Identification of FMRP target mRNAs in the developmental brain: FMRP might coordinate Ras/MAPK, Wnt/ß-catenin, and mTOR signaling during corticogenesis

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Abstract

Corticogenesis is one of the most critical and complicated processes during embryonic brain development. Any slight impairment in corticogenesis could cause neurodevelopmental disorders such as Fragile X syndrome (FXS), of which symptoms contain intellectual disability (ID) and autism spectrum disorder (ASD). Fragile X mental retardation protein (FMRP), an RNA-binding protein responsible for FXS, shows strong expression in neural stem/precursor cells (NPCs) during corticogenesis, although its function during brain development remains largely unknown. In this study, we attempted to identify the FMRP target mRNAs in the mouse cortical primordium using RNA immunoprecipitation sequencing analysis in the mouse embryonic brain. We identified 865 candidate genes as targets of FMRP involving 126 and 118 genes overlapped with ID and ASD-associated genes, respectively. These overlapped genes were enriched with those related to chromatin/chromosome organization and histone modifications, suggesting the involvement of FMRP in epigenetic regulation. We further identified a common set of 17 "core" genes involved in neurogenesis/FXS/ID/ASD, containing factors associated with Ras/mitogen-activated protein kinase, Wnt/ß-catenin, and mTOR pathways. We indeed showed overactivation of mTOR signaling via an increase in mTOR phosphorylation in the Fmr1 knockout (Fmr1 KO) neocortex. Our results provide further insight into the critical roles of FMRP in the developing brain, where dysfunction of FMRP may influence the regulation of its mRNA targets affecting signaling pathways and epigenetic modifications.

Introduction

The neocortex is an important region in higher cognitive functions, and its formation, i.e., corticogenesis, is an extremely complicated process during embryonic brain development During corticogenesis, neural stem/precursor cells (NPCs), or in another name, radial glial cells (RGCs) proliferate and differentiate to immature neurons. These immature neurons migrate towards the basal side and stack from inside-to-outside, and further differentiate into glutaminergic excitatory neurons, produce neurotransmitters or neurotrophic factors, and begin to form neural networks [1, 2]. These processes are precisely programmed at the genetic level; therefore, any slight impairment in the developmental program could result in severe functional defects in the brain.

Intensive genetic analyses of patients with neurodevelopmental disorders have identified various molecules critical for the neuropathogenesis [3, 4]. *Fragile X mental retardation 1* (*FMR1*) encoding fragile X mental retardation protein (FMRP) is a well-characterized gene related to a typical neurodevelopmental disorder, Fragile X syndrome (FXS) [5, 6]. The FXS patients have moderate intellectual disability (ID), and a third of the patients show features of autism spectrum disorder (ASD) [7–9]. Therefore, elucidating the FMRP function is critical to understand the molecular mechanism relating also to ID and ASD.

FMRP is a polyribosome-associated RNA binding protein (RBP) [10, 11]. In the matured neuron of the adult brain, FMRP is localized at cell body, proximal dendrites, and axons [12, 13]. FMRP plays profound regulatory roles in the synaptic function and neuronal plasticity through the interaction with transcripts of

pre- and postsynaptic proteins [14, 15] and by regulation of mRNA trafficking into the dendrite [11, 16]. On the other hand, FMRP is also expressed in the RGCs and immature neurons of the developing brain [17, 18]. Within the RGCs, FMRP is localized at the apical and basal endfeet [17, 18]. Previous studies have suggested that FMRP regulates the transition from RGCs to intermediate progenitors in the embryonic brain [18] and that its deficit affects neuronal migration and cortical circuitry [19]. Altogether, FMRP has multiple roles at distinct time points in brain development.

Since the discovery of FMRP, various studies have been conducted to identify FMRP target genes using RNA-binding protein immunoprecipitation (RIP) [17, 20], crosslinking immunoprecipitation [10], photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation [21] and ribosome profiling [22], all of which are based on high-throughput sequencing. While the role of FMRP and its target genes have been most highlighted in the adult brain [10, 20, 23, 24], only limited studies have reported its role in the embryonic brain [17, 18]. In this study, we performed RIP high-throughput sequencing (RIP-seq) analysis using mouse embryonic brain samples and identified FMRP target genes that are also associated with ID and ASD. We also found “core” genes shared with our data, neurogenesis, ID, and ASD, which were involved in the Ras/mitogen-activated protein kinase (MAPK), Wnt/β-catenin, and mTOR pathways. Our data may contribute to understand the role of FMRP in corticogenesis and may serve as important resources for future studies of neurodevelopmental disorder.

Results

FMRP is expressed in the mouse embryonic cortex

We first confirmed the FMRP expression in the cortical primordium of wild type (WT) mice at embryonic day (E) 14.5 when massive neurogenesis occurs. The immunostaining signal of FMRP was present in the cortical plate (CP), including immature neurons, and at apical (ventricular) and basal (pial) surface areas (Fig. 1A, B). The accumulation of FMRP at the apical and basal endfeet of the RGCs was confirmed by the GFP-labeling of RGCs using *in utero* electroporation with an EGFP reporter gene (pCAG-EGFP) (Fig. 1C); FMRP was overlapped with GFP fluorescence in both the apical and basal endfeet of the RGCs (Fig. 1D-E). Therefore, our data are consistent with findings in the previous literature [17–19].

Identification of FMRP target mRNAs in embryonic mouse cortex

To explore the target mRNAs of FMRP during corticogenesis, we performed RIP-seq analyses using cortical samples isolated from the WT mice at E14.5 (Fig. 2A). In total, we found 2288 candidate FMRP target mRNAs that were significantly expressed (measured by fragment per kilobase of transcript per RNA-seq read mapped, FPKM) in the FMRP-IP compared to IgG-IP or the negative control (FMRP-IP FPKM > IgG-IP FPKM). Next, we selected a stringent set of 947 mRNAs from 865 FMRP target genes based on gene expression values, fold changes in the logarithmic scale with base 2 greater than 1 ($\log_2 FC > 1$), and FPKM greater than 10 against the FMRP-IP. (Fig. 2B; Table S1). The set of 865 FMRP target genes showed higher gene expression than the negative control, and therefore, could be validated as targets of FMRP in the developing neocortex.

To estimate the functions of the FMRP target mRNAs, we performed gene ontology (GO) analyses using the Visual Annotation Display (VLAD) – gene analysis and visualization analysis tool of the Mouse Genome Informatics (MGI) [25]. The top significant GO terms included biological processes related to early brain development, such as “Nervous system development,” “Generation of neurons,” and “Neurogenesis.” (Fig. 2C). This is quite reasonable because the identified FMRP target mRNAs were collected from developing cortices where massive neurogenesis is occurring (Fig. 1, Table S2).

Overlap among FMRP targets, neurogenesis, ID and ASD-associated genes

To obtain more insight for the significance of FMRP target candidate genes, we focused on the three criteria, i.e., neurogenesis, ID, and ASD since FXS patients often show ID and ASD symptoms [8, 9]. We first compared the identified 865 FMRP target genes with 1791 neurogenesis genes from MGI [25]. There was a highly significant overlap of 156 genes between the two groups, including those mainly assigned to GOs related to “Neuronal development”, “Generation of neurons,” “Neuron differentiation”, and “Cell morphogenesis involved in differentiation” (Fig. 3A). The results thus indicate that several targets of FMRP are important for neurogenesis during early brain development. We also found genes for “Axonogenesis” and “Neuron projection development”, i.e., the events after neuronal differentiation, as GOs for FMRP target genes, which may suggest the importance of FMRP in the establishment of neuronal networks.

We then examined the association between the identified FMRP target genes with 1088 ID genes based on Online Mendelian Inheritance in Man (OMIM) [26], which resulted in 126 genes (Fig. 3B), as expected, because ID is a core feature of FXS [27]. These overlapped genes included not only GOs such as “Brain development” and “Central nervous system development”, but also “Chromosome organization” and “Histone modification” unexpectedly. We also found 118 FMRP target genes that significantly overlapped with the 1025 ASD-associated genes using the public database Simons Foundation Autism Research Initiative (SFARI) [28] (Fig. 3C); again there came up with GOs such as “Histone modification” and “Chromatin organization”, as well as “Brain development”. As we expected, these GOs include several syndromic ASD-associated genes such as *paired box 6* (*PAX6*) [29, 30],*lysine acetyltransferase 6a* (*KAT6A*) [31], *mammalian target of rapamycin* (*mTOR*) [32], *Abelson’s helper integration 1* (*AHI1*) [33] and *ubiquitin-specific peptidase 9 X-linked* (*USP9X*) [34]. Overall, the overlap between FMRP target genes linked to ID and ASD could provide a correlation between loss of function of FMRP and the development of both ID and ASD.

Finally, we identified 17 genes as the “core” genes shared with neurogenesis, ID, and ASD gene sets (Fig. 3D, Table 1). The “core” gene set contained not only major transcription regulators such as Pax6, Myt1l, and Tcf4 but also components of Ras/MAPK (Nf1) [35], Wnt/β-catenin (Ahi1, Ctnna2, and Ctnnb1) [33, 36], and mTOR (mTOR, Ep300, Itpr1and Synj1) [36–40] signaling pathways (Fig. 3E). As mentioned above, the “core” gene set also included factors of the chromatin-remodeling complex [41], such as Nipbl, Smarcc2, and Smarca4. Besides, Usp9X has been thought to be involved in developmental processes

through Wnt/β-catenin and mTOR pathways [42]. These common pathways can cause shared symptoms among FXS, ID, and ASD.

Table 1. 17 FMRP target genes associated with neurogenesis, ID and ASD.

Gene symbol	Gene name
Ahi1	*Abelson Helper Integration Site 1*
Ank3	*Ankyrin 3*
Ctnna2	*Catenin Alpha 2*
Ctnnb1	*Catenin Beta 1*
Ep300	*E1A Binding Protein P300*
Itpr1	*Inositol 1,4,5-Trisphosphate Receptor Type 1*
Mtor	*Mechanistic Target Of Rapamycin Kinase*
Myt1l	*Myelin Transcription Factor 1 Like*
Nf1	*Neurofibromin 1*
Nipbl	*NIPBL Cohesin Loading Factor*
Pax6	*Paired Box 6*
Smarca4	*SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4*
Smarcc2	*SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin Subfamily C Member 2*
Synj1	*Synaptojanin 1*
Tbc1d23	*TBC1 Domain Family Member 23*
Tcf4	*Transcription Factor 4*
Usp9x	*Ubiquitin Specific Peptidase 9 X-Linked*

Expression of the “core” gene set in the developing cortex

To confirm the FMRP interaction with the mRNAs of the 17 “core” genes, we performed RIP-qPCR. All mRNAs were significantly enriched in the FMRP-IP, suggesting that these mRNAs included in 17 “core” gene set are targeted by FMRP (Fig. 4A). We further examined the mRNA amount of the genes in cortical primordial samples from E15.5 WT and *Fmr1* knockout (KO) male mice. We found a significant increase of *Nf1* mRNA and a significant decrease of *Ahi1* mRNA in the *Fmr1* KO mouse neocortex, while other genes showed no significant difference (Fig. 4B). These findings suggest that FMRP mainly functions as a post-transcriptional regulator of its target genes.

Among the “core” gene set, we highlighted three genes, *Nf1, Ctnnb1*, and *Mtor*, because these are involved in Ras/MAPK, Wnt/β-catenin, and mTOR pathways, respectively [40, 43, 44]. We first assessed the protein expression of Nf1, Ctnnb1, and mTOR in the cortical primordium at E15.5 (Fig. 5A, B). The Nf1 and mTOR proteins were widely expressed throughout the cortical primordium, while Ctnnb1 was concentrated at the apical surface. There seemed to be no change in these expression patterns in *Fmr1* KO mice compared to that of WT (Fig. 5A). Immunoblotting analyses also showed that Nf1, Ctnnb1, and mTOR showed normal expression levels in *Fmr1* KO mice corresponding to the immunostaining (Fig. 5A-D). This could imply that the translation of these targets was unaffected in the *Fmr1* KO neocortex. Although the protein level of mTOR was unchanged in the *Fmr1* KO, its phosphorylated form (p-mTOR) at Ser2448 was significantly elevated by 25.4% in the lysate of the *Fmr1* KO neocortex compared to that of WT (Fig. 5B, E, F). This result suggests that mTOR signaling might be enhanced in *Fmr1* KO mice during corticogenesis, which is similar to the result in the adult hippocampus [40] but the first evidence in the embryonic brain.

Discussion

Even though FMRP shows unique expression patterns in the cortical primordium, most of the previous studies have highlighted FMRP’s role in the post-transcriptional regulation of its mRNA targets in the adult brain. Here we focused on the FMRP target genes during corticogenesis and identified 865 genes including not only those related to neural and neuronal development, as expected, but also those involved in chromatin remodeling, histone modifications, and cell signaling.

Using independent databases, i.e., OMIM and SFARI, we showed that our FMRP target genes significantly overlapped with genes associated with ID and ASD. This may suggest that phenotypes shared among FXS, ID, and ASD patients may likely be caused by these common genes. In other words, these lists also represent common impaired pathways or molecular mechanisms observed in neurodevelopmental disorders.

We identified 17 “core” genes common in FXS, ID, ASD, and neurogenesis gene sets including critical components in Ras/MAPK, Wnt/β-catenin, and mTOR pathways. Nf1 is a negative regulator of the Ras/MAPK signaling pathway, and loss of Nf1 leads to an increase in the number of NPCs, but not of neurons, within the mouse cortex [35]. Several groups have previously shown Nf1 as a target of FMRP [10, 21, 22, 45] with some inconsistency. Knocking down *Fmr1* in oocytes increases *Nf1* at the mRNA level but decreased its protein level [45]. Ribosomal profiling of cultured adult NPCs shows normal *Nf1* mRNA and protein expression in the *Fmr1* KO [22]. In our study using *Fmr1* KO forebrain samples, the absence of FMRP caused an increased expression of *Nf1* mRNA but unaffected its protein amount. Thus, regulation of Nf1 expression at mRNA and protein levels may be highly context-dependent; the increase of *Nf1* mRNA level could be an indirect effect of FMRP on upstream transcriptional or epigenetic regulators of *Nf1* that might be different in distinct cell types.

It is well known that the Wnt/β-catenin pathway regulates NPC proliferation and neuronal differentiation in the developing mouse neocortex [46–48]. In this study, we identified a Wnt/β-catenin pathway

regulator, *Ctnnb1*, as a target of FMRP. However, *Ctnnb1* mRNA and protein amounts were normal in *Fmr1* KO embryonic brain. Although Ctnnb1 amount is reported to be reduced in adult NPCs derived from *Fmr1* KO mouse [49], NPCs derived from human embryonic stem cells established from FXS patients showed unchanged Ctnnb1 level compared to the nonaffected control [50]. These discrepancies might come from different stages of the NPCs, and our finding is rather close to the NPCs that may reflect FXS conditions during development.

mTOR signaling is a well-known pathway for its responsibility to not only for FXS [40], but also for ASD [51, 52]. Overactivation of mTOR signaling during neurogenesis can increase protein synthesis and induce neuronal differentiation [53, 54], leading to cortical malformation [55]. Several studies have explored the relationship between FMRP and mTOR signaling in the adult brain. In the hippocampus of *Fmr1* KO mice, increased phosphorylation of mTOR is observed in addition to increased expression of mTOR upstream and downstream molecules [40]. Here we found for the first time that the p-mTOR level was elevated also in the developing neocortex of *Fmr1* KO mice. Our results suggesting enhanced mTOR signaling activity in the embryonic brain in loss of FMRP function can explain the enlarged brain of *Fmr1* KO mice [18, 56].

Finally, FMRP may further affect gene expression during corticogenesis by regulating epigenetic (chromatin and histone) modifications (as seen in Fig. 3B, C, Table S2). One of the chromatin targets of FMRP, bromodomain-containing 4, Brd4, has been reported to be overactivated in *Fmr1* KO mice, and its inhibition alleviated phenotypes in the mouse associated with FXS [57]. The mechanism of regulation by FMRP on these epigenetic regulators was not investigated in this study. Whether or not the misregulation of epigenetic modifications is due to the absence of FMRP, it is evident that these modifications could modulate widespread changes in the expression of its downstream targets. For this reason, our findings could provide additional evidence that FMRP may modulate multiple regulations of gene expression during corticogenesis.

In summary, we discovered that 13 of 17 FMRP “core” target genes were involved in Ras/MAPK, Wnt/β-catenin, and mTOR signaling pathways, all of which are pivotal in brain development. Proper regulation of these genes by FMRP is thus believed to be essential for appropriate corticogenesis. There could be other modulations due to the loss of function of FMRP at the epigenetic level. Our study sheds light on the significance of genetic programs in early brain development, in addition to previously proven roles in the function of postnatal neurons, concerning the etiology of FXS, of which symptoms are shared with ID and ASD.

Methods

Animals

Animal experiments were carried out in accordance with the National Institutes of Health guidelines outlined in the Guide for the Care and Use Laboratory Animals. The Committee for Animal Experimentation of Tohoku University Graduate School of Medicine (2017-MDA-189) and the Animal care

and Use Committee of Yokohama City University (TA-16-006) approved all the experimental procedures. Male WT (C57BL/6J) and *Fmr1* KO (B6.129P2-*Fmr1*^{tm1Cgr}/J, stock #003025) [5] mice were used in this study. Hemizygote (*Fmr1*$^{-/y}$) male and heterozygote (*Fmr1*$^{+/-}$) female mice were mated to obtain WT (*Fmr1*$^{+/y}$) and *Fmr1* KO (*Fmr1*$^{-/y}$) male embryos.

DNA extraction and *Fmr1* genotyping

Genomic DNA was extracted from the tail of E15.5 mouse embryos, and a standard polymerase chain reaction was performed as previously described [58, 59]. Screening for the presence or absence of the wild-type allele was performed using primers S1m (5'- GTGGTTAGCTAAAGTGAGGATGATAAAGGGTG-3') and S2m (5'- CAGGTTTGTTGGGATTAACAGATCGTAGACG-3'). Primers N2c (5'- CGCCTCAGAAGCCATAGAGCC-3') and N3 (5'- CATCGCCTTCTATCGCCTTCTTGAC-3') were used to screen for the presence of the knockout allele. The amplified PCR products were visualized by electrophoresis on 1% agarose gels using the Gel Doc™ EZ Imager (Bio-Rad).

Immunohistochemistry

Immunohistochemistry was performed as described previously [58, 59]. The sections were incubated with primary antibodies diluted with 3% BSA/TBST (containing 0.1% Triton X100), including goat anti-FMRP (1:1000; LS-B3953; LifeSpan Biosciences Inc.), rabbit anti-mTOR (1:1000; 7C10; Cell Signaling Technology), rabbit anti-phospho-mTOR (Ser2448) (1:1000; 2971; Cell Signaling Technology), mouse anti-Ctnnb1 (1:2000; 610153; BD Biosciences), and rabbit anti-Nf1 (1:1000; ab17963; Abcam) overnight at 4 °C. The secondary antibodies used were Cy3-conjugated donkey anti-goat IgG (1:500; Life Technologies), Cy3-conjugated donkey anti-rabbit IgG (1:500; Life Technologies), and Alexa 488-conjugated donkey anti-mouse IgG (1:500; Life Technologies), and counterstained with 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI)/TBST (1:1000; Sigma). Images were visualized by a confocal laser microscope Zeiss LSM800 (Carl Zeiss).

In utero electroporation into the mouse embryonic brain

In utero electroporation was performed as described previously with minor modification [60, 61]. The expression vectors pCAG-EGFP plasmid (kindly gifted from Prof. Tetsuichiro Saito, Chiba University, Japan) and 1% Fast green in PBS were injected into the lateral ventricle of embryos at E13.5. The embryos were collected at E14.5 for analysis for FMRP localization.

Preparation of RNA libraries and sequencing

Following the manufacturer’s protocol, RIP assay was performed to extract FMRP-bound mRNAs (n = 3, FMRP-IP; n = 2, negative control (IgG-IP)) from E14.5 WT mice cortex by using RiboCluster Profiler™ RIP-Assay Kit with anti-FMRP human polyclonal antibody, RN016P (Medical and Biological Laboratories Co., Ltd.) and Dynabeads™ Protein beads G/A (Invitrogen™). The quality and quantity of the total RNA were evaluated using the Agilent 2100 Bioanalyzer with RNA 6000 Pico Kit (Agilent). Total RNA concentration greater than 50 ng with an RNA Integrity Number (RIN) value greater than or equal to 7.9 was sequenced.

Sequence Alignment And Estimation Of Gene Expression Levels

Raw reads were cleaned by removing adapter sequences and low-quality sequences (Phred quality score: 33; minimum threshold: 20; minimum length: 70) using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Using TopHat (http://tophat.cbcb.umd.edu/), cleaned reads were aligned to reference genome *Mus musculus* genome (mm10) with default parameter values, except for the distance between mate pairs ($r = 200$). Calculation of gene expression in FPKM and test of significance were calculated using Cuffdiff (http://cufflinks.cbcb.umd.edu/). FMRP mRNA targets were defined as transcripts showing significant difference at $q < 0.01$ (between FMRP-IP and IgG-IP), $\log_2$FC greater than 1, and FPKM value greater than 10 in the FMRP-IP samples.

Gene Ontology And Protein Association Network

Functional annotation of the differentially expressed genes was performed using the VLAD tool (v1.6.0) of the MGI [25] and Network Analyst – a visual analytics platform for comprehensive gene profiling and meta-analysis [62]. GO was determined *via* an enrichment analysis (biological process), and false discovery rate (FDR) less than 0.05 were considered as significantly enriched GO annotation.

Gene Sets Associated With Neurogenesis, ID, And ASD

The 1791 neurogenesis genes were retrieved from the MGI database (retrieved on September 24, 2019) using GO: neurogenesis [25]. The 1088 ID genes were retrieved from the OMIM database (retrieved on September 24, 2019) using the keyword ID [26]. The 1025 ASD-associated genes were retrieved from the SFARI database (updated on May 19, 2020) [28].

RNA Extraction And RT-QPCR

Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and inverse transcribed into complementary DNA (cDNA) using the SuperScipt III™ First-Strand Synthesis System for RT-PCR (Invitrogen). RT-qPCR was performed using 2x SsoAdvanced Universal SYBR®Green Supermix (Roche) and the Mastercycler® ep Gradient Realplex 2 (Eppendorf). The relative expression of each target was calculated (2^ΔΔCt) with *Rplp0* as normalizer. PCR sequences for RT-qPCR (Table S3) were obtained in the Primerbank [63] and from a previous report [64].

Immunoblotting

To assess protein levels, neocortical lysates from pooled (n = 2) dorsal telencephalon of WT and *Fmr1* KO embryos at E15.5 were prepared using cell lysis buffer containing 20 mM HEPES pH 7.5, 20% glycerol, 400 mM NaCl, 1 mM $MgCl_2$, 0.5 M DTT, 0.5 mM PMSF, 0.1% NP40, 1x protease and phosphatase inhibitor, and 1 mM EDTA pH 8.0. Following the manufacturer's protocol, protein concentration was measured by the Lowry Assay Method (Bio-Rad). The neocortical lysates (25 µg) were subjected to SDS/PAGE (7.5% TGX™ FastCast™ Acrylamide Kit; Bio-Rad) and transferred onto polyvinylidene difluoride membranes (Millipore) with 40 V at 4 °C for 4 hours. The membranes were then blocked in 10% TBS

blocking buffer (Licor) for 1 hour, and incubated with a primary antibody (as described above, Immunohistochemistry). The membrane was washed with TBST (containing 0.1% Tween 20) for 1 minute with three repeats and 5 minutes with three repeats and incubated with a secondary antibody, either donkey anti-rabbit 680 (1:10000; Licor), or donkey anti-mouse 680 (1:20000; Licor), diluted in 10% TBS blocking buffer for 1 hour at RT under a shaded condition. The signal was detected using the ODYSSEY infrared imaging system (Licor) and quantified using ImageJ 1.48v software (National Institute of Health) with Gapdh as normalizer.

Statistical analysis

Data were compiled using Microsoft Excel 2011, and Student's *t*-test was used to calculate statistical significance. Hypergeometric distribution was calculated using the webtool Hypergeometric Distribution Calculator (https://keisan.casio.com/exec/system/1180573201). Values of $p < 0.05$ were considered statistically significant.

Abbreviations

ASD: Autism spectrum disorder

BSA: Bovine serum albumin

cDNA Complementary DNA

CP: Cortical plate

DAPI 4',6-Diamidino-2-phenylindole dihydrochloride

DTT Dithiothreitol

FDR: False discovery rate

FMRP: Fragile X mental retardation protein

Fmr1: *Fragile X mental retardation 1*

Fmr1 KO: *Fmr1* knockout

FPKM: Fragment per kilobase of transcript per RNA-seq read mapped

FXS: Fragile X syndrome

GO: Gene ontology

HEPES (2-hydroxyethyl)-1-piperazineethanesulfonic acid

ID: Intellectual disability

Log_2FC: fold change on a logarithmic scale with base 2 fold-change

MGI: Mouse Genome Initiative

NPCs: Neural stem/precursor cells

OMIM: Online Mendelian Inheritance in Man

PCR: Polymerase chain reaction

PMSF Phenylmethylsulfonyl fluoride

RBP: RNA-binding protein

RGCs: Radial glial cells

RT-qPCR: Real-time quantitative PCR

RIP: RNA immunoprecipitation

RIP-seq: RNA immunoprecipitation-sequencing

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFARI: Simons Foundation Autism Research Initiative

TBST: Tris-buffered saline, 0.1% Tween 20 for Western blot; Tris-buffered saline, 0.1% Triton X for immunohistochemistry

VZ: Ventricular zone

WT: Wild type

Declarations

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Authors contribution

C.C., T.K., H.I., Y.S., N.O conceptualized this study and designed the experiments. C.C., T.K., and H.I. performed the experiments and analyzed and interpreted the data. C.C. wrote the manuscript draft and T.K., H.I., Y.S., N.O. reviewed and edited the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in public databases (MGI, OMIM, and SFARI) and are included in this published article. Additional inquiries can be directed to the corresponding author.

Ethics approval and consent to participate

The use of mice and all experimental procedures were reviewed and approved by the Committee for Animal Experimentation of Tohoku University Graduate School of Medicine (2017-MDA-189) and the Animal care and Use Committee of Yokohama City University (TA-16-006).

Consent for publication

Not applicable

Competing interest

The authors declare no competing or financial interests.

References

1. Agirman G, Broix L, Nguyen L. Cerebral cortex development : An outside-in perspective. FEBS Lett. 2017;591:3978–92.
2. Uzquiano A, Gladwyn-Ng I, Nguyen L, Reiner O, Götz M, Matsuzaki F, et al. Cortical progenitor biology: key features mediating proliferation versus differentiation. J Neurochem. 2018;146:500–25.
3. Homberg JR, Kyzar EJ, Scattoni ML, Norton WH, Pittman J, Gaikwad S, et al. Genetic and environmental modulation of neurodevelopmental disorders: Translational insights from labs to beds. Brain Res Bull. 2016;125:79–91.
4. Hu WF, Chahrour MH, Walsh CA. The diverse genetic landscape of neurodevelopmental disorders. Annu Rev Genomics Hum Genet. 2014;15:195–213.

5. Bakker CE, Verheij C, Willemsen R, van der Helm R, Oerlems F, Vermey M, et al. Fmr1 knockout mice: A model to study fragile X mental retardation. Cell. 1994;78:23–33.
6. Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DPA, Pizzuti A, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell. 1991;65:905–14.
7. Abrahams BS, Geschwind DH. Advances in autism genetics: On the threshold of a new neurobiology. Nat Rev Genet. 2008;9:341–55.
8. Garber K, Visootsaka J, Warren S. Fragile X syndrome. Eur J Hum Genet. 2008;16:666–72.
9. Dahlhaus R. Of men and mice: Modeling the fragile X syndrome. Front Mol Neurosci. 2018;11 March:1–38.
10. Darnell JC, Van Driesche SJ, Zhang C, Hung KYS, Mele A, Fraser CE, et al. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell. 2011;146:247–61.
11. Dictenberg JB, Swanger SA, Antar LN, Singer RH, Bassell GJ. A direct role for FMRP in activity-dependent dendritic mRNA transport links filopodial-spine morphogenesis to fragile X syndrome. Dev Cell. 2008;14:926–39.
12. Otero Y, Severijnen L-A, van Cappellen G, Schrier M, Oostra B, Willemsen R. Transport of fragile X mental retardation protein via granules in neurites of PC12 cells. Mol Cell Biol. 2002;22:8332–41.
13. Soden ME, Chen L. Fragile X protein FMRP is required for homeostatic plasticity and regulation of synaptic strength by retinoic acid. J Neurosci. 2010;30:16910–21.
14. Sidorov MS, Auerbach BD, Bear MF. Fragile X mental retardation protein and synaptic plasticity. Mol Brain. 2013;6:1–11.
15. Sears JC, Broadie K. Fragile X mental retardation protein regulates activity-dependent membrane trafficking and trans-synaptic signaling mediating synaptic remodeling. Front Mol Neurosci. 2018;10 January:1–15.
16. Bassell GJ, Warren ST. Fragile X syndrome: Loss of local mRNA regulation alters synaptic development and function. Neuron. 2008;60:201–14.
17. Pilaz LJ, Lennox AL, Rouanet JP, Silver DL. Dynamic mRNA transport and local translation in radial glial progenitors of the developing brain. Curr Biol. 2016;26:3383–92.
18. Saffary R, Xie Z. FMRP regulates the transition from radial glial cells to intermediate progenitor cells during neocortical development. J Neurosci. 2011;31:1427–39.
19. La Fata G, Gärtner A, Averna M, Poorthuis RB, Achsel T, Bagni C, et al. FMRP regulates multipolar to bipolar transition affecting neuronal migration and cortical circuitry. Nat Neurosci. 2014;17:1693–700.
20. Brown V, Jin P, Ceman S, Darnell JC, O'Donnell WT, Tenenbaum SA, et al. Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell. 2001;107:27710.

21. Ascano M, Mukherjee N, Bandaru P, Miller JB, Nusbaum JD, Corcoran DL, et al. FMRP targets distinct mRNA sequence elements to regulate protein expression. Nature. 2012;492:382–6.
22. Liu B, Li Y, Stackpole EE, Novak A, Gao Y, Zhao Y, et al. Regulatory discrimination of mRNAs by FMRP controls mouse adult neural stem cell differentiation. Proc Natl Acad Sci. 2018;115:E11397–405.
23. Miyashiro KY, Beckel-Mitchener A, Purk TP, Becker KG, Barret T, Liu L, et al. RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. Neuron. 2003;37:417–31.
24. Maurin T, Lebrigand K, Castagnola S, Jarjat M, Popa A, Grossi M, et al. HITS-CLIP in various brain areas reveals new targets and new modalities of RNA binding by fragile X mental. Nucl Acids Res. 2018;46:6344–55.
25. Smith CL, Blake JA, Kadin JA, Richardson JE, Bult CJ. Mouse Genome Database (MGD)-2018: Knowledgebase for the laboratory mouse. Nucleic Acids Res. 2018;46:D836–42.
26. Amberger JS, Bocchini CA, Scott AF, Hamosh A. OMIM.org: Leveraging knowledge across phenotype-gene relationships. Nucleic Acids Res. 2019;47:D1038–43.
27. Bailey DB, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with FMR1 gene variations: Findings from a national parent survey. Am J Med Genet Part A. 2008;146:2060–9.
28. Abrahams BS, Arking DE, Campbell DB, Mefford HC, Morrow EM, Weiss LA, et al. SFARI Gene 2.0: a community-driven knowledgebase for the autism spectrum disorders (ASDs). Mol Autism. 2013;:36.
29. Davis LK, Meyer KJ, Rudd DS, Librant AL, Epping EA, Sheffield VC, et al. Pax6 3' deletion results in aniridia, autism and mental retardation. Hum Genet. 2008;123:371–8.
30. Maekawa M, Iwayama Y, Nakamura K, Sato M, Toyota T, Ohnishi T, et al. A novel missense mutation (Leu46Val) of PAX6 found in an autistic patient. Neurosci Lett. 2009;462:267–71.
31. Kennedy J, Goudie D, Blair E, Chandler K, Joss S, McKay V, et al. KAT6A Syndrome: genotype–phenotype correlation in 76 patients with pathogenic KAT6A variants. Genet Med. 2019;21:850–60.
32. Mroske C, Rasmussen K, Shinde DN, Huether R, Powis Z, Lu HM, et al. Germline activating MTOR mutation arising through gonadal mosaicism in two brothers with megalencephaly and neurodevelopmental abnormalities. BMC Med Genet. 2015;16:1–11.
33. Lancaster MA, Gopal DJ, Kim J, Saleem SN, Silhavy JL, Louie CM, et al. Defective Wnt-dependent cerebellar midline fusion in a mouse model of Joubert syndrome. Nat Med. 2011;17:726–31.
34. Homan CC, Kumar R, Nguyen LS, Haan E, Raymond FL, Abidi F, et al. Mutations in USP9X are associated with x-linked intellectual disability and disrupt neuronal cell migration and growth. Am J Hum Genet. 2014;94:470–8. doi:10.1016/j.ajhg.2014.02.004.
35. Hegedus B, Dasgupta B, Shin JE, Emnett RJ, Hart-Mahon EK, Elghazi L, et al. Neurofibromatosis-1 regulates neuronal and glial cell differentiation from neuroglial progenitors in vivo by both cAMP- and ras-dependent mechanisms. Cell Stem Cell. 2007;1:443–57.
36. Choi SH, Estarás C, Moresco JJ, Yates JR, Jones KA. α-Catenin interacts with APC to regulate β-catenin proteolysis and transcriptional repression of Wnt target genes. Genes Dev. 2013;27:2473–88.

37. Herrera F, Chen Q, Fischer WH, Maher P, Schubert DR. Synaptojanin-1 plays a key role in astrogliogenesis: Possible relevance for Down's syndrome. Cell Death Differ. 2009;16:910–20.
38. Kania E, Roest G, Vervliet T, Parys JB, Bultynck G. IP3 receptor-mediated calcium signaling and its role in autophagy in cancer. Front Oncol. 2017;7 JUL:1–15.
39. Son SM, Park SJ, Lee H, Siddiqi F, Lee JE, Menzies FM, et al. Leucine Signals to mTORC1 via Its Metabolite Acetyl-Coenzyme A. Cell Metab. 2019;29:192-201.e7. doi:10.1016/j.cmet.2018.08.013.
40. Sharma A, Hoeffer CA, Takayasu Y, Miyawaki T, McBride SM, Klann E, et al. Dysregulation of mTOR Signaling in Fragile X Syndrome. J Neurosci. 2010;30:694–702. doi:10.1523/JNEUROSCI.3696-09.2010.
41. Crawley JN, Heyer WD, LaSalle JM. Autism and Cancer Share Risk Genes, Pathways, and Drug Targets. Trends Genet. 2016;32:139–46.
42. Bridges CR, Tan MC, Premarathne S, Nanayakkara D, Bellette B, Zencak D, et al. USP9X deubiquitylating enzyme maintains RAPTOR protein levels, mTORC1 signalling and proliferation in neural progenitors. Sci Rep. 2017;7:1–15.
43. Ehyai S, Miyake T, Williams D, Vinayak J, Bayfield MA, McDermott JC. FMRP recruitment of β-catenin to the translation pre-initiation complex represses translation. EMBO Rep. 2018;19:1–17.
44. Banerjee S, Crouse NR, Emnett RJ, Gianino SM, Gutmann DH. Neurofibromatosis-1 regulates mTOR-mediated astrocyte growth and glioma formation in a TSC/Rheb-independent manner. Proc Natl Acad Sci U S A. 2011;108:15996–6001.
45. Greenblatt EJ, Spradling AC. Fragile X mental retardation 1 gene enhances the translation of large autism-related proteins. Science (80-). 2018;361:709–12.
46. Hirabayashi Y, Itoh Y, Tabata H, Nakajima K, Akiyama T, Masuyama N, et al. The Wnt/β-catenin pathway directs neuronal differentiation of cortical neural precursor cells. Development. 2004;131:2791–801.
47. Qu Q, Sun G, Murai K, Ye P, Li W, Asuelime G, et al. Wnt7a Regulates Multiple Steps of Neurogenesis. Mol Cell Biol. 2013;33:2551–9.
48. Viti J, Gulacsi A, Lillien L. Wnt regulation of progenitor maturation in the cortex depends on Shh or fibroblast growth factor 2. J Neurosci. 2003;23:5919–27.
49. Luo Y, Shan G, Guo W, Smrt RD, Johnson EB, Li X, et al. Fragile X mental retardation protein regulates proliferation and differentiation of adult neural stem/progenitor cells. PLoS Genet. 2010;6.
50. Telias M, Mayshar Y, Amit A, Ben-Yosef D. Molecular mechanisms regulating impaired neurogenesis of Fragile X syndrome human embryonic stem cells. Stem Cells Dev. 2015;24:2353–65.
51. Winden KD, Ebrahimi-Fakhari D, Sahin M. Abnormal mTOR Activation in Autism. Annu Rev Neurosci. 2018;41:1–23.
52. Costa-Mattioli M, Monteggia LM. mTOR complexes in neurodevelopmental and neuropsychiatric disorders. Nat Neurosci. 2013;16:1537–43.

53. Easley IV CA, Ben-Yehudah A, Redinger CJ, Oliver SL, Varum ST, Eisinger VM, et al. mTOR-mediated activation of p70 S6K induces differentiation of pluripotent human embryonic stem cells. Cell Reprogram. 2010;3:263–73.
54. Licausi F, Hartman NW. Role of mTOR complexes in neurogenesis. Int J Mol Sci. 2018;19:1–16.
55. Subramanian L, Calcagnotto ME, Paredes MF. Cortical Malformations: Lessons in Human Brain Development. Front Cell Neurosci. 2020;13 January:1–17.
56. Castrén M, Tervonen T, Kärkkäinen V, Heinonen S, Castrén E, Larsson K, et al. Altered differentiation of neural stem cells in fragile X syndrome. Proc Natl Acad Sci U S A. 2005;102:17834–9.
57. Korb E, Herre M, Zucker-Scharff I, Gresack J, Allis CD, Darnell RB. Excess translation of epigenetic regulators contributes to fragile X syndrome and Is alleviated by Brd4 inhibition. Cell. 2017;170:1209-1223.e20.
58. Kikkawa T, Obayashi T, Takahashi M, Fukuzaki-Dohi U, Numayama-Tsuruta K, Osumi N. Dmrta1 regulates proneural gene expression downstream of Pax6 in the mammalian telencephalon. Genes to Cells. 2013;18:636–49.
59. Kikkawa T, Sakayori N, Yuuki H, Katsuyama Y, Matsuzaki F, Konno D, et al. Dmrt genes participate in the development of Cajal-Retzius cells derived from the cortical hem in the telencephalon. Dev Dyn. 2020;249:698–710.
60. Sato T, Muroyama Y, Saito T. Inducible gene expression in postmitotic neurons by an in vivo electroporation-based tetracycline system. J Neurosci Methods. 2013;214:170–6.
61. Saito T, Nakatsuji N. Efficient Gene Transfer into the Embryonic Mouse Brain Using in Vivo Electroporation. Dev Biol. 2001;240:237–46.
62. Xia J, Gill EE, Hancock REW. NetworkAnalyst for statistical , visual and network-based meta-analysis of gene expression data. Nat Protoc. 2015;10:823–44.
63. Spandidos A, Wang X, Wang H, Seed B. PrimerBank: A resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res. 2009;38:792–9.
64. Hawes JJ, Tuskan RG, Reilly KM. Nf1 expression is dependent on strain background: implications for tumor suppressor haploinsufficiency studies. Neurogenetics. 2007;8:121–30.

Figures

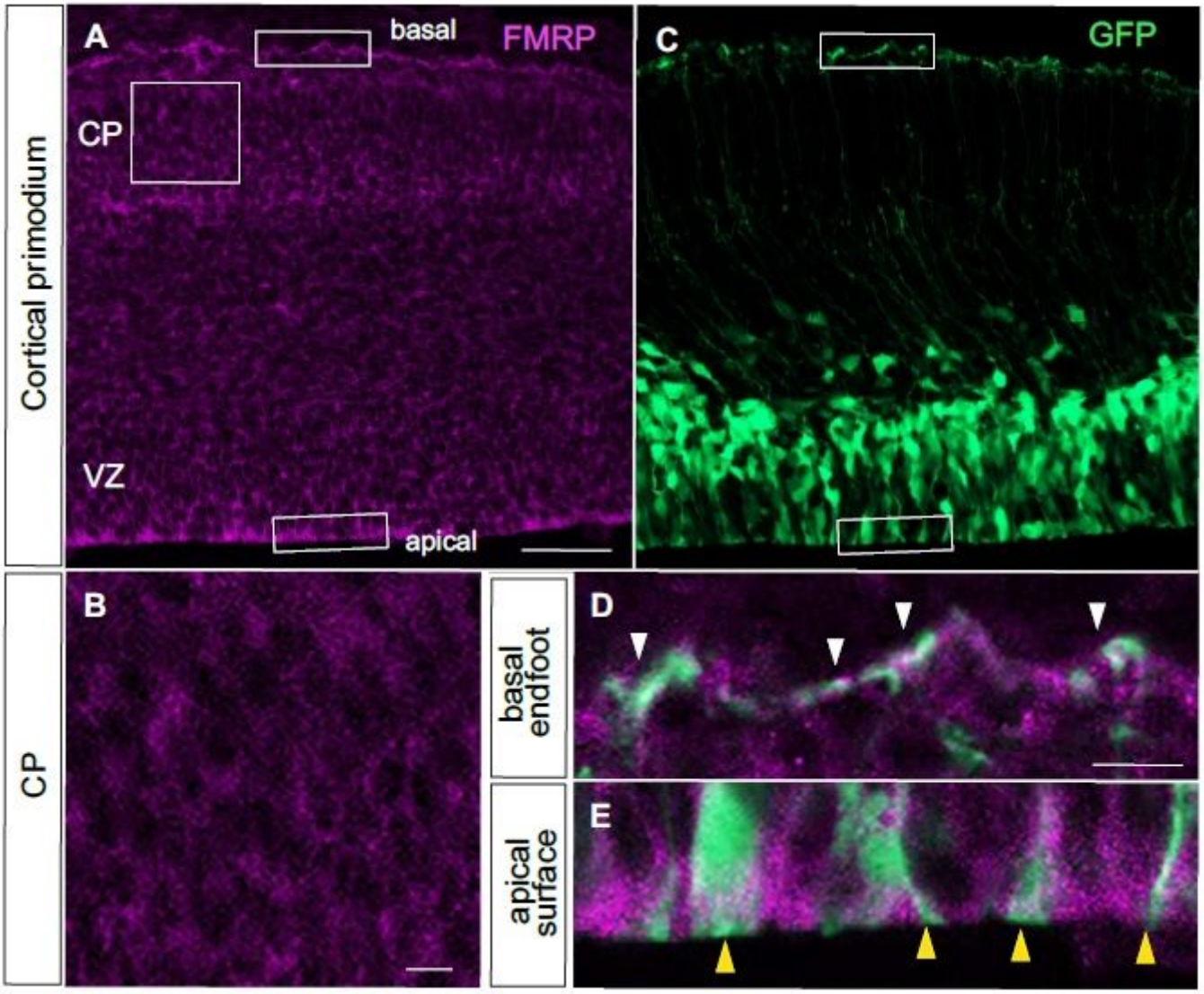

Figure 1

FMRP expression pattern in the coronal sections of the E14.5 WT mouse neocortex. (A, B) FMRP is expressed throughout the cortical primordium, showing the highest accumulation at both basal and apical endfeet of the RGCs. (C) Green fluorescent signal shows GFP-labeled RGCs. (D, E) Merged images showed that FMRP is highly localized in the basal (white arrowheads) and apical (yellow arrowheads) endfeet of the RGCs. CP, cortical plate: VZ, ventricular zone. Scale bars: 50 μm, 10 μm (inset).

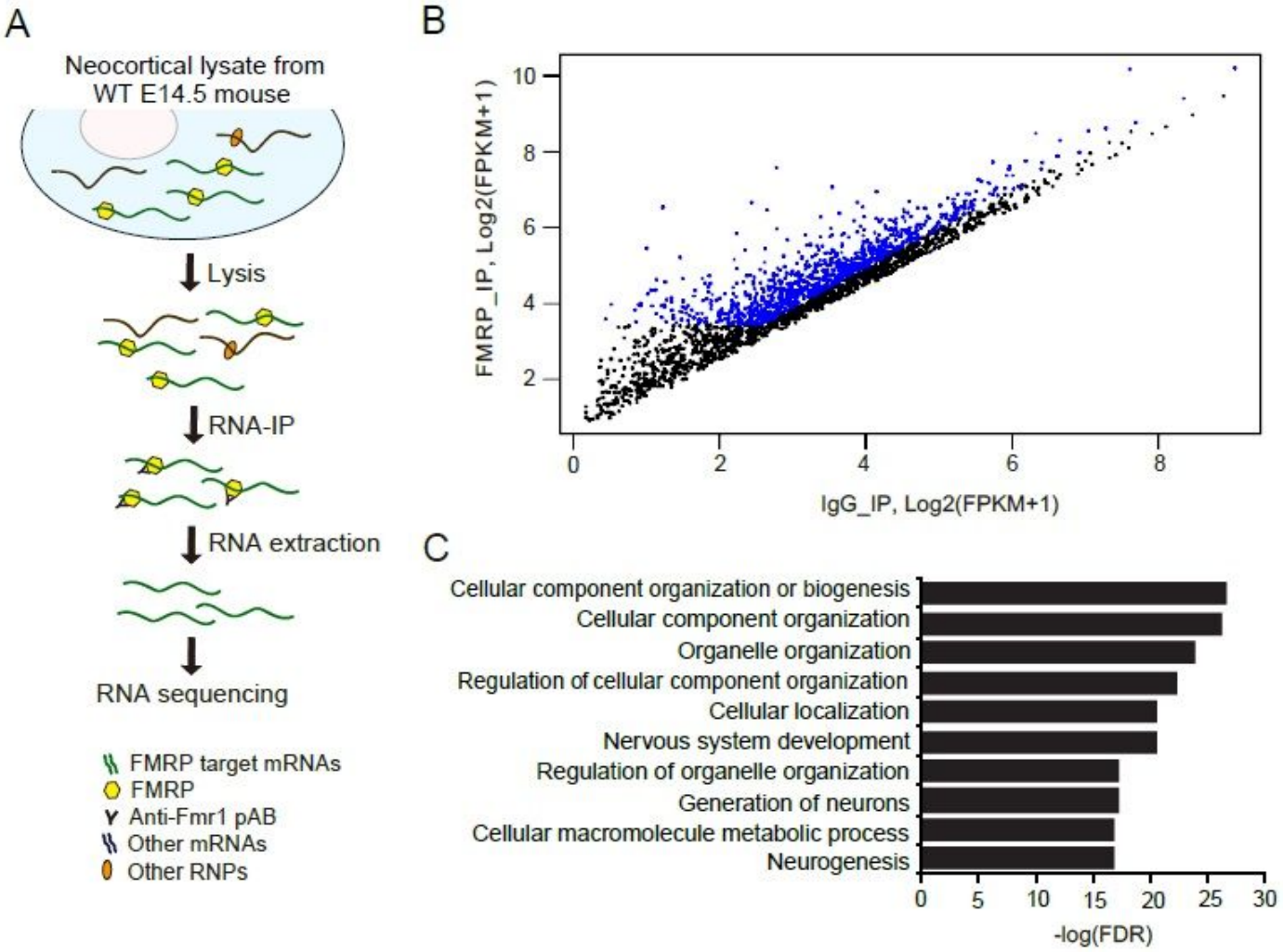

Figure 2

FMRP mRNA targets identified from the RIP-seq of the WT E14.5 cortex. (A) RIP-seq workflow. (B) Scatterplot comparing log2 ratios of significant (q<0.01) FPKM expressions values of FMRP-IP and IgG-IP. The vertical coordinates represent the log2-FPKM+1 values for each gene from FMRP-IP, and the horizontal coordinates represent the log2-FPKM+1 values for each gene IgG-IP. Highlighted in blue are the 865 FMRP target genes selected based on gene expression values, log2FC>1, and FPKM>10 for the FMRP-IP. (C) The top 10 GO (biological process) terms enriched in the 865 FMRP target genes.

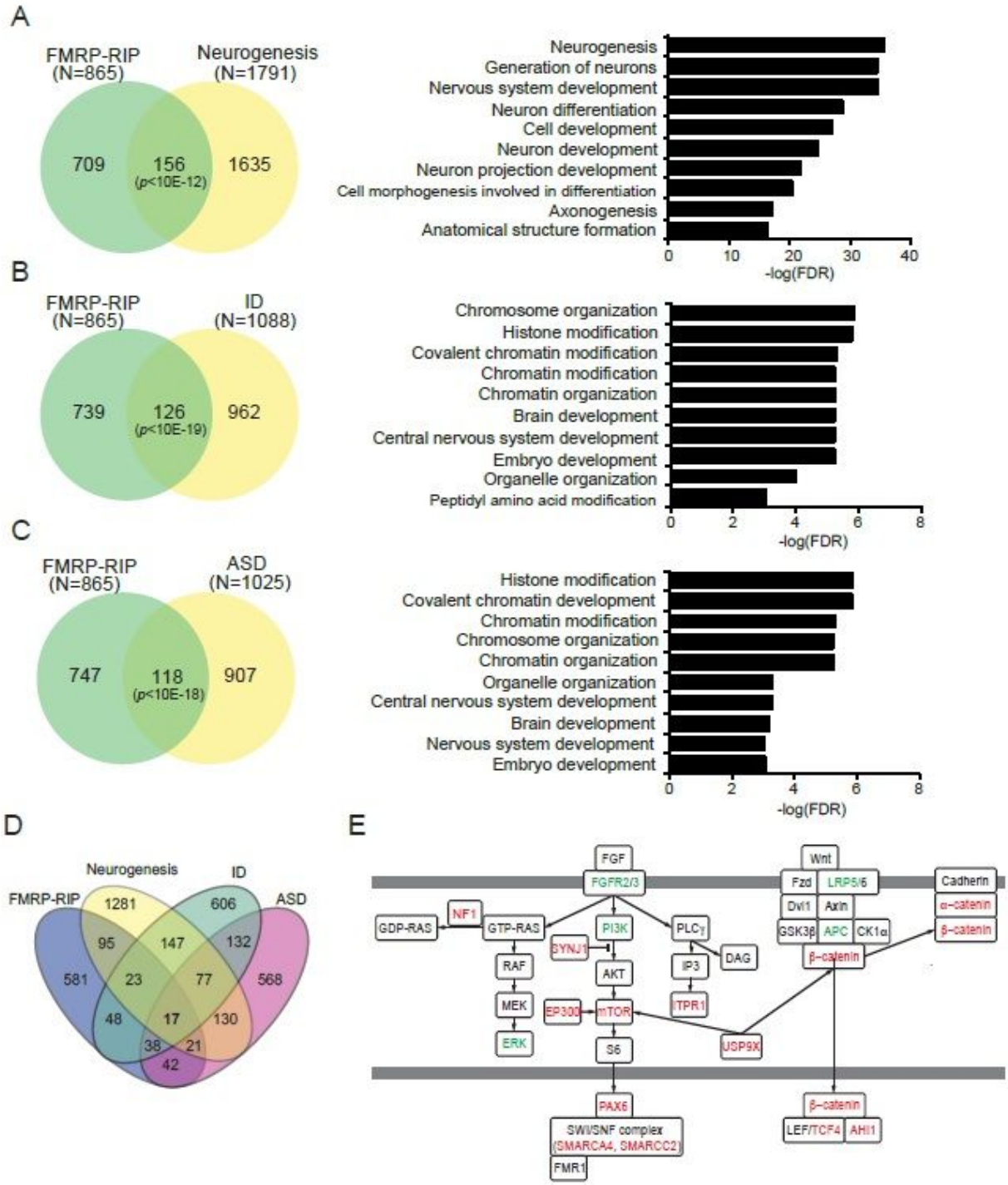

Figure 3

The overlap between our FMRP target genes and genes associated with neurogenesis, ID, and ASD and their involvement in signaling pathways. (A) Venn diagram depicting the overlap of our FMRP target genes and neurogenesis genes retrieved from the MGI (GO: neurogenesis) and its top 10 GO. (B) Venn diagram depicting the overlap of our FMRP target genes and ID-associated genes retrieved from the OMIM (keyword: ID) and its top 10 GO. (C) Venn diagram depicting the overlap of our FMRP target genes

and ASD-associated genes retrieved from the SFARI database. Statistical significance was determined by hypergeometric distribution analysis. (D) The 17 core FMRP target genes common in the four gene sets FMRP-IP, neurogenesis, ID, and ASD. (E) Signaling pathways in which the FMRP target genes were involved. The highlighted molecules were the identified “core” FMRP targets (red) and other FMRP targets in our data (green), which were involved in Ras/MAPK, Wnt/β-catenin, and mTOR signaling pathways.

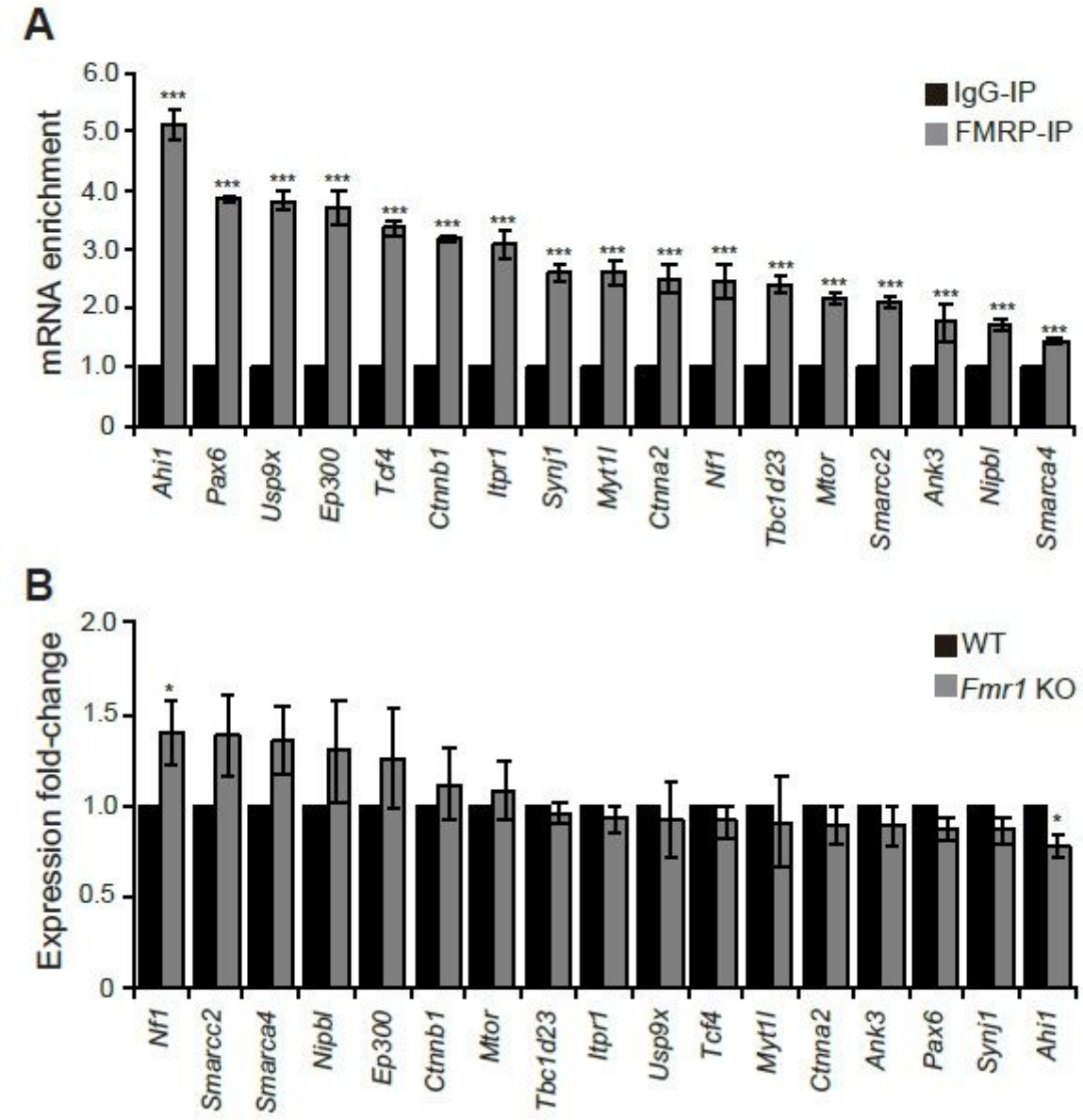

Figure 4

Validation of the 17 “core” FMRP targets by RIP-qPCR and their expression in the WT and Fmr1 KO mice. (A)The RNAs from WT (n=3) of the E15.5 mouse dorsal telencephalons were isolated and subjected to cDNA synthesis and RT-qPCR. All 17 FMRP “core” FMRP targets were significantly enriched in the FMRP-IP compared with that of IgG-IP (n=3). (B) The RNAs (n=7 WT; n=7 Fmr1 KO) from the E15.5 mouse

dorsal telencephalon were isolated and subjected to cDNA synthesis and RT-qPCR. Nf1 and Ahi1showed an increase and decrease in expression, respectively, in Fmr1 KO neocortex, compared to the WT. The Student's t-test was used to test statistical significance (*p<0.05, ***p<0.0001). Error bars represent the standard error of the mean (SEM).

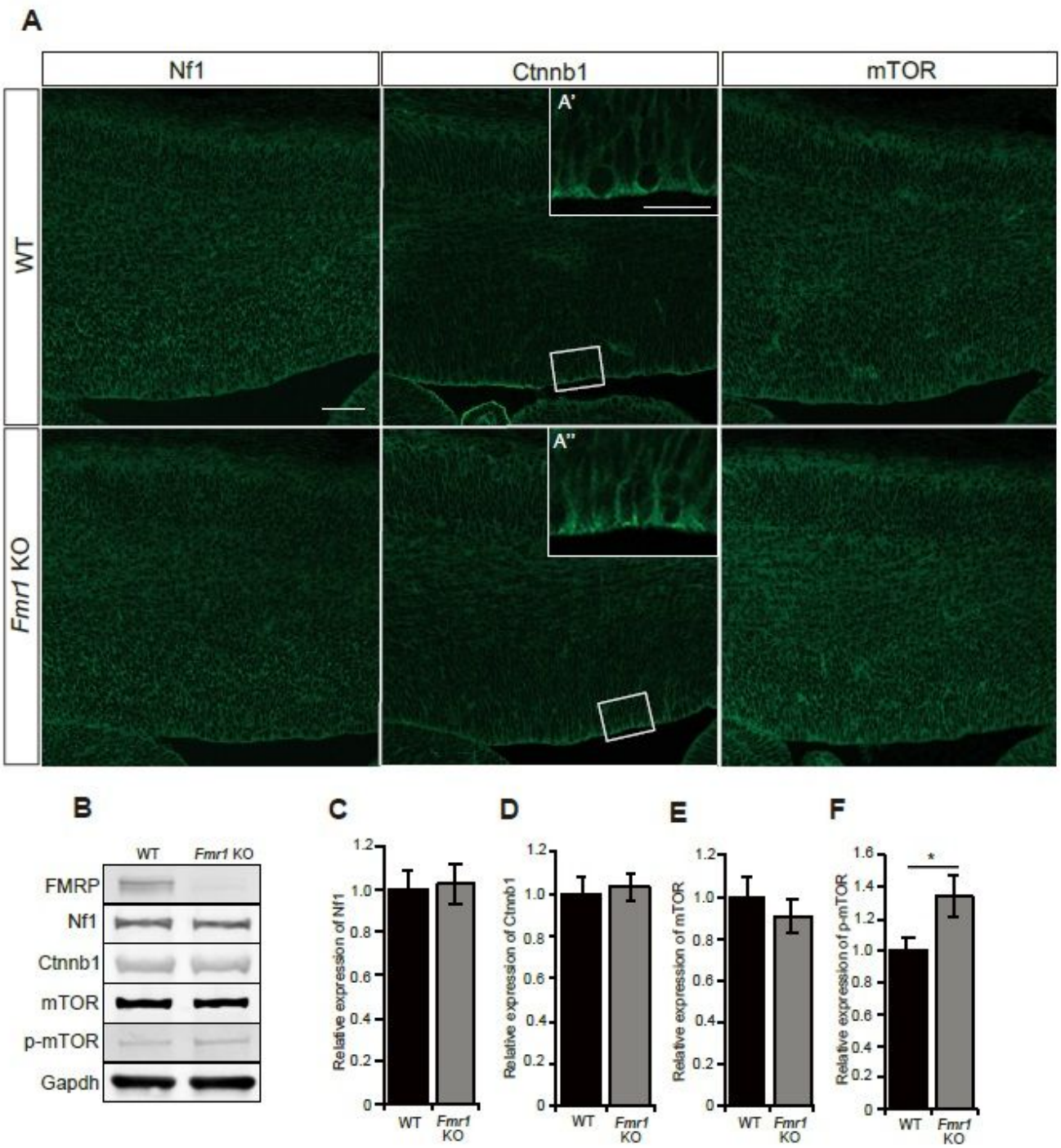

Figure 5

Nf1, Ctnnb1 and mTOR expression in mouse neocortical lysates. (A) Expression pattern of Nf1 (n=5 WT; n=5 Fmr1 KO), Ctnnb1 (n=6 WT; n=6 Fmr1 KO) and mTOR (n=5 WT; n=5 Fmr1 KO) in E15.5 neocortex

showed no difference between the WT and Fmr1 KO mice. (B) Representative western blots of selected FMRP target genes. (C-F) Relative expression of Nf1 (n=12 WT; n=12 Fmr1 KO), Ctnnb1 (n=7 WT; n=7 Fmr1 KO), mTOR (n=12 WT; n=12 Fmr1 KO) and p-mTOR (n=10 WT; n=10 Fmr1 KO) were measured using ImageJ software. The findings indicate no difference in Nf1, Ctnnb1 and mTOR expression between WT and Fmr1 KO neocortices. However, p-mTOR at Ser2448 showed a 25.4% increase in protein level in the Fmr1 KO. Scale bars: 50 μm, 20 μm (inset). Student's t-test was used to test statistical significance (*$p<0.05$). Error bars represents the SEM.

Supplementary Files

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- TableS1.xlsx
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- TableS3.xlsx