**TRANSLATIONAL REGULATION OF THE HUMAN ACHAETE-SCUTE HOMOLOGUE-1 (hASH1) BY FRAGILE X MENTAL RETARDATION PROTEIN (FMRP).**

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Running head: Translational control of hASH1 mRNA by FMRP

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Fragile X syndrome is a common inherited cause of mental retardation that results from loss or mutation of the fragile X mental retardation protein (FMRP). In this study, we identified the mRNA of the basic helix-loop-helix transcription factor human achaete-scute homologue-1 (\textit{hASH1}; \textit{ASCL1}), which is required for normal development of the nervous system and has been implicated in the formation of neuroendocrine tumors, as a new FMRP target. Using a double-immunofluorescent staining technique we detected an overlapping pattern of both proteins in the hippocampus, temporal cortex, subventricular zone and cerebellum of newborn rats. Forced expression of FMRP and gene silencing by siRNA transfection revealed a positive correlation between the cellular protein levels of FMRP and hASH1. A luciferase reporter construct containing the 5'-untranslated region (UTR) of \textit{hASH1} mRNA was activated by the full-length FMRP, but not by naturally occurring truncated FMR-proteins, in transient co-transfections. The responsible \textit{cis}-element was mapped by UV-cross-linking experiments and reporter mutagenesis assays to a (U)\textsubscript{10}-sequence located in the 5'-UTR of the \textit{hASH1} mRNA. Sucrose density gradient centrifugation revealed that \textit{hASH1} transcripts were translocated into a translationally active polysomal fraction upon transient transfection of HEK293 cells with FMRP, thus indicating translational activation of \textit{hASH1} mRNA. In conclusion, we identified \textit{hASH1} as a novel downstream target of FMRP. Improved translation efficiency of \textit{hASH1} mRNA by FMRP may represent an important regulatory switch in neuronal differentiation.

The \textit{FMR1} gene encodes the fragile X mental retardation protein (FMRP), an RNA-binding protein, which is expressed among various tissues with the highest levels in neurons of the developing brain and in spermatogonia in adult testis (1;2). Dysfunction of the fragile X mental retardation-1 (\textit{FMR1}) gene transcription is associated with neuronal disorders, such as Fragile X Syndrome (FXS) and Fragile X associated Tremor/ Ataxia Syndrome (FXTAS) (3-6). FMRP binds to \textit{cis}-regulatory mRNA elements that include G-quartet structures, poly-U sequences, and the so-called kissing complex (7). Beside direct FMRP/RNA binding, FMRP is part of messenger ribonucleoprotein (mRNP) complexes by interaction with further RNA-binding proteins, i.e. the poly-A binding protein (PABP), nucleolin and others (8). At least 12 different FMR-proteins are generated by alternative mRNA splicing (1;9) and may contribute to the functional diversity of the \textit{FMR1} gene.

FMRP has been suggested to play a role in synaptic development and plasticity through regulating mRNA transport and local protein synthesis at synapses (10-12). Accordingly, FMRP was found to gate the translation of a large set of mRNAs in dendrites that are involved in synaptic plasticity. Some of these transcripts are transported in mRNA granules together with FMRP. Activation of metabotropic glutamate receptors (mGlurR) has been shown to stimulate synaptic translation of FMRP (13;14). Since protein synthesis by activation of GluR was enhanced in the absence of FMRP (15), a model of mGluR-stimulated inhibition of local mRNA translation by FMRP has been developed (16). Reduced synaptic protein formation may explain at least some of the mental deficits in patients suffering from FXS due to FMRP deficiency.
FMRF may function not only as a translational repressor, but also as an activator of specific mRNA translation (8). Depending on its phosphorylation status (17), FMRF co-localizes with actively translating polysomes (18-20). In addition, FMRF shuttles between the nucleus and the cytoplasm (21-23). Thus, it has been proposed that FMRF might contribute to chromatin remodeling through the RNA interference (RNAi) pathway in the nucleus (8). Furthermore, FMRF has been implicated in mRNA splicing (24), mRNA stabilization (25) and as a component of RNA-granules like stress granules (26). Identification and validation of downstream targets may provide the key to understanding the role of FMRF in neuronal development and disease. Previous attempts to discover FMRF-regulated genes were based mostly on immunoprecipitation of FMRF in mouse tissue followed by extraction and micro-array analysis of co-precipitated RNA (8;12;27). Alternative approaches consisted in the use of random oligonucleotides linked to anti-FMRP antibody to reveal putative FMRF targets (28) and in differential display analysis (29;30). While these investigations yielded important information on the RNA-binding properties of FMRF, little overlap among the proposed candidate targets was found (8). Furthermore, the precise molecular mechanisms of FMRF function remain elusive (31), and the question of how abnormal regulation of a single gene can result in such a diversity of neuronal dysfunctions remains to be answered (32). Thus, the goal of this study was to identify potential new FMRF targets, which play a pivotal role in gene regulation during neuronal development. Starting with an unbiased combined approach of gene ontology and motif search followed by extensive experimental analyses we characterize the basic helix-loop-helix transcription factor, human achaete-scute homologue-1 (hASH1; ASCL1), as a new FMRF target and describe its molecular mechanism of regulation by FMRF in detail. Since hASH1 is crucial in generating neuronal diversity by regulating neuronal subtype specification and differentiation (33), our findings offer a new view of how FMRF influence neuronal development.

**EXPERIMENTAL PROCEDURES**

**Cell culture:** Human embryonic kidney (HEK)293 cells (ACC 305) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose; PAA Laboratories GmbH, Cölbe, Germany), supplemented with 10% fetal calf serum (Biochrom KG, Berlin, Germany), 50 U/ml penicillin, 50 µg/ml streptomycin, 15 mM Hepes and 2 mmol/l glutamine, at 37°C, 5% CO₂.

For primary hippocampal neurons animals were sacrificed according to the permit (LaGeSo, 0122/07) given by the Office for Health Protection and Technical Safety of the regional government of Berlin and in compliance with regulations laid down in the European Community Council Directive. Hippocampal cultures from E19 Wistar rats were prepared as previously described (34) and maintained for 8-14 days in vitro in B27- and 1 % FCS-supplemented Neurobasal medium (35).

**Plasmids:** For expression of the full-length FMRF, a construct described earlier (36) (kind gift of J. Darnell) served as a template for recloning of the FMR1 cDNA into the CMV promoter based pEGFP-C1 vector (BD Biosciences, Clontech, Heidelberg, Germany). FMRF-variant expression vectors (also under control of a CMV-promoter) were purchased by Deutsches Ressourcenzentrum für Genomforschung GmbH (RZPD, Berlin, Germany). For the generation of useful luciferase reporter constructs, the pGL3-promotor vector, which contains a constitutive SV40 promoter (Promega GmbH, Mannheim, Germany), was modified as follows: The vector specific 5’- and 3’UTRs of luciferase mRNA were replaced by the human hASH1 mRNA UTRs. The UTRs were amplified by PCR using a hASH1 full-length cDNA clone (RZPD GmbH) as template, and restriction sites were added by primer extension. The following cis-elements were deleted in the hASH1 5’UTR: U-rich element (pos. 223-232), G-quartet motif (pos. 304-330), AU-rich element (pos. 397-416) according to the ASCL1 sequence accession number gi:190343011 (NM_004316). The quality of processed vectors was confirmed by sequencing.

**Cell transfection experiments and reporter gene assays:** Cultured cells were grown to approximately 70% confluence in 96-well plates (µClear Platte 96K, Greiner BIO-ONE GmbH, Frickenhausen, Germany) and transiently co-transfected with a reporter construct containing the firefly luciferase gene flanked by the 5’- and/or 3’-UTRs of hASH-1 mRNA (pGL3-promoter vector; Promega, Mannheim, Germany), and the renilla luciferase phRL-TK vector (Promega, Mannheim, Germany). A ratio (DNA : transfection reagent) of 1:3 was used with the FuGENE
6 Transfection Reagent (Roche Diagnostics Corporation, Mannheim, Germany) according to the manufacturer’s protocol. Transfection of HEK293 cells with empty pGL3-promoter vector served as control. Co-transfection with the renilla luciferase reporter plasmid was performed for normalization of transfection efficiencies. The luciferase activities were measured in a luminometer (Labsystems Luminoscan RS, Helsinki, Finland) programmed with individual software (Luminoscan RII, Ralf Mrowka) 24 h after transfection as described in (37). Luciferase mRNA quantification was performed as described recently (38). For forced expression of FMR-proteins, cells were transfected in 6-well or 60 mm dishes for 36 h using the FuGENE 6 Transfection Reagent as described above.

For transfection of siRNAs human neuroblastoma-derived Kelly cells (ACC 355) were obtained from the American Type Culture Collection and grown to approx. 50% confluence in 60-mm dishes in RPMI medium (PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (FCS) (Biochrom KG, Berlin, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% glutamate (all from Invitrogen). The siRNA for targeting the human FMR1 gene (NCBI accession number NM_002024) was synthesized by Dharmacon (Lafayette, USA). A pool of non-targeting siRNAs (Dharmacon, Lafayette, USA; 200 pmoles per dish) was used as a negative control. To achieve maximum efficiency of gene silencing the cells were transfected with a mixture of 4 different siRNAs, each at 50 pmol per dish. For this purpose the siRNAs were diluted at a 1:20 volume ratio in 0.2 ml serum- and antibiotic-free RPMI. Likewise, the DharmaFECT® transfection reagent (Perbio Science, Bonn, Germany) was diluted (1:100) in 0.2 ml serum- and antibiotic-free RPMI. The diluted siRNAs and the transfection reagent were combined and kept at room temperature for 20 min to allow complex formation. In the meantime the culture medium was removed from the cells and replaced with 1.6 ml fresh RPMI/10% PCS. The siRNA transfection mixtures (0.4 ml) were carefully added to the cells, which were subsequently grown in the tissue culture incubator for 60 hours.

Determination of mRNA and protein levels: Total RNA was isolated from cultured cells with the Trizol® reagent (Invitrogen GmbH, Karlsruhe, Germany). First-strand cDNA synthesis was performed with 2 µg of total RNA using oligo(dT) primers and superscript II reverse transcriptase (Invitrogen GmbH, Karlsruhe, Germany). mRNA quantification was performed as described previously (39). The following intron bridging primers were used for the amplification reactions: hASH1-forward: 5’- CGACCTCAACACGTGTTCT, hASH1-reverse: 5’-CCGTAATGATTGAGTGC, β-actin-forward: 5’- TGAAGTGTACGTGAGACATC, β-actin-reverse: 5’-GTCATAGTCGCTAGAAGC.

Cytosolic protein extracts were prepared from cultured cells as described previously (40) and separated on a 10% polyacrylamide gel. The proteins were transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany) with the use of a semidry blotting apparatus (BioRad, München, Germany). Immunodetection of hASH1 and FMRP was performed according to our routine protocol with the following primary antibodies: anti-Mash1 antibody diluted 1:500 (#556604, BD Biosciences, Heidelberg, Germany), anti-FMR1, diluted 1:1,000 (H-120, sc28739, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), mouse monoclonal antibody against β-actin in a 1:1,000 dilution (#MAB1501R, Chemicon, Schwalbach/Ts, Germany). The anti-beta-actin antibody was applied after stripping of the membranes with 0.2 M NaOH at room temperature for 6 minutes to reveal possible differences in protein loading. Primary antibodies were detected with peroxidase-coupled secondary antibodies, and the reaction products were visualized with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Freiburg, Germany).

Antibodies, immunofluorescence and quantification: Mash1 and FMRP were stained using mouse monoclonal (1:500, clone 24B72D11.1, #556604, BD Biosciences, Heidelberg, Germany) and rabbit polyclonal (1:500, H-120, sc28739, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) antibodies, respectively. Immunofluorescence staining was performed on primary hippocampal neurons and on horizontal sections of postnatal day 2 (P2) rat brain fixed with ice-cold mixture of paraformaldehyde and sucrose (4% both) in PBS buffer. Primary neurons and 2-3 mm thick freshly isolated horizontal rat brain sections were fixed for 15 min at room temperature and 1 h at 4°C, respectively. After fixation slices were washed three times with PBS at room temperature and cryoprotected overnight at 4°C in PBS supplemented with 8% sucrose.
Brain sections were embedded in O.C.T TissueTek (Sakura Finetec, USA) and 12 µm cryosections were obtained (CM1850, Leica Microsystems, Wetzlar, Germany) and mounted on Superfrost Plus microscope slides (Menzel GmbH, Braunschweig, Germany). Sections were post-fixed for 5 min with ice-cold PFA/sucrose. Prior to incubation with antibodies (1 h at room temperature) primary neurons were permeabilized with 0.12% Triton X-100 for 4 min at room temperature. Antibody reaction with cryosections occurred over night at 4°C in PBS-gelatine supplemented with 0.12% Triton X-100. Appropriate control experiments were performed using species-matched normal sera instead of specific primary antibodies. Secondary antibodies (1:200 each) were coupled to carboxymethyl indocyanine (Cy3) or FITC (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Preparations were mounted in DAPI-containing Vectashield medium (Vector Laboratories, Burlingame, CA, USA). The slides were viewed under an epifluorescence microscope (Olympus BX51, Olympus Deutschland GmbH, Hamburg, Germany). Images were acquired with a 14-bit cooled CCD camera (Spot PURSUIT, Visitron Systems GmbH, Puchheim, Germany) and the software Metamorph (Universal Imaging Corp., Downingtown, PA, USA). Cell-matched signals were quantified within circular, nucleus-centered, regions of interest (Mash and FMRP: diameter 50 and 150 pixel, respectively). Integrated fluorescence intensities were obtained from min/max-thresholded images using Metamorph. Correlation analysis was performed using Spearman Rank Order algorithm.

RNA/ protein interaction studies: UV-cross-linking: In vitro transcripts representing the 5'- or 3'UTR of hASH1 mRNA were radioactively labeled using [alpha-32P]uridine-, [alpha-32P]cytosine-, [alpha-32P]adenine-, or [alpha-32P]guanosine-5'-triphosphate (800 Ci/mmol, MP Biomedicals GmbH, Heidelberg, Germany). In vitro transcripts were purified by BD Chroma Spin™-100 (DEPC) columns (BD-Bioscience, Heidelberg, Germany). One to 2 ng of the [alpha-32P]NTP labeled in vitro transcripts (corresponding to 100,000 cpm) were incubated with 35 µg cytosolic protein extract for 30 min at room temperature in 10 mM Hepes pH 7.2, 3 mM MgCl2, 5% glycerol, 1 mM DTT, 150 mM KCl, 2 U/µl RNaseOUT (Invitrogen™ life technologies), 0.5 µg/µl rabbit rRNA for 30 min at room temperature. The formed RNA/ protein complexes were incubated over night at 4°C with anti-FMRP antibody or anti-β-actin control antibody with gentle shaking. The complexes were separated by electrophoresis (0.5 x TBE buffer). Radioactive signals were detected using the Phospho-Imager-System (FUJI FILM FLA-3000).

Sucrose gradient centrifugation and polynomial profiles: Prior to lysis, cells were pre-treated with cycloheximide (100 µg/ml) for 10 min. Cells were lysed on ice for 10 min in the presence of 20 mM Tris (pH 7.4), 150 mM KCl, 30 mM MgCl2, 100 µg/ml cycloheximide, 1 mM DTT, 1 x proteinase inhibitor cocktail (Roche Diagnostics Corporation, Mannheim, Germany), 100 units/ml of RNase inhibitor (MBI Fermentas GmbH, St. Leon-Rot, Germany) and 0.5% Nonidet P-40. Cytosolic extracts were obtained after centrifugation at 10,000 g for 10 min at 4°C. The cytoplasmic supernatant was layered onto 11 ml of a linear 17 to 51% sucrose gradient (0.5 to 1.5 M sucrose, 20 mM Tris pH 7.4, 150 mM KCl, 5 mM MgCl2, 1 mM DTT) and centrifuged for 2 h at 36,000 rpm using a Beckman SW-41 rotor. Following sedimentation, the gradient solution was pumped out from the bottom with a peristaltic pump. The ribosomal profile was continuously determined at an absorbance of 254 nm using a 2138 UVICORD-S UV monitor (LKB Bromma). Sucrose gradients were split into 12 sub-fractions each, starting with 1 (bottom) to 12 (top). For protein isolation tri-
chloroacetic acid (TCA) was supplemented to a 10% final concentration. Precipitated proteins were sedimented, washed twice with acetone and solved in buffer (25 mM Tris, 1% SDS). RNA was isolated using the E.Z.N.A. RNA Total Kit (#OMEGR6834-02, VWR International, Darmstadt, Germany) according to the manufacturer’s protocol.

Selection of potential FMRP targets: We obtained UTR sequences from the 5’ and 3’ end of the RNA from the ENSEMBL database using the BIOMART tool. The underlying genomic data came from the human NCBI36 dataset representing 56,511 unique transcripts from 32,562 ENSEMBL genes. The dataset of the 3’ UTRs and 5’ UTRs consisted of 38,857 and 38,685 sequences, respectively. These numbers are smaller than the total number of transcripts since some genes did not have an UTR prediction. These data were the basis for the motif search for poly-U stretches (U\[\geq 8\]) and G-quartet motifs according to the consensus [DWGG (N\[0-2\]) DWGG (N\[0-1\]) DWGG (N\[0-1\]) DWGG] (D = A/G/T, W = A/T, N = A/T/C/G) (36). Both cis-elements are known FMRP binding sites.

An additional filter step was the selection based on known molecular function. The prediction of molecular function was obtained with BIOMART from the ENSEMBLE database. We used the Gene-Ontology classification identifiers for that purpose. The gene ontology is a rooted tree like structured classification. The database entries in ENSEMBLE do contain the last tree children only, but not the complete ontology tree. Therefore we remapped the complete tree structure for each ontology entry. From all genes we selected those having a positive “GO:0007399: nervous system development” in their complete remapped ontology trees.

Statistics: If not otherwise indicated, all values are presented as means ± SD. Students’ paired t-test was performed to reveal statistical significances. P values less than 0.05 were considered significant.

RESULTS

Selection of potential FMRP targets.

In order to identify potential FMRP target mRNAs with a role in neuronal development we selected all genes belonging to the gene ontology class “nervous system development” and searched within their mRNA untranslated regions (UTRs) for poly-U stretches (U[28]) and G-quartet motifs according to the consensus [DWGG (N[0,2]) DWGG (N[0,1]) DWGG (N[0,1]) DWGG] (D = A/G/T, W = A/T, N = A/T/C/G) (36). Both cis-elements are known FMRP binding sites.

We retrieved 5,545 unique genes that had a poly-U stretch or a G-quartet in their mRNA 5’- or 3’UTR. When all genes were filtered for the gene ontology “nervous system development” we obtained 336 genes. Within the dataset of all genes a total number of 682 transcription factors were identified. Applying all three criteria together we ended with a list of 17 genes. The complete relationships between the sets are presented in the Venn-diagram in figure 1. A summary of the genes matching all criteria is given in table S1 together with detailed motif information.

The hASH1 mRNA encoded by the ASCL1 gene was selected for detailed analysis of potential FMRP interaction, because the hASH1 mRNA UTRs indicated the strongest enrichment of potential FMRP binding sites. Moreover, we have previously found that hASH1 expression is regulated by a post-transcriptional mechanism during neuron development (41). The hASH1 mRNA contains an U-rich element (U 10) in the 5’UTR, as well as in the 3’UTR (U8). Furthermore, a G-quartet structure is present in the 5’UTR, which differed from the G-quartet consensus sequence described by Darnell et al. (36) in only one G as indicated in bold (DWGG is underlined): 5’-…G AAGG GAGG A GGG GAGG GAGG AG GAGG…-3’. Searching FMRP target mRNAs for G-quartet structures indicates that the canonical motif is rather variable (28) and may therefore permit FMRP-binding to the hASH1 mRNA G-quartet. Other candidates shown in table S1 did not contain further G-quartet like structures.

FMRP and hASH1 are co-localized in distinct areas of newborn rat brain.

Direct regulation of hASH1 by FMRP requires that both proteins share an overlapping cellular expression pattern. We applied a double-immunostaining technique to detect FMRP and the rat hASH1 homologue, Mash1, in the postnatal rat brain at day P2. For this purpose, a FITC-conjugated secondary antibody was used to visualize FMRP (green fluorescence), whereas Mash1 was stained with Cy3-conjugate (red fluorescence). Distinctive Mash1 signals were observed in the subventricular zone, the hippocampal formation, temporal cortex and the cerebellum (Fig. 2A). Consistent with previous reports (2;22;42), Mash1 immunoreactivity deco-
rated the nuclei of cells that often exhibited a granular cytosolic FMRP signal (Fig. 2B). Due to fluorescence signals in non-overlapping cell compartments a correlation between Mash1 and FMRP signals could not be established in brain slices. Therefore, we have analyzed primary hippocampal neurons (Fig. 2C) and found that Mash1 and FMRP expression was weakly (Rs = 0.23), but significantly (P = 0.01), correlated (Fig. 2D).

**FMRP expression is correlated with hASH1 protein levels in vitro.**

To test whether FMRP can regulate *hASH1* gene expression *in vitro* we transiently transfected human embryonic kidney (HEK)293 cells with an *FMR1* expression vector. HEK293 cells were chosen because of their higher transfection efficiencies (>80%) compared to most other cell lines. Moreover, HEK293 cells are derived from neuronal cells in human fetal kidney as they express a variety of marker proteins typically found in neurons (43). While the basal amounts of FMRP and hASH1 proteins were rather low in HEK293 cells, graded expression of FMRP to a level normally found in neuroblastoma-derived Kelly cells resulted in a stepwise increase in hASH1 protein (Fig. 3A, C). Trypan blue exclusion indicated that more than 95% of the cells were viable, no matter whether they had been transfected with FMRP expression construct or empty vector. Moreover, knock-down of FMR1 by siRNA transfection of Kelly cells significantly reduced hASH1 protein expression (Fig. 3B, D). Noteworthy, manipulating the cellular FMRP content did not significantly change *hASH1* transcripts (Fig. S1) suggesting a post-transcriptional mechanism for the action of FMRP.

**FMRP increases the hASH1 translation rate.**

Translational activation is mainly accomplished through enhanced initiation rate (44). Hence, the status of mRNA assembling with ribosomes to form polysomal complexes reflects the translational efficiency of the mRNA. Polysomes represent an actively translated cellular fraction. The high molecular weight of ribosomes enables a distinct separation of ribonucleoprotein complexes (RNPs) (translationally active, low molecular weight - top) and polysomal complexes (translationally active, high molecular weight - bottom) by sucrose gradient ultra-centrifugation. The polysomal profile (measured by absorbance at 254 nm [A_{254}]), indicates the percentage of ribosomes involved in translation (overall translation), as well as the ribosome density (45). Upon fractionating the sucrose gradient solution, specific mRNA or protein levels can be estimated in order to assess the translational efficiency status of specific mRNAs, or the involvement of certain proteins in polysomal complexes. Disaggregation of polysomal complexes is induced by EDTA treatment due to Mg\(^{2+}\) capturing.

Typical polysomal profiles (A_{254}) after sucrose gradient centrifugation of HEK293 cells transfected either with *FMR1* expression vector or mock are shown in figure 4A. The data indicate that FMRP neither causes an alteration in overall ribosome density, nor in the percentage of ribosomes involved in translation. This suggests that FMRP does not influence overall translation. The over-expressed FMR-protein was detected by immunoblot analysis in polysomes as well as in low molecular weight fractions associated with translationally inactive RNPs and free proteins (Fig. 4B). In contrast, a lower molecular weight splicing variant of FMRP was only seen in the non-polysomal fractions (Fig. 4B). Importantly, forced expression of FMRP resulted in a shift of the *hASH1* mRNA from low to high molecular weight fractions (Fig. 4C). Thus, the peak of *hASH1* mRNA in mock-transfected cells occurred in fraction 8, representing monosomes, whereas *hASH1* transcripts accumulated in fraction 5, which corresponds to an assembling of ~4-5 ribosomes/transcript according to the ribosomal profile. In contrast, the polysomal profile of β-actin mRNA, which was used as a control transcript, was not affected by FMRP expression (Fig. 4D). Polysome complexes were disrupted by EDTA treatment as expected (Fig. 4A, C, D). These data show that *hASH1* mRNA undergoes translational activation in response to elevated FMRP.

**FMRP stimulates reporter gene activity acting through the 5'UTR of hASH1 mRNA.**

Control of post-transcriptional gene expression, including changes in translational efficiency, is mainly established via mRNA untranslated regions (UTRs) (46). We therefore used a reporter gene expression system to assess the importance of the *hASH1* mRNA 5'- and/ or 3'UTR in FMRP-mediated translational control. The 5'- and/ or 3'UTRs of the luciferase transcript encoded by the pGL3-promoter vector were replaced by *hASH1* mRNA specific UTRs (Fig. 5A). The transcription rate in these constructs is controlled by the constitutive CMV-
promoter. Thus, alterations in luciferase activity indicate post-transcriptional control that is mediated by hASH1 UTRs. Co-transfection of HEK293 cells with FMRP increased reporter gene expression in the presence of the hASH1 5’UTR more than 2-fold, but no significant changes were seen with the 3’UTR (Fig. 5B). Although the hASH1 3’UTR reduced the basal reporter activity slightly compared to the original luciferase 3’UTR, FMRP still significantly increased luciferase activity of a construct carrying luciferase 3’UTR, FMRP still significantly increased reporter activity slightly compared to the original translational control of hASH1 mRNA (Fig. 5B). No significant differences in luciferase mRNA levels of the different reporter constructs were determined by real-time RT-PCR upon transfection into HEK293 cells (data not shown). These findings indicate that the translational control of hASH1 expression by FMRP is mediated by the 5’UTR.

The effect of FMRP on hASH1 expression is mediated through an U-rich element in the 5’UTR.

In a next step, we aimed at localizing the element(s) responsible for the FMRP effect in the 5’UTR of the hASH1 mRNA. For this purpose, potential FMRP binding sites, an U(10) element and the G-quartet structure, were deleted from the hASH1 mRNA 5’UTR. For control we also deleted an AU-rich regulatory element (ARE), which did not bind FMRP (Fig. 6A). HEK293 cells were transiently co-transfected with the modified hASH1 UTR-dependent reporter gene vectors and expression constructs encoding either a full-length FMRP-variant with both KH-domains and the RGG-box, or naturally occurring truncated FMR-proteins (Fig. 6B). We found that only the full-length FMRP variant but not the shorter proteins increased reporter gene activity (Fig. 6C). Furthermore, deletion of a poly-(U) tract abolished the effect of FMRP on hASH1 mRNA 5’UTR-dependent luciferase activity. In contrast, removal of a G-quartet motif did not significantly reduce the influence of FMRP (Fig. 6C). Interestingly, deletion of the ARE increased basal reporter gene activity, suggesting that hASH1 mRNA translation is inhibited through this element. However, this deletion did not change reporter gene activation by FMRP (Fig. 6C). These functional analyses indicate that an U-rich element located in the hASH1 mRNA 5’UTR is required for translational control by FMRP.

FMRP binds to the 5’UTR of hASH1 mRNA.

RNA-protein binding studies were performed to explore whether the effect of FMRP on hASH1 mRNA translation is mediated by direct interaction with hASH1 mRNA 5’UTR. For this purpose, RNA electrophoretic mobility super-shift assays (EMSA) were conducted using cytosolic extracts from FMRP-transfected HEK293 cells and anti-FMRP antibody. Cytosolic extracts from mock-transfected cells served as a negative control. We obtained a super-shift signal by incubating labeled hASH1 mRNA 5’UTR transcripts with FMRP containing cytosolic cellular extracts, but not with extracts from mock-transfected cells. Hence, the EMSA experiments confirmed the physical interaction of the over-expressed FMRP variant with the hASH1 mRNA 5’UTR. In contrast, FMRP did not bind to the 3’UTR, which also contains a potential FMRP binding site (Fig. 7A).

UV-cross-linking studies detecting label transfer from the transcripts to the interacting proteins were performed as described previously (40) to narrow-down the FMRP binding element in the 5’UTR of hASH1 mRNA. Label transfer experiments were carried out with cytosolic extracts from FMRP- or mock-transfected HEK293 cells and hASH1 mRNA 5’UTR probes that were labeled either with alpha-32P-C/A/G or UTP nucleotides. A marked UV-cross-linking signal at the molecular weight of over-expressed FMRP (migrating at ~120 kDa) was obtained only with the U-labeled transcripts (Fig. 7B). Consistent with the results from the reporter gene experiments (Fig. 6C) these findings indicate that FMRP directly binds to an U-rich element in the 5’UTR of hASH1 mRNA. Interestingly, contact formation of FMRP with the hASH1 mRNA 5’UTR changed the binding affinity of other trans-factors suggesting reorganization of the protein occupancy at the 5’UTR (Fig. 7B).

Label transfer to FMRP was reduced upon deletion of the U-rich element from the hASH1 mRNA 5’UTR (Fig. 7C). This finding is in line with the results obtained by reporter gene assays (Fig. 6C). To confirm that the binding of FMRP to the hASH1 mRNA 5’UTR affects interaction with other trans-factors, we performed FMRP immuno-depletion using anti-FMRP antibody prior to the label transfer. Immuno-depletion reduced the UV-cross-linking signal caused by FMRP in an antibody concentration dependent manner (Fig. 7D). Furthermore, several trans-factors were impaired in their binding ability, which - in general - is opposite to the response to enhanced FMRP binding. In the
same setting we found no changes of the UV-cross-linking pattern with the *hASH1* mRNA 3′UTR (Fig. 7E). In summary, the RNA/protein interaction studies indicate that FMRP binds directly to an U-rich motif in the *hASH1* mRNA 5′UTR resulting in complex changes of the binding pattern of other trans-factors.

**DISCUSSION**

FMRP is a multi-functional RNA-binding protein that affects gene expression rates of target mRNAs in different ways. The pivotal role of FMRP in neuronal development is indicated by several forms of mental disorder correlating with alterations in *FMR1* gene expression (3;47;48), or loss of function, ranging from single nucleotide mutations in the coding sequence to the deletion of extended parts of the gene (49-52). Consistently, mutant mice with targeted inactivation of *FMR1* (*FMR1*−/−) exhibited cognitive and behavioral deficits in addition to other abnormalities, i.e. macroorchidism (53). In this study, we asked whether FMRP might influence the expression of regulatory key factors in neuronal development. For this purpose, we performed a genome-wide search for 1) candidates that are involved in neuronal development (according to the gene ontology class “nervous system development”), 2) candidates that are known transcription factors and might therefore activate crucial signaling pathways according to criteria 1, and 3) candidates that contain potential FMRP binding sites (poly-U stretches (*U*≥8) and G-quartet motifs) within their 5′- or 3′UTR. Results yielded 17 genes meeting all these criteria. Among them was the neuronal transcription factor hASH1 (*ASCL1*). hASH1 belongs to the basic helix-loop-helix (bHLH) transcription factor family, which are involved in the generation of neuronal diversity by regulating neuronal subtype specification and differentiation (33). Mash1 lineage cells contribute to distinct cell types in different regions of the brain, including the cerebral cortex, hippocampus, and the cerebellum (54). Mash1 is expressed in proliferating precursor cells derived from neural stem cells in the developing central nervous system (55). It is required for oligodendrocyte development (56), formation of neuroendocrine cells (57;58), differentiation of GABAergic neurons (59;60), and for the early development of olfactory and autonomic neurons (61). Consistently, neuron formation is severely impaired in the absence of Mash1 (55;62) demonstrating the essential role of this molecule in neurogenesis (63;64). Furthermore, forced expression of Mash1 was demonstrated to re-specify the fate of adult hippocampal stem/progenitor progeny, which might be relevant for endogenous brain repair (65).

Co-immunostaining of FMRP and the rat homologue of hASH1, Mash1, revealed an overlapping cellular expression pattern in the hippocampal formation, temporal cortex, cerebellum as well as in the subventricular zone of newborn rat brain. Moreover, Mash1 and FMRP signals were positively correlated in primary hippocampal neurons. These findings indicate that indeed, FMRP may affect neural development by modulating hASH1 gene expression. This assumption is strengthened by forced *FMR1* expression in HEK293 cells, resulting in an increased level of native hASH1 protein. In contrast, *FMR1* overexpression did not influence the level of *hASH1* mRNA, suggesting that FMRP modulates the rate of *hASH1* mRNA translation. Consistently, knock-down of FMR1 by siRNA transfection resulted in a marked decrease of hASH1 protein in Kelly neuroblastoma cells indicating that a physiological level of FMRP is necessary for hASH1 expression. Applying UTR-dependent reporter gene assays we identified the *hASH1* mRNA 5′UTR as functional FMRP recognition site, although both, the 5′- and 3′UTR contain potential FMRP binding motifs. Furthermore, we found that only a specific full-length FMRP variant, but not naturally occurring truncated proteins without the RGG-box and/or the KH2 domain, stimulated the translation rate of *hASH1* mRNA. The observation that the RGG-box is necessary for *hASH1* mRNA interaction is not unusual, since it was shown that deletion of the RGG-box reduced the overall affinity of FMRP to nucleic acids (66). Performing super-shift assays with cytosolic extracts from FMRP-transfected HEK293 cells supported the view that only the over-expressed full-length protein, but no other endogenous FMR-proteins in HEK293 cells, bound to the hASH1 mRNA 5′UTR. The observation that the RGG-box is necessary for *hASH1* mRNA interaction is not unusual, since it was shown that deletion of the RGG-box reduced the overall affinity of FMRP to nucleic acids (66). Performing super-shift assays with cytosolic extracts from FMRP-transfected HEK293 cells supported the view that only the over-expressed full-length protein, but no other endogenous FMR-proteins in HEK293 cells, bound to the hASH1 mRNA 5′UTR. These findings are in line with UTR-dependent reporter gene assays. The stimulatory influence of FMRP on *hASH1* mRNA translation requires interaction of FMRP with an *U*<sub>10</sub>-element in the 5′UTR of *hASH1* mRNA, as indicated by functional reporter gene assays and a label transfer technique. Interestingly, FMRP favors interacts with the poly-U element, although a G-quartet sequence is also present in the 5′UTR of *hASH1* transcripts. The G-quartet motif is strongly conserved in a 28 species alignment (BLAST search at the UCSC genome browser, http://genome.ucsc.edu/), suggesting...
functional importance (data not shown). However, so far we found neither an alteration of basal reporter gene activity upon deletion of the G-quartet motif, nor a change in the pattern of hASH1 mRNA 5’UTR interacting proteins obtained by UV-cross-linking assays. However, this only indicates that function of the G-quartet motif is not activated in our setting. Notably, we used the same FMRP variant, which has earlier been reported to interact through its RGG-box with a G-quartet RNA loop structure (36). The finding that the same FMRP molecule can bind either to a G-quartet or U-rich elements supports the view that nucleic acid interaction of FMRP depends on a complex network of modulating factors. This may be attributed to mRNA secondary structures, cis-elements and other trans-factors surrounding the FMRP recognition site, as well as FMRP interacting proteins, which may influence the binding behavior of FMRP.

Applying the UV-cross-linking label transfer technique, contact formation of FMRP with hASH1 mRNA 5’UTR was found to change the binding behavior of other trans-factors. On the other hand, immunodepletion of FMRP using an anti-FMRP antibody resulted in a partly inverse pattern of protein bound to the 5’UTR of hASH1 transcripts. Most of the factors do not compete with FMRP for the same cis-element as indicated by different binding characteristics. Together, these findings suggest that FMRP may act as RNA-chaperone by modulating the configuration of trans-factors at the hASH1 mRNA 5’UTR. Indeed, it has been shown previously that FMRP has RNA-chaperone properties and influences nucleic acids folding and hybridization (67). We propose that hASH1 transcripts undergo translational activation in response to changes in the configuration of bound proteins due to interaction of FMRP with the 5’UTR. This is suggested by the increase in the hASH1 mRNA abundance in fractions of actively translating polyribosomes following forced FMRP expression. In contrast to the over-expressed FMR-protein, other FMRP variants that naturally occur in HEK293 cells, were found exclusively in non-polysomal fractions. Thus, it seems that not all FMRP variants can regulate mRNA translation, including translational control of hASH1.

The hASH1 mRNA has not been recognized as FMRP target in earlier studies, i.e. by using immunoprecipitation of FMRP-containing mRNP particles. Beside different settings, one explanation would be that the interaction of FMRP as RNA chaperone with hASH1 mRNA is only transient. On the other side, the hASH1-related transcriptional repressor Hairy and Enhancer of Split1 (HES1), was discovered as a potential FMRP target gene (12). HES1 was found to inhibit hASH1/ Mash1 transcription, as well as nerve growth factor (NGF)-mediated differentiation of hippocampal neurons (68;69). Recently it was shown that HES1 can determine the fate of quiescent cells towards terminal cell differentiation and/ or cell cycle arrest (70). Furthermore, HEY1 (Hairy/enhancer-of-split related with YRPW motif protein 1) as well as PHOX2B (Paired mesoderm homeobox protein 2B), which may also contribute to the HES1/ hASH1 pathway, also contains a potential FMRP binding site (Table S1). Hence, FMRP may regulate neuron differentiation by its post-transcriptional control of key neuronal molecules including hASH1 and others.

Finally, our findings raise the possibility that translational control of hASH1 mRNA by FMRP might be important in neuronal development. As our data are mainly based on in vitro findings, further studies are needed to explore the possible role of the regulatory link between FMRP and hASH1 in aberrant neuronal development in vivo, such as Fragile X Syndrome.
REFERENCES


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**FOOTNOTES**

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FIGURE LEGENDS

Figure 1: Selection of potential FMRP target genes involved in neuronal development. The set relationships of the genes meeting different filter criteria. A total number of 32,562 human ENSEMBL-genes were screened for the presence of a poly-U stretch or a G-quartet in the mRNA 5’- or 3’UTRs, for the gene ontology “nervous system development”, and for the property “transcription factor”. A group of 17 genes met all the criteria, and was the basis for appropriate candidate selection.

Figure 2: Double-immunostaining of FMRP and Mash1 in the postnatal rat brain and primary hippocampal neurons. An immunofluorescent double-labeling technique was applied to detect the rat hASH1 homologue, Mash1 (red), and FMRP (green) on cryostat sections from the postnatal rat brain. A: Overview of Mash1 expression in a horizontal P2 rat brain section. B: Cellular co-localization of both proteins in cells of the subventricular zone (LV), cerebellum (CB), and hippocampal formation (EC) at postnatal day P2. C, D: Mash1 (red) and FMRP (green) signals are weakly positively correlated in primary hippocampal neurons at 8 days in vitro. Cell nuclei were visualized with DAPI (blue). Abbreviations: LV, lateral ventricle; CB, cerebellum; EC, entorhinal cortex; TC, temporal cortex. No fluorescence signals were obtained when species-matched normal sera instead of primary antibodies were used (not shown).

Figure 3: Effect of FMRP on hASH1 expression. A: Representative Western blot analysis of total cell lysates from HEK293 cells that had been transfected either with FMRP expression vector (FMRP) or empty plasmid (mock). Graded levels of FMRP were adjusted by stepwise increasing the amount of transfected expression construct (0 µg, 2 µg, 4 µg or 8 µg DNA/ 60 mm dish) and proportionately reducing the quantity of empty vector such that the overall amount of transfected plasmid DNA was the same in all experiments. Note, that increasing FMRP expression to a level normally found in neuroblastoma-derived Kelly cells resulted in a graded increment of hASH1 protein in HEK293 cells. Detection of beta-actin served as loading control. B: Representative Western blot showing the effect of FMRP-silencing by siRNA transfection on hASH1 expression in Kelly cells. Transfection with a non-targeting siRNA served as control. Knock-down of FMR1 clearly reduced hASH1 protein without changing the level of beta-actin. C, D: Relative quantification of hASH1 and FMRP levels. Shown are mean values, and error bars represent the standard deviation. (N=3; *P < 0.05).

Figure 4: Analysis of sucrose density gradients. HEK293 cells were transiently transfected for 36 h using either FMRP expression vector or mock (empty vector control). A: Typical ribosomal profiles after sucrose gradient ultra-centrifugation monitored at 254 nm absorbance from the bottom (51% sucrose) to the top (17% sucrose). Each gradient was divided into 12 fractions for analysis. Addition of EDTA [50 mM] resulted in disaggregation of polysomal complexes. B: Western blot detecting FMRP and beta-actin in different fractions collected from sucrose gradient centrifugation. Note, that FMRP is found in polysomes (fractions 1 to 7), the monosome fraction (fraction 8) as well as in non-polysomal fractions (fractions 9 to 12). EDTA treatment resulted in a shift of FMRP from polysomal to non-polysomal fractions. A smaller FMRP splicing variant (FMRP var) and beta-actin are shown as controls. C: Quantification of hASH1 mRNA in polysomal fractions. Note, that forced expression of FMRP caused a shift of hASH1 transcripts into polysomal fractions, where mRNAs are actively translated. hASH1 mRNA was released from the polysomes in response to EDTA treatment. D: FMRP did not change the distribution of beta-actin mRNA. Shown are mean values, and error bars represent the standard deviation. (N=4).

Figure 5: hASH1 mRNA UTR-dependent reporter gene assays. HEK293 cells were co-transfected with FMRP expression vector and the pGL3-promoter plasmid containing a constitutive SV40 promoter. Additional co-transfections were performed with a pGL3-promoter derived plasmid, in which the 5’- and/ or 3’UTRs of luciferase mRNA had been replaced by the hASH1 mRNA UTRs. A: Schematic illustration of the reporter gene constructs used for transfection. Dotted lines indicate the original UTRs of firefly luciferase-mRNA. Boxes “5’UTR” and “3’UTR” represent the specific hASH1 mRNA UTRs. “Luciferase cds” marks the coding sequence of the luciferase transcript. B: Results of the reporter gene experiments demonstrate that FMRP stimulates luciferase activity in constructs harboring the hASH1 mRNA 5’UTR either alone or in combination with the 3’UTR. Shown are relative
mean values normalized to the values obtained by transfection of the original pGL3-promoter vector. Error bars represent the standard deviation. (N=12; **P < 0.01).

**Figure 6: Mapping of the functional cis-element in the 5’UTR of hASH1 mRNA.** For functional cis-element mapping we deleted potential FMRP-binding sites within the hASH1 mRNA 5’UTR including an U_{10}-element (poly-U del) and a G-quartet motif (GQ del). An AU-rich element (AU del), which does not represent a typical FMRP binding site, was deleted for control reasons. A: Schematic illustration of the hASH1 mRNA 5’UTR deletion variants. B: Scheme of the different FMR-proteins that were used for co-transfection. All FMRP molecules represent naturally occurring FMRP splice variants, but contain different sets of RNA-binding domains, i.e. two KH-domains (KH1 and KH2) and a RGG-box (RGG). Additional functional domains are the nuclear localization signal (NLS), and a cytosolic localization signal (CLS), also termed nuclear export signal. C: Effect of different FMRP variants on reporter activity of the constructs shown in panel A. Only the full-length FMRP stimulated hASH1 mRNA 5’UTR dependent luciferase activity. This effect of FMRP was abolished by deletion of the U-rich element. (N=12; **=P < 0.01).

**Figure 7: RNA/ protein interaction studies.** Alpha-^{32}P-NTP labeled in vitro transcripts of the 5’- or 3’UTR of hASH1 mRNA were incubated with cytosolic extracts from FMRP- and mock-transfected cells. A: Electrophoretic mobility super-shift assay: In vitro labeled transcripts were incubated with cytosolic extracts or buffer (free probe). Both, the 5’- and the 3’UTR of hASH1 mRNA interact with cytosolic proteins as indicated by the shift to higher molecular weight complexes. Incubation with anti-FMRP antibody, but not with anti-β-actin antibody, supershifted the 5’UTR-protein complex. B: UV-crosslinking: Separate labeling of hASH1 mRNA 5’UTR using alpha-^{32}P-C/ A/ G or UTP nucleotides. A FMRP related UV-crosslinking signal was only observed with alpha-^{32}P-UTP-labeled 5’UTR transcripts. Furthermore, the binding properties of other trans-factors were increased (+) or reduced (*) upon FMRP transfection. C: UV-cross-linking assays using alpha-^{32}P-UTP labeled transcripts corresponding to the hASH1 mRNA 5’UTR deletion variants described in figure 6. Transcripts were incubated with cytosolic extracts from FMRP-transfected cells. Deletion of the poly-U cis-element, but not of the G-quartet and the AU-rich element, reduced the FMRP-related UV-cross-linking signal. D: Immuno-depletion of FMRP in cytosolic extracts enriched in FMRP decreased the FMRP-related signal. Moreover, other trans-factors were also affected in their binding behavior, which is partially inverse to the findings shown in (B). E: No differences in the protein binding pattern between mock- and FMRP-transfected cells were seen with 3’UTR transcripts (D). Representative original data are shown. The experiments were repeated at least three times.
Fig. 3

A  HEK293 cells

B  Kelly cells

C  

D  

control siRNA  FMR1 siRNA

< FMRP  < hASH1  < beta-actin

< FMRP  < hASH1  < beta-actin

FMRF  0 µg  2 µg  4 µg  8 µg

FMRF  0 µg  2 µg  4 µg  8 µg

FMRF  0 µg  2 µg  4 µg  8 µg

FMRF  hASH1
Fig. 4

A

mock

FMRP

Bottom → Top

Bottom → Top

mock + EDTA

FMRP + EDTA

Bottom → Top

Bottom → Top

B

mock

< fraction

< FMRP

< FMRP var

< beta-actin

FMRP

< fraction

< FMRP

< FMRP var

< beta-actin

mock + EDTA

< fraction

< FMRP

< FMRP var

< beta-actin

FMRP + EDTA

< fraction

< FMRP

< FMRP var

< beta-actin

C

Δactin mRNA level (relative values)

mock

FMRP

Fraction

Fraction

Fraction

Fraction

+ EDTA

D

Δactin mRNA level (relative values)

mock

FMRP

Fraction

Fraction

Fraction

Fraction

+ EDTA

beta-actin mRNA level (relative values)
Fig. 5

A

B

mock
FMRP

**

1.00 1.01 2.35 0.55 0.46 1.20

pGL3p 5'UTR-Luc 3'UTR Luc-3'UTR 5'UTR-Luc-3'UTR
Fig. 6

A

cis-element: hASH1 - 5'UTR

hASH1 - 5'UTR
5' U GQ AU >>ods

hASH1 - 5'UTR_poly-U tract deletion (poly-U del)
5' U GQ AU >>ods

hASH1 - 5'UTR_G-quartet deletion (GQ del)
5' U AU >>ods

hASH1 - 5'UTR_AU-rich element deletion (AU del)
5' U GQ >>ods

B

trans-factor: FMRP variants

FMRP - KH1
NLS KH1

FMRP - KH1/2
NLS KH1 KH2

FMRP - KH1/2, RGG
NLS KH1 KH2 NLS RGG

C

mock
FMR1 (KH 1)
FMR1 (KH 1/2)
FMR1 (KH 1/2, RGG)

Luciferase activity (relative values normalized to pGL3-promoter)

0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00

pGL3p hASH1 5'UTR hASH1 5'UTR poly-U del hASH1 5'UTR GQ del hASH1 5'UTR AU del

1.00 0.66 0.72 0.78 1.38 1.57 1.59 3.18

**
Fig. 7

A

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Translational regulation of the human achaete-scute homologue-1 (hASH1) by fragile x-mental retardation protein (FMRP)

Michael Fähling, Ralf Mrowka, Andreas Steege, Karin M. Kirschner, Edgar Benko, Benjamin Förstera, Pontus B. Persson, Bernd J. Thiele, Jochen C. Meier and Holger Scholz

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