Fragile X Mental Retardation Protein is Required for Synapse Elimination by the Activity-Dependent Transcription Factor MEF2

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SUPPLEMENTARY DATA AND PROCEDURES

Supplementary Figure 1, related to Figure 1: Nuclear transport of MEF2 is required for functional synapse elimination. These are vehicle controls for the 4OHT data in Figure 1. ***A,B***, Transfection of slice cultures with MEF2-VP16-ER^{TM} and treatment with vehicle (0.1% EtOH) does not affect synaptic transmission. These experiments are the vehicle controls for the 4OHT treatment experiments in Fig. 1A-C. $\boldsymbol{A_1}$ Representative traces of mEPSCs (scale bar = 10 pA/500 ms.) and evoked AMPAR-mediated EPSCs ($\boldsymbol{A_2}$; scale bar = 50 pA/10 ms) from a simultaneous recording of untransfected and a neighboring MEF2-VP16-ER^{TM} transfected WT neuron treated with vehicle (0.1% EtOH). ***B.*** Average mEPSC frequency, mEPSC amplitude, evoked AMPAR-mediated EPSC amplitude, and paired-pulse ratio (amplitude $EPSC_2/EPSC_1$) from untransfected WT and MEF2-VP16-ER^{TM} transfected cells treated with 0.1% EtOH. Plotted are averages ± SEM and *n* (# of cell pairs) is indicated on each bar. ***D.*** **Acute transfection of MEF2-VP16-ER^{TM} does not affect resting membrane potential or input resistance.** Group data of resting membrane potential and input resistance of MEF2-VP16-ER^{TM} transfected WT or *Fmr1* KO neurons treated with 4OHT (same neurons as in Fig. 1). Plotted are averages ± SEM and *n* (# of cell pairs) is indicated on each bar.

Supplementary Figure 2, related to Figure 2: MEF2-EN plasmid inhibits endogenous MEF2 dependent transcription. This data demonstrates that the MEF2-EN construct used in Fig. 2 suppresses expression of MEF2 dependent transcriptional reporters. ***A.*** Dissociated hippocampal neurons were transfected with MRE-GFP alone or together with MEF2-EN. * $p < 0.05$, n = 15 cells per condition. ***B.*** Cultured striatal neurons were transfected with MRE-Luc alone or together with MEF2-EN. Cells were depolarized with isotonic 60 mM KCl to activate

endogenous MEF2. Data are expressed as relative luciferase data using normalization to co-transfected pTK-Renilla luciferase in dual luciferase assays (Promega). MEF2-EN significantly attenuates depolarization-induced MRE-luc activity (n=9, 3 independent experiments, ***p < 0.001, Students' t-test)..

Supplementary Figure 3, related to Figure 3: MEF2 isoforms and FMRP do not physically interact in cells. ***A,*** Endogenous FMRP does not associate with endogenous MEF2A or 2D in wildtype hippocampal slice cultures in the presence or absence of high KCl (1% Triton-X100 IP lysis buffer condition shown). Note that the IP conditions immunodeplete most of the endogenous MEF2A or MEF2D, but no FMRP is co-IPed in these mild buffer conditions. ***B,*** Neither endogenous FMRP (open arrow) nor exogenous expression of an FMRP-GFP fusion protein (closed arrow) associates with T7-tagged MEF2A, 2C or 2D (0.5% Triton-X100 IP lysis buffer condition shown) in HEK293T cells. ***C,*** In a separate experiment, under similar conditions, HDAC4 positively associates with T7-MEF2A. ***D,*** Average relative optical density of FMRP-GFP (Immunoprecipitated (IP)/ Total cell lysate (TCL)) from three separate experiments. FMRP protein levels in the IP fraction relative to the TCL fraction do not change when T7-MEF2 is present, compared to the FMRP-only condition.

Supplementary Figure 4, related to Figure 4: Working models of MEF2 and FMRP roles in synapse elimination. ***A,*** MEF2 and FMRP may function in a common single pathway regulating the same transcript(s) for synapse elimination. Because MEF2 activated transcription is normal in *Fmr1* KO neurons (Fig. 3), we place FMRP downstream of MEF2. In this model MEF2 activation generates an mRNA transcript whose transport or translation is controlled by FMRP. ***B,*** Alternatively, MEF2 and FMRP may function in distinct parallel, but convergent pathways each regulating distinct transcripts which are both necessary for synapse elimination. Another possibility, not schematized here, is that MEF2 generates a microRNA (miRNA) transcript(s) that interacts and suppresses translation of an FMRP target mRNA.

Experimental Procedures

Hippocampal Slice Cultures and Transfections: Organotypic hippocampal slice cultures were prepared from postnatal day (P) 6-7 WT or *Fmr1* KO mice bred from the congenic C57BL/6 mouse strain using previously published protocols (Pfeiffer and Huber, 2007; Stoppini et al., 1991). Cultures were biolistically transfected at 3 DIV. Biolistic transfection and gold bullet preparation were performed with the Helios Gene Gun system (BioRad) according to the manufacturer's protocols (McAllister, 2004).

Electrophysiology: Simultaneous whole-cell recordings were obtained from CA1 pyramidal neurons in slice cultures visualized using IR-DIC and GFP fluorescence to identify transfected and non-transfected neurons (Pfeiffer and Huber, 2007). Recordings were made at 30°C in a submersion chamber perfused at 3 ml/min with artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 26 NaHCO3, 1 NaH2PO4, 11 D-Glucose, 3 CaCl2, 2 MgCl2, 0.1 picrotoxin, 0.002 2-chloro-adenosine; 0.1% DMSO pH 7.28, 300 mOsm and saturated with 95% O2/5%CO2. For evoked EPSC and mEPSC recordings, neurons were voltage clamped at -60 mV through whole cell recording pipettes (~3-7 MΩ) filled with an intracellular solution containing (in mM): 0.2 EGTA, 130 K-Gluconate, 6 KCl, 3 NaCl, 10 HEPES, 2 QX-314, 4 ATP-Mg, 0.4 GTP-Na, 14 phosphocreatine-Tris, pH7.2 adjusted by KOH, 285 mOsm. To obtain isolated NMDAR mediated EPSCs, the ASCF was supplemented with 20 μM DNQX and 20 μM glycine and the neuron was clamped at +50 mV. The internal pipette solution for NMDAR EPSCs contained (in mM) 2.5 BAPTA, 125 Cs-Gluconate, 6 CsCl, 3 NaCl, 10 HEPES, 2 QX-314, 4 ATP-Mg, 0.4 GTP-Na, 14 phosphocreatine-Tris, pH7.2 adjusted by CsOH, 290 mOsm. Responses were confirmed to be NMDAR mediated by blockade with 100μM D,L-APV treatment.

For mEPSC measurements, the ACSF was supplemented with 1 μM TTX. Synaptic responses were evoked by single bipolar electrode placed in stratum radiatum of area CA1 (along the Schaffer collaterals) 50-200 μm from the recorded neurons with monophasic current pulses (1-60 μA, 0.2-1 ms). Series and input resistance 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 series resistance was less than 30 MΩ and was stable throughout the experiment. Input resistance ranged from 75-600 MΩ. Data were not corrected for junction potential. No significant difference was observed between transfected and untransfected neurons in resting membrane potential or input resistance, indicating that overall neuronal health and subthreshold membrane conductances were unaffected by expression of MEF2-VP16 ER^{TM} (Fig. S1C). We have previously reported that mEPSC frequency is not detectably different between WT and *Fmr1* KO slice cultures (Pfeiffer and Huber, 2007). This is likely due to the interculture variability in mEPSCs that makes modest changes difficult to detect. In addition, homeostatic or compensatory mechanisms that are independent of FMRP may function to maintain synapse number. This illustrates the necessity to perform dual simultaneous recordings from individual neighboring neurons to measure the effects of acute gene manipulations in a cell autonomous fashion.

Synaptic currents were filtered at 2 kHz, acquired and digitized at 10 kHz on a PC using custom software (Labview; National Instruments, Austin, TX). 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 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 figures the stimulation artifact has been digitally removed for clarity. Significant differences between transfected and non-transfected neurons were determined using a paired t-test.

Dissociated Culture and Fluorescent imaging: Dissociated CA3-CA1 hippocampal cultures (dentate gyrus was discarded) were prepared from P0-1 WT and *Fmr1* KO mice using modified, previously published protocols (Brewer et al., 1993) (Waung et al., 2008). Briefly, dissected hippocampi from P0-1 WT and *Fmr1* KO mice were trypsinized for 10 min, and dissociated by trituration. After centrifugation, neurons were plated in Neurobasal A medium (Invitrogen) containing with B27 (2%; Invitrogen), 0.5μM glutamine, and 1% fetal bovine serum (FBS) at a density of 450 neurons/mm^2 on glass coverslips coated overnight with 50 μg/ml poly-D-lysine and 25 μg/ml laminin. Cultures were fed at 1 day *in vitro* (DIV) and every 3 days afterwards by replacing half the media with serum-free glial-conditioned Neurobasal A media (containing B27, glutamine and cytosine arabinoside; 2μM). Glial cultures were prepared from the neocortex of

P0-P2 mouse pups and maintained in Neurobasal A containing 10% FBS and 50 μg/ml penicillin, 50 U/mL streptomycin, Sigma) for 3-4 weeks(Viviani, 2006). Neurobasal A media was conditioned for 48 hours, collected and stored at 4°C for no more than one week prior to addition to neuronal cultures.

At 7-8 DIV, cultures were transfected with MEF2-VP16-ER^{TM}, 3XMRE-GFP and mCherry (as a transfection marker) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. One to two hours post-transfection, neurons were treated with 4OHT (1 μM); live imaging was performed in Tyrode's solution 16-30 hr post-treatment. Fluorescence was detected using a Nikon TE2000 inverted microscope equipped with a cooled CCD camera. Background fluorescence was determined by a region (5 μm radius) in the field of view adjacent to the neuron. Transfected and MRE-GFP+ neurons were determined by measuring the red and green soma fluorescence, respectively, above a threshold value (2 times above background fluorescence; Fig. S3 C,D). To determine the effects of MEF2-EN on basal, endogenous MEF2 transcriptional activity at MRE-GFP (Fig. S2A), a lower threshold value was used. Cells were scored positive for GFP if soma fluorescence was 10% above background. Neuron images were analyzed and quantitated using Metamorph software (Molecular Devices). Significant differences between groups were determined with an unpaired t-test. For all group data, averages + SEM are plotted and n (# of cells) is on each bar. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Striatal neurons (Fig. S2B) were cultured from E18 rat embryos as described (Pulipparacharuvil et al., 2008). At 8 DIV, the neurons were co-transfected with MRE-Luc, pTK-Renilla Luciferase and either vector (pcDNA1) or pcDNA1-MEF2-EN by calcium phosphate method as described (Pulipparacharuvil et al., 2008). Forty-two hours later, the neurons were stimulated with isotonic stimulation solution at 60 mM KCl final for 8 hours. Cell lysates were analyzed for dual-luciferase activity using standard protocols (Promega). Data (Fig. S2B) is expressed as the ratio of MRE-luciferase activity divided by TK-Renilla luciferase activity (internal transfection control), and represent mean +/- SEM (n=3, $p<0.001$, students' t-test).

Quantitative real-time PCR: At 8 DIV (equivalent day P14), hippocampal slice cultures were transferred to isosmotic media containing 55 mM KCl for various time points indicated in Fig. 3E. Slices were snap-frozen and stored at -80°C until processing. PC12 cells were transfected

with pcDNA3-MEF2-VP16-ER^{TM} using Lipofectamine 2000 (Invitrogen) by manufacturers recommended conditions. After 2 days, cells were treated with 4OHT (1 μM in 0.1% EtOH) or EtOH as a control for 1, 2, 4 and 8 hours in 90% conditioned medium/ 10% fresh medium (DMEM (high glucose), 10% horse serum, 5% fetal bovine serum, 1X pen/strep, and 4 mM glutamine (Invitrogen)) (Fig. 3F).

RNA was extracted from slice cultures or PC12 cells using TriZol reagent (Invitrogen), followed by purification using RNAeasy micro columns (Qiagen). Equal amounts of RNA were prepared for reverse transcription reactions using the Superscript II reverse transcriptase enzyme (Invitrogen). The efficiency of each primer set used in real-time quantitative PCR experiments was first tested on 10-fold serial dilutions of hippocampal cDNA to ensure that the primers promoted specific, exponential amplification of the target cDNA. Optimal primer sets for each gene were then used to assess the abundance of the reverse-transcribed mRNA in cDNA samples. PCR reactions were run in triplicate using iQ SYBR Green supermix (BioRad) and 10-fold serial dilution standard curves were run along with the cDNA samples. In each experiment, a control cDNA sample prepared with no reverse transcriptase enzyme was also measured to ensure that the signal observed was not due to contamination by genomic DNA. Each reaction was quantified using the ΔΔCt method as previously described (Tsankova et al., 2006). Expression for each gene was normalized to GAPDH expression.

The primers used were: *GAPDH*- F: 5' AGGTCGGTGTGAACGGATTTG-3'; R: 5'-TGTAGACCATGTAGTTGAGGTCA-3'; *Nurr77*-F: 5'-CCTCCACATCTTCTTCCTCGTCC-3'; R: 5'-AGGGGCTTCCATAGTAATCAGAG-3'
FMR1-F: 5' GTGCCATTTCATGTCCTGTG-3'; R: 5'-CCCTGAACTCTGCATCCAA-3'

Co-immunoprecipitations (Fig. S3): HEK-293T cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum, 1.8 mM L-glutamine (Sigma-Aldrich, St. Louis, MO) and penicillin-streptomycin (Sigma-Aldrich) at 37°C (5% CO_2). Cells were cultured in six-well plates (1 million/well) and transiently transfected with 2 ug of total DNA/well of plasmids for FMRP-GFP and/or T7-tagged MEF2A, C or D (i.e. 1 μg of MEF2 and 1 μg FMRP or appropriate vector control) by calcium phosphate precipitation. Cells were lysed and harvested in one of three different IP lysis buffers ~42 hrs after transfection. IP lysis buffers used were:

10mM HEPES pH 7.4, 200 mM NaCl, 30 mM EDTA, 0.5% or 1% (v/v) Triton X-100, 2 mM activated Na_3V0_4, 10 mM NaF + 1X EDTA-free protease inhibitor cocktail (Roche) or RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.5% DOC, 0.1% SDS, 2 mM activated Na_3VO_4, 10 mM NaF + 1X EDTA-free protease inhibitor cocktail (Roche)). Immunoprecipitations were performed using anti-T7 tag agarose beads (Novagen) with a 2 hr incubation at 4°C. Beads were washed 3 x 1 ml with IP lysis buffer. Precipitated proteins were solubilized with sample buffer and boiled prior to SDS-PAGE.

Hippocampal slice cultures were prepared as described above. On equivalent postnatal day 15, slices were treated with either normal or depolarizing media (isotonic 55 mM KCl) for 1 h, then pooled according to treatment (~31 slices per condition) in 0.5 or 1.0% Triton-X100 IP lysis buffer. Slice proteins were solubilized using a glass-teflon homogenizer in a total volume of 1.5 ml of lysis buffer. Protein quantification was performed using the Lowry method. Protein concentrations were equalized between conditions using IP lysis buffer (0.94 mg/ml), and each condition was divided into three pre-chilled tubes (420 µl each) treated separately with rabbit anti-IgG antibody (3 µg), MEF2A antibody, 1:50 (Santa Cruz; sc-313, 1.7 µg) or MEF2D rabbit antibody (3 µg; (Flavell et al., 2006) and rotated for 70 min at 4°C prior to addition of Protein A-agarose resin. Samples were incubated for an additional hour prior to washing the beads 3 x 1 ml with IP lysis buffer. Precipitated proteins were solubilized with sample buffer and boiled prior to SDS-PAGE.

Western Blotting: For the slice culture co-IPs, 3% of the input volume for total cell lysate and supernatant after IP fractions and 15 ul of the IP fraction were loaded onto an 8 or 6% SDS-PAGE gel. For HEK cell co-IPs, 2% of the input volume for total cell lysate and 20 µl of the IP fraction were loaded onto 8% SDS-PAGE gels. PVDF membranes were blocked in 10% nonfat milk in 1X TBS-Tween 20 (TBS-T), then rinsed briefly in 1X TBS-T (except TBS without Tween was used prior to FMRP antibody incubation). Membranes were incubated with shaking either overnight at 4°C or 2 h at room temperature in 3% bovine serum albumin (unless noted otherwise) plus 0.02% NaN_3 with primary antibodies: T7 tag 1:10,000 (Novagen; 69522); MEF2A, 1:500 (Santa Cruz; sc-313); MEF2D, 1:2500 (Flavell et al., 2006) (3 mg/ml stock), FMRP 1:1500 or 1:2500 prepared in 0.1% nonfat milk in 1X TBS (no Tween) (Millipore, Billerica, MA; MAB2160). Membranes were washed in TBS-T, incubated with shaking for 1 h

at room temperature with HRP-conjugated anti-rabbit or mouse IgG (1:10,000; Calbiochem), washed and developed using standard enhanced chemiluminescence (ECL) and X-ray film.

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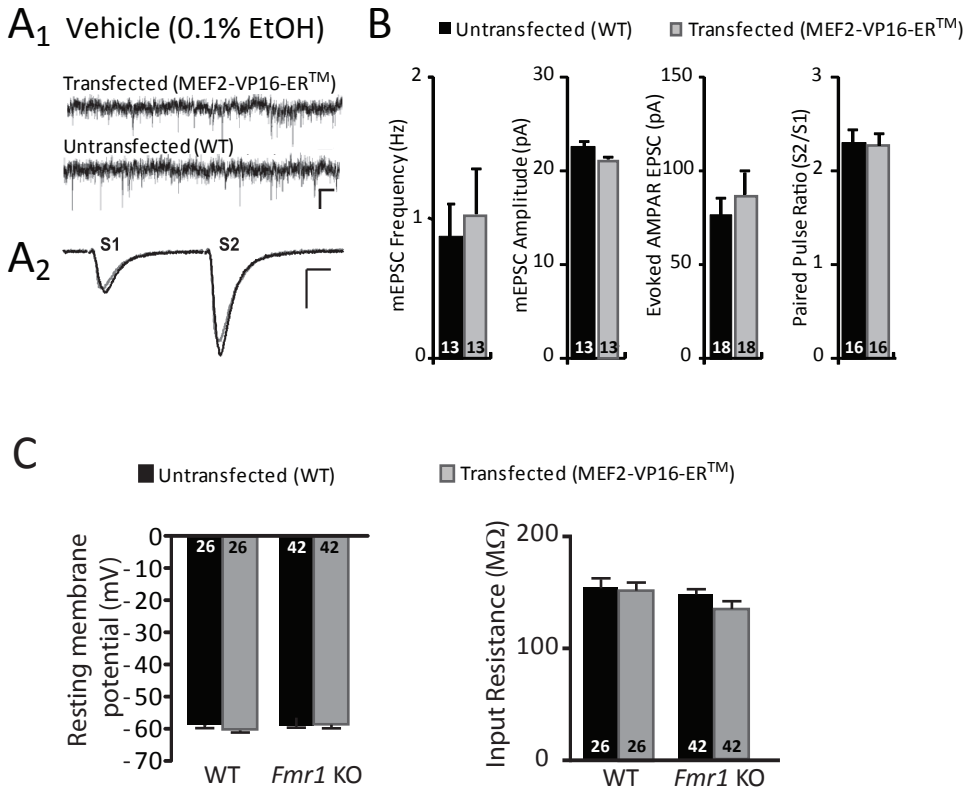
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Evoked AMPAR EPSC (pA)
Paired Pulse Ratio (S2/S1)
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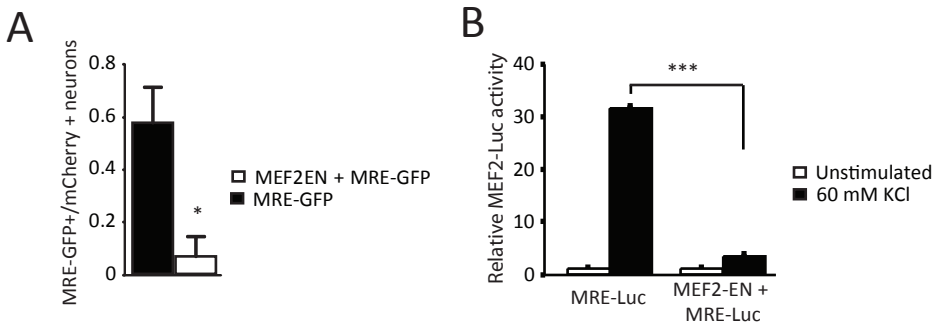
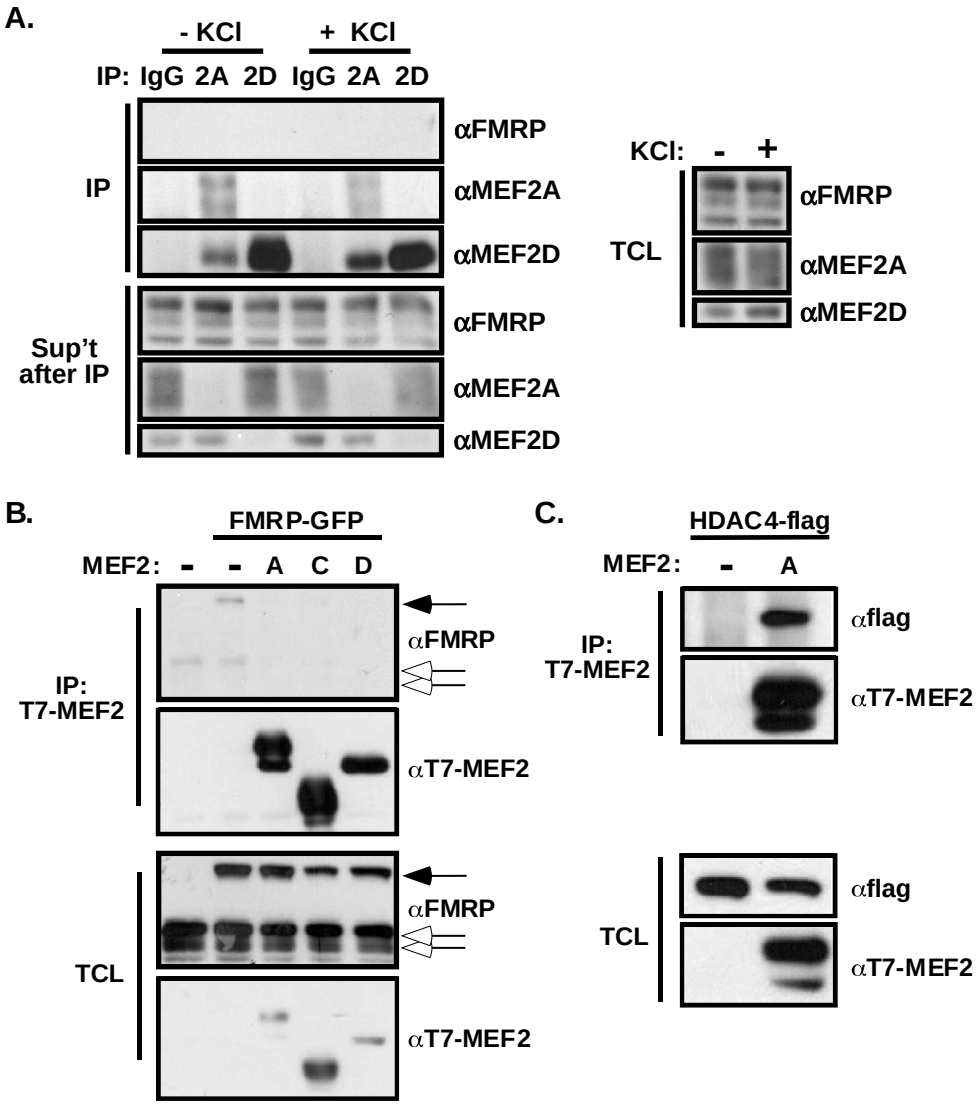
Pfeiffer et al., Supplementary Figure 2

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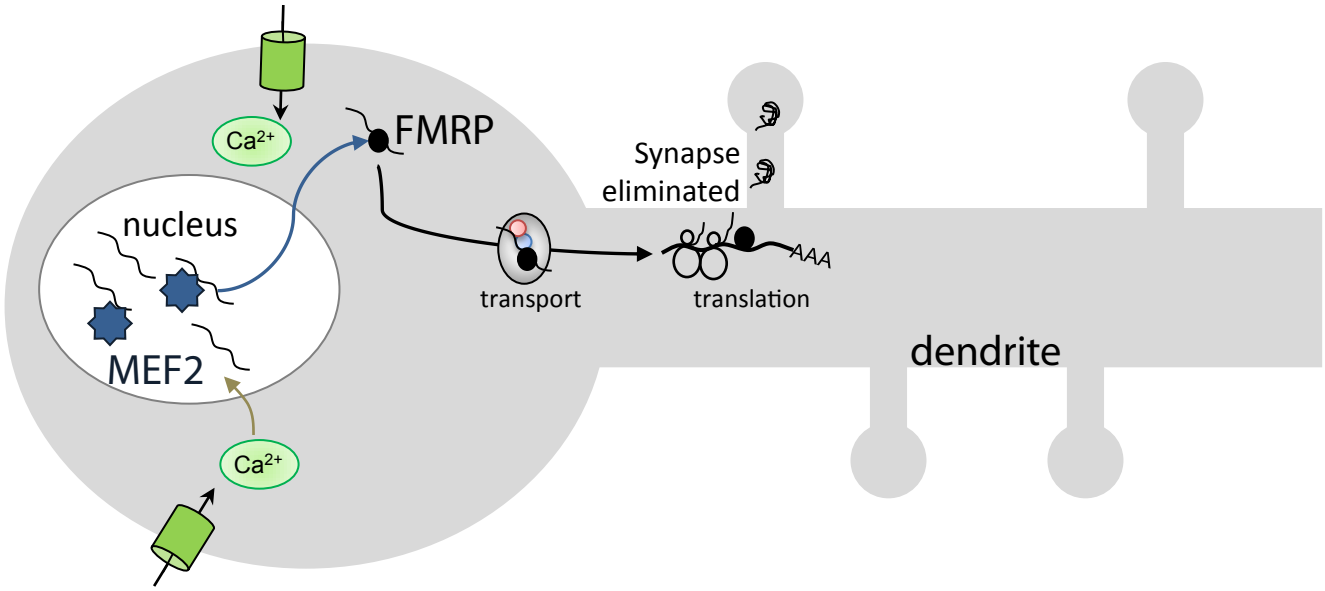
A. Common pathway and transcripts



B. Parallel, convergent pathways and distinct transcripts