

1 **Widespread Alterations in Translation Elongation in the Brain of**
2 **Juvenile *Fmr1* Knock-Out Mice**

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49 **Summary**

50 FMRP is a polysome-associated RNA-binding protein encoded by *Fmr1* that is lost in Fragile X
51 syndrome. Increasing evidence suggests that FMRP regulates both translation initiation and
52 elongation, but the gene-specificity of these effects is unclear. To elucidate the impact of *Fmr1*
53 loss on translation, we used ribosome profiling for genome-wide measurements of ribosomal
54 occupancy and positioning in the cortex of 24 day-old *Fmr1* knock-out mice. We found a
55 remarkably coherent reduction in ribosome footprint abundance per mRNA for previously
56 identified, high-affinity mRNA binding partners of FMRP, and an increase for terminal oligo-
57 pyrimidine (TOP) motif-containing genes canonically controlled by mTOR-4EBP-eIF4E
58 signaling. Amino acid motif- and gene-level analyses both showed a widespread reduction of
59 translational pausing in *Fmr1* knock-out mice. Our findings are consistent with a model of
60 FMRP-mediated regulation of both translation initiation through eIF4E and elongation that is
61 disrupted in Fragile X syndrome.

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100 Introduction

101 Fragile X syndrome (FXS) is a highly penetrant, heritable form of intellectual disability that is
102 associated with autism. The most common cause of FXS is epigenetic silencing of the *FMR1*
103 gene that encodes the fragile X mental retardation protein (FMRP). FMRP is an RNA binding
104 protein that regulates both translation initiation and elongation (Darnell et al., 2011; Khandjian,
105 1999; Napoli et al., 2008; Stefani et al., 2004). Translation of the majority of cellular mRNAs
106 begins with recognition of the of the 5' cap structure m⁷G(5')ppp(5')N by eukaryotic initiation
107 factor 4E (eIF4E). FMRP has been shown to repress translation initiation by interacting with
108 cytoplasmic FMRP-interacting protein 1 (CYFIP1) (Napoli et al., 2008), an eIF4E binding protein
109 which competes with eIF4G for interaction with eIF4E and prevents formation of the initiation
110 complex (Richter and Sonenberg, 2005).

111 FMRP co-sediments with actively translating ribosomes and polyribosomes in gradient
112 fractionation assays (Feng et al., 1997; Khandjian et al., 1996; Stefani et al., 2004). Recently, a
113 genome-wide analysis of RNA-FMRP interactions was undertaken in the murine brain with high
114 throughput cross-linking immunoprecipitation (HITS-CLIP) (Darnell et al., 2011). In this study,
115 FMRP was found to bind primarily to protein-coding sequences (CDS) of mRNAs, and no
116 specific binding motif was identified. The highest-affinity mRNA binding partners were enriched
117 in postsynaptic and autism-related genes, including components of the mGluR5 metabotropic
118 glutamate receptor complex and downstream PI3K signaling regulator PIKE, both of which are
119 dysregulated in Fragile X Syndrome (Bear et al., 2004; Gross et al., 2015). *In vitro* puromycin
120 run-off experiments on a set of nine high-affinity binding partners showed extensive, FMRP-
121 dependent ribosomal stalling compared to genes with lower HITS-CLIP signal. Furthermore, the
122 *in vitro* ribosome translocation rate was shown to be significantly higher in brain lysates of *Fmr1*
123 knock-out (*Fmr1*-KO) mice than wild-type mice (Udagawa et al., 2013). Studies have also
124 shown elevated rates of protein synthesis in brains of *Fmr1*-KO mice (Qin et al., 2005) and
125 increased protein expression of many FMRP high-affinity mRNA binding partners (Tang et al.,
126 2015). Taken together, these studies suggest that FMRP represses protein synthesis at the
127 level of translation elongation by acting as a ribosomal brake.

128 Despite this progress, important questions remain regarding the nature of translational
129 regulation by FMRP in the brain. While Darnell and colleagues have identified high-affinity
130 binding partners, it is unclear whether there is a relationship between FMRP affinity and
131 translational repression. Furthermore, it remains unknown whether FMRP represses translation
132 in the brain through a dominant mechanism or whether both initiation and elongation are
133 significantly affected. Ribosome profiling enables genome-wide measurement of ribosome
134 density on mRNAs with single-nucleotide resolution, allowing simultaneous analysis of the
135 overall ribosome density on each gene and ribosomal stalling. In this study, we conducted
136 ribosome profiling and RNA-Seq in wild type and *Fmr1*-KO mice to obtain an unbiased, high-
137 resolution assessment of the impact of *Fmr1* loss on protein synthesis in the brain.

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141 Results

142 Translational landscape of *Fmr1* knock-out mice

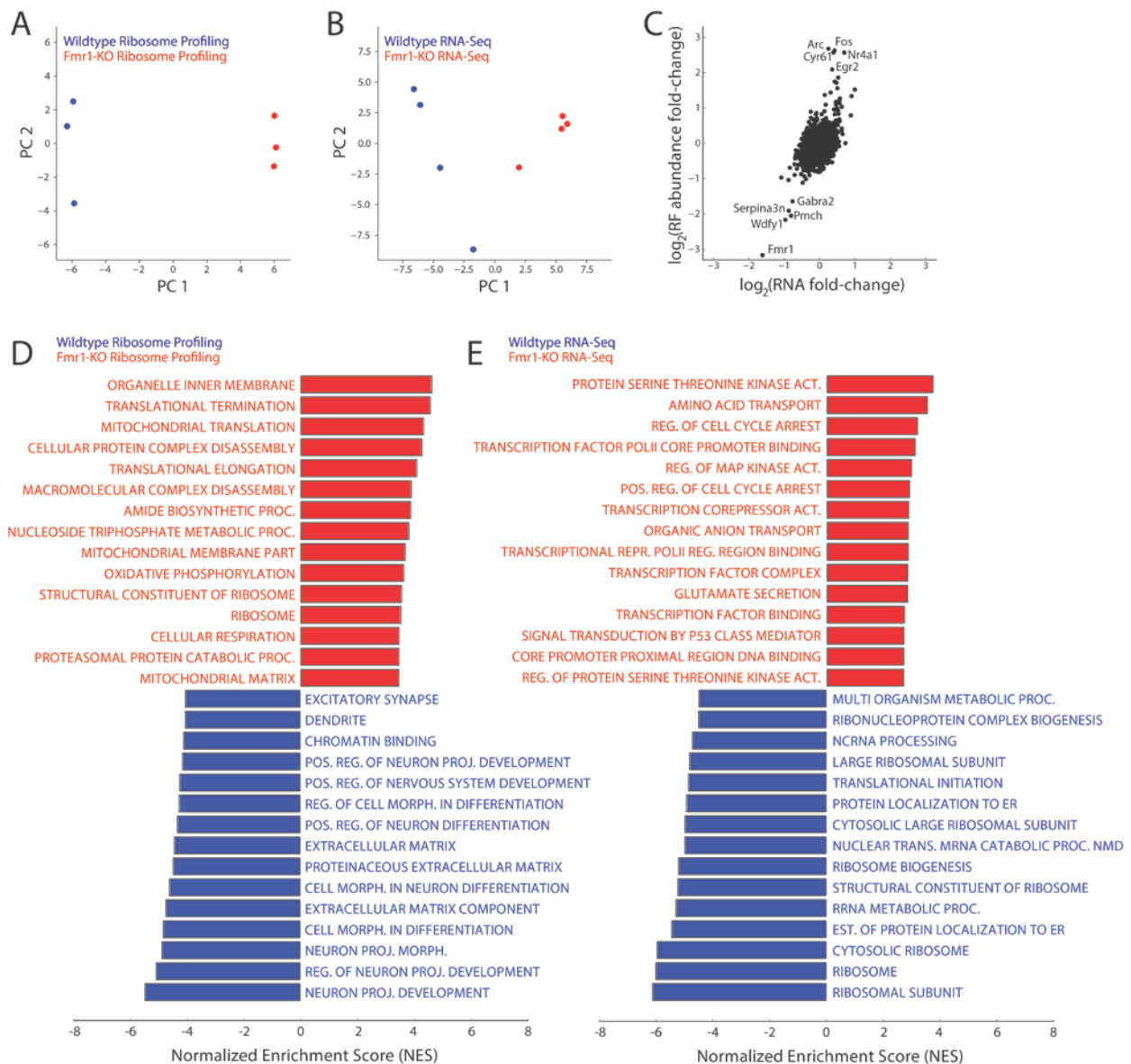


Figure 1: Principal component analysis (PCA) of both ribosome profiling (A) and RNA sequencing (B) libraries from *Fmr1*-KO and wild-type mice. Samples are segregated by genotype in principal component 1 (PC1), the axis representing the major source of variation in the data, in both plots. (C) Comparison of differential ribosome footprint abundance against differential RNA expression levels between genotypes at the level of individual genes. Though ribosome footprint abundance displays a greater range of changes than RNA expression level, these measurements are highly correlated. *Fmr1*, knocked out at the transcript level (by deletion of one exon), shows decreased RNA expression and ribosome density as expected, while the immediate early genes *Fos*, *Arc*, and *Egr2* show increased ribosome density and RNA expression. (D,E) Enrichment scores of the top 15 gene ontologies (GOs) enriched in the wild-type or *Fmr1*-KO brain, determined by GSEA on genes ranked by their fold-changes in ribosome footprint abundance (D) or RNA expression (E) as presented in (C). Genes related to protein synthesis are enriched in ribosome density in *Fmr1*-KO mice compared to wildtype but depleted in RNA expression level (and therefore enriched for in wild-type vs *Fmr1*-KO mice), while ontologies related to neuronal development and morphology show decreased ribosome density in *Fmr1*-KO mice.

143 To determine the effect of *Fmr1* loss on translation, we conducted ribosome profiling and RNA-Seq on the frontal cortex of *Fmr1*-KO and wild-type male mice at postnatal day 24 (P24).
 144 Genome-wide ribosome footprint (RF) and RNA-Seq data were highly reproducible across
 145 biological replicates with genotype as the principal source of variation (**Figure 1A-B**). As
 146 expected, alterations in RF abundance and RNA expression were generally correlated (**Figure**
 147 **1C, Supplementary Tables 1-2**), and a handful of genes exhibited particularly large differences
 148 in RF abundance between genotypes. For example, we found immediate early genes, including
 149 *Arc*, *Fos*, and *Egr2* to have significantly elevated RF abundance in *Fmr1*-KO mice, with much
 150 smaller alterations at the RNA level. To characterize the effects of *Fmr1* loss more broadly, we
 151 conducted differential RF abundance and RNA expression analyses. We used gene set
 152 enrichment analysis (GSEA) to assess differentially translated and expressed gene ontologies
 153 (GOs). Interestingly, while GOs associated with protein synthesis had higher RF abundance in
 154 *Fmr1*-KO mice compared to wildtype, translation-associated GOs exhibited lower expression at
 155 the RNA level in the *Fmr1*-KO mice (**Figure 1D-E**). In addition, GOs associated with neuronal
 156 projection development, morphology, and extracellular matrix have lower RF abundance in
 157 *Fmr1*-KO mice.
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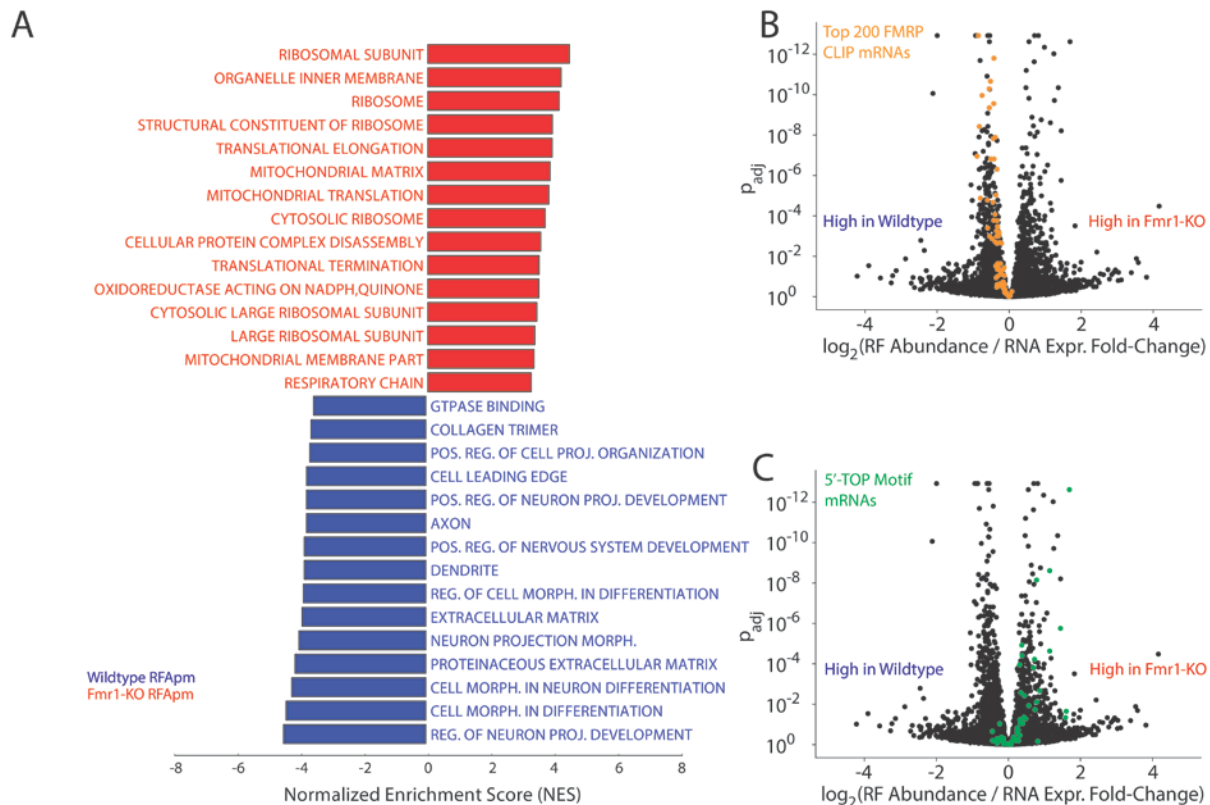


Figure 2: (A) GSEA performed on genes ranked by their differential ribosome footprint abundance per mRNA (RFAPm) between genotypes reveals increased RFAPm of genes related to protein synthesis (ribosome, translation elongation, mitochondrial translation) in *Fmr1*-KO mice with decreased RFAPm of genes involved in neuronal projection development and morphology. (B) and (C) are volcano plots comparing the observed effect size of log-fold change in RFAPm with adjusted p-values for all detected genes. (B) demonstrates a uniform, modest reduction in RFAPm ($p < 0.00001$, GSEA) across the top 200 highest-affinity binding partners for FMRP determined by HITS-CLIP in *Fmr1*-KO mice (orange), while (C) shows a trend towards increased RFAPm in the 5'-terminal oligopyrimidine motif-containing (5'-TOP) genes ($p < 0.00001$, GSEA), the canonical targets of mTOR (green). (B) and (C) together demonstrate the concerted dysregulation of distinct gene sets in opposite directions associated with FMRP loss.

159
160 To identify genes with significant alterations in ribosome footprint abundance per mRNA
161 (RFAPm), calculated as the ratio of RF abundance and RNA expression, we used the
162 generalized linear model (GLM) implemented in RiboDiff for joint statistical analysis of the
163 ribosome profiling and RNA-Seq data (**Supplementary Table 3**). This metric approximates the
164 number ribosomes bound per mRNA and is commonly referred to as “translation efficiency”
165 (Ingolia et al., 2009). However, RFAPm depends on complex relationships between the rates of
166 translation initiation, elongation, and termination that complicate its interpretation (Arava et al.,
167 2005). GSEA revealed that genes involved in protein synthesis have elevated RFAPm in *Fmr1*-
168 KO mice with concomitant reductions in genes associated with extracellular matrix and neuronal
169 function, differentiation, and projection (**Figure 2A**). Translation initiation for effectors of protein
170 synthesis such as ribosomal proteins and translation factors is regulated by mTOR signaling
171 through a cis-regulatory element known as the 5'-terminal oligopyrimidine (5'TOP) motif found in
172 the corresponding mRNAs (Hsieh et al., 2012; Thoreen et al., 2012). This regulation is
173 mediated by 4E-BPs, which, in their dephosphorylated state, sequester the initiation factor
174 eIF4E (Thoreen et al., 2012). FMRP can repress translation via an inhibitory FMRP-CYFIP1-
175 eIF4E complex (Napoli et al., 2008; Santini et al., 2017) and *Fmr1*-KO mice exhibit increased
176 eIF4E-dependent translation (Sharma et al., 2010). Therefore, we expected that the 5'TOP
177 motif-containing mRNAs would exhibit increased RFAPm in *Fmr1*-KO mice. Indeed, **Figure 2B**
178 shows that the 5'TOP transcripts exhibited significantly higher RFAPm in *Fmr1*-KO mice
179 ($p < 0.00001$, GSEA), consistent with a previously characterized mechanism through which
180 FMRP modulates translation initiation.

181
182 As described above, earlier work showed that FMRP binds to mRNAs that encode proteins
183 associated with synaptic activity and other neuronal functions. The GSEA in **Figure 2A**
184 suggests a reduction in RFAPm for genes with similar functions. Indeed, **Figure 2C** shows that
185 the top 200 highest-affinity FMRP binding partners exhibit significantly reduced RFAPm
186 ($p < 0.00001$, GSEA). This coherent reduction in apparent translation efficiency is surprising,
187 because many of these genes have been shown to be over-expressed at the protein level in the
188 brains of *Fmr1*-KO mice (Hou et al., 2006; Schutt et al., 2009; Zalfa et al., 2003; Zhang et al.,
189 2001). One possibility is that protein synthesis from these mRNAs is controlled at the level of
190 translation elongation. For example, a decrease in RFAPm could result from a reduction in
191 ribosomal stalling rather than in initiation efficiency (Ingolia et al., 2009).

192
193 *Alterations in translation elongation in Fmr1 knock-out mice*

194
195 Given the previous evidence of FMRP-dependent ribosomal pausing (Darnell et al., 2011) and
196 the results described above, we next quantified ribosomal pausing using the ribosome profiling
197 data. Specifically, we calculated the ribosome pause score at the level of encoded amino acid
198 sequences, averaging scores across all occurrences of codons corresponding to a given amino
199 acid residue. This metric allows the determination of pause activity due to encoded peptide
200 sequence. **Figure 3** compares the distributions of pause scores across mono-, di-, and tri-amino
201 acids between *Fmr1*-KO and wild-type ribosome occupancy profiles. With few exceptions,
202 sequences exhibited a lower mean pause score in *Fmr1*-KO than in wild-type profiles,
203 demonstrated by a downward shift away from the main diagonal in **Figure 3A-C**. This shift
204 indicates a global relief of pausing associated with *Fmr1* loss that is inconsistent with an effect
205 on a limited set of specific binding partners.

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207 While codon-level analysis suggests that alterations in translation elongation are widespread,
208 we further validated these changes directly at the gene-level. Gene-level analysis of
209 translational pausing is complicated by the large dynamic range in gene expression, which

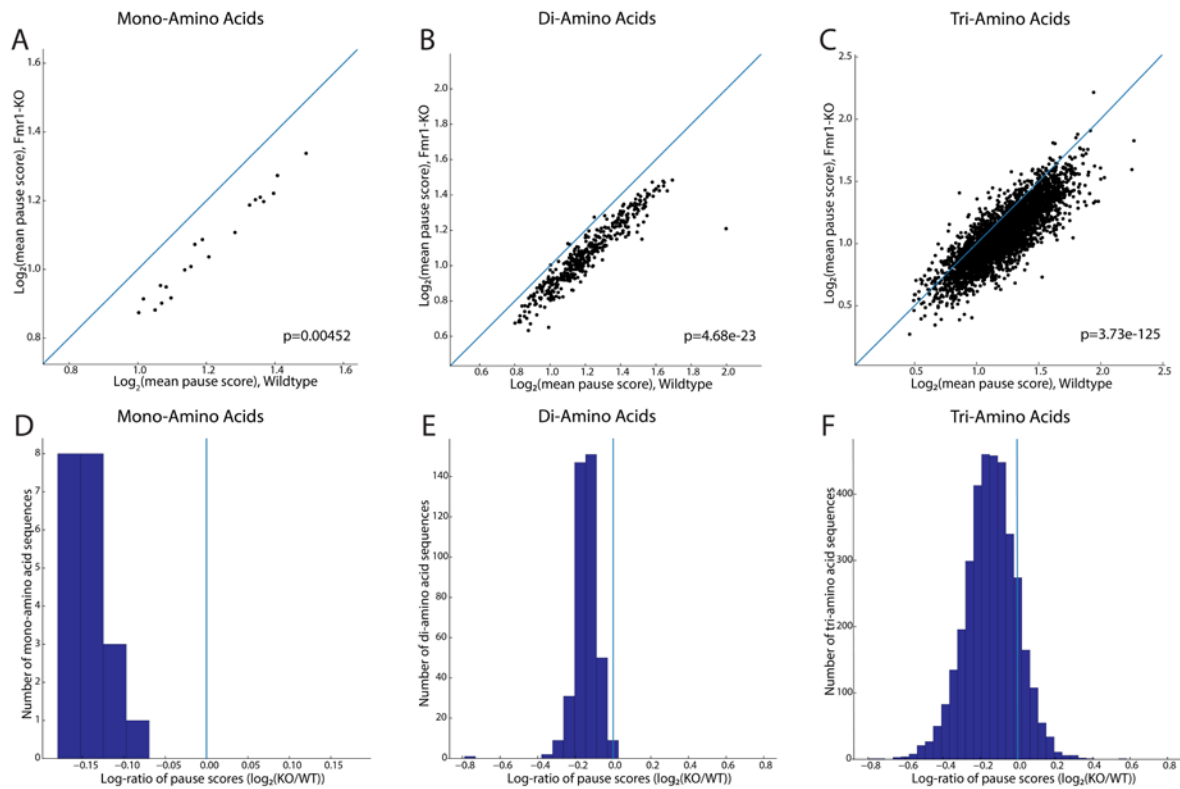


Figure 3: Log-log plots of mean pause scores calculated for single amino acid (A), di- (B), and tri-amino acid sequences (C) in *Fmr1-KO* and wild-type mice, with accompanying p-value for the significance of the difference in these two distributions (Mann-Whitney U-test). In each plot, the main diagonal is plotted as a blue line representing equal pausing in either genotype, highlighting the downward shift of the mass of individual sequences' scores and decrease in pause score in *Fmr1-KO* mice. This shift is visualized differently in (D-F), histograms of the log-ratios of mean pause scores for every mono- (D), di- (E), and tri-amino acid motif (F). The downward/rightward shift in (A-C) translates to a leftward shift away from the blue vertical line at $x=0$, showing decreased pausing for the majority of encoded amino acid motifs in *Fmr1-KO* vs wild-type mice.

210 results in a broad coverage distribution for ribosome profiling across genes. For example,
 211 consider two genes with similar translational pausing behavior where one gene is lowly
 212 expressed, resulting in a low-coverage ribosome profile. A naïve analysis might conclude that
 213 this lowly expressed gene has more translational pausing – an artifact of sparse coverage. At
 214 low coverage, it is challenging to differentiate noise (which scales inversely with coverage due
 215 to counting statistics) from real translational pausing. To address this issue, we developed an
 216 analytical method for gene-level analysis of translational pausing that explicitly models the
 217 dependence of noise in ribosome profiles on coverage.

218
 219 **Figure 4A-C** shows the dependence of the noise (expressed as coefficient of variation or CV) in
 220 the ribosome profile along the CDS of each gene on coverage (expressed as ribosome footprint
 221 reads per codon). As expected, the CV decreases with increasing coverage regardless of
 222 genotype (**Figure 4A-B, Supplementary Figure 1**). We fit the following two-parameter model to
 223 the data to accommodate a variety of statistical behaviors for counting noise:

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$$\log_2(CV) = \frac{1}{2} \log_2 \left(\frac{\beta}{\mu} + \alpha \right) \quad (1)$$

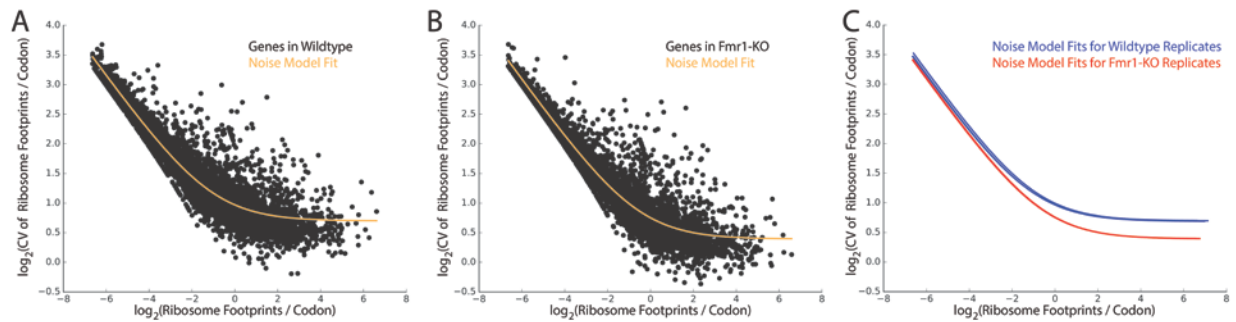
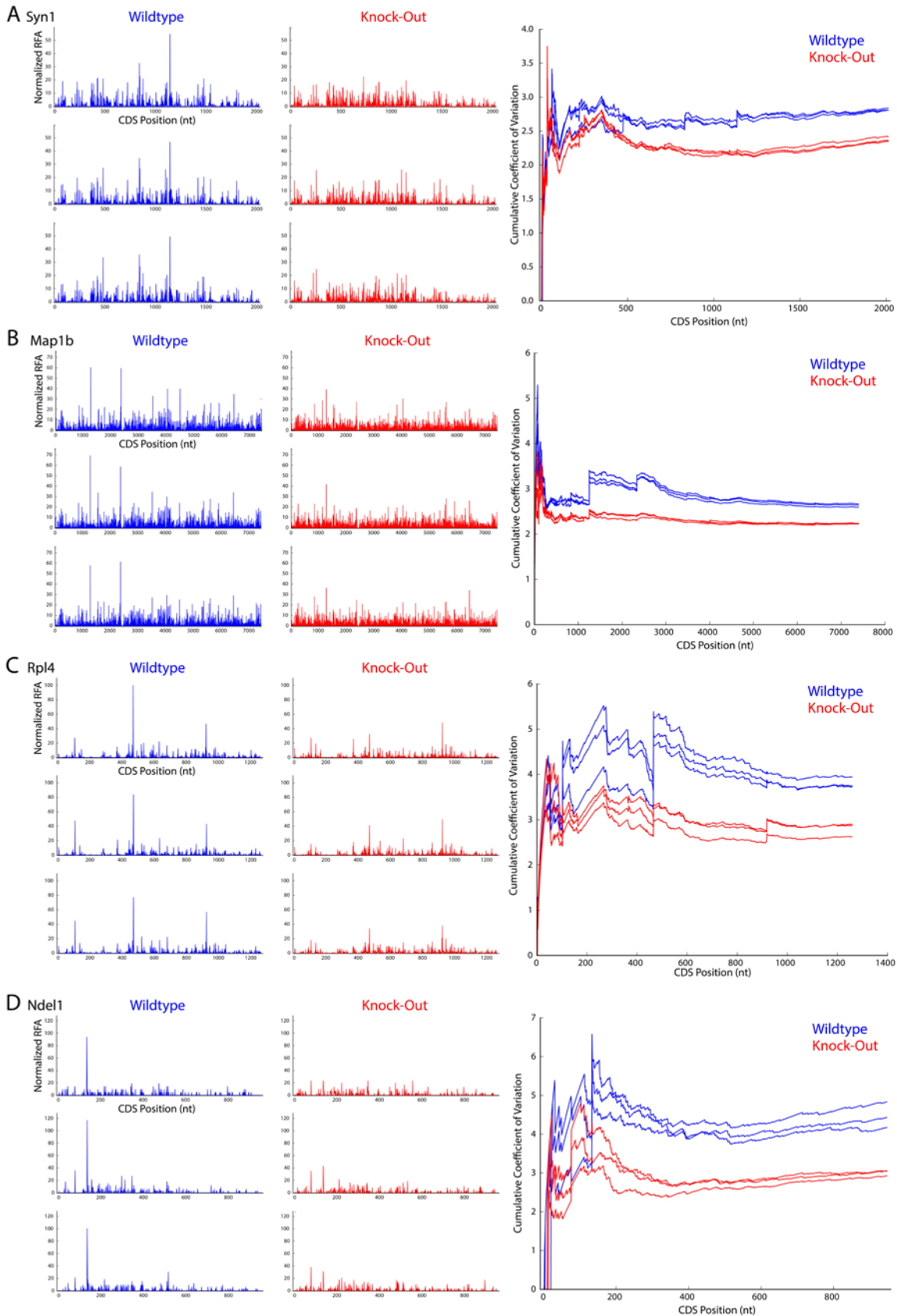


Figure 4: For gene-level analysis of ribosomal pausing, (A) and (B) plot the relationship between noise and coverage for a single wild-type and *Fmr1*-KO replicate, respectively. In these plots, coverage is the log-mean number of ribosome footprints aligned per codon of a given transcript, noise is represented by log-coefficient of variation, or standard deviation in the number of ribosome footprints per codon divided by mean, and the relationship of these values across each gene is summarized by regression to the two-parameter model in Equation 1, plotted in orange. (C) regression curves for each replicate on the same axes ($n=3$ for both genotypes), showing both the uniformity of this relationship across biological replicates, as well as the global downward shift in coefficient-of-variation of *Fmr1*-KO replicates relative to wild-type. This shift, representing a lower degree of coverage variation along the gene body, indicates a widespread reduction in pausing across transcripts.

226 where CV is the coefficient of variation in the ribosome profile of a given gene, μ is mean
227 coverage (ribosome footprint reads per codon), and α and β are fitting parameters. Importantly,
228 when $\alpha = 0$ and $\beta = 1$, Equation 1 results from a Poisson distribution whereas $\alpha > 0$ and $\beta = 1$
229 indicates a negative binomial distribution. **Figure 4C** shows the fits for all wild type ($n=3$) and
230 *Fmr1*-KO ($n=3$) ribosome profiling data sets. While biological replicates of each genotype are
231 highly reproducible, there is a clear difference between genotypes with the *Fmr1*-KO mice
232 exhibiting markedly lower CV at higher coverage. Over-dispersion is widely appreciated for RNA
233 counting data derived from high-throughput sequencing, and as expected, $\alpha > 0$ for all data
234 sets. For highly translated genes, where coverage is drawn from an over-dispersed distribution,
235 CV converges to $\alpha^{1/2}$. However, there is a strong genotype effect on α , (2.61 ± 0.02 for wildtype
236 and 1.720 ± 0.002 for *Fmr1*-KO, $p = 0.0001$). Taken together, these results indicate that the
237 ribosome profiles of genes in *Fmr1*-KO brains display less variability in coverage along the CDS
238 than in the wildtype. These findings are consistent with the codon-level analysis described
239 above, reflecting a global reduction in translational stalling in *Fmr1*-KO mice.

240
241 The analysis in **Figures 3-4** suggests that loss of *Fmr1* results in widespread alterations in
242 translation elongation. Although many of the high-affinity FMRP binding partners and 5'TOP
243 motif-containing mRNAs display decreased and increased RFAPm, respectively, nearly all of
244 these genes exhibit reduced pausing in *Fmr1*-KO mice (**Supplementary Figure 2**). **Figure 5**
245 shows specific examples of this among representative genes from a few different categories.
246 Importantly, there is not a large difference in coverage between wildtype and *Fmr1*-KO for any
247 of these genes. **Figure 5A-B** show the P-site ribosome profiles for all three wildtype and *Fmr1*-
248 KO mice for two genes with a significant reduction in RFAPm. *Syn1* is a high-affinity FMRP
249 binding partner (**Figure 5A**), while *Map1b* (**Figure 5B**) is not. There are two particularly notable
250 features of these data. First, the *Fmr1*-KO profiles display a clear reduction in the large,
251 reproducible pauses manifested as “spikes” in the wild type profiles. Second, as shown in the
252 rightmost panel of **Figure 5A-B**, there is a reproducible, overall reduction in the CV along the
253 gene body that is not explained simply by the reduction in large pauses. **Figure 5C-D** shows the
254 same analysis for two genes with a significant increase in RFAPm in *Fmr1*-KO mice. *Rpl4* is a
255 TOP-motif gene, which is enriched among genes with an apparent increase in translation

256 efficiency as described above, and the other (*Ndel1*) is not. For all four genes in **Figure 5**, we
257 detect stereotyped pauses in the wild type that are substantially ablated in the knock-out. We
258 also find a reproducible reduction in cumulative CV along the gene body, suggesting a smoother
259 overall translocation process for the ribosome in the brain of *Fmr1*-KO mice.
260



262 **Figure 5:** At left, nucleotide-resolution plots of P-site occupancy for genes *Syn1*, *Map1b*, *Rpl4*, and *Ndel1*
263 (A-D, respectively) for three replicates of both wild-type (blue) and *Fmr1*-KO (red) mice. These plots are
264 paired on the right with comparisons of the cumulative coefficient of variation (CV), calculated as the
265 coefficient of variation for the coding sequence up to a given nucleotide position in the CDS. While *Syn1*
266 and *Map1b* both exhibit decreased RFAPm in *Fmr1*-KO mice, *Syn1* is a high-affinity binding partner of
267 FMRP and *Map1b* is not; similarly, *Rpl4* and *Ndel1* both exhibit increased RFAPm in *Fmr1*-KO mice but
268 *Rpl4* is a 5'-TOP gene and target of mTOR. For all these genes, the magnitudes of the "spikes" of
269 reproducible, high-frequency P-site alignment, which represent pause sites, are significantly reduced in
270 *Fmr1*-KO occupancy plots compared to their wild-type counterparts, and this reduction is reflected in a
271 correspondingly diminished increase in cumulative CV at the pause site's coordinate for *Fmr1*-KO
272 replicates. The overall decrease in positional noise of aligned P-sites with FMRP loss, represented by the
273 consistent gap in cumulative CV between genotypes at nearly all coordinates, is larger than that which
274 can be explained by large pause-reductions alone.

275 276 **Discussion**

277
278 Previous studies have shown that FMRP associates with polysomes and the protein-coding
279 sequences of a large number of transcripts (Brown et al., 2001; Stefani et al., 2004). HITS-CLIP
280 data indicate that FMRP has particularly high affinity for mRNAs involved in synaptic activity and
281 appears to act as a translational brake, stalling ribosomes on these transcripts (Darnell et al.,
282 2011). Prior work has also revealed interactions between FMRP and the translation initiation
283 machinery (Napoli et al., 2008; Santini et al., 2017). Nonetheless, genome-wide measurements
284 of protein synthesis with the resolution to analyze both translation elongation and RFAPm have
285 not been undertaken in the brains of *Fmr1*-KO mice.

286
287 We characterized the translational landscape in the cortex of *Fmr1*-KO mice at a crucial time in
288 postnatal brain development. By P24, the mouse brain has reached its peak synaptic density
289 and significant pruning of excitatory synapses is taking place, a process known to be
290 dysregulated broadly in autism spectrum disorders (Tang et al., 2014) and specifically in FXS
291 (Comery et al., 1997; He and Portera-Cailliau, 2013). Loss of FMRP-mediated regulation of
292 protein synthesis may be critically linked to the synaptic plasticity and dendritic spine
293 phenotypes observed in FXS (Darnell and Klann, 2013). We discovered a remarkably uniform
294 trend in the RF abundance of FMRP's high affinity binding partners with nearly all of the top 200
295 FMRP-bound transcripts showing a significant reduction in RFAPm in *Fmr1*-KO mice (**Figure**
296 **2B**). This result is surprising because proteins encoded by many of these mRNAs have been
297 shown to be more highly expressed in *Fmr1*-KO mice (Tang et al., 2015). Importantly, reduction
298 in ribosome density was not a global effect. For example, the 5'TOP motif-containing mRNAs,
299 which are comprised mainly of ribosomal protein- and translation factor-encoding transcripts,
300 were enriched among genes with increased RFAPm (**Figure 2C**). These genes are known to be
301 controlled at the level of translation initiation by 4E-BP and eIF4E, the latter of which is
302 sequestered by an FMRP-mediated complex.

303
304 Despite these clear patterns, RFAPm is a complicated metric. In many studies, it is interpreted
305 as a measure of translation efficiency that primarily reflects translation initiation. However, this
306 interpretation assumes that initiation is rate-limiting and elongation rates are uniform (Arava et
307 al., 2005). Given the potential role of FMRP in regulating translation elongation (Darnell et al.,
308 2011), the apparent reduction in ribosome density for FMRP's high-affinity binding partners
309 (**Figure 2C**) may actually result from a relaxation of translational stalling in the absence of
310 FMRP. We took advantage of the nucleotide resolution of ribosome profiling and characterized
311 the noise in wild type and *Fmr1*-KO ribosome profiles with both codon motif- and gene-centric
312 analyses. In both cases, we found a significant and global reduction in translational pausing in
313 *Fmr1*-KO mice (**Figures 3-4**). As a genome-wide snapshot of translation in the cortex of *Fmr1*-

314 KO mice *in vivo*, our results expand on previous *in vitro* measurements of ribosome stalling on
315 select mRNAs using puromycin run-off (Darnell et al., 2011) and elongation rate using the
316 ribosome transit time assay (Udagawa et al., 2013). We observed decreases in ribosomal
317 pausing for the FMRP high-affinity binding partners, which exhibited a reduction in RFAPm, and
318 for the 5'TOP motif-containing mRNAs, which showed an increase in RFAPm (**Supplementary**
319 **Figure 2**). We note that our results do not formally rule out the possibility that the FMRP-
320 associated mRNAs are also differentially regulated at the level of translation initiation. However,
321 these results are consistent with a model in which FMRP loss dysregulates ribosomal pausing
322 across a large number of transcripts, and that competition between initiation- (e.g., through
323 FMRP-mediated sequestration of EIF4E) and elongation-level regulation results in disparate
324 alterations in RFAPm for certain genes.

325
326 We suggest that therapeutic strategies for FXS should carefully consider the consequences of
327 globally altered protein synthesis. Recent evidence suggests that enhanced translation of
328 certain mRNAs in *Fmr1*-KO mice may represent compensatory changes and that enhancing
329 their function may ameliorate disease phenotypes (Thomson et al., 2017). Importantly, our study
330 does not assess whether translational alterations in *Fmr1*-KO mice are caused by direct loss of
331 FMRP function or by secondary effects arising due to continued absence of FMRP during neural
332 development. A critical aspect is that neuronal activity may be tightly coupled to translational
333 regulation. Several recent studies found translational repression of neuronal mRNAs following
334 fear conditioning *in vivo* (Cho et al., 2015), and of FMRP binding partners following KCl
335 depolarization *in vitro* (Dalal et al., 2017). Given extensive evidence of cortical hyperexcitability
336 (Gibson et al., 2008; Hays et al., 2011) and dysregulation of GABAergic neurotransmission in
337 *Fmr1*-KO mice (Paluszkiwicz et al., 2011), it is possible that the downregulation of RFAPm we
338 observed in FMRP binding partners (**Figure 2C**) is linked to increased cortical activity. We found
339 enhanced translation of immediate early genes such as *Arc* and *Fos* as well as decreased
340 translation of *Gabra2* (**Figure 1C**), consistent with previous reports of decreased GABA_A
341 receptor expression and GABA dysfunction in FXS (Braat et al., 2015; D'Hulst et al., 2006).
342 Future studies using knockdown or conditional knockout of *Fmr1* may be necessary to
343 disentangle the primary effects of acute FMRP loss from secondary alterations in neuronal
344 physiology. Nonetheless, our study shows that *Fmr1* loss leads to widespread alterations in
345 mRNA translation, particularly at the level of elongation, during the developmental period of
346 cortical synaptic refinement.

347 348 **Acknowledgements**

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355 356 357 **Author Contributions**

358 P.A.S., D.S., and G.T. conceived the project and designed the experiments. S.D.S., H.L.,
359 B.D.H., and G.T. conducted the experiments. S.D.S., J.B.M., B.D.H., N.H. and P.A.S. analyzed
360 the data. All authors wrote and edited the manuscript.

361 362 **Declaration of Interests**

363 The authors declare no competing interests.

364

365 **Data Availability**

366 The ribosome profiling and RNA-Seq data have been deposited in the Gene Expression
367 Omnibus (GEO) under accession GSE114064.

368

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515 **Experimental Procedures**

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517 *Mice*

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519 *All mice were in C57BL/6J background. Camk2a-cre-RiboTag mice were generated by crossing*
520 *Camk2a-cre (JAX 005359) mice with RiboTag mice (JAX 011029) as reported previously*
521 *(Hornstein et al., 2016). Camk2a-cre heterozygotes were crossed to RiboTag mice to obtain*
522 *Rpl22^{flox/flox};Camk2a-cre^{+/-} mice, which were further crossed to Fmr1^{-y} mice (Jax 00325) to*
523 *generate Fmr1^{X+/X-};Rpl22^{flox/flox};Camk2a-cre^{+/-} females. The Fmr1^{X+/X-};Rpl22^{flox/flox};Camk2a-cre^{+/-}*
524 *females were then bred to Rpl22^{flox/flox} males to obtain Fmr1^{-y};Rpl22^{flox/flox};Camk2a-cre^{+/-} mice*
525 *and Rpl22^{flox/flox};Camk2a-cre^{+/-} control littermates.. Throughout the manuscript, we refer to the*
526 *Fmr1^{-y};Rpl22^{flox/flox};Camk2a-cre^{+/-} mice as Fmr1-KO and the Rpl22^{flox/flox};Camk2a-cre^{+/-} mice as*
527 *wild type. All experiments we conducted at postnatal day 24 (P24). All mouse experimental*
528 *procedures were reviewed and approved by Columbia University Medical Center Institutional*
529 *Animal Care and Use Committee.*

530

531 The mice were genotyped with the following primers for Cre: GCG GTC TGG CAG TAA AAA
532 CTA TC (transgene), GTG AAA CAG CAT TGC TGT CAC TT (transgene), CTA GGC CAC
533 AGA ATT GAA AGA TCT (internal positive control forward), GTA GGT GGA AAT TCT AGC
534 ATC ATC C (internal positive control reverse), and the following primers for RiboTag: GGG
535 AGG CTT GCT GGA TAT G (forward), TTT CCA GAC ACA GGC TAA GTA CAC (reverse).
536 The primers for Fmr1KO mice were: CAC GAG ACT AGT GAG ACG TG (mutant forward); TGT
537 GAT AGA ATA TGC AGC ATG TGA (wild type forward); CTT CTG GCA CCT CCA GCT T
538 (reverse)

539

540 *Tissue processing for RNA sequencing and Ribosome profiling*

541

542 Brain tissue was processed as described previously (Hornstein et al., 2016). Briefly, snap-frozen
543 frontal cortex (n=4 mice/genotype for RNA-Seq and n=3 mice/genotype for ribosome profiling,
544 sample weight ~25mg) was disrupted using a Dounce homogenizer in 1mL of polysome lysis
545 buffer (20 mM Tris-HCl pH 7.5, 250 mM NaCl, 15 mM MgCl₂, 1mM DTT, 0.5% Triton X-100,
546 0.024 U/ml TurboDNase, 0.48 U/mL RNasin, and 0.1 mg/ml cycloheximide). Homogenates were
547 clarified by centrifugation at 14,000 x g for 10 min at 4°C. Supernatant was collected and used
548 for RNA-Seq and ligation-free ribosome profiling.

549

550 *RNA-Seq library construction*

551

552 Total RNA was isolated from brain lysates using a Qiagen RNeasy kit (cat no. 74104) and
553 ribosomal RNA was depleted using the Ribo-Zero rRNA removal kit from Illumina (Cat no.
554 MRZH11124) according to the manufacturer's instructions. rRNA depleted total RNA samples
555 were converted to a strand-specific sequencing library using the NEBNext® Ultra™ Directional
556 RNA Library Prep Kit from Illumina (Cat no.E7420S). There were a total of four RNA-Seq
557 libraries generated for each genotype, with each library originating from a different animal. RNA-
558 Seq libraries were quantified using Qubit fluorometer (ThermoFisher) and library size was
559 measured using an Agilent Bioanalyzer.

560

561 Sequencing of eight RNA-Seq libraries was performed on an Illumina NextSeq 500 desktop
562 sequencer with a read length of 75 bases. Approximately 20 to 50 million demultiplexed, pass-
563 filtered, single-end reads for each sample were obtained.

564

565 *Ligation-free ribosome profiling*

566
567 Ligation-free ribosome profiling libraries were prepared from dephosphorylated foot-prints (~ 28-
568 34 nucleotides in length) using a commercially available kit (SMARTer small RNA-Seq Library
569 Preparation Kit, Clontech, Cat no. 635029) following manufacturer's instructions (Hornstein et
570 al., 2016) . We performed library purification with AMPure XP beads (Beckman Coulter).
571 Libraries were quantified using the Qubit dsDNA High-Sensitivity kit (Life Technologies) and
572 library size was verified with the High-Sensitivity Bioanalyzer DNA chip (Agilent Technologies).
573 Sequencing of six ribosome profiling libraries was done on an Illumina NextSeq 500 desktop
574 sequencer with a read length of 50 bases. We obtained between 20 to 50 million demultiplexed,
575 pass-filtered, single-end reads for each sample.

576 577 *High-Throughput Sequencing Data Processing*

578
579 Bioinformatics analysis was performed following a protocol from Hornstein et al 2016 (Ingolia et
580 al., 2012) with minor modifications. Ribosome profiling libraries were processed by removing the
581 first 4 and last 10 positions of each sequenced read with the following command to fastx-
582 trimmer:

```
583 fastx_trimmer -f 4 -l 40 -Q33 -i INFILE -o OUTLFILE
```

584 following which we trimmed remaining poly(A) sequence from the 3' end, discarding trimmed
585 reads shorter than 25 nucleotides. Libraries were then depleted of ribosomal RNA by alignment
586 to an rRNA reference library comprised of rRNA sequences from mm9 with bowtie2, allowing for
587 one alignment error. Unaligned reads were retained and aligned to the mm10 assembly of the
588 mouse genome and Gencode-annotated transcriptome with STAR (Dobin et al., 2013).
589 Alignments to the exons and coding sequences (CDS) of genes were counted with the
590 featureCounts (Liao et al., 2014) program from the subread suite, yielding between 4 and 10
591 million reads uniquely mapped to the CDS per ribosome profiling library.

592 593 *Statistical Analysis of RNA Expression, Ribosome Footprint Abundance, and Ribosome* 594 *Footprint Abundance per mRNA (RFAPM)*

595
596 We used DESeq2 (Love et al., 2014) to analyze differential expression from uniquely aligned
597 RNA-Seq reads and differential ribosome footprint abundance from ribosome profiling reads that
598 aligned uniquely to the CDS of each gene. We used the generalized linear model in RiboDiff
599 (Zhong et al., 2017) to analyze differential ribosome footprint abundance per mRNA (RFAPM).
600 For this analysis, only reads that aligned uniquely to the CDS were used for both RNA-Seq and
601 ribosome profiling. We used the Java implementation of gene set enrichment analysis (GSEA)
602 (Subramanian et al., 2005) to assess the statistical enrichment of gene ontologies. Specifically,
603 we pre-ranked each gene by fold-change and used "classic" mode to compute normalized
604 enrichment scores and corrected p-values for gene sets in the MSigDB C5 gene ontology
605 collection.

606 607 *Codon Motif-Level Analysis of Pausing*

608
609 Ribosome profiling libraries were first aligned to the transcriptome using the `-quantmode`
610 `TranscriptomeSAM` option in STAR v2.5 as follows:

```
611 STAR --readFilesCommand zcat --genomeDir STAR_INDEX --runThreadN 12 --outSAMtype  
612 BAM SortedByCoordinate --readFilesIn INFILE --outSAMprimaryFlag AllBestScore --
```

613 outSAMattrIHstart 0 --quantMode TranscriptomeSAM --outFileNamePrefix OUTFILE

614 Transcriptome-aligned libraries were then filtered by removing reverse-complemented (SAM
615 flag 272 or 16), suboptimal, and non-CDS-aligned reads.

616 We chose one representative transcript and coding sequence for each gene by summing counts
617 for all transcripts independently, then choosing the transcript with the highest sum of counts for
618 each gene. To reduce reads from ~28-30nt footprints to A-site locations, we used the *psite*
619 script from the plastid library for ribosome profiling analysis (Dunn and Weissman, 2016). This
620 script calculates the location of a ribosomal P-site relative to the 5' end of a footprint based on
621 its length; increasing the calculated P-site offset by 3 nucleotides yields the A-site offset. We
622 obtained codon occupancy profiles by summing over A-sites overlapping the 0, +1, and -1
623 nucleotide positions relative to the codon start, then merged them by summation across
624 samples within either condition (wild-type or *Fmr1*-KO), collapsing six samples to two overall
625 profiles with greatly increased coverage. We then limited the set of transcripts under
626 consideration to those with mean coverage of at least 0.1 A-sites per codon for the first 150
627 codons in both profiles, yielding 8,967 total transcripts, and calculated pause scores for all but
628 the first and last 10 codons within each.

629 Ribosome pause scores were calculated following the approach described by Woolstenhulme et
630 al (Woolstenhulme et al., 2015), modified to correct for potential differences in splicing across
631 profiles in line with Ishimura et al (Ishimura et al., 2014). We calculated context-specific pause
632 scores for every codon of every coding sequence by dividing the codon's ribosome occupancy
633 by the maximum of three values: the mean occupancy of the first 150 codons of the transcript
634 and the median occupancies of the five codons 5' and 3' to the codon in question. To obtain a
635 mean pause score for each amino acid, we averaged scores across all occurrences of codons
636 encoding that amino acid residue; di- and tri-amino acids with a minimum of 100 occurrences
637 across the transcripts considered were similarly summarized. For mono-, di-, and tri-amino acid
638 datasets, we performed a Mann-Whitney U-test to determine statistical significance of the
639 difference in the distributions of pause scores between genotypes.

640 *Gene-Level Analysis of Translational Pausing*

641 We used Ribo-TISH (Zhang et al., 2017) to determine the ribosome P-site offsets for each
642 fragment length and P-site ribosome profiles for each transcript in our ribosome profiling data.
643 For the initial quality control step, we used the following command:

```
644 ribotish quality -b BAMFILE -g GTF -p 16
```

645 followed by a prediction step with:

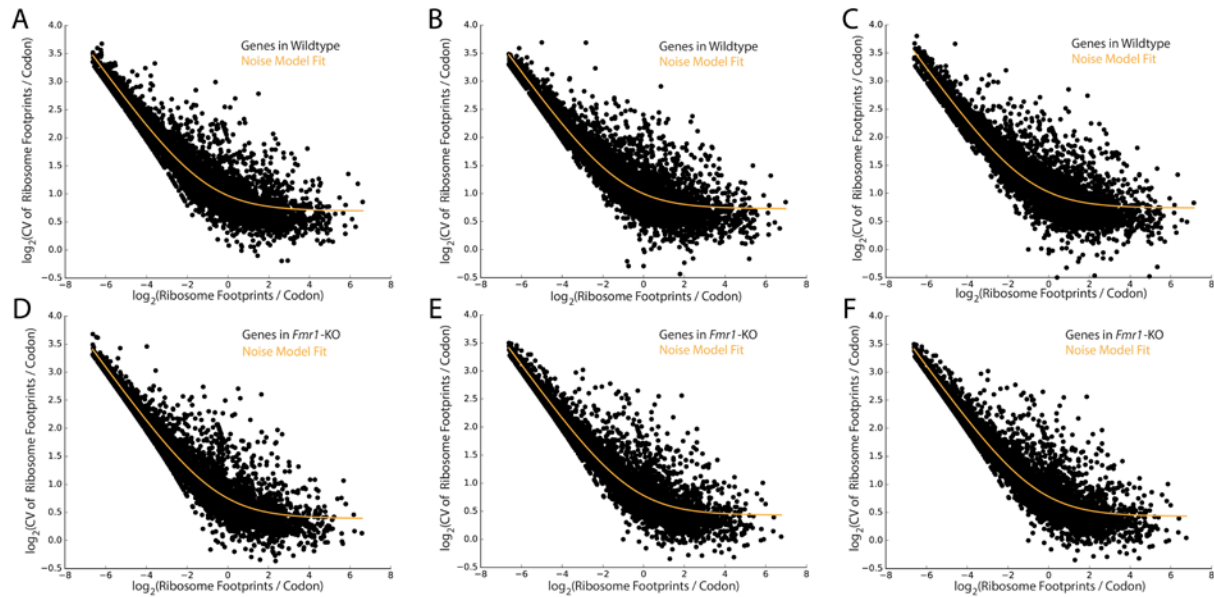
```
646 ribotish predict -b BAMFILE -g GTF -f GENOME_FASTA -o OUTPUT_FILE -p 16 --transprofile  
647 PROFILE_OUTPUT_FILE --framebest --seq --aseq
```

648 We then restricted our analysis to annotated ORFs, and for each isoform of each gene, we

649 computed the mean coverage (number of ribosome footprints per codon) and the coefficient of
650 variation (CV) in coverage (standard deviation in the number of ribosome footprints per codon
651 divided by mean). For each gene, we selected the isoform with the lowest CV. Isoforms with
652 extremely non-uniform coverage, which can result from low usage or exclusion of a subset of
653 exons, are typically not the dominantly expressed isoform. Finally, as described under Results,
654 we fit Equation 1 to a plot of $\log_2(\text{CV})$ vs. $\log_2(\text{mean coverage})$ to assess the genome-wide
655 dependence of noise along the CDS on coverage using the *curve_fit* function in SciPy.

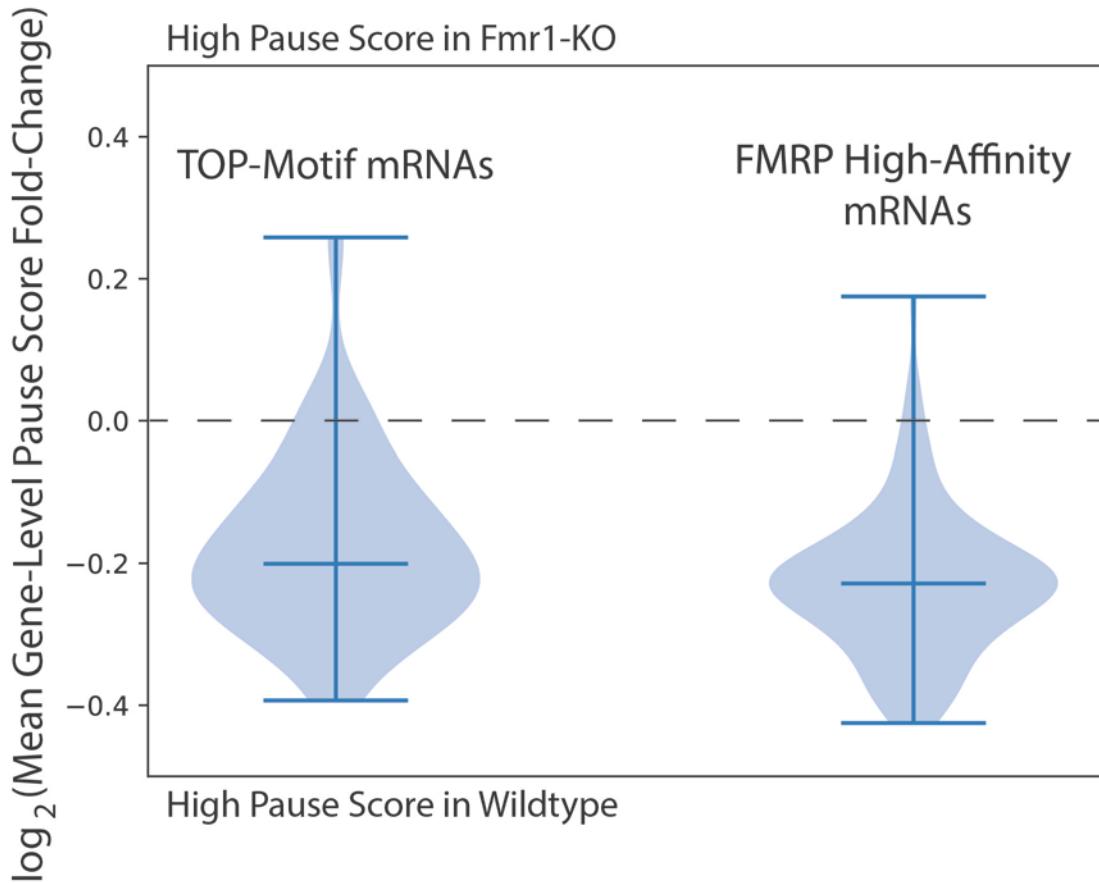
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697 **Supplementary Information for “Widespread Alterations in Translation Elongation**
698 **in the Brain of Juvenile *Fmr1* Knock-Out Mice”**
699



700 **Supplementary Figure 1.** Coefficient of variation vs. mean of the number of ribosome footprints
701 per codon across genes for A)-C) wildtype and D)-F) *Fmr1*-KO mice with fit to Equation 1.
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716 **Supplementary Figure 2.** Distributions of fold-change in gene-level pause scores averaged
717 across replicate experimental batches (each with a wildtype and Fmr1-KO mouse). The score is
718 defined as the coefficient of variation divided by its expectation value from the noise model of
719 the wildtype mouse (from the fit to Equation 1 shown in Supplementary Figure 1). We computed
720 distributions for the TOP motif-containing mRNAs and the top 200 high-affinity FMRP binding
721 partner mRNAs to show that nearly every gene in these two groups exhibits reduced pausing in
722 Fmr1-KO mice.

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737 **Supplementary Tables**

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739 **Supplementary Table 1.** Output of DESeq2 differential expression analysis comparing
740 CDS-aligned ribosome footprint abundances between *Fmr1*-KO and wildtype mice.

741 **Supplementary Table 2.** Output of DESeq2 differential expression analysis comparing
742 RNA-Seq profiles between *Fmr1*-KO and wildtype mice.

743 **Supplementary Table 3.** Output of RiboDiff differential translation analysis comparing
744 RFAPm between *Fmr1*-KO and wildtype mice.

745