miR-124 regulates adult neurogenesis in the SVZ stem cell niche
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Supplementary Figure 1. miR-124 expression in the adult and embryonic mouse brain

(a) In situ hybridization of miR-124 combined with immunostaining in the striatum of the adult brain. Split channels show miR-124 (left panel) and NeuN (right panel) of one field. miR-124 is present in NeuN+ mature neurons (arrows). Scale bar, 20 µm. (b–g) In situ hybridization of miR-124 in coronal sections of developing brain. Images correspond to the lateral telencephalic
ventricles at E11 (b, c), E14 (d, e) and E17 (f, g) with higher magnification of area in boxes shown below (c, e, g). miR-124 is largely absent from the ventricular zone (VZ, b) but is detectable in isolated individual cells at E11 (e, arrowheads). At E14, miR-124 is present in a subset of cells in the VZ (d, e) and is highly expressed in the subventricular zone (SVZ, d, e). By E17, miR-124 is absent from the VZ (f, g) and is abundantly expressed in the SVZ and cortical plate (CP, f). Scale bar, 100 µm.
Supplementary Figure 2. miR-124 knockdown in vitro

(a) Penetratin-conjugated antisense 2’OMe-RNAs against miR-124 were labeled with Cy3 fluorophore (AS-124-Cy3) to verify incorporation into SVZ cells. AS-124-Cy3 is efficiently taken up by cultured SVZ cells after 24 hours. Scale bar, 20 µm. (b) qRT-PCR of miR-124 on FACS purified neuroblasts. Continuous feeding of AS-124 for 5 DIV results in 10-fold down-regulation of endogenous miR-124 as compared to penetratin-only (Pen) and AS-194. Histogram shows ratio relative to untreated neurons. Data represent mean ± s.e.m. normalized to 5S rRNA from three independent experiments. Asterisk, p<0.01, two-tailed paired Students-t test. (c) qRT-PCR for miR-124 and miR-194 confirms that miR-194 expressed at low or non-detectable levels in the SVZ lineage. Histogram shows relative quantity normalized to 5S rRNA. (d) Quantification of total cell number. AS-124 treatment increases the total M2/M6+ cells
generated from mouse SVZ stem cells. Histogram shows ratio normalized to the total cell number in untreated culture. Data represent mean ± s.e.m. from six independent experiments. Asterisk, p<0.01, two-tailed paired Students-t test. (e) Quantification of total colonies formed in AS-124, AS-194 and Pen treated co-cultures. AS-124 treated culture shows highest number of colonies as compared to Pen or AS-194 controls. Histogram shows ratio normalized to the total colony number in untreated culture. Data represent mean ± s.e.m. from six independent experiments.
Supplementary Figure 3. miR-124 knockdown in purified adult SVZ neuroblasts in vitro
Representative micrographs of neuronal survival assay. FACS purified SVZ neuroblasts (mCD24low population) were maintained on laminin for 5 DIV (a) in the presence of penetratin-only (b), AS-194 (c), or AS-124 (d). Surviving neurons are identified by CellTracker (red, split channels to the right) and TuJ1 (green) together with ToPro3 nuclear staining (blue). Scale bar, 50 μm.
Supplementary Figure 4. miR-124 over-expression reduces colony size

Histogram shows the distribution of colony size of individual colony 5 DIV in co-culture after retroviral transduction with RV-GFP (n=53), RV-124 (n=50) or RV-124mt (n=71). RV-124 is significantly different than RV-GFP and RV-124mt (one-way ANOVA [F2, 171=9.1949, p=0.000161]). P value between each group was examined by Newman-Keuls test.
Supplementary Figure 5. Ectopic expression of miR-124 in the SVZ in vivo

(a) RV-124 transduced SVZ cells migrate and differentiate into NeuN+ granule neurons in the olfactory bulb. (b) Mis-expression of miR-124 with RV-124 in Olig2+ oligodendrocyte lineage cells. Scale bar, 20 µm. (c) Quantification of Olig2+ cells generated from retrovirally transduced SVZ precursors in vivo. Histogram shows percentage of Olig2+ cells with branched morphology from a total of n=2154 (RV-GFP), n=3490 (RV-124mt) and n=2985 (RV-124) retrovirally transduced cells counted from whole brain sections. Data represents mean ± s.e.m. from three independent experiments.
Supplementary Figure 6. Sox9 protein expression in the developing brain
(a–d) Immunostaining for Sox9 in coronal sections of the mouse brains across different developmental stages. Right panels show Sox9 channel. The onset of Sox9 in the developing telencephalon corresponds to the first appearance of miR-124 (shown in e–h). At E11, stem cells in the VZ express low or non-detectable levels of Sox9 (a). Sox9 expression increases in the VZ from E14 (b) to E17 (c) and is maintained in adult SVZ (d). Note that differentiating DCX+ neurons in E14 and E17 do not express Sox9. (e–h) In situ hybridization of miR-124 in coronal sections of the mouse brain corresponding to E11, E14, E17 and adult lateral ventricles. Scale bar, 100 µm.
**Supplementary Figure 7.** Sox9 knockdown by shRNA-coding retroviruses

(a–c) Representative micrographs of RV-shRNA transduced SVZ adherent cultures maintained with EGF for 6 DIV. Two shRNA constructs (shSox9-A and shSox9-C) targeting *sox9*
transcripts were examined for their knockdown efficiency by analyzing Sox9 protein levels under confocal microscopy (split channels of Sox9 immunostaining in right panels). shRan construct coding for scrambled sequences that do not target any genes. Retrovirus coding for shSox9-C successfully knocks down Sox9 protein in cultured SVZ precursors (arrows). Scale bar, 20 µm. (d) Western blotting of Sox9 protein upon shRNA knockdown. Sox9 antibodies detect two different phosphorylation forms. (e) Quantification of Sox9 in the western blot by densitometric analysis. Histogram shows ratio to shRan control (shSox9-A, 0.7; shSox9-C, 0.2).
Supplementary Figure 8. miR-124 regulates the timing of progression along the SVZ stem cell lineage. This is achieved by promoting neuronal differentiation and cell cycle exit, as well as by repressing genes involved in self-renewal and glial differentiation. Sox9 protein is expressed in SVZ stem cell astrocytes and at lower levels in a subset of transit amplifying cells. Although Sox9 protein is not detected in neuroblasts, they express sox9 mRNA. The onset of miR-124 at the transition from a dividing transit amplifying cell to a neuroblast mediates the repression of Sox9 translation. Blocking Sox9 expression allows the neurogenic progression of the lineage (arrow to the right), whereas over-expression of Sox9 maintains cells as astrocytes.
| Rank | mIR-124 targets                                                                 | Score |
|------|--------------------------------------------------------------------------------|-------|
| 1    | development, cell differentiation, morphogenesis, organ development            | 12.25 |
| 2    | organelle, intracellular organelle, intracellular membrane-bound organelle, intracellular membrane-bound organelle | 11.16 |
| 3    | metal binding, metal ion binding, ion binding, cation binding, zinc, zinc-finger, zinc ion binding, transition metal ion binding | 9.04  |
| 4    | nuclear protein, transcription regulation                                       | 9.04  |
| 5    | transport, establishment of localization, localization, transport, transport    | 7.8   |
| 6    | cellular physiological process, cellular process, primary metabolism, cellular metabolism, metabolism, macromolecule metabolism, physiological process | 6.87  |
| 7    | nervous system development, cell development, neurogenesis, neuron differentiation, axonogenesis, neuron morphogenesis during differentiation, neurite morphogenesis, neuron development, cellular morphogenesis during differentiation, cellular morphogenesis | 6.69  |
| 8    | membrane, transmembrane, glycocalyx, transmembrane glycoprotein, transmembrane region, membrane, glycosylation site N-linked [GlyXAc...) | 5.52  |
| 9    | positive regulation of transcription, positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism | 5.35  |
| 10   | cytoskeletal protein binding, actin binding, actin binding                       | 5.02  |
| 53   | cell cycle, regulation of progression through cell cycle, regulation of cell cycle | 1.29  |
Supplementary Table 1. Gene Ontology (GO) based functional clusters overrepresented in the miR-124 predicted target list
Table shows the top ten groups that obtained the highest EASE score from the DAVID-EASE analysis and “cell-cycle” group (No. 53 in the ranking). The first two GO terms from each group were used to confirm their over-representation among miR-124 targets with comparison to target lists of other miRNAs (in Supplementary Table 2).
**Supplementary Table 2.** Functional clusters over-represented in the miR-124 predicted target list compared to that of miR-1, miR-194, miR-181 and miR-9

The table shows the level of enrichment for each of the GO terms or swissprot database keywords (asterisk) among the miR-124 targets compared to the other miRNA target lists. The Fisher Exact p-value reflects the gene-enrichment for each annotation term.
Supplementary Methods

**Fluorescence activated cell sorting.** The different cell stages of the SVZ lineage were purified following procedures described in 1. Briefly, the SVZs from 2 month old adult hGFAP-GFP mice (Jackson labs), which express GFP under the control of the human GFAP promoter 2, or wild type CD-1 mice were dissected and dissociated as previously described 3. Cells were incubated for 10 minutes on ice with Phycoerythryn (PE)-conjugated rat anti-mCD24 (1:20, BD Pharmingen) and Alexa647-complexed EGF ligand (1:20, Molecular Probes) followed by three washes with ice-cold 1% BSA/0.1% glucose/1x HBSS. Vibrant DyeCycle Violet stain was added to the cells for 30 minutes at 37°C before sorting (1:1000, Molecular Probes). Cells were sorted using a Becton Dickinson FACS Aria. SVZ stem cells correspond to 1.49% of total SVZ cells and 9.3% of SVZ astrocytes, other SVZ astrocytes 13% of total SVZ cells, transit amplifying cells 5.43% of SVZ cells and neuroblasts 36.1% of SVZ cells. Only stem cell astrocytes (20-fold enrichment) and transit amplifying cells (7-fold enrichment) generate neurospheres.

**Immunohistochemistry.** 40 µm vibratome sections and whole mounts were prepared as described 3,4. Samples were fixed with 3% PFA overnight and fixed whole mounts were incubated in 100% methanol followed by 100% acetone (30 min each at -20°C) prior immunostaining except for EGFR. Samples were blocked in 10% normal serum/0.5% Triton-X100 for 1 hour (mCD24 and EGFR staining were performed without Triton-X100) and incubated with primary antibodies diluted in the blocking solution overnight (sections) or for 48 hours (whole-mounts) at 4°C. Secondary antibodies were applied for 2 hours at room temperature.

Antibody dilutions: guinea pig anti-DCX (1:300, Chemicon), goat anti-DCX (1:250, Santa Cruz), rabbit anti-BLBP (1:1500, gift from Dr. N. Heintz), rabbit anti-Ki67 (1:500, NovoCastra), mouse anti-NeuN (1:500, Chemicon), sheep anti-GFP (1:500, Biogenesis), mouse anti-TuJ1 (1:500, Covance), sheep anti-EGFR (1:250, Upstate), rabbit anti-Sox9 (1:2000, Chemicon), rabbit anti-GFP (1:1000, Molecular Probes), rat anti-mCD24 (1:100, BD Pharmingen), rabbit anti-Olig2 (1:500, Chemicon). Secondary antibodies conjugated to fluorophores Cy2, Cy3, Cy5 (1:250, Jackson Immuno) or Alexa488, Alexa568 (1:1000, Molecular Probes).
**Immunocytochemistry.** Cells were fixed for 10 minutes in a 1:1 mix of 3% PFA and culture medium, followed by 10 minute fixation in 3% PFA. Cells were washed with PBS, blocked in 10% normal serum/0.1% Triton-X100 (M2/M6 staining was performed without Triton-X100). Cells were incubated with primary antibodies overnight at 4°C. For BrdU staining, cells were fixed again for 10 minutes in 3% PFA and treated with 2 N HCl at 37°C for 10 minutes followed by 0.1 M sodium borate neutralization and incubated overnight with Alexa647-conjugated mouse anti-BrdU (1:200, Molecular Probes) at 4°C. Nuclear staining was performed by incubating with DAPI (1:1000, Sigma) or TOPRO-3 (0.5 μM, Molecular Probes) for 10 minutes at room temperature.

Antibody dilutions: mouse anti-TuJ1 (1:500, Covance), rat M2 and M6 (1:200 each, Developmental Studies Hybridoma Bank), rabbit anti-TuJ1 (1:500, Convance), rabbit anti-GFP (1:1000, Invitrogen), chicken anti-GFP (1:1000, Chemicon), mouse anti-Nestin (rat-401 clone, 1:1, Developmental Studies Hybridoma Bank), mouse IgM anti-Vimentin (40EC clone, 1:10, Developmental Studies Hybridoma Bank), guinea-pig anti GFAP (1:1000, Chemicon), rabbit anti-DsRed (1:100, Clontech). Secondary antibodies conjugated to fluorophores Cy2, Cy3, Cy5 (1:1000, Jackson Immuno) or Alexa488, Alexa568 (1:5000, Molecular Probes).

**Co-cultures.** Cultures of neonatal rat cortical astrocytes (passage 3 or 4) were trypsinized and plated in growth medium at 50,000 cells per cm² in 16-well LabTek chamber slides coated with 0.5 mg/ml PDK (Sigma). Upon confluency, monolayers were rinsed with 4 changes of DMEM-F12 and the medium was replaced by NB/B27 after the last wash. NB/B27 was conditioned by the monolayer for 24 hours. FACS purified SVZ cells were plated at clonal density (500 cells per cm²) on the monolayer for 5 days. 1 μg/ml of BrdU was added to the medium in the last 24 hours. Cells were fixed in 3% PFA for 20 minutes and immunostained.

In the miR-124 knockdown experiments, 200 nM of penetratin-conjugated 2′OMe-RNAs were added to the cultures every 24 hours by replacing the medium volume by half.

For ectopic miR-124 expression in the co-cultures, wild type mice were used instead of hGFAP-GFP mice as the retrovirus expresses GFP. EGFR expressing SVZ cells, which correspond to stem cell astrocytes and transit amplifying cells, were purified by FACS and infected with retrovirus for 4 hours at 37°C immediately after sorting. Retroviral infection was performed
before plating so as not to alter the bed of cortical astrocytes. Cells were then washed several times to remove viral particles and subsequently plated on rat astrocyte monolayers. After 5 days, co-cultures were processed as described above.

**Cell cultures.** HeLa, EcoPack 2-293 and NIH 3T3 cell lines were grown in DMEM supplemented with 10% fetal bovine serum and 2 mM glutamine, in an incubator with 5% CO₂ at 37 °C.

**Retroviruses.** pSUPER.Retro vector (pSR, OligoEngine) was modified to carry an enhanced GFP reporter driven by the PGK promoter (pSR-GFP). EGFP fragment from pEGFP-C1 (Clontech) was digested from Nhel-BspE sites and cloned into the pSR vector substituting the puromycin gene (Nhel-Nsil). Incompatible sites were blunted by Klenow fragment. mir-124-3 locus on chromosome 2 and its ~125 bp flanking sequences were PCR amplified from mouse genomic DNA. The PCR product was TOPO cloned (pCRII, Invitrogen), sequenced, and subcloned from BamHI-EcoRV into BglII-HincII sites of the second expression cassette on the pSR vector. The expression of the miRNAs was placed under the control of H1 promoter. Mutated mir-124 was modified by two steps PCR with QuikChange site-directed mutagenesis kit (Stratagene). See primer list for mutation sites.

Sox9 shRNA sequences were chosen based on previous reports. Hairpin sequences were designed (Ambion), annealed and cloned into BglII-XhoI sites of pSR-GFP. See primer list for hairpin sequences.

Sox9 retroviruses were generated with either mCherry or YFP reporter. mCherry fragment was PCR amplified from the pCMV-Cherry vector (provided by R. Tsien) and subcloned into XhoI site of pQCXIX retroviral vector (Clontech). EYFP fragment from pLP-EYFP-C1 vector (Clontech) was AgeI-XhoI digested and subcloned into MluI-XhoI site of pQCXIX. The open reading frame of the mouse sox9 gene was PCR amplified from the I.M.A.G.E. clone ID# 5351850. The PCR product was sequenced and subcloned into NotI-EcoRI sites of pQCXIX. See primer lists.

To prepare retrovirus, pSR or pQC constructs were transfected with Lipofectamine2000 (Invitrogen) into the EcoPack2-293 cell line (Clontech). The supernatants were harvested 24
hours post-transfection. Viral particles were concentrated at 20,000 rpm for two hours and resuspended overnight in DMEM-F12. The concentrated viral titers were ≥ 10^7 cfu/ml.

To analyze Sox9 protein levels under miR-124 overexpression, 70% confluent cultures were transfected with pSR constructs employing NeuroPORTER (Genlatis) following manufacturer’s protocol. After 3-4 days, Sox9 protein levels were determined by immunostaining.

To study the effects on differentiation, SVZ cells were transduced with retroviruses and maintained as adherent cultures for 7 days with EGF. After a further 7 days of EGF withdrawal, the cell fate of the GFP-positive retrovirally transduced progeny were determined by immunostaining.

**Luciferase reporter assays.** Sequences covering the putative miR-124 binding sites of *jagged1* and *sox9* 3’UTR were PCR amplified from mouse genomic DNA and cloned into the *XhoI-NotI* sites of the luciferase reporter vector pSiCHECK-2 (Promega). For the *dlx2* luciferase reporter, oligonucleotide containing the only putative miR-124 binding site was annealed to its complementary strand and cloned into pSiCHECK-2. Luciferase reporter constructs were co-transfected with pSR constructs over-expressing miR-124, reverse sequence of miR-124 (124rev) or mutated miR-124 (124mt) into HeLa cells by Lipofectamine2000 (Invitrogen). Luciferase activity was assayed 24 hours after transfection using the dual-luciferase reporter assay system (Promega).

**Western blot.** Three days after retroviral infection, RV-shRNA(+) and (-) cells were FACS purified gating on GFP expression. Cell lysates were processed in protein extraction buffer (1% SDS, 50mM Tris pH 7.4, 10mM EDTA, add 1x protease inhibitor before use) and 1-5µg of protein were electrophoresed on a 4-12% gradient NuPAGE gel (Invitrogen). After transfer to a PVDF mini-gel blot (QIAGEN), membranes were blocked in 1x Odyssey blocking buffer for 2 hours and stained with antibodies against Sox9 (rabbit, 1:5000, Chemicon) and GAPDH (mouse, 1:500, Santa Cruz) overnight at 4°C. Signals were revealed by anti-rabbit IRD800 (1:5000, Rockland) and anti-mouse Alexa680 (1:5000, Molecular Probe) secondary antibodies and quantified by Odyssey Infrared Imaging System and its densitometric analysis software (LI-COR Biosciences).
List of primers used:

For in situ hybridization

miR-124 5'-TTAAGGCACCGCGTGAATGCCAcctgtctc-3’
miR-194 5’-TGTAACAGCAACTCCATGTGAccgtctc-3’
scrambled 5’-AGATCCGACGTGTAAGACAGTTcctgtctc-3’

Lower case letters are the sequences complementary to the T7 promoter.

Retroviral constructs

pSR-124 forward 5’-GAAGCTGGAGCATTCGCGC-3’
reverse 5’-aaaaaaCCTTCTCGTGACGTCCTAGG-3’
pSR-124rev forward 5’-TAAGGCTACTTTTGCAGGCACTC-3’
reverse 5’-aaaaaaCATCTCATCTTTCTCCCCATCCC-3’
pSR-194 forward 5’-GAGGTTGGCGTCCTATGG-3’
reverse 5’- aaaaaaGCTCCTTCGAGTCGGTATGGC-3’
pSR-shRan
sense 5’-GATCCCCCTGGCCTTACACGTCGGATCCTCAAGAGA
GATCCGACGTGTAAGCCAGTTTTTTTA-3’
antisense 5’-TCGATAAAAAACTGGGCTTACACGTCGGATCTCTCTTTGAG
GATCCGACGTGTAAGCCAGGGG-3’
pSR-shSox9-A
sense  5’-GATCCCGAGCGACGTCATCTCCAAACATTCAAGAGA
           TGTTGGAGATGACGTCGCTGCTTTTTTA-3’
antisense 5’-TCGATAAAAAAGCAGCGACGTCATCTCCAACA
           TCTCTTGAATGTTGGAGATGACGTCGCTGGG-3’

pSR-shSox9-C
sense  5’ -GATCCCCGAACAGACTCACATCTCTTCAAGAGA
         GAGAGATGTGAGTCTGTTCCGTTTTTA-3’
antisense 5’-TCGATAAAAAACGGAACAGACTCACATCTCTC
           TCTCTTGAAGAGAGATGTGAGTCTGTTCGGG-3’

Oligo d(T)$_6$ was introduced as stop signal for polymerase III promoter $H1$ and the underlined letters are the sites used for sub-cloning.

pQC-Sox9

$mCherry$

forward 5’-GCCGGCCTCGAGGTCGCCACCACATGGTGAGCAAGG-3’
reverse 5’-GCCGGCCTCGAGGCCGCTTTACTTG-3’

$sox9$ open reading frame

forward 5’-CCCCCCGCGGCGGCGGAACTGGCCCTGGGAAACTTCTGTGGG-3’
reverse 5’-CCCCCCGGAATTCATAGCCTTTCTCTCTCAGGGTCTGG-3’

Underlined letters are the restriction sites used for subcloning.

*Mutagenesis to generate pSR-124mt*
Step 1:
forward 5’-GGAAGATTAAATGTCCATACAATTTTGGCAGGCAGTGAATGGCAAG-3’
reverse 5’-CTTGGCATTACCACCGCTGCCAAAATTGTATGGACATTAAATCTTCC-3’

Step 2:
forward 5’-GGAAGATTAAATGTCCATACAATTTTCCCAGGCAGTGAATGCC-3’
reverse 5’-GGCATTCACCGCTGGAAAAATTGTATGGACATTAAATCTTCC-3’

Underlined letters are the mutations introduced. The new 5’ seed region of the mutated miR-124 was confirmed by sequencing.

*Luciferase Assay*

*jagged1*  
forward 5’-CTCGAGTGATGGGCAGCCTTATTGATCATA-3’
reverse 5’-GCGGCCGCAACACCAAGTATCTCCCATGTC-3’

*sox9*  
forward 5’-CTCGAGCTTGGATTTCAAGAGTAGCTGCTT-3’
reverse 5’-GCGGCCGCTCCAGCACAGCGGATTTAAAGGC-3’

*dlx2*  
5’-CTCGAGATGGTCATCCGCAAAGGCACCTAAAACCTTTAAAAAGCGGCCGC-3’

Underlined letters are the restriction sites for subcloning and letters in bold are the putative miR-124 binding site.
Supplementary References

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