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Supplemental information

MARK2 variants cause autism spectrum disorder via the downregulation of WNT/β-catenin signaling pathway

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Supplementary Appendix

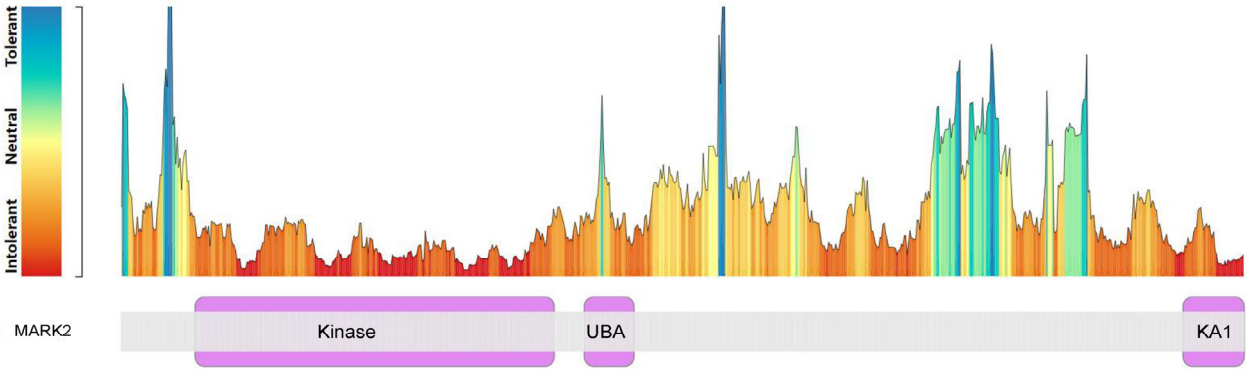


Figure S1. The missense variant tolerance landscape

Protein of MARK2 (GENCODE: ENST00000402010.2, RefSeq: NM_001039469.3, UniProt: Q7KZI7) was used. The ratios of missense over synonymous variant were calculated to indicate the regions that are intolerant to missense variation. The kinase and KA1 domain (purple parts) clearly show as intolerant compared with other parts.

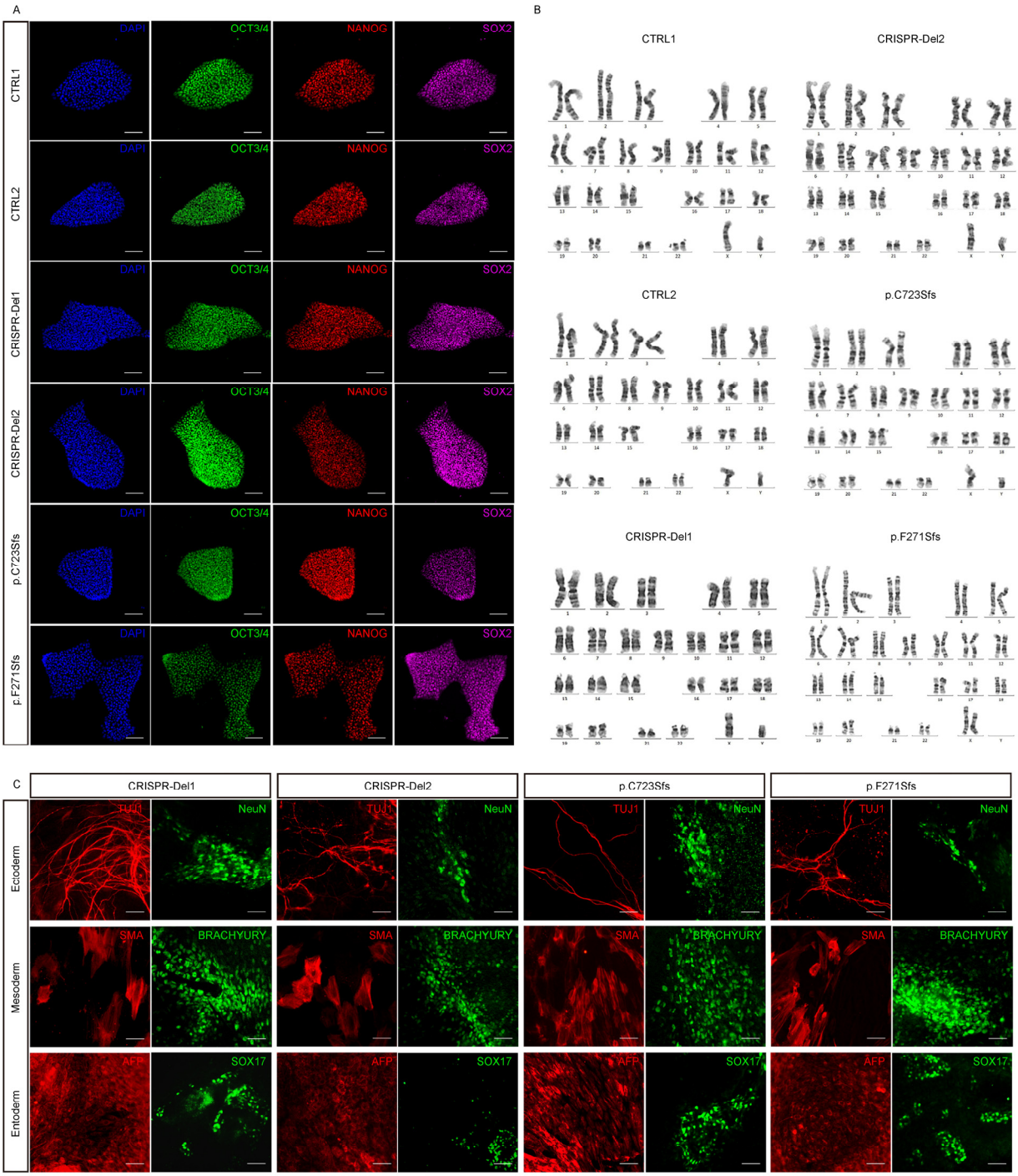


Figure S2. Representative validation images of iPSCs with different genotypes

A, Representative validation images of six iPSCs using several markers, OCT 3/4 (green), NANOG (red), and SOX2 (purple). Nuclei were stained with DAPI (blue). CTRL1 and CTRL2: two independent healthy adults without *MARK2* variant; p.C723Sfs and p.F271Sfs: two affected individuals with LoF *MARK2* variants; CRISPR-Del1 and CRISPR-Del2: two isogenic *MARK2* deletions produced by the CRISPR/Cas9 editing technology. scale bar = 50 μm. **B**, Representative karyotype images of six iPSCs separately. **C**,

Representative images of differentiated iPSC EBs using a Human pluripotent stem cell identification kit. Differentiated iPSCs were fixed for immunocytology analysis for the ectoderm (TUJ1, NeuN), mesoderm (SMA, BRACHYURY) and entoderm (AFP, SOX17) lineages. scale bar = 50 μm.

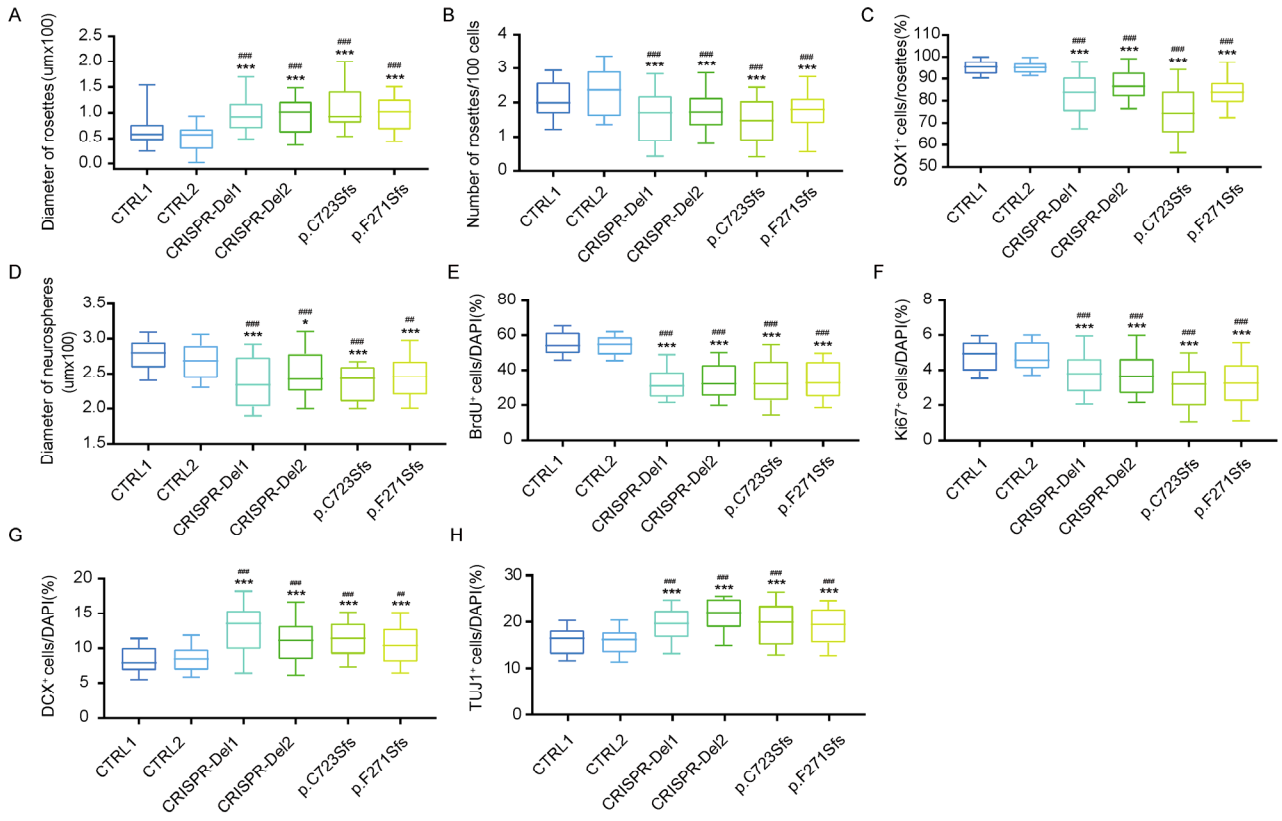


Figure S3. Quantification analysis of iPSC-derived neural rosettes and NPCs.

A-H the quantification analysis of rosette diameter (**A**, *n*=12 rosettes), rosette number (**B**) in **Fig 3A**, SOX1$^+$ cells (**C**) in **Fig 3B**, neurosphere diameter (**D**, *n*=12 neurospheres) in **Fig 3C**, BrdU$^+$ cells (**E**) and Ki67$^+$ cells (**F**) in **Fig 3D**, DCX$^+$ cells (**G**) and TUJ1$^+$ cells (**H**) in **Fig 3E**. The data of at least three independent experiments were analyzed by Student's t test; **p*<0.05 and ****p*<0.001(compared with CTRL1); $^{\#\#}$*p*<0.01 and $^{\#\#\#}$*p*<0.001(compared with CTRL2).

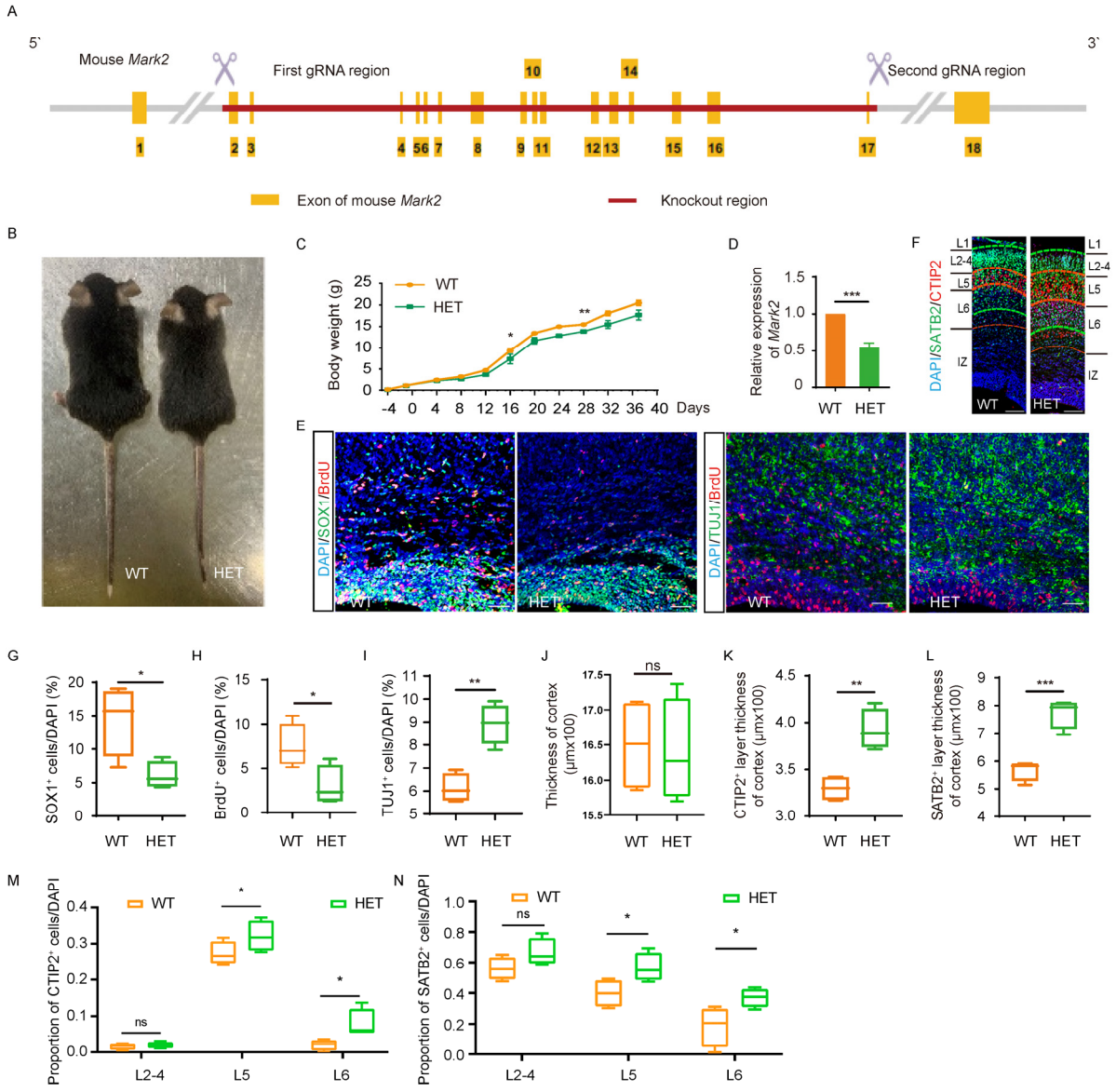


Figure S4. *Mark2* loss in mice affects the proliferation and differentiation of NPCs *in vitro.*

A, Schematic of *Mark2* knockout mice. **B-C**, Body size (**B**) and growth curve (**C**) of *Mark2*$^{+/+}$ (WT) mice and *Mark2*$^{+/-}$ (HET) mice in 6 weeks. **D,** Expression of *Mark2* of HET and WT mice. Total RNAs were isolated from the cortex in E18.5 mice, and GAPDH was used as the internal parameter. **E-F**, Representative images of immunofluorescence staining for BrdU (red) and SOX1 (green), BrdU (red) and TUJ1 (green), CTIP2 (red) and SATB2 (green) in the cortical region above the subventricular zone (SVZ) in E18.5 mice, L1, L2-4, L5, L6 and IZ were marked to point out layers 1-6 and intermediate zone of mouse neocortex, the thick red dotted line were used to point out the CTIP2^{+} layer in L5, the thick green dotted line were used to point out the SATB2^{+} layer in L2-6, and the thin red dotted lines was used to point out the border of L6 with farthest CTIP2^{+}

cells located. **G-I**, Quantification analysis of SOX1$^+$ cells (**G**), BrdU$^+$ cells (**H**), TUJ1$^+$ cells (**I**), **J-L**. Quantification analysis for the thickness of mice cortex. Total thickness of mice cortex from SVZ to the pial surface (**J**), thickness of CTIP2$^+$ layer (**K,** thick red dotted line), and thickness of SATB2$^+$ layer (**L,** thick green dotted line). **M-N**, Quantification analysis for proportion of CTIP2$^+$ or SATB2$^+$ cells in specifical mouse neocortex. The proportion were analysis using the number of CTIP2$^+$ or SATB2$^+$ cells divided by the number of DAPI, proportion of CTIP2$^+$ cells (**M**), and proportion of SATB2$^+$ cells (**N**), n=4. The data of at least three independent experiments were analyzed by Student's t test; *p<0.05, **p<0.01 and ***p<0.001. scale bar = 50 μm.

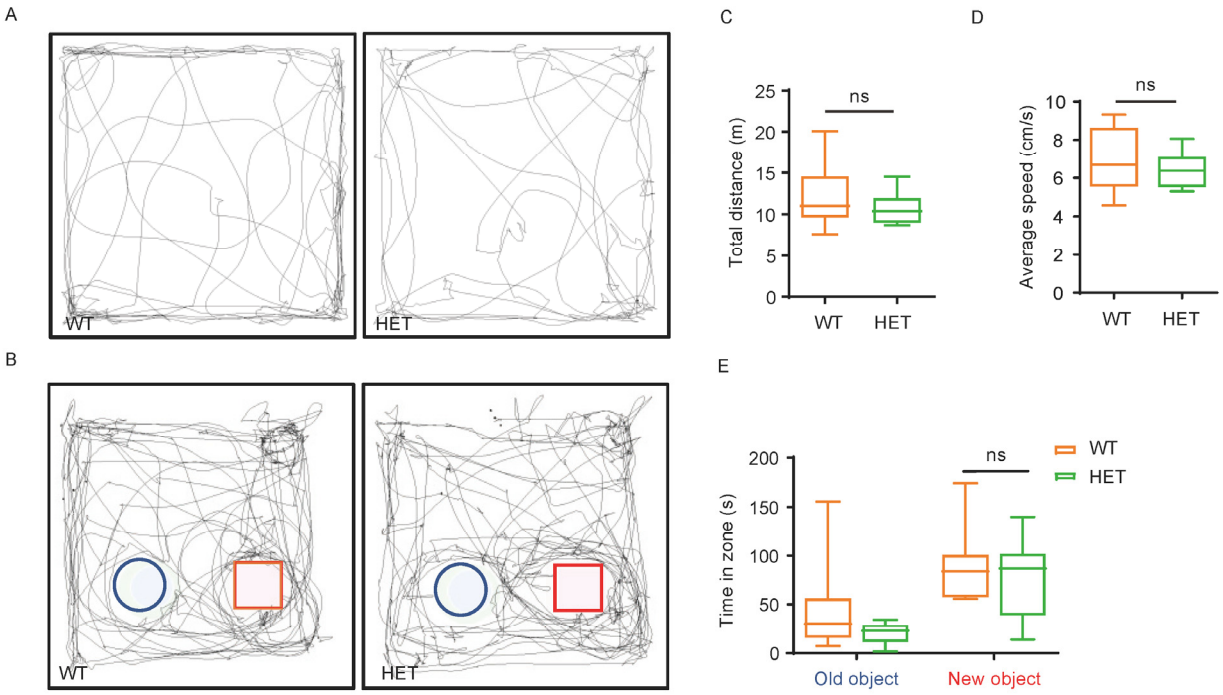
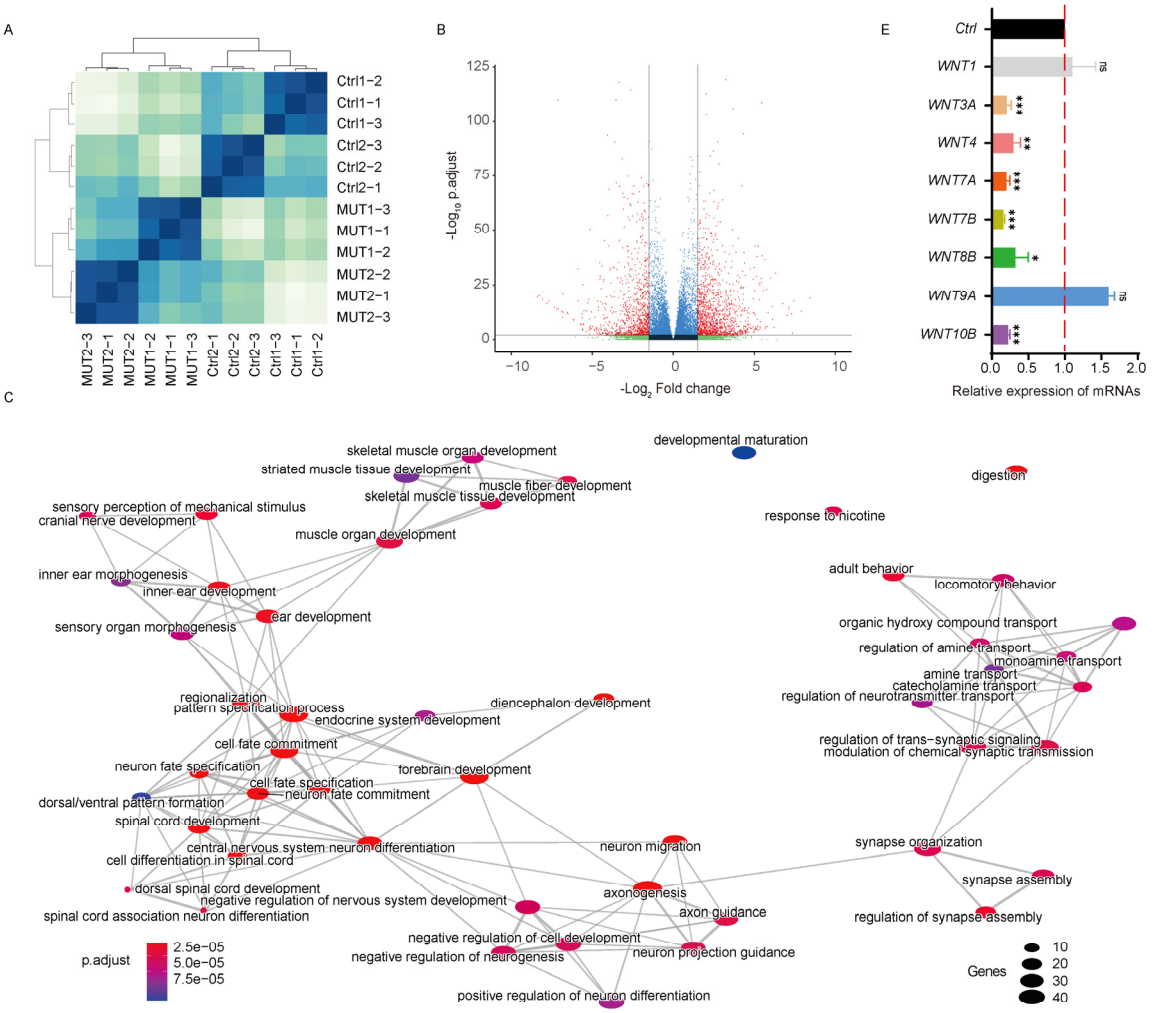


Figure S5. Open field and new object recognition tests in mice with *Mark2* loss

A-B, Trajectories of WT and HET mice in the open field test (**A**) and new object recognition test. (**B**). **C-D**, Quantification analysis of total distance (**C**) and average speed (**D**) between two genotypes in the open field test. **E**, Quantification analysis of time in zone in the new object recognition test (*n*=11). Time spend in contact with the object was used to represent for the new object recognition. The blue circle was used to point the location of the old object and red square was used to point the location of the new object. WT=11, HET=11. The data of at least three independent experiments were analyzed by Student's t test; ns: not significant.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Relative expression of mRNAs
C
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skeletal muscle tissue development
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cranial nerve development
muscle organ development
inner ear morphogenesis
inner ear development
ear development
sensory organ morphogenesis
regionalization
pattern specification process
endocrine system development
diencephalon development
cell fate commitment
neuron fate specification
forebrain development
cell fate specification
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spinal cord association neuron differentiation
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monoamine transport
amine transport
catecholamine transport
regulation of neurotransmitter transport
regulation of trans−synaptic signaling
modulation of chemical synaptic transmission
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synapse organization
synapse assembly
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axon guidance
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LRP5/6
Wif-1
SCGT
Dkk
BAMBI
APCL/DI
Idax
Daple
CKIε
GBP
Dvl
CK2
Axam
Scaffold
GSK-3β
+p
β-catenin
Axin
APC
CKIα
Alzheimer's disease
PS-1
PKA
MAPK signaling pathway
TAK1
Adherens junction
NLK
CBY1
Duplin
CBP
Pontin52
TCF/LEF
DNA
c-myc
c-jun
fra-1
cycD
WISP1
PPAR δ
Uterine
Cell cycle
ICAT
Xsox17
SMAD4
SMAD3
Gro/TLE
CtBP
δ-catenin
TGF-β signaling pathway
p53 signaling pathway
p53
Siah-1
SIP
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TBL1
APC
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Nkd
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β-TrCP
Skp1
Cul1
Rbx1
Ubiquitin mediated proteolysis
Phosphorylation independence
Proteolysis
Planar cell polarity (PCP) pathway
Sfbm
Prickle
Wnt11
Frizzled
Dvl
Daam1
RhoA
ROCK2
Focal adhesion
Cytoskeletal change
iNVS
Rac
JNK
Gene transcription
Knypek
ROR1/2
RYK
MAPK signaling pathway
Wnt/ Ca2+ pathway
Wnt5
Frizzled
RYK
G protein?
PLC
Ca2+
CaMKII
CaN
PKC
-p
NFAT
DNA

Figure S6. RNA-Seq analyses of iPSC-derived NPCs.

A, Correlation heatmap of RNA-Seq of control (CTRL1) and mutant iPSC-derived NPCs (Mut: p.C723Sfs, *n*=3 experiment). **B**, Example volcano plot. Points on top-right and top-left corners are considered the most promising genes (*P*<0.05, log_2Foldchange>1.5). **C**, Association network diagram between feature sets through GO analysis. All those feature sets of GO were enriched through downregulated genes (Log_2FoldChange<-1.5, *p* value>0.05) in mutant neurospheres. **D,** Pathview of WNT signaling pathway through GSEA analysis. Gene involved in WNT signaling pathway were showed in different colors, which the upregulated genes were marked with red, downregulated gene were labelled with green, and other genes were marked with gray. **E**, Decreased expression of several WNT genes (*WNT3A, WNT4, WNT7A, WNT7B, WNT8B, WNT10B*) in mutant hiPSC-derived NPCs were validated by quantitative RT-PCR. GAPDH was used as the internal reference and expressions were normalized by control iPSC-derived NPCs.

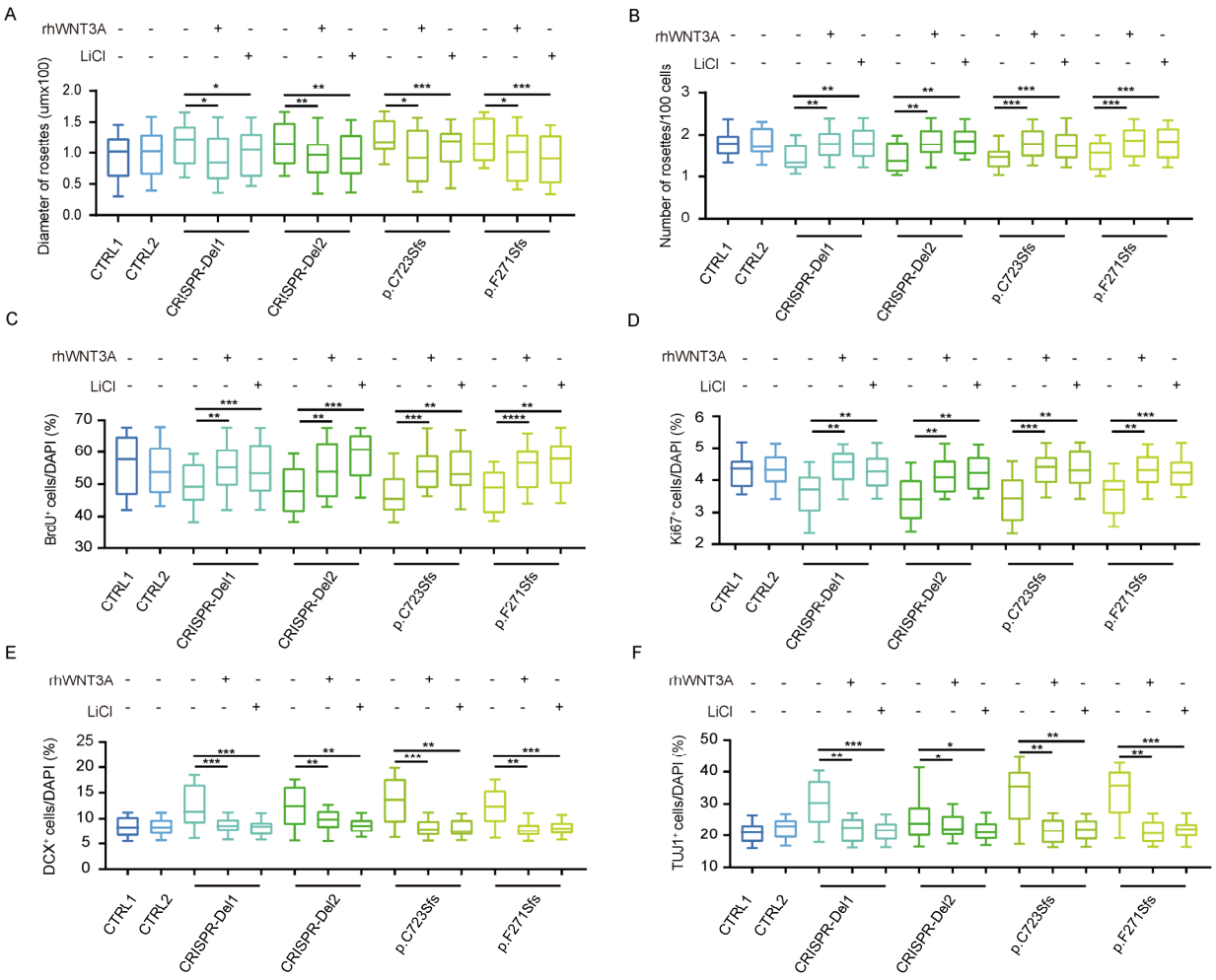

Figure S7. Quantification analysis of iPSC-derived neural rosettes and NPCs treated with LiCl or rhWNT3A.

A-F The quantification analysis of rosette diameter (**A**), rosettes number (**B**) in **Fig 6A**, BrdU$^+$ cells (**C**) and Ki67$^+$ cells (**D**) in **Fig 6B**, DCX$^+$ cells (**E**) and TUJ1$^+$ cells (**F**) in **Fig 6C**. The data of at least three independent experiments were analyzed by Student's t test; *p<0.05, **p<0.01 and ***p<0.001.

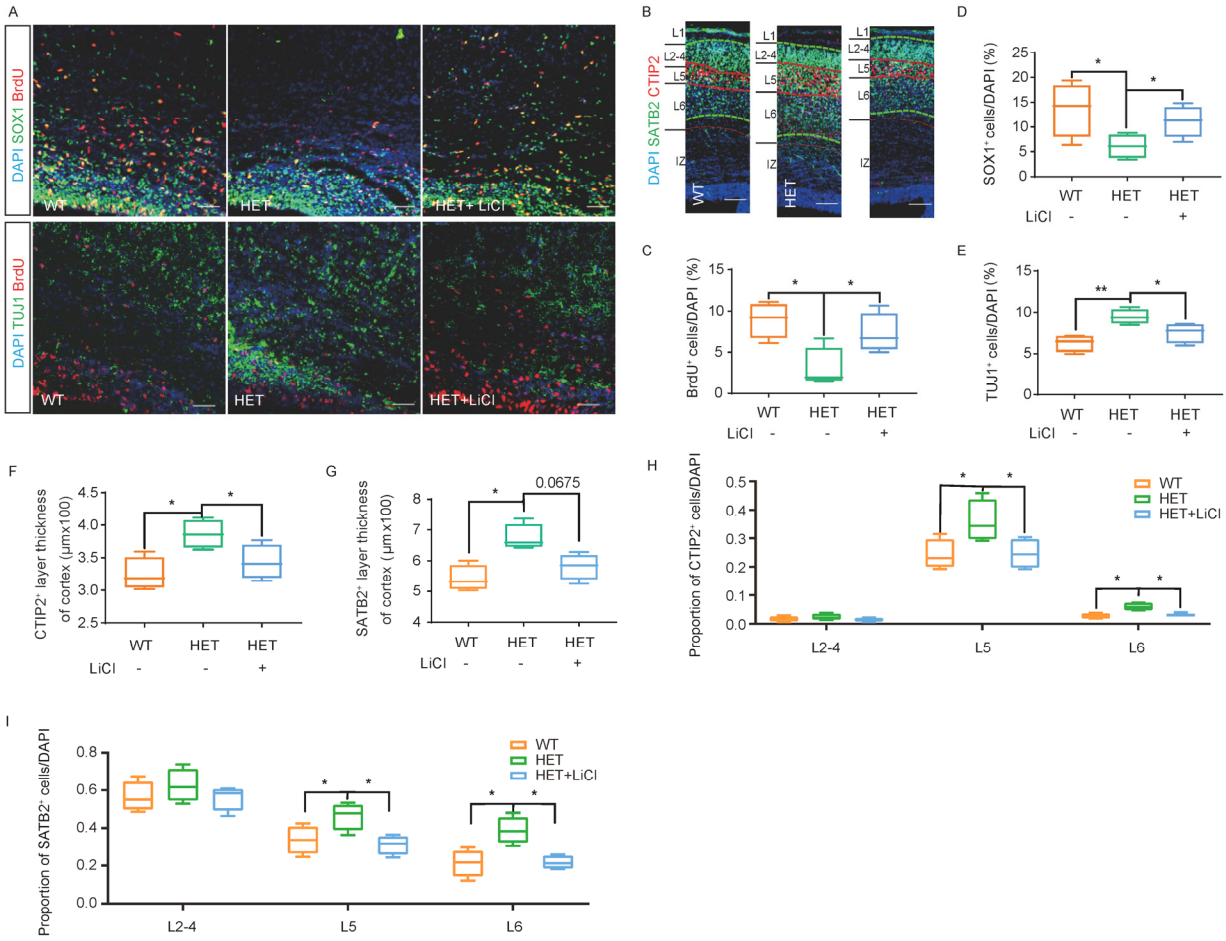

Figure S8. Abnormal cortical development in *Mark* $^{+/-}$ mice is rescued by LiCl

A-B, Representative images of immunofluorescence staining for BrdU/SOX1, BrdU/TUJ1 (**A**), and CTIP2/SATB2 (**B**) in the mouse embryonic cortex (E18.5) from three groups: *Mark2*$^{+/+}$ mice (WT=4), untreated *Mark2*$^{+/-}$ mice (HET=4) and LiCl-treated () *Mark2*$^{+/-}$ mice (HET+LiCl=4), L1, L2-4, L5, L6 and IZ were marked to point out layers 1-6 and intermediate zone of mouse neocortex. Thick red dotted line was used to point out the CTIP2^{+} layer in L2-4, and thick green dotted lines was used to point out the SATB2^{+} layer, and thin red dotted line was used to point out the border of L6 with farthest CTIP2^{+} cells located. **D-I,** Quantification analysis of the data **in A-B,** including the numbers of SOX1^{+} cells (**D**), BrdU^{+} cells (**C**), TUJ1^{+} cells (**E**), thickness of CTIP2^{+} layer (**F,** thick red dotted line), SATB2^{+} layer thickness (**G,** thick green dotted line), proportion of CTIP2^{+} cells (**H**), and proportion of SATB2^{+} cells (**I**). The data of at least three independent experiments were analyzed by Student's t test; *p<0.05, and **p<0.01. ns: not significant. scale bar = 50 μm.

METHODS

Behavioral and memory tests

All mice used for the behavioral tests were male mice aged 8-12 weeks (n≥8 per group), and all tests were performed between 09:00 and 17:00. Videos of the behavioral tests were analyzed by EthoVision XT 14 (Noldus).

Open field test

The open field test was conducted in a 50 x 50 x 50 cm box. A test subject was placed in the center of the box, and its behavior was video recorded for 5 min by a camera positioned directly above the box. Total distance and speed were quantified during video recording. A 30 cm square was delineated as the center zone.

Elevated plus maze test

Each mouse was placed in the central area of the elevated plus maze facing one of the open arms. The mice were allowed to explore the maze for 5 min, and the time spent in the open arm was calculated with EthoVision XT 14.

Three-chamber test

Two weeks before testing, subject animals and stimulus animals (female C57BL/6 mice aged 3-4 months) were housed alone in individual clean cages in the testing room. The test was performed in a novel, clean box (72 cm length x 72 cm width x 36 cm height) during the light phase. A subject mouse was placed in the empty apparatus and allowed to habituate to the three chambers, which contained two transparent bottles with holes (9 cm in diameter, 12 cm high), for 15 min. After habituation, one female stimulus mouse was placed in one of the transparent bottles in the right chamber, and the subject mouse was placed in the middle chamber and allowed to explore for 15 min. The subject mouse was then returned to its home cage. Then, another unknown female mouse was placed in the bottle in the other chamber (left) of the test box; the subject mouse was placed back in the test box equidistant from and facing the familiar and novel female mice. Interactions between the subject mouse with the familiar and novel female mice were videotaped for 15 min. Sniffing times were recorded and analyzed.

Novel object recognition test

A mouse was placed as in an open field test arena facing a wall and allowed to freely explore for 5 min. After a short rest in its home cage, the mouse was placed in the box again facing two identical objects (5 cm away

from the walls) and allowed to explore for another 10 min exploration (T1). After a 60-min rest period, the mouse was placed in the box again facing two objects (a novel one and one that was present in T1) and allowed to explore for another 10 min (T2). The recognition index was calculated and analyzed.

Marble-burying test

Mice were individually placed in Plexiglas cages containing 5-cm-deep fresh bedding, and then 20 black glass marbles (15 mm diameter) were gently placed in a 4 x 5 arrangement at equal distances. Testing was conducted for 30 min. After the test period, buried marbles were counted. Marbles were considered buried if at least one half was covered with bedding.

Grooming test

The grooming task consisted of 15 min of habituation followed immediately measurement of grooming behavior for 15 min. Mice were individually placed in novel Plexiglas cages (45 cm x 22 cm). The time spent grooming the genitals, tail, paw, leg, body and head was recorded in seconds.

Y-maze test

Mice were placed in one arm of the Y-maze apparatus (20 cm high, 50 cm long, and 10 cm wide at the bottom) and allowed to explore freely for 10 min. A correct spontaneous alternation was defined as the successive entry of a mouse into the three arms in overlapping triplet sets. The spontaneous alternation percentage (%) was calculated as the number of successive triplet sets (consecutive entries into three different arms)/total number of arm entries minus 2) x100.

Barnes maze test

The apparatus was a rotatable gray acrylic disc (1.22 m in diameter) elevated 0.58 m above the floor with 20 holes (5 cm diameter, 2 cm away from the edge) equally spaced along the perimeter. The apparatus was brightly lit (600 lux). Only one hole in the maze top led to a removable hiding box, which was situated directly below the escape hole. On the first day, a mouse was placed in a transparent cylindrical start chamber (10.5 cm), and 30 s after the onset of a buzzer sound (85 dB), the chamber was lifted, and the mouse was allowed to freely explore the maze. The trial ended when the mouse entered the hiding box or after 3 min had elapsed. Mice that did not find the hiding box by the end of the 3-min period were gently guided to the escape hole by the investigator. Immediately after the mouse entered the hiding box, the buzzer sound was turned off, and the mouse was allowed to stay in the hiding box for 1 min. On the second day, the mouse was placed in a black square-shaped start chamber (10.5 cm), and 15 s after the onset of a buzzer sound (85 dB), the chamber was lifted, and the mouse was allowed to freely explore the maze. The trial ended when the mouse

entered the hiding box or after 2 min had elapsed. Immediately after the mouse entered the hiding box, the buzzer sound was turned off, and the mouse was allowed to stay in the hiding box for 1 min. The experiment was repeated three times for each mouse. The procedure performed on the second day was conducted twice on the third day. On the fifth day, the subjects were placed in a black square-shaped start chamber (10.5 cm), and 15 s after the onset of a buzzer sound (85 dB), the chamber was lifted, and the mouse was allowed to freely explore the maze. The trial ended when the mouse entered the hiding box or after 2 min had elapsed. Immediately after the mouse entered the hiding box, the buzzer sound was turned off. The movements of the animals in the maze were digitally recorded.

Morris water maze test

A 120 cm diameter, 45 cm deep Morris water maze was filled with water, which was made with nontoxic white paint, to a depth of 25 cm. An escape platform (diameter 13 cm) was hidden 1 cm beneath the surface of the water in the center of one of the quadrants of the water tank. Four extra-maze cues, i.e., different shapes, were placed at equal distances on the wall surrounding the water tank. The water temperature was adjusted to 21±1°C. The mice were trained to find the escape platform in four trials per day for 6 consecutive days. In each trial, a mouse was placed in a randomly chosen quadrant and allowed to swim for up to 1 min to find and climb on the platform. If it failed to find the platform within that time, it was guided to the escape platform and kept there for 15 s. A probe test was conducted 24 h after completion of training. In the probe test, the platform was removed from the pool, and behavior was recorded for 60 s. Latency to reach the platform and time spent in the platform quadrant were recorded.