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FMRF phosphorylation and interactions with Cdhl regulate association with dendritic RNA granules and MEF2-triggered synapse elimination

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ABSTRACT

Fragile X Messenger Ribonucleoprotein (FMRF) is necessary for experience-dependent, developmental synapse elimination and the loss of this process may underlie the excess dendritic spines and hyperconnectivity of cortical neurons in Fragile X Syndrome, a common inherited form of intellectual disability and autism. Little is known of the signaling pathways that regulate synapse elimination and if or how FMRF is regulated during this process. We have characterized a model of synapse elimination in CA1 neurons of organotypic hippocampal slice cultures that is induced by expression of the active transcription factor Myocyte Enhancer Factor 2 (MEF2) and relies on postsynaptic FMRF. MEF2-induced synapse elimination is deficient in Fmr1 KO CA1 neurons, and is rescued by acute (24 h), postsynaptic and cell autonomous reexpression of FMRF in CA1 neurons. FMRF is an RNA binding protein that suppresses mRNA translation. Derepression is induced by posttranslational mechanisms downstream of metabotropic glutamate receptor signaling. Dephosphorylation of FMRF at S499 triggers ubiquitination and degradation of FMRF which then relieves translation suppression and promotes synthesis of proteins encoded by target mRNAs. Whether this mechanism functions in synapse elimination is not known. Here we demonstrate that phosphorylation and dephosphorylation of FMRF at S499 are both necessary for synapse elimination as well as interaction of FMRF with its E3 ligase for FMRF, APC/Cdh1. Using a bimolecular ubiquitin-mediated fluorescence complementation (UbFC) assay, we demonstrate that MEF2 promotes ubiquitination of FMRF in CA1 neurons that relies on activity and interaction with APC/Cdh1. Our results suggest a model where MEF2 regulates postranslational modifications of FMRF via APC/Cdh1 to regulate translation of proteins necessary for synapse elimination.

1. Introduction

Loss of function mutations in FMR1 cause Fragile X Syndrome in humans, a syndrome characterized by intellectual disability, autism, and epilepsy (Hagerman et al., 2017). Individuals with FXS and the mouse models of FXS, Fmr1 knockout (KO), display an increased number of dendritic spines on mature cortical neurons, suggesting there are deficits in the developmental elimination of excitatory synapses (Galvez and Greenough, 2005; Irwin et al., 2001; Pfeiffer and Huber, 2009). In support of this idea, experience dependent spine elimination is deficient in cortical neurons of the Fmr1 KO (Arroyo et al., 2019; Pan et al., 2010). There is also deficient developmental pruning of functional synaptic connections between local cortical neurons which results in hyperconnectivity of Fmr1 KO neurons (Patel et al., 2014; Testa-Silva et al., 2012).

FMR1 encodes Fragile X Messenger Ribonucleoprotein (FMRF), an RNA binding protein whose primary function is as a regulator of mRNA translation (Banerjee et al., 2018; Richter et al., 2015). Insight into the mechanisms by which FMRF regulates synapse elimination has come from work in hippocampal slice cultures. Acute expression (24 h) of the activity-dependent transcription factor Myocyte Enhancer Factor 2 (MEF2) in hippocampal CA1 neurons results in a rapid decrease in the number of functional excitatory synapses and dendritic spines, which fails to occur in Fmr1 KO neurons (Flavell et al., 2006; Pfeiffer et al., 2012).

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transcription to regulate translation of mRNAs that promote or allow elimination of synapses. If or how FMRP regulates translation during synapse elimination is unknown. It is also unknown if MEF2 regulates FMRP function or posttranslational modifications.

Current evidence indicates that FMRP suppresses translation of its mRNA targets through inhibition of translation initiation as well as elongation through inhibition of ribosomal movement (Banerjee et al., 2018; Richter et al., 2015). FMRP is highly expressed in neuronal processes, both axons and dendrites, where it associates with messenger ribonuclear protein (mRNP) complexes or RNA granules (Lai et al., 2020). These granules function to transport mRNAs to distal processes for localized translation near synapses. FMRP may function in the suppression as well as translational activation of mRNAs in granules to promote localized protein synthesis. Translation of mRNAs in dendritic granules can be activated by synaptic stimulation and in particular by activation of group 1 metabotropic glutamate receptors (mGluR1 and 5) (Lai et al., 2020). Translational activation of dendritic mRNAs such as Arc and PSD-95 require FMRP suggesting that FMRP functions as a translational switch to suppress translation under basal conditions and activate translation under stimulated conditions (Ifrim et al., 2015; Na et al., 2016; Niere et al., 2012). Such a property may be particularly important in dendrites and in the context of synaptic plasticity for rapid, localized protein expression. Post-translational modifications and rapid degradation of FMRP, via ubiquitination, have been implicated in such a translational switch mechanism (Prieto et al., 2020). Activation of mGluRs promote ubiquitination and proteasomal degradation of FMRP (Hou et al., 2006; Nalavadi et al., 2012; Zhao et al., 2011). Proteasome inhibitors block mGluR-stimulated translation of FMRP target mRNAs and associated forms of neuronal or synaptic plasticity, such as long-term synaptic depression (LTD) suggesting a requirement for FMRP degradation. FMRP is rapidly dephosphorylated at S499/500 (mouse/Fmr1) at the time of LTD induction suggesting a requirement for FMRP degradation. Inhibition of mGluR receptor stimulation is necessary for mGluR-induced ubiquitination and degradation of FMRP in neurons (Nalavadi et al., 2012; Narayan et al., 2007; Narayan et al., 2008). FMRP is ubiquitinated by the E3 ligase, Anaphase-promoting complex (APC) and interacts with APC through Cdh1, a regulatory and coactivating subunit of APC (Huang et al., 2015). Cdh1 deletion in neurons results in deficits in mGluR-induced ubiquitination and degradation of FMRP and LTD. Whether posttranslational modifications or degradation of FMRP occur in response to MEF2 or are necessary for MEF2-induced synapse elimination are unknown.

Here we demonstrate that acute pharmacological inhibition of Cdh1 blocks MEF2-induced synapse elimination in wildtype neurons and mutations in FMRP that prevent interaction with Cdh1, or dynamic phosphorylation at S499 fail to rescue synapse elimination in Fmr1 KO neurons. Consistent with a role for FMRP in translational control in RNA granules, we find that mutations in the Cdh1 interaction motif (D-box) of FMRP or S499 regulate the association or levels of FMRP in putative RNA granules in dendrites. Using a bimolecular fluorescent complementation assay to measure ubiquitination of FMRP in live CA1 neurons, we find that MEF2 triggers ubiquitination of FMRP which requires Cdh1 activity and FMRP interaction with Cdh1. These results suggest that MEF2 triggers degradation of FMRP via regulation of its phosphorylation to either de-repress or promote translation of mRNAs necessary for synapse elimination.

2. Materials and methods

2.1. Cell culture and transfection

Wildtype and Fmr1 KO C57B6/Cr mice were obtained from onsite breeding colony and new wildtype breeders were obtained from Charles River Laboratories. All experimental protocols involving mice were performed in accordance with the guidelines and regulations set forth by the Institutional Animal Care and Use Committee at The University of Texas Southwestern Medical Center and Emory University. Organotypic hippocampal slice cultures were prepared from postnatal day 6–7 mice and biologically transfected at 3–7 days in vitro (DIV) as previously described (McAllister, 2004; Pfeiffer et al., 2010). Dissociated hippocampal cultures were prepared and transfected as described (Ifrim et al., 2015). All experiment studies utilized two to three independent slice cultures (litters). The experimental design for electrophysiology and imaging in organotypic hippocampal slice culture utilizes two sister culture inserts prepared from one animal (~7–8 hippocampal slices per insert); each insert is transfected with a control or target set of constructs or drug (for example, WT-FMRP versus DBM-FMRP or vehicle versus drug). Each day, electrophysiology recordings or images were collected from sister inserts, creating an interleaved experimental design. For imaging studies, acquisition and analysis was conducted by an investigator blind to transfection construct and/or drug treatment.

2.2. Electrophysiology

Simultaneous whole-cell voltage-clamp recordings were obtained from transfected and neighboring non-transfected neurons under visual guidance using IR-DIC and GFP or mCherry-fluorescence to identify transfected neurons as described (Pfeiffer et al., 2010; Wilkerson et al., 2014). Recordings from slice cultures were made at 30 °C in a submersion chamber perfused at 2.3 ml/min with artificial cerebrospinal fluid (aCSF) containing (in mM): 119 NaCl, 2.5 KCl, 26 NaHCO3, 1 NaH2PO4, 11 d-Glucose, 4 CaCl2, 4 MgCl2, 0.002 2-chloro-adenosine; pH 7.28, 300 mOsm and saturated with 95%O2/5% CO2. For mEPSC measurements, aCSF was supplemented with 0.5 μM TTX. Whole cell recording pipettes (~4–7 MΩ) were filled with intracellular solution containing (in mM): 0.2 EGTA, 130 K-gluconate, 6 KCl, 3 NaCl, 10 HEPES, 10 sucrose, 4 ATP—MgO, 0.4 GTP-Na, 14 phosphocreatine-Tris; pH 7.2, 290 mM. EPSCs were evoked by a single bipolar electrode placed in stratum radiatum of area CA1 50–100 μm from the recorded neurons with monophasic current pulses (5–120 μA, 100–200 μs). For all recordings, input and series resistances were measured in voltage clamp with a 400-ms, –10 mV step from a –60 mV holding potential (filtered at 30 kHz, sampled at 50 kHz). Cells were only used for analysis if the following criteria were met: series resistance <25 MΩ and stable throughout the experiment, resting membrane potential <–35 mV; and input resistance >75 MΩ; evoked EPSC >20 pA and mEPSC freq >0.15 Hz (for the untransfected control neuron). Waveforms were filtered at 3 kHz, acquired and digitized at 10 kHz on a PC using custom software (LabView; National Instruments). Data analysis was performed using custom software in LabView. mEPSCs were detected off-line using an automatic detection program (MiniAnalysis; Synaptosoft Inc., Decatur, Ga.) with a detection threshold set at a value greater than at least 2 S.D. of the RMS noise values, followed by a subsequent round of visual confirmation. The detection threshold remained constant for the duration of each experiment. For evoked EPSCs shown in figure insets the stimulation artifact has been digitally removed for clarity.

2.3. Constructs

pcDNA1-MEF2-VP16 and mREx-mCherry constructs have been described previously (Flavell et al., 2006; Pfeiffer et al., 2010; Wilkerson et al., 2014). MEF2-VP16 encodes amino acids 1–117 of MEF2C fused to the activation domain of the viral activator, VP16 (Black et al., 1996). Venus-FMRP-3’UTR, containing human Fmr1 ORF and 3’UTR, and tagged with Venus at the N terminus was previously described (Subil et al., 2015). S499A and S499D FMRP mutants were generated by
substituting the Serine 499 (500 in human FMRP) with Alanine, or Aspartic acid, respectively, by using QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA) according to manufacturer’s instructions. DBM FMRP mutant was generated by substituting the R276 and L279 within the D box (aa276–284) with Alanine as previously described (Huang et al., 2015), using Quick-Change II XL Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA) according to manufacturer’s instructions.

For Ubiquitin mediated fluorescence (UbFC) experiments (Fang and Kerppola, 2004; Hu et al., 2002) we used Venus–155 and Venus156–239 fragments as described (Ifrim et al., 2022; Nagai et al., 2002; Shyu et al., 2006). To make split Venus-tagged FMRP (VC-FMRP), a single high-resolution snapshot through the center of the cell was acquired using a Plan-Apochromat 63× 1.4 oil objective mounted on a Zeiss LSM 880 inverted confocal microscope. Using of UbFC-Venus signal was unreliable in primary dendrites and not observed in secondary dendrites, even with increased expression time (up to 72 h post-transfection; data not shown). Therefore, high resolution stacked images of neuronal somas were acquired at 1024 × 1024 pixel resolution (x = y = 0.13 μm, z = 1 μm). Images were collapsed into a single, maximum intensity projection image and somas were traced in ImageJ and superimposed onto the Venus channel. A threshold intensity was determined based on the background Venus intensity of same image to quantify the % soma area above threshold. Soma Venus intensity was measured and normalized to background Venus intensity within the same image or normalized to mCherry as indicated. Representative images in some figures (Fig. 4, S4) control and experimental images were each scaled equally to improve visualization using the adjust levels function in Adobe Photoshop Creative Suite 6.

2.4. Live imaging of MEF2 activity using MRE-mCherry and analysis

Sixteen to 24 h post-transfection, slice cultures were submerged in warm Tyrode’s solution containing (in mM): 150 NaCl, 4 KCl, 4 CaCl2, 2 MgCl2, 10 glucose, 10 HEPES. A single image (1024 × 1024 pixel resolution) was acquired using a Plan-Apochromat 63×1.4 oil immersion objective mounted on a Zeiss LSM 880 inverted confocal microscope. Quantification of MRE-mCherry and PA1-GFP soma fluorescence intensity was performed using ImageJ software as previously described (Chang et al., 2017; Pfeiffer et al., 2010; Wilkerson et al., 2014). For each neuron, a ratio of MRE-mCherry to PA1-GFP intensity (transfection marker) was determined which was then normalized to the mean of the control group (vehicle-treated with pcDNA3 transfection). Data were then grouped and averaged.

2.5. Live imaging of FMRP-containing mRNP granules and analysis

Organotypic slice cultures underwent pharmacological treatment and/or biolistic transfection at 4–5 DIV as indicated in the main text. 24–30 h post-transfection, slices were imaged live as above. Images were acquired using a Plan-Apochromat 63×1.4 oil objective on a Zeiss LSM 880 inverted confocal microscope. mCherry served as a transfection marker and only mCherry-positive cells were imaged. For soma images, a single high-resolution snapshot through the center of the cell was acquired. High resolution stacked images of apical and secondary apical dendrites were acquired at 1024 × 1024 pixel resolution (x = y = 0.13 μm, z = 1 μm). Analysis of soma and dendritic images utilized ImageJ. For analysis, the soma was traced in the mCherry channel using freehand drawing tool, thresholded to the background of Venus image and the % area above threshold in the Venus channel was quantified. For secondary dendrites, 2 to 3 regions of interest were analyzed per cell. Dendritic images were collapsed into a single, maximum intensity projection image. Using the mCherry channel, dendrites were measured for length and the dendrite area was traced. Particles within the traced dendrite area in the Venus channel were thresholded to the background and detected with the “Analyze Particles” tool in ImageJ. The size of particles to be detected was set as >0.065 μm based on (1) within an image, 1 μm = 0.13 pixels and (2) preliminary, empirical analysis of puncta from neuronal dendrites expressing exogenous WT-FMRP. For each neuron, detected Venus-positive particles were counted and averaged for the total number of dendrites analyzed. Additionally, the percentage of dendrites positive for FMRP-containing puncta was calculated.

2.6. Ubiquitin-mediated Fluorescence Complementation (UbFC)

Slices cultures underwent pharmacological treatment and/or biolistic transfection at 4–7 DIV. As indicated in text, cultures were transfected with split Venus and mCherry-tagged constructs as a transfection marker, mCherry-positive cells were imaged 24–30 h post-transfection using a Plan-Apochromat 63×1.4 oil objective mounted on a Zeiss LSM 880 inverted confocal microscope. Detection of UbFC-Venus signal was unreliable in primary dendrites and not observed in secondary dendrites, even with increased expression time (up to 72 h post-transfection; data not shown). Therefore, high resolution stacked images of neuronal somas were acquired at 1024 × 1024 pixel resolution (x = y = 0.13 μm, z = 1 μm). Images were collapsed into a single, maximum intensity projection image and somas were traced in ImageJ and superimposed onto the Venus channel. A threshold intensity was determined based on the background Venus intensity of same image to quantify the % soma area above threshold. Soma Venus intensity was measured and normalized to background Venus intensity within the same image or normalized to mCherry as indicated. Representative images in some figures (Fig. 4, S4) control and experimental images were each scaled equally to improve visualization using the adjust levels function in Adobe Photoshop Creative Suite 6.

2.7. Statistics

Statistics were performed in GraphPad Prism. For electrophysiology, significant differences were determined using a paired t-test (for normally distributed data) or Wilcoxon matched pairs signed rank test (for non-normal distributed data). For imaging experiments, statistical significance between groups was determined with either an unpaired t-test (for normally distributed data) or Mann-Whitney test (for non-normally distributed data) or a two-factor ANOVA and Sidak’s post hoc multiple comparison test. Some UbfC data (Fig. 4-A-D) was log transformed to improve the normality of the data distribution.

3. Results

3.1. MEF2-induced synapse elimination requires APC/Cdh1 activity

To determine the role of the E3 ubiquitin ligase for FMRP, APC/Cdh1, in synapse elimination, we treated organotypic hippocampal slice cultures with apcin (10 μM), an inhibitor that blocks substrate binding to APC/Cdh1 (De et al., 2019; Wang et al., 2015), or vehicle, 30 min prior to biolistic transfection of MEF2PV16 and a MEF2-driven transcriptional reporter (MRE-mCherry). Sixteen to 30 h later, dual patch clamp recordings were made from transfected and untransfected neurons in apcin- and vehicle-treated slices. Compared to neighboring untransfected neurons, vehicle-treated transfected neurons showed a depression in evoked excitatory postsynaptic currents (eEPSC) and miniature EPSC (mEPSC) frequency without changes in paired pulse facilitation (PPF) or mEPSC amplitude (Fig. 1A, Table S1), which we have shown.
accompanies loss of dendritic spines (Pfeiffer et al., 2010). Although apcin had no effect on synaptic transmission in untransfected neurons, it blocked MEF2-induced depression of eEPSCs and mEPSC frequency (Fig. 1B). These results suggest a role for APC/Cdh1 in MEF2-induced synapse elimination. To determine if apcin blocked synapse elimination by inhibiting MEF2 transcriptional activity, we tested the effects of apcin on MEF2VP16 induction of a MEF2 transcriptional reporter (MRE-mCherry) in CA1. Apcin had no effect on basal nor MEF2VP16-induced MRE-mCherry, indicating that APC/Cdh1 likely regulates synapse elimination downstream of MEF2 transcriptional activity (Fig. 1C).

3.2. APC/Cdh1 interaction with FMRP is required for MEF2-induced synapse elimination

Because both APC/Cdh1 and FMRP are both required for MEF2-induced synapse elimination, we hypothesized that FMRP interactions with APC/Cdh1 are also necessary. To test this idea, we assessed the ability of an FMRP mutant that does not interact with APC/Cdh1 to rescue synapse elimination in Fmr1 KO neurons. We first replicated our original finding that MEF2VP16-induced depression of evoked EPSC amplitude and mEPSC frequency is deficient in Fmr1 KO hippocampal CA1 neurons (Fig. 2A; Table S1) and could be rescued by acute co-expression of a N-terminal Venus-tagged, wildtype (WT) FMRP with MEF2VP16 (Fig. 2B). FMRP expression alone for this short time (16–30 h) has no effect on synaptic function (Pfeiffer et al., 2010). Cdh1 recognizes substrates of the APC through interaction with a D-box motif (DBM) in FMRP at amino acid position (aa)276–284 (Huang et al., 2015; Peters, 2006). Mutation of the DBM in FMRP, from RSFLEFAED to ASPAFAEDA, abolishes interactions with Cdh1, reduces FMRP ubiquitination in brain and inhibits mGluR-LTD in hippocampal CA1 (Huang et al., 2015). In contrast to WT-FMRP, transfection of Fmr1 KO neurons with a DBM-mutated form of FMRP (DBM-FMRP) failed to rescue MEF2VP16-induced synaptic depression (Fig. 2D). DBM-FMRP had no effects on synaptic transmission when expressed alone, without MEF2VP16 (Table S1), indicating a specific role in MEF2-regulated synaptic depression. Taken together, these data suggest that APC/Cdh1 activity as well as its interaction with FMRP are necessary for MEF2-dependent synapse elimination.

3.3. D-Box motif mutations disrupt expression of FMRP in dendritic mRNP granules

Fig. 1. Endogenous APC/Cdh1 is required for MEF2-induced synapse elimination and functions downstream of MEF2 transcriptional activity.

Simultaneous whole cell recordings from wildtype CA1 neurons transfected (T) with MEF2-VP16 and MRE-mCherry and treated with vehicle (0.1%DMSO) shows a depression of evoked EPSC amplitude (A₁) and mEPSC frequency (A₂) compared to neighboring, untransfected (U) neurons in slice cultures. (n = 9–14 cell pairs). (B) Treatment of slices with 10 μM apcin, an APC/Cdh1 inhibitor, blocks MEF2-dependent depression of evoked EPSC (B₁) and mEPSC frequency (B₂) compared to neighboring, untransfected (U) neurons (n = 10–14 cell pairs). Dot plots of evoked EPSC and mEPSC frequency show individual cell pairs (gray dots) and mean ± SEM (black star) of the transfected versus untransfected neuron; the solid black line represents equality between pairs. Inset: Representative evoked EPSC traces of transfected (gray) and untransfected (black) cells. Scale bar = 25pA/10 msec. Right: Inset of representative mEPSCs from transfected and neighboring untransfected cells. Scale bar = 10pA/500 msec. Raw values of electrophysiology measurements are in Table S1. (C) Representative images of wildtype CA1 neurons in vehicle or apcin, transfected with vector (pcDNA3) or MEF2VP16 along with a MEF2 reporter (MRE-mCherry) and transfection marker (PA1-GFP). Reporter activity is measured as MRE-mCherry intensity and normalized to background fluorescence within each image and then normalized to the vector+vehicle group and presented as mean ± SEM (n = 16–43 cells/group). *p < 0.05, **p < 0.01, ****p < 0.0001.
expression in dendrites was diffuse, with few or no puncta (Figs. 2D and F). FMRP was expressed at similar levels to WT-FMRP in the cell soma, its and fill MEF2 transcriptionally active neurons (Fig. 2D). Although DBM- number of FMRP-positive puncta in secondary apical dendrites or per (Figs. 2C; S1C). To compare effects of the DBM mutation on FMRP expression, an independent set of cultures was transfected with Venus tagged WT- or DBM-FMRP, MEF2VP16 and MRE-mCherry, to identify expression is highly diffuse, with few detected FMRP-positive puncta and a low average percentage of FMRP-positive secondary dendrites (n = 19,20 cells/group). Grouped data are presented as mean ± SEM. Scale bar = 10 μm. ** p < 0.01, *** p < 0.001.

in RNA granules, we observed a punctate expression of Venus-tagged WT-FMRP in dendrites, but not the soma (Figs. 2C, S1A,C). Cotransfection with MEF2VP16 had no effect on soma levels of FMRP, nor the number of FMRP-positive puncta in secondary apical dendrites or percentage of secondary apical dendrites with FMRP-positive puncta (Figs. 2C; S1C). To compare effects of the DBM mutation on FMRP expression, an independent set of cultures was transfected with Venus tagged WT- or DBM-FMRP, MEF2VP16 and MRE-mCherry, to identify and fill MEF2 transcriptionally active neurons (Fig. 2D). Although DBM-FMRP was expressed at similar levels to WT-FMRP in the cell soma, its expression in dendrites was diffuse, with few or no puncta (Figs. 2D and S1D), suggesting a deficient association with RNA granules. Similar results were observed in dissociated hippocampal cultured neurons with transfection of Venus tagged WT- or DBM-FMRP without MEF2VP16 (Fig. S1A,B). Taken together, these results suggest that the D-box motif (DBM) of FMRP is necessary for formation and/or stability of dendritic FMRP granules. To determine if APC/Cdh1 acutely regulated dendritic FMRP expression and association with granules, we examined the effects of apcin on dendritic Venus tagged-WT-FMRP puncta in MEF2VP16 transfected neurons. Apcin treatment had no effect on FMRP soma or dendritic puncta expression, nor affected levels of endogenous FMRP in wildtype slice cultures (Fig. S1E-G). This finding suggests that acute inhibition of APC/Cdh1 activity does not alter association of FMRP with granules, whereas altered expression of DBM-FMRP in dendrites may imply a requirement for a role of the DBM in granule localization and/or stability. Importantly, both pharmacologic inhibition of APC/Cdh1 and inhibition of its biochemical association with FMRP impair synapse elimination. This motivated additional experiments to investigate mechanisms that further link FMRP granules to synapse elimination.

3.4. Phosphorylation of FMRP is required for MEF2-induced synapse elimination and bidirectionally regulates FMRP-containing mRNP granules

Phosphorylation at the S499 residue (S500 in humans) of FMRP (Ceman et al., 2003) regulates ubiquitination of FMRP (Huang et al., 2015; Nalavadi et al., 2012). Mutation of serine 499 to alanine (S499A) in FMRP generates a dephosphomimetic protein that is more strongly associated with Cdh1 and ubiquitinated in comparison to WT-FMRP (Huang et al., 2015; Nalavadi et al., 2012). Mutation of serine 499 to glutamine (S499D) creates a phosphomimetic FMRP (Huang et al., 2015; Nalavadi et al., 2012). In contrast, replacing serine 499 with glutamine (S499D) creates a phosphomimetic FMRP that is less associated with Cdh1 and less ubiquitinated (Huang et al., 2015; Nalavadi et al., 2012). Given our results implicating APC/Cdh1 in MEF2-induced synaptic depression, we tested the requirement for FMRP phosphorylation and dephosphorylation at S499. S499A-FMRP or S499D-FMRP were transfected into Fmr1 KO CA1 neurons together with MEF2VP16 and MRE-mCherry. As a positive control, interleaved, sister Fmr1 KO cultures were co-transfected with WT-FMRP and MEF2VP16
which showed the expected depression of evoked EPSC amplitude and mEPSC frequency in comparison to untransfected neurons (Fig. 3A). In contrast, transfection of either S499A-FMRP or S499D-FMRP failed to rescue MEF2VP16-induced depression of evoked or mEPSCs in Fmr1 KO neurons (Fig. 3B,C). Transfection of S499A FMRP or S499D FMRP alone, without MEF2VP16, had no effect on evoked EPSCs or mEPSCs (Table S1). These results indicate that phosphorylation and dephosphorylation of FMRP are necessary for MEF2-induced synapse elimination which suggests a dynamic regulation of FMRP phosphorylation during this process.

To determine if S499 mutants of FMRP have altered dendritic expression, we imaged Venus-tagged WT-, S499A-, or S499D-FMRP in the soma and secondary dendrites from Fmr1 KO CA1 neurons co-transfected with MEF2VP16. In comparison to WT-FMRP, neurons transfected with S499A-FMRP had fewer puncta and a reduced percentage of dendrites with FMRP puncta (Fig. 3B). In contrast, S499D-FMRP had more puncta and more dendrites with FMRP puncta in comparison to cells transfected with WT-FMRP (Fig. 3C). Expression of the S499 FMRP mutants in the soma was similar to WT-FMRP, as measured by % of soma area (Fig. S2A,B). These results are consistent with previous reports that dephosphorylation of FMRP at S499 promotes FMRP degradation and reduces association with RNA granules and suggests this regulates FMRP association with granules in dendrites (Huang et al., 2015; Nalavadi et al., 2012; Tsang et al., 2019).

Fig. 3. Phosphorylation and dephosphorylation of FMRP is necessary for MEF2-induced synapse elimination and bidirectionally regulates FMRP expression in dendrites. (A) Simultaneous recordings from Fmr1 KO neurons transfected (T) with MEF2-VP16, MRE-mCherry and WT-FMRP show depression of evoked EPSC and mEPSC frequency in MEF2VP16 transfected cells (n = 21–27 cell pairs). In contrast, co-transfection with either S499A-FMRP (B₁) or S499D-FMRP (C₁) fails to rescue MEF2-induced depression of evoked EPSCs or mEPSC frequency (n = 15–25 cell pairs). Group data is presented as normalized to untransfected neurons. *p < 0.05, **p < 0.01. Raw values are in Table S1. (B₂, C₂) Representative images of secondary apical dendrites from Fmr1 KO CA1 neurons transfected with MEF2VP16, MRE-mCherry and either Venus-tagged WT-FMRP or S499A-FMRP (B₂) or S499D FMRP (C₂). (B₃, C₃) Quantified group data (mean ± SEM) reveal changes in the number of Venus-FMRP puncta in dendrites and the % of FMRP-positive secondary dendrites with S499A-FMRP or S499D-FMRP in comparison to WT-FMRP. Scale bar = 10 μm. *p < 0.05.
3.5. MEF2 activation increases ubiquitination of FMRP that requires APC/Cdh1

Based on the requirement for APC/Cdh1 activity, as well as the interaction of FMRP with Cdh1 in MEF2-induced synaptic depression, we hypothesized that FMRP ubiquitination is regulated by MEF2. To test this idea, we used a Bimolecular Ubiquitin-mediated fluorescence complementation assay (UbFC) to visualize FMRP-ubiquitin interactions in neurons and its regulation by MEF2VP16 (Fang and Kerppola, 2004; Hu et al., 2002; Ifrim et al., 2022). CA1 neurons in slice culture were co-transfected with “split Venus” tagged versions of ubiquitin (VN-Ub) and WT-FMRP (VC-FMRPWT; Fig. 4 and S3). When co-expressed in cells, Venus fluorescence is observed when ubiquitin and FMRP interact in a macromolecular complex (Fig. 4 A1) (Ifrim et al., 2022; Kerppola, 2008). To confirm this, VC-FMRPWT was transfected into Fmr1 KO slice cultures with or without VN-Ub together with mCherry, as a transfection marker. Live neurons were imaged 24–36 h post-transfection. As expected, detectable Venus fluorescence in the soma, above background,

Fig. 4. MEF2 activation enhances ubiquitination of FMRP through the E3 ligase APC/Cdh1 (A1) Schematic of Bimolecular Ubiquitin-mediated fluorescence complementation assay (UbFC), adapted from (Miller et al., 2015). The C-terminus of Venus protein (VC) was conjugated to mouse WT-FMRP (VC-FMRPWT), whereas the N-terminus of Venus protein (VN) was fused to ubiquitin (VN-Ub). When two proteins interact, Venus fluorescence is observed. (A2) Left: Representative images of Fmr1 KO CA1 neurons transfected with VC-FMRPWT, VN-Ub, mCherry (transfection marker) and either MEF2VP16 or vector control (pcDNA3). Right: Quantified group data show increased Venus intensity (UbFC) in MEF2 transfected neurons expressed as a ratio of mCherry or Venus background (B) Left: Images from cells transfected with the same plasmids as in A2, except MEF2VP16 expressing neurons were co-transfected with MRE-mCherry. Right: Quantified group data show increased UbFC in MEF2 transfected neurons measured as a ratio of Venus/mCherry intensity, Venus intensity /background, or % of Venus-positive soma area (C) Left: Images from cells transfected with the same plasmids as in B, except slice cultures were incubated overnight in vehicle (0.1% DMSO) or apcin (10 μM). Right: Quantified group data show that apcin blocks MEF2-induced increases in UbFC measured as a ratio of Venus background intensity or % of soma area. (D) Left: Images from cells transfected with the same plasmids as in B, except VC-FMRPWT was substituted for VC-FMRPDBM. Right: Quantified group data show that MEF2 does not increase UbFC with the DBM-FMRP mutant measured as a ratio of Venus background intensity or % of soma area. Scale bar = 5 μm. *p < 0.05, ** p < 0.01, *** p < 0.001, ****p < 0.0001.
was observed when VC-FMRP<sup>WT</sup> was cotransfected with VN-Ub, but not alone, indicative of UbFC (Fig. S3). FMRP-UbFC was too dim in secondary dendrites to reliably quantify, so we limited our analysis to the soma. To determine whether MEF2VP16 regulates FMRP ubiquitination, we co-transfected slice cultures with VC-FMRP<sup>WT</sup> and VN-Ub along with MEF2VP16 and mCherry. Sister cultures were co-transfected with VC-FMRP<sup>WT</sup>, VN-Ub, a vector control (pcDNA) and mCherry. In comparison to empty vector transfected neurons, MEF2VP16 expressing neurons showed an increase in FMRP UbBC in the soma, as measured by Venus/mCherry fluorescence intensity, Venus/background fluorescence intensity or the % of the soma (defined by mCherry fluorescence) with Venus fluorescence (above a threshold; Fig. 4A). We performed experiments in the absence of proteasome inhibitors to avoid confounds of the known effects of these inhibitors to induce FMRP-containing RNA/stress granules (Aguilera-Gomez et al., 2017; Mazroui et al., 2007; Shelkovnikova et al., 2017). To measure FMRP-UbBC in neurons with high levels of MEF2 transcriptional activation, we co-transfected MEF2VP16 with the MEF2 transcriptional reporter, MRE-mCherry, in place of mCherry, together with VC-FMRP<sup>WT</sup> and VN-Ub (Fig. 4B). Vector control neurons were co-transfected with pcDNA, mCherry, VC-FMRP<sup>WT</sup> and VN-Ub. Comparable results were observed in MEF2VP16 neurons expressing either mCherry or MRE-mCherry; MEF2 enhanced FMRP-UbFC as compared to vector control neurons (Fig. 4B). For the remaining experiments, we used MRE-mCherry to identify MEF2-transcriptionally active cells.

We hypothesized that MEF2-induced increases in FMRP-UbFC were dependent on APC/Cdh1 and required an interaction of FMRP with APC/Cdh1. To test this hypothesis, we examined the effects of apcin on MEF2-induced FMRP-UbFC using the same experimental design as in Fig. 4B except cultures were treated with either vehicle (0.1% DMSO) or apcin (10 μM) immediately after transfection until imaging (24–36 h). Apcin blocked MEF2-induced increases in FMRP-UbFC, supporting the role of APC/Cdh1 in FMRP ubiquitination and reinforcing the specificity of this UbFC assay (Fig. 4C). Apcin had no effect on FMRP ubiquitination in vector control cells suggesting that APC/Cdh1-mediated ubiquitination of FMRP is recruited in response to MEF2.

To determine if APC/Cdh1 interaction with FMRP is required for MEF2-induced FMRP-UbFC, we tagged DBM-FMRP with split Venus (VC-FMRP<sup>DBM</sup>). Fmr1 KO neurons in slice cultures were co-transfected with VC-FMRP<sup>DBM</sup> and VN-Ub, along with MEF2VP16 and MRE-mCherry or vector and mCherry. MEF2 increased UbFC in sister cultures transfected with VC-FMRP<sup>WT</sup>. However, in neurons transfected with VC-FMRP<sup>DBM</sup> (Fig. 4D), MEF2 failed to increase UbFC. Basal levels UbFC in vector control neurons was not different between WT and DBM-

Fig. 5. Working model of FMRP function in MEF2-induced synapse elimination. Neuronal depolarization and Ca<sup>2+</sup> influx through voltage-dependent Ca<sup>2+</sup> channels stimulates MEF2 to activate transcription of target genes, such as Arc (Flavell et al., 2008; Kawashima et al., 2009; Wilkerson et al., 2014) whose mRNA is transported into dendrites and associates with dendritic RNA granules containing FMRP (Na et al., 2016). Evidence suggests that mRNAs associated with dendritic RNA granules are suppressed and phosphorylated FMRP may contribute to such translational suppression in granules (Anadolu et al., 2023; El Fatimy et al., 2016; Kim et al., 2019; Tsang et al., 2019). In response to mGluR5 activity, FMRP can be dephosphorylated by PP2A and associate with APC/Cdh1 through a D-box motif which ubiquitinates FMRP and promotes its degradation through the proteasome (Huang et al., 2015; Nalavadi et al., 2012; Narayanan et al., 2007; Narayanan et al., 2008). MEF2 promotes ubiquitination of FMRP either through regulation of phosphorylation, association with Cdh1 or both. Degradation of FMRP would be expected to release translation suppression of target mRNAs such as Arc that are necessary for MEF2-dependent synaptic depression and elimination, where it promote endocytosis of AMPA receptors (Chang et al., 2017; Chowdhury et al., 2006; Niere et al., 2012; Waung et al., 2008; Wilkerson et al., 2014).
FMRF mutants. These results suggest that MEF2 transcriptional activation promotes ubiquitination of FMRF in neurons through APC/Cdh1.

4. Discussion

Excitatory synapse weakening and elimination functions in the refinement and formation of stimulus-selective neuronal circuits during postnatal development as well as during learning in the adult (Faust et al., 2021; Holtmaat and Caroni, 2016). Evidence in both humans and mouse models indicate that deficits in developmental and experience-dependent synapse elimination occur in autism spectrum disorders, including Fragile X Syndrome (Arroyo et al., 2019; Faust et al., 2021; Irwin et al., 2000; Pan et al., 2010; Patel et al., 2014). We have demonstrated that FMRF and activity-dependent transcription factor MEF2 function in a cell autonomous manner in postsynaptic CA1 neurons to eliminate synapses and have used this paradigm to explore the cellular and molecular mechanisms by which FMRF regulates synapse elimination (Pfeiffer et al., 2010). Our results suggest a model where MEF2 transcriptional activation promotes ubiquitination of FMRF by APC/Cdh1, in a process that requires FMRF dephosphorylation (Fig. 5). Dephosphorylation and ubiquitination of FMRF by APC/Cdh1 leads to proteasomal degradation of FMRF, de-repression and translation of target mRNAs (Huang et al., 2015; Nalavadi et al., 2012). In the context of MEF2 activation, degradation of FMRF may de-repress translation of mRNAs necessary for synapse elimination. Phosphorylation and dephosphorylation of FMRF at S499 also regulates association of FMRF with RNA granules and translation of RNAs within granules (Kim et al., 2019; Tsang et al., 2019). Thus, a dynamic FMRF interaction with RNA granules may also be necessary for MEF2-induced synapse elimination. This interaction likely occurs in dendrites whereby FMRF phosphorylation in granules maintains a repressive function, which can be dissociated in a MEF2 dependent manner leading to FMRF dephosphorylation and ubiquitination by APC/Cdh1.

Our results show that Cdh1 and FMRF interactions are necessary for MEF2 dependent synapse elimination, suggesting an important interplay between transcriptional activation and translational control. The Cdh1 inhibitor, apcin, and a mutation in Cdh1 interaction domain of FMRF (DBM-FMRF) prevented MEF2-induced synaptic depression and ubiquitination of FMRF, as analyzed using UbcF. Because apcin did not affect MEF2-induced transcriptional activity, measured by the MRE-mCherry reporter, this suggests that Cdh1 functions downstream of MEF2 transcription and promotes ubiquitination of FMRF in response to MEF2. It is unclear if MEF2-induced transcripts directly promote FMRF ubiquitination and what these transcripts may be. MEF2 activation promotes PSD-95 ubiquitination and does so by stimulating nuclear export and synaptic localization of Mdm2 (Tsai et al., 2017; Tsai et al., 2012). MEF2 may regulate expression, localization, or activity of APC/Cdh1. Alternatively, or in addition, mGluR5 activation stimulates PP2A-mediated dephosphorylation of FMRF, ubiquitination of FMRF via Cdh1 and is necessary for MEF2-induced synapse elimination (Huang et al., 2015; Iirim et al., 2022; Wilkerson et al., 2014). Therefore, mGluR5 activity or signaling may be regulated by MEF2 to promote FMRF ubiquitination indirectly. Future experiments may explore whether mGluR5 is necessary for MEF2-induced ubiquitination of FMRF.

APC/Cdh1 and Gdc20 promote degradation of MEF2C in myoblasts and regulate MEF2A transcriptional activation in neurons by acting as an E3 ligase for Sentrin/SUMO-specific proteases (SENPs) (Badodi et al., 2015; Xu et al., 2014). Through degradation of SENPs, Cdh1 enhances SUMOylation of MEF2A and decreases its transcriptional activity. Because the SUMOylation site on MEF2A (Ser408) is not present in the MEF2-VP16 which only contains the MEF2/MADS domain (aa1–117) this alternative role for Cdh1 may not be evident in our study.

We find that both phosphorylation and dephosphorylation of FMRF at S499 are necessary for MEF2-induced synapse elimination. Given the role of phosphorylation and dephosphorylation of FMRF at S499 in the translational “switch” of FMRF from a repressor to de-repression, our results suggest that such a mechanism may promote synthesis of proteins necessary for synapse elimination (Fig. 5). There is evidence that some MEF2-induced transcripts required for synapse elimination are translationally regulated by FMRF, and this occurs in dendrites. MEF2VP16 induces Activity-regulated cytoskeletal associated protein (Arc; Arg 3.1) which is necessary for both MEF2- and activity-induced synapse elimination (Chang et al., 2017; Wilkerson et al., 2014). Arc mRNA is expressed in dendritic RNA granules where it is colocalized with FMRF and rapidly translated, within minutes, in response to glutamate and mGluR1/5 (Fritzsch et al., 2013; Na et al., 2016; Waung et al., 2008). mGluR5 activity in dendrites is necessary for MEF2-induced increases in Arc protein in dendrites (Wilkerson et al., 2014). In support of a translational switch model (Fig. 5), S499D FMRF suppresses Arc levels in dendrites but does not rescue mGluR-stimulated translation of Arc in Pmr1 KO neurons (Niere et al., 2012). In contrast, S499A FMRF does not suppress Arc levels, nor rescue mGluR1/5-stimulated synthesis (Niere et al., 2012). FMRF-mediated translational suppression of Elongation Factor 1ε (EF1ε) mRNA, is also implicated MEF2-induced synapse elimination (Tsai et al., 2017; Tsai et al., 2012). EF1ε interacts with Mdm2 a ubiquitin E3 ligase for PSD-95. Pmr1 KO neurons have elevated levels of EF1ε which sequester Mdm2, prevent MEF2 triggered ubiquitination and degradation of PSD-95 and synapse elimination. Interestingly, MEF2 stimulates dephosphorylation of Mdm2 by PP2A in dissociated hippocampal neuron cultures, and may similarly regulate FMRF (Tsai et al., 2017). Another possible player are microRNAs which might be induced by MEF2 dependent transcription and neuronal activity (Fiore et al., 2009). Indeed, FMRF can associate with Argonaute-2/RISC and microRNAs to regulate translation in a phosphorylation dependent manner (Muddashetty et al., 2011).

We observe that the number of dendritic puncta formed by phospho- and dephospho-mimics at S499 FMRF are increased and decreased, respectively, in comparison to WT-FMRF. This may be a result of the effects of phosphorylation on ubiquitination and degradation of FMRF. Alternatively, recent work demonstrated that phosphorylation of FMRF, within a 445–632, enhanced liquid-liquid phase separation of FMRF with RNA, a process involved in the formation of RNA granules as well as translational suppression of a reporter RNA (Kim et al., 2019; Tsang et al., 2019). Thus, FMRF phosphorylation at S499 may promote association with RNA granules and translational suppression through interactions with RNA and other translational repressors within the granules. Activity-dependent translation in dendrites is associated with disassembly of RNA granules and “unmasking” of RNA (Buxbaum et al., 2014; Park et al., 2014). Posttranslational modifications of FMRF, such as phosphorylation/dephosphorylation that regulate dynamic association/dissociation of FMRF with RNA granules may unmask and activate translation of RNAs in response to synaptic activity and MEF2. Whether the phosphorylation-dependent release of FMRF from RNA granules leads to its ubiquitination and degradation is unknown. Surprisingly, we observed that DBM-FMRF also has reduced dendritic punctate expression suggesting that this mutation, in addition to preventing interactions with Cdh1, also prevents RNA granule association or stability. Similarly, I304N-FMRF mutation in the KH2 RNA binding domain has reduced interactions with KH-domain interacting RNAs and polysomes, does not form dendritic puncta, nor rescue MEF2-induced synapse elimination (Darnell et al., 2005; Pfeiffer and Huber, 2007; Pfeiffer et al., 2010; Zang et al., 2009). The D-box (aa276–284) is within the KH1/KH2 RNA binding domains. Although the DBM FMRF has normal interactions with the mGluR5 receptor, including mGluR1/5 (Darnell et al., 2005), it may affect KH1/KH2 domain interactions with RNAs necessary for granule association. Another explanation for the reduced DBM-FMRF puncta is that the formation and/or stability of FMRF in granules may require an association with the regulatory subunit Cdh1, but the E3 ligase activity is inactive and requires signaling. Therefore, the inability of the S499A, S499D and DBM- FMRF mutants to rescue MEF2-induced synapse elimination may be due to differential deficits in FMRF dynamic association and dissociation from RNA granules and translational
regulation within the granule. The D-box mutant and S449A fail to rescue because they do not assemble in stable FMRP granules that are poised to respond to stimuli, whereas S499D may be hyperstable and insensitive to stimuli. Conversely, apcin treatment did not affect dendritic punctate expression of FMRP suggesting that overnight inhibition of Cdh1 and FMRP ubiquitination does not affect association with RNA granules. Of interest another report showed that apcin treatment of cultured neurons for 16 h. resulted in increased stress granule formation that was dependent on FMRP (Valdez-Simon et al., 2020). Apcin was also shown in that study to reduce protein synthesis. These findings are consistent with our observations that apcin does not impair FMRP granules. Taken together these studies reveal a role for Cdh1/APC to regulate FMRP granule dynamics and protein synthesis. Furthermore, a recent study has used a single molecule adaptation of Ub-FC to directly visualize FMRP granule ubiquitination in dendrites of cultured neurons in response to mGluR activation (Ifrim et al., 2022). This has implications for our observations of MEF2 dependent ubiquitination of FMRP granules as a switch for translation in response to cues.

MEF2 family members promote transcription in response to neuronal activity, novelty experience and environmental enrichment and their activity is associated with cognitive function (Barker et al., 2021; Flavell et al., 2008). The deficits in experience-dependent spine elimination and cortical neuron synaptic hyperconnectivity in the Fmr1 KO (Galvez and Greenough, 2005; Pan et al., 2016; Patel et al., 2014) support a role for Fmr1 in developmental synaptic refinement. Our study suggests that Fmr1 functions downstream and coordinates with MEF2 gene products in synapse elimination. The model system used here with constitutively active MEF2 should motivate further work on whether endogenous MEF2 is required in activity or experience-dependent synaptic refinement in the brain.

5. Conclusions

Here we show a role for posttranslational modifications of FMRP in MEF2 triggered synapse elimination. Our data support a model where MEF2 transcriptional activation promotes dephosphorylation and ubiquitination of FMRP through APC/Cdh1 which derepresses translation of mRNAs necessary for synapse elimination. This work provides a molecular mechanism for the function of FMRP in refinement of developing circuits.

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Appendix A. Supplementary data

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References

Black, B.L., et al., 1996. Cooperative transcriptional activation by the neurogenic basic helix-loop-helix protein MASH1 and members of the myocyte enhancer factor-2 (MEF2) family. J. Biol. Chem. 271, 26659–26663.

CRediT authorship contribution statement


Data availability

Data will be made available on request.

Appendix B. Supplementary data

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