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Promoter-Bound Trinucleotide Repeat mRNA Drives Epigenetic Silencing in Fragile X Syndrome

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Abstract

Epigenetic gene silencing is seen in several repeat-expansion diseases. In fragile X syndrome, the most common genetic form of mental retardation, a CGG trinucleotide–repeat expansion adjacent to the *fragile X mental retardation 1* (*FMR1*) gene promoter results in its epigenetic silencing. Here, we show that *FMR1* silencing is mediated by the *FMR1* mRNA. The *FMR1* mRNA contains the transcribed CGG-repeat tract as part of the 5′ untranslated region, which hybridizes to the complementary CGG-repeat portion of the *FMR1* gene to form an RNA·DNA duplex. Disrupting the interaction of the mRNA with the CGG-repeat portion of the *FMR1* gene prevents promoter silencing. Thus, our data link trinucleotide-repeat expansion to a form of RNA-directed gene silencing mediated by direct interactions of the trinucleotide-repeat RNA and DNA.

Fragile X syndrome (FXS) results from the absence of the fragile X mental retardation protein (FMRP), which is encoded by the *fragile X mental retardation 1* (*FMR1*) gene on the X chromosome (1). Impaired FMRP expression is caused by an inherited CGG trinucleotide–repeat expansion adjacent to the *FMR1* promoter (2). *FMR1* alleles that contain >200 CGG repeats undergo epigenetic silencing of the *FMR1* promoter at ~11 weeks of gestation (2–4). It remains unclear how the CGG-repeat expansion leads to gene silencing.

The mechanism of gene silencing in FXS has been particularly difficult to dissect. Transgenes containing expanded CGG-repeat *FMR1* alleles are not silenced in cell lines or mice (5). Furthermore, overexpression of expanded CGG-repeat sequences is complicated by sequence instability in plasmids (6). Recently, human embryonic stem cells (hESCs) harboring an *FMR1* allele with >200 CGG repeats (FXS hESCs) were shown to undergo

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FMR1 gene silencing upon differentiation (7). The switch from active *FMR1* gene expression to *FMR1* silencing in FXS hESCs resembles the switch that occurs in FXS embryos (4), which provides an in vitro system to study *FMR1* silencing.

To monitor *FMR1* silencing, we used two FXS lines, WCMC-37 and SI-214 (8, 9) (referred to throughout the text as FXS-1 and FXS-2, respectively); each contains >400 CGG repeats (fig. S1). We monitored *FMR1* silencing after inducing neuronal differentiation over 60 days (fig. S2). In neurons derived from FXS hESCs, FMRP and *FMR1* mRNA were readily detected until ~48 days, at which point the levels dropped and were absent by day 51 (fig. S3 to S5). The *FMR1* promoters in the undifferentiated FXS hESC lines contain high levels of histone H3 dimethylated on lysine 4 (H3K4me2, associated with gene expression) and low levels of histone H3 dimethylated on lysine 9 (H3K9me2, associated with gene repression) (fig. S6). However, in differentiated FXS cells, the *FMR1* promoter switched to the repressive H3K9me2 mark, with a concomitant reduction in the levels of H3K4me2 (fig. S6). Taken together, these data indicate that the loss of FMRP and *FMR1* mRNA in FXS hESC lines correlates with the epigenetic silencing of the *FMR1* promoter.

The expanded CGG repeat influences DNA structure, which may be recognized by pathways that induce epigenetic silencing (10). However, the expanded CGG repeat is also transcribed as the 5′ untranslated region (UTR) of the *FMR1* mRNA, which makes it possible that the mRNA could induce promoter silencing. We therefore tested whether the *FMR1* transcript is required for silencing. Knockdown of *FMR1* mRNA in FXS hESCs prevented differentiation-induced *FMR1* silencing, as measured by the retention of transcriptionally active histone marks at the *FMR1* promoter (Fig. 1A). The effect of the *FMR1*-specific short hairpin RNAs (shRNAs) was not due to knockdown of a previously described antisense *FMR1* transcript that partially overlaps with the *FMR1* mRNA (11) (fig. S7B). These data indicate that the *FMR1* transcript is required for *FMR1* silencing.

CGG repeats in mRNA form a hairpin structure (10) (fig. S7C), which may be unfolded and linearized in order to mediate *FMR1* silencing. To test the role of the mRNA CGG repeat in *FMR1* gene silencing, we used 1a, a small molecule that selectively binds the repeating G-G internal loops in the RNA hairpin and inhibits its thermal melting (12) (fig. S7D). Application of 1a (10 μM) throughout the differentiation prevented differentiation-induced *FMR1* silencing in FXS hESCs (Fig. 1, B to D, and fig. S8). Application of the structurally related control compound 1f (10 μM), which does not bind CGG repeats (12), did not affect *FMR1* silencing. The effect of 1a was not due to impaired differentiation, as the 1a-treated cells expressed the neuronal marker β-III tubulin (fig. S9). Together, these data suggest that linearization of the CGG-repeat hairpin in the *FMR1* transcript is required for silencing.

Dicer processes CGG-repeat RNAs into small RNAs in vitro (13) and may contribute to *FMR1* silencing (14). However, knockdown of *Dicer*, *Ago1*, or *Ago2* did not prevent differentiation-induced *FMR1* silencing (fig. S10). Thus, *FMR1* silencing does not require a Dicer-directed pathway.

We next asked if the *FMR1* transcript directs silencing by binding, directly or indirectly, to the promoter. To test this, we measured *FMR1* mRNA binding to the *FMR1* gene using the

chromatin isolation by RNA purification (ChIRP) technique (Fig. 2A) (15, 16). After cross-linking endogenous RNA to its binding partners, we pulled down *FMR1* mRNA using biotinylated oligonucleotides that hybridize along the length of the transcript. The amount of *FMR1* promoter pulled down was quantified by quantitative polymerase chain reaction (qPCR) with promoter-specific primers. In control hESC-derived neurons, there was minimal *FMR1* transcript bound to the *FMR1* promoter at any time point during differentiation (Fig. 2B and fig. S11). Similarly, in FXS hESC-derived neurons at 12, 24, 36, and 60 days, minimal *FMR1* transcript was bound to the promoter (fig. S11). However, at 45 days in FXS hESC-derived neurons, *FMR1* mRNA was readily detectable on the *FMR1* promoter (Fig. 2B). Linearization of the CGG hairpin is required for binding of the FXS *FMR1* mRNA to the FXS *FMR1* promoter, as treatment of both FXS hESC lines with 1a, but not 1f, reduced binding of the *FMR1* RNA to the promoter during differentiation (Fig. 2C).

The length of the CGG-repeat tract is the major determinant for *FMR1* gene silencing (17). Normal alleles (less than 55 repeats) and "premutation" alleles (55 to 200 repeats) do not lead to *FMR1* silencing (17). We therefore asked if the *FMR1* transcript binds to the *FMR1* promoter in normal and premutation lines. In normal (~30 repeats) and premutation (70 and 73 repeats) hESC lines (fig. S1), the *FMR1* transcripts were not bound to the promoter (Fig. 2B). Thus, the lack of *FMR1* silencing in normal and premutation hESC lines may reflect the absence of *FMR1* mRNA binding to the promoter.

To more precisely define the temporal sensitivity of *FMR1* silencing to 1a, we selectively applied 1a during days 1 to 30 (fig. S12) or 31 to 60 of differentiation (Fig. 3). Only application of 1a during days 31 to 60 blocked silencing (Fig. 3). Similarly, application of *FMR1*-specific shRNA during days 31 to 60 also prevented differentiation-induced silencing (fig. S13). These data suggest that *FMR1* mRNA does not trigger gene silencing during the first 30 days but is required in the second 30-day period when it binds to the *FMR1* gene and leads to gene silencing.

We next asked whether *FMR1* gene silencing can occur after the 48- to 51-day time point of the differentiation protocol. In these experiments, we inhibited *FMR1* silencing by culturing differentiating FXS hESCs in the presence of 1a for 60 days. Withdrawal of 1a for 10 days triggered *FMR1* silencing (fig. S14), which suggests that sustained 1a treatment is required to maintain *FMR1* in the transcriptionally active state. The small molecule 1a appears to function to prevent silencing, rather than reverse silencing, as application of 1a to cells with an already silenced *FMR1* promoter did not reverse silencing (fig. S15).

We next mapped the part of the *FMR1* transcript that binds to the *FMR1* gene. To do this, we performed ChIRP using biotinylated primers that hybridize to different regions of the *FMR1* transcript (Fig. 4A). Primers that hybridize adjacent to the CGG repeat pulled down the *FMR1* promoter, whereas primers directed elsewhere along the *FMR1* transcript resulted in markedly reduced pull down (Fig. 4B). Together with the finding that 1a blocks the binding of *FMR1* mRNA to the *FMR1* gene, these data suggest that the CGG-repeat region of the *FMR1* transcript interacts with the *FMR1* gene.

We next mapped the part of the *FMR1* gene that is bound to the *FMR1* mRNA. In our previous ChIRP experiments, we measured the binding of the *FMR1* mRNA to a portion of the *FMR1* promoter that lies 92 to 196 base pairs (bp) upstream of the CGG-repeat sequence. To more precisely define the binding site, we measured the ChIRP signal both upstream and downstream of the genomic CGG-repeat sequence in differentiating FXS hESCs. Because of the high G/C content of the repeat sequence, binding to this region cannot be tested. We found that the ChIRP signal was detectable on both sides of the ~1200-bp genomic CGG repeat and is markedly reduced at sites away from the repeat (Fig. 4C). This pattern of binding is consistent with the genomic CGG-repeat sequence being the binding site for the *FMR1* mRNA (see fig. S16).

Some noncoding RNAs have been shown to interact with promoters indirectly through a protein intermediate (18). To determine if *FMR1* mRNA binds to its promoter through a protein intermediate, we performed ChIRP experiments, except we treated the cross-linked lysate with trypsin to digest any protein intermediates. For the control *TERC* noncoding RNA (15), the binding to its target promoters was abolished after trypsin treatment (Fig. 4D). However, *FMR1* mRNA binding to the *FMR1* gene was not affected by trypsin (Fig. 4D).

We next asked if *FMR1* mRNA binds to the *FMR1* gene by forming an RNA•DNA heteroduplex. To test this, we used ribonuclease H (RNase H), which selectively degrades RNA hybridized to DNA. Treatment of the cross-linked DNA fragments with RNase H selectively prevented the pull down of the *FMR1* promoter but did not affect the *TERC* pull downs (Fig. 4D). These data indicate that *FMR1* mRNA binds the *FMR1* gene through a direct RNA·DNA duplex and does not require a protein intermediate.

The *FMR1* mRNA CGG repeat may bind to the complementary CCG portion of the DNA that becomes accessible while it is being transcribed. Indeed, transcription through G-rich sequences causes stalling in vitro and in vivo (19, 20). Conceivably, the nascent *FMR1* CGG-repeat RNA interacts with the template strand of the unwound DNA to form a RNA•DNA duplex that is highly stabilized by its G/C content. This would require that the CGG-repeat sequence in the RNA achieves a sufficient length to reach back and interact with the DNA and may contribute to the requirement for >200 CGG repeats for silencing. In addition, the length of the resulting RNA•DNA duplex may need to be of sufficient length to activate downstream pathways that induce *FMR1* silencing.

The initial step in *FMR1* silencing is the binding of the *FMR1* mRNA to the genomic repeat. The inability of the *FMR1* transcript to bind to the DNA before day 45 may relate to DNA accessibility during transcription. The expression of diverse DNA helicases, which are known to regulate DNA accessibility (21, 22), is reduced during hESC differentiation (23). Conceivably, helicase activity may contribute to the temporal interaction of *FMR1* mRNA and DNA. However, the exact mechanisms underlying the temporal nature of *FMR1* silencing remain unknown.

The formation of the *FMR1* RNA•DNA duplex coincides with the initiation of epigenetic silencing in the *FMR1* gene. Because this causes a drop in *FMR1* mRNA expression,

subsequent maintenance of *FMR1* silencing is unlikely to be *FMR1* mRNA–dependent. It remains to be determined which mechanisms maintain epigenetic silencing of *FMR1* throughout the patient's lifetime.

FXS hESCs allow the characterization of the endogenous *FMR1* transcript transcribed from the endogenous promoter. This is important because RNA-directed gene silencing frequently occurs in cis with the nascent transcript affecting a promoter within the gene locus (24). Indeed, only *FMR1* and not other CGG repeats in the genome are silenced in FXS (25). Thus, rather than overexpressing CGG-repeat RNAs, which is complicated by plasmid instability, pharmacologic targeting of the endogenous CGG repeat provides insight into its role in promoter silencing.

Our data demonstrate that an mRNA can mediate promoter silencing and links trinucleotide repeat expansion to a novel form of RNA-directed promoter silencing. Epigenetic changes are seen in diverse repeat-expansion diseases (26, 27). The prevalence of repeat expansion–associated epigenetic changes raises the possibility that aspects of the mRNA-directed gene silencing pathway described here may contribute to gene expression alterations in other repeat-expansion diseases as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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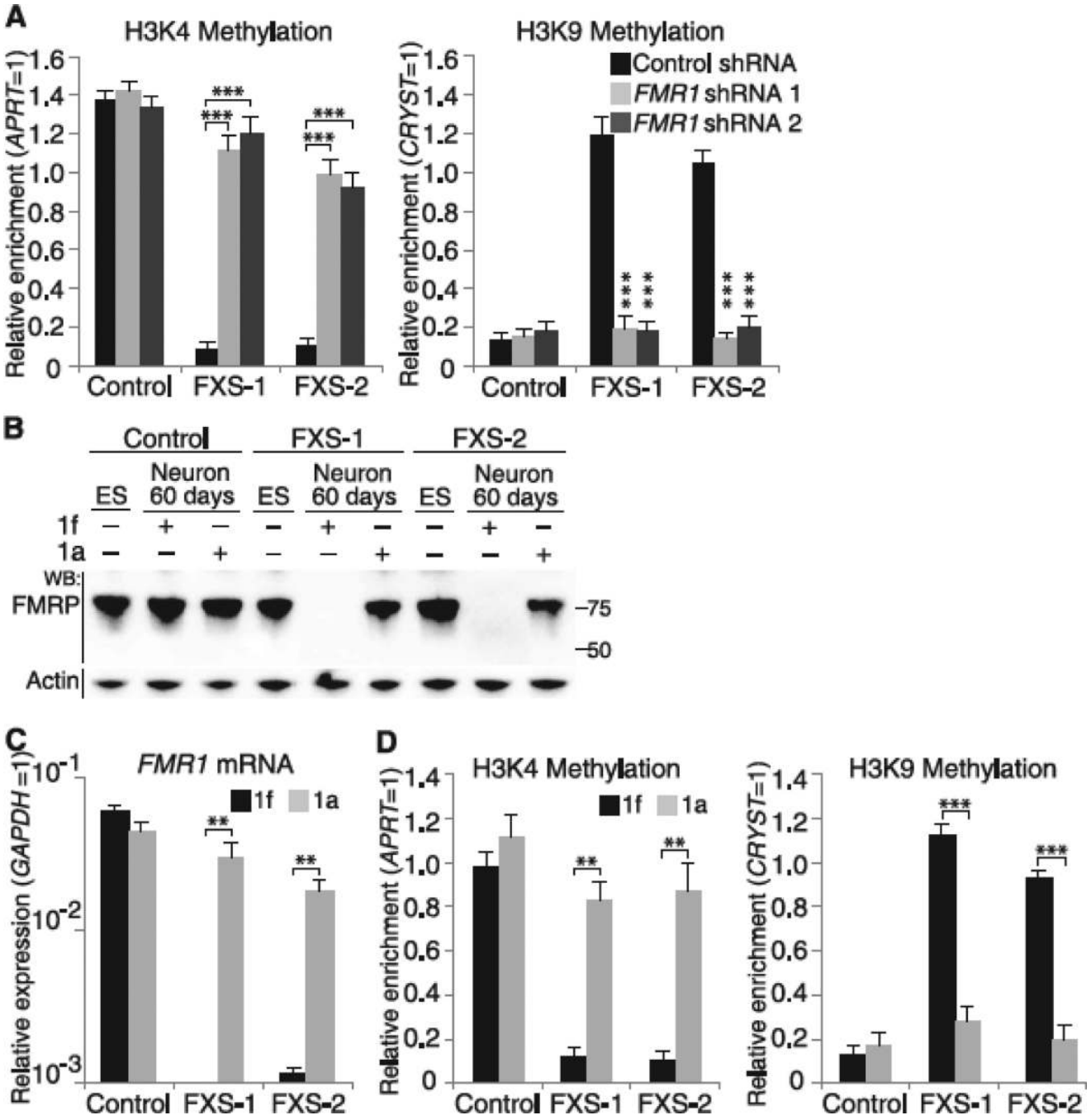

Fig. 1.

The *FMR1* transcript and its CGG-repeat tract are required for *FMR1* silencing. (**A**) *FMR1* mRNA is required for *FMR1* silencing in differentiating FXS hESCs. shRNA-expressing lentivirus was applied at day 1, and histone marks at *FMR1* promoters were measured at day 60. FXS hESCs expressing control shRNA showed high levels of transcriptionally repressive marks (H3K9me2) and low levels of transcriptionally active marks (H3K4me2). *FMR1*-specific shRNA prevented the appearance of repressive marks and maintained the expression of transcriptionally active marks ($n = 4$ per condition). ES, hESCs; WB, Western

blot; *APRT*, *adenine phosphoribosyltransferase*; *GAPDH*, *glyceraldehyde-3-phosphate dehydrogenase*; *CRYST*, *crystallin*. (**B** to **D**) The CGG-repeat RNA-binding small molecule 1a blocks *FMR1* silencing. Differentiating FXS hESCs treated with 10 μM 1a did not lose FMRP (B) or *FMR1* mRNA (C) ($n = 3$ per condition) and retained active *FMR1* promoters (D) ($n = 3$ per condition). Data are means ± SEM. Statistical analysis was performed using Student's *t* test (two-tailed distribution, $^{**}P < 0.01$, $^{***}P < 0.001$). When comparing different cell lines, we considered the samples as two samples with unequal variance. When comparing different conditions on the same cell line, we considered the samples as two samples with equal variance.

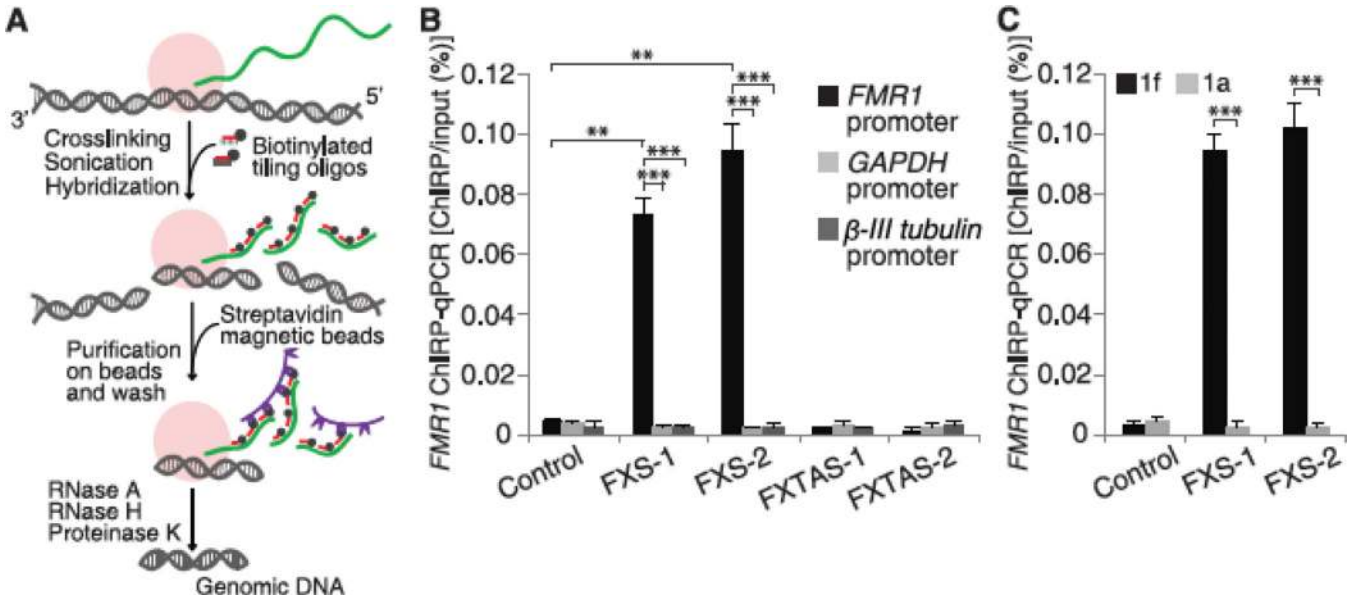

Fig. 2.

FMR1 mRNA interacts with the *FMR1* promoter in a CGG repeat–dependent manner. (**A**) Schematic representation of ChIRP technique [adapted from (15)]. RNA (green) and protein complexes (pink) are cross-linked to the DNA (gray) in cells by glutaraldehyde. The cell lysate is sonicated to shear DNA to ~500 bp. Streptavidin beads (purple) are used to pull down biotinylated oligonucleotides hybridized to RNA. Bound DNA sequences are detected by qPCR. (**B**) *FMR1* mRNA interacts with the *FMR1* promoter. *FMR1* mRNA bound to the *FMR1* gene was measured by ChIRP at day 45 of differentiation (see fig. S11 for other time points). *FMR1* mRNA was readily detectable on the *FMR1* promoter in FXS neurons but not control neurons ($n = 3$ per condition). *FMR1* mRNA does not bind to *FMR1* promoters in *FMR1* premutation lines, FXTAS-1 and FXTAS-2, that contain 70 and 73 CGG repeats, respectively. *GAPDH* and β-*III tubulin* promoters were used as controls. (**C**) The CGG-repeat portion of the transcript is required for the binding of *FMR1* mRNA to the *FMR1* gene. *FMR1* binding to the *FMR1* gene was markedly reduced in FXS hESC-derived neurons cultured in the presence of 1a. The control compound 1f did not block the *FMR1* transcript–*FMR1* gene interaction. Data are means ± SEM; Student's *t* test (two-tailed distribution, $^{**}P < 0.01$, $^{***}P < 0.001$). Different conditions on the same cell line were considered as two samples with equal variance; different cell lines were considered as two samples with unequal variance.

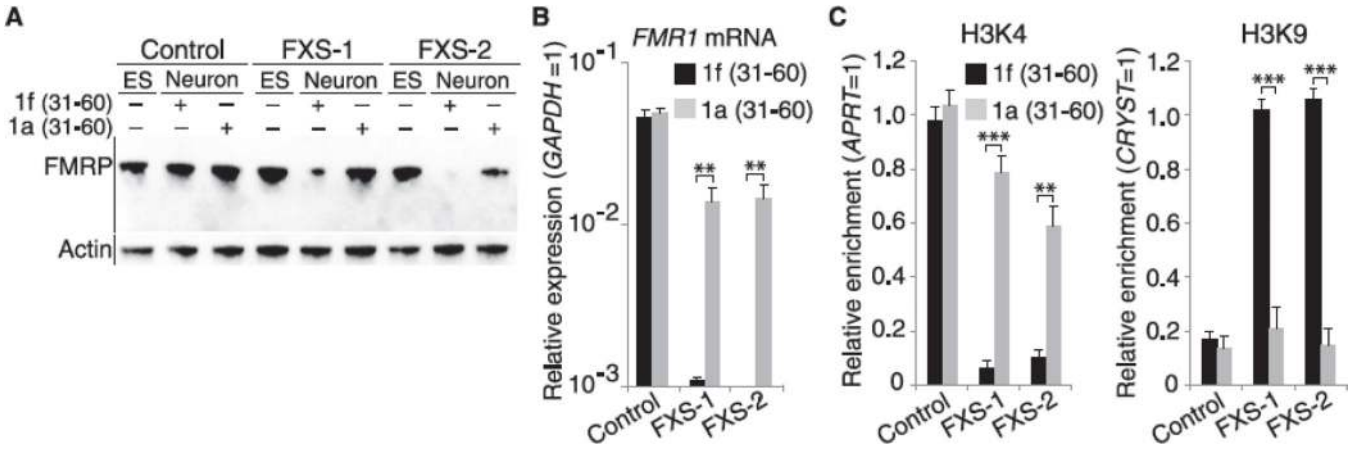

Fig. 3.

Temporal requirement for *FMR1* mRNA binding to the *FMR1* promoter. (**A**) The small molecule 1a blocks the drop in FMRP levels during days 31 to 60 of differentiation. To determine when *FMR1* mRNA is required for *FMR1* silencing, we applied 1a to FXS hESCs during days 31 to 60 of differentiation. 1a maintained FMRP expression in FXS neurons. (**B** and **C**) 1a prevents *FMR1* silencing. In FXS neurons, application of 1a during days 31 to 60 of differentiation was sufficient to maintain *FMR1* mRNA levels [(B), quantitative reverse transcription polymerase chain reaction (qRT-PCR), $n = 4$ per condition], high levels of H3K4me2 and low levels of H3K9me2 histone modifications [(C), chromatin immunoprecipitation, $n = 4$ per condition]. Control hESCs were unaffected by 1a. Application of 1a (10 μM) during days 1 to 30 of differentiation failed to prevent *FMR1* silencing (see fig. S12). Data are means ± SEM; Student's *t* test (two-tailed distribution, $^{**}P < 0.01$, $^{***}P < 0.001$). Different conditions on the same cell line were considered as two samples with equal variance.

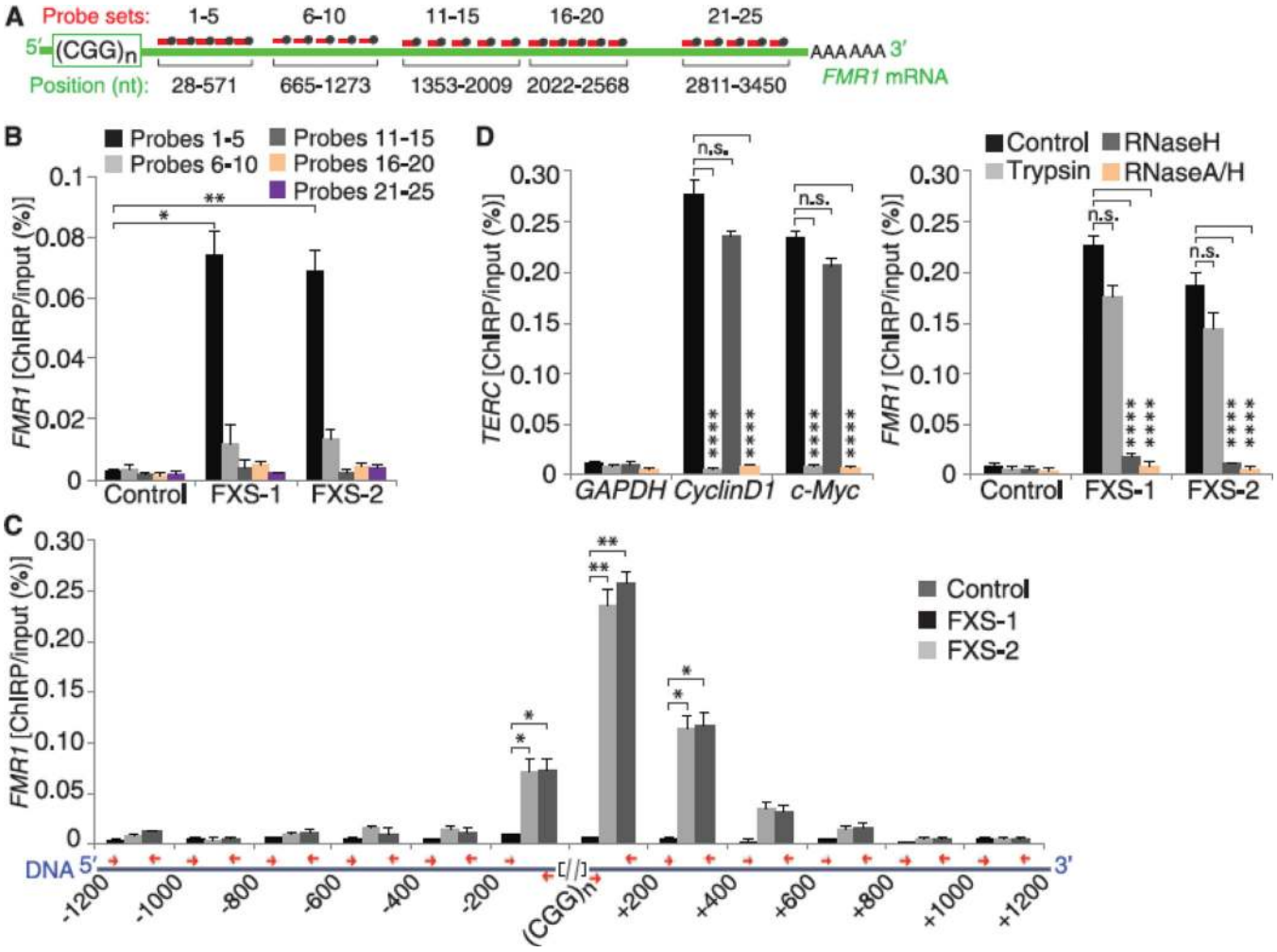

Fig. 4.

The CGG-repeat portion of the *FMR1* mRNA hybridizes to the complementary region of the *FMR1* DNA. (**A**) Schematic of the hybridization sites of the different biotinylated-oligonucleotide sets used to pull down sheared RNA in ChIRP experiments (see methods for further details on probe design). (**B**) The 5′ UTR portion of the *FMR1* transcript binds to the *FMR1* gene. ChIRP was performed using the probe sets shown in (A). Only probes that bind the CGG repeat–proximal portion of the *FMR1* transcript pulled down the *FMR1* promoter in FXS neurons ($n = 4$ per condition). (**C**) The *FMR1* transcript binds to the CGG-repeat portion of the *FMR1* gene. To determine where the *FMR1* mRNA binds on the *FMR1* gene, we measured the ChIRP signal along a 1200-bp region both upstream and downstream of the genomic CGG repeat. The positions of the primers used to amplify portions of the *FMR1* gene (blue) are indicated (red arrows) (referred to as bp relative to the 5′ or 3′ end of the CGG repeat). The ChIRP signal was highly enriched adjacent to the genomic CGG repeat ($n = 3$ per condition) (see also fig. S16). (**D**) The *FMR1* mRNA binds to the *FMR1* DNA in a protein-independent and RNase H–sensitive manner. The binding of the control noncoding-RNA *TERC* to its target promoters (*CyclinD1* and c-*Myc*) was abolished after trypsin treatment ($n = 3$), whereas the binding of the *FMR1* transcript to the *FMR1* gene was

unaffected by trypsin in FXS hESC-derived neurons ($n = 3$ per condition). In contrast, RNase H treatment only blocked the *FMR1* ChIRP signal ($n = 3$ per condition). RNase A–RNase H treatment, which digests all RNA, is used as a control to demonstrate the RNA-dependence of the ChIRP signal. Data are means ± SEM; Student's *t* test (two-tailed distribution, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$). Different conditions on the same cell line were considered as two samples with equal variance; different cell lines were considered as two samples with unequal variance.