

S6K1 Phosphorylates and Regulates Fragile X Mental Retardation Protein (FMRP) with the Neuronal Protein Synthesis-dependent Mammalian Target of Rapamycin (mTOR) Signaling Cascade^{*[S]}

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Fragile X syndrome is a common form of cognitive deficit caused by the functional absence of fragile X mental retardation protein (FMRP), a dendritic RNA-binding protein that represses translation of specific messages. Although FMRP is phosphorylated in a group I metabotropic glutamate receptor (mGluR) activity-dependent manner following brief protein phosphatase 2A (PP2A)-mediated dephosphorylation, the kinase regulating FMRP function in neuronal protein synthesis is unclear. Here we identify ribosomal protein S6 kinase (S6K1) as a major FMRP kinase in the mouse hippocampus, finding that activity-dependent phosphorylation of FMRP by S6K1 requires signaling inputs from mammalian target of rapamycin (mTOR), ERK1/2, and PP2A. Further, the loss of hippocampal S6K1 and the subsequent absence of phospho-FMRP mimic FMRP loss in the increased expression of SAPAP3, a synapse-associated FMRP target mRNA. Together these data reveal a S6K1-PP2A signaling module regulating FMRP function and place FMRP phosphorylation in the mGluR-triggered signaling cascade required for protein-synthesis-dependent synaptic plasticity.

Fragile X syndrome is the most common form of inherited mental retardation and is caused by a functional absence of the RNA-binding protein, fragile X mental retardation protein, FMRP.³ The protein, FMRP, is known to associate with ~3% of the mammalian brain mRNAs, repressing their translation; many of these target mRNAs indeed appear overtranslated in the absence of FMRP (1). Electrophysiology of the fragile X mouse model has revealed a synaptic deficit in the hippocampus with elevated group I metabotropic glutamate receptor (mGluR)-mediated long term depression. Accordingly, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization is enhanced in the absence of FMRP, all thought due to constitutive overtranslation of a long-term depression (LTD)-required FMRP target mRNA(s) (2–4). Group I mGluR activity is also known to influence FMRP transport and synthesis (5, 6); however, little is known about regulation of FMRP itself.

Post-translational modifications are known regulators of activity-dependent protein synthesis (7), and FMRP is known to be phosphorylated at a highly conserved serine at position 499. The effects of FMRP phosphorylation on translation by ribosomal run-off assays suggested that non-phosphorylated FMRP associates with actively translating ribosomes, whereas phosphorylated FMRP is found in the context of potentially stalled ribosomes (8). We recently determined that FMRP is dephosphorylated by protein phosphatase 2A (PP2A) and, immediately following mGluR stimulation, PP2A dephosphorylates FMRP, corresponding with the translation of SAPAP3, an FMRP target mRNA (9). SAPAP3 is a post-synaptic scaffolding protein associated with PSD95, whose message was previously identified as an FMRP target mRNA in the mouse brain following FMRP immunoprecipitation and microchip analyses (10, 35, 36). Less than 2 min following mGluR activation, FMRP is rephosphorylated in a PP2A- and mammalian target of rapamycin (mTOR)-dependent fashion, correlating with SAPAP3 translational repression and suggesting phosphorylation as a regulator of group I mGluR-mediated FMRP translational suppression.

Here we identify ribosomal protein S6 kinase 1 (S6K1) as a major FMRP kinase in the mouse hippocampus. Time course studies following group I mGluR stimulation find that mGluR-triggered ERK1/2 and mTOR signaling mediate FMRP phosphorylation by S6K1. The temporal dynamic of S6K1-mediated FMRP phosphorylation is also regulated by PP2A enzymatic activity, suggestive of an S6K1-PP2A signaling module in the presence mGluR stimulation. Finally, S6K1 K/O mouse hippocampal lysates revealed the absence phospho-FMRP along with an increased expression of SAPAP3, similar to *Fmr1* K/O mouse lysates.

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³ The abbreviations used are: FMRP, fragile X mental retardation protein; mTOR, mammalian target of rapamycin; PP2A, protein phosphatase 2A; S6K1, protein S6 kinase; IP, immunoprecipitation; K/O, knock-out; KD, kinase dead; WT, wild type; GST, glutathione S-transferase; DHPG, dihydroxyphenylglycol; P, phospho; mGluR, metabotropic glutamate receptor; OA, okadaic acid.

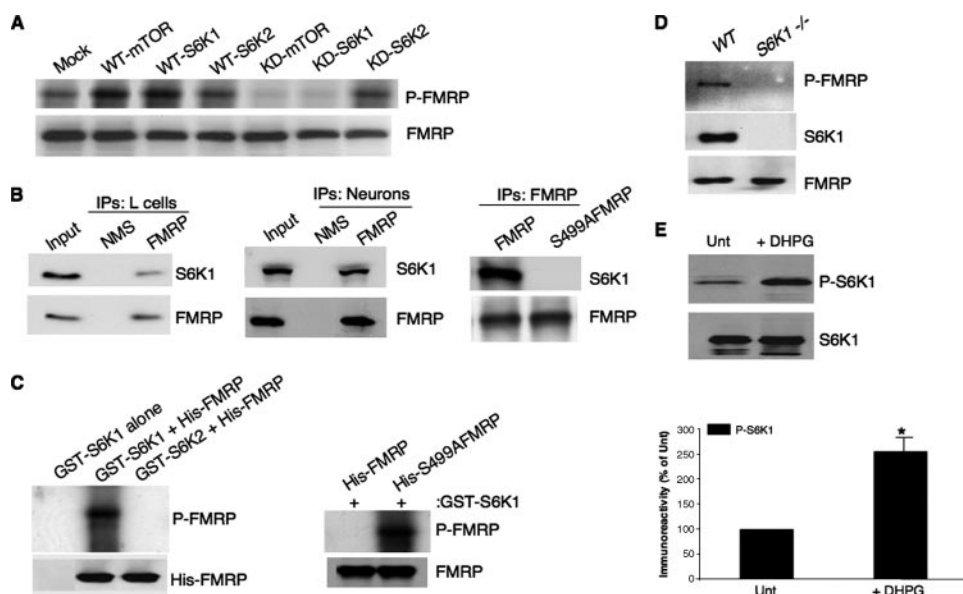


FIGURE 1. S6K1 is an FMRP kinase. *A*, WT or KD mTOR, S6K1, and S6K2 were transfected into L-cells stably expressing FLAG-FMRP; untransfected L-cells were used as control (*Mock*). P-FMRP was monitored by autoradiography following metabolic labeling in the presence of 0.5 nM OA and FLAG-IPs; FMRP was used as a loading control. *B*, S6K1 interacts with wild type but not non-phosphorylated FMRP. FMRP-IPs conducted from primary neuron/L-cell lysates were probed for S6K1 using normal mouse serum (NMS) as control; FMRP was used as a loading control for the Western blot (*left and middle panels*). FMRP-IPs conducted from lysates of wild type and S499A FMRP L-cells were probed for S6K1; FMRP was used as a loading control. *C*, S6K1 requires Ser-499 to phosphorylate FMRP *in vitro*. Recombinant His-FMRP was phosphorylated by purified, active GST-S6K1 but not by GST-S6K2 as scored by incorporation of [γ - 32 P]ATP; GST-S6K1 alone was used as a control. Recombinant His-FMRP but not His-S499A FMRP was phosphorylated by purified, active GST-S6K1 as scored by incorporation of [γ - 32 P]ATP. *D*, P-FMRP is not detected in the absence of S6K1. Hippocampal lysates from WT and *S6K1* K/O mice were probed for P-FMRP; FMRP was used as a loading control and S6K1 was used to verify the K/O. *E*, group I mGluR stimulation activates S6K1. Primary hippocampal neurons were treated with 100 μ M DHPG for 5 min and P-S6K1 at serine 421/424 was examined by Western blotting; total S6K1 was monitored as a control. P-S6K1 was normalized to total S6K1, calculated as \pm S.E., represented as a histogram, $n = 4$, * signifies $p < 0.05$ as calculated by a Student's *t* test. *Unt*, untreated.

EXPERIMENTAL PROCEDURES

Metabolic Labeling and Immunoprecipitation (IP) Analyses—Metabolic labeling and IPs were performed as in Ref. 6 with L cells and neurons plated in T75 flasks at a density of 5×10^6 and 3×10^5 cells/flask, respectively. The neurons were maintained in low serum medium prior to labeling, and each IP required cell lysates prepared from two T75 flasks plated at 3×10^5 cells/flask. The cytoplasmic lysates were generated and processed for FMRP IP as described (10).

Mice, Constructs, and Transfection—The *S6K1* and *S6K2* K/O mice have been characterized in Refs. 26 and 33. The wild type (WT) and kinase dead (KD) mTOR (FLAG-tagged), S6K1 (hemagglutinin (HA)-tagged), and S6K2 (hemagglutinin-tagged) were obtained from Addgene and used as in Refs. 11 and 12. The WT FLAG-FMRP construct and stable cell line are described in Ref. 6. Transient transfection was conducted using standard techniques (supplemental methods). GST-S6K1 and GST-S6K2 (kind gift of J. W. Hershey, McGill University) were purified from 293T cell lysates and used in kinase assays as described (13). Other constructs are described in the supplemental methods.

Drugs and Drug Treatments—Neuron cultures (supplemental methods) were treated with DHPG in Hanks' balanced salt solution (Invitrogen). At the end of the treatment, the cells were washed with ice-cold phosphate-buffered saline (pH = 7.4, Ca^{+2} free) and placed on ice. The cell monolayer was rapidly

scraped into ice-cold lysis buffer. Other drugs are described in the supplemental methods.

RESULTS

Group I mGluR activity is known to stimulate ERK1/2 and phosphatidylinositol 3-kinase (PI3K)-mTOR kinase signaling cascades (14–16), and we recently reported that mTOR influences the temporal pattern of FMRP phosphorylation through PP2A, a major FMRP phosphatase (22). Since kinases and phosphatases are known to form stable complexes regulating substrate phosphorylation in the context of synaptic activity (17–19), we evaluated serine-threonine kinases known to interact with PP2A and operational in activity-dependent mTOR and ERK1/2 signaling cascades as potential FMRP kinases (19–21).

Initially, we used known dominant-negative, catalytically inactive mutants of mTOR and S6K1 to study FMRP phosphorylation (Fig. 1A and Fig. S1A). Either WT or KD mTOR and S6K1 mutants were transiently transfected into L-cells stably expressing FLAG-FMRP, and

FMRP phosphorylation was monitored by metabolic labeling and FLAG-IPs. We used S6K2, a highly homologous S6 kinase (21–23), as a control, and the assays were conducted in the presence of 0.5 nM okadaic acid to suppress PP2A (24) and maximize detection of kinase activity. We found that both KD-mTOR and KD-S6K1 eliminated FMRP phosphorylation, whereas S6K2 did not (Fig. 1A and Fig. S1A). Likewise, only WT-mTOR and WT-S6K1 enhanced FMRP phosphorylation. The transfections were found to be efficient and relatively equivalent by Western blotting (Fig. S1B). Since S6K1 is known to function downstream of mTOR (25, 26), these data suggested S6K1 as a candidate FMRP kinase. Further, FMRP-IPs from both non-neuronal cells and primary hippocampal cultures revealed S6K1 complexed with WT FMRP (Fig. 1B, *left and middle panels*, and Fig. S1C), whereas non-phosphorylated FMRP (S499A FMRP) failed to associate with S6K1 (Fig. 1B, *right panel*, and Fig. S1D), confirming that serine 499 was required for the S6K1 interaction. However, since FMRP does not contain the canonical RXXXS S6K1 phosphorylation consensus site (27), we tested FMRP as a S6K1 substrate using *in vitro* kinase assays detecting phospho-FMRP only in the presence of purified, active GST-S6K1 but not GST-S6K2, indicating that FMRP is a S6K1 substrate *in vitro* (Fig. 1C, *left panel*). Importantly, serine 499 was required for FMRP phosphorylation *in vitro* as S499A FMRP was not phosphorylated by S6K1 (Fig. 1C, *right panel*, and Fig. S1E). These data confirm FMRP as

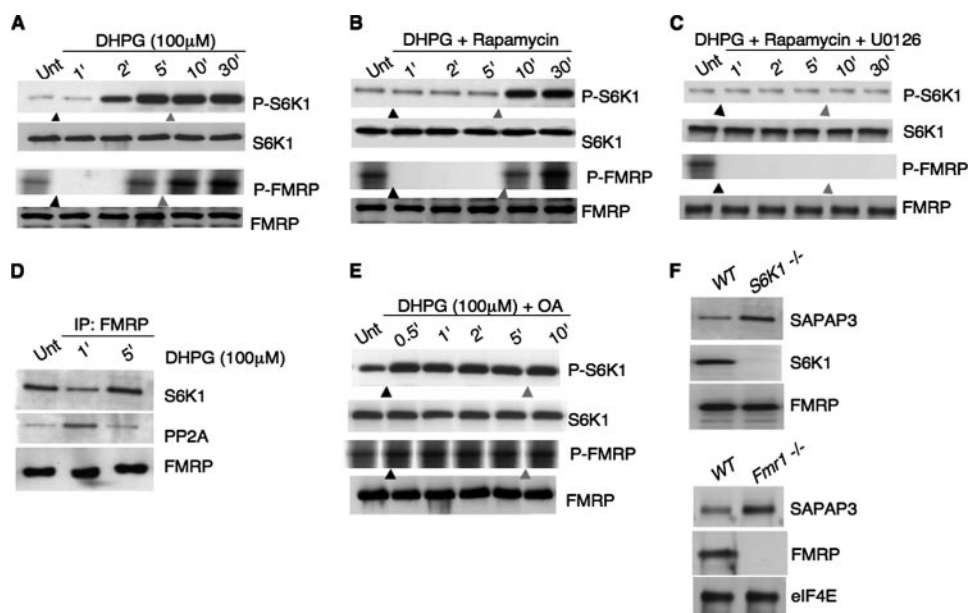


FIGURE 2. Activity-dependent S6K1 phosphorylation of FMRP requires mTOR, ERK1/2, and PP2A and influences downstream FMRP target mRNA translation. A–C, time course study of S6K1 activation in the presence of DHPG alone (A) or following preincubation with rapamycin (mTOR inhibitor) (B) or following preincubation with both rapamycin and U0126 (ERK1/2 inhibitor) (C). Primary neurons were treated with 100 μ M DHPG for 1, 2, 5, 10, and 30 min; an untreated (Unt) sample was used as control. DHPG was washed out after 5 min for physiological relevance as indicated by the gray arrowhead. P-S6K1 (serine 421/424) monitored S6K1 activation, and total S6K1 was used as control. Metabolic labeling at the same time points monitored P-FMRP; the DHPG-untreated sample was treated with 0.5 nM OA, and FMRP was used as a loading control. D, FMRP-IPs from neurons probed for S6K1 and PP2A in a DHPG time course reveal inverse associations between PP2A and S6K1 with FMRP at 1 and 5 min, respectively; FMRP was used as a loading control. E, time course study of S6K1 phosphorylation (serine 421/424) in the presence of 100 μ M DHPG applied for 0.5, 1, 2, 5, 10 min reveals an influence of PP2A activity in the presence of 100 nM OA. The assay was set up as panels A–C. F, upper panel, SAPAP3 shows increased expression in *S6K1* K/O hippocampal lysates as seen with *Fmr1* K/O hippocampal lysates (lower panel). Western blotting for S6K1/FMRP verified the K/Os.

an S6K1 catalytic substrate *in vitro*, albeit less efficient than the well known S6K1 substrate, ribosomal protein S6 (Fig. S2). To further validate FMRP as an *in vivo* S6K1 substrate, we used the *S6K1* K/O mouse (28–30). Using a previously described phospho-specific antibody recognizing phospho-serine499 (22), we found that hippocampal lysates from the *S6K1* K/O mouse lacked phospho-FMRP (Fig. 1D). Wild type mice revealed the presence of phospho-FMRP, and equivalent amounts of total FMRP were found between the two genotypes. Together, these data strongly support S6K1 as an FMRP kinase in the mouse hippocampus.

To understand the implications of S6K1 phosphorylation of FMRP in the context of neuronal activity, we first confirmed previous reports of S6K1 activation by group I mGluR-mediated ERK1/2 and mTOR pathways (15, 20, 31, 32). Indeed S6K1 phosphorylation on serine 421/424 (23) was observed in primary neurons treated with DHPG, a group I mGluR agonist using untreated neurons as control (Fig. 1E, upper and lower panels). To compare the temporal pattern of mGluR-dependent S6K1 activation with the FMRP phosphorylation pattern identified previously (22), we treated primary neurons with DHPG for 1, 2, 5, 10, and 30 min; an untreated sample was used as control (Fig. 2A and Fig. S3A). Under these conditions, S6K1 was modestly active at 2 min, becoming highly active at 10 and 30 min (as measured by serine 421/424 phosphorylation) with total S6K1 levels unchanged. Although S6K1 activation at 10 min of DHPG treatment was previously observed (13), the early

activation at 2 and 5 min was not previously reported (Fig. 2A and Fig. S3A). Further, FMRP-IPs found an increased S6K1-FMRP association upon 5 min of DHPG stimulation correlating with the increased FMRP phosphorylation seen at this time point (Fig. 2D and Fig. S3D), indicating that S6K1 mediates FMRP phosphorylation in an activity-dependent manner.

To examine the signaling that leads to FMRP phosphorylation by S6K1 in the presence of group I mGluR activity, we examined mTOR and ERK1/2, the two major mGluR-triggered pathways. Accordingly, we preincubated primary neurons with 20 μ M rapamycin, an mTOR inhibitor, either alone or in combination with 20 μ M U0126, an ERK1/2 inhibitor, followed by DHPG-mediated group I mGluR stimulation (Fig. 2B,C and Fig. S3, B and C). Strikingly, we found that S6K1 phosphorylation was delayed but not abolished in the presence of rapamycin at 10 min following DHPG stimulation, correlating with the delay in activity-dependent FMRP phosphorylation (Fig. 2B).

Given that rapamycin effectively inhibits S6K1 (21, 25), these data suggested that rapamycin-mediated S6K1 suppression by mGluR signaling might be delayed. This was confirmed by measuring S6K1 activity through phospho-S6 immunoreactivity and S6K1 kinase activity assays following DHPG stimulation alone or in the presence of rapamycin (Fig. S4, A, B, D, and E). ERK1/2, the other major mGluR-triggered signaling pathway, is also known to activate S6K1 (13, 23). In the presence of U0126 and rapamycin, S6K1 was completely inactivated, and FMRP phosphorylation was entirely abolished (Fig. 2C and Figs. S3C and S4, C and F). Rapamycin and U0126 treatment efficacy was confirmed by Western blotting for phospho-mTOR (P-mTOR) and P-ERK1/2 (Fig. S5). Since mGluR-triggered mTOR and ERK1/2 pathways are known to converge on S6K1 (20), these data suggest that mGluR-dependent S6K1 phosphorylation of FMRP occurs following the convergence of ERK1/2 and mTOR signals on S6K1.

To further position S6K1 phosphorylation of FMRP within the known mGluR-triggered pathway (13, 23, 24), we sought a link between S6K1 and PP2A, the FMRP phosphatase (22), since kinase-phosphatase modules regulate synaptic activity (41, 42) and PP2A inhibits S6K1 in mitogenic signaling (33, 34). We found that FMRP-IPs, at 1 or 5 min following DHPG stimulation, revealed an inverse correlation between the association of PP2A and S6K1 with FMRP in agreement with the pattern of FMRP phosphorylation (Fig. 2D and Fig. S3D). We next assessed the role of PP2A activity in S6K1-mediated FMRP

phosphorylation by monitoring S6K1 (serine 421/424) and FMRP phosphorylation in primary neurons preincubated with an excess of okadaic acid (100 nM) followed by DHPG treatment (Fig. 2E and Fig. S3E) with a DHPG-untreated sample as a control (Fig. S6). We included a 30-s time point as we had previously discovered an immediate-early burst in PP2A activity following DHPG exposure (22). As anticipated, an excess of okadaic acid revealed elevated and persistent S6K1 activation detected as phospho-S6K1 (serine 421/424), even at steady state (Fig. 2E and Fig. S3E), whereas DHPG alone yielded the previous pattern of S6K1 and FMRP phosphorylation (Fig. S6 and Fig. 2A). The increase in S6K1 phosphorylation at steady state that is potentiated in the presence of DHPG makes it likely that mGluR activity acts synergistically on PP2A inhibition of S6K1. Importantly, FMRP phosphorylation correlated tightly with the sustained increase in S6K1 activation mediated by PP2A inhibition (Fig. 2E and Fig. S3E), consistent with a possible mGluR-mediated PP2A-S6K1 signaling module regulating FMRP phosphorylation and perhaps downstream translation of FMRP target mRNAs.

To determine whether the lack of S6K1 and the consequential absence of phospho-FMRP influence FMRP-modulated translation, we studied SAPAP3 protein levels in the *S6K1* K/O mouse. SAPAP3 mRNA is an FMRP target (35–37), and SAPAP3 protein levels have been previously correlated inversely with FMRP phosphorylation (22). Hippocampal lysates from the *S6K1* K/O showed constitutively increased SAPAP3 expression in the absence of S6K1-mediated FMRP phosphorylation (Fig. 2F, upper panel) despite similar SAPAP3 mRNA levels in WT and *S6K1* K/O mice (Fig. S7A). This SAPAP3 difference in expression also remained unaffected in the presence of well known transcriptional and translational inhibitors, which were added to whole brain extracts to eliminate *de novo* protein synthesis that may occur during processing of samples (Fig. S7B). The efficacy of anisomycin was tested and quantitated using group I mGluR-mediated FMRP expression (Fig. S7C) since it is known that the increase in FMRP protein levels following mGluR stimulation is dependent on translation (2, 15). Interestingly, the observed increase in SAPAP3 in *S6K1* K/O mouse hippocampal lysates was similar to that seen in *Fmr1* K/O mouse (Fig. 2F, lower panel) and is consistent with the notion that phospho-FMRP negatively influences translation of target messages (6). Together, these data indicate that S6K1 activity mediates changes in FMRP phosphorylation and function.

DISCUSSION

FMRP is phosphorylated on a highly conserved serine residue, and it has been previously shown in *Drosophila* that *Drosophila* FMRP (dFMRP) is phosphorylated by casein kinase II (38). However, we failed to detect any effect of casein kinase II on FMRP phosphorylation in primary mammalian neurons in the presence of mGluR activity (Fig. S8A). Although it remains unclear whether the different kinases were found due to species differences, the data reported here find S6K1 as a major FMRP kinase using multiple approaches. Moreover, we tested PAK3 and eEF2K, kinases known to be downstream of S6K1 (39), and found no effect on FMRP phosphorylation, confirming S6K1

specificity for FMRP (Fig. S8, B and C). Furthermore, despite 80% sequence homology to S6K1 (22, 40), S6K2 did not affect FMRP phosphorylation, confirming that S6K1 was an FMRP kinase. Most significant, we failed to detect any phospho-FMRP in the hippocampus of *S6K1* K/O mice, validating S6K1 as the FMRP kinase.

The precise consequence of FMRP phosphorylation remains to be elucidated. However, data reported here and elsewhere (4, 22) suggest that changes in FMRP target mRNA expression concurred with FMRP phosphorylation suppressing target mRNA expression and FMRP dephosphorylation releasing/potentially activating target mRNA translation. One such message is SAPAP3, whose protein showed increased steady-state levels in the absence of FMRP (4). We show here that in the absence of phospho-FMRP, as in the *S6K1* K/O mouse, SAPAP3 protein levels were elevated, as seen in the *Fmr1* K/O mouse. This closely agrees with earlier suggestions that FMRP phosphorylation is coupled to translational suppression, whereas FMRP dephosphorylation releases target messages for translation (6). This overlap in the consequence of FMRP loss and S6K1 loss may also extend to similarities *Fmr1* and *S6K1* K/O mice in electrophysiological deficits and behavioral consequences (41, 42).

As described above, we observed that PP2A inhibition promotes S6K1 activation leading to FMRP phosphorylation at steady state, which is further enhanced in the presence of mGluR activity. These data are consistent with previous studies of mitogenic signaling where PP2A was found to inhibit S6K1 and mTOR was found to regulate both PP2A and S6K1 (33, 34). Our data extend this interplay to neurons, further indicating that mGluR acts synergistically on a pre-existing inhibition of S6K1 by PP2A. Together with prior reports of phosphorylation mediating protein folding (43), these data suggest a mechanistic model where changes in mGluR-mediated mTOR activity cause S6K1 and PP2A to compete for FMRP, perhaps modulated by FMRP phosphorylation. However, FMRP-IPs following mGluR stimulation revealed both S6K1-FMRP and PP2A-FMRP associations, suggesting that the binding between S6K1 and PP2A with FMRP may occur outside the FMRP phosphorylation pocket, thereby stabilizing the complexes and allowing detection, unlike other kinase-substrate interactions (44), which are transient and disrupted following substrate phosphorylation. These data would refine the model with a scaffold adapter protein(s) anchoring and coordinating the assembly/localization of an activity-dependent S6K1-PP2A-FMRP signaling complex. Such an arrangement would provide efficiency and specificity in signal transduction, as is seen with scaffold protein AKAP79 localized to excitatory neuronal synapses, securing a cAMP-dependent protein kinase A (PKA)-protein kinase C (PKC)-PP2B (PKA-PKC-PP2B) complex recruited to glutamate receptors by interactions with membrane-associated guanylate kinase (MAGUK) scaffold proteins (45, 46). Further investigation is required to validate such a scaffolding model, but it is an attractive hypothesis given the rapid dynamics of activity-dependent FMRP phosphorylation. This study places FMRP phosphorylation directly within the mGluR-triggered signaling cascade required for protein synthesis-dependent synaptic plasticity, a pathway central to the emerging function

of FMRP and the consequence of its absence in fragile X syndrome.

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