An autism-linked missense mutation in SHANK3 reveals the modularity of Shank3 function

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Abstract
Genome sequencing has revealed an increasing number of genetic variations that are associated with neuropsychiatric disorders. Frequently, studies limit their focus to likely gene-disrupting mutations because they are relatively easy to interpret. Missense variants, instead, have often been undervalued. However, some missense variants can be informative for developing a more profound understanding of disease pathogenesis and ultimately targeted therapies. Here we present an example of this by studying a missense variant in a well-known autism spectrum disorder (ASD) causing gene SHANK3. We analyzed Shank3’s in vivo phosphorylation profile and identified S685 as one phosphorylation site where one ASD-linked variant has been reported. Detailed analysis of this variant revealed a novel function of Shank3 in recruiting Abelson interacting 1 (ABI1) and the WAVE complex to the post-synaptic density (PSD), which is critical for synapse and dendritic spine development. This function was found to be independent of Shank3’s other functions such as binding to GKAP and Homer. Introduction of this human ASD mutation into mice resulted in a small subset of phenotypes seen previously in constitutive Shank3 knockout mice, including increased allogrooming, increased social dominance, and reduced pup USV. Together, these findings demonstrate the modularity of Shank3 function in vivo. This modularity further indicates that there is more than one independent pathogenic pathway downstream of Shank3 and correcting a single downstream pathway is unlikely to be sufficient for clear clinical improvement. In addition, this study illustrates the value of deep biological analysis of select missense mutations in elucidating the pathogenesis of neuropsychiatric phenotypes.

Introduction
Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders characterized by impaired social communication, poor language development, repetitive...
behaviors, and restricted interests. Genome sequencing has revealed an increasing number of genes responsible for ASD by identification of de novo likely gene-disrupting (LGD) mutations. Missense mutations, instead, have been considered far less informative because most of them are of unknown significance.

LGD mutations in SHANK3 are estimated to contribute to ~1% of all ASD cases [1–3]. Moreover, epigenetic dysregulation of SHANK3 has been reported in up to 15% of individuals with ASD [4]. Thus, SHANK3 is a well-established autism risk gene, and detailed mechanistic studies provide insights into ASD pathogenesis. Individuals with mutations in SHANK3 often manifest intellectual disability, autism, hypotonia, and motor delay [5, 6]. Shank3 serves as a scaffolding protein within the postsynaptic density (PSD) of dendritic spines, which are actin-rich protrusions from dendrites [7]. The protein has a PSD95/discs large/ZO-1 (PDZ) domain that binds guanylate kinase-associated proteins (GKAPs), which further bridge Shank3 to PSD-95, another major scaffold in the PSD [7]. Shank3 also has a large proline-rich domain containing AB11, IRSp53, Homer, and cortactin binding motifs [7–11]. Knockdown or knockout (KO) of Shank3 reduces dendritic spine density and maturation [12–16], whereas overexpression of Shank3 enhances spine development [12, 17–19]. Most excitatory synapses are built on dendritic spines. As expected, loss of Shank3 causes excitatory synaptic transmission deficits [13–16, 20–27]. Such synaptic dysfunction further leads to abnormal behaviors including altered social interaction, motor coordination deficits and repetitive behaviors in Shank3 KO mouse models [13–16, 20, 21, 23, 25–28].

Efforts have been made to elucidate the underlying molecular mechanisms for developing targeted treatments for individuals with ASD due to a SHANK3 mutation. Multiple pathways, including CLK2, Lq channel, mGlur5, and PAK signaling have been reported to be dysregulated upon loss of Shank3 in model systems [28–31]. However, it is unknown if individual dysregulated molecular pathways are independently responsible for a subset of physiological and behavioral phenotypes. If so, this would represent a modularity of Shank3 function. This information is particularly important regarding targeted treatment of SHANKopathies because if phenotypes are indeed independently mediated by distinct modules of Shank3 function, correcting a single downstream molecular defect is unlikely to be sufficient for improving all the symptoms. A modularity of Shank3 function has not been investigated because most, if not all, studies were done using Shank3 heterozygous or homozygous null models. Although these models retain construct validity of LGD mutations, it is difficult to pinpoint the specific contribution of each pathway due to the confounding effects of other dysregulated pathways in the same model. To understand the functional modularity of Shank3, missense variants, each only affecting one residue, could be informative.

Here, by analyzing a missense variant of SHANK3 found in an individual with autism, we identify a downstream effector that interacts with Shank3 in a phosphorylation-dependent manner. We describe generation and characterization of a knock-in (KI) mouse model of this variant with this interaction specifically disrupted. Using this model, we further determine how this effector and its associated pathway mediate a subset of SHANKopathy phenotypes. We demonstrate the modularity of Shank3 function in vivo and highlight the value of studying missense mutations in autism.

Materials and methods

Reagents and resources

For resources such as antibodies and primers, please see Supplementary Table 8 for more information.

Animals

Mice were housed in an AALAS-certified Level 3 facility on a 12-h light cycle. The following available mouse models were used: FVB-Tg(Shank3-EGFP)1Hzo/J (Inhouse, but mice with the same allele on the C57BL6/J background are available at Jackson Laboratory JAX024033), and B6.129-Shank33tm2Gfng/J (JAX: 017688).

To generate 2 × Flag-Shank3 transgenic mice, we used a BAC clone (RP23-278D8) containing a segment of mouse chromosome 15. This BAC clone was modified by recombinering techniques as described previously [17, 32]. Briefly, the sequence containing Kozak (ACCATGG) and 2 × Flag (ACCATGGATTATAAAGATCACGATATCGAG) was PCR amplified after primer annealing and inserted into the first start codon of the Shank3 gene (exon 1). IRES-EGFP sequence (cloned from a plasmid) was additionally inserted between the stop codon and 3′ UTR sequence (exon 22). The modified BAC clone was double digested with NotI/Swal, and the ~75 kb linearized segment with entire Shank3 gene plus ~12 kb 5′ and ~2 kb 3′ was injected into the FVB/N embryos. The primers used for genotyping were designed against EGFP sequence (forward: 5′-ATGGTGAACAGGGCGAGGA G-3′, reverse: 5′-GCGGACCTTGAAGAAGTGCAG-3′).

To generate S6851 KI mice, we employed the CRISPR/Cas9 gene editing method. Briefly, Donor DNAs expressing sgRNA under the T7 promoter were prepared by PCR using pX330 as a template, and two primers (forward: 5′-TTAATACGACTCACCTATAGGCGCAGGTCAGC-3′, reverse: 5′-TTAATACGACTCACCTATAGGCGCAGGTCAGC-3′).
AGAGAGCCCCA GTGGTGCGTTTTAGAGCTAGAAATAGC-3′. The sgRNA for injection were generated using the donor DNA and MEGAscript T7 Transcription kit (Invitrogen). ssODN for homologous recombination repair (5′-CTGGCCA CTCCGCGCCCTGCTGGACCGGAGGGCCAA GTGGTGCGTTTTAGAGCTAGAAATAGC-3′) was designed to have S685 codon (AGC) mutated to isoleucine (ATC, underlined). PAM site and several other synonymous mutations were also introduced into the ssODN for editing efficiency and genotyping convenience. A mixture of Cas9 protein (PNA Bio), sgRNA, and ssODN was injected into pronuclear stage eggs from C57BL/6 J female mice. The eggs were cultivated in KSOM overnight then transferred into the oviducts of pseudopregnant females. Two primers (forward: 5′-AGCCCCAATCACA ACACTCACA-3′, reverse: 5′-TGCCGATCTGTCTCCCAA ACC-3′) were used for genotyping. The founder mice from CRISPR/Cas9 microinjection were backcrossed to WT mice (C57BL/6 J) for three generations to get rid of potential off-target mutations before any experiments. KI-HET indicates mice heterozygous for the knock-in allele. KI-HOM indicates mice homozygous for the knock-in allele.

All procedures to maintain and use these mice were approved by the Institutional Animal Care and Use Committee for Baylor College of Medicine.

**Cell culture and transfection**

HEK293T cells and Neuro-2a cells (obtained and certified from ATCC) were cultured in DMEM (Invitrogen) containing 10% FBS and antibiotics (Penicillin/Streptomycin). siRNAs were transfected using DharmaFECT (Dharmacon) and incubated for 72 h prior to analysis by western blot. Plasmids were transfected using TransIT-293 (Mirus) and left to express for 48 h.

Mouse cortical neurons were prepared from postnatal day 0–1 FVB/N mice and plated in poly-D-lysine coated 12-well plates (5 × 10^5 per well) in Neurobasal medium supplemented with GlutaMAX (Invitrogen), B-27, antibiotics (Penicillin/Streptomycin).

Mouse hippocampal neurons were prepared from postnatal day 0–1 FVB/N mice with indicated genotypes and plated on poly-D-lysine/mouse laminin-coated coverslips (BD Biosciences) in 24-well plates (2 × 10^5 per well) in Neurobasal medium supplemented with GlutaMAX (Invitrogen), B-27, 1% FBS and antibiotics (Penicillin/Streptomycin). At days in vitro (DIV) 7, neurons were transfected with pEGFP-C1 (0.3 μg/well, Clontech) plus empty vector, vectors expressing HA-Shank3 variants (0.7 μg/well), or siRNAs (80 nM final concentration) with lipofectamine 2000.

Rat hippocampal neurons were prepared from E18 Long Evans rat embryos as previously described [33]. Rat neurons were plated at ~1.5 × 10^4 cells/cm^2 on coverslips coated with poly-D-lysine (20 μg/ml) and laminin (3.4 μg/ml) and cultured in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 2 mM glutamine and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). At DIV 6, rat hippocampal neurons were transfected with pEGFP-C1 plus either empty vector or vectors expressing HA-Shank3 variants using the calcium phosphate method [33].

**DNA constructs**

Rat Shank3 complementary DNA (cDNA) was described previously [34]. Full-length cDNA clones for human ABI1, ABI2, WASF1 (WAVE1), WASF3 (WAVE3), DLGAP1 (GKAP), HOMER1, BAIAP2 (IRSp53) were obtained from TransOMIC. Mouse Ctin cDNA was cloned from GFP-cortactin vector (Addgene #26722). Shank3 cDNA was cloned into pCMV-HA vector (Clontech) by EcoRI and BglII restriction enzymes for mammalian cell expression. ABI1, ABI2, CTTN, DLGAP1, HOMER1, BAIAP2 cDNAs were cloned into pcDNA3-cEGFP-DEST vector (a generous gift from Dr. Tiemo J. Klisch) by LR reaction for mammalian cell expression. Shank3 fragment 4 cDNA (aa 668–858) was cloned into a pDEST15 vector (Invitrogen) by LR reaction for bacterial expression. Internal point mutants were made by QuikChange Site-Directed Mutagenesis Kit (Agilent). All constructs were verified by sequencing.

**Mouse brain lysates preparation**

For whole lysates, whole brain or indicated brain regions were lysed in RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with phosphatase and protease inhibitors (GeneDEPOT). S2 (soluble fraction) and P2 (crude synaptosomal fraction) subcellular, and PSD fractions (synaptic fraction after one time Triton X-100 washout) in Supplementary Figure 1b were prepared as described [35]. LDS Sample buffer (Invitrogen) with a reducing agent was added to each lysate followed by a 10 min incubation at 95 °C. Samples were spun down and run on a 4–12% Bis-Tris gel, transferred to a nitrocellulose membrane and blocked for one hour with 5% non-fat milk prior to primary antibody incubation. Unless otherwise
indicated, the samples used for western blot were from whole lysates.

**Phosphorylation mapping of Shank3 in vivo**

Cortex, hippocampus and, striatum of 2xFlag-\textit{Shank3} transgenic mice were dissected out and lysed in TE buffer (10 mM Tris-HCl pH 7.4, 5 mM EDTA). The homogenates were then supplemented with 1\% sodium deoxycholate, sonicated and incubated with ANTI-FLAG M2 affinity gel (Sigma) for 1 h at 4 °C. The beads were eluted by boiling and subjected to mass spectrometry analysis to identify phosphorylation sites. The whole experiment was repeated for 11 times to detect as many phosphorylation sites as possible.

**LC-MS/MS analysis and data validation**

Samples were subjected to SDS-PAGE (NuPAGE 10\% Bis-Tris Gel, Invitrogen). Proteins were visualized with Coomassie Brilliant Blue stain and the target protein was excised from the gel according to molecular size. The individual gel piece was destained and subjected to in-gel digestion using trypsin (GenDepot T9600). The tryptic peptides were resuspended in 10\,\mu\text{L} of loading solution (5\% methanol containing 0.1\% formic acid) and subjected to nanoflow LC-MS/MS analysis with a nano-LC 1000 system (Thermo Scientific) coupled to Orbitrap Elite (Thermo Scientific) mass spectrometer. The peptides were loaded onto a Reprosil-Pur Basic C18 (1.9\,\mu\text{m}, Dr. Maisch GmbH, Germany) precolumn of 2 cm × 100 \mu\text{m} size. The precolumn was switched in-line with an in-house 50 mm × 150 \mu\text{m} analytical column packed with Reprosil-Pur Basic C18 equilibrated in 0.1\% formic acid/water. The peptides were eluted using a 35 min discontinuous gradient of 4–26\% acetonitrile/0.1\% formic acid at a flow rate of 800 nL/min. The eluted peptides were directly electro-sprayed into Orbitrap Elite mass spectrometer operated in the data-dependent acquisition mode acquiring fragmentation spectra of the top 25 strongest ions and under the direct control of Xcalibur software (Thermo Scientific). Obtained MS/MS spectra were searched against the target-decoy mouse RefSeq database in Proteome Discoverer 1.4 interface (Thermo Fisher) with Mascot algorithm (Mascot 2.4, Matrix Science). The precursor mass tolerance was confined within 20 ppm with fragment mass tolerance of 0.5 daltons and a maximum of two missed cleavage allowed. Dynamic modifications of Oxidation, protein N-terminal Acetylation, Destreak and Phosphorylation on serine, threonine and, tyrosine were allowed. The peptides identified from mascot result file were validated with 5\% false discovery rate (FDR) and subject to manual verifications for correct assignment.

**LC-MS/MS peptide quantification**

The PD1.4 result file and the RAW file from MS was then imported to Skyline software [36] in order to carry out relative quantification. Each individual peptide was validated by checking for its ID from PD1.4 result and the missing peptides were selected by the match-by-run approach. The area under the curve (AUC) for each peak was adjusted based on the retention time. Finally, the sum of AUC of top 3 strongest productions per each precursor ion (for each peptide) was calculated.

**λ phosphatase treatment**

Cortices of 2xFlag-\textit{Shank3} transgenic mice (or WT control) were dissected out and immediately lysed in lysis buffer (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.5\% TritonX-100) supplemented with protease inhibitor (GenDEPOT) by dounce homogenizer on ice. The lysate was spun down and divided into two tubes. One was supplemented with 1 mM MnCl\textsubscript{2}, 1 mM DTT, and 1200 U λ phosphatase (NEB). The other one was treated similarly but phosphatase inhibitor instead of λ phosphatase was added at the end. Both tubes were incubated at 30 °C for 60 min. After incubation, immunoprecipitation was performed with ANTI-FLAG M2 affinity gel (Sigma).

**Neuro-2a lysate preparation and western blot**

For total protein abundance analysis, cells were lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1\% NP-40, 0.5\% sodium deoxycholate, 0.1\% SDS) supplemented with phosphatase and protease inhibitors (GenDEPOT). Lysates were cleared by centrifugation (20 min, 15,000 rpm, 4 °C) followed by protein quantification via BCA assay (Pierce). LDS Sample buffer (Invitrogen) with a reducing agent was added to each lysate followed by a 10 min incubation at 95 °C. Samples were spun down and run on a 4–12\% Bis-Tris gel, transferred to a nitrocellulose membrane and blocked for one hour with 5\% non-fat milk prior to primary antibody incubation.

**Immunoprecipitation in HEK293T cells**

Since Shank3 overexpression caused toxicity and interfered with overexpression of other proteins in HEK293T cells, plasmids expressing HA-Shank3 were transfected separately and lysates were mix before immunoprecipitation. Most cell lysis was performed on ice with scraper using NP-40 lysis buffer (150 mM NaCl, 25 mM Tris-HCl pH 7.4, 1 mM EDTA, 1\% NP-40) supplemented with phosphatase and protease inhibitors (GenDEPOT). Cells expressing HA-Shank3 were lysed in deoxycholic acid (DOC) buffer.
(50 mM Tris-HCl pH 9.0, 5 mM EDTA, 1% sodium deoxycholate) supplemented with phosphatase and protease inhibitors (GenDEPOT), dialyzed against dialysis buffer (50 mM Tris-HCl pH 7.4, 0.1% TritonX-100) overnight. Cell debris was removed by centrifugation (20 min at 15,000 rpm, 4 °C). Lysates with different protein overexpressed were then mixed and added to anti-HA agarose beads (Sigma A2095) for 2 h. Beads were then washed 4 × 800 µL of lysis buffer before being eluted in LDS sample buffer (Invitrogen) at 95 °C for ten minutes.

In vitro kinase assay for mass spectrometry

Shank3 fragment 4 (aa 668–858) complementary DNA was cloned into a pDEST15 vector (GST-tagged, Invitrogen) and then transformed into BL21AI (Invitrogen). GST-Shank3 fragment 4 variants were purified through a GST column. 1 µg Shank3 fragment 4 and 0.3 µg of active kinase (ERK2 [Sigma], PKAα [Millipore], GSKβ [SignalChem] or CK2α [SignalChem]) were mixed in 15 µl kinase reaction buffer (40 mM Tris-HCl pH 7.5, 20 mM MgCl2, 0.1 mg/mL BSA, 2 mM dithiothreitol [DTT] or 100 µM ATP [Promega]) and incubated at 30 °C for 2 h. The kinase reactions were terminated by addition of NuPAGE LDS sample buffer and sample reducing agent (Invitrogen) and boiled for 15 min. The boiled samples were subjected to mass spectrometry analysis as mentioned above.

Radioactive in vitro kinase assay

BSA-conjugated Shank3 WT (H-[BSA]Cys-EEDGARR-RAPPPKRAPSTTLR-OH) and S685A mutant peptides (H-[BSA]Cys-EEDGARRRAPPAPRTTLR-OH) were synthesized by Biomatik. 10 µg of each peptide was combined with 250 ng of active PKα (Millipore) and incubated in kinase buffer (50 mM PO4 pH 7.4, 150 mM NaCl, 20 mM MgCl2, 0.1 mg/mL BSA, 1 mM dithiothreitol [DTT]), 1 x phosphatase inhibitor (Roche), 20 µM cold ATP (Invitrogen), and 1.2 µL of 10 mCi/ml 32P ATP (PerkinElmer) in a 50 µL reaction for 1 h at 30 °C. The kinase reaction was terminated by addition of NuPAGE LDS sample buffer and sample reducing agent (Invitrogen) and boiled for 15 min. The samples were run on NuPAGE 4–12% Bis-Tris Gel (Invitrogen). The gel was Coomassie stained (InstantBlue, VWR) for 20 min and exposed to x-ray film (GE) for 1 h.

Drug treatment in primary cortical neurons

On DIV14-18, cortical neurons were treated with 10 µM H89 (Selleckchem) or DMSO (Vehicle control) for 40 min to 1 h. After drug treatment, neurons were quickly washed with PBS and immediately lysed in lysis buffer (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.5% TritonX-100) supplemented with protease and phosphatase inhibitors (GenDEPOT) on ice. The lysate was spun down and the supernatant was pre-cleared by unconjugated Protein-G plus agaroase (Pierce) for 1 h at 4 °C. Monoclonal ABI1 antibody (MBL D147-3) was added at concentration 1:150 and the mixture was incubated at 4 °C overnight with agitation. Next day, Protein-G plus agaroase (Pierce) was added into the lysate-antibody mixture, and the new mixture was incubated at 4 °C for 4 h with agitation. After incubation, agaroase beads with immunoprecipitates were washed with lysis buffer for 4 times and eluted by boiling in NuPAGE LDS sample buffer with a reducing agent (Invitrogen) at 95 °C for 10 min.

Surface plasmon resonance assay

WT and S685I Shank3 fragment 4 (aa 668–858) complementary DNA were cloned into a pDEST15 vector (GST-tagged, Invitrogen) and then transformed into BL21AI (Invitrogen). GST-Shank3 fragment 4 variants were purified through a GST column. Kinetic parameters for binding of recombinant ABI1 (OriGene) to Shank3 fragment 4 variants were obtained using a Biacore3000 SPR instrument. ABI1 was immobilized by amine coupling to a CM5 sensor chip at pH 4.0 to total resonance units (RU) of ~1200. Experiments were performed at room temperature with the flow rate set at 20 µL/min. HBS-EP (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) buffer prepared by GE was used for all experiments. The values of binding constants determined were not dependent on the amount of ABI1 coupled to the chip or on the flow rate. All binding curves were baseline-corrected by subtracting the signal from the blank channel where no protein was coupled. Response curves were fit using the BiaEval program using a 1:1 Langmuir binding model with separate on- and off-rate determinations.

Immunoprecipitation of ABI1, GKAP, or Homer from mouse cortex or striatum

Cortices or striata were dissected out and immediately lysed in lysis buffer (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.5% TritonX-100) supplemented with protease and phosphatase inhibitors (GenDEPOT) by dounce homogenizer on ice. The lysate was spun down and the supernatant was pre-cleared by unconjugated Protein A agaroase (Millipore) or Protein-G plus agaroase (Pierce) for 30 min at 4 °C. Target antibodies were added at concentration 1:50 to 1:150 and the mixture was incubated at 4 °C overnight with agitation. Next day, Protein A agaroase beads or Protein-G plus agaroase beads were added into the lysate-antibody mixture, and the new mixture was incubated at 4 °C for 4 h with...
agitation. After incubation, agarose beads with immunoprecipitates were washed with lysis buffer for 3 times and eluted by boiling in NuPAGE LDS sample buffer with a reducing agent (Invitrogen) at 95 °C for 10 min.

**Behavioral assays**

For behavior assessment, all WT, KI-HET, and KI-HOM mice were derived from heterozygous breeding pairs, with the exception of ultrasonic vocalization test, in which WT and WT, WT and KI-HOM, or KI-HOM and KI-HOM breeding pairs were used to minimize handling and disturbance of the pups before the test. Mice were randomly assigned and group-housed, with two to five animals per cage, immediately after weaning. For majority of the test, more than 20 animals per genotype (male and female combined) were used which in our experience should have sufficient power to detect a moderate phenotypic difference. Experiments were performed during the light cycle, and mice were given inter-test intervals of at least one day in between each test. Before each test, mice were habituated in the test room for at least 30 min. For all the experiments and video analysis, the experimenter was blinded to the genotypes.

**Three-chamber sociability test**

Twelve-week-old mice were tested. Test mice were habituated for 30 min in their home cage in the test room (200 lx, 60 dB white noise). The three-chamber apparatus is a clear Plexiglass box (24.75 x 16.75 x 8.75 inches) with removable partitions separating the box into left, center and right chambers. The age-, sex-, and size-matched C57BL/6 J mice were used as novel partners. Two days before the test, the novel partner mice were habituated to the wire cages (3 inches diameter by 4 inches in height) for 1 h per day. The wire cage with an inanimate novel object inside served as a control. The test mouse was habituated in the chamber for 10 min. After that, the novel partner mouse was placed into one of the wire cages and located randomly in either the right or left side of the chamber. The novel object was placed in the wire cage located on the other side of the chamber. The test mouse was allowed to explore the three-chamber apparatus for another 10 min. The movements were recorded and the total amount of time spent in each chamber was automatically measured by ANY-maze software (Stoelting). The close interaction time, defined by rearing, sniffing or pawing at each wire cage, was measured manually.

**Resident-intruder**

Thirteen-week-old male mice were individually housed for 2 weeks prior to the test. Each singly-housed test male was confronted with an age, size, and sex-matched group-housed stranger WT opponent (C57BL/6 J or A/J) in its home cage for 20 min (C57BL/6 J) or 15 min (A/J). All experiments were video-recorded and analyzed later by BORIS (http://www.boris.unito.it/). Behaviors like allo-grooming, mounting, biting, and attacking were analyzed.

**Tube test**

Fourteen- to 36-week-old mice were tested. The tube test was performed according to a modified protocol from the previous literature [21, 37]. We used transparent plexiglass tubes with 30.5 cm length/3 cm inner diameter for male mice, and 30.5 cm length/2.5 cm inner diameter for female mice. Mice were habituated to walk through the tube two sessions per day for two consecutive days before testing. On the day of testing, mice with different genotypes (same age and gender) were pushed to the middle of the tube and released simultaneously. The mouse that completely retreated first from the tube within the first 6 min of the test was defined as the loser, and the other as the winner. In very rare cases, when no mice retreated within 6 min, the tests were repeated. Each mouse was tested against up to four different mice of a different genotype with a 30 min inter-trial interval. The tubes were cleaned with 75% ethanol between trials. A two-tailed binomial test was used to determine the significance of test score between mice.

**Pup ultrasonic vocalization**

Pup ultrasonic vocalization was recorded on P2, P4, P6, P8, P10, P12, P14, and P16. At the time of recording, a litter was separated from its parents and placed in an empty clean cage with beddings heated to 37 °C on a heat mat. For each recording, a pup was moved with minimal handling into a cup, which was placed into an anechoic, sound-attenuating chamber (Med Associates Inc.). The pup was then allowed to acclimate for 2 min. Subsequently, audio was recorded for 2 min using a CM16 microphone (Avisoft Bioacoustics) with a frequency range of 15–250 kHz. The signals were amplified/digitized using UltraSoundGate 416 H, at a 250-kHz sampling rate and a bit depth of 16. Ultrasonic events were detected by Avisoft RECORDER software (Avisoft Bioacoustics).

**Open field**

Nine-week-old mice were tested. After habituated in the test room (150 lx, 60 dB white noise), mice were placed in the center of a clear, open Plexiglass chamber (40 x 40 x 30 cm), and their behavior was tracked by laser photobeam breaks for 30 min. General locomotor activity was automatically analyzed using AccuScan Fusion software (Omnitech). Distance traveled, rearing activity, and time spent in the center of the arena were analyzed.
Rotarod

Nine- to 11-week-old mice were tested. After 30 min habituation in the test room (150 lx), motor coordination was measured using an accelerating rotarod apparatus (Ugo Basile). Mice were tested for 4 consecutive days, 4 trials each, with an interval of 60 min between trials to rest. Each trial lasted for a maximum of 10 min and the rod accelerated from 4 to 40 rpm in the first 5 min. “Latency to fall” was recorded either when the mouse fell from the rod or when the mouse had ridden the rotating rod for two revolutions without regaining control.

Elevated plus maze

Nine-week-old mice were tested. After 30 min habituation in the test room (150 lx, 60 dB white noise), mice were placed into the center of a Plexiglass cylinder (20 cm in diameter by 30 cm in height) and videotaped for 10 min. The amount of time spent grooming was measured from the videotape.

Self-grooming

Thirteen-week-old mice were tested. After 30 min habituation period in the test room (600 lx, 60 dB white noise), mice were placed into the center of a Plexiglass cylinder (20 cm in diameter by 30 cm in height) and videotaped for 10 min. The amount of time spent grooming was measured from the videotape.

Marble burying

Eleven- to 13-week old mice were tested. Mice were habituated for 30 min in the test room (170 lx, 60 dB white noise). A standard mouse housing cage was 50% filled with clean bedding material and 20 black glass marbles were placed in a 4 × 5 grid pattern on the surface of the bedding. Mice were placed individually into the prepared cage for 30 min. After the mouse was removed, the number of buried marbles were counted, with a marble considered buried if more than 75% of its surface was covered with bedding.

Acoustic startle and prepulse inhibition (PPI)

Eleven- to 12-week-old mice were tested. Mice were habituated for 30 min outside the test room. Each mouse was placed individually in an SR-LAB PPI apparatus (San Diego Instruments), which consists of a Plexiglass tube-shaped holder in a sound-insulated lighted box with 70 dB white noise and allowed to habituate for 5 min. The mouse was presented with eight types of stimulus, each presented six times in pseudo-random order with a 10–20 sec intertrial period: no sound; a 40 ms 120db startle burst; three 20 ms prepulse sounds of 74, 78, and 82 dB, each presented alone; and a combination of each of the three prepulse intensities presented 100 ms before the 120 dB startle burst. The acoustic startle response was recorded every 1 ms during the 65 ms period following the onset of the startle stimulus and was calculated as the average response to the 120 db startle burst normalized to body weight. Percent PPI was calculated using the following formula: (1 – (averaged startle response to prepulse before startle stimulus/averaged response to startle stimulus)) × 100.

Adult ultrasonic vocalization

The procedure is similar to previously described [15] with some modifications. In short, 38- to 40-week-old male mice were given 10–12 days of reproductive experience with a 7- to 8-week-old female partner before recording. The tested male mice were put into the chambers and then allowed to acclimate for 10 min. Next, a novel 7- to 8-week-old C57BL/6 J female was introduced and USVs were recorded for 5 min. USVs within 25–120 kHz range and with a duration more than 2 ms were recorded and analyzed as described for pups.

Mouse hippocampal neuron immunostaining

At DIV 13–14, mouse primary hippocampal neurons were fixed with 4% formaldehyde/4% sucrose in PBS and permeabilizedblocked by 2% goat serum and 0.1% Triton X-100 in PBS (blocking buffer). Samples were then stained with an anti-GFP antibody (Abcam, ab13970) and an anti-HA antibody (Biolegend, 901514) at 4 °C overnight, then secondary Alexa-conjugated antibodies at room temperature for 1 h. Z-stack images were acquired by LSM880 (Zeiss) confocal microscope under the same parameter settings. Dendritic spine analysis was performed using Neurolucida 360 (MBF Bioscience) with default spine category parameters in a blinded manner. Spine density and morphology were measured from secondary dendrites.

Prediction of S685 interacting amino acids on ABI2

While the experimental solution structure of the unbound SH3 domain of ABI2 was deposited into the Protein Data Bank (PDB:2ED0), a structure of the ABI2-SH3 domain bound to the Shank3 was not readily available. Therefore, to extract structural information about functionally relevant amino acid residues, SH3 homologs similar to the target ABI2-SH3 were analyzed. A sequence homology search with HHpred [38] was used to detect homologs with experimental structures bound to their corresponding binding motifs. Default settings were chosen with HHpred to
find homologs against both the sequence of ABI2-SH3 and the binding motif on Shank3 (PPPKRAP). From this search, two homologs were identified: the SH3 domain of β-p21-activated kinase-interactive exchange factor bound to E3 ubiquitin ligase atrophin-interacting protein 4 (PDB:2P4R) [39] and the SH3 domain of the C-terminal Src kinase bound to a proline-enriched tyrosine phosphatase (PDB:1JEG) [40]. The homolog PDB:2P4R had a root mean squared deviation (RMSD) of 0.65 Å against ABI2-SH3 and a sequence identity (SI) of 0.45, while the homolog PDB:1JEG had an RMSD of 0.77 Å and an SI of 0.28. The two homolog SH3 domains were structurally aligned with ABI2-SH3 in PyMOL (Version 1.8, Schrödinger, LLC.), and the Shank3 binding motif was threaded into the homolog binding motifs by replacing the backbone with the corresponding aligned amino acids from Shank3. The resulting overlaid structures of ABI2-SH3 and the threaded homolog binding motifs were used to determine the putative binding pocket and amino acids interacting with S685. Interacting residues were defined as having a heavy atom distance of less than 4 Å from the C-alpha atom of S685.

Whole-cell recording of primary hippocampal neurons

Neurons transfected with pEGFP-C1 (0.3 μg/well, Clontech) plus empty vector, or vectors expressing HA-Shank3 variants (0.7 μg/well) were recorded on DIV 12–15. On the day of recording, coverslips with neurons were transferred from culture media to artificial cerebrospinal fluid (ACSF, in mM) containing 119 NaCl, 26.2 NaHCO3, 11 D-glucose, 3 KCl, 2 CaCl2, 1 MgSO4, 1.25 NaH2PO4 and incubated at the room temperature for 30 min. The whole-cell recording was made from EGFP-positive neurons by using a patch-clamp amplifier (MultiClamp 700B, Molecular Devices, Union City, CA) under infrared differential interference contrast optics. Microelectrodes were made from borosilicate glass capillaries and had a resistance of 2.5–5 MΩ. Data was acquired with a digitizer (DigilData 1440A, Molecular Devices). The analysis software Minianalysis 6.0.3 (Synaptosoft Inc., Decatur, GA) was used for data analysis. Miniature EPSCs (mEPSCs, holding at −70 mV) were recorded in a voltage-clamp mode in the presence of 100 μM Picrotoxin and 1 μM Tetrodotoxin (TTX). The intrapipette solution contained (in mM) 140 potassium gluconate, 5 KCl, 10 HEPES, 0.2 EGTA, 2 MgCl2, 4 MgATP, 0.3 Na2GTP and 10 Na2-phosphocreatine, pH 7.2 (with KOH). Data were discarded when the change in the series resistance was >20% during the course of the experiment. The whole-cell recording was performed at 30 (±1) °C with the help of an automatic temperature controller (Warner Instruments, Hamden, CT).

Striatal slice preparation and whole-cell recording

Acute striatal slices were prepared from 11- to 13-week-old mice. Coronal striatal slices (350 μm thick) were cut with a vibratome (Leica Microsystems Inc., Buffalo Grove, IL) in a chamber filled with cutting solution containing (in mM) 110 choline-chloride, 25 NaHCO3, 25 D-glucose, 11.6 sodium ascorbate, 7 MgSO4, 3.1 sodium pyruvate, 2.5 KCl, 1.25 Na2HPO4 and 0.5 CaCl2. The slices were then incubated in artificial cerebrospinal fluid (ACSF, in mM) containing 119 NaCl, 26.2 NaHCO3, 11 D-glucose, 3 KCl, 2 CaCl2, 1 MgSO4, 1.25 NaH2PO4 at room temperature after the recovery at 37 (±1) °C for 30 min. The solutions were bubbled with 95% O2 and 5% CO2. Whole-cell recording of mEPSCs was done in a similar way as primary hippocampal neurons.

Golgi staining

Standard Golgi-Cox impregnation using the FD Rapid Golgi Stain kit (FD Neurotechnologies) was performed with brains from 18-week-old mice with indicated genotypes. Serial coronal sections (50 μm) were collected and images were acquired by LSM880 (Zeiss) confocal microscope. For of dorsal striatal medium spiny neurons, dendritic spines on the second and third order branches were analyzed. For hippocampal CA1 pyramidal neurons, dendritic spines on the secondary apical dendrites were analyzed. The dendritic spine densities were manually quantified (blinded to genotype) using ImageJ with the length of each dendrite segment determined by Simple Neurite Tracer.

Immunoprecipitation for Shank3 interactome

The whole procedure was essentially the same as described [17] except that only striatal tissue was used here and the sample volume was increased significantly. Briefly, Striata were dissected from 8 animals (5-week-old) per each genotype (WT and EGFP-Shank3 transgenic FVB/N male mice), and crude synaptosomal fraction solubilized with DOC buffer was prepared. Then, 10 mg of lysates were incubated with GFP-Trap beads (ChromoTek) for 2 h at 4 °C. The beads were briefly washed with binding/dialysis buffer (50 mM Tris-HCl, pH 7.4, 0.1% TritonX-100), boiled with NuPAGE LDS sample buffer (Invitrogen), and subjected to mass spectrometry analysis.

Building Shank3–ABI1 interactome

To build Shank3–ABI1 interactome, we first collected human protein interaction data from eight primary databases (BIND, BioGRID, DIP, HPRD, InnateDB, IntAct, MatrixDB and MPPI), nine integrated databases...
(ConsensusPathDB, GeneMANIA, HIPPIE, InWeb, mensa, OPHID, Pathway Commons, STRING and UniHI) and two publications [34, 41]. Shank3 interactors were defined as the union of the interactors detected in our two in vivo immunoprecipitations and the interactors found from the above database collection. ABI1 interactors were derived from the above database collection. Y2H interactions in supplementary tables are based on the iRefIndex database [42] and two publications [34, 41]. The human PSD protein list and mouse PSD protein list we used in supplementary tables are based on two previous publications [43, 44]. The statistical significance of the interactor overlap between Shank3 and ABI1 was measured using Fisher’s exact test. To further build an actin cytoskeleton-related sub-network of Shank3-ABI1 interactome, only the interactions with both protein pairs annotated under GO terms actin cytoskeleton organization (GO:0032956) and regulation of actin cytoskeleton organization (GO:0030036) and regulation of actin were included. The interactions among the interactors of Shank3 and ABI1 were extracted from the above database collection. The sub-network was visualized using Cytoscape [45].

**Gene ontology analysis**

To perform Gene Ontology (GO) enrichment analysis, the ontology and human annotation files were downloaded from the GO database (http://www.geneontology.org). The statistical significance P-values of GO enrichment analysis were computed using the hypergeometric test and corrected for multiple hypothesis testing using false discovery rate control procedure [46]. Only statistically significant GO terms with gene number more than 5 and less than 500 were selected.

**Size-exclusion chromatography and analysis**

Size-exclusion chromatography was performed as described previously with slight modifications [47, 48]. An AKTA purifier UPC10 system form GE with a Superose 6 GL300 column was used on soluble extracts from about 20-week-old WT mouse striatum. The protein extraction buffer used was 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 320 mM sucrose, complete protease inhibitor cocktail and phosphatase inhibitors. The column buffer was 0.1% Triton X-100, 50 mM Tris-HCl pH 8.0, 50 mM NaCl, Thryroglobulin (669 kDa), ADH (150 kDa), and Cytochrome C (12.4 kDa) were used for gel-filtration standards.

Striata from nine FVB/N mice were homogenized in 6 mL extraction buffer using a 10 mL tissue grinder (Wheaton# 358007) and power homogenizer at 900 rpm for 30 strokes. The homogenate was supplemented with 0.5% Triton X-100 and rotated end over end at 4 degrees for 15 min. The homogenate was then spun in a microfuge at 13,200 rpm for 10 min. The supernatant was filtered through a 0.45 μm filter and 700 μL was applied to the column (anywhere from 2–4 mg protein). The column was run at 0.3 mL/minute. 1 mL/fraction was collected with additional protease inhibitor cocktail in each fraction tube.

**Rat primary hippocampal neuron immunostaining**

Rat neurons were fixed and stained on DIV 21 using a similar protocol as mouse neurons described above, except that for ABI1 staining, 10% FBS was used as blocking reagent instead of 2% goat serum. For F-actin staining, Alexa Fluor 555 Phalloidin (Invitrogen, 1:30) was used per manufacturer’s protocol. Z-stack images were acquired by LSM710 (Zeiss) confocal microscope under the same parameter settings. Images were analyzed and quantified using Imaris (Bitplane) in a blinded manner. The ABI1 spine enrichment index was calculated by normalizing ABI1 concentration in the spine (ABI1 integrative fluorescence intensity/GFP intensity in the spine) to ABI1 concentration in the adjacent shaft (ABI1 integrative fluorescence intensity/GFP intensity in the adjacent shaft).

**Striatum subcellular fractionation**

Fractionation was done according to a procedure modified from previously described [49]. Briefly, pooled striata from 6–7 mice were homogenized using 10 mL tissue grinder (Wheaton# 358007) at 900 rpm for 12 strokes in 0.32 M sucrose, 2 mM EDTA and 4 mM HEPES, pH 7.4 with freshly added protease and phosphatase inhibitors (GenDEPOT). Postnuclear supernatants were obtained via 1000 × g spin. The supernatant (S1) was spun at 10,000 × g for 15 min and the pelleted membranes resuspended, re-homogenized, and spun again to obtain P2+. This pellet was lysed by hypoosmotic shock and then resuspended. This was followed by a spin at 25,000 × g for 20 min. The pellet (P3) was layered onto a 0.8/1.0/1.2 M discontinuous sucrose gradient and spun at 150,000 × g for 2 h. Synaptic plasma membranes (SPM) were recovered from the 1.0/1.2 M interface, pelleted, and resuspended. These membranes were extracted with 0.5% Triton X-100 for 15 min and pelleted with a 32,000 × g spin for 20 min (PSD I). Protein concentrations were determined using the BCA assay (Pierce).

**Statistical analysis**

All data in figures were presented as mean ± s.e.m. (standard error of the mean). All comparisons were two-sided. All statistical details and statistical significance, calculated using an unpaired t-test, paired t-test, Mann–Whitney U-test, one-way ANOVA, two-way ANOVA, or
Kruskal–Wallis test, were indicated in the figure legends. Tukey’s, Sidak’s, Dunnett’s, or Dunn’s post hoc tests were used following ANOVA or Kruskal–Wallis test as indicated in the figure legends. For the behavior tests, normality was determined using the D’Agostino and Pearson omnibus normality test and Shapiro–Wilk normality test. Statistical values for the behavioral tests are shown in Supplementary Table 6. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, NS, not significant.

Results

Phosphorylation of Shank3 at S685 modulates its interaction with ABI

Phosphorylation is a common regulatory mechanism to control key protein-effector interactions, and Shank3 phosphorylation has been reported before [50]. Therefore, to identify direct downstream effectors of Shank3, we first examined the phosphorylation state of Shank3 in vivo. As no commercial Shank3 antibody consistently worked for immunoprecipitation, we generated a 2 × Flag-Shank3 transgenic mouse to efficiently purify Shank3 in vivo (Supplementary Figure 1a). We characterized the 2 × Flag-tagged Shank3 and found that it, like endogenous Shank3, was enriched in synaptic fractions (P2 and PSD I) (Supplementary Figure 1b). The regional and temporal expression pattern of 2 × Flag-Shank3 paralleled those of endogenous Shank3 (Supplementary Figure 1c,d). Compared with wild-type (WT) mice, the transgenic mice had a 1.4-fold greater abundance of the α isoform and a 1.3-fold more in total Shank3 in the crude synaptosomal fraction (Supplementary Figure 1e).

We immunoprecipitated 2 × Flag-Shank3 with an anti-Flag antibody from the cortex, hippocampus, and striatum of the transgenic mice (Supplementary Figure 1f). Immunoprecipitation followed by mass spectrometry analysis identified 41 phosphorylated residues of Shank3 in vivo (Fig. 1a and Supplementary Table 1). We next evaluated whether any phosphorylated residues are in close proximity to known Shank3 protein-binding domains/motifs. We found that the phosphorylated site S685 is adjacent to a previously identified proline-rich motif (PRM) that is responsible for Shank3 interaction with the SH3 domain of ABI1, an indispensable subunit of the WAVE regulatory complex (WRC) [10, 51] (Fig. 1b and Supplementary Figure 2a). To further confirm that phosphorylation at S685 occurs in vivo, we generated a phospho-specific antibody to S685 (Supplementary Figure 2b). This antibody recognized purified Shank3 from mouse brain but failed to recognize Shank3 after it was treated with phosphatase (Supplementary Figure 2c). Given S685’s high degree of conservation among vertebrates (Fig. 1c), we considered it highly likely to have functional importance.

To determine whether phosphorylation of S685 modulates the Shank3–ABI interaction, we performed a co-immunoprecipitation assay in HEK293T cells. Consistent with previous findings [10], ABI1 co-immunoprecipitated with WT Shank3 (Fig. 1d). Mutation of the S685 residue of Shank3 to a non-phosphorylatable alanine (S685A) caused significantly less ABI1 to co-immunoprecipitate with Shank3 (Fig. 1d). The S685A mutation also decreased the interaction between Shank3 and the ABI1 homolog ABI2, to a similar extent (Fig. 1e). These results suggest that S685 phosphorylation is important for the Shank3–ABI interaction.

We next sought to identify kinases that can phosphorylate Shank3 at S685. We expanded our previously published datasets of the Shank3 interactome [17] by new immunoprecipitation-mass spectrometry experiments and public data curation, which resulted in a more comprehensive Shank3 interactome consisting of 793 proteins (see Methods and Supplementary Table 2). We identified 30 kinases among the 793 interactors. To narrow the list of candidate kinases, we searched the literature for their expression patterns and functions, which led us to four candidate kinases: Extracellular signal-regulated kinase 2 (ERK2), protein kinase A alpha catalytic subunit (PKAα), glycogen synthase kinase 3 beta (GSK3β), and casein kinase 2 (CK2). Because its large size makes full-length Shank3 difficult to purify from bacteria, we purified a partial fragment of Shank3 (fragment 4, aa 668–858) that included Shank3 PRM from bacteria, and used it in an in vitro kinase assay (Supplementary Figure 2d). Mass spectrometry analysis of the kinase reaction products showed that ~97% of all peptides covering S685 were phosphorylated by PKAα (Fig. 1f and Supplementary Figure 2e), and S685 is located within a consensus PKA phosphorylation motif (RXXS/T) [52]. None of the other three kinases phosphorylated this site.

Co-immunoprecipitation using the striatal tissue of EGFP-Shank3 transgenic mice revealed that Shank3 and PKAα interacted with each other (Fig. 1g). To further validate our mass spectrometry findings, we performed a second kinase assay using synthesized Shank3 peptides (aa 668–691) conjugated with BSA and radiolabeled ATP (see Methods). We found that PKAα phosphorylated WT peptides, while the negative control (BSA alone) and S685A mutant peptides were not phosphorylated (Fig. 1h). To examine whether PKA modulates Shank3–ABI interaction in neurons, we performed co-immunoprecipitation using dissociated cortical neurons while blocking PKA activity. Application of small molecule inhibitor H89 inhibited PKA in cortical
Fig. 1 S685 phosphorylation modulates Shank3–ABI interaction. a Diagram of Shank3 protein (NP_067398) with 41 in vivo phosphorylated residues identified by mass spectrometry. b Diagram of Shank3 protein with S685 (green highlighted) adjacent to a previously identified ABI1 binding site (red text) on Shank3. c Protein sequence alignment of Shank3 surrounding S685 among vertebrates. d, e Immunoblots of ABI1-GFP (d) or ABI2-GFP (e) and HA-Shank3 following immunoprecipitation of HA-Shank3 variants in the co-immunoprecipitation assay in 293T cells (n = 6, n = 6 experiments). f Percentage of peptides with S685 phosphorylation in Shank3 mass-spectrometry-based in vitro kinase assay by ERK2, PKAα, GSK3β, and CK2. g Immunoblots of PKA α-catalytic subunit following immunoprecipitation of EGFP-tagged Shank3 from EGFP-Shank3 transgenic mice striatal lysate. TG, EGFP-Shank3 transgenic mouse. h In vitro kinase assay with radiolabeled ATP using recombinant PKAα and Shank3 peptides including S685. Left, coomassie blue staining of BSA-conjugated peptides (aa 668–691). Right, autoradiography blot of kinase reaction product. i Immunoprecipitation of ABI1 in primary cortical neurons treated with vehicle (DMSO) or H89 (10 μM) followed by immunoblotting for Shank3, WAVE1, and ABI1 (n = 4 experiments). WAVE1-ABI1 co-immunoprecipitation was used as positive control. Veh, vehicle (DMSO). All data are presented as mean ± s.e.m. *p < 0.05, **p < 0.01; paired two-tailed Student’s t-test.
neurons, as indicated by a decrease in CREB S133 phosphorylation (Supplementary Figure 2f). Inhibition of PKA reduced the amount of Shank3 co-immunoprecipitated with ABI1, while not altering ABI1’s interaction with WAVE1, the core subunit of WRC (Fig. 1i). Together, these findings suggest that phosphorylation of S685 of Shank3 by PKA modulates Shank3–ABI interaction.

**An autism-linked S685I mutation specifically diminishes Shank3–ABI interaction**

Because *SHANK3* haploinsufficiency is strongly linked to ASD, we sought mutations in or around S685 that occur in ASD individuals. An existing ASD database, the Autism Sequencing Consortium, reported several missense mutations in *SHANK3* in autistic individuals [53]. We found a G>T substitution at chromosome 22: 51,144,527 (hg build 19), which resulted in an S686I (S685 in mouse) missense mutation. S686I has not been observed in the gnomAD database of over 120,000 individuals without neurologic diseases, indicating it is not a common variant [54]. Moreover, both Polyphen-2 and SIFT mutation analysis algorithms predicted this mutation to be damaging to disease, indicating it is not a common variant [54].

We tested whether the S685I mutation could alter Shank3–ABI interaction. In a co-immunoprecipitation assay, the S685I mutation diminished Shank3 interaction with ABI1 by at least 75% (Fig. 2a). The S685I substitution had a similar effect on Shank3–ABI2 interaction (Fig. 2b). It is worth noting that S685I did not alter Shank3 interaction with several other well-known binding partners, including cortactin, GKAP, Homer, and IRSp53 (Supplementary Figure 3a-d).

To better understand the effect of the S685I mutation, we performed kinetic analysis using surface plasmon resonance (Supplementary Figure 3e). Analysis of the binding curves of Shank3 fragment 4 with full-length recombinant ABI1 indicated that the S685I mutation significantly decreased the on-rate constant and caused a ~4-fold increase in the dissociation constant (Supplementary Figure 3f). Thus, the autism-linked Shank3 S685I variant appears to specifically disrupt Shank3–ABI interaction by changing a critical amino acid in the interacting motif.

**Loss of Shank3–ABI interaction causes altered social behaviors**

To validate our findings and understand the functional importance of Shank3–ABI interaction in vivo, we generated KI mice with the S685I substitution (Fig. 2c, d). Both heterozygous KI (KI-HET) and homozygous KI (KI-HOM) mice are viable and fertile. They also have a similar body weight compared with WT mice (Supplementary Figure 4a). In addition, both protein abundance and isoform-specific expression pattern of S685I Shank3 were not changed compared with WT control (Supplementary Figure 4b).

Next, to confirm that S685I mutation disrupts Shank3–ABI interaction in vivo, we performed a co-immunoprecipitation assay in KI mice. As expected, significantly less Shank3 was co-immunoprecipitated with ABI1 in the cortex of KI-HET and KI-HOM mice compared with WT mice, with KI-HOM showing more prominent effect (Fig. 2e). A similar reduction in Shank3–ABI1 co-immunoprecipitation was observed in the striatum (Fig. 2f).

In addition, Shank3–GKAP and Shank3–Homer interactions were not affected (Supplementary Figure 4c,d). These results indicate that S685I mutation specifically disrupts Shank3’s interaction with ABI1 in vivo.

Because S685I mutation is linked to autism, we next asked whether there are any social deficits in KI mice. We first tested sociability by the three-chamber test [17]. Both WT and KI mice showed a significant preference for a stranger mouse to a novel object (Supplementary Figure 4e), indicating normal sociability.

To determine if there are any specific abnormal social behaviors other than sociability in KI mice, we performed a resident-intruder assay using stranger C57BL/6J mice as intruders [57]. We quantified three categories of social behaviors: active social behavior (sniffing/allogrooming), threatening behavior (mounting), and aggressive behavior (attacking/biting). While there was no difference in threatening and aggressive behaviors, we found an increase in sniffing/allogrooming behavior in KI-HOM mice (Fig. 2g–i). The continuous moving and escaping of the C57 intruders made it difficult to differentiate allogrooming from sniffing. Thus, we repeated the assay using submissive A/J mice as intruders [58]. We found that KI-HOM mice displayed a robust increase in allogrooming behavior on A/J intruders (Fig. 2j and Supplementary Video 1 and 2).

Excessive allogrooming behavior has been associated with increased social dominance [59]. To evaluate social dominance, we performed the tube test. In this test, the dominant mouse will push its opponent out of the tube and “win” the match [21, 60]. We found that both male and female KI-HOM mice won most of the matches when tested against their WT opponents (Fig. 2k), indicating increased social dominance. Only male KI-HET mice displayed the same phenotype (Fig. 2k).

Social communication deficit is another major feature of ASD, especially those caused by mutations in *SHANK3*. To evaluate social communication phenotype in KI mice, we performed isolation-induced pup ultrasonic vocalization (USV) recording. As previously reported [61], the USVs of WT pups peaked around postnatal day 6 (P6) to P8 and stopped around P14 (Fig. 2l). KI-HOM pups, however,
emitted significantly fewer USVs on P2, P4, and P6. Interestingly, on P14, while most WT pups stopped emitting USVs, KI-HOM pups continued to do so. This is not due to eye-opening defects as both WT and KI-HOM pups opened their eyes around P13-P14. These data suggest that USV development is altered in KI-HOM mice.
Fig. 2 The autism-linked Shank3 S685I mutation specifically diminishes Shank3–ABI interaction and causes selective social phenotypes. a, b Immunoblots of ABI1-GFP (a) or ABI2-GFP (b) and HA-Shank3 following immunoprecipitation of HA-Shank3 variants in the co-immunoprecipitation assay in 293T cells (n=4, n=4 experiments). c Schematic diagram for S685I KI mice generation by CRISPR/Cas9. Top: single strand oligodeoxynucleotides used as a repair template; red: codon encoding isoleucine; blue: synonymous mutation for genotyping. Middle: genomic sequence spanning Shank3 S685; green: codon encoding S685; scissors: Cas9 cutting site. Bottom: structure of wild-type mouse Shank3 gene. d Sequencing electropherograms of the S685I allele. e, f Immunoprecipitation of ABI1 in cortical (e) or striatal (f) lysate of KI-HET or KI-HOM mice followed by immunoblotting for Shank3 and ABI1 (n=3, n=3 experiments). g Time spent sniffing or allogrooming the intruder C57 mice in the resident-intruder assay (n=10, 13 mice). h Number of mounting events on the intruder C57 mice in the resident-intruder assay (n=10, 13 mice). i Time spent attacking or biting the intruder C57 mice in the resident-intruder assay (n=10, 13 mice). j Time spent allogrooming the intruder AJ mice in the resident-intruder assay (n=20, 8, 14 mice). k Percentage of wins in test pairs between indicated genotypes in the tube test (n=36, 32, 30, 24 matches). l Number of ultrasonic calls from pups separated from their dams (n=53, 26, 27 pups). All data are presented as mean ± s.e.m. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, NS, not significant; a, b paired two-tailed Student's t-test; e, f paired and unpaired two-tailed Student’s t-test; g, h unpaired two-tailed Student’s t-test; i Mann–Whitney U test; j Kruskal–Wallis test with post hoc Dunn’s test; k two-tailed binomial test; l two-way ANOVA with post hoc Tukey’s test.

We also performed open field assay and rotarod to evaluate motor function; elevated plus maze to evaluate anxiety; self-grooming and marble burying to evaluate repetitive behaviors; acoustic startle and prepulse inhibition to evaluate sensory-gating; adult USV to evaluate social communication in the adult. All have previously been shown to be abnormal in Shank3 null mice [15, 20, 21]. We did not observe any difference between WT and KI mice in these tests (Supplementary Figure 5a-j and Supplementary Table 6). Together, these results show that loss of Shank3–ABI interaction causes abnormal social behavior/social communication that mimic a subset of phenotypes found in mice heterozygous or homozygous for Shank3 null alleles [15, 21].

Shank3–ABI interaction is critical for dendritic spine development and synaptic transmission

To understand how disruption of Shank3–ABI interaction impairs neuronal function at a cellular level, we first investigated the functional relationship between Shank3 and ABI1/2 in dendritic spine development. Loss of either ABI1 or ABI2 leads to reduced spine maturation and spine density [10, 62], similar to the effect of Shank3 deficiency [13, 14, 24]. We created distinct lines of mouse dissociated hippocampal neurons, either overexpressing Shank3, depleting Abi1/Abi2 using siRNAs, or combining the two (Fig. 3a and Supplementary Figure 6a). Analysis of the morphology of dendritic spines revealed that knockdown of Abi1 and Abi2 decreased stubby spine, mushroom-like spine (mature form), and total spine densities (Fig. 3a, b and Supplementary Figure 6b). Overexpression of Shank3 increased stubby spine, mushroom-like spine, and total spine densities, but concurrent Shank3 overexpression and Abi1/Abi2 depletion resulted in a spine morphology that was indistinguishable from Abi1/Abi2 depletion alone. These results demonstrate that Abi1/2 are downstream of Shank3 in spine development.

Next, the necessity of the Shank3–ABI protein interaction for Shank3’s function in spine development was assayed using a molecular replacement method. Neurons from Shank3B KO mice [13] had a greater number of immature thin spines and fewer mushroom-like spines (Fig. 3c, d and Supplementary Figure 6c). Overexpression of WT Shank3 in Shank3B KO neurons rescued all the altered spine characteristics and further increased the number of stubby spines, mushroom-like spines, and total spines compared with WT neurons. In contrast, both S685I and S685A Shank3 failed to rescue the decreased mushroom-like spines. These data indicate that Shank3 interaction with ABI requires phosphorylation at S685, which, in turn, is necessary for Shank3 to promote mushroom-like, more mature spine formation.

Conversely, the role of the Shank3–ABI interaction for ABI’s function in spine formation was assayed. To test this, the residues critical for ABI to bind Shank3 were identified. Based on the structure of the ABI2 SH3 domain (PDB: 2EDO) and sequence homology search with HHpred [38] (see Methods), N446 and L461 of ABI2 were predicted to bind S685 of Shank3. We confirmed the importance of these residues for Shank3–ABI interaction by co-immunoprecipitation (Supplementary Figure 6d). Overexpression of WT ABI1 significantly increased stubby spine, mushroom-like spine, and total spine densities (Supplementary Figure 6e). However, overexpression of the Shank3 binding-deficient ABI1 (N447A L462A) failed to increase spine densities of any category, which further confirmed the importance of Shank3–ABI interaction in spine development.

To test whether these morphological changes translate into functional consequences, we recorded miniature excitatory postsynaptic currents (mEPSCs) in dissociated pyramidal-like hippocampal neurons from WT and Shank3B KO mice. Compared with WT neurons, Shank3B KO neurons had reduced mEPSC frequency, without significant changes in mEPSC amplitude (Fig. 3e, f). This reduction in mEPSC frequency can be rescued by transfecting these neurons with WT Shank3, but not with S685 Shank3, suggesting that Shank3–ABI interaction contributes to the formation of functional synapses.
To validate these findings in vivo, we assessed spine development and synaptic transmission in KI-HOM mice. Because Shank3 is highly expressed in the striatum, and previous reports found both spine development and synaptic transmission deficits in the dorsal striatum of Shank3 KO mice [13, 15], we quantified dendritic spine densities of medium spiny neurons (MSNs) in the dorsal striatum. KI-HOM mice displayed a significant reduction in spine density (Fig. 3g). A reduction in spine density was not observed in hippocampal CA1 pyramidal neurons (Fig. 3h), probably...
due to compensation from Shank2 which is highly expressed in the hippocampus and interacts with ABI1/2. Recording mEPSCs of MSNs in the dorsal striatum determined that both mEPSC frequency and amplitude are reduced in KI-HOM mice (Fig. 3i, j), similar to what has been reported in Shank3 KO mice [13, 21]. Together, these results indicate that interaction with ABI is critical for Shank3 function in both spine maturation and excitatory synaptic transmission.

**Shank3–ABI interaction is critical for actin nucleation**

KI-HOM mice have reduced dendritic spines, impaired synaptic transmission, altered social behaviors, but intact motor function and lack repetitive self-grooming, indicating that only a subset of Shank3 function (a module) is mediated by Shank3–ABI interaction. To determine the module Shank3–ABI interaction belongs to, we compared the interactomes of Shank3 and ABI1 to find their shared functional partners. First, we applied a method similar to that which we used to build Shank3 interactome to build an ABI1 interactome consisting of 151 proteins (Supplementary Table 3). Next, we compared the two interactomes and found significant overlap (Fig. 4a; Fisher’s exact test, $p = 2.11 \times 10^{-13}$, odds ratio = 6.21). Gene ontology analysis of the 31 common interactors suggested that regulation of the Arp2/3 complex and actin nucleation may be one of their principal functions (Fig. 4b and Supplementary Table 7).

To pinpoint the specific roles of Shank3 and ABI1 in the actin nucleation pathway, we integrated the two interactomes to generate a sub-network of actin-related proteins (Fig. 4c and Supplementary Tables 2, 3, and 4). Most components of the Arp2/3-mediated actin nucleation pathway were found in the network. Rho GTPases such as RAC1 and CDC42 activate nucleation-promoting factors (NPF) such as WRC and N-WASP, which then activate the Arp2/3 complex to initiate actin nucleation and branching [63]. Cortactin is another type of NPF that facilitates Arp2/3 activation [63]. As shown in the network, Shank3 binds the Arp2/3 complex, its upstream regulators (Rho GTPases, WRC, cortactin), and its downstream effector actin, potentially serving as a hub for actin nucleation. In contrast, ABI1, as a subunit of WRC, interacts with other WRC components (WAVE1/2/3, CYFIP1/2, NCKAP1, BRK1) and cortactin but not with the Arp2/3 complex itself. Thus, ABI1 might participate in actin nucleation in a supercomplex centered on Shank3. Consistent with this, size-exclusion chromatography showed that some ABI1 and WAVE1 were present in the same high-molecular-weight fraction (fraction 8) as the Shank3-GKAP-PSD-95 complex (Fig. 4d).

To test if the Shank3–ABI1 interaction is critical for actin nucleation, we stained F-actin in rat primary hippocampal neurons overexpressing either WT or S685I Shank3. Overexpression of WT Shank3 greatly increased F-actin intensity in dendritic spines, but overexpression of the ABI1 binding-deficient S685I Shank3 had a minimum if any effect on F-actin intensity (Fig. 4e, f). Together, these results define ABI1-dependent actin nucleation as a module of Shank3 function.

**Shank3 recruits WRC to the PSD via ABI1 to promote spine maturation**

Finally, we investigated the molecular mechanism by which Shank3–ABI1 interaction promotes actin nucleation in dendritic spines. Because ABI1 is the only subunit of the WRC that has previously been shown to directly bind Shank3 [10], we hypothesized that it mediates the interaction between Shank3 and WAVE/WRC. To test this possibility, we performed co-immunoprecipitation assays between Shank3 and WAVE1 in HEK293T cells. Consistent with our hypothesis, S685I Shank3, which disables interaction with ABI1, also abolished Shank3 binding to WAVE1 (Fig. 5a). In a complementary experiment, a WAVE1 mutant, whose binding to ABI1 was abolished by two amino acid changes [51], failed to interact with Shank3 (Fig. 5b). We also tested the interaction between Shank3 and WAVE3, another WAVE family protein that is, like
WAVE1, enriched in brain and localized at dendritic spines [64]. Disruption of Shank3–ABI1 interaction also abolished Shank3 binding to WAVE3 (Fig. 5c).

Given that ABI1 mediates Shank3–WRC interaction, it is conceivable that Shank3 promotes F-actin formation in dendritic spines by recruiting ABI1 and its associated WRC into spines to activate Arp2/3. To test this possibility, we overexpressed Shank3 in rat hippocampal neurons and stained endogenous ABI1 to quantify its localization. Contrary to our hypothesis, Shank3 overexpression did not
An autism-linked missense mutation in SHANK3 reveals the modularity of Shank3 function

**Fig. 4** Shank3 and ABI1 functions converge on actin nucleation. a Venn diagrams showing the overlap between Shank3 interactome and ABI1 interactome. b Gene ontology analysis of the intersection of Shank3 and ABI1 interactomes. c Actin cytoskeleton-related sub-network of the Shank3-ABI1 interactome. Green nodes indicate Shank3 specific interactors, blue nodes indicate ABI1 specific interactors, and red nodes indicate the shared interactors between Shank3 and ABI1. Green edges indicate interactions within the Shank3 specific interactors, blue edges indicate interactions within the ABI1 specific interactors, red edges indicate interactions associated with the shared interactors, and gray edges indicate interactions between the Shank3 specific interactors and the ABI1 specific interactors. d Immunoblots following size-exclusion chromatography of mouse striatal lysate. V0, void volume; *, non-specific band. e Fluorescent images of dendrites from DIV 21 dissociated rat hippocampal neurons transfected with pEGFP-C1 plasmids and vectors expressing HA-Shank3 (empty, WT, or S685I) on DIV 6. Scale bar, 5 μm. OE, overexpression. f Quantification of normalized integrated spine F-actin intensity in e (n = 22, 22, 22 neurons). OE, overexpression. All data are presented as mean ± s.e.m. **p < 0.01, ***p < 0.001, NS, not significant; f one-way ANOVA with post hoc Tukey’s tests

change the enrichment of ABI1 in dendritic spines (Supplementary Figure 7a,b). The spine localization of ABI1 was also preserved in Shank3B KO neurons (Supplementary Figure 7c, d). These results indicate that ABI1 localization to spines might be independent of Shank3.

Since both Shank3 and ABI1 are expressed at the tip of the spine in the post-synaptic density or PSD, which occupies ~10% of the surface area of spine [65], Shank3 could interact with, recruit, and stabilize ABI1 and WRC to the PSD from other parts of the dendritic spine. Indeed, in the striatal PSD fraction from Shank3B KO mice, but not in the total homogenate, the abundance of ABI1 was dramatically lower than in WT (Fig. 5d, e). WAVE1 was also lower in the PSD of Shank3B KO mice. We noticed that PSD-95 abundance was also slightly reduced, which implies a change in the PSD architecture upon loss of Shank3. Thus, it is possible that reduced WRC in the PSD is caused by some secondary effects other than loss of Shank3–ABI1 interaction. To rule out this possibility, we performed PSD fractionation on striatal tissues from KI-HOM mice. Again, we found that ABI1 and WAVE1 abundances were reduced in the PSD, but not in the total homogenate of KI-HOM mice (Fig. 5f, g). Note that both Shank3 and PSD-95 abundances in the PSD fraction of KI-HOM mice remain unchanged, indicating their proper localization and intact PSD organization. These results demonstrate that recruitment of WRC to the PSD requires Shank3–ABI1 interaction.

To determine if WRC-dependent actin nucleation mediates the effect of Shank3 on spine maturation, we either overexpressed Shank3, depleted Wave1/Wave3 using siRNAs (Supplementary Figure 7e, f), or combined the two in dissociated hippocampal neurons and then analyzed the morphology of spines. Similar to Abi1/Abi2 depletion, Wave1/Wave3 depletion completely abolished the effect of Shank3 overexpression (Fig. 5h, i and Supplementary Figure 7g), demonstrating that WAVE proteins are also critical downstream effectors for Shank3.

**Discussion**

This study was initiated to identify critical protein interactions and downstream effectors of Shank3 for its function at the synapse. By combining protein interaction domain data, post-translational modification data, and ASD exome sequencing data, we discovered a residue of Shank3 critical for its interaction with ABI1 that is mutated in autism. We further validated ABI1/WRC as a bona fide, direct downstream effector of Shank3 in vivo. Shank3 recruits WRC to the PSD through phosphorylation-dependent interaction with ABI1 to facilitate actin nucleation and spine development, disruption of which impairs synaptic transmission and multiple social behaviors (Supplementary Figure 7h). The most critical finding is that the S685I mutation, which specifically disables Shank3 to promote actin nucleation, only causes a subset of phenotypes found in constitutive Shank3 KO condition. This sort of functional and phenotypic modularity was unexpected.

**Phosphorylation-dependent regulation of Shank3**

We explored Shank3 phosphorylation pattern in vivo and found that it is extensively phosphorylated at dozens of residues. As phosphorylation can rapidly modulate a protein’s function by altering its stability, subcellular localization, or protein interactions, our work provides a valuable resource for future studies on the post-translational regulation of Shank3 in spine remodeling and synaptic plasticity. It is worth mentioning that the phosphorylation sites were identified on the full-length isoform of Shank3. Whether other Shank3 isoforms are phosphorylated on the same residues requires further investigation.

In this study, we focused on one specific in vivo phosphorylation site S685, because of its importance for the Shank3–ABI1 interaction. Because this interaction is essential for Shank3 function and S685A Shank3 failed to promote mature spine formation, S685 phosphorylation might serve as a mechanism to finely tune Shank3 function in actin nucleation at the PSD during spine maturation. We showed that PKA phosphorylates S685 and that inhibition of PKA decreased Shank3–ABI1 interaction in neurons. The involvement of PKA in Shank3 regulation is interesting, as short-term activation of PKA increases both spine size and spine density in hippocampal neurons [66, 67]. Our data suggest that Shank3 phosphorylation at S685 facilitate WRC recruitment and rapid actin cytoskeleton reorganization in dendritic spines upon activation of PKA signaling.
Shank3 as a hub for actin nucleation necessary for spine development

The role of Shank3 in spine development is well-known. Although Shank3 has long been proposed to regulate actin cytoskeleton [68], whether Shank3 promotes spine development through regulating actin cytoskeleton has not been established, especially in vivo. We show that ABI and WAVE, both known to primarily function as actin nucleation regulators, are downstream effectors of Shank3 in spine development.
An autism-linked missense mutation in SHANK3 reveals the modularity of Shank3 function

Moreover, the S685I mutation, which specifically disables Shank3 for actin nucleation while does not affect its other interactions, abundance or PSD localization, still causes spine defects in vivo. Together, these results demonstrate that Shank3 function in spine development largely relies on its actin nucleation module. Based on our integrated interactome analysis, Shank3 might serve as a hub in the deeper layer of the PSD, bringing together NPFs and Arp2/3 to cooperatively facilitate actin nucleation.

The involvement of Shank3 in actin nucleation and the association of the S685I variant with autism indicate a critical role of actin nucleation in the pathogenesis of SHANK3 deficiency. Beyond that, dysregulation of actin nucleation could be a general mechanism underlying ASD. Supporting this idea, de novo mutations in NCKAP1 and ABI2, which encode WRC subunits, have been linked to autism [69]. Moreover, copy number variation of CYFIP1, which encodes another WRC subunit, has been linked to both autism spectrum disorder and schizophrenia [70–72]. Further human genetic studies to explore genes critical for actin dynamics might prove fruitful in identifying new etiologies for autism or other neurodevelopmental disorders. Detailed studies of how variation in these genes contribute to pathology will undoubtedly provide insights into not only mechanisms of the disease, but also the regulation of actin dynamics and its role in synaptogenesis.

The S685I missense mutation demonstrates the modularity of Shank3 function

While displaying increased social dominance, increased allogrooming, and delayed USV development, our Shank3 KO mice do not express all the phenotypes reported in Shank3 KO mice. For example, motor defects, increased anxiety, abnormal self-grooming behaviors, and altered sensory gating that were repeatedly reported in Shank3 KO lines [15, 20, 21], are intact in KO mice, suggesting that they are mediated by other protein interactions/pathways. These results are consistent with previous findings that targeting Shank3 at different domains leads to different sets of physiological and behavioral abnormalities [23, 25–27]. This modularity further indicates that there is more than one independent pathogenic pathway downstream of Shank3 and correcting a single downstream pathway is unlikely to be sufficient for clear clinical improvement. An example similar to this is Rett syndrome where targeting the downstream dysregulated BDNF through IGF-1 only partially rescued Rett phenotypes [73]. Thus, rather than attempting to correct downstream deficits, it might be more fruitful to identify upstream regulators of dosage-dependent genes in neuropsychiatric disorders for therapeutic targeting.

Our study also highlights the value of missense variants in understanding the pathogenesis of neuropsychiatric disorders. Most mutations found in genome sequencing studies are missense mutations of unknown significance. While they are often less informative for identifying new neuropsychiatric disease-causing genes, missense mutations could lead to a greater understanding of protein functions that are linked to the disease. The difficulty lies in distinguishing informative missense mutations from non-pathogenic ones. Our strategy was to map missense variants onto post-translational modification sites and protein interaction domains to identify potentially critical residues. This approach could be used for other neuropsychiatric disease-causing genes to gain additional insight into mechanisms of the disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.
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