. The test lists included genes with ovary-biased

or testis-biased IRs (see *Results*). The reference list included all genes that either have sufficient expression (reads per kilobase million > 2) in both tissues or have at least 15 total reads mapped to the IR isoform and intron-splicing isoform-specific regions [$(I + J) \geq 15$] in both tissues. We outputted biological process GO terms with $P < 0.01$.

Permutation test for the overlaps of two sets

This procedure was performed to test whether the overlap of two sets (a and b) is significantly higher than random expectation, while the two sets themselves are derived from two larger groups (A and B ; i.e., a is a subset of A and b is a subset of B) that have shared elements. Specifically, for each of 10,000 iterations, we randomly picked a subset (or a_i) from A and a subset (or b_i) from B , requiring that a_i and b_i have the same numbers as a and b , respectively. We then counted the number of times the overlap between a_i and b_i is equal to or larger than that observed between a and b . The P -value was calculated as this count divided by 10,000.

Data availability

RNA-seq data of *D. melanogaster* strains (with or without Y-chromosome aneuploidy) were deposited on the Gene Expression Omnibus database (accession number: GSE93699). A list of all samples can be found in Table S1.

Results

Identifying IR events in *D. melanogaster*

To ascertain IR events in *D. melanogaster*, we combined 38 RNA-seq samples produced by the modENCODE project (Graveley *et al.* 2011; Brown *et al.* 2014) with 30 samples recently sequenced in our laboratory (Branco *et al.* 2017). The complete set comprises 2,722,187,589 reads from five tissue types (accessory glands, brain, ovary, testis, and whole body; Table S1). We mapped reads to the reference *Drosophila* genome (Figure 1A) and identified 21,664 IR events (Figure 1B) in 7546 genes. We used the metric PIR (see *Materials and Methods*) as previously implemented (Braunschweig *et al.* 2014; Pimentel *et al.* 2016) to represent the percentage of transcripts with the focal intron retained in each sample. From these IR events, 1304 had been annotated in FlyBase (i.e., both IR and intron-splicing isoforms were annotated), while the other 20,360 were newly identified. As expected, the annotated events had a much higher PIR (Figure 1C; median PIR, 44.0 vs. 2.0, $P < 2.2e^{-16}$) and shorter intron length (Figure 1D; median length in base pairs, 79 vs. 85, Wilcoxon rank sum test $P = 9.28e^{-9}$) than novel ones. On the other hand, novel retention events occurred preferentially in introns localized in CDS (Figure 1E; 87.2 vs. 43.1%, χ^2 test $P < 2.2e^{-16}$) and frequently introduced a PTC (Figure 1F; 87.2 vs. 33.3%, χ^2 test $P < 2.2e^{-16}$). Hence, most of the newly identified IR events are not expected to produce functional protein isoforms and might be more frequently targeted by the NMD pathway than annotated ones.

Figure 1 (A) Diagram shows the identification of IR events. RNA-seq reads mapped to the intron region (unique to the IR isoform, *I*) and to the exon-exon junction region (unique to the intron splicing isoform, *J*) were separately counted. The normalized abundances of the IR isoform (N_r) and the intron splicing isoform (N_s) were estimated (see *Materials and Methods*). The PIR metric was calculated as: $N_r / (N_r + N_s) \times 100$. (B) Number of annotated and novel IR events. (C–F) Novel IR events tend to (C) have lower PIR (median PIR 2.0 vs. 44.0, Wilcoxon rank sum test, $P < 2.2e-16$), (D) have longer length (median length in bp, 85 vs. 79, Wilcoxon rank sum test, $P = 9.28e-9$), (E) be more likely to occur in CDS regions (87.2 vs. 43.1%, χ^2 test, $P < 2.2e-16$), and (F) introduce a PTC (87.2 vs. 33.3%, χ^2 test, $P < 2.2e-16$) than annotated events. ncRNA, noncoding RNA.

A single gene can simultaneously have multiple retained introns. These cooccurring IR events might increase or decrease their PIR coordinately across tissues/conditions. To test this possibility, we compared ovary and testis tissues. Indeed, examining genes that have exactly two different IR events showed that their PIRs preferentially change in the same direction (Figure 2A; $346/600 = 57.67\%$ of the pairs with simultaneous increase or decrease in PIR in ovary compared to testis, binomial test $P = 0.0002$). The pattern becomes more evident ($77/123 = 62.6\%$, binomial test $P = 0.0066$) when we restricted the analysis to IRs with considerable changes ($|\Delta PIR| > 5$) and even more evident ($61/93 = 65.59\%$, binomial test $P = 0.0035$) when only IRs within CDS were considered ($|\Delta PIR| > 5$ and CDS location). Of note, this pattern remained unchanged if we used other pairs of tissues for comparison (Figure S1 in File S1). These observations suggest mechanisms that similarly influence IR events across multiple introns within an mRNA during transcription.

Introns that are differentially retained in ovary and testis

Previous studies have shown that sex-biased splicing in *Drosophila* is mainly due to AS events in the gonads (Brown *et al.* 2014; Gibilisco *et al.* 2016). To explore IR variation between sexes, we first aimed at identifying introns differentially retained between male and female gonadal tissues. From 6573 IRs with ≥ 15 mapped reads in both ovary and testis, we identified 807 events (12.28%) using a cutoff of $|\Delta PIR| > 5$ between the sexes and FDR < 0.05 (P -values from Fisher's exact test) (Table S2). Among them, 280 events had significantly increased PIR in ovary (referred to as ovary-biased IR events), while 527 events had significantly increased PIR in testis (referred to as testis-biased IR events). All these events were hereafter considered sex-biased IR events. For instance, the intron M1 of the *tra2* gene, a key member of the core pathway for sex determination in *Drosophila*, is strongly retained in testis but not in ovary (Figure

Figure 2 Differences in IR between ovary and testis. (A) For genes with exactly two IRs, both IR events tend to change in the same direction between tissues. “Informative” refers to genes with two IR events (with any PIR value); “ $\Delta\text{PIR} > 5$ percent” refers to genes with two IRs for which both events had $\Delta\text{PIR} > 5$ percent between tissues; “ $\Delta\text{PIR} > 5$ percent & CDS” refers to genes whose CDS regions have two IRs for which both events had $\Delta\text{PIR} > 5$ percent between tissues. Error bars are estimated 95% confidence intervals calculated by binomial distribution. (B) Coverage for retained intron M1 as well as adjacent regions of *tra2* (intron: 2R:14603502–14603733) is shown. (C) Both ovary-biased and testis-biased IRs are enriched in UTRs compared to nonsex-biased events (hypergeometric test, $P < 2.43e^{-8}$). Testis-biased IR events are particularly enriched in 5' UTR (hypergeometric test, $P = 5.71e^{-15}$) and ovary-biased IR events are enriched in 3' UTR (hypergeometric test, $P = 6.55e^{-12}$). (D) Over 80% of IR events located within CDS introduced a PTC. (E) Genes with ovary-biased IR ($|\text{PIR}_{\text{ovary}} - \text{PIR}_{\text{testis}}| > 5$ and $\text{FDR} < 0.05$) have higher testis vs. ovary expression FC, compared with genes with stable PIR between the two tissues ($|\Delta\text{PIR}| < 5$ and $\text{FDR} > 0.1$) (Wilcoxon rank sum test, $P = 1.92e^{-9}$), as well as genes with testis-biased IR ($|\text{PIR}_{\text{ovary}} - \text{PIR}_{\text{testis}}| < -5$ and $\text{FDR} < 0.05$) ($P < 2.2e^{-16}$). (F) For the two groups of IR events located in CDS, the PTC-containing ones (4846 events) tend to have more negative Spearman's correlation coefficients with gene expression levels than in-frame IR events (213 events for which the length of retained intron is multiple of 3 and do not introduce a PTC) (permutation test with 10,000 iterations, $P = 0.002$). ** $P < 0.01$, *** $P < 0.001$. Corr coeff, correlation coefficient; ncRNA, noncoding RNA.

2B); a pattern that is in agreement with previous reports (Mattox and Baker 1991; Chandler *et al.* 2003). Interestingly, we observed a mild enrichment of sex-biased IRs in UTRs (Figure 2C; hypergeometric test $P < 2.43e^{-8}$; Figure S2 in File S1): testis-biased events are particularly enriched in 5' UTR (Figure 2C; hypergeometric test $P = 5.71e^{-15}$; Figure S2 in File S1), while ovary-biased ones are more enriched in 3' UTR (Figure 2C; $P = 6.55e^{-12}$; Figure S2 in File S1).

It has been suggested that IR is deliberately used as a regulatory step to decrease the expression of target genes (Braunschweig *et al.* 2014; Jung *et al.* 2015). Our data showed that the majority of sex-biased IR events located in CDS introduced PTCs (>80%; Figure 2D). We therefore asked whether sex-biased IR events with PTC are associated with gene expression changes. As a comparison, we selected a group of IRs with stable PIRs in ovary and testis ($|\Delta\text{PIR}| < 5$ and $\text{FDR} > 0.1$). We found that genes with ovary-biased IRs also

displayed lower ovary expression as shown by a higher testis vs. ovary expression fold change (FC) than genes with stable PIRs (Figure 2E; Wilcoxon rank sum test $P = 1.92e^{-9}$) or testis-biased IRs ($P < 2.2e^{-16}$). To further explore the issue, we selected three gene groups based on their expression FC between ovary and testis: (i) testis-biased genes ($\log_2 \text{FC} > 1$, $\text{FDR} < 0.01$), (ii) ovary-biased genes ($\log_2 \text{FC} < -1$, $\text{FDR} < 0.01$), and (iii) genes stably expressed in ovary and testis ($|\log_2 \text{FC}| < 0.25$ and $\text{FDR} > 0.1$). We found that genes with testis-biased expression had lower testis vs. ovary ΔPIR than stably expressed genes (Figure S3, A and B, in File S1; $P < 0.0007$) or ovary-biased genes (Figure S3, A and B, in File S1; $P < 2.2e^{-16}$). Further, we compared two groups of IRs located in CDS: one group comprises 213 in-frame events whose intron length is a multiple of 3, and another group comprises 4846 events that introduced a PTC. For the in-frame IRs, no significant correlation was observed between IR change and expression FC (Figure 2F; Spearman's

$\rho = -0.005$, $P = 0.94$). For the PTC containing IRs we randomly selected sets of 213 IR events and then similarly checked the correlation between IR change and expression FC. Such a sampling process is iteratively performed 10,000 times (each with replacement). In 9980 of these samples with PTC containing IRs, we observed a higher negative correlation coefficient between change in IR and change in expression than that observed for the in-frame IRs (permutation test $P = 0.002$; Figure 2F). Together, these results suggest a role for IR in sex-biased expression, and are consistent with previous reports indicating that IR can negatively tune gene expression (Braunschweig *et al.* 2014; Dvinge and Bradley 2015; Pimentel *et al.* 2016). Finally, GO analysis showed that genes with testis-biased IR were enriched in oogenesis, neurogenesis, female gamete generation, and others (Table S3; hypergeometric test $P < 0.001$). These enrichments are consistent with their higher expression in the ovary. On the other hand, genes with ovary-biased IR were enriched in developmental processes, cell differentiation, and others (Table S4; $P < 0.01$). In both cases, genes with an increased PIR might have functions that are less consequential in the respective tissue/sex.

Y-chromosome modulation of IR rates

The Y chromosome affects the balance of euchromatin and heterochromatin states of the genome and shapes genome-wide gene expression in males (Lemos *et al.* 2008, 2010; Sackton *et al.* 2011). We hypothesized that the Y chromosome could modulate IR rates and contribute to sex differences in IR. We reasoned that if the Y chromosome is relevant to IR, then we might recover sex-biased IRs upon Y-chromosome introgression into females or upon the addition of an extra Y chromosome in males. To test this hypothesis, we interrogated IR in gonadal tissues of recently developed *Drosophila* lines that are genetically identical to the reference of the same sex, except for the presence and origin of the ectopic Y chromosome (male: $X^{\wedge}YY_{\text{congo}}$ or $X^{\wedge}YY_{\text{ohio}}$ vs. $X^{\wedge}Y$; female: $X^{\wedge}XY_{\text{congo}}$ or $X^{\wedge}XY_{\text{ohio}}$ vs. $X^{\wedge}X$).

We first compared the effects of the two Y chromosomes with different geographical origins (*Ycongo* and *Yohio*) and observed that the ΔPIR induced by these Y chromosomes is well correlated in both ovaries and testes (Figure 3, A and B; Spearman's $\rho = 0.60$ for $X^{\wedge}XY_{\text{congo}}$ and $X^{\wedge}XY_{\text{ohio}}$, and $\rho = 0.53$ for $X^{\wedge}YY_{\text{congo}}$ and $X^{\wedge}YY_{\text{ohio}}$, $P < 2.2 \times 10^{-16}$ in both cases). This indicates that the effects of *Ycongo* and *Yohio* on IR rates are largely consistent within each sex. Therefore, to identify a group of higher confidence IRs that are sensitive to Y aneuploidy (hereafter, Y-sensitive IRs), we combined data from both *Ycongo* and *Yohio* using Fisher's method. Using this approach, we identified 245 events with significantly different IR rates in ovaries isolated from $X^{\wedge}XY$ flies relative to $X^{\wedge}X$ control, and 144 events with significantly different IR rates in testes from $X^{\wedge}YY$ flies relative to $X^{\wedge}Y$ control [Table S5 and Table S6; FDR-corrected combined P -values < 0.05 ; $|\Delta\text{PIR}| > 5$ compared to their corresponding wild types (see Materials and Methods)]. For comparison, we also examined

Figure 3 Y-chromosome modulation of IR. (A and B) Positive correlation between ΔPIR induced by the *Yohio* and *Ycongo* in both ovaries and testes (Spearman's $\rho = 0.60$ for $X^{\wedge}XY_{\text{congo}}$ and $X^{\wedge}XY_{\text{ohio}}$ and 0.53 for $X^{\wedge}YY_{\text{congo}}$ and $X^{\wedge}YY_{\text{ohio}}$, both $P < 2.2 \times 10^{-16}$). (C) In both ovaries and testes, genes with increased PIR showed significant downregulation, compared with genes with decreased PIR (Wilcoxon's rank sum test, $** P = 0.0012$ in ovary and $*** P = 1.45 \times 10^{-9}$ in testis). *Yohio* and *Ycongo* are merged in both tissues. A cutoff of $|\Delta\text{PIR}| > 5$ and $P < 0.01$ was used in (C). WT, wild type.

other AS types (exon skipping and alternative 5'/3' splice sites) and observed fewer significant events (Table 1). Notably, the frequency of Y-sensitive exon skipping events is higher than the other AS types and genes with significant IR events also tend to have other types of significant AS events (Table S7). Interestingly, while the overlap between IR events modulated by the Y chromosome in ovaries and testes is minimal (seven IR events), the majority of Y-sensitive IRs showed lowered IR rates upon Y introgression in both tissues (binomial test, $P < 0.0008$). The pattern is more striking in ovaries in which 94.69% (232/245) of the events showed a lower PIR in $X^{\wedge}XY$ relative to $X^{\wedge}X$, while 68.75% (99/144) of the events in testes displayed a lower PIR in $X^{\wedge}YY$ relative to $X^{\wedge}Y$. Moreover, the negative association between gene expression and PIR can again be observed in both ovaries and testes (Figure 3C; Wilcoxon's rank sum test $P < 0.01$). These data indicate a role for the Y chromosome in the modulation of IR rates.

Presence of the Y chromosome in ovary recapitulates testis-biased IR

To address whether the Y chromosome contributes to sex-biased IR, we selected the groups of IRs that are both sex biased and sensitive to the Y chromosome. We found significant overlap between the two groups of events (92 and 59

Table 1 Summary of four types of AS events that are sex biased or Y sensitive

	Significant (total) events in each AS type			
	IR	Exon skipping	Alternative 5' SS	Alternative 3' SS
WT ovary vs. WT testis (sex biased)	807 (6573)	390 (973)	143 (977)	128 (959)
X ^{XY} vs. X ^{XX} in ovary (Y sensitive)	245 (6515)	78 (979)	25 (1188)	27 (1087)
X ^{YY} vs. X ^{XY} in testis (Y sensitive)	144 (7873)	85 (1061)	29 (1137)	41 (1412)

Significant events were defined by a Δ PIR for IR > 5 or Δ percent splicing rate > 5 (for other AS types), and an FDR-corrected Fisher's combined P -value < 0.05 . SS, splice sites; WT, wild-type chromosome number.

Y-sensitive IR events identified in ovary and testis, respectively, are also sex biased; permutation test P -values = 0 for both cases of overlap between Y sensitivity and sex bias). Intriguingly, for the 92 IRs that are both Y sensitive in ovary and sex biased, we observed strong positive correlations between the Δ PIR induced by Y chromosomes in ovaries and the Δ PIR in the testis vs. ovary contrast (Figure 4A; Spearman's $\rho = 0.87$ and 0.81 for $X^{XYcongo}$ and X^{XYohio} , $P < 2.2e^{-16}$). This suggests that, for IR events that are sensitive to the Y chromosome in females, the Δ PIR between the sexes might be partially attributable to the presence of the Y chromosome in males. Lower but still notably significant correlations can be obtained by replacing the 92 Y-sensitive events with all available IRs (Figure S4A in File S1; $\rho = 0.40$ and 0.31 for $X^{XYcongo}$ and X^{XYohio} , $P < 2.2e^{-16}$). Therefore, the presence of the Y chromosome in the female germline recapitulated the differences in IR rates that are naturally observed between ovaries and testis.

Contrasting IR responses to Y-chromosome aneuploidy in testes and ovaries

We also addressed the consequence of an extra Y chromosome in the male testis. Surprisingly, for the 59 IRs that are both Y sensitive in testis and sex biased, we observed strong negative correlations between the Δ PIR induced by the extra Y chromosome in testes and the Δ PIR in the testis vs. ovary contrast (Figure 4B; $\rho = -0.51$ and -0.66 for $X^{YYcongo}$ and X^{YYohio} , $P = 5.37e^{-5}$ and $4.01e^{-8}$), which is also consistent for all available IRs (Figure S4B in File S1; $\rho = -0.35$ and -0.40 for $X^{YYcongo}$ and X^{YYohio} , $P < 2.2e^{-16}$). The contrasting effect of the extra dose/presence of the Y chromosome is further evident when directly comparing the Δ PIR induced by the Y chromosome in ovaries with the Δ PIR induced by the extra Y chromosome in testes (Figure S5 in File S1). These data indicate contrasting responses to Y introgression in ovaries and testes.

The expression of splicing factors differs between ovary and testis but is insufficient to explain the Y-chromosome influence on IR

The Y chromosome's modulation of IR could be mediated through changes in the expression of splicing factors. To address this, we examined the expression of 278 splicing factors (see *Materials and Methods*). We first compared their expression in wild-type ovary and testis tissues, and found higher overall abundances of splicing factors in ovary than in testis (paired Wilcoxon rank sum test, $P = 2.98e^{-10}$), with more

genes in the set displaying ovary- rather than testis-biased expression (Figure 5A). This is particularly noteworthy because it contrasts with the general pattern of all expressed genes, with more genes displaying testis- rather than ovary-biased expression (Table S8; see also Parisi *et al.* 2003; Assis *et al.* 2012). In agreement with the higher abundance of splicing factors in ovary, we also observed an overall lower IR rate in ovary than in testis (Figure S6 in File S1; $P < 2.2e^{-16}$). We also inspected 15 EJC factors, which are crucial for intron removal (Hayashi *et al.* 2014), and verified that nine of them had significantly lower mRNA abundance in testis (Figure 5B). Among these nine genes, *mago* and *Acn* are indispensable for splicing the fourth intron of *Piwi* transcripts (Hayashi *et al.* 2014). As expected, we observed that this intron is significantly retained in testis compared to ovary (Figure S7 in File S1; 51.3 vs. 4.9 , $FDR < 2.2e^{-16}$). We further examined AS of splicing factors and observed tens of sex-biased AS events, most of which are IR events (Table S9). Consistently, these sex-biased IR events also tend to have a lower PIR in ovary compared to testis (Wilcoxon rank sum test, $P < 0.005$). Together, these data indicate that ovary and testis differ in mRNA abundances and compositions for splicing factors.

On the other hand, when comparing X^{XY} ovaries and X^{YY} testes with their respective wild-type genotypes of the same sex, we observed that the abundances and splicing of splicing factors, including EJC genes, is largely unaffected by the Y chromosome (Figure 5, C and D, Figure S8 in File S1, and Table S9). This indicates that the mechanism of Y-chromosome modulation of IR may not require modifying the expression of splicing factors. However, these data should be interpreted with caution, because the activity of splicing factors might not be accurately indexed by their mRNA abundances. Other processes, including various post-transcriptional modifications, could also be influential.

Discussion

Recent studies have identified numerous differentially regulated AS events in a variety of conditions (McIntyre *et al.* 2006; Wang *et al.* 2008, 2014; Telonis-Scott *et al.* 2009; Gan *et al.* 2010; Chang *et al.* 2011; Hartmann *et al.* 2011; Sturgill *et al.* 2013) and highlighted the role of IR as a cellular mechanism to influence mRNA abundance and alter biological outcomes (Braunschweig *et al.* 2014; Jung *et al.* 2015; Edwards *et al.* 2016; Mauger *et al.* 2016; Ni *et al.* 2016; Middleton *et al.*

Figure 4 Contrasting effects of the introgressed Y chromosome in ovaries and testes. (A) Positive correlation between the Δ PIR induced by Y-chromosome presence in $X^A X Y$ ovaries and the Δ PIR between testis vs. ovary with wild-type chromosome number (Spearman's $\rho = 0.87$ and 0.81 for $X^A X Y_{congo}$ and $X^A X Y_{ohio}$, $P < 2.2e^{-16}$; 92 IR events that are both Y sensitive in ovaries and sex biased). (B) Negative correlation between the Δ PIR induced by extra Y chromosome in $X^A Y Y$ testes and the Δ PIR between testis vs. ovary with wild-type chromosome number ($\rho = -0.51$ and -0.66 for $X^A Y Y_{congo}$ and $X^A Y Y_{ohio}$, $P = 5.37e^{-5}$ and $4.01e^{-8}$; 59 IR events that are both Y sensitive in testis and sex biased). The titles of the four panels in (A and B) indicate the tissue types and the introgressed Y chromosomes. WT, wild type.

2017; Naro *et al.* 2017). However, the occurrence and extent of sex-biased IR has not been addressed and the role of sex chromosomes in the modulation of splicing has not been investigated. Our results expanded the catalog of IR in *Drosophila* and suggested a role for sex-dependent IR in the modulation of gene expression differences between males and females. Specifically, in either males or females, transcripts with lower abundances in one sex also displayed increased IR rates in the same sex (Figure 2E). This suggests IR as a mechanism that affects genes with a sex-specific function in one of the sexes. *Piwi*, for example, is a gene that is critical for stem cell renewal in *Drosophila* during oogenesis (Cox *et al.* 1998). The retention of *Piwi*'s fourth intron can greatly reduce the abundance of the PIWI protein and consequently hinder its function (Hayashi *et al.* 2014). As expected, *Piwi* expression is higher in the ovary and matched by a lower rate of retention of the fourth intron, whereas *Piwi* expression is lower in testis and accompanied by a much higher IR rate. We also observed that some other interesting genes important for oogenesis such as *Medea* (Xie and Spradling 1998) and *Tho2* (Jagut *et al.* 2013) had lower IR rates and higher abundance in the ovary; conversely, genes important for spermatogenesis

such as *Boule* (Castrillon *et al.* 1993) and *ana1* (Blachon *et al.* 2009) had lower IR rates and higher abundance in the testis. Hence, IR negatively affects the abundance of transcripts in one of the sexes and contributes to sex-biased gene expression. Preferential IR in one sex could be evolutionarily adaptive: the mechanism could be used by a gene to lower its impact in one of the sexes while retaining a sex-specific function in the other sex where it has lower IR rates. On the other hand, increased IR rates in one sex could reflect a lowered ability to maintain efficient splicing when gene function is under relaxed stabilizing selection to maintain faithful splicing in the focal sex.

In addition to negatively tune gene expression, regulated IR events could have additional biological significance. For instance, a fraction of the sex-biasedly retained introns do not constrain a PTC and could be competent to produce functional proteins. It is also noteworthy that many PTC-containing mRNAs are not affected by the NMD process (Hansen *et al.* 2009; Sauliere *et al.* 2010; Chapin *et al.* 2014), suggesting the existence of PTC-containing IR transcripts that are not subject to NMD. We also observed a mild enrichment for sex-biased IR events in UTR regions (Figure 2C) compared to the overall distribution. IR in the 5' UTR could affect the export of mRNA from the nucleus (Cenik *et al.* 2011) or its translation (Remy *et al.* 2014), whereas IR in the 3' UTR could affect microRNA target sites (Tan *et al.* 2007). The various roles of IR across genes and genomes are of interest for further research.

Our data indicated that the presence of the Y chromosome contributed to sex-biased IR. Introgressing the Y chromosome into females recapitulated natural differences in IR rates that were observed between wild-type testis and ovary of *Drosophila* (Figure 4A). The observations are also reminiscent of a study reporting differentially spliced transcripts in mice strains with the same genetic background but different origins of the Y chromosome (Case *et al.* 2013). Collectively, the observations indicate that the Y chromosome has the ability to modulate AS in both *Drosophila* and mammals and point to a Y-chromosome contribution to sexual dimorphism in the composition of transcription isoforms. What are the potential mechanisms for Y-chromosome regulation of IR? First we note that Y-chromosome modulation of splicing did not appear to be mediated by changes in the expression of the splicing machinery. This is because the mRNA abundance of splicing factors remained largely unaffected by the Y chromosome (Figure 5, C and D, and Figure S8 in File S1). Nevertheless, such data should be interpreted with caution, because the activity of splicing might not be accurately gauged by the expression level of splicing factors. On the other hand, the regulatory role of Y-linked variation on genome-wide gene expression has been documented in multiple studies (Lemos *et al.* 2008, 2010; Sackton *et al.* 2011). Such regulation need not involve the expression of Y-linked, protein-coding genes but might be mediated by Y-linked repetitive elements. In particular, the *Drosophila* Y chromosome is notorious for its heterochromatic state and the extensive

Figure 5 Splicing factors are differentially expressed between ovary and testis but remain stable in Y-chromosome aneuploids. The expression of (A) all splicing factors and (B) EJC complex genes are shown; factors with biased expression ($|\log_2 FC| > 1$, $FDR < 0.01$) are shown with navy (ovary biased) and orange (testis biased) colors. (C and D) The expression of splicing factors remains largely unchanged upon Y introgression compared to wild-type strains. No gene reached the cutoff ($FDR < 0.01$) in either tissue upon Y introgression. $X^{\wedge}XY_{congo}$ ovary and $X^{\wedge}YY_{congo}$ testis were used in (C and D) when comparing to their relative wild types. FPKM, fragments per kilobase million.

amounts of repetitive sequences that it harbors, including microsatellites, transposable elements, and ribosomal DNA arrays with variable copy number. According to one model, the large amounts of Y-linked repetitive sequences may serve as a sink that sequester chromatin-modifying proteins and factors that would otherwise participate in the transcription process and epigenetic states elsewhere in the genome (Locke *et al.* 1988; Dimitri and Pisano 1989; Lemos *et al.* 2010; Francisco and Lemos 2014; Kelsey and Clark 2017). Hence, the balance of euchromatin and heterochromatin on the X chromosome and autosomes is modified by reducing the availability of chromatin-binding proteins across the genome. It is also known that local changes in chromatin structure could affect the accessibility of splicing factors to the nascent transcripts (Allemand *et al.* 2016) and/or RNA polymerase II elongation rate during transcription (Fong *et al.* 2014). Thus, one possibility is that Y-chromosome modulation of IR may be mediated by Y-chromosome modulation of chromatin states, which subsequently affects the splicing machinery. This could explain the positive correlation between changes in IR rates induced by the presence of the Y chromosome in ovaries and differences in IR rates that occurred naturally between wild-type testis and ovary. One prediction is that introducing

a Y chromosome into females ($X^{\wedge}XY$ genotype) might induce chromatin states that are more similar to those of wild-type XY males.

Collectively, our observations showed that IR is a major form of AS that can be differentially regulated between males and females of *Drosophila*. Most sex-biased IR events introduced a PTC in CDS and are associated with lower gene expression in the sex in which it is most predominant. Intriguingly, the Y chromosome is able to modulate IR and can partially explain natural differences in IR between ovaries and testes. Y-linked modulation of IR could involve the Y chromosome's ability to influence epigenetic states across the genome and the local balance of euchromatin and heterochromatin factors.

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