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

Jian Guo[a,b], Hong-Wen Tang[c], Jing Li[a,b], Norbert Perrimon[c,d,1], and Dong Yan[a,1]

[a]Key Laboratory of Insect Developmental and Evolutionary Biology, Chinese Academy of Sciences Center for Excellence in Molecular Plant Sciences, Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, 200032 Shanghai, China; [b]University of Chinese Academy of Sciences, 100049 Beijing, China; [c]Department of Genetics, Harvard Medical School, Boston, MA 02115; and [d]Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115

Contributed by Norbert Perrimon, February 14, 2018 (sent for review December 6, 2017; reviewed by James W. Erickson and Helen Salz)

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, Fl(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, Fl(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, Fl(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 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.

Author contributions: J.G., H.-W.T., N.P., and D.Y. designed research; J.G., H.-W.T., J.L., and D.Y. performed research; N.P. and D.Y. contributed new reagents/analytic tools; J.G., H.-W.T., N.P., and D.Y. analyzed data; and N.P. and D.Y. wrote the paper.

Reviewers: J.W.E., Texas A&M University; and H.S., Case Western Reserve University.

The authors declare no conflict of interest.

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).

[1]To whom correspondence may be addressed. Email: perrimon@receptor.med.harvard.edu or yandong@sibs.ac.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1720945115/-/DCSupplemental.

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 m^{6}A writer complex.

Results

Xio Interacts and Colocalizes with Known m^{6}A 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 m^{6}A methyltransferase complex, we hypothesized that Xio is a new factor of the m^{6}A 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 m^{6}A writer complex.

Xio Is a Ubiquitously Expressed Nuclear Protein, and Its Expression Patterns Mimic Other m^{6}A 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 m^{6}A 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 m^{6}A 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 m^{6}A 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 m^{6}A modification is the *Drosophila* sex determination gene *Sxl* (10–12). Activation of Sxl in female embryos requires the coordination of two promoters, the

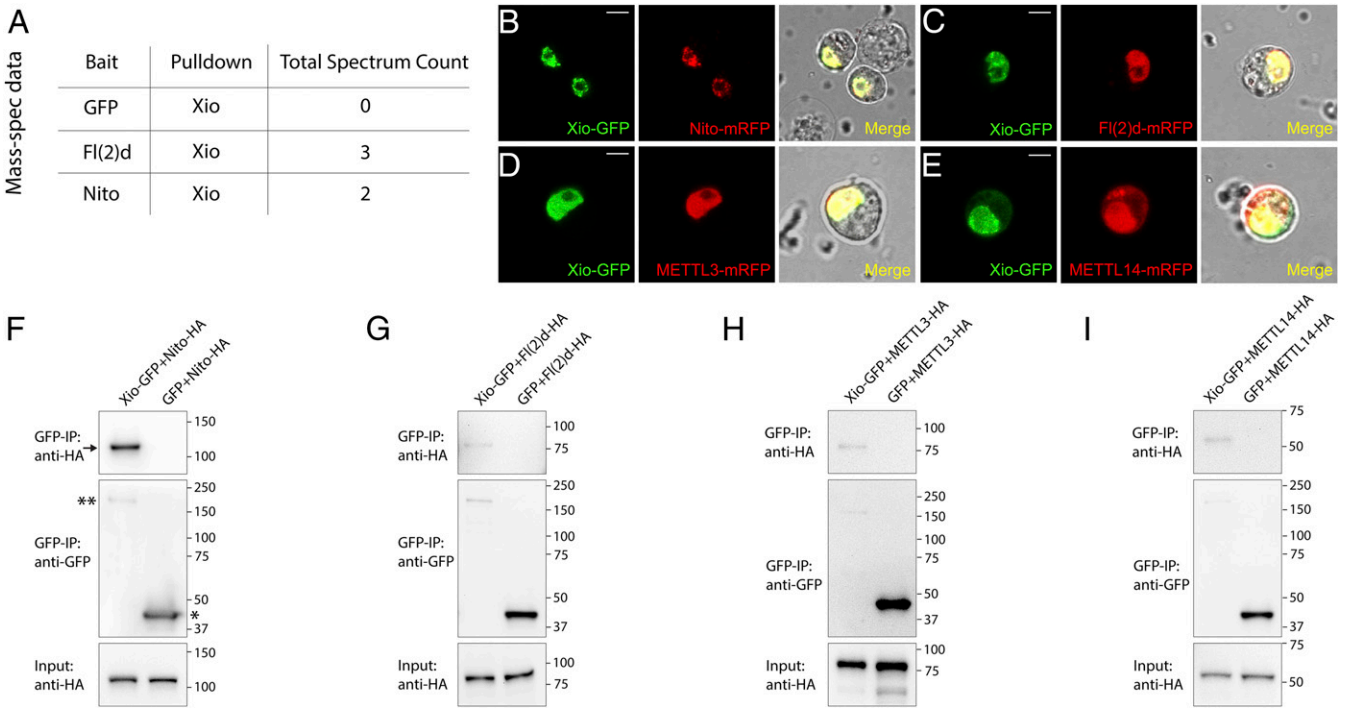

Fig. 1. Xio colocalizes and interacts with other m^{6}A writer components. (*A*) From mass-spec experiments, Fl(2)d and Nito, but not GFP, can pull down Xio. (*B–E*) Xio-GFP and Nito-mRFP, Fl(2)d-mRFP, METTL3-mRFP, or METTL14-mRFP were cotransfected into S2 cells, and their subcellular localization was examined in live cells. (Scale bars: 5 μm.) (*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 autoregulatory 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. 2*A*). 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. 2*A*), 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. 2*B*). 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. 2 *C*–*E*). In addition, *dome-Gal4/xio-RNAi* females showed strong abnormalities in the genitalia. Typical female structures, such as vaginal bristles (Fig. 2 *G*, white arrow), disappear, and structures resembling the male penis apparatus can be found (Fig. 2 *F* 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. 3 *A* 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 disk using *ap-Gal4* led to a significant reduction of Sxl levels (Fig. 3 *C* and *D*). Second, Sxl levels were almost absent from xio^A or xio^C mitotic mutant clones (Fig. 3 *E*–*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. 3 *G*–*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. 4*A*) (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. 4*A*) (24). In *ap-Gal4/xio RNAi* female wing discs, a large band corresponding to the male-specific spliced form was clearly detected (Fig. 4*B*). 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. 4*C*). 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. 4*C*).

We then analyzed how the m^{6}A 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

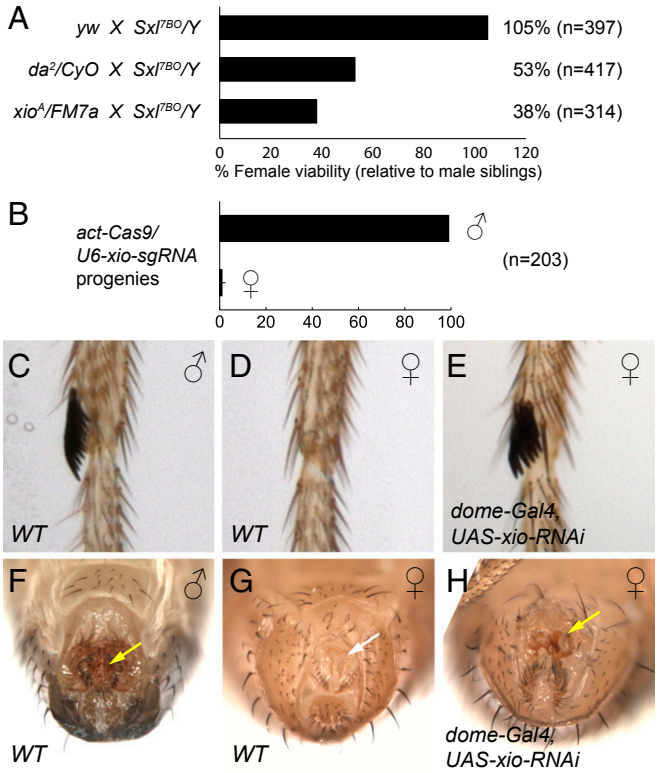

Fig. 2. Xio is required for sex determination in *Drosophila*. (*A*) Females of the indicated genotypes were crossed to Sxl^{7BO}/Y males, and the resulting progeny were scored. For *yw* and da^2/CyO crosses, the viability of total female adults relative to sibling male adults was quantified. For the $xio^A/FM7a$ 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 combs. (*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 m^{6}A 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. 4*D*, GFP-Xio, but not GFP alone, can pull down Sxl-HA. These data are consistent with recent findings that m^{6}A 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 m^{6}A writer complex physically interacts with the spliceosome. In S2 cells where Sxl is absent, we performed co-IP experiments between SNF and five m^{6}A writer subunits (Fig. 4*E*). 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 m^{6}A writer complex can interact with the spliceosome independent of Sxl and Nito serves as a bridge between the spliceosome and other m^{6}A subunits. Together, we propose a model that both Sxl and the m^{6}A writer complex interact with the spliceosome and they also interact with each other to repress the inclusion of the male-specific exon (Fig. 4*A*).

***xio* Mutant Phenocopies *Mettl3* Adult Defects, and *xio* Is Required for m^{6}A Levels.** Other than sex determination phenotype, m^{6}A writer

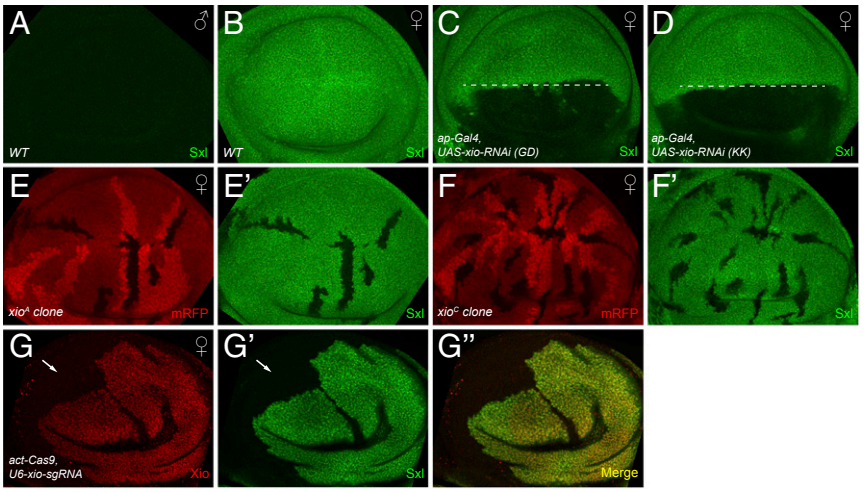

Fig. 3. Xio is required for Sxl levels in somatic tissues. Sxl stainings in *WT* male (*A*) and female (*B*) wing discs. (*C* and *D*) Expressing *xio* RNAi in the dorsal half of the disk (below the dashed line) using *ap-Gal4* results in strong reduction of Sxl stainings. (*C*) GD35212; (*D*) KK110253. (*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 m^6A modifications in the nervous system (11). If Xio is a component of the m^6A 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. S4*C*).

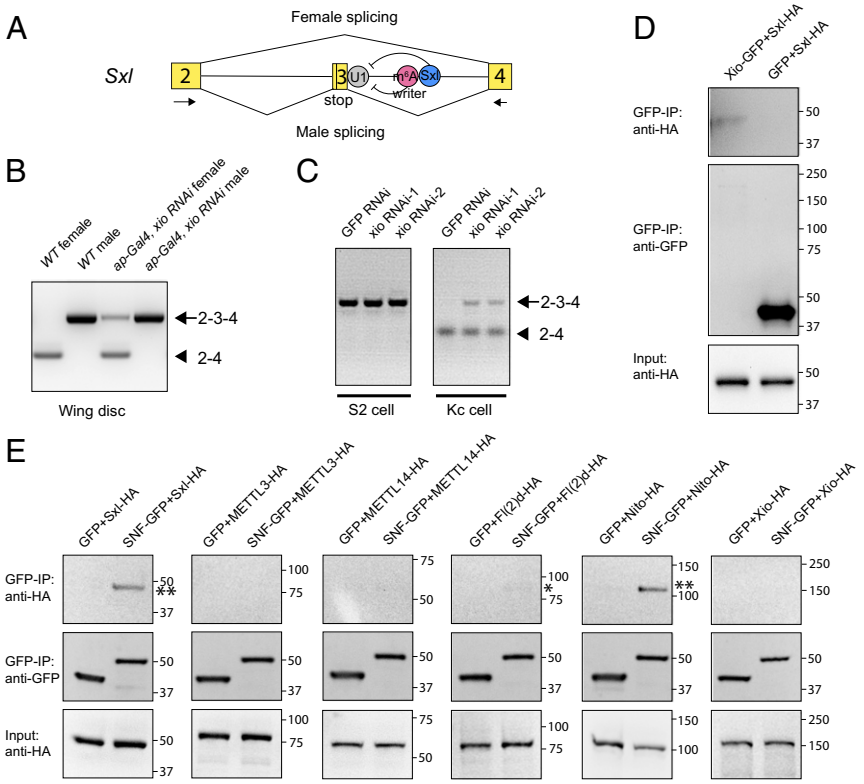

Fig. 4. Xio regulates *Sxl* alternative splicing and interacts with Sxl. (*A*) Model showing female- or male-specific *Sxl* alternative splicing mediated by the m^6A writer and Sxl protein. Only the regulatory events in the intron downstream of exon 3 are shown. The arrows indicate the primers used for 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, Fl(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 Fl(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^A 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^A mutant flies, as well as *actin-Cas9/U6-xio-sgRNA* flies, were flightless (Fig. 5*F*). Together, these results indicate that *xio* mutants resemble m^{6}A pathway mutants in terms of sex determination, held-out wings, and flight abilities, strongly arguing that Xio is a key component of the m^{6}A methylation pathway.

Next, we directly measured N^6-methyladenosine levels in *xio* mutants by quantitative liquid chromatography–mass spectrometry (LC-MS). We used an external calibration curve prepared with A and m^{6}A 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 m^{6}A 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 m^{6}A 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 m^{6}A methyltransferase complex (Fig. 5*H*).

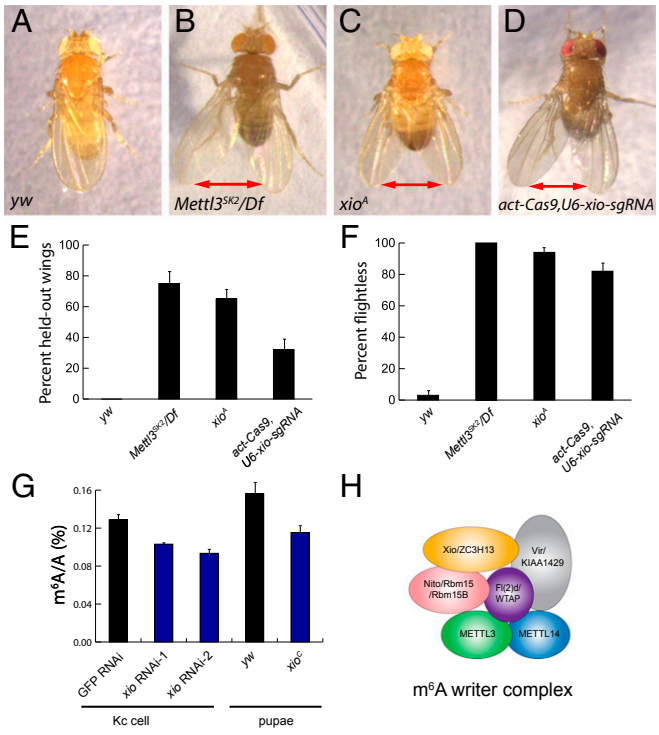

Fig. 5. *xio* mutants show adult defects, and Xio is required for proper m^{6}A levels. (*A*) *yw* flies have their wings properly folded. (*B*) $Mettl3^{SK2}/Df$, (*C*) xio^A, (*D*) *actin-Cas9/U6-xio-sgRNA* flies cannot fold their wings and exhibit a held-out wing phenotype (marked by the double arrows). 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 m^{6}A relative to A in Kc cells and pupae. Compared with controls, *xio* RNAi cells and xio^C mutant pupae showed reduced m^{6}A levels in their RNA after one round of polyA purification. Error bars represent SD. (*H*) A model of the m^{6}A writer complex comprised of six core components.

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^A hemizygous males are sterile, we were unable to generate xio^A homozygous females and thus performed RNA-Seq in xio^A 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 eclosure, as this is the period with very high m^{6}A levels and most xio^A 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, ΔPSI (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 m^{6}A methyltransferase complex.

m^{6}A modification has been known for more than 40 y (32) but recently gained attention due to the emergence of new technologies to map m^{6}A 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 m^{6}A 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, METTL14, 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 m^{6}A writer complex. We further show that *xio* mutants phenocopy *Mettl3* mutant adult defects

GENETICS

and that the m^{6}A 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 m^{6}A factor (Fig. 5*H*). Our study also shed light on how the m^{6}A modification is involved in *Sxl* splicing regulation. Similar to Fl (2)d and Nito (6, 8), we show that Xio can interact with Sxl in a co-IP experiment. Furthermore, we tested whether the m^{6}A 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 m^{6}A-mediated splicing in which the m^{6}A writer complex can interact with the spliceosome independently of Sxl and Nito may serve as a bridge between the spliceosome and the m^{6}A catalytic core (Fig. 4*A*).

Besides Sxl-mediated sex determination, m^{6}A mutants in *Drosophila* exhibit several characteristic phenotypes. *Mettl3* and *Mettl14* 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*^{A} mutants resemble *Mettl3* held-out wing and flightless phenotypes, strongly arguing that Xio is a core subunit of the m^{6}A 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 m^{6}A-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, m^{6}A has been shown to regulate embryonic stem cell differentiation, somatic cell differentiation, maternal-to-zygotic transition, circadian rhythm, and spermatogenesis (17); whether m^{6}A 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 m^{6}A levels by LC-MS, and RNA-Seq can be found in *SI Methods*.

ACKNOWLEDGMENTS. We thank Eric Lai, Jean-Yves Roignant, Helen Salz, and Matthias Soller for fly stocks. This work was supported by National Natural Science Foundation of China Grant 31771586, the Shanghai Pujiang Program, and a start-up fund from the Chinese Academy of Sciences (to D.Y.). N.P. is an investigator of the Howard Hughes Medical Institute.

1. Cline TW, Meyer BJ (1996) Vive la différence: Males vs females in flies vs worms. *Annu Rev Genet* 30:637–702.
2. Salz HK, Erickson JW (2010) Sex determination in Drosophila: The view from the top. *Fly (Austin)* 4:60–70.
3. Camara N, Whitworth C, Van Doren M (2008) The creation of sexual dimorphism in the Drosophila soma. *Curr Top Dev Biol* 83:65–107.
4. Lucchesi JC, Kuroda MI (2015) Dosage compensation in Drosophila. *Cold Spring Harb Perspect Biol* 7:a019398.
5. Hilfiker A, Amrein H, Dübendorfer A, Schneiter R, Nöthiger R (1995) The gene virilizer is required for female-specific splicing controlled by Sxl, the master gene for sexual development in Drosophila. *Development* 121:4017–4026.
6. Yan D, Perrimon N (2015) spenito is required for sex determination in Drosophila melanogaster. *Proc Natl Acad Sci USA* 112:11606–11611.
7. SchuLtt C, Hilfiker A, Nothiger R (1998) virilizer regulates Sex-lethal in the germline of Drosophila melanogaster. *Development* 125:1501–1507.
8. Penn JK, et al. (2008) Functioning of the Drosophila Wilms'-tumor-1-associated protein homolog, Fl(2)d, in Sex-lethal-dependent alternative splicing. *Genetics* 178: 737–748.
9. Granadino B, Campuzano S, Sánchez L (1990) The Drosophila melanogaster fl(2)d gene is needed for the female-specific splicing of Sex-lethal RNA. *EMBO J* 9:2597–2602.
10. Haussmann IU, et al. (2016) m^{6}A potentiates Sxl alternative pre-mRNA splicing for robust Drosophila sex determination. *Nature* 540:301–304.
11. Lence T, et al. (2016) m^{6}A modulates neuronal functions and sex determination in Drosophila. *Nature* 540:242–247.
12. Kan L, et al. (2017) The m^{6}A pathway facilitates sex determination in Drosophila. *Nat Commun* 8:15737.
13. Roundtree IA, Evans ME, Pan T, He C (2017) Dynamic RNA modifications in gene expression regulation. *Cell* 169:1187–1200.
14. Hongay CF, Orr-Weaver TL (2011) Drosophila Inducer of MEiosis 4 (IME4) is required for notch signaling during oogenesis. *Proc Natl Acad Sci USA* 108:14855–14860.
15. Meyer KD, Jaffrey SR (2017) Rethinking m^{6}A readers, writers, and erasers. *Annu Rev Cell Dev Biol* 33:319–342.
16. Patil DP, et al. (2016) m(6)A RNA methylation promotes XIST-mediated transcriptional repression. *Nature* 537:369–373.
17. Hsu PJ, Shi H, He C (2017) Epitranscriptomic influences on development and disease. *Genome Biol* 18:197.
18. Roignant JY, Soller M (2017) m^{6}A in mRNA: An ancient mechanism for fine-tuning gene expression. *Trends Genet* 33:380–390.
19. Guruharsha KG, et al. (2011) A protein complex network of Drosophila melanogaster. *Cell* 147:690–703.
20. Graveley BR, et al. (2011) The developmental transcriptome of Drosophila melanogaster. *Nature* 471:473–479.
21. Kumar S, et al. (2017) FlyExpress 7: An integrated discovery platform to study coexpressed genes using in situ hybridization images in Drosophila. *G3 (Bethesda)* 7: 2791–2797.
22. Port F, Chen HM, Lee T, Bullock SL (2014) Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in Drosophila. *Proc Natl Acad Sci USA* 111: E2967–E2976.
23. Yamamoto S, et al. (2014) A drosophila genetic resource of mutants to study mechanisms underlying human genetic diseases. *Cell* 159:200–214.
24. Johnson ML, Nagengast AA, Salz HK (2010) PPS, a large multidomain protein, functions with sex-lethal to regulate alternative splicing in Drosophila. *PLoS Genet* 6: e1000872.
25. Bell LR, Horabin JI, Schedl P, Cline TW (1991) Positive autoregulation of sex-lethal by alternative splicing maintains the female determined state in Drosophila. *Cell* 65: 229–239.
26. Flickinger TW, Salz HK (1994) The Drosophila sex determination gene snf encodes a nuclear protein with sequence and functional similarity to the mammalian U1A snRNP protein. *Genes Dev* 8:914–925.
27. Nagengast AA, Stitzinger SM, Tseng CH, Mount SM, Salz HK (2003) Sex-lethal splicing autoregulation in vivo: Interactions between SEX-LETHAL, the U1 snRNP and U2AF underlie male exon skipping. *Development* 130:463–471.
28. Oliver B, Perrimon N, Mahowald AP (1988) Genetic evidence that the sans fille locus is involved in Drosophila sex determination. *Genetics* 120:159–171.
29. Salz HK, et al. (2004) The Drosophila U1-70K protein is required for viability, but its arginine-rich domain is dispensable. *Genetics* 168:2059–2065.
30. Chaouki AS, Salz HK (2006) Drosophila SPF45: A bifunctional protein with roles in both splicing and DNA repair. *PLoS Genet* 2:e178.
31. Lallena MJ, Chalmers KJ, Llamazares S, Lamond AI, Valcárcel J (2002) Splicing regulation at the second catalytic step by Sex-lethal involves 3′ splice site recognition by SPF45. *Cell* 109:285–296.
32. Desrosiers R, Friderici K, Rottman F (1974) Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. *Proc Natl Acad Sci USA* 71:3971–3975.
33. Dominissini D, et al. (2012) Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. *Nature* 485:201–206.
34. Meyer KD, et al. (2012) Comprehensive analysis of mRNA methylation reveals enrichment in 3′ UTRs and near stop codons. *Cell* 149:1635–1646.
35. Bokar JA, Shambaugh ME, Polayes D, Matera AG, Rottman FM (1997) Purification and cDNA cloning of the AdoMet-binding subunit of the human mRNA (N6-adenosine)-methyltransferase. *RNA* 3:1233–1247.
36. Jia G, et al. (2011) N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nat Chem Biol* 7:885–887.
37. Zheng G, et al. (2013) ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell* 49:18–29.
38. Bokar JA, Rath-Shambaugh ME, Ludwiczak R, Narayan P, Rottman F (1994) Characterization and partial purification of mRNA N6-adenosine methyltransferase from HeLa cell nuclei. Internal mRNA methylation requires a multisubunit complex. *J Biol Chem* 269:17697–17704.
39. Liu J, et al. (2014) A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. *Nat Chem Biol* 10:93–95.
40. Zhong S, et al. (2008) MTA is an Arabidopsis messenger RNA adenosine methylase and interacts with a homolog of a sex-specific splicing factor. *Plant Cell* 20:1278–1288.
41. Horiuchi K, et al. (2013) Identification of Wilms' tumor 1-associating protein complex and its role in alternative splicing and the cell cycle. *J Biol Chem* 288:33292–33302.
42. Church C, et al. (2010) Overexpression of Fto leads to increased food intake and results in obesity. *Nat Genet* 42:1086–1092.