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Supplemental Information

Reciprocal Regulation between Bifunctional miR-9/9* and its Transcriptional Modulator Notch in Human Neural Stem Cell Self-Renewal and Differentiation

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Supplemental Information

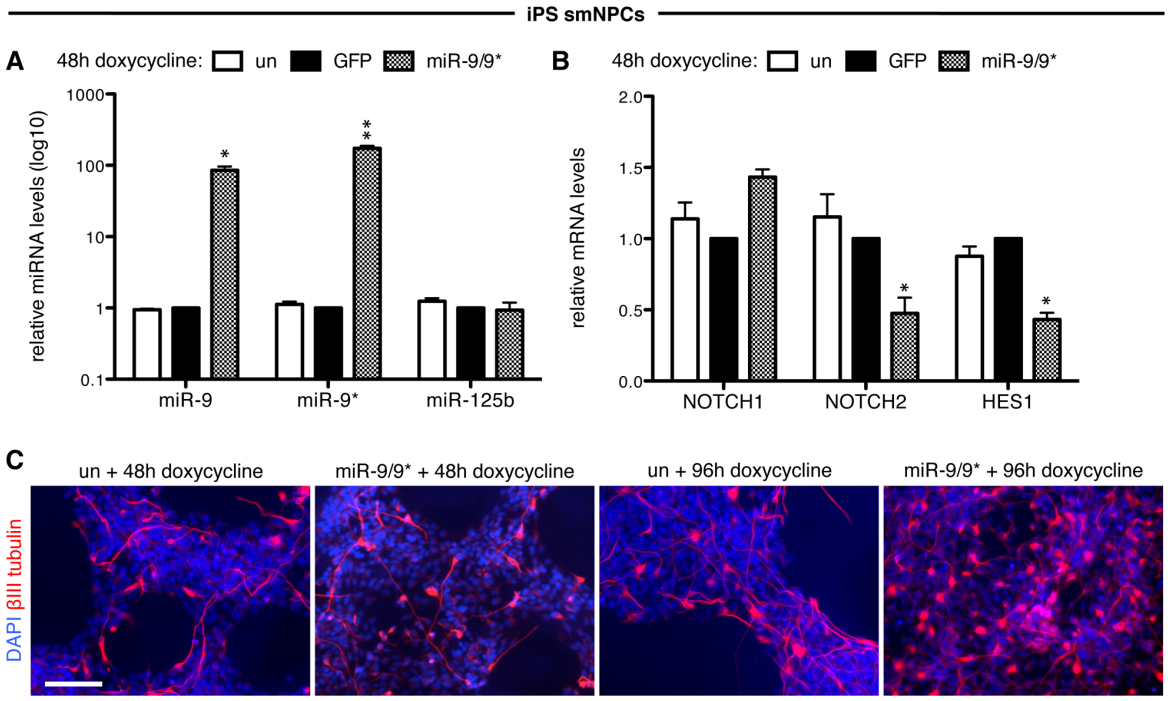

Figure S1. Overexpression of miR-9/9* in proliferating smNPCs, related to Figure 1.
(A, B) QRT-PCR analyses of miR-9, miR-9* & miR-125b levels (A) and *NOTCH1*, *NOTCH2* & *HES1* levels (B) in hiPS cell derived smNPCs overexpressing the miR-9_1 genomic sequence (9/9*), untransduced (un) cells and cells expressing GFP (used as control) after 48 hours of doxycycline treatment. Data are normalized to *RNU5A* (A) or *18S* rRNA (B) reference levels and are presented as average changes + SEM relative to expression in GFP-expressing smNPCs (GFP, equal to 1; n = 4; *, $p \leq 0.05$; **, $p \leq 0.01$; Student's t-test). **(C)** Immunofluorescence stainings for βIII tubulin in untransduced (un) smNPCs and in miR-9/9*-overexpressing smNPCs cultures treated for 48 or 96 hours with doxycycline. DAPI labels nuclei, scale bar = 50 µm. All experiments were performed in cells cultured under self-renewing conditions, i.e. in the presence of growth factors.

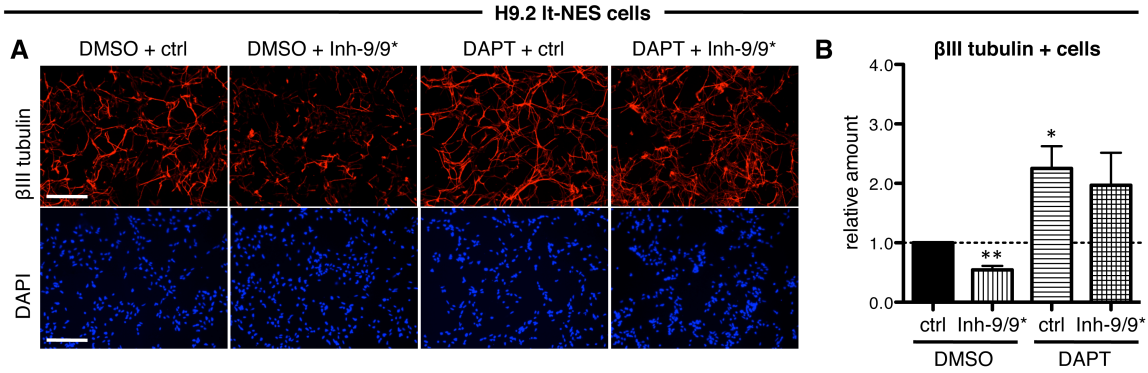

Figure S2. The impact of miR-9/9* on lt-NES cell differentiation via the Notch signaling pathway can be recapitulated in H9.2 lt-NES cells, related to Figure 2.

(A) Immunostainings for βIII tubulin in H9.2 lt-NES cells transfected with synthetic inhibitors for miR-9 and miR-9* (Inh-9/9*) or a short scrambled control RNA (ctrl) and treated with DAPT or DMSO (vehicle control), after 5 days of *in vitro* differentiation. DAPI stains nuclei. Scale bars indicate 100 μm. **(B)** Corresponding quantifications of the relative number of βIII tubulin-positive cells in lt-NES cultures after 5 days of *in vitro* differentiation in the conditions described above. All data are presented as mean + SEM (n = 4; *, $p \leq 0.05$; **, $p \leq 0.01$; Student's t-test).

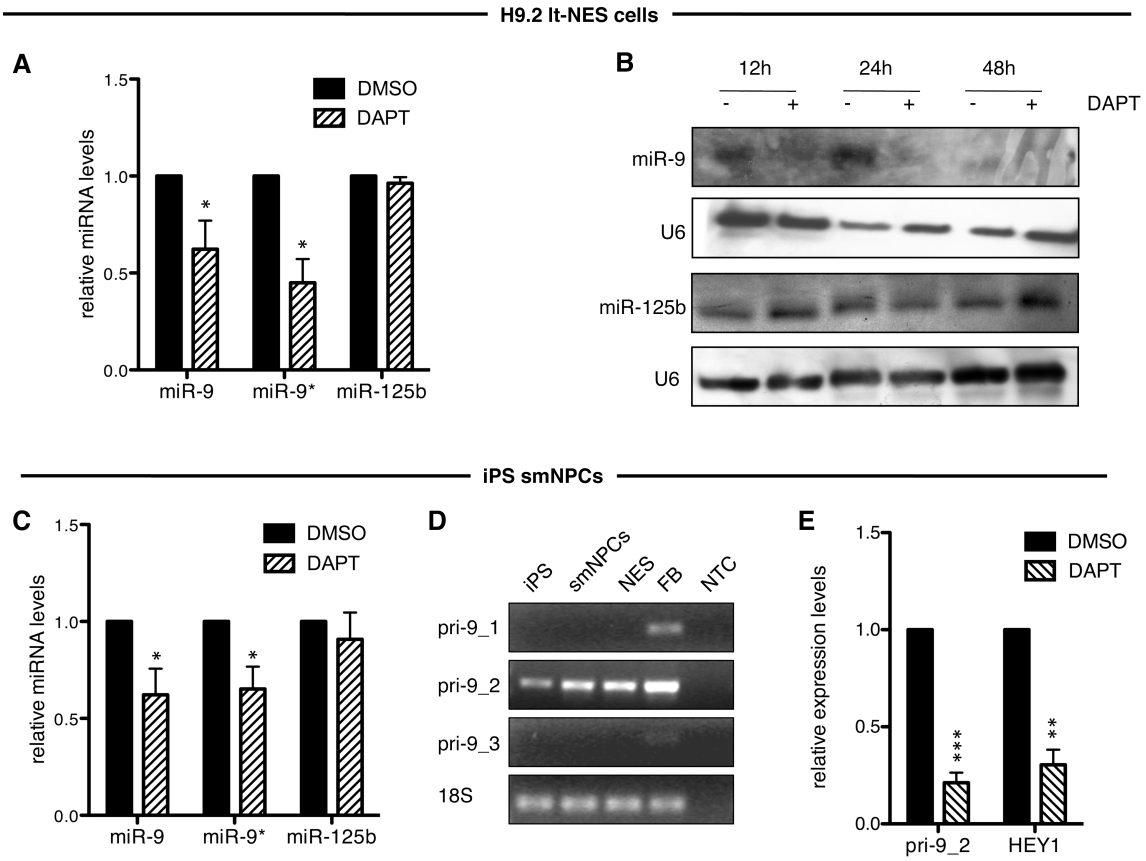

Figure S3. Analysis of miR-9/9* expression in H9.2 lt-NES cells and smNPCs treated with the γ-secretase inhibitor DAPT, related to Figure 3.

(A, C) QRT-PCR analyses monitoring mature miR-9, miR-9* and miR-125b in H9.2 lt-NES cells (A) or smNPCs (C) treated with DAPT or DMSO (vehicle control) for 24 hours. Data are normalized to *RNU5A* reference levels and presented as average changes + SEM relative to expression in the DMSO-treated samples (equal to 1; n = 4; *, $p \leq 0.05$; Student's t-test). **(B)** Northern blot analyses showing the expression of miR-9 and miR-125b in H9.2 lt-NES cells treated with DAPT (+) or DMSO (-; vehicle control) for 12, 24 and 48 hours. U6 snRNA was used as loading control. **(D)** Representative images of PCR analyses for expression of the three pri-miR-9 forms (pri-9_1, pri-9_2 and pri-9_3) in pluripotent human iPS cells (iPS) and in smNPCs, lt-NES cells derived from these hiPS cells and fetal brain mRNA (FB). *18S* rRNA levels were used as loading control, NTC stands for no template control. **(E)** QRT-PCR analyses monitoring HEY1 and pri-miR-9_2 expression levels in smNPCs treated with DAPT or DMSO for 24 hours. Data are normalized to 18S rRNA reference levels and presented as average changes + SEM relative to expression in DMSO-treated cells (equal to 1; n = 4; **, $p \leq 0.01$;***, $p \leq 0.0001$; Student's t-test). All experiments were performed in cells cultured under self-renewing conditions, i.e. in the presence of growth factors.

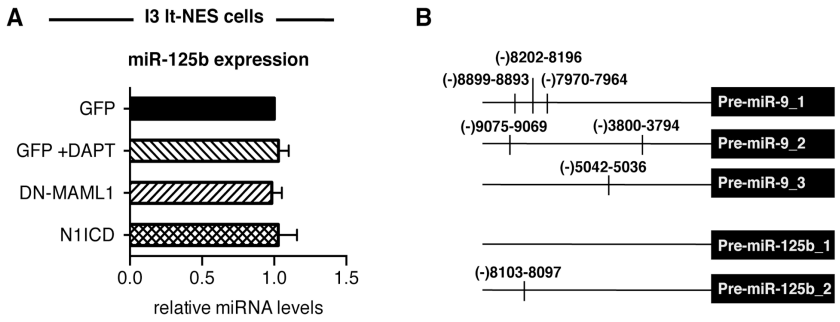

Figure S4. Predicted binding sites for RBPj upstream the miR-9/9* and miR-125b genomic loci, related to Figure 4.

(A) QRT-PCR analyses of miR-125b in lt-NES cells transduced with lentiviral vectors overexpressing in a doxycycline-dependent manner GFP, N1ICD or DN-MAML1, respectively, after 4 days of doxycycline treatment in presence or absence of DAPT. Data are normalized to miR-16 reference levels and presented as average changes + SEM relative to expression in GFP-expressing lt-NES cells (GFP, equal to 1; $n \geq 4$). **(B)** Scheme of the genomic loci encoding miR-9/9* and miR-125b including the region 10 kb upstream of the pre-miRNAs. Strikes indicate predicted binding sites for RBPj.

Table S1. Predicted targeting of members of the Notch signaling pathway by miR-9 and miR-9*, related to Figure 1.

The right columns present the number of algorithms predicting binding of each microRNA to components of the Notch signaling pathway, as analyzed by the miRWALK algorithm.

	miR-9	miR-9*
NOTCH1	1	0
NOTCH2	4	2
PSEN1	3	1
MAML3	2	0
DLL1	0	1
DLL4	1	0
JAG1	0	2
JAG2	1	0
HES1	4	0
HEY1	0	2
HEY2	1	2

Table S2. Primers used to generate recombinant DNA sequences, related to Figure 1 and Figure 3.

Target	Forward Primer	Reverse Primer
miR-9 binding site	AATTCGAGTAGAGCTCTAGTACTTCATACAGCTAGA TAACCAAAGATCATACAGCTAGATAACCAAAGAG	GATCCTCTTTGGTTATCTAGCTGTATGATCTTTGGTT ATCTAGCTGTATGAAGTACTAGAGCTCTACTCG
NOTCH1 3'UTR	ACTACGGCGCGCCCCACGA	CATATGCATCTACAGTTCCTCATGTAGATCAC
NOTCH2 3'UTR	TGAGAGAGTCCACCTCCAGT	GACTTATATCCCAGTTCCCAATTC
HES1 3'UTR	TCAGGCCACCCCTCCTC	CAAAAGAGTCAATTCCTGAATTACCA
miR-9-1 locus	TGTCTCGGACTTCATTTCTCTCTT	TGAAATGTCGCCCGAACCAGT

Table S3: Primers used to monitor expression of pri-miR-9 forms by semi-quantitative PCR, related to Figure 3.

Target	Forward Primer	Reverse Primer
Pri-miR-9_1	TGTCCCTTCCCTCCTACTCC	ATCCTCTGGTGCTGGTCAGT
Pri-miR-9_2	CTTCGGTACTGCCAGAAAGG	GCAACAACCCCTCTCAAGAC
Pri-miR-9_3	GTGTCTGTCCATCCCCTCTG	CTCGGCTCCTCTGGCTCT
18s rRNA	TTCCTTGGACCGGCGCAAG	GCCGCATCGCCGGTCGG

Table S4: Primers used for ChIP-qPCR analysis, related to Figure 4.

Target	Forward Primer	Reverse Primer
9_1 locus site1	TAGGAGCTGGGGGGAGAGA	TGGGGACCCCCCTAAATCT
9_1 locus site2	GGTGGAGACCAAAATTGGGA	CCTCCATTAGATGGGTGTGAAG
9_1 locus site3	TCCTACGGAAGGCCAGGA	CACAGCCCAGCAGGCA
9_2 locus site1	TAGAGTCTAGACCCGGCTGAGG	GAGCCTCCGGTCTAACTTCTGA
9_2 locus site2	TGGTAGTCTTGACTGTACTAGTGCACTG	GGTTTGCCTGCTCATTCACTAATA
9_3 locus	TGGGCAGCTCAGGCAG	CTGTCTGCAGCCCCACAA
HES1	CAAGACCAAAGCGGAAAGAA	GGATCCTGTGTGATCCCTAGG
125b_2	TCCCCAGTGCCTATGCC	TGTACCATTTCACATTAGCTGCA

Supplemental Experimental Procedures

Generation of lt-NES cells

Human ES cells (I3 or H9.2 ES cell lines) were harvested as clumps with 1 mg/ml collagenase (Invitrogen) to form embryoid bodies (EBs) in GMEM medium supplemented with 10 % KnockOut Serum Replacement, 0.1 mM β-mercaptoethanol, l-glutamine, sodium pyruvate, non-essential amino acids (all from Invitrogen), 5 µM SB431542 and 1 µM dorsomorphin (both from Tocris). Culture medium was changed every second day and after five days EBs were plated on polyornithine (Sigma-Aldrich)/fibronectin (Invitrogen) coated TC dishes (BD Bioscience) containing DMEM/F12 with N2-supplement (both Invitrogen), 20 µg/ml additional insulin (Sigma–Aldrich) and 1.6 g/l glucose (Sigma–Aldrich) (thereafter referred to as N2-medium) supplemented with 10 ng/ml FGF2 (Invitrogen). After 7 - 10 days neural rosettes were selectively detached by the addition of 0.15 mg/ml dispase (Invitrogen) for 3 - 10 minutes and carefully rinsed off the plate. Rosettes were maintained as spheres in non-adhesive dishes for 2 days in N2-medium containing 20 ng/mL FGF2, thereafter plated on polyornithine/laminin (Sigma-Aldrich) coated dishes and switched to 0.8 µM purmorphamine (Merck) and 10 ng/ml FGF2 for additional 5 - 7 days. Dishes consisting of pure rosettes were dissociated with trypsin (Invitrogen) to single cells and plated at high density on polyornithine/laminin coated dishes in proliferation medium composed of N2-medium containing 1:1000 B27 supplement (Invitrogen), 10 ng/ml FGF2 and 10 ng/ml EGF (both R&D systems).

Generation of smNPCs

SmNPCs were generated following an established protocol (Reinhardt et al., 2013). Briefly, in-house generated hiPS cells (iLB-C-31F-r1) were aggregated to form embryoid bodies (EB) and neuralized using 10 µM SB431542 (Sigma-Aldrich), 400 nM LDN193189 (Axon Medchem; both dual SMAD inhibitors), 3 µM GSK3-inhibitor CHIR99021 (Miltenyi Biotech) and 0.5 µM SHH agonist Purmorphamine (Merck). After 6 days of EB culture, cells were triturated to single cells and further propagated in the presence of CHIR99021 and Purmorphamine until passage 10.

Cloning and production of lentiviral constructs

Lentiviral overexpression constructs were generated by cloning the genes of interest into the multiple cloning site of the pTight vector from the Lenti-X™ Tet-On® Advanced Inducible Expression System (Clontech). The human NOTCH1-ICD and NOTCH2-ICD constructs (Capobianco et al., 1997) were kindly donated by Prof. A. J. Capobianco. The DN-MAML1-GFP fusion construct (Weng et al., 2003) was kindly donated by Prof. J. C. Aster. Overexpression of GFP alone was used as control. For miR-9/9* overexpression, the miR-9_1 locus (including the precursor and flanking sequences) was amplified from genomic DNA of lt-NES cells. We chose to introduce the miR-9_1 locus, which is not active in human neural stem cells to be able to discriminate between endogenous and exogenous pri-miR-9/9* expression. A modified version of the pLVX-tetOn Advanced vector with an EF1 α promoter replacing the CMV promoter, named pLVX-EtO vector and previously described in (Ladewig et al., 2012), was used. The primers used for cloning are listed in Table S2.

Lentiviral transduction

Lentiviral particles were produced using HEK293FT cells (Invitrogen) and helper plasmids as previously described (Szulc et al., 2006). Culture medium was supplemented with 5% FCS (Invitrogen) on the day of transfection, and 25 µM chloroquine (Sigma-Aldrich) was added 10 minutes before transfection. 1st and 2nd day-harvested medium from 15 cm tissue culture dishes was pooled, centrifuged at 19.600 rpm at 4°C for 1.5 hours in a Sorvall Surespin 630 rotor and resuspended in 1 ml HBSS (Invitrogen). Lt-NES cells were transduced with 0.2 ml virus dissolved in HBSS per 10 cm tissue culture dish, and cultured over night at 37°C and 5% CO2 in medium supplemented with 5 µg/ml polybrene (Sigma-Aldrich). For lentiviral transduction of smNPCs, cells were detached by Accutase treatment, replated as single cell suspension and supplemented with 10 nM ROCK inhibitor (Y-27632, Tocris9) and the appropriate amount of viral supernatant. Medium was changed after 24 hours. Starting from 72 hours post-transduction transduced cells were selected by puromycin (10 µg/ml, PAA Laboratories) treatment for at least 2 days. Transgene expression was induced by addition of 2.5 µg/ml doxycycline diluted in the respective cell culture medium.

Detection of miRNA expression levels by qRT-PCR

To monitor miRNA expression, cDNA was produced from total RNA samples using the miScript Reverse Transcription (RT) Kit (Qiagen). Quantitative real-time RT-polymerase chain reactions (qRT-PCR) were performed using the miScript SYBR Green PCR Kit (Qiagen) and run on an Eppendorf Mastercycler. DNA oligonucleotides with sequence corresponding to the mature miRNA forms were used as forward primers. As reverse primer, the miScript Universal Primer provided by the miScript SYBR Green PCR Kit was used. Data were normalized to miR-16 or *RNU5A* (fw: GTGGAGAGGAACAACTCTGAGTC) levels and analyzed using the ΔΔCt method.

Detection of mRNA expression levels by qRT-PCR
To monitor mRNA expression, cDNA was produced from total RNA samples using the iScript Reverse Transcription (RT) Kit (Biorad) and qRT-PCR analyses were performed on an Eppendorf Mastercycler using SYBR Green detection method. Primers used for mRNA detection were previously described in (Borghese et al., 2010). Data were normalized to *18S* rRNA levels and analyzed using the ΔΔCt method.

Image analysis and marker quantification
For image analysis, at least three random pictures were taken per condition. The number of cells (marked by DAPI) was determined by blind manual counting, whereby at least 700 nuclei were counted in each condition. For quantification of neurons (expressing βIII tubulin), only cells with morphologically intact neurites longer than the cell soma were counted.

Western Blot
40 micrograms of protein extracts were loaded onto a 8% (NOTCH1, NOTCH2) or 10% (HES1) SDS-PAGE gel, run at 100V for 2 hours and blotted onto a nitrocellulose membrane (Carl-Roth) at 70V for 2 hours. The membrane was blocked with 5% milk powder in TBST for 1 hour at room temperature and incubated with primary antibody in 2,5 % milk powder in TBST at 4°C over night (human HES1, Sigma-Aldrich, SAB3300102, 1:500; human NOTCH1, DHSB, bTAN 20, 1:500; human NOTCH2, DHSB, C651.6DbHN, 1:500; human β-actin, Millipore, A1978, 1:2000). Detection was carried out with PO-mouse-anti-rat (Jackson Immuno Research, 111-035-144, 1:500) for NOTCH1, NOTCH2 or PO-rabbit-anti-mouse (Jackson Immuno Research, 115-035-003, 1:500) for HES1 and Millipore ECL solutions according to manufacturer's protocol.

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