



Drosophila YBX1 homolog YPS promotes ovarian germ line stem cell development by preferentially recognizing 5-methylcytosine RNAs

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5-Methylcytosine (m⁵C) is a RNA modification that exists in tRNAs and rRNAs and was recently found in mRNAs. Although it has been suggested to regulate diverse biological functions, whether m⁵C RNA modification influences adult stem cell development remains undetermined. In this study, we show that Ypsilon schachtel (YPS), a homolog of human Y box binding protein 1 (YBX1), promotes germ line stem cell (GSC) maintenance, proliferation, and differentiation in the *Drosophila* ovary by preferentially binding to m⁵C-containing RNAs. YPS is genetically demonstrated to function intrinsically for GSC maintenance, proliferation, and progeny differentiation in the *Drosophila* ovary, and human YBX1 can functionally replace YPS to support normal GSC development. Highly conserved cold-shock domains (CSDs) of YPS and YBX1 preferentially bind to m⁵C RNA in vitro. Moreover, YPS also preferentially binds to m⁵C-containing RNAs, including mRNAs, in germ cells. The crystal structure of the YBX1 CSD-RNA complex reveals that both hydrophobic stacking and hydrogen bonds are critical for m⁵C binding. Overexpression of RNA-binding-defective YPS and YBX1 proteins disrupts GSC development. Taken together, our findings show that m⁵C RNA modification plays an important role in adult stem cell development.

m⁵C | GSC | RNA methylation | YPS | YBX

5-Methylcytosine (m⁵C) is one of the most abundant RNA modifications in eukaryotic cells (1). Such an RNA modification was once thought to be only common on rRNAs and tRNAs (2, 3), but with the development of high-throughput and next-generation sequencing technologies, the transcriptome-wide mapping of m⁵C on RNAs has shown that the majority of m⁵C sites are localized on mRNAs of animal and plant cells (4–9). Interestingly, m⁵C is situated mainly in the coding region and the vicinity of the translational start site in vertebrate and mammalian cells (4–6, 8, 9). The deposition of m⁵C in mRNAs is catalyzed primarily by the methyltransferase Misu/NSUN2 (10). ALYREF is the protein that recognizes m⁵C sites in mRNAs for their cytoplasmic export (6). Although Misu/NSUN2 was previously reported to be required for embryonic stem cell differentiation (11, 12), little is known about the functions of m⁵C RNA modification in adult stem cell development.

In *Drosophila*, Ypsilon schachtel (YPS) is the closely related homolog of human Y box binding protein 1 (YBX1) (13) (*SI Appendix, Fig. S1*). YBX1 has been shown to be important for late mouse embryonic development and tumorigenesis in humans (14, 15). YPS has been shown to bind to *osk* mRNA and regulate its localization and translation during late *Drosophila* oogenesis (16); however, whether YPS also regulates germ line stem cell (GSC) development in the *Drosophila* ovary has been unclear. In this study, we show that YPS promotes GSC maintenance, proliferation, and differentiation in the *Drosophila* ovary, and that its

human homolog YBX1 can replace YPS function in supporting GSC development. More importantly, the cold-shock domains (CSDs) of YBX1 and YPS can preferentially bind to m⁵C-containing RNA in vitro using hydrophobic stacking interactions, and YPS also preferentially binds to m⁵C-containing RNAs in germ cells, including those mRNAs encoding known GSC self-renewal and differentiation factors. Therefore, our results show that the m⁵C modification in mRNAs plays an important role in GSC development.

Results

YPS Promotes GSC Maintenance, Proliferation, and Differentiation in the *Drosophila* Ovary. To determine whether YPS regulates GSC development in the *Drosophila* ovary, we immune-stained control and *yps*^{ΔM2} mutant ovaries for Hts, a fusome component, to quantify

Significance

5-Methylcytosine (m⁵C) is one of the most abundant RNA modifications, but its function in adult stem cell development remains poorly defined. This study shows that Ypsilon schachtel (YPS) promotes germ line stem cell (GSC) maintenance, proliferation, and differentiation in the adult *Drosophila* ovary by preferentially binding to m⁵C-containing RNAs. Highly conserved cold-shock domains (CSDs) of YPS and its human homolog Y box binding protein 1 (YBX1) exhibit preferential binding to m⁵C-containing RNAs through hydrophobic interactions. Human YBX1 can functionally replace YPS to promote GSC development in the *Drosophila* ovary, and overexpressing RNA-binding-defective YBX1 and YPS mutant proteins disrupts normal GSC development. Thus, this study provides insight into the importance of m⁵C RNA modification in adult stem cell development.

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Data deposition: X-ray structures have been deposited in the RCSB Protein Data Bank (ID codes 6KTC for YBX1-m⁵C RNA and 6KUG for YBX1-C RNA) and original RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no. GSE139828). Original data underlying this manuscript can be accessed from the Stowers Original Data Repository at <http://www.stowers.org/research/publications/libpb-1489>.

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GSCs and cystoblasts (CBs). *yps*^{JM2} is a null mutation deleting the initiation ATG and CSD (16). In the ovary, two or three GSCs at the tip of the gerarium continuously divide to generate CBs and cysts (17) (Fig. 1A). GSCs and CBs carry a spherical fusome known as the spectrosome, but only GSCs directly contact cap cells, whereas cysts contain a branched fusome (Fig. 1A). Interestingly, 2-wk-old *yps* mutant geraria contain significantly fewer GSCs and accumulate significantly more CBs than the control geraria (Fig. 1B–D). The CB accumulation is indicative of the delayed differentiation into cysts (18, 19). In addition, 5-

ethynyl-2'-deoxyuridine (EdU) labeling results show that *yps* mutant GSCs proliferate slower than normal (Fig. 1E–G). Taken together, our results indicate that YPS is important for promoting GSC maintenance, proliferation, and progeny differentiation.

Our mRNA in situ hybridization results show that *yps* mRNA is expressed primarily in germ cells, including GSCs and CBs (Fig. 1H). We used *c587-Gal4* and *nos-gal4* to express a shRNA against *yps* to knock down its expression in niche cells and germ cells, respectively (20, 21), and found that germ line-specific, but not niche-specific, knockdown of *yps* can recapitulate the *yps*

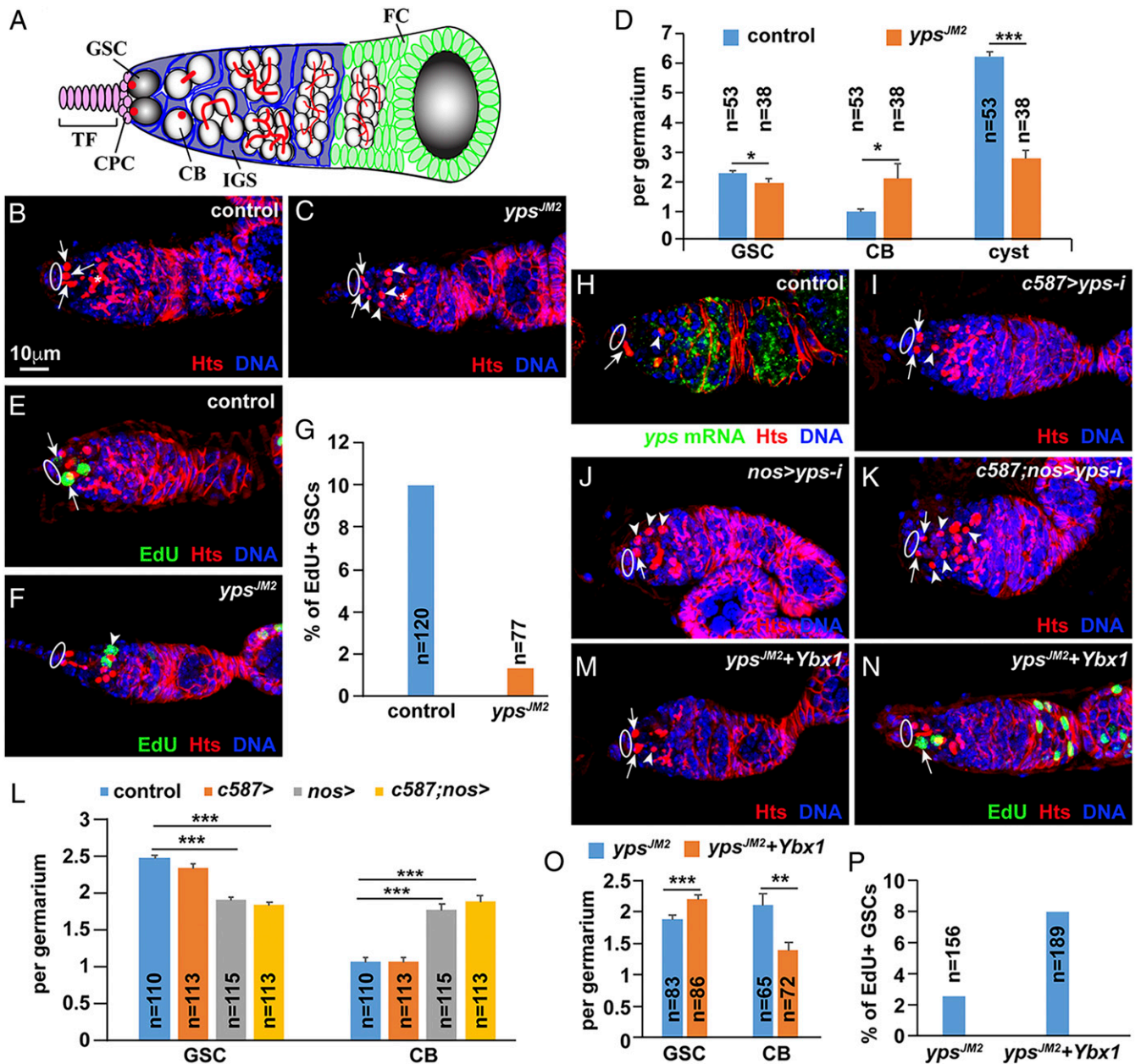


Fig. 1. YPS is required intrinsically to promote GSC maintenance, proliferation, and differentiation in the *Drosophila* ovary. (A) Schematic diagram of the gerarium: spectrosome (red dot) and fusome (red line). TF, terminal filament; CPC, cap cells; IGS, inner gerarial sheath cells; FC, follicle cell. Ovals and arrows indicate cap cells and spectrosomes in GSCs, respectively, whereas arrowheads and asterisks denote spectrosomes in CBs and fusomes in the cysts, respectively. (B–D) Control gerarium carries three GSCs and no CBs (B), while *yps* mutant geraria contains two GSCs and four CBs (C). (D) Quantification results. (E–G) *yps* mutant geraria contain fewer EdU-positive GSCs than controls. G shows quantification results. Arrows in E indicate EdU+ GSCs; arrowhead in F indicates EdU+ CB. (H) mRNA in situ hybridization results show that *yps* mRNA (green) is expressed primarily in GSCs, CBs, and cysts. (I–L) Germ line-specific, but not niche-specific, knockdown significantly decreases GSCs and increases CBs. L presents quantification results. (M–P) Ubiquitous expression of human *Ybx1* can rescue the defects in GSC maintenance, proliferation, and differentiation in the *yps* mutant.

mutant GSC phenotypes (Fig. 1 I–L). These results indicate that YPS is required intrinsically for promoting GSC maintenance, proliferation, and differentiation.

Human YBX1 Can Functionally Replace YPS to Support GSC Development. *Drosophila* YPS and human YBX1 share high homology in the CSD domain (SI Appendix, Fig. S1). We used *Actin5c-gal4* to ubiquitously express human *Ybx1* in the *yps* mutant ovary to explore whether YBX1 can functionally replace YPS. Interestingly, although it does not cause any discernible phenotypes in wild-type ovaries, ubiquitous *Ybx1* expression significantly and fully rescues the GSC maintenance, proliferation, and differentiation defects in *yps* mutant ovaries (Fig. 1 M–P). These results indicate that human YBX1 can functionally replace YPS to support GSC development in the *Drosophila* ovary.

YPS Preferentially Binds to m⁵C-Containing RNAs in Germ Cells. YBX1 and m⁵C methylase NSUN2 are required for loading tRNAs and rRNAs into exosomes, raising the possibility that YBX1 might bind to m⁵C-containing RNAs (22). To test this possibility, we used an anti-m⁵C antibody and germ line-expressed Flag-YPS to immunoprecipitate m⁵C-containing RNAs and YPS-bound mRNAs from adult ovaries, respectively (SI Appendix, Fig. S2). Interestingly, Flag-YPS and m⁵C antibody brought down 5,887 and 4,933 RNAs, respectively, which share 4,283 RNA species, suggesting that YPS binds to m⁵C-containing RNAs (Fig. 2A). In addition to rRNAs and tRNAs known to carry m⁵C modifications, other noncoding RNA species, such as long noncoding RNAs (lncRNAs), microRNA precursors (premiRNAs), antisense RNAs (asRNAs), small nucleolar RNAs (snoRNAs), and small nuclear RNAs (snRNAs), also carry m⁵C modifications based on Flag-YPS and anti-m⁵C pulldown results (Fig. 2 B–G). Interestingly, >85% of the m⁵C-containing RNAs are mRNAs, indicating that most of m⁵C modification sites are on mRNAs (Fig. 2H). These results indicate that YPS preferentially bind to m⁵C-containing RNAs in *Drosophila*.

Since RNAs were fragmented before the pulldown experiments, each peak likely represents one m⁵C site (SI Appendix, Fig. S2). Consistently, the RNA peaks pulled down by YPS and anti-m⁵C are almost perfectly correlated on individual mRNAs (Fig. 2 I and J'). For the mRNAs encoding known GSC self-renewal factors, *mad* carries m⁵C modifications in the coding sequence (CDS) only, while *elf4A* has m⁵C modifications in the 3' UTR and CDS (Fig. 2 I and I'). For the mRNAs encoding known differentiation factors, *mei-P26* carries m⁵C sites in the 5' UTR, whereas *sxl* contains m⁵C sites in the intron, CDS, and 3' UTR (Fig. 2 J and J'). The majority of m⁵C sites on mRNAs are located in the CDS, and the remaining sites reside in the intron, 5' UTR, and 3' UTR (Fig. 2 K and L). Based on KEGG pathway analysis, the m⁵C-containing mRNAs are significantly enriched for those encoding the proteins involved in protein synthesis, mRNA splicing, endocytosis, and other functions (Fig. 2M). These results indicate that m⁵C sites are abundantly located in the CDS and intron, suggesting m⁵C's potential importance in translation and splicing.

YBX1 CSD Exhibits Preferential Binding toward the m⁵C-Containing RNA Primarily through Hydrophobic Stacking Interactions. Since highly conserved CSDs in YBX family proteins are known to bind RNAs (23), we used isothermal titration calorimetry (ITC) to determine the binding affinity of YBX1 and YPS CSDs for m⁵C-containing and unmethylated RNAs. The 8-mer m⁵C and unmethylated RNA oligos 5'-A₁C₂C₃A₄G₅m⁵C₆C₇U₈-3' and 5'-A₁C₂C₃A₄G₅C₆C₇U₈-3' were chosen based on the previously reported YBX1 target sequence (22). YPS and YBX1 CSDs exhibited ~1.5-fold and 2-fold higher affinity, respectively, for the m⁵C-containing RNA than for the unmethylated RNA (Fig. 3 A–D). Along with the in vivo YPS results, these results indicate that YBX1/YPS CSDs function as the reader for m⁵C RNA.

Since the YPS CSD is highly similar to the YBX1 CSD (SI Appendix, Fig. S1), we solved the crystal structure of the YBX1 CSD in complex with the 8-mer methylated or unmethylated RNA oligo at a resolution of 2.0 Å and 1.4 Å, respectively. Both of the overall structures belong to the *P*₆₂ space group and adopt a canonical five-stranded antiparallel β-barrel CSD fold, which is similar to the previously determined bacterial CSD-RNA crystal structure (24). As the RNA wraps around the CSD, four bases of either the methylated or unmethylated RNA, (G₅, C₆, C₇, and U₈) establish π-stacking interactions with the aromatic side chains of CSD, including Trp65, Phe74, Phe85, and His87, which are conserved in the *Drosophila* YPS (Fig. 3 E and F and SI Appendix, Fig. S1). In addition, multiple hydrogen bonds were detected between the RNA and the CSD (Fig. 3G). Interestingly, Phe85 stacks with the base of m⁵C₆, and a weak cation–π interaction between residue Lys118 and m⁵C₆ also exists. Therefore, our structural results suggest that the methyl group in m⁵C likely strengthens hydrophobic interactions with the CSD, thereby enhancing YBX1/YPS binding to m⁵C-containing RNAs.

We next determined whether Trp65, Phe74, Phe85, His87, and Lys118 in the YBX1 CSD are important for m⁵C-containing RNA binding by mutating them to alanine. The ITC binding data show that the YBX1 CSD carrying a mutation in W65, F74, F85, and H87 has diminished binding affinity to both the m⁵C-containing and unmethylated RNAs, suggesting that they contribute to general RNA binding (SI Appendix, Fig. S3 A and B). Interestingly, the K118A mutant decreases RNA binding only slightly, suggesting that Lys118 contributes less to RNA binding (SI Appendix, Fig. S3 A and B). Consistently, the W68A and F88A mutant YPS CSDs (equivalent to W65A and F85A, respectively, in YBX1) also lose RNA binding (SI Appendix, Fig. S3 C and D'). Taken together, these results further confirm the importance of the aromatic amino acid residues in the CSD for RNA binding.

Overexpression of RNA-Binding-Defective Mutant YBX1 and YPS Proteins Severely Disrupts GSC Development. We next used *c587-Gal4* and *nos-gal4* to test the functions of RNA-binding-defective *yps* and *Ybx1* mutants by overexpressing them in niche cells and germ cells, respectively. Overexpressed wild-type *yps* and *Ybx1* in either germ cells or niche cells as well as overexpressed *Ybx1*^{W65A}, *Ybx1*^{F74A}, and *Ybx1*^{F85A} in niche cells behaved like the controls (Fig. 4 A and B and SI Appendix, Fig. S4). In contrast, germ line-specific overexpression of *yps*^{W68A}, *yps*^{F88A}, *Ybx1*^{W65A}, *Ybx1*^{F74A}, and *Ybx1*^{F85A} caused a significant loss of GSC, a decrease in EdU-positive GSCs, and an increase in CBs, indicating that these mutants can dominantly disrupt GSC maintenance, proliferation, and differentiation (Fig. 4 C–K). Germ line-expressed Flag-YPS, Flag-YPS^{W68A}, and Flag-YPS^{F88A} could still bind similar protein partners in germ cells, suggesting that the GSC developmental defects likely arise from titrating out the interacting partners from endogenous wild-type YPS protein (SI Appendix, Fig. S5). Surprisingly, overexpression of *Ybx*^{H87A} in germ cells did not produce any obvious GSC developmental defects, suggesting that His87 does not contribute to the function of YBX1 in vivo, even though it is essential for RNA binding in vitro (Fig. 4 F, F', and K). Taken together, our results indicate that Trp65, Phe74, and Phe85 in YBX1 and YPS are critical for their functions in vivo.

Discussion

The m⁵C RNA modification has recently been shown to be widespread in plant and human cells (4–9). This modification is involved in regulating various biological processes, including embryonic development, maternal-to-zygotic transition, and tumorigenesis, by modulating mRNA stability and translation (4–6, 8); however, whether it regulates adult stem cell development has been unclear. In this study, we show that YPS promotes GSC maintenance, proliferation, and differentiation in the *Drosophila* ovary by preferentially binding to m⁵C-containing RNAs. Human

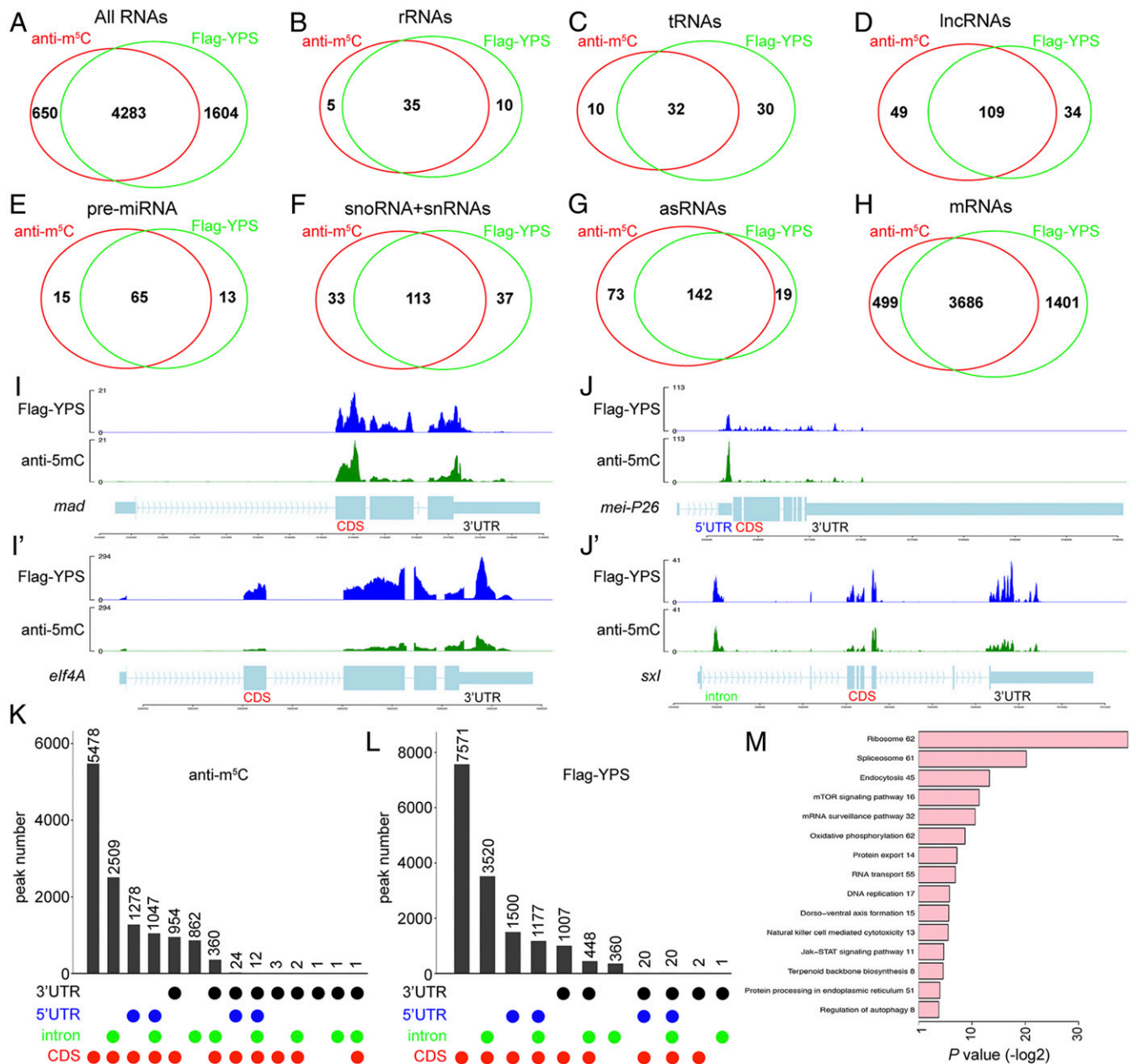


Fig. 2. *Drosophila* YPS preferentially binds to m⁵C-containing RNAs in germ cells. (A–H) Germ line-specific Flag-YPS and anti-m⁵C antibody pull down mostly overlapped total RNAs, rRNAs, tRNAs, lncRNAs, premiRNAs, asRNAs, snoRNAs, snRNAs, and mRNAs (numbers represent different RNAs). (I and J') YPS-binding sites are closely correlated with m⁵C sites in *mad* and *elf4A* mRNAs (I and I') and in *mei-P26* and *sxl* mRNAs (J and J'). (K and L) m⁵C sites and YPS-binding sites are more prevalent in the CDS and intron than in the 5' UTR and the 3' UTR. (M) Top 15 significantly overrepresented pathways for the mRNAs sharing YPS-binding sites and m⁵C sites based on KEGG pathway analysis.

YBX1 can functionally replace YPS to support GSC development in the *Drosophila* ovary. In germ cells, YPS preferentially binds to m⁵C-containing RNAs, most of which are mRNAs, including those encoding known GSC self-renewal and differentiation factors.

In addition, we used crystal structures to show that the YBX1 CSD binds to the RNA primarily through hydrophobic stacking interactions, and that m⁵C helps strengthen such hydrophobic interactions. Finally, forced expression of RNA binding-defective YPS or YBX1 mutants in germ cells sufficiently dismantles GSC development in a dominant-negative manner, indicating that m⁵C binding is important for YPS to regulate GSC development. Therefore, our findings provide insight into the importance of

YBX family protein YPS in adult GSC development by preferentially recognizing m⁵C-containing RNAs.

This study provides important structural information on how YPS and YBX1 proteins preferentially recognize m⁵C-containing RNAs. First, our RNA-immunoprecipitation results show that YPS preferentially binds to m⁵C-containing RNAs, including mRNAs. Approximately 88% of mRNAs and 75% of noncoding RNAs bound by YPS are m⁵C-containing RNAs, indicating that YPS preferentially binds to m⁵C-containing RNA in vivo. This finding is consistent with the two studies on YBX1 in zebrafish and humans, which were published during the revision of this current study (4, 5) and challenges the previous notion that YBX family proteins are low-specificity RNA chaperones (25). Second,

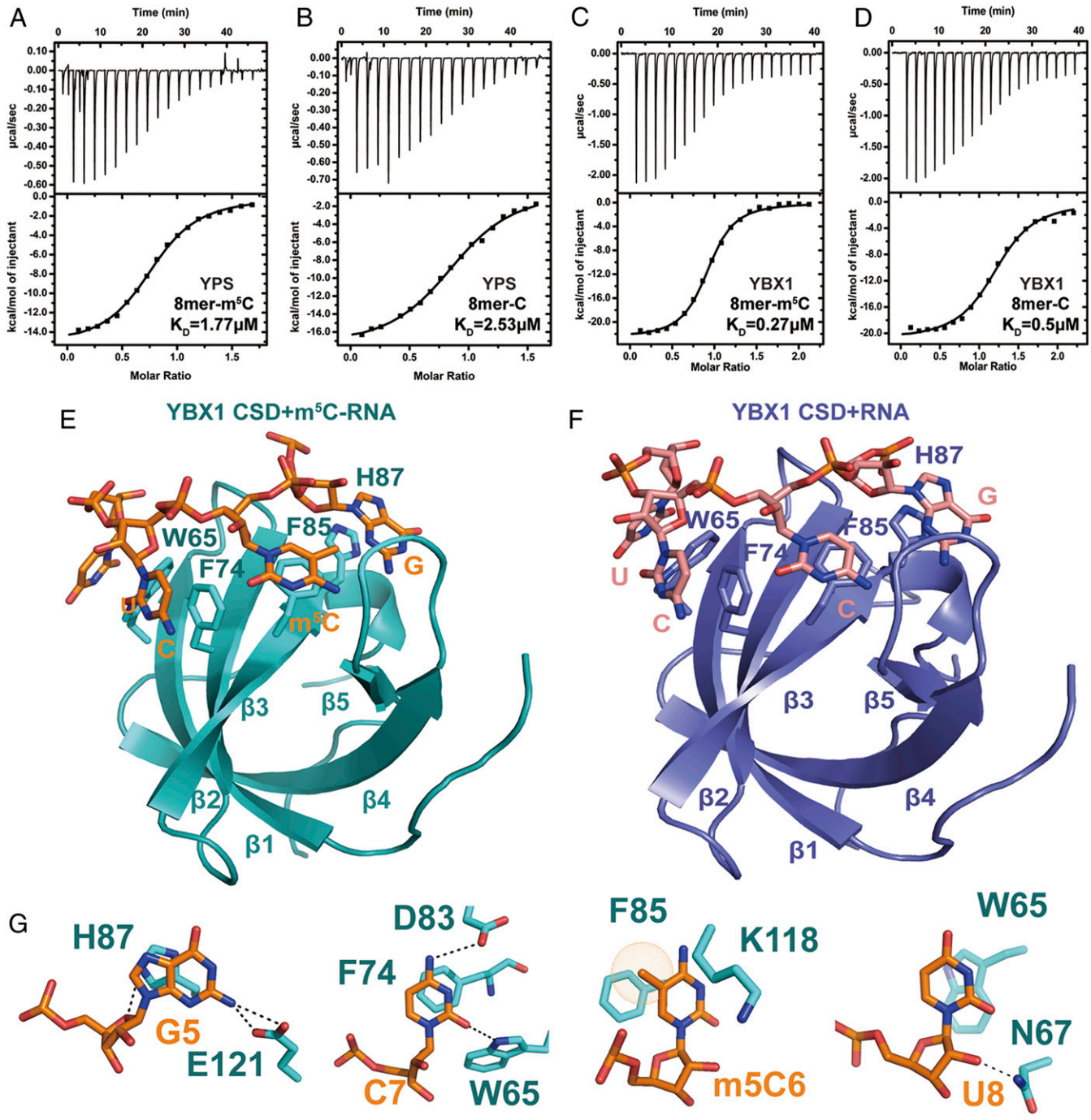


Fig. 3. The CSDs of *Drosophila* YPS and human YBX1 preferentially bind to the m⁵C-containing RNA. (A and B) ITC analysis of *Drosophila* YPS binding to m⁵C-containing and RNA oligos. (C and D) ITC analysis of human YBX1 binding to m⁵C-containing and RNA oligos. (E and F) Overall structure of the human YBX1 CSD domain in complex with to m⁵C-containing and RNA oligos. The m⁵C RNA is shown in sticks, while the YBX1 CSD is shown in cartoon. The aromatic residues that interact with the RNA bases are indicated. (G) Detailed presentation of the interactions between YBX1 CSD domain aromatic residues and individual m⁵C RNA bases. The residues in the CSD domain and the m⁵C RNA bases are shown in cyan and orange, respectively.

our ITC results show that both YBX1 and YPS CSDs preferentially bind to m⁵C-containing RNAs over unmethylated RNAs. Third, crystal structures show that four aromatic amino acid residues form hydrophobic stacking interactions with four RNA bases, and that m⁵C can further strengthen the stacking interactions. Consistently, mutating one of the four amino acid residues involved in hydrophobic stacking interactions (Trp65, Phe74, Phe85, or His87) completely abolishes the RNA binding of the YBX1 CSD. Finally, YBX1 mutants changing Trp65, Phe74, or Phe85

into alanine behave dominant-negatively as the corresponding YPS mutants interfere with YPS function in the *Drosophila* ovary. Since YBX family CSDs are highly conserved, our results can help explain how YBX family proteins bind to m⁵C-containing RNAs.

This study also provides important insight into how YPS regulates adult GSC development. YPS has previously been shown to bind to *osk* mRNA and regulate its localization and translation in the oocyte (16). Our findings reveal that YPS is required intrinsically to promote GSC maintenance, proliferation, and differentiation in

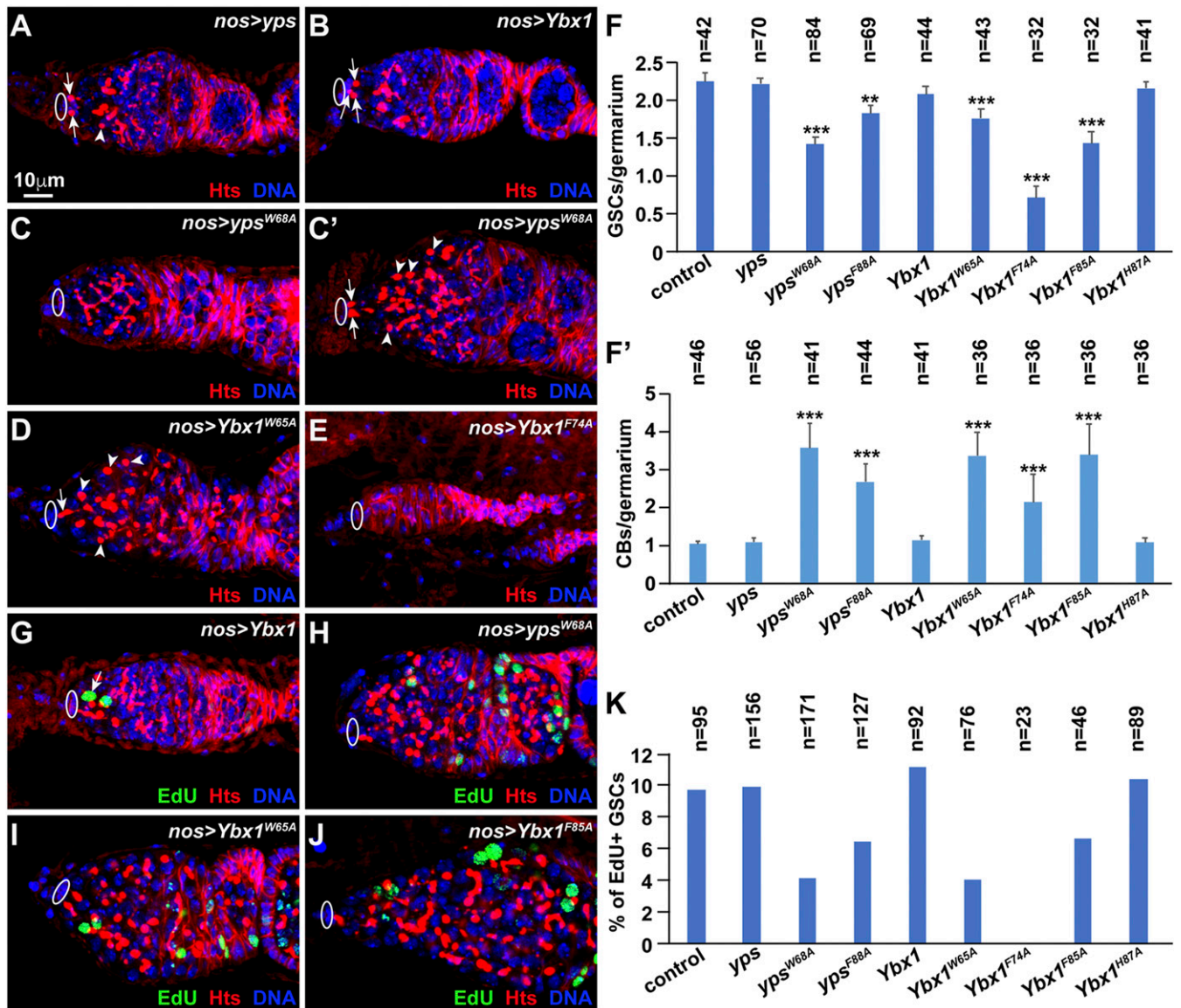


Fig. 4. Germ line-specific expression of RNA binding-defective *yps* and *Ybx1* mutants severely compromises GSC development. Ovals indicate cap cells, whereas arrows and arrowheads point to spectrosomes in GSCs and CBs, respectively. (A–F) Germ line-specific expression of *yps*^{W68A}, *Ybx1*^{W65A}, and *Ybx1*^{F74A}, but not of *Ybx1*^{H87A}, significantly decreases GSCs and increases CBs compared with overexpression of wild-type *yps* and *Ybx1*. F and F' show results. Some *yps* and *Ybx1* mutant-expressing germaria contain no GSCs (C and E) or one GSC (D), whereas other germaria accumulate many CBs (C' and D). (G–K) Germ line-specific expression of *yps* or *Ybx1* mutants except *Ybx1*^{H87A} decreases EdU⁺ GSCs compared with controls. In G, the arrow indicates EdU⁺ GSC. K shows quantification results.

the *Drosophila* ovary, since *yps* null mutant germaria have significantly fewer GSCs and EdU-positive GSCs and more CBs compared with control germaria. In addition, cell type-specific shRNA-mediated knockdown indicates that YPS is required intrinsically to support GSC development. Moreover, human YBX1 can functionally replace YPS to support GSC development in the ovary, since ubiquitous expression of human *Ybx1* in the *yps* mutant can fully rescue all the GSC developmental defects. In addition, germ line-specific expression of RNA binding-defective YPS and YBX1 mutant proteins can similarly disrupt GSC development in the ovary. Interestingly, YBX2 and YBX3 are required for mouse spermatogenesis, suggesting that the functions of YBX proteins in germ cell development are conserved (26).

Another important finding is that YPS promotes GSC development through binding to m⁵C-containing RNAs, including

mRNAs and noncoding RNAs. Some of YPS-bound mRNAs encode known self-renewal factors (e.g., Mad, eIF4A) and differentiation-promoting factors (e.g., Mei-P26, Sxl), which could potentially explain its requirement for GSC development. Interestingly, YPS more frequently binds to the CDS and intron in mRNAs than to the 5' UTR and 3' UTR, suggesting that YPS preferentially regulates mRNA translation and splicing. YPS-bound noncoding RNAs include tRNAs, rRNAs, miRNA precursors, lncRNAs, snoRNAs, and snRNAs, which are known to regulate translation, RNA splicing, or transcription. Although YBX1 is proposed to bind to DNA in mammalian cells, YPS protein is present almost exclusively in the cytoplasm of *Drosophila* ovarian germ cells, and there is little m⁵C DNA modification and DNA methylase Dmrt2 protein expression in adult *Drosophila* females based on the published results (16, 27), suggesting that YPS regulates GSC development primarily through binding to m⁵C-containing RNAs. In addition, it

will be interesting to investigate whether YPS binding influences m⁵C modification on RNAs.

Taken together, our findings suggest that YPS regulates translation, splicing, and stability by preferentially recognizing m⁵C-containing RNAs, thereby promoting GSC development in the *Drosophila* ovary. Since YPS/YBX family protein functions have been conserved from *Drosophila* to humans, the *Drosophila* ovary will be an effective system for dissecting the functions of human YBX family proteins and m⁵C modifications in germ cell development.

Materials and Methods

Drosophila Stocks. Flies were maintained and crossed at room temperature on standard cornmeal/molasses/agar media unless specified otherwise. To maximize the expression levels of shRNAs and transgenes, newly eclosed flies were cultured at 29 °C for 2 wk before the analysis of ovarian phenotypes. Further details on the *Drosophila* mutant and transgenic strains can be found in *SI Appendix, Materials and Methods*.

Immunostaining, EdU Labeling and mRNA In Situ Hybridization. Ovaries were dissected at room temperature in Grace's medium and fixed with 4% paraformaldehyde. Immunostaining, EdU detection, and mRNA in situ hybridization were performed as described in *SI Appendix, Materials and Methods*.

Identification of m⁵C-Containing and YPS-Bound RNAs. For each RNA pulldown experiment, 400 pairs of ovaries were dissected from the females of proper genotypes for RNA immunoprecipitation by anti-m⁵C and anti-Flag antibodies,

RNA sequencing, and bioinformatic analysis, as described in *SI Appendix, Materials and Methods*.

Protein Purification, ITC Measurement, and Crystal Structure Determination. *Drosophila* YPS (amino acids 58 to 132) and human YBX1 (amino acids 52 to 129) CSD domains were cloned, expressed in *Escherichia coli*, and purified for the ITC measurement for RNA binding and for the determination of crystal structures of human CSD complexed with methylated and unmethylated RNAs, as described in *SI Appendix, Materials and Methods*.

Data Availability. X-ray structures have been deposited in the RCSB Protein Data Bank (ID codes 6KTC for YBX1-m⁵C RNA and 6KUG for YBX1-C RNA). Original RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no. GSE139828). Original data underlying this manuscript can be accessed from the Stowers Original Data Repository at <http://www.stowers.org/research/publications/libpb-1489>.

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