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^6^A), 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^6^A 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^6^A factors, such as sexual transformations, Sxl splicing defect, held-out wings, flightlessness, and reduction of m^6^A levels. Thus, *Xio* encodes a member of the m^6^A methyltransferase complex involved in mRNA modification. Since its ortholog ZC3H13 (or KIAA0853) also associates with several m^6^A writer factors, the function of *Xio* in the m^6^A pathway is likely evolutionarily conserved.

CG7358 | *Xio* | sex determination | m^6^A 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^6^A) methyltransferase complex, revealing that the m^6^A pathway modulates sex determination in *Drosophila* (10–12). m^6^A is the most abundant chemical modification in mRNA, and its level is dynamically regulated (13). m^6^A 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^6^A modification sites have been mapped to Sxl introns, thus facilitating Sxl pre-mRNA alternative splicing. Importantly, m^6^A methylation is required in human dosage compensation by modifying the long noncoding RNA XIST, suggesting that m^6^A-mediated gene regulation is an ancient mechanism for sex determination (16).

Recent emerging studies suggest that m^6^A 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^6^A methylated mRNAs have been identified, Sxl pre-mRNA is arguably one of the best understood examples for m^6^A 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^6^A 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^6^A) is the most common internal modification in eukaryotic mRNA. m^6^A 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^6^A is installed by a large methyltransferase complex called the m^6^A 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^6^A writer subunits, and its loss of function shows typical phenotypes associated with other m^6^A factors, such as Sxl splicing misregulation, adult defects, and reduced m^6^A levels. Therefore, we conclude that *Xio* is a member of the m^6^A 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 m^A writer complex.

**Results**

**Xio Interacts and Colocalizes with Known m^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 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. S1B). Altogether, these data suggest that Xio is a new component of the m^A writer complex.

Xio Is a Ubiquitously Expressed Nuclear Protein, and Its Expression Patterns Mimic Other m^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. S2A). 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^A writers and readers, with high expression in the CNS and high expression in the ovary, imaginal discs, and fat tissues (Fig. S3A) (mod-ENCODE 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. S3A), which coincides with the reported m^A levels (11). *xio* expression showed enrichment in the neuroectoderm at later stages of embryogenesis (Fig. S3B) (FlyExpress) (21), which is highly similar to known m^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. S2A–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. 3G). 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^175Y* flies died at the pupal stage while *xio^175Y* flies died as pharate, with some flies halfway out of the pupal case (Fig. S4C). 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^A modification is the *Drosophila* sex determination gene Sxl (10–12). Activation of Sxl in female embryos requires the coordination of two promoters, the

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**Fig. 1.** Xio colocalizes and interacts with other m^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+ heterozygous mothers were crossed to Sxl+/Y (a null allele of Sxl) fathers, only 55% of the expected Sxl+/+ females survived to adulthood (Fig. 2A). Importantly, when xio+ heterozygous mothers were crossed to Sxl+/Y fathers, only 38% of the expected Sxl+/+ 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 act-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. 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 investigate 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. 3 C and D). Second, Sxl levels were almost absent from xio+ or xio2 mitotic mutant clones (Fig. 3 E–F). Third, crossing of act-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 frame includes exon 3 that contains a stop codon and leads to early termination of Sxl protein, the female frame 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. 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 SFN, a protein component of the U1 and U2 small nuclear ribonucleoproteins (snRNPs) (26, 27). Since the m6A writer components Fli(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 SFN and five m6A writer subunits (Fig. 4E). As a positive control, SFN-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 SFN-GFP, indicating that these two proteins interact strongly with each other. However, a very low amount of Fli(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. 4F).

**xio Mutant Phenocopies Mettl3 Adult Defects, and xio Is Required for m6A Levels.** Other than sex determination phenotype, m6A writer
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. S4C).

**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.

**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) S2-HA, Xio-GFP, or GFP were transfected 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, xio6 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 xio6 mutant flies, as well as actin-Cas9/U6-xio-sgRNA flies, were flightless (Fig. 5F). 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 xio6 mutant pupae (Fig. 5G). 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. 5H).

**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 xio6 hemizygous males are sterile, we were unable to generate xio6 homozygous females and thus performed RNA-Seq in xio4 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 xio6 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. S7A 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. S7C 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. S7B). In addition, we also analyzed the alternative splicing changes in these mutants and found that 105 alternative splicing events in 96 genes in xio mutants phenocopy xio A pathway mutants (Fig. S7D). The key methyltransferase METTL3 was identified in a recent proteomic study to identify WTAP interacting proteins in human cells (38). 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, 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 m6A writer complex. We further show that xio mutants phenocopy Mettl3 mutant adult defects.
and that the m^A level is reduced in xio mutant cells and fly. As ZC3H13 was found in the WTAP-associated protein complex (41), Xio/ZC3H13 is likely an evolutionarily conserved m^A factor (Fig. 5E). Our study also shed light on how the m^A modification is involved in 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^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^A-mediated splicing in which the m^A writer complex can interact with the spliceosome independently of Sxl and Nito may serve as a bridge between the spliceosome and the m^A catalytic core (Fig. 4A).

Besides Sxl-mediated sex determination, m^A mutants in Drosophila exhibit several characteristic phenotypes. Metl3 and Metl14 mutants are homozygous viable and show hold-out wing and flightless phenotypes (10–12). Fl(2)d, vir, and nito 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 maintain its adult phenotype. m^A mutants resemble Metl3 hold-out wing and flightless phenotypes, strongly arguing that Xio is a core subunit of the m^A writer complex. These phenotypes are likely due to the function of m^A 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 m^A RNA species that are m^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 Metl3 mutant flies (10). These genes are of particular interest since overexpression of the m^A demethylase Fto in mice leads to increased food intake and obesity (42). In vertebrates, m^A has been shown to regulate embryonic stem cell differentiation, somatic cell differentiation, maternal-to-zygotic transition, circadian rhythm, and spermatogenesis (17); whether m^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^A levels by LC-MS, and RNA-Seq can be found in SI Methods.

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