Sex-specific transcript diversity is regulated by a maternal pioneer factor in early *Drosophila* embryos

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Abstract

Maternally deposited factors play a crucial role in initiating zygotic transcription. However, the mechanisms by which maternal factors regulate early zygotic transcript diversity through alternative splicing remain unclear. Furthermore, how early in development widespread sex-specific transcript diversity occurs is not known. We show that widespread sex-specific transcript diversity occurs much earlier than previously thought and present a new pipeline called time2splice to quantify splicing changes over time. Using the powerful *Drosophila* model, we define several mechanisms by which a maternal factor regulates sex-specific zygotic transcriptome diversity: 1) In both males and females, GA-binding pioneer factor CLAMP (**C**hromatin **l**inked **a**dapter for **M**SL **p**roteins) links the DNA of gene bodies of sex-specifically spliced genes directly to the RNA of target genes and physically interacts with snRNA and protein components of the splicing machinery; 2) In males, CLAMP regulates the distribution of the spliceosome component **M**ale**l**ess (MLE) to prevent aberrant sex-specific splicing; 3) In females, CLAMP binds to the DNA and RNA encoded by the *sxl* gene, the master regulator of sex determination, to directly regulate its splicing which also modulates downstream targets. Overall, we provide key insight into how maternal factors influence sex-specific transcript diversity.

Introduction

One of the greatest challenges in modern biology is understanding the mechanism and significance of widespread transcript diversity between sexes and different developmental stages, tissues, and cell-types. **A**lternative **S**plicing (**AS**), a mechanism of selective inclusion or exclusion of introns and exons, drives widespread transcript diversity[1,2]. In addition to basic development and physiology, transcriptome diversity is critical for disease biology, especially in neurodegenerative diseases and developmental disorders that often show sex or tissue-specific differences in progression and severity[3-6]. Across species, precise regulation of genes to produce specific splice variants is critical for all developmental decisions, including sex determination. A key to understanding how transcript diversity drives biological processes lies in the events that shape the initial few hours of an organism's existence.

During early development, protein and RNA deposited by the mother into the embryo shape early embryonic milestones across metazoans[7,8]. Initially, cell number increases, followed by cellular differentiation into specific cell types. Sexual identity is then established, driving the fundamental physiological differences between sexes. However, the mechanisms by which maternally deposited proteins and RNAs regulate this process of sexual differentiation remains poorly understood. Moreover, maternal factors are often essential regulators that can have a lasting impact on gene regulation later in the life of an organism. Thus, it is essential to define the influence of maternal factors on transcriptome diversity during the early stages of embryonic development. Therefore, the key question is: How do maternally deposited products regulate transcript diversity including sex-specific splice variants?

The *Drosophila* embryo is an excellent tool to study the role of maternally deposited proteins and RNA in early development as it is easy to perform genetic manipulation to remove maternal factors to define how they regulate splicing and transcription. Also, embryos can be sexed before zygotic genome activation due to the recent application of a meiotic drive system [9].

During *Drosophila* embryogenesis, **Z**ygotic **G**enome **A**ctivation (**ZGA**) occurs shortly after the first two hours of development. Concurrently, maternal transcripts gradually decrease in abundance, and zygotic transcription increases, a process called the **M**aternal to **Z**ygotic **T**ransition (**MZT**). ZGA starts approximately 80 min after egg laying and most maternal transcripts are degraded by 180 min after egg laying[10]. Even at these early stages of development, AS generates multiple variants of the same gene transcripts resulting in transcript diversity. Although the earliest genes transcribed from the zygotic genome are mainly intron-less, almost 30% of early zygotic transcripts do have introns[11,12]. Furthermore, genes involved in sex determination use AS to drive male versus female-specific development [13]. Hence, during early embryonic development, AS is important for shaping cell and tissue-specific transcriptomes and sexual differentiation.

The master regulator of *Drosophila* sex determination is the *sxl* gene, which undergoes alternative splicing such that exon three is retained in males but not in females[14,15]. Therefore, functional Sxl protein is made only in the female embryo. Early expression of the Sxl protein made from a transcript driven by Sxl_{Pe}, an early *sxl* promoter[16], autoregulates splicing of *sxl*. Next, the Sxl protein causes splicing of exon three from pre-mRNA of *sxl* transcripts initiated from the late maintenance promoter (Sxl_{Pm}) specifically in females[17,18]. The early activation of the Sxl_{Pe} promoter is regulated by a group of X-linked genes called X signal elements (XSE). A higher dosage of XSE gene products drives Sxl_{Pe} expression in XX females and not XY males[19].

The Sxl protein is an RNA binding protein that binds to *sxl* pre-mRNA and the pre-mRNA of downstream target genes such as *transformer* (*tra*) and *male-specific lethal protein 2* (*msl-2*) to regulate their splicing and stability, giving rise to female-specific splice variants[20]. The female-specific Tra isoform then regulates sex-specific splicing of the *doublesex* (*dsx*) gene resulting in different Dsx protein isoforms in females and males which directly regulate sexual dimorphism in both sexes[14,15].

Sxl regulation of *msl-2* RNA splicing and stability links the sex determination pathway to another important early embryonic event: dosage compensation, which equalizes gene dosage of X-linked genes between females with two X-chromosome and males with a single X-chromosome. MSL-2 is an essential core component of the MSL complex, present only in the male embryo and

responsible for dosage compensation[21,22]. The binding of the Sxl protein to the *msl-2* transcript specifically in females results in skipping of the first exon, thereby hindering translation of the protein and preventing MSL complex formation in females[23].

Once formed specifically in males, the MSL complex targets the male X-chromosome at high-affinity binding sequences called **C**hromatin **E**ntry **S**ites (**CES**) that are enriched for clustered long GA-repeat sequences[24,25]. The maternally deposited **C**hromatin **l**inked **a**dapter for **MSL** **p**roteins (CLAMP) opens chromatin[26,27] and directly contacts MSL-2[28] to promote MSL complex recruitment. Subsequently, the MSL complex increases transcript levels on the male X-chromosome by promoting transcription elongation[29]. Interestingly, the MSL complex shares a key highly conserved component MLE (RNA helicase A) with the spliceosome complex[30-32], suggesting that there is likely to be an interplay between dosage compensation and splicing that has not yet been defined.

CLAMP is an essential pioneer **T**ranscription **F**actor (**TF**) that opens chromatin[26,27]. Loss of CLAMP results in complete loss of MSL complex from the male X-chromosome causing male lethality[33]. Unlike MSL complex component mutants, which cause only male-specific lethality, *clamp* mutants are lethal in both males and females early in embryogenesis after losing maternal CLAMP[27]. Several lines of evidence lead us to hypothesize that CLAMP regulates alternative splicing: 1) CLAMP is bound to both intronic and promoter regions on chromatin[34], 2) Intronic regions are rich in polypyrimidine tracts, which have similar GA-rich sequences to CES and often contain CLAMP binding sites[35], 3) GA-rich repeat sequences within CES are thought to have evolved from polypyrimidine tracks that regulate splicing[35], and 4) MALDI-mass spectrometry data identifying putative CLAMP interactors identified association with 33 RNA binding proteins, including 6 that regulate alternative splicing[36]. Therefore, we hypothesized that CLAMP is a maternally deposited factor that shapes transcriptome diversity in early embryos by regulating RNA transcript splicing.

To date, there are no reports defining alternative splicing globally in early *Drosophila melanogaster* sexed embryos. Moreover, how maternal TFs regulate alternative splicing in any organism is not known. Here, we determine that 16-18% of transcripts undergo alternative splicing

126 during the first four hours of development in *Drosophila melanogaster* embryos. Although sex-
127 specific isoforms have been identified for several candidate genes in early development[37-40], a
128 comprehensive analysis of all sex-specific isoforms forms has not yet been performed in early
129 embryos and is only available in fully developed tissues like ovaries, testis and brain[38,41].
130 Therefore, we used a meiotic drive system to sex embryos and measured total AS and sex-specific
131 splicing genome-wide before (0-2 Hrs) and after (2-4 Hrs) the major wave of MZT. For ease of
132 understanding, we have defined 0-2 Hr embryos as pre-MZT and 2-4 Hr embryos as post-MZT
133 even though a small number of early zygotic genes are activated during the 0-2 Hr time period.
134
135 To reveal new mechanisms by which maternal factors shape early embryonic transcriptome
136 diversity, we have focused on analyzing how the maternal transcription factor CLAMP regulates
137 sex-specific alternative splicing during the first few hours of development. We have identified
138 male-specific and female-specific genes whose splicing requires maternal CLAMP and are
139 important regulators of early development with diverse functions form including chromatin
140 regulation and splicing.
141
142 Moreover, we defined multiple mechanisms by which sex-specific transcript diversity is regulated
143 during early embryonic development: 1) In both males and females, CLAMP links the DNA of
144 gene bodies of sex-specifically spliced genes directly to the RNA of target genes and physically
145 interacts with U1-U6snRNAs and protein components of the splicing machinery; 2) In males,
146 CLAMP regulates the distribution of the spliceosome component **M**ale**l**ess (MLE) to prevent
147 aberrant sex-specific splicing; 3) In females, CLAMP binds to the DNA and RNA encoded by the
148 *sxl* gene, the master regulator of sex determination, to directly regulate its splicing which also
149 modulates downstream targets. Overall, we demonstrate that sex-specific transcriptome variation
150 is established very early in development and is regulated by maternally deposited CLAMP via
151 diverse mechanisms including direct binding to the spliceosome and the DNA and RNA of sex-
152 specifically spliced genes.
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Results

1. Alternative splicing plays an essential role in shaping the early embryonic transcriptome.

To determine when during early *Drosophila melanogaster* development sex-specific splicing begins, we analyzed RNA-sequencing data generated from sexed embryos at two-time points: 1) 0-2 Hrs (pre-MZT); 2) 2-4 Hrs (post-MZT)[9] (#GSE102922). Embryos were sexed using a meiotic drive system that produces sperm with either only X or only Y chromosomes[9], resulting in progeny of either only female or only male genotypes. Next, we measured the amount of AS in early *Drosophila* development using a pipeline that we developed and made publicly available called time2Splice (https://github.com/ashleymaeconard/time2splice). Time2Splice implements the commonly used SUPPA2 algorithm to identify splice variants and provides additional modules to integrate time, sex, and chromatin localization data (See **Supplemental method**) (Fig S1). SUPPA2 measures the percent spliced in (PSI) for each exon to calculate the differential alternative splicing between samples, represented as ΔPSI[42].

AS events can be classified into seven categories: 1) Alternative Last Exon (AL); 2) Skipping Exon (SE); 3) Alternative 5' Splice Site (A5SS); 4) Alternative 3' Splice Site (A3SS); 5) Mutually Exclusive Exon (MXE) 6) Retained Intron (RI) and 7) Alternative First Exon (AF) (**Fig1A**). We measured the total number of splicing events that belong to each of the seven different categories at the 0-2 Hr and 2-4 Hr embryonic stages in both female and male embryos. We found that 16-18% of total transcripts are alternatively spliced in early embryos. **A**lternative **F**irst Exon (AF) is the most common type of alternative splicing, constituting almost one-fourth of total AS. In contrast, **A**lternative **L**ast Exon (AL) is the least common alternative splice type. The AS transcript distribution across categories was similar between the two time points and sexes (**Fig 1B**).

We hypothesized that CLAMP regulates AS in early embryos for the following reasons: 1) CLAMP is a maternally deposited pioneer factor enriched at intronic regions ; 2) Proteomic data identified a physical association between spliceosome components and CLAMP[36]; 3) CLAMP binding sites evolved from polypyrimidine tracts that regulate splicing[35]. We tested our hypothesis in early staged and sexed embryos by measuring differences in splicing in RNA-sequencing data

188 generated from male and female 0-2 Hr and 2-4 Hr (pre- and post-MZT) embryos with and without
189 maternal CLAMP[9]. The maternal triple driver GAL4, *MTD-GAL4* was used to drive *UAS-*
190 *CLAMPRNAi[val22]* that strongly reduce maternal CLAMP levels, validated by qPCR and
191 Western blot [9].
192
193 First, we asked whether CLAMP alters AS and we found 200-400 transcripts where AS is regulated
194 by CLAMP depending on the time point and sex (**Fig 2A, B** and **Fig S2B, C, D, E**). Next, we
195 asked which type of alternative splicing is most affected by depleting CLAMP. The overall
196 distribution of AS transcripts into the seven splicing types remains mostly unaffected in the
197 absence of maternal CLAMP. However, at the 0-2 Hr (pre-MZT) stage, loss of maternal CLAMP
198 results in a more substantial decrease in **M**utually E**x**clusive **E**xon (**MXE**) splicing in both males
199 and females compared with all of the other types of splicing (males: p-value < 3.21e-21; females:
200 p-value < 6.26e-87 chi-squared test) (**Fig 1B**). At the 2-4 Hr (post-MZT) stage, only male embryos
201 have a significant percentage of MXE splicing affected in the absence of maternal CLAMP (p-
202 value < 1.95e-137 chi-squared test) (**Fig 1B**). Therefore, CLAMP alters AS and has a stronger
203 effect on MXE splicing than other types of splicing.
204
205 During MXE splicing one isoform of the transcript retains one of the alternative exons and
206 excludes another exon, which is retained by another isoform (schematic **Fig 1A**). Interestingly,
207 MXE alternative splicing occurs in many transcripts that encode components of the sex
208 determination pathway[43]. Sex determination pathway components undergo MXE alternative
209 splicing that regulates translation and influences the splicing of downstream genes to drive
210 transcriptome variation and sexual dimorphism. Furthermore, CLAMP has a sex-specific role in
211 dosage compensation[21,44]. Therefore, we next asked whether CLAMP regulates sex-specific
212 splicing.
213
214 **2. Maternal CLAMP regulates sex-specific alternative splicing in early *Drosophila* embryos**
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216 To determine whether CLAMP-dependent alternative splicing events are enriched for **s**ex-**s**pecific
217 **s**plicing (SSS) events, we first identified all of the transcripts undergoing female and male sex-
218 specific splicing (**Fig S2A, D**) (Supplementary **Method**). We measured alternative splicing using

an exon-centric approach to quantify individual splice junctions by measuring **p**ercent **s**pliced **i**n (**PSI**) for a particular exon using the established SUPPA algorithm within the time2splice pipeline[42]. Exon inclusion is represented as positive PSI, and exclusion events are defined as negative PSI (equation in **Supplementary Method**). In this way, we identified sex-specific splicing events (**Fig S2A, D**) and CLAMP-dependent splicing events (**Fig S2B-C, E-F**) in female and male 0-2 Hr and 2-4 Hr embryos. We did not see any statistically significant enrichment for MXE within the CLAMP-dependent sex-specifically spliced events (see Supplementary **Table S1**). Although we found substantial evidence of CLAMP-dependent sex-specific splicing, we think the gene list is likely to be too small to determine significance for a particular class of splicing event.

Next, we measured the percentage of total alternatively spliced and sex-specifically spliced transcripts that are CLAMP-dependent in males and females at both pre- and post-MZT stages. While only 2-3% of total AS is CLAMP-dependent, ~30-60% of sex-specific splicing is CLAMP-dependent (**Fig 2A**). Therefore, CLAMP regulates sex-specific splicing more strongly than total AS. Overall, more genes show CLAMP-dependent splicing in females (~150-250) than males (~100) (**Fig 2B and Supplementary Table S1**). However, the magnitude of the effect of CLAMP on splicing was stronger in males than in females (**Fig S3**). We found that before MZT, the CLAMP-dependent known male sex-specifically spliced genes have more exon inclusion events than their female counterparts since ΔPSI values for males is significantly higher than in females (**Fig S3**). This was further supported by validating a randomly selected set of 12 sex-specific splicing events we identified as described in more detail below (**Fig S4-6; Table S2**).

We then divided all CLAMP-dependent alternatively spliced events into two categories: 1) sex specifically spliced (SSS) events; and 2) non-sex specifically spliced (non-SSS) events (**Fig 2B**). We quantified CLAMP-dependent sex-specifically spliced events by comparing the change in PSI (ΔPSI) for two classes of transcripts which we defined as follows: 1) **known** sex-specifically spliced isoforms are different from each other in control samples ($p<0.05$) (**Fig. S2A, D**); 2) **new** sex-specifically spliced isoforms are those not present in control samples (**Fig 2B**). We identified widespread CLAMP-dependent sex-specific splicing, especially in female embryos (**Fig 2B**). Interestingly, the majority of CLAMP-dependent SSS events are **new** sex-specific splicing events

that did not occur in the presence of maternal CLAMP (~70%) (**Fig 2C**). Furthermore, at the post-MZT stage, both CLAMP-dependent exon inclusion and exclusion were significantly enriched in male new SSS genes compared to their female-specific counterparts (**Fig S3**). Thus, in the absence of CLAMP new aberrant sex-specific spliced isoforms are generated. Therefore, we hypothesized that CLAMP normally inhibits aberrant sex-specific splicing events.

During the first few hours of their development, *Drosophila* embryos have predominantly maternal transcripts. Therefore, we asked whether CLAMP-dependent female and male specifically-spliced genes are maternally deposited or zygotically transcribed. We compared our list of CLAMP-dependent sex-specifically spliced genes with known maternally expressed genes from previous studies [45,46]. We found very low levels of overlap with maternally deposited transcripts (**Fig 2D**). Therefore, most of the sex-specifically spliced genes we observed are likely to be zygotic transcripts, consistent with the function of CLAMP as a pioneer TF in the early embryo [27].

Next, we divided our CLAMP-dependent sex-specifically spliced genes into female-specific or male-specific categories. Here, it is important to note that many of these genes have multiple AS events, and thus multiple isoforms regulated by CLAMP (**Table S1**). Then, we performed Gene Ontology (GO) analysis to determine which biological processes are enriched within CLAMP-dependent sex-specifically spliced genes at pre- and post-MZT time points using time2splice. At the pre-MZT time point, female-specifically spliced genes are primarily mRNA regulatory factors such as spliceosome components and TFs (**Fig 2E**). Therefore, in females CLAMP alters the splicing of genes that can regulate the transcription and splicing of other genes to amplify its regulatory role. In contrast, the male specifically-spliced genes pre-MZT are not enriched for any specific biological function or process likely due to the small number of genes in the gene list. At the post-MZT stage in both sexes, CLAMP regulates the splicing of genes that drive development including organogenesis, morphogenesis, cell proliferation, signaling, and neurogenesis (**Fig 2E**).

We also selected 12 random genes for validation that we identified computationally as having CLAMP-dependent splicing events of diverse types such as alternative 5' splice site (A5) or alternative first exon (AF) (**Fig S4-5**) using qRT-PCR or RT-PCR. These genes perform diverse biological functions and provide new insights into how CLAMP regulates early development (**Fig.**

2E). We have summarized the results and functions of the validated target genes at which splicing is regulated by CLAMP (**Table S2**). Several of the target genes have functional links to CLAMP including *iab4*, *psq,* and *pep* suggesting that we have identified relevant target genes[36,47,48]. Furthermore, many genes that are sex-specifically spliced by CLAMP are themselves involved in splicing and chromatin regulation (**Table S2**).

3. CLAMP is highly enriched along gene bodies of sex-specifically spliced genes after MZT

Because maternal CLAMP regulates sex-specific alternative splicing, we next asked: How does CLAMP regulate female and male sex-specific splicing? If CLAMP directly regulates sex-specific splicing, we hypothesized that it would directly bind to the genes that it regulates. Therefore, we defined the binding pattern of CLAMP at the CLAMP-dependent female and male sex-specifically spliced genes in sexed embryos using CLAMP ChIP-seq data (#GSE133637).

We generated average profiles for CLAMP occupancy at genes showing CLAMP-dependent splicing in females and males at pre- and post-MZT time points in males and females (**Fig 3A, B**). We found that CLAMP occupies the gene bodies of many sex-specifically spliced genes that require CLAMP for their splicing. Specifically, 43.8% percent of all CLAMP-dependent sex-specifically spliced genes are bound by CLAMP: 21.9% in 0-2 Hr female embryos, 8.2% in 0-2 Hr male embryos, 65.2% in 2-4 hr female embryos and 59.43% in 2-4 hr male embryos (**Table S3**). The increase in percentage of genes bound in 2-4 Hr embryos compared with 0-2 Hr embryos is consistent with an increased number of CLAMP binding sites at the later time point[48]. Overall, these data are consistent with a direct role for CLAMP in regulating splicing of sex-specifically spliced genes.

Next, we compared the average CLAMP binding pattern at sex-specifically spliced genes to the CLAMP binding pattern at genes whose expression but not splicing is both sex-biased and dependent on CLAMP. We defined CLAMP-dependent sex-biased genes as differentially expressed genes in males in females that are dependent on CLAMP. At sex-specifically spliced genes, CLAMP occupancy is present over gene bodies which is a dramatically different binding profile from genes that are expressed but not spliced in a CLAMP-dependent and sex-biased

manner where CLAMP is enriched at the TSS and TES instead of at gene bodies (**Fig 3C and D**, rectangular box in **Fig 3A-D**). Furthermore, CLAMP binding is also modestly enriched at the TSS of female-biased expressed genes in females, consistent with enhanced CLAMP occupancy at the TSS of expressed genes [33]. As a control, we used a random set of active genes that are not regulated by CLAMP (green lines in **Fig 3A-D**) and we observed lower occupancy than at CLAMP-dependent genes. Overall, we found preferential binding of CLAMP along the gene bodies of genes that have CLAMP-dependent splicing in both females and males in contrast to TSS and TES binding at genes where expression requires CLAMP.

To determine whether the binding of CLAMP to gene bodies occurs close to splice junctions, we measured the distance of CLAMP peaks from the nearest splice junction (**Fig S7**). We found that CLAMP peaks are most frequently within 200-400bp of either the start or the end of a splice junction, especially in sex-specifically spliced genes. The resolution of these measurements is also limited by sonication and therefore it is possible that binding occurs even closer to splice junctions. We also found that CLAMP binds to chromatin closer to splice junctions at sex-specifically spliced genes compared to sex-biased genes in 2-4 hr female embryo samples that have the most target genes and CLAMP binding events. The results were similar for all CLAMP peaks (**Fig S7C**) compared to peaks only present in introns (**Fig S7G**). We did not observe a difference in proximity of CLAMP peaks to the nearest splice junction between sex-specifically spliced genes and sex-biased genes in other samples but this negative result is not conclusive due to the decreased number of peaks and target genes in the other samples. Together these data support a direct role for CLAMP in co-transcriptional RNA processing that we hypothesize is due to direct contact with target RNA transcripts and altering the recruitment of spliceosome components.

4. CLAMP binds to RNA on chromatin of many sex-specifically spliced genes and is a component of the mature spliceosome complex specifically in males

To test our hypothesis that CLAMP directly regulates co-transcriptional RNA splicing by contacting both the DNA and RNA of sex-specifically spliced genes and altering spliceosome recruitment, we first asked whether to CLAMP directly binds to RNA. Although CLAMP do not have a canonical **R**NA **r**ecognition **m**otifs (**RRM**), it has a prion-like intrinsically disordered

343 domain, present typically in many RNA binding proteins[49,50]. Using individual nucleotide
344 resolution UV crosslinked immunoprecipitation (iCLIP) which defines direct protein-RNA
345 interactions[51], we determined that most CLAMP RNA binds directly to hundreds of RNAs and
346 most targets are sex-specific with only 15% of the targets RNAs shared between males and females
347 (**Fig S8A, Table S5**). iCLIP was conducted on fractionated embryonic male and female cultured
348 cells because it was not possible to generate enough starting material from sexed embryos.
349 Interestingly, most CLAMP interaction with RNA occurs on chromatin (91.9% of male RNA
350 targets; 58.4% of female RNA targets) (**Fig S8A**). Even though iCLIP was conducted in embryos
351 cell lines and not embryos, we still found 47 target genes where CLAMP regulates sex-specific
352 splicing and interacts with both the DNA and RNA including but not only the key regulator of sex-
353 specific splicing *sxl*. Therefore, CLAMP sex-specifically and directly interacts with RNA targets
354 on chromatin including the RNA encoded by genes at which it regulates sex-specific splicing.
355
356 In addition to target RNAs, CLAMP also directly interacts with spliceosomal RNAs. Specifically,
357 we found that CLAMP binds to U1-U6 snRNAs (**Table S5**) more strongly in males (N=23)
358 compared to females (N=9) (**Fig S8B, C**). In the male chromatin fraction CLAMP interacts with
359 the catalytic step 2 spliceosome consisting of U2, U5, U6 snRNAs (FDR:1.7E-3). In contrast, the
360 female chromatin fraction is enriched for transcripts that encode proteins that bind to the U1-U2
361 snRNAs (FDR:1.1E-2) suggesting a different type of regulation of splicing in males and females.
362 Although these data establish that CLAMP interacts directly with some of its RNA targets to
363 regulate co-transcriptional splicing and interacts with the spliceosomes, it still does not explain
364 how CLAMP regulates splicing in a sex-specific manner.
365
366
367 **4. CLAMP interacts with components of the spliceosome complex and influences their**
368 **occupancy on chromatin**
369
370 CLAMP occupies the gene bodies of many CLAMP-dependent sex-specifically spliced genes
371 (**Fig 3**), physically associates with protein and RNA spliceosome components[36] , and directly
372 interacts with the RNA of a subset of target genes. Therefore, we hypothesized that CLAMP
373 regulates recruitment of components of the splicing machinery to chromatin differentially in males

and females. To test this hypothesis, we first examined how CLAMP regulates the occupancy of the MLE RNA helicase, a component of both the MSL complex and the spliceosome[30,32,52-54] for two reasons: 1) MLE is a component of both the MSL complex which is present only in males (ref) and the spliceosome in both sexes[54] and 2) CLAMP physically associates with MLE[28,55,56]. Therefore, we hypothesized that CLAMP regulates the distribution of MLE between the spliceosome and MSL complex to modulate sex-specific alternative splicing in males.

To determine whether CLAMP modulates MLE distribution on chromatin, we measured MLE distribution at the genomic level using CUT&RUN[57,58]. We performed CUT&RUN in the presence (*MTD-GAL4>UAS-GFPRNAi*) and absence (*MTD-GAL4>UAS-CLAMP [val22] RNAi*) of maternal CLAMP at the pre-MZT and post-MZT embryonic stages in males. Next, we identified MLE peaks in the presence or absence of maternally deposited CLAMP. MLE binds to chromatin both in males and females, with stronger binding in males (**Fig 4A**). The absence of CLAMP results in loss of MLE male peaks in males compared to no change in female MLE peaks (**Fig 4A**). This supports our hypothesis that CLAMP regulates MLE recruitment to chromatin in males.

We next compared the distribution and location of MLE peaks with that of CLAMP peaks previously identified in control embryos at the same time points[21] and classified MLE peaks into two groups: 1) MLE peaks overlapping with CLAMP peaks (**Fig 4B,C and Fig S9**) and 2) unique MLE peaks which do not overlap with CLAMP peaks (**Fig 4B,C and Fig S9**) at both 0-2 Hr pre-MZT and 2-4 Hr post-MZT stages. Also, independent of developmental stage, MLE peaks which overlap with CLAMP peaks are largely at promoters with fewer peaks localizing to introns. In contrast, unique MLE peaks that do not overlap with CLAMP peaks are primarily localized to introns (**Fig S9**). This distribution is similar for X-chromosomal and autosomal peaks.

In the absence of CLAMP, there is in a considerable loss and redistribution of both overlapping and non-overlapping MLE peaks. We found that overall ~60% of MLE peaks were lost at the 2-4 Hr post-MZT stage in the absence of CLAMP. Moreover, 26% (pre-MZT) and ~35% (post-MZT) of the MLE peaks observed in the absence of CLAMP were new and not present in control embryos (**Fig 4B-C**). After the loss of maternal CLAMP, ~23% of MLE peaks overlapping with CLAMP are also lost at the pre-MZT stage, which increases to ~51% at the post-MZT stage (**Fig 4B-C**).

Overall, our data suggest that MLE is redistributed in the absence of CLAMP suggesting that CLAMP normally prevents aberrant recruitment of MLE in addition to the formation of aberrant splice isoforms (**Fig 2**). Furthermore, we hypothesize that MLE at the new peaks is part of the spliceosome complex and not MSL complex because MSL complex is not present on chromatin in the absence of CLAMP[33].

To provide insight into the differences between MLE peaks which overlap with CLAMP and those which do not, we identified sequence motifs which are enriched within each class of peaks using MEME within the time2splice pipeline. The known CLAMP motif [33], a stretch of (GA)n repeats, is enriched at regions that are bound by both MLE and CLAMP independent of stage and chromosome type as expected. In contrast, MLE peaks which do not overlap with CLAMP have motifs with stretches of GTs, GCTs, and GTAs but not (GA)n repeats (**Fig S9**). In the absence of CLAMP, the remaining MLE peaks (red circle) were most enriched for (GT)n motifs (**Fig S9C, D**) which have known roles in splicing through forming secondary RNA structures[59-61]. Therefore, CLAMP prevents MLE from redistributing to sequence motifs that are known regulators of splicing.

We also found that CLAMP alters the distribution of MLE from introns to promoters. MLE peaks that overlap with CLAMP (intersection between green circle with red and grey circles, **Fig S9**) on the X-chromosome (**Fig S9A, C**) or autosomes (**Fig S9B, D)** are enriched at promoters (**Fig S9A, C**(X-chromosome), **Fig S9B, D** (Autosomes). In contrast, **new** unique MLE peaks not overlapping with CLAMP (grey area in Venn diagrams, **Fig S9**) and those that are gained after CLAMP RNAi (red area in Venn diagrams, **Fig S9**) are enriched at introns (**Fig S9A, C** (X-chromosome), **Fig S9B, D** (Autosomes). These results support a role for CLAMP in sequestering MLE at (GA)n rich sequences within promoters that prevents it from binding to GT motifs within introns that are known regulators of splicing[59-61]. Thus, in the absence of CLAMP, MLE is redistributed and aberrantly binds to intronic sequences with known motifs that regulate splicing irrespective of whether present on X-chromosome or autosomes.

To determine how MLE redistribution could alter sex-specific splicing, we plotted the distribution of MLE binding on CLAMP-dependent female and male-specifically spliced genes in the presence

and absence of CLAMP (**Fig S10A, B**). Pre-MZT, MLE binds near the TSS of male-specifically spliced genes independent of maternal CLAMP (**Fig S9A**). At the post-MZT stage, loss of maternal CLAMP in male embryos causes MLE to change its binding distribution along the gene body (rectangle with dotted lines: **Fig S10B**) of CLAMP-dependent male-specifically spliced genes (blue line) relative to CLAMP-dependent female-specifically spliced genes (red line). These profiles are consistent with a model in which CLAMP is essential for MLE distribution at male-specifically spliced genes which regulates male sex-specific splicing. In males, increased MLE binding at female sex-specifically spliced genes (red line, enclosed within rectangle with dotted lines: **Fig S10B**) in absence of CLAMP may result in differential splicing of these genes. Thus, we hypothesize that CLAMP inhibits mis-localization of MLE to female sex-specifically spliced genes in males.

Next, we asked whether CLAMP associates with spliceosome complex protein components other than MLE, which is also a component of the MSL complex. We have shown that CLAMP directly binds to snRNAs (Fig. S) and previously reported that CLAMP physically associates with several spliceosome complex components based on mass spectrometry analysis[36]. To validate these associations, we performed co-immunoprecipitation (coIP) experiments to assess association between CLAMP and two spliceosome components with known functions in sex-specific splicing, the conserved hrb27C and Squid proteins [54,62,63]. We found that in both S2 (male) and Kc (female) cells, CLAMP interacts with hrb27C (**Fig S11A, B**). In contrast, CLAMP only associates with Squid in female Kc cells and not in male S2 cells (**Fig S11A, B**), consistent with mass spectrometry data. In contrast to MLE and CLAMP which are enriched on the male X-chromosome, Squid occupancy on polytene chromosomes is decreased on the male X chromosome compared with the female X chromosome (**Fig S11C-E**). Therefore, it is possible that there is a competition between CLAMP recruitment of MSL complex to the male X chromosome and CLAMP recruitment of the spliceosome complex containing Squid that contributes to sex-specific splicing.

Overall, CLAMP differentially associates with spliceosome components in males and females, providing a potential mechanism by which CLAMP can regulate sex-specific splicing. Specifically, it is possible that CLAMP influences the distribution of spliceosome components like MLE between different protein complexes such as MSL complex and the spliceosome itself. We

467 hypothesize that differential binding of CLAMP to components of spliceosome like Squid in
468 females and MLE in males confers sex-specific recruitment of the spliceosome, thus generating
469 transcriptome diversity between female and male animals.

5. CLAMP regulates the chromatin accessibility and splicing of the *sxl* gene and directly interacts with the *sxl* RNA and alters splicing of other sex determination pathway component genes

In *Drosophila*, sex-specific alternative splicing is regulated by the sex-determination pathway. Sex-lethal (Sxl) is the master regulator of sex determination[15] and drives subsequent sex-specific splicing in females[64]. Therefore, we asked whether CLAMP regulates *sxl* gene splicing and Sxl protein levels in addition to directly regulating sex-specific splicing by binding to the DNA and RNA of target genes as we have demonstrated (**Fig 3, S8 and Table S3, S5**).

Functional Sxl protein is produced specifically in females because exon three in the *sxl* transcript contains a premature stop codon which is spliced out in females but retained in males [14]. Therefore, the Sxl protein is non-functional in males and becomes degraded[15,65]. In females, Sxl protein inhibits the splicing of the *msl-2* transcript which prevents aberrant MSL-2 expression and MSL complex formation in females (**Fig 5A**). Furthermore, Sxl promotes female specific-splicing of the downstream effector called transformer (*tra*). The female-specific Transformer protein regulates splicing of another downstream transcript doublesex (*dsx*), which is sex-specifically spliced. In contrast, the male-specific *transformer* transcript is not translated into functional Transformer protein in males. Therefore, in the absence of any functional male-specific Transformer protein, the *doublesex* transcript undergoes male-specific splicing (**Fig 5A**). The sex-specific isoforms of the Doublesex protein drive sex-specific splicing of many downstream genes, shaping sexual variation between males and females [66,67].

Because CLAMP regulates sex-specific splicing and is maternally deposited, we hypothesized that CLAMP regulates the canonical sex determination pathway described above. To test this hypothesis, we used our previously described recessive *clamp* null mutant $clamp^2$ line [44], the heterozygous mutant $clamp^2/CyO$-*GFP* line, and our previously reported rescue line which is homozygous for the $clamp^2$ allele and contains an insertion of the wild type *CLAMP* gene. Sxl is strongly regulated via alternative splicing of its mRNA[68-70]. Therefore, we designed an RT-PCR assay to distinguish between the female-specific (excluding exon 3) and male-specific (including exon 3) versions of the *sxl* transcript (**Fig 5B**). We used this assay to measure CLAMP-dependent changes in alternative splicing and found that in homozygous $clamp^2$ female animals, there is a

small but detectable amount of the longer male-specific *sxl* transcript (**Fig 5B**, lane c). This mis-regulation of *sxl* splicing is rescued by our *CLAMP* containing rescue construct (**Fig 5B**, lane d). Our iCLIP data shows that CLAMP directly binds to *sxl* transcripts in females and not males (**Table S5**) emphasizing CLAMP's direct role in *sxl* splicing in females.

To test whether defects in splicing altered Sxl protein levels, we performed western blots to quantify Sxl protein in wild type females and males and *clamp*^{2}null females (**Fig 5C**). We observed a reduction in Sxl protein levels in females in the *clamp*^{2} null background when compared with controls. Also, homozygous *clamp*^{2} mutant males die before the third instar larval stage, and therefore it was not possible to measure the splicing of transcripts in male *clamp*^{2} mutant larvae. Overall, we determined that CLAMP promotes female-specific splicing of the *sxl* transcript to ensure that normal Sxl protein levels are produced.

To determine a possible mechanism by which CLAMP regulates splicing of *sxl*, we mined our previously generated Micrococcal Nuclease sequencing data[26], which measures chromatin accessibility in S2(male) and Kc(female) cells in the presence and absence of CLAMP. Regions of the genome that are accessible have a positive MACC score and are shown in blue and regions of the genome that are inaccessible have a negative score and are shown in red (range is between -0.33(red) and +1.33 (blue). We found that after the loss of CLAMP in female Kc cells, chromatin accessibility at exon 3 of *sxl* increases significantly (**Fig 5D**). As shown in the Fig 5D boxed rectangular inset, *sxl* exon3 shows a strong and statistically significant peak[26] indicative of open chromatin specifically in females in which CLAMP has been depleted compared to control females. Therefore, CLAMP normally promotes a closed chromatin environment at exon 3 in females but not males.

Our results suggest that increased chromatin accessibility in males compared to females results in retention of exon3 in the male *sxl* transcript. Consistent with our results, recent reports provide strong evidence that increased chromatin accessibility contributes substantially to the retention of introns during AS[71]. In addition, splicing-associated chromatin signatures have recently been identified[72]. Consistent with our results in males, open chromatin marks such as H3K4me1& H3K4me2 are enriched just upstream of the start site of retained exons[72]. In contrast, histone marks

associated with condensed chromatin such as H4K20me1&2, H3K9me3, and H3K27me3 are highly enriched at excluded exons[72], consistent with our results in females.

Next, we examined the splicing of other components of the sex determination pathway for defects in alternative splicing in the absence of CLAMP for which we were able to design effective RT-PCR primers (**Fig 5E-F**). In embryos which lack CLAMP (**Fig 5E**, lane 2), the *dsx* female-specific transcript is aberrantly produced in males (**Fig 5E**, lanes 4 and 5). In contrast, the male-specific *dsx* transcript is not expressed in male embryos which lack CLAMP, similar to wild type female embryos (**Fig 5E**, lane7-10). We also observed male-specific *dsx* transcripts in female *clamp*^{2} mutant larvae (**Fig 5E**, lane c). Therefore, *dsx* splicing is regulated by CLAMP and CLAMP binds to the *dsx* gene (**Fig S11**). However, *dsx* splicing is also regulated by Sxl whose protein levels are regulated by CLAMP (**Fig 5C**).

Furthermore, we found that CLAMP regulates splicing of the male-specific lethal-2 (*msl-2*) transcript, which is present only in males because Sxl regulates its splicing and stability. In the *clamp*^{2} mutants: 1) levels of the nonfunctional female *msl-2* isoform (**Fig 5F**, lane 5) increased in male embryos (**Fig 5F**, lane 2); 2) levels of the functional male-specific *msl-2* isoform increased in females (**Fig 5F**, lane c). To determine whether these splicing defects also cause dysregulation of MSL-2 protein expression and localization, we performed polytene immunostaining from female *clamp*^{2} mutant salivary glands. In the absence of CLAMP, ectopic MSL2 protein (in red) is present at several locations on female chromatin compared with controls (*clamp*^{2}*/CyOGFP* heterozygous females) where MSL-2 protein is not present on chromatin (**Fig 5G**). Similar to *dsx*, the gene is also bound by CLAMP (**Fig S12**) and regulated by Sxl and therefore could be regulated through both direct and indirect mechanisms. Since CLAMP does not binds to the *dsx* or *msl-2* transcripts directly it is possible that it indirectly regulates *dsx* and *msl-2* splicing through Sxl. CLAMP does binds to the RNA that encodes another sex-specific splicing regulator and Sxl target gene *fru* (Fruitless) in both females and males (**Table S5**). Together, these data reveal that CLAMP regulates the splicing and protein expression of multiple components of the sex determination pathway.

564 To further define how CLAMP and Sxl function together to regulate sex-specific splicing, we
565 asked whether CLAMP nuclear RNA targets in males and females have known Sxl binding motifs
566 (**Fig S13A**). Therefore, we compared CLAMP RNA targets from female embryonic cell lines with
567 available data identifying Sxl RNA targets in adult female heads (#**GSE98187**). Even though the
568 data sets are from different cell types, we still identified 61 overlapping targets in females (**Fig**
569 **S13B, Table S6**) which include the *sxl* transcripts and snRNAU5, a component of the U5snRNP
570 complex involved in splicing. Interestingly, 153 CLAMP RNA targets from males overlapped with
571 Sxl female RNA targets, indicating that CLAMP may function in males at genes that are Sxl targets
572 in females (**Fig S13B, C Table S6**). Overall, we found ~20% of CLAMP RNA targets have Sxl
573 motifs and similarly ~25% of CLAMP targets are Sxl targets as well. Also, 55 (19.6%) of the
574 CLAMP-dependent female sex-specific spliced genes are Sxl targets (**Table S7**). These results
575 further support our hypothesis that CLAMP functions together with Sxl to regulate sex-specific
576 splicing both via directly interacting with the DNA and RNA of sex-specifically spliced genes and
577 indirectly through regulating Sxl.
578
579
580 **Discussion:**
581
582 Alternative splicing (AS) is a highly conserved mechanism that generates transcript and protein
583 diversity[3,73,74]. Several studies have reported highly dynamic RNA bound proteomes (RBPs)
584 during the Maternal Zygotic Transition (MZT) across diverse phyla, with widespread alternative
585 splicing events occurring during early embryonic development[1,2,39,41,46]. Furthermore, different
586 isoforms are present in maternal and zygotic transcripts[45,46]. However, the mechanisms that
587 regulate transcript diversity during the early hours of an organism's life remain elusive.
588
589 Maternally-deposited pioneer transcription factors drive zygotic genome activation, but their role
590 in generating transcription diversity in the early embryo was unknown. Here, we define sex-
591 specific alternative spliced isoforms in pre- and post- MZT *Drosophila melanogaster* female and
592 male embryos genome-wide for the first time. Furthermore, we identify a maternally-deposited
593 pioneer TF, CLAMP, which regulates sex-specific transcript diversity in early embryos. Prior work
594 on sex-specific transcript diversity[24,37-39,41,62,75-78] either examined sex-biased differences in gene

595 expression or sex-specific transcript diversity much later in development in adult gonads or brain.
596 To overcome the challenge of sexing early embryos before zygotic genome activation, we used a
597 meiotic drive system that generates sperm with either X or only Y chromosomes[9] and measured
598 sex specific transcript diversity caused by alternative splicing.
599
600 Splice variants occur at different frequencies over time and between sexes. To date, we lack
601 pipelines to characterize these how isoforms change over time. Therefore, we developed
602 time2splice, which identifies mechanisms to regulate temporal and sex-specific alternative
603 splicing by combining RNA-seq and protein-DNA interaction data from CUT&RUN and ChIP-
604 seq experiments. Time2splice has three parts: 1) temporal splicing analysis based on the SUPPA
605 algorithm; 2) temporal protein-DNA analysis, and 3) temporal multi-omics integration. The
606 pipeline and analysis steps can be accessed at https://github.com/ashleymaeconard/time2splice.
607
608 Even following the initial few hours of its existence, there is a clear difference between a male and
609 female *Drosophila* embryo's transcript variation that was not previously identified (**Fig1, 2**).
610 Because the RNA transcript variants in both males and females encode genes involved in
611 developmental processes, sex-specific developmental distinctions may occur earlier than
612 previously thought. We demonstrate that a fundamental developmental trajectory differs between
613 males and females from the initial hours of their existence long before gonad formation. Such early
614 sex-specific transcript diversity provides insight into how developmental disorders that originate
615 before gonad formation can exhibit variable penetrance between sexes.
616
617 We defined groups of genes in both males and females that undergo alternative splicing events
618 which are regulated by maternally deposited CLAMP. Thus, the maternal environment regulates
619 both transcription initiation and shapes RNA processing. The key question is: How does CLAMP,
620 a ubiquitously expressed pioneer TF, regulate sex-specific splicing? We identified several
621 mechanisms by which CLAMP regulates sex-specific splicing.
622
623 CLAMP binds directly to intronic regions of approximately half of the sex-specifically spliced
624 genes that it regulates in both males and females. CLAMP also directly binds to different RNA
625 transcripts on chromatin in males and females and associates with U1-U6 snRNAs providing a

626 mechanism by which it regulates, co-transcriptional splicing. Therefore, our data supports a model
627 in which direct CLAMP binding to DNA and RNA regulates splicing of its target genes.
628
629 Furthermore, CLAMP regulates chromatin as a pioneer TF[26,48] and recent literature links
630 chromatin and splicing[71,72]. For example, closed chromatin marks have recently been linked to
631 exon exclusion and open chromatin has been linked to exon inclusion[71,72]. Proteomic analysis [36]
632 and coIPs (**Fig S11 A,B**) show that CLAMP is associated with spliceosome complex components,
633 including Squid and Hrb27C, further supporting a direct role for CLAMP in splicing. Interestingly,
634 CLAMP associates with Squid specifically in females. In contrast to Squid, MLE, a component of
635 both the spliceosomes and MSL complex[32] physically interacts with CLAMP only in males.
636 Because Squid is already known to regulate sex-specific splicing[62], these data support a model in
637 which differential association between CLAMP and spliceosome complex components in males
638 and females regulates sex-specific splicing. Our results also indicate that CLAMP is present in
639 functional spliceosome complex in males but not in females (**Table S5**). Thus, we hypothesize that
640 CLAMP may recruit spliceosome complex components to regulate splicing by altering the
641 chromatin environment or/and directly binding to target RNA transcripts (**Fig 6A**).
642
643 Based on our results, we hypothesize that there are two separate mechanisms by which CLAMP
644 can regulate splicing in males and females (**Fig 6).** We show that CLAMP inhibits aberrant splicing
645 events in males, especially at the post-MZT stage (**Fig 2**). In males, we found that MLE distribution
646 on chromatin is CLAMP dependent (**Fig 4A**) Also, MLE colocalizes with CLAMP at promoter
647 regions but in contrast, MLE alone is more often bound to intronic sequences, a prerequisite for
648 its splicing function, (**Fig 6B**). However, in the absence of CLAMP, the promoter bound MLE is
649 reduced, and MLE re-localizes from its normal intronic binding sites to new intronic regions that
650 contain GT sequence motifs (**Fig S9**) that are known to regulate splicing[59-61]. Therefore, we
651 hypothesize that CLAMP regulates the localization of MLE to prevent the aberrant formation of
652 female-specific transcripts in males that we observed in the absence of CLAMP.
653 In males, another function of CLAMP is to recruit the MSL ribonucleoprotein complex to the X
654 chromosome to regulate dosage compensation. CLAMP physically interacts directly with MSL-
655 2[28] and associates with MLE, an RNA helicase that acts on the *roX* non-coding RNA components
656 of MSL complex[31,44,52,55,79]. Furthermore, RNA transcript splicing is regulated by the spliceosome,

657 another ribonucleoprotein complex, consisting of many protein and RNA components[54]. Because
658 MLE is part of the MSL complex only in males and the spliceosome complex in both sexes, we
659 hypothesize that CLAMP influences the relative distribution of MLE between the MSL complex
660 and the spliceosome to co-regulate sex-specific splicing and male dosage compensation (**Fig 6B**).
661 Without CLAMP, the MSL complex does not localize to the X-chromosome and becomes
662 destabilized [33]; thus, MLE is no longer part of the MSL complex and is available to redistribute to
663 new spliceosome binding sites. Therefore, we provide evidence to support a model in which
664 CLAMP sex-specifically inhibits aberrant binding of MLE to motifs that regulate splicing which
665 alters sex-specific transcript diversity.
666
667 To provide mechanistic insight into how a pioneer factor like CLAMP regulates sex-specific
668 splicing, we also examined the splicing of sex determination pathway components. In females but
669 not in males, CLAMP binds near the early promoter of the *sxl* gene (SxlPe) and regulates the
670 chromatin environment at exon 3 of *sxl* which is normally spliced out in females (**Fig 6C**). Also,
671 only in females and not in males CLAMP directly binds to *sxl* transcript. Consistent with recent
672 literature[71,72], we hypothesize that closed chromatin at exon 3 induces exclusion of this exon from
673 female *sxl* transcripts whereas more open chromatin in males result in its inclusion in the male *sxl*
674 transcript. In the absence of CLAMP in females, the chromatin becomes more open, and *sxl*
675 transcript is not bound by CLAMP. Therefore, exon 3 is included in *sxl* transcripts which prevents
676 translation due to the incorporation of a stop codon. In the absence of CLAMP, exon 3 is retained
677 in females similar to wild type males, resulting in the formation of untranslatable *sxl* transcript in
678 females, significantly reducing levels of Sxl protein, thus dysregulating downstream splicing
679 events (**Fig 6C**). Because CLAMP binding sites are present near the promoter region of the *sxl*
680 gene, we hypothesize that CLAMP regulates chromatin at exon 3 from a distance consistent with
681 literature. CLAMP can mediate long-range chromatin interactions [80,81] and act on chromatin
682 accessibility at a distance [26].
683
684 In *Drosophila*, the master regulator Sxl initiates the sex-specific splicing cascade very early in
685 development to drive sex determination [14,20,69]. Decreased Sxl protein levels in female *clamp*^{2}
686 mutants and mis-expression of female and male-specific *dsx* transcripts suggest that CLAMP may
687 regulate sexual differentiation because sex-specific Dsx protein isoforms are known determinants

of sexual dimorphism[15]. Also, CLAMP directly binds to the DNA of *sxl, dsx,* and *msl-2* target genes. Furthermore, CLAMP binds to the DNA and RNA of *sxl* and *fru*. Fruitless (*fru*) encodes a BTB zinc finger transcription factor that contributes to sexual differentiation of the neural circuits [82,83] and many of the CLAMP dependent sex-specifically spliced genes belong to neural development pathway (**Fig 2E**). Since CLAMP and Sxl have both overlapping and distinct targets, we hypothesize that CLAMP regulates sex-specific splicing both via the Sxl-mediated sex-determination pathway as well as independent of it.

Therefore, CLAMP may regulate splicing through at least two possible mechanisms that are not mutually exclusive: 1) CLAMP directly regulates the splicing of many sex-specifically spliced genes by linking the RNA to the DNA and altering the recruitment of the spliceosome; 2) CLAMP regulates the sex-specific of genes such as *msl-2* and *dsx* indirectly by regulating Sxl which is a known regulator of their splicing and thus downstream splicing events.

Overall, we hypothesize that both different composition of the spliceosome and differential recruitment to chromatin drive sex-specific changes in splicing. We identify CLAMP as a maternal factor that regulates sex-specific alternative splicing through its sex-biased association with the DNA and RNA of target genes and recruitment of spliceosome components and its ability to influence the sex determination pathway. Identifying the factors that regulate this sex-biased association of CLAMP with spliceosome complex components will be a key future direction.

Here, we show for the first time that a maternal factor controls sex-specific splicing during early embryonic development, highlighting how the maternal environment influences transcript diversity in the zygote from activation of the zygotic genome to the processing of zygotic RNA products. Consistent with recent literature linking chromatin accessibility and splicing, our results suggest that CLAMP could be one example of a more general splicing regulatory mechanism controlled by the interaction between pioneer TFs that alter chromatin accessibility and components of the RNA processing machinery to generate spatial-temporal transcript diversity. While we analyzed sex-specific transcriptome diversity in this study and linked it to the sex-

specific dosage compensation process, similar mechanisms could drive cell-type specific variation. For example, cell fate-determining transcription factors could regulate the chromatin occupancy of splicing complex components to promote the formation of cell-type-specific isoforms. We also present time2splice, a new pipeline to uncover mechanisms which drive such spatial-temporal transcript diversity by integrating splicing and chromatin occupancy data.

Materials and Methods:

Fly strains and rearing

Drosophila melanogaster fly stocks were maintained at 24°C on standard corn flour sucrose media. Fly strains used: *MTD-GAL4* (Bloomington, #31777), *UAS-CLAMPRNAi[val22]* (Bloomington, #57008), Meiotic drive fly stocks +; SD72/CyO and 19-3, yw, Rsp[s]-B[s]/Dp(2:y)CB25-4, y+, Rsp[s]B[s]; SPSD/CyO (Bloomington, #64332) (both gifts from Cynthia Staber). These were crossed to obtained male and female embryo of desired genotypes according to Rieder et al 2017.

Sample collection and Western blotting

Salivary glands from third instar larvae were dissected in cold PBS and samples frozen in liquid nitrogen. Total protein from the samples was extracted by homogenizing tissue in the lysis buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% SDS, 0.5X protease inhibitor) using a small pestle. After a five-minute incubation at room temperature, cleared the samples by centrifuging at room temperature for 10 minutes at 14,000xg. To blot for CLAMP and Actin, 5 micrograms of total protein was run on a Novex 10% Tris-Glycine precast gel (Life technologies). To measure Sex-lethal protein levels, 20 micrograms of total protein was run on a Novex 12% Tris-Glycine precast gel (Life technologies). Protein was transferred to PVDF membranes using the iBlot transfer system (ThermoFisher Scientific) and probed the membranes for CLAMP (1:1000, SDIX), Actin (1:400,000, Millipore), and SXL (1:500, a gift from Fatima Gebauer) antibodies using the Western Breeze kit following the manufacturer's protocol (ThermoFisher Scientific). We quantified the relative expression of protein for SXL using the gel analysis tool in ImageJ software following the website's guidelines [84]. For each genotype, we first internally normalized the amount of SXL

protein to Actin. Next, we determined the protein's relative expression by comparing the Actin normalized quantities to y[1], w[1118] female samples.

Polytene chromosome squashes and immunostaining

Polytene chromosome squashes were prepared as previously described in Reider et al. 2017. We stained polytene chromosomes with rabbit anti-CLAMP (1:1000, SDIX), mouse anti-Squid (1:50, 1B11, DSHB), rabbit anti-MLE (1:1000, gift from Mitzi Kuroda), rat anti-MSL2 (1:500, gift from Peter Becker) antibodies. For detection, we used all Alexa Fluor secondary antibodies against rabbit and mouse at a concentration of 1:1000 and visualized slides at 40X on a Zeiss Axioimager M1 Epifluorescence upright microscope with the AxioVision version 4.8.2 software.

Splicing assays for male and female-specific transcripts

To test for the male and female splice forms of *sex-lethal, transformer, doublesex*, and *msl2*, total RNA was extracted from ten third instar larvae from each genotype. We reverse-transcribed two micrograms of total RNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies) following the manufacturer's protocol. We amplified target sequences by PCR using primers designed to span Alternatively spliced junctions. Alternative splicing primer sequences for sxl FP-TGCAACTCACCTCATCATCC, sxl RP- GATGGCAGAGAATGGGACAT, for tra FP-TGAAAATGGATGCCGACAG, tra RP- CTCTTTGGCGCAATCTTCTC, for dsx female transcript dsxFFP-CTATCCTTGGGAGCTGATGC, dsxF RP-TCGGGGCAAAGTAGTATTCG, for dsx male transcript dsxM FP-CAGACGCCAACATTGAAGAG, dsxM RP- CTGGAGTCGGTGGACAAATC, for msl2 FP-GTCACACTGGCTTCGCTCAG and msl2 RP- CCTGGGCTAGTTACCTGCAA were used.

Immunoprecipitation

Nuclear extract preparation: Male (S2) and female (Kc) cells were grown to a cell concentration of 2X 10^6 cells/mL in T25 tissue culture flasks. Cells were scraped from the flask, centrifuged for 5min at 2500rpm at 4°C. Supernatant was removed and cell pellets were washed twice in 5ml of cold PBS. The washed cell pellets were then resuspended in 5X volume of Buffer A (10mM HEPES pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mMDTT, 1X Protease inhibitors). Cells were incubated on ice for 15 minutes before dounce homogenization with an A pestle. Cytoplasmic

fraction was collected after centrifugation at 4°C for 20 min at 700xg. The remaining nuclear pellet was re-suspended in 3 times volume in Buffer B (20mM HEPES pH7.9, 20%Glycerol, 0.5%NP 40, 200mMKCl, 0.5mMEDTA, 1mMEGTA, 1X protease inhibitors). Nuclei after re-suspension were dounce homogenized with a B pestle. Nuclear debris was then pelleted by centrifugation at 10,000xg for 10 min at 4°C. 1ml aliquots of both cytoplasmic and nuclear fractions were prepared in 1.5mL Protein LoBind Eppendorf tubes and flash frozen in liquid nitrogen for storage at -80 °C.

Immunoprecipitation: Magnetic anti-CLAMP beads were prepared to a final concentration of 10mg/mL by coupling rabbit anti-CLAMP antibody (SDIX) to magnetic beads, according to Dynabeads Antibody coupling kit (ThermoFisher Scientific) instructions. Both prepared anti-CLAMP and purchased anti-IgG (anti-rabbit IgG M-280 Dynabeads) were blocked to reduce background the night before the immunoprecipitation. First, the beads were washed 3 times for 5 minutes in 500L Tris-NaCl Wash (50mM Tris, 500mM NaCl, 0.1% NP-40) by rotating at 4C. The beads were next suspended in block buffer (3.3mg/mL of yeast tRNA extract prepared in 20mM HEPES, pH7.9, 20% Glycerol, 0.5% NP-40, 200mM KCl, 1mM EDTA, and 2mM EGTA) and rotated overnight at 4C. The next day, beads were washed 3 times for 5 minutes in the block buffer without yeast tRNA by rotating at 4°C. After the final wash, beads were resuspended in the same amount of block buffer as the starting volume.

To 1mL of previously prepared nuclear extract, 100uL of blocked anti-CLAMP or anti-IgG magnetic Dynabeads were added. The nuclear extracts and beads were then rotated for 1 hour at 4°C. Afterward, the beads were collected and the supernatant discarded. The beads were then washed three times in Tris-NaCl wash (50mM Tris, 500mM NaCl, 0.1% NP-40) by rotating for 5 minutes at 4°C and cleared by using a magnetic rack. To elute proteins from the beads, 100uL of 1% SDS was added, and the beads were boiled for 10 minutes at 95C. To the eluate, 300uL of ultrapure water was added, and the tubes gently vortexed. After collecting the beads on a magnetic rack, the eluate was saved in a clean Protein LoBind Eppendorf tube.

Western blotting: Squid and Hrb27C were detected in IP-CLAMP and IGG-rabbit protein samples using mouse anti-Squid (1:500, 1B11, DSHB) and rabbit anti-Hrb27C (1:5000, Fatima Gebauer), performed as mentioned above under western blotting protocol.

CUT&RUN

0-2 hr and 2-4 hr male and female embryos of desired genotypes (~50 each) were collected on standard grape juice agar medium and washed with water. The embryos were dechorionated in 6% bleaching solution for 2 min and washed twice in ice cold 1XPBS. Centrifuged at 12,000g for 10 min at 4°C. Supernatants were discarded and embryos resuspended in 200µl Dig-Wash buffer with EDTA (20mM HEPES-NaOH, 150mM NaCl, 2mM EDTA, 0.5mM Spermidine, 10mM PMSF, 0.05% digitonin) and washed twice. Embryos were incubated in 200µl primary antibody overnight at 4°C on a tube rotator. Next, embryos were centrifuged at 12,000g for 10 min at 4°C and liquid removed and embryos were washed twice in Dig-Wash buffer with EDTA. Then, embryos were incubated for 3 hours at 4°C in ~700 ng/ml pAMNase solution in Dig-Wash buffer with EDTA. Embryos were washed twice in Dig-Wash buffer without EDTA and resuspended in 150µl of Dig-Wash buffer without EDTA. Samples were equilibrated to 0°C on a heat block maintained on ice-bath. 2µl of 100mm $CaCl_2$ added to each sample to initiate MNase activity and digestion was performed for 30 min before adding 150µl of 2X RSTOP Buffer (200mM NaCl, 20mM EDTA, 4mM EGTA, 50ug/ml RNase, 40ug/ml glycogen, 10pg/ml yeast spike-in DNA) to stop the reaction. Incubated at 37°C for 10 minutes to release the DNA fragments. Samples were spun at 12,000g for 10 minutes and aqueous layer transferred to a fresh 1.5 ml microfuge tube and centrifuged at 16,000g for 5 minutes. Cleared liquid was again transferred to a fresh tube, 1µl of 20% SDS and 2.5µl proteinase K (20ng/ml) added, incubated at 70°C for 10 minutes. 300µl PCI was added to each tube, mixed and total solution was transferred to phase lock tubes and centrifuged at 16,000g for 5 minutes. After adding 300µl of chloroform and mixing gently, samples were centrifuged at 16,000g for 5 minutes at RT. The aqueous layer was transferred to a DNA low binding tube. 1µl glycogen (5mg/ml) and 750µl ethanol added to precipitate DNA at -80°C. Samples were centrifuged at 16,000g for 10 min at 4°C and washed in ethanol twice. Pellet air dried and dissolved in 15µl of 1mM TrisHCl + 0.1mM EDTA pH 8.0 [57,58]. 1ng of Cut and Run DNA was used to make libraries using the KAPA Hyper prep kit and SeqCap adapters A &B (Roche) according to manufacturer's protocol. For library amplification 14 cycles were used and a 1.0X SPRI bead cleanup was performed using Agencourt Ampure XP beads. The following

antibody concentrations were used: rabbit anti-CLAMP (5µg/sample, SDIX); 1:200 anti-rabbit (MilliporeSigma); rat anti-MLE (1:50, 6E11); 700ng/ml pA-MNase (from Steven Henikoff).

iCLIP

Kc and S2 cells were maintained at 25°C in Schneider's media supplemented with 10% Fetal Bovine Serum and 1.4X Antibiotic-Antimycotic. Cells were passaged every 3 days to maintain an appropriate cell density. Cells were allowed to grow to confluency and UV crosslinked using 254nm UV light in Stratalinker 2400 on ice (Stratagene, USA). UV treated cells were lysed to get different cellular fractions (Cytoplasmic, Nucleoplasmic and Chromatin) according to Fr-iCLIP (fractionation-iCLIP) protocol from Brugiolo et al 2017[85]. Chromatin and Nucleoplasmic fractions were sonicated with a Branson digital sonicator at 30% amplitude for 30 s total (10 sec on and 20 sec off) to disrupt DNA before IP. All three fractions were separately centrifuged at 20,000 xg for 5 min at 4℃. Fractions were tested by Western blotting using RNApolI for Chromatin Fraction, Actin for Cytoplasmic Fraction. Protein quantification for each fraction was done using manufacturer's protocol for Pierce 660nm protein assay reagent (Thermo Scientific, USA). Each Fraction was subjected to iCLIP protocol as described in Huppertz et al 2014[51] using rabbit-CLAMP antibody to immuniprecipitate bound RNAs which were extracted using proteinase K and phenol:chloroform. Custom cDNA libraries prepared according to Huppertz et al 2014[51] using distinct primers Rt1clip-Rt16clip for separate samples containing individual 4nt-barcode sequences that allow multiplexing of samples. cDNA libraries for each sample amplified separately using 31 cycles of PCR, mixed together later and sequenced using standard illumina protocols. Heyl et al. 2020[86] methods using the Galaxy CLIP-Explorer were followed to preprocess, perform quality control, post-process and perform peak calling. Data deposited in GEO (awaiting accession number).

Computational Methods:

Time2splice tool: see Supplementary methods

Sex-specific splicing event analysis

RNA sequencing data from Rieder et al 2017 (#GSE102922) was analyzed using time2splice to determine sex-specifically splicing events dmel-all-r6.29.gtf from BDGP6 in genomes [87] was used to map each transcript identifier (ID) to gene ID and symbol, for .bed creation data for the associated chromosome, transcription start site (TSS) and transcription end site (TES), and strand information were imported from Illumina (https://support.illumina.com/sequencing/sequencing_software/igenome.html). From the raw data after quality control i.e, FastQC[88], Salmon[89] was used to quantify transcript expression for treatment and control samples. Calculated transcripts per million (TPM) values from SUPPA[42] were used for all four replicates of female and male controls at both time points (before and after MZT). Each sample was filtered to include transcripts where the mean value is less than or equal to 3 TPMs per gene. The number of transcripts included at various thresholds were plotted from 1 to 10 and the fraction of genes filtered out begins to plateau around threshold 3. The percent of spliced in (PSI) transcripts between females and males were compared at both 0-2 Hr (pre-MZT) and 2-4 Hr (post-MZT) (p-value of 0.05), thereby resulting in delta PSI values and p-values for each transcription in each experimental condition comparison. Given these resulting delta transcript PSI values, significantly alternatively splice genes (p-value 0.05) were found between females vs. males 0-2 Hr (pre-MZT) controls to show which genes are normally sex-specifically spliced pre-MZT. The same process was followed at 2-4 Hr (post-MZT). To then determine the sex-specifically spliced genes, the female RNAi experiment compared with the control delta PSI gave the number of total alternative spliced transcripts pre-MZT, then considering those that are not shared with males, and are only expressed in females normally, this defined our sex specifically spliced set of genes for females pre-MST. This process was also performed for males pre-MZT. The same process was followed for post-MZT samples.

ChIP-seq: Data analysis

We used preprocessed ChIP-seq data from Rieder et al 2019 (#GSE133637), specifically the .bw and .broadPeak.gz files in our analysis using ChIPseeker [90] and deeptools [91]. Specifically, when plotting the average profiles using deeptools, we achieved a baseline signal representing genome-wide binding taking into consideration the number of genes in other groups by the following procedure: of all genes that are on (no zero read-count genes), we sampled the number of the

largest other group (to which we are comparing), and ran computeMatrix on that subset. This process was repeated 500 times and the resulting 500 matrices were averaged to produce a representative signal. For motif analysis MEME [92] suite was used.

Cut and Run: Data analysis

Sequenced reads were run through FASTQC[88](fastqc replicate_R1_001.fastq.gz replicate_R2_001.fastq.gz) with default parameters to check the quality of raw sequence data and filter out any sequences flagged for poor quality. Sequences were trimmed and reassessed for quality using TrimGalore (https://github.com/FelixKrueger/TrimGalore/issues/25) and FastQC [88], respectively. All Illumina lanes of the same flow cell .fastq files were merged, and sequenced reads were then mapped to release 6 *Drosophila melanogaster* genome (dm6). We compared Bowtie2[93], HISAT2[94], and BWA[95]. We found the best alignment quality with BWA and thus used this method's results downstream. Next, we performed conversion to bam and sorting (e.g. using: bowtie2 -x dm6_genome -1 replicate_R1_001.fastq.gz -2 replicate_R2_001.fastq.gz -S out.sam > stout.txt 2> alignment_info.txt; samtools view -bS out.sam > out.bam; rm -rf out.sam; samtools sort out.bam -o out.sorted.bam). We removed reads (using samtools) with a MAPQ less than 30 and any reads with PCR duplicate reads (identified using MarkDuplicates Picard -2.20.2). Peaks identified using MACS2[96](macs2 callpeak -t out.sorted.bam -B -f BAM --nomodel --SPMR --keep-dup all -g dm --trackline -n outname --cutoff-analysis --call-summits -p 0.01 --outdir outdir) and keep duplicates separate**.** To calculate fold-enrichment macs2 is run again (macs2 bdgcmp -t $treat -c $control -o $out.sorted.bam_FE.bdg -m FE 2> $ out.sorted.bam_FE.log; macs2 bdgcmp -t $treat -c $control -o $out.sorted.bam_logLR.bdg -m logLR -p 0.00001 2). For motif analysis MEME [92] suite was used. Data submitted in GEO repository (#GSE174781).

iCLIP: Data analysis

Heyl et al. 2020[86] methods using the Galaxy CLIP-Explorer were followed to preprocess, perform quality control, post-process and perform peak calling. For preprocessing UMI-Tools was used, and then UMI-tools and Cutadapt used for Adapter, Barcode and UMI-removal. Cutadapt (Galaxy version 3.5) was used for filtering with a custom adapter sequence

AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCGTCTTCT GCTTG. All other settings followed the Heyl et al 2020 Galaxy iCLIP-explorer workflow. UMI-Tools Extract (Galaxy Version 1.1.2+galaxy2) was then used with a barcode pattern of NNNXXXXNN. No unpaired reads were allowed. The barcode was on the 3' end. Je-Demultiplex (Galaxy Version 1.2.1) was then used for demultiplexing. FastQC was used for quality control. Mapping was done by RNA STAR (Galaxy version 2.5.2b-2) using dm6. All settings were chosen based on the existing parameters from the iCLIP-explorer settings. We selected FALSE for the option to use end-to-end read alignments with no soft-clipping. bedtools used for Read-Filtering, and UMI-Tools (Galaxy version 0.5.3.0) for de-duplication. PEAKachu was used for Peak Calling to generate bed files. The PEAKachu settings were followed using the Galaxy CLIP-explorer workflow. The maximum insert size was set to 150, the minimum cluster expression fraction was set to 0.01, the minimum block overlap set to 0.5, the minimum block expression set to 0.1. The Mad Multiplier was set to 0.0, the Fold Change Threshold was set to 2.0, and the adjusted p-value threshold was set to 0.05. Peaks were annotated using RCAS[97] (RNA Centric Annotation System), a R package using Rstudio. MEME Suite used for motif detection. RCAS was used for functional analysis of the transcriptomes isolated by iCLIP, such as transcript features. ShinyGO 0.76[98] was used to perform Gene Ontology Analysis of the iCLIP data.

Competing Interest Statement

The authors declare no conflicting interests.

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962 **Author Contributions**

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964 M.R., A.M.C. and E.N.L. planned experiments, analyzed results and wrote the manuscript. A.M.C
965 did all the computational analysis. M.R. carried out the experimental work and collected data for
966 Cut and Run, Polytene squashes and IF, splicing assays and IP. J.U. carried out sex determination
967 pathway splicing assays and WB. JA analyzed the MLE cut and run data. A.H analyzed the iCLIP-
968 seq data.

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Fig 1. Alternative splicing during early *Drosophila melanogaster* embryonic development

A Schematic diagram showing 7 different types of Alternative splicing (AS).

B Pie chart showing the distribution of different types of AS at 0-2 Hr pre-MZT and 2-4 Hr post-MZT for female (red) and male (Blue) embryos in the presence (top row) and absence (bottom row) of maternal CLAMP. The total percentage of AS in each category in control embryos is noted at the top of the schematic. A Chi-square test was performed to determine if there is a significant difference between the percentage of each type of AS including MXE splicing (grey region) in the presence vs. absence of CLAMP in each class of sample: females/males 0-2 Hr, and 2-4 Hr embryos. A significant difference ($p<0.001$ marked by ***) was found between categories connected by solid black lines.

Fig 2. Maternal CLAMP regulates sex-specific alternative splicing during early embryonic development.

A Bar graph showing the percentage of transcripts (values noted at the top of each bar) out of total AS events or sex-specific splicing events (number of events noted within parentheses at the top of each bar) regulated by maternal CLAMP, at the pre-MZT and post-MZT stages, in females (red bars) and males (blue bars). A Fischer's Exact Test was performed, with significance at $p<0.001$.

B Bar plot showing the total number of splicing events undergoing CLAMP-dependent AS (**N**) in females and males at 0-2 Hr pre-MZT and 2-4 Hr post-MZT embryonic stages. Spliced genes are divided into non-sex specific (grey) and sex-specific (orange shades) sub-categories of genes. CLAMP-dependent female and male sex-specifically spliced (SSS) genes are divided into known (darker orange) and new (lighter orange) sub-categories identified in 0-2 Hr pre-MZT and 2-4 Hr post-MZT embryos.

C Percentage of **new** female (red) and male (blue) CLAMP-dependent sex-specifically spliced genes in 0-2 Hr pre-MZT and 2-4 Hr post-MZT embryos that were not identified in control samples.

D Male (blue) and female (brown) CLAMP-dependent sex-specific spliced genes compared with maternal genes (**N=841**, magenta) at 0-2 Hr pre-MZT (female, **N=153** and male, **N=99**) and 2-4 Hr post-MZT stages (female, **N= 270** and male, **N=116**).

E Gene ontology results for genes showing CLAMP-dependent female sex-specific splicing in the embryo at 0-2 Hr pre-MZT stage and for genes exhibiting CLAMP-dependent female as well as male sex-specific splicing in the embryo at the 2-4 Hr post-MZT stage. The size of the circle increases as the number of the genes in that category increases. The color of the circle represents significance (p-value).

Fig 3. CLAMP binds along the gene body of female and male sex-specifically spliced genes at the post-MZT embryonic stage

A-B Average profiles for CLAMP binding at pre-MZT and post-MZT embryonic stages in females and males for genes spliced female-specifically (red line) and male-specifically (blue line) during the pre-MZT (**A**) and post-MZT (**B**) stages.

C-D Average profiles for CLAMP binding to genes expressed in a sex-biased manner in females (red line) and males (blue line) during pre-MZT (**C**) and post-MZT (**D**) stage.

Green lines in **A-D** represent CLAMP binding at a random set of active genes used as a control (see **Material and Methods** for details). Stippled regions in **A, B** (female, 0-2 Hr pre-MZT) denote chromatin around the TSS with more CLAMP binding in female sex-specifically spliced genes vs. male sex-specifically spliced genes. The dotted box in **A-D** marks the gene body region in sex-specifically spliced and biased expressed genes.

Fig 4. CLAMP regulates the distribution of MLE on chromatin in males

A-B. Heat maps showing the distribution of MLE at the male (**A**) and female (**B**) specific control MLE peaks on the X chromosome and autosomes in male and female 0-2 Hr pre-MZT embryos with maternal CLAMP (*MTD-GAL4>GFPRNAi*) and after the loss of maternal CLAMP (*MTD-GAL4>CLAMPRNAi)*.

C-D. Venn diagrams and bar plots showing loss and gain of MLE peaks in the presence and absence of maternal CLAMP in male 0-2 Hr pre-MZT (**B**) and 2-4 Hr post-MZT (**C**) embryos. CLAMP peaks were identified only under control conditions (green circle), whereas MLE peaks were identified in the presence (grey circle) and absence (red circle) of maternal CLAMP protein depleted using the *MTD-GAL4>CLAMPRNAi* system.

Fig 5. Alternative splicing of components of the sex determination pathway is regulated by CLAMP in females

A The sex determination pathway in *Drosophila* is regulated by master regulator SXL.

B Electrophoresis gel image (inverted colors) showing splicing of *sxl* transcripts in third instar larvae of females and males of genotypes listed in the key (**a-g**) with a representative schematic at the top of the gel image.

C Western blot showing the level of SXL protein in genotypes (3 replicates for each) mentioned below each lane. Tubulin levels were used for protein loading control. Below the blot is the relative density of SXL protein compared with Tubulin, with each genotype represented by separate colored bars.

D Chromatin accessibility measured by the MNase Accessibility (MACC) score is shown across the *sxl* gene in male (S2) and female (Kc) cells under control and CLAMP RNAi conditions. The MACC score is a previously reported (Urban et al 2017) quantification of chromatin accessibility at each locus in the genome. Positive/high accessibility values (blue) indicate high chromatin accessibility, and negative/low (red) accessibility values indicate low chromatin accessibility. Each window covers MACC values ranging from -0.333 to +1.33. MACC values increase in females after CLAMP RNAi, specifically at exon 3 (red box) and are shown in the inset to the right. Green boxes represent CLAMP binding peaks in the *sxl* gene just below the schematic for the *sxl* gene itself.

E-F Electrophoresis gel image from 0-2 Hr embryonic (lane 2-5 & 7-10) and third instar larval samples (a-g) showing splicing of *dsx* (E) and *msl2* (F) transcripts in females (lane 3,5,8,10, a-d) and males (lane 2,4,7,9, e-g). Embryos were from *MTD-GAL4>GFPRNAi* control (lane 4,5,9,10) and *MTD-GAL4>CLAMPRNAi* (lane 2,3,7,8) females. a-g genotypes are the same as in B. The schematic at the top of each gel image shows female and male splice variants of *dsx* (E) and *msl2* (F) transcripts.

G Fluorescent microscopy images of polytene chromosomes from third instar salivary gland in the genotypes listed to the left of each panel show the distribution of CLAMP (green) and MSL2 (red) on chromatin (blue, DAPI)

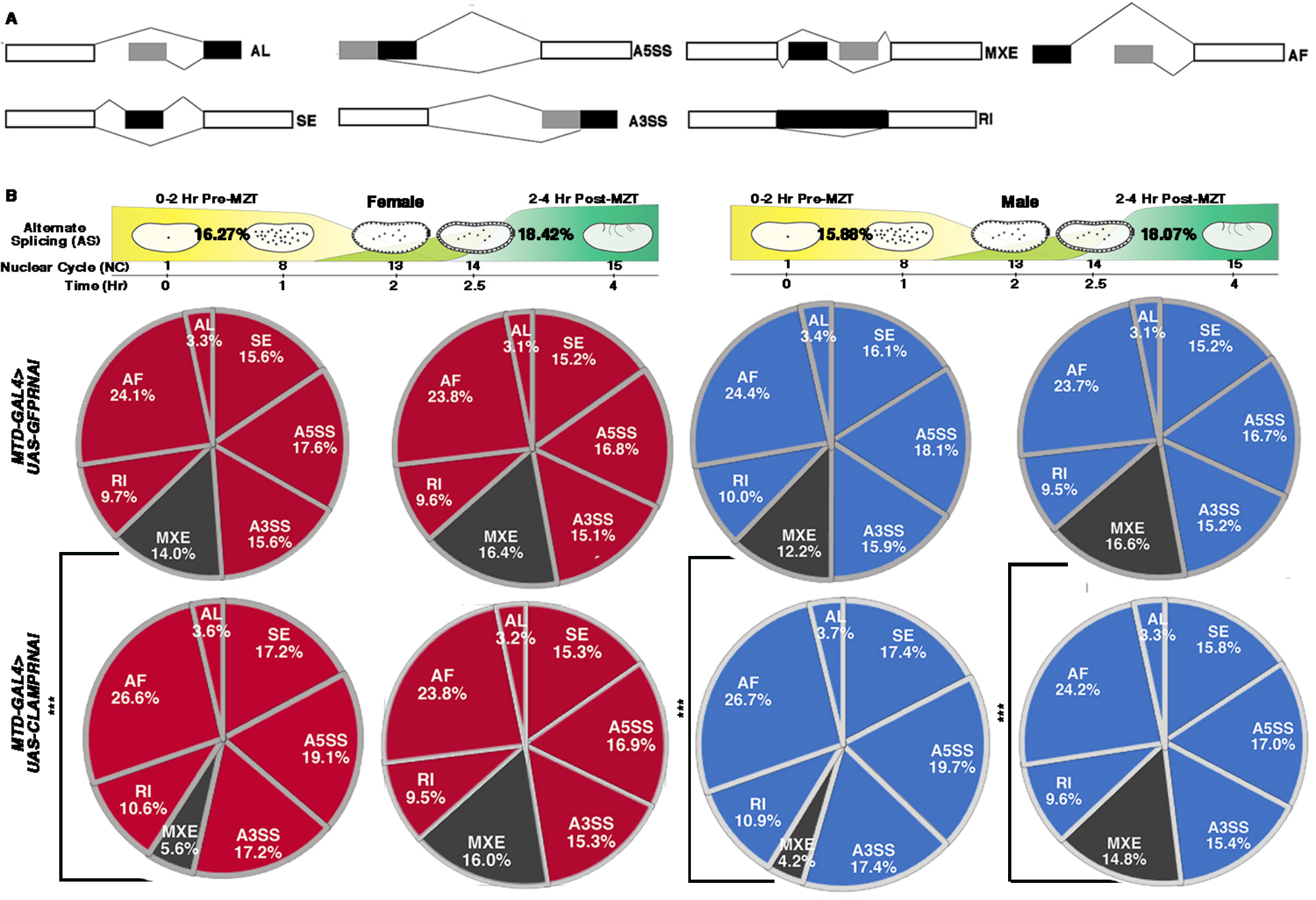
Fig 6. Mechanisms by which CLAMP regulates sex-specific splicing in females and males

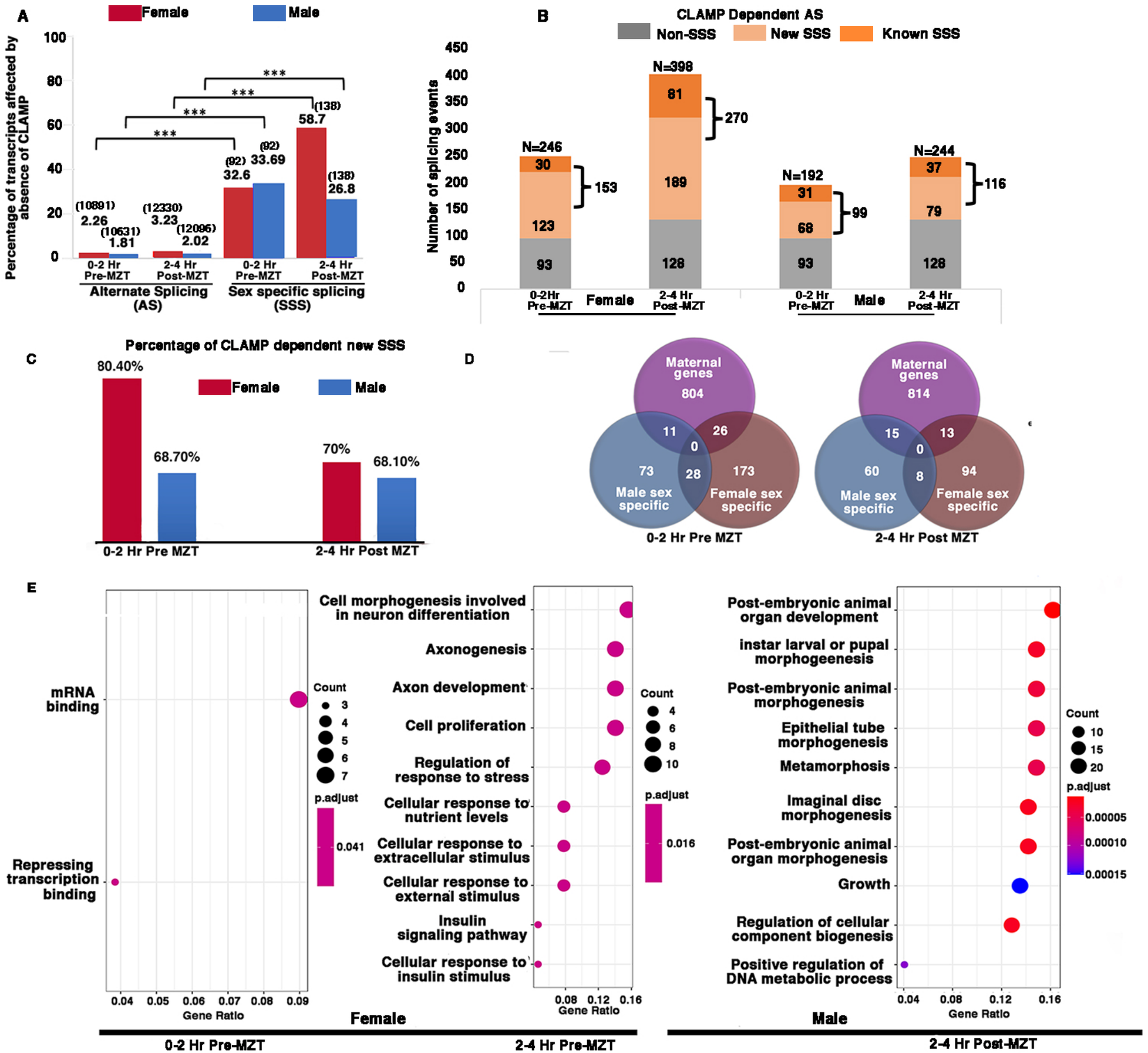
A CLAMP regulates splicing in both males and females via directly binding to intronic DNA sequences of CLAMP-dependent sex-specifically spliced genes and interaction with some sex-specifically spliced genes and spliceosomal RNAs.

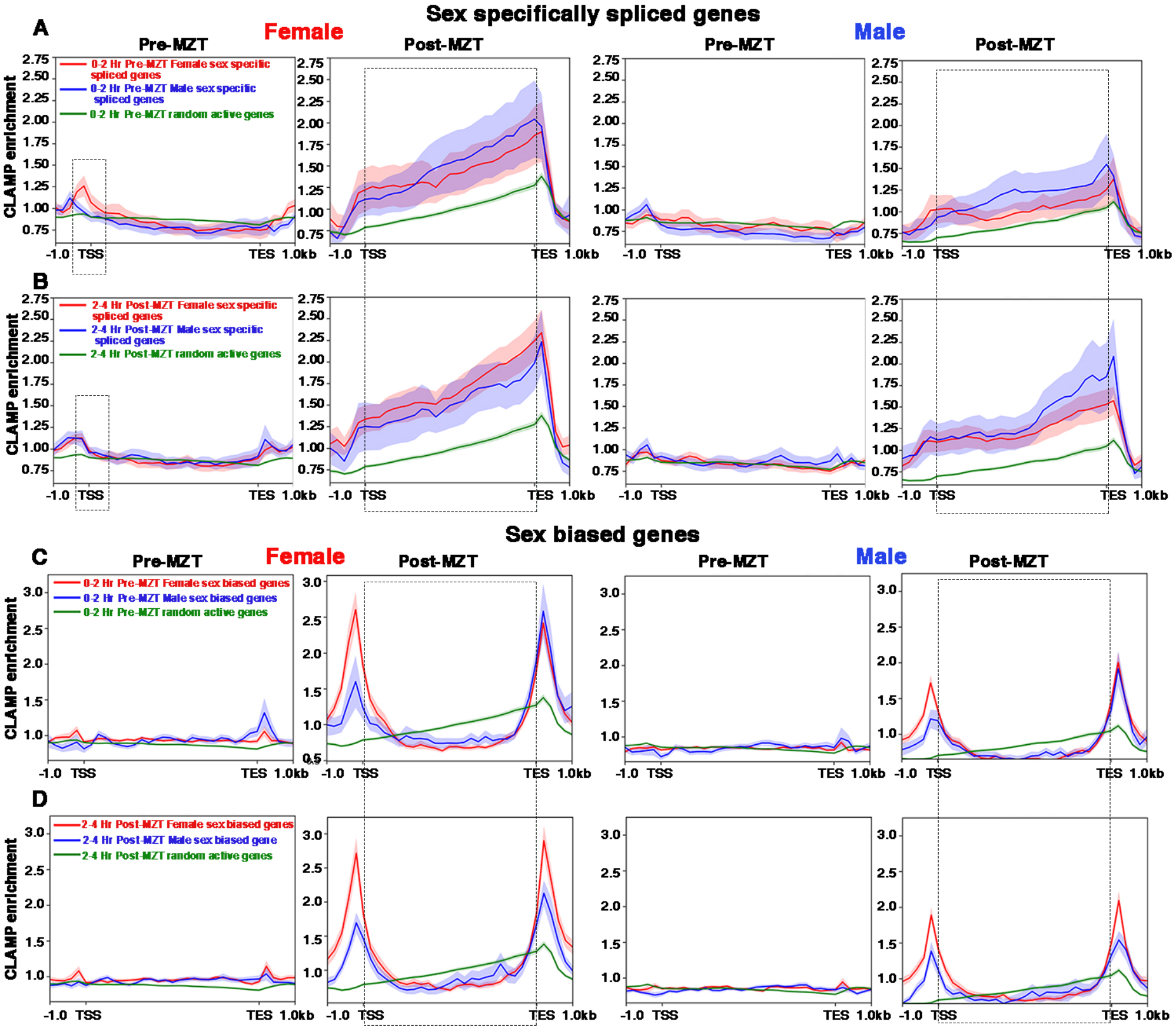
B CLAMP regulates MLE distribution between the spliceosome and the male X-chromosome specific MSL complex in males. CLAMP increases the occupancy of MLE at promoters and CES. In the absence of CLAMP, MLE is lost from many sites, including CES and promoters, and is gained at new intronic sequences which contain motifs that regulate splicing, resulting in aberrant sex-specific splicing in males.

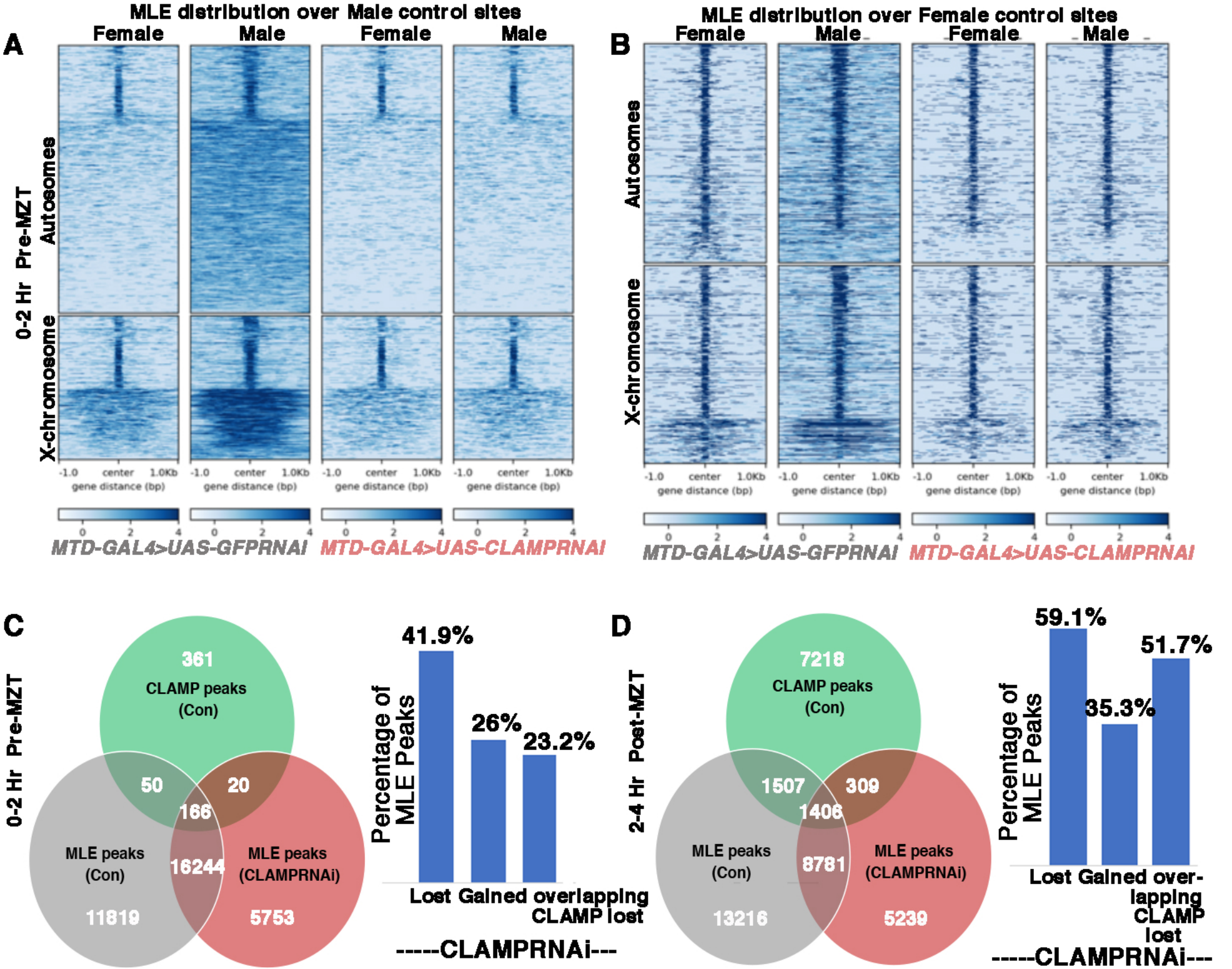
C In females, CLAMP binds near the SxlPe promoter and regulates chromatin accessibility at exon three (blue square) of the *sxl* gene and to the *sxl* mRNA. In this way, CLAMP promotes the splicing out of exon3 such that functional Sxl protein is formed, which drives female-specific splicing events. The absence of CLAMP in females thus results in the aberrant production of non-functional male-specific *sxl* transcripts which retain exon3, reducing levels of functional Sxl protein. CLAMP and Sxl have shared and distinct RNA targets suggesting that they function by both dependent and independent mechanisms.

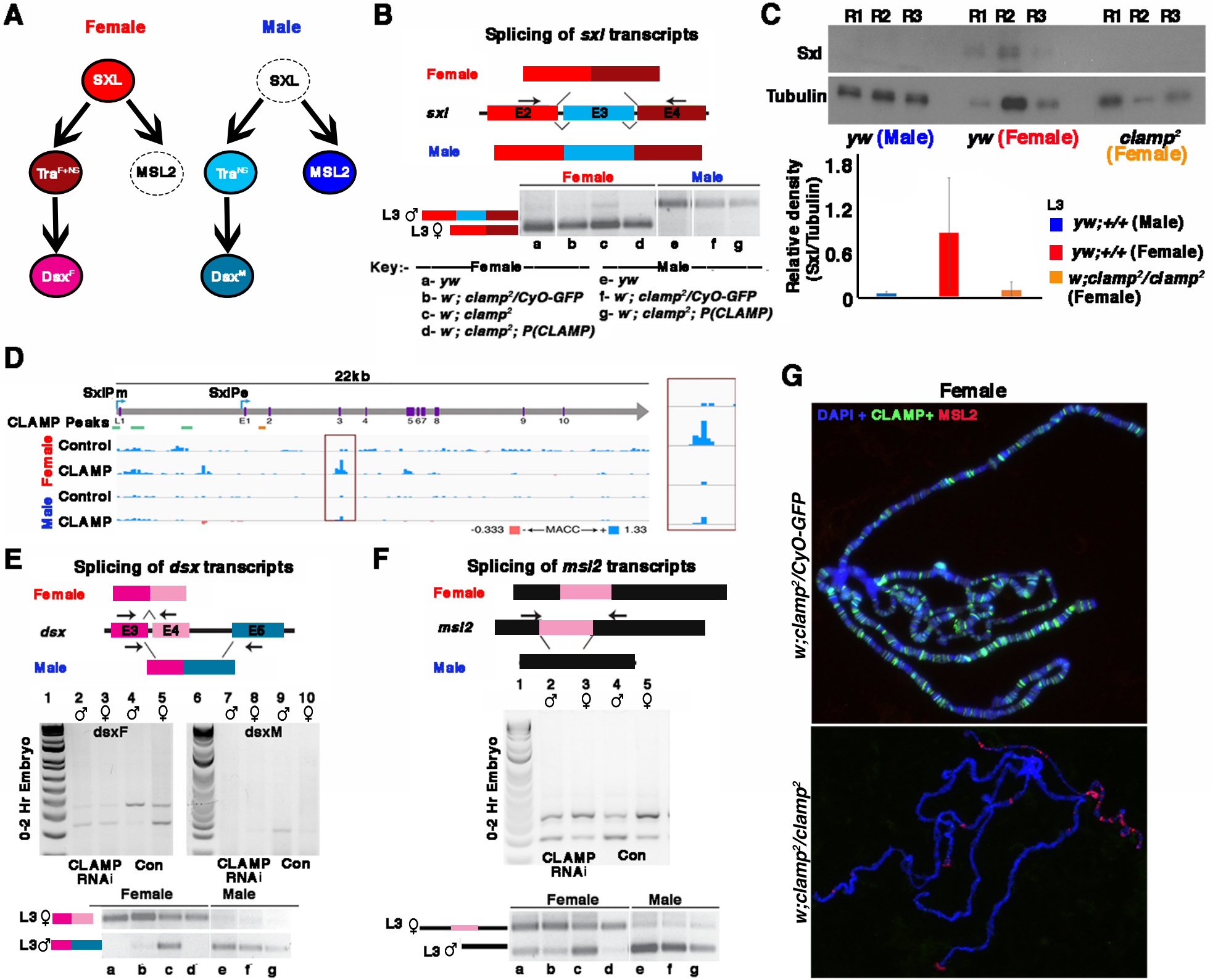
The three mechanisms proposed in parts **A**, **B**, and **C** are not mutually exclusive and are likely to occur simultaneously.

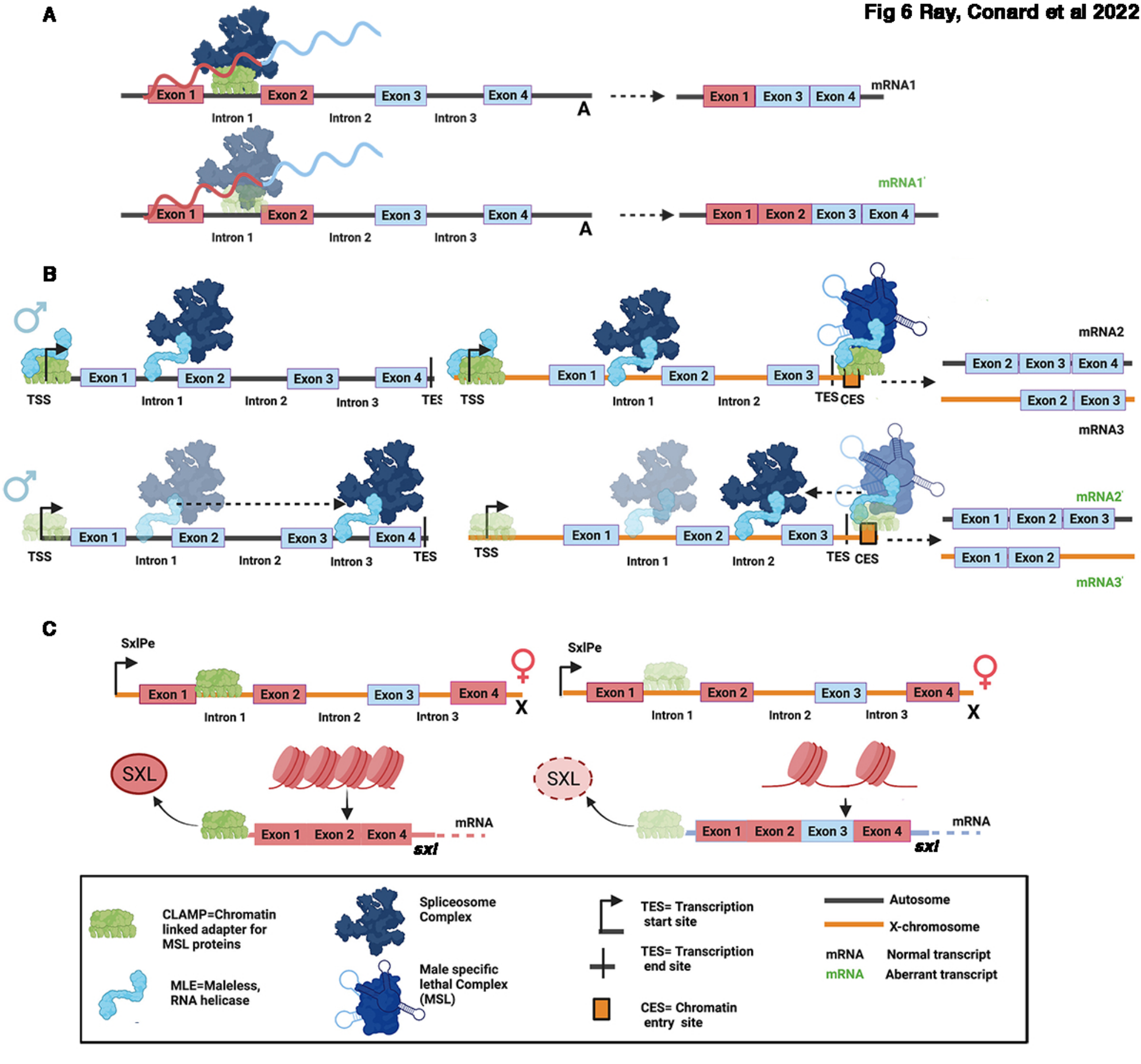

A
AL
A5SS
MXE
AF
SE
A3SS
RI
B
0-2 Hr Pre-MZT
Female
2-4 Hr Post-MZT
Alternate Splicing (AS)
16.27%
18.42%
Nuclear Cycle (NC)
1
8
13
14
15
Time (Hr)
0
1
2
2.5
4
0-2 Hr Pre-MZT
Male
2-4 Hr Post-MZT
15.88%
18.07%
MTD-GAL4> UAS-GFPRNAi
AL 3.3%
SE 15.6%
A5SS 17.6%
A3SS 15.6%
MXE 14.0%
RI 9.7%
AF 24.1%
AL 3.1%
SE 15.2%
A5SS 16.8%
A3SS 15.1%
MXE 16.4%
RI 9.6%
AF 23.8%
AL 3.4%
SE 16.1%
A5SS 18.1%
A3SS 15.9%
MXE 12.2%
RI 10.0%
AF 24.4%
AL 3.1%
SE 15.2%
A5SS 16.7%
A3SS 15.2%
MXE 16.6%
RI 9.5%
AF 23.7%
MTD-GAL4> UAS-CLAMPRNAi
***
AL 3.6%
SE 17.2%
A5SS 19.1%
A3SS 17.2%
MXE 5.6%
RI 10.6%
AF 26.6%
AL 3.2%
SE 15.3%
A5SS 16.9%
A3SS 15.3%
MXE 16.0%
RI 9.5%
AF 23.8%
***
AL 3.7%
SE 17.4%
A5SS 19.7%
A3SS 17.4%
MXE 4.2%
RI 10.9%
AF 26.7%
***
AL 3.3%
SE 15.8%
A5SS 17.0%
A3SS 15.4%
MXE 14.8%
RI 9.6%
AF 24.2%


A
Female
Male
Percentage of transcripts affected by absence of CLAMP
100
80
60
40
20
0
***
***
***
***
(10891)
2.26
(10631)
1.81
(12330)
3.23
(12096)
2.02
(92)
32.6
(92)
33.69
(138)
58.7
(138)
26.8
0-2 Hr Pre-MZT
2-4 Hr Post-MZT
0-2 Hr Pre-MZT
2-4 Hr Post-MZT
Alternate Splicing (AS)
Sex specific splicing (SSS)
B
CLAMP Dependent AS
Non-SSS
New SSS
Known SSS
Number of splicing events
450
400
350
300
250
200
150
100
50
0
N=246
30
123
93
153
N=398
81
189
128
270
N=192
31
68
93
99
N=244
37
79
128
116
0-2Hr Pre-MZT
Female
2-4 Hr Post-MZT
0-2 Hr Pre-MZT
Male
2-4 Hr Post-MZT
C
Percentage of CLAMP dependent new SSS
80.40%
Female
Male
68.70%
70%
68.10%
0-2 Hr Pre MZT
2-4 Hr Post MZT
D
Maternal genes
804
11
26
0
73
28
173
Male sex specific
Female sex specific
0-2 Hr Pre MZT
Maternal genes
814
15
13
0
60
8
94
Male sex specific
Female sex specific
2-4 Hr Post MZT
E
mRNA binding
Repressing transcription binding
Count
3
4
5
6
7
p.adjust
0.041
0.04
0.05
0.06
0.07
0.08
0.09
Gene Ratio
Cell morphogenesis involved in neuron differentiation
Axonogenesis
Axon development
Cell proliferation
Regulation of response to stress
Cellular response to nutrient levels
Cellular response to extracellular stimulus
Cellular response to external stimulus
Insulin signaling pathway
Cellular response to insulin stimulus
Count
4
6
8
10
p.adjust
0.016
0.08
0.12
0.16
Gene Ratio
Female
0-2 Hr Pre-MZT
2-4 Hr Pre-MZT
Post-embryonic animal organ development
instar larval or pupal morphogeenesis
Post-embryonic animal morphogenesis
Epithelial tube morphogenesis
Metamorphosis
Imaginal disc morphogenesis
Post-embryonic animal organ morphogenesis
Growth
Regulation of cellular component biogenesis
Positive regulation of DNA metabolic process
Count
10
15
20
p.adjust
0.00005
0.00010
0.00015
0.04
0.08
0.12
0.16
Gene Ratio
Male
2-4 Hr Post-MZT


Sex specifically spliced genes
A
Female
Pre-MZT
Post-MZT
Male
Pre-MZT
Post-MZT
0-2 Hr Pre-MZT Female sex specific spliced genes
0-2 Hr Pre-MZT Male sex specific spliced genes
0-2 Hr Pre-MZT random active genes
CLAMP enrichment
B
2-4 Hr Post-MZT Female sex specific spliced genes
2-4 Hr Post-MZT Male sex specific spliced genes
2-4 Hr Post-MZT random active genes
CLAMP enrichment
-1.0 TSS TES 1.0kb
Sex biased genes
C
Female
Male
0-2 Hr Pre-MZT Female sex biased genes
0-2 Hr Pre-MZT Male sex biased genes
0-2 Hr Pre-MZT random active genes
CLAMP enrichment
D
2-4 Hr Post-MZT Female sex biased genes
2-4 Hr Post-MZT Male sex biased gene
2-4 Hr Post-MZT random active genes
CLAMP enrichment


A
MLE distribution over Male control sites
Female
Male
Female
Male
0-2 Hr Pre-MZT
Autosomes
X-chromosome
-1.0
center
1.0Kb
gene distance (bp)
0
2
4
MTD-GAL4>UAS-GFPRNAI
MTD-GAL4>UAS-CLAMPRNAI
B
MLE distribution over Female control sites
Female
Male
Female
Male
Autosomes
X-chromosome
MTD-GAL4>UAS-GFPRNAI
MTD-GAL4>UAS-CLAMPRNAI
C
0-2 Hr Pre-MZT
361
CLAMP peaks
(Con)
50
20
166
MLE peaks
(Con)
16244
MLE peaks
(CLAMPRNAi)
11819
5753
Percentage of
MLE Peaks
41.9%
26%
23.2%
Lost
Gained overlapping
CLAMP lost
-----CLAMPRNAi---
D
2-4 Hr Post-MZT
7218
CLAMP peaks
(Con)
1507
309
1406
MLE peaks
(Con)
8781
MLE peaks
(CLAMPRNAi)
13216
5239
Percentage of
MLE Peaks
59.1%
35.3%
51.7%
Lost
Gained over-
lapping
CLAMP
lost
-----CLAMPRNAi---


A
Female
Male
SXL
SXL
Tra F+NS
MSL2
Tra NS
MSL2
Dsx F
Dsx M
B
Splicing of sxl transcripts
Female
sxl
E2
E3
E4
Male
Female
Male
L3 ♂
L3 ♀
a b c d e f g
Key:-
Female
a- yw
b- w; clamp2/CyO-GFP
c- w; clamp2
d- w; clamp2; P(CLAMP)
Male
e- yw
f- w; clamp2/CyO-GFP
g- w; clamp2; P(CLAMP)
C
R1 R2 R3 R1 R2 R3 R1 R2 R3
Sxl
Tubulin
yw (Male)
yw (Female)
clamp2 (Female)
Relative density (Sxl/Tubulin)
1.8
1.2
0.6
0
L3
yw;+/+ (Male)
yw;+/+ (Female)
w;clamp2/clamp2 (Female)
D
22kb
SxlPm
SxdPe
CLAMP Peaks
L1
E1
2
3
4
5 6 7
8
9
10
Female
Control
CLAMP
Male
Control
CLAMP
-0.333
MACC
1.33
E
Splicing of dsx transcripts
Female
dsx
E3
E4
E5
Male
1 2 3 4 5 6 7 8 9 10
♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀
dsxF
dsxM
0-2 Hr Embryo
CLAMP RNAi
Con
CLAMP RNAi
Con
Female
Male
L3 ♀
L3 ♂
a b c d e f g
F
Splicing of msl2 transcripts
Female
msl2
Male
1 2 3 4 5
♂ ♀ ♂ ♀
0-2 Hr Embryo
CLAMP RNAi
Con
Female
Male
L3 ♀
L3 ♂
a b c d e f g
G
Female
DAPI + CLAMP+ MSL2
w;clamp2/CyO-GFP
w;clamp2/clamp2


A
Exon 1
Exon 2
Exon 3
Exon 4
Intron 1
Intron 2
Intron 3
A
mRNA1
mRNA1'
B
TSS
TES
CES
mRNA2
mRNA3
mRNA2'
mRNA3'
C
SxlPe
X
SXL
mRNA
sxl
CLAMP=Chromatin linked adapter for MSL proteins
MLE=Maleless, RNA helicase
Spliceosome Complex
Male specific lethal Complex (MSL)
TES= Transcription start site
TES= Transcription end site
CES= Chromatin entry site
Autosome
X-chromosome
Normal transcript
Aberrant transcript

A. Retrieve raw data, quality control, trim, and alignment

Run as needed. Note enumeration follows script names.

1. Retrieve data
Creates time2splice/ folder structure, metadatafile.csv and SraAccList.txt. .fastqs retrieved using SraAccList.txt.

2. Run quality control
Run *FastQC* for all .fastq files in time2splice/ directory.

3. Trim data
Run *Trim Galore!*, then *FastQC* to trim reads below quality threshold. Merge lanes of the same flow cell .fastq files.

4/5. Run & plot alignment
Run *Bowtie2*, *BWA*, or *HISAT2* on .fastq data in time2splice directory. Plot alignment for one or two aligners.

B. Temporal expression analysis

Run as needed. Note enumeration follows script names.

1. Run transcript quantification
Quantify transcript treatment and control expression with *Salmon*.

2. Run differential splicing analysis
Run *SUPPA* differential splicing analysis across case and controls.

3. Format results
Converts NM_ gene names to flybase name. Merges outputs.

4. Identify differential splicing forms
SUPPA identifies forms of differential splicing (e.g. using PSI and DTU).

5. Calculate total control alternative splicing
Calculate and plot the proportions of alternative splicing in control samples.

6. Calculate total case alternative splicing
Calculate and plot the proportions of alternative splicing in case samples.

7. Get bias genes
Retrieve male and female biased genes. Create .beds to plot average profiles.

8/9. Plot splicing events
Plots alternative splicing (PSI and DTU), and events in categories (e.g. female sex specific, male new sex specific).

C. Temporal protein-DNA analysis

Run as needed. Note enumeration follows script names.

1. Mark duplicate reads
Run Run *Picard's MarkDuplicates* in for all .sorted.bam files in a given directory.

2. Call peaks
Run *MACS2* to call peaks for all .sorted.bam files in a given directory.

3. Find fold enrichment
Generate signal track using *MACS2* to profile transcription factor modification enrichment levels genome-wide.

time2splice

D. Temporal multi-omics integration

Run as needed. Miscellaneous tests at README end plot peak intensity and perform chi-squared test

Peak intersections
Run *Intervene* to view intersection of each narrowpeak file.

Gene ontology
Perform gene ontology analysis with *ClusterProfiler* given a list of genes.

Find motifs *denovo*
Get coordinates of bed file and run through *MEME*.

Fig S2. Ray, Conard et al 2022

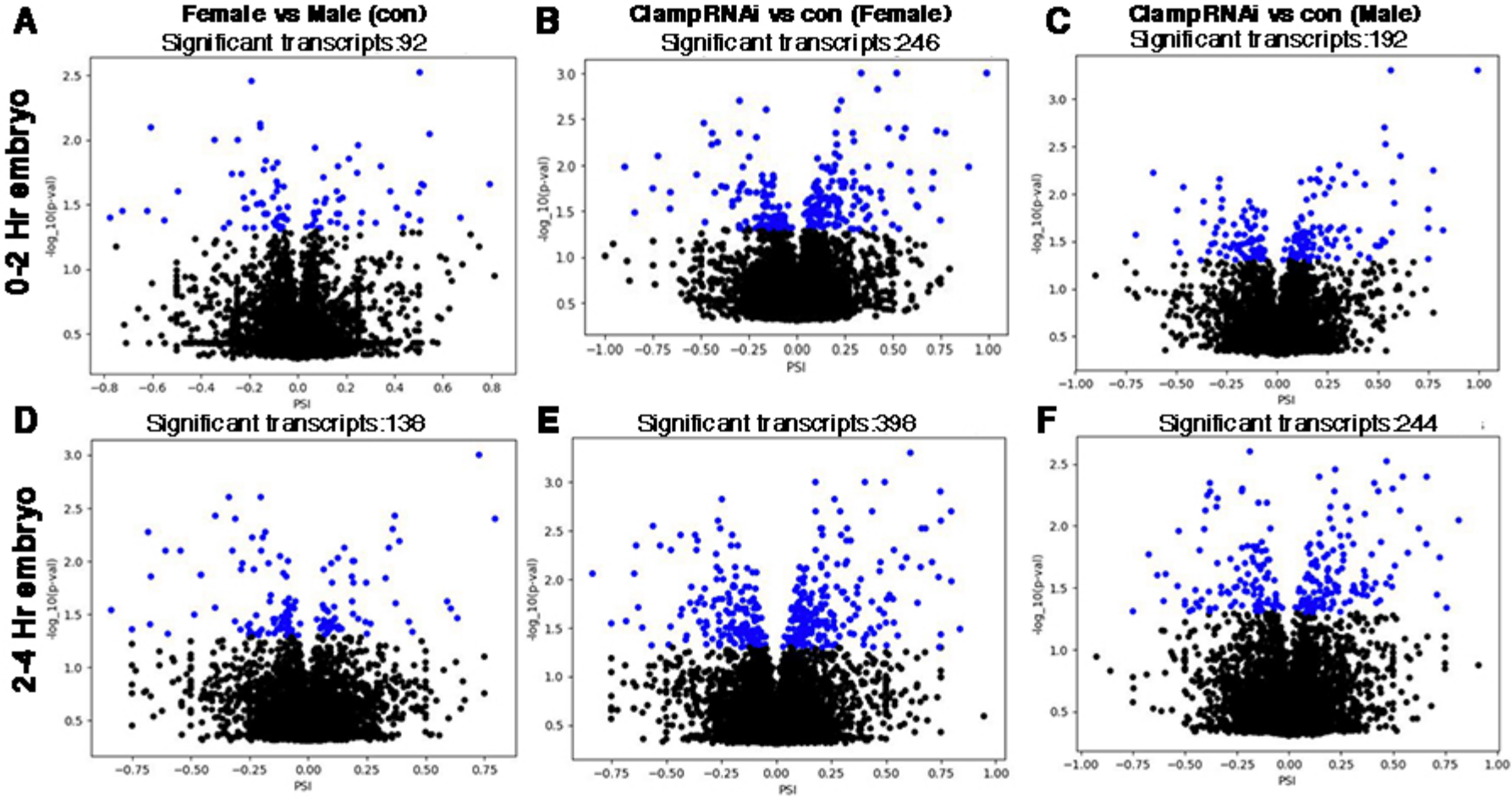
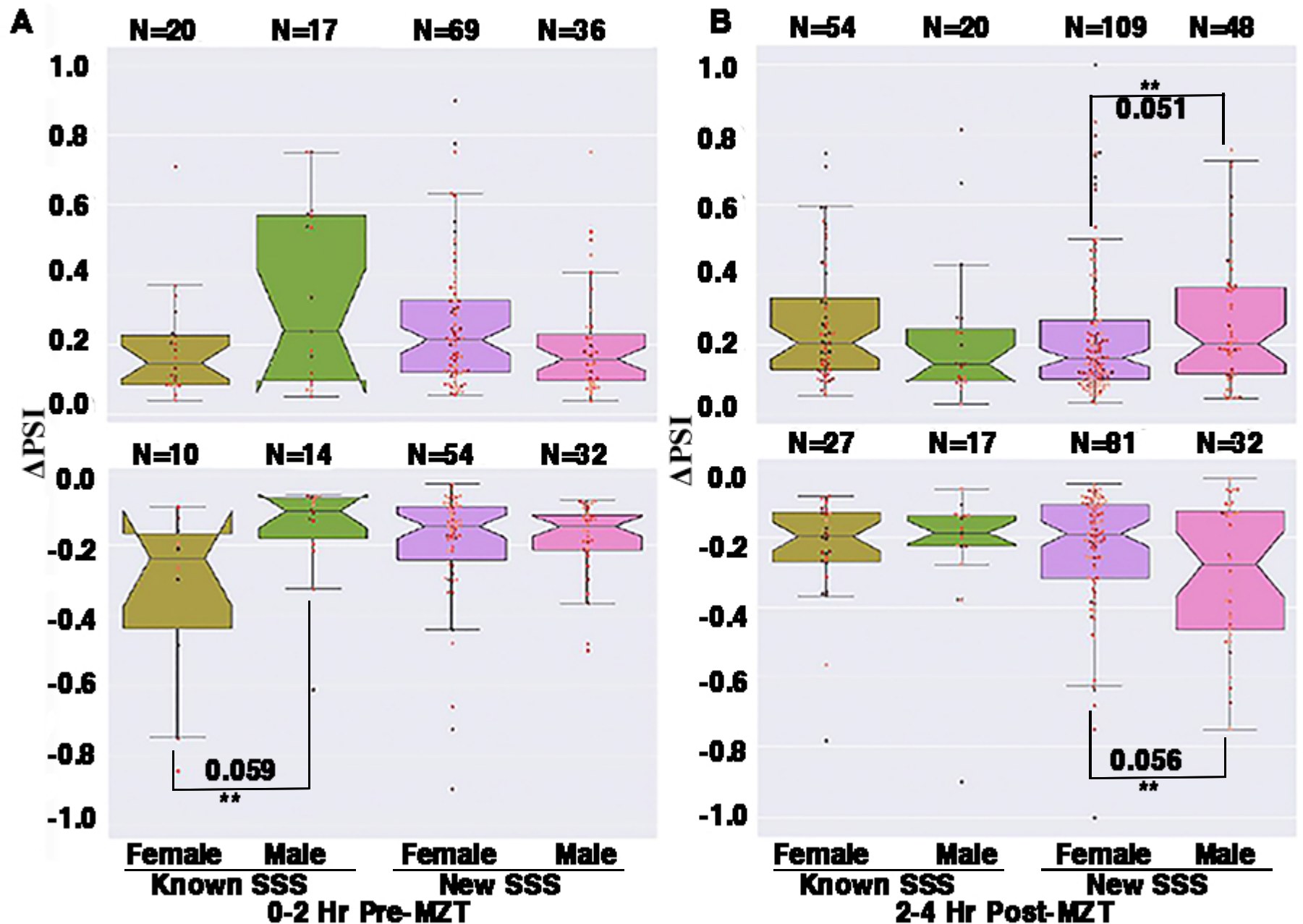
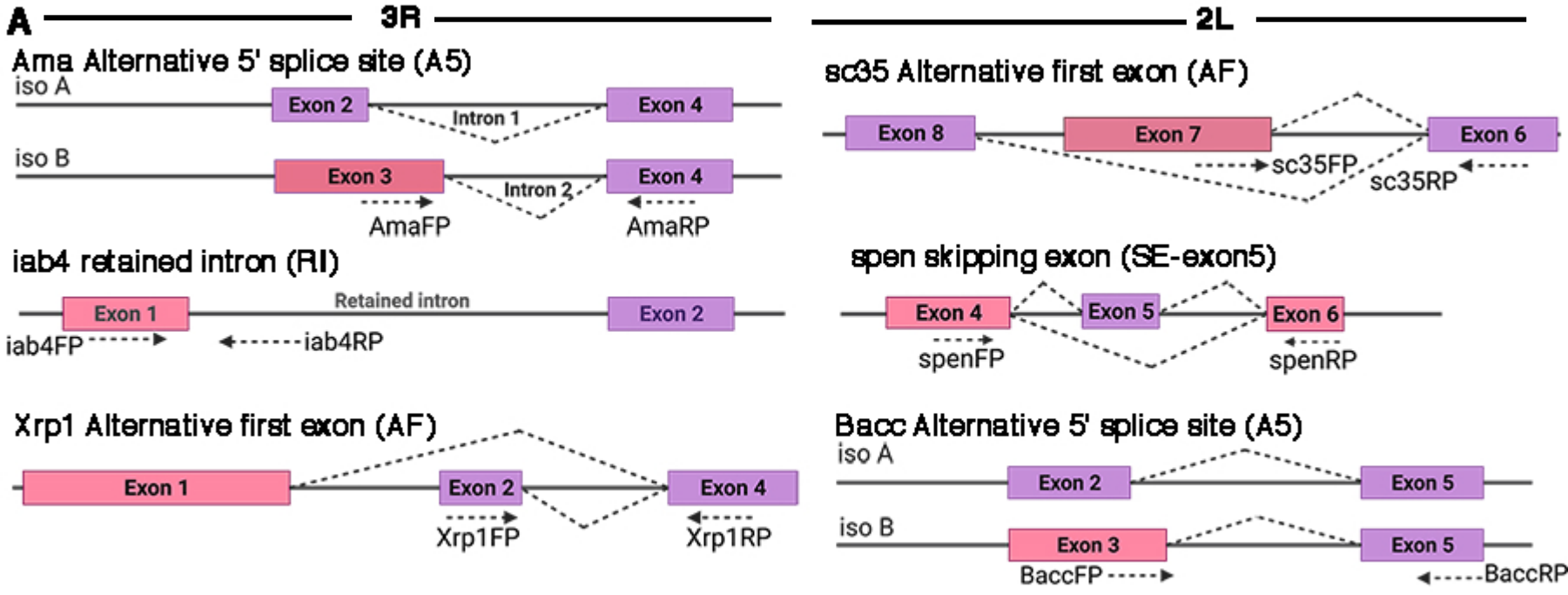
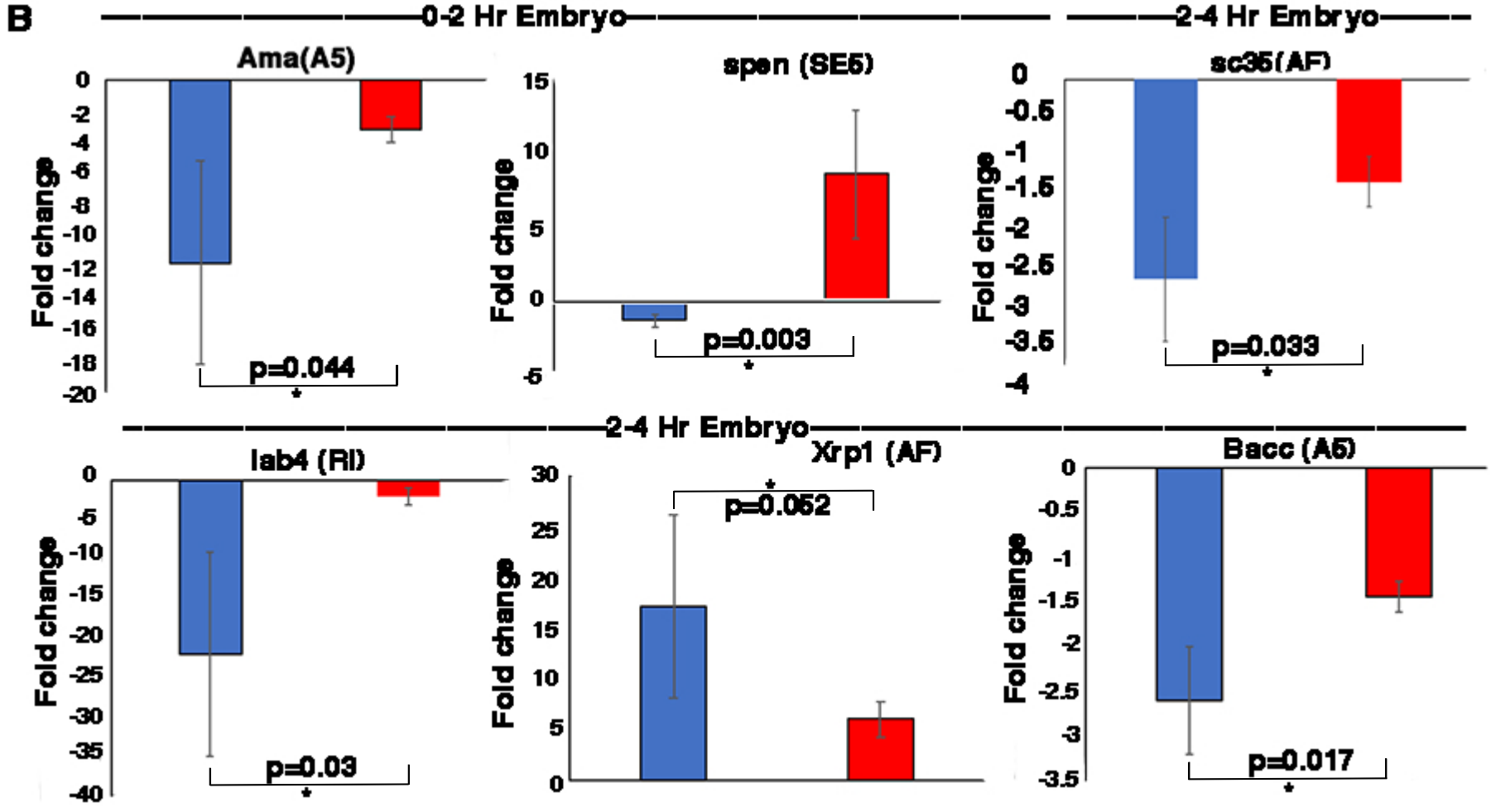


Fig S3. Ray, Conard et al 2022




A
3R
2L
Ama Alternative 5' splice site (A5)
iso A
Exon 2
Intron 1
Exon 4
iso B
Exon 3
Intron 2
Exon 4
AmaFP
AmaRP
sc35 Alternative first exon (AF)
Exon 8
Exon 7
Exon 6
sc35FP
sc35RP
iab4 retained intron (RI)
Exon 1
Retained intron
Exon 2
iab4FP
iab4RP
spen skipping exon (SE-exon5)
Exon 4
Exon 5
Exon 6
spenFP
spenRP
Xrp1 Alternative first exon (AF)
Exon 1
Exon 2
Exon 4
Xrp1FP
Xrp1RP
Bacc Alternative 5' splice site (A5)
iso A
Exon 2
Exon 5
iso B
Exon 3
Exon 5
BaccFP
BaccRP


B
0-2 Hr Embryo
2-4 Hr Embryo
Ama(A5)
Fold change
p=0.044
*
spen (SE5)
Fold change
p=0.003
*
sc35(AF)
Fold change
p=0.033
*
2-4 Hr Embryo
Iab4 (RI)
Fold change
p=0.03
*
Xrp1 (AF)
Fold change
*
p=0.052
Bacc (A5)
Fold change
p=0.017
*

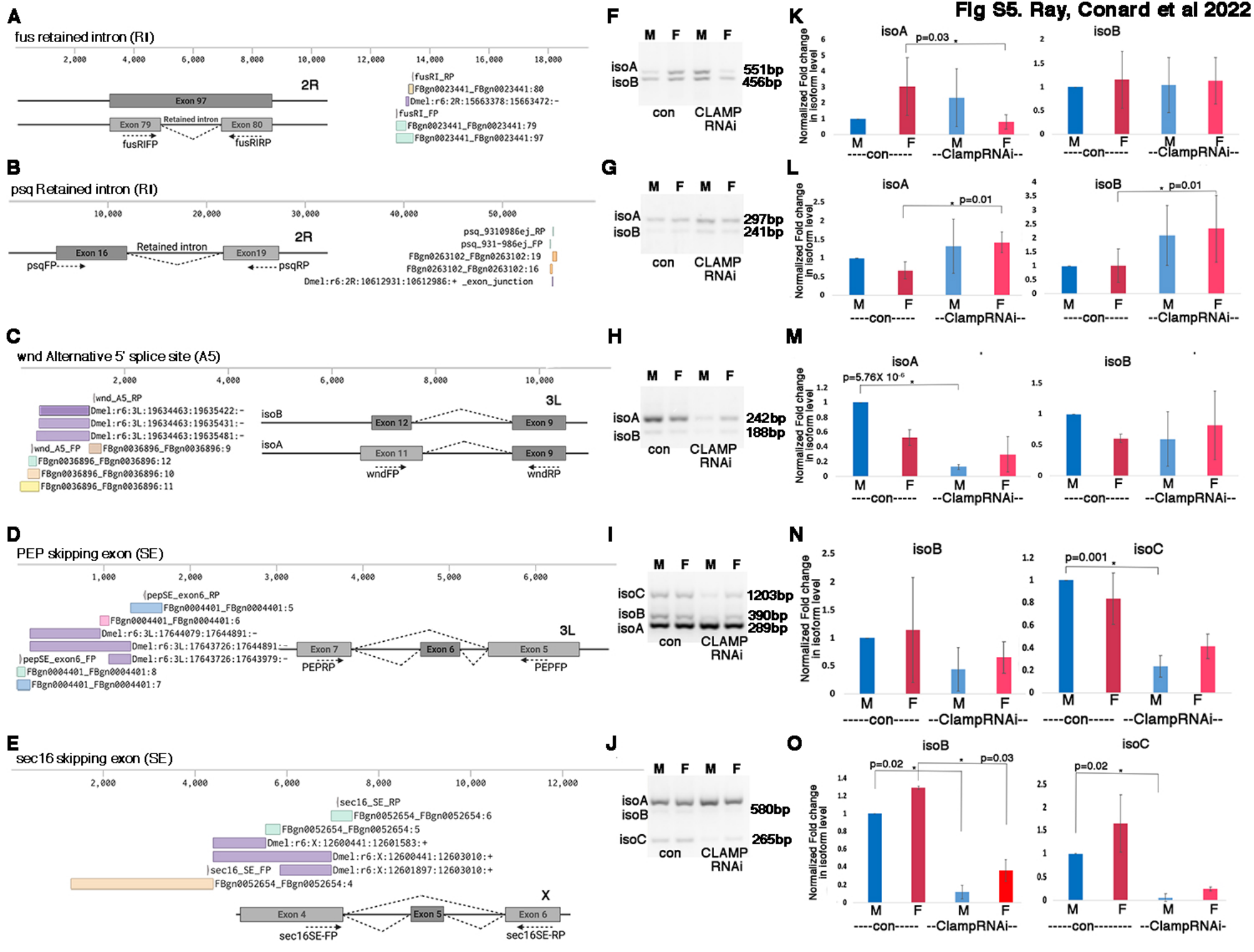
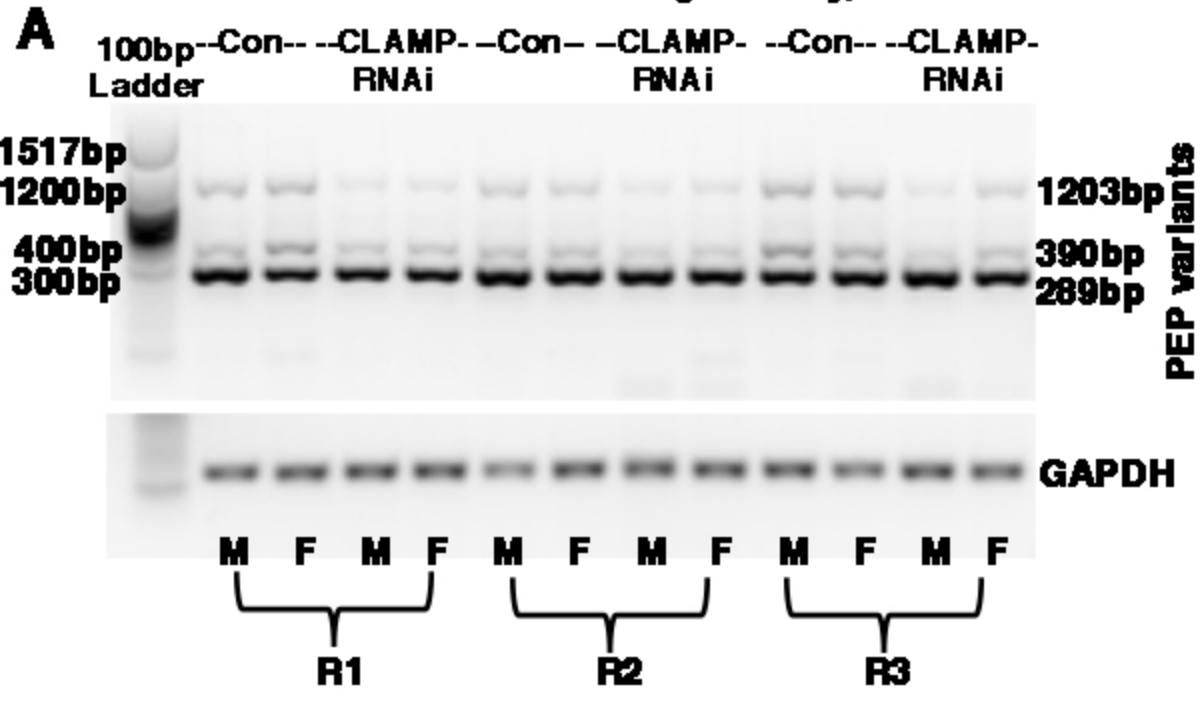
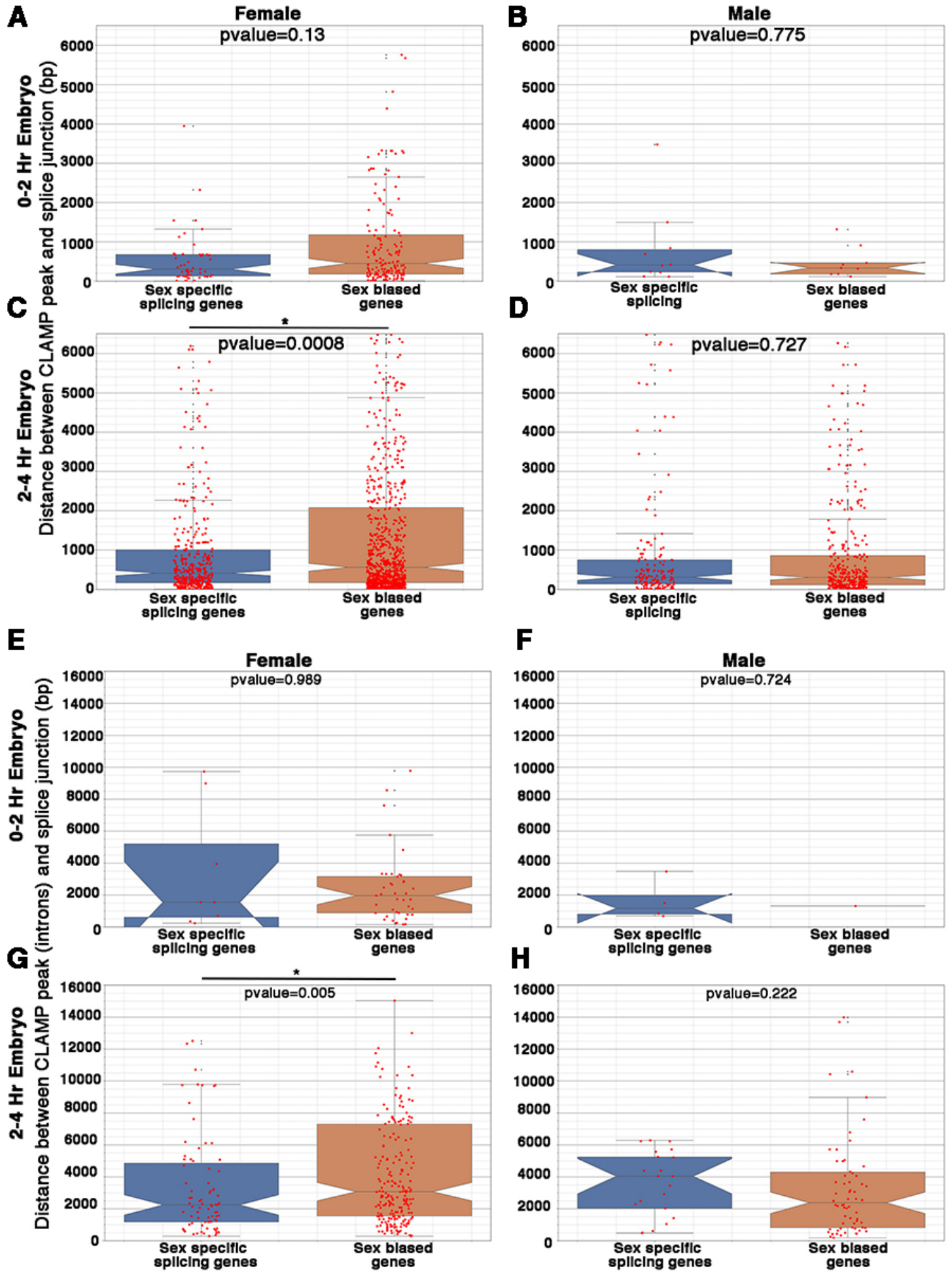
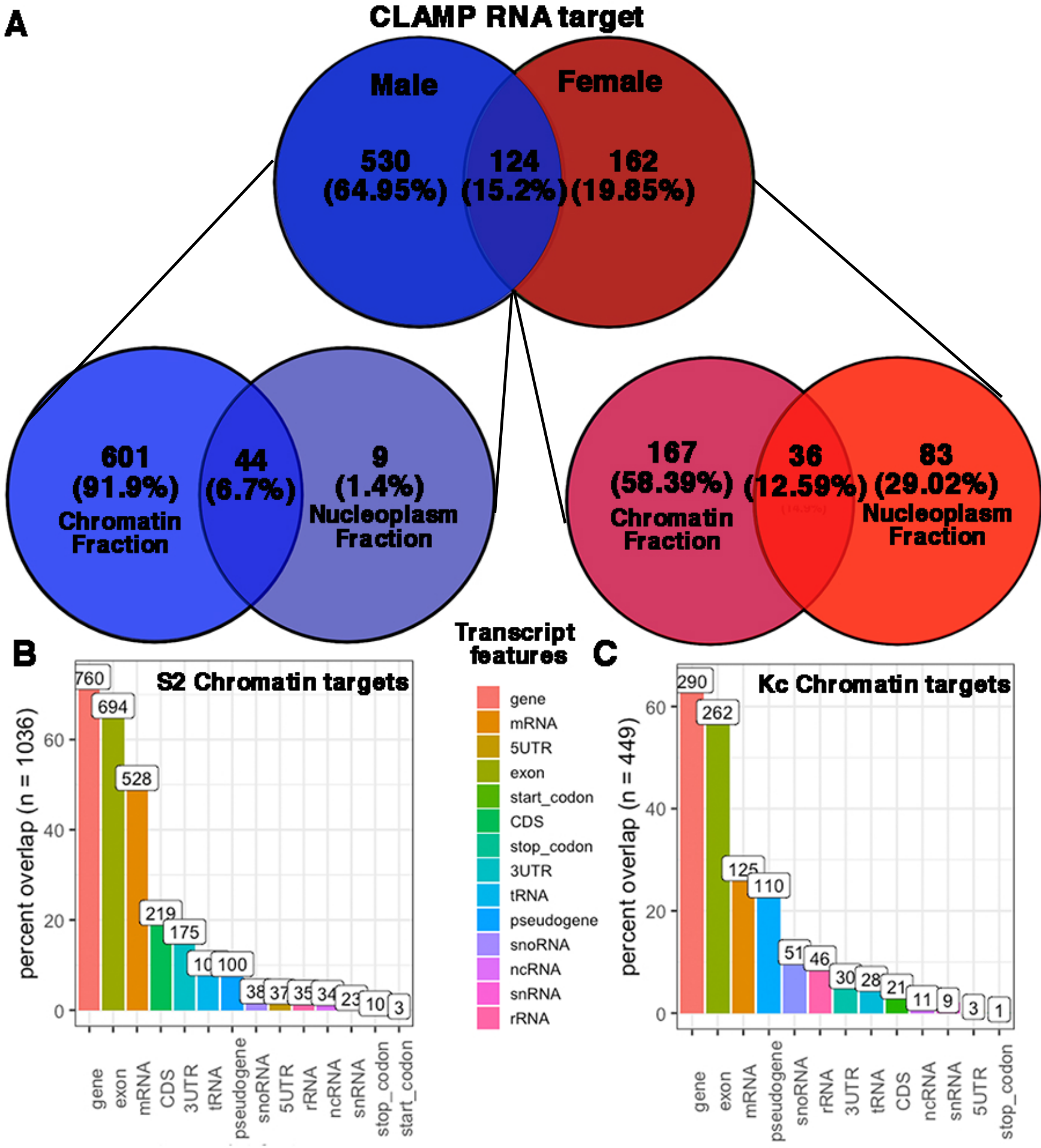


Fig S5. Ray, Conard et al 2022
A
fus retained intron (RI)
2R
Exon 97
Exon 79
Retained intron
Exon 80
fusRIFP
fusRIRP
fusRI_RP
FBgn0023441_FBgn0023441:80
Dmel:r6:2R:15663378:15663472:-
fusRI_FP
FBgn0023441_FBgn0023441:79
FBgn0023441_FBgn0023441:97
B
psq Retained intron (RI)
2R
Exon 16
Retained intron
Exon19
psqFP
psqRP
psq_9310986ej_RP
psq_931-986ej_FP
FBgn0263102_FBgn0263102:19
FBgn0263102_FBgn0263102:16
Dmel:r6:2R:10612931:10612986:+ _exon_junction
C
wnd Alternative 5' splice site (A5)
3L
wnd_A5_RP
Dmel:r6:3L:19634463:19635422:-
Dmel:r6:3L:19634463:19635431:-
Dmel:r6:3L:19634463:19635481:-
wnd_A5_FP
FBgn0036896_FBgn0036896:9
FBgn0036896_FBgn0036896:12
FBgn0036896_FBgn0036896:10
FBgn0036896_FBgn0036896:11
isoB
Exon 12
Exon 9
isoA
Exon 11
Exon 9
wndFP
wndRP
D
PEP skipping exon (SE)
3L
pepSE_exon6_RP
FBgn0004401_FBgn0004401:5
FBgn0004401_FBgn0004401:6
Dmel:r6:3L:17644079:17644891:-
Dmel:r6:3L:17643726:17644891:-
pepSE_exon6_FP
Dmel:r6:3L:17643726:17643979:-
FBgn0004401_FBgn0004401:8
FBgn0004401_FBgn0004401:7
Exon 7
Exon 6
Exon 5
PEPRP
PEPFP
E
sec16 skipping exon (SE)
X
sec16_SE_RP
FBgn0052654_FBgn0052654:6
FBgn0052654_FBgn0052654:5
Dmel:r6:X:12600441:12601583:+
Dmel:r6:X:12600441:12603010:+
sec16_SE_FP
Dmel:r6:X:12601897:12603010:+
FBgn0052654_FBgn0052654:4
Exon 4
Exon 5
Exon 6
sec16SE-FP
sec16SE-RP
F
M F M F
isoA
isoB
551bp
456bp
con
CLAMP RNAi
G
M F M F
isoA
isoB
297bp
241bp
con
CLAMP RNAi
H
M F M F
isoA
isoB
242bp
188bp
con
CLAMP RNAi
I
M F M F
isoC
isoB
isoA
1203bp
390bp
289bp
con
CLAMP RNAi
J
M F M F
isoA
isoB
isoC
580bp
265bp
con
CLAMP RNAi
K
isoA
p=0.03
isoB
Normalized Fold change in isoform level
M F M F
----con-----
--ClampRNAi--
L
isoA
p=0.01
isoB
p=0.01
Normalized Fold change in isoform level
M F M F
----con-----
--ClampRNAi--
M
isoA
p=5.76X 10-6
isoB
Normalized Fold change in isoform level
M F M F
----con-----
--ClampRNAi--
N
isoB
p=0.001
isoC
Normalized Fold change in isoform level
M F M F
-----con-----
--ClampRNAi--
O
isoB
p=0.02
p=0.03
isoC
p=0.02
Normalized Fold change in isoform level
M F M F
----con-----
--ClampRNAi--
-----con--------
--ClampRNAi-

Fig S6. Ray, Conard et al 2022




A
Female
pvalue=0.13
0-2 Hr Embryo
Distance between CLAMP peak and splice junction (bp)
Sex specific splicing genes
Sex biased genes
B
Male
pvalue=0.775
Sex specific splicing
Sex biased genes
C
*
pvalue=0.0008
2-4 Hr Embryo
Distance between CLAMP peak and splice junction (bp)
Sex specific splicing genes
Sex biased genes
D
pvalue=0.727
Sex specific splicing
Sex biased genes
E
Female
pvalue=0.989
0-2 Hr Embryo
Distance between CLAMP peak (introns) and splice junction (bp)
Sex specific splicing genes
Sex biased genes
F
Male
pvalue=0.724
Sex specific splicing genes
Sex biased genes
G
*
pvalue=0.005
2-4 Hr Embryo
Distance between CLAMP peak (introns) and splice junction (bp)
Sex specific splicing genes
Sex biased genes
H
pvalue=0.222
Sex specific splicing genes
Sex biased genes


A
CLAMP RNA target
Male
Female
530
(64.95%)
124
(15.2%)
162
(19.85%)
601
(91.9%)
Chromatin
Fraction
44
(6.7%)
9
(1.4%)
Nucleoplasm
Fraction
167
(58.39%)
Chromatin
Fraction
36
(12.59%)
83
(29.02%)
Nucleoplasm
Fraction
B
S2 Chromatin targets
percent overlap (n = 1036)
760
694
528
219
175
100
38
37
35
34
23
10
3
gene
exon
mRNA
CDS
3UTR
tRNA
pseudogene
snoRNA
5UTR
rRNA
ncRNA
snRNA
stop_codon
start_codon
Transcript
features
gene
mRNA
5UTR
exon
start_codon
CDS
stop_codon
3UTR
tRNA
pseudogene
snoRNA
ncRNA
snRNA
rRNA
C
Kc Chromatin targets
percent overlap (n = 449)
290
262
125
110
51
46
30
28
21
11
9
3
1
gene
exon
mRNA
pseudogene
snoRNA
rRNA
3UTR
tRNA
CDS
ncRNA
snRNA
5UTR
stop_codon

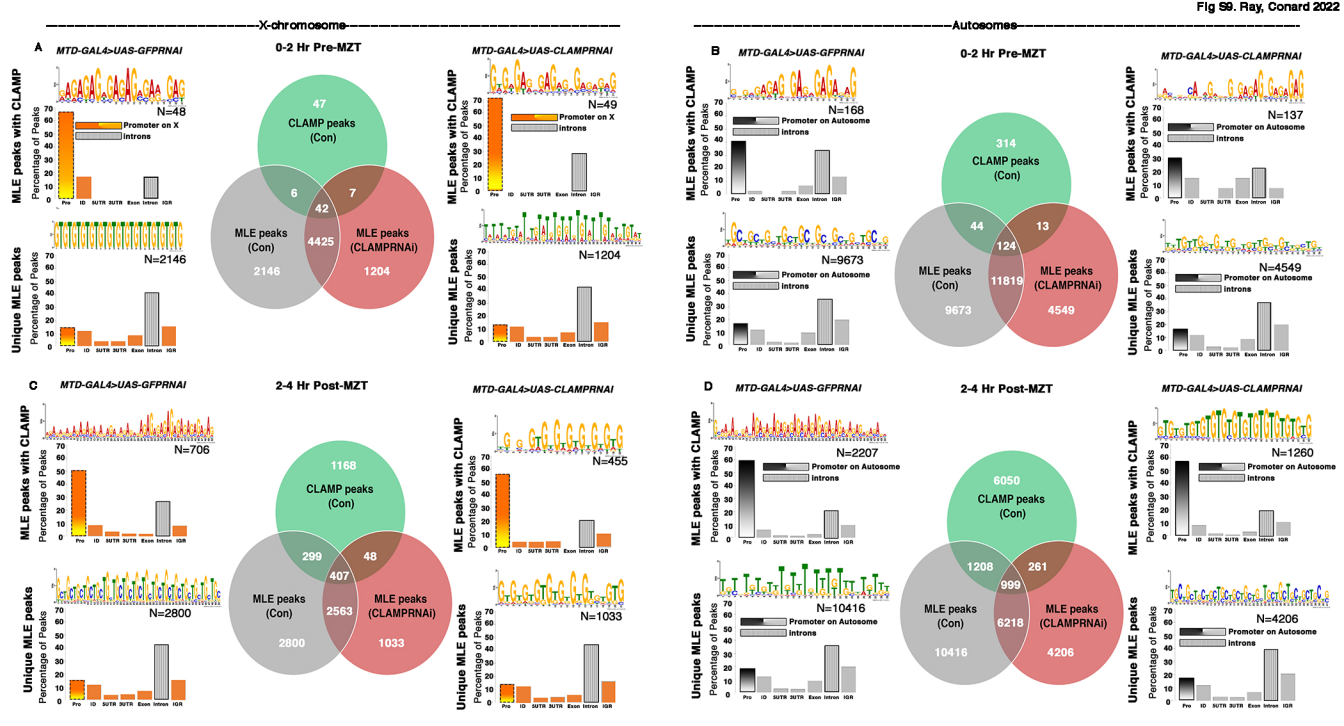
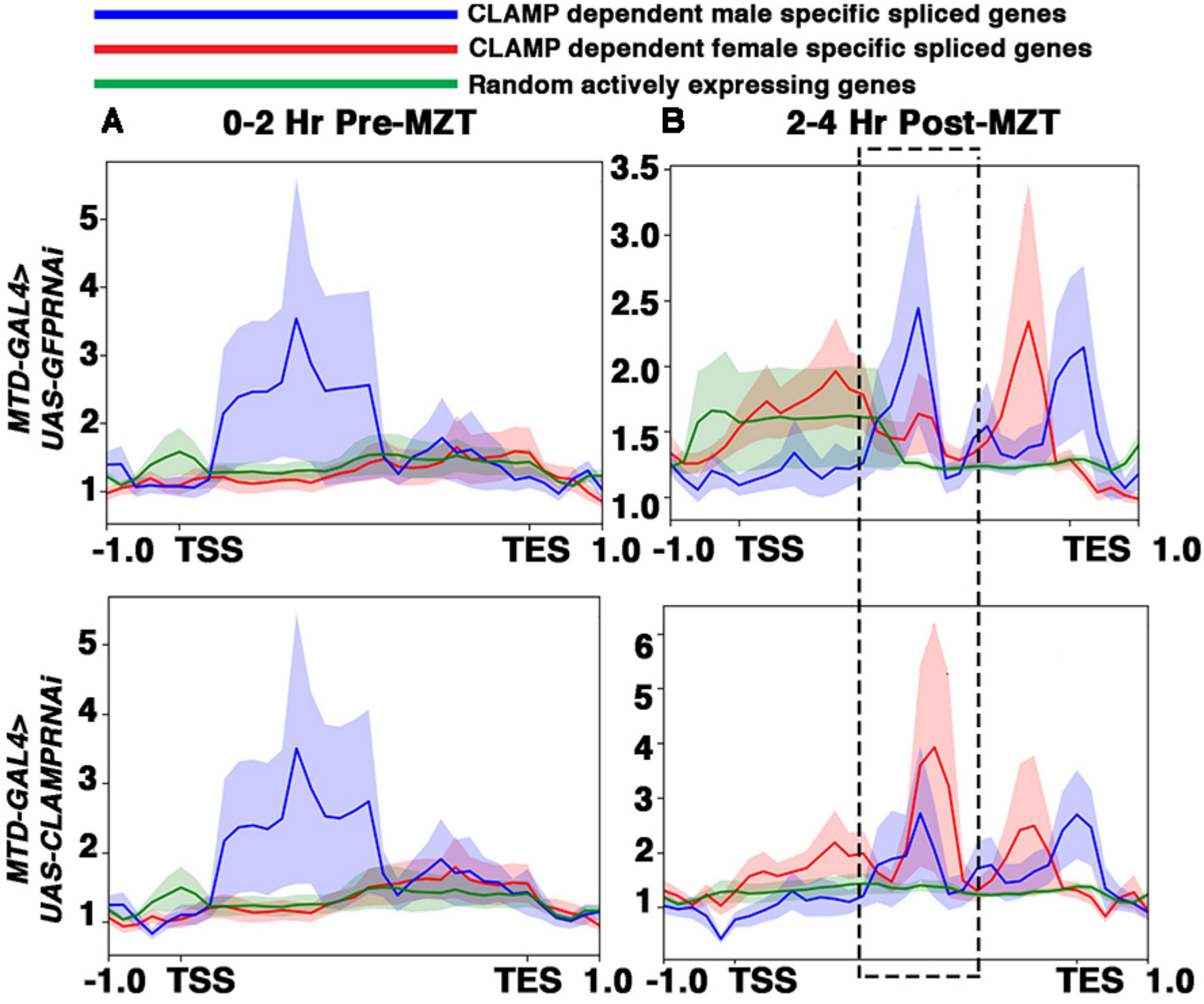
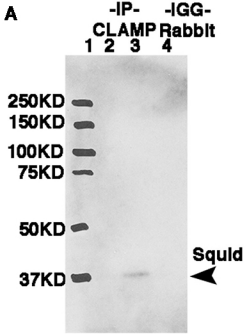
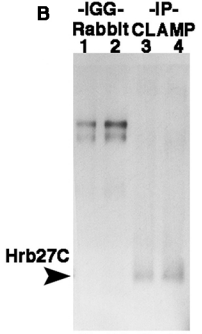
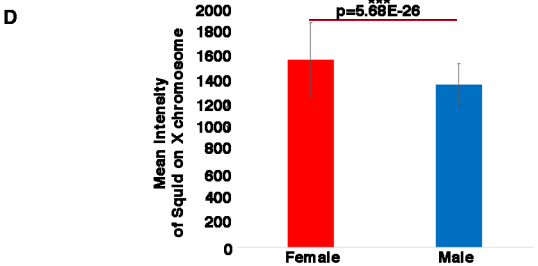

X-chromosome
Autosomes
A
B
C
D
MTD-GAL4>UAS-GFPRNAi
MTD-GAL4>UAS-CLAMPRNAi
0-2 Hr Pre-MZT
2-4 Hr Post-MZT
MLE peaks with CLAMP
Unique MLE peaks
Percentage of Peaks
Promoter on X
Promoter on Autosome
Introns
N=48
N=2146
N=49
N=1204
N=168
N=9673
N=137
N=4549
N=706
N=2800
N=455
N=1033
N=2207
N=10416
N=1260
N=4206
47
CLAMP peaks (Con)
6
42
7
MLE peaks (Con)
2146
4425
MLE peaks (CLAMPRNAi)
1204
314
CLAMP peaks (Con)
44
124
13
MLE peaks (Con)
9673
11819
MLE peaks (CLAMPRNAi)
4549
1168
CLAMP peaks (Con)
299
407
48
MLE peaks (Con)
2800
2563
MLE peaks (CLAMPRNAi)
1033
6050
CLAMP peaks (Con)
1208
999
261
MLE peaks (Con)
10416
6218
MLE peaks (CLAMPRNAi)
4206
Pro
ID
5UTR
3UTR
Exon
Intron
IGR

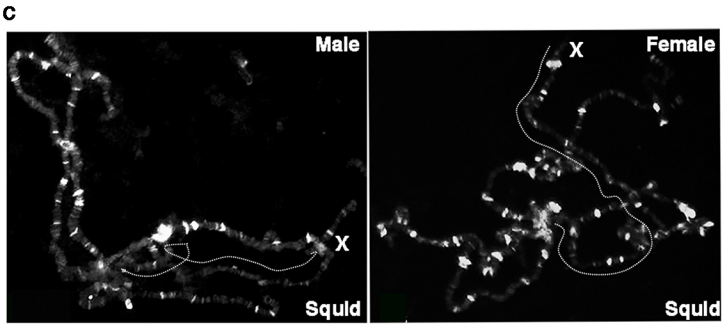
Fig S10. Ray, Conard 2022




A
-IP-
CLAMP
-IGG-
Rabbit
1 2 3 4
250KD
150KD
100KD
75KD
50KD
37KD
Squid
S2 Kc Kc
----Nuclear----
Fraction


B
-IGG-
Rabbit
-IP-
CLAMP
1 2 3 4
Hrb27C
Kc S2 Kc S2
----Nuclear----
Fraction


D
***
p=5.68E-26
Mean Intenelty
of Squid on X chromosome
2000
1800
1600
1400
1200
1000
800
600
400
200
0
Female
Male


C
Male
X
Squid
X
Female
Squid

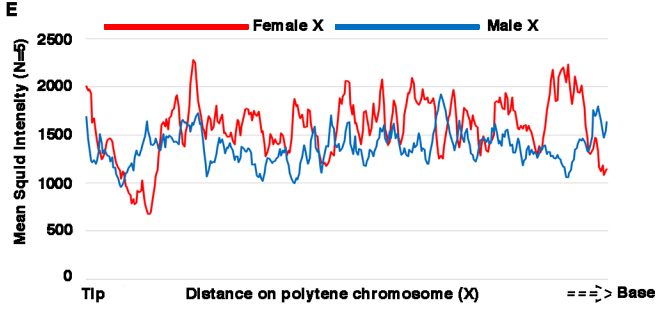
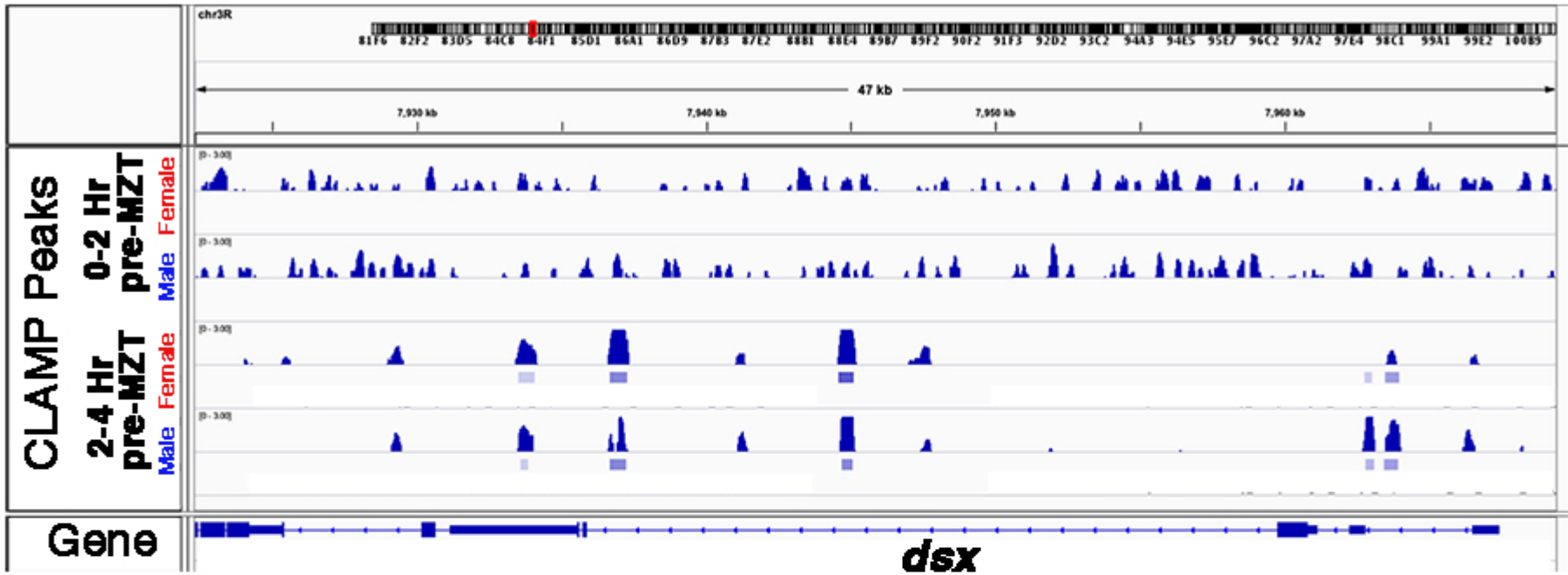
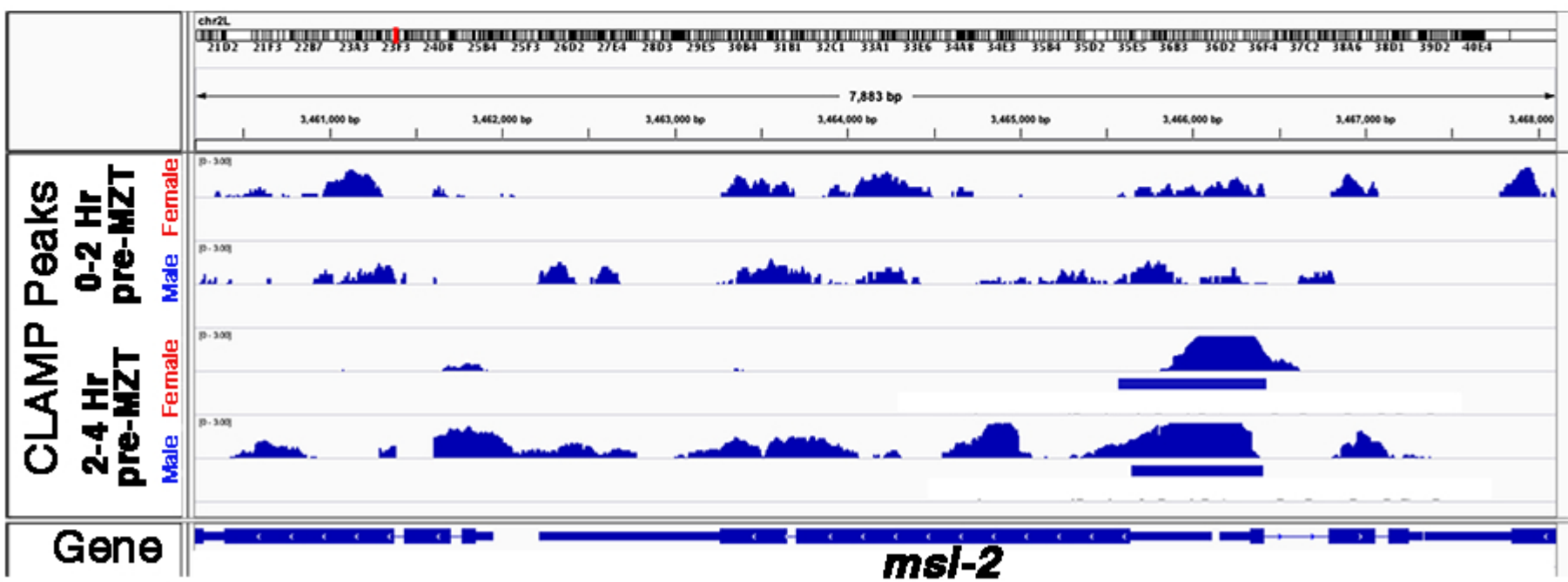

E
Female X
Male X
Mean Squid Intenelty (N=5)
2500
2000
1500
1000
500
0
Tip
Distance on polytene chromosome (X)
==> Base

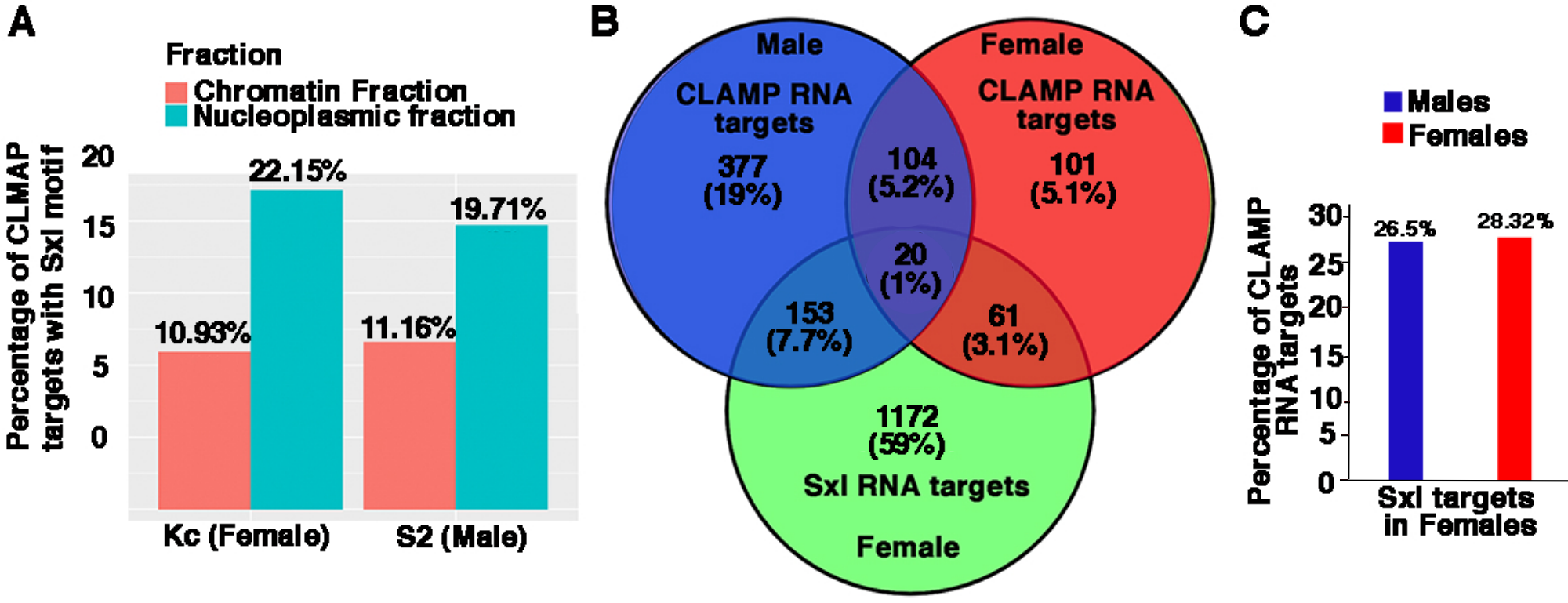
Fig S12. Ray, Conard 2022

A



B




A
Fraction
Chromatin Fraction
Nucleoplasmic fraction
Percentage of CLMAP targets with Sxl motif
20
15
10
5
0
22.15%
19.71%
10.93%
11.16%
Kc (Female)
S2 (Male)
B
Male
CLAMP RNA targets
377 (19%)
Female
CLAMP RNA targets
101 (5.1%)
104 (5.2%)
20 (1%)
153 (7.7%)
61 (3.1%)
1172 (59%)
Sxl RNA targets
Female
C
Males
Females
Percentage of CLAMP RNA targets
30
25
20
15
10
5
0
26.5%
28.32%
Sxl targets in Females