Stem Cell Reports, Volume *8*

Supplemental Information

miR-342-5p Regulates Neural Stem Cell Proliferation and Differentiation Downstream to Notch Signaling in Mice

Fang Gao, Yu-Fei Zhang, Zheng-Ping Zhang, Luo-An Fu, Xiu-Li Cao, Yi-Zhe Zhang, Chen-Jun Guo, Xian-Chun Yan, Qin-Chuan Yang, Yi-Yang Hu, Xiang-Hui Zhao, Ya-Zhou Wang, Sheng-Xi Wu, Gong Ju, Min-Hua Zheng, and Hua Han

Supplemental Information

Supplemental data items

FigS1

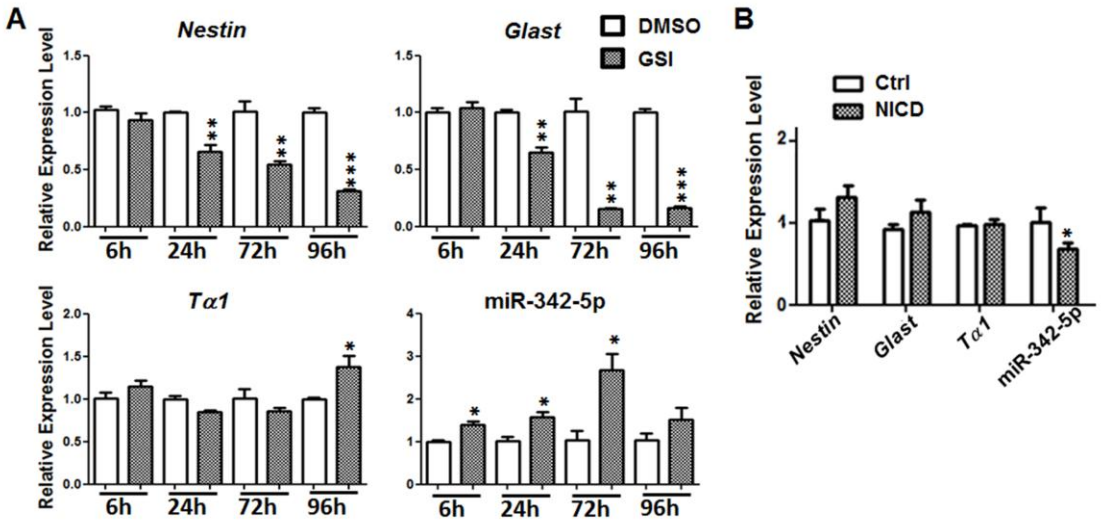

Figure S1 (Related to Figure 1). The expressions of *Nestin*, *Glast*, *Tα1* and miR-342-5p in neurospheres. (A) Primary neurospheres were cultured by using cells derived from wild type embryos (E15.5). Cells were treated with 75 μM GSI for 6, 24, 72, and 96 h, and the expressions of *Nestin*, *Glast*, *Tα1* and miR-342-5p were examined by qRT-PCR. DMSO was used as a control. (RNA samples of each time point were derived from 3 different pairs of GSI and DMSO treated neurospheres.) (B) Neurospheres cultured from E15.5 *NesCre-ROSA-Stop*$^{f/+}$-NIC (NICD) and *ROSA-Stop*$^{f/+}$-NIC embryos (Ctrl) were harvested and total RNAs were extracted (RNA samples were derived from 3 different pairs of embryos). The expressions of *Nestin*, *Glast*, *Tα1* and miR-342-5p were determined by qRT-PCR. Bars = means ± SD, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

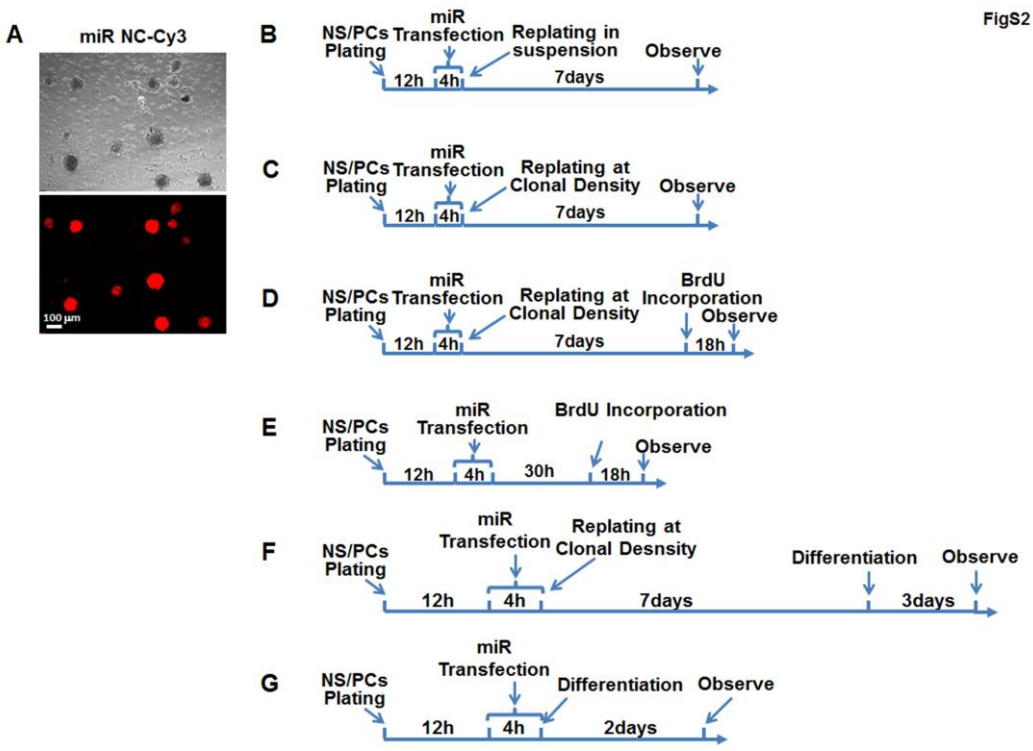

Figure S2 (Related to Experimental Procedures). Transfection efficiency and diagrams of neurosphere assay and NS/PCs differentiation assay after miRNA transfection. (A) Control nucleotide labeled with Cy3 was transfected into neurospheres to verify the transfection efficiency in our system. (**B**) For neurosphere assay, NS/PCs were replated in adherence and cultured for 12 h. Then after 4 h transfection of miR-342-5p mimics or the control oligonucleotides, adherent NS/PCs were replated in suspension, and the number of neurospheres were counted on the 7th day of culture. **(C)** For adherent NS/PC clone assay, NS/PCs were plated and transfected as above. Then NS/PCs were replated at clonal density described in Supplemental experimental procedures, and the numbers of adherent NS/PC colonies were counted on the 7th day of culture. **(D)** For BrdU incorporation in NS/PC colonies, transfection and replating methods were the same with adherent NS/PC clone assay. BrdU was incorporated for 18 h before analyses of the colonies. **(E)** For BrdU incorporation on dissociated NS/PCs, NS/PCs were transfected as above. BrdU was incorporated for 18 h before analyses, and cells were collected 48 h after transfection. **(F)** For NS/PC clone differentiation assay, transfection and replating method were the same with adherent NS/PC clone assay. After transfection, NS/PC clones were cultured for 7 days, and differentiation conditional medium was added for 72 h before analyses of the clones. **(G)** For dissociated NS/PC differentiation assay, NS/PCs were transfected as above. After transfection, differentiation conditional medium was added for 48 h before analyses of the cells.

FigS3

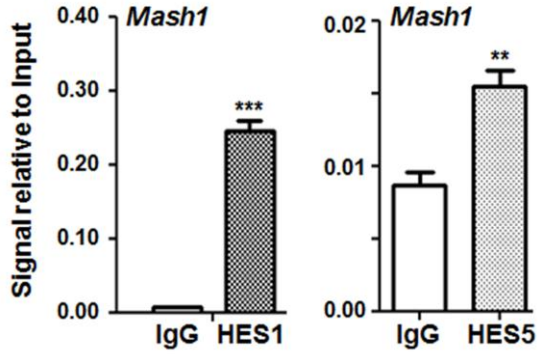

Figure S3 (Related to Figure 2). ChIP assays. Crosslinked chromatins were prepared from cultured primary NS/PCs (crosslinked chromatin samples derived from 3 independently cultured neurospheres), and immunoprecipitated with IgG and anti-HES1 or anti-HES5 antibody. After washing, the co-precipitated DNA was extracted and amplified by PCR primers targeting N-box sequence in the *Mash1* promoter as a positive control. Bars = means ± SD, **, P < 0.01, ***, P < 0.001.

FigS4

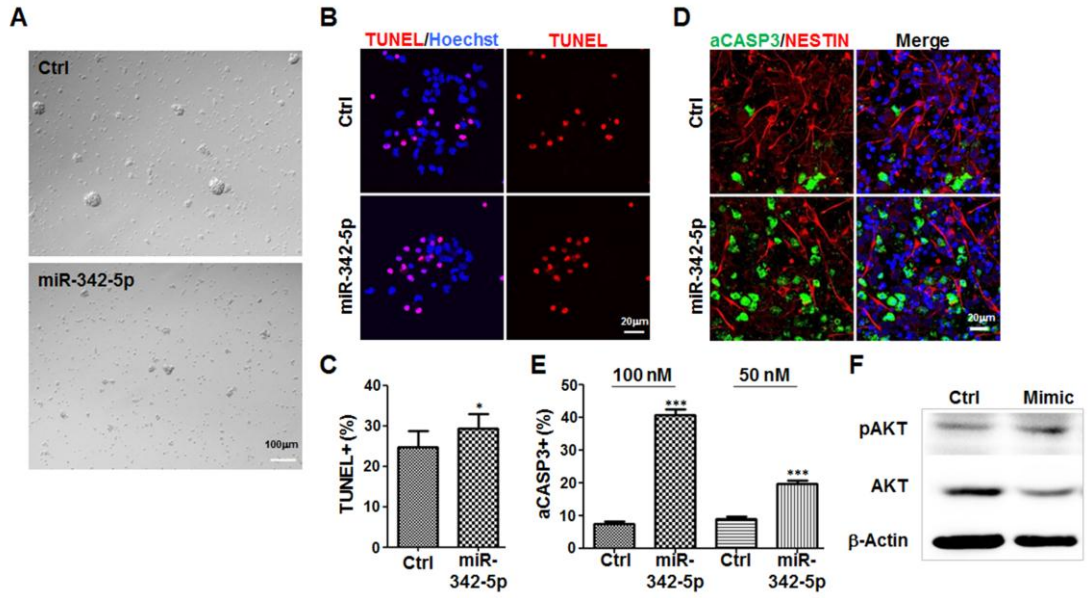

Figure S4 (Related to Figure 3). Overexpression of miR-342-5p repressed the formation of neurospheres and induced apoptosis of NS/PCs. (A) Single cell suspensions were prepared from GE of E15.5 embryos and cultured on PLL-coated dishes in the medium of DF12 supplemented with N2,

20 ng/ml bFGF and 20 ng/ml EGF. After 12 h, NS/PCs were transfected with 50 nM or 100 nM of miR-342-5p mimics or control oligonucleotides (Ctrl) (5 independent transfections performed). Cells were detached 4 h later and cultured under the neurosphere condition for 7 days. The cultures were photographed. **(B, C)** miR-342-5p increased the number of cells that underwent apoptosis. NS/PCs were transfected with miR-342-5p mimics and the control separately (5 independent transfections performed), and cultured adherently at clonal density for 7 days. Cells were performed TUNEL staining. Nuclei were counterstained with Hoechst. The percentage of TUNEL+ cells within each colony was determined (50 colonies counted in each group). **(D, E)** The number of activated CASPASE3 (aCASP3) positive cells increased, but few of them showed NESTIN positive. NS/PCs were transfected with miR-342-5p mimics and the control as above (5 independent transfections performed), and cultured at normal density under the adherent condition for 48 h. Cells were stained with the anti-aCASP3 and anti-NESTIN antibodies. The percentage of aCASP3+ cells was determined (10 fields counted in each group). The anti-NESTIN staining was used to outline NS/PCs specifically. **(F)** After miR-342-5p overexpression in NS/PCs (4 independent transfections performed), the total protein level of AKT was attenuated, and the phosphorylated protein level of AKT was approximately stable. Bars = means ± SD, *, P < 0.05, ***, P < 0.001.

FigS5

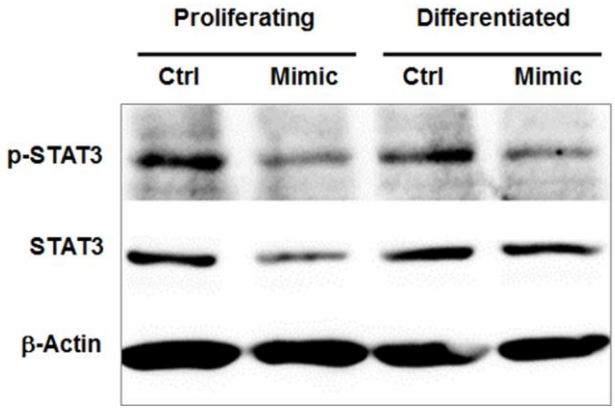

Figure S5 (Related to Figure 6). Differential protein levels of STAT3 after miR-342-5p overexpression. NS/PCs were cultured in proliferation condition or induced to differentiate for 48 hours (protein samples derived from 4 independent transfections). The protein level of STAT3 reduced in proliferating NS/PCs but not in differentiated cells after miR-342-5p overexpression. But the phosphorylated protein level of STAT3 decreased after miR-342-5p overexpression whether in proliferating or differentiated NS/PCs.

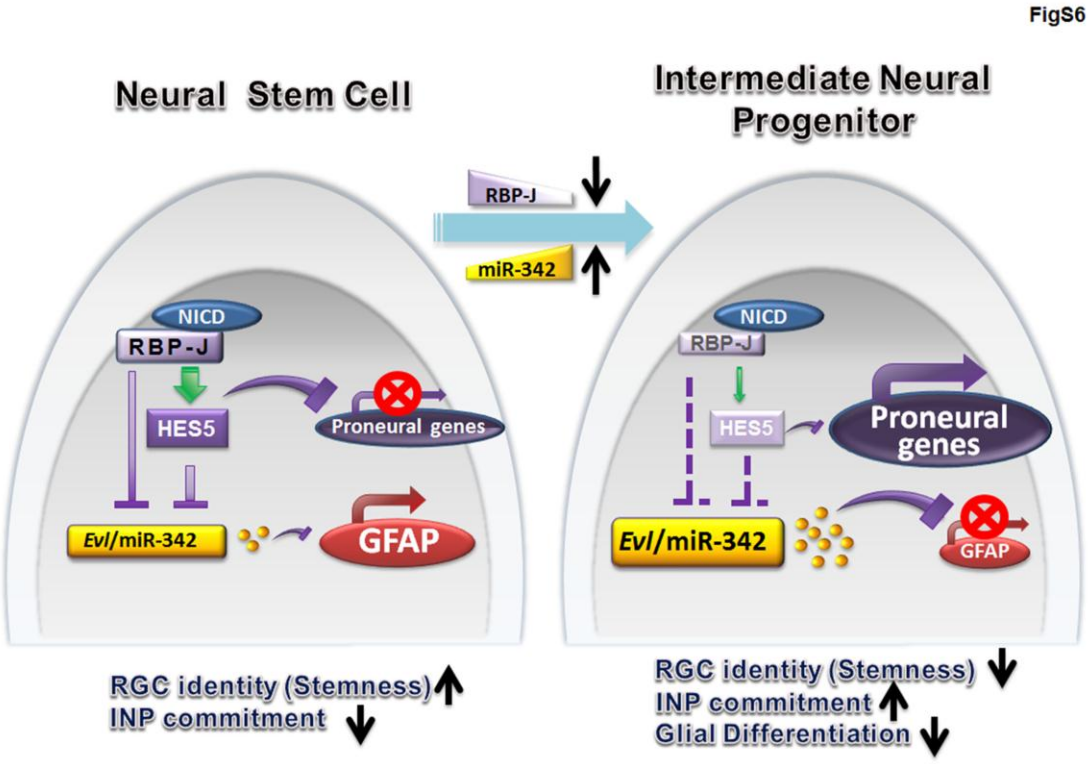

Figure S6. Diagram of the functions of Notch/RBP-J signaling and miR-342-5p in NSCs and INPs. In NSCs where Notch signaling is activated, the level of *Evl*/miR-342-5p is repressed by RBP-J and HES5, and NSCs maintain undifferentiated RGC identity. As RBP-J dependent Notch signaling is becoming attenuated in NSCs with their development, miR-342-5p rises up and targets GFAP, which might result in NSCs losing their identity of radial glia and the potential into astrocytes. Meanwhile, the blunted RBP-J dependent Notch signaling releases the expression of proneural genes, and thus promotes the switch from multipotent NSCs into neurogenic INPs.

Supplemental experimental procedures

Primary cell culture and miRNA transfection

NS/PCs were cultured by using the floating neurosphere culture and the adherent NS/PC culture (Currle et al., 2007; Sun et al., 2011). Single cell suspensions were obtained by mechanical dissociation of E15.5 embryonic brains under a stereomicroscope. For the neurosphere culture, cells were plated at a density of 1×10^5 cells/ml in 24-well plates (0.4 ml/well). Otherwise for the adherent culture, cells

were plated at a density of 5×10^4 cells/ml in poly-L-lysin (PLL)-coated 24-well plates (0.4 ml/well). In both of the conditions, cells were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 20 ng/ml recombinant human basic fibroblast growth factor (bFGF, R&D Systems), 20 ng/ml mouse submaxillary epidermal growth factor (EGF, R&D Systems), and the N-2 supplement (Gibco). To differentiate NS/PCs, the medium was changed to DMEM/F12 supplemented with 0.5 × N2, 5 ng/ml bFGF, 5 ng/ml EGF and 5% fetal bovine serum (FBS, Gibco). Otherwise, to differentiate NS/PC colonies, cells were plated on PLL-coated 35-cm^2 culture dishes at a very low density (Molofsky et al., 2003) (fewer than 100 clones per dish for a 7-day culture) and the medium was changed as described above. Primary oligodendrocytes, astrocytes, and neurons from neonatal or fetal rats were cultured as previously described (Zhao et al., 2012). To treat neurospheres with gamma-secretase inhibitor (GSI), DAPT (Abcam, Cambridge, MA) was added into the culture medium at a dosage of 75 μM, with dimethyl sulfoxide (DMSO) as a control.

Cells were transfected with synthetic miR-342-5p mimics (aggggugcuaucugugauugag), inhibitors (cucaaucacagauagcaccccu) or their scrambled control oligonucleotides respectively (Ribobio) by using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions. Scrambled control oligonucleotides labeled with Cy3 (Ribobio) were transfected to test the transfection efficiency. And the different designs of experiments after the miRNA transfections were showed in Figure S2. For small interfering RNA (siRNA) transfection, primary NS/PCs were transfected with synthetic siHes5 (gucagcuaccugaaacacatt), or the scrambled control oligonucleotides respectively (Genepharma).

miRNA expression profiling

Total RNA was extracted from cells and purified by using miRNeasy micro kit (QIAGEN) following the manufacturer's instructions. Microarray hybridization, data collection, and analyses were fulfilled by custom service provided by ShanghaiBio, using an Agilent miRNA chip (Sanger miRBase v.12.0). Raw data were normalized by using MAS 5.0 algorithm (Gene Spring Software 11.0, Agilent Technologies). Hierarchical clustering of samples was performed by using Cluster 3.0 software.

miRNA *in situ* hybridization

The *in situ* hybridization of miR342-5p was performed by using locked nucleic acid (LNA) miRNA probe (Exiqon). Briefly, frozen sections of E14.5 brain were warmed up at room temperature, dried at 50 ℃ for 15 min and fixed in 4% paraformaldehyde (PFA) for 20 min. Sections were then treated with 10 μg/mL proteinase K (Exiqon) for 12 min at room temperature and post-fixed with 4%

PFA for 15 min. After washing in phosphate-buffered saline (PBS), sections were prehybridized at 55 ℃ for 30 min in hybridization buffer (Exiqon). Then sections were hybridized for 3 h with 40 nM digoxigenin (DIG)-labeled LNA-modified probes (Exiqon) for miR-342-5p or a scrambled control. Sections were then washed in 5× SSC (once at hybridization temperature), 1× SSC (twice at hybridization temperature) and 0.2× SSC (twice at hybridization temperature then once at room temperature) sequentially for 5 min each. Sections were blocked with 2% bovine serum albumin (BSA) in PBS and incubated with alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche, 11093274910) overnight at 4 ℃. The AP indicated miRNA signals were finally detected by freshly prepared AP substrate (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate, NBT/BCIP).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated with miRNeasy micro kit (QIAGEN), and was used to synthesize cDNA with miScript II RT Kit (QIAGEN). Real-time PCR was performed using different kits on the ABI PRISM 7300 real-time PCR system. The miScript SYBR Green PCR Kit (QIAGEN) was used for the detection and quantification of miRNA, and the SYBR Premix EX Taq kit (Takara) for mRNA.

The primers used in this article for real-time PCR were as follows: miR-342-5p, 5’-CGGAGGGGTGCTATCTGTGATTGAG and the universal primer (QIAGEN); miR-342-3p, 5’-TCTCACACAGAAATCGCACCCGT and the universal primer (QIAGEN); RNU6, 5’-GGATGACACGCAAATTCGTGAAGC and the universal primer (QIAGEN); *Hes5*, 5’-AGTCCCAAGGAGAAAAACCGA and 5’-GCTGTGTTTCAGGTAGCTGAC; *Hes1*, 5’-GCAGACATTCTGGAAATGACTGTGA and 5’-GAGTGCGCACCTCGGTGTTA; *Evl*, 5’-AGCCTGCTGGGAGTGTGAATG and 5’-ACCTTGTGCAGCTCCCGAAC; *Tuj1*, 5’-CCCAGCGGCAACTATGTAGG and 5’-CCAGACCGAACACTGTCCA; *Gfap*, 5’-TCTCGAATGACTCCTCCACTC and 5’-AAGCTCCGCCTGGTAGACAT; *Stat3*, 5’-CAATACCATTGACCTGCCGAT and 5’-GAGCGACTCAAACTGCCCT; *Pdgfrα*, 5’-GGAGACTCAAGTAACCTTGCAC and 5’-TCAGTTCTGACGTTGCTTTCAA; *Olig1*, 5’-GCAGCCACCTATCTCCTCATC and 5’-CGAGTAGGGTAGGATAACTTCGC; *Olig2*, 5’-GGGAGGTCATGCCTTACGC and 5’-CTCCAGCGAGTTGGTGAGC; *Tα1*, 5’-GTCCTGGACAGGATTCGCAAG and 5’-GCTCAACCACAGCAGTGGAAAC; *Nestin*, 5’-TGAAAAGTTCCAGCTGGCTGT and 5’-AGTTCTCAGCCTCCAGCAGAGT; *Glast*, 5’-ACCAAAAGCAACGGAGAAGAG and 5’- GGCATTCCGAAACAGGTAACTC; *Gapdh*,

5’-TCGACAGTCAGCCGCATCTTCTT and 5’-GCGCCCAATACGACCAAATCC.

***In utero* electroporation**

In utero electroporation of mouse embryos was performed as described (Saito, 2006). Five square pulses of 50 milliseconds duration with 1 second intervals were applied using a BTX ECM830 pulse generator. Pulses with 40 V were applied to E15.5 embryos. pcDNA6.2-GW/EmGFP-miR-342-5p was constructed on the basis of linearized pcDNA6.2-GW/EmGFP-miR (Invitrogen). Negative control plasmid (pcDNA6.2-GW/EmGFP-negative control) coming with the kit was used as the control vector. Electroporated mouse embryos were harvested at E18.5 for further analyses.

Immunofluorescence

For immunofluorescence, mice were perfused with 2 ml of 4% PFA in 0.1 M phosphate buffer (PB), pH7.4. The brain was removed and postfixed in 4% PFA for 1 h at 4 ℃ before being moved into 20% sucrose in 0.1 M PB overnight at 4 ℃ for cryoprotection. Frozen sections (14 μm) were cut with a cryostat and mounted on gelatinized slides. Cells on coverslips were fixed in 4% PFA for 30 min at room temperature. Samples were incubated with primary antibodies overnight at 4 ℃, followed by Alexa Fluor® 488-conjugated or Alexa Fluor® 594-conjugated second antibodies (1:500, Jackson ImmunoResearch, 712-585-150, 711-545-152, 711-585-152, 705-545-147, 715-585-150). Samples were mounted in glycerol and visualized under a fluorescence microscope (BX51, Olympus) or a laser scanning confocal microscope (FV-1000, Olympus). Primary antibodies included rat anti-BrdU (1:250, Abcam, ab6326), goat anti-NESTIN (1:100, Santa Cruz, sc-21248), rabbit anti-Ki67 (1:200, Thermo Scientific, MA5-14520), rabbit anti-GFAP (1:1000, DAKO, Z0334), mouse anti-TUJ1 (1:500, R&D system, MAB1195), rabbit anti-PAX6 (1:50, Proteintech, 12323-1-AP), and rabbit anti-TBR2 (1:800, Abcam, ab23345).

For BrdU incorporation into cells, BrdU was added with a final concentration of 10 μM into the culture medium 18 h before the fixation of the cells. After the fixation, 2 N HCl was applied for 30 min at room temperature for permeabilization.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

TUNEL staining was carried out following the instructions of the kit (Promega). Briefly, cells cultured on slides were fixed in 4% PFA. After washing with PBS, fixed cells were permeabilized with 0.2% Triton X-100. Then cells were equilibrated for 10 minutes at room temperature using the equilibration buffer within the kit. The reaction mix that contained the fluorescein-labeled nucleotide

mix and the terminal deoxynucleotidyl transferase was prepared and applied onto the cells for 60 minutes at 37 ℃ in a humidified chamber. Then the reaction was stopped and cells were washed with 2x SSC solution. After the nuclei were counterstained with Hoechst, slides were mounted and observed under a fluorescence microscope (BX51, Olympus) or a confocal microscope (FV-1000, Olympus). For CASPASE3 staining, adherent cells were stained with immunofluorescence methods as stated above. The primary antibodies used in Figure S4 were rabbit anti-active CASPASE3 (1:100, Sigma-Aldrich, C8487) and goat anti-NESTIN (1:100, Santa Cruz, sc-21248) antibodies.

Reporter assay

pEFBOS-NICD and pCMV-RBP-J^{R218H} (a dominant-negative RBP-J) were constructed previously (Chung et al., 1994; Kuroda et al., 2003). The *Evl* promoter fragment and its truncates were cloned by PCR, and inserted into pGL3-basic (Promega) to construct reporter genes (*Evl*-RepL, *Evl*-RepM, and *Evl*-RepS, Figure 2A). The two fragments of 3'-untranslated region (3'-UTR) of the mouse *Gfap* cDNA containing the two miR-342-5p recognition sites respectively, were amplified by PCR and subcloned into pMIR-REPORT (Ambion) to construct two pmiR-*Gfap* reporters. Reporters with mutations (pmiR-*Gfap*-mt) in the 3'-UTR corresponding to the seed sequence of miR-342-5p were generated by PCR.

NIH3T3 cells, HEK293 and PC12 cells were cultured in DMEM supplemented with 2 mM glutamine and 10% FBS. Cells were transfected with different combinations of reporters and expression vectors by using Lipofectamine 2000^{TM} (Invitrogen), with a Renilla luciferase vector (phRL-TK, Promega) as an internal control. Cells were harvested 48 h after the transfection, and luciferase activity was measured with Dual Luciferase Reporter Assay using a Gloma X^{TM} 20/20 Luminometer (Promega).

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed by using a kit from Millipore (Merck Millipore) according to the manufacturer's instructions. Briefly, cells were fixed in 1% formaldehyde, and cross-linked protein-DNA complexes were fragmented into 200 bp to 500 bp fragments by ultrasonic sound waves. Immunoprecipitation was performed by using an anti-HES1 antibody (H-20, Santa Cruz, sc-13844) and anti-HES5 antibody (M-104, Santa Cruz, sc-25395). Immune complexes were collected using protein G-Sepharose beads, washed, eluted and incubated for 5 h at 65 ℃ to reverse the cross-linking. DNA was extracted and analyzed by real-time PCR using the primers targeting the four putative

HES-binding sites as illustrated in Figure 2A: site 1, 5'-CACACCTCCCAATAGTGCCCTG and 5'-CCTCAGGAATGTAATAAAGCCACAGG; site 2, 5'-AATGTCTGAGGCCAAGTAATGTATAATG and 5'-ATCTGGTGCCCTCAACAGAACTAG; site 3, 5'-CTAGCCATTTGAAGCCAACCTCTC and 5'-CAGAAGAGTGGGAAACACCAAGTTC; and site 4, 5'-GAAAGCCGGTTCAGTAGGGCTC and 5'-AGAATCCCTGGTGGTGGAGGAG. Primers targeting the N-box sequence of *Mash1* promoter were used as a positive control: 5'-GAGGACTCAAGTTCTCATACAGAG and 5'-AACTCTTGGAAGTGGAGGG.

Western blot

Whole cell lysates were extracted with the RIPA buffer (Beyotime) containing a protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was determined with the BCA Protein Assay (Pierce). Samples were separated by SDS-PAGE, blotted onto polyvinylidene fluoride (PVDF) membranes and probed with primary antibodies, followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Boster Biotech, BA1050) or HRP-conjugated goat anti-rabbit IgG (Boster Biotech, BA1054). The primary antibodies used in this article included mouse anti-GFAP (1:2000, Sigma-Aldrich, G3893), mouse anti-TUJ1 (1:500, R&D system, MAB1195), rabbit anti-AKT (Ab-308) antibody (1:500, Signalway Antibody, 21055), rabbit anti-AKT (Phospho-Thr308) antibody (1:500, Signalway Antibody, 11055), rabbit anti-STAT3 (ab-727) antibody (1:500, Signalway Antibody, 21046), rabbit anti-STAT3 (Phospho-Tyr705) antibody (1:500, Signalway Antibody, 11045), and mouse anti-β-Actin (1:5000, Sigma-Aldrich, A5441). Signals were developed with chemo-luminescent reagents (Pierce).

Statistics

Statistical evaluation was performed with the Image Pro Plus 6.0 and Graph Pad Prism 5.0 softwares. Data were compared using Student's *t*-test or Paired *t*-test for two-group analysis, and using one-way ANOVA in conjunction with Bonferroni correction for multiple-group analysis. $P< 0.05$ was considered statistically significant. Sections were collected from at least five pairs of mouse embryos. Similar sections between control and transfected groups were identified according to specific anatomical structures.

Supplemental references

Currle, D.S., Hu, J.S., Kolski-Andreaco, A., and Monuki, E.S. (2007). Culture of mouse neural stem

cell precursors. Journal of visualized experiments : JoVE, *152*.

Sun, T., Wang, X.J., Xie, S.S., Zhang, D.L., Wang, X.P., Li, B.Q., Ma, W., and Xin, H. (2011). A comparison of proliferative capacity and passaging potential between neural stem and progenitor cells in adherent and neurosphere cultures. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience *29*, 723-731.

Molofsky, A.V., Pardal, R., Iwashita, T., Park, I.K., Clarke, M.F., and Morrison, S.J. (2003). Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. Nature *425*, 962-967.

Zhao, X., Wu, J., Zheng, M., Gao, F., and Ju, G. (2012). Specification and maintenance of oligodendrocyte precursor cells from neural progenitor cells: involvement of microRNA-7a. Molecular biology of the cell *23*, 2867-2878.

Saito, T. (2006). In vivo electroporation in the embryonic mouse central nervous system. Nature protocols *1*, 1552-1558.

Chung, C.N., Hamaguchi, Y., Honjo, T., and Kawaichi, M. (1994). Site-directed mutagenesis study on DNA binding regions of the mouse homologue of Suppressor of Hairless, RBP-J kappa. Nucleic acids research *22*, 2938-2944.

Kuroda, K., Han, H., Tani, S., Tanigaki, K., Tun, T., Furukawa, T., Taniguchi, Y., Kurooka, H., Hamada, Y., Toyokuni, S., *et al.* (2003). Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. Immunity *18*, 301-312.