Fragile X mental retardation 1 gene enhances the translation of large autism-related proteins

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Mutations in the fragile X mental retardation 1 gene (FMR1) cause the most common inherited human autism spectrum disorder. FMR1 influences messenger RNA (mRNA) translation, but identifying functional targets has been difficult. We analyzed quiescent Drosophila oocytes, which, like neural synapses, depend heavily on translating stored mRNA. Ribosome profiling revealed that FMR1 enhances rather than represses the translation of mRNAs that overlap previously identified FMR1 targets, and acts preferentially on large proteins. Human homologs of at least 20 targets are associated with dominant intellectual disability, and 30 others with recessive neurodevelopmental dysfunction. Stored oocytes lacking FMR1 usually generate embryos with severe neural defects, unlike stored wild-type oocytes, which suggests that translation of multiple large proteins by stored mRNAs is defective in fragile X syndrome and possibly other autism spectrum disorders.

FMR1 is a polysome-associated RNA binding protein required for the nervous system and ovary to develop and function normally in humans, mice, and Drosophila (1, 2). Both tissues translationally control stored mRNAs associated with FMR1-containing ribonucleoprotein particles (RNPs) (3–6), which suggests that FMR1 has a specific function in using stored mRNAs. However, the challenge of obtaining highly enriched FMR1-containing RNPs from neural tissue may have contributed to difficulties in defining FMR1 target genes (7–9). We reasoned that FMR1 function could be studied in a physiologically relevant context using mature Drosophila oocytes, which lack transcription and depend entirely on ongoing translation.

Drosophila oogenesis is highly amenable to such studies because each female can hold up to 30 mature oocytes per ovary for several weeks (Fig. 1A). In the ovary, each oocyte is surrounded by 800 somatic cells constituting a follicle. We found a reliable method (see supplementary materials) to maintain completed follicles in the ovary for a known period of time in the absence of new follicle maturation (Fig. 1B). Ovulation and fertilization could then be induced by adding males to test the stored oocyte’s ability to support embryogenesis.

We tested the function of specific genes during oocyte storage by depleting their transcripts with germline-specific GAL4-driven RNA interference (RNAi), which is produced throughout oogenesis in the germline stem cell. Disrupting a gene required during oocyte storage would cause oocytes to at first develop normally, and then to lose developmental capacity more quickly than the wild type during further storage. To screen for such genes, we depleted Fmr1 mRNA or several other candidate gene transcripts and analyzed oocytes after a storage period of 1 day or 10 days (Fig. 1C). In most gene knockdown lines and in wild-type controls, the hatch rate was nearly 100% after 1 day of storage, and after 10 days only dropped to 80%. In contrast, Fmr1-depleted oocytes hatched normally at first, but after 10 days of storage, only 20 to 30% of them completed development (Fig. 1C). Germline Fmr1 RNAi drastically reduced Fmr1 mRNA levels, and antibody staining confirmed that FMR1 protein was effectively depleted throughout oogenesis specifically in germ cells but not in somatic cells (fig. S1).

We validated the Fmr1 requirement in stored oocytes by showing that oocyte viability drops continuously over time without Fmr1 (Fig. 1D). The defects were specific to the old oocytes themselves. Refeeding the mother caused the remaining stored oocytes to be laid and new oocytes to mature, which were fully functional (Fig. 1D). Thus, germline Fmr1 RNAi does not observably impair stem cells, follicle development, or any nonautonomous aspects of female germline function. This differs from Fmr1 mutants, which lose stem cells and produce fewer follicles because mutants affect niche cells and other somatic cells in addition to germ cells (10).

We analyzed embryos derived from control and Fmr1-deficient oocytes to investigate what processes are affected by FMR1 depletion. Embryos from control oocytes developed a normal nervous system regardless of prior storage, as shown by staining with broadly expressed neural markers (Fig. 2, A and B). The same was true of embryos derived from Fmr1 RNAi oocytes after 1 day of storage (Fig. 2A). However, in embryos derived from follicles lacking FMR1 during 10 days of storage, ventral nerve cord–specific labeling showed missing commissures and breaks in the longitudinal connectives (Fig. 2C), in contrast to the wild type. These neuronal defects were not due to a generalized deterioration of the FMR1-depleted oocyte’s ability to support embryonic development (fig. S2). We never observed comparable neural defects in embryos derived from

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Fig. 1. Fmr1 is specifically required during the storage of mature, quiescent stage 14 oocytes in the ovary. (A) Schematic of a Drosophila ovariole with immature precheckpoint follicles and two stored mature stage 14 follicles. (B) Arrested mature follicle stability (red) following feeding protocol as described (see supplementary materials). (C) Fmr1 knockdown (lines 1 and 2), but not knockdowns of controls or other indicated genes, specifically reduces 10-day stored (red) but not 1-day stored (black) oocytes from developing into hatching larvae. (D) Fmr1 germline RNAi during storage progressively reduces the fraction of mature oocytes competent after 1, 4, 7, or 10 days of storage to support development. Refeeding females to promote maturation of fresh stage 8 follicles restores full developmental potential. Error bars in (B) and (D) denote SD; n.s., not significant. GFP, green fluorescent protein.
wild-type oocytes, even when they were stored for 14 days when fewer than 20% of their embryos developed to hatching (Fig. 2D). In contrast, more than 50% of embryos derived from FMR1-deficient oocytes stored in the ovary for 10 days developed a severely abnormal nervous system (Fig. 2E). Thus, disrupting FMR1 function while oocytes are fully dependent on translational regulation specifically compromised their ability to support neural development relative to controls.

To determine how FMR1 disruption affects oocyte translation and to identify FMR1 translational target genes that may be important for sustaining nervous system development, we developed a ribosome-profiling protocol based on (11) (see supplementary materials) to quantify oocyte translation in an unbiased, genome-wide manner. Flies induced for oocyte storage were analyzed after only 1 or 2 days to identify initial translational changes in healthy Fmr1 knockout oocytes prior to viability reduction. Although whole ovaries were needed to obtain enough material for profiling, most of the analyzed ribosomes should still derive from stored stage 14 follicles, because they are larger than the total size of earlier follicles. Interestingly, the ribosome footprints and transcript levels of most mRNAs were unaffected by germline Fmr1 knockdown (Fig. 3A); this result indicates that FMR1 does not generally control translation or mRNA stability.

From 11 independent, highly reproducible ribosome profiling experiments (Fig. S3), we identified 421 genes whose germline expression significantly declined and 14 genes that significantly increased expression in Fmr1 RNAi (Fig. 3, B and C, and tables S1 and S2). Except for Fmr1 itself, translation of the significantly altered targets generally decreased by a factor of 1.3 to 2.5, which we verified by Western blotting (Fig. S4), whereas their RNA levels were unchanged (Fig. 3B). Many down-regulated genes, at least 56 of 421, are orthologs of human genes that have been implicated in human neurodevelopmental syndromes (Fig. 3C and table S3)—a proportion greater than expected by chance ($P = 1.1 \times 10^{-5}$; fig. S5A). For example, the neurofibromatosis gene Nf1, which is associated with cognitive and behavioral disorders and neural tumors (22), was reduced by a factor of 2.5. Several E3 ubiquitin ligases, including Ctrtip/TRIP21, POE/UBR4, and HUWE1, whose human homologs are associated with intellectual disability, autism, early-onset dementia, and schizophrenia (13–15), were reduced by about a factor of 2. In total, homologs of at least 20 dominant autism/intellectual disability genes were significantly reduced (table S2). Because mutations in these genes are dominant (16), a factor of 2 reduction in expression has potential consequences, even for a single target.

To determine whether FMR1 acted on target transcripts through direct binding, we compared our candidate FMR1 targets with a previous report, which used proximity-based strategies to cross-link mRNAs in brain tissue before immunoprecipitation of FMR1 (7). We found significant overlap between both datasets ($P = 1.2 \times 10^{-25}$; fig. S5B), which suggests that FMR1 directly binds many affected transcripts. However, our results differed from prior studies in two important ways. First, the great majority of targets decreased in expression, indicating that FMR1 usually enhances rather than represses translation, in contrast to most (17, 18) but not all (9, 19) previous reports. This difference might arise because multiple FMR1 targets act negatively on protein stability, translation, or cell growth, including nine ubiquitin ligases, NSF1, and Not1 (table S1). Down-regulation of these negative regulators after Fmr1 loss might substantially increase protein levels, simulating the direct effects of a translational repressor.

Second, almost all of the affected proteins are much larger in size than the average Drosophila protein. Dividing mRNAs undergoing translation into size classes showed that FMR1 strikingly affects translation in proportion to protein size (Fig. 3, C and D) and to some extent UTR length (fig. S6A), but not by transcript level (fig. S6D). The translation of nearly half (46%) of expressed proteins longer than 2000 amino acids, 13% of proteins 750 to 1000 amino acids in length, but only 1% of proteins shorter than 250 amino acids was significantly reduced with Fmr1 knockdown (Fig. 3C). Fmr1 RNAi did not always impair the translation of large proteins, as there was a broad response to Fmr1 knockdown (Fig. 3C). Fmr1 RNAi did not always impair the translation of large proteins, as there was a broad response to Fmr1 knockdown (Fig. 3C). Fmr1 RNAi did not always impair the translation of large proteins, as there was a broad response to Fmr1 knockdown (Fig. 3C). Fmr1 RNAi did not always impair the translation of large proteins, as there was a broad response to Fmr1 knockdown (Fig. 3C).

The preferential effect on large mRNAs is likely mediated by direct FMR1 binding, because the average size of target proteins common to both this study and (7) was 1841 amino acids. Furthermore, genes linked to autism as a group are significantly longer than average (16, 20).

The Poe/Ubri4 gene, encoding one of the longest Drosophila proteins (5322 amino acids), was investigated as an FMR1 target with potentially large effects, something not previously identified. Both Poe and Fmr1 mutant Drosophila are male sterile, cannot fly, and show increased neuromuscular junction synaptic excitability (21–23). Stored Poe mutant oocytes lost developmental competence at the same rate as Fmr1 germline RNAi oocytes (Fig. 4A), and these embryos also developed a high frequency of neural defects (Fig. 4B and fig. S7A). Beginning in near-mature follicles, POE protein formed 0.5- to 2-µm spherical particles in germ cells (Fig. 4C), which were distinct from RNP granules containing the P body marker TRAL (fig. S8). POE antibody staining was lost in Poe germline RNAi and Poe mutants (Fig. 4D). As predicted, POE protein levels were reduced and particles were reduced or eliminated in Fmr1 germline RNAi and in Fmr1-null egg chambers (Fig. 4D). These observations demonstrate that Poe is a major functional target of FMR1, and that Poe is itself essential to maintain the oocyte’s ability to support neural development. However, overexpression of POE using a duplication in an Fmr1 RNAi background restored POE granule expression (Fig. 4D) but did not rescue Fmr1 RNAi lethality upon storage (fig. S7F).

FMR1’s function in maintaining an oocyte’s ability to support neural development may only be revealed during oocyte storage because some FMR1 targets act catalytically. For example, E3 ligases whose levels are reduced in FMR1-deficient...
germline granules that arise in maturing follicles. Scale bars, 20 μm.

(D) Many POE granules are seen in wild-type stage 10 follicles but not in Poe RNAi, PoeOE/m, Fmr1 RNAi, or null Fmr1−/−/− follicles. Fmr1 RNAi combined with POE overexpression (OE) recovers POE granules. Scale bars, 20 μm.
not only underlies fragile X syndrome but is relevant to other neurodevelopmental conditions. The same challenges likely exist in oocytes, spermatocytes, and non-neural somatic cells that require regulated translation from stored mRNAs. Because the challenges of translating large proteins are likely to increase in adult neurons under the influence of aging, the pathways and targets assayed here may contribute to adult-onset neural impairments such as schizophrenia and dementia. Improved knowledge of how FMR1 preserves target genes such as target translation, and of the identities of major improved knowledge of how FMR1 preserves target translation, and of the identities of major target genes such as Poe/UBR4, may open new opportunities to monitor susceptible cells and to intervene to mitigate declining levels of the most critical targets. Small-molecule agents that counteract the tendency of large mRNAs to be segregated into inactive granules represent potentially valuable therapeutics. Continued study of these highly conserved pathways in Drosophila represents one powerful and efficient means to further address both the fundamental and applied implications of these findings.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS
www.sciencemag.org/content/361/6403/709/suppl/DC1
Materials and Methods
Figs. S1 to S8
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References (25–27)
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Fragile X and fragile translation in flies

Mutations in the fragile X mental retardation 1 (FMR1) gene underlie fragile X syndrome and fragile X–associated primary ovarian insufficiency, which are prominent intellectual disability and reproductive disorders, respectively. FMR1 is thought to reduce protein synthesis (translation) at synapses. In Drosophila oocytes, Greenblatt and Spradling found that Fmr1 loss leads to oocytes that generate embryos exhibiting neural defects (see the Perspective by Aryal and Klann). Ribosome profiling of oocytes identified a specific role for FMR1 in enhancing the translation of large proteins, including many associated with autism. FMR1 seems to help maintain translation of large mRNAs that otherwise condense into inactive ribonucleoprotein particles. This mechanism may underlie other causes of autism and mental dysfunction.

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