

1 **m6A potentiates *Sxl* alternative pre-mRNA splicing for robust *Drosophila* sex**
2 **determination**

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26 *N6*-methyladenosine (m6A) is the most common internal modification of eukaryotic
27 messenger RNA (mRNA) and is decoded by YTH domain proteins¹⁻⁷. The mammalian
28 mRNA m6A methylosome is a complex of nuclear proteins that include METTL3
29 (Methyltransferase-like 3), METTL14, WTAP (Wilms tumour 1 associated protein) and
30 KIAA1429. *Drosophila* has corresponding homologues named dIME4 and dKAR4 (Inducer
31 of meiosis-4 and Karyogamy protein-4), and Female-lethal(2)d (Fl(2)d) and Virilizer (Vir)⁸⁻
32 ¹². In *Drosophila*, *fl(2)d* and *vir* are required for sex-dependent regulation of alternative
33 splicing (AS) of the sex determination factor *Sex-lethal* (*Sxl*)¹³. However, the functions of
34 m6A in introns in the regulation of AS remain uncertain³. Here we show that m6A is
35 absent in mRNA of *Drosophila* lacking *dIME4*. In contrast to mouse and plant knock-out
36 models^{5,7,14}, *Drosophila dIME4* null mutants remain viable, though flightless and show a
37 sex bias towards maleness. This is because m6A is required for female-specific AS of *Sxl*,
38 which determines female physiognomy, but also translationally represses *male-specific*
39 *lethal2* (*msl-2*) to prevent dosage compensation normally occurring in males. We further
40 show that the m6A reader protein YT521-B decodes m6A in the sex-specifically spliced
41 intron of *Sxl*, as its absence phenocopies *dIME4* mutants. Loss of m6A also affects AS of
42 additional genes, predominantly in the 5'UTR, and has global impacts on the expression of
43 metabolic genes. Requirement of m6A and its reader YT521-B for female-specific *Sxl* AS
44 reveal that this hitherto enigmatic mRNA modification constitutes an ancient and specific
45 mechanism to adjust levels of gene expression.

46 In mature mRNA the m6A modification is most prevalently found around the stop codon as well
47 as in 5'UTRs and in long exons in mammals, plants and yeast^{2,3,6,7,15}. Since methylosome
48 components predominantly localize to the nucleus it has been speculated that m6A localized in

49 pre-mRNA introns could have a role in AS regulation in addition to such a role when present in
50 long exons^{9-12,16}. This prompted us to investigate whether m6A is required for *Sxl* AS, which
51 determines female sex and prevents dosage compensation in females¹³. We generated a null
52 allele of the *Drosophila* *METTL3* methyltransferase homologue *dIME4* by imprecise excision of
53 a *P-element* inserted in the promoter region. The excision $\Delta 22-3$ deletes most of the protein-
54 coding region including the catalytic domain and is thus referred to as *dIME4*^{null} (Fig 1a). These
55 flies are viable and fertile, but flightless, and this phenotype can be rescued by a genomic
56 construct restoring *dIME4* (Fig 1a and b). *dIME4* shows increased expression in the brain, and
57 like in mammals and plants¹⁷, localizes to the nucleus (Fig 1c,d).

58 Following RNase T1 digestion and ³²P end-labeling of RNA fragments we detected m6A after G
59 in polyA mRNA of adult flies at relatively low levels compared to other eukaryotes (m6A/A
60 ratio: 0.06%, Fig. 1g)^{2,3,5}, but higher in unfertilized eggs (0.18%, Extended Data Fig. 1). After
61 enrichment with an anti-m6A antibody m6A is readily detected in polyA mRNA, but absent
62 from *dIME4*^{null} (Fig. 1h-j).

63 As found in other systems and consistent with a potential role in translational regulation¹⁸⁻²¹,
64 m6A was detected in polysomal mRNA (0.1%, Fig. 1k), but not in the poly(A)-depleted
65 ribosomal RNA (rRNA) fraction. This also confirmed that any m6A modification in rRNA is not
66 after G in *Drosophila* (Fig. 1l).

67 Consistent with our hypothesis that m6A plays a role in sex determination and dosage
68 compensation, the number of *dIME4*^{null} females was reduced to 60% compared to the number of
69 males (p<0.0001), while in the control strain female viability was 89% (Fig. 2a). The key
70 regulator of sex determination in *Drosophila* is the RNA binding protein *Sxl*, which is
71 specifically expressed in females. *Sxl* positively auto-regulates expression of itself and its target

72 *transformer* (*tra*) through AS to direct female differentiation¹³. In addition, *Sxl* suppresses
73 translation of *msl-2* to prevent up-regulation of transcription on the X-chromosome for dosage
74 compensation (Fig. 2b); full suppression also requires maternal factors²². Accordingly, female
75 viability was reduced to 13% by removal of maternal m6A together with zygotic heterozygosity
76 for *Sxl* and *dIME4* (*dIME4*^{Δ22-3} females crossed with *Sxl*^{7B0} males, a *Sxl* null allele, p<0.0001).
77 Female viability of this genotype is completely rescued by a genomic construct (Fig. 2a) or by
78 preventing ectopic activation of dosage compensation by removal of *msl-2* (*msl-*
79 *2²²⁷/Df(2L)Exel7016*, Fig. 2a). Hence, females are non-viable due to insufficient suppression of
80 *msl-2* expression resulting in up-regulation of gene expression on the X-chromosome from
81 reduced *Sxl* levels. In the absence of *msl-2*, disruption of *Sxl* AS resulted in females with sexual
82 transformations (32%, n=52) displaying male-specific features such as sex combs (Fig. 2c-e),
83 which were mosaic to various degrees indicating that *Sxl* threshold levels are affected early
84 during establishment of sexual identities of cells and/or their lineages¹³. In the presence of
85 maternal *dIME4*, *Sxl* and *dIME4* do not genetically interact (*Sxl*^{7B0}/*FM7* females crossed with
86 *dIME4*^{null} males, 103% female viability, n=118). In addition, *Sxl* is required for germline
87 differentiation in females and its absence results in tumorous ovaries²³. Consistent with this we
88 detected tumorous ovaries in *Sxl*^{7B0/+}; *dIME4*^{null/+} daughters from *dIME4*^{null} females (22%,
89 n=18, Extended Data Fig. 2), but not in homozygous *dIME4*^{null} or heterozygous *Sxl*^{7B0} females
90 (n=20 each).
91 Furthermore, levels of the *Sxl* female-specific splice form were reduced to ~50% consistent with
92 a role for m6A in *Sxl* AS (Fig. 2f and Extended Data Fig. 3a). As a result, female-specific splice
93 forms of *tra* and *msl-2* were also significantly reduced in adult females (Fig. 2f and Extended
94 Data Fig. 3b,c).

95 To obtain more comprehensive insights into *Sxl* AS defects in *dIME4^{null}* females, we examined
96 splice junction reads from RNA-seq. Besides the significant increase in inclusion of the male-
97 specific *Sxl* exon in *dIME4^{null}* females (Fig. 2f- h, and Extended Data Fig. 3a), cryptic splice sites
98 and increased numbers of intronic reads were detected in the regulated intron. Consistent with
99 our RT-PCR analysis of *tra*, the reduction of female splicing in the RNA sequencing is modest,
100 and as a consequence, AS differences of Tra targets *dsx* and *fru* were not detected in whole flies,
101 suggesting cell-type specific fine-tuning required to generate splicing robustness rather than
102 being an obligatory regulator (Extended Data Fig. 4a-c). In agreement with dosage compensation
103 defects as main consequence of *Sxl* miss-regulation in *dIME4^{null}* mutants, X-linked, but not
104 autosomal, genes are significantly up-regulated in *dIME4^{null}* females compared to the control
105 ($p < 0.0001$, Extended Data Fig. 4d,e).

106 Further, we also find enrichment of *Sxl* mRNA in pull-downs with an m6A antibody compared to
107 m6A-deficient yeast mRNA added for quantification (Fig. 2i). This enrichment is comparable to
108 what was observed for m6A-methylated mRNA in yeast²⁴.

109 To further map m6A sites in the intron of *Sxl* we employed an in vitro m6A methylation assay
110 using *Drosophila* nuclear extracts and labeled substrate RNA. m6A methylation activity was
111 detected in the vicinity of alternatively spliced exons (Fig. 2j, RNAs B, C, and E). Further fine-
112 mapping localized m6A in RNAs C and E to the proximity of *Sxl* binding sites (Extended Data
113 Fig. 5). Likewise, the female-lethal single amino acid substitution alleles *fl(2)^{d1}* and *vir^{2F}*
114 interfere with *Sxl* recruitment, resulting in impaired *Sxl* auto-regulation and inclusion of the
115 male-specific exon²⁵. Female lethality of these alleles can be rescued by *dIME4^{null}*
116 heterozygosity ($p < 0.0001$, Fig. 2k), further demonstrating involvement of the m6A methylosome
117 in *Sxl* AS.

118 Next, we globally analyzed AS changes in *dIME4^{null}* females compared to the wild-type control
119 strain. As described earlier (Fig. 2h), a statistically significant reduction in female-specific AS of
120 *Sxl* ($\Delta\psi=0.34$, $q=9\times 10^{-8}$) was observed. In addition, 243 AS events in 163 genes were
121 significantly different in *dIME4^{null}* females ($q<0.05$, $\Delta\psi>0.2$), equivalent to ~2% of
122 alternatively spliced genes in *Drosophila* (Suppl. Table 1). Six genes for which the AS products
123 could be distinguished on agarose gels were confirmed by RT-PCR (Extended Data Fig. 6).
124 Interestingly, lack of *dIME4* did not affect global AS and no specific type of AS event was
125 preferentially affected. However, alternative first exons (18% vs 33%) and mutually exclusive
126 exon (2% vs 15%) events were reduced mostly to the extent of retained introns (16% vs 6%),
127 alternative donor (16% vs 9%) and unclassified events (14% vs 6%) compared to a global
128 breakdown of AS in *Drosophila* (Extended Data Fig. 7a). Interestingly, the majority of affected
129 AS events in *dIME4^{null}* were located to the 5'UTR, and these genes had a significantly higher
130 number of AUGs in their 5'UTR compared to the 5'UTRs of all genes (Extended Data Fig.
131 7b,c). Such feature had been shown relevant to translational control under stress conditions²⁶.
132 The majority of the 163 differentially alternatively spliced genes in *dIME4* females are broadly
133 expressed (59%), while most of the remainder are expressed in the nervous system (33%),
134 consistent with higher expression of *dIME4* in this tissue (Extended Data Fig. 7d). Accordingly,
135 gene ontology (GO) analysis revealed a highly significant enrichment for genes in synaptic
136 transmission ($p<7\times 10^{-7}$, Suppl. Table 1).
137 Since the absence of m6A affects AS, m6A marks are probably deposited co-transcriptionally
138 before splicing. Co-staining of polytene chromosomes with antibodies against HA-tagged *dIME4*
139 and RNA Pol II revealed broad co-localization of *dIME4* with sites of transcription (Fig. 3a-e),
140 but not with condensed chromatin visualized with antibodies against histone H4 (Fig. 3f-i).

141 Furthermore, localization of dIME4 to sites of transcription is RNA-dependent, as staining for
142 dIME4, but not for RNA Pol II, was reduced in an RNase-dependent manner (Fig. 3j,k).

143 Although m6A levels after G are low in *Drosophila* compared to other eukaryotes, broad co-
144 localization of dIME4 to sites of transcription suggests profound effects on the gene expression
145 landscape. Indeed, differential gene expression analysis revealed 408 differentially expressed
146 genes (≥ 2 -fold change, $q \leq 0.01$) where 234 genes were significantly up- and 174 significantly
147 down-regulated in neuron-enriched head/thorax of adult *dIME4^{null}* females ($q < 0.01$, at least two-
148 fold, Suppl. Table 2). Cataloguing these genes according to function reveals prominent effects on
149 gene networks involved in metabolism including reduced expression of 17 genes involved in
150 oxidative phosphorylation ($p < 0.0001$, Suppl. Table 2). Notably, overexpression of the m6A
151 mRNA demethylase FTO in mice leads to an imbalance in energy metabolism resulting in
152 obesity²⁷.

153 Next, we tested whether either of the two substantially divergent YTH proteins, YT521-B and
154 CG6422 (Fig. 4a) decodes m6A marks in *Sxl* mRNA. When transiently transfected into male S2
155 cells, YT521-B localizes to the nucleus, whereas CG6422 is cytoplasmic (Fig. 4b-d, Ext. Data
156 Fig. 8). Nuclear YT521-B can switch *Sxl* AS to the female mode and also binds to the *Sxl* intron
157 in S2 cells (Fig. 4e,f). In vitro binding assays with the YTH domain of YT521-B indeed
158 demonstrate increased binding of m6A-containing RNA (Ext. Data Fig. 9). In vivo, YT521-B
159 also localizes to sites of transcription (Ext. Data Fig. 10).

160 To further examine the role of YT521-B in decoding m6A we analyzed *Drosophila* strain
161 *YT521-B^{MI02006}* where a transposon in the first intron disrupts *YT521-B*. This allele is also viable
162 (*YT521-B^{MI02006}/Df(3L)Exel6094*; Fig. 4g,h,j), and phenocopies the flightless phenotype and the
163 female *Sxl* splicing defect of *dIME4^{null}* (Fig. 4h,i). Likewise, removal of maternal *YT521-B*

164 together with zygotic heterozygosity for *Sxl* and *YT521-B* reduced female viability ($p < 0.0001$,
165 Fig. 4j) and resulted in sexual transformations (57%, $n=32$) such as male abdominal
166 pigmentation (Fig. 4k-m). In addition, overexpression of *YT521-B* results in male lethality,
167 which can be rescued by removal of *dIME4* further reiterating the role of m6A in *Sxl* AS
168 ($p < 0.0001$, Fig. 4n). Since *YT521-B* phenocopies *dIME4* for *Sxl* splicing regulation it is the main
169 nuclear factor for decoding m6A present in the proximity of the *Sxl* binding sites. *YT521-B*
170 bound to m6A assists *Sxl* in repressing inclusion of the male-specific exon, thus providing
171 robustness to this vital gene regulatory switch (Fig. 4o).

172 Nuclear localization of m6A methylome components suggested a role for this “fifth”
173 nucleotide in AS regulation. Our discovery of the requirement of m6A and its reader *YT521-B*
174 for female-specific *Sxl* AS has important implications for understanding the fundamental
175 biological function of this enigmatic mRNA modification. Its key role in providing robustness to
176 *Sxl* AS to prevent ectopic dosage compensation and female lethality, together with localization of
177 the core methylome component *dIME4* to sites of transcription, indicates that the m6A
178 modification is part of an ancient, yet unexplored mechanism to adjust gene expression. Hence,
179 the recently reported role of m6A methylome components in human dosage compensation^{28,29}
180 further support such role and suggests that m6A-mediated adjustment of gene expression might
181 be a key step to allow for development of the diverse sex determination mechanisms found in
182 nature.

183

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189

190 **Author contributions**

191 I.U.H. and M.S. performed biochemistry, cell biology and genetic experiments, E.S.M. stained
192 chromosomes, and Z.B., N.A. and R.F. performed biochemistry experiments. N.M. analyzed
193 sequencing data. I.U.H., R.F. and M.S. conceived the project and wrote the manuscript with help
194 from N.M. and Z.B.

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268

269 **Figure legends**

270 **Figure 1: Analysis of *dIME4* null mutants and m6A methylation in *Drosophila*.** **a**, Genomic
271 organization of the *dIME4* locus depicting the transposon (black triangle) used to generate the
272 deletion $\Delta 22-3$, which is a *dIME4* null allele and the hemagglutinin (HA)-tagged genomic rescue
273 fragment. **b**, Flight ability of *dIME4*^{null}/*Df(3R)Exel6197* shown as mean \pm SE ($n=3$). *gdIME4*:
274 genomic rescue construct. **c** and **d**, Nuclear localization of *dIME4*::HA in eye discs and brain
275 neurons expressed from *UAS*. Scale bars: 50 and 1 μ m. **e**, Schematic diagram of a 2D thin layer
276 chromatography (TLC). **f**, TLC from an *in vitro* transcript containing m6A. **g**, TLC from mRNA
277 of adult flies. **h** and **i**, TLC of fragmented mRNA after enrichment with an anti-m6A antibody

278 from wild type (**h**) and *dIME4^{null}* (**i**, overexposed). **j**, Quantification of immunoprecipitated ³²P
279 label shown as normalized mean (*n*=2). **k** and **l**, TLC from mRNA (**k**) or rRNA (**l**) from
280 polysomes from wild-type flies.

281

282 **Figure 2: m6A methylation is required for *Sex-lethal* AS in sex determination and dosage**

283 **compensation.** **a**, Female viability of indicated genotypes devoid of maternal m6A (*n*: total

284 number of flies). **b**, Schematic depicting Sxl control of female differentiation. **c-e**, Front legs of

285 indicated genotypes. Scale bar: 100 μm. The arrowhead points towards the position of the sex

286 comb normally present only in males. **f**, Ratio of sex-specific splice isoforms from adult females

287 from RT-PCR shown as mean±SE (*n*=3, *p*<0.01). **g**, RT-PCR for male-specific *Sxl* splicing in

288 control and *dIME4^{null}* females. **h**, Sashimi plot depicting Tophat-mapped RNA sequencing reads

289 and exon junction reads from control and *dIME4^{null}* females below the annotated gene model.

290 Male-specific splice junction reads are circled and cryptic splice sites are boxed. RNA fragments

291 used for m6A *in vitro* methylation assays are indicated at the bottom. **i**, Presence of m6A in *Sxl*

292 transcripts detected by m6A immunoprecipitation followed by qPCR from nuclear mRNA of

293 early embryos (shown as mean, *n*=2). **j**, 1D-TLC of *in vitro* methylated, [³²P]-ATP-labeled

294 substrate RNAs shown in **g**. Nucleotide markers from *in vitro* transcripts in the absence (M1) or

295 presence (M2) of m6A. The right part shows an overexposure of the same TLC. **k**, Rescue of

296 female lethality of female-lethal *fl(2)^{d1}* and *vir^{2F}* alleles by removal of one copy of *dIME4*.

297

298 **Figure 3: dIME4 co-localizes to sites of transcription.** **a-e**, Polytene chromosomes from

299 salivary glands expressing dIME::HA stained with anti-Pol II (red, **c**), anti-HA (green, **d**) and

300 DNA (DAPI, blue, **e**), or merged (yellow, **a** and **b**). **f-i**, Polytene chromosomes stained with anti-

301 Pol II (red, **h**), anti-histone H4 (green, **g**) and DNA (blue, **i**), or merged (yellow, **f**). Polytene
302 chromosomes treated with low (**j**, 2 µg/ml) and high (**k**, 10 µg/ml) RNase A concentration prior
303 to staining with anti-Pol II, anti-histone H4 and DNA. Scale bars in **a**, **j** and **k** are 20 µm and in **e**
304 and **i** are 5 µm.

305

306 **Fig 4: YTH protein YT521-B decodes m6A methylation in *Sxl*.** **a**, Domain organization of
307 *Drosophila* YTH proteins (YTH domain in green). n: nuclear, c: cytoplasmic **b-d**, Cellular
308 localization and size of HA-tagged YT521-B and CG6422 in S2 cells. Scale bar: 1 µm. **e**,
309 Suppression of male-specific *Sxl* AS upon expression of *Sxl* and YT521-B, but not CG6422 in
310 male S2 cells. **f**, Binding of YT521-B to pre-mRNA of the regulated *Sxl* intron. **g**, Genomic
311 organization of the *YT521-B* locus depicting the transposon (black triangle) disrupting the ORF.
312 **h**, Flight ability of *YT521-B*^{M102006}/*Df(3L)Exel6094* shown as mean±SE (*n*=3). **i**, *Sxl* AS in female
313 wild-type and *YT521-B*^{M102006}/*Df(3L)Exel6094* flies. **j**, Female viability of indicated genotypes
314 (*n*: total number of flies) reared at 29° C. **k-m**, Abdominal pigmentation of indicated genotypes
315 reared at 29 °C. The arrowhead points towards the position of the dark pigmentation normally
316 present only in males. Scale bar: 100 µm. **n**, YT521-B was overexpressed from a *UAS* transgene
317 with *tubulinGAL4* (2nd) in wild type or *dIME4*^{null} at 27 °C. **o**, Model for female-specific *Sxl* AS
318 by *Sxl*, m6A and its reader YT521-B in co-operatively suppressing inclusion of the male-specific
319 exon.

320

321 **Online Methods**

322 **Data reporting**

323 No statistical methods were used to predetermine sample size. The experiments were not
324 randomized and the investigators were not blinded to allocation during experiments and outcome
325 assessment.

326

327 ***Drosophila* genetics, generation of constructs and transgenic lines**

328 The deletion allele *dIME4*^{Δ22-3} was obtained from imprecise excision of the transposon *P{SUPor-*
329 *P}{KrT95D* and mapped by primers 5933 F1 (CTCGCTCTATTTCTCTTCAGCACTCG) and
330 5933 R9 (CCTCCGCAACGATCACATCGCAATCGAG). To obtain a viable line of *dIME4*^{null},
331 the genetic background was cleaned by out-crossing to *Df(3R)Exel6197*. Flight ability was
332 scored as number of flies capable of flying out of a petri-dish within 30 sec for groups of 15-20
333 flies for indicated genotypes. Viability was calculated from the numbers of females compared to
334 males of the correct genotype and statistical significance was determined by a χ^2 test (GraphPad
335 Prism). Unfertilized eggs were generated by expressing sex-peptide in virgin females as
336 described³⁰.

337 The genomic rescue construct was retrieved by recombineering (Genebridges) from BAC clone
338 *CH321-79E18* by first cloning homology arms with *SpeI* and *Acc65I* into *pUC3GLA* separated
339 by an *EcoRV* site for linearization
340 (CTCCGCCGCCGGAACCGCCGCCTCCTCCGCCACTTTGCAGGTTGAGCGGACCGCCT
341 CCAGGGCCGCTGCCGCCGGTGCCGCTGATATCCCAGCATGGTAGCTGCGGCCACTCC
342 TAGTCCCGCCTTTAACCACAGCTTGGGGTCCTCCGTCATCAGGCCGAATTGCCTCGA
343 G). An HA-tag was then fused to the end of the ORF using two PCR amplicons and *SacI* and
344 *XhoI* restriction sites. This construct was the inserted into *PBac{y+-attB-3B}VK00002* at 76A as
345 described³¹.

346 The dIME4 UAS construct was generated by cloning the ORF from fly cDNA into a modified
 347 *pUAST* with primers *Adh* dMT-A70 F1 EI
 348 (GCAGAATTCGAGATCtAAAGAGCCTGCTAAAGCAAAAAAGAAGTCACCATGGCAGA
 349 TCGTGGGACATAAAATCAC) and dMT-A70 HA R1 Spe
 350 (GGTAACTAGTCTTTTGTATTCCATTGATCGACGCCGATTGG) by adding a translation
 351 initiation site from the *Adh* gene and two copies of an HA tag to the end of the ORF. This
 352 construct was then also inserted into *PBac{y+attB-3B}VK00002* at 76A.
 353 For transient transfection in S2 cells, *YT52B-1* and *CG6422* ORFs were amplified from fly
 354 cDNA by a combination of nested and fusion PCR incorporating a translation initiation site from
 355 the *Adh* gene using primers *CG6422* *adh* F1
 356 (GCCTGCTAAAGCAAAAAAGAAGTCACCACATGTCAGGCGTGGATCAGATGAAAAT
 357 ACCAG), *pact* *adh* *CG6422* F1
 358 (CCAGAGACCCCGGATCCAGATATCAAAGAGCCTGCTAAAGCAAAAAAGAAGTCAC
 359 CAC), *CG6422* *adh* R1, (GATTCCTGCGAACAGGTCCCGTGGGCGAAAC) and *CG6422* 3'
 360 F1 (CCCACGGGACCTGTTCGCAGGAATCTAG), *CG6422* 3' R1
 361 (CATTGCTTCGCATTTTATCCTTGTCCGTGTCCTTAAAGCGCACGCCGATTTTAATTTG
 362), *pact* *CG6422* 3xHA R1
 363 (GTGGAGATCCATGGTGGCGGAGCTCGAGGAATATTCATTGCTTCGCATTTTATCCTT
 364 GTC) for *CG6422* and primers *YT521* *adh* F1,
 365 (AAGCAAAAAAGAAGTCACATGCCAAGAGCAGCCCGTAAACAAACGCTGCCGATGC
 366 GCGAG), *pact* *adh* *YT521* F1
 367 (CCAGAGACCCCGGATCCAGATATCAAAGAGCCTGCTAAAGCAAAAAAGAAGTCAC
 368 ATGCC), *YT521* *adh* R1

369 (TGCCATCCGGGCGAATCCTGCAAATTTACCACTCTCGTTGACCGAGAAAATGAGCA
370 GGAC) and YT521 3' F1(GCAGGATTCGCCCCGGATGGCAGCCCCCTCAC), Pact YT521 R1
371 (GGTGGAGATCCATGGTGGCGGAGCTCGAGCGCCTGTTGTCCCGATAGCTTCGCTG)
372 for *YT521-B*, and cloned into a modified *pACT* using Gibson Assembly (NEB) also incorporating
373 HA epitope tags at the C terminus. Constructs were verified by Sanger sequencing. The Sxl-HA
374 expression vector was a gift from N. Perrimon³².
375 The YT521-B UAS construct was generated by sub-cloning the ORF from the pACT vector into
376 a modified *pUAST* with primers YT521 adh F1
377 (AAGCAAAAAGAAGTCACATGCCAAGAGCAGCCCGTAAACAAACGCTGCCGATGC
378 GCGAG), YT521 adh F2
379 (TAGGGAATTGGGAATTCGAGATCTAAAGAGCCTGCTAAAGCAAAAAGAAGTCAC
380 ATGCC) and YT521 3' R1
381 (GGGCACGTCGTAGGGGTACAGACTAGTCTCGAGGCGCCTGTTGTCCCGATAGCTTC
382 GCTG) by adding a translation initiation site from the *Adh* gene and two copies of an HA tag to
383 the end of the ORF. This construct was then also inserted into *PBac{y+-attB-3B}VK00002* at
384 76A.

385 Essential parts of all DNA constructs were sequence verified.

386

387 **Cell culture, transfections and immune-staining of S2 cells**

388 S2 cells (ATCC) were cultured in Insect Express medium (Lonza) with 10% heat-inactivated
389 FCS and 1% penicillin/streptomycin. The *Drosophila* S2 cell line was verified to be male by
390 analysing *Sxl* alternative splicing using species-specific primers Sxl F2
391 (ATGTACGGCAACAATAATCCGGGTAG) and Sxl R2

392 (CATTGTAACCACGACGCGACGATG) to confirm species and gender (Ext. Data Fig 8).

393 Transient transfections were done with Mirus Reagent (Bioline) according to the manufacturer's
394 instruction and cells were assayed 48 h after transfection for protein expression or RNA binding
395 of expressed proteins. To adhere S2 cells to a solid support, Concanavalin A (Sigma) coated
396 glass slides (in 0.5 mg/ml) were added 1 d prior to transfection, and cells were stained 48 h after
397 transfection with antibodies as described. Transfections and follow up experiments were repeated
398 at least once.

399

400 **RNA extraction, RT-PCR, qPCR, immune-precipitations and Western blots**

401 Total RNA was extracted using Tri-reagent (SIGMA) and reverse transcription was done with
402 Superscript II (Invitrogen) according to the manufacturer's instructions using an oligodT17V
403 primer. PCR for *Sxl*, *tra*, *msl2* and *ewg* was done for 30 cycles with 1 µl of cDNA with primers
404 *Sxl* F2, *Sxl* R2 or *Sxl* NP R3 (GAGAATGGGACATCCCAAATCCACG), *Sxl* M F1
405 (GCCCAGAAAGAAGCAGCCACCATTATCAC), *Sxl* M R1
406 (GCGTTTCGTTGGCGAGGAGACCATGGG), *tra* FOR (GGATGCCGACAGCAGTGGAAC),
407 *tra* REV (GATCTGGAGCGAGTGCGTCTG), *msl-2* F1
408 (CACTGCGGTCACACTGGCTTCGCTCAG), *msl-2* R1
409 (CTCCTGGGCTAGTTACCTGCAATTCCTC), *ewg* 4F and *ewg* 5R and quantified with
410 ImageQuant (BioRad)²². Experiments included at least three biological replicates.

411 For qPCR reverse transcription was carried out on input and pull-down samples spiked with
412 yeast RNA using ProtoScript II reverse transcriptase and random nanomers (NEB). Quantitative
413 PCR was carried out using 2x SensiMix Plus SYBR Low ROX master mix (Quantace) using
414 normalizer primers *ACT1* F1 (TTACGTCGCCTTGGACTTCG) and *ACT1* R1

415 (TACCGGCAGATTCCAAACCC) and for Sxl, Sxl ZB F1 (CACCACAATGGCAGCAGTAG)
416 and Sxl ZB R1 (GGGGTTGCTGTTTGTGAGT). Samples were run in triplicate for technical
417 repeats and duplicate for biological repeats. Relative enrichment levels were determined by
418 comparison with yeast *ACT1*, using the $2^{-\Delta\Delta C_T}$ method³³.
419 For immunoprecipitations of *Sxl* RNA bound to Sxl or YTH proteins, S2 cells were fixed in PBS
420 containing 1% formaldehyde for 15 min, quenched in 100 mM glycine and disrupted in IP-
421 Buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% NP-40, 5% glycerol). After IP with anti-
422 HA beads (Sigma) for 2 h in the presence of Complete protein inhibitors (Roche) and 40 U
423 RNase inhibitors (Roche), IP precipitates were processed for *Sxl* RT-PCR using gene-specific RT
424 primer SP NP2 (CATTCCGGATGGCAGAGAATGGGAC) and PCR primers Sxl NP intF
425 (GAGGGTCAGTCTAAGTTATATTCG) and Sxl NP R3 as described³¹. Western blots were
426 done as described using rat anti-HA (1:50, clone 3F10, Roche) and HRP coupled secondary goat
427 anti-rat antibodies (Molecular Probes)³⁴. All experiments were repeated at least once from
428 biological samples.

429

430 **Analysis of m6A levels**

431 PolyA mRNA from at least two rounds of oligo dT selection was prepared according to the
432 manufacturer (Promega). For each sample, 10-50 ng of mRNA was digested with 1 μ l of
433 Ribonuclease T1 (1000 U/ μ l; Fermentas) in a final volume of 10 μ l in polynucleotide kinase
434 buffer (PNK, NEB) for 1 h at 37 °C. The 5' end of the T1-digested mRNA fragments were then
435 labeled using 10 U T4 PNK (NEB) and 1 μ l [γ -³²P]-ATP (6000 Ci/mmol; Perkin-Elmer). The
436 labeled RNA was precipitated, resuspended in 10 μ l of 50 mM sodium acetate buffer (pH 5.5),
437 and digested with P1 nuclease (Sigma-Aldrich) for 1 h at 37 °C. Two microliters of each sample

438 was loaded on cellulose TLC plates (20x20 cm; Fluka) and run in a solvent system of isobutyric
439 acid: 0.5 M NH₄OH (5:3, v/v), as first dimension, and isopropanol:HCl:water (70:15:15, v/v/v),
440 as the second dimension. TLCs were repeated from biological replicates. The identification of
441 the nucleotide spots was carried out using m⁶A-containing synthetic RNA. Quantification of ³²P
442 was done by scintillation counting (Packard Tri-Carb 2300TR). For the quantification of spot
443 intensities on TLCs or gels, a storage phosphor screen (K-Screen; Kodak) and Molecular Imager
444 FX in combination with QuantityOne software (BioRad) were used.
445 For immunoprecipitation of m⁶A mRNA, polyA mRNA was digested with RNase T1 and 5'
446 labeled. The volume was then increased to 500 µl with IP buffer (150 mM NaCl, 50 mM Tris–
447 HCL, pH 7.5, 0.05% NP-40). IPs were then done with 2 µl of affinity-purified polyclonal rabbit
448 m⁶A antibody (Synaptic Systems) and protein A/G beads (SantaCruz).

449

450 **Polysome profiles**

451 Whole fly extracts were prepared from 20-30 adult *Drosophila* previously frozen in liquid N₂ and
452 ground into fine powder in liquid N₂. Cells were then lysed in 0.5 ml lysis buffer (0.3 M NaCl,
453 15 mM MgCl₂, 15 mM Tris-HCl pH 7.5, cycloheximide 100 µg/ml, heparin (sodium salt) 1
454 mg/ml, 1% Triton X-100). Lysates were loaded on 12 ml sucrose gradients and spun for two h at
455 38 000 rpm at 4 °C. After the gradient centrifugation 1 ml fractions were collected and
456 precipitated in equal volume of isopropanol. After several washes with 80% ethanol the samples
457 were resuspended in water and processed. Experiments were done in duplicate.

458

459 **Nuclear extract preparation and *in vitro* m⁶A methylation essays**

460 *Drosophila* nuclear extracts were prepared from Kc cells as described³⁵. Templates for *in vitro*
461 transcripts were amplified from genomic DNA using the primers listed below and *in vitro*
462 transcribed with T7 polymerase in the presence of [α -³²P]-ATP. DNA templates and free
463 nucleotides were removed by DNase I digestion and Probequant G-50 spin columns (GE
464 Healthcare), respectively. Markers were generated by using *in vitro* transcripts with or without
465 m⁶ATP (Jena Bioscience), which were then digested with RNase T1, kinased with PNK in the
466 presence of [γ -³²P]-ATP. After phenol extraction and ethanol precipitation, transcripts were
467 digested to single nucleotides with P1 nuclease as above. For *in vitro* methylation, transcripts
468 (0.5-1x10⁶ cpm) were incubated for 45 min at 27 °C in 10 μ l containing 20 mM potassium
469 glutamate, 2 mM MgCl₂, 1 mM DTT, 1 mM ATP, 0.5 mM S-adenosylmethionine disulfate
470 tosylate (Abcam), 7.5% PEG 8000, 20 U RNase protector (Roche) and 40% nuclear extract.
471 After phenol extraction and ethanol precipitation, transcripts were digested to single nucleotides
472 with P1 nuclease as above, and then separated on cellulose F TLC plates (Merck) in 70%
473 ethanol, previously soaked in 0.4 M MgSO₄ and dried³⁶. *In vitro* methylation assays were done
474 from biological replicates at least in duplicates.

475 Primers to amplify parts of the *Sxl* alternatively spliced intron from genomic DNA for *in vitro*
476 transcription with T7 polymerase were *Sxl* A T7 F
477 (GGAGCTAATACGACTCACTATAGGGAGAGGATATGTACGGCAACAATAATCCGGGT
478 AG) and *Sxl* A R (CGCAGACGACGATCAGCTGATTCAAAGTGAAAG), *Sxl* B T7 F
479 (GGAGCTAATACGACTCACTATAGGGAGAGCGCTCGCATTATCCACAGTCGCAC)
480 and *Sxl* B R (GGGTGCCCTCTGTGGCTGCTCTGTTTAC), *Sxl* C T7 F
481 (GGAGCTAATACGACTCACTATAGGGGTCGTATAATTTATGGCACATTATTCAG) and
482 *Sxl* C R (GGGAGTTTTGGTTCTTGTTTATGAGTTGGGTG), *Sxl* D T7 F

483 (GGAGCTAATACGACTCACTATAGGGAGAAAACCTTCCAGCCCACACAACACACAC)
 484 and Sxl D R (GCATATCATATTCGGTTCATACATTTAGGTCTAAG), Sxl E T7 F
 485 (GGAGCTAATACGACTCACTATAGGGAGAGGGGAAGCAGCTCGTTGTAAAATAC)
 486 and Sxl E R (GATGTGACGATTTTGCAGTTTCTCGACG), Sxl F T7 F
 487 (GGAGCTAATACGACTCACTATAGGGAGAGGGGGATCGTTTTGAGGGTCAGTCTAAG
 488) and Sxl NP2, Sxl C T7 F and Sxl C1 R (GTAGTTTTGCTCGGCATTTTATGACCTTGAGC),
 489 Sxl C2 F
 490 (GGAGCTAATACGACTCACTATAGGGAGACTCTCATTCTCTATATCCCTGTGCTGACC
 491) and Sxl C2 R (CTAATTCGTGAGCTTGATTTTATTTTGCACAG), Sxl C3 F
 492 (GGAGCTAATACGACTCACTATAGGGAGACTGTGCAAAATGAAATCAAGCTCACGA
 493 AATTAG) and Sxl C R, Sxl E T7 F and Sxl E1 R
 494 (AAAAAATCAAAAAATAATCACTTTTGGCACTTTTTCATCAC), Sxl E2 F
 495 (GGAGCTAATACGACTCACTATAGGGAGATGAAAAAGTGCCAAAAGTGATTATTTTT
 496 TTG), Sxl E2 R (AAAAGCATGATGTATTTTTTTTTTTTTTTGTACTTTCGAATCACCG), Sxl
 497 E3 F
 498 (GGAGCTAATACGACTCACTATAGGGAGACGGTGATTTCGAAAGTACAAAAAAAAAAAA
 499 AAATAC) and Sxl E R, Sxl C4 F
 500 (GAGCTAATACGACTCACTATAGGGAGAAATACTAAAACATCAAACCGCAAGCAGA
 501 GCAGC) and Sxl C4 R (GAGTGCCACTTCAAATCTCAGATATGC), Sxl C5 F
 502 (CTAATACGACTCACTATAGGGAGACTCTTTTTTTTTTTCTTTTTTTTACTGTGCAAAA
 503 TG) and Sxl C5 R
 504 (AAAAAATATGCAAAAAAAGGTAGGGCACAAAGTTCTCAATTAC), Sxl C6 F
 505 (GAGCTAATACGACTCACTATAGGGAGACTGTGCAAAATGAAATCAAGCTCACGAA

506 ATTAG) and Sxl C6 R (CAATTTCACTATATGTACGAAAACAAAAGTGAG), Sxl E4 F
507 (GGAGCTAATACGACTCACTATAGGGAGAACCAAATTCGACGTGGGAAGAAAC)
508 and Sxl E4 R (TAATCACTTTTGGCACTTTTTCATCACATTAAC), Sxl E5 F
509 (GGCTAATACGACTCACTATAGGGAGATTTTTTTTGATTTTTTTTAAAGTGAAAATGTGC
510 TCC) and Sxl E5 R (CACCGAAAAAAAATAAAAAAAAATAATCATGGGACTATACTAG),
511 Sxl E6 F
512 (GGCTAATACGACTCACTATAGGGAGACTTAAGTGCCAATATTTAAAGTGAAACCAA
513 TTG) and Sxl E6 R (CCCCCAGTTATATTCAACCGTGAAATTCTGC).

514

515 **Illumina sequencing and analysis of differential gene expression and AS**

516 Total RNA was extracted from 15 pulverized head/thoraces previously flash frozen in liquid
517 nitrogen, using Trizol reagent from *white* (*w*) control and *w*; *dIME4*^{A22-3} females that have been
518 outcrossed for several generations to *w*; *Df(3R)Exel6197* to equilibrate genetic background. Total
519 RNA was treated with DNase I (Ambion) and stranded libraries for Illumina sequencing were
520 prepared after polyA selection from total RNA (1 µg) with the TruSeq stranded mRNA kit
521 (Illumina) using random primers for reverse transcription according to the manufacturer's
522 instructions. Pooled indexed libraries were sequenced on an Illumina HiSeq2500 to yield 40-46
523 million paired-end 100 bp reads, and in a second experiment 14-19 million single-end 125 bp
524 reads for three controls and mutants each. After demultiplexing, sequence reads were aligned to
525 the *Drosophila* genome (dmel-r6.02) using Tophat2.0.6³⁷. Differential gene expression was
526 determined by Cufflinks-Cuffdiff and the FDR-correction for multiple testings to raw P values
527 with $q < 0.05$ considered significant³⁸. AS was analysed by SPANKI³⁹ and validated for selected
528 genes based on length differences detectable on agarose gels. Illumina sequencing, differential

529 gene expression and AS analysis was done by Fasteris (Switzerland). For dosage compensation
 530 analysis, differential expression analysis of X-linked genes versus autosomal genes in *dIME4^{null}*
 531 mutant was done by filtering Cuffdiff data by a p value expression difference significance of
 532 $p < 0.05$, which corresponds to a false discovery rate of 0.167 to detect subtle differences in
 533 expression consistent with dosage compensation. Visualization of sequence reads on gene
 534 models and splice junctions reads in Sashimi plots was done using Integrated Genome Viewer⁴⁰.
 535 For validation of AS by RT-PCR as described above, the following primers were used: Gprk2 F1
 536 (CCAACCAGCCGAAACTCACAGTGAAGC) and Gprk2 R1
 537 (CAGGGTCTCGGTTTCAGACACAGGCGTC), fl(2)d F1
 538 (GCAGCAAACGAGAAATCAGCTCGCAGCGCAG) and fl(2)d R1
 539 (CACATAGTCCTGGAATTCTTGCTCCTTG), A2bp1 F3
 540 (CTGTGGGGCTCAGGGGCATTTTCCTTCCTC) and A2bp1 R1
 541 (CTCCTCTCCCGTGTGTCTTGCCACTCAAC), cv-c F1
 542 (GGGTTTCCACCTCGACCGGGAAAAGTCG) and cv-c R1
 543 (GCGTTTGCGGTTGCTGCTCGCGAAGAGAG), CG8312 F1
 544 (GCGCGTGGCCTCCTTCTTATCGGCAGTC) and CG8312 R1
 545 (GCGTGGCCACTATAAAGTCCACCTCATC), Chas F2
 546 (CCGATTCGATTCGATTCGATCCTCTCTTC) and Chas R1
 547 (GTCGGTGTCTCGGTGGTGTGGTGGAG). GO enrichment analysis was done with
 548 FlyMine. For the analysis of uATGs, a custom R script was used to count the uATGs in 5'UTRs
 549 in all ENSEMBL isoforms of those genes which are differentially spliced in *dIME4* mutants, that
 550 were then compared to the mean number of ATGs in all *Drosophila* ENSEMBL 5'UTRs using a
 551 t-test. Gene expression data were obtained from flybase.

552 **Custom R Script**

```
553 > fasta_file <-read.fasta("Soller_UTRs.fa", as.string=T)# read fasta file
554 > pattern <- "atg" # the pattern to look for
555 > dict <-PDict(pattern, max.mismatch = 0)#make a dictionary of the pattern to look for
556 > seq <- DNAStringSet( unlist(fasta_file)[1:638])#make the DNAstringset from the
557 DNAsequences ie all 638 UTRs related to the 156 genes identified in spanki
558 > result <-vcountPDict(dict,seq)#count the pattern in each of the sequences
559 > write.csv2(result, "result.csv")
```

560

```
561 > fasta_file <-read.fasta("dmel-all-five_prime_UTR-r6.07.fa", as.string=T)# read fasta file
562 > pattern <- "atg" # the pattern to look for
563 > dict <-PDict(pattern, max.mismatch = 0)#make a dictionary of the pattern to look for
564 > seq <- DNAStringSet( unlist(fasta_file)[1:29822])#make the DNAstringset from the
565 DNAsequences ie all UTRs
566 > result <-vcountPDict(dict,seq)#count the pattern in each of the sequences
567 > write.csv2(result, "result_allutrs.csv")
```

568

569 **Polytene chromosome preparations and stainings**

570 dIME4 or YT521-B were expressed in salivary glands with *C155-GAL4* from a *UAS* transgene.
571 Larvae were grown at 18 °C under non-crowded conditions. Salivary glands were dissected in
572 PBS containing 4% formaldehyde and 1% Triton X-100, and fixed for 5 min, and then for
573 another 2 min in 50% acetic acid containing 4% formaldehyde, before placing them in
574 lactoacetic acid (lactic acid:water:acetic acid, 1:2:3). Chromosomes were then spread under a

575 siliconized cover slip and the cover slip removed after freezing. Chromosome were blocked in
576 PBT containing 0.2% BSA and 5% goat serum and sequentially incubated with primary
577 antibodies (mouse anti-PolIII H5, 1:1000, Abcam, or rabbit anti-histone H4, 1:200, Santa-Cruz,
578 and rat anti-HA MAb 3F10, 1:50, Roche) followed by incubation with Alexa488- and/or
579 Alexa647-coupled secondary antibodies (Molecular Probes) including DAPI (1 µg/ml, Sigma).
580 RNase A treatment (4 and 200 µg/ml) was done before fixation for 5 min. Ovaries were analyzed
581 as previously described⁴¹.

582

583 **RNA binding assays**

584 The YTH domain (aa 207-423) was PCR amplified with oligos YTHdom F1
585 (CAGGGGCCCTGTCTGACTAGTCCCGGGAATGGTGGCGGCAACGGCCG) and R1
586 (CACGATGAATTGCGGCCGCTCTAGATTACTTGTAGATCACGTGTATACCTTTTTCTC
587 GC) and cloned with Gibson assembly (NEB) into a modified pGEX expression vector to
588 express a GST-tagged fusion protein. The YTH domain was cleaved while GST was bound to
589 beads using Precession protease. Electrophoretic mobility shift assays and UV cross-linking
590 assays were performed as described^{35,42}. Quantification was done using ImageQuant (BioRad) by
591 measuring free RNA substrate to calculate bound RNA from input. All binding assays were done
592 at least in triplicates.

593

594 **Data availability statement:** RNA-seq data that support the findings of this study have been
595 deposited at GEO under the accession number GSE79000
596 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79000>), combining the single-end
597 (GSE78999) and paired-end (GSE78992) experiments

598 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78999> and
599 <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE78992>, respectively). All other data
600 generated or analysed during this study are included in this published article (and its
601 Supplementary Information files).

602

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634

635 **Extended Data figure legends**

636 **Extended Data Figure 1: m6A levels in unfertilized eggs.** **a** and **b**, TLC from maternal total
637 RNA (**a**) and mRNA (**b**) present in unfertilized eggs. The arrow indicates m6A.

638

639 **Extended Data Figure 2: dIME4 supports Sxl in directing germline differentiation.** **a-c**,
640 Representative ovarioles of wild type (**a**), *dIME4^{null}/dIME4^{null}* (**b**) and *Sxl/+; dIME4^{null}/+* (**c**), and
641 a tumorous ovary of a *Sxl/+; dIME4^{null}/+* female (**d**). The tumorous ovary consisting mostly of

642 undifferentiated germ cells in (d) is indicated with a bracket and the oviduct with an asterisk.
643 The scale bar in (d) is 100 μm .

644

645 **Extended Data Figure 3: *dIME4* is required for female-specific splicing of *Sxl*, *tra* and *msl-***
646 **2. a-c**, RT-PCR of *Sxl* (a), *tra* (b) and *msl-2* (c) sex-specific splicing in wild-type males and
647 females, and *dIME4*^{null} males and females. 100 bp markers are shown on the left.

648

649 **Extended Data Figure 4: AS of sex determination genes and differential expression of X-**
650 **linked genes in *dIME4*^{null} females. a-c**, Sashimi plot depicting Tophat-mapped RNA
651 sequencing reads and exon junction reads below the annotated gene model for sex-specific AS of
652 *tra*, *fru* and *dsx*. The thickness of lines connecting splice junctions corresponds to the number of
653 junction reads also shown. ss: splice site. **d**, Significantly ($p < 0.05$, $q < 0.166853$) differentially
654 expressed gene expression values expressed as reads per kb of transcript per million mapped
655 reads (RPKM) were +1 log transformed and Spearman r correlation values determined for X-
656 linked and autosomal genes in wild-type and *dIME4*^{null} *Drosophila*. **e**, The proportion of
657 autosomal and X-linked genes that were significantly either up- or down-regulated in *dIME4*^{null}
658 as compared to wild-type *Drosophila* were statistically compared using χ^2 with Yates' continuity
659 correction. GraphPad Prism was used for statistical comparisons. Similar results as for the
660 single-read RNA-seq experiment were obtained for the pair-end RNA sequencing experiment.

661

662 **Extended Data Figure 5: m6A methylation sites map to the vicinity of *Sxl* binding sites. a**,
663 Schematic of the *Sxl* alternatively-spliced intron around the male specific exon depicting
664 substrate RNAs used for *in vitro* m6A methylation. Solid lines depict fragments containing m6A

665 methylation and dashed lines fragments where m6A was absent. **b** and **c**, 1D-TLC of *in vitro*
666 methylated [³²P]-ATP-labeled substrate RNAs shown in **(a)**. Markers are *in vitro* transcripts in
667 the absence (M1) or presence (M2) of m6A ³²P-labeled after RNase T1 digestion. The right part
668 in **(b)** and **(c)** shows an overexposure of the same TLC.

669

670 **Extended Data Figure 6: RT-PCR validation of differential AS in *dIME4*^{null}. a-f**, Sashimi
671 plot depicting Tophat-mapped RNA sequencing reads and exon junction reads below the
672 annotated gene model of indicated genes on the left, and RT-PCR of AS shown on the right
673 using primers depicted on top. The thickness of lines connecting splice junctions corresponds to
674 the number of junction reads also shown.

675

676 **Extended Data Figure 7: *dIME4* affects AS predominantly in 5'UTRs in genes with a higher**
677 **than average number of upstream AUGs. a** and **b**, Classification of differential AS in
678 *dIME4*^{null} according to splicing event **(a)** and location of the event in the mRNA **(b)**. **c**,
679 Quantification of upstream AUGs in all annotated 5'UTRs (white) or in alternative isoforms
680 differentially spliced between wild type and *dIME4*^{null}. All *Drosophila* UTRs were accessed in
681 fasta format from Flybase (version r6.07),
682 (ftp://ftp.flybase.net/genomes/Drosophila_melanogaster/current/fasta/). A custom R script was
683 used to count the number of ATG sequences in all *Drosophila* 5'UTRs and from the genes
684 identified by the Spanki analysis comprising 638 5'UTRs. A *T* test then used to statistically
685 compare the number of ATGs present in the 638 5'UTRs of the differentially-spliced genes as
686 compared to all 29822 *Drosophila* 5'UTRs. **d** and **e**, Classification of differentially alternative
687 spliced genes in *dIME4*^{null} according to expression pattern **(d)** or function **(e)**.

688

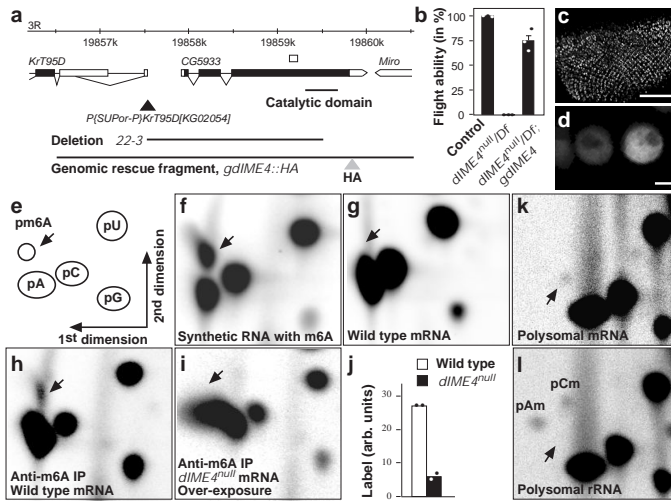
689 **Extended Data Figure 8: *Drosophila* S2 cells are male.** RT-PCR of *Sxl* AS in females, males
690 and S2 cells. 100 bp markers are shown on the left.

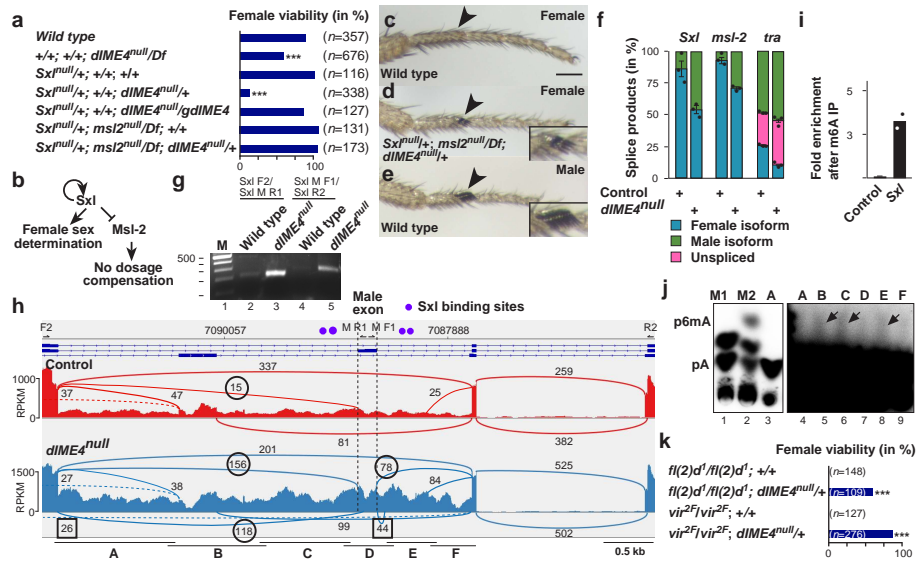
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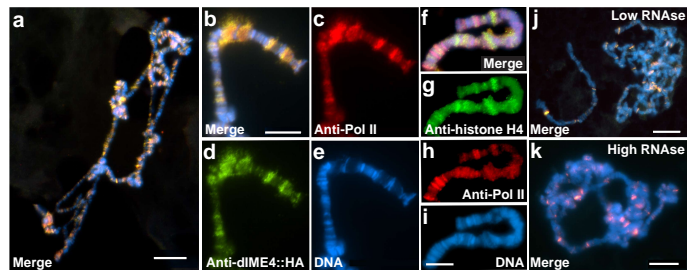
692 **Extended Data Figure 9: Preferential binding of the YTH domain of YT521-B to m6A-**
693 **containing RNA. a,** Coomassie-stained gel depicting the recombinant YTH domain (aa 207-
694 423) of YT521-B. **b** and **c,** Electrophoretic mobility shift assay of YTH domain binding to *Sxl*
695 RNA fragment C with or without m6A (50%) and quantification of RNA bound to the YTH
696 domain shown as mean±SE ($n=3$). Note that the YTH domain does not form a stable complex
697 with RNA (asterisk) and that this complex falls apart during the run or forms aggregates in the
698 well. **d,** In solution UV crosslinking of the YTH domain to *Sxl* RNA fragment C at 0.25 μ M, 1
699 μ M, 4 μ M and 16 μ M (lanes 1-4).

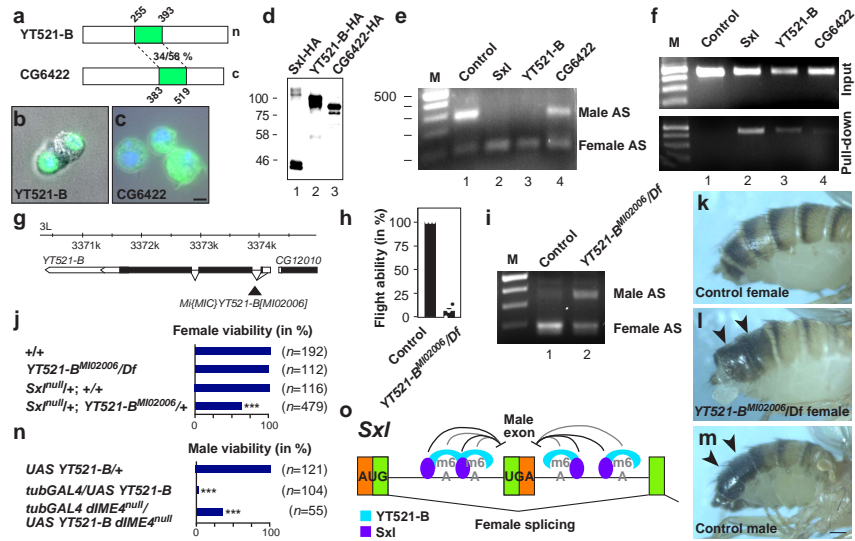
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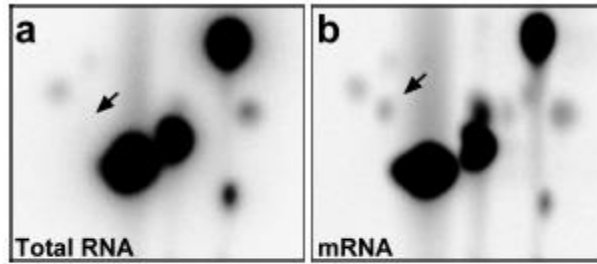
701 **Extended Data Figure 10: YT521-B co-localizes to sites of transcription. a-d,** Polytene
702 chromosomes from salivary glands expressing YT521-B::HA stained with anti-Pol II (red, **b**),
703 anti-HA (green, **c**) and DNA (DAPI, blue, **d**), or merged (yellow, **a**). Scale bars are 5 μ m.



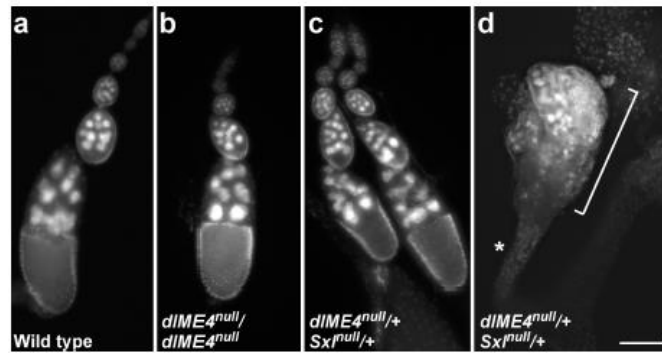




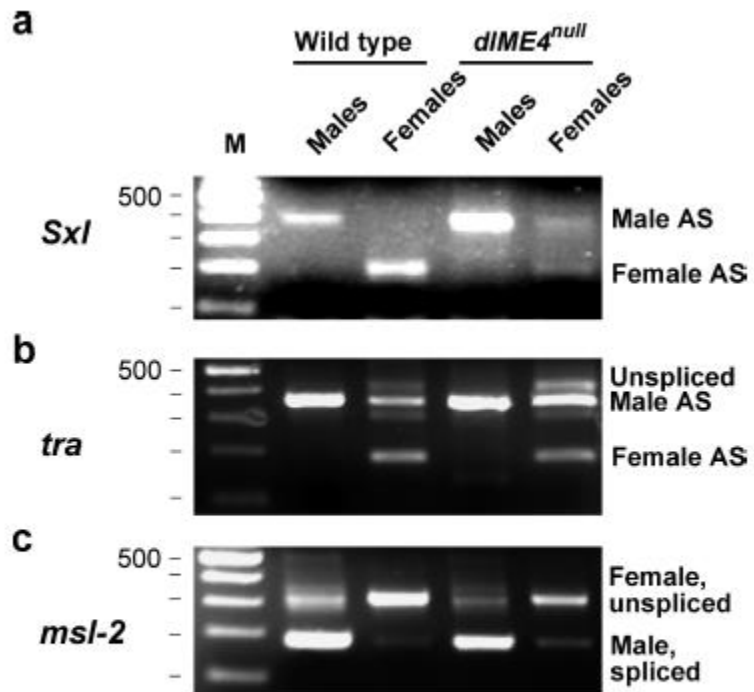




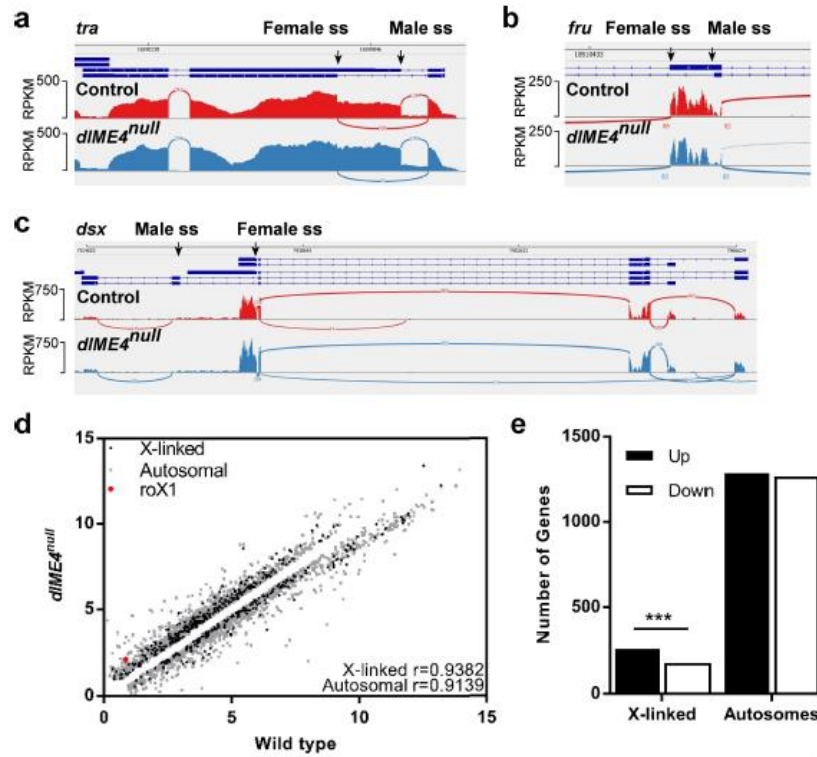
Extended Data Figure 1 | m⁶A levels in unfertilized eggs. a, b, Thin-layer chromatography from maternal total RNA (a) and mRNA (b) present in unfertilized eggs. The arrow indicates m⁶A.



Extended Data Figure 2 | *Ime4* supports *Sxl* in directing germline differentiation. a–c, Representative ovarioles of wild-type (a), *Ime4^{null}/Ime4^{null}* (b) and *Sxl/+;Ime4^{null}/+* females (c), and a tumorous ovary of a *Sxl/+;Ime4^{null}/+* female (d). The tumorous ovary consisting mostly of undifferentiated germ cells in d is indicated with a bracket and the oviduct with an asterisk. Scale bar, 100 μ m (applies to all panels).



Extended Data Figure 3 | *Ime4* is required for female-specific splicing of *Sxl*, *tra* and *msh-2*. a–c, RT-PCR of *Sxl* (a), *tra* (b) and *msh-2* (c) sex-specific splicing in wild-type males and females, and *Ime4^{null}* males and females. 100-bp markers are shown on the left. AS, alternative splicing.



Extended Data Figure 4 | Alternative splicing of sex-determination genes and differential expression of X-linked genes in *Ime4^{null}* females. a–c, Sashimi plot depicting Tophat-mapped RNA sequencing reads and exon junction reads below the annotated gene model for sex-specific alternative splicing of *tra*, *fru* and *dsx*. The thickness of lines connecting splice junctions corresponds to the number of junction reads also shown. ss, splice site. d, Significantly ($P < 0.05$, $q < 0.166853$) differentially expressed gene expression values expressed as reads per kb of transcript per million mapped reads (RPKM) were $\log[x + 1]$ -transformed and

Spearman r correlation values determined for X-linked and autosomal genes in wild-type and *Ime4^{null}* *Drosophila*. e, The proportion of autosomal and X-linked genes that were significantly either up- or downregulated in *Ime4^{null}* as compared to wild-type *Drosophila* were statistically compared using χ^2 with Yates' continuity correction. GraphPad Prism was used for statistical comparisons. Similar results as for the single-read RNA-seq experiment were obtained for the paired-end RNA sequencing experiment.