A human forebrain organoid model of fragile X syndrome exhibits altered neurogenesis and highlights new treatment strategies

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Fragile X syndrome (FXS) is caused by the loss of fragile X mental retardation protein (FMRP), an RNA-binding protein that can regulate the translation of specific mRNAs. In this study, we developed an FXS human forebrain organoid model and observed that the loss of FMRP led to dysregulated neurogenesis, neuronal maturation and neuronal excitability. Bulk and single-cell gene expression analyses of FXS forebrain organoids revealed that the loss of FMRP altered gene expression in a cell-type-specific manner. The developmental deficits in FXS forebrain organoids could be rescued by inhibiting the phosphoinositide 3-kinase pathway but not the metabotropic glutamate pathway disrupted in the FXS mouse model. We identified a large number of human-specific mRNAs bound by FMRP. One of these human-specific FMRP targets, CHD2, contributed to the altered gene expression in FXS organoids. Collectively, our study revealed molecular, cellular and electrophysiological abnormalities associated with the loss of FMRP during human brain development.

FXS (OMIM #300624) is the most common inherited form of intellectual disability and a leading genetic cause of autism spectrum disorder (ASD). CGG trinucleotide repeat expansion within the 5′-untranslated region of the FMR1 gene has been identified as the most prevalent cause of FXS. This CGG expansion beyond 200 repeats, referred to as the full mutation, leads to hypermethylation of the FMR1 promoter, thereby preventing FMR1 expression. FXS is caused by the loss of functional FMRP.

Over the course of nearly three decades of research, a great deal has been learned about the function of FMRP and the consequence of its absence in FXS, primarily using mouse and fruit fly model systems. FMRP is a selective RNA-binding protein associated with translating polyribosomes that appears to be involved in the regulation of local protein synthesis at synapses. The loss of FMRP leads to abnormal translation of selective mRNAs, many of which are autism-linked genes. Substantial progress in characterizing the underlying disease mechanisms in animal models has led to highly successful preclinical studies of drugs modulating metabotropic glutamate and GABA receptors. However, follow-up clinical trials in humans have been largely unsuccessful, suggesting that mouse models might not fully recapitulate human disease, and highlighting the need for human cell-based models.

Recent technical advances have enabled longer neuroepithelium formation and support growth of large, complex three-dimensional (3D) organoids to recapitulate key features of human brain development, such as progenitor zone organization, neurogenesis, gene expression profile and, notably, a distinct human-specific outer radial glia cell layer. Thus, the human brain organoid model provides a new platform to investigate human brain development in a dish, otherwise inaccessible to experimentation.

In this study, we developed FXS forebrain organoids from patient-derived induced pluripotent stem cells (iPSCs) and observed reduced proliferation of neural progenitor cells, dysregulated neural differentiation, increased synapse formation and neuronal hyperexcitability and a deficit in the production of GABAergic neurons. Bulk and single-cell transcriptome analyses revealed that loss of FMRP could lead to pervasive gene expression alterations in human brain development and an altered developmental trajectory of the forebrain organoids in a cell-type-specific manner. Furthermore, mechanism-guided pharmacological inhibition of the phosphoinositide 3-kinase (PI3K) pathway, but not the mGluR5 pathway that was previously identified in mouse models, rescued the defects in neurodevelopment and synapse formation of FXS forebrain organoids. To identify the mRNAs bound by FMRP, we performed enhanced crosslinking and FMRP immunoprecipitation followed by high-throughput sequencing using human forebrain organoids and mouse embryonic forebrain at similar developmental stages. Our comparative analyses identified a large number of mRNAs that were bound by FMRP in a human-specific manner. Furthermore, many FMRP mRNA targets involved in neurogenesis...
and synaptic plasticity were found to be upregulated at the protein level. We showed that one of the human-specific FMRP targets, chromodomain helicase DNA-binding protein 2 (CHD2), contributes to the altered gene expression in FXS organoids. Our analyses revealed molecular, cellular and electrophysiological abnormalities associated with the loss of FMRP during human brain development. Our study identified human-specific mRNA targets of FMRP which have the potential to serve as human-specific druggable targets for FXS and autism in general.

Results

FMRP regulates neurogenesis and neuronal differentiation in human forebrain organoids. To model FXS and study the consequence of FMRP deficiency in the context of neurodevelopment, we generated iPSC lines from dermal fibroblasts of three male patients with FXS and three age- and sex-matched healthy and unaffected controls (Supplementary Table 1). We also used the previously generated isogenic correction line by excising the expanded CGG-repeat in one of the FXS iPSC lines (FX51) through CRISPR–Cas9 gene editing’ (Supplementary Table 1). All iPSC lines exhibited normal karyotypes (Supplementary Table 1) and were positive for pluripotency markers Tra-1–60 and NANOG (Supplementary Fig. 1a). Transcriptome analysis demonstrated that these iPSC lines express pluripotency-associated genes, including NANOG, KLF4, OCT4, SOX1, SOX2, DPPA3 and PODOXL, at similar levels, further confirming their pluripotency (Supplementary Fig. 1d).

To determine whether loss of FMRP dysregulates human cortical development, we derived 3D forebrain organoids from the iPSCs using a recently established protocol with miniature bioreactors’ (Supplementary Fig. 1f). We focused on Day 56 (D56) forebrain organoids, which strongly correlate with fetal human brain development during the mid-fetal period, a critical period for human cortical neurogenesis and FMRP depletion in patients with FXS. We confirmed that FMRP was absent in iPSCs, embryoid bodies (EBs) and D28 and D56 forebrain organoids derived from FXS patient lines (Supplementary Fig. 1c).

To examine the effect of FMRP loss on neural progenitor cell (NPC) proliferation, we co-immunostained Ki67, a proliferation marker, with NPC markers SOX2 or PAX6 (refs. 52) (Fig. 1a and Extended Data Fig. 1b). Although the loss of FMRP did not affect the overall size of forebrain organoids in general (Extended Data Fig. 1a), we found a significant reduction of Ki67+ cells among all SOX2+ or PAX6+ cells (Fig. 1a,b and Extended Data Fig. 1b,c), indicating a reduction in NPC proliferation at Day 56. In addition, we pulsed D56 forebrain organoids with 5-ethyl-2′-deoxyuridine (EdU; 10 μM) for 2h to label proliferating cells in S phase (Extended Data Fig. 1d). Quantitative analysis showed that loss of FMRP in FXS forebrain organoids led to reductions in the percentage of EdU+ cells among all SOX2+ or PAX6+ cells (Fig. 1a,b and Extended Data Fig. 1b,c), indicating a reduction in NPC proliferation at Day 56. We also noticed that significantly more SOX2+ or PAX6+ NPCs detached from the apical VZ (SOX2+ or PAX6+ layer) in FXS organoids (Extended Data Fig. 1b,c), further suggesting an altered neural differentiation caused by loss of FMRP, as well as impaired cortical plate (CP)-like layering in FXS organoids (Fig. 2a,b and Extended Data Fig. 2b,c), further suggesting an altered neural differentiation caused by loss of FMRP, Doublecortin (DCX), a marker of neuronal precursor and immature neurons, is involved in neuronal migration via stabilizing microtubules53. The DCX expression is abnormally stronger in PAX6+ NPCs in FXS organoids compared to controls (Fig. 2c,d), further confirming the dysregulated differentiation of FXS forebrain organoids.

To further assess the neuronal differentiation, we divided the entire span of the neuroepithelium into five equal portions (bins) and measured the distributions of specific cell types in each bin as described previously54. We observed that the distribution of CTIP2+ or TBR1+ cortical neurons was altered in the neuroepithelium of FXS organoids (Fig. 2e,f), suggesting dysregulated neuronal differentiation and cortical layer formation in FXS organoids. Interestingly, we also noticed that significantly more SOX2+ or PAX6+ NPCs detached from the apical VZ (SOX2+ or PAX6+) layer and were dispersed into the expanded neuroepithelium in FXS organoids (Fig. 2b,g), further confirming that the structure of FXS organoids is less organized. We also evaluated the neuronal differentiation by quantifying the relative thickness of the VZ (SOX2+ or SOX2+ layer) and CP (CTIP2+ or MAP2+ or TBR1+ layer) between apical and basal surfaces at D56 (Extended Data Fig. 3a–f), likely due to the dysregulated differentiation of FXS NPCs. In addition, the percentages of CTIP2+ or TBR1+ cortical neurons in total cells were significantly increased (Extended Data Figs. 2a and 3g). We also analyzed the distribution of the SOX2+, PAX6+, CTIP2+ and TBR1+ cell populations in D56 forebrain organoids (Extended Data Fig. 3i–l). The distributions of SOX2 and PAX6 were significantly reduced in the VZ layer and increased in the CP layer of the FXS organoids, indicating the dysregulated neuronal differentiation caused by the loss of FMRP. We further assessed the expression of marker genes for each cortical layer (l-RELN, I-CUX1, III-POU3F2, IV:SATB2, V:BC111B (also known as CTIP2) and VI:TBR1). The markers for deeper layers (POU3F2, SATB2 and BC111B) were increased in FXS (Supplementary Table 1), suggesting a change in layer predominance toward deeper layers in FXS organoids. To confirm the neuronal fates after differentiation, we quantified the proportion of glutamatergic and GABAergic neurons by co-immunostaining with CaMKIIα, indicative of glutamatergic neuronal differentiation, and the GABAergic interneuron marker GABA (Fig. 2i). Intriguingly, we found that the percentage and number of GABAergic neurons were dramatically decreased in FXS organoids compared to the controls (Fig. 2k and Extended Data Fig. 4a,b), indicating a reduction of GABAergic neuronal fate. Furthermore, RNA in situ hybridization for markers of ganglionic eminence (GE) NPCs (DLX2), dorsal telencephalic NPCs (PAX6) and pan-NPCs (SOX2) revealed a
reduction of DLX2+ ventral NPCs, which give rise to GABAergic interneurons, in both D28 and D56 FXS organoids (Extended Data Fig. 4c,d). This suggests that the loss of FMRP might deplete the pool of GE NPCs at an early stage, which further leads to reduction of GABAergic neuronal fate.

Loss of FMRP increases synapse formation and enhances neuronal excitability. We next characterized the synapse formation and neuronal function in FXS forebrain organoids. To investigate the effect of FMRP loss on synapse formation, we quantified the density of synaptic puncta by immunocytochemistry of presynaptic vesicle protein SYNAPSIN1 (SYN1), postsynaptic protein PSD95 and dendrite marker MAP2 (Fig. 3a). The density of SYN1+PSD95+ synaptic boutons was significantly increased in FXS neurons compared to

Fig. 1 | FMRP regulates cortical neurogenesis in a human forebrain organoid model. a,b, Loss of FMRP reduces NPC proliferation. Shown are representative images (a) and stereological quantification (b) of the proportion of Ki67+ proliferating NPCs in both control and FXS-derived forebrain organoids at Day 56. Yellow dashed lines indicate the borders of VZ-like structures. Data are presented as mean ± s.e.m. (n=10 organoids from each line with 15–20 cortical structures analyzed per organoid; ***P=0.0002 (FXS2 versus CTRL1) or 0.0001 (FXS3 versus CTRL1), ****P<0.0001 (FXS1 versus CTRL1), one-way ANOVA). Scale bars, 50 μm. c,d, FMRP deficiency accelerates NPC cell cycle exit during the 24-h EdU exposure in FXS forebrain organoids. Shown are representative images (c) and quantification (d) of the proportion of Ki67− NPCs in total EdU+ cells in both control and FXS-derived forebrain organoids after 24-h EdU exposure. Yellow dashed lines indicate the borders of VZ-like structures. Data are presented as mean ± s.e.m. (n=29 cortical structures from at least ten organoids each condition; ****P<0.0001, one sided Student’s t-test). Scale bars, 50 μm. e,f, Loss of FMRP accelerates neural differentiation. Day 49 control and FXS forebrain organoids were infected with retroviruses expressing GFP and analyzed 7 d later (Day 56). Shown are sample images for immunostaining for GFP, SOX2 and MAP2 (e) and quantifications of percentages of MAP2+GFP+ cells among all GFP+ cells (f). Values represent mean ± s.e.m. (n=6 organoids from control or FXS lines each with 10–12 cortical structures analyzed per organoid; ****P<0.0001; one-sided Student’s t-test). Scale bars, 50 μm.
Fig. 2 | Loss of FMRP impairs cortical neurodevelopment.  

a, b, Loss of FMRP dysregulates distribution of TBR2+ IPCs. Shown are representative images (a) and quantification (b) of the proportion of TBR2+ IPCs in CTIP2+ layer of both control and FXS-derived forebrain organoids. Yellow dashed lines indicate the borders of VZ-like structures. Data are presented as mean ± s.e.m. (n = 10 organoids from each line with 15–20 cortical structures analyzed per organoid; ***P = 0.0001, ****P < 0.0001, one-way ANOVA). Scale bars, 50 µm.  

c, d, FMRP deficiency induces premature neural differentiation. Shown are sample images for immunostaining for PAX6, DCX and MAP2 (c) and quantifications of relative expression level of DCX in PAX6+ NPCs in the VZ-like structures (d). Yellow dashed lines indicate the borders of VZ-like structures. Values represent mean ± s.e.m. (n = 10 organoids from control or FXS lines each with 15–20 cortical structures analyzed per organoid; ****P < 0.0001; one-sided Student’s t-test). Scale bars, 50 µm.  

e–i, Loss of FMRP alters cortical layer formation. Shown in e are sample images of the expanded neuroepithelium in D56 control and FXS organoids stained with SOX2 and CTIP2. Scale bars, 50 µm. Shown in f–i are quantifications of distributions of SOX2 (f), PAX6 (g), CTIP2 (h) and TBR1 (i) in the entire span of the neuroepithelium, which was divided into five equal portions (bins). Data are presented as mean ± s.e.m. (n = 10 organoids from control or FXS lines each with 15–20 cortical structures analyzed per organoid; *P = 0.0240 (h) or 0.0488 (i), **P = 0.0014 (h), ****P < 0.0001; one-way ANOVA).  

j, k, Loss of FMRP prevents differentiation of GABAergic interneurons. Shown are sample images for immunostaining for CaMKIIα, GABA and MAP2 (j) and quantification (k) of percentages of GABA+ inhibitory neurons (left) and CaMKIIα+ excitatory neurons (right) in total DAPI+ cells in both control and FXS-derived forebrain organoids. Data are presented as mean ± s.e.m. (n = 10 sections from ten organoids each line; ****P < 0.0001, one-way ANOVA). Scale bars, 50 µm.
control neurons in D56 forebrain organoids (Fig. 3b), suggesting dysregulated formation of morphological excitatory synapses in FXS neurons.

To examine the physiological properties of these FXS neurons, we performed electrophysiological whole-cell recordings in slices acutely sectioned from organoids. Only CTIP2+ cortical glutamatergic neurons were analyzed, the identity of which was confirmed by injection of Alexa Fluor 594 dye into neurons after the recording and co-immunostaining of CTIP2 (Extended Data Fig. 5a). The passive membrane properties, including the resting membrane potential, membrane capacitance and input resistance, were not altered by the loss of FMRP expression (Extended Data Fig. 5b–d). We next examined the neuronal excitability under current-clamp mode with steps of current injection. Interestingly, we observed a significant increase in action potential firing frequency (Fig. 3c), suggesting the hyperexcitability of FXS neurons. We further characterized the
Loss of FMRP leads to pervasive gene expression alterations in human brain development. Given the observed cellular and electrophysiological phenotypic changes in FXS organoids, we determined how the loss of FMRP could affect gene expression during human brain development. We performed bulk RNA sequencing (RNA-seq) using both control and FXS organoids at different developmental stages—D28, D56 and D84—and identified several differentially expressed genes (DEGs) in the absence of FMRP in a developmental stage-dependent manner (Fig. 4a, Extended Data Fig. 7 and Supplementary Table 3). The relative consistency across each line of the control and FXS organoids is shown in the heat map of DEGs at D56 showing reasonable reproducibility across the organoids within the same group (Supplementary Fig. 2). To determine whether the changes that we observed also occurred in human fetal brain, we generated RNA-seq data using two human fragile X fetal brain cortex tissues that we obtained previously, one full mutation male (23 weeks after conception) and one full mutation female (24 weeks after conception) (Supplementary Table 4). We identified 2,723 DEGs from the fetal brain data and 810, 1,194 and 3,839 DEGs in D28, D56 and D84 organoids, respectively. Comparing the RNA-seq data of FXS fetal brain tissues and forebrain organoids, we discovered an overlap between fragile X fetal brain tissue DEGs and FXS organoid DEGs in the same direction at different stages (P < 2.2 × 10−10), in particular the D84 forebrain organoids, which are estimated to be at a similar stage of human fetal brain at ~18–23 weeks after conception (Fig. 4b and Supplementary Fig. 3). Thus, our data indicate that FXS organoids could potentially mimic the developmental alterations in human fragile X fetal brain.

Given the significant number of genes with altered expression in FXS organoids, we performed similar RNA-seq analyses using mouse brain at embryonic day 13.5 (E13.5), which is a similar developmental stage to human D56 organoids. Interestingly, we observed that only three genes were differentially expressed in the absence of Fmrp in mouse, one of which is the 

$$\text{Fmr1}$$

gene, whereas we found a markedly greater number of DEGs in human forebrain organoids. Genes with false discovery rate (FDR) < 0.20 were defined as DEGs, and a volcano plot was drawn using the same criteria (Fig. 4c and Supplementary Table 5). We further examined the genes that displayed human-specific alteration in the absence of FMRP in forebrain organoids. The DEGs are associated with neurodevelopment, many of which have been implicated in neuronal migration, axonogenesis, neurogenesis, regulation of membrane potential and neuron differentiation (Fig. 4d,e and Supplementary Figs. 4 and 5). We did observe that both GE NPC markers DLX1 and DLX2 were decreased in FXS organoids (Supplementary Fig. 6), which is consistent with the reduction of GE NPCs and GABAergic neurons in FXS organoids (Fig. 2g,h and Extended Data Fig. 4). Together, these data suggest that the loss of FMRP in human causes a more pervasive gene expression alteration during brain development compared to mouse.

Loss of FMRP leads to altered developmental trajectory in forebrain organoids. Bulk RNA-seq is unable to provide detailed insights, such as the distinct cell population alteration, the presence of specific cell types and developmental variation. To more comprehensively understand the specificity and complexity of the effect of loss of FMRP on human brain development, we performed single-cell RNA sequencing (scRNA-seq) on three pairs of control and FXS forebrain organoids, including the isogenic line. After quality control and filtering, a total of 30,550 single cells were analyzed, and ontology analyses on DEGs between control and FXS revealed specific enrichment in selective pathways (Fig. 5a)19. Among these pathways, the PANTHER analyses on downregulated DEGs revealed the enrichment of the genes involved in neuronal development, such as neurogenesis, differentiation and neuron projection morphogenesis, whereas upregulated DEGs were enriched in pathways of protein translation and targeting to the membrane and oxidative phosphorylation, which is required for the function of a neuron. Uniform manifold approximation and projection (UMAP) plots were visually adopted to identify 14 clusters, which were systematically classified as representing neural lineages based on the cell-type-specific molecular signatures in combination with Gene Ontology (GO) analysis20 (Fig. 5b, Extended Data Fig. 8a–c and Supplementary Tables 6–8). After the assessment of the overlap between human fetal brain DEGs and cell-type-specific DEGs, C7 overlap was highest (Extended Data Fig. 9). The overlapped genes are highly enriched in neuron generation and development, as seen in the cellular phenotype change (Extended Data Fig. 9b and Figs. 1–3). The neural stem cell/radial glia (C3, C5 and C6) are expanded in FXS organoids, which could be the consequence of increased proliferation earlier in D28 organoids12. Cyclin D2 (CCND2), a cortical progenitor proliferation promoter and differentiation blocker21, decreased in clusters of neural stem and progenitors and inhibitory neurons in FXS organoids (Supplementary Table 9), supporting the notion of the reduced NPC proliferation in D56 FXS organoids. In FXS organoids, marker genes specific for certain developmental stage are ambiguously expressed. Some undifferentiated cells express mature markers too early, and some differentiated cells express abnormally stronger immature markers but less or no expression of some mature markers. In particular, C7 inhibitory neurons are digressing from the proper maturation.
toward functional inhibitory neurons (Fig. 5 and Extended Data Fig. 8). We found that several important molecules in GABAergic synapse development and function are decreased in FXS C7 compared to control (Supplementary Fig. 7). The gene expression profile in Seurat clusters of FXS organoids clearly suggests that they undergo distinct developmental progression from control, and the development and differentiation of FXS organoids are not properly orchestrated and are perturbed, especially in inhibitory neurons.

To investigate the developmental progression alteration caused by the loss of FMRP, we used statistical methods based on minimal spanning tree to obtain pseudotime trajectory to investigate the developmental stages of cell populations. Twenty-two pseudotime trajectory clusters were identified (Fig. 5d, left)\(^22,23\). We located branches, bypasses and break points that were distinct in the FXS trajectory (blue line) compared to the control organoid time trajectory (red line) (Fig. 5d, right). The shared path between control and
FXS is shown in black. Cells of these subpopulations with altered neuronal states are strongly enriched in FXS. The characteristic transcriptional features of each branch cluster (yellow marks in Fig. 5d, right) define the differential dynamics of gene regulation in the developmental progression along the distinguishing developmental trajectory (Extended Data Fig. 8d and Supplementary Table 10). The scRNA-seq and time trajectory analyses suggest that the loss of FMRP leads to alternative and differential developmental progression, possibly related to aberrant neuronal differentiation.

**PI3K inhibitors, not mGluR5 antagonists, rescue neurodevelopmental defects in FXS forebrain organoids.** Given the developmental and synaptic deficits that we observed in FXS forebrain organoids, we explored pharmacological approaches to rescue these phenotypes. Previously, the group 1 metabotropic glutamate receptors (mGlurRs) theory of FXS has postulated that excessive mGlur signal causes synaptic defects of FXS, affecting translation at the synapse<sup>24,25</sup>, which was supported in animal models where genetic or pharmacological inhibition of mGlur5 could rescue both behavioral and synaptic abnormalities associated with the loss of Fmrp<sup>26,27</sup>. To test whether inhibition of the mGlur5 pathway could rescue the developmental phenotypes of FXS organoids, we treated the organoids with 2-methyl-6-(phenylethynyl)-pyridine (MPEP, 10 μM), an mGlur5 antagonist, from Day 42 to Day 56. No significant differences in proportion of Ki67<sup>+</sup> NPCs (Fig. 6a,b) and SYN1<sup>+</sup>PSD95<sup>+</sup> synaptic bouton density (Fig. 6c,d) between the MPEP-treated and vehicle-treated FXS organoids were seen, suggesting that MPEP fails to rescue the developmental defects in FXS forebrain organoids. These results are consistent with recent clinical trials with several mGlur5 antagonists in humans that showed a lack of efficacy<sup>28</sup>.

In addition to MGlur5, PI3K has also drawn attention owing to its specific dysregulation in FXS. Intriguingly, previous studies reported elevated PI3K signaling in FXS models, and genetic reduction or pharmacological inhibition of PI3K has been shown to ameliorate FXS-associated phenotypes in animal models<sup>29-31</sup>. Therefore, we treated FXS forebrain organoids with a pan-PI3K inhibitor, LY294002 (10 μM), or a selective inhibitor of PI3Kβ, GSK2636771 (1 μM), from Day 42 to Day 56. Quantitative analyses showed that the 2-week treatment of LY294002 (10 μM) or GSK2636771 (1 μM) largely normalized the proportion of Ki67<sup>+</sup> NPCs (Fig. 6a,b) and SYN1<sup>+</sup>PSD95<sup>+</sup> synaptic bouton densities (Fig. 6c,d) to similar levels as seen in control. Our results support that PI3K, but not MGlur5, could be a potential therapeutic target for intervention in human.

**Identification of human FMRP mRNA targets.** Given the specific neurodevelopmental deficits associated with the loss of FMRP and more pervasive gene expression alteration in fragile X organoids compared to mouse, we performed enhanced crosslinking and immunoprecipitation followed by high-throughput sequencing (eCLIP-seq) to identify the specific mRNAs that could be bound by FMRP in human forebrain organoids. As a comparison, cerebral cortices of mouse embryonic brain E13.5 were analyzed in parallel. We conducted eCLIP-seq using an FMRP antibody that was used previously for FMRP eCLIP-seq in non-neuronal cells (Fig. 7a). For all eCLIP-seq experiments, we identified significant peaks by comparing read density between eCLIP and input, excluding PCR duplications and considering only uniquely mapped reads, as previously described<sup>32</sup>. We used a stringent peak threshold of at least eight-fold enrichment in immunoprecipitation over the input and identified the consistent peaks across the biological replicates.

Most FMRP binding sites are located in the coding sequence and introns in both human and mouse analyses (Fig. 7b and Supplementary Fig. 8). Overall, our analyses identified more than 3,700 mRNAs bound by FMRP in human organoids (Fig. 7c). Of the 3,726 mRNAs, 1,651 overlap with the mRNAs bound by Fmrp in mouse embryonic cortex. Approximately, 80% of the mRNAs bound by both human and mouse FMRP overlap with the previously identified Fmrp mRNA targets in mouse adult brain (Supplementary Table 11). GO analyses of different groups of FMRP targets revealed different terms. The mRNA targets that overlap between human and mouse are enriched for genes involved in neurodevelopment, neurogenesis and synaptic plasticity. The human-specific targets are more involved in synaptic signaling and the Wnt pathway. Intriguingly, the most enriched pathway among mouse-specific mRNA targets is glutamate receptor signaling pathway (Fig. 7d). Among 1,197 D56 DEGs, we found 170 genes overlapped with human-specific targets, whereas 78 genes overlapped with shared targets. We next assessed whether FMRP targets are enriched for neurodevelopmental disease genes (Fig. 7e). We obtained the genes associated with ASD, schizophrenia (SCZ), depression, aortic lesion and obesity and overlapped the disease-related genes with FMRP targets of both human and mouse<sup>30,32</sup>. FMRP mRNA targets, particularly those from human, are significantly enriched among ASD-associated genes (Fig. 7e and Extended Data Fig. 10). These data are consistent with previous studies showing overlap between the genes implicated in autism and FMRP mRNA targets and further expand the FMRP targets with the relevance to the developing human brain.

Given the defects of neurogenesis and synaptic plasticity associated with FXS organoids, we further examined the protein level of selective mRNA targets identified above. Indeed, most also displayed augmented protein levels, which suggests that FMRP could suppress the translation of these mRNA targets (Fig. 8a).

**CHD2 is a human-specific FMRP mRNA target.** One of the human-specific mRNA targets of FMRP is CHD2, which belongs to the CHD family, characterized by presence of chromatin organization modifier domains (chromo domain) and SNF2-related helicase/ATPase domains<sup>33</sup>. CHD2 mutations have been associated with intellectual disability and ASD<sup>34</sup>. CHD2 could play critical roles in neurogenesis and the generation of GABAergic neurons. To further validate CHD2 mRNA as a human-specific FMRP target, we performed parallel FMRP immunoprecipitation followed by qRT–PCR and confirmed that only human FMRP could bind to CHD2 mRNA (Fig. 8b). Furthermore, we examined the expression of CHD2 at both mRNA and protein levels. CHD2 mRNA levels are similar without a significant difference in either mouse embryonic...
cortex or human forebrain organoids in the presence or absence of FMRP (Fig. 8c and Supplementary Fig. 9). However, at the protein level, whereas no significant change was detected in Fmr1 knockout (KO) mouse embryonic cortex, a significant increase of CHD2 protein was observed in FXS forebrain organoids compared to unaffected control (Fig. 8d). These results suggest that human...
FMRP, but not mouse Fmrp, could regulate the expression of CHD2 post-transcriptionally. A previous study showed that Chd2 is necessary for neural circuit development and long-term memory in mice, and the DEGs in embryonic cortex of E13.5 were also identified. To determine whether the misregulation of CHD2 in FXS forebrain organoids could have any functional consequence, we compared the DEGs between FXS organoids and E13.5 Chd2+/− mice and observed significant overlap (408 of 1,004 genes were altered in both FXS organoids and E13.5 Chd2+/− mice, \( P < 2.2 \times 10^{-16} \)) (Fig. 8e and Supplementary Table. 12). Furthermore, using the available chromatin immunoprecipitation followed by sequencing data of CHD2 in human cells generated by ENCODE, we found that 255 of 408 genes could be bound by CHD2 (\( P = 2.97 \times 10^{-5} \); Fig. 8e and Supplementary Table. 12). These analyses suggest that the loss of FMRP could lead to the increased expression of CHD2 protein, which, in turn, alters the expression of its downstream targets. Thus, the identification of these human-specific FMRP mRNA targets will provide new insights into the molecular pathogenesis of FXS in a human context.

**Discussion**

In this study, we developed FXS forebrain organoids from patient-derived iPSCs and observed dysregulated neurodevelopment in these FXS organoids. Transcriptomic analyses revealed that loss of FMRP led to more pervasive gene expression alterations in human brain development and an altered developmental trajectory in forebrain organoids in a cell-type-specific manner. Mechanistically, we demonstrated that FMRP could bind to several human-specific mRNA targets, which are significantly enriched in ASD-associated genes. Furthermore, we have shown that pharmacological inhibition of the PI3K pathway, but not the mGluR5 pathway, could largely rescue the developmental defects in FXS forebrain organoids. These data together provide new insights into mechanisms underlying FXS in human context and identify potential human-specific druggable targets for FXS and autism in general.
Major progress into the understanding of the molecular mechanisms underlying FXS in animal models led to several clinical trials, such as modulating metabotropic glutamate and GABA receptors; however, these clinical trials have been unsuccessful so far. One potential explanation for the lack of successful clinical trials could be the fundamental developmental, biochemical and physiological differences between animal models and humans, which motivated us in the current study. Previous studies using two-dimensional culture of FXS human neurons showed impaired differentiation and hyperexcitability1−45. Recently, 3D organoids have been generated from human iPSCs via 3D culturing methods46,47. Human organoids resemble critical organ and tissue-specific features of cell assembly, integration and organization, and they exhibit unique properties not observed in animal models. Thus, 3D organoids provide a unique opportunity to model human organ structure and function under healthy and disease conditions. Studies have shown that human organoids derived from patient-specific iPSCs, such as patients with autism and other diseases, recapitulate specific disease phenotypes, respond to drug treatments and are valuable in mechanistic studies48−50.

In the present study, we observed dysregulated neurodevelopment in FXS forebrain organoids, much of which was not observed in the FXS mouse model. Furthermore, our transcriptome analyses revealed a large number of overexpressed genes differentially expressed in both FXS forebrain organoids and fetal brain tissues, with only a few genes dysregulated in FXS mouse brain, suggesting that human brain organoids could better mimic the FXS-related phenotypes in a human context. Our comparative analyses have led to the identification of a large number of mRNAs that were bound by FMRP in human but not mouse. Interestingly, manipulating mGluR5 signaling, one well-studied pathway that is dysregulated in FXS animal models leading to impairments in protein synthesis, synaptic structure and function51, showed no effect on rescuing neurodevelopmental defects in FXS forebrain organoids. However, we should note here that the mGluR5 clinical trial was done in adolescents and adults, not during early development. Dysregulated PI3K signaling has also been linked to FXS52−54, and our data presented here suggest that pharmacological inhibition of the PI3K pathway could rescue some of the defects in neurodevelopment and synapse formation in FXS forebrain organoids. Future comprehensive evaluation of the effect of PI3K inhibitors on fragile X organoids is certainly warranted. Our findings also indicate that human brain organoids might provide a human-specific preclinical model for studying disease mechanisms underlying FXS and identifying human-specific therapeutic targets.

FMRP is widely expressed throughout the embryonic brain development, and its expression increases during neuronal differentiation, indicating the importance of FMRP for neurogenesis55−61. Importantly, there is evidence that, in chorionic villi samples taken from FXS fetuses, FMRP is absent at the early fetal period (12 post-conception weeks)62, a critical period for neurogenesis53. Indeed, examination of the postmortem brain tissues from patients with FXS revealed an increased density of long and tortuous dendritic spines63. In addition, previous studies showed neuroanatomical abnormalities in children with FXS, which are likely due to altered neurogenesis during early development6. Consistent with previous analyses, we observed dysregulated neurodevelopment in FXS forebrain organoids at both the molecular and cellular level. Furthermore, our transcriptome analyses on FXS forebrain organoids revealed human-specific molecular signatures associated with FXS, which largely overlapped with signatures of FXS fetal brain tissues but not with mouse brains. These results highlight the unique effect of loss of functional FMRP on human brain development and provide mechanistic insights into the molecular pathogenesis of FXS. One potential mechanism underlying the striking difference of FMRP-dependent gene expression changes in human organoids and mouse embryonic brains could be due to the differential m6A RNA modification. Earlier works showed that FMRP could use the m6A pathway to regulate gene expression, and that the m6A modification is more prevalent in human brain tissues than mouse64,65.

Emerging evidence suggests that FMRP plays a critical role during earlier stages of neural development. Our data suggest that absence of FMRP leads to fewer actively proliferating (Ki67+) NPCs, coinciding with an increase in TBR2+ intermediate progenitors/neuroblasts and accelerated cortical layer formation, which is consistent with previous reports in mouse and human models43,44. Intriguingly, other groups and our group have also shown that loss of FMRP increases the proliferation of adult neural stem cells in mouse hippocampus66 and early stage of human iPSC-derived neural stem cells67. Thus, the effect of loss of FMRP could be developmental stage specific: loss of FMRP increases NPC proliferation at early stages, whereas it decreases NPC proliferation and promotes neural differentiation at the later stages, and its overall effect is to accelerate premature neural differentiation.

The GABAergic inhibitory neurons are ~10% of total cells in our control forebrain organoids, which is similar to organoids generated by other groups68. Interestingly, although they found that GABAergic inhibitory neurons were overproduced in organoids from patients with idiopathic autism, we observed significant reduction of GABAergic inhibitory neurons in FXS organoids. To further investigate how loss of FMRP affects the development of GABAergic neurons, a model system with enrichment of GABAergic neurons, such as the ventral forebrain organoids or the forebrain assemblies69, will be needed in future studies.

To identify the druggable targets for FXS therapeutic interventions, it is important to identify the mRNA targets regulated by FMRP in human brain. Given the complexity of the CLIP assay itself, it was technically challenging to perform the CLIP assay using postmortem tissues. Accordingly, development of human brain organoids has provided us the first opportunity to identify the FMRP mRNA targets during human brain development. By performing eCLIP-seq on both human forebrain organoids and mouse brains, we identified a large number of mRNAs that were bound by FMRP only in human, many of which are ASD-associated genes. The identification of these human-specific FMRP mRNA targets might provide new insights into the molecular pathogenesis of FXS in human context and reveal potential therapeutic targets for interventions. Intriguingly, one of the human-specific targets of FMRP is CHD2, which is a well-known risk factor for intellectual disability and ASD that plays critical roles in neurogenesis and generation of...
GABAergic neurons. Increased expression of CHD2 in FXS forebrain organoids not only provides a potential mechanistic link to the neurodevelopmental deficits associated with loss of functional FMRP but also suggests that CHD2 could serve as a converging point of ASD and a potential druggable target for FXS and autism in general.
In summary, we developed a human forebrain organoid model of FXS and showed that loss of functional FMRP could lead to neurodevelopmental defects and alter the developmental trajectory. The identification of human FMRP mRNA targets could provide potential druggable targets for the therapeutic development of FXS. The human forebrain organoid model of FXS presented here could serve as a human-specific preclinical model and a resource for further studying the molecular pathogenesis of FXS.

Fig. 8 | CHD2 is a human-specific mRNA target of FMRP. a, FMRP target genes identified by eCLIP and related to neurogenesis and synaptic plasticity showed alteration at the protein expression level. b, Shown is the fold enrichment of CHD2 mRNA by FMRP immunoprecipitation followed by qRT-PCR in human forebrain organoids and mouse brain. Data are presented as mean ± s.e.m. (n = 3, **P = 0.0024, two-tailed t-test). c, CHD2 mRNA level did not display significant alteration in D28, D56 and D84 fragile X forebrain organoids based on chi-square test. NS indicates that it is not statistically significant (P > 0.05). d, Western blots are presented for comparing the CHD2 protein level in human organoids (Control versus FXS) and mouse embryonic brains (WT versus Fmr1 KO) using GAPDH as loading control. Upper panel shows western blot of CHD2 and GAPDH, and the lower panel is quantification of the western blot in human (lower left, n = 3, **P = 0.0016, two-tailed t-test) and mouse (lower right, n = 3, NS P > 0.05, two-tailed t-test). Data are presented as mean ± s.e.m. e, Shown is overlap of the DEGs between FXS organoids and E13.5 Chd2+/− mice. Approximately half of the common DEGs (408) between human forebrain organoids and Chd2+/− mice were also verified to be bound by CHD2 in human cell (255). NS, not significant; WT, wild type.
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Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41593-021-00913-6.

Received: 12 April 2020; Accepted: 15 July 2021; Published online: 19 August 2021

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Methods

Human forebrain-specific organoid cultures. The human iPSC lines were previously generated from skin biopsy samples of three male patients with FXS and three age-matched healthy males at Emory University. The isogenic line was generated by CRISPR edited expanded FXS iPSC lines (FXS1) using CRISPR-Cas9 gene editing. All iPSC lines have been fully characterized and cultured on irradiated mouse embryonic fibroblasts in human iPSC medium consisting of DMEM/F12 (Invitrogen), 20% KnockOut Serum Replacement (Invitrogen), 1x GlutaMAX (Invitrogen), 1x MEM non-essential amino acids (Invitrogen), 100µM β-mercaptoethanol (Invitrogen) and 10 ng ml⁻¹ of human basic FGF (PepTroTech) as described. Forebrain-specific organoids were generated using established protocols as previously described. Briefly, human iPSC colonies were detached from the feeder layer with 1 mg ml⁻¹ of collagenase treatment for 1 h and suspended in EB medium, consisting of FGF-2-free iPSC medium supplemented with 2µM dorsomorphin and 2µM A-83 in non-treated poly-L-lysine-coated plates prior to initiation with a daily medium change. On Days 5–6, EB medium was replaced with induction medium consisting of DMEM/F12, 1× N2 supplement (Invitrogen), 10 µg ml⁻¹ of heparin (Sigma-Aldrich), 1x penicillin/streptomycin, 1x non-essential amino acids, 1x GlutaMAX, 4 ng ml⁻¹ of WNT-3A (R&D Systems), 1µM CHIR99021 (Tocris) and 1µM SB-431542 (Tocris). On Day 7, organoids were embedded in Matrigel (BD Biosciences) and continued to grow in induction medium for six more days. On Day 14, embedded organoids were mechanically dissociated from Matrigel by pipetting up and down onto the plate with a 5-ml pipette tip. Typically, 10–20 organoids were transferred to each well of a 12-well spinning bioreactor (Spindbi) containing differentiation medium, consisting of DMEM/F12 (Invitrogen), 1x N2 and B27 supplements (Invitrogen), 1x penicillin/streptomycin, 100µM β-mercaptoethanol (Invitrogen), 1x MEM NEAA and 2.5 µg ml⁻¹ of insulin (Sigma-Aldrich). At Day 71, differentiation medium was exchanged with maturation medium, consisting of neurobasal (Gibco), 1x B27 supplement, 1x penicillin/streptomycin, 1x β-mercaptoethanol, 0.2 mM ascorbic acid, 20 ng ml⁻¹ of BDNF (PepTroTech), 20 ng ml⁻¹ of GDNF (PepTroTech), 1 ng ml⁻¹ of TGFβ (PepTroTech), 0.5 mM CAMP (Sigma-Aldrich). All media was changed every other day. LY294002 and MPEP were purchased from Millipore Sigma, and GS2636771 was purchased from Cayman Chemical.

Tissue infection and immunocytochemistry. For retroviral infection, retroviruses expressing GFP were prepared by Emory Viral Core, and forebrain organoids were infected at D49. For immunocytochemistry, forebrain organoids were processed at Day 56 as previously described. Briefly, whole organoids were fixed in 4% paraformaldehyde (PFA) in PBS for 30–60 min at room temperature. Organoids were washed three times with PBS and then incubated in 30% sucrose solution overnight. Organoids were embedded in Tissue Freezing Medium (General Data) and sectioned with a cryostat (Leica). For immunostaining, freezing medium was washed with PBS before permeabilization with 0.2% Triton X-100 in PBS for 1h. Tissues were then blocked with blocking medium consisting of 10% donkey serum in PBS with 0.1% Tween-20 (PBS) for 30 min. The following primary antibodies were used: anti-Ki67 (mouse, 1:500, BD Pharmingen), anti-SOX2 (goat, 1:500, R&D Systems), anti-PAX6 (rabbit, 1:500, Thermo Fisher Scientific), anti-CTIP2 (rat, 1:400, Abcam), anti-TBR1 (rabbit, 1:500, Abcam), anti-TBR2 (rabbit, 1:500, Abcam), anti-CaMKIIα (mouse; 1:800, Cell Signaling), anti-GABA (rabbit, 1:800, Sigma-Aldrich), anti-SYN1 (mouse, 1:500, Synaptic Systems), anti-PSD95 (rabbit, 1:500, Thermo Fisher Scientific), anti-DCX (rabbit, 1:500, Cell Signaling Technology), anti-MAP2 (chicken, 1:500, Novus), anti-TRIT-1 (rabbit, 1:500, Millipore) and anti-NANOG (goat, 1:500, R&D Systems). Primary antibodies diluted in blocking solution were applied to the sections overnight at 4°C. After washing with PBS, secondary antibodies diluted in blocking solution were applied to the sections for 1 h at room temperature. Finally, sections were washed with PBS1 and stained with DAPI. For EdU labeling, forebrain organoids were exposed to 10µM EdU for 2 h or 4 h on Day 56, followed by fixation and immunostaining. All images were captured by a Nikon Eclipse Ti-E microscope. Quantitative analyses were conducted on randomly selected cortical structures in a blinded fashion. The age-matched control cortex tissues were obtained from local brain banks. All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee at Emory University. Human fetal brain cortex tissues, human organoids and mouse embryonic whole forebrain were obtained in accordance with guidelines in Thiel-fixed (30% formaldehyde) and processed for immunofluorescence. RNA was then reverse transcribed using a SuperScript III First-Strand Synthesis System (Invitrogen). qPCR quantified cDNA via TaqMan assay (Invitrogen). Each reaction was run in triplicate and analyzed following the ΔΔCT method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control.

RNA isolation, qRT–PCR and RNA-seq. The study of human fetal tissue was approved by the Central South University Medical Genetics National Laboratory Ethics Review Committee (2013051202) and consented to by the patients and their families. After prenatal diagnosis confirmed the FXS mutation, the patients decided to terminate the pregnancy and donate the fetal tissues for research. The age-matched control cortex tissues were obtained from local brain banks. All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee at Emory University. Human fetal brain cortex tissues, human organoids and mouse embryonic whole forebrain were obtained in accordance with guidelines in Thiel-fixed (30% formaldehyde) and processed for immunofluorescence. RNA was then reverse transcribed using a SuperScript III First-Strand Synthesis System (Invitrogen). qPCR quantified cDNA via TaqMan assay (Invitrogen). Each reaction was run in triplicate and analyzed following the ΔΔCT method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a normalization control.

RNA-seq library construction was performed following the instructions of Illumina mRNA sample prep kit (cat. no. RS-100-0801). In brief, the poly-A-containing mRNA was purified using poly-T-oligo-attached magnetic beads. The mRNA was then fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first-strand cDNA using reverse transcriptase and random primers. This was followed by second-strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments went through an end repair process, the addition of a single ‘A’ base and then ligation of the adapters. These products were gel purified and enriched with PCR to create the final cDNA libraries. The library constructs were sequenced on the bioanalyzer to determine the size and concentration before sequencing on the Illumina HiSeq 2500 machine. Raw reads were examined for quality issues to ensure that library generation and sequencing were suitable for further analysis. As necessary, adapter sequences, other contaminant sequences such as poly-A tails and low-quality sequences with PHRED quality scores lower than 5 were trimmed from reads using cutadapt. RNA-seq reads were aligned using STAR v2.7.2, reference genome hg19 for human samples and mm9 for mouse samples, and differential expression values were extracted using DESeq2 v1.26.0 (ref. [3]). Genes with FDR <0.20 are defined as DEGs. All downstream analysis, including plotting and statistical tests, were conducted using R 3.6.2.
scRNA-seq. Three batches of human D56 forebrain organoids generated from three sets of unaffected and FXS iPSCs, including an isogenic line, were dissociated with Accutase solution (Sigma-Aldrich) and the single-cell suspension was subjected to 10× Genomics Chromium Chromium Controller (10x Genomics Core) with 5,000 target cell numbers and HiSeq 4000 sequencing (Novogenene). Briefly, the captured cells were processed for barcoding and generation of a single-cell library with Chromium Next GEM Single Cell 3′ Library kit, and the resulting single-cell libraries were sequenced with HiSeq4000. Using CellRanger 3.0.2 from 10x Genomics, raw Chromium scRNA-seq output was processed to align reads, generate feature–barcode matrices and perform clustering and gene expression analysis.

The GRCh38 human genome assembly was adopted to map the single-cell sequence reads. The median number of genes detected per cell is 1,995, and the fraction reads in cells is 76.3%. The median unique molecular identifier (UMI) counts is 1,964. We except the genes expressed in at least 20% of cells, we filtered out cells expressing high levels of genes mapped to the mitochondrial genome. To preserve high-quality cells, we removed cells with more than 30% of mitochondrial sequence reads. After filtering, normalization and scaling were performed. We analyzed 30,550 single cells with 1,528,965,000 unique mRNAs reads in downstream analysis with Seurat 3.1 and Monocle 3 packages in R.

scRNA-seq data analysis. Using all 29,025 genes, we identified anchor genes to integrated FXS and control samples for comparison in Seurat. To acquire the integrated Seurat object, we performed for detecting cell types on the original Seurat object and performed normalization as well as variable feature selection. The objects were normalized using LogNormalize, and the scale factor was set as 10,000. We identified a set of features exhibiting high cell-to-cell variability, using variance-stabilizing transformation. The integration anchors were found using the two objects and employed 1:20 dimensions using canonical correlation analysis. After dimension reduction, we performed principal component analysis using the previously identified variable genes. Fourteen cell types (clusters) were identified using the UMAP dimensional reduction technique. We used the top 20 principal components to retain sufficient information for dimension reduction. Using known marker genes in combination with GO analysis, we were able to identify each cell type representing neural lineages, such as neural progenitors, astrocytes, immature neurons, inhibitory neurons and excitatory neurons.

We used the ‘FindMarkers’ function in Seurat to identify the DEGs comparing FXS with control in each cluster using the Wilcoxon rank-sum test. Analyses were controlled for multiple testing. Adjusted P value and FDR were calculated. FDR < 0.05 was defined as statistical significance.

Cell types were assigned to the clusters as modiﬁed from previous studies with scRNA-seq. Briefly, all genes expressed in each cell are ranked by relative expression to the average of all cells, and the clusters were first annotated by collectively weighing the expression of markers of each speciﬁc cell type, such as NPC markers (SOX2, NEURO2, NOTCH1, HES1 and CDH1), radial glia markers (HES5, PAX6, VIM, FABP5, FABP7, CDH2, LGR, FAM107A, PTPRZ1, BMP7 and HOX2), glial oligodendrocyte and mature astrocyte markers (SLC1A2, SLC1A3, SLC1A4, GLUD1, OLIG1, OLIG2, GFAP and S100B), intermediate progenitor markers (NEURO2, DLX1 and ASCL1), migrating neuronal committed cell markers (DCX and GAP43), cortical neuronal marker (TBR1), cerebellar markers (STMN1, NEUROD1, NEUROD2, NEUROD4 and TUBB3A), mature neuron markers (B2F3, MAP2, SYN, SNAP25 and NEFM), inhibitory neuron markers (GAD1, GAD2, GAD45, GABBR1, GABBR2 and SLC32A1), excitatory neuron markers (SLC17A6, SLC17A7, GLUT2, GLUT1 and GLUL) and mesoderm markers (MYL1 and MYH3). First, any intermediate mesodermal cell cluster was identiﬁed with myosin and myofibril (MYL1 and MYH3), mesoderm markers (GRIN2B, GLS and GLUL) and mesoderm markers (MYL1 and MYH3). Then, the annotation term of each cluster was veriﬁed with GO term analysis to correlate the enriched GO term to the identity of each cluster.

Time trajectory analysis in Monocle 3. We ﬁrst created a new Seurat object containing all the samples using their indexes from the original object. It should be noted that the cell cycle stage and pre-cycle processes are retained in the expression matrix, cell metadata and gene metadata generated previously. We retained the top 50 principal components for downstream analysis. We performed the dimension reduction using the reduce ‘dimension’ function using UMAP. Then, the clusters were identiﬁed through ‘cluster_cells’ with a resolution of 10−1. To observe the marker genes expressed by each cluster for cell dataset objects, the marker genes were identiﬁed using the function ‘top_markers.’ Then, we grouped the cells by cluster to ﬁnd the cluster-speciﬁc marker genes. Next, we used the ‘learn_graph’ function to investigate the overall pseudotime trajectory of cells and labeled them in the trajectory path.

Enhanced crosslinking and eCLIP-seq. To ﬁx the interactions between the RNA-binding proteins and their related RNA permanently, UV crosslinking was conducted at 254 nm and 400 nm cm−2 three times on an iced waterbed using the D56 organoids of both unaffected and fragile X as well as the mouse embryonic brains of E13.5. The UV crosslinked samples were lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and protease inhibitor) on ice for 10–30 min, followed by digestion with RNase I and DNase (Invitrogen) at 37 °C for 5 min. After digestion, RNase inhibitor was added immediately to avoid further degradation of RNA, and centrifugation was carried out at 14,000 rpm for 10 min at 4 °C. The supernatant was used for immunoprecipitation of FMRP/RNA complexes with speciﬁc anti-FMRP primary antibody (Abcam) and normal IgG as negative immunoprecipitation control using Dynabeads Protein G (Invitrogen).

The chromatin immunoprecipitated by FMRP was digested by Proteinase K (Promega) and the supernatant was used for enrichment using immunoprecipitation of FMRP/RNA complexes with speciﬁc anti-FMRP primary antibody (Abcam) and normal IgG as negative immunoprecipitation control using Dynabeads Protein G (Invitrogen) for dephosphorylation using a 37 °C Thermomixer at 1,200 rpm to avoid bead precipitation followed by washing with the ice-cold wash buffer, the cold high salt buffer and the 1x lysis buffer, respectively. To enhance the ligation efﬁciency and to wash unincorporated adapters, the on-beads ligation was carried out to ligate the RNA adapter R119 to the 3′ end of the bound RNA by FMRP using T4 RNA Ligase (NEB), PEG8000 (NEB) and DMSO (Sigma–Aldrich). After stringent washes, the washed/ligated beads for each sample were resuspended in 100 μl of 1X PBS buffer followed by dividing the beads into two tubes: one for 20% and the other for 80%, respectively. After the PBS buffer was completely removed from both tubes for each sample, the FMRP/RNA complex samples were then separated on NuPAGE gels and transferred to nitrocellulose membranes. The membrane containing the elution from 20% of the beads was cut out and used for Western blotting for both the coinmunoprecipitation and crosslink via western blot, whereas the membrane for 80% of the eluted was used for recovery of the FMRP/RNA complexes.

A region starting from the FMRP protein size (RNA fragment-free FMRP) to above about 75 kDa (FMRP bound with around 220 nt of RNA fragments) on the membrane was recovered. The recovered region of the membrane was digested with Proteinase K (NEB), and the RNA fragments were puriﬁed by Zipym column (Zymo Research). Using AR17 as the primer, the recovered RNA fragments were reverse transcribed with AffinityScript (Agilent). The first-strand cDNA was puriﬁed by treatment with ExoSAP-IT (Affymetrix) and binding to MyONE Silane beads (Thermo Fisher Scientiﬁc). After the MyONE Silane beads were stringently washed to remove the excess AR17 oligos, ligations was carried out on beads to ligate the DNA oligo rand3Tr3 adapter to the 3′ end of the cDNA at room temperature overnight using the same RNA ligase as mentioned above, followed by a stringent wash to get rid of the primer dimers. After puriﬁcation, both 3′- and 5′-ligated cDNAs were ﬁrst applied as templates for qPCR to determine the proportion of ﬁnal PCR cycle for enrichment of the cDNA-seq library using Q5 high-fidelity DNA polymerase and 1:57 primer pairs. Finally, the ampliﬁed cDNA-seq library size range from 175 bp to 300 bp was selected by PAGE puriﬁcation, followed by elution and precipitation with glycogen (Ambion), 3 M sodium acetate (pH 5.2) and ethanol. The library constructions were run on the bioanalyzer to verify the size and concentration before sequencing on the Illumina HiSeq 2500 machine.

eCLIP-seq bioinformatical analyses. After standard HiSeq demultiplexing, reads were aligned using STAR 2.7 with human genome (hg19) for human forebrain organoids and mouse genome (mm9) for mouse fetal brain. Candidate binding sites were identiﬁed using CLIPper. To normalize peak-level read counts, Input was used as the standard for each sample. The read counts were log2 transformed and quantiﬁed using R. A set of peaks was identiﬁed using CLIPper with a minimum of 10,000. We added an M approaches to transcriptome analysis. The gene interactome plot was obtained using GeneMANIA (https://genemania.org/). GO analyses were performed using the PANTHER overrepresentation test and GO database annotation with Fisher’s test and Bonferroni correction.

Western blotting. Protein samples were separated on SDS-PAGE gels and then transferred to polyvinylidene ﬂuoride membranes (Millipore). Membranes were probed according to the ECL Western Blotting Protocol (GE Healthcare). The membranes were probed with anti-FMRP, anti-CHD2 and anti-GAPDH, respectively, at the dilution of 1:1,000 for anti-FMRP, 1:2,000 for anti-CHD2 and 1:100,000 for GAPDH. Horseradish peroxidase-labeled secondary antibodies were obtained from Cell Signaling Technology (7074S and 7076S) and were used at a 1:10,000 dilution. The anti-GAPDH (AM4309, Thermo Fisher Scientific) were used for loading controls. All western blot quantiﬁcations were performed using ImageJ software.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
We have deposited the scRNA-seq, RNA-seq and eCLIP-seq data into the Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/. The accession number is GSE146878. Source data are provided with this paper.

Code availability
RNA-seq data were analyzed following the standard pipeline with STAR 2.7 software (https://hbctraining.github.io/Intro-to-rnaseq-hpc-O2/lessons/03_alignment.html) and DESeq2 (https://bioconductor.org/packages/release/bioc/vignettes/DESeq2/inst/doc/DESeq2.html). CLIP-seq data were analyzed following the pipeline with STAR 2.7 software and CLIPper (https://github.com/YeoLab/clipper). scRNA-seq data were analyzed following the pipeline with CellRanger 3.0.2 software (https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/using/tutorial_ov), Seurat 3.1 (https://satijalab.org/seurat/articles/pbmc3k_tutorial.html) and Monocle 3 (https://cole-trapnell-lab.github.io/monocle3/docs/starting/). Analysis code is available upon reasonable request.

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Acknowledgements
This work is dedicated to the late S. Warren and was supported, in part, by the National Institutes of Health (NS091859 to S.T.W. and P.J.; HD104458 to S.T.W., G.B. and Z.W.; HD082013 to G.B.; AI131130 to Z.W. and P.J.; MH123711 and MH121102 to Z.W.; and NS051630 and NS111602 to P.J.), the Department of Defense (W81XWH1910068 to E.G.A. and W81XWH1910353 to Z.W.), the Edward Mallinckrodt, Jr. Foundation (Z.W.) and the FRAXA Research Foundation (Y.K.). We would like to thank S. Sloan at Emory University for help with scRNA-seq analyses. This study was supported, in part, by the Emory Integrated Genomics Core, which is subsidized by the Emory University School of Medicine and is one of the Emory Integrated Core Facilities. Additional support was provided by the Georgia Clinical & Translational Science Alliance of the National Institutes of Health under Award Number UL1TR002378. This work was performed with the support of the Georgia Genomics and Bioinformatics Core (GGBC) at the University of Georgia. The scRNA-seq work was performed at the GGBC at the University of Georgia, Athens. We thank M. Alabady and his team at the GGBC for their support and contribution to this work.

Author contributions
Y.K. led the molecular aspects of the project, and Y.Z led the cellular aspects of the project. Y.L. performed the eCLIP-seq analysis. Y.H. performed electrophysiology analyses. Z.L., S.L., H.F., F.Z. and H.W. performed bioinformatic analyses. J.X., W.N., J.D. and C.X. helped with data collection. G.J.B. and N.R. provided FXS iPSC lines. G.J.B., J.P., S.T.W. and E.G.A. helped with data analyses and interpretation. Z.W., P.J. and Y.K. designed the project and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41593-021-00913-6.
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41593-021-00913-6.
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Peer review information Nature Neuroscience thanks Rudolf Jaenisch and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | FMRP regulates cortical neurogenesis in a human forebrain organoid model. a, Quantification of the size of control and FXS forebrain organoids. Data are presented as mean ± s.e.m. (n = 30 organoids from each line; one-way ANOVA). b, c, Loss of FMRP reduces NPC proliferation. Shown are representative images (b) and quantification (c) of the proportion of Ki67+ proliferating neuronal progenitor cells in total PAX6+ dorsal forebrain neuronal progenitor cells of both control and FXS-derived forebrain organoids at day 56. Data are presented as mean ± s.e.m. (n = 6 organoids from each line with 15–20 cortical structures analyzed per organoid; ****P < 0.0001, one-way ANOVA). Scale bars: 50 μm. d, e, D56 forebrain organoids were pulsed with EdU (10 μM) for 2 hr. Shown are representative images (d) and quantification (e) of the proportion of EdU+ proliferating cells in total SOX2+ NPCs in both control and FXS-derived forebrain organoids at day 56. Data are presented as mean ± s.e.m. (n = 62 cortical structures from at least ten organoids each condition; ****P < 0.0001, one-way ANOVA). Scale bars: 50 μm.
Extended Data Fig. 2 | Loss of FMRP impairs cortical neurodevelopment. 

**a.** Quantification of the proportions of TBR2^+^ IPCs, CTIP2^+^ cortical neurons, and SOX2^+^ NPCs in total DAPI^+^ cells in control and FXS-derived forebrain organoids at day 56. Data are presented as mean ± s.e.m. (n = 6 organoids from each line with 15–20 cortical structures analyzed per organoid; ****P < 0.0001, one-way ANOVA).

**b, c.** Loss of FMRP dysregulates distribution of TBR2^+^ intermediate neural progenitor cells. Shown are representative images (b) and quantification (c) of the proportion of TBR2^+^ IPCs in MAP2^+^ layer of both control and FXS-derived forebrain organoids. Yellow dashed lines indicate the borders of VZ-like structures. Data are presented as mean ± s.e.m. (n = 6 organoids from each condition with 15–20 cortical structures analyzed per organoid; ****P < 0.0001, one-way ANOVA). Scale bars: 50 µm.
Extended Data Fig. 3 | Loss of FMRP accelerates cortical layer formation. a, b, Shown are sample images (a) and quantification (b) of relative thickness of SOX2\textsuperscript{+}CTIP2\textsuperscript{−} VZ layer and CTIP2\textsuperscript{+} CP layer in day 56 forebrain organoids. Yellow dashed lines indicate the borders between VZ and CP layers. Data are presented as mean ± s.e.m. (n = 15 cortical structures per organoid from at least 12 organoids each line; ***P = 0.0005, ****P < 0.0001, one-way ANOVA). Scale bars: 50 µm. c, d, Shown are sample images (c) and quantification (d) of relative thickness of SOX2\textsuperscript{+}MAP2\textsuperscript{−} VZ layer and MAP2\textsuperscript{+} CP layer in day 56 forebrain organoids. Yellow dashed lines indicate the borders between VZ and CP layers. Data are presented as mean ± s.e.m. (n = 15 cortical structures per organoid from at least 12 organoids each line; ****P < 0.0001, two-way ANOVA). Scale bars: 50 µm. e, f, Shown are sample images (e) and quantification (f) of relative thickness of SOX2\textsuperscript{+}TBR1\textsuperscript{−} VZ layer and TBR1\textsuperscript{+} CP layer in day 56 forebrain organoids. Yellow dashed lines indicate the borders between VZ and CP layers. Data are presented as mean ± s.e.m. (n = 15 cortical structures per organoid from at least 12 organoids each line; ***P = 0.0009, ****P < 0.0001, one-way ANOVA). Scale bars: 50 µm. g, h, Quantification of the proportions of TBR1\textsuperscript{+} cortical neurons (g) and SOX2\textsuperscript{+} NPCs (h) in total DAPI\textsuperscript{+} cells in control and FXS-derived forebrain organoids at day 56. Data are presented as mean ± s.e.m. (n = 6 organoids from each line with 15–20 cortical structures analyzed per organoid; ****P < 0.0001, one-way ANOVA). i–l, Analysis of marker distribution across the VZ/CP layers. Data are presented as mean ± s.e.m. (n = 10 organoids from control or FXS lines each with 15–20 cortical structures analyzed per organoid; ****p < 0.0001; one-way ANOVA).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Loss of FMRP prevents differentiation of GABAergic interneurons.  

a, b, Quantifications of the numbers of GABA\(^+\) inhibitory neurons (a) and CaMKII\(\alpha^+\) excitatory neurons (b) in a field of 588\(\mu\)m \(\times\) 588\(\mu\)m in both control and FXS-derived forebrain organoids. Data are presented as mean \(\pm\) s.e.m. (n = 10 sections from 10 organoids each line; \(**p = 0.0012\) (FXS2 v.s. CTRL1 in b) or \(0.0097\) (FXS3 v.s. CTRL1 in b), ***p = 0.0008 (b), ****P < 0.0001, one-way ANOVA).  

c, Sample images of RNA expression of DLX2, PAX6 and SOX2 by RNAscope in control and FXS forebrain organoids at day 56. Blue staining represents DAPI. Scale bars: 50\(\mu\)m.  

d, Quantification of ratio of DLX2\(^+\) MGE-like NPC area v.s. PAX6\(^+\) dorsal forebrain NPC area in D28 and D56 control and FXS-derived forebrain organoids. Data are presented as mean \(\pm\) s.e.m. (n = 5 organoids from each condition with 15-20 sections analyzed per organoid; ****P < 0.0001, one-way ANOVA).
Extended Data Fig. 5 | Basic electrophysiological characterization of FXS neurons in forebrain organoids. a, Shown are sample images of a CTIP2+ cortical neuron that was filled with Alexa Fluor-594 dye after the electrophysiological recording. Scale bars: 20 µm. Experiment was repeated at least 13 times independently for each condition with similar results. b–d, Characterization of passive membrane properties, including the resting membrane potential (RMP; b), input resistance (RIN; c), and membrane capacitance (d). Data are presented as mean ± s.e.m. (two-tailed unpaired t test or one-way ANOVA). e–h, Basic properties of action potentials, including the amplitude (e), threshold (f), half-width (g), and the rise time (h) of the first action potentials. Data are presented as mean ± s.e.m. (two-tailed unpaired t test or one-way ANOVA). i–k, Characterization of transient inward currents and sustained outward currents of FXS neurons. Shown are sample tracings of transient inward and sustained outward currents (i), quantification of transient inward current-voltage curve (j) and peak density of transient inward currents (k). Data are presented as mean ± s.e.m. (two-tailed unpaired t test or one-way ANOVA). Cell number (n) recorded and analyzed in each condition is indicated.
Extended Data Fig. 6 | Expression of Kv4.2 voltage-gated potassium channel in human forebrain organoids. Sample images (a) and quantification (b) of Western blots are presented for comparing Kv4.2 protein level in D56 control and FXS forebrain organoids using GAPDH as loading control. Data are presented as mean ± s.d. (n = 3 cultures; **P = 0.0085 (FXS1 v.s. CTRL1) or 0.0033 (FXS2 v.s. CTRL1), ***P = 0.0003, one-way ANOVA).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | The PANTHER overrepresentation test on the upregulated genes in FXS organoid at each stage show enrichment in distinct pathways. The upregulated genes in FXS at a given developmental stage, D28, D56, or D84 show specific pathway enrichment. The upregulated genes at D28 in FXS organoids are enriched in ciliary locomotion of neuron, axoneme assembly, and other synaptogenesis related pathways while the up-regulated genes at D56 in FXS organoids show more relevance to the pathways associated with synaptic function. Interestingly, genes with higher expression in FXS than in control organoids at the more developed D84 are concentrated in DNA replication, cell division and cell cycle pathways. This suggests aberrant developmental manifestation in FXS organoids. The numbers on the bars indicate the two-sided p values by Fisher’s exact test. The p values have been adjusted for multiple testing using Bonferroni correction.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Lack of FMRP causes altered neural differentiation and aberrant developmental trajectory in forebrain organoids. 

**a.** A heat map of expression of annotation reference genes in 14 cell type specific clusters present during human forebrain organoids shows the differential expression of various marker genes for specific cell types in each cluster. (C1: fate determining stage neurons toward excitatory neuron, C2: excitatory neuron, C3: neural stem cell / radial glia2, C4: immature neuron, C5: neural stem cell / radial glia1 cell, C6: glial progenitor, C7: inhibitory neuron, C8: astrocyte, C9: radial glia, C10: astrocyte, C11: immature neuron very early stage, C12: oligodendrocyte, C13: ectodermal origin non-neuronal cells, C14: non-neuronal cells) **b.** The expression of neural stem cell/progenitor marker, SOX2 (red) and differentiated cortical plate neuron marker, BCL11B (CTIP2, green) were presented simultaneously in the UMAP plot. Compared to control, cells in FXS organoids expressing BCL11B/CTIP2 at low level were increased and widely distributed spanning various cell types regardless of differentiation status and cell function. Many of these are accompanied by the expression of SOX2. Significantly high co-expression rate of the NPC marker, SOX2, and cortical plate marker, BCL11B in the C7, young inhibitory neuron cluster (19% in FXS forebrain organoids compared to 0% in control forebrain organoids), suggest that the spatiotemporal regulation of SOX2 and BCL11B expression critical for proper specification and lamination of neurons is severely perturbed in FXS organoids. Data are presented as mean ± s.e.m. (n=3 single cell RNAseq of 3 independent culture sets, **P=0.0025, two-tailed unpaired t test) **c.** Among the 14 clusters, the highest number of DEGs were detected in the young inhibitory neuron cluster, C7. PANTHER analyses show high relevance to regulation of synapse organization, learning and memory, and forebrain development with down-regulated DEGs and protein targeting. mRNA stability and regulation of cell cycle. Yellow represents up-regulated genes and blue represents down-regulated genes. The numbers on the bars are the associated two-sided p-values by Fisher’s Exact test. The p values have been adjusted for multiple testing using Bonferroni correction. **d.** Transcriptional features of the cluster 6 at the developmental break point between FXS and control (arrow in red) in the time trajectory was assessed. The Monocle cluster 4, one of the major break point in the time trajectory, has marker genes associated with cell proliferation and regulation of DNA methylation, (for example, KMT2A), neuron migration and regulation of neuron projection development (ACAP3), synapse organization and axon guidance (NFASC).
Extended Data Fig. 9 | The overlap between human fetal brain DEGs and cell type specific DEGs. (a) all single cell cluster specific DEGs were compared with human fragile X fetal brain RNAseq DEGs. The highest overlap is marked with an asterisk above the bar.  

**b** PANTHER gene ontology revealed that they are involved in GABAergic neuron differentiation, forebrain neuron generation and differentiation. Downregulated genes are enriched in regulation of neural precursor cell, neurogenesis and proliferation, cerebral cortex and forebrain development, gliogenesis, and cell differentiation. The numbers on the bars are two-sided p-values by Fisher’s exact test. The p values have been adjusted for multiple testing using Bonferroni correction.
Extended Data Fig. 10 | An overlap between disease risk genes and the subset of human and mouse FMRP binding genes are shown. The percentage of overlap between Schizophrenia and ASD risk genes and human-specific, mouse-specific or human-mouse shared FMRP binding genes are indicated. Statistical significance was calculated by Pearson’s χ² tests, and p-values are indicated.
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Software and code

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Data collection
- Imaging: NIS-Elements AR 4.51 (Nikon).
- Electrophysiology: pClamp 11 software (Molecular Devices)

Data analysis
- STAR 2.7, CLIPper, DESeq2, CellRanger 3.0.2, Seurat 3.1, Monocle 3, GENEMANIA 3.5.2, Homer v4.10, Panther 16.0, and enrichR 3.0 were used for data analysis. Analysis code is available upon reasonable request.

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Data to support the finding of this study is available at GEO under accession number GSE146878. GO Biological Process and KEGG pathway databases have been used for enrichment analysis through softwares Panther 16.0 and enrichR 3.0.
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Life sciences study design

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**Sample size**

Sample size was determined based on our previous experience and the work of other groups using human iPSC-derived neurons and mouse as experimental model systems. Sample sizes are reported in Figure legends or Figures.

**Data exclusions**

No data was excluded for the analyses.

**Replication**

All the experimental data were replicated two or three independent experiments. The experimental findings were reliably reproduced.

**Randomization**

Samples were randomly allocated to control and experimental groups. The control and FXS organoids, wildtype and Fmr1 KO mice were chosen randomly for all experiments including RNA-seq, single cell RNA-seq, and eCLIP-seq.

**Blinding**

Investigators were blinded to groups/genotypes.

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### Materials & experimental systems

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|-----|-----------------------|
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☑ | Palaeontology |
| ☑ | Animals and other organisms |
| ☑ | Human research participants |
| ☑ | Clinical data |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑ | ChIP-seq |
| ☑ | Flow cytometry |
| ☑ | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

- anti-Ki67 (mouse; 1:500; BD-Pharming #550609), anti-SOX2 (goat; 1:500; R&D Systems #AF2018), anti-PAX6 (rabbit; 1:500; Thermofisher #42-6600), anti-CTIP2 (rat; 1:400; Abcam #ab18465), anti-TBR1 (rabbit; 1:500; Abcam #ab31940), anti-TBR2 (rabbit; 1:500; Abcam #ab33345), anti-CaMKII (mouse; 1:800; Cell Signaling #50049), anti-GABA (rabbit; 1:800; Sigma #A2052), anti-SYN1 (mouse; 1:500; Synaptic System #106011), anti-PSD95 (rabbit; 1:500; Thermofisher #51-6900), anti-MAP2 (chicken; 1:500; Novus #nb300-213), anti-DCX (rabbit; 1:500; Cell Signaling #4604), anti-Tra-1-60 (mouse; 1:500; Millipore #MAB4360), anti-NANOG (goat; 1:500; R&D Systems #AF1997), anti-FMRP (rabbit; 1:1000; Abcam #ab17722), anti-CHD2 (rabbit; 1:2000; Thermofisher #PA5-96471), anti-GAPDH (mouse; 1:10000; Santa Cruz #sc-32333), Donkey anti-Mouse Alexa Fluor 488 (1:1000; Thermofisher #R37114), Donkey anti-Rabbit Alexa Fluor 568 (1:1000; Thermofisher #A10042), Donkey anti-Rat Alexa Fluor 488 (1:1000; Thermofisher #A21208), Donkey anti-Mouse Alexa Fluor 568 (1:1000; Thermofisher #A10037), Donkey anti-Rabbit Alexa Fluor 488 (1:1000; Thermofisher #A-2106), Donkey Anti-Chicken Cy5 (1:1000; Jackson ImmunoResearch #703-175-155), HRP-linked Goat Anti-rabbit IgG (1:5000; Cell Signaling Technology #7074), HRP-linked Horse Anti-mouse IgG (1:5000; Cell Signaling Technology #7076).

**Validation**

Antibody validation was performed by the manufacturers and ourselves, and we used antibodies that have been validated for each assay (i.e. immunostaining and western blotting).

- anti-Ki67 (Antibody ID: AB_393778; https://antibodyregistry.org/search.php?q=AB_393778),
- anti-SOX2 (Antibody ID: AB_355110; https://antibodyregistry.org/search.php?q=AB_355110),
- anti-PAX6 (Antibody ID: AB_2533534; https://antibodyregistry.org/search.php?q=AB_2533534),
- anti-CTIP2 (Antibody ID: AB_2064130; https://antibodyregistry.org/search.php?q=AB_2064130),
- anti-TBR1 (Antibody ID: AB_2200219; https://antibodyregistry.org/search.php?q=AB_2200219),
- anti-TBR2 (Antibody ID: AB_778267; https://antibodyregistry.org/search.php?q=AB_778267),
- anti-CaMKII (Antibody ID: AB_2067938; https://antibodyregistry.org/search.php?q=AB_2067938),
- anti-GABA (Antibody ID: AB_477652; https://antibodyregistry.org/search.php?q=AB_477652),
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  Please see Supplementary Table 1.
1. CTRL1: derived from a healthy control with wild type FMR1 (generated by Dr. Zhexing Wen’s lab);
2. CTRL2: derived from a healthy control with wild type FMR1 (generated by Dr. Zhexing Wen’s lab);
3. CTRL3: derived from a healthy control with wild type FMR1 (generated by Dr. Zhexing Wen’s lab);
4. FXS1: derived from a FXS patient with FMR1 full mutation (generated by Dr. Stephen Warren’s lab);
5. FXS2: derived from a FXS patient with FMR1 full mutation (generated by Dr. Stephen Warren’s lab);
6. FXS3: derived from a FXS patient with FMR1 full mutation (generated by Dr. Gary Bassell’s lab);
7. FXS1R: Isogenic correction line of FXS1 by CRISPR/Cas9 (generated by Dr. Stephen Warren’s lab);

Authentication  All cell lines were characterized using RT-PCR and immunofluorescence for markers of pluripotency. All of them had a normal karyotype and were confirmed to be a full mutation line by showing absence of FMRP protein and FMR1 mRNA using Western blotting and qRT-PCR.

Mycoplasma contamination  Negative

Commonly misidentified lines (See ICLAC register)  None of the cell lines used are listed in the ICLAC database.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  Wild type (C57BL/6; Jackson Laboratory #000664) and FMR1 KO (Fmr1tm1Cgr; Jackson Laboratory #003025) mice were obtained from Jackson Laboratory. Mice were housed in a vivarium with a 12-h light/dark cycle, housing groups of 5 maximum, with food/water ad libitum. At embryonic day 13.5, 3 pairs of wildtype and Fmr1 KO mice litter mates were randomly chosen for RNAseq and eCLIP experiments.

Wild animals  No wild animals were used in the study.

Field-collected samples  No field collected samples were used in the study.

Ethics oversight  All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics  Fetuses with fragile X full mutation

Recruitment  After the prenatal diagnosis confirmed that the fetus was fragile X full mutation, the patients decided to terminate the pregnancy and donate the fetal tissues for research.

Ethics oversight  The study of human fetal tissue was approved by Central South University Medical Genetics National Laboratory Ethics Review Committee (2013051202) and consented by the patients and their families.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

anti-SYN1 (Antibody ID: AB_2619772; https://antibodyregistry.org/search.php?q=AB_2619772),
anti-PSD95 (Antibody ID: AB_2533914; https://antibodyregistry.org/search.php?q=AB_2533914),
anti-MAP2 (Antibody ID: AB_2138178; https://antibodyregistry.org/search.php?q=AB_2138178),
anti-DCX (Antibody ID: AB_561007; https://antibodyregistry.org/search.php?q=AB_561007),
anti-Tra-1-60 (Antibody ID: AB_2119183; https://antibodyregistry.org/search.php?q=AB_2119183),
anti-NANOG (Antibody ID: AB_355097; https://antibodyregistry.org/search.php?q=AB_355097),
anti-FMRP (Antibody ID: AB_2278530; https://antibodyregistry.org/search.php?q=AB_2278530),
anti-CHD2 (Antibody ID: AB_2808273; https://antibodyregistry.org/search.php?q=AB_2808273),
anti-GAPDH (Antibody ID: AB_627679; https://antibodyregistry.org/search.php?q=AB_627679).