The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development

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The fragile X mental retardation protein (FMRP), a selective mRNA-binding protein implicated in regulating translation of its mRNA ligands, is a neuronal MAP playing critical roles in controlling cytoskeleton organization during neuronal development, and the abnormal microtubule dynamics is a conceivable underlying factor for the pathogenesis of fragile X mental retardation.

The absence of fragile X mental retardation protein (FMRP), a messenger ribonucleoprotein (mRNP) associated with a subclass of brain mRNAs, results in fragile X mental retardation, affecting 1 in 4,000 males and 1 in 8,000 females (1–3). The lack of FMRP results in delayed dendritic spine maturation in fragile X patients as well as in Fmr1 knockout (KO) mice, indicating the essential role of FMRP in synapse development. How FMRP governs normal synapse development in the brain remains elusive. Here we report that the developmentally programmed FMRP expression represses the translation of microtubule-associated protein 1B (MAP1B) and is required for the accelerated decline of MAP1B during active synaptogenesis in neonatal brain development. The lack of FMRP results in misregulated MAP1B translation and delayed MAP1B decline in the Fmr1 KO brain. Furthermore, the aberrantly elevated MAP1B protein expression leads to abnormally increased microtubule stability in Fmr1 KO neurons. Together, these results indicate that FMRP plays critical roles in controlling cytoskeleton organization during neuronal development, and the abnormal microtubule dynamics is a conceivable underlying factor for the pathogenesis of fragile X mental retardation.

Methods

Animals and Tissue Collection. WT and Fmr1 KO mice were raised at the Emory University animal facility and treated in accordance with National Institutes of Health regulations and under approval of the Emory University Institutional Animal Care and Use Committee. WT and Fmr1 KO littersmates were produced by breeding heterozygous females with Fmr1 KO males in congenic background of C57BL/6. The genotype of each animal was initially mapped by PCR (25) and confirmed by immunoblot analysis of FMRP. For tissue collection, hippocampi were dissected on ice, followed by total RNA isolation using TRIZol extraction (Invitrogen), or subjected to preparation of whole tissue lysate (17).

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Abbreviations: FMRP, fragile X mental retardation protein; mRNP, messenger ribonucleoprotein; KO, knockout; MAP1B, microtubule-associated protein 1B; Pn, postnatal day; eIF5, translation initiation factor 5; RPA, RNase protection analysis.

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concentration and duration indicated in the corresponding figure legends.

**Immunoprecipitation and RT-PCR.** Cytoplasmic extracts derived from WT and *Fmr1* KO P7 brain were subjected to immunoprecipitation using the monoclonal antibody 7G1-1 (18). RNA was extracted followed by RT-PCR analysis (Invitrogen). The sequence of primers used to detect MAP1B, MAP1A, and hypoxanthine phosphoribosyltransferase are available upon request.

**Linear Sucrose Gradient Fractionation.** Primary cultures of cortical neurons were incubated with cycloheximide (100 µg/ml) for 15 min to arrest polyribosome migration. Cytoplasmic extracts loaded on 15–45% (wt/vol) sucrose gradient were centrifuged at 39,000 rpm in a SW41 rotor for 60 min at 4°C and fractionated as described (9). EDTA-lysate was centrifuged on a parallel gradient lacking MgCl2 but containing 1 mM EDTA to dissociate polyribosomes into subunits. Total RNA was extracted from each fraction by phenol-chloroform extraction.

**Antibodies and Immunodetection.** For immunoblot analysis, the protein quantity of each sample was estimated by Bradford assay (Bio-Rad) before subjected to SDS/PAGE. Blots were stained by Ponceau S (Sigma) to confirm equal protein loading before immunoblot analysis. The primary antibodies were diluted as follows: 1C3, 1:1,000; translation initiation factor 5 (eIF5α), 1:5,000 (Santa Cruz Biotechnology); acetylated α-tubulin, 1:2,000 (Sigma); MAP1B, 1:100,000 (a gift from I. Fischer, Drexel University, Philadelphia); and SMI-31, 1: 5,000 (Steinberg Monoclonals).

For indirect immunofluorescent staining, cells raised on coverslips were fixed in 3.7% paraformaldehyde, 0.05% glutaraldehyde, and 0.5% Triton X-100 in PHEMO buffer (0.068 M Pipes/0.025 M Hepes acid/0.015 M EGTA Na2/0.003 M MgCl2/10% DMSO) at room temperature, and incubated with the antibody against α-tubulin (Chemicon) and conjugated secondary antibody. Fluorescence was detected at room temperature by a Zeiss LSM510 confocal microscopic imaging system.

**RNA Extraction and Analysis.** Total RNA was extracted by using TRIzol (Invitrogen). The quantity of RNA was determined by OD260 reading and further confirmed by ethidium bromide-stained gel electrophoresis. RNase protection analysis (RPA) was performed following standard protocols. The templates for RPA probes of MAP1B and GAP-43 were generated by RT-PCR of mouse brain RNA. Each RT-PCR fragment was cloned into pDrive (Qiagen, Valencia, CA), and sequence was confirmed to be 100% identical to those published previously. The GAPDH template was purchased from Ambion. Riboprobe templates were generated by in vitro transcription (Stratagene) in the presence of [32P]-UTP (Amersham Pharmacia).

**Results**

**FMRP Is Up-Regulated in the Neonatal Brain, Selectively Associated with the MAP1B mRNA.** To understand the role of FMRP in brain development, we first examined the expression profile of FMRP in the developing hippocampus. As shown in Fig. 1A, the level of FMRP reached a peak at the end of the first postnatal week, gradually declined thereafter, and was maintained at a moderate level through later development. As expected, FMRP was completely absent in the *Fmr1* KO littermates at all ages examined. A similar expression profile of FMRP was also observed in the cerebellum during neonatal development (data not shown). The peak of FMRP expression coincides with the maximal polyribosome localization beneath the dendritic spine in the normal brain (27) and with the most severe dendritic spine abnormality in the *Fmr1* KO brain (6), suggesting a functional requirement for high levels of FMRP in translation-dependent synapse maturation.

Among the proteins encoded by FMRP-associated brain mRNAs, MAP1B undergoes vigorous regulation when FMRP expression reaches the peak (28), therefore is a candidate for FMRP-dependent translation regulation during active synaptogenesis. As shown in Fig. 1B, abundant MAP1B mRNA was detected in the immunoprecipitated FMRP–mRNP complexes isolated from WT P7 brain. In contrast, no MAP1B mRNA was detected in the immunoprecipitates derived from age-matched *Fmr1* KO brain in parallel experiments. Interestingly, the mRNA encoding MAP1A, a neuronal MAP harboring high levels of sequence and functional similarity to MAP1B (29), was negligible in the FMRP–mRNP complexes. In addition, as a negative control, the mRNA encoding the house keeping protein hypoxanthine phosphoribosyltransferase (HPRT) mRNA are marked on the left.
The Absence of FMRP Results in Delayed MAP1B Decline in the Developing Hippocampus. To determine how FMRP may influence the developmentally programmed MAP1B expression, we compared the level of MAP1B gene expression in the hippocampus derived from WT and Fmr1 KO mice. By using an RPA, we detected a gradual decline in the steady-state levels of MAP1B mRNA in WT hippocampus within the first two postnatal weeks (Fig. 2A). The quantity of hippocampal MAP1B mRNA was nearly identical in Fmr1 KO mice as that in the age-matched WT controls, demonstrating that the lack of FMRP did not affect the steady-state level of the MAP1B mRNA. On immunoblot, MAP1B protein started to decline after P5 in the WT hippocampus (Fig. 2B), coinciding with the up-regulated expression of FMRP (Fig. 1A). However, the decline of MAP1B protein was delayed in the Fmr1 KO hippocampus, resulting in higher levels of MAP1B protein, with the most obvious difference detected at P10 (Fig. 2B). Immunofluorescent staining further revealed that the abnormally elevated MAP1B expression in the Fmr1 KO hippocampus was more obviously visualized in the hilus area and the mossy fiber terminals (Fig. 2C) that undergo vigorous neuronal network construction at this stage of development. These data support the hypothesis that FMRP functions to suppress MAP1B translation, and the absence of FMRP leads to misregulated MAP1B translation when MAP1B is subjected to the developmentally programmed down-regulation.

MAP1B Translation Is Abnormally Elevated in Primary Cultured Fmr1 KO Neurons. An important issue is to determine whether MAP1B is indeed misregulated in Fmr1 KO neurons, because expression of MAP1B is also found in the developing glia (30). To address this question, we compared MAP1B expression in primary cultured cortical neurons derived from embryonic day 16 WT and Fmr1 KO brain in parallel experiments. We found that, despite the normal quantity of MAP1B mRNA expression in the Fmr1 KO neurons (Fig. 3A), the level of MAP1B protein was significantly elevated (Fig. 3B).

Because the activity for MAP1B to modulate microtubule stability is regulated by both the quantity and the phosphorylation of MAP1B (31, 32), we next examined the GSK-3b kinase-dependent regulation of mode-1 phosphorylation of MAP1B (MAP1B-P1), which modulates the microtubule binding activity of MAP1B, in WT and Fmr1 KO neurons. When treated with lithium under concentrations that inhibit GSK-3b kinase activity (32), a comparable decrease of MAP1B-P1 was detected in both WT and Fmr1 KO neurons (Fig. 3C). Therefore, the regulation of MAP1B phosphorylation by GSK-3b appears unaffected by the absence of FMRP.

To test whether the abnormally increased MAP1B protein levels in Fmr1 KO neurons is indeed caused by misregulated translation, we analyzed the polyribosome profile of MAP1B mRNA in WT and Fmr1 KO primary cultured cortical neurons. As shown in Fig. 4A and B, the majority of MAP1B mRNA was associated with translating polyribosomes (fraction 4–10) in both WT and Fmr1 KO neurons. EDTA-treatment dissociated polyribosomes into ribosomal subunits and released MAP1B mRNA into the mRNP fractions (Fig. 4C), supporting this interpretation. Noticeably, there was a clear shift of the MAP1B mRNA to the most actively translating polyribosomes in the Fmr1 KO neurons (fraction 9 and 10, Fig. 4B and D). In contrast to MAP1B mRNA, the GAP43 mRNA that does not associate with FMRP (17) displayed nearly identical patterns of polyribosome association in WT and Fmr1 KO neurons (Fig. 4A and B). These data are consistent with enhanced MAP1B translation caused by the absence of FMRP.

Fmr1 KO Neurons Are More Resistant to Microtubule Depolymerization Caused by Nocodazole. The primary function of MAP1B in neuronal development is to modulate microtubule dynamics (23, 33). Increased MAP1B expression is associated with microtubule stabilization (29, 34). Therefore, we next questioned whether the elevated MAP1B expression in Fmr1 KO neurons is indeed misregulated in Fmr1 KO neurons, because expression of MAP1B is also found in the developing glia (30). To address this question, we compared MAP1B expression in primary cultured cortical neurons derived from embryonic day 16 WT and Fmr1 KO brain in parallel experiments. We found that, despite the normal quantity of MAP1B mRNA expression in the Fmr1 KO neurons (Fig. 3A), the level of MAP1B protein was significantly elevated (Fig. 3B).

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neurons may result in aberrantly increased microtubule stability, which could limit microtubule dynamics in synaptic formation and remodeling. MAP1B has been shown to protect microtubule polymers from disassembly against the microtubule destabilization drug nocodazole (29), which can be used as an experimental index to assess the biological consequence of elevated MAP1B expression in Fmr1 KO neurons. We treated parallel cultures of WT and Fmr1 KO cortical neurons with low concentrations of nocodazole for 2 h, and evaluated the nocodazole-mediated microtubule disruption by indirect immunofluorescence confocal microscopy. As shown in Fig. 5A, nocodazole-treatment resulted in a significant increase in the number of WT neurons that harbor disrupted microtubule network, marked by broken or dissolved microtubules. In contrast, such treatment did not have any effect on Fmr1 KO neurons. The increased resistance to nocodazole in Fmr1 KO neurons suggests an increase in microtubule stability, a predicted consequence of elevated MAP1B expression. High concentrations of nocodazole resulted in a severe disruption of microtubule networks and a blockade of neurite extension in both WT and Fmr1 KO neurons, indicating that the alterations in microtubule stability in Fmr1 KO neurons did not completely abrogate microtubule dynamics.

To more quantitatively evaluate the abnormal alterations of microtubule stability caused by the lack of FMRP, we measured the nocodazole-induced reduction of stable microtubule polymers in WT and Fmr1 KO neurons by immunoblot. In living cells, stable microtubule polymers are enriched with acetylated α-tubulin, whereas unstable microtubule polymers and soluble tubulin dimers are devoid of acetylated α-tubulin (35). Nocodazole inhibits microtubule polymerization, consequentially leading to microtubule depolymerization and reduced acetylated α-tubulin. As shown in Fig. 5B, exposing WT...
cortical neurons to nocodazole overnight resulted in a significant reduction of acetylated α-tubulin. However, such treatment resulted in only a minimal reduction of acetylated α-tubulin with no statistical significance in Fmr1 KO neurons. The increased resistance to nocodazole-mediated microtubule depolymerization in Fmr1 KO neurons leads us to conclude that microtubule stability is abnormally increased in fragile X neurons, and the elevated MAP1B translation caused by the loss of FMRP-dependent translation suppression is an obvious underlying mechanism for the aberrant microtubule dynamics.

**Discussion**

Our study demonstrates that FMRP binds to the MAP1B mRNA and acts to control the translation efficiency of MAP1B in brain neurons during neonatal development. The lack of FMRP-dependent translation suppression results in overproduction of MAP1B and aberrantly increased microtubule stability in Fmr1 KO neurons. This evidence suggests that FMRP is involved in regulating microtubule dynamics during brain neuron development. The activity-regulated localization of FMRP into dendrites and spines (36) further implicates the possibility for FMRP to regulate translation of MAP1B and microtubule dynamics within the individual dendrite and/or synapse, perhaps in response to neurotransmitter release. Considering the critical roles of microtubule dynamics in the development of neuronal networks, particularly in growth cone dynamics and synapse formation (32, 37–40), abnormal microtubule dynamics caused by FMRP deficiency provides a conceivable mechanism underlying the abnormalities in structural synaptic plasticity in fragile X mental retardation, represented by the delayed dendritic spine maturation in vivo (6) and the deficits in synapse formation in primary cultured neurons (41).

The essential role of MAP1B in supporting neurite extension has been well documented (34, 42). On the other hand, down-regulation of MAP1B in the neonatal brain coincides with the completion of the vigorous extension of neuronal processes and the initiation of active synaptogenesis (28). It is not understood whether the developmentally programmed down-regulation of MAP1B is physiologically important or simply represents a disused phenomenon. A gradual reduction of hippocampal MAP1B mRNA was observed during the first two postnatal weeks (Fig. 2A), most likely caused by reduced MAP1B transcription (43), which was unaffected by the absence of FMRP. Interestingly, a sharp decrease of MAP1B protein levels was observed after the elevated expression of FMRP (Fig. 1A), suggesting a role of FMRP in accelerating the decline of MAP1B by suppressing MAP1B translation. This hypothesis is further supported by the delayed decline of MAP1B protein in the Fmr1 KO hippocampus (Fig. 2B). The elevated MAP1B expression in the hilus of dentate gyrus and mossy fiber pathway (Fig. 2C) is consistent with the previously reported abnormal mossy fiber distribution in adult Fmr1 KO brain (44), suggesting a possibility for abnormal neuronal network formation that may contribute to the increased susceptibility to seizures in the Fmr1 KO mice and fragile X patients (45, 46). The fact that the delayed decline of MAP1B temporally associates with the most severe dendritic spine abnormality in the Fmr1 KO neurons (6) and the deficits in synapse formation in vivo also indicates the functional importance of MAP1B down-regulation in synapse development. At the adult stage, low levels of MAP1B expression is limited to brain neurons that are prone to drug and/or lesion induced plasticity (47, 48). In this regard, although misregulated MAP1B expression was more obviously detected in Fmr1 hippocampus in neonates (Fig. 2B), FMRP-dependent MAP1B regulation is likely used to govern the dynamic reconstruction of neuronal network throughout life. The fact that the Drosophila homologue of Fmr1 (dFmr1) regulates the microtubule-associated protein Futsch to control synapse development at the neuromuscular junction (24) further suggests that such regulation is evolutionarily conserved.

The enhanced polyribosome association of MAP1B mRNA in Fmr1 KO neurons (Fig. 4) indicates a loss of translation suppression by FMRP. The normal translation profile of mRNAs that are incapable for FMRP-association, represented by GAP43 mRNA (17), suggests that translation suppression of MAP1B mRNA depends on its biochemical association with FMRP, as demonstrated in the neonatal (Fig. 1B) and adult mouse brain (18). In addition, MAP1B mRNA harbors a G-quartet structure in the 5′ UTR, which has been shown to interact with FMRP.
directly in vitro (19). More recent studies raised the possibility that association of FMRP with its mRNA ligands may also involve micro-RNAs and other small noncoding RNAs (13, 21). Identification of sequence elements in MAP1B mRNA that mediate FMRP-dependent translation regulation in neuronal development is the next challenge for future studies.

An increasing number of recent reports indicate the critical role of MAP1B in modulating microtubule dynamics in the growing neurites, especially in the developing growth cones (23, 37, 38, 49) essential for neural network development. The role of MAP1B in modulating microtubule dynamics in the development is the next challenge for future studies. The elevated expression of MAP1B in Fmr1 KO neurons leads to aberrant microtubule dynamics (Fig. 5), which is expected to affect structural synaptic development. Abnormalities in dendritic spine maturation and synapse formation in the Fmr1 KO neurons (4–6, 41) are consistent with this view. Besides synaptic development, microtubule dynamics also controls the trafficking of neurotransmitter receptors to the synaptic junction (50). Thus, altered microtubule dynamics in Fmr1 KO neurons could affect neurotransmitter-dependent synaptic communication, as indicated by the abnormalities in glutamate receptor-mediated long-term plasticity (51). Moreover, interaction of MAP1B with actin (52) raises an intriguing possibility that MAP1B may be a link for the coordinated organization of microtubules and microfilaments to modulate growth cone dynamics and synaptic plasticity (23, 53).

In summary, our study demonstrates the functional requirement of FMRP in regulating the production of MAP1B in neonatal brain neurons, which governs the cytoskeleton dynamics for normal neuronal development and function. The absence of FMRP in fragile X neurons leads to elevated MAP1B expression due to the loss of translation suppression of the MAP1B mRNA. This, in turn, may result in more rigid cytoskeleton, which could account for the deficits of synaptic plasticity in fragile X mental retardation.

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