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Supplemental Information

ESC-sEVs Rejuvenate Aging Hippocampal NSCs by Transferring SMADs to Regulate the MYT1-Egln3-Sirt1 Axis

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Supplemental Information

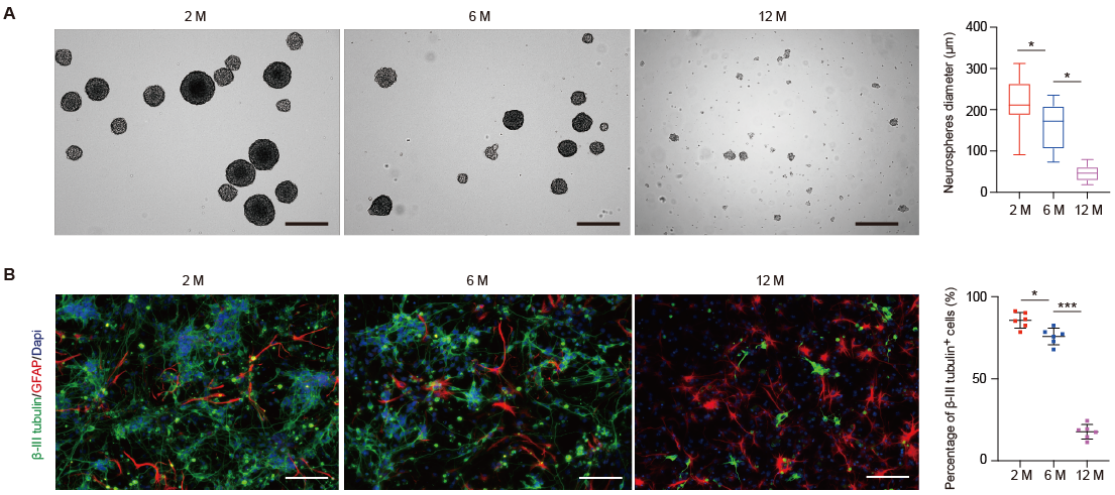

Figure S1. H-NSCs proliferation and neuronal-differentiation decreased with aging. (A) Neurospheres formation and quantification of neurospheres diameter in 2M, 6M, and 12M mice. Scale bar=300 μm; n=6 per group, *P<0.05. (B) IF images for β-III tubulin^{+} (green) and GFAP^{+} cells and the percentage of β-III tubulin^{+} cells in whole cells in 2M, 6M, 12M mice. Scale bar=100 μm, n=6 per group, *P<0.05, ***P<0.001.

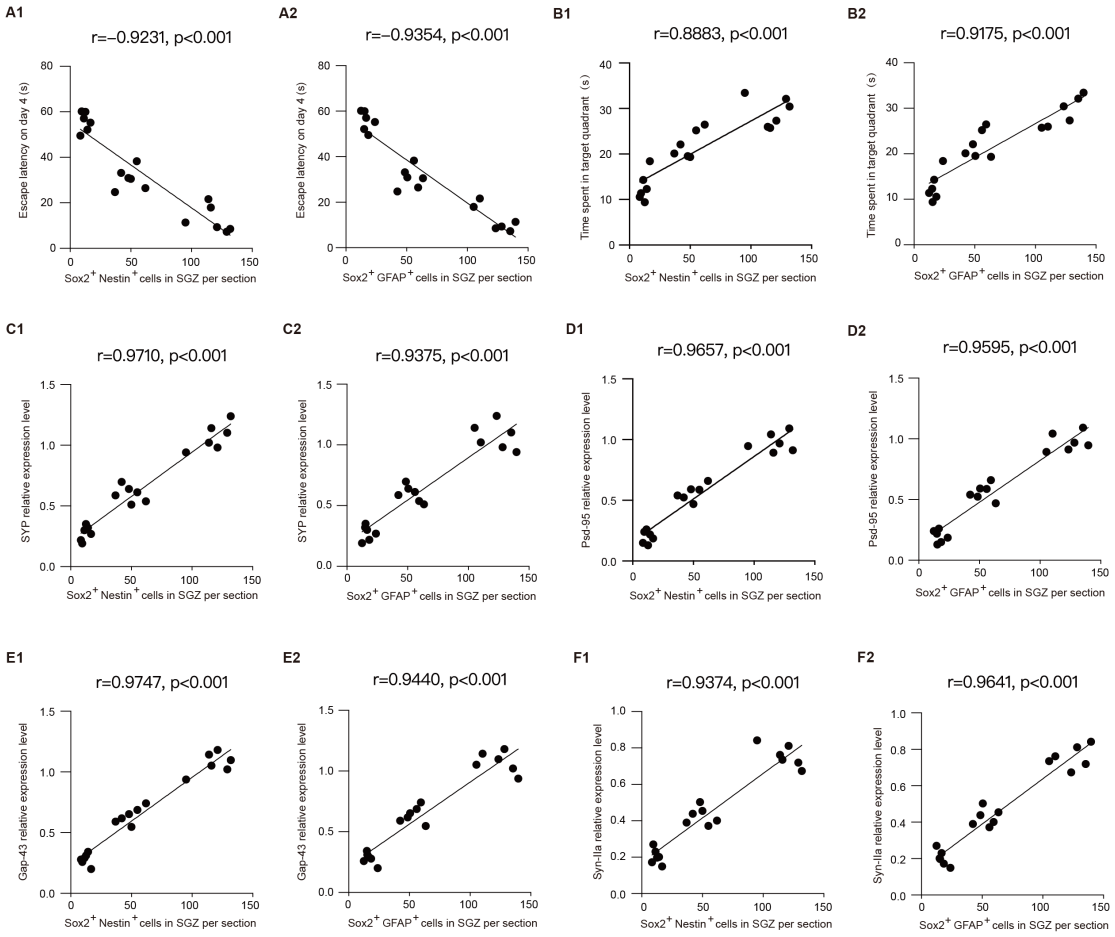

Figure S2. Correlation between the number of H-NSCs and cognitive function. (A) Correlation between the number of H-NSCs ($Sox2^+/Nestin^+$ cells and $Sox2^+/GFAP^+$ cells) in SGZ and the escape latency on day 4. n=6 per group. (B) Correlation between the number of H-NSCs in SGZ and the time spent in the target quadrant on day 5. n=6 per group. (C-F) Correlation between the number of H-NSCs in SGZ and the expression level of hippocampal synapse-related proteins (Syp, Psd-95, Gap-43, Syn-IIa). n=6 per group.

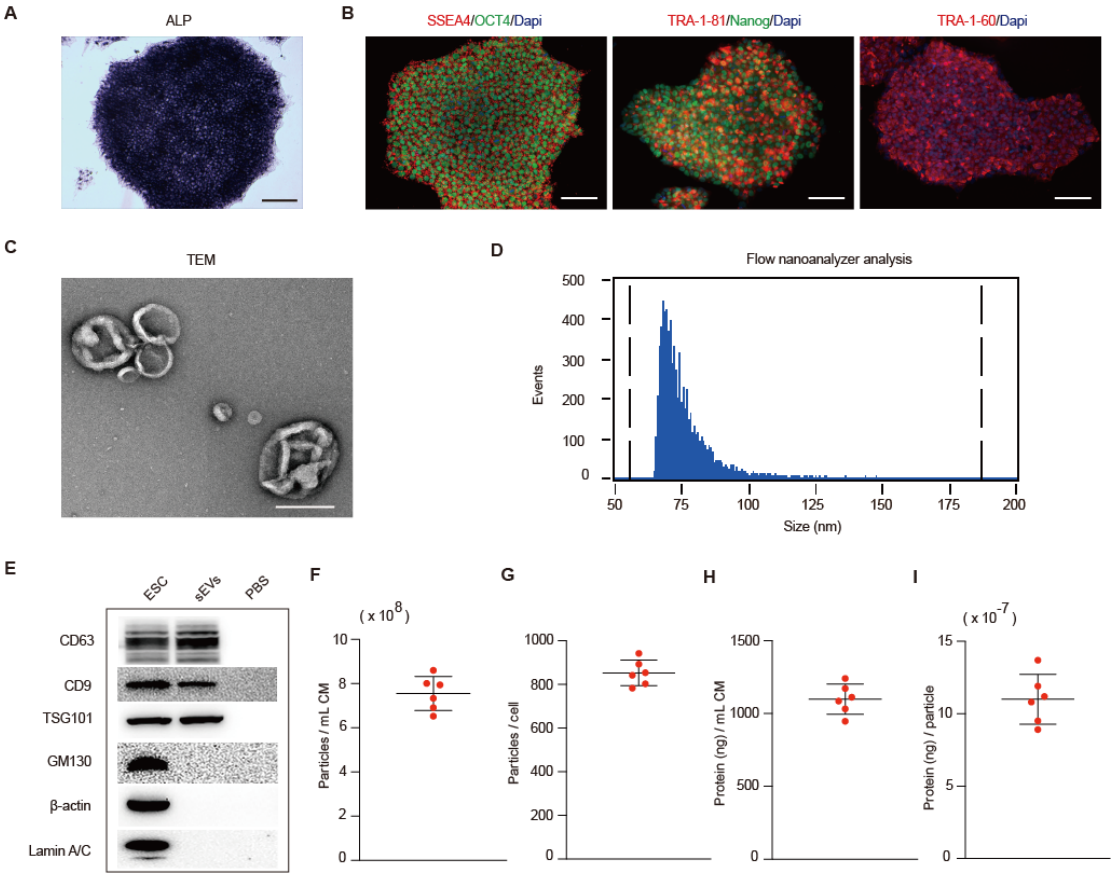

Figure S3. Characterization of ESCs and ESC-sEVs. (A) ALP staining of ESCs. Scale bar=150 μm. (B) IF images for OCT4, Nanog, TRA-1-81, TRA-1-60, and SSEA4 staining in ESCs. Scale bar=100 μm. (C) Morphology of ESC-sEVs observed by TEM. Scale bar=200 nm. (D) Particle size distribution of ESC-sEVs measured by nano-flow cytometer. (E) Western blot showed the presence of sEVs markers including CD63, CD9, and TSG101, and negative for GM130, β-actin, and Lamin A/C. (F)-(I) The yield of ESC-sEVs was evaluated in terms of particle concentration and protein concentration. The mean particle concentration of ESC-sEVs in condition medium (CM) was $7.06\times10^{8}\pm0.93\times10^{8}$ (particles/mL) (F) and 817.92±94.13 (particles/cell) (G), the mean protein concentration of ESC-sEVs in CM was 1127.49±81.47 (ng/mL) (H) and $11.43\times10^{-7}\pm1.57\times10^{-7}$ ng per particle (I). n=6 per group.

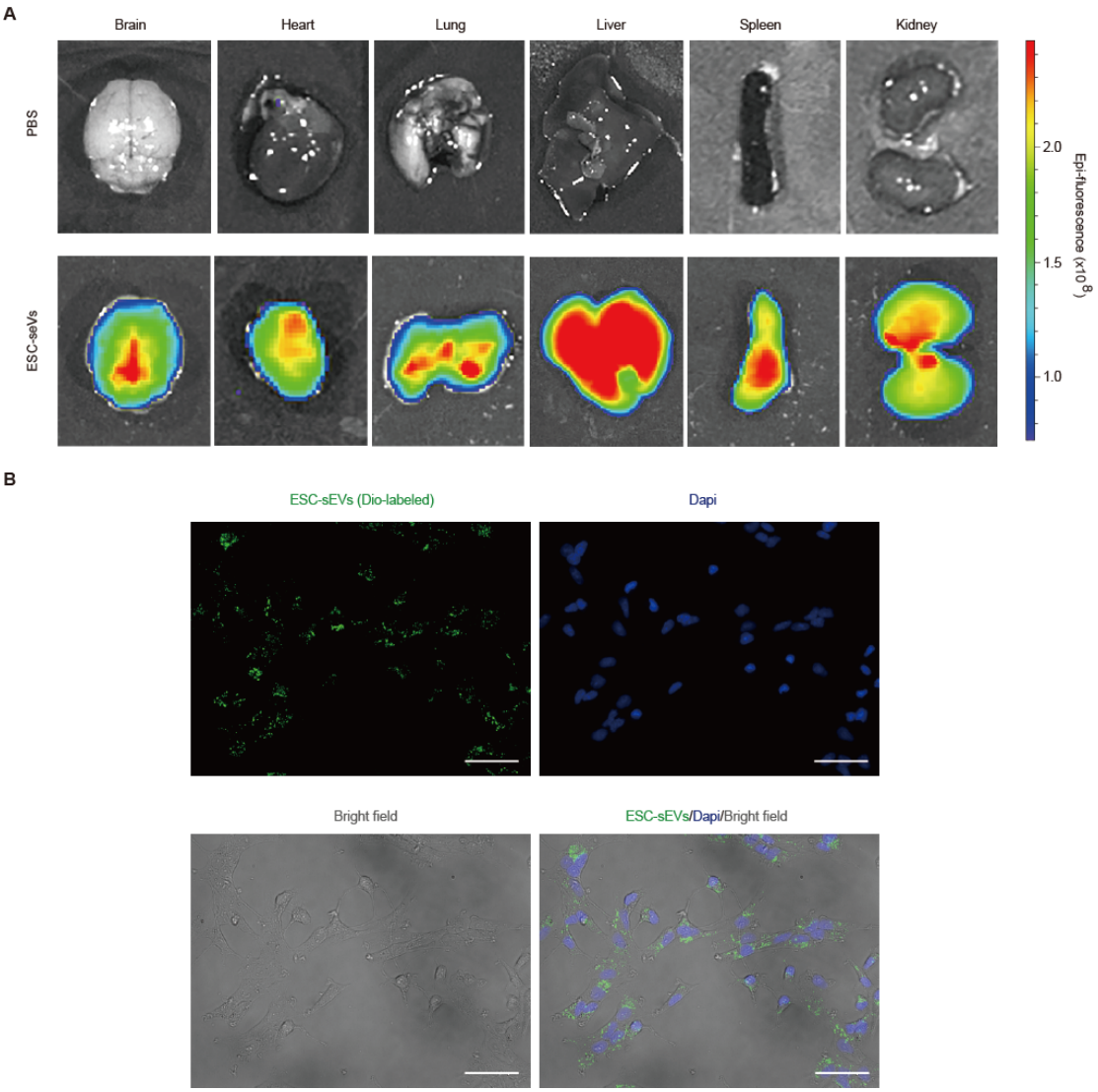

Figure S4. (A) *In vivo* fluorescence images of the distribution of DiR-labeled ESC-sEVs. DiR-labeled sEVs can distribute into brain, heart, lung, liver, spleen, and kidney at 24 hours after injection. n=3 per group. (B) IF for Dio-labeled ESC-sEVs (green) internalized by H-NSCs. Scale bar=50 μm, n=3 per group.

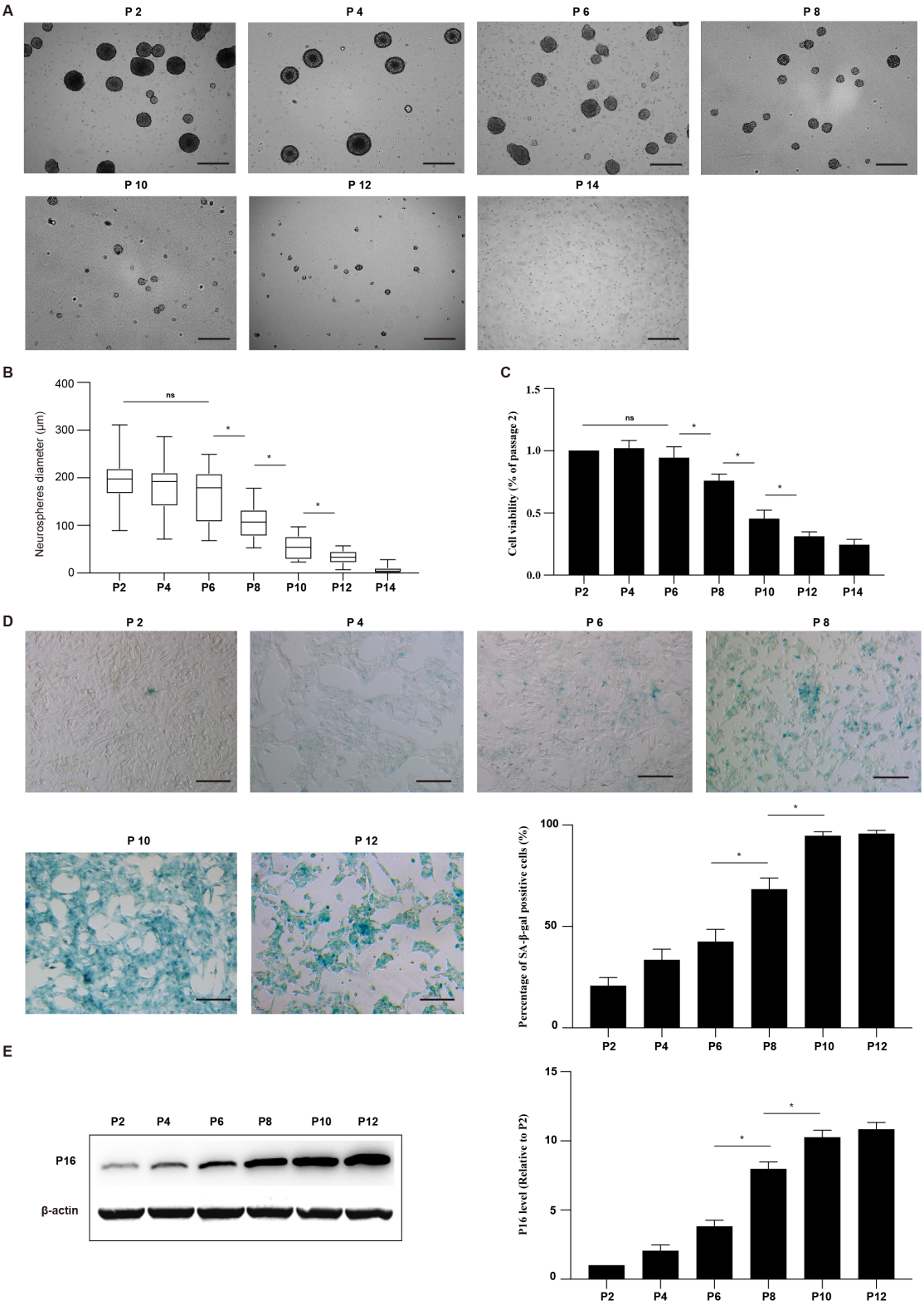

Figure S5. H-NSCs proliferation and senescence from passage 2 to 14 in vitro. (A) and (B) Neurospheres formation and quantification of neurospheres diameter in H-NSCs of

passage 2, 4, 6, 8, 10, 12, 14. Scale bar=300 μm; n=3 per group, *P<0.05. (C) Cell Counting Kit-8 (CCK-8) assay for H-NSCs of passage 2, 4, 6, 8, 10, 12, 14. n=3 per group, *P<0.05. (D) SA-β-gal staining and the percentage of SA-β-gal-positive cells in H-NSCs of passage 2, 4, 6, 8, 10, 12. Scale bar=100 μm, n=3 per group, *p<0.05. (E) Western blot analysis and quantification of $p16^{INK4a}$ in H-NSCs of passage 2, 4, 6, 8, 10, 12. n=3 per group, *p<0.05.

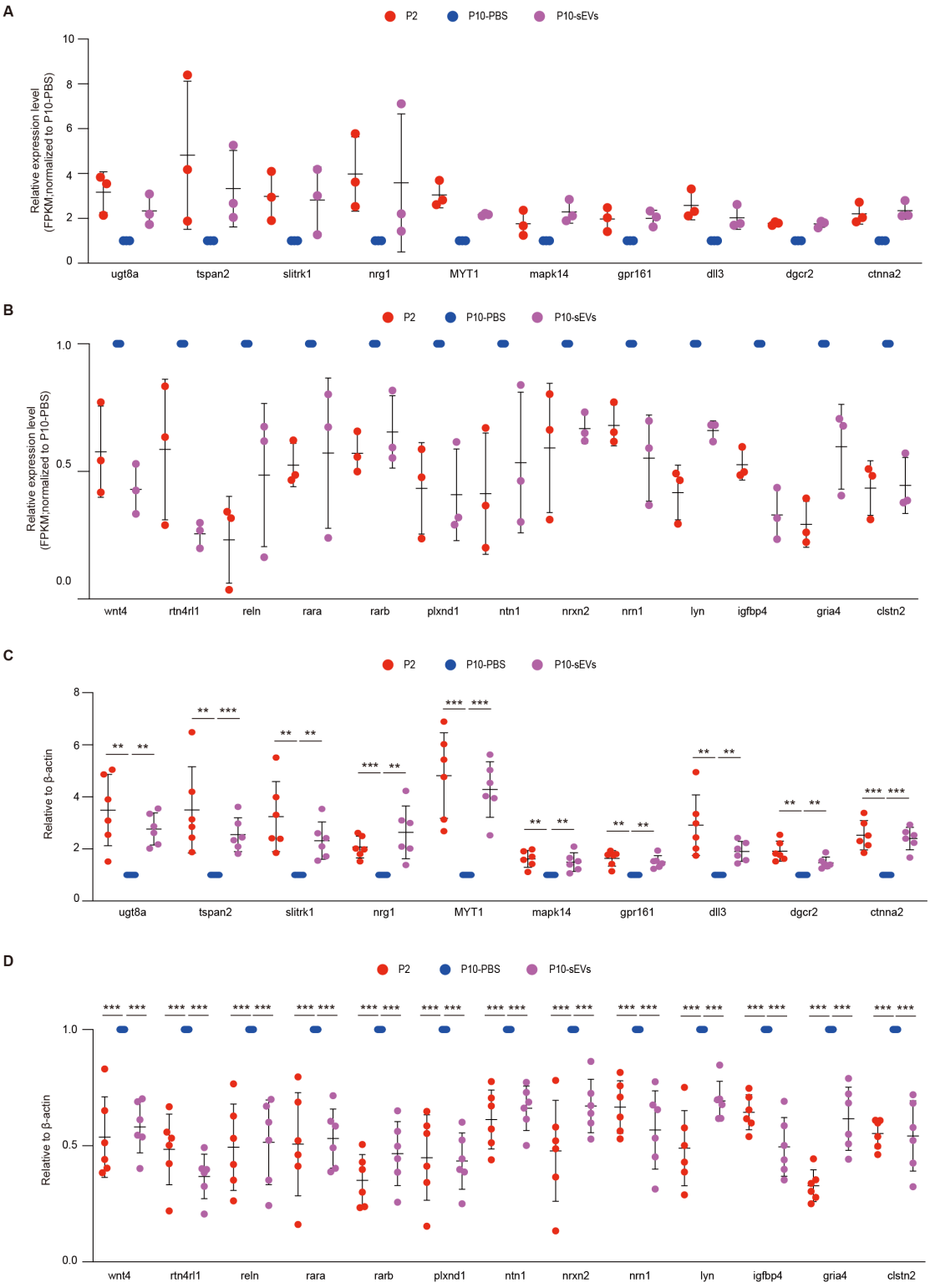

Figure S6. (A) and (B) Summary of the nervous system related 10 co-upregulated and 13 co-downregulated genes according to RNA-seq data from H-NSCs of P2 vs. P10-PBS and P10-sEVs vs. P10-PBS. (C) and (D) The relative expression of nervous system

related 10 co-upregulated and 13 co-downregulated genes were verified by RT-qPCR. n=6 per group, **P<0.01, ***P<0.001.

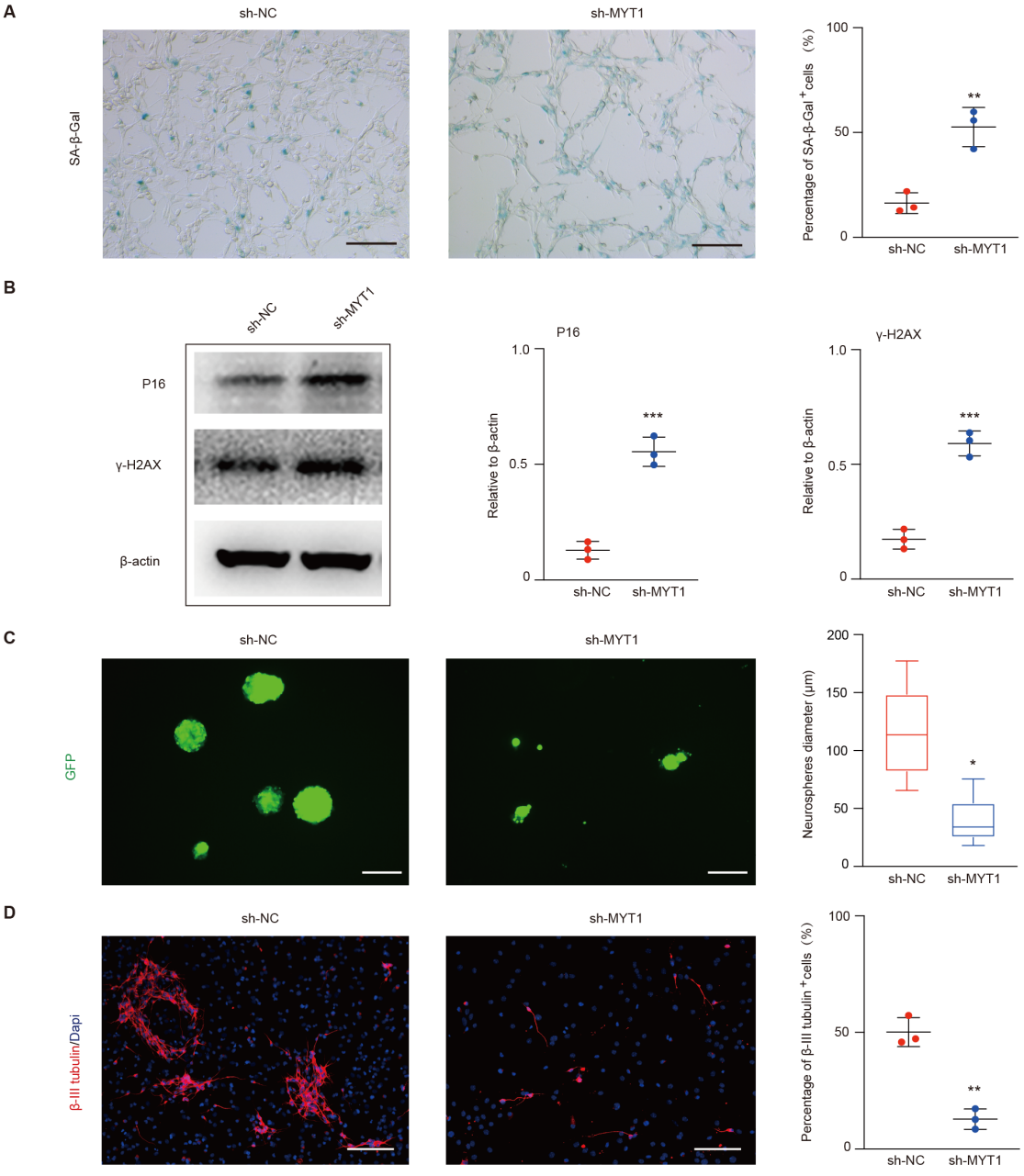

Figure S7. Knockdown of MYT1 induce H-NSCs senescence and impair proliferation and neuronal differentiation capacities. (A) Representative images of SA-β-gal staining and quantification of the percentage of SA-β-gal-positive cells in s hsh-NC NSCs and

sh-MYT1 NSCs. Scale bar=100 μm, n=3 per group, **P<0.01. (B) Western blot analysis and quantification of $p16^{INK4a}$ and γ-H2AX in sh-NC NSCs and sh-MYT1 NSCs. n=3 per group, ***P<0.001. (C) IF images for GFP labeled neurospheres and quantification of the diameter of neurospheres in sh-NC NSCs and sh-MYT1 NSCs. Scale bar=200 μm, n=3 per group, *P<0.05. (D) IF images for β-III $tubulin^+$ cells and the percentage of β-III $tubulin^+$ cells in whole cells in sh-NC NSCs and sh-MYT1 NSCs. Scale bar=100 μm, n=3 per group, **P<0.01.

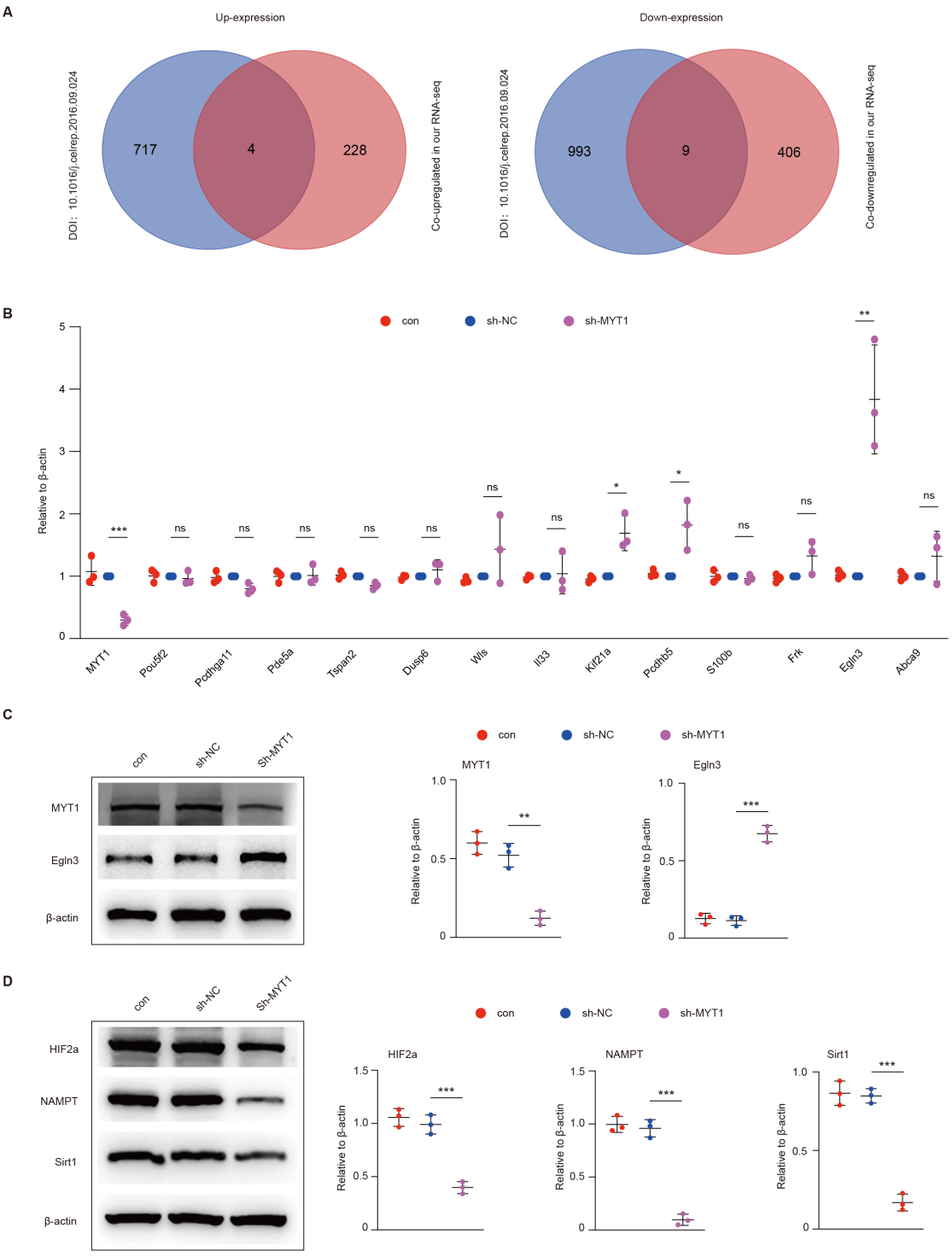

Figure S8. MYT1 inhibit Egln3 and upregulate HIF-2α, NAMPT, Sirt1 in H-NSCs. (A) Comparison of the co-upregulated and co-downregulated genes in H-NSCs of P2 vs. P10-PBS, P10-sEVs vs. P10-PBS with the published data (DOI : 10.1016/j.celrep.2016.09.024). (B) RT-qPCR verified the relative expression of Pou5f2,

Pcdhga11, Pde5a, Tspan2, Dusp6, Wls, Il33, Kif21a, Pcdhb5, S100b, Frk, Egln3, and Abca9 in sh-MYT1 NSCs and sh-NC NSCs. n=3 per group, *P<0.05, **P<0.01, ***P<0.001. (C) Western blot analysis and quantification of MYT1 and Egln3 in control NSCs, sh-NC NSCs, sh-MYT1 NSCs. n=3 per group, **P<0.01, ***P<0.001. (D) Western blot analysis and quantification of HIF-2α, NAMPT and Sirt1 in control NSCs, sh-NC NSCs, and sh-MYT1 NSCs. n=3 per group, ***P<0.001.

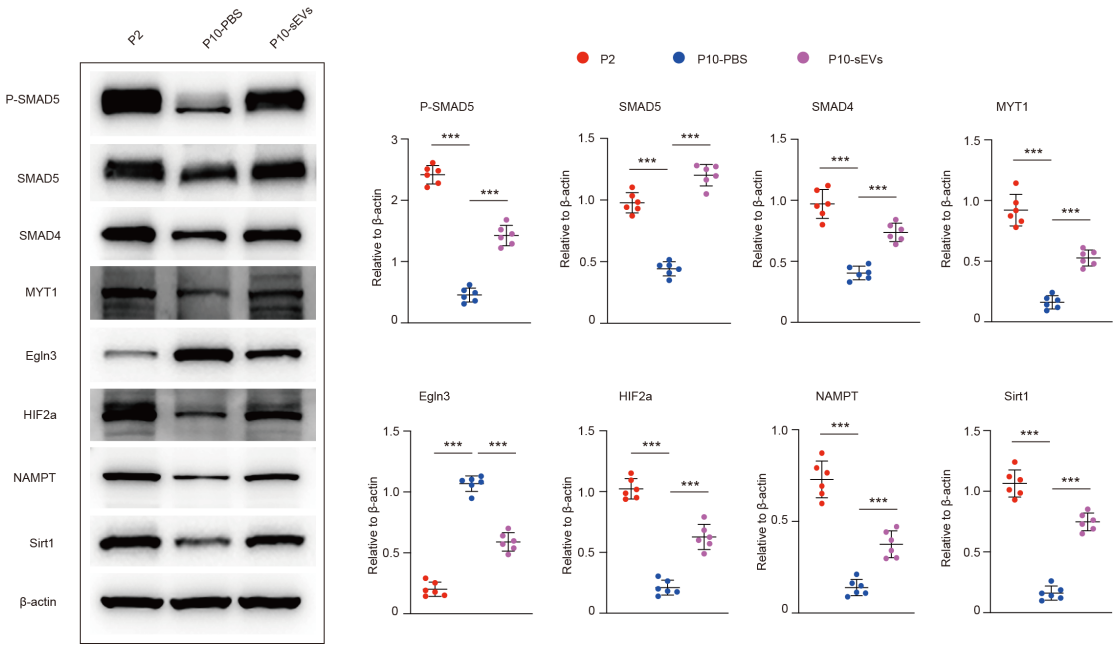

Figure S9. Western blot analysis and quantification of P-SMAD5, SMAD5, SMAD4, MYT1, Egln3, HIF-2α, NAMPT, and Sirt1 in H-NSCs of P2, P10-PBS, and P10-sEVs. n=6 per group, ***P<0.001.

Table S1. MYT1 gene interference sequence.

Interference fragment name	Sequence	
shRNA1	Top strand：	GATCCGCCCAGAGCTATCTAGTCCTAATTCAAGAGAT
		TAGGACTAGATAGCTCTGGGTTTTTTG
	Bottom	AATTCAAAAAACCCAGAGCTATCTAGTCCTAATCTCT
	strand：	TGAATTAGGACTAGATAGCTCTGGGCG
shRNA2	Top strand：	GATCCGAAGCAGCTTGAGGTTCCACCTTATTTCAAGA
		GAATAAGGTGGAACCTCAAGCTGCTTCTTTTTTG
	Bottom	AATTCAAAAAAGAAGCAGCTTGAGGTTCCACCTTATT
	strand：	CTCTTGAAATAAGGTGGAACCTCAAGCTGCTTCG
shRNA3	Top strand：	GATCCGAGGCAGAAAGAGGGAGCTCTCAATTTCAAG
		AGAATTGAGAGCTCCCTCTTTCTGCCTCTTTTTTG
	Bottom	AATTCAAAAAAGAGGCAGAAAGAGGGAGCTCTCAAT
	strand：	TCTCTTGAAATTGAGAGCTCCCTCTTTCTGCCTCG

Table S2. Sequences of primers used in RT-qPCR.

Gene name	Forward primer (5’ → 3’)	Reverse primer (5’ → 3’)
ugt8a	ACTCCATATTTCATGCTCCTGTG	AGGCCGATGCTAGTGTCTTGA
tspan2	TATCTGCTGCTCGGCTTCAAC	GTCCAAATGCAATAACGGCTG
slitrk1	GAAGGGGACTTACACGTAGACT	AGTGAGGGAATTGCCATGCAG
nrg1	ATGGAGATTTATCCCCCAGACA	GTTGAGGCACCCTCTGAGAC
MYT1	TGCAGACCTCAGTTGTCCTAC	TCCTCTTGGATACCAGGTGCT
mapk14	GGGACACCCCCTGCTTATCT	TCCCTGCTTTCAAAGGACTGG
gpr161	CTCACGCTTGGGGTCATTG	GAGCCAGATGTAGACGAGAGC
dll3	CTGGTGTCTTCGAGCTACAAAT	TGCTCCGTATAGACCGGGAC
dgcr2	ATGCTGCACAGACCTGTCAG	TCCTGGGCTAGGACGAAGC
ctnna2	TTCAGAGCACTTCCACTACCT	CTGCTTCCACTCCCGATTGG
wnt4	AGACGTGCGAGAAACTCAAAG	GGAACTGGTATTGGCACTCCT
rtn4rl1	CTGCCAGGCACACAACTTTG	TGTTGGAGTAGATCCAGAGGG
reln	TTACTCGCACCTTGCTGAAAT	CAGTTGCTGGTAGGAGTCAAAG
rara	ATGTACGAGAGTGTGGAAGTCG	ACAGGCCCGGTTCTGGTTA
rarb	GCAGTGCGTGGACACATGA	GGCAGGGAGAGTCCTCTGAT
plxnd1	TCGCTGCCAATCCCTAATAAGA	TGACCTGGTTTGGAACTGTTG
ntn1	CAGCCTGATCCTTGCTCGG	GCGGGTTATTGAGGTCGGTG
nrxn2	TTAAACAGCGAAGTAGGGTC	GCCAGTGTGATCTCGTCA
nrn1	GCGGTGCAAATAGCTTACCTG	CGGTCTTGATGTTCGTCTTGTC

lyn	GTGACATTGTGGTGGCCTTAT	ACCATTCCCCATGCTCTTCTA
igfbp4	AGAAGCCCCTGCGTACATTG	TGTCCCCACGATCTTCATCTT
gria4	GTTTTCTGGATTTTGGGGACTCG	AAGAGACCACCTATTTGAACGC
clstn2	CCTCCTGCGGAAGGACTTTG	TGGGCCAGTCATTAGTCACCA
Pou5f2	GGTCTTCAAACGTCTTCCCTC	CCCATACTTGGTCGCACCAT
Pcdhga11	CTGATCCCGCTGTGCATTTTC	TGCCTACGAAGAAGCCTTTATCT
Pde5a	CGGCCTACCTGGCATTCTG	GCAAGGTCAAGTAACACCTGATT
Dusp6	ATAGATACGCTCAGACCCGTG	ATCAGCAGAAGCCGTTCGTT
Wls	ATGGCTGGGGCAATTATAGAAAA	GGGTGCTGGAGCGATCAAG
Il33	TCCAACTCCAAGATTTCCCCG	CATGCAGTAGACATGGCAGAA
Kif21a	AAGATCGAAGGTTGCCATATCTG	CAGCTCCGGTTTGTCCATAGG
Pcdhb5	CAGGCAAGTGATTCCTTTCCT	ATTGCTGTTTGTCATGGTCAGA
S100b	TGGTTGCCCTCATTGATGTCT	CCCATCCCCATCTTCGTCC
Frk	ACCCGAAGCCATTCGTACTAA	AGCACCTGTCATACCACTGTA
Egln3	AGGCAATGGTGGCTTGCTATC	GCGTCCCAATTCTTATTCAGGT
Abca9	TCGATAGATGCAGTGAGAGTCA	CACAAGGAGCTGAATGGTCTTT
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Table S3. Models and methods in article.

	Models	Methods
In vivo	SAMP8 mice: (1) 2M, 6M, 12M; (2)6M, 12M-PBS, 12M-sEVs	(1) Morris water maze; (2) Western blot for Syp, Psd-95, Gap-43, Syn-IIa; (3) IF staining for Sox2, Nestin, GFAP, DCX, PCNA, $p16^{INK4a}$; (4) SA-β-gal staining
	C57/BL mice: 20M-PBS, 20M-sEVs	(1) Morris water maze; (2) Western blot for Syp, Psd-95, Gap-43, Syn-IIa; (3) IF staining for Sox2, GFAP, DCX, Edu, $p16^{INK4a}$; (4) SA-β-gal staining
Ex vivo	SAMP8 mice: H-NSCs isolated from 6M, 12M-PBS, 12M-sEVs	(1) SA-β-gal staining; (2) Western blot for $p16^{INK4a}$, γ -H2AX, P21, and P53; (3) proliferation assay: IF staining for Edu; (4) neuronal-differentiation assay: IF staining for β-Tubulin III and GFAP

		(5) Western blot for P-SMAD5, SMAD5, SMAD4, and MYT1, Egln3, HIF-2α, NAMPT, Sirt1
	C57/BL mice: H-NSCs isolated from 20M-PBS, 20M-sEVs	(1) SA-β-gal staining; (2) Western blot for $p16^{INK4a}$, γ -H2AX, P21, and P53; (3) Western blot for P-SMAD5, SMAD5, SMAD4, and MYT1, Egln3, HIF-2α, NAMPT, Sirt1
In vitro	H-NSCs isolated from 2-weeks C57/BL mice, replicative senescence model （passage 2 -10）	(1) SA-β-gal staining; (2) Western blot for $p16^{INK4a}$, γ -H2AX, P21, and P53; (3) proliferation assay: IF staining for Edu; neurospheres formation assay; (4) neuronal-differentiation assay: IF staining for β-Tubulin III and GFAP; (5) RNA-seq analysis; (6) RT-qPCR; (7) Lentiviral knockdown; (8) chromatography-tandem mass spectrometry (LC-MS/MS) analysis; (9) Western blot for P-SMAD5, SMAD5, SMAD4, and MYT1, Egln3, HIF-2α, NAMPT, Sirt1

Supplemental Materials & Methods

ESC culture

The human embryonic stem cells (ESC; H9) were provided by the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (Shanghai, China). The ESC were cultured in ncEpic hPSC medium (NO. RP01001; Nuwacell Biotechnologies, China) on a Nuwacell™-Vitronectin (NO. RP01002-A; Nuwacell) coated plate under the standard conditions of 5%CO2 and 37°C in humidified incubator. The medium was changed daily and cells were passaged at 80~90% confluence. ESCs identification was performed by ALP staining and immunofluorescence staining (IF) for markers (Nanog, OCT4, SSEA4, TRA-1-60, and TRA-1-81) in the ESC colonies.

ESC-sEVs isolation and identification

ESC-sEVs isolation

Embryonic stem cells derived small extracellular vesicles (ESC-sEVs) were isolated by differential ultracentrifugation protocols from ESCs condition medium (ESC-CM) according to the MISEV2018 guideline. Briefly, ESC-CM was centrifuged at 300×g for 10 min to remove dead cells and 2000×g for 20 min to remove cellular debris and apoptotic bodies. Then, the supernatant was centrifuged at 10,000×g for 30 min and filtered through 0.22 μm sterilized filter (Millipore, Bedford, MA, USA) to remove large EVs. Next, the supernatant was subjected to ultracentrifugation at 100,000×g for 114 min by using a SW 32 Ti Rotor Swinging Bucket rotor (K factor of 256.8, 28536 rpm; Beckman Coulter, Fullerton, CA) to pellet ESC-sEVs. After removing the

supernatant, the ESC-sEVs pellet was washed with PBS and followed by a second ultracentrifugation at 100,000×g for 114 min. All centrifugation steps were performed at 4°C. Finally, the pellet was re-suspended in PBS and stored at -80°C.

Transmission Electron Microscope (TEM)

The morphology of ESC-sEVs was observed by TEM (JEM 1400, Tokyo, Japan). Briefly, a total of 10μL ESC-sEVs enriched solution was placed on a formvar-carbon coated grid (300 meshes) and left to dry at room temperature for 20min. Then, ESC-sEVs was washed with PBS and fixed in 1% glutaraldehyde for 5 min. Next, ESC-sEVs was washed with water and stained with saturated aqueous uranyl oxalate for 5 min. Finally, the grid was dried at room temperature for 10 min and then imaged.

Size distribution and particle concentration

The size distribution and particle concentration of ESC-sEVs were measured by using the nano-flow cytometer (N30 Nanoflow Analyzer, NanoFCM Inc., Xiamen, China) as described previously[1]. Briefly, the side scatter intensity (SSI) was measured by the loading of the standard polystyrene nanoparticles (200 nm) with a concentration of 1.58 $\times 10^8$/mL to the nano-flow cytometer. Next, isolated ESC-sEVs sample diluted with 1000-fold PBS (for a nanoparticle concentration of approximately 5×10^9/mL) was loaded to the nano-flow to measure the SSI. Finally, the concentration of EVs was calculated according to the ratio of SSI to particle concentration in the standard polystyrene nanoparticles. For size measurement, standard silica nanoparticles with mixed size (68nm, 91nm, 113nm, 155nm) were load to the nano-flow cytometer to

generate a standard cure, followed by the loading of sEVs sample. The size distribution was calculated according to the standard cure.

Protein concentration

The protein concentration of ESC-sEVs was quantified by Pierce BCA Protein Assay Kit (Thermo Scientific) according to the product manual. Briefly, 200 μL of the WR solution was loaded into each well of a 96-well plate. Next, 10 μL of the ESC-sEVs sample was added. Finally, the plate was incubated at 37°C for 30 min and the absorbance was detected at 562 nm. A standard curve was used to determine the protein concentration of each ESC-sEVs sample.

sEVs markers

The expression of sEVs markers such as CD9, CD63, and TSG-101 was analyzed by western blot. GM130, β-actin, and Lamin A/C were also detected to determine the purity of ESC-sEVs. Briefly, the ESC-sEVs pellet was routine ultracentrifugation as described above. ESC-sEVs protein was harvested by using RIPA lysis buffer supplemented with protease inhibitor cocktail (Roche). Then, the protein concentration of ESC-sEVs was detected by using the Pierce BCA Protein Assay Kit as described above. Next, protein was separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore). The membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4 °C with the following antibodies: rabbit monoclonal anti-β-actin (1:1,000; Abcam, NO. ab179467), rabbit monoclonal anti-CD9 (1:1,000; Abcam, NO. ab92726), rabbit monoclonal anti-CD63 (1:1,000; Abcam, NO. ab134045), mouse monoclonal anti-

TSG-101 (1:1,000; Abcam, NO.ab83), mouse polyclonal anti-GM130 (1:500; Abcam, NO.ab169276), Rabbit polyclonal anti-Lamin A/C (1:1000; Servicebio, NO. GB11407). Membranes were then incubated with HRP-conjugated secondary antibodies (1:3,000; Cell Signaling Technology) at room temperature for 1 h. The immunoreactive bands were visualized using ECL (Thermo Fisher Scientific) and imaged with a FluorChem M Fluorescent Imaging System (ProteinSimple, Santa Clara, CA, USA).

In vivo ESC-sEVs migration into brain

To determine the migration of ESC-sEVs into the brain, ESC-sEVs were labeled with the Molecular Probes' Vybrant (Life Technologies, NY, Cat No. D12731) 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) according to the protocol as previously described with small modification[2]. Briefly, ESC-sEVs were incubated with DiR fluorescent dye under room temperature for 15 minutes, then were loaded at the bottom of an iodixanol cushion (0%, 20%, 30%, and 50%) and centrifuged at 100,000×g for 114 min (SW 32-Ti Rotor) to separate them from the free unbound dye. The DiR-labeled ESC-sEVs were collected from the interphase between 20% and 30% and washed in PBS and centrifuged at 100,000×g for 114 min (SW 32-Ti Rotor). SAMP8 mice (n = 3) were intravenously administered with fresh purified DiR-labeled ESC-sEVs (1×10^{10} particles/ml,100μl), followed by euthanasia and ex vivo imaging of dissected organs at 24 hours after application. Bio-distribution of DiR-labeled ESC-sEVs was examined by using IVIS Spectrum (Perkin Elmer). The harvested organs were imaged for 1-2 seconds (excitation 710 nm, emission 760 nm). The data were analyzed with the IVIS software (Living Image Software for IVIS).

Morris Water Maze

Morris water maze (MWM) test was employed to assess spatial learning and memory abilities of rats as described previously[3]. The latency to escape onto the platform was recorded as the performance of spatial learning. Mice were trained once a day over four consecutive days. In each trial, mice were gently released into the water with their head facing opposite of the platform, and were given a maximum of 60s to find the submerged platform. For mice that could not find the platform within 60s, they were guided to stay on it for 15s, and the score of 60s was given to such mice. To assess spatial memory, a spatial probe trial was performed on day 5 of the training trial. The platform was removed and mice were placed in water opposite to the target quadrant, and allowed to swim freely for 60s. The percentage of time that mice spent in the target quadrant within 60s was recorded. The Shanghai Xinran Mdt InfoTech Ltd (Shanghai, China) SuperMaze animal behavior record and analysis system was used for data collection and analysis.

Immunofluorescence (IF) staining

Mice were anesthetized and perfused transcardially with saline, then followed by 4% paraformaldehyde (PFA; 4.3 g/L NaOH, 40 g/L paraformaldehyde, 18.8 g/L NaH2PO4.H20). The brains were removed and post-fixed overnight in 4% PFA, and then dehydrated in 20% and 30 % sucrose at 4 °C, respectively. Each brain was embedded in OCT and cut every sixth 20-μm-thick coronal sections on a freezing microtome (Leica CM 1950; Leica Biosystem, Heidelberg, Germany). Cells were fixed by 4% PFA for 15 min. Brain sections or cultured cells were incubated with 0.3% Triton

X-100 for 30 min and 5 % bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) for 1 h, and then incubated overnight at 4 °C with the following antibodies: rabbit polyclonal anti-Sox2 (1:100; Cell Signaling Technology, NO. cst2748), mouse monoclonal anti-GFAP (1:100; Abcam, NO. ab10062), rabbit monoclonal anti-β-Tubulin III (1:100; Abcam, NO.ab18207), mouse monoclonal anti-Nestin (1:100; Abcam, NO. ab6142), rabbit polyclonal anti-DCX (1:100; Abcam, ab18723), mouse monoclonal anti-$p16^{INK4a}$ (1:200; Invitrogen, NO.MA5-17142), mouse monoclonal anti-PCNA (1:100; Abcam, NO. ab29), rabbit polyclonal anti-Nanog (1:100; Abcam, NO.ab109250), rabbit polyclonal anti-OCT4 (1:100; Abcam, NO.ab19857), mouse monoclonal anti-SSEA4 (1:100; Abcam, NO.ab16287), mouse monoclonal anti-TRA-1-60 (1:100; Abcam, NO.ab16288), mouse monoclonal anti-TRA-1-81 (1:100; Abcam, NO.ab16289). Followed by three times of rinse in 0.1 M PBS (pH 7.4) before the incubation with secondary antibodies (1:400; Invitrogen) including Alexa Fluor® 594 goat anti-rabbit or anti-mouse IgG (H+L) or Alexa Fluor® 488 goat anti-rabbit or anti-mouse IgG (H + L) for 1 h at room temperature, protected from light. EdU^+ cells were stained by using Click-iT Edu Alexa Fluor 488 or 594 Imaging Kit (Life Technologies) following the manufacturer's protocol. Nuclei was visualized by using 2 μM DAPI (Sigma). Images were acquired using the Leica DM6B microscope (Leica Microsystems, Milan, Italy). The number of positively stained cells were performed using the Image J software (National Institutes of Health, Bethesda, MD, USA).

H-NSCs isolation and cultivation

H-NSCs were isolated from mice hippocampus as described previously. Briefly, hippocampus was separated from brain and minced by scissors, then treated with enzyme cocktail solution (papain (2.5 U/mL), DNase (250 U/mL) and dispase (1U/mL)) at 37°C for 1 h. Later mixed with an equal volume of percoll solution and centrifuged at 20,000 g for 30 min at 18°C. H-NSCs located in the lower layer fraction were harvested and washed three times with DMEM/F12, and resuspended in complete H-NSCs medium: DMEM/F12 medium supplemented with 2% B27 (Gibco Life Technologies), 1% penicillin/streptomycin (Gibco Life Technologies), 20 ng/ml epidermal growth factor (EGF, ProSpec), 20 ng/ml basic fibroblast growth factor (bFGF, ProSpec), and 5 μg / ml heparin (Sigma-Aldrich). Cells were seeded in a culture plate and the medium was fifty percent replaced every 2 days.

In vitro ESC-sEVs uptake assay

ESC-sEVs were labeled with fluorescent carbocyanine dye (Dio, Life Technologies) for 30 min at 37 °C as our previously described. The labeled ESC-sEVs were loaded at the bottom of an iodixanol cushion (0%, 20%, 30%, and 50%) and centrifuged at 100,000×g for 114 min (SW 32-Ti Rotor) to separate them from the free unbound dye. The DiR-labeled ESC-sEVs were collected from the interphase between 20% and 30% and washed in PBS and centrifuged at 100,000×g for 114 min (SW 32-Ti Rotor). H-NSCs were incubated with Dio-labeled ESC-sEVs (1×10^{10} particles/mL) for 12 h. After that, culture medium was discarded and the cells were rinsed twice with PBS. H-

NSCs were fixed, nucleus was stained with DAPI. Images were acquired using the Leica DM6B microscope.

Senescence-associated β-galactosidase (SA-β-gal) staining

SA-β-gal staining of brain sections and NSCs were performed using the SA-β-gal staining kit (Beyotime Biotechnology, Cat No.C0602). According to manufacturer's protocol, brain sections or cell cultures were fixed and then stained with SA-β-gal staining solution for 16-18 h at 37 °C (without CO_2), ice-cold PBS was used to stop the enzymatic reaction. Images of brain sections were acquired using the Leica DM6B microscope, the activity of SA-β-gal for hippocampus was evaluated by means of a ROD (relative optical density) value. ROD of SA-β-gal positive cells was obtained after transformating mean gray values (obtained by Image Pro Plus software) into ROD via the formula: ROD = log (256/mean gray). In cell cultures, neurospheres were dissociated with Accutase (Sigma-Aldrich), 20,000 cells were plated into individual wells of poly-L-lysine (Sigma-Aldrich) coated 24-well plates and incubated in complete NSCs medium for 6 hours, then H-NSCs were fixed and stained for SA-β-gal activity, at least ten images of NSCs were acquired under the phase-contrast microscope (Leica Microsystems). The activity of SA-β-gal was defined as the ratio of SA-β-gal positive cells, which determined by counting the blue cells and dividing by the total number of observed cells.

Effects of ESC-sEVs on H-NSCs senescence, proliferation, and differentiation

We applied replicative senescence model to detect effects of ESC-sEVs on H-NSCs senescence. Briefly, H-NSCs were incubated with 1×10^{10} particles/mL ESC-sEVs or

an equal volume of PBS from passage 2 to passage 10. NSCs senescence was detected by SA-β-gal staining and western blot. For Edu incorporation assay, neurospheres were dissociated and 20,000 cells were plated into individual wells of ultralow-binding 24-well plates and incubated in complete NSCs medium for 4 days, and then administered EdU (10uM) for 4 hours. Neurospheres were plated on poly-L-lysine (Sigma-Aldrich) coated 24-well plates and adhered for 6 hours. Then, Neurospheres were fixed and immunofluorescence staining to calculate the percentage of EdU^+ cells in whole cells, at least ten images of neurospheres were acquired. For CCK8 assay, 20,000 cells were plated into individual wells of ultralow-binding 24-well plates and incubated in complete NSCs medium for 4 days, CCK-8 solution (per 10 μL in 100 μL medium) was added into medium and incubated for 2 h at 37°C. The amount of formazan dye generated by cellular dehydrogenase activity was measured for absorbance at 450 nm by using a microplate reader. The optical density values of each well represented the survival/proliferation of H-NSCs. For neurospheres formation assay, 20,000 cells were plated into individual wells of ultralow-binding 24-well plates and incubated in complete NSCs medium for 6 days, the diameter of neurospheres were counted under a phase-contrast microscope or fluorescent microscope, at least ten images of neurospheres were acquired. For the differentiation assay, neurospheres were dissociated and 50,000 cells were plated on poly-L-lysine coated 48-well plates. Cells were cultured with the differentiation medium: neural basal (NB) medium (Gibco Life Technologies) supplemented with 2% B27 and 1% fetal bovine serum (Gibco Life Technologies), and cultured for 5 days, the medium was half-changed every other day.

Cells were fixed and immunofluorescence staining for β-Tubulin III and GFAP to calculate the percentage of β-Tubulin III $^{+}$ cells in whole cells. Images were acquired using the Leica DM6B microscope, and at least ten images were acquired.

RNA-sequencing analysis

RNA-seq analysis was performed by Shanghai Biotechnology Corporation (Shanghai, China). In brief, total RNA was isolated using RNeasy mini kit (Qiagen, Germany). Paired-end libraries were synthesized by using the TruSeq™ RNA Sample Preparation Kit (Illumina, USA) following TruSeq™ RNA Sample Preparation Guide. The poly-A containing mRNA molecules were purified using Poly-T oligo-attached magnetic beads. Following purification, the mRNA is fragmented into small pieces using divalent cations under 94°C for 8 min. The cleaved RNA fragments are copied into first strand cDNA using reverse transcriptase and random primers. This is followed by second strand cDNA synthesis using DNA polymerase I and RNase H. These cDNA fragments then go through an end repair process, the addition of a single 'A' base, and then ligation of the adapters. The products are then purified and enriched with PCR to create the final cDNA library. Purified libraries were quantified by Qubit® 2.0 Fluorometer (Life Technologies) and validated by Agilent 4200 bioanalyzer (Agilent Technologies, USA) to confirm the insert size and calculate the mole concentration. Cluster was generated by cBot with the library diluted to 10 pM and then were sequenced by using the Next Generation Sequencing (NGS) on the Illumina HiSeq 2500. Raw Reads were checked Seqtk to select the clean Reads. Genome mapping was carried out by using Hisat2 (version: 2.0.4). Using Stringtie (version:1.3.0) to calculate genes's fragments,

and perl was used to calculate Fragments Per Kilobase of exon model per Million mapped reads (FPKM) of every gene.

Proteomic analysis of ESC-sEVs

The proteomic analysis was performed by the Shanghai Applied Protein Technology Company (Shanghai, China) as our previous described. In brief, sEVs samples were suspended in SDT lysis buffer. The proteins were separated on 12.5% SDS-PAGE gel and protein bands were visualized by Coomassie Blue R-250 staining. 200 μg of proteins for each sample were incorporated into 30 μl SDT buffers. The detergent, DTT and other low-molecular-weight components were removed using UA buffer by repeated ultrafiltration. Then 100 μl iodoacetamide was added to block reduced cysteine residues and the samples were incubated for 30 min in darkness. The filters were washed with 100 μl UA buffer three times and then 100 μl 25mM NH4HCO3 buffer twice. Finally, the protein suspensions were digested with 4 μg trypsin (Promega) overnight at 37 °C, and the resulting peptides were collected as a filtrate. The peptides of each sample were desalted on C18 Cartridges, concentrated by vacuum centrifugation and reconstituted in 40 μl of 0.1% (v/v) formic acid. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Thermo Fisher Scientific) for 240 min. The mass spectrometer was operated in positive ion mode. The MS data were analyzed using MaxQuant software version 1.5.3.17.

Real-time quantification polymerase chain reaction (RT-qPCR) analysis

Targeted gene expression analyses were performed by RT-qPCR analysis as described before[4]. Briefly, total RNA was isolated using QIAzol Lysis Reagent and RNeasy Mini Columns (QIAGEN, Valencia, CA). DNase treatment was applied to degrade contaminating genomic DNA using an on-column RNase-free DNase solution (QIAGEN, Valencia, CA). RNA quantity and purity were confirmed with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Reverse transcriptase was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Life Technologies, Foster City, CA). PCR reactions were run using the ABI Prism 7900HT Real Time System (Applied Biosystems, Carlsbad, CA) with SYBR green (QIAGEN, Valencia, CA). The data was analyzed using the cycle threshold (Ct) value. Each experiment was performed in triplicate. The primer sequences used in this study are listed in Table S2.

Western blot

H-NSCs whole protein was harvested by using RIPA lysis buffer supplemented with protease inhibitor cocktail (Roche) and Phosphatase inhibitor cocktail (Roche). The protocols for protein concentration mesurement and western blot were described above. The following antibodies were usde: $p16^{INK4a}$ (1:1,000), rabbit monoclonal anti-P21 (1:1,000; Abcam, NO.ab109199), rabbit polyclonal anti-P53 (1:1,000; Abcam, NO.ab131442), rabbit monoclonal anti-Phospho-Histone H2A.X (Ser139) (γ-H2AX; 1:200; Cell Signaling Technology, NO. cst9718), mouse monoclonal anti-SYP (1:1,000; Abcam, NO.ab8049), mouse monoclonal anti-Psd-95 (1:1,000; Abcam, NO.ab13552),

mouse monoclonal anti-Gap-43 (1:500; Santa Cruz, NO.sc-17790), mouse monoclonal anti-Syn-IIa (1:500; Santa Cruz, NO.sc-136086), rabbit monoclonal anti-SMAD5 (phospho S463 + S465) (P-SAMD5; 1:2000; Abcam, NO.ab92698), rabbit monoclonal anti-SMAD5 (1:2000; Abcam, NO.ab40771), rabbit monoclonal anti-SMAD4 (1:5000; Abcam, NO.ab40759), rabbit monoclonal anti-NAMPT (1:1000; Abcam, NO.ab236874), rabbit monoclonal anti-Egln3 (1:2000; Abcam, NO.ab184714), rabbit polyclonal anti-MYT1 (1:1,000; Abcam, NO.ab82844), rabbit polyclonal anti-HIF2α (1:1,000; Abcam, NO.ab199), rabbit monoclonal anti-Sirt1 (1:1000; Abcam, NO.ab189494), rabbit monoclonal anti-β-actin (1:1,000; Abcam, NO.ab179467). The bands were imaged with a FluorChem M Fluorescent Imaging System and the gray value was analyzed by Image J software.

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