New Perspectives on the Biology of Fragile X Syndrome

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Abstract

Fragile X syndrome (FXS) is a trinucleotide repeat disorder caused by a CGG repeat expansion in *FMR1*, and loss of its protein product FMRP. Recent studies have provided increased support for the role of FMRP in translational repression via ribosomal stalling and the microRNA pathway. In neurons, particular focus has been placed on identifying the signaling pathways such as PI3K and mTOR downstream of group 1 metabotropic glutamate receptors (mGluR1/5) that regulate FMRP. New evidence also suggests that loss of FMRP causes presynaptic dysfunction and abnormal adult neurogenesis. In addition, studies on FXS stem cells especially induced pluripotent stem (iPS) cells and new sequencing efforts hold out promise for deeper understanding of the silencing process and mutation spectrum of *FMR1*.

Keywords

fragile X syndrome; intellectual disability (ID); translational regulation; ribosomal stalling; microRNAs; long term depression (LTD); group 1 metabotropic glutamate receptors; presynapse; adult neurogenesis; human embryonic stem cell (hESC); induced pluripotent stem cell (iPS); missense mutation

Introduction

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability (ID) and the leading monogenic cause of autism spectrum disorders [1]. In almost all known cases of FXS, the causative mutation is a trinucleotide CGG expansion in the 5'-untranslated region of the fragile X mental retardation gene, *FMR1*. In humans, the number of CGG repeats is highly polymorphic. Normal individuals have between 6–54 repeats, with 29 or 30 repeats being the most common allele. When the number of repeats expands to between 60–200, it is referred to as a premutation allele. When the repeat number reaches over 200, it is known as a full mutation and leads to hypermethylation and epigenetic silencing of *FMR1*, resulting in the loss of its protein product, fragile X mental retardation protein, FMRP, which in turn causes FXS [2, 3]. FMRP is a selective RNA-binding protein found to have a major role in negatively regulating the translation of bound mRNAs, especially at synapses

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in neurons. Loss of FMRP impairs normal synaptic plasticity, which is believed to be the molecular basis for ID in FXS patients [4]. Recent studies are continuing to uncover new aspects of FMRP function in translational regulation and neural function. Particular focus has also been placed on identifying the signaling pathways that regulate FMRP in hopes of revealing new therapeutic targets. Furthermore, research on induced pluripotent stem (iPS) cells and next-generation sequencing efforts hold out promise for deeper understanding of the silencing and mutation spectrum of the *FMR1* gene.

Mechanisms of FMRP-Mediated Translational Regulation

Loss of FMRP results in increased translation of FMRP-bound transcripts, implying that FMRP normally acts as a translational repressor. The exact mechanisms of translational regulation by FMRP are not entirely clear, although mounting evidence suggests that FMRP inhibits translation of its target mRNAs by stalling ribosomes and via association with microRNAs (miRNAs). Recent findings are providing further support that these mechanisms are critical for the translational function of FMRP.

The majority of cytoplasmic FMRP is associated with polyribosomes and evidence that FMRP causes ribosome stalling was observed by treating cells with the translational inhibitors sodium azide or puromycin, which cause actively translocating ribosomes to be released or "run-off" the transcript [5–7]. Interestingly, following treatment, some FMRP was still associated with polyribosomes, suggesting that sodium azide and puromycin-resistant ribosomes were "stalled" in an inactive state. By using an *in vitro* translation system with endogenous brain polyribosomes, Darnell *et al.*[8] recently uncovered more evidence of ribosome stalling. They found that the presence of FMRP increased the number of ribosomes associated with FMRP-target transcripts following puromycin run-off, but did not affect the number of ribosomes on non-target mRNAs. The increased number of ribosomes stalled on FMRP-target transcripts implies that FMRP associated with stalled polyribosomes is a major mechanism of translational control.

Notably, high throughput sequencing of RNA transcripts co-immunoprecipitated with crosslinked FMRP revealed that FMRP is closely associated with transcripts throughout both the coding and non-coding regions and does not appear limited to known RNA binding motifs such as G-quadruplexes [8]. It is unclear how FMRP mediates such broad binding patterns, since RNA binding domains (KH domains and an RGG box) of FMRP are believed to only recognize RNA secondary structures such as "kissing complexes" and G-quadruplexes. It is possible that FMRP binds specific recognition sequences in its targets and then spreads along the transcript by associating with ribosomes as they are loaded on the transcript. Or perhaps, FMRP is merely part of a complex whose specificity is determined by one or more of the other components. Creating mutations in known G-quadruplex motifs and seeing how they might affect these results would be instructive. Despite the unanswered questions, the study by Darnell *et al.* provides strong support that FMRP regulates translation by stalling ribosomal translocation (Figure 1a).

In Drosophila and mammalian cells, FMRP is also found to interact with miRNAs and members of the RISC complex, including Dicer and Argonaute 2/eIF2C2 [9, 10]. FMRP has no miRNA/siRNA-binding domain, which indicates that miRNAs are likely associated with FMRP via interaction with other members of the RISC complex. In mice, this interaction is also evident in the postsynapse underscoring its relevance for synaptic translational control. Several miRNAs, such as miR-125b and miR-132, are selectively associated with the FMRP-RISC RNP complex in the mouse brain. Interestingly, regulation of a known FMRP target, NR2A, was recently shown to depend in part on miR-125b [11]. This study provides direct evidence that specific miRNAs facilitate the selection and repression of target

mRNAs by FMRP. In another recent study, the FMRP-miR-125a complex was shown to bind the 3'UTR of PSD-95 mRNA, inhibiting translation of the PSD-95 protein, a key component of postsynaptic organization. The authors further revealed that the translation inhibition of PSD-95 by FMRP-miR-125a is dependent on the phosphorylation of FMRP at serine 499 (human serine 500). Stimulation of mGluR, which leads to dephosphorylation of FMRP, or substitution of an unphosphorylatable mutant FMRP, S499A, relieved the translational repression and caused the dissociation of the RISC complex from PSD-95 mRNA [12]. Interestingly, FMRP does not disassociate from the message following the loss of the RISC complex suggesting that FMRP may not itself suppress ranslation but rather utilizes this function of the RISC complex (Figure 1b). Phosphorylation of FMRP was also reported to inhibit its association with Dicer, while increasing its affinity for pre-miRNA complexes [13]. Together, these studies suggest that microRNAs function as a critical component to modulate FMRP-mediated translation and that phosphorylation of FMRP acts as a switch in this pathway.

How ribosome stalling and miRNA-directed translational repression are temporally and spatially coordinated with each other remains to be determined. It seems reasonable to speculate that these mechanisms can occur separately in spatially distinct locations or at different times of the mRNA life-cycle, for example, prior to transport to the synapse or after synaptic stimulation. In other situations, the mechanisms may be coordinated on a single transcript, such that miRNAs facilitate the ability of FMRP to stall ribosomes. In recent findings with miRNA-mediated repression and in early studies of ribosome stalling, phosphorylation of FMRP appears to be the primary trigger for releasing the translation repression of FMRP-bound transcripts.

Besides the well-established ribosome stalling and miRNA-directed translational repression model, Napoli *et al.* suggest that FMRP can also suppress translation via inhibition of translation initiation [14, 15]. Recently, FMRP was also found to behave as a translational activator of the Sod1 mRNA, with the absence of FMRP resulting in decreased expression of Sod1 [16]. In addition, FMRP is shown to be involved in regulating mRNA stability [17, 18]. All these studies demonstrate that much remains to be learned about the role of FMRP in translational regulation.

Neuronal Dysfunction in FXS

Many FMRP target transcripts are localized in neuronal dendrites and play important roles in synaptic structure and function. The current working model is that FMRP accompanies specific target mRNAs to dendritic spines, where it regulates their translation in response to synaptic stimuli. In FXS, loss of FMRP leads to misregulation of activity-dependent local protein synthesis, which is evidenced by impaired synaptic plasticity. Unraveling the neuronal signaling pathways that are regulated by FMRP is a main focus for developing treatments to rescue FXS cognitive phenotypes. In wild-type neurons, activation of group I mGluR receptors rapidly increases protein synthesis of synaptic transcripts, including FMRP-bound transcripts, via mTOR and ERK-dependent pathways. Both pathways converge to increase eIF4E activity and initiate the assembly of the initiation complex 4F (eIF4F), the first step in the initiation of mRNA cap-dependent translation [19–21]. This group I mGluR-dependent protein synthesis induces long-term depression (LTD), a molecular basis of learning and memory, which is impaired in FXS [22, 23]. Recently, different observations on how the loss of FMRP affects the relative levels of mTOR and ERK signaling molecules have emerged. In one set of studies, increased activities of PI3K, Akt, and mTOR have been detected in cortical synaptoneurosomes and hippocampal lysates from *Fmr1* KO mice [19, 21]. Additionally, the inhibition of PI3K, but not inhibition of ERK, specifically rescued excess translation and subsequent AMPA receptor endocytosis

seen in the KO [19]. However, another study failed to observe any increased levels of mTOR pathway components in cultured brain slices from *Fmr1* KO mice and additionally showed that inhibition of ERK, but not mTOR, could rescue excess protein synthesis in the KO slices [24]. Differences in experimental procedures may cause such discrepancies; therefore, it remains to be determined how those results explain the *in vivo* status of the mGluR downstream signals in the absence of FMRP. Nevertheless, these studies suggest that FMRP modulates translation of its mRNA targets in an activity-dependent manner such as in response to mGluR stimulation.

Amygdala dysfunction is also a hallmark characteristic in FXS. It has been implicated that alterations in the GABA system, including dramatic changes in levels of expression of GABA receptors and the defects in GABAergic neurotransmission could contribute to circuit dysfunction in FXS [25, 26]. Initial findings of exaggerated LTD in FXS mouse models have largely focused on the postsynaptic function of FMRP. However, several studies now report that the loss of FMRP causes morphological and functional presynaptic abnormalities. Quantitative proteomic analysis shows that many presynaptic proteins involved in presynaptic specialization, vesicle recycling, excitability and neurotransmitter release are affected when FMRP is absent [27, 28]. High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) results also reveal that FMRP directly binds mRNAs encoding nearly one-third of the presynaptic proteome [8]. In addition, the loss of FMRP leads to altered short-term plasticity in excitatory synapses and excessive calcium influx in the presynaptic neurons during spike trains. Furthermore, faster vesicle recycling and enlarged vesicle pools are also observed in the absence of FMRP, which leads to reduced short-term depression (STD) [27]. Additionally, FMRP also directly interacts with the membrane protein Slack-B, a sodium-activated potassium channel, which is presumably expressed presynaptically. Further data also support the idea that FMRP functions as a potent activator of Slack-B to modulate neuronal firing patterns [29]. All these studies point to presynaptic dysfunction as a potential contributor to neurologic impairments in FXS.

Recent studies also reveal an unexpected regulatory role for FMRP in adult neurogenesis. Adult neurogenesis occurs mainly at the subgranular zone of the dentate gyrus in hippocampus and the subventricular zone of the lateral ventricles, both of which contain adult neural stem cells/progenitor cells (aNSCs), generating new neurons and glia. Although the precise role of adult neurogenesis in learning and memory is unclear, mounting evidence suggests that it plays a role in adult neuroplasticity and hippocampus-dependent learning [30, 31]. Deletion of FMRP from adult neural stem cells in mice leads to impaired performance in hippocampus-dependent learning tasks and these defects can be rescued by restoring FMRP expression [32]. FMRP-deficient aNSCs also display increased proliferation, decreased neuronal differentiation, and increased glial differentiation, which in turn alters the fate specification of aNSCs. The altered aNSC function is partially dependent on CDK4 and GSK3β signaling, which are both known FMRP targets of translational repression [33].

Silencing of the *FMR1* full mutation

Understanding how and when the expanded CGG repeat is methylated and silenced in FXS is critical for a broader understanding of the disease etiology and potential development of therapies aimed at rescuing *FMR1* expression. The identification of high-functioning fragile X males who have near normal intelligence and carry unmethylated full mutation alleles holds out additional promise that preventing or rescuing *FMR1* silencing is a viable pursuit [34, 35].

Studies investigating the developmental timing of *FMR1* silencing, using human embryonic stem cells (hESCs) and chorionic villi (CV) samples from FXS pregnancies, have found that, at early embryogenesis, when extra embryonic tissue separates from the embryo proper, the FXS full mutation alleles are still active. Thus, in early development, *FMR1* remains unsilenced, presumably producing transcripts with long CGG tracts [36, 37]. The mechanisms behind this unique epigenetic event in response to DNA sequence variation remains unclear. Use of hESCs may be useful, although the continuous generation of hESCs presents an ethical challenge. Moreover, incorporation of the CGG repeat expansion in mice fails to recapitulate the human hypermethylation seen in FXS. One emerging solution may lie in continuing advances to reprogram somatic cells into iPS cells. Generating iPS cells bypasses the ethical problems with creating hESCs and offers a great opportunity to dissect changes in methylation during development and differentiation. Recent studies reported the derivation of the first FXS-iPSC lines from FXS patients and surprisingly found that the CGG repeat region remained hypermethylated, which differs from findings in FXS hESC [38, 39]. A possible explanation for the discrepancy may be that FMRP-dependent signaling pathways, dysfunctioning in FXS cells, are required for *FMR1* reactivation. In this scenario, FMRP may directly regulate the silencing of its own transcript or indirectly modulate its methylation via translational control of target mRNAs. Another, perhaps more plausible, explanation for the difference in *FMR1* methylation between iPSCs and hESCs could be that human iPS cells represent a later stage of development in which silencing of the full mutation has already occurred; thus, FXS-iPS cells may not have all the full characteristics of early pluripotency. FXS-iPS cells show very similar pluripotent characteristics with hESCs [36, 38], nevertheless, studies have found that different stages of pluripotency are critical for certain epigenetic events, such as X-inactivation. Investigators have since discovered how to create iPSCs that represent an earlier developmental stage, called the ground state/naïve pluripotent stage, which shares more similar characteristic of mouse ES cells, such as both active X chromosomes in females [40, 41]. The generation of iPS cells to a state before X-inactivation is encouraging because CV sampling from *FMR1* full mutation carriers suggests that X-inactivation occurs prior to inactivation of the full mutation allele. In these samples, X-inactivation was evident by 10 weeks gestational age but FMRP silencing did not occur until 10–12.5 weeks [37]. It is worth noting that in humans, the epigenetic regulation in extra-embryonic tissues such as CV is different compared with the embryo, thus, the exact time for full mutation silencing in embryos remains unclear. Nevertheless, the successful generation of naïve FXS-iPS cells should allow new investigations into the epigenetic status of the full mutation in this naïve pluripotent stage.

Successful generation of naïve/ground state FXS-iPS cells with reactivated *FMR1* will allow the exploration of many hypotheses including one intriguing mechanism of transcriptional silencing. RNA-directed transcriptional gene silencing (RDTS), which occurs in both plants and animals, uses small 22–26nt RNA fragments processed by the RNAi machinery to induce methylation of the target transcript [42]. This is interesting because FMRP is known to bind its own *FMR1* transcript and also associates with Argonaute and Dicer in the RISC complex. In addition, transcripts with long CGG tracts are known to be cut by Dicer in vitro [43]. Thus, FMRP might direct binding of the RISC complex on the *FMR1* transcript and lead to production of 22–26nt CGG fragments, which then facilitate *FMR1* methylation and silencing by directing histone modifying proteins to the locus. This is just one example of how studying FXS-iPS cells may give new insight into understanding these processes.

FMR1 mutations in FXS

Currently, clinical testing of the CGG repeat size is the standard of care for the diagnosis of FXS. However, identification of point mutations, insertions, or deletions at the *FMR1* locus, could increase the overall diagnostic yield and help account for a portion of undiagnosed ID.

Indeed, several cases have been reported for these non-conventional mutations in FXS patients. Many examples of *FMR1* deletion have been reported in patients with FXS like phenotypes [44, 45]. Also, a missense mutation of I304N in the second FMRP KH-type RNA-binding domain which alters the biological function of FMRP was found in a patient with severe ID [46–48].

To find additional pathogenic sequence variants, a recent study systematically screened 963 males who were clinically referred for FXS testing but showed normal CGG-repeat length. This study discovered several additional mutations in *FMR1*. One of the mutations, R138Q, occurs in a highly conserved residue within the nuclear localization signal of FMRP. Three additional mutations were found in the promoter region, all of which were shown to reduce reporter transcription *in vitro*. The authors postulate that the frequency of *FMR1* sequence variants causing developmental delay would be up to 0.8%. However, thorough functional testing will be needed to investigate the causality of these variants [49]. Regardless, it remains surprising that in the 20 years since the cloning of *FMR1* only two missense mutations have been uncovered while other X-linked ID loci, such as MECP2, have revealed over a hundred missense mutations [50]. While this paradox maybe partly due to the exclusive testing of the CGG repeat length, rather than DNA sequences, the study described above still suggests a deficiency of *FMR1* missense mutations. Such mutations must exist in the population and, indeed, the NHLBI Exome Variant Project has found seven additional missense mutations of *FMR1* (by Oct, 2011) in a heterogeneous group of samples drawn largely from adult onset common disease [51]. Therefore it is reasonable to conclude that missense mutations in *FMR1* may either have less impact on the protein function than missense mutations in many other proteins or that the correct population has not been sampled.

Conclusion

There has been great progress in our understanding of the role of FMRP in neurogenesis, presynaptic signaling, and translational regulation, as well as the *FMR1* mutation spectrum. In addition, identifying pathways up- and downstream of FMRP activation have revealed several possible targets for drug intervention. This avenue of research has been the impetus for several current clinical trials aimed at down-regulating the exaggerated mGluR activity seen in FXS by using mGluR antagonists or GABA agonists. Other downstream signals, such as PI3K, may also be potential targets. An alternative area of therapeutic potential is to identify the mechanisms for rescuing the silencing of *FMR1*. However, the translational repression caused by expanded CGG repeats needs to be overcome. Pluripotent stem cells derived from FXS patients may provide invaluable model systems for studying *FMR1* epigenetic silencing mechanism, as well as drug screening and creating *in vitro* neuronal model. With new discoveries will undoubtedly also come new complexities and the study of FXS has been replete with both.

Acknowledgments

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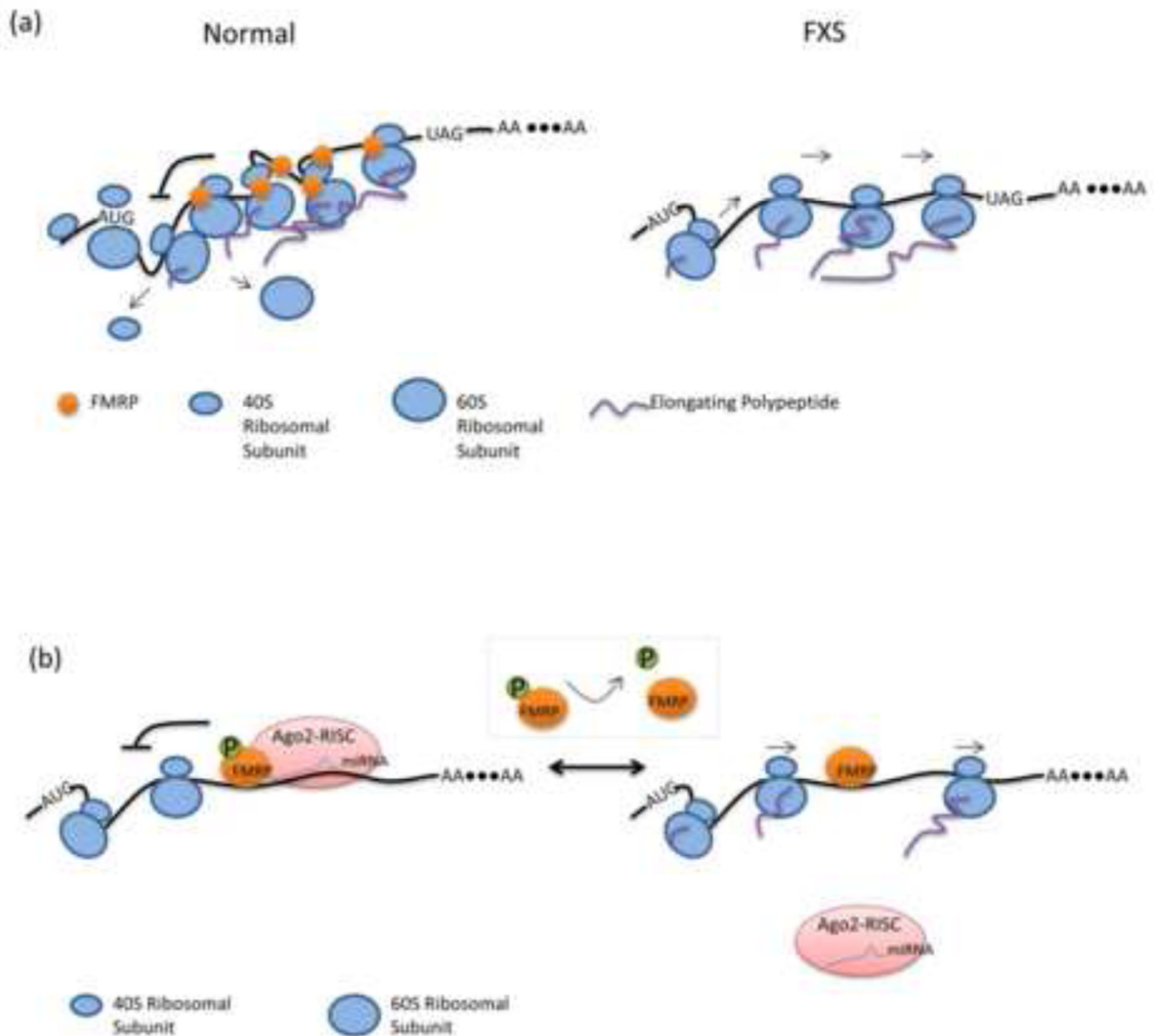
Figure 1.
Mechanisms of FMRP mediated translational regulation. (a) FMRP inhibits translation of target mRNAs by ribosome stalling. In normal cells, increased number of ribosomes are stalled on FMRP-target transcripts, which results in reduced translation. Darnell *et al.*[8] shows that a predominance of tags among FMRP target transcripts are distributed within the coding sequence by HITS-CLIP experiment. The stalling event occurs not only on target mRNAs bearing known secondary structures such as kissing complex and G-quadruplexes, but also on some mRNAs without these structures. In FXS cells, translational repression by FMRP via ribosome stalling is absent. (b) MiRNA-mediated translational repression by FMRP. FMRP is found to interact with miRNAs and members of the RISC complex. MiRNAs such as miR-125a, miR-125b and miR-132 are selectively associated with the FMRP-RISC RNP complex. These miRNAs in turn facilitate the selection and repression of target mRNAs by FMRP. Phosphorylation of FMRP acts as a switch for this mechanism.

Dephosphorylation of serine 499 causes the dissociation of the RISC complex from target mRNAs and relieves the translational repression. However, dephosphorylated FMRP remains associated with target mRNAs.
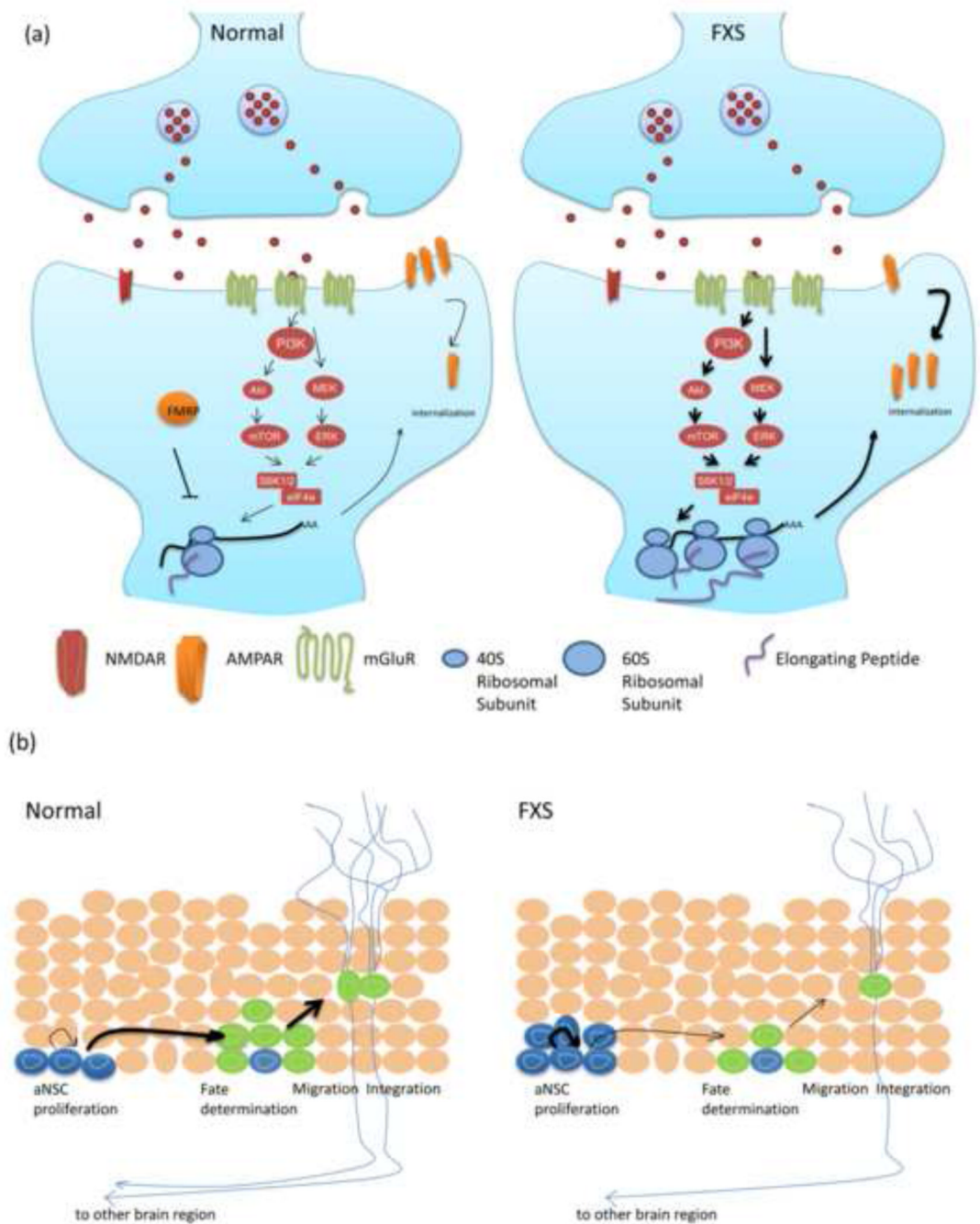
Figure 2.
(a) FMRP is a negative regulator in Group I mGluR-dependent protein synthesis. In wild-type synpase, activation of Group I mGluR receptors increases protein synthesis via the mTOR and ERK signaling pathways. Both pathways converge to increase eIF4E activity and initiate the assembly of eIF4F, the first step in the initiation of mRNA cap-dependent translation. This group I mGluR-dependent protein synthesis induces long-term depression (LTD), a molecular basis of learning and memory, which is impaired in FXS. In FXS, stimulation of Group I mGluR receptors causes excessive protein synthesis via increased mTOR and ERK signaling pathways, which leads to abnormal synaptic plasticity such as increased AMPAR internalization. (b) The role of FMRP in adult neurogenesis. The panel

shows the cells from subgranular zone of the dentate gyrus in hippocampus in mice. Compared with wild-type cells, Fmrp-deficient aNSCs display increased proliferation, decreased neuronal differentiation, and increased glial differentiation, which in turn alter the fate specification of aNSCs. Subsequently, the abnormal adult neurogenesis leads to impaired hippocampus-dependent learning.
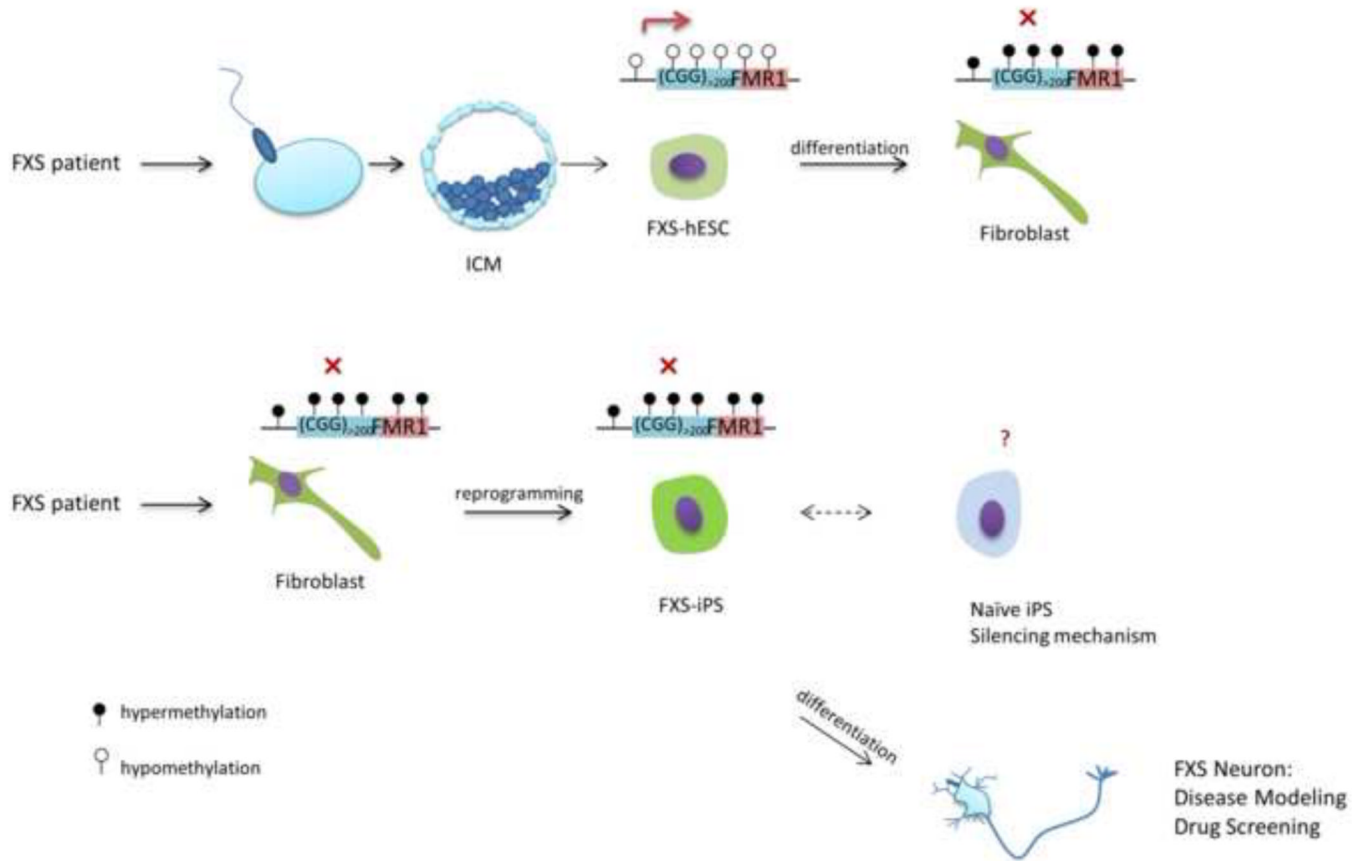
Figure 3.
The paradox of *FMR1* full mutation epigenetic status between FX-iPSC and FX-hESC. In FXS, during early embryonic development, the *FMR1* full mutation allele remains unsilenced. Analysis from one FXS-hESC line shows that the full mutation allele is still active, while during *in vitro* differentiation, *FMR1* undergoes epigenetic silencing. In contrast, the full mutation allele remains methylated in iPS cells reprogrammed from FXS patients' fibroblasts. The successful generation of naïve/ground state FXS-iPS cells will allow new investigations into the epigenetic status of the full mutation. These pluripotent stem cells derived from FXS patients provide invaluable model systems for studying *FMR1* epigenetic regulation, drug screening and *in vitro* neuronal modeling.