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Knockdown of *Ddx3x* in mPFC induces autistic-like phenotype in mice *via* altered synaptic plasticity

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DDX3X, a DEAD-box RNA helicase, has been identified as a risk gene for autism spectrum disorder (ASD). To elucidate the role of *DDX3X* mutations in ASD pathogenesis, HT22 cell models and mouse models with *Ddx3x* knockdown specifically in the medial prefrontal cortex were established. *Ddx3x* knockdown in HT22 cells resulted in slower growth, while in mice, it induced autism-like behaviors. Proteomic analysis in cortex revealed that many down-regulated proteins were involved in synaptic plasticity. The differentially expressed proteins (DEPs) were associated with long-term potentiation, glutamatergic and GABAergic synapses, postsynaptic density, branched-chain amino acid degradation, and the oxytocin pathway. Integrating cellular and cortical omics studies revealed overlaps in the ubiquitin–proteasome system and the ‘de novo’ protein folding pathway. These pathways were inhibited in the cortex of the model mice. The expression of several important proteins was validated. Confocal microscopy and transmission electron microscopy demonstrated a significant reduction in dendritic spine density and postsynaptic density of mouse models. Electrophysiological experiments showed that the frequency of miniature excitatory postsynaptic currents was significantly reduced. These results suggest that *DDX3X* deficiency impairs synaptic function, thereby affecting neurodevelopment and social abilities. Abnormal synaptic plasticity may contribute to the pathogenesis of ASD with *DDX3X* gene mutations.

Translational Psychiatry (2026)16:216; <https://doi.org/10.1038/s41398-026-03945-3>

INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental syndromes in which individuals have deficits in social communication and the manifestation of limited and repetitive behaviors [1, 2]. A significant proportion of ASD individuals may have complications such as intellectual disability (ID), global developmental delay, and epilepsy, accompanied by ultra-rare genetic variants [3]. In the past two decades, the prevalence of ASD has continued to rise, and it has become a serious global public health problem.

ASD is highly heritable, as evidenced by its increased prevalence among relatives of individuals with ASD compared to the general population [4, 5]. With the advancement of genomics research, over 1200 genes have been identified as susceptibility genes for ASD in the SFARI Gene database (<https://www.sfari.org/resource/sfari-gene/>). More than 100 risk genes have been confirmed, and the discovery of new genes is ongoing [6]. Despite these significant achievements, several challenging questions remain. Firstly, the precise relationship between these identified genes and the pathogenesis of ASD requires further elucidation [7, 8]. Secondly, given the genetic heterogeneity of ASD, it is crucial to identify common characteristics or underlying mechanisms that may link these diverse genetic factors [9–11]. Moreover, the occurrence of ASD is also associated with environmental factors, the interaction between genetic and environmental factors, and epigenetic regulation [9, 12, 13].

In the previous studies, we identified an ASD child carrying a *DDX3X* risk variant via targeted sequencing [14, 15]. *DDX3X*, also known as *DBX* or *DDX3*, is a DEAD-box helicase involved in translation initiation and multiple stages of RNA metabolism, with critical roles in neurite growth, synaptogenesis, and cortical neurogenesis [16–18]. Mutations in *DDX3X* occur in ~1–3% of individuals with unexplained ID [19–21], and large-scale exome sequencing studies have implicated *DDX3X* in severe developmental disorders [19, 22, 23]. *DDX3X* syndrome is now recognized as a neurodevelopmental disorder associated with ASD, ID, and various comorbidities [24], with loss-of-function mutations classified as ASD risk variants [25].

Existing studies have confirmed that *DDX3X* is a core risk gene for ASD. Deficiency in *DDX3X* disrupts neurodevelopment through multiple mechanisms. These include impaired RNA helicase activity [18], altered translation of synaptic mRNAs [18, 26], and reduced crebbp mRNA stability [16]. Additionally, deletion of *Ddx3x* leads to embryonic lethality in mice, which is associated with its critical role in placental and embryogenesis [17]. At the cellular level, *Ddx3x* depletion prolongs neural progenitor cell cycles, reduces neurogenic divisions, and disrupts the excitatory/inhibitory balance [16, 27], while knockout in neural stem cells impairs hindbrain development [28]. Phenotypically, *DDX3X*-deficient mice and zebrafish exhibit ASD behaviors and brain abnormalities (e.g., microcephaly) [16, 29].

Notably, although *DDX3X* syndrome is more common in females, males with hypomorphic missense mutations also show

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Received: 21 June 2025 Revised: 5 February 2026 Accepted: 6 March 2026

Published online: 30 March 2026

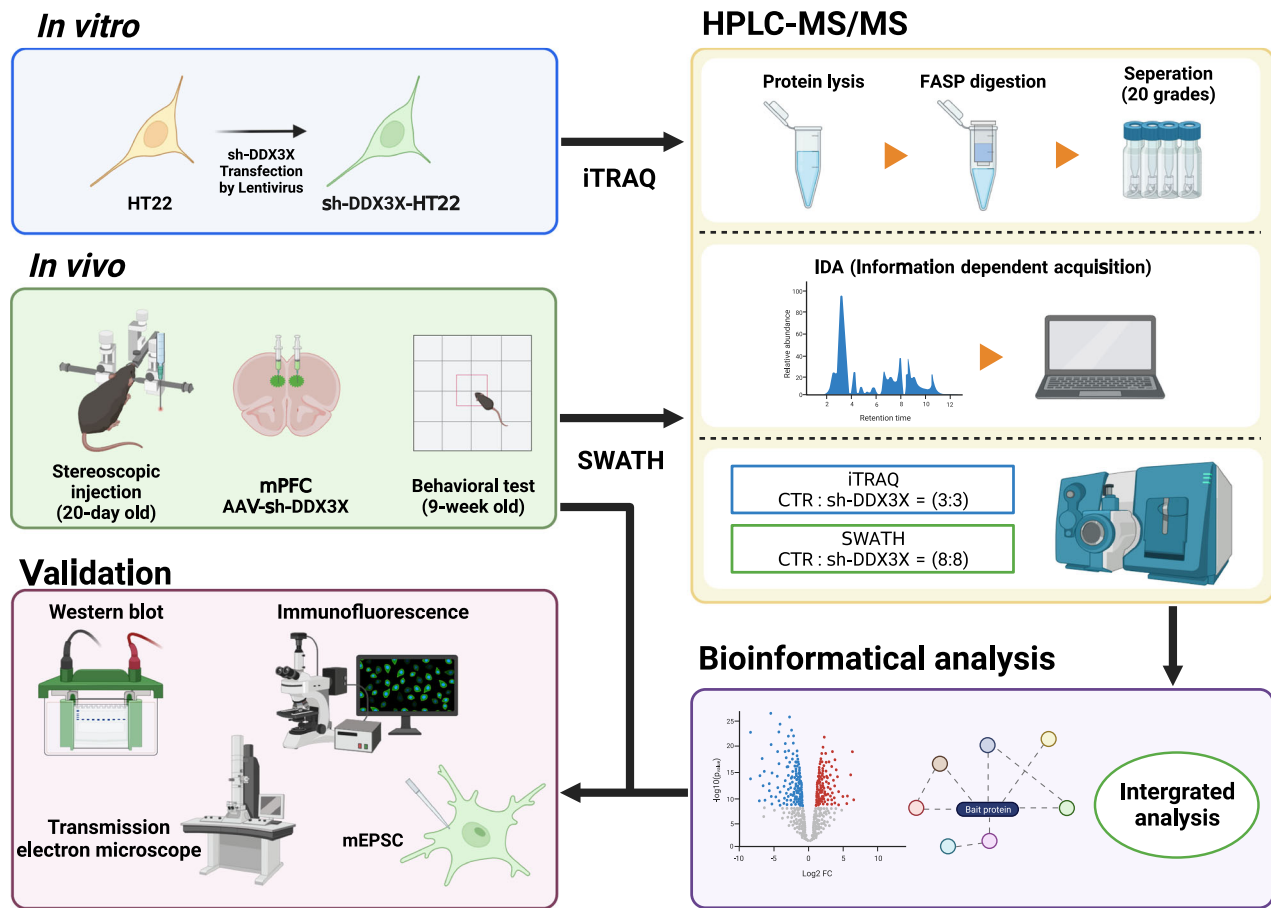


Fig. 1 Study flow diagram. FASP: filter-aided sample preparation, mPFC: medial prefrontal cortex, iTRAQ: isobaric tags for relative and absolute quantitation, SWATH: sequence window to obtain all theoretical fragment ions, TEM: transmission electron microscope, mEPSC: miniature excitatory postsynaptic currents. This figure panel was created using BioRender.

neurodevelopmental deficits (e.g., ID, autistic features) [17, 30]. Critically, *Ddx3y* (Y-homolog) is lowly expressed in the mouse brain and unable to fully compensate for *Ddx3x* loss [28, 30], while *DDX3X*'s partial escape from X-chromosome inactivation (XCI) leads to mosaic expression in female tissues [17, 29]. Combined with female estrous cycle-induced behavioral variability [31, 32], we thus used male mice and targeted the mPFC—a key region for ASD-related core functions including social behavior, cognition, and memory [33, 34]—to establish a *Ddx3x* knockdown mouse model. We also constructed an in vitro *Ddx3x* knockdown cellular model. Subsequently, we combined behavioral, proteomic, biochemical, and electrophysiological approaches to investigate the link between *DDX3X* deficiency and ASD as well as the underlying mechanisms.

MATERIAL AND METHODS

Cell culture and lentivirus transfection

The experimental flow of this study is shown in Fig. 1. The mouse hippocampal neuron cells (HT22) purchased from Wuhan university cell bank (Wuhan, China). HT22 cells were cultured in high-glucose DMEM medium with 10% fetal bovine serum and placed in a stable-temperature incubator at 37°C, 5% CO₂, and saturated humidity. The *Ddx3x*-knockdown and empty lentivirus expressed green fluorescent protein (GFP) were purchased from Jikai Gene (Shanghai, China). The sequence of RNA interference screen for the knockdown of *Ddx3x* and GCAAGCAAAGGGCGTTATATC was selected as targeted sequence (Supplementary Fig. 1). The *Ddx3x*-knockdown lentivirus was seeded in HT22 cells with an MOI (multiplicity of infection) of 8 as *sh-DDX3X* group, and the empty lentivirus was used as control group. The cell fluorescence was

observed with a fluorescence microscope after 24–48 h. The cells were counted manually. The cells were harvested after 96 h.

Animals and stereotaxic surgeries

Since previous neuroimaging studies on functional connectivity suggested that social task-related is lack of activation of medial prefrontal cortex (mPFC) [35, 36]. Here, the mPFC brain region of C57/B6J mice was treated with adeno-associated virus (AAV) to reduce *DDX3X* levels and generate an ASD-like phenotype mouse. Wild type (WT, C57BL/6J strain) were obtained from Bestest Biotechnology company (Zhuhai, China). All the animals were housed in an air-conditioned room (22 ± 2°C, 12-h light, and 12-h dark cycle) with food and water ad libitum. The recombinant AAV including AAV-hSyn-EGFP-*Ddx3x*-RNAi (103688-1) and AAV-hSyn-EGFP-MCS were manufactured by Jikai Gene (Shanghai, China). The target sequence for siRNAs is GCAAGCAAAGGGCGTTATATC. The hSyn promoter, derived from the human *SYN1* gene, is known for its specificity in driving gene expression in neurons [37, 38]. Male 20-days-old WT mice were randomly divided into two groups. The mice (n = 18, male) in control group were injected AAV-hSyn-EGFP-MCS and the mice (n = 20, male) in *sh-DDX3X* group were injected AAV-hSyn-EGFP-*Ddx3x*-RNAi (103688-1). The mice were anesthetized with isoflurane (3% initial dose, 1.5% maintenance dose) and the mouse brains were injected with 2 µL of the AAV at a rate of 0.25 µL/min in mPFC (from Bregma: AP, +1.75 mm; ML, ±0.30 mm; DV, −1.75 mm). Before behavioral tests, mice were allowed to grow to 9-weeks-old of age. Injection sites were confirmed at the end of the behavior experiments and incorrect injection placement were excluded.

Mouse behavioral tests

The open field test. For the open field test, mice were placed in an open field apparatus. Mice were recorded with a video camera for 10 min, and the total distance traveled was calculated using a camera and further

analyzed. This was to exclude confounding factors like motor dysfunction and excessive anxiety, ensuring subsequent observed social abnormalities were specific to ASD-related deficits.

Three-chamber social test

The three-chamber social test was performed as previously described with minor modifications [39, 40], which is a classic assay for evaluating core social deficits in ASD rodent models. Time spent in close interaction with a stranger mouse were recorded for analysis. Target subjects (stranger 1 and stranger 2) were 9-week-old male mice habituated to being placed inside the cages for a week before the test. (A) Habituation: the test mouse was placed in the middle chamber and allowed to explore for 10 min. (B) Sociability: an age- and sex-matched stranger 1 was introduced to the wire cage located on one side. An inanimate object was introduced on opposite side. The test mouse was placed in the center chamber and allowed to explore the entire apparatus for 10 min. (C) Social novelty: a new stranger 2 was replaced to object. The test mouse spent another 10 min in exploring the entire apparatus.

Y-maze test

This assay was used to assess spatial recognition memory, a common cognitive comorbidity in ASD. Mice were food-deprived to 85% of their free-feeding body weight before undergoing the Y-maze test [41]. The Y-maze apparatus included a start arm and two new arms enclosed by a 10 cm high wall. (A) Mice were habituated for 2 days to obtain food from the ends of the two arms. (B) Training trials were conducted three times daily for 4 days. During forced runs, mice were placed in the start arm with one arm blocked and the other open for food access. In test runs, mice chose between the two arms after the block was removed, with correct choices rewarded by food consumption and incorrect choices unrewarded. (C) In tested trials, the interval between forced and test runs was extended to 1 min and 3 min.

Social interaction with a juvenile mouse

This assay was used to quantify micro-level social behaviors and further confirm ASD-related social recognition impairments. An individual tested mouse was placed in a clean and empty arena; stimulus animals were 20 days old male wild-type C57BL/6 J mice. Subjects and stimulus partners were individually housed in a clean cage for 1 h before the test. They were allowed to freely interact for 10 min and recorded behaviors. Parameters were routinely evaluated include nose-to-nose sniffing, nose-to-anogenital sniffing, and following.

Marble burying test (MBT)

To assess ASD's core restricted, repetitive behaviors, the marble burying test was conducted. Experiments were conducted in a 29 × 18 × 15 cm cage with a uniform layer of cereal bedding to a depth of 5 cm. Each mouse was first allowed to explore the cage for 10 min without marbles, and then 15 glass marbles were spaced evenly in a 3 × 5 grid. Each mouse was recorded for 30 min and the glass marbles buried in the bedding (to 2/3 of their depth) were counted [42].

Proteomics analysis

In proteomics research, we used iTRAQ (isobaric tags for relative and absolute quantitation) and SWATH (sequence window to obtain all theoretical fragment ions) proteomics methods for the study of *Ddx3x* knockdown HT22 cells and mouse mPFC tissues, respectively.

Proteomic study of *DDX3X* gene interference cell model based on iTRAQ labeling

iTRAQ analysis was performed as previously described [43]. HT22 cell lysates were prepared using RIPA buffer (Beyotime, China). Proteins from control ($n = 3$) and *sh-DDX3X* ($n = 3$) groups were digested with trypsin, labeled with iTRAQ tags (controls: 115, 116, 117; *sh-DDX3X*: 118, 119, 121), and mixed. The samples were analyzed using a Triple TOF 6500 system (AB Sciex, Foster City, CA) in data-dependent acquisition (DDA) mode. Protein identification and quantification were conducted using ProteinPilot v4.5 (AB Sciex) against the UniProt *Mus musculus* database, with a false discovery rate (FDR) < 1 and a 95% confidence level. Proteins were identified with at least one unique peptide and quantified with at least two peptides.

Proteomic analysis of mPFC tissue by SWATH-MS: protein extraction and filter-aided sample preparation (FASP) digestion

The mouse from *sh-DDX3X* ($n = 8$) and control group ($n = 8$) were anesthetized by isoflurane at 14-weeks-old after behavior test. Sequentially, they were euthanized by cervical dislocation and the brains were collected. The mPFC tissue was dissected from coronal sections using anatomical landmarks and snaped frozen in liquid nitrogen. The mPFC tissue were lysed with RIPA lysis buffer (Beyotime, No. P0013B) and were homogenized with a freeze grinder (Jingxin, Shanghai, China) for 3 min, 90 hz. After incubation in 4 °C for 1 h, the samples were centrifuged at 12,000 × g and collect the supernatant to determine protein concentration with a BCA kit (KeyGen Biotech, China, No. KGP903). Trypsin digestion was performed using a previously described FASP method with slight modifications [44].

Information dependent acquisition (IDA) mode to create reference spectral library for SWATH

A library of reference spectra was required for SWATH-mass spectrum (MS) analysis. We collected 20 µg of peptides from each sample to obtain a pooled sample of all samples for quality control (QC). A liquid phase system was used to carry out liquid chromatography (LC) to collect peptides at 1-min intervals in 20 fractions. The data were acquired by IDA mode in Triple TOF 6600 (AB Sciex) mass spectrometer. Following each survey scan the top 40 most intense ions with multiple charged ions (+2– +5) sequentially.

SWATH-MS analysis

A SWATH-MS analysis was performed in IDA mode, and peptides were separated after enzymatic hydrolysis for each sample [45, 46]. During the IDA detection process, samples from two groups were tested alternately. In subsequent quality control analyses, QC samples were detected every five samples. Following the acquisition process, the Paragon™ algorithm process IDA data files by ProteinPilot version 5.0 (AB Sciex), while SWATH data files undergo processing via PeakView software (SWATH acquisition Micro APP 2.0).

Analysis of proteomic characteristics and identification of differentially expressed proteins (DEPs) between groups

Data were analyzed using ProteinPilot v4.5 (AB Sciex). Principal coordinates analysis (PCoA) was conducted by R package Vegan (2.6–4) and partial least squares discriminant analysis (PLS-DA), orthogonal partial least squares discriminant analysis (OPLS-DA), and permutation test were performed using the SIMCA-P software (version 14.1, Sartorius Stedim Data Analytics AB, Umea, Sweden). The DEPs were calculated using the R package Limma (v3.48.3). The cutoff value for up-regulation was 1.2-fold change and for down-regulation was 0.83-fold change, and P -value < 0.05 was established for DEPs between *sh-DDX3X* and control group.

Bioinformatic analysis

Gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and protein-protein interaction (PPI) network were analyzed by R package clusterProfiler (v4.2.1), STRING (<https://string-db.org/>) and Cytoscape software (V 3.8.2). The Volcano plot and heatmaps were drawn using the ggplot2 (v3.3.5) and R package pheatmap (v1.0.12). The sunburst plots were generated based on synaptic gene ontologies (<https://syngoportal.org>). The hub proteins were calculated by CytoHubba plugin and R package RobustRankAggreg (v1.1).

Western blot analysis

Protein samples were boiled for 10 min in loading buffer and separated by electrophoresis. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, No. IPVH00010, USA) and blocked with 5% non-fat milk in tris-buffered saline (TBS: 100 mM Tris, and 1.5 M NaCl, pH 7.6) containing 0.4% (v/v) tween 20 (TBST) for 1 h. The membrane was incubated overnight at 4 °C with primary antibodies against DDX3X (Abcam, No. ab271002, 1:2000, UK), MAPK1 (Abclonal, No. A0229, 1:1000, China), CAMKII-delta (Proteintech, No. 20667-1-AP, 1:1000, China), NMDAR (Huabio, No. JM11-26, 1:600, China), PSD95 (Proteintech, No. 20665-1-AP, 1:1000), PRRT1 (Proteintech, No. 66031-1-Ig, 1:600), GAPDH (Abmart, No. M20050, 1:5000, China), TUBA (Proteintech, No. 66031-1-Ig, 1:10000), and

VCL (Proteintech, No. 66305-1-Ig, 1:15000). Secondary antibodies included anti-rabbit (Beyotime, No. A0208, 1:1000, China) and anti-mouse (Abmart, No. M21001, 1:5000, China), incubated for 1 h at room temperature. Protein expression levels were normalized to α -tubulin or vinculin. Specific proteins were detected using chemiluminescence with horseradish peroxidase (HRP) substrate (Millipore, No. WBKLS0100), and band intensity was quantified by densitometry using ImageJ software (Bio-Rad, Hercules, CA).

Immunofluorescence detection of representative proteins, morphological analysis of dendritic spines, and the detection of synaptic ultrastructure

Mice were perfused with PBS (phosphate buffered saline) and fixed with 4% PFA (paraformaldehyde) under anesthesia. Brains were postfixed in 4% PFA overnight at 4 °C and dehydrated in 30% sucrose. Coronal sections (20 μ m) were prepared from OCT (optimal cutting temperature compound (Sakura, Japan))-embedded brains using a cryostat (Leica CM 3050S). Sections were washed with PBS, permeabilized with 0.3% Triton-X 100 for 30 min, and blocked with PBS containing 1% BSA, 0.1% Triton-X 100, and 10% normal goat serum. Sections were incubated with primary antibodies against DDX3X, MAPK1, and PSD95 overnight at 4 °C, followed by incubation with a fluorescence-conjugated secondary antibody (Abcam, No. ab150088, 1:200) for 2 h at room temperature. Nuclei were counterstained with DAPI (4',6-Diamidino-2-phenylindole; Beyotime, No. C1002, 1:2000) for 3 min. Confocal images were captured using a Zeiss LSM880 microscope.

For the spine analysis, dendritic were randomly chosen from AAV infected neurons expressing GFP and spines on dendrites were identified and counted in the orthogonal projection images using software Zen blue (ZEISS). Counting of dendritic spines along traced dendrites was performed manually. Stubby spines were defined as short protrusions without a neck, thin as long protrusions without a wide head, and mushroom spines as protrusions with a short neck and a distinguishable head.

To observe ultramicroscopic structure, samples were fixed in 2.5% glutaraldehyde (pH 7.4) for 2 h, washed with 0.1 M phosphate buffer (pH 7.2), and postfixed in 1% osmium tetroxide at 4 °C for 2 h. After gradient ethanol dehydration, samples were embedded in Epon-Araldite resin, sectioned, and stained with 3% uranyl acetate and 2.7% lead citrate. Ultrathin sections were analyzed using a JEM1400 transmission electron microscope (Hitachi, Japan).

Electrophysiology for miniature excitatory postsynaptic currents (mEPSCs)

In brief, mice were deeply anesthetized and transcardially perfused with 95% O₂ and 5% CO₂ oxygenated ice-cold cutting solution. The brain was quickly removed and immersed in ice-cold cutting solution. Coronal brain slices containing mPFC (250 μ m) were cut with a Leica VT1200s vibratome and then maintained at 25 °C in oxygenated artificial cerebrospinal fluid (ACSF) for 1 h until electrophysiological recordings.

Slices were transferred to the recording chamber with oxygenated ACSF (~3 mL/min). Neurons in the mPFC were identified by their location and fluorescence. For whole-cell recording of mEPSCs, pipettes were filled with a solution containing: 135 mM K-gluconate, 5 mM KCl, 0.5 mM CaCl₂, 10 mM HEPES, 2 mM Mg-ATP, 0.1 mM GTP, and 5 mM EGTA, 300 mOsm (pH adjusted to 7.3 with KOH). Bicuculline (10 μ M) and TTX (1 μ M) were used to block the GABAA receptors and Na⁺ channel, respectively. Peak events were detected automatically using an amplitude threshold of 10 pA. The holding potential was -70 mV during mEPSC recording.

All signals were acquired with a MultiClamp 700B amplifier (Molecular Devices, USA), filtered at 1 kHz, and sampled at 5 kHz with a Digidata 1440A interface using Clampex 10.2 (Molecular Devices). Data were accepted when series resistance fluctuated within 15% of initial values (25–35 M Ω). mEPSCs were analyzed using MiniAnalysis 6.07 software (Synaptosoft, Decatur, GA). A 5-min recording duration was used for frequency and amplitude analyses.

Statistical analysis

Statistical analysis was calculated with GraphPad Prism Version 9.0 (GraphPad Software, San Diego, USA). Data were presented as the mean $\pm$ standard deviation (SD). The non-paired and 2-tailed of *t* test was applied to determine whether a significant (*p* < 0.05), and have ensured that the homogeneity of variance and normal distribution status of the data were in accordance with the testing requirements.

RESULTS

The knockdown of *Ddx3x* decreased the growth rate of HT22 cells

The sh-*Ddx3x* and control lentivirus infected HT22 cells successfully and expressed GFP (Fig. 2A). The protein expression level of DDX3X was significantly decreased in sh-DDX3X group (Fig. 2B). As shown in Fig. 2C, the processes of the cells in the sh-DDX3X group developed more slowly than those in the control group, and at the same incubation time, the cells in the control group already appeared to be connected, whereas the cells in the sh-DDX3X group still existed singly. The DDX3X deficiency induced cell growth rate significantly decreased compared to the control group.

iTRAQ-based proteomic analysis of sh-*Ddx3x* HT22 cells and controls

iTRAQ analysis identified a total of 7861 proteins, of which 5451 were quantified (Supplementary Table 1). Unsupervised PCoA analysis revealed a general trend towards separation between the control and sh-DDX3X groups (Fig. 2D). The PLS-DA analysis showed that the two groups were clearly distinguishable, and the model exhibited robustness without overfitting (Figs. 2E and 2F). A total of 242 DEPs were identified between sh-*Ddx3x* HT22 cells and controls, including 114 up-regulated and 128 down-regulated proteins (Figs. 2G, 2H and Supplementary Table 2). Clustering analysis of these DEPs completely distinguished the sh-DDX3X group from the control group (Fig. 2I).

GO analysis indicated that the DEPs were primarily involved in biological processes (BPs) such as protein folding, tRNA metabolic process, Golgi organization, and chaperone cofactor-dependent protein refolding. In terms of molecular function (MF), significant changes were observed in catalytic activity, single-stranded telomeric DNA binding, chaperone binding, and aminoacyl-tRNA ligase activity (Supplementary Fig. 2). KEGG analysis revealed enrichment in pathways including metabolic pathways, mTOR signaling pathway, ErbB signaling pathway, aminoacyl-tRNA biosynthesis, and adherens junction (Supplementary Fig. 3).

Validation of shRNA AAV against *Ddx3x* gene in mPFC of C57/B6J mice

The *Ddx3x* shRNA AAV was delivered to mPFC bilaterally of C57/B6J mice (Fig. 3A). In vivo knockdown was confirmed by the significantly lower level of DDX3X protein in mPFC of sh-DDX3X group by Western blot analysis (Figs. 3B and C). The lower overlap DDX3X and GFP was found in neuron indicating the decrease of DDX3X (Figs. 3D and E).

Behavior test to study the sociability

To investigate the relationship between decreased DDX3X and social deficits, we conducted behavioral tests on the two groups. In the open field test, sh-DDX3X mice showed no significant differences in total distance traveled, number of zone entries, edge time, or edge distance compared to controls (Figs. 3F and G), indicating that reduced DDX3X levels did not affect mouse mobility or anxiety.

In the three-chamber social test, control mice exhibited a preference for a stranger mouse (S1) over a novel object, whereas sh-DDX3X mice showed equivalent interaction times with both (Fig. 4A–D). When a new stranger mouse (S2) replaced the object to test social novelty preference, control mice interacted more with S2 than with the familiar S1, while sh-DDX3X mice did not show a significant preference for S2 (Fig. 4E–G). These results suggest that both social preference and social novelty preference were impaired in the sh-DDX3X group.

Additionally, a food-restricted Y-maze test was used to assess spatial recognition memory. The correct rate between control and sh-DDX3X groups was similar within a 1-min interval, but sh-DDX3X mice exhibited decreased accuracy within a 3-min interval (Fig. 4H).

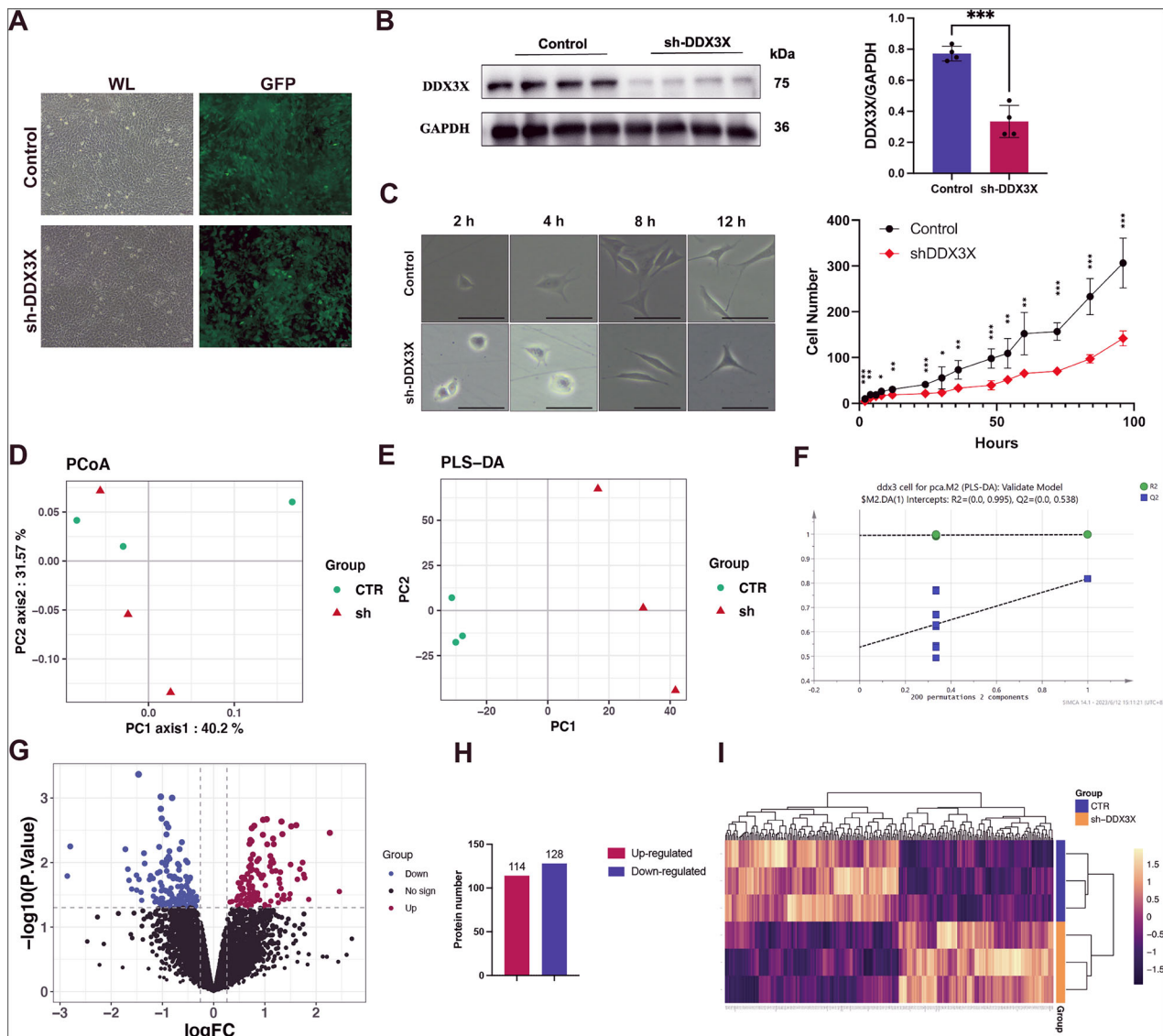


Fig. 2 Lower growth rate in HT22 cells with knockdown of *Ddx3x* and its iTRAQ proteomics results. **A** Images of HT22 cells infected by lentivirus and expressed GFP. **B** Representative images of HT22 cells growth for different time periods and quantification analysis. scale bar: 100 μ m. **C** Western blot bands showing DDX3X protein levels in HT22 cells compared to control group. **D** PcoA plot between control and *sh-DDX3X* group. **E** PLS-DA score plot between control and *sh-DDX3X*. **F** PLS-DA validation plot (permutation = 200). **G** The volcano plot of DEPs from iTRAQ analysis. **H** The number of up-regulated and down-regulated DEPs from iTRAQ analysis. **I** The cluster heatmap of DEPs from iTRAQ analysis. WL: white light. sh: *sh-DDX3X*, CTR: control.

and I), indicating impaired spatial recognition memory in *sh-DDX3X* mice over longer intervals.

In social interaction tests with juvenile mice, *sh-DDX3X* mice displayed a significant reduction in nose-to-anogenital contacts compared to controls (Fig. 4J) and tended to initiate fewer such interactions (Fig. 4K). However, no significant differences were observed in social sniffing, time spent sniffing, following behavior, or time spent following between the two groups (Fig. 4L–O). Finally, in the marble burying test, *sh-DDX3X* mice buried more marbles than controls (Fig. 4P), suggesting increased repetitive behaviors in this group.

The results of proteomic analysis the medial prefrontal cortex in *Ddx3x* gene knockdown mice

A total of 3087 quantitative proteins were identified by SWATH-MS analysis, of which 2480 quantitative proteins met the quality control requirements after QC correction (Supplementary Table 3). In unsupervised PcoA analysis, there was some overlap between

sh-DDX3X and control groups despite the general trend towards separation of protein profiles, and QC samples cluster tightly together indicating the reliability and stability of the data (Fig. 5A). The contribution of variables was characterized using the supervised OPLS-DA model, which showed that there were significant differences between proteins of *sh-DDX3X* and control group and the model was validated by 200 times permutation tests, which showed low Q^2 values and a negative intercept of the regression line on the y-axis, indicating that the model was robust and not over-fitted (Figs. 5B and 5C).

Volcano plot analysis revealed that a total of 285 DEPs were identified including 265 down-regulated proteins, and up-regulated 20 proteins (Figs. 5D, E, and Supplementary Table 4). These DEPs were used for clustering analysis, the results showed that the majority of *sh-DDX3X* group could distinguished from the control group (Fig. 5F).

GO analysis showed that DEPs, in BP network, have significant alteration in small molecule catabolic process, carboxylic acid catabolic process, response to oxidative stress, peptidyl-serine

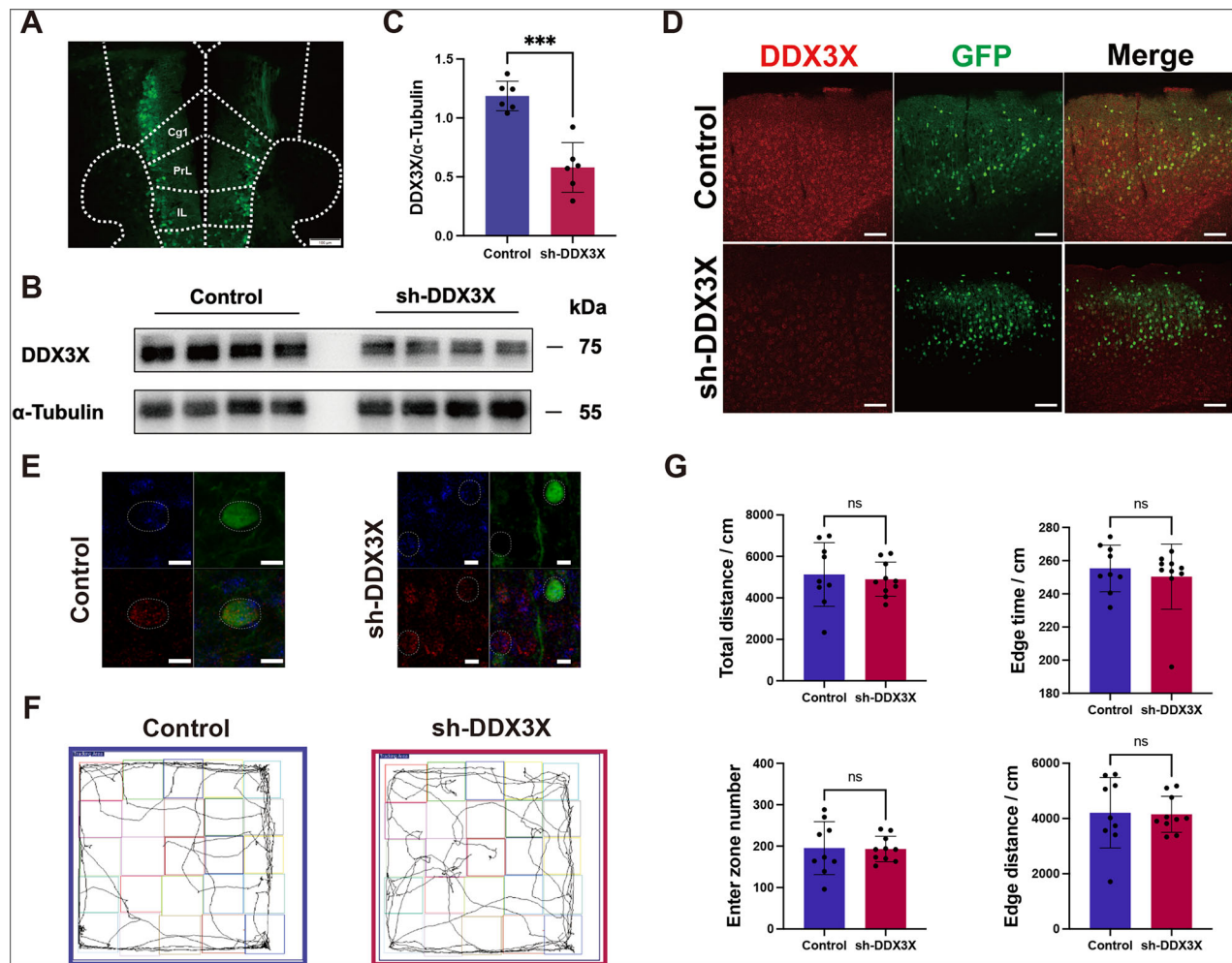


Fig. 3 DDX3X expression is significantly decreased in mPFC of mouse which exhibited social deficiency. **A** An image showing the viral-infected mPFC region (expressed GFP) from a mouse with the stereotaxic injection of *Ddx3x* shRNA AAV. Scale bar: 100 μ m. **B** and **C** Western blot data showing DDX3X protein levels in mPFC of mice in *sh-DDX3X* group compared to control group. **D** The confocal images of immunostaining of DDX3X (red) in mPFC neurons infected with *Ddx3x* shRNA AAV. **E** The representative image of DDX3X in cell between *sh-DDX3X* and control group. Scale bar: 10 μ m. **F** Representative motion map of control and *sh-DDX3X* group mice in open field test. **G** Results of total distance, enter zone number, edge time, and edge distance in open field. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed t test.

phosphorylation, and generation of precursor metabolites and energy, etc. Several network clusters in cellular components (CC) showed presynapse, growth cone, postsynaptic density, proteasome accessory complex, actomyosin, and mitochondrial matrix, etc. In MF network, the pathways including lyase activity, scaffold protein binding, ubiquitin protein ligase binding, and endopeptidase regulator activity, were significantly enriched in the *sh-DDX3X* group (Supplementary Fig. 4).

KEGG analysis showed that the DEPs were mostly enriched in valine, leucine and isoleucine degradation, carbon metabolism, long-term potentiation (LTP), Parkinson disease, and pathways of neurodegeneration - multiple diseases, cholinergic synapse, oxytocin signaling pathway, and GABAergic synapse, etc. The network clusters showed that LTP has strong correlation with other significant pathways (Fig. 5G).

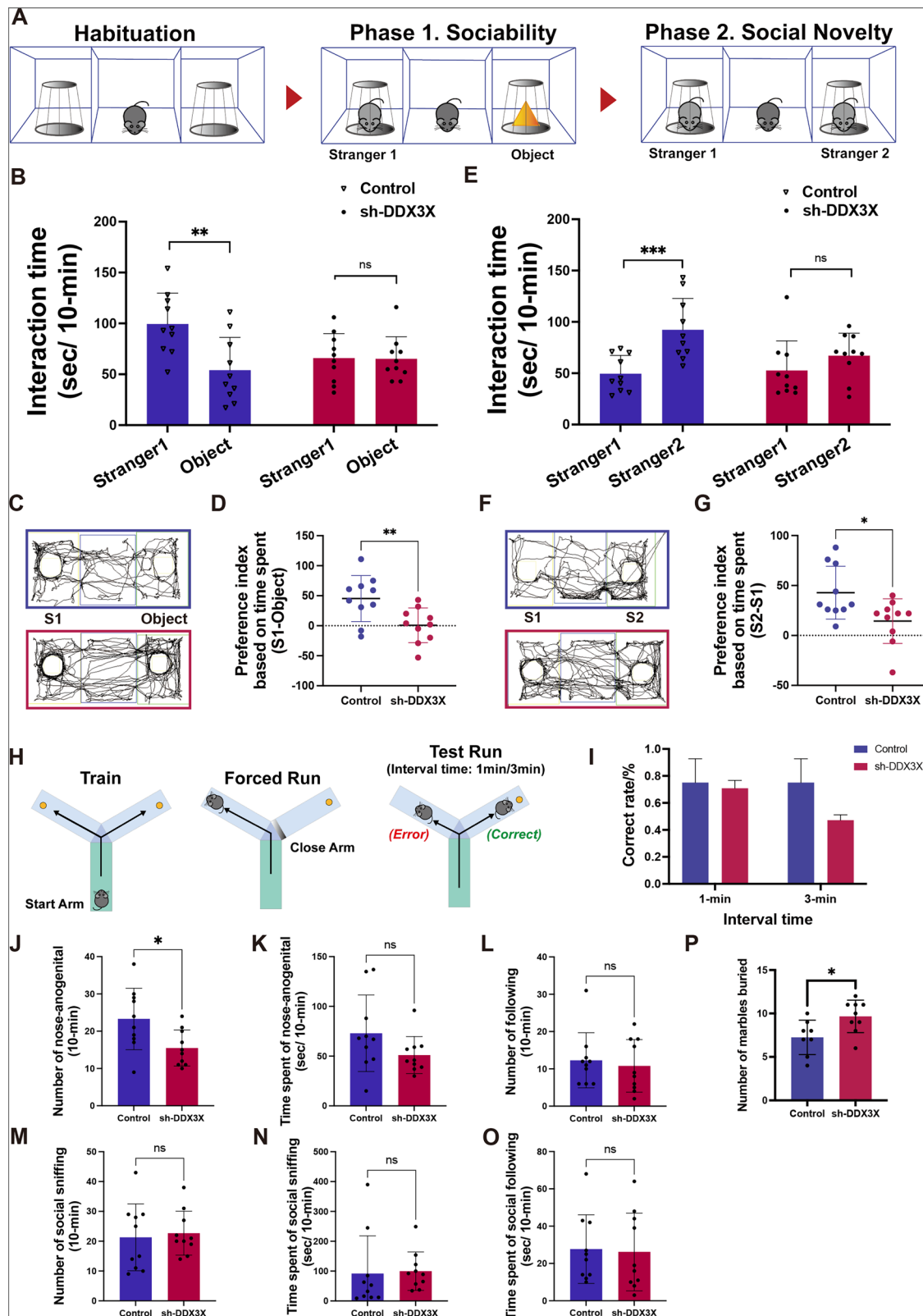
We calculated the z-scores of KEGG pathways related to ASD, including valine, leucine, and isoleucine degradation, LTP, oxytocin signaling pathway, GABAergic synapse, long-term depression, ErbB signaling pathway, glutamatergic synapse, mTOR signaling pathway, synaptic vesicle cycle, and autophagy-animal pathway. These pathways were found to be inhibited in the *sh-DDX3X* group (Fig. 5H). The relationship of proteins involved in LTP, glutamatergic synapse, and GABAergic synapse was showed

in PPI network, and PKCA, PRKACA, MAPK1, MAPK3, CACNA1A, and GNG4 were repeated among these pathways (Fig. 5I). The results suggest that a series of pathways were not sufficient activation with deficiency of DDX3X.

We further annotated the DEPs obtained from the mouse cortex using the synaptic protein annotation database. The results showed that 49 DEPs were mapped to 49 unique SynGO annotated genes. The sunburst plot showed that 23 DEPs were categorized into presynapse, and 9 DEPs were enriched in synaptic vesicle compared to control group (Fig. 5J). Twenty-eight (28) DEPs were categorized into postsynapse, and 11 DEPs were enriched in postsynaptic density (PSD) by DDX3X deficiency (Fig. 5K). The PPI network revealed the interplay between DEPs originating from the PSD, integral of postsynaptic membrane, postsynaptic ribosome, and postsynaptic actin cytoskeleton (Fig. 5L). These results indicated that the deficiency of *Ddx3x* expression in the mPFC impacted synaptic signaling and structures, which may underlie the abnormal behavioral phenotypes observed.

The shared dysregulated pathways observed in mouse brain and HT22 cells with decreased DDX3X expression

An integrated analysis of the proteomic data from HT22 cells and mouse cortex revealed that 9 proteins were commonly



dysregulated, including *Pebp1*, *Osbp18*, *Echdc1*, *Pdap1*, *Ppia*, *Sod1*, *Prdx5*, *Atp6v1g1*, and *Myg1*. Among them, the expression of *Echdc1*, *Osbp18*, and *Myg1* exhibited consistent down-regulation (Figs. 6A and B). These overlapping DEPs were enriched in acetylation and antioxidant pathways (Fig. 6C).

Consequently, we calculated z-scores to assess the activation or inhibition of shared and significant pathways in both proteomics results. Based on GO analysis, 16 overlapping pathways were identified, of which 7 pathways including chaperone-mediated protein folding requiring cofactor, chaperone-mediated protein

Fig. 4 The results of behavior test involved sociability between control and *sh-DDX3X* group. **A** The flow diagram of three-chamber social test. **B** The interaction time in testing sociality. **C** Representative motion map of control and *sh-DDX3X* groups mice in testing sociality. **D** The preference index in testing sociality. **E** The interaction time in testing social novelty. **F** Representative motion map of control and *sh-DDX3X* groups mice in testing social novelty. **G** The preference index in testing social novelty. **H** The flow diagram of appetitively-motivated Y-maze test. **I** The correct rate of Y-maze test in differently interval time. **J–O** Results of number of nose-anogenital, time spent of nose-anogenital, number of social sniffing, time spent of social sniffing, number of followings, and time spent of following in juvenile mouse interaction test. **P** Results of marble burying test. In the three-chamber social test (**B–G**), the Y-maze (**I**), the social interaction with a juvenile mouse (**J–O**), the number of mice was Control:*sh-DDX3X* = 10:10, whereas in the marble burying test (**P**) this number was 8:9. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed t test.

folding, 'de novo' posttranslational protein folding, 'de novo' protein folding, unfolded protein binding, hydro-lyase activity, and carbon-oxygen lyase activity were inhibited in the context of deficiency of DDX3X (Fig. 6D). Moreover, four overlapping pathways were found in KEGG analysis, i.e., ubiquitin-mediated proteolysis, EGFR tyrosine kinase inhibitor resistance, mTOR signaling, and ErbB signaling pathway. Among these, the ErbB signaling pathway and the ubiquitin-mediated proteolysis pathway were both inhibited compared to the control group (Fig. 6E). The PPI network analysis showed that DEPs involved mTOR signaling pathway, EGFR tyrosine kinase inhibitor resistance, and ErbB signaling pathway exhibited stronger interaction. Additionally, DEPs from the ubiquitin-mediated proteolysis pathway consistently showed significant inhibition in both mouse and HT22 cells (Fig. 6L).

The decreased expression of proteins related to synaptic plasticity

The top 20 hub proteins identified by CytoHubba and robust rank aggregation included MAPK3, MAPK1, MAP2K1, PSD95, PRKCA, and PRKACA, which were belonged to LTP and glutamatergic synapse pathways (Fig. 7A). Seven key proteins, namely MAPK1, PKCA, PSD95, PRRT1, GRIA2, GRIN1, and CAMKIID, were selected for Western blot analysis validation. Among these, MAPK1, PKCA, GRIA2, GRIN1, and CAMKIID were involved in the LTP pathway, while PSD95 and PRRT1 were associated with the postsynapse according to SynGO analysis. Their expression levels were down-regulated compared to the control group, consistent with the proteomics data (Figs. 7B and C). Furthermore, immunofluorescence staining was employed to examine the expression of MAPK1 and PSD95. Their fluorescence intensity in the *sh-DDX3X* group was significantly lower than that in the control group, which corroborated the proteomics findings (Figs. 7D and E).

The alteration of synapse structure and decreased excitatory induced by the deficiency of DDX3X

Confocal microscopy revealed a significant decrease in total spine density in the *sh-DDX3X* group compared to controls (Fig. 7F–G), with reductions specifically observed in stubby and mushroom-type spines (Fig. 7H–I), but not in thin spines (Fig. 7J). This suggests that DDX3X deficiency selectively impacts mature spines. Transmission electron microscopy showed that DDX3X deficiency in excitatory synapses reduced PSD thickness and area (Fig. 7K–L), while PSD length and synaptic cleft width were unaffected (Fig. 7M–O), indicating a modest impact on synaptic structure. Moreover, mEPSC recordings in the mPFC demonstrated a significant reduction in frequency but not amplitude in the *sh-DDX3X* group compared to controls (Fig. 7P–R). These findings collectively suggest that DDX3X deficiency impairs synaptic structure and function, leading to synaptic dysplasia.

DISCUSSION

The DDX3X gene encodes a conservatively expressed ATP-dependent DEAD-box RNA helicase that play an important role in mammalian development [17, 47]. The mutation of DDX3X

contributes to a significant portion of ASD patients associated with ID, global developmental delay, and epilepsy [3]. At present, there are rare reports about DDX3X deficiency in animals and cultured neuronal cells in vitro. In this study, we selected an effective targeted sequence to knockdown *Ddx3x* expression. In HT22 cells, DDX3X deficiency significantly inhibited cell growth. In mice, *Ddx3x* knockdown did not affect open field behavior but decreased social preference and social novelty preference in the three-chamber test. Additionally, the *sh-DDX3X* group exhibited impaired spatial recognition memory in the Y-maze test and increased anxiety and repetitive behaviors in the marble burying test. These results indicate that knockdown of the *Ddx3x* gene in the mPFC of male mice induces social deficits and repetitive behaviors. These phenotypes are similar to the reduced social interaction observed in *Ddx3x* insufficient mice [29] and the decreased social cohesion reported in *ddx3x*-deficient zebrafish [16] in previous studies, suggesting that DDX3X deficiency may contribute to ASD-like behaviors and moderate social deficits in mice.

The proteomic analysis of *sh-Ddx3x* HT22 cells revealed that DEPs between the *sh-DDX3X* group and the control group were predominantly linked to pathways associated with protein folding, viral genome replication, tRNA metabolism, and DNA binding. These findings are consistent with previous studies on DDX3X-related RNA transcriptional dysregulation [21, 48, 49]. Proteomic analysis of the mPFC in *Ddx3x* knockdown mice revealed that DEPs were primarily enriched in pathways associated with neurological diseases, including valine, leucine, and isoleucine degradation, long-term potentiation, Parkinson's disease, GABAergic synapses, and glutamatergic synapses.

Valine, leucine, and isoleucine are branched-chain amino acids (BCAAs) that are essential for protein synthesis and various physiological processes. They serve as key metabolic intermediates in mitochondrial metabolism, influencing glucose and energy metabolism, fatty acid metabolism, and acting as amino acid donors for glutamate synthesis in the brain [50]. BCAA degradation involves a series of enzymatic reactions that ultimately produce ATP. The initial step of BCAA catabolism is catalyzed by two isoenzymes, BCAT1 and BCAT2, with BCAT2 deficiency linked to developmental delays and ASD [51]. In this study, BCAT1 was down-regulated in the *Ddx3x* knockdown group. Elevated levels of valine, leucine, and isoleucine have been observed in the brains of individuals with ASD [52] and in the hippocampus of valproate (VPA) and thalidomide (THAL)-induced ASD mouse models [53], potentially due to blocked metabolic pathways leading to BCAA accumulation. Our results indicate that reduced DDX3X levels in the mPFC can alter the expression of proteins involved in valine, leucine, and isoleucine degradation pathways, causing BCAA metabolic abnormalities and contributing to social deficits and memory decline in mice.

In the LTP pathway, intracellular kinases, including MAPK1, MAP2K1, MAPK3, CAMKIID, CAMKIIIB, PKCA, and PRKACA, were significantly reduced, while gated channel proteins and ion receptors, such as CACNA1A, GRIA2, and GRIN1, were down-regulated. Numerous studies have suggested that MAPK signaling is broadly altered in ASD [54–58]. Dysfunction of the MAPK

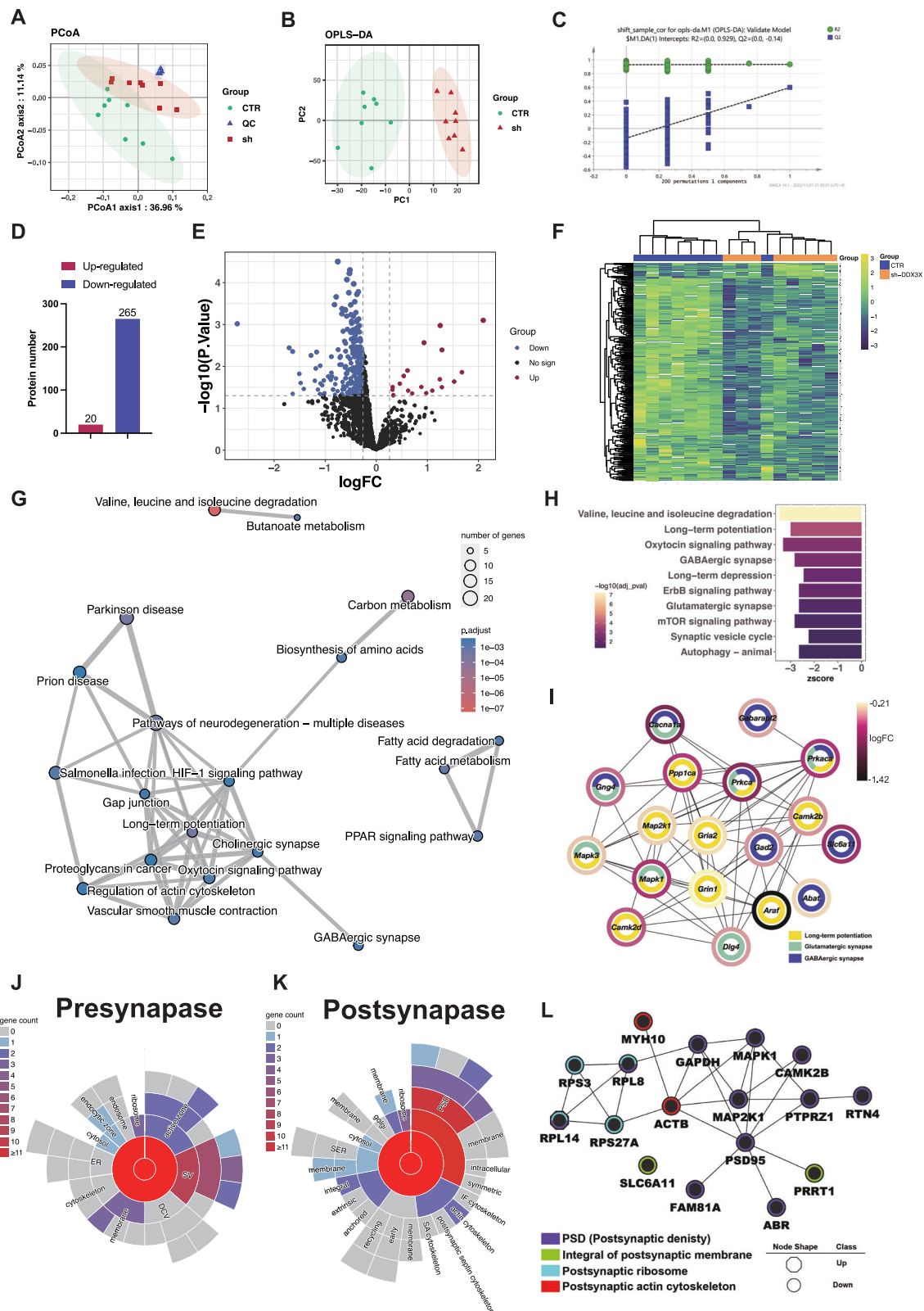


Fig. 5 Proteomics analysis identified proteins profile of DDX3X deficiency in mPFC. **A** PcoA plot among QC, control and *sh-DDX3X* group. **B** OPLS-DA score plot between control and *sh-DDX3X*. **C** OPLS-DA validation plot (permutation = 200). **D** The number of up-regulated and down-regulated DEPs. **E** The volcano plot of DEPs. **F** The cluster heatmap of DEPs. **G** The network plot of enriched pathways based on KEGG analysis. **H** The z-score of ASD-related pathways based on KEGG analysis. The positive and negative z-score means the pathway is activated and inhibited, respectively. **I** The PPI network among LTP, glutamatergic synapse, and GABAergic synapse. **J** The sunburst plot of DEPs in presynapse. **K** The sunburst plot of DEPs in postsynapse. **L** The PPI network from four term based on SynGO. CTR: control, sh:*sh-DDX3X*.

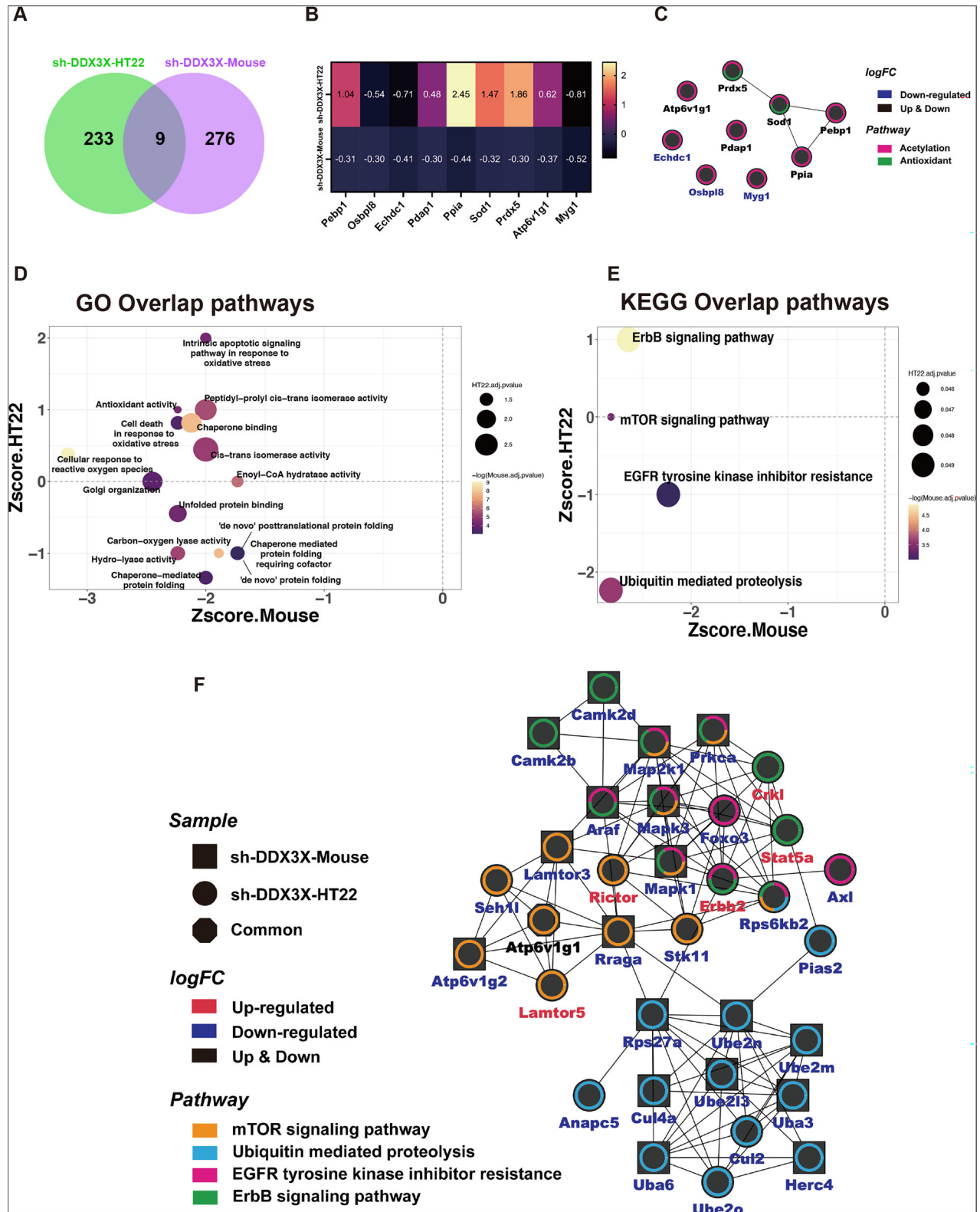


Fig. 6 Combined analysis of proteomics between mouse and HT22 cells with decreased DDX3X. **A** The Venn diagram of overlapping DEPs between mouse and HT22 cells. **B** The $\log_2FC$ value of overlapping DEPs. **C** The PPI network of overlapping DEPs. **D** The scatter plot based on the z-score of overlapping pathways from GO analysis. **E** The scatter plot based on the z-score of overlapping pathways from KEGG analysis. **F** The PPI network of DEPs from four overlapping pathways from KEGG analysis. The positive and negative z-score means the pathway is activated and inhibited, respectively.

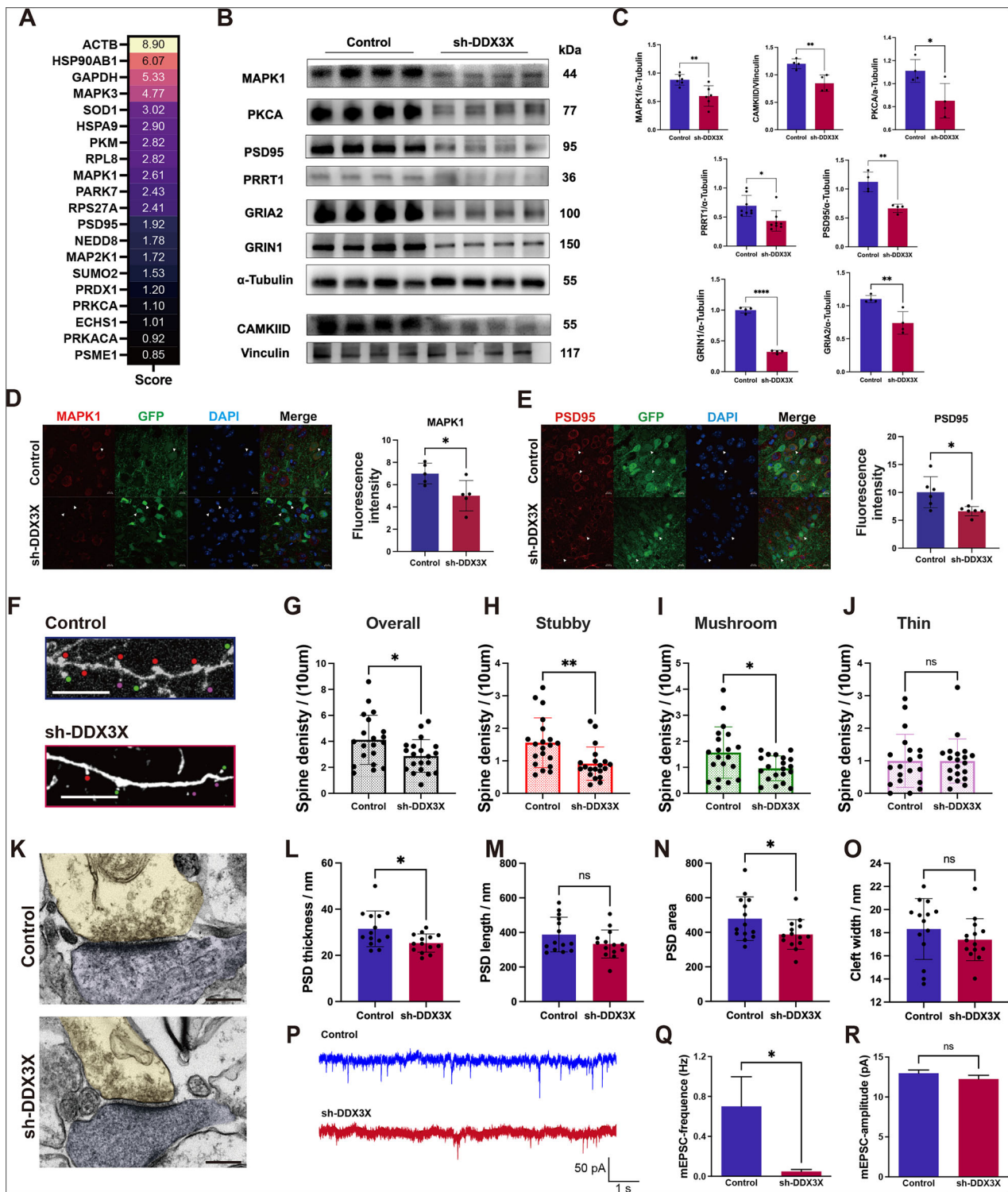


Fig. 7 Validation of SWATH proteomics and morphological changes of the synapse. **A** The rank of hub proteins identified by robust rank aggregation. **B** Representative images of Western blot. **C** Quantification of Western blot bands. **D** The representative confocal images of MAPK1 staining and quantification of fluorescence intensity. Scale bar: 10 μ m. **E** The representative confocal images of PSD95 staining and quantification of fluorescence intensity. Scale bar: 10 μ m. **F** The representative images of dendrite spine. Scale bar: 10 μ m. **G** The quantitative analysis of the total spine density. **H–J** The density of the stubby-, mushroom-, and thin-shaped spines. **K** Representative transmission electron microscopy image of the synapse ultrastructure. Scale bar: 200 nm. **L–O** The quantitative analysis of PSD thickness, PSD length, PSD area, and cleft width. **P** Representative samples of mEPSC from control and sh-DDX3X group. **Q–R** Quantitative analyses of the mEPSC frequency and amplitude ($n = 10$ from 3 mice per group) from control and sh-DDX3X group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-tailed t test.

pathway may contribute to synaptic pathology in ASD-like phenotypes [59, 60]. In addition, several physiologically relevant MAPK substrates have been identified at synaptic sites, including key synaptic scaffold proteins (PSD-95 and PSD-93), Kv4.2 K⁺ channels, and metabotropic glutamate receptors [61]. Activation of the Ras-ERK pathway by NMDA induces a series of transmissions that influence postsynaptic scaffolding proteins, such as PSD-95 [62]. In this study, the down-regulation of MAPK1 and PSD95 in the *sh-DDX3X* group suggests an underlying relationship between kinase activity and scaffold protein function.

The CaMKII family is a central molecule involved in organizing crucial events for synaptic plasticity, including receptor trafficking, localization and activity, and actin cytoskeletal dynamics [63]. While CAMKII β has been implicated in neurodevelopmental disorders, CAMKII δ has rarely been reported in this context [64, 65]. Maintaining the activity of these protein kinases is essential for synaptic excitation and dendritic spine remodeling, including increased spine volume, stability, and clustering [55, 63, 66–69].

Previous studies have shown that DDX3X deficiency alters the balance of glutamate neurons in the developing mouse cortex [34]. The interplay among glutamate receptors, CaMKII, postsynaptic density, and MAPK is critical for regulating learning and memory, as well as synaptic plasticity [59]. Additionally, polymorphisms in CACNA1A have been associated with ASD in Chinese populations [60]. Here, the reduced expression of MAPK1, CAMKII δ , CAMKII β , and PKCA in the *sh-DDX3X* group, which are involved in LTP, was validated by immunoblotting or immunofluorescence. Collectively, these results demonstrate that DDX3X deficiency is associated with a widespread down-regulation of proteins involved in synaptic plasticity. This finding further highlights the key role of the CaMKII family, or kinase-like proteins, in neuroregulation.

Eight DEPs in the GABAergic signaling pathway were significantly down-regulated, namely GABARAPL2, SLC6A11, PRKCA, GNG4, PRKACA, CACNA1A, GAD2, and ABAT. Among them, GAD2 and ABAT are associated with GABA production and metabolism. GAD2 is a regulatory target for cellular activity in various brain functions and GABA-related neurological diseases, catalyzing the conversion of glutamate to GABA. ABAT is a key enzyme responsible for GABA catabolism [70]. This suggests that *Ddx3x* knockdown may lead to GABA concentration dysregulation and metabolic disturbances in cortical neurons. Changes in GABAergic neurons in the cerebral cortex and striatum are common in autism and schizophrenia [71]. However, in the present study, GABA-related receptors, such as GABRA1 and GABRA3, showed no significant changes. This is consistent with a previous study indicating that GABAA receptors are normal in number and function in individuals with ASD and three types of autism model mice. The autism phenotype may result from other disruptions in the GABAergic pathway, such as reduced GABA release [72]. Therefore, the reduced social behavior due to down-regulated *Ddx3x* gene expression is likely more related to impaired GABA release than to GABAergic receptors [73].

SynGO analysis revealed that DEPs were enriched in PSD, integral of postsynaptic membrane, postsynaptic ribosome, and postsynaptic actin cytoskeleton. In the context of DDX3X deficiency, the protein expression of PSD95, PRRT1, and SIPA1L1 was significantly decreased. PSD95 is crucial for the formation and function of dendritic spines. It can anchor receptors on the postsynaptic membrane, thereby regulating the quantity, transport, and localization of NMDAR and AMPAR in the postsynaptic membrane [74–78]. PSD95 mutations have been implicated in ASD [79, 80]. PRRT1 regulates AMPAR levels and membrane trafficking [81], while SIPA1L1 is essential for spine growth, synaptic contraction, and the PSD-95/NMDAR complex [82]. The reduced levels of NMDAR and AMPAR in the postsynaptic membrane due to *Ddx3x* knockdown may decrease neuronal

excitability and impair synaptic plasticity [83, 84]. This aberrant synaptic function could disrupt synaptic homeostasis and increase ASD risk.

Indeed, many ASD-related genes encode synaptic proteins or are involved in chromatin remodeling, transcription, protein turnover, and cytoskeleton dynamics. Changes in these proteins can alter synaptic strength and structure, reduce plasticity, and impact neuronal connectivity in the brain [85], and inadequate neuronal connectivity and synaptic development in specific brain regions are considered significant pathogenic factors in ASD [86]. Our results suggest that DDX3X deficiency impairs neurodevelopment and synaptic plasticity by altering the protein profile. Consistently, a previous study showed that silencing *Ddx3x* expression reduced dendritic spine density, decreased mature mushroom-like spines, and increased immature filamentous and irregular spines [17]. These changes may trigger ASD by disrupting synapse formation and neuronal connectivity. Previous studies have shown that the pathological characteristics of ASD are not caused by a single change in synaptic excitation or a single change in synaptic inhibition, but rather by the combined effect of an imbalance between excitation and inhibition (E/I) resulting from alterations in glutamatergic or GABAergic synapses [9, 87–89]. Disruption of the *Ddx3x* gene, which results in a decrease in excitatory synapses and potential impairment of GABA release, may lead to an E/I imbalance and thereby contribute to the development of ASD.

Over all, proteomic analysis of the mPFC revealed that *Ddx3x* knockdown significantly impacts postsynaptic signaling pathways. To verify functional synaptic abnormalities, we conducted electrophysiological recordings and confocal microscopy, observing reduced mEPSC frequency, decreased mature dendritic spine density, and smaller PSD size. These findings align with previous studies showing that *Ddx3x* mutations disrupt LTP-related kinase activity [13], *Ddx3x* regulates Rac1-mediated axonal growth and synaptogenesis [14], and *Ddx3x* haploinsufficient mice exhibit reduced cortical PSD95 and impaired dendritic spine maturation [15]. Collectively, these results confirm that *Ddx3x* deficiency inhibits excitatory synaptic transmission by disrupting postsynaptic structure and function. Importantly, the reduced mEPSC frequency correlates with decreased spine density and may reflect impaired neuronal activity, which is crucial for learning and memory [86].

Integrated analysis of cellular and mouse brain proteomics suggests that ubiquitin-related pathways play a crucial role in ASD pathogenesis. The ubiquitin–proteasome system (UPS) is essential for regulating protein degradation, synapse composition, assembly, and elimination [76], as well as neural development processes such as axonal initiation, dendritic maturation, and synaptic pruning, which are often impaired in ASD [77]. As a regulatory hub intersecting with pathways like mTOR [78], UPS dysfunction can broadly disrupt neural development. Supporting this, brain-specific *Uba6* knockout mice exhibit ASD-like behaviors, including increased anxiety, reduced social interaction, and impaired communication [79], while our model mice showed down-regulated cortical UBA3, UBA5, and UBA6 (Supplementary Table 4).

De novo protein synthesis is critical for synaptic plasticity and memory [80], and the UPS mediates most eukaryotic protein turnover [80, 81]. Protein chaperones prevent misfolding by directing proteins to the UPS or autophagy [82]. Abnormalities in postsynaptic ribosomal proteins, such as RPS27A (which mediates eEF1A ubiquitination for translation termination [83]), may disrupt this balance. Consistent with this, *sh-DDX3X* mice had reduced cortical RPS27A (Supplementary Table 4), and existing studies confirm that DDX3X regulates cortical mRNA translation [13, 34, 84, 85].

These results indicate that *Ddx3x* deficiency coordinately suppresses the ubiquitin-mediated proteolytic pathway (via

UBA3/5/6 downregulation) and de novo protein folding pathway (via RPS27A downregulation). This aligns with previous findings of UPS protein abnormalities in *Ddx3x*-knockdown mice [35] and the disruption of embryonic protein folding pathways by germline *Ddx3x* inactivation [14]. Collectively, these findings suggest that inhibition of the UPS and protein folding pathways may represent a conserved mechanism of *Ddx3x* deficiency, contributing to ASD pathogenesis by disrupting synaptic protein homeostasis.

In summary, this study established a male mouse model with mPFC neuron-specific *Ddx3x* knockdown, suggesting that *Ddx3x* deficiency in the mPFC is associated with abnormal synaptic plasticity and core ASD phenotypes. The findings revealed that *Ddx3x* knockdown can regulate the structure and function of the PSD by affecting the expression of key proteins in postsynaptic signaling pathways; meanwhile, it may exacerbate abnormalities in synaptic protein quality control by inhibiting the UPS and protein folding pathways, a conserved mechanism. These results provide new insights into understanding the potential role of *Ddx3x* in ASD and the pathological significance of synaptic protein homeostasis imbalance.

However, this study has limitations: the absence of rescue experiments makes it difficult to fully establish the causal relationship between *DDX3X* deficiency and ASD-like phenotypes; *Ddx3x*'s role in non-neuronal cells (e.g., astrocytes and microglia) is unexplored; and other ASD-related regions (e.g., the hippocampus and amygdala) lack systematic study. Future work will verify causality via conditional knockout or re-expression experiments, analyze *Ddx3x* across cell types, and extend to female mice and key regions to deepen understanding of *DDX3X*'s mechanisms.

CONCLUSION

In this study, an HT22 cell model with *Ddx3x* gene knockdown and a mouse model with *Ddx3x* gene knockdown in the mPFC were successfully established. The results demonstrated that *Ddx3x* gene knockdown led to retarded growth of HT22 cells and induced autism-like behaviors in mice. Proteomic, morphological, and electrophysiological analyses revealed: DEPs enriched in synaptic plasticity, PSD, and UPS pathways; reduced dendritic spine density/PSD size; and decreased mEPSC frequency. These confirm *Ddx3x* deficiency impairs postsynaptic structure/function, inhibits excitatory synaptic transmission, and disrupts synaptic protein homeostasis (via suppressed UPS and protein folding)—key mechanisms linking *Ddx3x* mutation to ASD. Future studies will verify causality via knockout/re-expression, explore *Ddx3x* in non-neuronal cells and female mice, and clarify how *DDX3X* specifically regulates the expression and function of synaptic plasticity-related proteins as well as their impacts on neuronal communication and network function—deepening insights into *DDX3X*-related ASD pathogenesis.

DATA AVAILABILITY

All raw data and corresponding parameter have been deposited as online resource to the Figshare database with the name: "The Knockdown of *Ddx3x* in mPFC Causes Autistic-like Phenotype in Mice with Altered Synaptic Plasticity". (10.6084/m9.figshare.23684220).

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ACKNOWLEDGEMENTS

We thank the Center for Instrumental Analysis, Shenzhen University. We also grateful to the BioRender website and PowerPoint for providing the image materials.

AUTHOR CONTRIBUTIONS

HZ and XC wrote the original draft and contributed to visualization, methodology, and investigation. XT contributed to writing—review & editing, methodology, and visualization. ZL contributed to writing—review & editing and visualization. HW contributed to writing—review & editing and methodology. QL contributed to writing—review & editing and investigation. XY, MW, and YH contributed to writing—review & editing. XL and XS contributed to writing—review & editing and methodology. DL and YW contributed to methodology. LS contributed to writing—original draft, writing—review & editing, methodology, investigation, supervision, and funding acquisition. All authors read and approved the final manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grant No. 31870825), the Shenzhen Bureau of Science, Technology and Information (No. JCYJ20170412110026229), the Shenzhen-Hong Kong Institute of

Brain Science-Shenzhen Fundamental Research Institutions (2023SHBS0003), and the Graduate Student Innovation Cultivation Foundation of Shenzhen University.

COMPETING INTERESTS

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All methods were performed in accordance with the relevant guidelines and regulations. The study was approved by the Animal Ethical and Welfare Committee of Shenzhen University (Permit Number: AEW-20140615-002). This study did not involve human participants or identifiable human images, so informed consent and consent for publication are not applicable.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41398-026-03945-3>.

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