Supporting Information

Guo et al. 10.1073/pnas.1720945115

SI Methods

Fly Strains. The following stocks were used in this study: w^{1118} (used as WT), *yw*, *ap-Gal4*, *dome-Gal4, UAS-2xEYFP* (6), *xio* RNAi [GD35212; Vienna Drosophila Resource Center (VDRC)], *xio* RNAi (KK110253; VDRC), Sxl^{7BO}, da^{2} (24), *actin-Cas9* [54590; Bloomington Drosophila Stock Center (Bloomington)], *U6-xio-sgRNA* (68063; Bloomington), $Mettl3^{SK2}$ (12), *Df(3R)Exel6197* (*Mettl3* deficiency, 7676; Bloomington), *Ubi-mRFP, hsFlp FRT19A* (31418; Bloomington), xio^{A} *FRT19A* (57081; Bloomington), and xio^{C} *FRT19A* (57082; Bloomington). Resequencing of these two alleles showed that xio^{A} has a frameshift mutation at amino acid 782 (Fig. S4*A*) and that xio^{C} has a point mutation that results in a truncated protein at amino acid 729 (Fig. S4*B*). Xio antibody staining was strongly reduced in these clones although the epitope is not supposed to be truncated in these mutations.

To generate mutant clones in discs, xio^{A} *FRT19A* and xio^{C} *FRT19A* females were crossed to *Ubi-mRFP*, *hsFlp FRT19A* males. Progeny were heat-shocked at 37 °C for 1 h twice at first- and second-instar larval stage. To generate mutant clones in ovaries, progeny were heat-shocked at 37 °C for 2 h twice at the end of third-instar larvae. To test flight ability, cohorts of 10 flies were tapped down into a Petri dish, and the number of flies that flew away within 2 min was recorded.

Antibody Stainings in Discs and Ovaries. Larval wing discs and female ovaries were stained as previously described (6). Briefly, tissues were dissected in PBS and fixed in 4% formaldehyde in PBST (PBS + 0.1% Triton X-100). After blocking in 1% normal donkey serum in PBST for 1 h, the samples were incubated with the primary antibody in the same solution at 4 °C overnight. After three washes in PBST, samples were incubated with the secondary antibody for 2 h at room temperature, washed in PBST three times, and subsequently mounted in Antifade Mounting Medium (Beyotime). All images were taken on a Zeiss LSM 880 microscope.

The following antibodies were used: mouse anti-Sxl (1:10) [M18; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Fl(2)d (1:10) (9G2; DSHB), rabbit anti-Xio (1:200), Alexa 488-conjugated (1:1,000) (ThermoFisher) or Cy3-conjugated (1:400) (Jackson Immuno) secondary antibodies, and DAPI (1:1,000) (Beyotime). Xio antibody was generated in rabbits against a peptide containing amino acids 486 to 500 (TEDTLYPDERERLIR) and affinity-purified at Youke Biotech.

Adult legs were mounted in a 1:1 (vol/vol) mixture of Permount (Fisher Scientific) and xylene. The genitalia images were taken in stacks and rendered with HeliconFocus software.

Cell Culture and RNA Interference. *Drosophila* S2 and Kc cells were maintained in Schneider's medium (Gibco) supplemented with 10% FBS (Sigma) and 1% penicillin–streptomycin (Sigma). For the RNAi experiments, PCR templates for the dsRNA were prepared using Phusion Hot Start High-Fidelity DNA Polymerase (NEB), and the dsRNA were synthesized using T7 MEGAscript Kit (Invitrogen). dsRNA against GFP was used as a control for all RNAi experiments. S2 and Kc cells were resuspended at a density of 1 to 5 × 10^{6} cells per mL in serum-free media, and 1 mL of resuspended cells were plated into wells of a six-well plate, followed by addition of 30 μg of dsRNA per well and incubated with dsRNA for 30 min. Subsequently, 3 mL of complete media with 10% FBS was added to each well. After 72 h of incubation at 25 °C, cells were harvested for analysis. The amplicons used for *xio* are DRSC25501 and DRSC35494.

Coimmunoprecipitation. To generate the GFP-, mRFP-, or HA-tagged plasmids, full-length cDNAs for Xio (GH14795), Nito (GH11110), Fl(2)d (LD21616), METTL3 (AT20169), METTL14 (LD06016), and SNF (LD45302) were cloned into the pENTR vector (Invitrogen) and transferred into the *Drosophila* gateway vector pAGW, pARW, and pAHW. Sxl-HA was from ref. 6. GFP was cloned into pAWM as a control.

One microgram of total DNA was transfected into S2 cells in a single well of six-well plates with Effectene (QIAGEN). After 48 h, cells were lysed in IP lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1× protease inhibitor; Beyotime] on ice for 30 min and cleared at 20,000 × *g* for 10 min at 4 °C. Supernatants were incubated with anti-GFP nanobody agarose beads (Allele Biotechnology) for 2 h at 4 °C. The beads were washed three to four times with 1 mL of lysis buffer and resuspended in 2× SDS sample buffer. Eluted proteins were detected by Western blotting using anti-GFP antibody (A6455; Molecular Probes) or anti-HA antibody (3F10; Roche). For colocalization, S2 cells were grown on Lab-Tek chamber slides and imaged in live conditions 2 d after transfection.

RT-PCR. Total RNAs were extracted using TRIzol (Invitrogen) and purified through the RNeasy Mini Kit (QIAGEN). cDNA was generated from 1 μg of purified RNA using the iScript cDNA Synthesis Kit (Bio-Rad). TaKaRa Taq was used for the PCR. Sxl primers (GTGGTTATCCCCCATATGGC and GATGGCAGAGAATGGGAC) and PCR conditions are described in ref. 24.

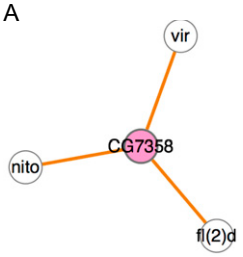
Analyzing m^{6}A Levels by LC-MS. Total RNAs were extracted from *yw* and xio^{A} pharate pupae or dsRNA-treated Kc cells and then subjected to one round of polyA selection using the GenElute mRNA Miniprep kit (Sigma). Before LC-MS analysis, all RNA samples were hydrolyzed enzymatically to ribonucleosides. Briefly, 10 μg of RNA of each sample was digested by nuclease P1 (Sigma) and snake venom phosphodiesterase (Sigma) at 37 °C for 2 h and followed by digestion with fast alkaline phosphatase (ThermoFisher) for another 1 h. Then, the digested samples were used for the following LC-MS analysis.

Quantitative LC-MS analyses of m^{6}A and adenosine were achieved using a Waters UPLC coupled to a Thermo Q Exactive mass spectrometer in positive ion mode using dynamic multiple reaction monitoring. The ribonucleosides in the hydrolyzed RNA samples were resolved on an Acquity UPLC HSS T3 column (1.8-μm particle size, 100-Å pore size, 2.1 mm × 50 mm, 25 °C) at 300 μL·min^{-1} using a solvent system of 0.1% formic acid in H_2O (A) and acetonitrile (B). The elution profile was 2% B for 2 min, 2 to 11% B over 4 min, then to 11 to 80% B over 4 min, followed by a column washing at 80% B and column equilibration. The operating parameters for the mass spectrometer were as follows: gas temperature 350 °C; gas flow 35 L per min. The quantification of a ribonucleoside can be achieved using *m/z* of the parent ribonucleoside ion and *m/z* of the deglycosylated ion product. Nucleosides were quantified based on the transition of the parent ribonucleoside to the deglycosylated base ion: *m/z* 282.1 to 150.1 for m^{6}A and *m/z* 268.1 to 136.1 for A. Absolute quantities of each ribonucleoside were determined using an external calibration curve prepared with A standards (Sigma) and m^{6}A standards (Selleck).

RNA-Seq and Analysis of Differential Gene Expression and Alternative Splicing. Total RNA was extracted from *xio* mutants and w^{1118} control pupae using TRIzol (Invitrogen) and purified through an RNeasy Mini Kit (Qiagen). It was then used to prepare stranded

libraries using a NEXflexTM Rapid Directional RNA-Seq Kit (Bioo Scientific). The quality of the prepared libraries was analyzed using an Agilent 2100 Bioanalyzer. Pooled libraries were sequenced on an Illumina HiSeq2500 to yield around 240 million paired-end 150-bp reads for three controls and mutants. Reads were then aligned to the *Drosophila* genome (dmel-r6.02) using Tophat, and differential gene expression was determined by Cufflinks. Alternative splicing analysis was achieved by Mixture of Isoforms (MISO), and GO enrichment analysis was done with the Database for Annotation, Visualization and Integrated Discovery (DAVID). RNA-Seq data were deposited in the Gene Expression Omnibus (GEO) (accession no. GSE110047).

A



B

DPiM mass-spec data

Bait	Pulldown	Unique peptides	Total peptides
CG7358	CG7358	49	90
CG7358	Nito	37	64
CG7358	Vir	17	17
CG7358	Fl(2)d	8	10

Fig. S1. Interactions between Xio/CG7358 with Nito, Fl(2)d, and Vir from the DPiM database. (*A*) Physical interactions between Xio/CG7358 with Nito, Fl(2)d, and Vir based on DPiM data shown as an esyN diagram. (*B*) From DPiM mass-spec dataset, CG7358 as a bait can pull down Nito, Vir, and Fl(2)d. Unique and total peptides pulled were shown.

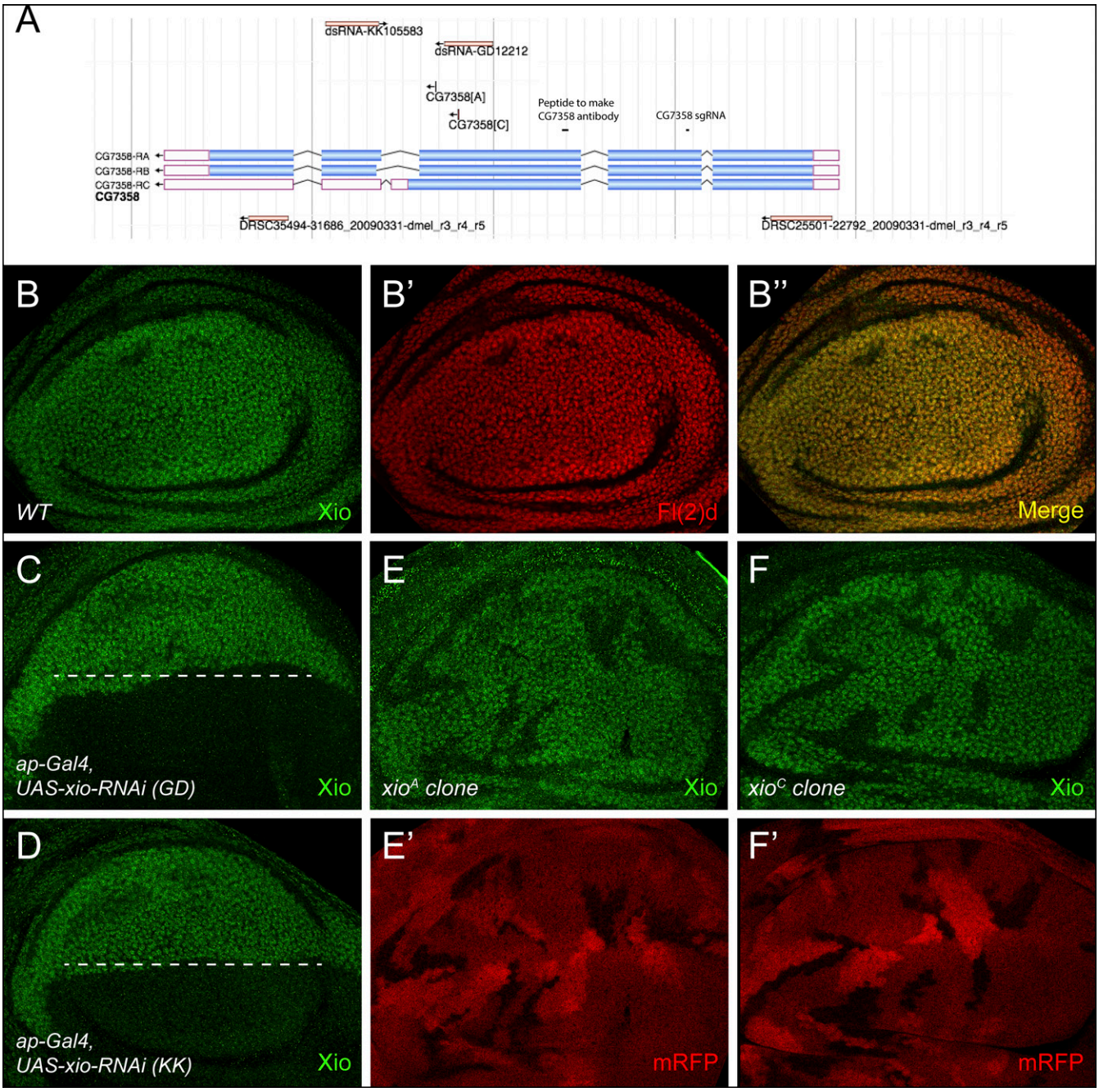


Fig. S2. Characterization of *xio* mutants, RNAi strains, and antibody. (*A*) Flybase JBrowse view of the *CG7358/xio* gene locus. *CG7358* has three transcripts due to alternative splicing that generate proteins of 1,150 aa, 1,139 aa, and 842 aa. The position of the VDRC RNAi amplicons, *Drosophila* RNAi Screening Center (DRSC) RNAi amplicons, $CG7358^{A}$ mutation, $CG7358^{C}$ mutation, *CG7358* sgRNA, and the peptide used to generate a CG7358 antibody are indicated. (*B*–*B*″) Xio and Fl(2)d antibody staining in WT wing discs showing a high degree of colocalization. (*C* and *D*) Expression of *xio* RNAi (GD35212 in *C* and KK110253 in *D*) in the dorsal half of the wing disk (below the dashed line) using *ap-Gal4* leads to strong reduction of Xio protein. (*E*–*F*′) Xio antibody staining in wing discs containing xio^{A} (*E* and *E*′) or xio^{C} (*F* and *F*′) mutant clones, marked by the absence of mRFP. Note the reduction of Xio staining in these clones.

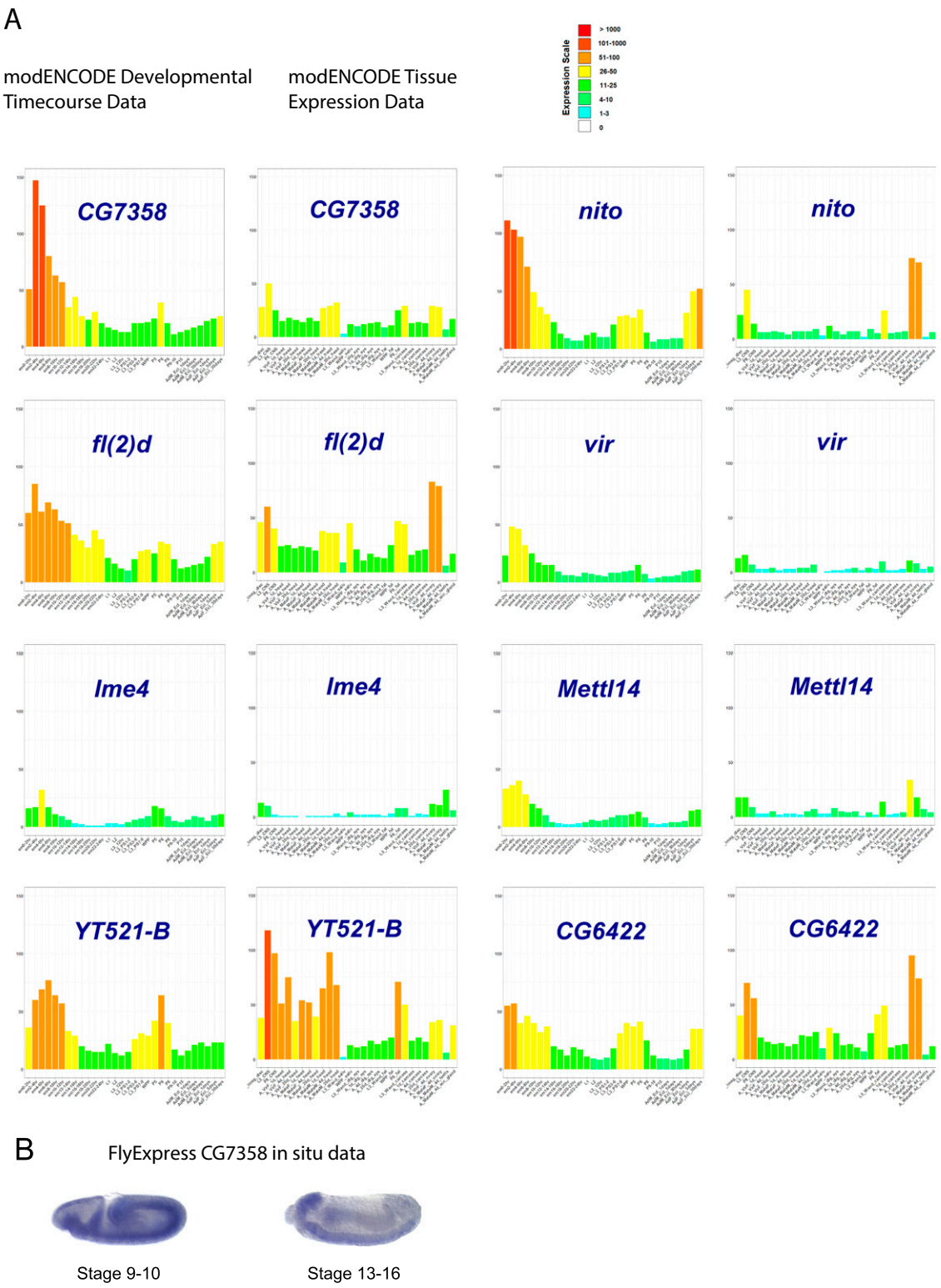
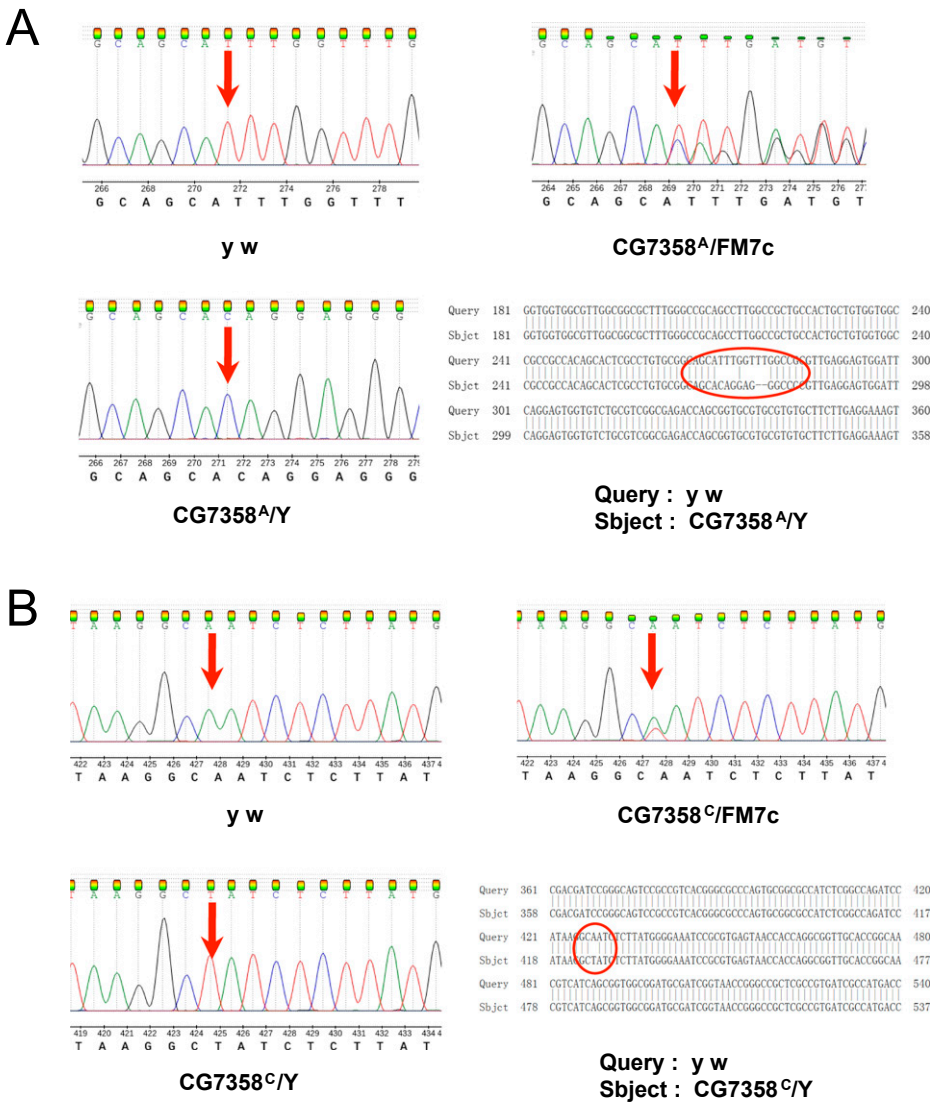


Fig. S3. Expression of *xio* and other m6A factors across modENCODE data and in situ hybridization of xio from FlyExpress data. (*A*) RPKM (reads per kilobase per million mapped reads) measurements of *xio* and other m6A factors from modENCODE developmental time course and tissue expression dataset. (*B*) RNA in situ hybridization of *xio* in stage 9 to 10 and 13 to 16 embryos. Adapted with permission from ref. 1.

1. Tomancak P, et al. (2007) Global analysis of patterns of gene expression during *Drosophila* embryogenesis. *Genome Biol* 8:R145.



C

♀	X ♂	Phenotype of CG7358/Y progeny
CG7358^{C}/FM7c	FM7c/Y	pupal lethal
CG7358^{A}/FM7c	FM7c/Y	pharate lethal, some can get half out of pupal case
CG7358^{A}/FM7c	w^{1118}/Y	pharate lethal, some can get half out of pupal case
CG7358^{A}/FM7c	y w/Y	some pharate lethal, some can grow to adults

Fig. S4. Analysis of *xio*^{A} and *xio*^{C} mutant alleles. Sequencing results of xioA (*A*) and xioC (*B*) and the alignment with WT controls. Red arrows and circles indicate the nucleotide change in the mutants. (*C*) Phenotype of *xio* hemizygous mutant males from various crosses indicated on the left.

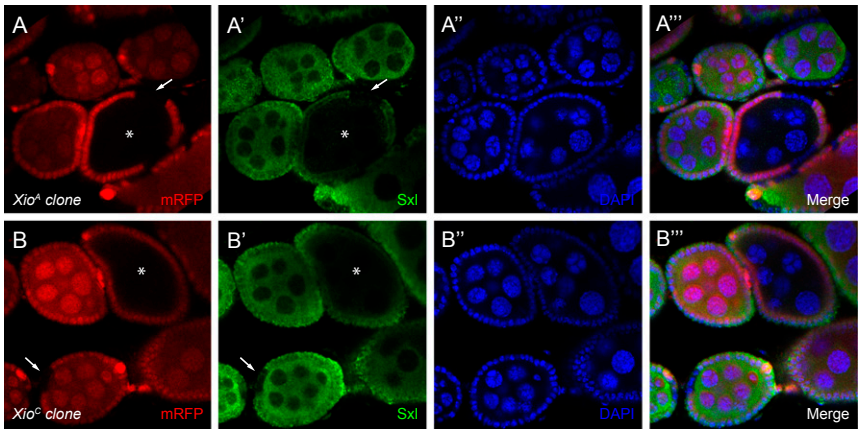


Fig. S5. *xio* mutant germline and follicle cell clones show reduced Sxl levels. Ovaries containing xio^{A} (*A*–*A*‴) or xio^{C} (*B*–*B*‴) mutant germline clones (asterisks) and follicle cell clones (arrows) were stained for Sxl and DAPI. Clones were marked by the absence of mRFP expression.

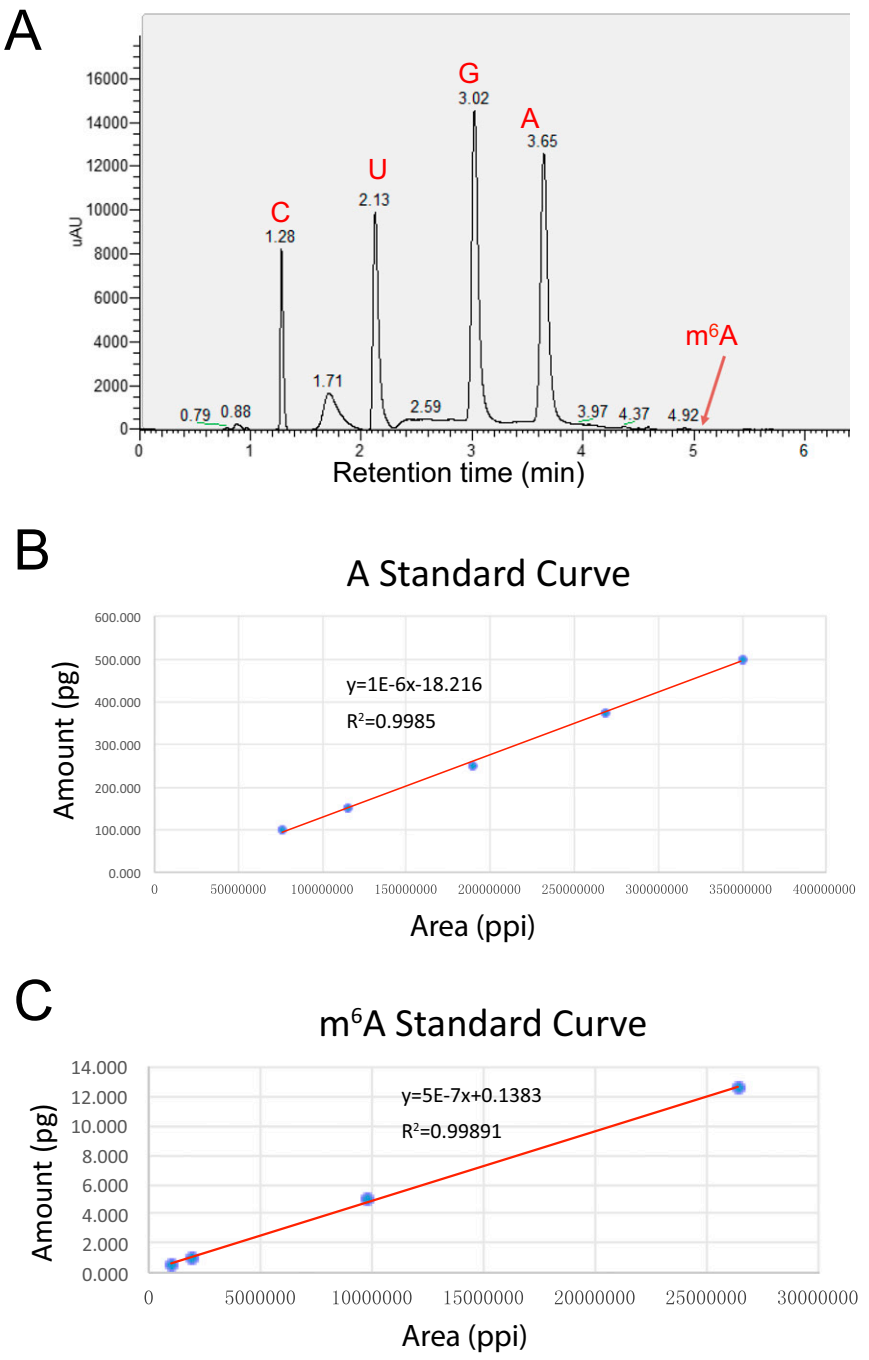


Fig. S6. Calibration curves for m6A quantification. (*A*) UV chromatogram of the four canonical nucleosides and m^6A. uAU, microabsorbance unit. Standard calibration curves for detection of adenosine (*B*) and N^6-methyladenosine (*C*) used for absolute quantifications. ppi, pixels per inch.

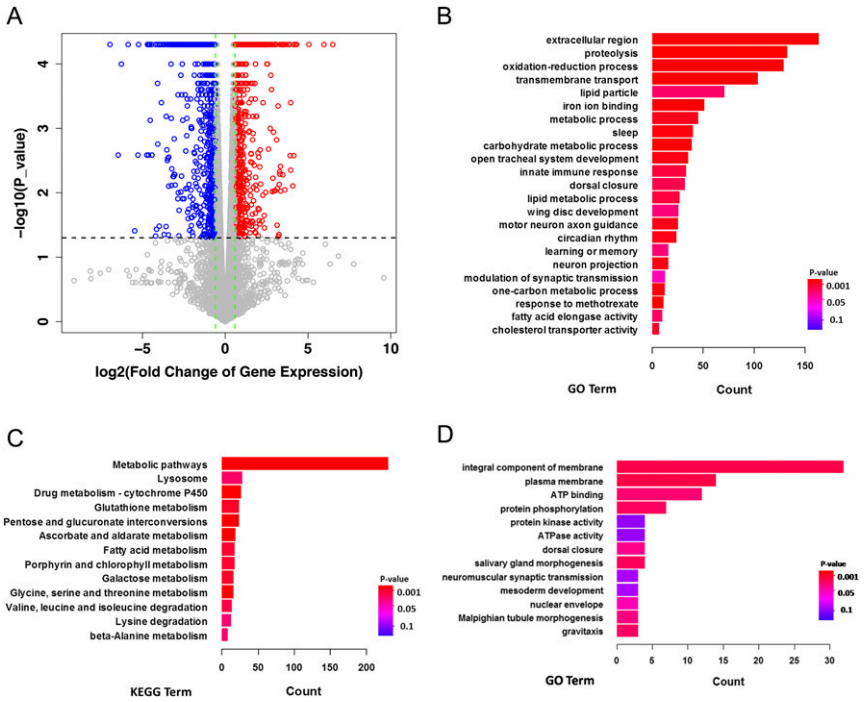


Fig. S7. RNA-Seq analysis of *xio* mutants. (*A*) Volcano plot showing the differentially expressed genes (fold change > 1.5 and *P* value < 0.05). (*B*) Gene Ontology analysis and (*C*) KEGG pathway enrichment analysis of the differentially expressed genes. (*D*) Gene Ontology analysis of the 140 differentially alternatively spliced genes in *xio* mutants.

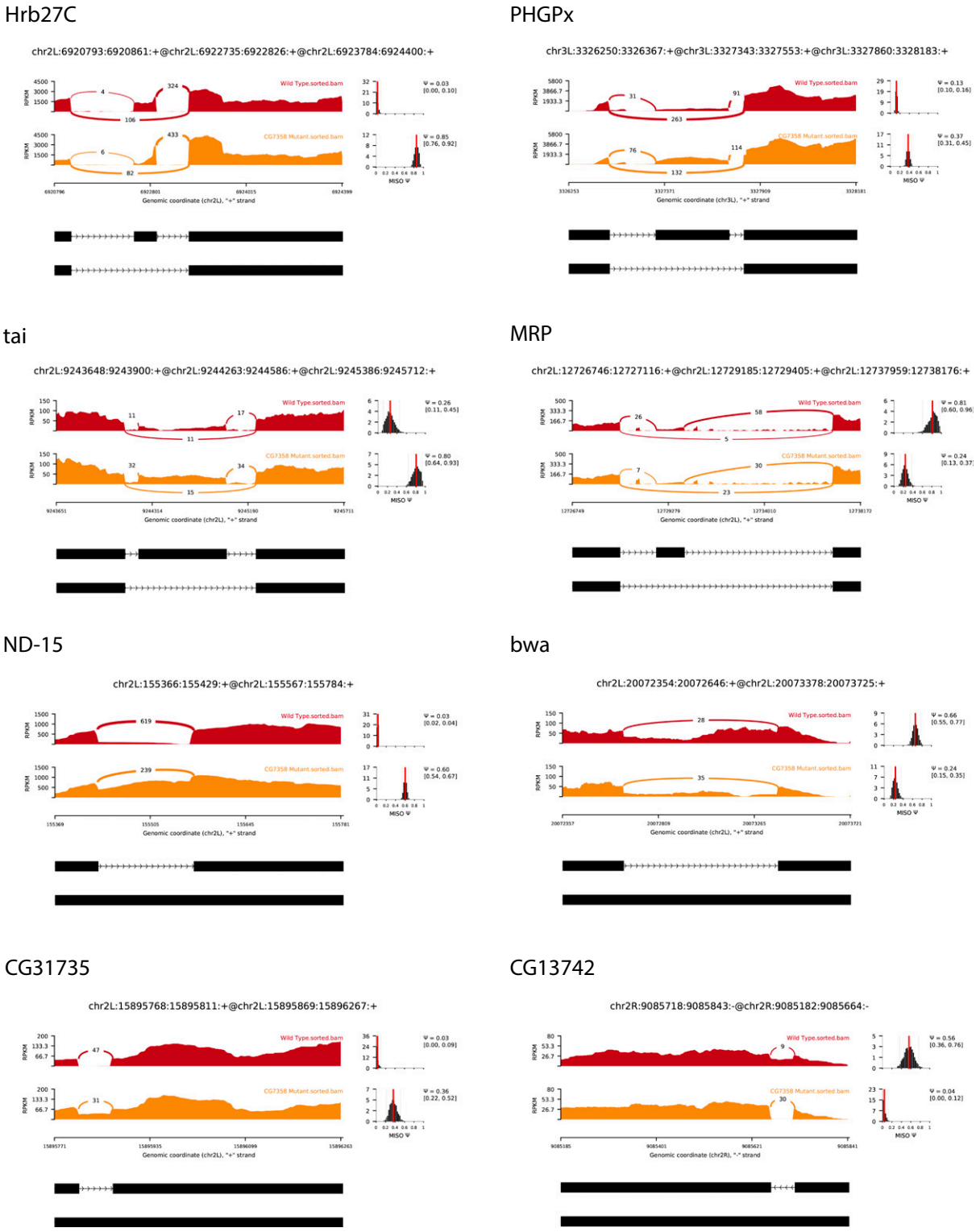


Fig. S8. Examples of differential alternative spliced genes in *xio*^{A} mutants. Sashimi plots depicting mapped RNA-Seq reads and exon junction reads with the annotated gene models of indicated genes below. Ψ denotes percentage spliced in.

Dataset S1. Mass-spec results using GFP, Fl(2)d, and Nito as baits

Dataset S1

Dataset S2. Differential gene expression analysis in *xio* mutant

Dataset S2

Dataset S3. Alternative splicing analysis in *xio* mutant

Dataset S3