The Drosophila hnRNP F/H homolog Glorund recruits dFMRP to inhibit nanos translation elongation

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ABSTRACT
Translational control of maternal mRNAs generates spatial and temporal patterns of protein expression necessary to begin animal development. Translational repression of unlocalized nanos (nos) mRNA in late-stage Drosophila oocytes by the hnRNP F/H homolog, Glorund (Glo), is important for embryonic body patterning. While previous work has suggested that repression occurs at both the translation initiation and elongation phases, the molecular mechanism by which Glo regulates nos translation remains elusive. Here, we have identified the Drosophila fragile X mental retardation protein, dFMRP, as a Glo interaction partner with links to the translational machinery. Using an oocyte-based in vitro translation system, we confirmed that Glo regulates both initiation and elongation of a nos translational reporter and showed that dFMRP specifically represses translation elongation and promotes ribosome stalling. Furthermore, we combined mutational analysis and in vivo and in vitro binding assays to show that Glo’s qRRM2 domain specifically and directly interacts with dFMRP. Our findings suggest that Glo regulates nos translation elongation by recruiting dFMRP and that Glo’s RNA-binding domains can also function as protein-protein interaction interfaces critical for its regulatory functions. Additionally, they reveal a mechanism for targeting dFMRP to specific transcripts.

INTRODUCTION
Translational control, coupled with other types of post-transcriptional regulation including mRNA localization and degradation, is a key mechanism for establishing and maintaining protein asymmetries. Translational control plays a particularly important role in early embryonic development in organisms like Drosophila (1,2), Caenorhabditis elegans (3) and Xenopus (1), where transcription of the zygotic genome is delayed for many cell divisions. The embryo therefore relies on spatial and temporal control over the translation of a stockpile of maternally synthesized mRNAs to execute the earliest cell fate determination and axial patterning programs.

The Drosophila anterior-posterior body axis is patterned by opposing protein gradients of Bicoid (Bcd) and Nanos (Nos) in the early embryo. Both gradients originate from localized translation of maternal bcd and nos mRNAs at the anterior and posterior poles of the embryo, respectively (4). Unlike bcd, which is tightly sequestered at the anterior pole (5), only a small proportion (4%) of nos resides at the posterior and is translated (6). Therefore, whereas RNA localization is sufficient to sequester Bcd protein synthesis at the anterior (7), an additional level of control is required to restrict production of Nos to the posterior. This is accomplished by maintaining nos in a translationally repressed state that is alleviated only by its posterior localization (6,8,9). Failure to repress unlocalized nos mRNA and the consequent accumulation of Nos throughout the embryo result in loss of anterior structures (10,11).

As a maternally supplied mRNA, nos is synthesized in the ovarian nurse cells (12) and then transferred to the oocyte during the latter part of oogenesis, where localization ensues. nos is translated initially in the nurse cells but becomes repressed when it enters the oocyte. Upon localization to the posterior of the oocyte, translation is reactivated (9). Translational repression of the unlocalized nos is mediated primarily by a 90-nucleotide cis-acting translational control element (TCE) in the nos 3′ untranslated region (3′ UTR) (13–15). The nos TCE consists of a base stem, TCEI, connected to two stem loops, termed TCEII and TCEIII, which have temporally distinct activities (9,16). TCEI and TCEII together confer translational regulation on nos during late oogenesis through their interaction with the hnRNP F/H protein Glorund (Glo) (16–18). TCEII confers repression on unlocalized nos during early embryogenesis through its interaction with a different RNA-binding protein, Smaug (Smg) (15,19).

Translational repression of unlocalized nos during late stages of oogenesis appears to be bipartite. Previous
vitro translation experiments showed that TCE-mediated repression of a reporter RNA in embryo extract was abolished if the reporter RNA was unable to bind eukaryotic translation initiation factor 4E (eIF4E) (20). This cap-dependence of repression suggests that the TCE acts to block initiation in the embryo and is consistent with the ability of Smg to recruit the Drosophila 4E-binding protein (4E-BP), Cup, and disrupt the formation of the translation initiation complex eIF4F (21). By contrast, TCE-mediated repression of the same reporter in ova-ry extract was only partially abrogated (20), indicating that repression in the ovary is only cap-dependent in part. Furthermore, sucrose density gradient sedimentation showed that approximately 50% of nos is poly somese associated whereas only 4% of nos is posteriorly localized and translationally active (22), consistent with repression at the elongation step. Compared to initiation control, an elongation-based mechanism could afford a more rapid on/off switch to shut down translation as nos enters the oocyte and then reactivate translation as it becomes localized. Glo is the only known repressor of nos in the ovary, suggesting that it might mediate both the initiation and elongation-based repression mechanisms. Like its mammalian counterparts, Glo is a multi-functional RNA-binding protein. In addition to translationally repressing nos, Glo has been shown to repress the translation of nuclear-encoded mitochondrial respiratory chain complex mRNAs (23). Glo also impacts ovarian nurse cell chromatin organization and dorsal-ventral patterning, possibly acting as a regulator of alternative splicing of ovarian tumor mRNAs through its interaction with Hrp48 and Hfp (17,24). Most recently, Glo has been implicated in triglyceride homeostasis in adults by regulating mRNAs encoding lipid transport proteins (25). Finally, Glo is required for viability to adulthood (17), although the target RNAs and regulatory mechanism used in this capacity are not known.

How a single protein, Glo, regulates multiple mRNA metabolic pathways and how it distinguishes regulatory needs among its RNA targets remain largely elusive. One possibility is suggested by Glo’s dual modes of RNA recognition. Glo contains 3 highly conserved quasi-RNA recognition motifs (qRRMs), each of which harbors 2 RNA-binding interfaces with distinct sequence specificities, allowing different combinations of RNA-binding modes to select subsets of target mRNAs (18). Apart from the qRRMs, Glo contains no known protein domains. Thus, the qRRMs may also act as protein-protein interaction interfaces to recruit different effector proteins that diversify Glo’s regulatory activities. To gain further insight into Glo as a multi-faceted translational repressor and into qR RM function beyond RNA binding, we isolated Glo-interacting proteins from late-stage oocytes and investigated whether the interaction is mediated by one or more of Glo’s qRRMs.

Here, we identify the Drosophila fragile X mental retardation protein, dFMRP, as a Glo-interacting protein required for translational repression of nos mRNA during late stages of oogenesis. We show that dFMRP negatively regulates nos translation in late-stage oocytes and that dFMRP associates with polysome-bound nos, consistent with its proposed role as a translation elongation repressor. Using an in vitro translation run-off assay that distinguishes between effects on translation initiation and elongation, we show that Glo represses a reporter containing the nos TCE at both the initiation and elongation phases and that dFMRP specifically represses translation elongation. Puromycin release and [35S]-methionine incorporation assays demonstrated that dFMRP stalls ribosomes on nos and decreases elongation rate, respectively. Analysis of engineered Glo variants lacking individual qRRMs revealed that qR RM2 is specifically required for dFMRP binding to Glo and consequently to nos in vivo. Furthermore, an in vitro binding assay using purified qRRMs and dFMRP showed that qR RM2 interacts directly with dFMRP. Taken together, these results support a model in which Glo represses translation elongation of nos by directly recruiting dFMRP through qR RM2.

MATERIALS AND METHODS

Construction of transgenes and transgenic lines

The gfp-glo transgene was previously described (18). The transgene was maintained in flies homozygous for the glo162x null allele (17), so that GFP-Glo constituted the only source of Glo protein. This transgene fully rescues glo162x phenotypes (24). qR RM and CTX deletions were generated in the glo coding sequence by overlap extension PCR mutagenesis. All transgenes were inserted into the attP40 landing site by phiC31-mediated integration. glo162x mutant germline clones expressing gfp-glo or gfp-gloΔqR RM transgenes were generated using the dominant female-sterile method (26). The gfp-dfmr1 transgenic line was previously described (27).

Collection of ovary and oocyte samples

To obtain late-stage-oocyte-enriched ovaries (late-ovaries), female flies were fed for 4 days at 25°C in bottles of corn meal agar food supplemented with yeast paste and then starved in bottles with only wet Kimwipes (Kimtech) for 18 h at 25°C. Ovaries were hand-dissected on ice in PBS and used immediately to prepare extract. For late-stage oocyte isolation, hand-dissected ovaries were teased apart, and late-stage oocytes were separated out according to morphology, pooled in PBS, and used immediately. The Oregon-R strain was used for preparation of wild-type tissues. dfmr1 RNAi tissues were produced by mat-tub-Gal4 > UAS-dfmr1RNAi (TrRP GL0075) females.

Immunoprecipitation and mass spectrometry (IP-MS)

Late-ovaries expressing GFP-tagged proteins were homogenized in IP buffer (10 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% IGEPAL-CA630, 1x cComplete EDTA-Free Protease Inhibitor Cocktail (Roche), 1 mM PMSF) supplemented with 20 µl of RNase A/T1 Mix (Thermo Scientific) and 20 U of RNase ONE (Promega) per 1 ml of IP buffer and cleared by centrifugation at 13,200 rpm for 30 min at 4°C. The supernatant was incubated with GFP-Trap_A beads (Chromotek) at 4°C for 1.5 h, and the beads were then washed with IP buffer without IGEPAL-CA630.
Immunoprecipitates were eluted in 0.1% (v/v) TFA. For crosslinked IP-MS, dissected oocytes were incubated in PBS containing 1 mM DSP (Thermo Scientific) or in PBS without DSP (control) for 30 min at room temperature, quenched by addition of Tris–HCl pH 7.5 to a final concentration of 20 mM, and incubated for 15 min. Extract was prepared and immunoprecipitation with GFP-Trap A beads was performed as described above, except that the beads were washed with IP buffer containing 1 M NaCl and no IGEPAL-CA630. Immunoprecipitates were eluted by boiling the beads in SDS-PAGE sample buffer (2% SDS, 62.5 mM Tris base, 10% glycerol, 100 mM DTT) which also cleaves the disulfide linker in DSP to reverse-crosslink. For both native and crosslinked IP-MS, LC–MS/MS analysis was performed at Proteomics and Mass Spectrometry Core at Princeton University. The resulting MS datasets were analyzed using Scaffold 4 (Proteome Software). Fold enrichment was defined as follows.

Native IP-MS:

\[
\text{Fold enrichment} = \frac{\text{total spectral counts in } GFP\text{-Glo IP}}{\text{total spectral counts in } MCP\text{-GFP IP}}
\]

Crosslinked IP-MS:

\[
\text{Fold enrichment} = \frac{\text{# total spectra count in crosslinked (+ DSP) IP sample}}{\text{# total spectra count in un-crosslinked (DSP) control}}
\]

**Immunoprecipitation of endogenous Glo**

10 µg of anti-Glo antibody (SB7) (17), or anti-GFP antibody (JL-8; Clontech) was crosslinked to 50 µl of Dynabeads Protein G (Thermo Fisher) by dimethyl pimelimidate. Wild-type *Drosophila* late-ovary lysate was prepared, incubated with beads, and washed as described above (native IP-MS), except that the IP buffer contained 200 mM NaCl.

**RNA co-immunoprecipitation**

Late-ovary extracts were prepared as follows. 50 pairs of freshly dissected late-stage-oocyte-enriched ovaries were homogenized on ice in 100 µl of RNA-IP buffer containing 10 mM HEPES–NaOH pH 7.0, 100 mM KCl, 5 mM MgCl₂, 0.5% IGEPAL CA-630, 1 mM DTT, 200 U/ml SUPERase-In (Ambion), and 1 x cOmplete EDTA-Free Protease Inhibitor Cocktail. The resulting MS datasets were analyzed using Scaffold 4 (Proteome Software). Fold enrichment was defined as follows.

\[
\text{Fold enrichment} = \frac{\text{total spectral counts in } GFP\text{-Glo IP}}{\text{total spectral counts in } MCP\text{-GFP IP}}
\]

**Fold enrichment** = \[\frac{\text{total spectral counts in crosslinked (+ DSP) IP sample}}{\text{total spectral counts in un-crosslinked (DSP) control}}\]

**Polysome fractionation**

Late-ovary extracts were prepared by homogenizing 200 pairs of freshly dissected late-stage-oocyte-enriched ovaries on ice in a lysis buffer containing 50 mM Tris–HCl pH 7.5, 250 mM NaCl, 50 mM Mg(OAc)₂, 0.2% Triton X-100, 2 mg/ml heparin, 1 mM DTT, 200 U/ml SUPERase-In, 0.5 mg/ml cycloheximide (Sigma-Aldrich), and 1 x cOmplete EDTA-Free Protease Inhibitor Cocktail. The extract was cleared by centrifugation at 13,200 rpm for 15 min at 4°C, and the supernatant was layered onto a 12 ml 10–50% sucrose gradient in 50 mM Tris–HCl pH 7.5, 250 mM NaCl and 50 mM Mg(OAc)₂. Gradients were centrifuged in a Beckman SW41Ti rotor at 36,000 rpm for 3 h at 4°C. Following centrifugation, the gradient was fractionated using a Piston Gradient Fractionator (BioComp) to record UV absorbance at 260 nm and collect 500 µl fractions.

For RNA extraction, fractions were treated first with 10 µl of RQ1 RNase-free DNase for 30 min at 37°C and then with 150 µg/ml proteinase K in the presence of 1% SDS and 50 mM EDTA for 30 min at 55°C, followed by phenol:chloroform extraction and isopropanol precipitation, and re-dissolved in 20 µl of nuclease-free water.

**Puromycin release and polysome analysis**

100 pairs of late-ovaries were dissected in PBS (–Puro) or PBS containing 3 mM puromycin (+Puro; Sigma-Aldrich) on ice. Dissected ovaries were transferred to 500 µl of lysis buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 5 mM Mg(OAc)₂, 0.2% Triton X-100, 2 mg/ml heparin, 1 mM DTT, 200 U/ml SUPERase-In and 1 x cOmplete EDTA-Free Protease Inhibitor Cocktail; –Puro) or lysis buffer with 2 M urea. Washed beads were treated first with 100 µl of wash buffer containing 8 µl of RQ1 RNase-free DNase (Promega) for 15 min at 37°C and then with 150 µg/ml proteinase K (NEB) in the presence of 1% SDS for 30 min at 55°C. RNA was extracted with phenol:chloroform, precipitated with isopropanol, and re-dissolved in 20 µl of nuclease-free water.

**Immunoblotting**

For analysis of Nos levels, late-ovary extract preparation and immunoblotting were performed as previously described (9) except that nitrocellulose membrane was used. For IP and co-IP experiments, immunoprecipitates were eluted by boiling the beads in SDS-PAGE sample buffer (2% SDS, 62.5 mM Tris base, 10% glycerol, 100 mM DTT), resolved on a 10% SDS-PAGE gel, and transferred to nitrocellulose membrane. The following primary antibodies were used: 1:1,000 rabbit anti-Nos (gift of A. Nakamura), 1:1,000 mouse anti-Glo (SB7) (17), 1:10,000 rabbit anti-Kinesin heavy chain (Khc; Cytoskeleton), 1:1,000 mouse anti-GFP (JL-8; Clontech), and 1:1,000 mouse anti-dFMRP (6A15; Abcam). Results were visualized by enhanced chemiluminescence (Roche) and autoradiographic film exposure. Relative Nos and Glo levels were quantified in ImageJ by normalizing band intensity to Khc.
containing 3 mM puromycin (+Puro) and incubated at 37°C for 15 min. Ovaries were then homogenized and incubated at 4°C for 15 min with constant mixing, incubated at 37°C for 10 min, and supplemented with cycloheximide (1 mg/ml final concentration) and Mg(OAc)₂ (50 mM final concentration). The extract was cleared by centrifugation at 13,200 rpm for 15 min at 4°C, and the supernatant was layered onto a 12 ml, 10–50% sucrose gradient in 50 mM Tris–HCl pH 7.5, 250 mM NaCl, and 50 mM Mg(OAc)₂. Gradients were centrifuged in a Beckman SW41Ti rotor at 36,000 rpm for 1.5 h at 4°C and were fractionated and RNA extracted as described in Polyson fractionation, except that 2 μl of 1 mM Renilla luciferase RNA was added to each fraction prior to RNA extraction as internal control for subsequent RT-qPCR analysis.

RT-qPCR

For analysis of oocyte samples, RNA was extracted by homogenizing late-stage oocytes in TRIzol (Invitrogen) followed by chloroform extraction and isopropanol precipitation. 1 μg of total RNA was used for reverse transcription as described above. For both oocyte and gradient samples, 2 μl of cDNA was combined with 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM forward and reverse primers (final concentration) in a total volume of 20 μl. qPCR was performed on a StepOnePlus Real-Time PCR System (Applied Biosystems). Two (gradient samples) or three (oocyte samples) biological replicates were analyzed with three technical replicates each, all using auto thresholding and the following parameters: Holding, 95°C for 10 min; Cycling, 95°C for 15 s, 60°C for 1 min, repeated for 39 cycles; Melt curve, 95°C for 15 s, 60°C for 1 min, + 0.3°C step & hold, 95°C for 15 s. rpl32 and rluc were used as internal control for oocyte and gradient samples, respectively, unless otherwise stated. Relative levels of other target transcripts were calculated as follows (Ct of un-detected sample was set as 40).

\[
\text{Relative level} = \frac{2^{-(C_{\text{target transcript}} - C_{\text{internal control}})\times\text{fractional transcript level}}}{\sum \text{relative transcript level}}
\]

For polysome and 80S formation analysis, the following primers were used: rp32-F: 5′-dGGATCGATATGCTAAGCTGT-3′, rp32-R: 5′-dGGCATTTGTTGATCCGTA-3′, nos-qPCR-F: 5′-dCACCAGAATTCGCTCCTTAT-3′, nos-qPCR-R: 5′-dGCTGGACTCGCACTAGC-3′, act5c-F: 5′-dAGTACCCCCATTGAGCACGG-3′, act5c-R: 5′-dACATACTGCGGAGGTGTGTT-3′, ha-fluc-qPCR-F: 5′-dCcCGGGAGGCCAACTAAAAG-3′, ha-fluc-qPCR-R: 5′-dCTGATCTTCATAGCCT-3′, rluc-qPCR-F: 5′-dGATACTGCGGAGGTGTGTT-3′, rluc-qPCR-R: 5′-dACACAGATTTGCGTTTGC-3′.

Generation of reporter and competitor RNAs for in vitro translation assay

The luciferase reporter RNAs were constructed in a derivative of a BSκ-A vector containing the firefly luciferase coding sequence followed by 73 adenosine residues (kindly provided by F. Gebauer and M. W. Hentze). For firefly luciferase (Fluc) reporters, nos 5′ UTR sequence was sub-cloned from the pA25-nos5′ UTR plasmid (9) and inserted between the SacI and Smal sites. 3xTCE, 3xTEIIIA, 3xSRE and 3xUTR plasmids were sub-cloned from respective plasmids (9) and inserted between the BglII and BamHI sites. For the Renilla luciferase (Rluc) reporter, the Smal-BamHI fragment containing the firefly luciferase coding sequence in the BSκ-A derivative plasmid was replaced by the Renilla luciferase coding sequence. HA-tagged Fluc reporters were generated as follows. The nos 5′UTR sequence was sub-cloned from the pA25-nos5′ UTR plasmid (9) and inserted between the SacI and Neoi sites of a FLAG-3xHA vector (kindly provided by T. Aoki and P. Schedl). Firefly luciferase coding sequence with 3xTCE or 3xTEIIIA sequences followed by 73 adenosine residues was sub-cloned from the respective Fluc reporter plasmids (described above) and inserted between the Smal and HindIII sites. Capped reporter RNA was transcribed from 1 μg of HindIII-linearized plasmid DNA using.
the mMessage mMachine T3 Transcription kit (Ambion). TCEII and TCEIII competitor RNAs were generated from BamHI-linearized plasmids containing individual TCE stem-loop II and TCE stem-loop III sequences (17), respectively, using the MEGAscript T7 Transcription kit (Ambion). In all cases, \textit{in vitro} transcription reactions were treated with 1 \mu M of TURBO DNase (Ambion) for 15 min at 37\degree C, and RNA was extracted with phenol/chloroform and precipitated first with isopropanol and then with ethanol. Prior to use, the competitor RNA was de-natured at 65\degree C for 5 min and slowly cooled to room temperature (17).

\textbf{In vitro translation and translation run-off assay}

Late-ovary translation extract was prepared as previously described (20) except that 1 \times Complete EDTA-Free Protease Inhibitor Cocktail was used in place of 0.5 mM PMSF. Extract was made, aliquoted, flash frozen in liquid nitrogen, and stored at \(-80\degree C\) for no more than 2 weeks before use.

The \textit{in vitro} translation reaction (20 \mu l) contained 50\% late-ovary translation extract, 1 \times translation mix (25 mM HEPES–NaOH pH 7.5, 3 mM Mg(OAc)\textsubscript{2}, 2.5 mM DTT, 25 \mu M Complete Amino Acid Mixtures (Promega), 1.2 mM ATP, and 0.3 mM GTP), energy regeneration system (15 mM creatine phosphate and 0.1 mg/ml creatine phosphokinase (Sigma-Aldrich)), 0.1 nM \textit{Renilla} luciferase reporter RNA (internal control), and 1 nM firefly luciferase reporter RNA. Reactions were incubated at 28\degree C for 2 h. Additional external control reactions (25 \mu l) were assembled as follows. The same firefly-\textit{Renilla} luciferase reporter pair (0.1 nM each, final concentration) was incubated in 17.5 \mu l of Nuclease-Treated Rabbit Reticulocyte Lysate (Promega), 40 \mu M Complete Amino Acid Mixtures, and 1 \mu l of Human Placenta RNase Inhibitor (NEB). The reaction was allowed to proceed for 90 min at 28\degree C. Luciferase activity was assayed using the Dual-Luciferase Reporter Assay System (Promega), and luminescence was measured by a Glomax 20/20 Luminometer (Promega) using the built-in DLR protocol. The relative reporter activity and fold repression were defined as follows.

\begin{align*}
\text{Relative reporter activity} &= \frac{[\text{Fluc activity}]_{\text{ovary lysate}}}{[\text{Fluc activity}]_{\text{rabbit reticulocyte lysate}}} \\
\text{Normalized reporter activity} &= \frac{[\text{relative reporter activity}]_{\text{any 3'UTR}}}{[\text{relative reporter activity}]_{\text{18S rRNA}}} \\
\text{Fold repression} &= \frac{1}{\text{Normalized reporter activity}}
\end{align*}

Data were averaged over three biological replicates using different preparations of extract.

For the translation run-off assay, the reaction mixture was assembled without the energy regeneration system, pre-incubated at 16\degree C for 5 min. Either 0.1 \mu l of 10 mg/ml 4E1RCat (in DMSO; Sigma-Aldrich) (the final concentration of 4E1RCat is 50 \mu g/ml which effectively blocks 80S ribosome formation as determined by the 80S formation assay below) or 0.1 \mu l of DMSO was added, followed by 1 \mu l of TCEII or TCEIII competitor RNA (2 \mu g/\mu l) or nuclease-free water. Finally, the energy regeneration system was added, and the reaction was incubated at 28\degree C for 2 h. Fold repression was calculated as described above except that the value was normalized to the value of 3xTCEIIA without 4E1RCat or competitor RNA treatment.

\begin{align*}
\text{Elongation repression} &= \frac{[\text{fold repression}]_{\text{TCE1 + 4E1RCat + TCEII}}}{[\text{fold repression}]_{\text{TCE1 + TCEIII}}}
\end{align*}

\begin{align*}
\text{Initiation repression} &= \frac{[\text{fold repression}]_{\text{4E1RCat + TCEII}}}{[\text{fold repression}]_{\text{TCE1 + TCEIII}}}
\end{align*}

The data were averaged over three biological replicates using different preparations of extract.

\textbf{80S formation assay}

10 \mu l of 10 nM Rluc reporter was incubated in a total volume of 100 \mu l of \textit{in vitro} translation reaction mixture containing 50\% Drosophila ovary translation extract (as described above) in the presence of 1 mg/ml cycloheximide. 0.5 \mu l of 10 mg/ml 4E1RCat (+4E1RCat) or DMSO (\(-4E1RCat\)) was added, and the mixture was incubated at 25\degree C for 30 min followed by polysome fractionation. 2 \mu l of 1 nM HA-Fluc reporter RNA was added to each fraction as an internal control for subsequent RT-qPCR analysis, and RNA from each fraction was prepared as described in Puromycin release and polysome analysis.

\textbf{\textsuperscript{35}S]-Methionine incorporation assay}

Reactions containing 50 \mu l of \textit{Drosophila} late-ovary extract, 10 \mu l of 10 \times translation mix (250 mM HEPES–NaOH pH 7.5, 30 mM Mg(OAc)\textsubscript{2}, 25 mM DTT, 250 \mu M Amino Acids Mixture Minus Methionine (Promega), 12 mM ATP and 3 mM GTP), 10 \mu l of 5 nM HA-Fluc reporter RNA, 2.5 \mu l of 190 mM methionine (Sigma-Aldrich), 25 mM \textsuperscript{35}S]-methionine (2.5 \mu l of \sim 10 \mu M equivalent; PerkinElmer NEG709A001MC), and 10 \mu l of nuclease-free water were pre-incubated at 25\degree C for 10 min. The concentration of unlabelled methionine was optimized to sustain efficient elongation while retaining sufficient \(\textsuperscript{35}S\)-Met incorporation. 10\% of initial volume was removed at 0 and 5 min for anti-HA IP and scintillation counting. At 10 min, 2.5 \mu l of 10 mg/ml 4E1RCat, 15 \mu l of 100 mM creatine phosphate, and 2 \mu l of 5 mg/ml creatine phosphokinase were added to the reaction mixture to block \textit{de novo} initiation and start translation run-off. For experiments involving addition of dFMRP or BSA, 10 \mu l of 1 mg/ml purified \Delta TdT-dFMRP (see Protein expression and purification below) or 1 mg/ml BSA (Sigma-Aldrich) was also added to the reaction mixture at 10 min. Additional aliquots equivalent to 10\% of initial volume were removed at 10, 15, 20, 30, 45, 60, 90 and 120 min for anti-HA IP and scintillation counting. For anti-HA IP, aliquots were incubated with 5 \mu l of Pierce Anti-HA Magnetic Beads (Thermo Fisher) in 250 \mu l of RIPA buffer (25 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM ethylene diaminetetraacetic acid (EDTA), 10\% glycerol, 1\% Nonidet P-40, 1 \times Complete EDTA-Free Protease Inhibitor Cocktail (Ambion)) and 5 \mu l of Pierce Magnetic Beads (Thermo Fisher) for 15 min at 4\degree C. For experiments involving addition of dFMRP, BSA, or dFMRP + BSA, 25 \mu g of Pierce Anti-HA Magnetic Beads (Thermo Fisher) was added to the reaction mixture at 10 min.
Protein expression and purification

The pET15b plasmids encoding N-terminal hexahistidine (His6)-tagged qRRM domains of Glo (qRRM1, residues 45–141; qRRM2, residues 142–234; qRRM3, residues 475–562) were previously described (18). The ΔNT-dFMRP (residues 220–681) coding sequence was sub-cloned from a pTYB1 NT-dFMRP construct (28) into pET15b (Novagen) with N-terminal His6-tag. Individual qRRM domains and ΔNT-dFMRP were expressed in E. coli strain Rosetta (DE3)-pLysS (Novagen).

For purification of individual qRRM domains, E. coli cultures were grown in LB medium to an OD600 of 0.6–0.7 and induced overnight with 0.5 mM IPTG at 16°C. The cells were pelleted, resuspended in buffer containing 50 mM Tris–HCl pH 8.0, 500 mM NaCl and 2 mM PMSF, and lysed using a cell disruptor. Cell lysate was cleared by centrifugation and the qRRM proteins were purified by Ni-NTA agarose (Qiagen) chromatography. Eluates were further purified by size-exclusion chromatography (Superdex 200 Increase 10/300, GE Healthcare), concentrated, flash-frozen, and stored at –80°C in 20 mM HEPES–NaOH pH 8.0 and 200 mM NaCl (qRRM1 and qRRM3) or 20 mM HEPES–NaOH pH 8.0 and 250 mM NaCl (qRRM2).

For purification of ΔNT-dFMRP, E. coli culture was grown in LB medium to an OD600 of 0.4–0.5 and induced overnight with 0.5 mM IPTG at 16°C. The cells were pelleted, re-suspended in buffer containing 50 mM Tris–HCl pH 8.0, 500 mM NaCl, 20 mM imidazole, 0.1% CHAPS, 5 mM β-mercaptoethanol, and 2 mM PMSF. The cells were lysed using a cell disruptor, and the lysate was cleared by centrifugation and applied to Ni-NTA agarose chromatography. The eluted protein was further purified by size-exclusion chromatography (Superdex 200 Increase 10/300), concentrated, flash-frozen, and stored at –80°C in 20 mM HEPES–NaOH pH 8.0, 250 mM NaCl and 2 mM DTT.

His6-tag pull-down assay

Purified individual qRRMs (50 μg each) were immobilized on Ni-NTA agarose beads separately, washed in 20 mM Tris–HCl pH 7.5, 500 mM NaCl and 0.5% IGEPAL CA-630, and incubated with late-ovary extract (prepared as described in the IP-MS section) expressing GFP-dFMRP for 1.5 h at 4°C. The beads were washed three times with IP buffer (above) and eluted in boiling SDS-PAGE sample buffer. The IP products were resolved on a 15% SDS-PAGE gel, transferred onto nitrocellulose membrane, and detected by Ponceau S staining (qRRMs) or anti-GFP immunoblotting (GFP-dFMRP).

Analytical size-exclusion chromatography binding assay

Proteins were diluted to 2.5 μM (ΔNT-dFMRP) or 20 μM (individual qRRM domains) in 20 mM HEPES–NaOH pH 8.0, 150 mM NaCl, and 5 mM TCEP in a volume of 60 μl, incubated at 4°C for 48 h, and then loaded onto a Superdex 200 Increase 3.2/300 column. The fractions were collected, and the proteins were concentrated by TCA-precipitation, resolved on a 15% SDS-PAGE gel, and visualized by staining with Coomassie Brilliant Blue R-250.

Statistics

All statistical analyses were performed in GraphPad Prism 9.0.2. A two-tailed Welch t-test was used for comparisons of two means, and post hoc Holm-Sidak test was used for multiple comparison correction when necessary. Brown-Forsythe and Welch one-way ANOVA with post hoc Dunnett T3 test was used for one-dimensional comparisons of more than two means. Two-way full-model ANOVA with post hoc Tukey test was used for two-dimensional comparisons of means within and between genotype groups. Linear regressions of [35S]-methionine incorporation data were performed using the Simple Linear Regression function which also tested the significance of the difference between slopes. Data were plotted as mean ± SEM from two or three biological replicates as indicated in the figure legends. Individual data points are shown unless for visibility reasons.

RESULTS

Identification of dFMRP as an RNA-independent Glo-interacting protein

To understand how Glo – which contains only annotated RNA-binding domains – represses nos translation, we isolated Glo interacting proteins from ovaries enriched for late-stage oocytes (late-ovaries) (20) by immunoprecipitation coupled to mass spectrometry (IP-MS). Anti-GFP immunoprecipitation was performed using late-ovaries expressing functional GFP-tagged Glo (GFP-Glo) in the absence of endogenous, native Glo. Late-ovaries expressing GFP-tagged bacteriophage MS2 coat protein (MCP-GFP) were used as a control (Figure 1A). Ovary extracts were treated with RNase prior to immunoprecipitation to eliminate RNA-dependent protein interactions. Mass spectrometry (MS) analysis identified 94 proteins, including Glo itself, that were at least 2-fold enriched in GFP-Glo immunoprecipitates relative to MCP-GFP immunoprecipitates (Supplementary Table S1). To facilitate recovery of potentially weak and transient Glo-interactors, we also performed crosslinking-based IP-MS under stringent conditions (Figure 1B) and detected 170 proteins that were at least 2-fold enriched in
Figure 1. Identification of dFMRP as a RNA-independent Glo-interacting protein by IP-MS. (A) Schematic representation of the native IP-MS experiment. The total peptide count in GFP-Glo immunoprecipitates was compared to that in MCP-GFP immunoprecipitates. (B) Schematic representation of the crosslinked IP-MS experiment. The total peptide count in DSP-crosslinked immunoprecipitates was compared to that in un-crosslinked immunoprecipitates. (C) Top: Venn diagrams comparing native and crosslinked IP-MS results from (A, B). Bottom: Heat map showing examples of high-confidence IP-MS interactors. Heat-map scale indicates fold enrichment. (D) Venn diagrams comparing the >2-fold enriched set of IP-MS interactors identified in the Bansal dataset (34) with our >2-fold enriched native IP-MS interactors (middle) or with our pooled high-confidence interactors (bottom). (E) Immunoblots of extract prior to anti-GFP immunoprecipitation (input lanes) and anti-GFP immunoprecipitates (α-GFP lanes) from late-ovaies expressing GFP, GFP-Glo or GFP-dFMRP probed with anti-dFMRP antibody (top left panel), anti-Glo antibody (top right panel), or anti-GFP antibody (bottom panels). Endogenous dFMRP co-immunoprecipitated with GFP-Glo, and endogenous Glo co-immunoprecipitated with GFP-dFMRP.

the GFP-Glo immunoprecipitates from the crosslinked sample compared to those from the un-crosslinked control (Supplementary Table S1). A single experiment was performed for each condition. 23 interactors were common to both the native and the crosslinked IP-MS experiments and were therefore considered high-confidence Glo-interacting proteins (Figure 1C; Supplementary Table S1). Among the high-confidence interactors are eIF4E and ribosomal subunits, consistent with Glo's implicated functions in regulating translation initiation and elongation of nos, respectively. The list also contains known translation regulators, including dFMRP (29,30), Caprin (31) and Lingerer (32,33), consistent with Glo's function of Glo as a translational repressor more generally. Our high-confidence interactors (11 out of 23) overlap those identified in a recently reported Glo native IP-MS experiment using non-staged ovaries (34) (Figure 1D; Supplementary Table S1), further supporting the validity of the interactors identified here.

We focused on dFMRP because studies of dFMRP and its mammalian homologs have identified multiple modes of action in translational control, including inhibition of translation initiation (35,36) and ribosome stalling (28,37). Interaction of Glo and dFMRP in late-ovaies was further tested by reciprocal in vivo co-immunoprecipitation (co-IP) in the presence of RNase. Endogenous dFMRP and Glo were detected in anti-GFP immunoprecipitates from late-ovaies expressing GFP-dFMRP or GFP-Glo, respectively, but were not detected in control immunoprecipitates (Figure 1E). Endogenous dFMRP also co-immunoprecipitated with endogenous Glo (Supplementary Figure S1), confirming that dFMRP is an RNA-independent Glo-interacting protein during late oogenesis.

dFMRP negatively regulates Nos protein levels

We next sought to determine whether dFMRP, like Glo, participates in nos regulation. To test whether dFMRP interacts with nos mRNA in vivo, we performed RNA co-IP experiments using wild-type, GFP-Glo, or GFP-dFMRP late-ovaies. nos could be detected by RT-PCR in both GFP-Glo and GFP-dFMRP immunoprecipitates, whereas a control maternal transcript, his3.3b, was not detected (Figure 2A). To probe a requirement for dFMRP in translational repression of nos, we knocked down dfmr1 expression in the female germline. Expression of either of two independent UAS-dfmr1 RNAi transgenes with a maternal-tubulin-Gal4 driver resulted in almost complete knock-down (KD) of dFMRP protein expression (un-
dFMRP negatively regulates Nos levels. (A) Ethidium bromide-stained gel analysis of RT-PCR for nos and control his3.3b RNA in anti-GFP immunoprecipitated from wild-type (WT), GFP-Glo expressing, and GFP-dFMRP expressing late-ovaries. Nos, but not his3.3b RNA co-immunoprecipitated with Glo and dFMRP and was not detected in the control WT IP. (B) Immunoblots detecting dFMRP, Nos, Glo, and Khc (loading control) in wild-type (WT) and dFMRP KD (TRIP GL0075 and TRIP 34944 RNAi lines) in hand-dissected stage 13/14 oocytes. Both RNAi lines effectively reduced dFMRP expression. Nos level relative to wild-type: 4.3-fold in GL0075 and 4.5-fold in 34944; Glo level relative to wild-type: 1.1-fold in GL0075 and 1.2-fold in 34944. (C) Relative quantification of Nos RNA levels in wild-type and dFMRP KD late-stage oocytes by quantitative RT-PCR. dfmr1 RNAi did not impact Nos RNA level in late-stage oocytes. Data are plotted as mean ± SEM from 3 biological replicates each with three technical replicates; \( P > 0.05 \) (ns) by Brown-Forsythe and Welch one-way ANOVA with post hoc Dunnett T3 test.

dFMRP reduces ribosome load on nos

To determine how dFMRP regulates nos translation, we first performed sucrose density gradient sedimentation to monitor ribosome load on nos in wild-type and in dFMRP KD late-ovaries. We hypothesized that if dFMRP only affects translation initiation of nos mRNA, dFMRP KD would increase polysome association of nos but leave its distribution within the polysomal fractions unchanged. In contrast, if dFMRP plays a role in regulating translation elongation, dFMRP KD would affect the number of ribosomes on nos mRNA, hence the relative level of nos in light (fewer ribosomes) versus heavy (more ribosomes) polysome fractions. Consistent with previous studies (22,38), the overall polysome signal was low (Figure 3A), presumably due to generally low translational activity during the later stages of oogenesis. While the overall polysome profile did not show an obvious change upon dFMRP KD, there was a redistribution of nos mRNA toward the heavier fractions (Figure 3B). Specifically, the shift of nos mRNA from lighter to heavier polysome fractions suggests that dFMRP regulates translation elongation of nos mRNA. In addition, we observed a moderate increase in the percentage of polysome-associated nos, from ~50% in wild-type, which is consistent with our previous findings (20,22), to ~65% in dFMRP KD late-ovaries (Figure 3C), indicating either that dFMRP also represses nos translation initiation or that initiation-based repression plays a secondary role and, by itself, does not completely eliminate ribosome loading onto nos mRNA. Together with the results presented above (Figure 2), these data provide further evidence that dFMRP is a translational repressor of nos.
Figure 3. dFMRP KD increases polysome association of *nos*. (A) UV$_{260}$ absorbance profile from representative 10–50% sucrose density gradients. *A$_{260}$* values are shown at 5-fold in polysome fractions for visualization purposes. Light polysomes (fractions 12–15) contains 2–4 ribosomes per transcript; heavy polysomes (fractions 16–24) contains five or more ribosomes per transcript. (B) Analysis of *nos* RNA distribution in sucrose gradient fractions. Quantification of relative *nos* levels in gradient fractions from wild-type (WT) and dFMRP KD late-ovaries (top) as determined by RT-PCR (bottom). Individual data points are plotted and their mean values (n = 3) are shown by trend lines. *nos* levels were calculated as a fraction of total *nos* band intensity. Representative ethidium bromide-stained agarose gels of *nos* RT-PCR products are shown. (C) Fraction of *nos* RNA associated with polysomes in WT (54%) and dFMRP KD (66%) late-ovaries. Values are mean ± SEM from three biological replicates; **P < 0.01 by two-tailed Welch *t*-test. (D) RT-PCR analysis of RNA isolated following anti-GFP IP of sucrose gradient fractions shown in (A) for WT, GFP-Glo expressing, and GFP-dFMRP expressing late-ovaries: RNP (RNP, fractions 1–5), monosomal (Mono, fractions 6–11), and polysomal (Poly, fractions 12–24) fractions. GFP-dFMRP co-immunoprecipitated with *nos*, but not *his3.3b*, RNA in both RNP and polysomal fractions.

regeneration system to allow the assembly of the repression complex and the initial loading of ribosomes (39). Subsequently, the translation initiation inhibitor 4E1RCat, which competitively disrupts the interaction of eIF4G with eIF4E and hence the formation of the eIF4F complex (40), was added to block *de novo* initiation on the reporter RNA. 4E1RCat reduced the absolute expression level of luciferase reporters in a dosage-dependent manner (Supplementary Figure S2A) but did not affect TCE-mediated translational repression (Supplementary Figure S2B). Sucrose gradient analysis confirmed that 4E1RCat effectively prevented 80S ribosome formation on the reporter RNA in late-ovary extract (Supplementary Figure S2C, D). Competitor TCEIII RNA was then added to titrate the TCE-bound repression complex from the reporter RNA. Finally, the ATP/GTP regeneration system was provided to resume
Figure 4. Biochemical dissection of TCE-mediated nos regulation. (A) Schematics of the reporters used. The firefly luciferase (Fluc) coding sequence was fused to either 3xTCE (TCE), 3xTCEIIIA (TCEIIIA), 3xTCE[SRE+], or α-tubulin 3' UTR (tub3'UTR) sequences, as indicated by 3'UTR*. The Renilla luciferase coding sequence did not contain specific 3' UTR sequence and was not subject to translational regulation. Both reporters contained a 5' 7-methylated guanosine (m7G) cap and 3' poly(A) tail. (B) Schematic representation of the nos TCE. Glo binds to both TCEI and TCEIII, whereas Smg binds to TCEII. The TCEIIIA and SRE- mutations disrupt Glo and Smg binding, respectively. (C) In vitro translation assay using reporters shown in (A). For each indicated reporter, fold repression is the inverse of the normalized relative luciferase activity, which was calculated as the ratio of firefly to Renilla luciferase activity in late-ovary extract normalized first to activity in rabbit reticulocyte lysate and then to the value for the tub3'UTR reporter. (D) Experimental setup of the translation run-off assay. Reporter RNAs were pre-incubated in late-ovary extract without the energy regeneration system (t0). The translation initiation inhibitor 4E1RCat (50 μg/ml) was then added to block de novo initiation (t1), followed by the addition of competitor RNAs (t2; control TCEII or specific TCEIII). Finally, the energy regeneration system was added back to restore translation (t3), and luciferase activity was assayed (t4). (E) Results of the translation run-off assay in wild-type (WT) and dFMRP KD ovary extracts. Normalized relative luciferase activity and fold repression were calculated as in (C), except that the luciferase activity was normalized to the value for TCEIIIA (without 4E1RCat or competitor RNA). (F) Contributions of translation initiation and elongation to fold repression. [Fold repression]_elongation = [Fold repression]_TCE+/[Fold repression]_TCE+4E1RCat+TCEIII. [Fold repression]_initiation = [Fold repression]_TCE+4E1RCat+TCEII+/[Fold repression]_TCE+TCEIII. In (C), (E) and (F), values are mean ± SEM from three biological replicates from different ovary extract preparations. Statistical significance was analyzed using two-way full-model ANOVA with post hoc Tukey test (in C), Brown-Forsythe and Welch one-way ANOVA with post hoc Dunnett T3 test (in E, significance symbol for wild-type/dFMRP KD), and two-tailed Welch t-test with post hoc Holm-Sidak test (in F); *P < 0.05, **P < 0.01 and ***P < 0.001, not all tested P-values are shown.
translation. In this way, we were able to directly test if the nos TCE imposes blocks at both translation initiation and elongation, manifested by partial de-repression by TCEIII in the presence of 4E1R Cat, and to de-couple the two repression modes to assess the impact of dFMRP on each independently.

As expected, addition of TCEIII competitor RNA to the wild-type extract almost fully relieved repression of the Fluc-3xTCE reporter (1.1-fold repression for Fluc-3xTCE + TCEIII versus 9.0-fold for Fluc-3xTCE), whereas a control competitor, TCEII RNA, had no effect as expected (20) (Figure 4E). In the presence of 4E1R Cat, addition of the TCEIII competitor, but not TCEII, partially de-repressed Fluc-3xTCE RNA (2.6-fold repression for Fluc-3xTCE + TCEIII + 4E1R Cat versus 9.0-fold for Fluc-3xTCE, 3.6-fold up-regulated; Figure 4E). Since de novo initiation was inhibited, the 3.6-fold increase in reporter activity reflects the elimination of a TCE-mediated elongation block. Maximal de-repression was observed only when initiation was allowed to occur (-4E1R Cat), however, indicating that full de-repression resulted from elimination of both an initiation and an elongation block (Figure 4E). Therefore, the remaining repression when initiation was prevented (2.6-fold for + TCEIII, +4E1R Cat versus 1.1-fold for + TCEIII, -4E1R Cat, 2.3-fold repressed) corresponds to a TCE-mediated initiation block (Figure 4E). In summary, TCE-based regulation in wild-type extract comprises 2.3-fold repression of initiation and 3.6-fold repression of elongation (Figure 4F).

In parallel, we assessed the behavior of the dFMRP KD extract. Similarly to the wild-type extract, the addition of TCEIII, but not TCEII, competitor RNA alone almost completely eliminated repression (1.1-fold versus 4.4-fold repression; Figure 4E). In the presence of 4E1R Cat, TCEIII, but not TCEII, partially de-repressed Fluc-3xTCE RNA (2.4-fold versus 4.4-fold repression, 1.9-fold up-regulated; Figure 4E). Therefore, whereas repression at initiation remained 2.2-fold (2.4-fold for + TCEIII, +4E1R Cat versus 1.1-fold for + TCEIII, -4E1R Cat, 2.2-fold repressed, not statistically different from the 2.4-fold repression in wild-type extract), repression at elongation was attenuated by 50% (Figure 4F), suggesting that dFMRP specifically represses translation elongation in a Glo/TCE-dependent manner. Consistent with dFMRP acting at the elongation step, nos RNA was present in dFMRP immunoprecipitates from polysomal fractions after sucrose density gradient sedimentation (Figure 3D).

dFMRP stalls ribosomes and decreases elongation rate on nos

The substantial polysome association of nos in late-ovaries and the specific inhibition of nos translation elongation by dFMRP led us to hypothesize that dFMRP stalls elongating ribosomes on nos. To test if dFMRP promotes ribosome stalling on nos, we first measured puromycin release of ribosomes on endogenous nos in wild-type and dFMRP KD late-ovaries. Puromycin releases actively elongating, but not stalled ribosomes and caused an overall loss of polysomes in both wild-type (Figure 5A) and dFMRP KD (Figure 5B) late-ovaries. The polysome profile of nos, however, responded differently to puromycin treatment in the presence or absence of dFMRP. In wild-type late-ovary extract, puromycin treatment did not cause significant loss of polysomal nos (Figure 5C, +Puro, Fractions 10 to 24), as compared to the mock-treated extract (Figure 5C, -Puro). In contrast, significant redistribution of nos from heavier to lighter polysomal fractions was observed in puromycin-treated dFMRP KD extract (Figure 5D, +/−Puro). The differential puromycin release of ribosomes from nos in wild-type and dFMRP KD extract was not a global effect of dFMRP KD, as ribosomes on a control maternal transcript, act5c, were released to a similar extent in both wild-type (Supplementary Figure S3A–C) and dFMRP KD (Supplementary Figure S3D–F) late-ovary extracts. Thus, these results indicate that dFMRP stalls elongating ribosomes on nos.

Next, we sought to directly assess if the elongation rate on nos is affected by dFMRP in a TCE-dependent manner. To do this, we modified the reporter RNAs to encode an N-terminal triple HA epitope tag (HA-Fluc-3xTCE and HA-Fluc-3xTCEIIIA), allowing immunoprecipitation of nascent polypeptide chains with anti-HA antibody. We then performed an in vitro translation run-off assay with each reporter RNA in the presence the initiation inhibitor 4E1R Cat and [35S]-Met. Following anti-HA immunoprecipitation, incorporation of [35S]-Met into nascent polypeptide chains was monitored by liquid scintillation counting. Because of the low total Met concentration necessary for labeling with [35S]-Met (see Materials and Methods), the absolute elongation rate could not be easily estimated. However, we were able to compare qualitatively the elongation rates on the HA-Fluc-3xTCE and HA-Fluc-3xTCEIIIA RNAs and to determine if the difference in elongation rates depends on dFMRP. Translation run-off with HA-Fluc-3xTCE RNA resulted in ∼50% less [35S]-Met incorporation than with HA-Fluc-3xTCEIIIA in the wild-type late-ovary extract (Figure 5E) but not in the dFMRP KD extract (Figure 5F). This is consistent with the luciferase assay results (Figure 4). We fitted the relative [35S]-Met incorporation during first 20 min of the run-off, when neither [35S]-Met nor elongating ribosomes were rate-limiting, to a linear model. After regression, the slope for HA-Fluc-3xTCEIIIA RNA in the wild-type extract was significantly steeper than that for HA-Fluc-3xTCE, indicating a faster elongation rate for the mutant reporter RNA (Figure 5G). However, the elongation rates for the two reporters were indistinguishable in the dFMRP KD extract (Figure 5H), consistent with dFMRP impeding elongation. Moreover, adding purified dFMRP, but not BSA, to the dFMRP KD extract slowed [35S]-Met incorporation during translation run-off and led to an earlier plateau for HA-Fluc-3xTCE, but not for HA-Fluc-3xTCEIIIA RNA (Figure 5I, S4). Taken together, results from polysome profiling and [35S]-Met incorporation analyses demonstrate that dFMRP stalls elongating ribosomes on nos via the TCE.

qRRM2 is necessary and sufficient for direct recruitment of dFMRP by Glo

Given that Glo lacks known functional domains other than the qRRMs, we hypothesized that Glo interacts with dFMRP through one or more of its qRRMs. To test this,
Figure 5. dFMRP stalls ribosomes and decreases elongation rate on nos. (A, B) UV260 absorbance profile from representative puromycin release experiment in wild-type (A) and dFMRP KD (B) late-ovary extract after polysome fractionation. The A260 range is scaled to better visualize the polysomal fractions. Overall polysome signal decreased when wild-type and dFMRP KD extract were treated with puromycin, which releases elongating but not stalled ribosomes. The same profiles are also used in Supplementary Figure S2A, D. (C, D) Distribution of nos RNA in sucrose gradient fractions of wild-type (C) and dFMRP KD (D) late-ovaries as determined by RT-qPCR. nos RNA was partially released from polysomes when treated with puromycin in dFMRP KD but not in wild-type late-ovaries. Relative nos level is calculated as 2 to the power of Ct minus Ct of the internal control. The amount of nos in each fraction is plotted as a fraction of total nos level. Individual data points from two independent puromycin release experiments, each with 3 qPCR technical replicates, are plotted, and their mean values are shown by trend lines. (E, F) Relative [35S]-Met incorporation during translation run-off in wild-type (E) and dFMRP KD (F) in vitro translation extract. Translation run-off of HA-Fluc-3xTCE RNA resulted in less and slower [35S]-Met incorporation than did the translation run-off of HA-Fluc-3xTCEIIIA RNA in wild-type, but not in dFMRP KD extract. Run-off began at 10 min (dotted vertical line) with the addition of the initiation inhibitor 4E1RCat (250 μg/ml). Individual data points are plotted and their mean values (n = 3) are shown by trend lines. (G, H) Linear regression of relative [35S]-Met incorporation from 10 to 30 min in wild-type (G) and dFMRP KD (H) extract. The difference in rate of [35S]-Met incorporation between the two reporters is significant in wild-type, but not dFMRP KD extract. Regression results are plotted as best-fit line (solid) centered in 95% confidence intervals (shaded area between dotted lines). Slopes are best-fit value ± Std. Error. 95% confidence intervals: 0.0087 to 0.016 (TCE, G); 0.020 to 0.030 (TCEIIIA, G); 0.013 to 0.023 (TCE, H); and 0.011 to 0.022 (TCEIIIA, H). Relative [35S]-Met incorporation during translation run-off in dFMRP KD extract supplemented with purified dFMRP. Purified ΔNT-dFMRP was added to the reaction mixture when translation run-off started (10 min, dotted vertical line). Addition of purified dFMRP decreased both the level and rate of [35S]-Met incorporation in the TCE, but not the TCEIIIA, reporter. Relative [35S]-Met incorporation is calculated as in (E) and (F). Individual data points are plotted and their mean values (n = 2) are shown by trend lines.
a series of Glo deletion variants were made by removing individual qRRMs. Two of Glo’s qRRMs, qRRM1 and qRRM2, have additional alpha helical extensions (CTX) C-terminal to the core RRM fold (18). Since similar CTX structures have been shown to serve as protein-protein interaction platforms in other RRM-containing proteins (41), we also generated variants lacking the CTX residues either individually or in combination (Figure 6A). The deletions were engineered in a functional GFP-Glo transgene, and the resulting transgenes were each integrated at the same chromosomal location.

We then performed anti-GFP IPs from late-ovary extracts to determine whether any of the deletions affected the ability of Glo to interact with dFMRP. Western blot analysis showed that dFMRP co-immunoprecipitated with all Glo variants except GloΔqRRM2 (Figure 6B), indicating that Glo specifically requires qRRM2 to bind to dFMRP. Notably, RNA co-IP analysis showed that all of the deletion variants including qRRM2 bound nos (Supplementary Figure S5), confirming that protein structure is maintained and that, consistent with previous biochemical analysis (18), two qRRMs are sufficient for Glo-dFMRP interaction.

Next, we asked if qRRM2 is sufficient for dFMRP interaction. We purified recombinant hexahistidine-tagged qRRMs (18) (Figure 6A) from E. coli using Ni-NTA agarose chromatography followed by size-exclusion chromatography. Following purification, qRRMs were immobilized on Ni-NTA agarose and incubated with late-ovary extract expressing GFP-dFMRP. qRRM2, but not qRRM1 or qRRM3, was able to pull down GFP-dFMRP (Figure 6C), indicating that qRRM2 alone is sufficient for Glo-dFMRP interaction.

We then sought to determine if dFMRP binds directly to qRRM2. We expressed and purified a hexahistidine-tagged N-terminally truncated dFMRP protein (ΔNT-dFMRP), which lacks the first 219 amino acid residues containing the nuclear localization signal but is folded and retains full translational repression capability as determined by both cryo-EM and in vitro translation (28). Purified ΔNT-dFMRP was incubated with each of the purified qRRM proteins and the mixture was subjected to size-exclusion chromatography. When chromatographed alone, each Glo qRRM behaved as a monomer. However, when mixed with dFMRP, a proportion of the Glo qRRM2 protein eluted in higher molecular weight fractions, together with ΔNT-dFMRP (Figure 6D). Neither Glo qRRM1 nor qRRM3 proteins were shifted to higher molecular weight fractions (Supplementary Figure S6). Together, our results show that interaction of Glo with dFMRP is mediated directly and specifically by qRRM2.

Finally, we tested if Glo is required for recruitment of dFMRP to nos by RNA co-IP. dFMRP was immunoprecipitated from glo null mutant late-ovaries expressing either GFP-Glo or individual GFP-GloΔqRRM variants, using anti-FMRP antibody. nos mRNA was detected by RT-PCR in all dFMRP immunoprecipitates except when the only Glo protein expressed was GFP-GloΔqRRM2 (Figure 6E). The failure of nos to co-immunoprecipitate with dFMRP when Glo lacks qRRM2 indicates that qRRM2, and thus the interaction between Glo and dFMRP, is required for dFMRP binding to nos.

**DISCUSSION**

The Drosophila hnRNP F/H homolog, Glo, represses the translation of unlocalized nos during late stages of oogenesis to ensure proper anterior-posterior patterning of the embryo. Previous work suggested that Glo imposes both an initiation and a post-initiation block on nos translation (20, 22), but the molecular mechanism and the nature of the post-initiation block remained unknown. Moreover, how a single protein composed primarily of RNA-binding domains can confer repression by two different mechanisms is unclear. Here, we have begun to fill these gaps by identifying dFMRP as a Glo-interacting protein during late stages of oogenesis and biochemically dissecting its role in nos regulation. Our results lead to a new model wherein Glo recruits dFMRP specifically through qRRM2 to repress elongating ribosomes on nos (Figure 7) and suggest that Glo’s distinct activities are dictated by different effector proteins recruited through different qRRMs.

Since the discovery of FMRP, numerous high-throughput analyses along with transcript-specific studies have uncovered a multiplicity of mechanisms by which it is thought to regulate translation in both neuronal and non-neuronal contexts. These include repression of cap-dependent translation initiation (35, 36), modulation of microRNA activity (42, 43), and stalling of elongating ribosomes (28, 37). Among its large set of targets, FMRP is capable of binding to a wide variety of sequence motifs (44), further complicating the understanding of its target selectivity. In this work, we show that an FMRP-interacting protein recognizing an mRNA signature specific to an individual target mRNA can provide the required target selectivity.

A recent ribosome profiling study found that dFMRP activates translation of a collection of stored mRNAs, particularly those encoding large autism-related proteins, in Drosophila oocytes (45). Our work, however, suggests that dFMRP, recruited by specificity factors, can also function as translational repressor of individual targets in oocytes by blocking translation elongation. Indeed, dFMRP has been reported to interact with the 60S ribosomal protein L5, which may prevent tRNA binding during elongation (28). The Glo/TCE-dependent elongation-repressing activity of dFMRP together with the increased ribosome load following dFMRP KD leads to two non-exclusive hypotheses: 1) dFMRP can repress nos translation initiation independently of Glo/TCE and/or 2) in the absence of the dFMRP-mediated elongation block, the initiation block, regardless of its dFMRP dependency, is insufficient for complete shutdown of translation. Although we cannot yet distinguish among these possibilities, the latter is consistent with the relative magnitude of the initiation versus elongation-based repression (2.4-fold versus 3.6-fold) measured by our translation run-off assay (Figure 4F).

Whereas Glo-RNA interactions have been characterized in some detail (18), much less is known about Glo-protein interactions and how they contribute to Glo’s functional
Figure 6. Glo qRRM2 is necessary and sufficient for direct recruitment of dFMRP. (A) Schematic representation of Glo protein variants analyzed. Green: GFP tag; yellow: qRRM1; blue: qRRM2; orange: qRRM3; black: His6 tag. ΔqRRM1: deletion of residues 47–136; ΔqRRM2: deletion of residues 478–559; ΔqRRM3: deletion of residues 145–232; ΔCTX1: deletion of residues 129–136; ΔCTX2: deletion of residues 226–232; ΔCTX1 + 2: deletion of residues 129–136 and 226–232. His6-qRRM1: Glo residues 45–141; His6-qRRM2: Glo residues 142–234; His6-qRRM3: Glo residues 475–562. (B) Anti-dFMRP and anti-GFP immunoblots of input extracts (top two panels) and anti-GFP immunoprecipitates (bottom two panels) from late-ovaries expressing GFP or GFP-Glo variants. dFMRP co-immunoprecipitated with all Glo variants except Glo-ΔqRRM2. (C) Pull-down assay with His6-qRRMs. Top panel, anti-GFP immunoblot to detect GFP-dFMRP (dFMRP) in input extract and after pull-down with qRRM1, qRRM2, or qRRM3. Bottom panel, Ponceau S staining of His6-qRRMs in input extract and after pull-down. Only qRRM2 was able to pull down GFP-dFMRP. (D) Analytical size-exclusion chromatography binding assays. For chromatograph (top), red profile: 2.5 μM His6-ΔNT-dFMRP (ΔNT-dFMRP) alone (the same chromatograph is also used in Supplementary Figure S6A, B); blue profile: 20 μM His6-qRRM2 (qRRM2) alone; green profile: 2.5 μM ΔNT-dFMRP + 20 μM qRRM2 (ΔNT-dFMRP + qRRM2). Coomassie stained SDS-PAGE gels below show corresponding fractions from the chromatograph. Mixing ΔNT-dFMRP and qRRM2 resulted in elution of qRRM2 in higher molecular weight fractions. Because Coomassie binding is roughly proportional to the size of the protein (~53 kDa for ΔNT-dFMRP and ~10 kDa for qRRM2), the qRRM2 bands in the higher molecular weight fractions represent a significant amount of qRRM2/ΔNT-dFMRP complex. The same ΔNT-dFMRP gel image is also used in Supplementary Figure S6A, B. (E) Ethidium bromide-stained gel analysis of RT-PCR for nos RNA in anti-dFMRP immunoprecipitates from glo null mutant ovaries expressing GFP-Glo and GFP-Glo-ΔqRRM variants. nos RNA co-immunoprecipitated with dFMRP when GFP-Glo, GFP-Glo-ΔqRRM1, or GFP-Glo-ΔqRRM3, but not GFP-Glo-ΔqRRM2, was expressed.
the G-tract binding mode is required for Glo’s function.

unknown targets required for viability. By contrast, only nos
hence

binding modes are required for Glo-TCE interaction, and
repertoire of Glo. Both the G-tract and UA-rich motif
of 3 qRRMs are thought to diversify the RNA target
binding modes into a single qRRM and the combination
sequence motifs: a single-stranded G-tract and a double-
interfaces that allow Glo to recognize two different
that each Glo qRRM harbors 2 distinct RNA-binding
residues on the latter (52). Previously, we showed
U11
SF3b155 to p14, a human RRM-containing U2 and
BCL-X
binding to
hnRNP F qRRM2 interaction prevents hnRNP F from
binding residues to RNA targets. While the FOXP3-interacting protein could affect the accessibility of RNA-
on which part of qRRM is used for interaction, a qRRM-
interactors to influence RNA target selectivity. Depending
platforms into a single qRRM may enable specific protein
capacity among Glo’s qRRMs.

further evidence for differential protein-protein interaction
proteins identified by our IP-MS analysis may provide
Followup studies of other high-confidence Glo-interacting
the 3 Glo qRRMs differin their protein binding specificity.

Figure 7. Model of Glo/dFMRP-mediated nos regulation. Glo recruits dFMRP to the nos TCE via qRRM2 to repress translation elongation of nos mRNA.

diversity. The lack of apparent functional domains apart
from the qRRMs raises the possibility that these RNA-
binding domains also mediate protein-protein interactions.
Notably, the RRM and its variants have been implicated in
RRM-RRM inter-domain packing (46,47) and RRM-
protein interactions (48,49). Among these protein-binding
RRM family members, the U2AF homology motif (UHM) has emerged as a specialized protein-interacting
platform rather than an RNA-interacting platform (50).
Interestingly, the mammalian hnRNP F qRRM2 was recently shown to physically interact with FOXP3,
which modulates the activity of hnRNP F in regulating
alternative splicing (51). Our demonstration that the interaction between Glo and dFMRP also involves
qRRM2 suggests that qRRM2 might be an important
protein-interaction platform as well as an RNA-binding
platform for the hnRNP F/H family more generally. This
finding also indicates that despite their structural similarity,
the 3 Glo qRRMs differ in their protein binding specificity.
Followup studies of other high-confidence Glo-interacting
proteins identified by our IP-MS analysis may provide
further evidence for differential protein-protein interaction
capacity among Glo’s qRRMs.

The incorporation of protein-binding and RNA-binding
platforms into a single qRRM may enable specific protein
interactors to influence RNA target selectivity. Depending
on which part of qRRM is used for interaction, a qRRM-
interacting protein could affect the accessibility of RNA-
binding residues to RNA targets. While the FOXP3-
hnRNP F qRRM2 interaction prevents hnRNP F from
binding to BCL-X pre-mRNA (51), the interaction of
SF3b155 to p14, a human RRM-containing U2 and
U11/12 snRNP component, exposes secondary RNA-
binding residues on the latter (52). Previously, we showed
that each Glo qRRM harbors 2 distinct RNA-binding
interfaces that allow Glo to recognize two different
sequence motifs: a single-stranded G-tract and a double-
stranded UA-rich motif (18). The integration of both
binding modes into a single qRRM and the combination
of 3 qRRMs are thought to diversify the RNA target
reertoire of Glo. Both the G-tract and UA-rich motif
binding modes are required for Glo-TCE interaction, and
hence nos regulation, as well as for regulation of as yet
unknown targets required for viability. By contrast, only
the G-tract binding mode is required for Glo’s function
as a putative splicing regulator in dorsal-ventral patterning
and ovarian nurse cell chromatin structure remodeling. It
remains to be determined if and how binding of dFMRP
affects the RNA-binding affinity and specificity of qRRM2.
Regardless, the choice of protein interactor could influence
the combinatorial use of the 6 RNA-binding interfaces of
Glo. For example, binding of Glo to dFMRP or other nos
regulatory factors may leave both RNA-binding surfaces
exposed, favoring nos TCE recognition and translational
repression, whereas binding of Glo to Hrp48 and Hfp may
leave only the G-tract binding residues exposed, favoring
a different subset of targets for alternative splicing.

Using nos regulation as a model, we have shown that
Glo represses translation elongation by interacting
with a polysome-associated protein, dFMRP, and that
Glo’s RNA-binding qRRM domains also mediate protein-
protein interaction. Since Glo is a multi-functional post-
transcriptional regulator, the same principles could also
be applied to its other activities where integration of
multiple regulatory modes into a single node is required.
Identification of such targets and additional regulatory
factors involved will forward our understanding of how
qRRMs of Glo contribute to its functional diversity and,
more generally, how RNA-binding proteins confer post-
transcriptional gene regulation.

MATERIALS AND CORRESPONDENCE

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DATA AVAILABILITY

The datasets generated or analyzed during this study are included in this published article (and its supplementary information files). Data are available via ProteomeXchange with identifier PXD033174 and PXD033175.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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