Dynamic FMR1 granule phase switch instructed by m6A modification contributes to maternal RNA decay

Guoqiang Zhang1, Yongru Xu1,2,4, Xiaona Wang2,4, Yuanxiang Zhu1,2,4, Liangliang Wang1, Wenxin Zhang1, Yiru Wang1, Yajie Gao1,2, Xuna Wu3, Ying Cheng1, Qinmiao Sun2 & Dahua Chen1,2

Maternal RNA degradation is critical for embryogenesis and is tightly controlled by maternal RNA-binding proteins. Fragile X mental-retardation protein (FMR1) binds target mRNAs to form ribonucleoprotein (RNP) complexes/granules that control various biological processes, including early embryogenesis. However, how FMR1 recognizes target mRNAs and how FMR1-RNP granule assembly/disassembly regulates FMR1-associated mRNAs remain elusive. Here we show that Drosophila FMR1 preferentially binds mRNAs containing m6A-marked “AGACU” motif with high affinity to contributes to maternal RNA degradation. The high-affinity binding largely depends on a hydrophobic network within FMR1 KH2 domain. Importantly, this binding greatly induces FMR1 granule condensation to efficiently recruit unmodified mRNAs. The degradation of maternal mRNAs then causes granule de-condensation, allowing normal embryogenesis. Our findings reveal that sequence-specific mRNAs instruct FMR1-RNP granules to undergo a dynamic phase-switch, thus contributes to maternal mRNA decay. This mechanism may represent a general principle that regulated RNP-granules control RNA processing and normal development.

1 Institute of Biomedical Research, Yunnan University, Kunming, China. 2 State Key Laboratory of Membrane Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, China. 3 School of Life Sciences, Yunnan University, Kunming, China. 4 These authors contributed equally: Guoqiang Zhang, Yongru Xu, Xiaona Wang, Yuanxiang Zhu. **Email: qinmiosun@ioz.ac.cn; chendh@ynu.edu.cn

https://doi.org/10.1038/s41467-022-28547-7
The m6A controls normal embryogenesis and maternal RNA decay in Drosophila. The m6A modification of mRNAs undergos
dynamic changes during Drosophila early embryonic development. We quantified levels of m6A of mRNAs in early embryos at multiple stages by performing mass spec analysis and obtained consistent results (Fig. S1a). The dynamic changes of m6A appeared to be associated with the event of maternal RNA degradation (Fig. S1b). Because the proper degradation of maternal RNAs is critical for embryogenesis, we sought to ask whether the m6A plays a role in normal embryogenesis. Given that Mettl3 and Mettl14 form the methyltransferase complex to establish m6A modification on mRNAs, we employed the CRISPR/Cas9 system and generated multiple null mutant alleles of the mettl3 and mettl14 genes (Fig. S1c–f). To determine the maternal role of the m6A modification, we generated mettl3 and mettl14 single maternal mutant, and mettl3–mettl14 double maternal mutant embryos, which displays much lower levels of m6A, compared to the wild-type control (Fig. 1a). According to the method we described previously, we then performed egg-hatching rate analysis and found that ~20% of embryos failed to hatch into larvae when maternal mettl3 or mettl14 was depleted (Fig. 1b). Moreover, nearly 40% of embryos did not reach the larval stage when maternal mettl3 and mettl14 were simultaneously removed (Fig. 1b), suggesting that the m6A modification is important for normal embryogenesis in Drosophila.

To ask whether the Mettl3–Mettl14 complex is involved in maternal RNA degradation, we generated transcriptome RNA-seq datasets of wild type and mettl3–mettl14 double maternal mutant at multiple embryonic stages (0–0.5, 2–3, and 5–6 h stages). Based on the wild-type datasets, we clustered the mRNAs into three groups, including degraded maternal mRNAs, stable maternal mRNAs, and zygotically expressed mRNAs (Fig. 1c). By comparing the RNA-seq datasets between the wild type and mettl3–mettl14 double maternal mutants, we found that a significant portion of maternal transcripts (686 mRNAs, q < 0.05) degraded in wild-type embryos was aberrantly stable in mettl3–mettl14 mutant embryos at the 5–6 h stage (Fig. 1d and Fig. S1g, h). We next generated the m6A datasets of embryos at the 0–1 h and 5–6 h stages and identified 834 transcripts that were marked by m6A, including 380 degraded maternal transcripts, and 454 stable maternal transcripts (Fig. S1i). Interestingly, we found that the m6A signal strengths for 380 degraded maternal mRNAs were decreased markedly from the 0–1 h stage to the 5–6 h stage (Fig. S1j), whereas no apparent difference was observed in levels of m6A modification of the 454 stable maternal transcripts between the two stages (Fig. S1k). These findings suggest that the Mettl3–Mettl14 complex-mediated m6A modification contributes to normal embryogenesis and likely participates in the degradation of a portion of maternal mRNAs.

**FMRI is an m6A-RNA binding protein in Drosophila embryos.** YTH domain-containing proteins have been reported to function as the canonical “m6A readers” to regulate RNA fate. For example, in zebrafish, Ythdf2 acts as an “m6A reader” to regulate the decay of a set of maternal RNAs. Drosophila genome harbors two genes encoding YTH homologs, Ythdf and Ythdc. To ask whether the YTH proteins contribute to embryogenesis, we generated a number of null mutant alleles for ythdf and ythdc, respectively (Fig. S2a–d). Genetic analysis revealed that while ythdc mutant flies showed phenotypes described previously (Fig. S2e), loss of maternal Ythdf or Ythdc, or double maternal Ythdf and Ythdc led to no effect on egg hatching (Fig. S2f), raising a possibility that a YTH-independent factor regulates early embryogenesis by recognizing m6A marks on mRNAs in Drosophila.

To search for the functional “m6A readers” specifically in the context of Drosophila early embryos, we synthesized a pair of bion-
labeled RNA probes containing multiple repeated “recognition motifs”, including “GGACU”, “AGACU” and “GAACU”, based on the m6A recognition motifs reported in the previous studies and the motifs identified in Drosophila embryos (Fig. S3a). These probes were validated by human Ythdf2 (hYthdf2, an “m6A reader” reported previously) in an electrophoretic mobility shift assay (EMSA) (Fig. S3b, c). After the validation, we incubated the m6A-modified or unmodified RNA probes with cytosolic lysates from early embryos and performed immunoprecipitation experiments followed by mass spectrometry analysis (Fig.1e). We identified 12 proteins that were either preferentially or specifically associated with m6A-modified RNA probes (Fig. S3d), and found that Drosophila FMRP

Fig. 1 The m6A modification regulates maternal RNA decay via preferentially binding m6A-marked mRNAs. a Abundances of m6A on mRNAs from indicated embryos were measured by mass spectrometry. The mole ratios of m6A vs. A are shown on the y-axis. Data expressed as means of two independent samples. b Relative hatching rate of embryos with indicated genotypes (methyl3(M−), Z+), P = 2.5e−05; methyl14(M−), Z+, P = 0.0005; methyl3–14(M−), Z+, P = 4.6e−06; methyl3–14(M−), Z−, P = 1.6e−05). Data expressed as means of three independent experiments. The two-sided Student’s t test was used to analyze statistical variance. Error bars indicate mean ± SD. ***P < 0.001. c Expression profile of clustered gene groups by RNA sequencing. d The degraded maternal mRNAs (686 overlapped mRNAs shown in Fig. S1h) in methyl3–methyl14 double maternal mutant embryos displayed aberrant stable, when compared to wild-type at the 5–6-h stage (0–0.5 h, P = 0.33; 2–3 h, P = 0.48; 5–6 h, P = 3e−35). P-values were calculated by a one-sided Student’s t test. The boxplot shows median (the horizontal line in the box), 1st and 3rd quartiles (lower and upper bounds of box, respectively), minimum and maximum (lower and upper whiskers, respectively). e Schematic for RNA pulldown and proteomics analysis. f Western blot assays were performed by using the anti-Drosophila FMR1, anti-Ythdf, and anti-Ythdc antibodies against complexes purified by the m6A modified or unmodified RNA probes. Representative figures of three independent replicates are shown. g EMSAs showing the complex formation of FMR1 with m6A modified or unmodified probes. Representative figures of three independent replicates are shown. h, i Surface plasmon resonance (SPR) assays of the interaction of FMR1 protein with unmodified probe (h) or with m6A-modified probe (i). Equilibrium and kinetic constants were calculated by a global fit to the 1:1 Langmuir binding model. RU resonance units. Source data are provided as a Source Data file and Supplementary Data 1 and 2.
(FMR1) was highly abundant in the complexes immuno-precipitated by the m6A-modified probe (Fig. S3d). Further western blot assays revealed that the m6A-modified complex exhibited a much stronger FMR1 signal than the unmodified probe complex isolated from early embryos, but Ythdf was not detectable in these complexes when anti-Ythdf antibody was used (Fig. 1f and Fig. S3e). Moreover, we found that Ythdc was strongly associated with the m6A-modified probe, but not the unmodified probe (Fig. 1f). In this study, we focused on FMR1, because the loss of maternal FMR1, but not Ythdc, caused embryonic lethal phenotype. To further confirm the binding of FMR1 to m6A modified RNA, we then purified full-length FMR1 protein fused to an MBP tag from a bacterial expression system and performed EMSAs to conduct a detailed investigation of in vitro FMR1 binding to the RNA probes. EMSAs results showed that, although FMR1 could bind both the m6A-modified and the unmodified probes, signals from “shift complexes” were much stronger for the m6A-modified probe than for the unmodified probe (Fig. 1g and Fig. S3c). To quantify the binding affinities of FMR1 to the RNA probes, we performed the surface plasmon resonance (SPR) kinetic assays, found that FMR1 bound much more strongly to the RNA probes, we performed the surface plasmon resonance (SPR) kinetic assays, found that FMR1 bound much more strongly to the m6A-modified RNA probe ($K_a = 80.7 \text{nM}$) than to the unmodified probe ($K_a = 3.6 \mu \text{M}$) (Fig.1h, i). Thus, our results suggest that FMR1 preferentially binds the m6A-modified RNAs in Drosophila.

FMR1 regulates embryogenesis at least partly via binding m6A-marked RNAs. FMR1 is required for normal embryogenesis in Drosophila35–37. To test whether FMR1 regulates early embryonic development through binding m6A-modified RNAs, we performed genetic experiments using fmri null alleles (Fig. S4a), as described previously38. Consistent with previous findings37, we found that more than 50% of fmri maternal mutant embryos failed to reach the larval stage, and this embryonic lethal phenotype could be significantly rescued by maternal expression of FMR1 driven by the Vas-Gal4:VP16 driver (Fig. 2a, b). To understand the mechanism of how FMR1 regulates early embryonic development, we generated RNA-seq datasets for fmri maternal mutant embryos at multiple stages (0–0.5, 2–3, and 5–6 h stages). By comparing these RNA-seq datasets to those from wild-type embryos, we again found that, like that in mettl3–mett14 double mutant embryos, a significant proportion of maternal transcripts that were degraded in wild-type embryos were aberrantly upregulated in the fmri maternal mutant embryos at the 5–6 h stage (Fig. 2c and Fig. S4b, c). Intriguingly, we found that majority (~70%) of degraded maternal transcripts aberrantly upregulated in mettl3–mett14 double mutant embryos were also aberrantly stable in fmri maternal mutant at the 5–6 h stage (Fig. S4d), suggesting that FMR1 and the m6A modification share the common mRNA targets in regulating early embryogenesis. Of note, we examined the length of these stabilized maternal mRNAs at the 5–6 h stage, and found that most of them have a length of 1000–3000 nt (Fig. S4e). To ask whether FMR1 coordinates with the m6A pathway to regulate early embryonic development, we performed a series of genetic experiments. As shown in Fig. S4f–h, eggs produced by heterozygous fmri mothers, in which maternal FMR1 expression was partly reduced, fertilized by wild-type sperms displayed normal embryogenesis. The fmri heterozygous embryos carrying either mettl3 or mettl14 maternal mutant showed much stronger embryonic lethal phenotypes, compared to maternal depletion of either mettl3 or mettl14 alone (Fig. 2d). These results suggested that the reduced m6A modification by depletion of either mettl3 or mettl14 causes embryos to be sensitive to the dose of maternal FMR1. Of note, the fmri heterozygous embryos carrying mettl3–mett14 double maternal mutant embryos failed to enhance the embryonic lethality of mettl3–mett14 double maternal mutant (Fig. 2d). Collectively, our findings support the notion that FMR1 regulates embryonic development, at least in part, through binding m6A-marked mRNAs. Of note, although YTH-domain-containing proteins are dispensable for Drosophila embryonic development, we do not rule out a possibility that YTH proteins could act in concert with FMR1 to regulate other biological processes. Additionally, for other potential m6A-binding factors identified in our mass spec assays (Fig. S3d), it would be interesting to examine their potential role in the future.

FMR1 preferentially binds the m6A marked “AGACU” motif. A long-standing yet unsolved question in the field is how FMR1 recognizes its target mRNAs with high affinity35. Of note, in our original screen designed to identify the m6A-modified RNA binding proteins in early embryos, we used a pair of RNA probes (51 nucleotides in length) containing repeated “GGACU”, “AGACU”, and “GAACU” sequences with or without m6A modification. To determine which sequence contributes to the preferential binding of FMR1 with m6A-modified RNA, we synthesized additional RNA probes, containing nine repeats of the “GGACU”, “AGACU”, “GAACU” or “AAACU” sequences, with or without m6A modification, and then performed EMSAs. As shown in Fig. 2c, the m6A-modified RNA probe containing “AGACU” sequence formed a complex with FMR1 with the highest binding affinity, whereas all unmodified probes and probes containing m6A-modified “GGACU”, “GAACU”, or “AAACU” displayed relatively low binding affinities. To test whether the central A in “AGACU” is important for high-affinity binding of the probe to FMR1, we synthesized a mutant probe containing repeated “AGCCU” sequences and found that mutation of the central A to C markedly reduced the affinity of the RNA probe for FMR1 (Fig. 5a). We then synthesized an additional probe containing repeats of “AGmCU” or “AAmGACU”, in which only the central A or the first A was modified. EMSAs results suggested that m6A modification in “AGACU” could support the high affinity of FMR1-probe binding (Fig. S5b). In addition, we noted that besides the “U”, the “A” and the “C” are also present at the fifth position of the “RRACH” motif with high frequency that we identified in early embryos (Fig. S3a). By performing the EMSAs, we found that the probes containing m6A-modified “AGACC” and “AGAGA” motif displayed a relatively low affinity for FMR1, compared to the probe containing m6A-modified “AGACU” (Fig. S5c). Collectively, our findings suggest that the m6A-modified “AGACU” sequence is the highest affinity motif recognized and bound by FMR1. Next, we sought to determine the mechanism by which FMR1 preferentially binds the m6A-modified “AGACU”-containing RNAs. KH domains in many RBPs interact selectively with target mRNAs in a sequence-specific manner59. We asked whether the KH domains in FMR1 contribute to its high binding affinity for mRNAs containing the specific m6A-modified motif, and found that the deletion of KH domains abolished the formation of the complex by FMR1 with the tested probes (Fig. S6a–c). Previous structural studies have suggested that the “GxxG” loop within KH domains is critical for KH-containing proteins to recognize their target RNAs39. To test whether the GxxG loop is required for high-affinity binding of FMR1 to the m6A-modified “AGACU”-containing RNAs, we generated an FMR1 mutant (FMR1KH2-GDDG), EMSAs experiments revealed that the “GDDG” mutation abolished FMR1–RNA complex formation (Fig. S6d).

Selective binding of “m6A readers” to m6A-modified RNAs largely depends on a specific structure formed by certain residues in “m6A reader” proteins50,41. For example, in the case of hYthd2 binding to m6A-modified RNAs, the m6A mononucleotide is
tightly locked in a hydrophobic pocket formed by residues from the α1 helix, β2 strand, and β4–β5 loop. Because FMR1 can distinguish between the m6A-modified and unmodified RNAs, and given the critical role of the “GxxG” loop for FMR1 binding to RNAs, we speculated that the high affinity of FMR1 binding to RNA containing the m6A-modified “AGACU” motif might be attributable to specific residues around the “GxxG” loop. Of interest, we noted that the KH2 domain in FMR1 contains a hydrophobic network around the “GxxG” loop, as reported in the previous structure study and confirmed by our alignment and modeling analysis (Fig. 2f and Fig. S7a). Thus, we asked whether this hydrophobic network is critical for differential recognition of the m6A-modified and unmodified RNAs by FMR1. To test this possibility, we generated a series of FMR1 mutant proteins by...
Fig. 2 FMRI preferentially binds RNAs with the m6A marked “AGACU” motif in a residue-dependent manner. a Relative hatching rate of embryos with indicated genotypes (WT vs. fmr1(M−/−)), P = 2.6e−05; fmr1(M−/−) vs. vasP + myc::fmr1, fmr1(M−/−), P = 0.0008). b Western blot assays were performed to show expression levels of endogenous FMRI or overexpressed Myc-FMRI in the 0–1 h embryos with indicated genotypes. The experiment was performed once. c The degraded maternal mRNAs (S70 overlapped mRNAs shown in Fig. S4c) in fmr1 maternal mutant embryos displayed aberrant stable, when compared to wild-type at the 5–6 h stage (0.5–5 h, P = 0.49; 2–3 h, P = 5.5e−05; 5–6 h, P = 3.4e−48). P-values were calculated by a one-sided Student’s t test. The boxplot shows median (the horizontal line in the box), 1st and 3rd quartiles (lower and upper bounds of box, respectively), minimum and maximum (lower and upper whiskers, respectively). d Relative hatching rate of embryos with indicated genotypes (WT vs. mettl4(M−/−)), P = 4.9e−05; WT vs. mettl4(M−/−) vs. vasP + myc::fmr1, fmr1(M−/−), P = 0.0003; mettl4(M−/−) vs. mettl4(M−/−), fmr1+/−, P = 0.00098; mettl3-1d(M−/−) vs. mettl3-1d(M−/−) fmr1+/−, P = 0.56). e EMASAs showing the binding capability of FMRI to RNA probes containing either “GGACU,” “AGACU,” “GAACU,” or “AAACU” sequences (9×) with or without m6A modification. Representative figures of three independent replications are shown. f Structure modeling of a hydrophobic network within the KH2 domain of FMRI. g EMASAs were performed to detect the binding affinities of FMRI proteins carrying indicated site mutation to RNA probes with or without m6A modification. Representative figures of three independent replications are shown. h, i SPR assays showing binding affinity of FMRIV311K protein with RNA probes with unmodified RNA probes (h) or with m6A-modified probes (i). Equilibrium and kinetic constants were calculated by a global fit to the Langmuir binding model. RU resonance units. j Relative hatching rate of embryos with indicated genotypes (WT vs. fmr1(M−/−)), P = 3.5e−06; fmr1(M−/−) vs. vasP + myc::fmr1, fmr1(M−/−), P = 0.0002; fmr1(M−/−) vs. vasP + myc::fmr1V311K, fmr1(M−/−), P = 0.014). In a, d, j, data were expressed as means of three independent experiments, and the two-sided Student’s t test was used to analyze statistical variance. Error bars indicate mean ± SD. *P < 0.05, **P < 0.001. n.s. not significant. Source data are provided as a Source Data file and Supplementary Data 2.

mutating individual hydrophobic or aromatic amino acid residues around the “GxxG” loop to hydrophilic amino acids (Asn or Lys) (Fig. S7b, c). Using these mutants, we searched for the residue(s) responsible for the preferential binding of FMRI to m6A-modified RNAs by performing an EMSA screen. As shown in Fig. 2g, while changing F340 to Asn had no apparent effect on FMR1 RNA binding, mutation of V291, V299, I307 (corresponding to I304 in human FMR1) or F340 almost completely blocked FMR1 binding to both the m6A-modified and unmodified RNA probes. Surprisingly, mutating V311 to Lys did not affect FMR1 binding to m6A-modified RNA, but significantly increased the affinity of FMR1 for the unmodified probe (Fig. 2g–i), indicating that the change of V311 residue to K affects the preferential binding of FMRI to RNAs containing the m6A-modified “AGACU” motif. Next, we examined embryos with an expression of maternal FMRIV311K. As shown in Fig. S7g, neither detectable FMR1V311K protein expression was detected on Western blot assays performed with indicated genotypes (WT, fmr1(M−/−), fmr1(M−/−) vs. vasP + myc::fmr1, fmr1(M−/−), P = 0.0003; fmr1(M−/−) vs. mettl4(M−/−), fmr1+/−, P = 0.00098; mettl3-1d(M−/−) vs. mettl3-1d(M−/−) fmr1+/−, P = 0.56). e EMASAs showing the binding capability of FMRI to RNA probes containing either “GGACU,” “AGACU,” “GAACU,” or “AAACU” sequences (9×) with or without m6A modification. Representative figures of three independent replications are shown. f Structure modeling of a hydrophobic network within the KH2 domain of FMRI. g EMASAs were performed to detect the binding affinities of FMRI proteins carrying indicated site mutation to RNA probes with or without m6A modification. Representative figures of three independent replications are shown. h, i SPR assays showing binding affinity of FMRIV311K protein with RNA probes with unmodified RNA probes (h) or with m6A-modified probes (i). Equilibrium and kinetic constants were calculated by a global fit to the Langmuir binding model. RU resonance units. j Relative hatching rate of embryos with indicated genotypes (WT vs. fmr1(M−/−)), P = 3.5e−06; fmr1(M−/−) vs. vasP + myc::fmr1, fmr1(M−/−), P = 0.0002; fmr1(M−/−) vs. vasP + myc::fmr1V311K, fmr1(M−/−), P = 0.014). In a, d, j, data were expressed as means of three independent experiments, and the two-sided Student’s t test was used to analyze statistical variance. Error bars indicate mean ± SD. *P < 0.05, **P < 0.001. n.s. not significant. Source data are provided as a Source Data file and Supplementary Data 2.

FMRI granules undergo a dynamical assembly in early embryos in an LC domain- and RNA binding-dependent manner. RBPs execute their function to control the targeted RNA fate by forming RNP complexes/granules. To study the behavior of FMRI RNP complexes/granules, we performed live-cell imaging assays to trace the FMRI–RNP complex in embryos, in which GFP-FMRF1 was maternally expressed. As shown in Fig. 3a, only a few FMRI granules (less than 200 nm diameter) with weak GFP signals were present in the 0–1 h wild-type embryos, whereas many relatively larger FMRI granules (0.5–1 μm diameter) with strong GFP signals were found in the 2–3 h embryos. Interestingly, we noted that the number of relatively large-sized granules was reduced with development, and only a few small granules were observed in the 5–6 h embryos. Similar results were obtained when an anti-FMRI antibody was used to perform immunostaining assays (Fig. S8a–c). Thus, our findings suggest that FMRI granules have a highly dynamic property. To obtain additional evidence to confirm this observation, we then performed semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) analysis, a method for detecting high-molecular-weight (HMW) protein aggregates43,44. As shown in Fig. 3b, relatively weak HMW FMRI polymer signals were detected in wild-type embryos at the 0–1 h stage, and these signals became much stronger at the 2–4 h stage but decreased significantly at the 5–6 h stage. These findings together suggest that FMRI granules undergo an assembly/disassembly process during early embryonic development.

We next sought to determine the requirement for FMRI RNP-granule assembly in early embryos. LC domain has been reported to be important for many RBPs to form RNP-granules15,46. Given that FMRI contains an LC domain, we, therefore, asked whether the LC domain is required for FMRI to form RNP granules and thus regulate Drosophila embryogenesis. We evaluated early embryos maternally expressing the full-length FMRI (GFP-FMRF1FL) or a form of FMRI lacking the LC domain (GFP-FMRF1ALC). As shown in Fig. 3c, d, GFP-FMRF1FL, but not GFP-FMRF1ALC, formed cytoplasmic granules in the 2–3 h embryos. Moreover, we found that maternal expression of GFP-FMRF1FL, but not GFP-FMRF1ALC, significantly rescued embryonic lethal phenotypes induced by loss of maternal fmr1 (Fig. 3e, f), suggesting the functional importance of LC domain for FMRI in supporting embryogenesis.

In addition to the LC domain, FMRI also contains KH domains that enable FMRI to selectively bind its targeted mRNAs. To test whether RNA-binding is required for FMRI granule assembly and its dynamics, we removed the RNAs by treating the lysates at the 2–4 h embryos with RNase. By performing SDD-AGE analysis, we found that RNase treatment markedly reduced signals of HMW FMRI polymers at the 2–4 h stage (Fig. 3g). This result indicated that the binding of RNAs to FMRI stimulates the condensation of FMRI granules. We then asked whether the RNA-binding activity of FMRI contributes to dynamical assembly and disassembly of FMRI granules during early embryonic development. Given that the “GxxG” loop is critical for FMRI to selectively bind their target RNAs, we generated fmr1 maternal mutant embryos with an expression of fmr1 mutant, GFP-FMRF1KH2-GDDG, and traced the behavior of this mutant at multiple embryonic stages. As shown in Fig. 3i, unlike the wild-type FMRI, GFP-FMRF1KH2-GDDG failed to form large size granules at the 2–3 h stage. Importantly, in contrast to the wild-type FMRI, maternal expression of FMRF1KH2-GDDG was unable to rescue the embryonic lethal phenotype induced by loss of maternal FMRI (Fig. 3j). Collectively, our results support the
notion that specific interaction between FMR1 and RNAs dynamically regulates the assembly/disassembly switch of FMR1 granules.

The m6A modification regulates the assembly and disassembly of FMR1 granules. To simulate in vivo processes of FMR1 granule formation regulated by its LC domain, we purified GFP-FMR1^{FL} and GFP-FMR1^{ΔLC} (Fig. S9a), and studied the behavior of FMR1 in solution. In vitro phase separation assays revealed that GFP-FMR1^{FL}, but not GFP-FMR1^{ΔLC}, could form micrometer-sized droplets within several minutes under low-salt conditions (110 mM NaCl) (Fig. S9b), and these small droplets then underwent fusion to form larger ones (Fig. S9c). Interestingly, we found that the droplets became smaller and finally
disappeared, when salt concentrations were increased (Fig. S9d), suggesting that FMR1 droplet formation is a reversible process regulated by phase separation.

Given that RNA binding stimulates FMR1 granule assembly and controls the dynamic of FMR1 granules in embryos, and that FMR1 has a high affinity for the m6A-modified “AGACU” motif-containing RNAs, we speculated that FMR1 binding to the m6A-modified “AGACU” probe might influence dynamics of FMR1 droplets. To test this possibility, we incubated the GFP-FMR1\textsubscript{FL} with the m6A-modified “AGACU” probe or the unmodified probe. As shown in Fig. 4a–c and Fig. S9e, the m6A-modified probe only showed co-phase separation with GFP-FMR1\textsubscript{FL} (but not GFP-FMR1\textsubscript{ΔLC}), it also significantly increased the size of FMR1\textsubscript{FL}-RNA droplets. By contrast, the unmodified RNA probes only displayed a weak co-phase separation with FMR1\textsubscript{FL}, and this weak interaction did not apparently change the size of FMR1–RNA granules (Fig. 4a–c and Fig. S9e). Of note, neither the m6A-modified nor the unmodified probe alone showed any sign of phase separation (Fig. S9e). We next performed fluorescence recovery after photo-bleaching analysis and found that the FMR1–m6A-modified RNA droplets showed minimal recovery after photo-bleaching (Fig. 4d, e), compared with FMR1-unmodified RNA droplets or droplets containing only FMR1 without mixed with RNAs, suggesting that binding to m6A-modified RNA significantly changes the FMR1 condensate fluidity.

Of note, based on our datasets, loss of maternal FMR1 not only affected m6A-marked mRNA degradation, but it also caused the decay of a portion of non-m6A-marked mRNAs in early embryos. Similar results were observed in mettl3–mettl14 double mutant embryos. We reasoned that the incorporation of m6A-marked RNAs might affect the ability of FMR1 granules to further sequester unmodified RNAs. We thus performed additional in vitro phase separation assays. As shown in Fig. 4f, g, the presence of m6A-marked RNAs in solution induced much stronger co-phase separation of FMR1 and unmodified RNAs, compared with the absence of m6A-marked RNAs. Of note, when we used the negative control RNA probes that did not bind the FMR1 protein (Fig. S9f), we found that these probes failed to be recruited into FMR1 droplets, even in the presence of m6A-marked RNAs containing “AGACU” motif (Fig. 4h). These findings provide an important line of evidence indicating that the presence of m6A-modified RNA increases the size of FMR1 granules and enhances the partitioning of non-methylated RNA into the granules. To gain in vivo evidence for supporting our conclusion, we performed live-cell imaging assays to trace the behavior of GFP-FMR1\textsubscript{FL} in mettl3–mettl14 double maternal mutant embryos and found that number of the relatively large-sized granules was significantly reduced (Fig. 4i and Fig. S9g, h). In support of this, the SDD-AGE assay showed a dynamic change of high molecular weight of FMR1 in wild-type and mettl3–mettl14 maternal embryos from different developmental stages (Fig. S9i). Collectively, these findings emphasize the importance of m6A modification in enhancing FMR1 granule size and partitioning of RNA molecules into the granules.

FMR1 regulates decay of its target maternal mRNAs in an m6A-dependent manner. FMR1 has been previously proposed to repress target gene expression through translational repression\textsuperscript{47,48}. In this study, we found that loss of maternal FMR1 led to the aberrant stability of a portion of the degraded maternal mRNAs. Thus, there are two possibilities that can explain our results: (1) FMR1 directly regulates control target gene expression through RNA decay; (2) aberrant stable of maternal mRNAs induced by loss of maternal FMR1 could be a consequence (a byproduct) of defective translational repression. To distinguish these two possibilities, we performed data-independent acquisition (DIA) quantitative proteomics on the wild-type and fmr1 mutant maternal embryos at multiple developmental stages, including 0–1-, 2–3–, and 5–6–h stages (each sample with three biological replicates) (Fig. S10a). We identified 4987 protein hits (high confident hits) shared with each sample. It is worth noting that if the “byproduct” mode were correct, aberrant stable of degraded maternal mRNAs should be highly associated with up-regulation of corresponding protein products. To address this important issue, we analyzed the proteomic datasets together with RNA-seq datasets and identified the 4814 hits (Fig. S10b). Because FMR1 is a well-known translation repressor and potentially regulates RNA decay, we then focused on analyzing the upregulated genes and no significant changed genes at either mRNA or protein levels. As shown in Fig. 5a and Fig. S10c, we classified these genes into three major groups: genes in group 1 showed no significant change at both protein and mRNA levels; genes in group 2 displayed an increase at protein levels, but no significant change in levels of mRNAs; and genes in the group 3 showed an increase at mRNA levels, but no significant change in levels of proteins. Interestingly, we found that the majority of targets with aberrant upregulation at mRNA levels showed a decrease or no change in levels of proteins (group 3), suggesting that FMR1 has a role in regulating maternal mRNA decay. Additionally, we also found that genes in group 3 were significantly correlated with m6A modification, when compared to group 2 (Fig. 5b). Collectively, our findings suggest that in addition to translation repression,
FMR1 indeed plays a role in maternal mRNA decay through m6A modification.

To determine the mechanism of how FMR1 regulates maternal RNA decay, we collected the 2–3-h embryos and performed immunoprecipitation using the anti-Drosophila FMR1 antibody, followed by mass spectrometric analysis. As shown in Fig. S10d, in addition to ribosomal proteins and the Caprin protein, which are known FMR1-associated partners, poly(A)-binding protein (pAbp) was also highly enriched in the FMR1 complex in early embryos. pAbp has been shown to be involved in mRNA deadenylation, thus contributing to mRNA degradation 49,50.

We reasoned that interaction between FMR1 and pAbp could provide a mechanistic explanation for the action of FMR1 to regulate mRNA decay. In order to address the issue, we performed immunostaining and co-immunoprecipitation (co-IP) experiments. Immunostaining assays showed that pAbp was significantly overlapped with a portion of condensed FMR1 granules with a high Pearson’s correlation coefficient ($r = 0.81$) (Fig. 5c, d). The co-IP analysis suggested that FMR1 and pAbp could form a complex in wild-type early embryos (the 2–4-h stage) (Fig. S10e), suggesting that pAbp is associated with condensed FMR1 granules. Interestingly, the further co-IP...
Fig. 4 The m6A modification regulates assembly/disassembly switch of FMR1 granules. a Droplet formation assays for GFP-FMR1FL(10 μM) mixed with Cy3-labeled m6A-modified or unmodified probes, or alone. Scale bars, 10 μm. b The quantitative area of green droplet signal in (a) (GFP-FMR1FL + Cy3-ss-A, P = 0.41; GFP-FMR1FL + Cy3-ss-m6A, P = 0.008). c Quantitative intensity of RNA probes (red) droplet signal in (a) (P = 1.7e−4). d Fluorescence recovery after photo-bleaching (FRAP) assays to study the in vitro behavior of FMR1 droplets. Representative figures of four independent replicates are shown. Scale bars, 1 μm. e Changes in the fluorescence intensity of droplets after photobleaching were plotted over time. The curve represents the mean of the fluorescence intensity in the photobleached region of interest in distinct droplets (n = 4). Error bars indicate mean ± SD. f Droplet formation assays for the ability of GFP-FMR1FL(10 μM) to sequester unmodified RNAs (Cy3-labeled) with m6A-modified RNA probes or alone. Scale bars, 10 μm. g Quantitative intensity of RNA probes (red) droplet signal in (f) (P = 4.4e−5). h Droplet formation assays for testing the ability of GFP-FMR1FL (10 μM) to sequester unmodified mutant RNAs (Cy3-labeled) with m6A-modified RNA probes or alone. Representative figures of three independent replicates are shown. Scale bars, 10 μm. i Confocal imaging of live embryos with maternally expressing GFP-FMR1FL in wild-type or mettl3-mettl14 double maternal mutant embryos at the 0–1, 2–3, and 5–6-h stages. Representative figures of three independent replicates are shown. Scale bars, 10 μm. j In b, c, g, data were expressed as means of three independent experiments, and the two-sided Student's t test was used to analyze statistical variance. Error bars indicate mean ± 3D (b) or SEM (c, g). **P < 0.01, ***P < 0.001. n.s. not significant. Source data are provided as a Source Data file.

analysis showed that loss of maternal Mettl3–Mettl14 markedly reduced the association of FMR1 and pAbp (Fig. 5e). As mentioned above, pAbp affects mRNA stability through mRNA polyadenylation/de-adenylation49,50, we reasoned that aberrant upregulation of maternal mRNAs induced by loss of maternal FMR1 could be attributable to inefficient deadenylation. To test this possibility, we measured the poly(A) tail length of FMR1 targets, such as RASSF8, hdc, and HIP in wild-type and fmr1 maternal mutant embryos at multiple stages. We chose these targets for the further assays based on two observations. First, these targets contain the m6A-modified “AGACU” motif (Fig. S11a–c) and undergo degradation during early embryonic development, as tested by q-RT-PCR (Fig. S11d–f). Second, by using anti-FMR1 antibody, we performed RNA-IP followed by q-RT-PCR assays and found that these targets were indeed associated with FMR1 (Fig. S11g). By carrying out poly(A) tail length (PAT) assays, we found that RASSF8, hdc, and HIP transcripts underwent normal deadenylation in wild-type embryos, but were less deadenylated in fmr1 maternal mutants (Fig. 5f). These findings further suggest that loss of maternal fmr1 leading to aberrant up-regulation of these m6A-modified transcripts was due to inefficient deadenylation. Moreover, we obtained similar results, when these targets were analyzed in the mettl3–mettl14 double maternal mutant embryos (Fig. 5f). Interestingly, in agreement with our argument that binding of m6A-modified RNAs efficiently sequesters unmodified RNAs into FMR1 granules, we found that FMR1 regulated genes (Crag, Gdh, and Dah) without m6A modification, which underwent degradation (Fig. S11h–j) were also less deadenylated in embryos when maternal FMR1 was depleted (Fig. S11k).

Discussion

During early embryogenesis, a significant proportion of the maternal transcriptome is degraded and gradually replaced by zygotic transcripts, thus allowing normal embryonic development. However, little is known about how maternal RNA decay is triggered and how maternal RNAs (but not zygotic RNAs) are selectively cleared by RNP complexes. In this study, we report that a subset of m6A-modified mRNAs instructs FMR1–RNP granules to undergo a dynamic phase-switch, thus contributing to maternal RNA degradation during early embryogenesis. We show that while the LC domain initiates FMR1 granule self-assembly, KH domains facilitate the high-affinity binding of FMR1 to the mRNAs containing m6A-modified “AGACU” motif. This binding leads to significant condensation of FMR1 granules and increases the granules’ ability to sequester unmodified RNAs. Accompanying with degradation of the maternal mRNAs including m6A-modified RNAs, the FMR1 granules then undergo a de-condensation process, which allows normal embryogenesis to proceed (Fig. 5g). Our findings demonstrate the mechanism by which a subset of sequence-specific RNAs regulates a functional dynamical phase-switch of certain RNP–granules, thus contributing to RNA processing and normal development.

FMR1 regulates a subset of maternal mRNA decay, at least in part, via m6A. It has been reported previously that FMR1 and Caprin form an RNP-complex to control Drosophila early embryogenesis by directly mediating the translational repression of maternal cycB, suggesting a role of the FMR1/Caprin complex in early embryos through translation control36. By performing the hatching-rate analysis, we found that loss of maternal Caprin led to relatively weak phenotypes (~20% of embryonic lethality) (Fig. S12a–c). Given that the loss of maternal FMR1 caused a relatively strong phenotype (~55% of embryonic lethality), our results suggest that FMR1 may have other functions to control embryogenesis, in addition to its role via Caprin.

Given that maternal RNA decay is a hallmark of early embryogenesis, in this study, we investigate whether FMR1, as a typical RBP, contributes to maternal RNA decay in Drosophila early embryos. Several lines of evidence support that FMR1 is involved in maternal RNA decay. First, RNA-seq analyses showed that the loss of maternal FMR1 led to aberrant upregulation of a portion of the degraded maternal RNAs in early embryos. Second, loss of maternal Mettl3–Mettl14 also caused aberrant stability of a portion of the degraded maternal RNAs. Interestingly, we found that aberrantly upregulated maternal RNAs from fmr1 maternal mutant embryos were highly overlapped with those from mettl3–mettl14 mutant maternal embryos, suggesting that FMR1 and the m6A pathway share common targets in early embryos. Third, by performing DIA mass spec experiments, we found that loss of maternal FMR1 caused a defect of RNA decay in addition to translational repression. In order to link FMR1 to the deadenylation pathway, we performed immunostaining and co-immunoprecipitation experiments. Our immunostaining assays showed that signals of the pAbp protein were significantly detected in a portion of condensed FMR1 granules. The co-IP analysis suggested that FMR1 and pAbp could form a complex in wild-type early embryos (the 2–3-h stage), suggesting that pAbp is associated with condensed FMR1 granules. These findings together support the notion that FMR1 regulates maternal RNA decay, at least in part, via m6A modification.

How does FMR1 preferentially bind the “AGACU” motif in maternal mRNA? It has been reported that several missense mutations within the KH domains in FMR1 are associated with FXS, suggesting that FMR1 KH domains play roles in the pathophysiology of FXS12,51,52. Indeed, in this study we found that KH domains are essential for FMR1–RNA binding and critical for preferential binding of FMR1 to m6A-modified RNAs in


a sequence-specific manner. Consistent with previous findings, we found that the “GxxG” loop in KH domains is critical for FMR1 to interact with RNA targets. Structural studies of hYthdf2 have suggested that the m6A mononucleotide is tightly locked in a hydrophobic pocket. In this study, we tested whether the preferential recognition of m6A-modified RNA by FMR1 can be attributed to specific residues around the “GxxG” loop. By performing alignment and modeling analysis, we found that Droso-

Phase-switch of FMR1 granules contributes to maternal RNA decay. Of note, FMR1 not only binds to m6A-modified RNA with high affinity, but it also associates with unmodified RNA with low affinities in vitro, thus the question becomes what the biological
Fig. 5 FMR1 regulates the decay of its target maternal mRNAs in an m6A-dependent manner. a Scatterplot integrating protein and mRNA levels were generated by using wild-type and fmr1 maternal mutant embryos at the 5–6-h stage. By comparing fold change of fmr1 maternal mutant versus wild-type embryos, three major groups were classified: Group 1 (gray), including the genes without any change in both protein and mRNA level; Group 2 (blue), including the genes with upregulated protein level, but limited mRNA level change; Group 3 (red), including the genes with upregulated mRNA level, but limited or decreased protein level change. b Group 2 and Group 3 were further analyzed to overlap with m6A marked genes. We identified that 537 out of 4814 genes (used for proteomic and transcriptomic analyses) were marked with m6A. In total, m6A modification (highlighted with black circle) was found in as few as 5 genes in Group 2 (two-sided chi-square test, P = 0.93), but in a significant portion of genes (116) in Group 3 (two-sided chi-square test, P = 0.0066). c Wild-type embryos (at the 2–3-h stage) were stained with anti-FMR1 (red) and anti-pAbp (green) antibodies. Representative figures of three independent experiments are shown. Scale bars, 10 μm. d Pearson’s correlation coefficients of line profiles of FMR1 and the indicated proteins (n = 10). Error bars indicate mean ± SEM. e Comunoprecipitation of FMR1 with pAbp in wide-type and mettl3-mettl4 double maternal mutants at 2–4-h embryonic stage. The lysates were immunoprecipitated with anti-FMR1 antibody, and western blot assays were performed to detect pAbp protein in each immunoprecipitation. Representative figures of two independent replicates are shown. f PAT assay results showing changes in poly(A)-tail length for the indicated transcripts in the 0-1 and 5-6-h embryos with indicated genotypes. Representative figures of three independent replicates are shown. g Model for dynamic condensation/de-condensation of FMR1 granules to regulate maternal mRNA decay in fly early embryos. Source data are provided as a Source Data file and Supplementary Data 4.

significance of the differential binding of FMR1 to target RNAs is. In addition to other RNA-binding domains, FMR1 also contains a large LC domain at its C-terminus. We showed that the LC domain and KH domains coordinate to regulate FMR1 granule condensation and de-condensation in Drosophila early embryos through high-affinity binding of FMR1 with m6A-modified RNAs and the dynamically regulated m6A modification, respectively. Thus, the dynamic phase-switch likely confers the function of FMR1 granules in selectively regulating the degradation of maternal mRNAs, but not newly synthesized zygotic transcripts in the later stage embryos, thus ensuring proper embryogenesis. These findings support a model that a subset of sequence-specific RNAs can instruct a symmetric phase-switch of FMR1 granules, thus regulating maternal RNA decay in early embryos.

Methods

Drosophila strains. Fly stocks used in this study were maintained under standard culture conditions. The Drosophila w1118 strain was used for collecting the wild-type embryos and the host for P element-mediated transformations in this study. The fmr1 null flas, fmr1 P[2.5]) and fmr1 P[1.3]) were described previously. The following fly strains were generated in this study: (1) The mutant strains: mettl3, mettl4, mettl14, yhdi1, yhdi2, yhdi3, yhdi4, and caprin mutant alleles, were generated according to the method described previously. Briefly, the sequences targeted by gRNAs were chosen according to the CRISPER/Cas9 target software (http://sidf.partners.org/ZiFiT). The gRNA was in vitro transcribed and injected into embryos expressing Cas9 driven by the vasa promoter. The mutant alleles were screened and finally identified by sequencing (2) the transgenic strains include P[uasp-myc-fmr1], P[uasp-myc-fmr1(1-314)], P[uasp-myc-fmr1(1-232,520)], P[uasp-gfp-fmr1], P[uasp-gfp-fmr1(1-232,520)], and P[uasp-gfp-fmr1(1-314)] in which the full-length, mutant or truncated fmr1 tagged by myc or gfp were placed under the control of the UAS promoter; (3) P[vasa-gal4·p16] is a knock-in strain, in which the gal4·p16 sequence was placed at the downstream of the vasa promoter. This strain was used as a maternal driver. The detailed information of primers is described in Supplementary Table 1.

Embryo preparation. Drosophila embryo samples were collected according to the method described recently. Briefly, the well-fed flies were used to lay eggs in bottles, each of which was covered by a petri dish with agar gel. Embryos were carefully collected and examined under light microscopes. The older embryos were removed using the light microscope and the embryos were cleaned with washing buffer (1× PBS) to avoid contamination. Then the samples were immediately frozen in liquid nitrogen and stored at −80 °C.

RNA sequencing (RNA-seq). Total RNAs were isolated from embryos at the indicated stages using TRIzol reagent (ThermoFisher, 15596026). The mRNAs were purified using a NEBNext Poly(A) mRNA Magnetic Isolation Kit (NEB, E7490) according to the user manual. Illumina Truseq libraries were constructed according to the manufacturer’s instructions and sequenced using NovaSeq platform.

MeRIP-m6A-Seq. Firstly, 5 μg of fragmented mRNAs were incubated with 5 μg of the anti-m6A monoclonal antibody (1:100; Millipore, ABES572) in IP buffer (150 mM NaCl, 10 mM Tris-HCl, 0.1% NP-40, pH 7.4) in two independent biological replicates for 2 h at 4 °C. The mixture was incubated with Pierce protein A/G agarose (ThermoFisher, 20421) at 4 °C for an additional 2 h. Beads with captured RNA fragments were then immediately washed 3 times with ice-cold IP buffer. Then, the bound mRNAs were eluted from the beads with N6-methyladenosine (Bioneer & Ace) in IP buffers, and were exchanged with TRIzol reagent. Finally, both of the input samples and the m6A-Iped samples were prepared for RNA-seq library construction using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB, E7760). Library sequencing was subsequently performed on an Illumina HiSeq 4000 sequencer with 150 bp paired-end reads.

m6A quantification by UHPLC-MRM-MS/MS. The polyadenylated RNA from indicated embryos was isolated using the NEBNext Poly(A) mRNA Magnetic Isolation Module Kit (NEB, E7490). In brief, 100 ng isolated mRNAs were digested by nucleases (NEB, M0660S) in IP buffer, and were exchanged with TRIzol reagent. Finally, both of the input samples and the m6A-Iped samples were prepared for RNA-seq library construction using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB, E7760). Library sequencing was subsequently performed on an Illumina HiSeq 4000 sequencer with 150 bp paired-end reads.

Analysis of hatching rate. Embryos were collected for 24 h at 25 °C, then removed from the adults and allowed to develop for another 36 h. Then, unhatched embryos and larvae were calculated for the hatching rate.

Antibody preparation and western blot analysis. The antibodies were generated by immunizing mice or rabbits with recombinant proteins that were produced in Escherichia coli. The recombinant proteins were used as follows: GST-Metll3 (amino acids 1–200 aa), GST-Metll4 (amino acids 52–202 aa), GST-Yhdf (amino acids 501–700 aa), MBP-Yhdc (amino acids 351–721 aa), MBP-pAbp, and MBP-Caprin (amino acids 351–600 aa). Western blots were performed by using standard protocols. The antibodies were used as follows: mouse anti-β-Tubulin (1:2000; Cwbio, CW0098M); rabbit anti-Myc (1:2000; MBL, 562); mouse anti-Drosophila FMR1 (1:2000; Abcam, ab10299); mouse anti-Mettl3 (1:1000); mouse anti-Mettl14 (1:1000); mouse anti-Yhdc (1:1000); rabbit anti-pAbp (1:2000); rabbit anti-Caprin (1:2000). The quantification of band intensity was measured using ImageJ (1.46e) software.

Recombinant protein expression and purification. All recombinant proteins for EMSA and phase separation assay were expressed and purified from Escherichia coli BL21 (DE3) using PET28a vector. In brief, The MBP or GFP tag was added on the N terminus of FMR1 to the constructs. BL21 cells were grown in LB medium supplemented with 50 μg/mL kanamycin at 37 °C to an optical density (OD600 = 0.6). Then cells were induced with 0.5 mM IPTG for 18 h at 16 °C. Cells were harvested at 1500g for 5 min in 4 °C and resuspended in lysis buffer (20 mM Tris-Cl, pH8.0, 500 mM NaCl, 5 mM imidazole, 1% Triton-X100). The cells were lysed by sonication and then centrifuged at 15,800 g for 20 min. The supernatants were collected and purified using Ni SepharoseHigh-Performance beads (GE Healthcare, 17-5208-01). The beads were washed twice with wash buffer 1 (20 mM Tris-Cl, pH8.0, 500 mM NaCl, 20 mM imidazole) and once with wash buffer 2 (20 mM Tris-Cl, pH8.0, 500 mM NaCl, 50 mM imidazole). The recombinant proteins were eluted using elution buffer (20 mM Tris-Cl, pH8.0, 500 mM NaCl, 800 mM NaCl, 50 mM imidazole).
500 mM imidazole, 10% glycerol). All protein purification steps were performed at 4°C.

Synthesis of RNA probes. Biotin-3′-C- and biotin-5′-C-modified RNA probes were synthesized using Riboprobes in vitro transcription Systems (Promega, P1440). Biotinylated RNA (Roche, 11388890910) or Aminoallyl-UTP-Cy3 (Jena, NU-821-CY3), and other reaction components in a 20 μl reaction volume following the kit’s instructions. The RNA products were purified by ethanol precipitation. 5′-biotinylated "A*GACU" and "AGA*CU" RNA probes, in which only the first "A" or the central "A" was modified by m6A, were synthesized by Ribobiotechnology Company. The primers used for the generation of DNA templates are shown in Supplementary Table 2.

Electrophoretic mobility shift assays (GMSAs). In vitro synthesized biotin-5′-m6A RNA probes and purified proteins were used to perform this assay. Brieﬂy, the indicated recombinant proteins were expressed in E. coli and puriﬁed by His-tag afﬁnity puriﬁcation. The biotin-5′-m6A RNA probes were incubated with puriﬁed protein in 10 μl binding buffer (10 mM HEPES, pH 7.4, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 0.05% TritonX-100, 5% glycerol, 10 ng/μl salmon DNA, and 2 U/ml RNase inhibitor) at 25°C for 30 min. Then, 1 μl glutaethylene (0.2% ﬁnal concentration) was added to the mixture and incubated at 25°C for 15 min. Totally, 11 μl RNA-protein mixture was subjected to 6% native polyacrylamide gel and run for 40 min at 90 V in 0.5× TBE buffer. The gel was then transferred to a positively charged nylon transfer membrane (GE Healthcare, RPN303B) and the membranes were washed by UV irradiation (150 mJ/cm2) using a commercial UV-light crosslinking instrument. The membrane was blocked with blocking buffer (50 mM HEPES, pH 7.5, 0.5% NP-40, and protease inhibitors). The resuspended embryos were transferred to DNAne with homogenized with tight 50 times and centrifuged at 400g for 1 min at 4°C. The supernatant was centrifuged at 15,800g for 15 min at 4°C. The protein concentration of cytosolic lysates was examined by BCA protein assay kit (ThermoFisher, 23227). Biotinylated RNA pull-down assay was performed using the described method17. Brieﬂy, 10 μl streptavidin sepharose high-performance beads (GE Healthcare, 17-5113-01) were used for each pull-down assay. Beads were washed twice in 1 ml of RNA binding buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% NP40, 10 mM MgCl2) and incubated in 10 μM RNA probe (120 μM KCl, 1.5 mM MgCl2, and protease inhibitors). The beads were then washed using lysis buffer for 1 h and incubated at 95°C for 10 min to elute the complexes. For RNA-dependent droplet-formation experiments, samples were performed with corresponding primary antibodies by using standard protocols. For LC-MS/MS analysis, samples were separated using 10% SDS-PAGE and digested in-gel using Trypsin. Tryptic peptides were analyzed using the same parameters as described in the biotinylated RNA pull-down assay followed by the LC-MS/MS analysis.

MaxQuant analysis. We used label-free quantification for performing the relative quantiﬁcation in MS-based biotinylated RNA pull-down and co-IP experiments. Brieﬂy, all of their raw data of protein LC-MS/MS were analyzed using the MaxQuant software (v1.6.1.0). Tandem mass spectra were further searched against UniProt database containing 309323 sequences (uniprot_taxonomy_7215), released in March 2019 for protein identiﬁcation. Mass tolerance was set to 4.5 ppm (precursor ions) and 20 ppm (fragment ions). The maximum missed tryptic cleavages was set to 2. Variable modiﬁcations were methionine oxidation and protein N-terminal acetylation. The ﬁxed modiﬁcation was cysteine carbamidomethylation. The minimum length of amino acids per peptide was 7. A target decoy search approach was applied for the peptide and protein identiﬁcation with the default MaxQuant setting of 1% FDR. The post MaxQuant analysis included filtering the generated “protein groups.txt” table for contaminants. Proteins that were all identiﬁed in three replicates were used for the later analysis. We used the ratio of LEQ intensity (m6A vs. A or FMR1 antibody vs. IgG) to screen signiﬁcantly changed proteins. Proteins with signiﬁcant differential levels were identiﬁed by a one-tailed t-test for preferentially m6A interacting proteins, they were deﬁned as those whose at least twofold upregulated (q-value < 0.05) in m6A-modified probe samples. For speciﬁcally m6A interacting proteins, they were deﬁned as those only appeared in m6A-modiﬁed probe samples. Similarly, proteins that were upregulated more than twofold (q-value < 0.05) in three biological replicates compared with control IgG were considered as FMR1-associated proteins.

SPR measurements. SPR experiments were performed using Biacore 8K instrument (GE Healthcare). His-MBP tagged proteins were captured on sensor chip CM5 (2000–5000 response units). Biotin-5′-m6A RNA probes with increasing concentrations were injected into the protein surface for 1 min at a ﬂow rate of 30 μl/min, dissociated for 1 min in running buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 1 mM MgCl2, and 0.1% NP-40). Equilibrium dissociation ﬁts were calculated by a global ﬁt to the 1:1 Langmuir binding model (Biacore 8K evaluation software).

Quantitative real-time PCR analysis (q−RT−PCR). Embryos were collected with indicated stages and genotypes for RNA extraction. Total RNA was extracted using TRIzol reagent and cDNA was generated with a FastQuant RT Kit (Tiangen, KR106). Actin5C was used as the constitutive control for the normalization of candidate gene expression. For RIP-q-RT-PCR, the RNAs were immunoprecipitated by anti-Drosophila FMR1 antibody or IgG. q-RT-PCR reactions were performed in triplicate on a CFX Connect Real-time System (Bio-Rad) using the UltraSYBR mixture (Cwbio, CW0957). The detailed information of primers is described in Supplementary Table 3.

Poly(A)-tail (PAT) assay. Total RNAs were isolated from embryos with indicated stages and genotypes using TRIzol reagent. The poly(A) tail length was tested with Poly(A) Tail-Length Assay Kit (ThermoFisher, 76455) following the manufacturer's instructions. PCR products were analyzed on a 2% agarose gel. The detailed information of primers is described in Supplementary Table 3.

Embry staining and ﬂuorescence analysis. For immunohistochernistry, embryos were ﬁxed in ﬁxation buffer (4% formaldehyde and 0.3% Tween-20 in PBS) for 30 min and washed in PBT (0.3% Tween-20 in PBT) for 15 min. Fixed samples were incubated with FMR1 (1:2000) or pAbp (1:2000) antibodies at 4°C overnight and washed 3 times. Then the samples were incubated with secondary antibody at room temperature for 2 h, followed by washing for 3 times (10 min per time) in PBT. For tracing the GFP ﬂuorescence in living embryos, the embryos at the indicated stages were collected and immediately examined under Nikon Eclipse Ti microscope. All the images were acquired using Nikon software on a Nikon Eclipse Ti microscope. The granule size was calculated with the analyzed particle tools in Image J 1.64r. Line proﬁle analysis was used to analyze the correlation of FMR1 and pAbp proteins. Fluorescence intensity along the plasma line of FMR1 and pAbp proteins was calculated with the plot proﬁle tool in Image J 1.64r. The Pearson’s correlation coeﬃcient r values of two ﬂuorescence signals were calculated with Excel.

Phase separation assay. Recombinant GFP-FMR1FL and GFP-FMR1ΔC were expressed and puriﬁed from Escherichia coli. The phase separation was generated by mixing diluted GFP-FMR1FL or GFP-FMR1ΔC protein (10 μM) with NaCl buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, pH 7.5, with a dose gradient of NaCl) on a glass-bottom cell culture dish. For RNA-dependent droplet-formation experiments, proteins were incubated with Cy3-labeled A/m6A ssRNA or m6A probes in the buffer.
Fluorescence recovery after photobleaching. FMRI droplets were photobleached and imaged with a 488 nm laser using Nikon Eclipse Ti microscopy. At each time point, fluorescence intensity within the bleaching spot was divided by the intensity of a neighboring unbleached area of the same size to correct the changes. The ratio of recovery was analyzed from three independent biological replicates.

SDD-Age assay. The indicated embryos were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Triton X-100, 5% glycerol with protease inhibitors) for 30 min, followed by centrifugation at the speed of 13,500 g at 4 °C. The supernatant was then diluted with loading buffer (0.5X TBE, 10% glycerol, 2% SDS, 0.0025% bromophenol blue) at room temperature for 30 min, and loaded onto a newly-prepared 15% agarose gel with 0.1% SDS. After electrophoresis in the running buffer (1X TBE and 0.1% SDS) for 40 min with a constant voltage of 100 V at 4 °C, the proteins were transferred to BioTrace NT nitrocellulose (PALL, 66485) for immunoblotting with indicated antibodies.

RNA-seq data analysis. We performed RNA-seq for wild-type, mettl3–mettl14 double maternal mutant and fmr1 maternal mutant embryo samples at the indicated stages using sequencing adapters and 150bp-end sequencing. Low-quality reads and adapter sequences were trimmed for all raw reads using fastq2fmapping with default parameters except for “–q 3 –a 50-length required 150”. The FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to confirm the high quality of the sequencing data. The clean reads were mapped to the December 2014 assembly of the D. melanogaster genome (UCSC version dm6, BDGP Release 6, unlocalized scaffolds excluded) with TopHat version 2.0.9 with parameter “–keep-fasta-header”. The FPKM (per kilobase of coding exon per million fragments mapped) value for gene expression and differential expression analysis was carried out by cuffdiff from the cufflinks package (version 2.2.1). K-means clustering was performed on differentially expressed genes in the five developmental stages was evaluated using Cluster 3.0 (clustering method: K-means clustering, Distance metric: Euclidean distance)68. The clustered file was identified as (1) degraded maternal genes: if their original FPKM values >10 at 0–0.5-h stage and expression levels were consistently reduced thereafter; (2) stable maternal genes: if their original FPKM values >10 >0–0.5-h stage and expression levels were relatively stable upon a time; (3) zygotic genes: if their original FPKM values <10 at 0–0.5-h stage and expression levels were induced after 2–3-h stage. The clustered gene groups with original FPKM values were imported to Excel and R for statistical processing and visualizing. The medians of FPKM values of each group were used to visualize expression data.

MeRIP–m6A-Seq data analysis. After performing RNA immunoprecipitation (RNA-IP) experiments using specific anti-m6A antibodies, all samples were sequenced on an Illumina HiSeq 4000 platform with paired-end 150bp read length. For alignment of reads, adapter-trimming and low quality read filtering were firstly performed by the Cutadapt software (version 1.12)69. Then the remaining reads were aligned to Drosophila reference genome (UCSC version dm6, BDGP Release 6, unlocalized scaffolds excluded) by HISAT2 (version 2.1.0)69 with default parameters. After discarding reads that were mapped to more than one genomic region, the uniquely mapped reads were processed to peak calling analysis. The call peak module in MACS2 (version 2.2.1)70 was used to identify enrichment peaks with default parameters except for “–max-model”, and the corresponding reads were serving as control. The two replicates of m6A peaks were intersected with each other by Bedtools package (version 2.25.0)62 with the minimum overlap required as a fraction of them (80%). The overlapped peaks between two replicates were identified as high-confident m6A peaks. Differentially methylated sites on transcripts were identified by diffReps (version 1.53.3). These peaks were added to transcripts using bamreadaligner and the motifs enriched in m6A peaks were detected using Find Individual Motif Occurrences (FIMO)69 on the peak summits 200 bp of sequences.

We normalized m6A changes between conditions by using ngs.plot Two-step Normalization. In brief, we calculated the number of reads of m6A reads in the m6A region of reads in the m6A region of each sample that mapped to each window or region by normalizing the number of reads in the m6A region to the number of reads in the corresponding input sample. The normalized m6A RIP read number was divided by the read number of the corresponding input as enrichment score of that window or region. Significant differences of normalized m6A coverage between the conditions were determined with a 95% confidence interval and Wilcoxon rank-sum test of the R stats library package. In order to get the 95% confidence interval of enrichment score for each window or region, we sampled the peaks 100 times by using the -s parameter of ngs.plot software, and the resulting enrichment scores were subjected to seaborn (a Python data visualization library) to plot coverage with 95% confidence interval for the specific regions in the study. To evaluate the statistical significance of m6A coverage, we divided the range (~11000 bp around the peak summit) into 60 bins. Then the statistical significance was estimated by comparing the values representing the coverage of m6A after normalization in the ten bins around the summit (~3300 bp) using the Wilcoxon rank-sum test.

Data-dependent acquisition (DDA) and data analysis. Embryos from different stages were lysed using a buffer containing 8 M urea, 50 mM Tris-HCl (pH 8.0), and protease inhibitor cocktail, and the tissue was centrifuged (12,000g, 15 min). To build a spectral library for following DIA analysis, we firstly used an equal amount of samples applied in DIA to generate a data-dependent acquisition (DDA) spectral library. The samples were mixed and then subjected to SDS-PAGE gel, which was further cut into 10 pieces from high to low molecular weight. The proteins in each piece of gel were digestion by trypsin overnight at 37 °C, and then dissolved in a solution of 0.1% formic acid in water containing proportionally added iRT reagent and then further analyzed by Orbitrap Experior 480 MS system. Proteome Discoverer ver. 2.4 (ThermoFisher) was used to search the above 10 groups of DDA mass spectrometry results. Peptide/protein entries identified were used to build the final DDA spectral libraries using Skyline software (Skyline 21. 1. 0. 146) for the following DIA data analysis61. The Drosophila reference proteome was downloaded from UniProt on January 14, 2021.

DIA quantitative proteomics and analysis. Embryos from different stages were lysed using a buffer containing 8 M urea, 50 mM Tris-HCl (pH 8.0), and protease inhibitor cocktail, and the tissue was centrifuged (12,000g, 15 min). The supernatant was harvested and digested by trypsin (1:80 weight/weight (w/w)) in the solution overnight at 37 °C. The peptides were desalted with a C18 column. The concentrations of the peptides obtained after trypsin digestion were determined using nanodrop. Digested samples that were added with a proportion of iRT reagents (Biogynosys, Ki-3002-2) were analyzed on an Orbitrap Experior 480 mass spectrometry system equipped with an Easy nLC 1200 ultra-high performance liquid chromatography system (ThermoFisher).

The DIA data was analyzed using Skyline software (Skyline 21. 1. 0. 146), which used iRT peptides to calibrate retention time. The default parameters were used for the DIA data analysis, in which the protein and peptide FDRs were set to less than 5%. The protein expression levels of fmr1 maternal mutant groups were compared with those of the wild-type group, and the ratio between groups was regarded as the expression change of a protein or peptide, which was also the basis for further data analysis. Quantified proteins with at least an expression change value >1.5fold, q-value <0.05 were considered to have significant expression changes.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data supporting the findings of this study are available from the corresponding authors upon reasonable request. The raw sequencing data generated in this study have been deposited in the NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE143821. The mass spectrometry proteomics data generated in this study have been deposited to the ProteomeXchange Consortium via the iProX partner repository65 with the dataset identifier PXD026356. Source data are provided with this paper.

Received: 12 May 2021; Accepted: 18 January 2022; Published online: 14 February 2022.

References
1. Despic, V. & Neugebauer, K. M. RNA tales—embryos read and discard messages from mom. J. Cell Sci. https://doi.org/10.1242/0x.201996 (2018).
2. Palfy, M., Joseph, S. R. & Vastenhouw, N. L. The timing of zygotic genome activation. Curr. Opin. Genet. Dev. 43, 53–60 (2017).
3. Tadros, W. & Lipshitz, H. D. The maternal-to-zygotic transition: a play in two acts. Development 136, 3033–3042 (2009).
4. Schier, A. F. The maternal-zygotic transition: death and birth of RNAs. Science 316, 406–407 (2007).
5. Kato, M. et al. Cell-free formation of RNA granules: low complexity sequence domains form dynamic fibers within hydrogels. Cell 149, 753–767 (2012).
6. Lunde, B. M., Moore, C. & Varani, G. RNA-binding proteins: modular design for efficient function. Nat. Rev. Mol. Cell Biol. 8, 479–490 (2007).
7. Hyman, A. A., Weber, C. A. & Julicher, F. Liquid-liquid phase separation in biology. Annu. Rev. Cell Dev. Biol. 30, 39–53 (2014).
8. Youn, J. Y. et al. Properties of stress granule and P-body proteomes. Mol. Cell 76, 286–294 (2019).
9. Nicastro, G., Taylor, I. A. & Ramos, A. KH-RNA interactions: back in the groove. Curr. Opin. Struct. Biol. 30, 65–70 (2015).
10. Valverde, R., Edwards, L. & Regan, L. Structure and function of KH domains. FEBS J. 275, 2712–2716 (2008).

11. Verkerk, A. J. et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65, 905–914 (1991).

12. Drozd, M., Bardoni, B. & Capovilla, M. Modeling fragile X syndrome in Drosophila. Front. Mol. Neurosci. 11, 124 (2018).

13. Astuto, C. T., Strohmeyer, K. D., Reines, D. & Warren, S. T. FMR1 protein: conserved RNP family domains and selective RNA binding. Science 262, 563–566 (1993).

14. Myrick, L. K., Hashimoto, H., Cheng, X. & Warren, S. T. Human FMRP contains an integral tandem Agnet (Tudor) and KH motif in the amino terminal domain. Hum. Mol. Genet. 24, 1733–1740 (2015).

15. Acsan, M. Jr. et al. FMRP targets distinct mRNA sequence elements to regulate protein expression. Nature 492, 382–386 (2012).

16. Greenblatt, E. J. & Spradling, A. C. Fragile X mental retardation 1 gene defect in Drosophila. Proc. Natl Acad. Sci. USA 60, 231–241 (1993).

17. Edupuganti, R. R. et al. N(6)-methyladenosine (m6A) recruits and repels proteins to regulate mRNA homeostasis. Nat. Struct. Mol. Biol. 24, 870–878 (2017).

18. Edens, B. M. et al. FMRP modulates neural differentiation through m(6)A-Nature. Commun. 8, 395–405 (2017).

19. Arguello, A. E., DeLiberto, A. N. & Kleiner, R. E. RNA chemical proteomics reveals that N6-methyladenosine (m(6)A)-regulated protein-RNA interactor. J. Am. Chem. Soc. 139, 17249–17252 (2017).

20. Zhang, F. et al. Fragile X mental retardation protein modulates the stability of its m6A-marked messenger RNA targets. Hum. Mol. Genet. 27, 3956–3960 (2018).

21. Wu, B., Li, L., Huang, Y., Ma, J. & Min, J. Readers, writers and erasers of N(6)-methylated adenosine modification. Curr. Opin. Struct. Biol. 47, 67–76 (2017).

22. Zaccara, S., Ries, R. J. & Jaffrey, S. R. Reading, writing and erasing mRNA methylation. Nat. Rev. Mol. Cell Biol. 20, 608–624 (2019).

23. Roundtree, I. A., Evans, M. E., Pan, T. & He, C. Dynamic RNA modifications in gene expression regulation. Cell 169, 1187–1200 (2017).

24. Lence, T. et al. m6A modulates neuronal functions and sex determination in Drosophila. Nature 540, 242–247 (2016).

25. Hamm, D. C. & Harrison, M. M. Regulatory principles governing the maternal-to-zygotic transition: insights from Drosophila melanogaster. Open Biol. 8, 180183 (2018).

26. Liu, J. et al. A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. Nat. Chem. Biol. 10, 93–95 (2014).

27. Schwartz, S. et al. Perturbation of m6A writers reveals two distinct classes of mRNA methylation at internal and 5' sites. Cell Rep. 8, 284–296 (2014).

28. Mali, P. et al. RNA-guided human genome engineering via Cas9. Science 339, 823–826 (2013).

29. He, S. et al. 6mA-DNA-binding factor Jumbl controls maternal-to-zygotic transition upstream of Zelula. Nat. Commun. 10, 2219 (2019).

30. Zhao, B. S. et al. m6A-dependent maternal mRNA clearance facilitates zebrafish maternal-to-zygotic transition. Nature 542, 475–478 (2017).

31. Kan, L. et al. The m6A pathway facilitates sex determination in Drosophila. Nat. Commun. 8, 15737 (2017).

32. Meyer, K. D. et al. Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. Cell 149, 1635–1646 (2012).

33. Dominissini, D. et al. Topology of the human and mouse m6A RNA methylation by m6A-seq. Nature 485, 201–206 (2012).

34. Wang, X. et al. N6-methyladenosine-dependent regulation of messenger RNA stability. Nature 505, 117–120 (2014).

35. Deshpande, G., Calhoun, G. & Schiedl, P. The drosophila fragile X protein DfMR1 is required during early embryogenesis for pole cell formation and rapid nuclear division cycles. Genetics 174, 1287–1298 (2006).

36. Papoulis, O. et al. dMRP and Caprin, translational regulators of synaptic plasticity, control the cell cycle at the Drosophila mid-blastula transition. Development 137, 4201–4209 (2010).

37. Mayer, K. et al. Fragile X mental retardation protein controls trailer hitch expression and cleavage furrow formation in Drosophila embryos. Proc. Natl Acad. Sci. USA 103, 18160–18165 (2006).

38. Yang, L. et al. Fragile X mental retardation protein modulates the fate of germine stem cells in Drosophila. Hum. Mol. Genet. 16, 1814–1820 (2007).

39. Hollingsworth, D. et al. KH domains with impaired nuclear acid binding as a tool for functional analysis. Nucleic Acids Res. 40, 6873–6886 (2012).

40. Li, F., Zhao, D., Wu, J. & Shi, Y. Structure of the YTH domain of human YTHDF2 in complex with an m(6)A mononucleotide reveals an aromatic cage for m(6)A recognition. Cell Res. 24, 1490–1492 (2014).

41. Xin, Y. Structural basis for the discriminative recognition of N6-methyladenosine RNA by the human YT521-B homology domain family of proteins. J. Biol. Chem. 290, 24902–24913 (2015).
Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-28547-7.

Correspondence and requests for materials should be addressed to Qinmiao Sun or Dahua Chen.

Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.