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

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

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

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

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

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

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

transformer (tra) through AS to direct female differentiation¹³. In addition, *Sxl* suppresses translation of *msl-2* to prevent up-regulation of transcription on the X-chromosome for dosage compensation (Fig. 2b); full suppression also requires maternal factors²². Accordingly, female viability was reduced to 13% by removal of maternal m6A together with zygotic heterozygosity for *Sxl* and *dIME4* (*dIME4*^{Δ22-3} females crossed with *Sxl*^{7B0} males, a *Sxl* null allele, $p < 0.0001$). Female viability of this genotype is completely rescued by a genomic construct (Fig. 2a) or by preventing ectopic activation of dosage compensation by removal of *msl-2* (*msl-2*^{227/Df(2L)Exel7016}, Fig. 2a). Hence, females are non-viable due to insufficient suppression of *msl-2* expression resulting in up-regulation of gene expression on the X-chromosome from reduced *Sxl* levels. In the absence of *msl-2*, disruption of *Sxl* AS resulted in females with sexual transformations (32%, $n=52$) displaying male-specific features such as sex combs (Fig. 2c-e), which were mosaic to various degrees indicating that *Sxl* threshold levels are affected early during establishment of sexual identities of cells and/or their lineages¹³. In the presence of maternal *dIME4*, *Sxl* and *dIME4* do not genetically interact (*Sxl*^{7B0}/*FM7* females crossed with *dIME4*^{null} males, 103% female viability, $n=118$). In addition, *Sxl* is required for germline differentiation in females and its absence results in tumorous ovaries²³. Consistent with this we detected tumorous ovaries in *Sxl*^{7B0/+}; *dIME4*^{null/+} daughters from *dIME4*^{null} females (22%, $n=18$, Extended Data Fig. 2), but not in homozygous *dIME4*^{null} or heterozygous *Sxl*^{7B0} females ($n=20$ each). Furthermore, levels of the *Sxl* female-specific splice form were reduced to ~50% consistent with a role for m6A in *Sxl* AS (Fig. 2f and Extended Data Fig. 3a). As a result, female-specific splice forms of *tra* and *msl-2* were also significantly reduced in adult females (Fig. 2f and Extended Data Fig. 3b,c).

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

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

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

Next, we globally analyzed AS changes in *dIME4^{null}* females compared to the wild-type control strain. As described earlier (Fig. 2h), a statistically significant reduction in female-specific AS of *Sxl* ($\Delta\psi=0.34$, $q=9\times 10^{-8}$) was observed. In addition, 243 AS events in 163 genes were significantly different in *dIME4^{null}* females ($q<0.05$, $\Delta\psi>0.2$), equivalent to ~2% of alternatively spliced genes in *Drosophila* (Suppl. Table 1). Six genes for which the AS products could be distinguished on agarose gels were confirmed by RT-PCR (Extended Data Fig. 6). Interestingly, lack of *dIME4* did not affect global AS and no specific type of AS event was preferentially affected. However, alternative first exons (18% vs 33%) and mutually exclusive exon (2% vs 15%) events were reduced mostly to the extent of retained introns (16% vs 6%), alternative donor (16% vs 9%) and unclassified events (14% vs 6%) compared to a global breakdown of AS in *Drosophila* (Extended Data Fig. 7a). Interestingly, the majority of affected AS events in *dIME4^{null}* were located to the 5'UTR, and these genes had a significantly higher number of AUGs in their 5'UTR compared to the 5'UTRs of all genes (Extended Data Fig. 7b,c). Such feature had been shown relevant to translational control under stress conditions²⁶. The majority of the 163 differentially alternatively spliced genes in *dIME4* females are broadly expressed (59%), while most of the remainder are expressed in the nervous system (33%), consistent with higher expression of *dIME4* in this tissue (Extended Data Fig. 7d). Accordingly, gene ontology (GO) analysis revealed a highly significant enrichment for genes in synaptic transmission ($p<7\times 10^{-7}$, Suppl. Table 1).

Since the absence of m6A affects AS, m6A marks are probably deposited co-transcriptionally before splicing. Co-staining of polytene chromosomes with antibodies against HA-tagged *dIME4* and RNA Pol II revealed broad co-localization of *dIME4* with sites of transcription (Fig. 3a-e), 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*

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

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

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Author contributions

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

Author information

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Figure legends

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

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

Figure 2: m6A methylation is required for *Sex-lethal* AS in sex determination and dosage compensation. **a**, Female viability of indicated genotypes devoid of maternal m6A (*n*: total number of flies). **b**, Schematic depicting Sxl control of female differentiation. **c-e**, Front legs of indicated genotypes. Scale bar: 100 μm. The arrowhead points towards the position of the sex comb normally present only in males. **f**, Ratio of sex-specific splice isoforms from adult females from RT-PCR shown as mean±SE (*n*=3, *p*<0.01). **g**, RT-PCR for male-specific *Sxl* splicing in control and *dIME4^{null}* females. **h**, Sashimi plot depicting Tophat-mapped RNA sequencing reads and exon junction reads from control and *dIME4^{null}* females below the annotated gene model. Male-specific splice junction reads are circled and cryptic splice sites are boxed. RNA fragments used for m6A *in vitro* methylation assays are indicated at the bottom. **i**, Presence of m6A in *Sxl* transcripts detected by m6A immunoprecipitation followed by qPCR from nuclear mRNA of early embryos (shown as mean, *n*=2). **j**, 1D-TLC of *in vitro* methylated, [³²P]-ATP-labeled substrate RNAs shown in **g**. Nucleotide markers from *in vitro* transcripts in the absence (M1) or presence (M2) of m6A. The right part shows an overexposure of the same TLC. **k**, Rescue of female lethality of female-lethal *fl(2)^{d^l}* and *vir^{2F}* alleles by removal of one copy of *dIME4*.

Figure 3: dIME4 co-localizes to sites of transcription. **a-e**, Polytene chromosomes from salivary glands expressing dIME::HA stained with anti-Pol II (red, **c**), anti-HA (green, **d**) and DNA (DAPI, blue, **e**), or merged (yellow, **a** and **b**). **f-i**, Polytene chromosomes stained with anti-

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

Fig 4: YTH protein YT521-B decodes m6A methylation in *Sxl*. **a**, Domain organization of *Drosophila* YTH proteins (YTH domain in green). n: nuclear, c: cytoplasmic **b-d**, Cellular localization and size of HA-tagged YT521-B and CG6422 in S2 cells. Scale bar: 1 µm. **e**, Suppression of male-specific *Sxl* AS upon expression of *Sxl* and YT521-B, but not CG6422 in male S2 cells. **f**, Binding of YT521-B to pre-mRNA of the regulated *Sxl* intron. **g**, Genomic organization of the *YT521-B* locus depicting the transposon (black triangle) disrupting the ORF. **h**, Flight ability of *YT521-B*^{MI02006}/*Df(3L)Exel6094* shown as mean±SE (*n*=3). **i**, *Sxl* AS in female wild-type and *YT521-B*^{MI02006}/*Df(3L)Exel6094* flies. **j**, Female viability of indicated genotypes (*n*: total number of flies) reared at 29° C. **k-m**, Abdominal pigmentation of indicated genotypes reared at 29 °C. The arrowhead points towards the position of the dark pigmentation normally present only in males. Scale bar: 100 µm. **n**, YT521-B was overexpressed from a *UAS* transgene with *tubulinGAL4* (2nd) in wild type or *dIME4*^{null} at 27 °C. **o**, Model for female-specific *Sxl* AS by *Sxl*, m6A and its reader YT521-B in co-operatively suppressing inclusion of the male-specific exon.

Online Methods

Data reporting

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

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

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

The genomic rescue construct was retrieved by recombineering (Genebridges) from BAC clone *CH321-79E18* by first cloning homology arms with *SpeI* and *Acc65I* into *pUC3GLA* separated by an *EcoRV* site for linearization (CTCCGCCGCCGGAACCGCCGCCTCCTCCGCCACTTTGCAGGTTGAGCGGACCGCCTCCAGGGCCGCTGCCGCCGGTGCCGCTGATATCCCAGCATGGTAGCTGCGGCCACTCC TAGTCCCGCCTTTAACCACAGCTTGGGGTCCTCCGTCATCAGGCCGAATTGCCTCGA G). An HA-tag was then fused to the end of the ORF using two PCR amplicons and *SacI* and *XhoI* restriction sites. This construct was the inserted into *PBac{y+-attB-3B}VK00002* at 76A as 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 (GCAGAATTCGAGATCtAAAGAGCCTGCTAAAGCAAAAAGAAGTCACCATGGCAGA
 349 TCGTGGGACATAAAATCAC) 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 (GCCTGCTAAAGCAAAAAGAAGTCACCACATGTCAGGCGTGGATCAGATGAAAAT
 357 ACCAG), *pact* *adh* *CG6422* F1
 358 (CCAGAGACCCCGGATCCAGATATCAAAGAGCCTGCTAAAGCAAAAAGAAGTCAC
 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 (AAGCAAAAAGAAGTCACATGCCAAGAGCAGCCCGTAAACAAACGCTGCCGATGC
 366 GCGAG), *pact* *adh* *YT521* F1
 367 (CCAGAGACCCCGGATCCAGATATCAAAGAGCCTGCTAAAGCAAAAAGAAGTCAC
 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

(CATTGTAACCACGACGCGACGATG) 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 (TTACGTGCGCTTGGACTTCG) and *ACT1* R1

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

Analysis of m6A levels

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

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

Polysome profiles

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

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 (GGAGCTAATACGACTCACTATAGGGAGAGCGCTCGCATTTATCCCACAGTCGCAC)
 480 and *Sxl* B R (GGGTGCCCTCTGTGGCTGCTCTGTTTAC), *Sxl* C T7 F
 481 (GGAGCTAATACGACTCACTATAGGGGTCGTATAATTTATGGCACATTATTTCAG) 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 (CTAATTCGTGAGCTTGATTTTCATTTTGCACAG), Sxl C3 F
 492 (GGAGCTAATACGACTCACTATAGGGAGACTGTGCAAAATGAAATCAAGCTCACGA
 493 AATTAG) and Sxl C R, Sxl E T7 F and Sxl E1 R
 494 (AAAAAATCAAAAAAATAATCACTTTTGGCACTTTTTCATCAC), 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 (GAGTGCCACTTCAAAATCTCAGATATGC), Sxl C5 F
 502 (CTAATACGACTCACTATAGGGAGACTCTTTTTTTTTTTCTTTTTTTTACTGTGCAAAA
 503 TG) and Sxl C5 R
 504 (AAAAAATATGCAAAAAAAAAAAGGTAGGGCACAAAGTTCTCAATTAC), 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 (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*^{Δ22-3} 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 (GTCGGTGTCTCGGTGGTGTGTTGGTGGAG). 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

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

RNA binding assays

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

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

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

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Extended Data figure legends

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

Extended Data Figure 2: dIME4 supports Sxl in directing germline differentiation. **a-c**, Representative ovarioles of wild type (**a**), *dIME4^{null}/dIME4^{null}* (**b**) and *Sxl/+; dIME4^{null}/+* (**c**), and a tumorous ovary of a *Sxl/+; dIME4^{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.
The scale bar in (d) is 100 μ m.

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

Extended Data Figure 4: AS of sex determination genes and differential expression of X-linked genes in *dIME4*^{null} females. a-c, Sashimi plot depicting Tophat-mapped RNA sequencing reads and exon junction reads below the annotated gene model for sex-specific AS 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 +1 log transformed and Spearman r correlation values determined for X-linked and autosomal genes in wild-type and *dIME4*^{null} *Drosophila*. e, The proportion of autosomal and X-linked genes that were significantly either up- or down-regulated in *dIME4*^{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 pair-end RNA sequencing experiment.

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

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

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

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

691

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 $\pm$ 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).

700

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.