Supporting Information

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A Multi-target Small Molecule for Targeted Transcriptional Activation of Therapeutically Significant Nervous System Genes

Yulei Wei,[a] Ganesh N. Pandian,[b] Tingting Zou,[a] Junichi Taniguchi,[a] Shinsuke Sato,[b] Gengo Kashiwazaki,[a] Thangavel Vaijayanthi,[a] Takuya Hidaka,[a] Toshikazu Bando,[a] and Hiroshi Sugiyama*[a, b]

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Experimental Section

Synthesis of PIP conjugates

The synthesis of the SAHA-L, L-ββAc were performed on a PSSM-8 peptide synthesizer and purity were checked by HPLC (elution with 0-50% MeCN/0.1% TFA for 20 min under254 nm, figure S1 A,B) and MS(figure S1 C,D); All the procedure were followed to the published procedures [1].

For L-ββAc, ESI-TOF-MS (positive) m/z calcd for C62H78N24O12, $[M+2H]^{2+}$ 676.31; found in 676.31. 1H NMR (600 MHz, DMSO-d6): δ 10.43 (s, 1H), 9.92 (s, 1H), 9.90 (s, 3H), 9.83 (s, 1H), 9.68 (s, 1H), 9.62 (S, 1H), 8.31 (t, J = 6.2 Hz, 1H), 8.15 (t, J = 5.5 Hz, 1H), 7.96 (t, J = 5.2 Hz, 1H), 7.83 (brt, 1H), 7.66 (s, 1H), 7.63 (s, 1H), 7.53 (s, 1H), 7.30 (s, 1H), 7.22 (s, 1H), 7.18 (s, 1H), 7.17 (s, 1H), 7.16 (s, 1H), 7.09 (s, 1H), 7.07 (s, 1H), 7.01 (s,1H), 6.95 (s, 1H), 6.88 (s, 1H), 4.02 (s, 3H), 4.00 (s, 3H), 3.96 (s, 3H), 3.85 (s, 9H), 3.83 (s, 3H), 3.82 (s, 3H), 3.30 (m, 4H), 3.24 (m, 4H), 3.06 (m, 2H), 2.42 (t, J = 7.2 Hz, 2H), 2.28 (t, J = 7.2 Hz, 2H), 2.21 (t, J = 6.9 Hz, 2H), 1.83 (m, 4H);

For SAHA-L, ESI-TOF-MS (positive) m/z calcd for $C_{75}H_{94}N_{26}O_{15}$, $[M+2H]^{2+}$ 800.37; found in 800.38. 1H NMR (600 MHz, DMSO-d6): δ 10.42 (s, 1H), 10.31 (s, 1H), 10.05 (s, 1H), 9.91 (s, 1H), 9.90 (s, 1H), 9.895 (s, 1H), 9.885 (s, 1H), 9.82 (s, 1H), 9.69 (s, 1H), 9.63 (s, 1H), 8.35 (t, J = 5.6 Hz, 1H), 8.31 (t, J = 6.2 Hz, 1H), 8.15 (t, J = 6.0 Hz, 1H), 8.00 (t, J = 5.8 Hz, 1H), 7.76 (d, J = 8.9 Hz, 2H), 7.65 (d, J = 9.2 Hz, 2H), 7.63 (s, 2H), 7.53 (s, 1H), 7.28 (d, J = 1.4 Hz, 1H), 7.21 (m, 2H), 7.17 (d, J = 1.7 Hz, 1H), 7.15 (d, J = 1.7 Hz, 1H), 7.07 (m, 2H), 6.95 (m, 2H), 6.88 (d, J = 1.7 Hz,1H), 4.02 (s, 3H), 4.00 (s, 3H), 3.96 (s, 3H), 3.85 (s, 6H), 3.83 (s, 3H), 3.82 (s, 3H), 3.81 (s, 3H), 3.33 (m, 2H), 3.27 (m, 2H), 3.24 (m, 2H), 3.08 (m, 2H), 2.79 (d, J = 5.0 Hz, 6H), 2.52 (m, 2H), 2.43 (t, J = 7.2 Hz, 2H), 2.35 (t, J = 7.4 Hz, 2H), 2.30 (m, 2H), 2.28 (m, 2H), 1.93 (t, J = 7.5 Hz, 2H), 1.83 (m, 4H), 1.56 (m, 2H), 1.48 (m, 2H), 1.27 (m, 4H).

Human BJ fibroblast culture and compound treatment

BJ foreskin fibroblasts (BJ fibroblast) were purchased from American type culture collection, and all the cell experiments were performed in passage 6 to 7. BJ fibroblasts were cultured in Dulbecco's modified eagle medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Hyclone), and 0.5% penicillin and streptomycin (P/S, Nacalai Tesque). BJ fibroblasts were passaged with 0.25% trypsin (Nacalai Tesque) in PBS and seeded at a concentration of $4x10^4$ cells per well at 12 well plate. One day after, when the cell confluent to about 40%, treatment was done with the effectors and DMSO was used as the negative control. Optimization of incubation time studies (6h, 12h, 24h, 48 h and 72 h) showed that by our previous reports [1, 3], a notable bioactivity was not attained with the earlier time window and that consistent result could be obtained only with 48 h incubation. Likewise, the effect of concentration studies showed one μM as the optimal concentration to have a notable effect on the endogenous expression of brain and nervous system genes.

Culture human 201B7-iPS cells and compound treatment

Human induced pluripotency stem cells 201B7 were purchased from RIKEN Cell Bank [2], and cultured as per the protocol from CiRA homepage

(http://www.cira.kyoto-u.ac.jp/e/research/protocol.html). IPS cells were maintained on mouse fibroblast SNL 76/7 (Applied StemCell) and treated with Mitomycin C solution (Wako) before use. The SNL cell were seeded at $1.0x10^6$/well in 60mm dishes after coated in gelatin (Wako) in 37℃ for 30min. The iPS cell culture medium used were as follows: DMEM/F12 (Gibco), 20% KSR (Gibco), 1% L-glutamine (Wako), 1% Non-essential amino acids solution (Gibco), 1% P/S, 2-mercaptoethanol (sigma), 4ng/ml recombinant basic fibroblast growth factor (βFGF, Gibco). Passages 1 to 6 were done with the confluency of about 80% with CTK solution, and the component is 0.25% trypsin, 0.1% collagenase type IV (Gibco), 20% KSR, 1mM $CaCl_2$ in PBS(-).

For compound treatment, 0.1% DMSO was used to make various concentrations (1 µM, 5µM, 10 µM) of compounds. Initial optimization studies showed 10 µM as the optimal concentration. When treated with the effectors, the iPS cells were passaged in feeder free condition with the method as shown below. Conditional SNL medium were prepared ahead of time by using βFGF lacking iPS medium and were coated in the SNL cell for 24 hours, Then the coated medium were collected and filtered by 0.25µM filter for feeder-free culture. CTK solution was used to detach the feeder cell out of iPS, passage was done in a matrigel coated 12 well plate. After 24 h the treatment was done for 4, 15 days to the cells having 40% confluency. Medium is changed everyday. All the experiment were performed in p35 to p50.

Quantitative real time PCR

Total RNA were extracted by RNeasy Mini Kit (Qiagen), cDNA was synthesized from 500ng total RNA by ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo), and SYBR green real-time PCR amplifications were carried out with THUNDERBIRD® SYBR qPCR Mix (Toyobo) and analyzed by using ABI 7300 Real-time PCR System (Applied Biosystems, USA). The primer information is in the supplementary data table S3.

Microarray

BJ fibroblasts were treated with 1µM of the compound for 2 days and the iPS cells were treated with 10µM of the compound for 4 days. Then the total RNA were isolated by RNeasy Mini Kit (QIAGEN). Examined the RNA quality by Agilent RNA 6000 Pico Kit (Agilent), then use the Genechip WT PLUS Reagent KIT (Agilent), to amplification the RNA into cRNA, after cRNA purification and quantitation, synthesis the ss-cDNA. Then the Genechip WT Terminal Labeling Kit to fragment and labeling the ss-cDNA was used, the hybridization were performed in human gene 2.1 ST array strip (Agilent).

IPA

The raw data of the genes up-regulated after effector treatment by more than two fold, p-value less than 0.05 were subjected to the network analysis by QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN, www.qiagen.com/ingenuity) software. Gene ontology analysis was done by GENEMANIA as mentioned before [3]. And the venn diagram was drawn using VENNY2.1 online[4].

Immunostaining

Immunostaining of Nestin (Abcam) was performed in 201B7-iPS cells treated for 15 days with 10μM of SAHA-L and L-ββAc (negative control). 0.1% DMSO, was used as the vehicle control. For differentiation, we used the conditional medium without the stem cell maintaining factor βFGF, and changed the medium every days. After 15 days culture, the cells were fixed using 4% paraformaldehyde (Sigma) in PBS for 30 min at room temperature, then the cells were permeabilized by 0.5% Triton X-100 (Nacalai tesque) in PBS for 20 minutes. 5% BSA (Nacalai tesque) was used for blocking for 1 hour at room temperature. We incubated the cell with 1st antibody Nestin mouse anti human at 1:500 in 1% BSA solutions at 4 ℃ overnight. The next day, we incubated with 2nd antibody alex488 goat anti mouse (Invitrogen) in 1:500 for 1 hour at room temperature. Nuclear staining was done with 1μg/mL DAPI/PBS solution for 15 minutes at room temperature. The immunostaining result was checked by confocal microscope.

Prediction of DNA-binding specificities for Cys2His2 zinc finger proteins

The protein sequence of human zinc finger protein 521(ZFP 521) (GenBank: EAX01201.1) were acquired from NCBI database. DNA sequence logo generator were used to predict the DNA binding site of the zinc finger protein [5].

Supplementary Figures

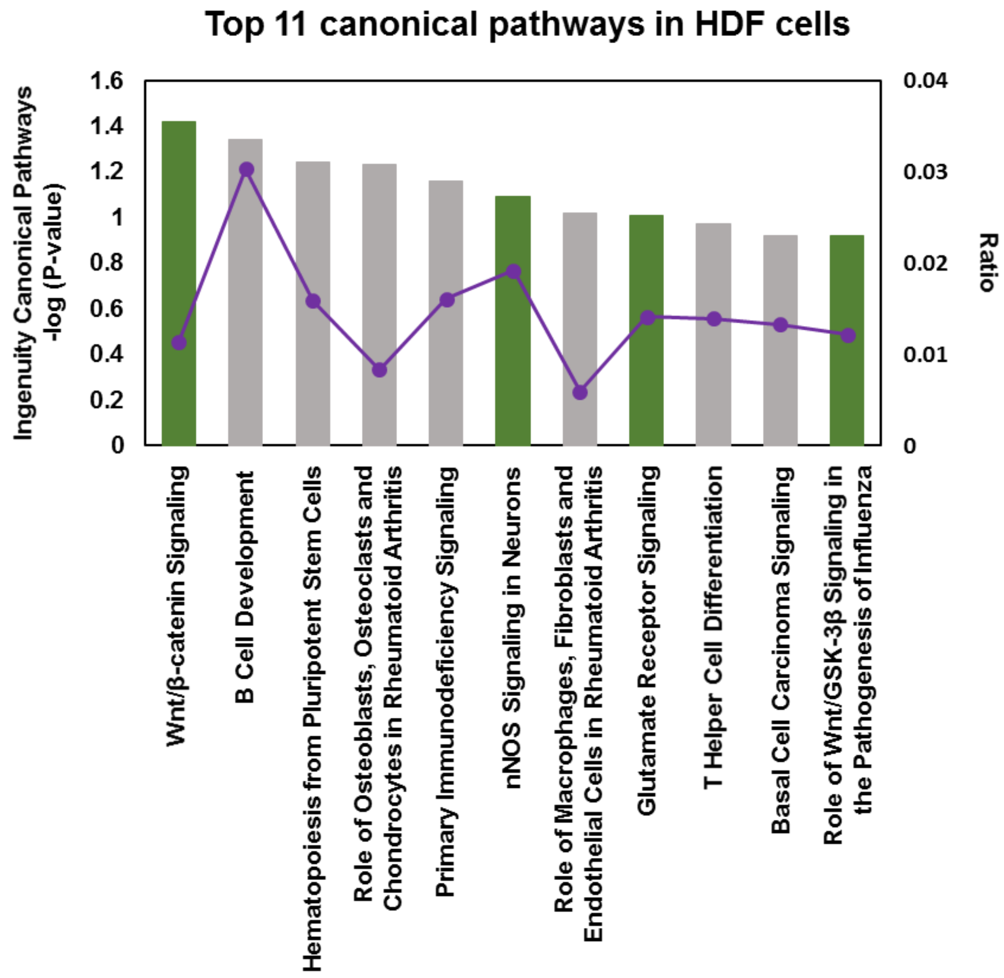

Fig.S1 Ingenuity pathway analysis of SAHA-L treated 54-yr old breast HDFs (> 5-fold upregulated).

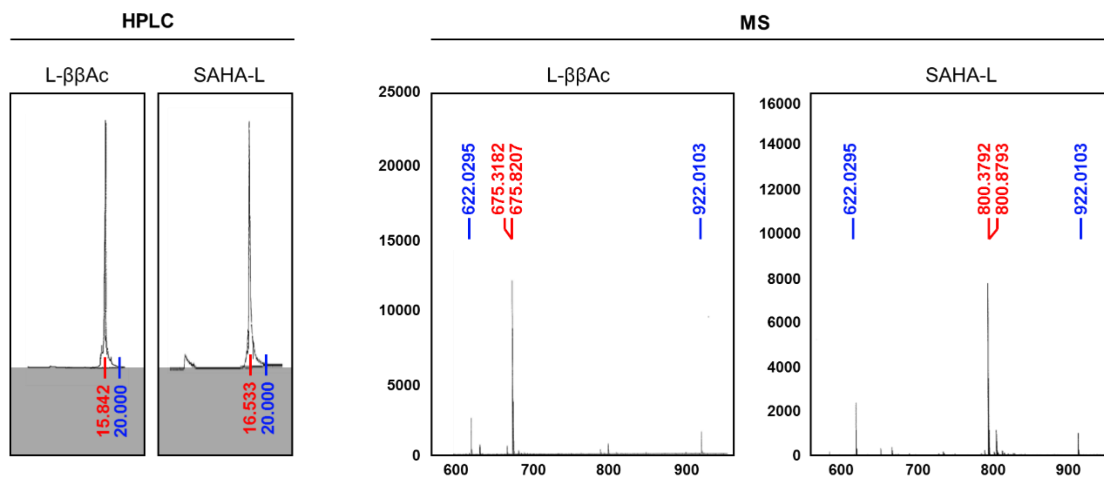

Fig.S2 HPLC and MS of L-ββAc and SAHA-L.

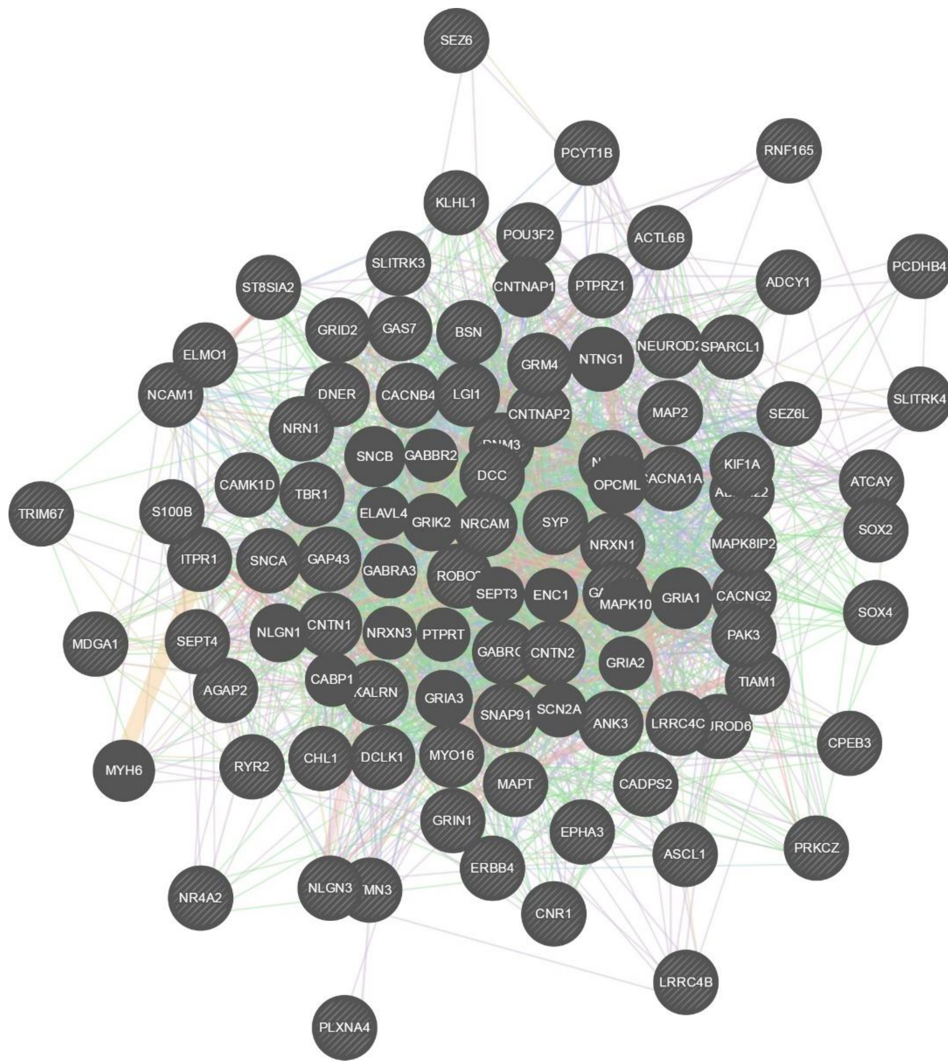

Fig. S3 GENEMANIA analysis of the major SAHA-L activation gene with the neural central system.

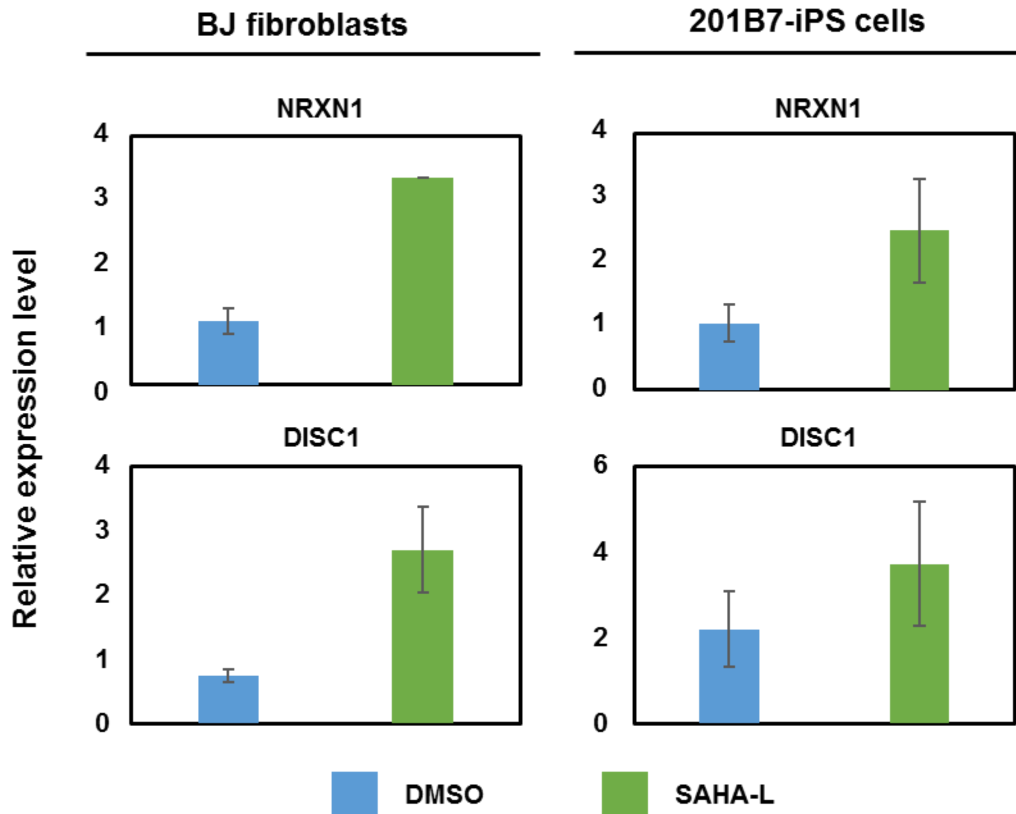

Fig.S4 Gene expression in BJ fibroblasts and 201B7-iPS cells. Quantitative real-time PCR analysis of A) NRXN1, B) DISC1 in BJ fibroblasts and C) NRXN1, D) DISC1 in 201B7-iPS cells. The relative gene expression was normalized to the house-keeping gene β-ACTIN, Three biological replicates were performed and the means ± SD are indicated.

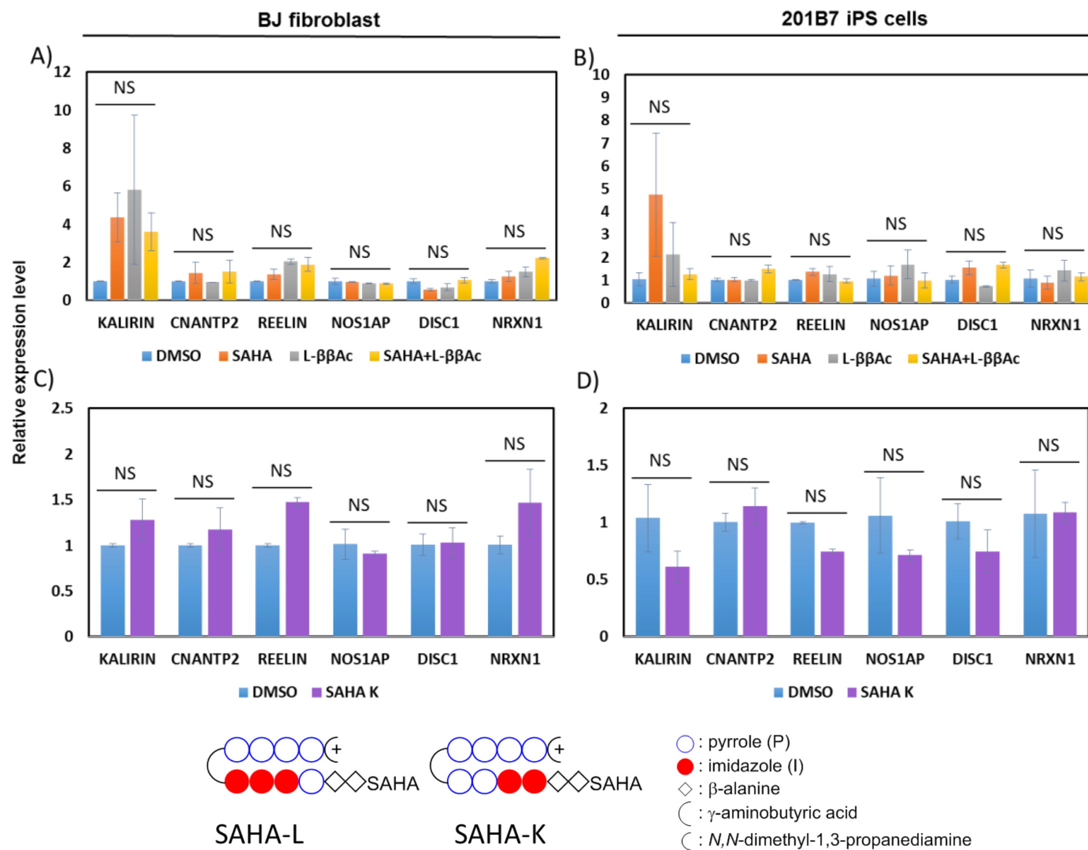

Fig.S5 Gene expression of the SAHA, L-ββAc, SAHA plus L-ββAc and SAHA-K in BJ fibroblasts and 201B7-iPS cells. Quantitative real-time PCR analysis of KALIRIN, CNTNAP2, REELIN, NOS1AP, DISC1, NRXN1 expression in DMSO, SAHA, L-ββAc, SAHA plus L-ββAc treated A) BJ fibroblasts and B) 201B7-iPS cells. The gene expression of SAHA-K treated C) BJ fibroblasts and D) 201B7-iPS cells. The relative gene expression was normalized to the house-keeping gene β-ACTIN, Three biological replicates were performed and the means ± SD are indicated. NS indicates not significant ($p>0.05$).

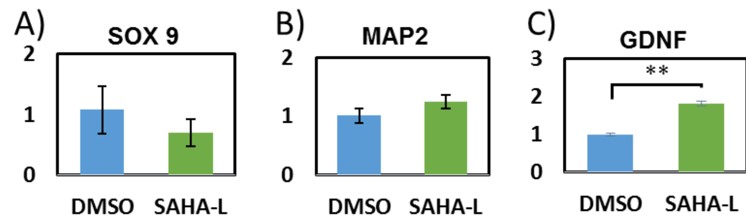

Fig.S6 The expression of neural markers in human 201B7-iPS cells A: SOX 9, B: MAP2, C: GDNF is displayed as determined by q-PCR analysis. The relative gene expression was normalized to the house-keeping gene β-ACTIN, Three biological replicates were performed and the means ± SD are indicated; * * $p<0.01$.

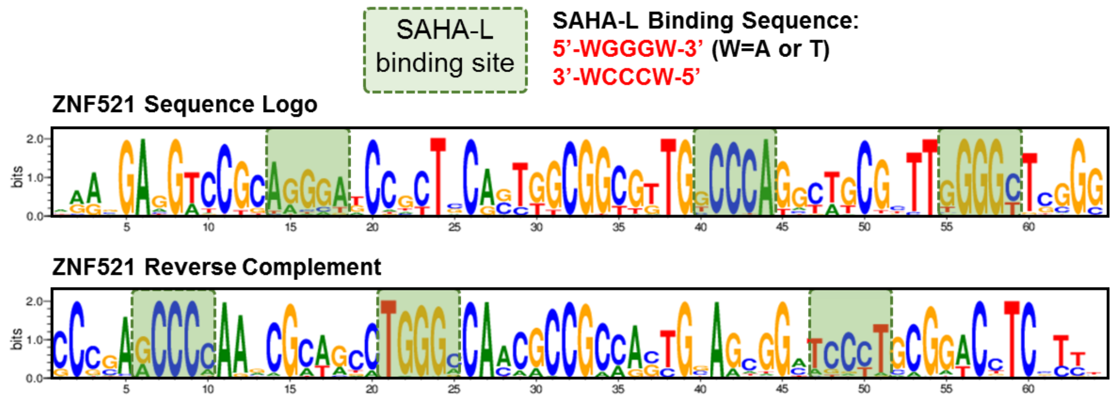

Fig.S7 Predicted binding site of ZFP521 and SAHA-L. SAHA-L Binding site should be 5'-WGGGW-3'. W stand for A or T.

Top Networks of SAHA-PIP L	Score
1. Nervous System Development and function, Cellular Development, Tissue Development	48
2. Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cellular Assembly and Organization	43
3. Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Cellular Function and Maintenance	41
4. Neurological Disease, Psychological Disorder, Nervous System development and function	41
5. Nervous System Development and function, Organ Morphology, Organismal Development	41

Table S1 Top Networks of SAHA-L in BJ fibroblasts

Nervous System Development and Function	p-Value	Molecules (numbers)
neurotransmission	2.13E-54	103
synaptic transmission	1.46E-47	87
development of neurons	1.32E-44	128
morphology of nervous system	1.17E-42	128
abnormal morphology of nervous system	2.06E-40	119
synaptic transmission of cells	1.14E-34	63
morphology of nervous tissue	5.85E-34	84
morphology of neurons	9.08E-34	82
morphology of central nervous system	1.36E-32	89
abnormal morphology of central nervous system	2.73E-30	83
excitatory postsynaptic potential	3.07E-30	41
abnormal morphology of neurons	1.17E-29	71
morphology of brain	5.16E-29	80
abnormal morphology of brain	3.22E-28	77
long-term potentiation	4.30E-27	54
neuritogenesis	2.59E-26	86
quantity of neurons	3.53E-26	70
excitatory postsynaptic current	4.80E-26	30
development of central nervous system	1.22E-22	85
coordination	8.95E-22	44
developmental process of synapse	5.91E-21	41
morphology of neurites	2.70E-20	36
action potential of neurons	2.70E-20	33

Table S2 Nervous System Development and Function of SAHA-L treated BJ fibroblasts

	Forward sequence	**Reverse Sequence**
DISC 1	GCTCTTAGCAGTTTCCTGGGTC	TGTCCTGAGCAGTGGGTTCCAA
REELIN	TCACGTGAGAGGCTACCACA	TTGGAGGTTCCAGTGCTTTC
NRXN 1	GCTATCTTGGCAGGTCCTGTGA	ACATCCTCAGCCTCCGTATGCA
CNTNAP2	CTGTGAGCATGGTGGAAAGTGC	GCTTCACAGGAAGGCTCGTAGA
KALIRIN	GAGCAGACTCATAAGCGGCTAG	TCTGCTTCCGACAAAGAGCTGG
NOS1AP	AGAGACACCGCTGTCCACTCAC	AGCCAACTGGTCCTTCAGCAAG
NESTIN	GGGAAGAGGTGATGGAACCA	AAGCCCTGAACCCTCTTTGC
PAX6	CTGAGGAATCAGAGAAGACAGGC	ATGGAGCCAGATGTGAAGGAGG
NGN2	TCAGACATGGACTATTGGCAG	GGGACAGGAAAGGGAACC
MAP2	TAACCAACCACTGCCAGACCTGAA	GCCACATTTGGATGTCACATGGCT
GDNF	TTATGGGATGTCGTGGCTGT	GATCAGGATAATCCTCTGGC
SOX9	ACATCAAGACGGAGCAGCTGA	AGTAGGAGCTGGAGTTCT
β-ACTIN	CAATGTGGCCGAGGACTTTG	CATTCTCCTTAGAGAGAAGTGG

Table S3 Primer sequence used in this paper

Reference

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