



Xio is a component of the *Drosophila* sex determination pathway and RNA N^6 -methyladenosine methyltransferase complex

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N^6 -methyladenosine (m^6A), the most abundant chemical modification in eukaryotic mRNA, has been implicated in *Drosophila* sex determination by modifying *Sex-lethal* (*Sxl*) pre-mRNA and facilitating its alternative splicing. Here, we identify a sex determination gene, *CG7358*, and rename it *xio* according to its loss-of-function female-to-male transformation phenotype. *xio* encodes a conserved ubiquitous nuclear protein of unknown function. We show that Xio colocalizes and interacts with all previously known m^6A writer complex subunits (METTL3, METTL14, FI(2)d/WTAP, Vir/KIAA1429, and Nito/Rbm15) and that loss of *xio* is associated with phenotypes that resemble other m^6A factors, such as sexual transformations, *Sxl* splicing defect, held-out wings, flightless flies, and reduction of m^6A levels. Thus, Xio encodes a member of the m^6A methyltransferase complex involved in mRNA modification. Since its ortholog ZC3H13 (or KIAA0853) also associates with several m^6A writer factors, the function of Xio in the m^6A pathway is likely evolutionarily conserved.

CG7358 | Xio | sex determination | m^6A writer complex | alternative splicing

Sex determination is one of the most fundamental problems in biology and affects all aspects of life, such as morphology, metabolism, aging, and behavior (1). For more than 90 y, *Drosophila* has remained a major model organism to study sex determination genes and mechanisms (2). Similar to humans, *Drosophila* males have XY chromosomes, and females have XX chromosomes. *Sex-lethal* (*Sxl*), the master regulatory gene in the *Drosophila* sex determination pathway, is activated in females by the X-chromosome counting system while it is not expressed in males. Once activated, *Sxl* maintains its own expression by controlling the alternative splicing of its own pre-mRNA. *Sxl* also regulates the alternative splicing of the downstream gene *transformer* (*tra*), which, together with *transformer2* (*tra2*), controls the alternative splicing of *doublesex* (*dsx*) and *fruitless* (*fru*), generating male- and female-specific transcription factors (3). In addition, *Sxl* prevents the activation of the male-specific dosage compensation system by repressing *male-specific lethal 2* (*msl-2*) at the level of splicing and translational control (4).

In addition to these genes, three factors encoded by *female-lethal-2-d* (*fl(2)d*), *virilizer* (*vir*), and *spenito* (*nito*) have been shown to be involved in the sex determination pathway and to be required for *Sxl* alternative splicing regulation (5–9). Recently, FI(2)d, Vir, and Nito were shown to encode components of the N^6 -methyladenosine (m^6A) methyltransferase complex, revealing that the m^6A pathway modulates sex determination in *Drosophila* (10–12). m^6A is the most abundant chemical modification in mRNA, and its level is dynamically regulated (13). m^6A pathway factors include the methyltransferase complex (or writers), demethylases (or erasers), and readers. Both in *Drosophila* and mammals, known writer complex subunits include METTL3 (or Ime4) (14), METTL14, FI(2)d (WTAP), Vir (KIAA1429), and Nito (Rbm15/15B) (15). These writer components, as well as the

reader YT521-B, are required for *Drosophila* sex determination and *Sxl* splicing regulation. Further, m^6A modification sites have been mapped to *Sxl* introns, thus facilitating *Sxl* pre-mRNA alternative splicing. Importantly, m^6A methylation is required in human dosage compensation by modifying the long noncoding RNA *XIST*, suggesting that m^6A -mediated gene regulation is an ancient mechanism for sex determination (16).

Recent emerging studies suggest that m^6A is involved in numerous key biological processes, such as development, disease, stem cell differentiation, immunity, and behavior, by controlling various aspects of RNA metabolism, such as splicing, stability, folding, export, and translation (17). Although many m^6A methylated mRNAs have been identified, *Sxl* pre-mRNA is arguably one of the best understood examples for m^6A modification and is useful for mechanistic studies. Importantly, *Drosophila* sex determination provides a unique system to screen for new components as all previously identified writers and readers show unambiguous sex transformation phenotypes (18).

Here, we identified a component in the *Drosophila* sex determination pathway as well as a m^6A modification pathway. As this gene, *CG7358*, has not been studied before, we named it *Xiong* (*Xio*, Chinese character for maleness) since its loss of function shows female-to-male transformations. We demonstrate that Xio interacts with other methyltransferase factors and that

Significance

RNAs contain over 100 types of chemical modifications, and N^6 -methyladenosine (m^6A) is the most common internal modification in eukaryotic mRNA. m^6A is involved in a variety of important biological processes, including sex determination in *Drosophila*, by modifying *Sxl* pre-mRNA and regulating its alternative splicing. m^6A is installed by a large methyltransferase complex called the m^6A “writer.” We have identified *xio* as a component of the *Drosophila* sex determination pathway based on its female-to-male transformation phenotypes. Xio interacts with other m^6A writer subunits, and its loss of function shows typical phenotypes associated with other m^6A factors, such as *Sxl* splicing misregulation, adult defects, and reduced m^6A levels. Therefore, we conclude that Xio is a member of the m^6A writer complex.

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Data deposition: RNA-Seq data have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no. GSE110047).

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loss of Xio activity phenocopies other methyltransferase mutants in terms of *Sxl* splicing regulation and adult defects. Altogether, our study identifies and characterizes a conserved component of the m⁶A writer complex.

Results

Xio Interacts and Colocalizes with Known m⁶A Writer Complex Subunits. From the *Drosophila* protein–protein interaction database (DPiM), we identified one interesting protein, CG7358/Xio, which, as a bait, can pull down Nito, Vir, and Fl(2)d in affinity purification and mass spectrometry (mass-spec) experiments (Fig. S1) (19). Similarly, in our own mass-spec studies, Nito or Fl(2)d as a bait can reciprocally pull down Xio (Fig. 1*A* and Dataset S1). As these proteins are core components of the RNA m⁶A methyltransferase complex, we hypothesized that Xio is a new factor of the m⁶A writer complex. To confirm these interactions, we performed both colocalization and coimmunoprecipitation (co-IP) experiments. Xio-GFP localized to nuclei in live S2 cells and colocalized with Nito-mRFP, Fl(2)d-mRFP, METTL3-mRFP, and METTL14-mRFP (Fig. 1*B–E*). Next, we transfected Xio-GFP and different HA-tagged constructs in S2 cells and used GFP alone as a control. Although GFP was expressed at a much higher amount, only Xio-GFP was able to pull down Nito-HA, Fl(2)d-HA, METTL3-HA, and METTL14-HA (Fig. 1*F–I*). Interestingly, the pull-down between Xio and Nito is particularly strong compared with other factors, suggesting that Xio may directly interact with Nito, which is consistent with the published mass-spec data where most Nito peptides were pulled down using Xio as a bait (Fig. S1*B*). Altogether, these data suggest that Xio is a new component of the m⁶A writer complex.

Xio Is a Ubiquitously Expressed Nuclear Protein, and Its Expression Patterns Mimic Other m⁶A Pathway Members. *xio* is located on the

X chromosome, and its transcript is alternatively spliced, producing proteins of 1,150, 1,139, and 842 amino acids, respectively (Fig. S2*A*). Xio protein has no obvious domains, and its human ortholog is ZC3H13. The biological function of Xio and its ortholog have not been studied in any organism. *xio* expression patterns are very similar to those of other m⁶A writers and readers, with highest expression in the CNS and high expression in the ovary, imaginal discs, and fat tissues (Fig. S3*A*) (modENCODE developmental and tissue expression database) (20). Developmentally, *xio* expression was enriched in early embryos, decreased during larval stages, and rose again at pupal stages (Fig. S3*A*), which coincides with the reported m⁶A levels (11). *xio* expression showed enrichment in the neuroectoderm at later stages of embryogenesis (Fig. S3*B*) (FlyExpress) (21), which is highly similar to known m⁶A writers and readers (11).

To study Xio function, we generated and evaluated tools. We raised an antibody against Xio, and, as expected, Xio was a ubiquitously expressed nuclear protein that colocalizes with Fl(2)d (Fig. S2*A–B'*). Two nonoverlapping RNAi lines effectively knocked down Xio when induced by *ap-Gal4* (Fig. S2*C* and *D*). In addition, we constructed a transgenic *xio-sgRNA* under U6:3 promoter (22), and, when crossed with *actin-Cas9*, this line can generate random clones that showed no detectable Xio protein (Fig. 3*G*). Finally, we obtained two ethyl methanesulfonate (EMS) mutants of *xio* previously generated in a mosaic screen of lethal mutations on the X chromosome (23). *xio*^{C/Y} flies died at the pupal stage while *xio*^{A/Y} flies died as pharate, with some flies half way out of the pupal case (Fig. S4*C*). We induced mosaic clones of these mutants and found that Xio proteins are significantly reduced (Fig. S2*E–F'*).

Xio Is Required for Sex Determination in *Drosophila*. One of the major targets regulated by m⁶A modification is the *Drosophila* sex determination gene *Sxl* (10–12). Activation of *Sxl* in female embryos requires the coordination of two promoters, the

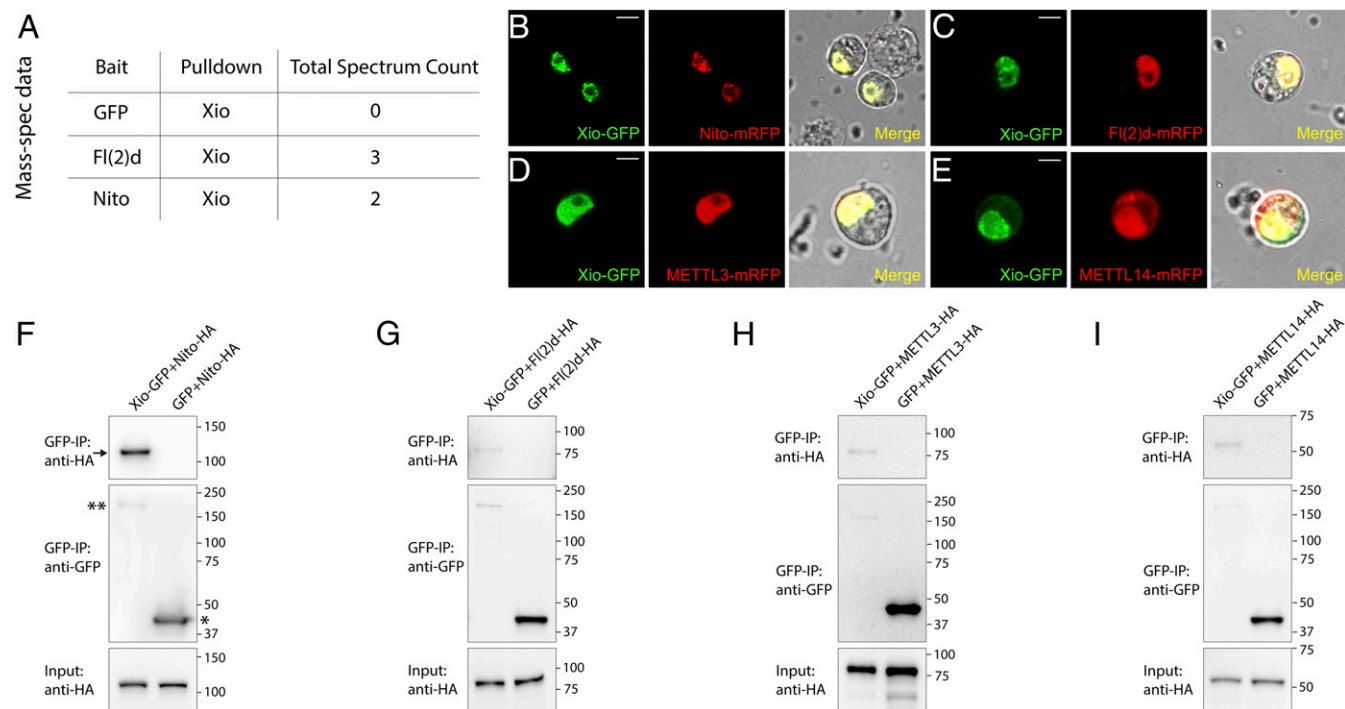


Fig. 1. Xio localizes and interacts with other m⁶A writer components. (*A*) From mass-spec experiments, Fl(2)d and Nito, but not GFP, can pull down Xio. (*B–E*) Xio-GFP and Nito-mRFP, Fl(2)d-mRFP, METTL3-mRFP, or METTL14-mRFP were cotransfected into S2 cells, and their subcellular localization was examined in live cells. (Scale bars: 5 μ m.) (*F–I*) Xio-GFP or GFP and Nito-HA, Fl(2)d-HA, METTL3-HA, METTL14-HA were cotransfected into S2 cells. Cell lysates were immunoprecipitated using a GFP nanobody and analyzed by Western blot. Although GFP (asterisk) is expressed at a much higher amount than Xio-GFP (double asterisk), only Xio-GFP can pull down Nito-HA, Fl(2)d-HA, METTL3-HA, and METTL14-HA. Note that much more Nito-HA (arrow) was co-IPed than other factors.

establishment promoter *SxlPe* and the maintenance promoter *SxlPm*. *SxlPe* is transiently activated by maternal factors in females, and *Sxl* produced from *SxlPe* subsequently drives the autor-regulatory splicing loop for *SxlPm*-derived transcripts (2). Any perturbation of these processes can lead to female-specific lethality, as manifested by progeny sex imbalance. This system is particularly sensitive when one copy of *Sxl* is removed (24): as shown, for example, for *daughterless* (*da*), which encodes a maternal factor required for *Sxl* activation. When *da*² heterozygous mothers were crossed to *Sxl*^{7BO}/Y (a null allele of *Sxl*) fathers, only 53% of the expected *Sxl*^{7BO}/+ females survived to adulthood (Fig. 2A). Importantly, when *xio*^A heterozygous mothers were crossed to *Sxl*^{7BO}/Y fathers, only 38% of the expected *Sxl*^{7BO}/+ female adults were observed (Fig. 2A), clearly implicating *Xio* in *Sxl* regulation.

Next, we used the CRISPR/Cas9 system to perturb *xio* function. Surprisingly, crossing *actin-Cas9* with *U6-xio-sgRNA* produced progeny that are almost exclusively males (Fig. 2B). This result, together with the genetic interaction experiments, implies that *Xio* is involved in sex determination. To directly look at the sexual phenotype, we expressed *xio* RNAi using *dome-Gal4* and observed striking transformation of female tissues into males, as evidenced by the appearance of sex combs in the forelegs of *xio* RNAi females (Fig. 2C–E). In addition, *dome-Gal4/xio-RNAi* females showed strong abnormalities in the genitalia. Typical female structures, such as vaginal bristles (Fig. 2G, white arrow), disappear, and structures resembling the male penis apparatus can be found (Fig. 2F and H, yellow arrow). Together, these data suggest that *Xio* is a new component of the *Drosophila* sex determination pathway.

Xio Regulates Sxl Protein Levels in the Soma and Germline. The phenotype associated with loss of *Xio* function suggests that *Xio* may regulate *Sxl* activity. In somatic tissues such as wing discs, *Sxl* protein was expressed ubiquitously in females but absent in males (Fig. 3A and B). We used three different approaches to inactivate *Xio* function for examination of *Sxl* expression. First, expression of *xio* RNAi in the dorsal half of the wing disc using *ap-Gal4* led to a significant reduction of *Sxl* levels (Fig. 3C and D). Second, *Sxl* levels were almost absent from *xio*^A or *xio*^C mitotic mutant clones (Fig. 3E–F'). Third, crossing of *actin-Cas9* with *U6-xio-sgRNA* generated random *xio* loss-of-function clones. These clones were marked by the loss of *Xio* staining, and *Sxl* was completely depleted in these clones as well (Fig. 3G–G', arrows).

Finally, we asked whether *Xio* regulates *Sxl* levels in ovaries by inducing mitotic clones in both germline cells and follicle cells. Consistent with the disk results, *Sxl* was strongly reduced in either *xio* mutant germline or follicle cell clones (in Fig. S5, arrows indicate follicle cell clones, and asterisks indicate germline clones). Altogether, we conclude that *Xio* regulates *Sxl* levels in both germline and somatic tissues.

Xio Controls Sxl Alternative Splicing both in Vivo and in Cell Lines. *Sxl* transcripts are alternatively spliced. While the male form includes exon 3 that contains a stop codon and leads to early termination of *Sxl* protein, the female form skips exon 3 and thus produces a functional *Sxl* protein (Fig. 4A) (25). To monitor *Sxl* splicing pattern, we used a pair of primers flanking exon 3 that detects the small female and large male spliced *Sxl* products by RT-PCR (Fig. 4A) (24). In *ap-Gal4/xio RNAi* female wing discs, a large band corresponding to the male-specific spliced form was clearly detected (Fig. 4B). We further analyzed *Sxl* splicing regulation in *Drosophila* cell lines. The S2 cell is a male cell line, and *Sxl* is spliced in the male form, while the Kc cell is a female cell line, and *Sxl* is spliced in the female form (Fig. 4C). While *xio* dsRNA had no effect on the male-specific splicing of *Sxl* in S2 cells, treating Kc cells with *xio* dsRNAs led to *Sxl* splicing shifted from the female form to the male form (Fig. 4C).

We then analyzed how the m⁶A writer complex contributes to *Sxl* splicing regulation. In females, *Sxl* itself is the key protein that binds its pre-mRNA and inhibits splicing of the male-specific exon 3 by interacting with components of the spliceosome, such as

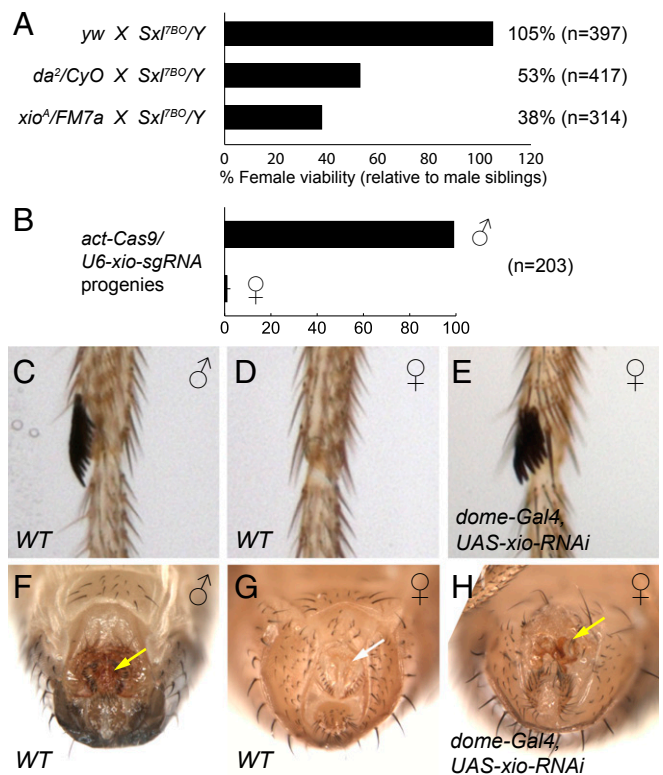


Fig. 2. *Xio* is required for sex determination in *Drosophila*. (A) Females of the indicated genotypes were crossed to *Sxl*^{7BO}/Y males, and the resulting progeny were scored. For *yw* and *da*²/*CyO* crosses, the viability of total female adults relative to sibling male adults was quantified. For the *xio*^A/*FM7a* cross, the viability of *Sxl*^{7BO}/*FM7a* females relative to *FM7a*/Y males was quantified. n, total number of adult progeny counted. (B) *actin-Cas9* flies were crossed to *U6-xio-sgRNA* flies, and the number of male and female progeny was counted. n, total number of adult progeny counted. (C) Foreleg of a WT male showing the sex comb. (D) Foreleg of a WT female. (E) Foreleg of a female fly expressing *xio* RNAi driven by *dome-Gal4*. Genitalia of WT male (F) and female (G) flies show distinct morphology. (H) *xio* RNAi driven by *dome-Gal4* transforms female genital morphology into male-like. The white arrow indicates vaginal bristles, and the yellow arrows indicate penis apparatus.

SNF, a protein component of the U1 and U2 small nuclear ribonucleoproteins (snRNPs) (26, 27). Since the m⁶A writer components Fl(2)d and Nito interact with *Sxl* (6, 8), we examined whether *Xio* interacts with *Sxl* using a co-IP assay in S2 cells. As shown in Fig. 4D, GFP-*Xio*, but not GFP alone, can pull down *Sxl*-HA. These data are consistent with recent findings that m⁶A sites have been mapped in introns on both sides of exon 3 and in the vicinity of *Sxl* binding sites (10, 12). We next asked whether the m⁶A writer complex physically interacts with the spliceosome. In S2 cells where *Sxl* is absent, we performed co-IP experiments between SNF and five m⁶A writer subunits (Fig. 4E). As a positive control, SNF-GFP pulled down a large amount of *Sxl*-HA, in agreement with a previous report (27). Interestingly, we found that even more Nito-HA was co-IPed by SNF-GFP, indicating that these two proteins interact strongly with each other. However, a very low amount of Fl(2)d-HA was pulled down, and there were no detectable METTL3-HA, METTL14-HA, and *Xio*-HA from the co-IP. These results indicate that the m⁶A writer complex can interact with the spliceosome independent of *Sxl* and Nito serves as a bridge between the spliceosome and other m⁶A subunits. Together, we propose a model that both *Sxl* and the m⁶A writer complex interact with the spliceosome and they also interact with each other to repress the inclusion of the male-specific exon (Fig. 4A).

***xio* Mutant Phenocopies *Mettl3* Adult Defects, and *xio* Is Required for m⁶A Levels.** Other than sex determination phenotype, m⁶A writer

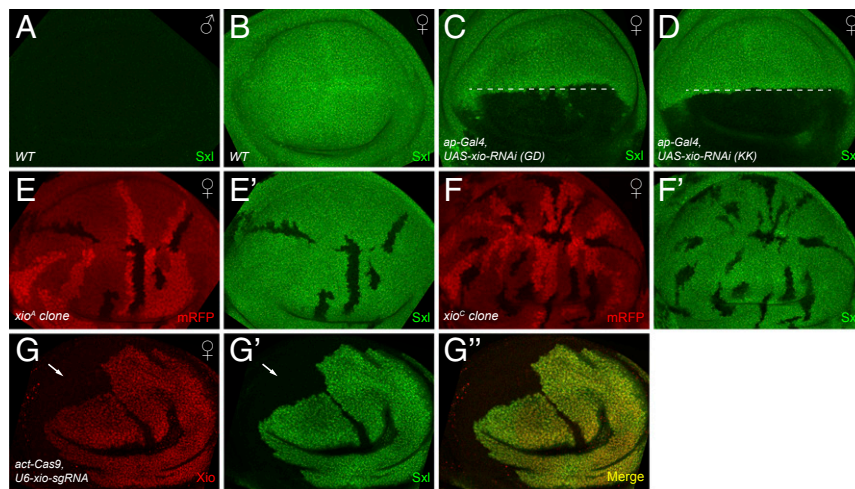


Fig. 3. Xio is required for Sxl levels in somatic tissues. Sxl stainings in WT male (A) and female (B) wing discs. (C and D) Expressing *xio* RNAi in the dorsal half of the disk (below the dashed line) using *ap-Gal4* results in strong reduction of Sxl stainings. (C) GD35212; (D) KK110253. (E–F) Sxl staining is abolished in *xio*^A (E) or *xio*^C (F) mitotic mutant clones that are marked by the absence of mRFP (E and F). (G and G') Both Xio and Sxl staining are completely abolished in *xio* mutant clones (arrows) generated by *actin-Cas9/U6-xio-sgRNA*.

and reader mutants exhibit characteristic adult defects. The most prominent ones are held-out wings and flightless phenotypes in both adult males and females (10, 12), likely due to functions of m⁶A modifications in the nervous system (11). If Xio is a component of

the m⁶A writer complex, one would expect to see similar defects associated with *xio* mutant. By crossing *xio*^A heterozygous females to males of different backgrounds, we were able to recover *xio*^A hemizygous males and analyze their adult phenotypes (Fig. S4C).

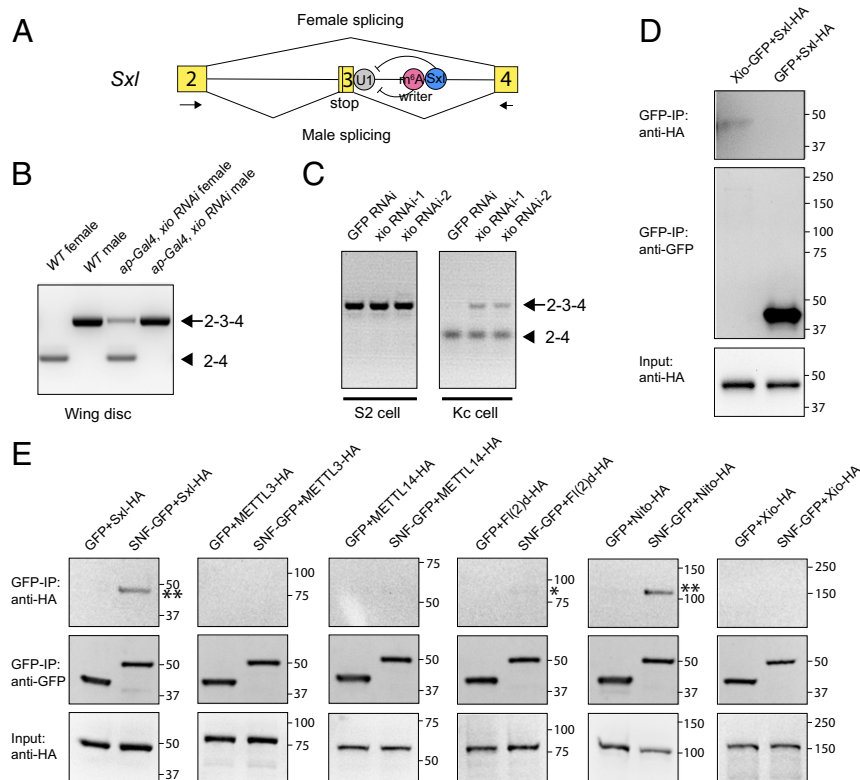


Fig. 4. Xio regulates *Sxl* alternative splicing and interacts with *Sxl*. (A) Model showing female- or male-specific *Sxl* alternative splicing mediated by the m⁶A writer and *Sxl* protein. Only the regulatory events in the intron downstream of exon 3 are shown. The arrows indicate the primers used for RT-PCR. (B) *Sxl* splicing was analyzed by RT-PCR using RNA extracted from wing discs of indicated genotypes. Male-specific bands: 2-3-4. Female-specific bands: 2-4. (C) S2 or Kc cells were treated with *xio* RNAi or GFP RNAi, and *Sxl* splicing was analyzed by RT-PCR. (D) *Sxl*-HA, *Xio*-GFP, or GFP were transfected into S2 cells. Cell lysates were IPed and analyzed by Western blot. (E) SNF-GFP or GFP and *Sxl*-HA, METTL3-HA, METTL14-HA, FI(2)d-HA, Nito-HA, and *Xio*-HA were cotransfected into S2 cells. Cell lysates were IPed and analyzed by Western blot. While large amounts of *Sxl*-HA and Nito-HA were co-IPed by SNF-GFP (double asterisk), a very low amount of FI(2)d-HA was observed (asterisk), and there are no detectable METTL3-HA, METTL14-HA, and *Xio*-HA from the pull down.

WT flies normally keep their wings in a folded position (Fig. 5 *A* and *E*); however, the majority of *Mettl3* mutant flies cannot fold their wings correctly and exhibited held-out wings (Fig. 5 *B* and *E*). Interestingly, *xio*⁴ mutant flies showed strong held-out wing phenotypes (Fig. 5 *C* and *E*). In addition, progeny of *actin-Cas9* crossed with *U6-xio-sgRNA* exhibited similar held-out wings (Fig. 5 *D* and *E*), further confirming that this defect is specifically due to loss of *xio*.

We further tested these flies for their flight ability. While 100% of *Mettl3* mutant flies could not fly, the majority of *xio*⁴ mutant flies, as well as *actin-Cas9/U6-xio-sgRNA* flies, were flightless (Fig. 5 *F*). Together, these results indicate that *xio* mutants resemble m⁶A pathway mutants in terms of sex determination, held-out wings, and flight abilities, strongly arguing that Xio is a key component of the m⁶A methylation pathway.

Next, we directly measured N⁶-methyladenosine levels in *xio* mutants by quantitative liquid chromatography–mass spectrometry (LC-MS). We used an external calibration curve prepared with A and m⁶A standards to determine the absolute quantities of each ribonucleoside (Fig. S6). After one round of polyA selection, we detected a modest but consistent reduction of m⁶A levels in *xio* RNAi cells and in *xio*^c mutant pupae (Fig. 5 *G*). The amount of reduction was comparable with that seen in *nito* mutants (12), and the modest reduction was likely due to the contamination of rRNA in the sample and/or incomplete loss of function of *xio* in these conditions. Nevertheless, these results validate that Xio is required for proper m⁶A levels and, together with our phenotypic analysis and biochemical interaction data,

strongly support the model that Xio is a new bona fide subunit of the m⁶A methyltransferase complex (Fig. 5 *H*).

Xio Regulates a Broad Spectrum of Gene Expression and Alternative Splicing Events. To gain a global view of Xio-mediated gene expression, we performed RNA-Seq experiments in control and *xio* mutant animals. As *xio*⁴ hemizygous males are sterile, we were unable to generate *xio*⁴ homozygous females and thus performed RNA-Seq in *xio*⁴ males. Since *Sxl* regulates the expression of numerous genes in females, using males has the advantage of dissecting *Sxl* independent events. We used the pharate stage, just before eclosion, as this is the period with very high m⁶A levels and most *xio*⁴ mutants cannot develop beyond this stage. Differential gene expression analysis revealed 2,002 down-regulated genes and 842 up-regulated genes (fold change ≥ 1.5 and *P* value < 0.05) (Fig. S7*A* and Dataset S2). Kyoto Encyclopedia of Genes and Genomes (KEGG)-pathway analysis indicated that metabolic pathways, including fatty acid, carbohydrate, and amino acid metabolism genes, are strongly enriched (Fig. S7*C* and Dataset S2). Consistent with the adult phenotypes, Gene Ontology (GO) analysis found a significant enrichment of neuron-related categories, such as sleep, neuron projection, circadian rhythm, and motor neuron axon guidance (Fig. S7*B*). In addition, we also analyzed the alternative splicing changes in these mutants and found that 105 alternative splicing events in 96 genes were significantly different (Bayes factor > 10 , Δ PSI (difference in percentage spliced in) > 0.2) (Dataset S3). GO term enrichment revealed similar neuron-related categories, such as synaptic growth, gravitaxis, axon guidance, and neuromuscular synaptic transmission, as well as other developmental processes (Fig. S7*D* and Dataset S3). A few examples of differentially alternative spliced genes are shown in Fig. S8. In summary, our data revealed a broad range of genes and splicing events regulated by Xio and provide an important dataset for further mechanistic studies.

Discussion

The *Drosophila* sex determination pathway, comprised of a hierarchy of alternative splicing events, remains a textbook paradigm for sex determination mechanisms and alternative splicing. Here, we describe the characterization of Xio as a component of the *Drosophila* sex determination pathway. Xio loss of function results in female-specific lethality and striking sexual transformation phenotypes. We further show that Xio regulates the master sex determination gene *Sxl* by controlling its alternative splicing. Such function is reminiscent of previously reported genes, such as *snf* (26, 28), *U2AF* (27), *U1-70K* (29), *SPF45* (30, 31), *PPS* (24), *fl(2)d* (8, 9), *vir* (5, 7), and *nito* (6), that play similar roles in *Sxl* splicing regulation. While several of these genes mainly act as splicing factors, Fl(2)d, Vir, Nito, and Xio are now known as the core subunits of the RNA m⁶A methyltransferase complex.

m⁶A modification has been known for more than 40 y (32) but recently gained attention due to the emergence of new technologies to map m⁶A sites throughout the transcriptome (33, 34), as well as the identification of the writers, erasers, and readers of this pathway (35–37). The key methyltransferase METTL3 was first discovered in 1994 (38); then, other subunits of the writer complex were identified mainly through biochemical interaction experiments and genetic screens (39, 40). For example, a proteomic study to identify WTAP interacting proteins in human cells revealed Vir/KIAA1429, RBM15, and the ortholog of Xio, ZC3H13 (or KIAA0853) (41). *Drosophila* sex determination provides an unambiguous phenotype to screen and characterize m⁶A pathway components. Fl(2)d and Vir have been known to be involved in sex determination for more than two decades, and recently we identified Nito as a new component of the sex determination pathway in an RNAi screen (6). Finally, METTL3, METTL4, and the reader YT521-B were shown to be also required for sex determination (10–12).

The sexual phenotype associated with *xio* and the biochemical interactions between Xio and other m⁶A factors indicate that Xio is a new component of the m⁶A writer complex. We further show that *xio* mutants phenocopy *Mettl3* mutant adult defects

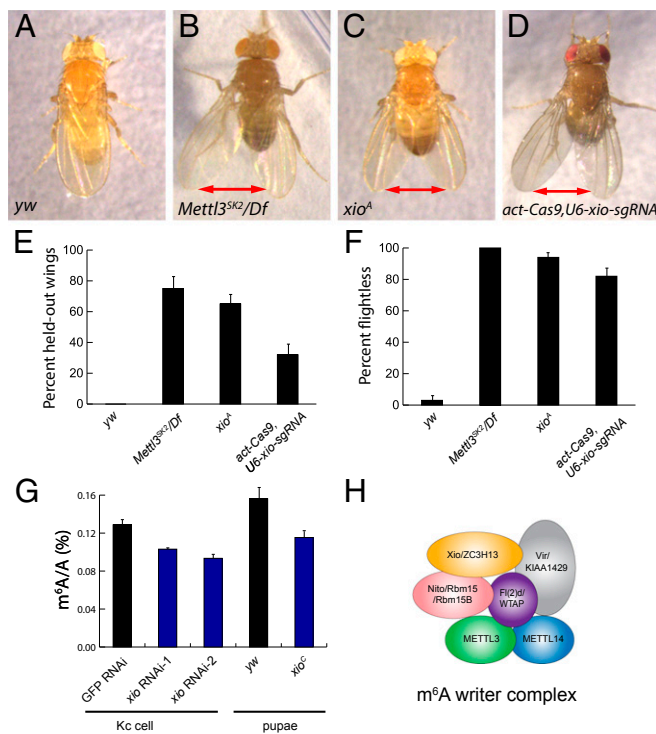


Fig. 5. *xio* mutants show adult defects, and Xio is required for proper m⁶A levels. (*A*) *yw* flies have their wings properly folded. (*B*) *Mettl3*^{SK2/Df}, (*C*) *xio*⁴, (*D*) *actin-Cas9/U6-xio-sgRNA* flies cannot fold their wings and exhibit a held-out wing phenotype (marked by the double arrows). The frequency of flies that show held-out wings was quantified in *E*; error bars represent SEM. (*F*) Flies of the indicated genotypes were tested for their flight abilities, and the number of flightless flies was quantified; error bars represent SEM. All flies used from *A*–*F* are males. (*G*) Quantifications of m⁶A relative to A in Kc cells and pupae. Compared with controls, *xio* RNAi cells and *xio*^c mutant pupae showed reduced m⁶A levels in their RNA after one round of polyA purification. Error bars represent SD. (*H*) A model of the m⁶A writer complex comprised of six core components.

and that the m⁶A level is reduced in *xio* mutant cells and fly. As ZC3H13 was found in the WTAP-associating protein complex (41), Xio/ZC3H13 is likely an evolutionarily conserved m⁶A factor (Fig. 5H). Our study also shed light on how the m⁶A modification is involved in *Sxl* splicing regulation. Similar to Fl (2d) and Nito (6, 8), we show that Xio can interact with Sxl in a co-IP experiment. Furthermore, we tested whether the m⁶A writer complex physically interacts with the spliceosome and found that Nito is the major component that strongly interacts with SNF in the absence of Sxl. This is not particularly surprising since Nito has three RRM domains, compared with two RRMs in the Sxl protein. These results suggest a new mechanism for m⁶A-mediated splicing in which the m⁶A writer complex can interact with the spliceosome independently of Sxl and Nito may serve as a bridge between the spliceosome and the m⁶A catalytic core (Fig. 4A).

Besides Sxl-mediated sex determination, m⁶A mutants in *Drosophila* exhibit several characteristic phenotypes. *Mettl3* and *Mettl4* mutants are homozygous viable and show held-out wing and flightless phenotypes (10–12). *fl(2)d*, *vir*, and *nito* mutants die during larval stages, preventing the analysis of their adult phenotypes. A strong allele of *xio* also causes lethality during the pupal stage; however, by using a slightly weaker allele of *xio*, we were able to examine its adult phenotype. *xio*⁴ mutants resemble *Mettl3* held-out wing and flightless phenotypes, strongly arguing that Xio is a core subunit of the m⁶A writer complex. These phenotypes are likely due to the function of m⁶A modifications

in the nervous system where its level is highest in both fly and mammals. Finally, RNA-Seq analysis revealed that many neuronal genes are differentially expressed and/or alternatively spliced in *xio* mutants, suggesting that it will be important to pinpoint the critical mRNA species that are m⁶A-modified in the nervous system. Another group of genes that were found from the RNA-Seq analysis are metabolic genes, which are also significantly enriched in *Mettl3* mutant flies (10). These genes are of particular interest since overexpression of the m⁶A demethylase Fto in mice leads to increased food intake and obesity (42). In vertebrates, m⁶A has been shown to regulate embryonic stem cell differentiation, somatic cell differentiation, maternal-to-zygotic transition, circadian rhythm, and spermatogenesis (17); whether m⁶A plays similar roles in *Drosophila* remains to be determined.

Methods

Details on the fly strains and antibodies used in this study, as well as how *xio* clones were generated and how Xio antibody was generated, can be found in *SI Methods*. Protocols used for antibody staining, cell culture and RNA interference, coimmunoprecipitation, RT-PCR, analyzing m⁶A levels by LC-MS, and RNA-Seq can be found in *SI Methods*.

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