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

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

2 **determination**

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22

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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 proteins[1-7]. 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 uncertain[3]. 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 females[13]. 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 *Δ22-3* 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 plants[17], localizes to the nucleus (Fig 1c,d).

58   Following RNAse T1 digestion and ^{32}P 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 regulation[18-21],

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 differentiation[13]. 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 factors[22]. Accordingly, female

75   viability was reduced to 13% by removal of maternal m6A together with zygotic heterozygosity

76   for *Sxl* and *dIME4* (*dIME4*$^{\Delta 22-3}$ 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   *2*^{227}*/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 lineages[13]. 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 ovaries[23]. 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 yeast[24].

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)d[1]* and *vir[2F]*

114 interfere with Sxl recruitment, resulting in impaired *Sxl* auto-regulation and inclusion of the

115 male-specific exon[25]. 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* (Δpsi=0.34, q=9x10^-8^) was observed. In addition, 243 AS events in 163 genes were

121   significantly different in *dIME4^null^* females (q<0.05, Δpsi>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 conditions[26].

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<7x10^7^, 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 ($\geq$2-fold change, q$\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 obesity[27].

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 methylosome 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 methylosome 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 methylosome 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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198   declare no competing financial interests. Correspondence and requests for materials should be

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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 Δ*22-3*, which is a *dIME4* null allele and the hemaglutinin (HA)-tagged genomic rescue

273   fragment. **b**, Flight ability of *dIME4[null]/Df(3R)Exel6197* shown as mean±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 μm. **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 ^{32}P

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, [^{32}P]-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)d^1^* 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^{MI02006}/Df(3L)Exel6094* shown as mean±SE (*n*=3). **i**, *Sxl* AS in female

313 wild-type and *YT521-B^{MI02006}/Df(3L)Exel6094* flies. **j**, Female viability of indicated genotypes

314 (*n*: total number of flies) reared at 29º C. **k-m**, Abdominal pigmentation of indicated genotypes

315 reared at 29 ºC. 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 ºC. **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*$^{\Delta 22-3}$ 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 described[30].

337 The genomic rescue construct was retrieved by recombineering (Genebridges) from BAC clone

338 *CH321-79E18* by first cloning homology arms with *Spe*I and *Acc*65I into *pUC3GLA* separated

339 by an *EcoR*V 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 *Sac*I and

344 *Xho*I restriction sites. This construct was the inserted into *PBac{y+-attB-3B}VK00002* at 76A as

345 described[31].

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   TGCGTGGGACATAAAATCAC)   and   dMT-A70   HA   R1   Spe

350   (GGTAACTAGTCTTTTGTATTCCATTGATCGACGCCGCATTGG) 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(GCAGGATTCGCCCGGATGGCAGCCCCCTCAC), 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. Perrimon[32].

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   (AAGCAAAAAAGAAGTCACATGCCAAGAGCAGCCCGTAAACAAACGCTGCCGATGC

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

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

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

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

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

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

415 (TACCGGCAGATTCCAAACCC) and for Sxl, Sxl ZB F1 (CACCACAATGGCAGCAGTAG)

416 and Sxl ZB R1 (GGGGTTGCTGTTTGTTGAGT). 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 $2^{-\Delta\Delta C'_T}$ method[33].

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-specifc RT

424 primer SP NP2 (CATTCCGGATGGCAGAGAATGGGAC) and PCR primers Sxl NP intF

425 (GAGGGTCAGTCTAAGTTATATTCG) and Sxl NP R3 as described[31]. 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 µl of

433 Ribonuclease T1 (1000 U/µl; Fermentas) in a final volume of 10 µl in polynucleotide kinase

434 buffer (PNK, NEB) for 1 h at 37 ºC. The 5' end of the T1-digested mRNA fragments were then

435 labeled using 10 U T4 PNK (NEB) and 1 µl [γ-^{32}P]-ATP (6000 Ci/mmol; Perkin-Elmer). The

436 labeled RNA was precipitated, resuspended in 10 µl of 50 mM sodium acetate buffer (pH 5.5),

437 and digested with P1 nuclease (Sigma-Aldrich) for 1 h at 37 ºC. 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 NH_4OH (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 ^{32}P

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 N_2 and

452   ground into fine powder in liquid N_2. Cells were then lysed in 0.5 ml lysis buffer (0.3 M NaCl,

453   15 mM $MgCl_2$, 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 $^{\circ}$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 described[35]. 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 $[\alpha\text{-}^{32}P]$-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 $[\gamma\text{-}^{32}P]$-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   $(0.5\text{-}1\text{x}10^6$ cpm) were incubated for 45 min at 27 ºC in 10 µl containing 20 mM potassium

469   glutamate, 2 mM MgCl$_2$, 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 MgSO$_4$ and dried[36]. *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   (GGAGCTAATACGACTCACTATAGGGAGAGCGCTCGCATTTATCCCACAGTCGCAC)

480   and   Sxl   B   R   (GGGTGCCCTCTGTGGCTGCTCTGTTTAC),   Sxl   C   T7   F

481   (GGAGCTAATACGACTCACTATAGGGGTCGTATAATTTATGGCACATTATTCAG)   and

482   Sxl   C   R   (GGGAGTTTTGGTTCTTGTTTATGAGTTGGGTG),   Sxl   D   T7   F

483   (GGAGCTAATACGACTCACTATAGGGAGAAAACTTCCAGCCCACACAACACACAC   )

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 (CTAATTTCGTGAGCTTGATTTCATTTTGCACAG), Sxl C3 F

492   (GGAGCTAATACGACTCACTATAGGGAGACTGTGCAAAATGAAATCAAGCTCACGA

493   AATTAG)   and   Sxl   C   R,   Sxl   E   T7   F   and   Sxl   E1   R

494   (AAAAAAATCAAAAAAATAATCACTTTTGGCACTTTTTCATCAC),   Sxl   E2   F

495   (GGAGCTAATACGACTCACTATAGGGAGATGAAAAGTGCCAAAAGTGATTATTTTT

496   TTG), Sxl E2 R (AAAAGCATGATGTATTTTTTTTTTTTGTACTTTCGAATCACCG), Sxl

497   E3   F

498   (GGAGCTAATACGACTCACTATAGGGAGACGGTGATTCGAAAGTACAAAAAAAAAA

499   AAATAC)   and   Sxl   E   R,   Sxl   C4   F

500   (GAGCTAATACGACTCACTATAGGGAGAAATACTAAAACATCAAACCGCAAGCAGA

501   GCAGC) and Sxl C4 R (GAGTGCCACTTCAAAATCTCAGATATGC), Sxl C5 F

502   (CTAATACGACTCACTATAGGGAGACTCTTTTTTTTTTTCTTTTTTTTACTGTGCAAAA

503   TG)   and   Sxl   C5   R

504   (AAAAAAATATGCAAAAAAAAAAAGGTAGGGCACAAAGTTCTCAATTAC), Sxl C6 F

505   (GAGCTAATACGACTCACTATAGGGAGACTGTGCAAAATGAAATCAAGCTCACGAA

506 ATTAG) and Sxl C6 R (CAATTTCACTATATGTACGAAAACAAAAGTGAG), Sxl E4 F

507 (GGAGCTAATACGACTCACTATAGGGAGAACCAAAATTCGACGTGGGAAGAAAC)

508 and Sxl E4 R (TAATCACTTTTGGCACTTTTTCATCACATTAAC), Sxl E5 F

509 (GGCTAATACGACTCACTATAGGGAGATTTTTTTGATTTTTTTAAAGTGAAAATGTGC

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*$^{\Delta 22-3}$ 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 Tophat2.0.6[37]. 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 significant[38]. AS was analysed by SPANKI[39] 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 Viewer[40].

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 (CTGTGGGGCTCAGGGGCATTTTTCCTTCCTC)   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 (GTCGGTGTCCTCGGTGGTGTTGGTGGAG). 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 DNAstrinset 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 DNAstrinset 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 ºC 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-PolII 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 described[41].

582

583   **RNA binding assays**

584   The YTH domain (aa 207-423) was PCR amplified with oligos YTHdom F1

585   (CAGGGGCCCCTGTCGACTAGTCCCGGGAATGGTGGCGGCAACGGCCG)   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

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 tumerous 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 [^{32}P]-ATP-labeled substrate RNAs shown in (**a**). Markers are *in vitro* transcripts in

667   the absence (M1) or presence (M2) of m6A ^{32}P-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 avarage 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

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.

691

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 µM, 1

699   µM, 4 µM and 16 µM (lanes 1-4).

700

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 µm.

a

3R

19857k   19858k   19859k   19860k

KrT95D   *CG5933*   *Miro*

Catalytic domain

P(SUPor-P)KrT95D[KG02054]

Deletion   *22-3*

Genomic rescue fragment, *gdIME4::HA*

HA

b

Flight ability (in %)

100
75
50
25
0

Control

dIME4^{null}/Df

dIME4^{null}/Df; gdIME4

c

d

e

pm6A

pU

pA   pC

pG

2nd dimension

1st dimension

f   Synthetic RNA with m6A

g   Wild type mRNA

k   Polysomal mRNA

h   Anti-m6A IP   Wild type mRNA

i   Anti-m6A IP   *dIME4^{null}* mRNA   Over-exposure

j

□ Wild type
■ *dIME4^{null}*

Label (arb. units)

30
20
10

l   pCm   pAm   Polysomal rRNA

a

Female viability (in %)

Wild type (*n*=357)
+/+; +/+; *dIME4^null*/Df (*n*=676) ***
Sxl^null/+; +/+; +/+ (*n*=116)
Sxl^null/+; +/+; *dIME4^null*/+ (*n*=338) ***
Sxl^null/+; +/+; *dIME4^null*/gdIME4 (*n*=127)
Sxl^null/+; *msl2^null*/Df; +/+ (*n*=131)
Sxl^null/+; *msl2^null*/Df; *dIME4^null*/+ (*n*=173)

b

Sxl

Female sex determination   ⊣ Msl-2

No dosage compensation

c Female — Wild type

d Female — *Sxl^null*/+; *msl2^null*/Df; *dIME4^null*/+

e Male — Wild type

f

Splice products (in %)

Sxl   *msl-2*   *tra*

Control / *dIME4^null*

Female isoform
Male isoform
Unspliced

g

Sxl F2/Sxl M R1   Sxl M F1/Sxl R2

M   Wild type *dIME4^null*   Wild type *dIME4^null*

500

1   2   3   4   5

i

Fold enrichment after m6A IP

Control   *Sxl*

h

Male exon   ● Sxl binding sites

F2   7090057   M R1 M F1   7087888   R2

Control
RPKM   337   37   47   (15)   25   259   81   382

dIME4^null
RPKM   201   27   38   (156)   (78)   84   525   99   44   502   (26)   (118)

A   B   C   D   E   F   0.5 kb

j

M1 M2 A   A B C D E F

p6mA

pA

1   2   3   4 5 6 7 8 9

k

Female viability (in %)

fl(2)d^1/*fl(2)d^1*; +/+ (*n*=148)
fl(2)d^1/*fl(2)d^1*; *dIME4^null*/+ (*n*=109) ***
vir^2F/*vir^2F*; +/+ (*n*=127)
vir^2F/*vir^2F*; *dIME4^null*/+ (*n*=278) ***

a Merge

b Merge

c Anti-Pol II

d Anti-dIME4::HA

e DNA

f Merge

g Anti-histone H4

h Anti-Pol II

i DNA

j Low RNAse — Merge

k High RNAse — Merge

a

YT521-B   255 393
                        n
            34/58 %
CG6422
            383 519   c

b YT521-B   **c** CG6422

d
Sxl-HA   YT521-B-HA   CG6422-HA
100 —
75 —
58 —
46 —
        1   2   3

e
M   Control Sxl YT521-B CG6422
500 —
                            Male AS
                            Female AS
        1   2   3   4

f
M   Control Sxl YT521-B CG6422
500 —
                            Input
                            Pull-down
        1   2   3   4

g
3L
        3371k   3372k   3373k   3374k
YT521-B   CG12010
            Mi{MIC}YT521-B[MI02006]

h
Flight ability (in %)
100
Control   YT521-B^MI02006/Df

i
M   Control   YT521-B^MI02006/Df
                    Male AS
                    Female AS
        1   2

k Control female

l YT521-B^MI02006/Df female

m Control male

j
Female viability (in %)
+/+   (n=192)
YT521-B^MI02006/Df   (n=112)
Sxl^null/+; +/+   (n=116)
Sxl^null/+; YT521-B^MI02006/+ *** (n=479)
0   100

n
Male viability (in %)
UAS YT521-B/+   (n=121)
tubGAL4/UAS YT521-B   ***   (n=104)
tubGAL4 dIME4^null/   *** (n=55)
UAS YT521-B dIME4^null
0   100

o
Sxl
                    Male
                    exon
AUG   m6 m6   UGA   m6   m6
         A A   A   A
    YT521-B
    Sxl
            Female splicing