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**less** (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.

34

35 **Introduction**

36

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

47

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

58

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

63

64 During *Drosophila* embryogenesis, **Z**ygotic **G**enome **A**ctivation (**ZGA**) occurs shortly after the
65 first two hours of development. Concurrently, maternal transcripts gradually decrease in
66 abundance, and zygotic transcription increases, a process called the **M**aternal to **Z**ygotic **T**ransition
67 (**MZT**). ZGA starts approximately 80 min after egg laying and most maternal transcripts are
68 degraded by 180 min after egg laying[10]. Even at these early stages of development, AS generates
69 multiple variants of the same gene transcripts resulting in transcript diversity. Although the earliest
70 genes transcribed from the zygotic genome are mainly intron-less, almost 30% of early zygotic
71 transcripts do have introns[11,12]. Furthermore, genes involved in sex determination use AS to drive
72 male versus female-specific development [13]. Hence, during early embryonic development, AS is
73 important for shaping cell and tissue-specific transcriptomes and sexual differentiation.
74
75 The master regulator of *Drosophila* sex determination is the *sxl* gene, which undergoes alternative
76 splicing such that exon three is retained in males but not in females[14,15]. Therefore, functional Sxl
77 protein is made only in the female embryo. Early expression of the Sxl protein made from a
78 transcript driven by Sxl_{Pe}, an early *sxl* promoter[16], autoregulates splicing of *sxl*. Next, the Sxl
79 protein causes splicing of exon three from pre-mRNA of *sxl* transcripts initiated from the late
80 maintenance promoter (Sxl_{Pm}) specifically in females[17,18]. The early activation of the Sxl_{Pe}
81 promoter is regulated by a group of X-linked genes called X signal elements (XSE). A higher
82 dosage of XSE gene products drives Sxl_{Pe} expression in XX females and not XY males[19].
83
84 The Sxl protein is an RNA binding protein that binds to *sxl* pre-mRNA and the pre-mRNA of
85 downstream target genes such as *transformer* (*tra*) and *male-specific lethal protein 2* (*msl-2*) to
86 regulate their splicing and stability, giving rise to female-specific splice variants[20]. The female-
87 specific Tra isoform then regulates sex-specific splicing of the *doublesex* (*dsx*) gene resulting in
88 different Dsx protein isoforms in females and males which directly regulate sexual dimorphism in
89 both sexes[14,15].
90
91 Sxl regulation of *msl-2* RNA splicing and stability links the sex determination pathway to another
92 important early embryonic event: dosage compensation, which equalizes gene dosage of X-linked
93 genes between females with two X-chromosome and males with a single X-chromosome. MSL-2
94 is an essential core component of the MSL complex, present only in the male embryo and

95 responsible for dosage compensation[21,22]. The binding of the Sxl protein to the *msl-2* transcript
96 specifically in females results in skipping of the first exon, thereby hindering translation of the
97 protein and preventing MSL complex formation in females[23].
98
99 Once formed specifically in males, the MSL complex targets the male X-chromosome at high-
100 affinity binding sequences called **C**hromatin **E**ntry **S**ites (**CES**) that are enriched for clustered long
101 GA-repeat sequences[24,25]. The maternally deposited **C**hromatin **l**inked **a**dapter for **M**SL **p**roteins
102 (CLAMP) opens chromatin[26,27] and directly contacts MSL-2[28] to promote MSL complex
103 recruitment. Subsequently, the MSL complex increases transcript levels on the male X-
104 chromosome by promoting transcription elongation[29]. Interestingly, the MSL complex shares a
105 key highly conserved component MLE (RNA helicase A) with the spliceosome complex[30-32],
106 suggesting that there is likely to be an interplay between dosage compensation and splicing that
107 has not yet been defined.
108
109 CLAMP is an essential pioneer **T**ranscription **F**actor (**TF**) that opens chromatin[26,27]. Loss of
110 CLAMP results in complete loss of MSL complex from the male X-chromosome causing male
111 lethality[33]. Unlike MSL complex component mutants, which cause only male-specific lethality,
112 *clamp* mutants are lethal in both males and females early in embryogenesis after losing maternal
113 CLAMP[27]. Several lines of evidence lead us to hypothesize that CLAMP regulates alternative
114 splicing: 1) CLAMP is bound to both intronic and promoter regions on chromatin[34], 2) Intronic
115 regions are rich in polypyrimidine tracts, which have similar GA-rich sequences to CES and often
116 contain CLAMP binding sites[35], 3) GA-rich repeat sequences within CES are thought to have
117 evolved from polypyrimidine tracks that regulate splicing[35], and 4) MALDI-mass spectrometry
118 data identifying putative CLAMP interactors identified association with 33 RNA binding proteins,
119 including 6 that regulate alternative splicing[36]. Therefore, we hypothesized that CLAMP is a
120 maternally deposited factor that shapes transcriptome diversity in early embryos by regulating
121 RNA transcript splicing.
122
123 To date, there are no reports defining alternative splicing globally in early *Drosophila*
124 *melanogaster* sexed embryos. Moreover, how maternal TFs regulate alternative splicing in any
125 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**less** (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.
153
154
155
156

157 **Results**

158

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

160

161 To determine when during early *Drosophila melanogaster* development sex-specific splicing
162 begins, we analyzed RNA-sequencing data generated from sexed embryos at two-time points: 1)
163 0-2 Hrs (pre-MZT); 2) 2-4 Hrs (post-MZT)[9] (#GSE102922). Embryos were sexed using a meiotic
164 drive system that produces sperm with either only X or only Y chromosomes[9], resulting in progeny
165 of either only female or only male genotypes. Next, we measured the amount of AS in early
166 *Drosophila* development using a pipeline that we developed and made publicly available called
167 time2Splice (https://github.com/ashleymaeconard/time2splice). Time2Splice implements the
168 commonly used SUPPA2 algorithm to identify splice variants and provides additional modules to
169 integrate time, sex, and chromatin localization data (See **Supplemental method**) (Fig S1).
170 SUPPA2 measures the percent spliced in (PSI) for each exon to calculate the differential alternative
171 splicing between samples, represented as ΔPSI[42].

172

173 AS events can be classified into seven categories: 1) Alternative Last Exon (AL); 2) Skipping
174 Exon (SE); 3) Alternative 5' Splice Site (A5SS); 4) Alternative 3' Splice Site (A3SS); 5) Mutually
175 Exclusive Exon (MXE) 6) Retained Intron (RI) and 7) Alternative First Exon (AF) (**Fig1A**). We
176 measured the total number of splicing events that belong to each of the seven different categories
177 at the 0-2 Hr and 2-4 Hr embryonic stages in both female and male embryos. We found that 16-
178 18% of total transcripts are alternatively spliced in early embryos. **A**lternative **F**irst Exon (AF) is
179 the most common type of alternative splicing, constituting almost one-fourth of total AS. In
180 contrast, **A**lternative **L**ast Exon (AL) is the least common alternative splice type. The AS transcript
181 distribution across categories was similar between the two time points and sexes (**Fig 1B**).

182

183 We hypothesized that CLAMP regulates AS in early embryos for the following reasons: 1)
184 CLAMP is a maternally deposited pioneer factor enriched at intronic regions ; 2) Proteomic data
185 identified a physical association between spliceosome components and CLAMP[36]; 3) CLAMP
186 binding sites evolved from polypyrimidine tracts that regulate splicing[35]. We tested our hypothesis
187 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**
215
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

219 an exon-centric approach to quantify individual splice junctions by measuring **p**ercent **s**pliced **i**n
220 (**PSI**) for a particular exon using the established SUPPA algorithm within the time2splice
221 pipeline[42]. Exon inclusion is represented as positive PSI, and exclusion events are defined as
222 negative PSI (equation in **Supplementary Method**). In this way, we identified sex-specific
223 splicing events (**Fig S2A, D**) and CLAMP-dependent splicing events (**Fig S2B-C, E-F**) in female
224 and male 0-2 Hr and 2-4 Hr embryos. We did not see any statistically significant enrichment for
225 MXE within the CLAMP-dependent sex-specifically spliced events (see Supplementary **Table**
226 **S1**). Although we found substantial evidence of CLAMP-dependent sex-specific splicing, we think
227 the gene list is likely to be too small to determine significance for a particular class of splicing
228 event.
229
230 Next, we measured the percentage of total alternatively spliced and sex-specifically spliced
231 transcripts that are CLAMP-dependent in males and females at both pre- and post-MZT stages.
232 While only 2-3% of total AS is CLAMP-dependent, ~30-60% of sex-specific splicing is CLAMP-
233 dependent (**Fig 2A**). Therefore, CLAMP regulates sex-specific splicing more strongly than total
234 AS. Overall, more genes show CLAMP-dependent splicing in females (~150-250) than males
235 (~100) (**Fig 2B and Supplementary Table S1**). However, the magnitude of the effect of CLAMP
236 on splicing was stronger in males than in females (**Fig S3**). We found that before MZT, the
237 CLAMP-dependent known male sex-specifically spliced genes have more exon inclusion events
238 than their female counterparts since ΔPSI values for males is significantly higher than in females
239 (**Fig S3**). This was further supported by validating a randomly selected set of 12 sex-specific
240 splicing events we identified as described in more detail below (**Fig S4-6; Table S2**).
241
242 We then divided all CLAMP-dependent alternatively spliced events into two categories: 1) sex
243 specifically spliced (SSS) events; and 2) non-sex specifically spliced (non-SSS) events (**Fig 2B**).
244 We quantified CLAMP-dependent sex-specifically spliced events by comparing the change in PSI
245 (ΔPSI) for two classes of transcripts which we defined as follows: 1) **known** sex-specifically
246 spliced isoforms are different from each other in control samples ($p<0.05$) (**Fig. S2A, D**); 2) **new**
247 sex-specifically spliced isoforms are those not present in control samples (**Fig 2B**). We identified
248 widespread CLAMP-dependent sex-specific splicing, especially in female embryos (**Fig 2B**).
249 Interestingly, the majority of CLAMP-dependent SSS events are **new** sex-specific splicing events

250 that did not occur in the presence of maternal CLAMP (~70%) (**Fig 2C**). Furthermore, at the post-
251 MZT stage, both CLAMP-dependent exon inclusion and exclusion were significantly enriched in
252 male new SSS genes compared to their female-specific counterparts (**Fig S3**). Thus, in the absence
253 of CLAMP new aberrant sex-specific spliced isoforms are generated. Therefore, we hypothesized
254 that CLAMP normally inhibits aberrant sex-specific splicing events.
255
256 During the first few hours of their development, *Drosophila* embryos have predominantly maternal
257 transcripts. Therefore, we asked whether CLAMP-dependent female and male specifically-spliced
258 genes are maternally deposited or zygotically transcribed. We compared our list of CLAMP-
259 dependent sex-specifically spliced genes with known maternally expressed genes from previous
260 studies [45,46]. We found very low levels of overlap with maternally deposited transcripts (**Fig 2D**).
261 Therefore, most of the sex-specifically spliced genes we observed are likely to be zygotic
262 transcripts, consistent with the function of CLAMP as a pioneer TF in the early embryo [27].
263
264 Next, we divided our CLAMP-dependent sex-specifically spliced genes into female-specific or
265 male-specific categories. Here, it is important to note that many of these genes have multiple AS
266 events, and thus multiple isoforms regulated by CLAMP (**Table S1**). Then, we performed Gene
267 Ontology (GO) analysis to determine which biological processes are enriched within CLAMP-
268 dependent sex-specifically spliced genes at pre- and post-MZT time points using time2splice. At
269 the pre-MZT time point, female-specifically spliced genes are primarily mRNA regulatory factors
270 such as spliceosome components and TFs (**Fig 2E**). Therefore, in females CLAMP alters the
271 splicing of genes that can regulate the transcription and splicing of other genes to amplify its
272 regulatory role. In contrast, the male specifically-spliced genes pre-MZT are not enriched for any
273 specific biological function or process likely due to the small number of genes in the gene list. At
274 the post-MZT stage in both sexes, CLAMP regulates the splicing of genes that drive development
275 including organogenesis, morphogenesis, cell proliferation, signaling, and neurogenesis (**Fig 2E**).
276
277 We also selected 12 random genes for validation that we identified computationally as having
278 CLAMP-dependent splicing events of diverse types such as alternative 5' splice site (A5) or
279 alternative first exon (AF) (**Fig S4-5**) using qRT-PCR or RT-PCR. These genes perform diverse
280 biological functions and provide new insights into how CLAMP regulates early development (**Fig.**

281 **2E**). We have summarized the results and functions of the validated target genes at which splicing
282 is regulated by CLAMP (**Table S2**). Several of the target genes have functional links to CLAMP
283 including *iab4*, *psq,* and *pep* suggesting that we have identified relevant target genes[36,47,48].
284 Furthermore, many genes that are sex-specifically spliced by CLAMP are themselves involved in
285 splicing and chromatin regulation (**Table S2**).
286
287 **3. CLAMP is highly enriched along gene bodies of sex-specifically spliced genes after MZT**
288
289 Because maternal CLAMP regulates sex-specific alternative splicing, we next asked: How does
290 CLAMP regulate female and male sex-specific splicing? If CLAMP directly regulates sex-specific
291 splicing, we hypothesized that it would directly bind to the genes that it regulates. Therefore, we
292 defined the binding pattern of CLAMP at the CLAMP-dependent female and male sex-specifically
293 spliced genes in sexed embryos using CLAMP ChIP-seq data (#GSE133637).
294
295 We generated average profiles for CLAMP occupancy at genes showing CLAMP-dependent
296 splicing in females and males at pre- and post-MZT time points in males and females (**Fig 3A,**
297 **B**). We found that CLAMP occupies the gene bodies of many sex-specifically spliced genes that
298 require CLAMP for their splicing. Specifically, 43.8% percent of all CLAMP-dependent sex-
299 specifically spliced genes are bound by CLAMP: 21.9% in 0-2 Hr female embryos, 8.2% in 0-2
300 Hr male embryos, 65.2% in 2-4 hr female embryos and 59.43% in 2-4 hr male embryos (**Table**
301 **S3**). The increase in percentage of genes bound in 2-4 Hr embryos compared with 0-2 Hr embryos
302 is consistent with an increased number of CLAMP binding sites at the later time point[48]. Overall,
303 these data are consistent with a direct role for CLAMP in regulating splicing of sex-specifically
304 spliced genes.
305
306 Next, we compared the average CLAMP binding pattern at sex-specifically spliced genes to the
307 CLAMP binding pattern at genes whose expression but not splicing is both sex-biased and
308 dependent on CLAMP. We defined CLAMP-dependent sex-biased genes as differentially
309 expressed genes in males in females that are dependent on CLAMP. At sex-specifically spliced
310 genes, CLAMP occupancy is present over gene bodies which is a dramatically different binding
311 profile from genes that are expressed but not spliced in a CLAMP-dependent and sex-biased

312 manner where CLAMP is enriched at the TSS and TES instead of at gene bodies (**Fig 3C and D**,
313 rectangular box in **Fig 3A-D**). Furthermore, CLAMP binding is also modestly enriched at the TSS
314 of female-biased expressed genes in females, consistent with enhanced CLAMP occupancy at the
315 TSS of expressed genes [33]. As a control, we used a random set of active genes that are not regulated
316 by CLAMP (green lines in **Fig 3A-D**) and we observed lower occupancy than at CLAMP-
317 dependent genes. Overall, we found preferential binding of CLAMP along the gene bodies of
318 genes that have CLAMP-dependent splicing in both females and males in contrast to TSS and TES
319 binding at genes where expression requires CLAMP.
320
321 To determine whether the binding of CLAMP to gene bodies occurs close to splice junctions, we
322 measured the distance of CLAMP peaks from the nearest splice junction (**Fig S7**). We found that
323 CLAMP peaks are most frequently within 200-400bp of either the start or the end of a splice
324 junction, especially in sex-specifically spliced genes. The resolution of these measurements is also
325 limited by sonication and therefore it is possible that binding occurs even closer to splice junctions.
326 We also found that CLAMP binds to chromatin closer to splice junctions at sex-specifically spliced
327 genes compared to sex-biased genes in 2-4 hr female embryo samples that have the most target
328 genes and CLAMP binding events. The results were similar for all CLAMP peaks (**Fig S7C**)
329 compared to peaks only present in introns (**Fig S7G**). We did not observe a difference in proximity
330 of CLAMP peaks to the nearest splice junction between sex-specifically spliced genes and sex-
331 biased genes in other samples but this negative result is not conclusive due to the decreased number
332 of peaks and target genes in the other samples. Together these data support a direct role for
333 CLAMP in co-transcriptional RNA processing that we hypothesize is due to direct contact with
334 target RNA transcripts and altering the recruitment of spliceosome components.
335
336 **4. CLAMP binds to RNA on chromatin of many sex-specifically spliced genes and is a**
337 **component of the mature spliceosome complex specifically in males**
338
339 To test our hypothesis that CLAMP directly regulates co-transcriptional RNA splicing by
340 contacting both the DNA and RNA of sex-specifically spliced genes and altering spliceosome
341 recruitment, we first asked whether to CLAMP directly binds to RNA. Although CLAMP do not
342 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

374 and females. To test this hypothesis, we first examined how CLAMP regulates the occupancy of
375 the MLE RNA helicase, a component of both the MSL complex and the spliceosome[30,32,52-54] for
376 two reasons: 1) MLE is a component of both the MSL complex which is present only in males
377 (ref) and the spliceosome in both sexes[54] and 2) CLAMP physically associates with MLE[28,55,56].
378 Therefore, we hypothesized that CLAMP regulates the distribution of MLE between the
379 spliceosome and MSL complex to modulate sex-specific alternative splicing in males.
380
381 To determine whether CLAMP modulates MLE distribution on chromatin, we measured MLE
382 distribution at the genomic level using CUT&RUN[57,58]. We performed CUT&RUN in the presence
383 (*MTD-GAL4>UAS-GFPRNAi*) and absence (*MTD-GAL4>UAS-CLAMP [val22] RNAi*) of
384 maternal CLAMP at the pre-MZT and post-MZT embryonic stages in males. Next, we identified
385 MLE peaks in the presence or absence of maternally deposited CLAMP. MLE binds to chromatin
386 both in males and females, with stronger binding in males (**Fig 4A**). The absence of CLAMP
387 results in loss of MLE male peaks in males compared to no change in female MLE peaks (**Fig 4A**).
388 This supports our hypothesis that CLAMP regulates MLE recruitment to chromatin in males.
389
390 We next compared the distribution and location of MLE peaks with that of CLAMP peaks
391 previously identified in control embryos at the same time points[21] and classified MLE peaks into
392 two groups: 1) MLE peaks overlapping with CLAMP peaks (**Fig 4B,C and Fig S9**) and 2) unique
393 MLE peaks which do not overlap with CLAMP peaks (**Fig 4B,C and Fig S9**) at both 0-2 Hr pre-
394 MZT and 2-4 Hr post-MZT stages. Also, independent of developmental stage, MLE peaks which
395 overlap with CLAMP peaks are largely at promoters with fewer peaks localizing to introns. In
396 contrast, unique MLE peaks that do not overlap with CLAMP peaks are primarily localized to
397 introns (**Fig S9**). This distribution is similar for X-chromosomal and autosomal peaks.
398
399 In the absence of CLAMP, there is in a considerable loss and redistribution of both overlapping
400 and non-overlapping MLE peaks. We found that overall ~60% of MLE peaks were lost at the 2-4
401 Hr post-MZT stage in the absence of CLAMP. Moreover, 26% (pre-MZT) and ~35% (post-MZT)
402 of the MLE peaks observed in the absence of CLAMP were new and not present in control embryos
403 (**Fig 4B-C**). After the loss of maternal CLAMP, ~23% of MLE peaks overlapping with CLAMP
404 are also lost at the pre-MZT stage, which increases to ~51% at the post-MZT stage (**Fig 4B-C**).

405 Overall, our data suggest that MLE is redistributed in the absence of CLAMP suggesting that
406 CLAMP normally prevents aberrant recruitment of MLE in addition to the formation of aberrant
407 splice isoforms (**Fig 2**). Furthermore, we hypothesize that MLE at the new peaks is part of the
408 spliceosome complex and not MSL complex because MSL complex is not present on chromatin
409 in the absence of CLAMP[33].
410
411 To provide insight into the differences between MLE peaks which overlap with CLAMP and those
412 which do not, we identified sequence motifs which are enriched within each class of peaks using
413 MEME within the time2splice pipeline. The known CLAMP motif [33], a stretch of (GA)n repeats,
414 is enriched at regions that are bound by both MLE and CLAMP independent of stage and
415 chromosome type as expected. In contrast, MLE peaks which do not overlap with CLAMP have
416 motifs with stretches of GTs, GCTs, and GTAs but not (GA)n repeats (**Fig S9**). In the absence of
417 CLAMP, the remaining MLE peaks (red circle) were most enriched for (GT)n motifs (**Fig S9C,**
418 **D**) which have known roles in splicing through forming secondary RNA structures[59-61]. Therefore,
419 CLAMP prevents MLE from redistributing to sequence motifs that are known regulators of
420 splicing.
421
422 We also found that CLAMP alters the distribution of MLE from introns to promoters. MLE peaks
423 that overlap with CLAMP (intersection between green circle with red and grey circles, **Fig S9**) on
424 the X-chromosome (**Fig S9A, C**) or autosomes (**Fig S9B, D)** are enriched at promoters (**Fig S9A,**
425 **C**(X-chromosome), **Fig S9B, D** (Autosomes). In contrast, **new** unique MLE peaks not overlapping
426 with CLAMP (grey area in Venn diagrams, **Fig S9**) and those that are gained after CLAMP RNAi
427 (red area in Venn diagrams, **Fig S9**) are enriched at introns (**Fig S9A, C** (X-chromosome), **Fig**
428 **S9B, D** (Autosomes). These results support a role for CLAMP in sequestering MLE at (GA)n rich
429 sequences within promoters that prevents it from binding to GT motifs within introns that are
430 known regulators of splicing[59-61]. Thus, in the absence of CLAMP, MLE is redistributed and
431 aberrantly binds to intronic sequences with known motifs that regulate splicing irrespective of
432 whether present on X-chromosome or autosomes.
433
434 To determine how MLE redistribution could alter sex-specific splicing, we plotted the distribution
435 of MLE binding on CLAMP-dependent female and male-specifically spliced genes in the presence

436 and absence of CLAMP (**Fig S10A, B**). Pre-MZT, MLE binds near the TSS of male-specifically
437 spliced genes independent of maternal CLAMP (**Fig S9A**). At the post-MZT stage, loss of
438 maternal CLAMP in male embryos causes MLE to change its binding distribution along the gene
439 body (rectangle with dotted lines: **Fig S10B**) of CLAMP-dependent male-specifically spliced
440 genes (blue line) relative to CLAMP-dependent female-specifically spliced genes (red line). These
441 profiles are consistent with a model in which CLAMP is essential for MLE distribution at male-
442 specifically spliced genes which regulates male sex-specific splicing. In males, increased MLE
443 binding at female sex-specifically spliced genes (red line, enclosed within rectangle with dotted
444 lines: **Fig S10B**) in absence of CLAMP may result in differential splicing of these genes. Thus, we
445 hypothesize that CLAMP inhibits mis-localization of MLE to female sex-specifically spliced
446 genes in males.
447
448 Next, we asked whether CLAMP associates with spliceosome complex protein components other
449 than MLE, which is also a component of the MSL complex. We have shown that CLAMP directly
450 binds to snRNAs (Fig. S) and previously reported that CLAMP physically associates with several
451 spliceosome complex components based on mass spectrometry analysis[36]. To validate these
452 associations, we performed co-immunoprecipitation (coIP) experiments to assess association
453 between CLAMP and two spliceosome components with known functions in sex-specific splicing,
454 the conserved hrb27C and Squid proteins [54,62,63]. We found that in both S2 (male) and Kc (female)
455 cells, CLAMP interacts with hrb27C (**Fig S11A, B**). In contrast, CLAMP only associates with
456 Squid in female Kc cells and not in male S2 cells (**Fig S11A, B**), consistent with mass spectrometry
457 data. In contrast to MLE and CLAMP which are enriched on the male X-chromosome, Squid
458 occupancy on polytene chromosomes is decreased on the male X chromosome compared with the
459 female X chromosome (**Fig S11C-E**). Therefore, it is possible that there is a competition between
460 CLAMP recruitment of MSL complex to the male X chromosome and CLAMP recruitment of the
461 spliceosome complex containing Squid that contributes to sex-specific splicing.
462
463 Overall, CLAMP differentially associates with spliceosome components in males and females,
464 providing a potential mechanism by which CLAMP can regulate sex-specific splicing.
465 Specifically, it is possible that CLAMP influences the distribution of spliceosome components like
466 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.

470 **5. CLAMP regulates the chromatin accessibility and splicing of the *sxl* gene and directly**
471 **interacts with the *sxl* RNA and alters splicing of other sex determination pathway component**
472 **genes**
473

474 In *Drosophila***,** sex-specific alternative splicing is regulated by the sex-determination pathway.
475 Sex-lethal (Sxl) is the master regulator of sex determination[15] and drives subsequent sex-specific
476 splicing in females[64]. Therefore, we asked whether CLAMP regulates *sxl* gene splicing and Sxl
477 protein levels in addition to directly regulating sex-specific splicing by binding to the DNA and
478 RNA of target genes as we have demonstrated (**Fig 3, S8 and Table S3, S5**).
479

480 Functional Sxl protein is produced specifically in females because exon three in the *sxl* transcript
481 contains a premature stop codon which is spliced out in females but retained in males [14]. Therefore,
482 the Sxl protein is non-functional in males and becomes degraded[15,65]. In females, Sxl protein
483 inhibits the splicing of the *msl-2* transcript which prevents aberrant MSL-2 expression and MSL
484 complex formation in females (**Fig 5A**). Furthermore, Sxl promotes female specific-splicing of the
485 downstream effector called transformer (*tra*). The female-specific Transformer protein regulates
486 splicing of another downstream transcript doublesex (*dsx*), which is sex-specifically spliced. In
487 contrast, the male-specific *transformer* transcript is not translated into functional Transformer
488 protein in males. Therefore, in the absence of any functional male-specific Transformer protein,
489 the *doublesex* transcript undergoes male-specific splicing (**Fig 5A**). The sex-specific isoforms of
490 the Doublesex protein drive sex-specific splicing of many downstream genes, shaping sexual
491 variation between males and females [66,67].
492

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

502 small but detectable amount of the longer male-specific *sxl* transcript (**Fig 5B**, lane c). This mis-
503 regulation of *sxl* splicing is rescued by our *CLAMP* containing rescue construct (**Fig 5B**, lane d).
504 Our iCLIP data shows that CLAMP directly binds to *sxl* transcripts in females and not males
505 (**Table S5**) emphasizing CLAMP's direct role in *sxl* splicing in females.
506
507 To test whether defects in splicing altered Sxl protein levels, we performed western blots to
508 quantify Sxl protein in wild type females and males and *clamp*^{2}null females (**Fig 5C**). We
509 observed a reduction in Sxl protein levels in females in the *clamp*^{2} null background when compared
510 with controls. Also, homozygous *clamp*^{2} mutant males die before the third instar larval stage, and
511 therefore it was not possible to measure the splicing of transcripts in male *clamp*^{2} mutant larvae.
512 Overall, we determined that CLAMP promotes female-specific splicing of the *sxl* transcript to
513 ensure that normal Sxl protein levels are produced.
514
515 To determine a possible mechanism by which CLAMP regulates splicing of *sxl*, we mined our
516 previously generated Micrococcal Nuclease sequencing data[26], which measures chromatin
517 accessibility in S2(male) and Kc(female) cells in the presence and absence of CLAMP. Regions
518 of the genome that are accessible have a positive MACC score and are shown in blue and regions
519 of the genome that are inaccessible have a negative score and are shown in red (range is between
520 -0.33(red) and +1.33 (blue). We found that after the loss of CLAMP in female Kc cells, chromatin
521 accessibility at exon 3 of *sxl* increases significantly (**Fig 5D**). As shown in the Fig 5D boxed
522 rectangular inset, *sxl* exon3 shows a strong and statistically significant peak[26] indicative of open
523 chromatin specifically in females in which CLAMP has been depleted compared to control
524 females. Therefore, CLAMP normally promotes a closed chromatin environment at exon 3 in
525 females but not males.
526
527 Our results suggest that increased chromatin accessibility in males compared to females results in
528 retention of exon3 in the male *sxl* transcript. Consistent with our results, recent reports provide
529 strong evidence that increased chromatin accessibility contributes substantially to the retention of
530 introns during AS[71]. In addition, splicing-associated chromatin signatures have recently been
531 identified[72]. Consistent with our results in males, open chromatin marks such as H3K4me1&
532 H3K4me2 are enriched just upstream of the start site of retained exons[72]. In contrast, histone marks

533 associated with condensed chromatin such as H4K20me1&2, H3K9me3, and H3K27me3 are
534 highly enriched at excluded exons[72], consistent with our results in females.
535
536 Next, we examined the splicing of other components of the sex determination pathway for defects
537 in alternative splicing in the absence of CLAMP for which we were able to design effective RT-
538 PCR primers (**Fig 5E-F**). In embryos which lack CLAMP (**Fig 5E**, lane 2), the *dsx* female-specific
539 transcript is aberrantly produced in males (**Fig 5E**, lanes 4 and 5). In contrast, the male-specific
540 *dsx* transcript is not expressed in male embryos which lack CLAMP, similar to wild type female
541 embryos (**Fig 5E**, lane7-10). We also observed male-specific *dsx* transcripts in female *clamp*^{2}
542 mutant larvae (**Fig 5E**, lane c). Therefore, *dsx* splicing is regulated by CLAMP and CLAMP binds
543 to the *dsx* gene (**Fig S11**). However, *dsx* splicing is also regulated by Sxl whose protein levels are
544 regulated by CLAMP (**Fig 5C**).
545
546
547 Furthermore, we found that CLAMP regulates splicing of the male-specific lethal-2 (*msl-2*)
548 transcript, which is present only in males because Sxl regulates its splicing and stability. In the
549 *clamp*^{2} mutants: 1) levels of the nonfunctional female *msl-2* isoform (**Fig 5F**, lane 5) increased in
550 male embryos (**Fig 5F**, lane 2); 2) levels of the functional male-specific *msl-2* isoform increased
551 in females (**Fig 5F**, lane c). To determine whether these splicing defects also cause dysregulation
552 of MSL-2 protein expression and localization, we performed polytene immunostaining from
553 female *clamp*^{2} mutant salivary glands. In the absence of CLAMP, ectopic MSL2 protein (in red) is
554 present at several locations on female chromatin compared with controls (*clamp*^{2}*/CyOGFP*
555 heterozygous females) where MSL-2 protein is not present on chromatin (**Fig 5G**). Similar to *dsx*,
556 the gene is also bound by CLAMP (**Fig S12**) and regulated by Sxl and therefore could be regulated
557 through both direct and indirect mechanisms. Since CLAMP does not binds to the *dsx* or *msl-2*
558 transcripts directly it is possible that it indirectly regulates *dsx* and *msl-2* splicing through Sxl.
559 CLAMP does binds to the RNA that encodes another sex-specific splicing regulator and Sxl target
560 gene *fru* (Fruitless) in both females and males (**Table S5**). Together, these data reveal that CLAMP
561 regulates the splicing and protein expression of multiple components of the sex determination
562 pathway.
563

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

688 of sexual dimorphism[15]. Also, CLAMP directly binds to the DNA of *sxl, dsx,* and *msl-2* target
689 genes. Furthermore, CLAMP binds to the DNA and RNA of *sxl* and *fru*. Fruitless (*fru*) encodes a
690 BTB zinc finger transcription factor that contributes to sexual differentiation of the neural circuits
691 [82,83] and many of the CLAMP dependent sex-specifically spliced genes belong to neural
692 development pathway (**Fig 2E**). Since CLAMP and Sxl have both overlapping and distinct targets,
693 we hypothesize that CLAMP regulates sex-specific splicing both via the Sxl-mediated sex-
694 determination pathway as well as independent of it.
695
696
697 Therefore, CLAMP may regulate splicing through at least two possible mechanisms that are not
698 mutually exclusive: 1) CLAMP directly regulates the splicing of many sex-specifically spliced
699 genes by linking the RNA to the DNA and altering the recruitment of the spliceosome; 2) CLAMP
700 regulates the sex-specific of genes such as *msl-2* and *dsx* indirectly by regulating Sxl which is a
701 known regulator of their splicing and thus downstream splicing events.
702
703
704 Overall, we hypothesize that both different composition of the spliceosome and differential
705 recruitment to chromatin drive sex-specific changes in splicing. We identify CLAMP as a maternal
706 factor that regulates sex-specific alternative splicing through its sex-biased association with the
707 DNA and RNA of target genes and recruitment of spliceosome components and its ability to
708 influence the sex determination pathway. Identifying the factors that regulate this sex-biased
709 association of CLAMP with spliceosome complex components will be a key future direction.
710
711 Here, we show for the first time that a maternal factor controls sex-specific splicing during early
712 embryonic development, highlighting how the maternal environment influences transcript
713 diversity in the zygote from activation of the zygotic genome to the processing of zygotic RNA
714 products. Consistent with recent literature linking chromatin accessibility and splicing, our results
715 suggest that CLAMP could be one example of a more general splicing regulatory mechanism
716 controlled by the interaction between pioneer TFs that alter chromatin accessibility and
717 components of the RNA processing machinery to generate spatial-temporal transcript diversity.
718 While we analyzed sex-specific transcriptome diversity in this study and linked it to the sex-

719 specific dosage compensation process, similar mechanisms could drive cell-type specific variation.
720 For example, cell fate-determining transcription factors could regulate the chromatin occupancy
721 of splicing complex components to promote the formation of cell-type-specific isoforms. We also
722 present time2splice, a new pipeline to uncover mechanisms which drive such spatial-temporal
723 transcript diversity by integrating splicing and chromatin occupancy data.
724
725 **Materials and Methods:**
726
727 **Fly strains and rearing**
728 *Drosophila melanogaster* fly stocks were maintained at 24°C on standard corn flour sucrose
729 media. Fly strains used: *MTD-GAL4* (Bloomington, #31777), *UAS-CLAMPRNAi[val22]*
730 (Bloomington, #57008), Meiotic drive fly stocks +; SD72/CyO and 19-3, yw, Rsp[s]-
731 B[s]/Dp(2:y)CB25-4, y+, Rsp[s]B[s]; SPSD/CyO (Bloomington, #64332) (both gifts from
732 Cynthia Staber). These were crossed to obtained male and female embryo of desired genotypes
733 according to Rieder et al 2017.
734
735 **Sample collection and Western blotting**
736 Salivary glands from third instar larvae were dissected in cold PBS and samples frozen in liquid
737 nitrogen. Total protein from the samples was extracted by homogenizing tissue in the lysis buffer
738 (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% SDS, 0.5X protease inhibitor) using a small pestle.
739 After a five-minute incubation at room temperature, cleared the samples by centrifuging at room
740 temperature for 10 minutes at 14,000xg. To blot for CLAMP and Actin, 5 micrograms of total
741 protein was run on a Novex 10% Tris-Glycine precast gel (Life technologies). To measure Sex-
742 lethal protein levels, 20 micrograms of total protein was run on a Novex 12% Tris-Glycine precast
743 gel (Life technologies). Protein was transferred to PVDF membranes using the iBlot transfer
744 system (ThermoFisher Scientific) and probed the membranes for CLAMP (1:1000, SDIX), Actin
745 (1:400,000, Millipore), and SXL (1:500, a gift from Fatima Gebauer) antibodies using the Western
746 Breeze kit following the manufacturer's protocol (ThermoFisher Scientific). We quantified the
747 relative expression of protein for SXL using the gel analysis tool in ImageJ software following the
748 website's guidelines [84]. For each genotype, we first internally normalized the amount of SXL

749 protein to Actin. Next, we determined the protein's relative expression by comparing the Actin
750 normalized quantities to y[1], w[1118] female samples.
751
752 **Polytene chromosome squashes and immunostaining**
753 Polytene chromosome squashes were prepared as previously described in Reider et al. 2017. We
754 stained polytene chromosomes with rabbit anti-CLAMP (1:1000, SDIX), mouse anti-Squid (1:50,
755 1B11, DSHB), rabbit anti-MLE (1:1000, gift from Mitzi Kuroda), rat anti-MSL2 (1:500, gift from
756 Peter Becker) antibodies. For detection, we used all Alexa Fluor secondary antibodies against
757 rabbit and mouse at a concentration of 1:1000 and visualized slides at 40X on a Zeiss Axioimager
758 M1 Epifluorescence upright microscope with the AxioVision version 4.8.2 software.
759
760 **Splicing assays for male and female-specific transcripts**
761 To test for the male and female splice forms of *sex-lethal, transformer, doublesex*, and *msl2*, total
762 RNA was extracted from ten third instar larvae from each genotype. We reverse-transcribed two
763 micrograms of total RNA using the SuperScript VILO cDNA Synthesis Kit (Life Technologies)
764 following the manufacturer's protocol. We amplified target sequences by PCR using primers
765 designed to span Alternatively spliced junctions. Alternative splicing primer sequences for sxl FP-
766 TGCAACTCACCTCATCATCC, sxl RP- GATGGCAGAGAATGGGACAT, for tra FP-
767 TGAAAATGGATGCCGACAG, tra RP- CTCTTTGGCGCAATCTTCTC, for dsx female
768 transcript dsxFFP-CTATCCTTGGGAGCTGATGC, dsxF RP-
769 TCGGGGCAAAGTAGTATTCG, for dsx male transcript dsxM FP-
770 CAGACGCCAACATTGAAGAG, dsxM RP- CTGGAGTCGGTGGACAAATC, for msl2 FP-
771 GTCACACTGGCTTCGCTCAG and msl2 RP- CCTGGGCTAGTTACCTGCAA were used.
772
773 **Immunoprecipitation**
774 *Nuclear extract preparation:* Male (S2) and female (Kc) cells were grown to a cell concentration
775 of 2X 10^6 cells/mL in T25 tissue culture flasks. Cells were scraped from the flask, centrifuged for
776 5min at 2500rpm at 4°C. Supernatant was removed and cell pellets were washed twice in 5ml of
777 cold PBS. The washed cell pellets were then resuspended in 5X volume of Buffer A (10mM
778 HEPES pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5mMDTT, 1X Protease inhibitors). Cells were
779 incubated on ice for 15 minutes before dounce homogenization with an A pestle. Cytoplasmic

780 fraction was collected after centrifugation at 4°C for 20 min at 700xg. The remaining nuclear pellet
781 was re-suspended in 3 times volume in Buffer B (20mM HEPES pH7.9, 20%Glycerol, 0.5%NP
782 40, 200mMKCl, 0.5mMEDTA, 1mMEGTA, 1X protease inhibitors). Nuclei after re-suspension
783 were dounce homogenized with a B pestle. Nuclear debris was then pelleted by centrifugation at
784 10,000xg for 10 min at 4°C. 1ml aliquots of both cytoplasmic and nuclear fractions were prepared
785 in 1.5mL Protein LoBind Eppendorf tubes and flash frozen in liquid nitrogen for storage at -80 °C.
786
787 *Immunoprecipitation:* Magnetic anti-CLAMP beads were prepared to a final concentration of
788 10mg/mL by coupling rabbit anti-CLAMP antibody (SDIX) to magnetic beads, according to
789 Dynabeads Antibody coupling kit (ThermoFisher Scientific) instructions. Both prepared anti-
790 CLAMP and purchased anti-IgG (anti-rabbit IgG M-280 Dynabeads) were blocked to reduce
791 background the night before the immunoprecipitation. First, the beads were washed 3 times for 5
792 minutes in 500L Tris-NaCl Wash (50mM Tris, 500mM NaCl, 0.1% NP-40) by rotating at 4C. The
793 beads were next suspended in block buffer (3.3mg/mL of yeast tRNA extract prepared in 20mM
794 HEPES, pH7.9, 20% Glycerol, 0.5% NP-40, 200mM KCl, 1mM EDTA, and 2mM EGTA) and
795 rotated overnight at 4C. The next day, beads were washed 3 times for 5 minutes in the block buffer
796 without yeast tRNA by rotating at 4°C. After the final wash, beads were resuspended in the same
797 amount of block buffer as the starting volume.
798
799 To 1mL of previously prepared nuclear extract, 100uL of blocked anti-CLAMP or anti-IgG
800 magnetic Dynabeads were added. The nuclear extracts and beads were then rotated for 1 hour at
801 4°C. Afterward, the beads were collected and the supernatant discarded. The beads were then
802 washed three times in Tris-NaCl wash (50mM Tris, 500mM NaCl, 0.1% NP-40) by rotating for 5
803 minutes at 4°C and cleared by using a magnetic rack. To elute proteins from the beads, 100uL of
804 1% SDS was added, and the beads were boiled for 10 minutes at 95C. To the eluate, 300uL of
805 ultrapure water was added, and the tubes gently vortexed. After collecting the beads on a magnetic
806 rack, the eluate was saved in a clean Protein LoBind Eppendorf tube.
807
808 *Western blotting:* Squid and Hrb27C were detected in IP-CLAMP and IGG-rabbit protein samples
809 using mouse anti-Squid (1:500, 1B11, DSHB) and rabbit anti-Hrb27C (1:5000, Fatima Gebauer),
810 performed as mentioned above under western blotting protocol.

811

812 **CUT&RUN**

813 0-2 hr and 2-4 hr male and female embryos of desired genotypes (~50 each) were collected on
814 standard grape juice agar medium and washed with water. The embryos were dechorionated in 6%
815 bleaching solution for 2 min and washed twice in ice cold 1XPBS. Centrifuged at 12,000g for 10
816 min at 4°C. Supernatants were discarded and embryos resuspended in 200µl Dig-Wash buffer with
817 EDTA (20mM HEPES-NaOH, 150mM NaCl, 2mM EDTA, 0.5mM Spermidine, 10mM PMSF,
818 0.05% digitonin) and washed twice. Embryos were incubated in 200µl primary antibody overnight
819 at 4°C on a tube rotator. Next, embryos were centrifuged at 12,000g for 10 min at 4°C and liquid
820 removed and embryos were washed twice in Dig-Wash buffer with EDTA. Then, embryos were
821 incubated for 3 hours at 4°C in ~700 ng/ml pAMNase solution in Dig-Wash buffer with EDTA.
822 Embryos were washed twice in Dig-Wash buffer without EDTA and resuspended in 150µl of Dig-
823 Wash buffer without EDTA. Samples were equilibrated to 0°C on a heat block maintained on ice-
824 bath. 2µl of 100mm $CaCl_2$ added to each sample to initiate MNase activity and digestion was
825 performed for 30 min before adding 150µl of 2X RSTOP Buffer (200mM NaCl, 20mM EDTA,
826 4mM EGTA, 50ug/ml RNase, 40ug/ml glycogen, 10pg/ml yeast spike-in DNA) to stop the
827 reaction. Incubated at 37°C for 10 minutes to release the DNA fragments. Samples were spun at
828 12,000g for 10 minutes and aqueous layer transferred to a fresh 1.5 ml microfuge tube and
829 centrifuged at 16,000g for 5 minutes. Cleared liquid was again transferred to a fresh tube, 1µl of
830 20% SDS and 2.5µl proteinase K (20ng/ml) added, incubated at 70°C for 10 minutes. 300µl PCI
831 was added to each tube, mixed and total solution was transferred to phase lock tubes and
832 centrifuged at 16,000g for 5 minutes. After adding 300µl of chloroform and mixing gently, samples
833 were centrifuged at 16,000g for 5 minutes at RT. The aqueous layer was transferred to a DNA low
834 binding tube. 1µl glycogen (5mg/ml) and 750µl ethanol added to precipitate DNA at -80°C.
835 Samples were centrifuged at 16,000g for 10 min at 4°C and washed in ethanol twice. Pellet air
836 dried and dissolved in 15µl of 1mM TrisHCl + 0.1mM EDTA pH 8.0 [57,58]. 1ng of Cut and Run
837 DNA was used to make libraries using the KAPA Hyper prep kit and SeqCap adapters A &B
838 (Roche) according to manufacturer's protocol. For library amplification 14 cycles were used and
839 a 1.0X SPRI bead cleanup was performed using Agencourt Ampure XP beads. The following

840 antibody concentrations were used: rabbit anti-CLAMP (5µg/sample, SDIX); 1:200 anti-rabbit
841 (MilliporeSigma); rat anti-MLE (1:50, 6E11); 700ng/ml pA-MNase (from Steven Henikoff).
842
843 **iCLIP**

844 Kc and S2 cells were maintained at 25°C in Schneider's media supplemented with 10% Fetal
845 Bovine Serum and 1.4X Antibiotic-Antimycotic. Cells were passaged every 3 days to maintain an
846 appropriate cell density. Cells were allowed to grow to confluency and UV crosslinked using
847 254nm UV light in Stratalinker 2400 on ice (Stratagene, USA). UV treated cells were lysed to get
848 different cellular fractions (Cytoplasmic, Nucleoplasmic and Chromatin) according to Fr-iCLIP
849 (fractionation-iCLIP) protocol from Brugiolo et al 2017[85]. Chromatin and Nucleoplasmic fractions
850 were sonicated with a Branson digital sonicator at 30% amplitude for 30 s total (10 sec on and 20
851 sec off) to disrupt DNA before IP. All three fractions were separately centrifuged at 20,000 xg for
852 5 min at 4°C. Fractions were tested by Western blotting using RNApolI for Chromatin Fraction,
853 Actin for Cytoplasmic Fraction. Protein quantification for each fraction was done using
854 manufacturer's protocol for Pierce 660nm protein assay reagent (Thermo Scientific, USA). Each
855 Fraction was subjected to iCLIP protocol as described in Huppertz et al 2014[51] using rabbit-
856 CLAMP antibody to immuniprecipitate bound RNAs which were extracted using proteinase K and
857 phenol:chloroform. Custom cDNA libraries prepared according to Huppertz et al 2014[51] using
858 distinct primers Rt1clip-Rt16clip for separate samples containing individual 4nt-barcode
859 sequences that allow multiplexing of samples. cDNA libraries for each sample amplified
860 separately using 31 cycles of PCR, mixed together later and sequenced using standard illumina
861 protocols. Heyl et al. 2020[86] methods using the Galaxy CLIP-Explorer were followed to
862 preprocess, perform quality control, post-process and perform peak calling. Data deposited in GEO
863 (awaiting accession number).

864 **Computational Methods:**

865 **Time2splice tool: see Supplementary methods**
866
867 **Sex-specific splicing event analysis**

868 RNA sequencing data from Rieder et al 2017 (#GSE102922) was analyzed using time2splice to
869 determine sex-specifically splicing events dmel-all-r6.29.gtf from BDGP6 in genomes [87] was used
870 to map each transcript identifier (ID) to gene ID and symbol, for .bed creation data for the
871 associated chromosome, transcription start site (TSS) and transcription end site (TES), and strand
872 information were imported from Illumina
873 (https://support.illumina.com/sequencing/sequencing_software/igenome.html). From the raw data
874 after quality control i.e, FastQC[88], Salmon[89] was used to quantify transcript expression for
875 treatment and control samples. Calculated transcripts per million (TPM) values from SUPPA[42]
876 were used for all four replicates of female and male controls at both time points (before and after
877 MZT). Each sample was filtered to include transcripts where the mean value is less than or equal
878 to 3 TPMs per gene. The number of transcripts included at various thresholds were plotted from 1
879 to 10 and the fraction of genes filtered out begins to plateau around threshold 3. The percent of
880 spliced in (PSI) transcripts between females and males were compared at both 0-2 Hr (pre-MZT)
881 and 2-4 Hr (post-MZT) (p-value of 0.05), thereby resulting in delta PSI values and p-values for
882 each transcription in each experimental condition comparison. Given these resulting delta
883 transcript PSI values, significantly alternatively splice genes (p-value 0.05) were found between
884 females vs. males 0-2 Hr (pre-MZT) controls to show which genes are normally sex-specifically
885 spliced pre-MZT. The same process was followed at 2-4 Hr (post-MZT). To then determine the
886 sex-specifically spliced genes, the female RNAi experiment compared with the control delta PSI
887 gave the number of total alternative spliced transcripts pre-MZT, then considering those that are
888 not shared with males, and are only expressed in females normally, this defined our sex specifically
889 spliced set of genes for females pre-MST. This process was also performed for males pre-MZT.
890 The same process was followed for post-MZT samples.
891
892 **ChIP-seq: Data analysis**
893
894 We used preprocessed ChIP-seq data from Rieder et al 2019 (#GSE133637), specifically the .bw
895 and .broadPeak.gz files in our analysis using ChIPseeker [90] and deeptools [91]. Specifically, when
896 plotting the average profiles using deeptools, we achieved a baseline signal representing genome-
897 wide binding taking into consideration the number of genes in other groups by the following
898 procedure: of all genes that are on (no zero read-count genes), we sampled the number of the

899 largest other group (to which we are comparing), and ran computeMatrix on that subset. This
900 process was repeated 500 times and the resulting 500 matrices were averaged to produce a
901 representative signal. For motif analysis MEME [92] suite was used.
902
903 **Cut and Run: Data analysis**
904
905 Sequenced reads were run through FASTQC[88](fastqc replicate_R1_001.fastq.gz
906 replicate_R2_001.fastq.gz) with default parameters to check the quality of raw sequence data and
907 filter out any sequences flagged for poor quality. Sequences were trimmed and reassessed for
908 quality using TrimGalore (https://github.com/FelixKrueger/TrimGalore/issues/25) and FastQC [88],
909 respectively. All Illumina lanes of the same flow cell .fastq files were merged, and sequenced reads
910 were then mapped to release 6 *Drosophila melanogaster* genome (dm6). We compared Bowtie2[93],
911 HISAT2[94], and BWA[95]. We found the best alignment quality with BWA and thus used this
912 method's results downstream. Next, we performed conversion to bam and sorting (e.g. using:
913 bowtie2 -x dm6_genome -1 replicate_R1_001.fastq.gz -2 replicate_R2_001.fastq.gz -S out.sam >
914 stout.txt 2> alignment_info.txt; samtools view -bS out.sam > out.bam; rm -rf out.sam; samtools
915 sort out.bam -o out.sorted.bam). We removed reads (using samtools) with a MAPQ less than 30
916 and any reads with PCR duplicate reads (identified using MarkDuplicates Picard -2.20.2). Peaks
917 identified using MACS2[96](macs2 callpeak -t out.sorted.bam -B -f BAM --nomodel --SPMR --
918 keep-dup all -g dm --trackline -n outname --cutoff-analysis --call-summits -p 0.01 --outdir outdir)
919 and keep duplicates separate**.** To calculate fold-enrichment macs2 is run again (macs2 bdgcmp -t
920 $treat -c $control -o $out.sorted.bam_FE.bdg -m FE 2> $ out.sorted.bam_FE.log; macs2 bdgcmp
921 -t $treat -c $control -o $out.sorted.bam_logLR.bdg -m logLR -p 0.00001 2). For motif analysis
922 MEME [92] suite was used. Data submitted in GEO repository (#GSE174781).
923
924 **iCLIP: Data analysis**
925
926 Heyl et al. 2020[86] methods using the Galaxy CLIP-Explorer were followed to preprocess, perform
927 quality control, post-process and perform peak calling. For preprocessing UMI-Tools was used,
928 and then UMI-tools and Cutadapt used for Adapter, Barcode and UMI-removal. Cutadapt (Galaxy
929 version 3.5) was used for filtering with a custom adapter sequence

930 AGATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCTCGTATGCCGTCTTCT
931 GCTTG. All other settings followed the Heyl et al 2020 Galaxy iCLIP-explorer workflow. UMI-
932 Tools Extract (Galaxy Version 1.1.2+galaxy2) was then used with a barcode pattern of
933 NNNXXXXNN. No unpaired reads were allowed. The barcode was on the 3' end. Je-Demultiplex
934 (Galaxy Version 1.2.1) was then used for demultiplexing. FastQC was used for quality control.
935 Mapping was done by RNA STAR (Galaxy version 2.5.2b-2) using dm6. All settings were chosen
936 based on the existing parameters from the iCLIP-explorer settings. We selected FALSE for the
937 option to use end-to-end read alignments with no soft-clipping. bedtools used for Read-Filtering,
938 and UMI-Tools (Galaxy version 0.5.3.0) for de-duplication. PEAKachu was used for Peak Calling
939 to generate bed files. The PEAKachu settings were followed using the Galaxy CLIP-explorer
940 workflow. The maximum insert size was set to 150, the minimum cluster expression fraction was
941 set to 0.01, the minimum block overlap set to 0.5, the minimum block expression set to 0.1. The
942 Mad Multiplier was set to 0.0, the Fold Change Threshold was set to 2.0, and the adjusted p-value
943 threshold was set to 0.05. Peaks were annotated using RCAS[97] (RNA Centric Annotation System),
944 a R package using Rstudio. MEME Suite used for motif detection. RCAS was used for functional
945 analysis of the transcriptomes isolated by iCLIP, such as transcript features. ShinyGO 0.76[98] was
946 used to perform Gene Ontology Analysis of the iCLIP data.
947
948 **Competing Interest Statement**
949
950 The authors declare no conflicting interests.
951
952 **Acknowledgments**
953
954 This work and funding to M.R. was supported by R35GM126994 to E.N.L. from NIH. A.M.C is
955 funded by the NSF Graduate Research Fellowship and CCMB, Brown University. We thank
956 Bloomington stock center for fly lines. We thank Leila Rieder for SD and Rsp^s stocks, Peter Becker
957 for MSL2 and MLE antibodies, Steve Henikoff for pAMNase protein and spike-in DNA for Cut
958 and Run, and Daniel J McKay for sharing his Cut and Run protocol for tissues.
959
960

961

962 **Author Contributions**

963

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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1247 **Fig 1. Alternative splicing during early *Drosophila melanogaster* embryonic development**
1248 **A** Schematic diagram showing 7 different types of Alternative splicing (AS).
1249 **B** Pie chart showing the distribution of different types of AS at 0-2 Hr pre-MZT and 2-4 Hr post-
1250 MZT for female (red) and male (Blue) embryos in the presence (top row) and absence (bottom
1251 row) of maternal CLAMP. The total percentage of AS in each category in control embryos is noted
1252 at the top of the schematic. A Chi-square test was performed to determine if there is a significant
1253 difference between the percentage of each type of AS including MXE splicing (grey region) in the
1254 presence vs. absence of CLAMP in each class of sample: females/males 0-2 Hr, and 2-4 Hr
1255 embryos. A significant difference ($p<0.001$ marked by ***) was found between categories
1256 connected by solid black lines.
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1277 **Fig 2. Maternal CLAMP regulates sex-specific alternative splicing during early embryonic**
1278 **development.**
1279 **A** Bar graph showing the percentage of transcripts (values noted at the top of each bar) out of total
1280 AS events or sex-specific splicing events (number of events noted within parentheses at the top of
1281 each bar) regulated by maternal CLAMP, at the pre-MZT and post-MZT stages, in females (red
1282 bars) and males (blue bars). A Fischer's Exact Test was performed, with significance at $p<0.001$.
1283 **B** Bar plot showing the total number of splicing events undergoing CLAMP-dependent AS (**N**) in
1284 females and males at 0-2 Hr pre-MZT and 2-4 Hr post-MZT embryonic stages. Spliced genes are
1285 divided into non-sex specific (grey) and sex-specific (orange shades) sub-categories of
1286 genes. CLAMP-dependent female and male sex-specifically spliced (SSS) genes are divided into
1287 known (darker orange) and new (lighter orange) sub-categories identified in 0-2 Hr pre-MZT and
1288 2-4 Hr post-MZT embryos.
1289 **C** Percentage of **new** female (red) and male (blue) CLAMP-dependent sex-specifically spliced
1290 genes in 0-2 Hr pre-MZT and 2-4 Hr post-MZT embryos that were not identified in control
1291 samples.
1292 **D** Male (blue) and female (brown) CLAMP-dependent sex-specific spliced genes compared with
1293 maternal genes (**N=841**, magenta) at 0-2 Hr pre-MZT (female, **N=153** and male, **N=99**) and 2-4
1294 Hr post-MZT stages (female, **N= 270** and male, **N=116**).
1295 **E** Gene ontology results for genes showing CLAMP-dependent female sex-specific splicing in the
1296 embryo at 0-2 Hr pre-MZT stage and for genes exhibiting CLAMP-dependent female as well as
1297 male sex-specific splicing in the embryo at the 2-4 Hr post-MZT stage. The size of the circle
1298 increases as the number of the genes in that category increases. The color of the circle represents
1299 significance (p-value).
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1307 **Fig 3. CLAMP binds along the gene body of female and male sex-specifically spliced genes**
1308 **at the post-MZT embryonic stage**
1309 **A-B** Average profiles for CLAMP binding at pre-MZT and post-MZT embryonic stages in females
1310 and males for genes spliced female-specifically (red line) and male-specifically (blue line) during
1311 the pre-MZT (**A**) and post-MZT (**B**) stages.
1312 **C-D** Average profiles for CLAMP binding to genes expressed in a sex-biased manner in females
1313 (red line) and males (blue line) during pre-MZT (**C**) and post-MZT (**D**) stage.
1314 Green lines in **A-D** represent CLAMP binding at a random set of active genes used as a control
1315 (see **Material and Methods** for details). Stippled regions in **A, B** (female, 0-2 Hr pre-MZT) denote
1316 chromatin around the TSS with more CLAMP binding in female sex-specifically spliced genes vs.
1317 male sex-specifically spliced genes. The dotted box in **A-D** marks the gene body region in sex-
1318 specifically spliced and biased expressed genes.
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1338 **Fig 4. CLAMP regulates the distribution of MLE on chromatin in males**

1339 **A-B.** Heat maps showing the distribution of MLE at the male (**A**) and female (**B**) specific control
1340 MLE peaks on the X chromosome and autosomes in male and female 0-2 Hr pre-MZT embryos
1341 with maternal CLAMP (*MTD-GAL4>GFPRNAi*) and after the loss of maternal CLAMP (*MTD-*
1342 *GAL4>CLAMPRNAi)*.

1343 **C-D.** Venn diagrams and bar plots showing loss and gain of MLE peaks in the presence and
1344 absence of maternal CLAMP in male 0-2 Hr pre-MZT (**B**) and 2-4 Hr post-MZT (**C**) embryos.
1345 CLAMP peaks were identified only under control conditions (green circle), whereas MLE peaks
1346 were identified in the presence (grey circle) and absence (red circle) of maternal CLAMP protein
1347 depleted using the *MTD-GAL4>CLAMPRNAi* system.

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1368 **Fig 5. Alternative splicing of components of the sex determination pathway is regulated by**
1369 **CLAMP in females**
1370 **A** The sex determination pathway in *Drosophila* is regulated by master regulator SXL.
1371 **B** Electrophoresis gel image (inverted colors) showing splicing of *sxl* transcripts in third instar
1372 larvae of females and males of genotypes listed in the key (**a-g**) with a representative schematic at
1373 the top of the gel image.
1374 **C** Western blot showing the level of SXL protein in genotypes (3 replicates for each) mentioned
1375 below each lane. Tubulin levels were used for protein loading control. Below the blot is the relative
1376 density of SXL protein compared with Tubulin, with each genotype represented by separate
1377 colored bars.
1378 **D** Chromatin accessibility measured by the MNase Accessibility (MACC) score is shown across
1379 the *sxl* gene in male (S2) and female (Kc) cells under control and CLAMP RNAi conditions. The
1380 MACC score is a previously reported (Urban et al 2017) quantification of chromatin accessibility
1381 at each locus in the genome. Positive/high accessibility values (blue) indicate high chromatin
1382 accessibility, and negative/low (red) accessibility values indicate low chromatin accessibility.
1383 Each window covers MACC values ranging from -0.333 to +1.33. MACC values increase in
1384 females after CLAMP RNAi, specifically at exon 3 (red box) and are shown in the inset to the
1385 right. Green boxes represent CLAMP binding peaks in the *sxl* gene just below the schematic for
1386 the *sxl* gene itself.
1387 **E-F** Electrophoresis gel image from 0-2 Hr embryonic (lane 2-5 & 7-10) and third instar larval
1388 samples (a-g) showing splicing of *dsx* (E) and *msl2* (F) transcripts in females (lane 3,5,8,10, a-d)
1389 and males (lane 2,4,7,9, e-g). Embryos were from *MTD-GAL4>GFPRNAi* control (lane 4,5,9,10)
1390 and *MTD-GAL4>CLAMPRNAi* (lane 2,3,7,8) females. a-g genotypes are the same as in B. The
1391 schematic at the top of each gel image shows female and male splice variants of *dsx* (E) and *msl2*
1392 (F) transcripts.
1393 **G** Fluorescent microscopy images of polytene chromosomes from third instar salivary gland in the
1394 genotypes listed to the left of each panel show the distribution of CLAMP (green) and MSL2 (red)
1395 on chromatin (blue, DAPI)
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1399 **Fig 6. Mechanisms by which CLAMP regulates sex-specific splicing in females and males**
1400 **A** CLAMP regulates splicing in both males and females via directly binding to intronic DNA
1401 sequences of CLAMP-dependent sex-specifically spliced genes and interaction with some sex-
1402 specifically spliced genes and spliceosomal RNAs.
1403 **B** CLAMP regulates MLE distribution between the spliceosome and the male X-chromosome
1404 specific MSL complex in males. CLAMP increases the occupancy of MLE at promoters and CES.
1405 In the absence of CLAMP, MLE is lost from many sites, including CES and promoters, and is
1406 gained at new intronic sequences which contain motifs that regulate splicing, resulting in aberrant
1407 sex-specific splicing in males.
1408 **C** In females, CLAMP binds near the SxlPe promoter and regulates chromatin accessibility at exon
1409 three (blue square) of the *sxl* gene and to the *sxl* mRNA. In this way, CLAMP promotes the
1410 splicing out of exon3 such that functional Sxl protein is formed, which drives female-specific
1411 splicing events. The absence of CLAMP in females thus results in the aberrant production of non-
1412 functional male-specific *sxl* transcripts which retain exon3, reducing levels of functional Sxl
1413 protein. CLAMP and Sxl have shared and distinct RNA targets suggesting that they function by
1414 both dependent and independent mechanisms.
1415 The three mechanisms proposed in parts **A**, **B**, and **C** are not mutually exclusive and are likely to
1416 occur simultaneously.
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Fig 1. Ray, Conard et al 2022

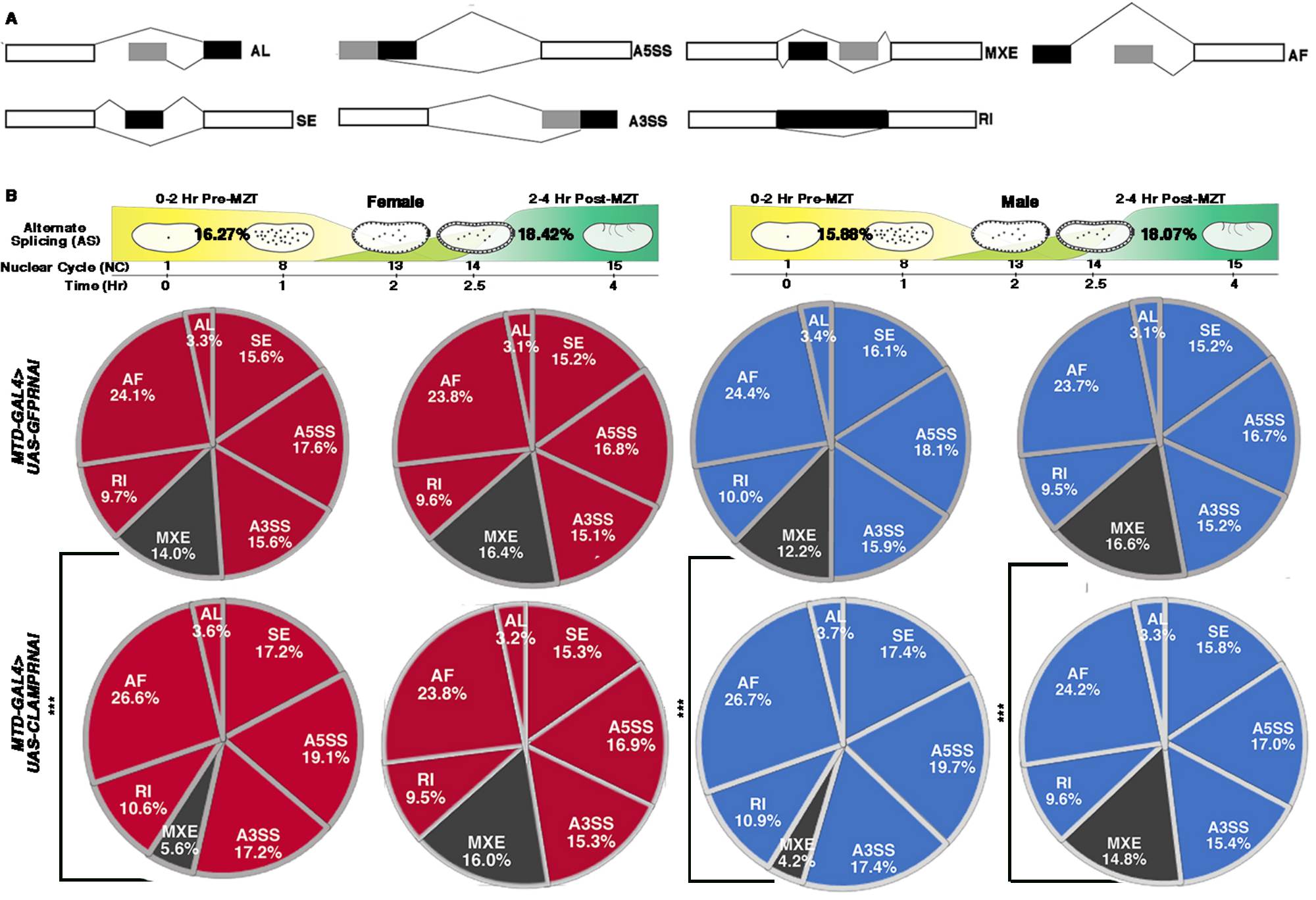
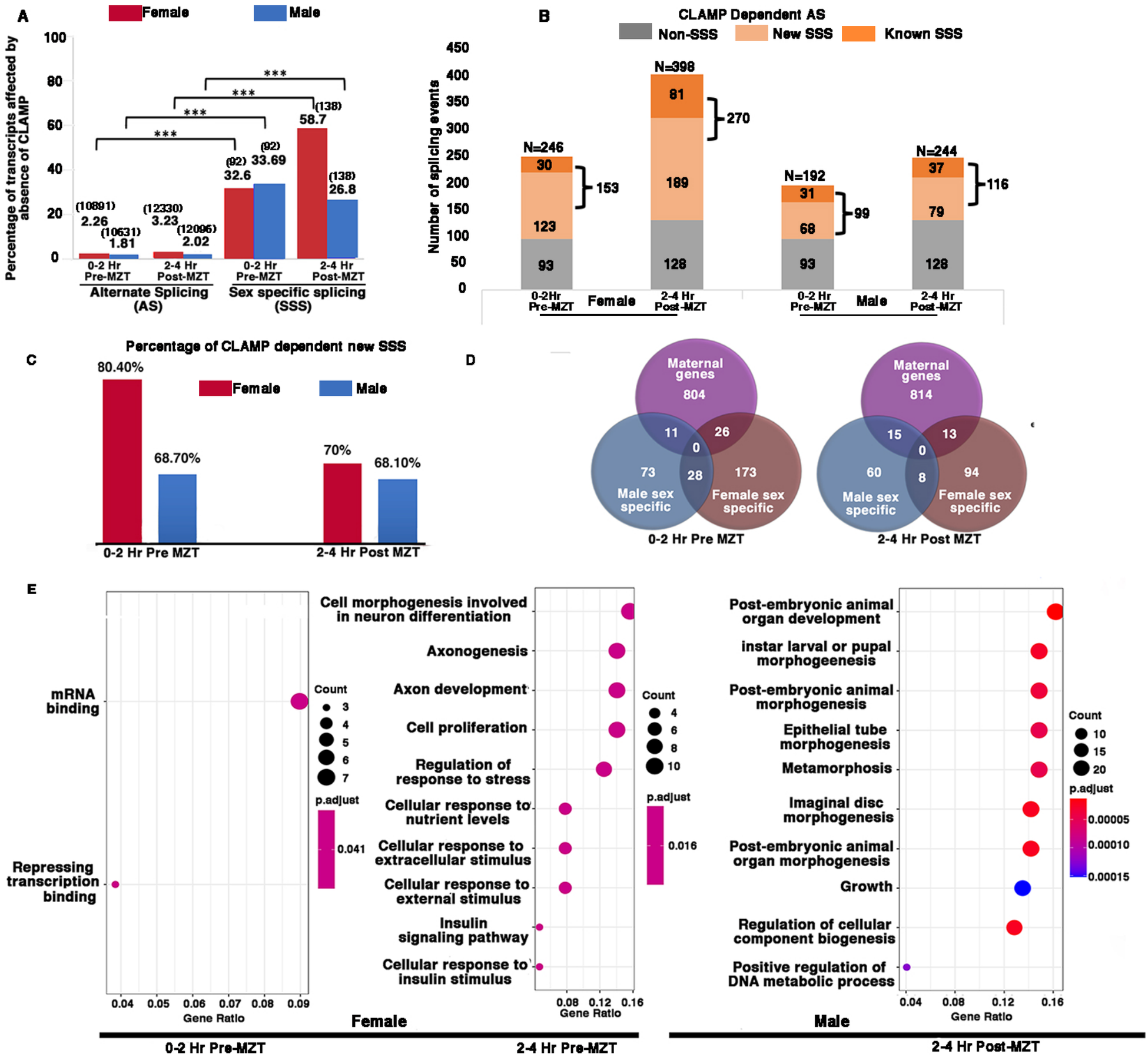


Fig 2. Ray, Conard et al 2022



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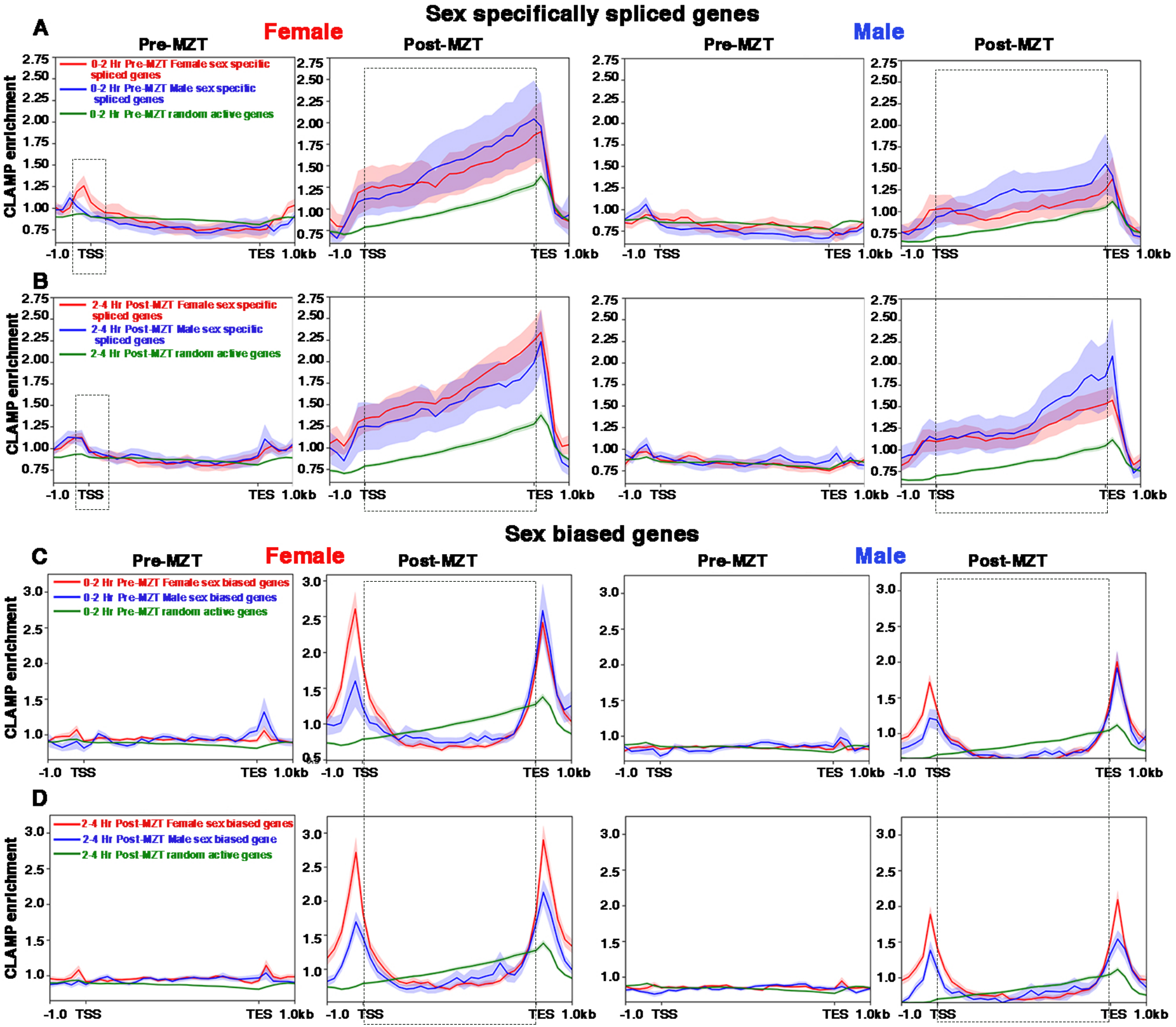

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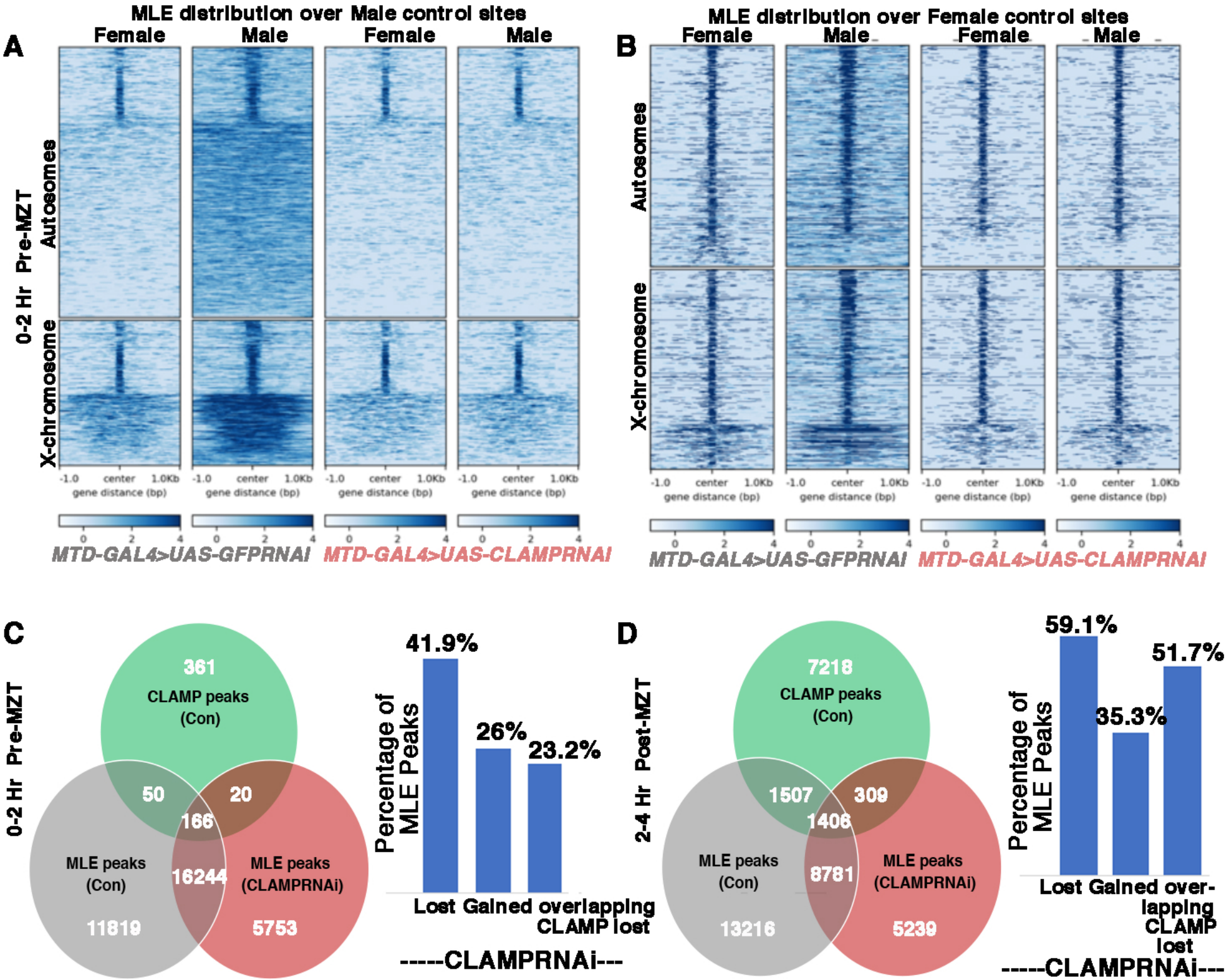

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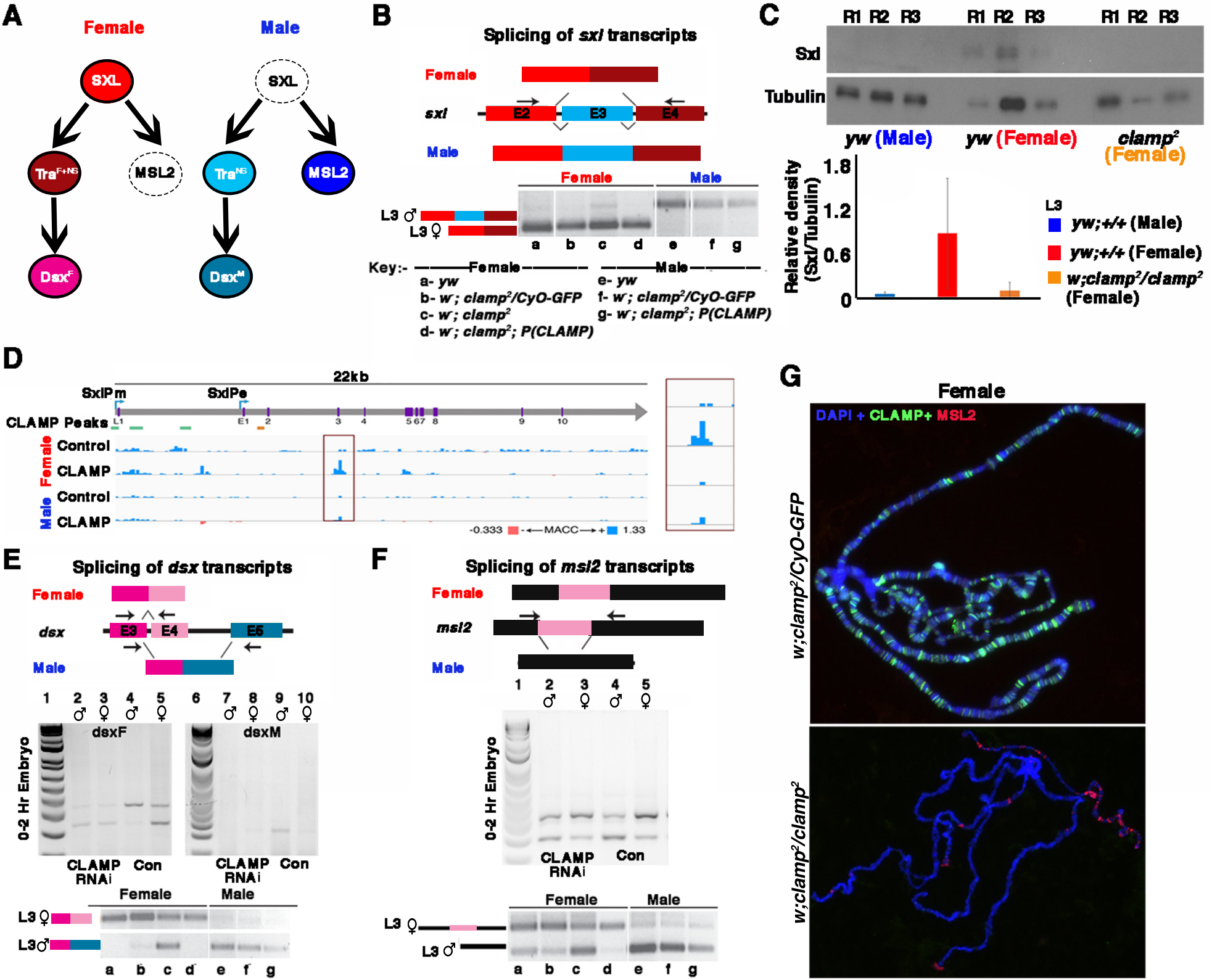

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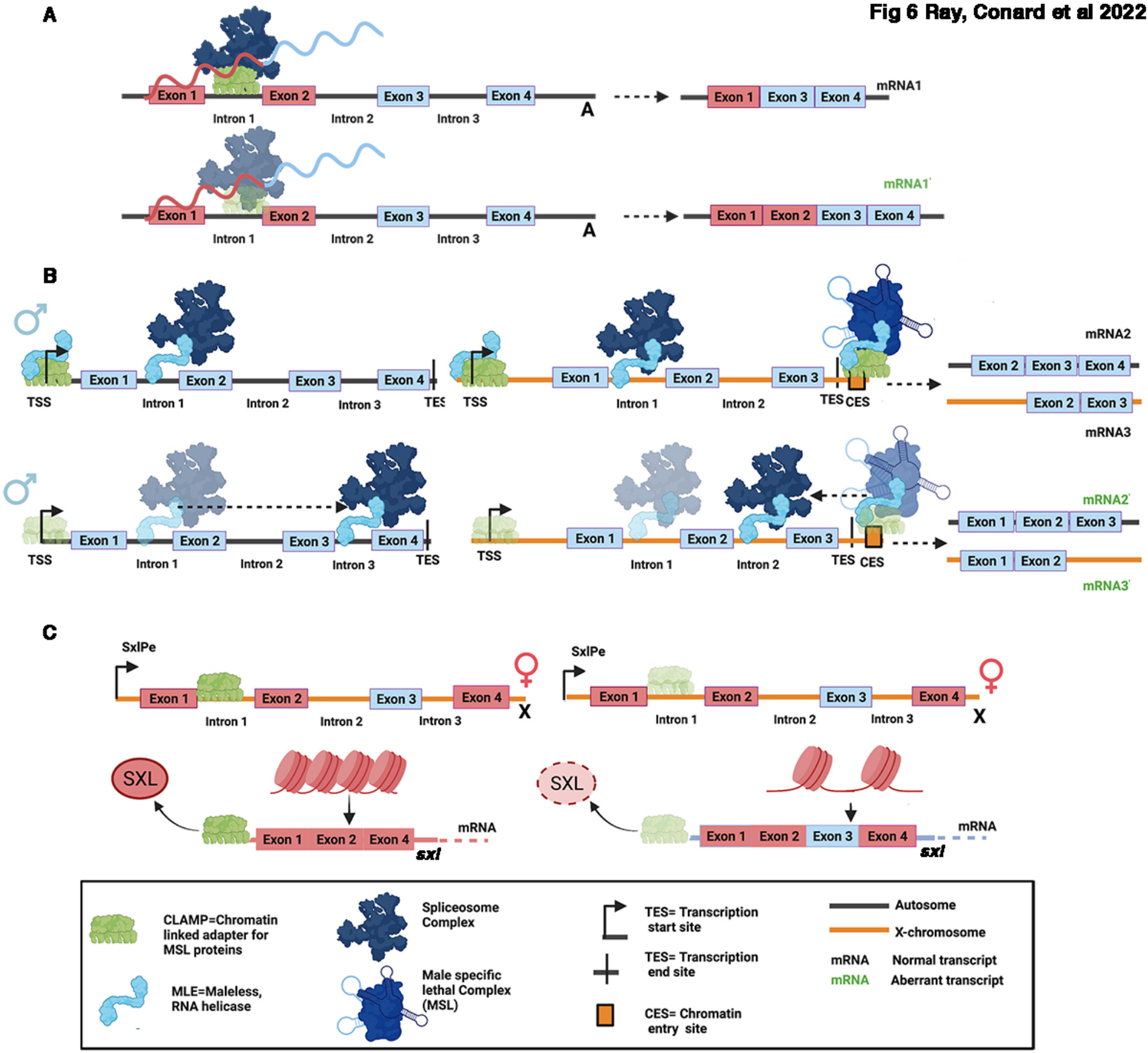


Fig S1. Ray, Conard et al 2022

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

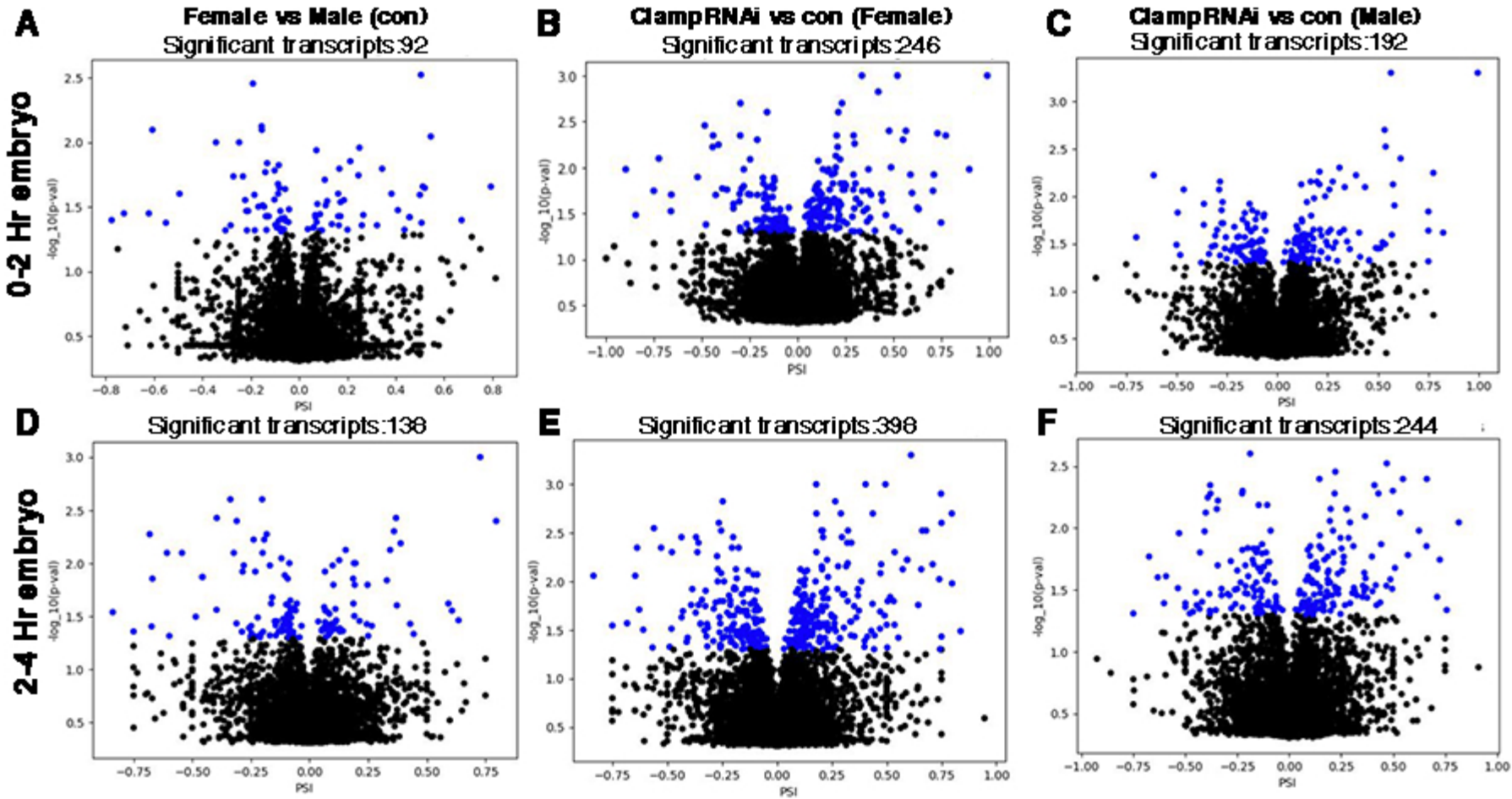


Fig S3. Ray, Conard et al 2022

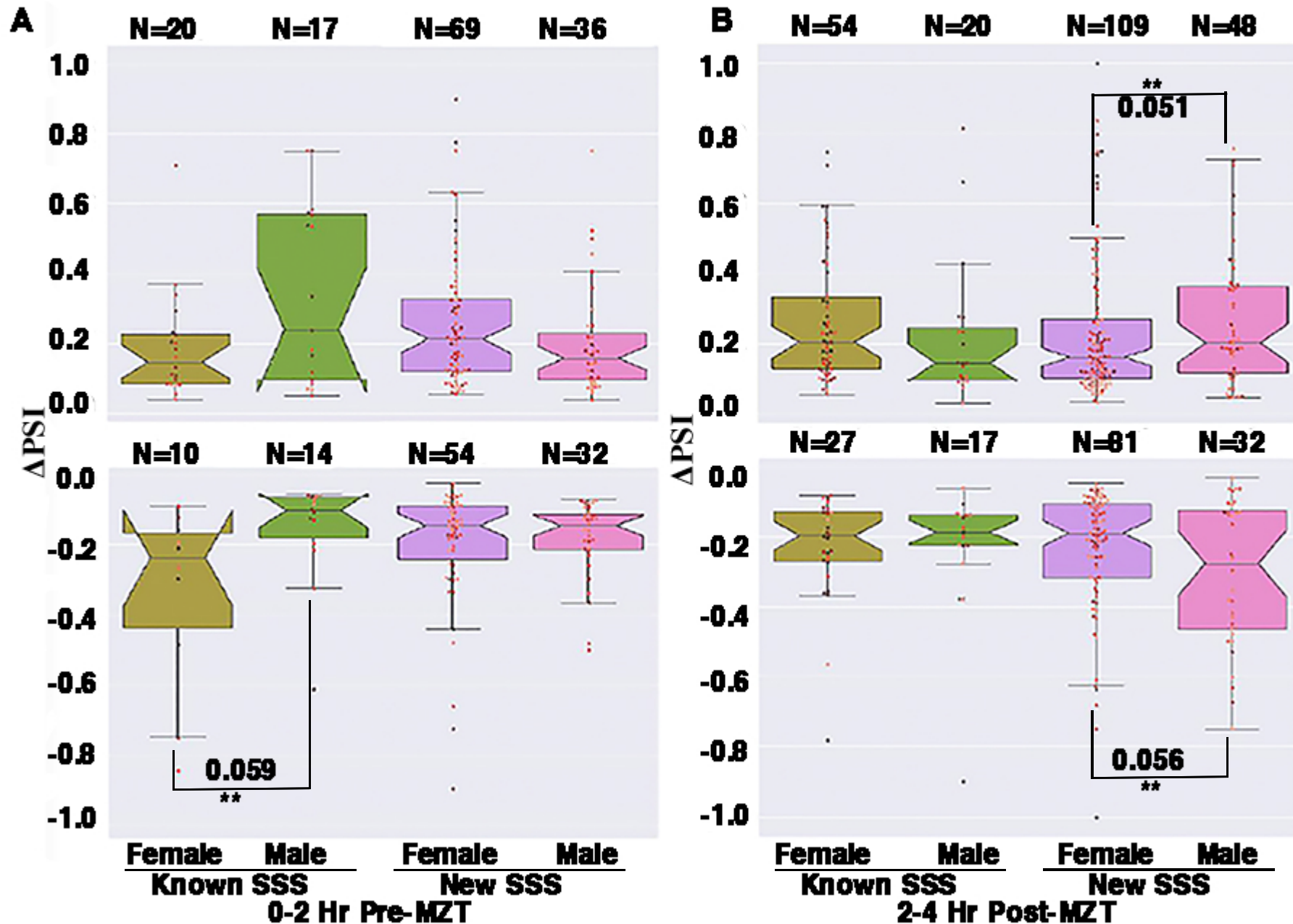


Fig S4 Ray, Conard et al 2022

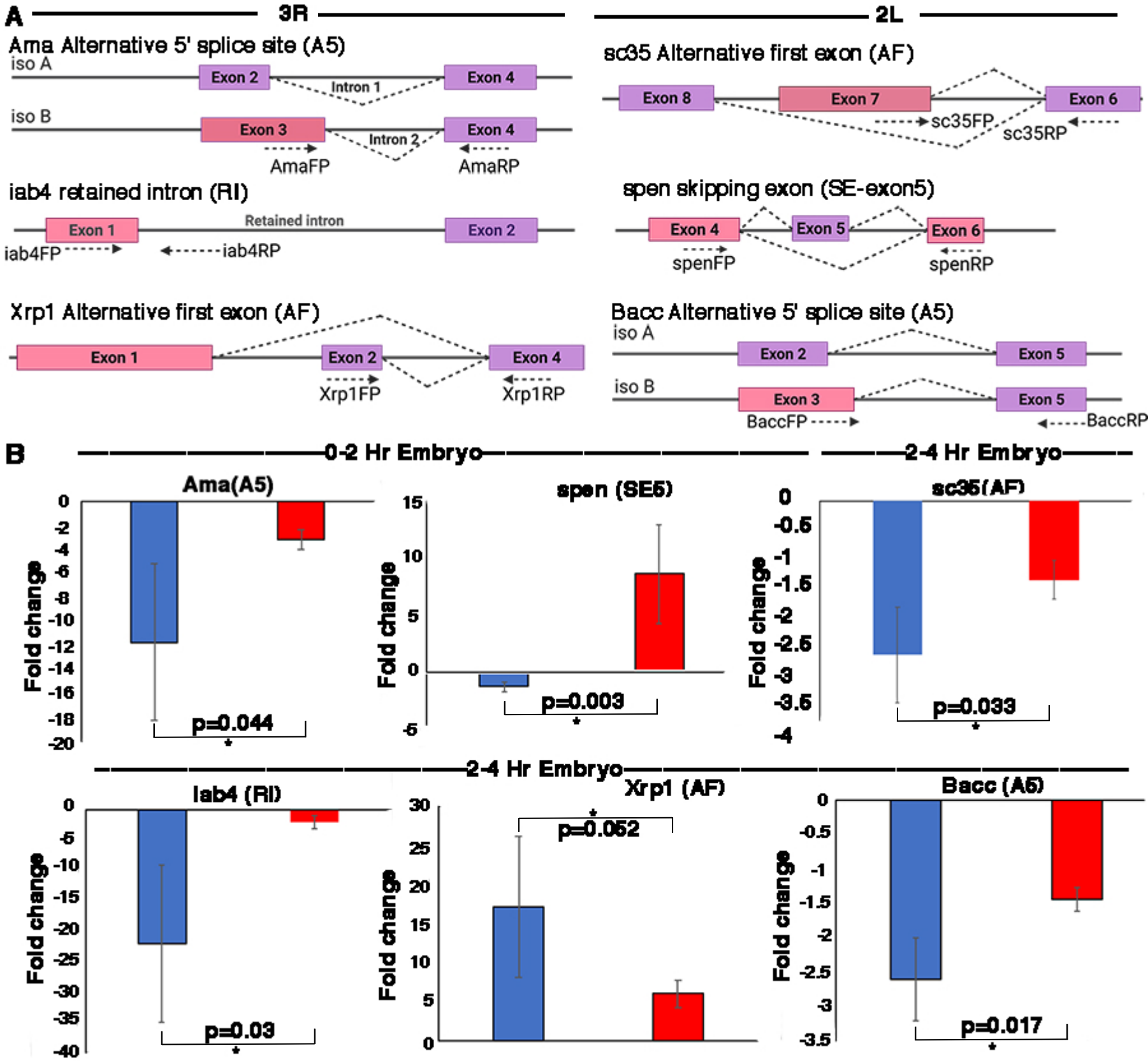


Fig S5. Ray, Conard et al 2022

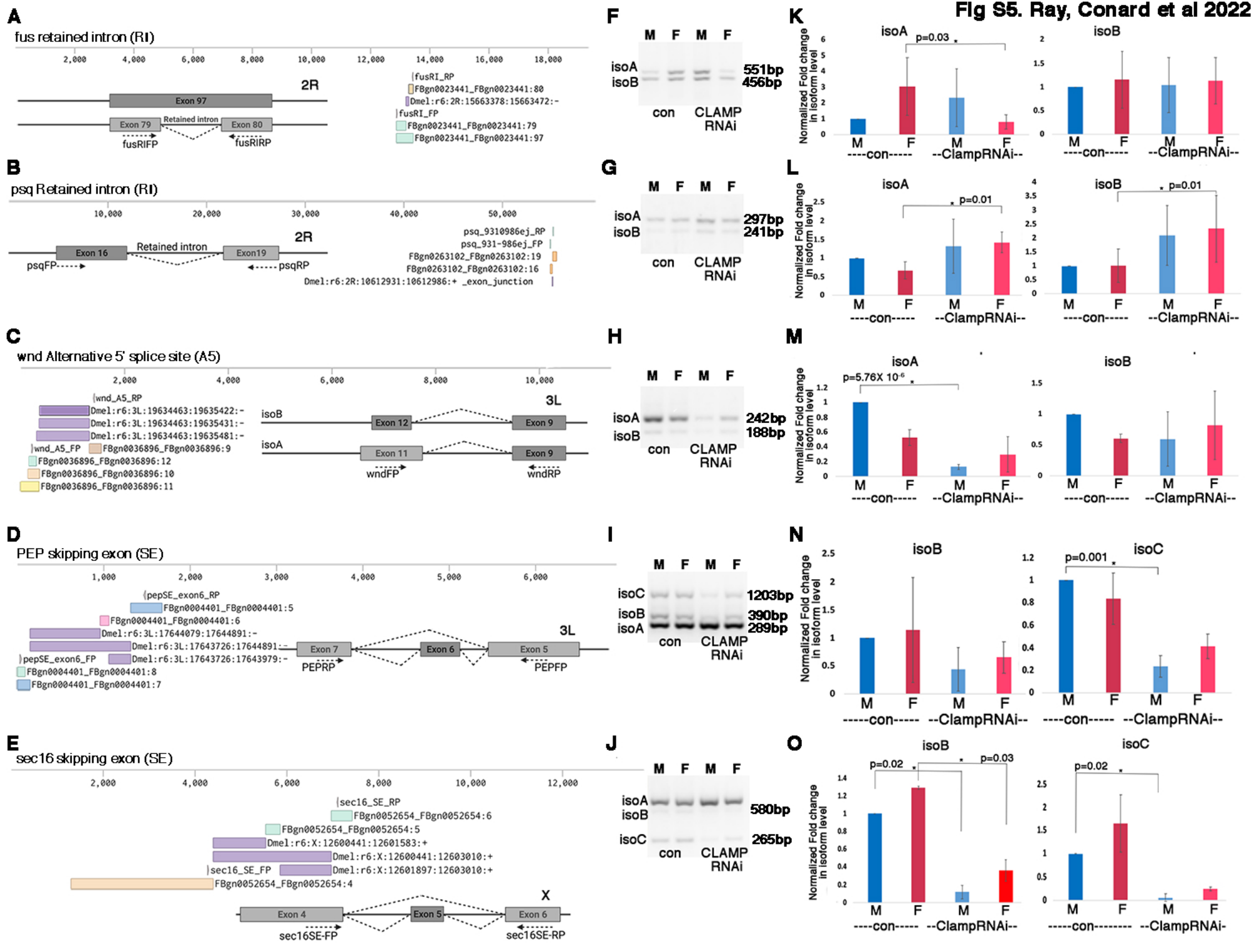


Fig S6. Ray, Conard et al 2022

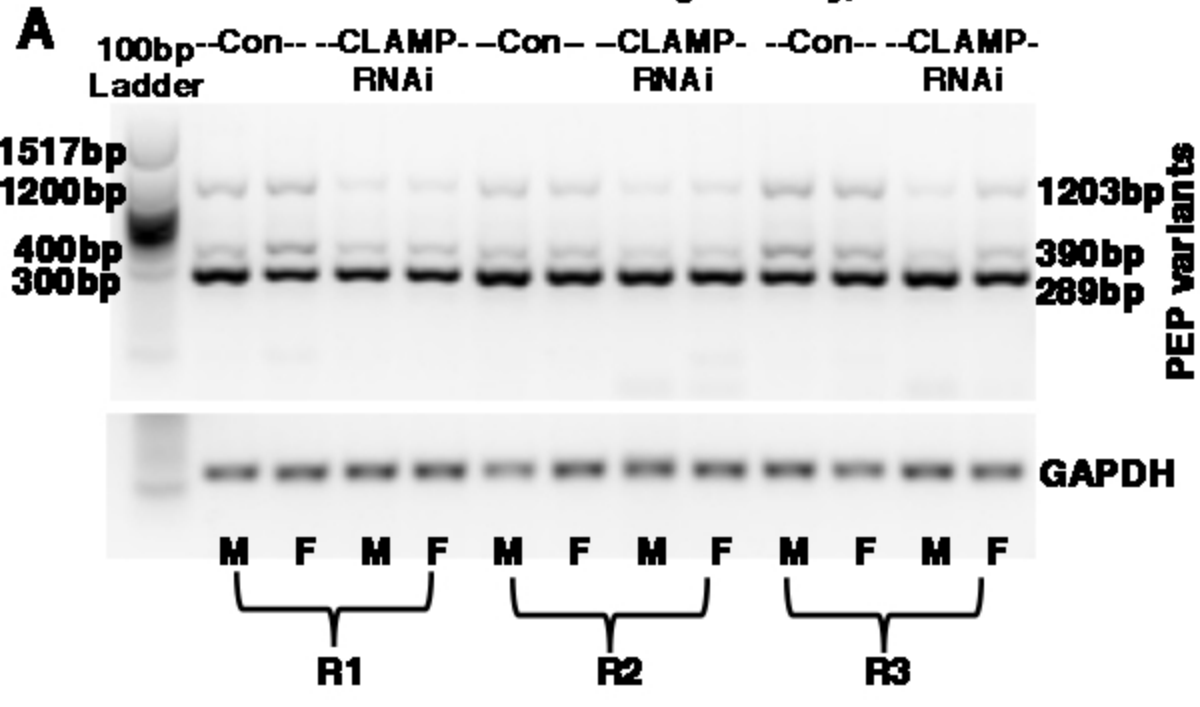


Fig S7. Ray, Conard et al 2022

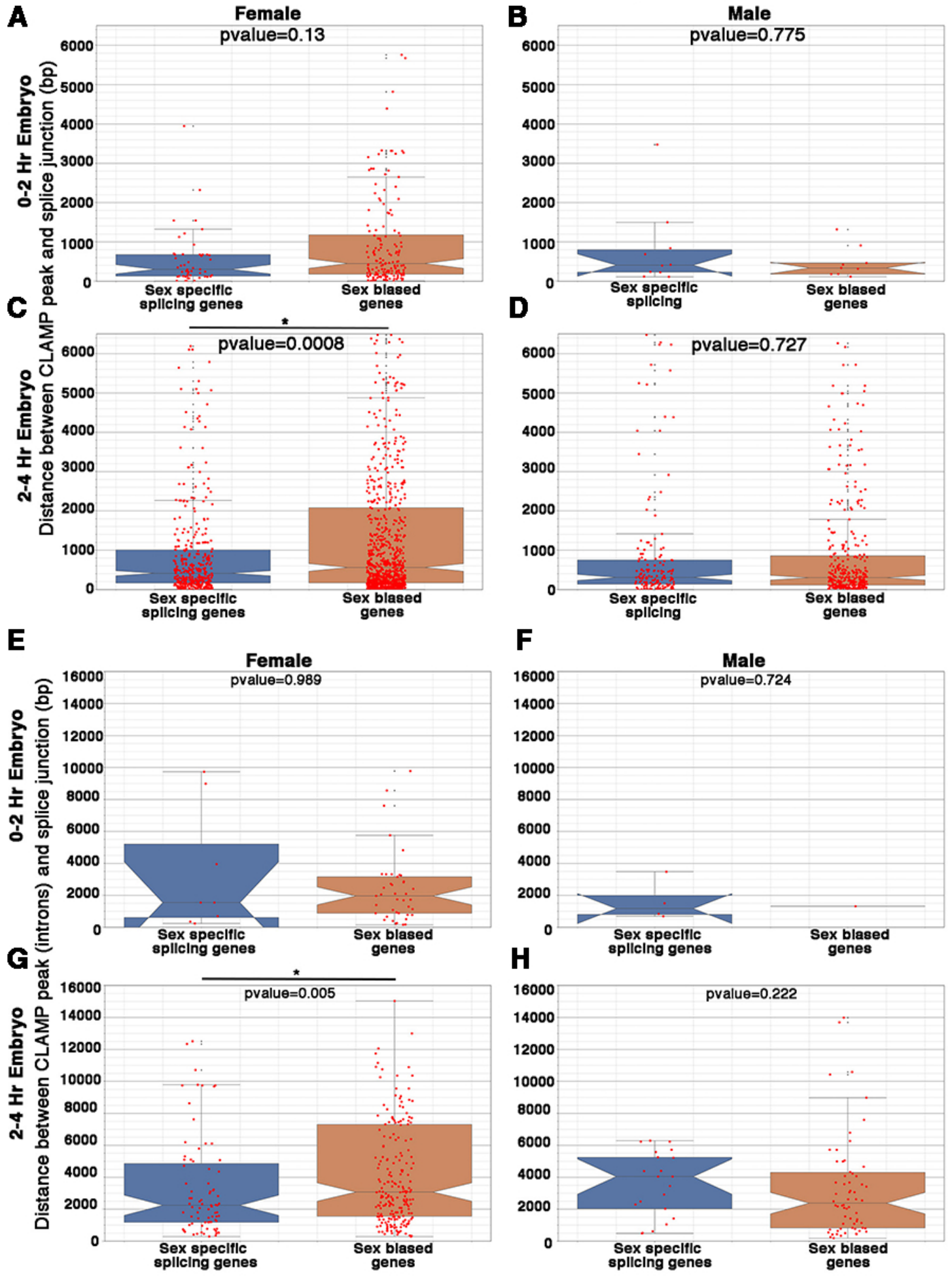
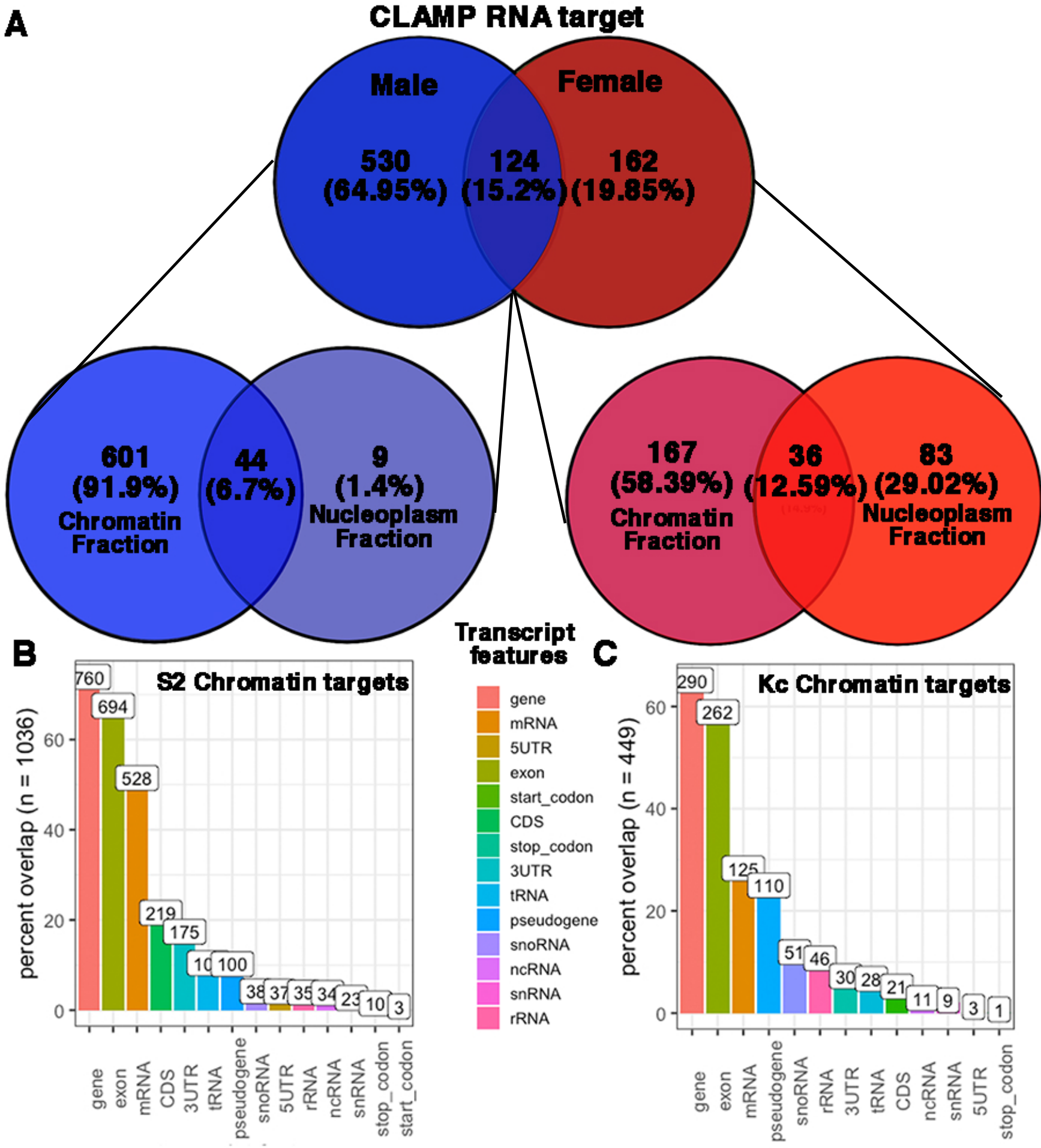
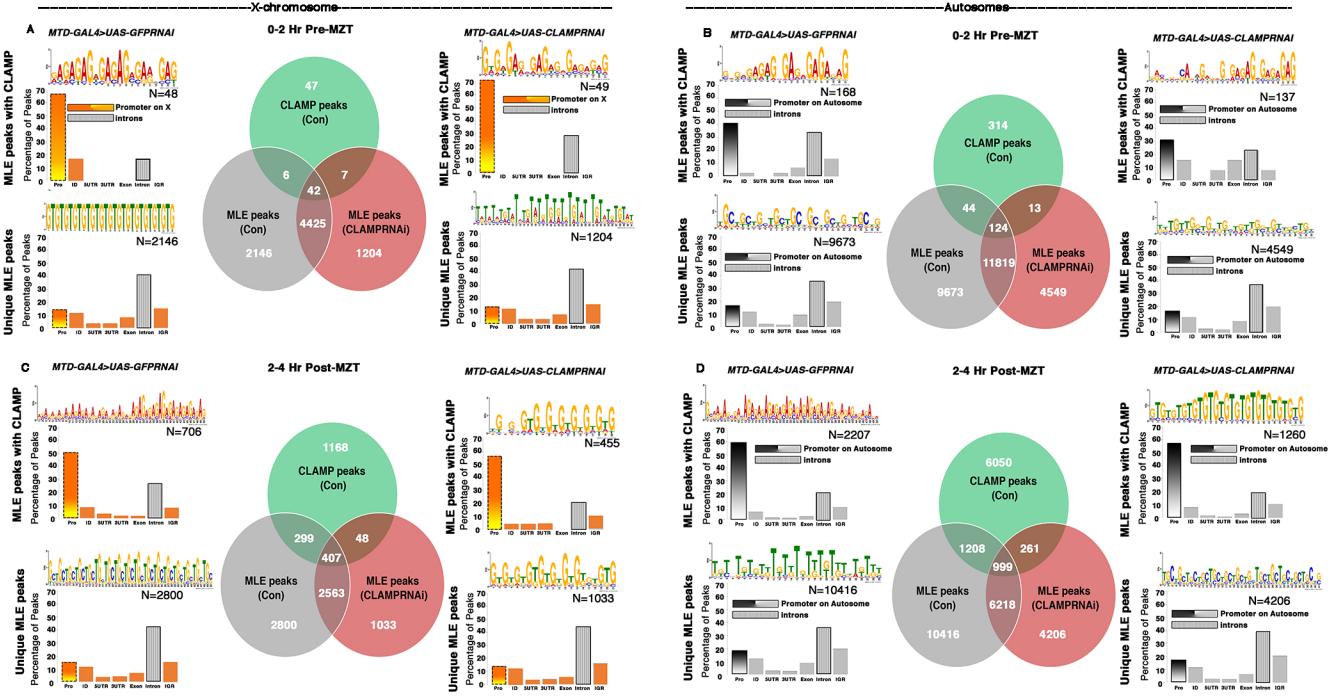


Fig S8 Ray, Conard et al 2022




X-chromosome
Autosomes
A
MTD-GAL4>UAS-GFPRNAI
0-2 Hr Pre-MZT
MTD-GAL4>UAS-CLAMPRNAI
MLE peaks with CLAMP
Unique MLE peaks
Percentage of Peaks
N=48
Promoter on X
Introns
N=2146
47
CLAMP peaks
(Con)
6
7
42
MLE peaks
(Con)
2146
4425
MLE peaks
(CLAMPRNAi)
1204
N=49
N=1204
B
N=168
Promoter on Autosome
N=9673
314
44
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124
11819
9673
4549
N=137
N=4549
C
2-4 Hr Post-MZT
N=706
N=2800
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Fig S10. Ray, Conard 2022

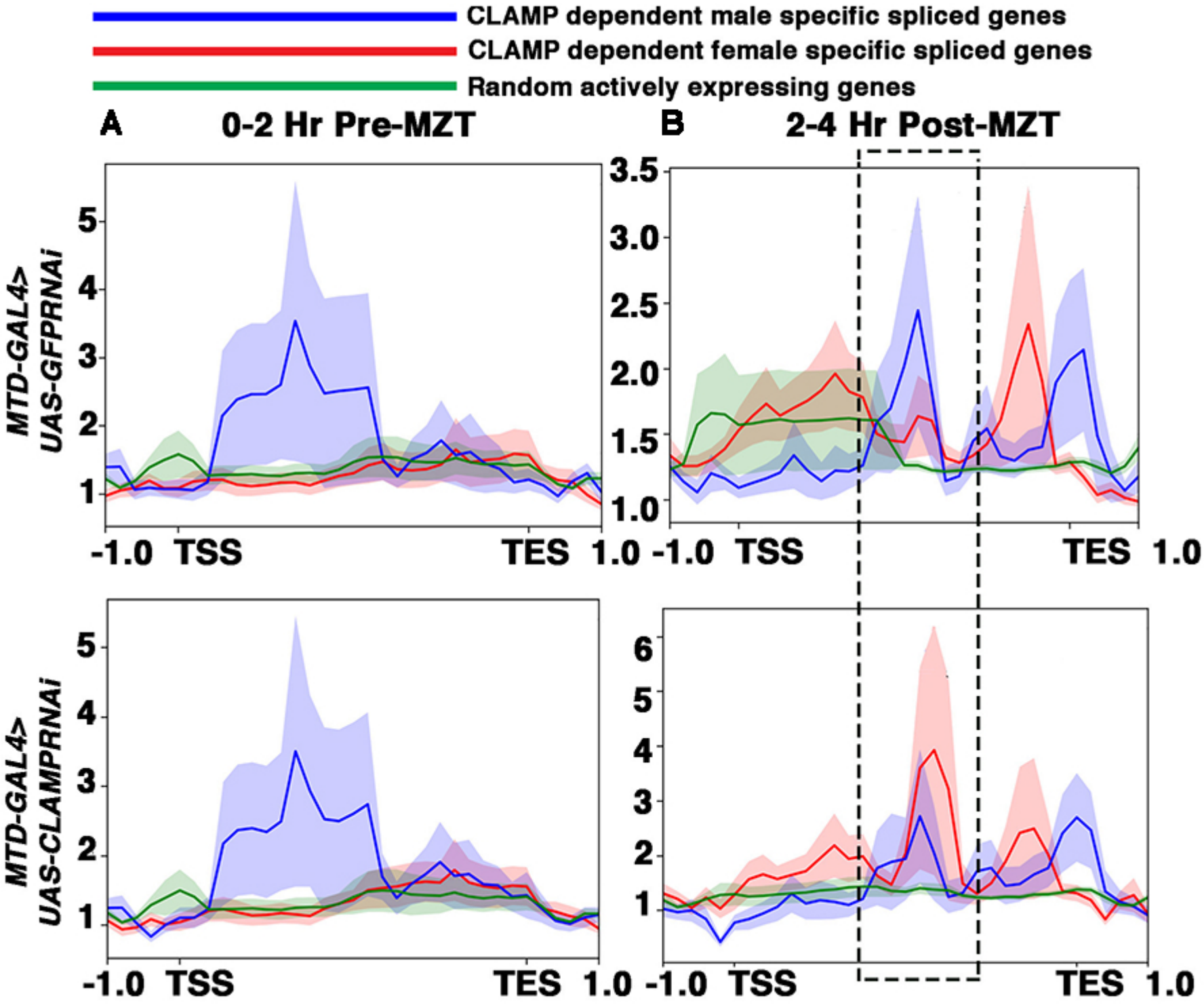
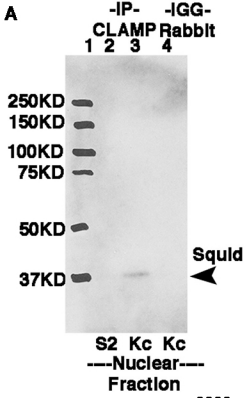
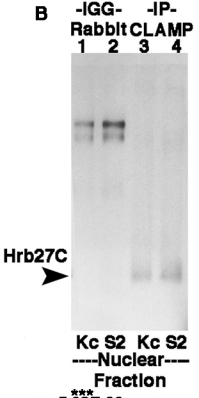
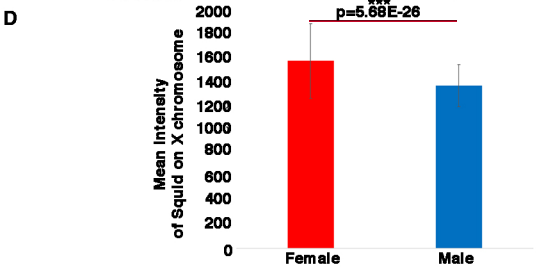
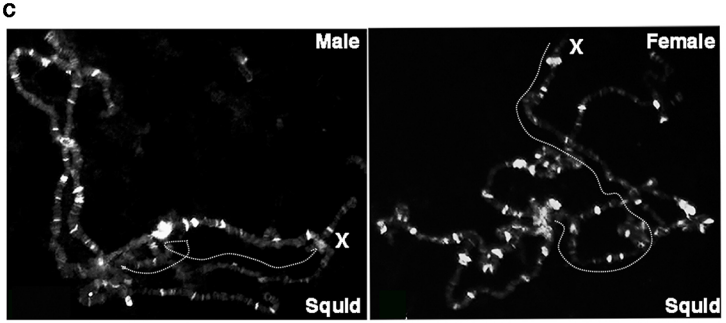


Fig S11. Ray, Conard 2022









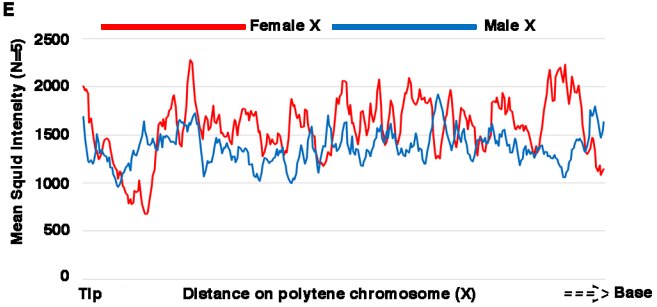
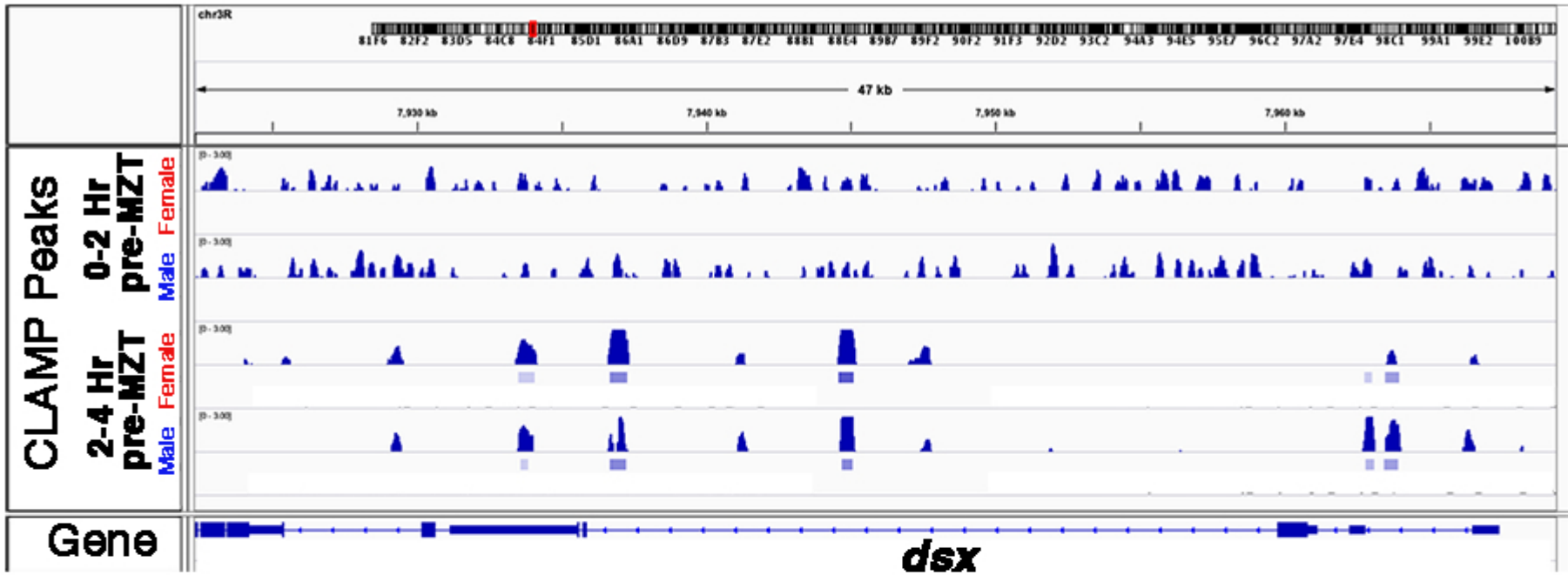


Fig S12. Ray, Conard 2022



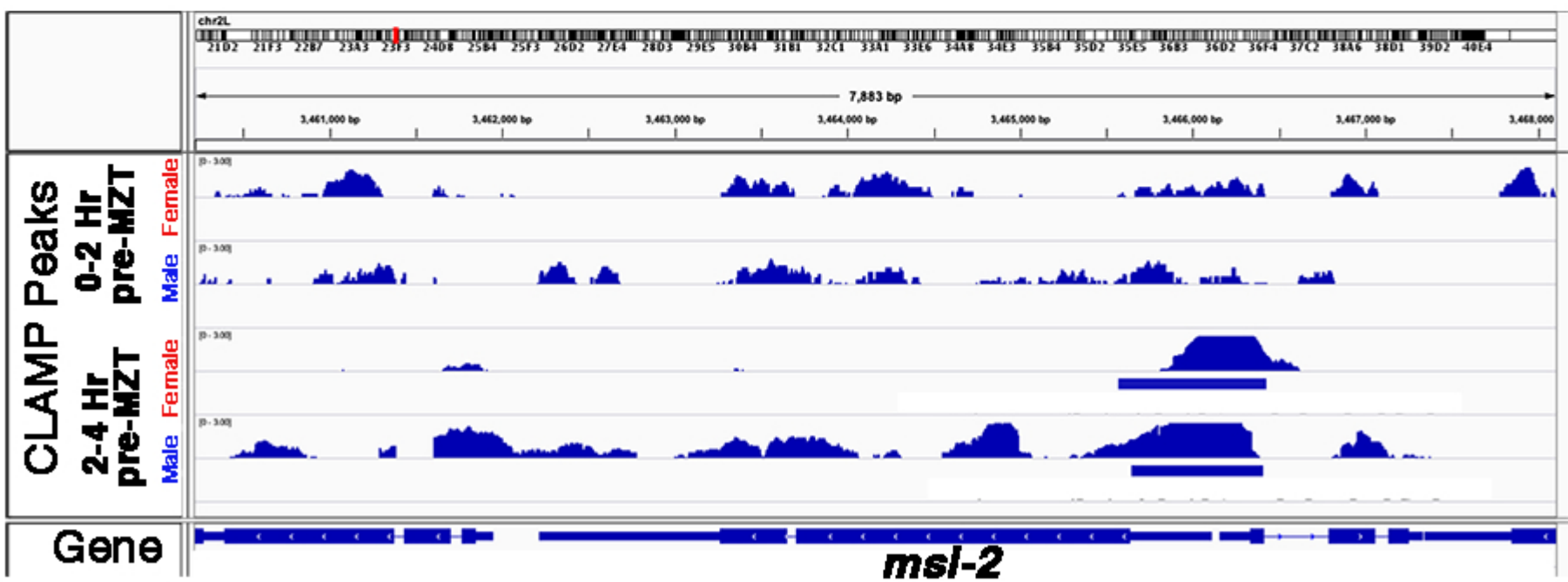
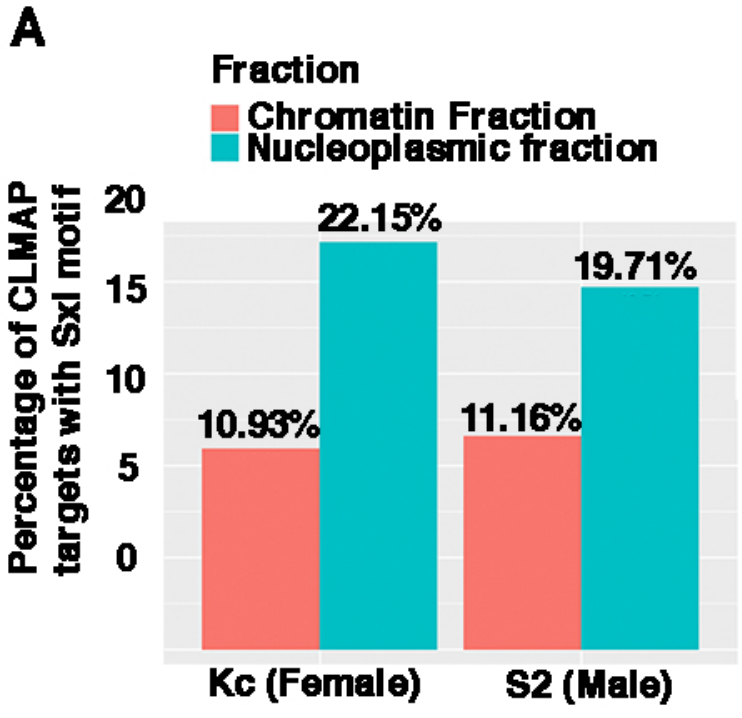
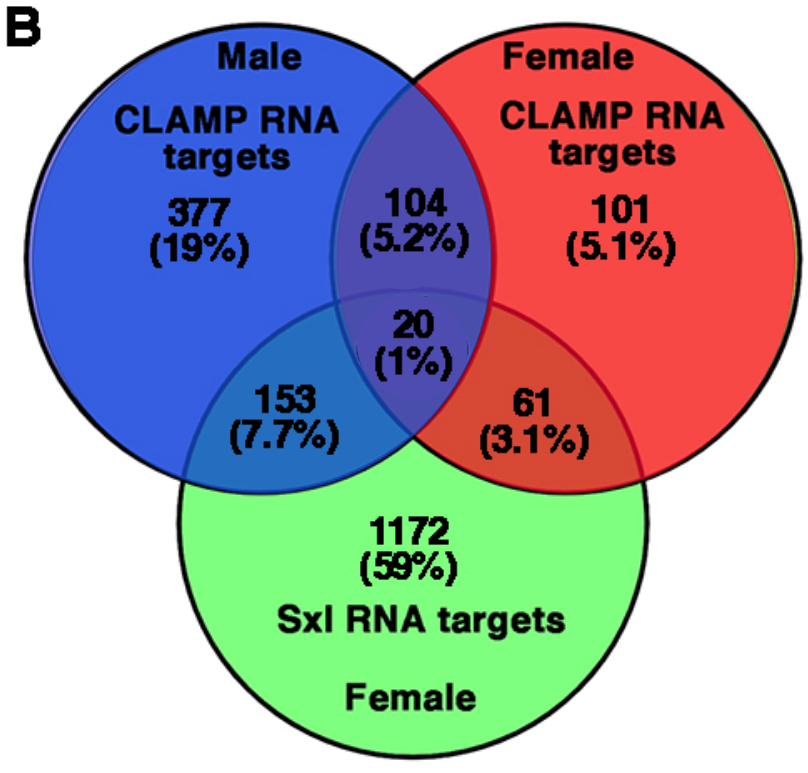
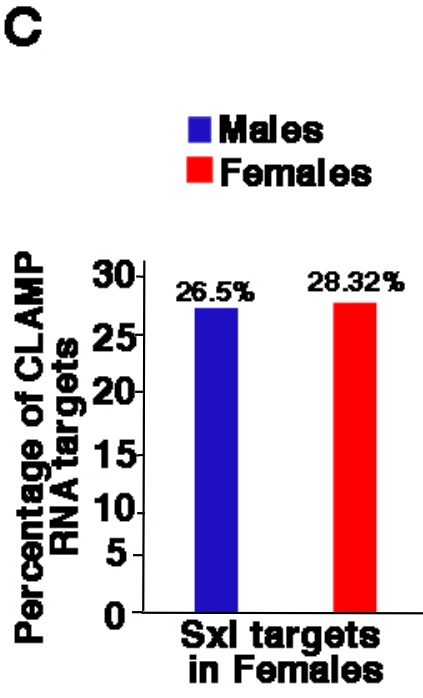



Fig S13 Ray, Conard et al 2022