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Tyson E. Graber[1,3], Erika Freemantle[2], Mina Anadolu[1], Sarah Hébert-Seropian[2], Robyn MacAdam[1], Unkyung Shin[1], Huy-Dung Hoang[3], Tommy Alain[3], Jean-Claude Lacaille[2] and Wayne S. Sossin[1]

[1]*Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada*

[2]*Department of Neurosciences, GRSNC, Université de Montréal, Montreal, Canada*

[3]*Children's Hospital of Eastern Ontario Research Institute, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada.*

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Correspondence should be addressed to correspondence: Wayne Sossin, Montreal Neurological Institute, Room BT110, 3801 University Avenue, Montreal, Quebec, Canada, wayne.sossin@mcgill.ca

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UPF1 governs synaptic plasticity through association with a STAU2 RNA granule

Abbreviated Title: UPF1 regulates synaptic plasticity.

Tyson E. Graber[1,3], Erika Freemantle[2], Mina Anadolu[1], Sarah Hébert-Seropian[2], Robyn MacAdam[1], Unkyung Shin[1], Huy-Dung Hoang[3], Tommy Alain[3], Jean-Claude Lacaille[2#] and Wayne S. Sossin[1#*]

[1]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, Canada

[2]Department of Neurosciences, GRSNC, Université de Montréal, Montreal, Canada

[3]Children's Hospital of Eastern Ontario Research Institute, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada.

[#] equal contributors

*correspondence: Wayne Sossin
Montreal Neurological Institute
Room BT110
3801 University Avenue
Montreal, Quebec, Canada
wayne.sossin@mcgill.ca

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Abstract

Neuronal mRNAs can be packaged in reversibly stalled polysome granules prior to their transport to distant synaptic locales. Stimulation of synaptic metabotropic glutamate receptors (mGluRs) reactivates translation of these particular mRNAs to produce plasticity-related protein; a phenomenon exhibited during mGluR-mediated long-term depression (mGluR-LTD). This form of plasticity is deregulated in Fragile X Syndrome, a monogenic form of autism in humans, and understanding the stalling and reactivation mechanism could reveal new approaches to therapies. Here, we demonstrate that UPF1, known to stall peptide release during nonsense-mediated RNA decay, is critical for assembly of stalled polysomes in rat hippocampal neurons derived from embryos of either sex. Moreover, UPF1 and its interaction with the RNA binding protein STAU2 are necessary for proper transport and local translation from a prototypical RNA granule substrate and for mGluR-LTD in hippocampal neurons. These data highlight a new, neuronal role for UPF1, distinct from its RNA decay functions, in regulating transport and/or translation of mRNAs that are critical for synaptic plasticity.

Significance Statement

The elongation and/or termination steps of mRNA translation are emerging as important control points in mGluR-LTD, a form of synaptic plasticity that is compromised in a severe monogenic form of autism, Fragile X Syndrome. Deciphering the molecular mechanisms controlling this type of plasticity may thus open new therapeutic opportunities. Here, we describe a new role for the ATP-dependent helicase UPF1 and its interaction with the RNA localization protein STAU2 in mediating mGluR-LTD

through the regulation of mRNA translation complexes stalled at the level of elongation and/or termination.

Introduction

In neurons, mRNA transport and localized translation are crucial for synaptic plasticity (Jung et al., 2014). Proper spatiotemporal control of translation in dendrites or developing axons necessitates reliable suppression of protein synthesis during mRNA transport and a mechanism for subsequent reactivation at the appropriate time and destination. Most described repression mechanisms target translation initiation on particular mRNAs (Jung et al., 2014) and it would follow that their transport precedes polysome formation. In contrast, we and others have described a neuronal ribonucleoprotein complex (a neuronal RNA granule) consisting of stalled polysomes, where initiation of mRNAs has already occurred and repression is at the level of either elongation or termination (Elvira et al., 2006; Darnell et al., 2011; Graber et al., 2013b). The composition of the neuronal RNA granule seems to be particularly suited for the fast bursts of protein synthesis that have been observed in metabotropic glutamate receptor-dependent long-term depression (mGluR-LTD). This type of localized synaptic plasticity requires protein synthesis yet paradoxically occurs even in the absence of translation initiation, presumably due to the reactivated translation of pre-existing polysomes (Graber et al., 2013b).

One protein implicated in stalled polysomes is the fragile X mental retardation protein, FMRP (Darnell et al., 2011). A phosphorylated form of FMRP has been previously linked with stalled polysomes (Ceman et al., 2003). Indeed, FMRP is

dephosphorylated upon mGluR stimulation and this is required for the proper synthesis of proteins during mGluR-LTD (Niere et al., 2012). However, in the absence of FMRP, mRNA transport is largely unaffected (Steward et al., 1998). LTD proteins in this context are instead constitutively synthesized in dendrites independently of mGluR stimulation, leading to enhanced LTD upon mGluR stimulation (Hou et al., 2006; Nosyreva and Huber, 2006). Thus, FMRP appears to be necessary for stabilization of stalled polysomes but not their initial formation. In contrast, the mRNA binding protein STAU2 is required for mGluR-LTD and has been associated with the transport and translation of at least one FMRP and LTD target mRNA, *Map1b,* via its 3' untranslated region (UTR) (Lebeau et al., 2011).

STAU2 interacts with the RNA decay factor UPF1 (Miki et al., 2011; Park et al., 2013) and these proteins were found in the RNA granule proteome (Elvira et al., 2006; El Fatimy et al., 2016). UPF1 is a critical ATPase/helicase in nonsense-mediated RNA decay (NMD) pathways, where it is recruited to the stop codon through association with eRF1 and is involved in recognition of improper stop codons that are often present in mis-spliced mRNAs (Chang et al., 2007). One aspect of UPF1 function is to stall translation through interactions with eIF3, another abundant component of RNA granules (Elvira et al., 2006; Isken et al., 2008, El Fatimy et al., 2016), and to stall peptide release through binding to eRF3 (Ivanov et al., 2008). These characteristics made UPF1 an attractive candidate to play a mechanistic role in stalling polysomes.

Our results highlight a new role for UPF1 in regulating mRNA targets at the level of transport and translation, distinct from its RNA decay functions and provide

mechanistic insights into how stalled polysomes can be formed prior to transport and re-activated locally at synapses during synaptic plasticity.

Materials & Methods

Animals and cell culture

Sprague–Dawley rats were obtained from Charles River Canada. All animal experiments were approved by the Animal Ethics Committees of the Montreal Neurological Institute and Université de Montréal, and abided by the guidelines of the Canadian Council on Animal Care. Rat primary hippocampal neurons were dissected from embryonic day 18 Sprague–Dawley embryos of either sex and supplemented with new medium (neurobasal media supplemented with 1% (v/v) N2 and penicillin/streptomycin, 2% (v/v) B27 and 0.5 mM Gluta-MAX (Thermo Fisher Scientific)) every 6-8 d as previously described (Lebeau et al., 2011). Electrophysiology experiments were performed with 18-25 days *in vitro* (DIV) dissociated hippocampal neuron cultures while all other experiments were performed with 8-10 DIV cultures. Neurons were plated on 12 mm poly-L-lysine-coated #1.5 German glass coverslips (Neuvitro) at a density of 100,000 cells per well of a 12-well tissue culture plate. For super-resolution experiments, neurons were plated on poly-L-lysine-coated LAB-TEK II chambered #1.5 coverglass slides (Thermo Fisher Scientific). For knockdown experiments with single RNAi transduction, cultures were transduced with an MOI of 3-10. For rescue experiments with double transduction, cultures were transduced with *Stau2* RNAi with an MOI of 3 and with STAU2 constructs, each with an MOI of 5 or with *Upf1* RNAi with an MOI of 3 and with UPF1 rescue at an MOI of 0.5. While only a

subset of neurons was transduced with this MOI, they could be detected by the mCHERRY expressed from a separate promoter on the lentivirus. A minimum of three days was allowed for transduction and knockdown. Transduction efficiency was assessed in each experiment by GFP or mCHERRY expression. HEK293T cells were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% (v/v) FBS, sodium pyruvate, penicillin, and streptomycin.

DNA constructs and lentiviruses

The GFP.STAU2.flag plasmid (expressing the human 59 kDa isoform of STAU2) has been previously described (Graber et al., 2013b). C-terminally tagged UPF1.myc plasmid (expressing human UPF1) was a kind gift from Dr. Luc Desgroseillers, and RIP1.myc plasmid was a kind gift from Dr. Phil Barker. STAU2 C-terminal deletion and domain mutation constructs were created by PCR amplification of regions using GFP.STAU2.flag as template and inserting in-frame with GFP and the Flag tag. The F207A ribosome binding mutation was generated by site-directed mutagenesis. Plasmid sequences were verified by Sanger sequencing.

To efficiently knockdown STAU2 or UPF1 in rat hippocampal neurons, lentiviruses were generated that express short hairpin RNA (shRNA) driven by a CMV promoter as previously described (Thomas et al., 2009). A non-targeting, “scrambled” shRNA sequence (AATTCTCCGAACGTGTCACGT), a sequence targeting all of the rat isoforms of STAU2 (ACTAGTGGACGCTTTATAGCC), a sequence targeting rodent UPF1 isoforms (AGCAGCTTGTGGTAAATATAC), or a sequence targeting rodent PNRC2 (TGGTGATGGTGGTTCACTAAA), designed using the Invitrogen Block-iT™

RNAi designer (thus minimizing the possibility of off-target designs) were cloned into a lentiviral expression vector downstream of an emGFP ORF (pRRL.emGFP.shRNA). For rescue experiments, the RNAi-resistant GFP.STAU2.flag or the GFP.C351.flag (UPF1 binding mutation) ORFs were excised by restriction digest from the pcDNA plasmids used in the co-IP experiments and sub-cloned into the lentiviral expression plasmid. The UPF1 rescue construct was purchased from Vectorbuilder (Santa Clara, CA) and expressed mCHERRY from a viral promoter and human UPF1 from an EF1A promoter (pLV[Exp]-mCherry/Neo-EF1A>hUPF1[ORF003793]*/FLAG pLV[Exp]). All clones were verified by Sanger sequencing.

VSV G-pseudotyped virus was packaged by transient transfection of HEK293T helper cells with Lipofectamine 2000 (Thermo Fisher Scientific) pMD2.g, pRSV-Rev, and pMDLg/pRRE packaging plasmids together with the pRRL.emGFP.shRNA. Virus was collected over 48 hours with cell debris removed by microfiltration. Virus was then concentrated by centrifugation, resuspended in PBS, pH 7.3 and stored at -80 ºC. Virus was titered based on GFP expression in HEK293T cells. Transduction efficiency was assessed in each experiment by GFP or mCHERRY expression.

Co-Immunoprecipation and Immunoblotting

For experiments to determine if DHPG induced changes in phosphorylation of UPF1, hippocampal cultures (10 DIV) were incubated in the presence or absence of 50 μM (S)-3,5-DHPG (Tocris Bioscience) for 10 min and then harvested, lysed in Laemmli buffer, and the proteins separated by SDS-PAGE and immunoblotted first for phospho-UPF1 (1:500; anti-Ser1127; #0701016; Millipore) followed by anti-rabbit coupled to HRP, then

stripped and reblotted with anti-UPF1 (1:10,000; #ab133564, Epitomics) followed by anti-rabbit coupled to HRP. To determine loading, the membrane was stripped one additional time and probed with anti-GAPDH (1:1000; #sc-25778, Santa Cruz Biotechnology) followed by anti-rabbit coupled to HRP.

For experiments to determine the effectiveness of knockdown and rescue, hippocampal neurons were transduced at 7 DIV, and 3-7 days later were harvested and lysed in Laemmli buffer. Antibodies described above were used along with anti-STAU2 (kind gift of Dr. Michael Kiebler) and anti-PNRC2 (1:1000, #NBP1-74252, Novus Biologicals).

HEK293T cells were seeded in 6-well plates and transfected 24 h later with 1 µg each of the appropriate plasmid. Cells were lysed in non-denaturing co-ip buffer (150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 7.4) supplemented with Roche Complete EDTA-free protease inhibitor cocktail (Roche). 200 µg of lysate was incubated overnight with Flag antibody-conjugated beads (Sigma-Aldrich) followed by extensive washing with lysis buffer. Immunoprecipitated Flag-tagged species were eluted from beads with 2 µg/ml of Flag peptide (Sigma-Aldrich), resuspended in Laemmli buffer, resolved by SDS-PAGE and transferred to PVDF membrane for Western blotting. For co-IPs, membranes were probed with rabbit polyclonal anti-myc or mouse anti-Flag M2 monoclonal antibodies (Sigma-Aldrich) followed by anti-rabbit and anti-mouse secondary antibodies conjugated to infrared dyes CF680 and CF770 (Biotium), respectively. Secondary antibody detection was performed using the Licor Odyssey system (LI-COR Biosciences), or standard horseradish peroxidase detection with film was employed.

209 Ribopuromycylation and STAU2, UPF1 immunocytochemistry

210 To enrich for stalled polysomes, rat hippocampal neurons at 8–10 DIV were incubated
211 with 5 µM homoharringtonine (HHT; Tocris Bioscience) in 1 ml of supplemented
212 neurobasal media for 10 min. Ribopuromycylation has been described before (Graber et
213 al., 2013b). Briefly, puromycin (100 µM) and emetine (200 µM) (both from Sigma-
214 Aldrich) were then added and the cells were incubated at 37°C for an additional 5 min.
215 Free puromycin was removed with a digitonin wash on ice and cells were fixed at room
216 temperature for 15 min with 4% paraformaldehyde/sucrose. Cells were permeabilized
217 with 0.1% Triton X-100 and blocked with 5% BSA. Puromycin was detected with 1:1,000
218 of mouse anti-puromycin for 1 hr (clone 2A4, (David et al., 2012)), UPF1 was detected
219 with 1:500 rabbit monoclonal anti-RENT1/UPF1 (Epitomics), and STAU2 was detected
220 with 1:500 rabbit anti-STAU2 (Dr. Michael Kiebler). 1:1,000 of anti-mouse or anti-rabbit
221 Alexa 568 secondary antibody was incubated with samples for 1 h (Thermo Fisher
222 Scientific). For super-resolution imaging, primary antibodies were detected by
223 incubation with 1:1,000 or 1:10,000 of anti-mouse Alexa 647 and anti-rabbit Alexa 568
224 for 1 h. To reduce antibody drift during single molecule localization, antibody-antigen
225 pairs were post-fixed for 10 min at room temperature with 4%
226 paraformaldehyde/sucrose.
227
228 AHA labelling
229 Neurons were first incubated with methionine-free HEPES-buffered solution (120 mM
230 NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM HEPES, 30 mM glucose) for 30 min
231 at 37 °C followed by addition of 5 µM HHT and 25 µM AHA (Thermo Fisher Scientific)

232 for 10 min. before induction with 50 μM (S)-3,5-DHPG (Tocris Bioscience) for a further
233 10 min. DHPG was removed from the cells with one wash of PBS, and incubated for a
234 final 10 minutes with fresh AHA and HHT to resolve any DHPG-mediated increases in
235 protein synthesis. Cells were fixed as above. Subsequent detection of AHA has been
236 described previously (Graber et al., 2013b). For quantification of AHA, AHA intensity
237 was measured between 50-60 μM from the soma boundary. All values were normalized
238 to the average intensity of the scrambled control from that experiment.
239
240 MAP1B induction
241 Neurons were incubated with HHT for 10 min before co-incubation with 50 μM DHPG
242 for an additional 10 min. Cells were fixed as above and standard immunocytochemistry
243 was performed with 1:200 mouse anti-MAP1B clone AA6 (Santa Cruz Biotechnology)
244 and 1:1000 anti-mouse Alexa 568, both for 1 h. For quantification of MAP1B, MAP1B
245 intensity was measured between 50-60 μM from the soma boundary. All values were
246 normalized to the average intensity of the scrambled control from that experiment.
247
248 Droplet digital RT-PCR
249 Total RNA was isolated from $2x10^6$ hippocampal neurons using RNAqueous Total RNA
250 isolation kit following the manufacturer's protocol (Thermo Fisher Scientific). cDNA was
251 synthesized from 1 μg of total RNA using Superscript II reverse transcriptase with dT_{18}
252 primer (Thermo Fisher Scientific). Standard curves and the appropriate annealing
253 temperatures were obtained by quantitative PCR (Eppendorf realplex) for the following
254 rat-specific primer sets (listed as forward, reverse) that were designed to span an intron:

Map1b: CACACGAGGGGAAGAGAAGG, TTAGTGGTTCCTGGTCCTGC; *Upf1:* GTCACAGACTCAAGATAACATCACG, GACTAAATCCTCATTACCAGAGTCA; *Stau2:* ACTCCCACCTCTTCCTGTGA, CATACTCGGGTCCAGCCTTT; *Gapdh*: TCATGACCACAGTCCATGCC, ATCACGCCACAGCTTTCCAG; *Rgs4:* CATCGGCTGGGATTTCTGCT, GCTCACCCTCTGGCAAGTTA; *Cplx1:* GGCATACGAGATAAGTATGGCATC, CTTCTTGGGTCGAGTCAGGC; *Arc:* GCCAGTCTTGGGCAGCATAG, ACTGGTATGAATCACTGCTGGG; *Dexi:* AGAGGCCCGCCTGCTC, GGATCTGGCAACTCCCCATC; *Arf1:* GGCGGCCTGAGGTCTCT, TTGCCAAAAAGGCCCTTGAA; *Gap43:* AGGAGGAGAAAGAAGCTGTAGAT, GTTCTTGGTCAGCCTCGGG. Droplet digital PCR was performed with varying amounts of cDNA using QX200 ddPCR EvaGreen Supermix (Bio-Rad) together with the QX200 Droplet Digital PCR system (Bio-Rad) according to the manufacturer's protocol. Following droplet generation, the PCR step was performed using a Bio-Rad C1000 thermal cycler (Bio-Rad) with the following parameters: 1 cycle at 94 ^{o}C for 1 minute, 45 cycles at 94 ^{o}C for 15 seconds followed by 58 ^{o}C for 1 minute with a ramp rate of 2 ^{o}C/second, 1 cycle at 4 ^{o}C for 5 minutes, and 1 cycle at 90 ^{o}C for 5 minutes. Droplets were flagged as positive or negative for PCR product using the QX200 Droplet Reader and Quantasoft software v1.7.4 was used to determine absolute copy number per microliter.

Single molecule RNA-FISH (smRNA-FISH)

Neurons were fixed on glass coverslips with formaldehyde as above, washed with PBS and permeablized with 70% ethanol for at least 1 h. Neurons were re-hydrated with 10%

(v/v) formamide and 2X SSC (300 mM NaCl, 30 mM sodium citrate). Neurons were then incubated with 25 pmols of *Map1b* Stellaris FISH probes (Biosearch Technologies) This set of 48, 21-mer oligos each of which are conjugated to Quasar 570 fluorophores and designed to target nucleotides 93-1726 of the 7386 nucleotide rat *Map1b* mRNA (Genbank accession NM_019217). The probe sequences are as follows (5'-3'): GAGGAAGGCAATTTCTGCTG,

,GCAGCATGTTCAAAGTCTTC,CCAGTCTTTCTGATCTTTTG,CATGGGAGAGTCAAA CTGCT,TGACTCTCTCATCTTTCACA,CTCTCTTTGGAAGTAGCTGA,GGTGTCGAAG TCTCTTTTTT,TGGTTCAGTTTCAGTGAGTG,GATGTCTCTTCAAGCTTTGT,AGTCTG AATTACACCTGCTT,AACTGCCTGTTCTAGACTTA,CTCTTCAAAAAGTCCCTGGA,CA TCCCTGATTTATCTTCAT,CACTCAGCTCGTAGTAATCA,TTCATCTTGTTGGTTCTTG G,CTTGGTTCTTCAGGTAACTC,CGGCCAAAGTTAAACCCAAG,CCAGAGGGGAAAG ATCATGG,CAGGTAATCATCTCCTTCAT,GAGGATGTTTCAACTTGGGT,AGAAGCTA ATCTGGACCTGG,TTGACTGTCTGGTTTTACGG,TTGTTGAACACACAGTACCC,AAA GGTCAGTGGCCAAATCT,GAGGTTTGCTCATAGGTTAC,AGGTGCTGTTTCTTTAGTT G,TTTCTTGGCAGCAACATCTA,CTGTCCAAAGTCACTGACTT,CTGGGTGACATCAG AAGGTA,CTACTTCCTGGAACAAGCAG,ATCAGTCGTGGTTTGTACTA,AATCGTCGT AGGTTTCTGTT,ATCAATGGTGGTCTCATCTT,CTCTCTCCTCTTTAGGAATA,TTTTTC CTTCTCACTTCATC,TTTTCCGCTTAACACAGGAG,ATTCCATCAGTGACTTTGTC,TC TTGGGAGGGAAGAACGTT,GGAGCTGTTCAGAGAGAACG,GTGAAGAGTAGCTTGG AGGA,CTTTGGAGGAGTGCGAATGA,GGTTGGTTAATGAGCCGAAG,GTTGATCCGA TTTTGGACTT,GATGTTCTTTAGAGAGCCAC,CATGGTGAGCATTGTCAAGT,TGTCA ATCTTCACGTTACCA,TCTCGGAAGTTCAGCTTTTG. To detect rat *Map2* mRNA

(Genbank accession NM_013066.1), a probe-set was synthesized with the following Quasar 570-conjugated DNA sequences: GAGGAAGGCAATTTCTGCTG,GCAGCATGTTCAAAGTCTTC,CCAGTCTTTCTGATC TTTTG,CATGGGAGAGTCAAACTGCT,TGACTCTCTCATCTTTCACA,CTCTCTTTGGA AGTAGCTGA,GGTGTCGAAGTCTCTTTTTT,TGGTTCAGTTTCAGTGAGTG,GATGTC TCTTCAAGCTTTGT,AGTCTGAATTACACCTGCTT,AACTGCCTGTTCTAGACTTA,CT CTTCAAAAAGTCCCTGGA,CATCCCTGATTTATCTTCAT,CACTCAGCTCGTAGTAAT CA,TTCATCTTGTTGGTTCTTGG,CTTGGTTCTTCAGGTAACTC,CGGCCAAAGTTAAA CCCAAG,CCAGAGGGGAAAGATCATGG,CAGGTAATCATCTCCTTCAT,GAGGATGT TTCAACTTGGGT,AGAAGCTAATCTGGACCTGG,TTGACTGTCTGGTTTTACGG,TTG TTGAACACACAGTACCC,AAAGGTCAGTGGCCAAATCT,GAGGTTTGCTCATAGGTT AC,AGGTGCTGTTTCTTTAGTTG,TTTCTTGGCAGCAACATCTA,CTGTCCAAAGTCAC TGACTT,CTGGGTGACATCAGAAGGTA,CTACTTCCTGGAACAAGCAG,ATCAGTCGT GGTTTGTACTA,AATCGTCGTAGGTTTCTGTT,ATCAATGGTGGTCTCATCTT,CTCTC TCCTCTTTAGGAATA,TTTTTCCTTCTCACTTCATC,TTTTCCGCTTAACACAGGAG,AT TCCATCAGTGACTTTGTC,TCTTGGGAGGGAAGAACGTT,GGAGCTGTTCAGAGAGA ACG,GTGAAGAGTAGCTTGGAGGA,CTTTGGAGGAGTGCGAATGA,GGTTGGTTAAT GAGCCGAAG,GTTGATCCGATTTTGGACTT,GATGTTCTTTAGAGAGCCAC,CATGGT GAGCATTGTCAAGT,TGTCAATCTTCACGTTACCA,TCTCGGAAGTTCAGCTTTTG,AT GCTTCCAGAAGAGGAGAC. The probes were incubated with the cells in hybridization buffer (1 mg/ml yeast tRNA, 200 µg/ml BSA, 10 % [w/v] dextran sulphate, 2 mM vandal ribonucleoside complex, 10% [w/v] formamide, 2X SSC) overnight in a humidified chamber at 37 °C. Neurons were washed twice with 10 % (v/v) formamide, 2X SSC at

37 °C. Fresh GLOX buffer (0.37 mg/ml glucose oxidase, 300 AU catalase, 0.4 % (w/v) glucose, 2X SSC, 10 mM Tris-HCl, pH 8.0) was added prior to mounting the coverslips on glass slides for imaging.

Confocal imaging and quantitation of *Map1b* mRNA and puromycylated puncta

Neurons were imaged using a Zeiss LSM-710 confocal microscope with a 63X oil immersion objective (NA=1.4). ImageJ was used for image post-processing (e.g., neurite straightening using the "Straighten" plugin) including quantitation. For quantitation of puromycylated puncta, straightened images of neurites were thresholded so that only high intensity puncta were visible, and their numbers counted and normalized to neurite length. For smRNA-FISH, a Laplacian of Gaussian transformation was applied to confocal images using the ImageJ plugin "LoG3D" to enhance edges of individual puncta. Images were then thresholded and outline masks of puncta were generated and quantitated in ImageJ using the "Analyze particles" tool.

Super-resolution imaging

Single-molecule localization was performed using a Vutara SR-350 STORM microscope in the presence of a photo-switching buffer (20 mM MEA, 144 mM 2-mercaptoethanol, 2 mM cyclooctatetraene (COT), 170 AU glucose oxidase, 2000 AU catalase; all sourced from Sigma-Aldrich). At least 5,000 exposure frames were taken and localizations were calculated using Vutara's proprietary algorithms. Point clouds with ball size diameter reflecting localization accuracy were plotted using Vutara visualization software. A stringent confidence cut-off of 0.9 was used together with post-hoc de-noising by

Delaunay Tessellation Field Interpolation (DTFE) to remove diffuse background localizations. Localization data points (x, y, z coordinates) were exported and individual RPM/UPF1 puncta were plotted with the `scatterplot3d` R package. Euclidean distance between localization points in three-space were calculated using a custom MATLAB script with the formula, $\sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2}$, and kernel density estimates were plotted using the geom_density function of the `ggplot2` R package.

Electrophysiology and mGluR-LTD

Coverslips with cultured hippocampal neurons were placed in a recording chamber mounted on an inverted microscope equipped with epifluorescence (Nikon Eclipse Ti-S). Cultures were perfused at 1 ml/min with Tyrode's solution at room temperature containing (in mM) 150 NaCl, 4 KCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 2 $CaCl_2$, 0.1 picrotoxin, 0.001 tetrodotoxin (pH 7.37-7.41; adjusted to 295-305 mOsmol with KOH). Whole-cell recordings were obtained from hippocampal neurons with borosilicate micropipettes (3-7 MΩ) filled with intracellular solution containing (in mM) 125 K-gluconate, 2.6 KCl, 1.3 NaCl, 10 HEPES, 4 ATP-Mg, 0.3 GTP-Na, 0.1 EGTA, 14 phosphocreatine-Tris, (pH 7.2-7.3; 275-280 mOsmol). Neurons were selected for recordings by visual identification in phase contrast in experiments without transduction and by GFP expression in transduction experiments. Recordings were made in voltage-clamp mode using a Multiclamp 700B amplifier (Molecular Devices). The cell holding potential was maintained at -60mV and series resistance was routinely monitored. Recorded signals were low-pass filtered at 2 kHz, digitized at 20 kHz, and stored on a

PC using Digidata 1440A acquisition board and pClamp10 software (Molecular Devices). Data were only included if the holding current (<200 pA) was stable and series resistance (<32 MΩ) varied <25% of initial value.

Long-term depression was induced by bath application of the group I mGluR agonist (S)-3,5-DHPG (100 μM; Abcam) for 5 min. The translation inhibitors emetine (40 μM) and HHT (20 μM) were bath-applied 10 min before, during and 30 min after DHPG application. Miniature EPSCs (mEPSCs) were recorded in 5 min episodes throughout the experiments. For analysis, mEPSCs were detected and measured over two 5 min time periods, before (-5-0 min) and after (25-30 min) DHPG application using MiniAnalysis program (Synaptosoft) after lowpass Gaussian filtering of traces at 1 kHz. Detection threshold was set at 7 pA and all detected events were verified by visual inspection. Cells with mEPSC frequency ≤0.5 Hz were excluded from analysis.

Experimental Design and Statistical analysis

In general, all experiments were planned to have at least three replicates from separate hippocampal cultures. Since for most of the experiments, the amount of error was not known *a priori*, it was difficult to accomplish effective power analysis to pre-plan the number of experiments. Unless otherwise stated in figure legends, statistical P-values were determined using a one- (where appropriate) or two-tail, unpaired Student's t-test for two group comparisons and one-way ANOVA followed by Šidák post-hoc multiple comparison correction for >2 groups using Graphpad Prism v.6. For electrophysiology experiments, one-tailed, paired t-tests on mEPSC parameters pre- versus post-DHPG application were performed using Graphpad Prism. Any distinct analysis is described in

the figure legends. Bar plots are presented as the mean ± SEM and in-text values are stated as the mean ± SD. Asterisks indicate p-values as follows: *p<0.05, **p<0.01, ***p<0.001 and non-significant associations are denoted by “ns”. The experimenter was blinded for the analysis of neurites labelled for AHA, RPM, MAP1B protein and *Map1b* mRNA.

Results

UPF1 is required for the presence of stalled polysomes in neurites.

To examine the role of UPF1 in the generation of stalled polysomes we constructed a lentivirus encoding GFP and short-hairpin RNAi targeted to *Upf1*. Three days after infection of hippocampal neuron cultures, levels of UPF1 were significantly decreased (50%) compared to neurons transduced with a lentivirus expressing a non-targeting (scrambled) RNAi as measured by immunofluorescence (Figure 1A, B). Western blotting indicated a similar decrease in UPF1 expression in total cell lysates (Figure 1C). Next, we assessed the number of stalled polysomes in neurites with lowered UPF1 expression using an in-situ run-off assay in hippocampal cultures. Briefly, translating polysomes are allowed to complete translation in the presence of an inhibitor (homoharringtonine; HHT) that prevents formation of new polysomes (Graber et al., 2013b). The remaining stalled polysomes are detected by ribopuromycylation (RPM); where puromycin is covalently attached to polysome-bound nascent peptide chains that are fixed in position with the elongation inhibitor emetine (David et al., 2012). Reducing the expression of UPF1 was sufficient to decrease the number of stalled polysomes seen in distal dendrites (>50 µm from soma) by approximately 50% (Figure 1D).

Stimulation of mGlu receptors with DHPG can reactivate translation of stalled polysomes encoding proteins required for mGluR-LTD (Graber et al., 2013b). This burst of protein synthesis can be measured after first blocking initiation-dependent protein synthesis with HHT, allowing ribosome run-off and then measuring new, initiation-independent protein synthesis by pulsing with the methionine analogue azidohomoalanine (AHA) (Graber et al., 2013b). A fluorophore is then chemically attached to AHA and detected by fluorescence microscopy. Using this assay, we found that decreasing expression of UPF1 blocked induction of initiation-independent translation by DHPG (Figure 1E). To rule out off-target effects of our *Upf1* RNAi, we rescued DHPG-induced initiation-independent protein synthesis with a lentivirus expressing human UPF1, which lacks the sequence targeted by the RNAi. This vector also expresses mCHERRY on a separate cistron. Expression of this lentivirus rescues the knockdown of UPF1 (Figure 1F) and the activation of initiation-independent protein synthesis by DHPG (Figure 1G), demonstrating that the effect of UPF1 on blocking initiation-independent protein synthesis was not an off-target effect of the RNAi.

Knocking-down UPF1 should increase levels of mRNAs normally regulated by NMD. Previously, the protein ARC, whose mRNA is a constitutive target for NMD due to an intron in its 3' UTR, was shown to be upregulated when NMD is disrupted (Giorgi et al, 2007). Indeed, droplet digital RT-PCR results from our UPF1 knockdowns are consistent with this upregulation, with *Arc* mRNA increasing to 191 $\pm$ 8.85 percent over scrambled controls, n=2. Could the effects we observe in UPF1 knockdowns be due to upregulation of NMD targets? To assess this, we knocked-down expression of Proline-rich Nuclear Receptor Coregulatory protein 2 (PNRC2), a protein that interacts with

UPF1 and that is required for NMD (Cho et al., 2009). While we observed an efficient knockdown of PNRC2 in hippocampal neuronal cultures (Figure 2A), we saw no significant change in the induction of AHA incorporation in neurites following DHPG treatment in neurons lacking PNRC2 (Figure 2B). These data indicate that the observed decrease in translation from reactivated stalled polysomes in UPF1 knockdowns appears to be independent of its primary function in NMD.

One explanation for the paucity of stalled polysomes in distal dendrites lacking UPF1 is that their mRNA cargo might be degraded in the soma. We therefore chose to assess *Map1b* mRNA expression in this context as it encodes an essential protein for the expression of mGluR-LTD and whose synthesis occurs in a rapid and highly localized manner (Davidkova and Carroll, 2007; Lebeau et al., 2011; Graber et al., 2013b). We have previously shown that *Map1b* mRNA is present in ribosome-containing RNA granules (Lebeau et al., 2011) and undergoes initiation-independent translation upon stimulation of mGlu receptors (Graber et al., 2013b) all features that are consistent with stalled polysomes. Knockdown of UPF1 had no effect, however, on total *Map1b* mRNA levels in neurons measured using droplet digital RT-PCR (Figure 2C), demonstrating that UPF1 does not directly regulate levels of *Map1b* mRNA through NMD. This left the possibility that *Map1b* stalled polysomes do not form in the context of decreased UPF1 expression, or form but become mis-localized. Alternatively, these mRNAs may still be properly localized to the dendrite but undergo leaky, dispersed translation that could explain the decrease in the number of stalled polysome puncta. To test whether *Map1b* mRNA is still localized to dendrites, we looked at the sub-cellular distribution of endogenous *Map1b* mRNA using single-molecule RNA fluorescence in

462 situ hybridization (smRNA-FISH) (Raj et al., 2008). In neurons expressing non-targeting
463 RNAi we were able to detect many copies of *Map1b* throughout the cell soma, with
464 some also visible in neurites (Figure 2D). Strikingly, in neurons expressing *Upf1* RNAi
465 there was a significant decrease in the number of *Map1b* messages per micron of
466 neurite length in both proximal (<50 μm) and distal (>50 μm from the soma boundary)
467 neurites, while somatic *Map1b* did not significantly change (Figure 2D).

468 Thus, we conclude that in the absence of UPF1, reactivation of stalled
469 polysomes in neuronal dendrites is inhibited, most likely due to the lack of formation
470 and/or transport of these structures into dendrites.

471 If UPF1 is critical for the stalling process, one might expect that post-translational
472 modification of UPF1 could play a role in release of the stall. Indeed, we found that
473 DHPG induced a significant dephosphorylation of UPF1 (Fig. 2E), consistent with the
474 known activation of phosphatases by DHPG (Niere et al, 2012). Thus, dephoshorylation
475 of UPF1 could play a role in the release of stalled polysomes following DHPG treatment.

476

477 <u>UPF1 is co-localized with stalled polysomes in distal neurites.</u>

478 UPF1 has been associated with RNA transport granules in previous studies
479 (Barbee et al., 2006; Giorgi et al., 2007) and is present in the proteomics of RNA
480 granules (Elvira et al., 2006; El Fatimy et al., 2016). We took advantage of our ability to
481 identify stalled polysomes using RPM and then assayed their association with UPF1 in
482 super-resolution using dual color, three-dimensional stochastic optical reconstruction
483 microscopy (Dual Color 3D-STORM; Figure 3) (Juette et al., 2008). HHT-
484 ribopuromycylated neurons were fixed and probed for puromycin and UPF1 using

485 appropriate primary antibodies, incubated with standard secondary antibodies and
486 followed by an additional fixation step to minimize movement of antibody complexes.
487 Ribopuromycylated puncta in hippocampal neurons were first located by low resolution
488 wide-field immunofluorescence (Figure 3A) prior to surveying individual fluorophore-
489 conjugated secondary antibody localizations at super-resolution (Figure 3B, C). We
490 collected super-resolution localizations of both Alexa568 (labelling puromycin) and
491 Alexa647 (labelling UPF1) puncta in samples saturated with secondary antibodies (high
492 density probes at 1:1000 dilution; Figure 3B) and samples labelled with limited
493 secondary antibody (low density probes at 1:10000 dilution; Figure 3C) to minimize the
494 chances of detecting non-specific antibody complexes. Intriguingly, we found that the
495 topography of localizations, when plotted with Gaussian blur (point-splatting), highlight
496 structures that are suggestive of multiple UPF1 molecules (or a single molecule
497 complexed with multiple secondary antibodies) in complex with the apex of a stalled
498 polysome (Figure 3B, C). Supporting this supposition, the sizes of the densely-
499 puromycylated structures were found to be generally uniform and consistent with the
500 expected diameter of a puromycylated ribosome with associated primary and secondary
501 antibodies (approximately 25-50 nm). We used a commercially available monoclonal
502 antibody against UPF1 whose epitope remains proprietary, therefore it is difficult to
503 determine if the granules we observed contained one or more UPF1 molecules.
504 However, we did observe, in general, fewer UPF1 relative to puromycin localizations
505 (individual localizations for a typical granule are plotted in Figure 3D). While the number
506 of puromycin localizations appears too large for a single polysome, uncertainties
507 concerning the number of secondary antibody fluorophores and the possible recounting

of fluorophores due to movement during the lengthy imaging procedure, preclude definitive conclusions on this point. If multiple polysomes are present in a granule it would suggest some tertiary organizing feature in RNA granules as has recently been suggested by EM micrographs of purified RNA granules (El-Fatimy et al, 2016). Further, we found that puromycin and UPF1 localizations resided in close proximity to each other. We extended this qualitative analysis by calculating the point-to-point distances for each localization species (UPF1-UPF1, puromycin-puromycin and UPF1-puromycin) and plotting their densities as a function of distance in nanometres. Figure 3E describes the distribution of the localizations found in the single puncta represented in Figure 3D. By surveying the maxima of these densities for each species across multiple RPM puncta from independent experiments, we observed that peak hetero-molecular (UPF1-puromycin) distances were significantly different from peak homo-molecular (UPF1-UPF1 or puromycin-puromycin) distances, consistent with a specific molecular complex rather than non-specific association of secondary antibodies (Figure 3F). Thus, by using a super-resolution approach, we were able to sufficiently resolve sub-micron, anisotropic structures that are consistent with helical polysomes bound to UPF1.

Characterization of a STAU2 mutation that fails to interact with UPF1.

We have previously shown that STAU2, but not its paralogue STAU1, is required for production of MAP1B during mGluR-LTD in rat primary hippocampal neurons and slices (Lebeau et al., 2011). Consistent with this, we also found that STAU2 colocalizes with ribosomes and stalled polysomes (Lebeau et al., 2011; Graber et al., 2013b). We now find that similar to our results with *Upf1* RNAi, reduction of STAU2 expression

using RNAi targeting all major rat isoforms (Figure 4A, B), reduces the number of stalled polysomes (Figure 4C) and decreases the amount of *Map1b* mRNA found in distal neurites (Figure 4D). Importantly, no effect on localization was seen for *Map2* mRNA in either *Stau2* or *Upf1* RNAi-expressing neurons, indicating that the mis-localization effect for *Map1b* cannot be generalized to all mRNAs (Figure 4E). Similar to UPF1 (Figure 2C), there was no effect of knocking-down STAU2 on the total level of Map1b mRNA despite a strong reduction (>80%) in *Stau2* mRNA (Figure 4F).

Together, these results suggest a functional relationship between STAU2 and UPF1 in the context of RNA transport, translation, or both. Interestingly, both STAU1 and STAU2 have been previously implicated in RNA decay together with UPF1 where their interactions were found to enhance exon-junction complex-independent RNA decay (so-called Staufen-mediated RNA decay; SMD) (Park et al., 2013). This interaction triggers UPF1 phosphorylation that facilitates the recruitment of RNA decapping factors, although the exact mechanisms and proteins involved are still not well understood (Kim et al., 2005). However, the loss of Staufen-mediated decay is unlikely to explain our results as (i) in our cultures we do not observe upregulation of mRNAs known to be targets of SMD after STAU2 knockdown (Figure 4F) and (ii) knockdown of PNRC2 does not affect the DHPG-induced upregulation of initiation-independent RNA translation (Figure 2B), even though PNRC2 is also required for SMD (Cho et al., 2012). It should also be noted that neither *Upf1* mRNA, nor NMD targets (*Arc, Dexi)* are upregulated after knockdown of STAU2, although one mRNA previously shown to be regulated by STAU2, *Rgs4* (Heraud-Farlow et al, 2013), was slightly

553 downregulated, albeit non-significantly (Figure 4F). UPF1 knockdown also did not alter
554 *Stau2* mRNA levels (93.6 ± 8.7 percent of scrambled controls).

555 Using transiently expressed epitope-tagged versions of human UPF1 and the 59
556 kilodalton isoform of STAU2 (an RNA granule-associated isoform that is highly
557 expressed in hippocampal neurons (Duchaine et al., 2002)), we were able to confirm
558 their co-immunopurification in HEK293T (Figure 5A, B). Importantly, we were unable to
559 pull-down another myc-tagged protein, RIP-myc, with FLAG-tagged STAU2 indicating
560 that the STAU2-UPF1 interaction is specific (Figure 5B). Miki et al have previously
561 shown that STAU2 and UPF1 directly interact with each other in a cell-free system (Miki
562 et al, 2011). However, this interaction could depend on indirect binding through RNA or
563 RNA-ribosome complexes in HEK293T cells. To address this, we treated lysates with
564 RNase A prior to immunoprecipitation and found that despite the loss of the ribosomal
565 protein S6 (RPS6; indicative of ribosome-RNA association) in the nuclease-treated IP,
566 STAU2 complexes still contained UPF1, although we cannot say that RNA-ribosome
567 complexes do not facilitate this interaction (Figure 5C).

568 To begin to understand how STAU2-UPF1 interactions might affect mRNA
569 translation, we attempted to define the specific region(s) in the 59 kilodalton isoform of
570 human STAU2 that is necessary for binding to UPF1. We created several Flag-tagged
571 constructs that expressed STAU2 domains (incorporating the STAU2 nuclear export
572 signal to ensure cytoplasmic expression; Figure 5A). We found that on their own, none
573 of the defined double-stranded RNA binding domains (dsRBD) or the C-terminal tubulin
574 binding domain (TBD) were sufficient for interaction with UPF1 (data not shown).

However, a C-terminal deletion mutation that lacked the TBD (C351), but retained dsRBDs 1-4 was sufficient to abrogate binding to UPF1 (Figure 5D).

We next asked whether the loss of UPF1 binding with the STAU2 C351 mutation could be indirect, resulting from impaired STAU2 interaction with ribosome/RNA complexes that are presumably pulled-down in our immunoprecipitations lacking RNase A treatment. We detected the presence of ribosomal protein S6 (RPS6) in our immunoprecipitated STAU2-UPF1 complexes suggesting the presence of mono/polysome complexes. To confirm this, we introduced a point mutation in dsRBD3 of STAU2 (F207A; numbering based on Uniprot ID: Q9NUL3-2) that is analogous to the F135A mutation in its paralogue STAU1 that has previously been reported to ablate RNA binding and curtail binding to the ribosome, presumably by altering the secondary structure of the former (Luo et al., 2002). We observed decreased affinity of this mutation construct for RPS6 relative to wildtype (Figure 5D), likely due to a decrease in associated mono/polysomes. Importantly however, the ability to bring down RPS6 is unaffected in immunoprecipitants from cells expressing the C351 mutation vs. wildtype STAU2 (Figure 5D). These data suggest that the C351 mutation does not impair STAU2 interactions with mono/polysomes, but does affect the interaction with UPF1, consistent with an independent interaction between STAU2 and UPF1. It should be noted that the C351 mutation could still affect STAU2 function(s) other than binding UPF1, such as hetero-dimerization (Gleghorn et al., 2013).

To better define the region in the C-terminus that is necessary for UPF1 binding, we created several additional C-terminal deletion mutations. Full binding, indistinguishable from the wildtype, was observed in the C430 mutation which contained

598 the entire tubulin binding domain but lacked the remaining C-terminal residues (Figure
599 5E). Critically, we found that the middle portion of the tubulin binding domain (TBD) was
600 necessary, but not sufficient for efficient binding to UPF1. Interestingly, this binding
601 region has very low amino acid homology to Staufen 1 (Figure 5F) perhaps suggesting
602 that Staufen 1 and 2 interact with UPF1 in different ways, although the functional
603 significance of this, if any, remains unclear.
604
605 STAU2-UPF1 interactions are necessary for DHPG induction of MAP1B protein in distal
606 neurites
607 STAU2 is required for DHPG induction of MAP1B protein (Lebeau et al., 2011).
608 This occurs independently of translation initiation (Graber et al., 2013b) and is
609 presumably the result of peptide release from polysomes that have been reactivated. To
610 determine if the STAU2-UPF1 interaction is important for this functional release of
611 stalled polysomes, we used a rescue paradigm where STAU2 levels were reduced by
612 our lentiviral RNAi in hippocampal rat neurons and then rescued using a separate
613 lentivirus expressing either human full-length STAU2 or human STAU2 lacking the
614 UPF1 binding domain (C351) which are both resistant to rat-specific RNAi. Both
615 constructs expressed at equivalent levels (reported by GFP immunodetection in Figure
616 5G) and the full-length STAU2 construct expressed at similar levels to the endogenous
617 59 kilodalton STAU2 (top panel of Figure 5G; note the Stau2 antibody does not detect
618 C351 expression). As previously shown using transient siRNA transfection by Lebeau et
619 al, knockdown of STAU2 in this context blocked the DHPG-mediated induction of
620 MAP1B protein in distal neurites (Figure 5H). Demonstrating that this is not an off-target

621 effect, we were able to rescue induction of MAP1B with co-expression of RNAi-resistant
622 human STAU2, but not the STAU2 harbouring the C351 mutation (Figure 5H). These
623 results suggest that DHPG induction of MAP1B expression requires the STAU2-UPF1
624 interaction, although we cannot rule out the possibility that the loss of the DHPG-
625 induced increase was due to a separate function of STAU2 that was also lost with the
626 C351 mutation.
627
628 mGluR-LTD in dissociated hippocampal neurons does not require translation initiation
629 We next examined the role of UPF1 and STAU2 in regulating synaptic plasticity
630 mediated by reactivated translation from stalled polysomes. We first determined if
631 mGluR-LTD recorded at the single cell level in cultured hippocampal neurons was
632 dependent on re-activation of stalled polysomes using voltage clamp recordings of
633 miniature excitatory postsynaptic currents (mEPSCs). In cultured hippocampal neurons,
634 mGluR-LTD is associated with an internalization of GluA receptors resulting in a
635 reduction of mEPSC frequency with no change in amplitude (Waung et al., 2008;
636 Sanderson et al., 2011). Consistent with these previous reports, DHPG-induced LTD in
637 long-term hippocampal neuron cultures was associated with a decrease in mEPSC
638 frequency, but not amplitude, following bath application of DHPG (Figure 6A, B).
639 We recently showed that mGluR-LTD at Schaffer collateral synapses is
640 insensitive to HHT, an inhibitor of translation initiation or, more precisely, of the first
641 round of elongation. Furthermore, mGluR-LTD was prevented by emetine, an inhibitor
642 of translation elongation (Graber et al., 2013b). Thus, we first verified if mGluR-LTD was
643 similarly independent of translation initiation in dissociated cultured neurons. Application

644 of the elongation inhibitor emetine blocked the decrease in mEPSC frequency induced
645 by DHPG, while incubation with the initiation inhibitor HHT did not (Figure 6A, B). Mean
646 amplitude of mEPSCs was not significantly changed by DHPG in these experiments.
647 These results, showing a block of mGluR-LTD by the translation elongation inhibitor
648 emetine confirm that long-term synaptic plasticity in cultured hippocampal neurons is
649 dependent on *de novo* protein synthesis. Moreover, the lack of inhibition of mGluR-LTD
650 by the translation initiation inhibitor HHT, suggests that this plasticity is dependent on
651 regulation downstream of initiation, through reactivated translation from stalled
652 polysomes.
653
654 UPF1 and its interaction with STAU2 are required for mGluR-LTD in hippocampal
655 neurons
656 Having established that cultured neurons show mGluR-LTD independent of
657 translation initiation, but dependent on translation elongation in response to DHPG, we
658 next wanted to determine if UPF1, and its interaction with STAU2 on stalled polysomes,
659 were required for mGluR-LTD. We assessed DHPG-induced LTD of mEPSCs in
660 neurons expressing *Upf1* or scrambled RNAi lentivirus and found that the depression of
661 mEPSC frequency, without change in amplitude, was prevented in neurons with
662 reduced UPF1 expression relative to those expressing scrambled RNAi (Figure 7A, B).
663 We previously reported that mGluR-LTD induced by DHPG was impaired in cultured
664 hippocampal slices transiently transfected with *Stau2* siRNA (Lebeau et al., 2011). Next,
665 we verified the importance of STAU2 in mGluR-LTD in dissociated neurons using *Stau2*
666 RNAi lentivirus. Neurons expressing *Stau2* RNAi did not show DHPG-induced

depression of mEPSC frequency, consistent with our previous observations in slices (Figure 7A, B). There was, however an increase in EPSP amplitudes when Stau2 was knocked-down, that was not seen with UPF1. This may be due to regulation of additional messages by STAU2 independent of its role in stalled polysomes (Heraud-Farlow et al., 2013). Indeed, our droplet digital RT-PCR results revealed that one of the previously identified STAU2 mRNA targets was slightly downregulated in STAU2 knockdowns (*Rgs4*; Fig 4F). Decreases in this and presumably other proteins stabilized by STAU2 may underlie the changes in amplitude seen with its loss, but not with that of UPF1.

Next, we attempted to rescue the deficit in mGluR-LTD in neurons expressing *Stau2* RNAi with lentiviruses that express RNAi-resistant human STAU2. Infection with STAU2-expressing virus restored the DHPG-induced LTD of mEPSC frequency indicating that the *Stau2* RNAi phenotype was not due to off-target effects (Figure 7A, B). Finally, we sought to determine whether the UPF1-STAU2 interaction was necessary for the STAU2 rescue of mGluR-LTD by expressing the STAU2 C351 mutation which cannot interact with UPF1 into the *Stau2* RNAi background. Expression of the mutated STAU2 failed to rescue the DHPG-induced LTD of mEPSC frequency (Figure 7A, B). Interestingly, the mutated STAU2 did rescue the change in mEPSC amplitude, consistent with this effect of STAU2 being mediated through a UPF1-independent role of STAU2 and also demonstrating that the C351 mutation does not perturb all STAU2 functions.

Together, these data demonstrate that UPF1, as well as STAU2, are required for mGluR-LTD in hippocampal neurons and moreover, highlight the importance of their

690 direct interaction in the transport and/or translation of RNA granules that participate in
691 protein synthesis-dependent synaptic plasticity.
692
693 **Discussion**
694 A new role for UPF1 in the formation of neuronal granules comprising stalled polysomes
695 We've characterized a novel function for the RNA helicase UPF1 in the
696 regulation of transport RNA granules consisting of stalled polysomes in neurons. UPF1
697 is present in stalled polysomes by proteomic analysis (Elvira et al., 2006; El Fatimy et
698 al., 2016) and we demonstrate it occupies a polarized position in neuronal RNA
699 granules determined using super-resolution microscopy (Figure 3). Knockdown of UPF1
700 in hippocampal neurons results in: i) fewer stalled polysomes in neurites, ii) reduced
701 transport of *Map1b*, an mRNA stored in stalled polysomes, iii) ablation of DHPG-
702 induced, initiation-independent protein synthesis (Figure 1), and iv) a block in mGluR-
703 LTD expression, which is known to involve stalled polysomes (Figure 7). Together,
704 these data strongly argue for the importance of UPF1 in this form of regulated transport
705 and translation in neurons.
706 There are a number of possible functions for UPF1 in regulating stalled
707 polysomes: UPF1 may be important in their formation, stability, ability to be
708 translationally activated, or some combination thereof. One attractive model would be
709 that when a ribosome translating an mRNA reaches the stop codon, UPF1 is recruited
710 together with the peptide release factors eRF1 and eRF3, and instead of signalling
711 decay or stimulating release as in non-neuronal cells, a process initiating the formation
712 of a stalled polysome would ensue. Such a mechanism would also require STAU2,

713 mediating either UPF1 recruitment or stabilization and may require additional, neuron-
714 specific trans-acting proteins or regulatory RNAs, or cis-acting sequence factors such
715 as alternative 3' UTRs.

716 It is possible that the effect of knocking-down UPF1 indirectly regulates stalled
717 polysomes; mRNAs normally targeted for NMD through the presence of introns in their
718 3' UTRs may be longer-lived, thus exhibiting enhanced translation in this context
719 (Kurosaki and Maquat, 2013). However, the best-known LTD-related protein that is also
720 a NMD target, ARC (Giorgi et al., 2007), would have been expected to facilitate mGluR-
721 LTD by its increased levels in neurons lacking sufficient UPF1, not lead to a loss of
722 mGluR-LTD like we observed. Moreover, decreases in *Map1b* mRNA localization to
723 dendrites would be difficult to explain by a loss of NMD-mediated *Map1b* degradation.
724 Finally, in our effort to dissect disparate UPF1 functions, knockdown of PNRC2, a
725 protein that mediates decapping as part of the NMD/SMD function of UPF1 had no
726 effect on translation from reactivated stalled polysomes (new Figure 2). These data
727 strongly support the concept that the canonical NMD/SMD functions of UPF1 do not
728 play a role in DHPG-mediated changes in translation of plasticity-related proteins. It
729 remains to be seen whether these roles can be dissected in the context of synaptic
730 plasticity and in particular mGluR-LTD.

731 Perhaps the most parsimonious conclusion drawn from the observed reduction in
732 *Map1b* mRNA copy number and translation in neurites with reduced UPF1 expression,
733 is that the latter functions in *Map1b* RNA transport rather than translation *per se*. This
734 could occur in a number of ways. First, UPF1 interactions might be a pre-requisite for
735 proper transport of a *Map1b* stalled polysome. Second, UPF1 interactions might be

736 required for stalled polysome formation, without which *Map1b* becomes mis-localized.
737 CLIP analysis has shown that UPF1 interacts with mRNA in both EJC-dependent and
738 independent manners such that many copies of UPF1 proteins are scattered throughout
739 the message (Hurt et al., 2013; Zund et al., 2013). Importantly, Zund et al showed that
740 UPF1 is preferentially displaced from coding regions on mRNAs with associated
741 ribosomes while binding in 3' UTRs is retained. This observation is consistent with our
742 super-resolution data showing a large number of UPF1 localizations at the apical end of
743 a polysomal structure. UPF1 does indeed specifically interact with the *Map1b* 3' UTR
744 (Hurt et al., 2013) so it is certainly possible that downregulation of the former could
745 affect the transport of *Map1b* stalled polysomes, possibly by disturbing Staufen binding
746 sites. Importantly however, UPF1 (or STAU2) knockdown does not appear to adversely
747 affect steady-state levels of *Map1b* in neurons (Figure 2C, 4F). These data suggest that
748 the NMD functions of UPF1, whether direct or indirect are not involved in this context
749 and highlight a tantalizing new function for this protein.
750
751 Implications of a role for STAU2-UPF1 interactions in RNA granules
752 The interaction between STAU2 and UPF1 has now been seen in a number of
753 independent studies (Miki et al., 2011; Park et al., 2013; Flury et al., 2014). A previous
754 study, however, identified the dsRBD2 and dsRBD3 domains as sufficient to bind to
755 UPF1 and did not require C-terminal domains (Miki et al., 2011). In contrast, we found
756 that deletion of the C-terminus up to and including the TBD almost completely removed
757 UPF1 binding without affecting STAU2 interactions with the ribosome. These results are
758 not necessarily contradictory and suggest the possibility of two interacting domains, one

759 including dsRBD2 and dsRBD3 and the second involving the TBD, both of which are
760 required for full binding. For the purposes of our study, the loss of interaction with the
761 removal of the C-terminal (C351 mutation) allowed us to more specifically examine the
762 role of STAU2-UPF1 interactions, since disrupting dsRBD3 would confound
763 interpretation by removing most dsRNA binding for STAU2, including association with
764 the ribosome (Luo et al., 2002). The TBD and dsRBD4 were sufficient for binding of
765 STAU1 to UPF1 (Kim et al., 2005), although we show that removing the TBD was
766 sufficient to prevent binding even in the presence of dsRBD4. Previous reports indicate
767 that STAU2 binds to UPF1 more effectively than STAU1 (Park et al., 2013; Flury et al.,
768 2014), and this may be explained either by the additional binding site in dsRBD2 or
769 dsRBD3, or differences in the TBD involved in UPF1 binding (see Figure 5F for a
770 clustalW alignment of STAU2 vs. STAU1 that shows the low percent identity of residues
771 in this region).
772
773 Implications for the release of stalled polysomes
774 mGluR-LTD requires fast, local translation (Huber et al., 2000). We have
775 previously shown that this translation is independent of translation initiation (Graber et
776 al., 2013b), suggesting that LTD proteins are generated by finishing translation of
777 previously initiated mRNAs. Based on our data and previous studies, we propose that at
778 least three interlocked mechanisms could be implicated in releasing this form of stalled
779 translation. Phosphorylated FMRP is necessary for stalling or stabilization of stalled
780 polysomes and FMRP dephosphorylation is required for mGluR-LTD, presumably by
781 releasing the FMRP-mediated “brake” on stalled polysomes (Richter and Coller, 2015).

Second, eEF2 phosphoryation is required for mGluR-LTD and although the underlying reasons are still unclear, it is possible that this is due to a requirement for release of stalled polysomes (Graber et al., 2013a). Indeed, other forms of plasticity that require eEF2 phosphorylation also appear to be due to a need for reactivation of stalled polysomes (McCamphill et al., 2015). If release of mRNAs is stalled through UPF1-STAU2 interactions, then this would also be subject to signal transduction mechanisms induced by mGluR-LTD. Interestingly, we also observe dephosphorylation of UPF1 after induction of mGluR-LTD (Figure 2). Alternatively, once initially stalled through UPF1-STAU2 interactions, FMRP- and eEF2-mediated controls may predominate in the transported RNA granule. The role of UPF1-STAU2 interactions in release of a stalled polysome will be difficult to dissect if, as we suggest in this study, the latter do not form in the absence of this interaction.

A new role for UPF1 in long-term synaptic plasticity

It has been suggested that specific transport complexes are involved in distinct forms of plasticity. We have previously shown that STAU2 is required for mGluR-LTD (Lebeau et al., 2011), while the paralogous STAU1 is necessary for the late phase of NMDA receptor–dependent long-term potentiation (late-LTP) (Lebeau et al., 2008). Interestingly, while late-LTP and mGluR-LTD both require protein synthesis, late-LTP is blocked by *either* translation initiation *or* elongation inhibitors in contrast to mGluR-LTD, which is blocked only by elongation inhibitors (Graber et al., 2013b). Since both STAU1 and STAU2 can bind to UPF1 (Park et al., 2013; Flury et al., 2014), our finding that mGluR-LTD requires the UPF1-STAU2 interaction raises the intriguing possibility that

UPF1-STAU1 complexes might function in late-LTP. However, it remains to be determined how such a differential interaction by UPF1 may regulate the translation of different subsets of mRNAs in mGluR-LTD and late-LTP. Thus, it would be of interest to explore how a differential interaction of UPF1 with either STAU1 or STAU2 may regulate the synthesis of distinct plasticity related proteins through stalled polysomes, and direct the switch between long-term synaptic depression and potentiation.

Author Contributions

T.E.G., E.F., J.-C.L. and W.S.S conceived the experiments and wrote the manuscript. T.E.G. performed the super-resolution, co-IP, RNA-FISH and RPM experiments. E.F. and S.H.-S performed the electrophysiology experiments. M.A., R.M. and U.S. performed AHA, Western blotting and helped with cloning and lentivirus construction. H.H. and T.A. contributed the ddPCR expertise and experiments.

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945 **Legends**

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947 **Figure 1. UPF1 is required for DHPG-mediated reactivation of translation from**
948 **stalled polysomes in neurites.** (**A**) Schema of the lentivirus transduction protocol to
949 efficiently express shRNA targeting *Upf1* or a non-targeting control (scrambled RNAi) in
950 primary neurons. Lentivirus expression was monitored with an emGFP ORF fused
951 upstream of the shRNA cassette. Neurons were cultured for an additional 72 h prior to
952 fixation. (**B**) Representative confocal images (left) and quantitation (right) of UPF1
953 protein in neurons at Day 10 expressing indicated RNAi. Data is from 12 individual
954 neurons from three independent experiments. See Materials and Methods for
955 explanatory notes regarding statistics used throughout the article. (**C**) Immunoblot (IB)
956 showing steady-state levels of UPF1 in neurons expressing RNAi. GAPDH was used as
957 a loading control. Apparent molecular weight is indicated on left in kilodaltons (kDa).
958 Data is representative of three independent experiments. (**D**) Reduced UPF1
959 expression decreases the number of stalled polysomes in neurites. Representative
960 confocal images of straightened neurites (left) and quantitation (right) of stalled
961 polysomes more than 50 μm from the cell soma detected by ribopuromycylation in the
962 presence of HHT (see text for details). Data are from 15-24 neurons from three different
963 cultures. (**E**) UPF1 is required for mGluR-mediated, initiation-independent protein
964 synthesis. AHA incorporation (Click-AHA) into nascent peptides was measured in distal
965 neurites in the presence of HHT following activation of mGluR with 50 μm DHPG in
966 neurons expressing indicated RNAi. Data are from 30-49 neurons from four different

967 cultures. See methods for quantification. (**F**) Rescue of UPF1 levels in hippocampal
968 cultures. Neurons were transduced with scrambled, *Upf1* RNAi, or *Upf1* RNAi plus a
969 lentivirus expressing mCHERRY and human UPF1 ORFs (resistant to rat *Upf1* RNAi)
970 from separate promoters. Neurons from three independent cultures were fixed and
971 stained with anti-UPF1 and imaged for emerald GFP (emGFP; reporting RNAi cassette)
972 and mCHERRY (reporting UPF1 rescue cassette). Average levels of UPF1 from 10 cell
973 bodies transduced with *Upf1* RNAi or *Upf1* RNAi + UPF1 rescue lentiviruses were
974 standardized to average UPF1 levels in 10 scrambled RNAi-transduced neurons from
975 cultures transduced at the same time. Results are from three independent neuron
976 cultures. (**G**) Same as (E) but with expression of both *Upf1* RNAi plus UPF1 rescue
977 plasmid. Expression of UPF1 rescued the ability of DHPG to induce increased
978 incorporation of AHA. Data are from 15-26 neurons from three different cultures.
979
980 **Figure 2. DHPG-mediated translation occurs independently of NMD and requires**
981 **UPF1 for proper localization of a target mRNA.**
982 (**A**) Cartoon depicting function of PNRC2, acting as a bridge between the decapping
983 and NMD (or SMD) machineries on a mRNA substrate (top). Representative
984 immunoblot of PNRC2 in neurons expressing indicated RNAi (bottom). (**B**) PNRC2-
985 dependent NMD is not required for mGluR-mediated, initiation-independent protein
986 synthesis. The AHA incorporation assay was performed as in Figure 1E on neurons
987 expressing scrambled or *Pnrc2* RNAi. Data are from 19-29 neurons from three
988 independent cultures. (**C**) Droplet digital RT-PCR of *Map1b* (left) or *Upf1* (right) mRNAs
989 normalized to *Gapdh* mRNA from total lysates of hippocampal neurons expressing

990 RNAi. Data are from three independent experiments. A one-tailed t-test was used to
991 determine significance of *Upf1* knockdown. (**D**) Reduced UPF1 expression decreases
992 the number of *Map1b* mRNA copies in neurites determined using single molecule RNA-
993 FISH. Representative confocal images of straightened neurites after application of a
994 Laplacian of Gaussian transformation to highlight mRNA puncta edges are shown.
995 Segmentation and thresholding of individual puncta ("particle mask") allowed
996 quantitation of mRNA in the soma, proximal, and distal neurites (lower panels). Data are
997 from 20-39 neurons obtained from three different cultures. (**E**) Immunoblot of total
998 neuronal lysates showing effect of DHPG on total and phospho-UPF1 (P-UPF1) levels
999 (top) and quantitation from 4 independent experiments showing change in
1000 phospho:total-UPF1 and UPF1:GAPDH expression following 50 μM DHPG treatment for
1001 10 minutes (bottom). A one-sample, two-tailed t-test was used to determine
1002 significance.
1003
1004 **Figure 3. Super-resolution microscopy reveals stalled polysomes colocalized with**
1005 **UPF1 in distal neurites.** (**A**) Stalled polysome puncta were first detected in widefield
1006 fluorescence mode on the Vutara Stochastic Optical Reconstruction Microscope
1007 (STORM) using ribopuromycylation (RPM) in the presence of HHT (the location of an
1008 RPM puncta is indicated by arrowheads in each channel) prior to acquisition of the
1009 super-resolution image in both channels. Note that UPF1 signal was below detection
1010 during widefield acquisition due to the short exposure times needed to minimize
1011 bleaching. (**B**) Reconstructed image of a RPM puncta based on two-color localizations
1012 in three dimensions (Dual Color 3D STORM) using high density secondary antibodies to

1013 detect anti-puromycin and anti-UPF1 primary antibodies. Individual fluorophore
1014 localizations on conjugated secondary antibodies plotted using the point-splatting
1015 feature of the Vutara software in order to highlight structure. Top, side, and angled
1016 views shown. (**C**) As in (B) but using 1:10,000 instead of 1:1,000 secondary antibody
1017 dilution. (**D**) Individual fluorophore localizations of the polysome-UPF1 complex in (C).
1018 (**E**) Kernel density plot summarizing point-to-point distances of localizations shown in
1019 (D). Vertical lines indicate density maxima at distance x ("peak density distance"). (**F**)
1020 Peak density distances for a population of polysome-UPF1 complexes (n=13) with the
1021 mean indicated by a horizontal bar. A Kruskal-Wallis non-parametric ANOVA was
1022 performed with Dunn's multiple comparisons post-hoc test. Data were collected from
1023 three different neuronal cultures.
1024
1025 **Figure 4. STAU2 is required for the presence of stalled polysomes in neurites**. (**A**)
1026 STAU2 protein was knocked-down with the same lentivirus RNAi strategy as outlined in
1027 Figure 1A. Representative confocal images (left) and quantitation (right) of STAU2
1028 protein in neurons expressing indicated RNAi. (**B**) Representative Immunoblot showing
1029 steady-state levels of STAU2 isoforms in neurons expressing RNAi. GAPDH was used
1030 as a loading control. (**C**) Reduced STAU2 expression decreases the number of stalled
1031 polysomes in neurites. Representative confocal images of straightened neurites (left)
1032 and quantitation (right) of stalled polysomes more than 50 μm from the cell soma
1033 detected by ribopuromycylation (see text for details) in the presence of HHT. Data were
1034 obtained from 15-24 neurons from three different cultures. (**D**) Reduced STAU2
1035 expression decreases the number of *Map1b* mRNA copies in neurites determined using

single molecule RNA-FISH. Representative confocal images of straightened neurites after application of a Laplacian of Gaussian transformation to highlight mRNA puncta edges are shown. Segmentation and thresholding of individual puncta ("particle mask") allowed quantitation of mRNA in the soma, proximal, and distal neurites (lower panels). Data were obtained from 20-39 neurons from three different cultures. (**E**) Quantitation of *Map2* single molecule RNA-FISH puncta in neurons expressing indicated RNAi and analyzed as in (D). Data are from 3-4 neurons from 2 different cultures. (**F**) Expression of target mRNAs in total hippocampal neuron lysates expressing *Stau2* vs. scrambled RNAi measured by droplet digital RT-PCR. Expression of each indicated PCR target was normalized to *Gapdh*. A one-sample, two-tailed t-test was used to determine significance, n=3-4 independent cultures.

Figure 5. UPF1 interacts with the tubulin binding domain of STAU2. (**A**) Schematic of DNA constructs used for co-immunoprecipitation (co-IP) assays. NES, Nuclear Export Signal; dsRBD, double-stranded RNA binding domain; TBD, tubulin binding domain. (**B**) Co-IP of Flag-tagged STAU2 and Myc-tagged UPF1 in HEK293T cells. Myc-tagged RIP1, a protein not involved in this complex, was not immunoprecipitated with Flag-tagged STAU2 (right panel), indicating that the interaction seen with UPF1 and STAU2 was not due to promiscuous binding or specific to the Myc and Flag peptide tags. (**C**) Flag co-IP as in (B) but incubated with 100 µg/ml RNase A. Note loss of endogenous RPS6 from complex with RNase A treatment and retention of UPF1. (**D**) Co-IP demonstrating that removal of the STAU2 C-terminus along with the TBD (C351) abrogates binding of UPF1 but has no effect on binding to the ribosome (RPS6). In

contrast, a mutation in dsRBD3 of STAU2 (F207A), but not the full-length or C351 mutation, disrupts ribosomal interactions as indicated by reduced IP of endogenous RPS6. (**E**) Co-IP of STAU2 proteins harbouring C-terminal deletion mutations. (**F**) Putative UPF1 binding region in STAU2 based on the co-IP experiments. The homology of this region compared to STAU1 is low (ClustalW alignment), indicating that the interaction with UPF1 might be different between these two proteins. (**G**) Immunoblot of STAU2 and GFP expression in hippocampal neurons expressing scrambled or *Stau2* RNAi (expressing GFP on a separate cistron; "RNAi virus GFP") or RNAi plus a rescue lentivirus expressing the GFP-STAU2 or the C351 mutation constructs that are both resistant to the rat-specific RNAi. Note the STAU2 antibody used does not detect the STAU2 C351 species. GAPDH was used a loading control. (**H**) mGluR-mediated induction of MAP1B protein synthesis requires the UPF1 binding region. MAP1B protein levels were assessed by immunofluorescence in distal neurites of hippocampal neurons after 10 minutes following stimulation with 50 μm DHPG or with vehicle. Neurites from 25-36 neurons and four independent cultures were analyzed. A one-tailed, one-way ANOVA with Šidák multiple hypothesis correction was used.

Figure 6. mGluR-LTD in cultured neurons is protein synthesis-dependent yet translation initiation-independent. (**A**) Representative traces of miniature excitatory post-synaptic currents (mEPSCs; denoted by dots above traces) over time during whole cell recording from cultured (18-25 DIV) rat hippocampal neurons. (**B**) Summary bar graphs for all cells, showing LTD of mEPSC frequency, but not amplitude, 30 minutes after application of DHPG. LTD was prevented by the translation elongation inhibitor

emetine (40 μM) but not by the translation initiation inhibitor homoharringtonine (HHT; 20 μM). Data are accumulated from 4-9 neurons from 4-5 independent experiments.

Figure 7. mGluR-LTD is impaired by STAU2 and UPF1 knockdown, and its rescue requires UPF1-STAU2 interaction. (**A**) Representative traces of mEPSCs during whole-cell recording from cultured rat hippocampal neurons transduced with indicated lentiviruses (expressing GFP and/or mCHERRY). (**B**) Summary bar graphs for all cells, showing that LTD of mEPSC frequency, but not amplitude, induced by DHPG (scrambled RNAi-treated cells) is impaired in cells with knockdown of UPF1 or STAU2, and rescued in cells with RNAi-resistant STAU2 overexpression but not in cells with overexpression of STAU2 lacking the UPF1 binding region (C351 mutation). Data are accumulated from 5-9 neurons from 3-6 independent experiments.

Figure 8. Model illustrating how UPF1-STAU2 interactions might contribute to local protein synthesis from stalled polyribosomes in synaptic plasticity. Target mRNAs such as *Map1b* harbour STAU2-UPF1 complexes in their 3' UTRs that may prevent proper translation termination (indicated by eRF1 at the stop codon) and/or stall elongation, thus creating a stalled polysome complex. In the absence of either STAU2 or UPF1, *Map1b* mRNA fails to be transported into neurites. After transport of the stalled polysome complex to synapses, its reanimation is dependent on UPF1-STAU2 interactions, as mGluR-LTD fails to be expressed in the presence of HHT and a STAU2 mutant protein that still interacts with ribosomes but cannot form a complex with UPF1.

This interaction depends on the tubulin binding domain (TBD) present in STAU2 which likely mediates interaction with the cysteine-histidine-rich (CH) domain of UPF1.

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